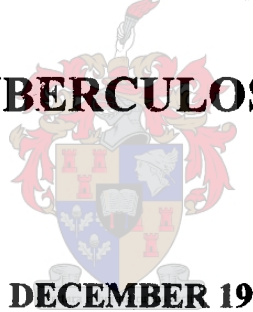
IMMUNE REGULATION IN CHILDREN AND ADULTS IN A COMMUNITY WITH A HIGH INCIDENCE OF TUBERCULOSIS



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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not, to my knowledge, previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

There is a progressive maturation of the immune system from infancy to adulthood. The immature immune system in early life is characterised by impaired macrophage function and antigen presentation as well as a higher naïve to memory T cell ratio with subsequent diminished IFN-γ production. Children with tuberculosis often present with lymphadenopathy, the complications thereof or with systemic spread of the organisms. Adults generally manifest with pronounced systemic effects (such as weight loss and high fever) and immunopathology (such as cavitation and fibrosis). We hypothesised that the immunopathology in adults may be due to enhanced cytokine production in comparison to children. The first aim of this study was therefore to measure cytokine responses in healthy children and adults. Cytokine responses in patients with tuberculosis will be examined in future studies. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from 9 healthy children and 9 healthy adults. The cells were cultured in serum-free medium, unstimulated or polyclonally stimulated with Phytohaemagglutinin (PHA). Supernatants were harvested after which IFN-γ, IL-2, TNF-α, IL-4 and IL-10 production was determined by means of ELISA analysis. RNA was subsequently extracted from the cells followed by RT-PCR analysis for the semi-quantitative determination of mRNA levels of these cytokines. PBMC isolated from healthy children produced significantly less IFN-γ protein than adults. Futhermore, IFN-γ production in the adults seemed to be trimodally distributed. No significant differences could be found in the production of IL-2, TNF-α, IL-4 and IL-10. Although children produced low levels of IFN-γ protein, their IFN-γ, TNF-α, IL-2, IL-4 and IL-10 mRNA levels were comparable to that of adults.

Tuberculosis is a major cause of mortality and morbidity, particularly in the third world. Ravensmead and Uitsig, two adjacent suburbs in the Western Cape, have a tuberculosis incidence of > 1 000/100 000 population. Also, up to 90 % of the children in the Western Cape have been reported to be infested by intestinal parasites such as *Ascaris lumbricoides* and *Trichurius trichuria*. Infection with *M. tuberculosis* induces a Th1

immune response, while intestinal parasites elicit a Th2 immune response. Th2 dominance induced by intestinal parasite infestations could predispose individuals to an enhanced susceptibility to *M. tuberculosis*. The second aim of this study was to investigate serum IgE levels, surrogate markers for Th2 activation, in the community. The serum IgE levels were subsequently correlated to the tuberculosis incidence per enumerator sub-district (ESD), crowding, female literacy and socio-economic levels. Similarly, the tuberculosis incidence per ESD was correlated with the above mentioned parameters. A significant positive correlation was found between tuberculosis incidence and the serum IgE levels in the community. However, further studies are needed to determine if intestinal parasites are the main cause of the high IgE levels in the community and to determine if parasite loads or Th2 dominance are causally linked to the incidence of tuberculosis. Correlation between serum IgE levels and tuberculosis incidence with the other parameters were significant, except in the case of crowding.

The third aim of this study was to measure serum IgE and specific IgE levels against Ascaris and common allergens on presentation of tuberculosis and again after completion of successful treatment. Significant declines in serum IgE and Ascaris specific IgE levels were observed after completion of tuberculosis treatment. This down regulation of IgE levels may be due to an up regulation of Th1 responses in patients following successful treatment for tuberculosis.

OPSOMMING

Die immuunsisteem matureer toenemend vanaf kinderjare tot en met volwassewording. Die onvolwasse immuunsisteem van jong kinders word gekenmerk deur verswakte makrofaag-funksionering en antigeenpresentering, sowel as 'n verhoogde naïwe tot geheue T-sel verhouding met gevolglike verminderde IFN-γ produksie. Kinders met tuberkulose presenteer gewoonlik met limfadenopatie, komplikasies daarvan of met gedissemineerde siekte. Volwassenes presenteer met sistemiese gevolge (soos gewigsverlies en hoë koors) en immunopatologie (soos kavitasie en fibrose). Ons hipotese is dat die immunopatologie in volwassenes die gevolg is van 'n verhoogde sitokienproduksie in vergelyking met kinders. Die eerste doelwit van die studie was om sitokienproduksie in gesonde kinders en volwassenes te meet. Sitokienproduksie in tuberkulose pasiente sal in 'n opvolgstudie bepaal word. Perifere bloed mononukleêre selle was geïsoleer vanuit heel bloed verkry vanaf 9 gesonde kinders en 9 gesonde volwassenes. Die selle was gekweek, ongestimuleer of gestimuleer met Phytohaemagglutinien (PHA). Supernatante was geoes vir die bepaling van IFN-γ, IL-2, IL-4, IL-10 en TNF-α produksie, deur gebruik te maak van ELISA analise. RNA was gevolglik vanaf die selle ge-ekstraheer vir die tru-transkriptase polimeerketting reaksie analise, waartydens sitokien mRNA vlakke op 'n semi-kwantitatiewe wyse bepaal was. Perifere bloed mononukleêre selle geïsoleer vanaf die kinders het minder IFN-γ geproduseer as dié van volwassenes. Hierdie verminderde produksie was hoogs betekenisvol. Dit wou voorkom asof die IFN-γ produksie deur volwassenes trimodaal versprei was. Geen betekenisvolle verskille tussen kinders en volwassenes kon gevind word in die produksie van IL-2, IL-4, IL-10 en TNF-α nie. Alhoewel kinders minder IFN-γ proteïen geproduseer het, het hulle IFN-γ, IL-2, IL-4, IL-10 en TNF-α mRNA produksie met vlakke van volwassenes ooreengestem.

Tuberkulose speel 'n groot rol in morbiditeit en mortaliteit in veral die derde wêreld. Ravensmead en Uitsig, twee aangrensende voorstede in die Wes-Kaap, het 'n tuberkulose voorkomssyfer van > 1 000/1 00 000 populasie. Verder, is tot 90 % van die kinders in die

Wes-Kaap geïnfesteer met intestinale parasiete soos *Ascaris lumbricoides* en *Trichurius trichuria*. *M. tuberculosis* infeksie induseer 'n Th1 immuunrespons, terwyl intestinale parasiete 'n Th2 immuunrespons uitlok. 'n Dominante Th2 respons mag moontlik individue predisponeer tot 'n verhoogde vatbaarheid vir *M. tuberculosis*. Gevolglik was die tweede doelwit van die studie om serum IgE vlakke as surrogaat merkers vir Th2 aktivering in die gemeenskap bestudeer. Die serum IgE vlakke was gevolglik gekorreleer met die tuberkulose voorkoms per opnemerssensusgebied (OSG), saamdringing, vroulike geletterdheid en sosio-ekonomiese vlakke. Die tuberkulose voorkoms per OSG, is op dieselfde wyse gekorreleer met die bogenoemde parameters. 'n Betekenisvolle positiewe korrelasie is gevind tussen tuberkulose voorkoms en serum IgE vlakke in die gemeenskap. Verdere studies is egter nodig om te bepaal of intestinale parasiete wel die oorsaak van die hoë IgE vlakke in die gemeenskap is en of parasiet ladings of Th2 dominansie oorsaaklik verbind kan word aan die tuberkulose voorkoms.

Die derde doelwit van die studie was om serum IgE en spesifieke IgE vlakke teen Ascaris en algemene allergene te meet met presentering van tuberkulose en weer na voltooing van suksesvolle behandeling. 'n Betekenisvolle afname in serum IgE en Ascaris spesifieke IgE vlakke is waargeneem na voltooing van tuberkulose behandeling. Die afregulering van IgE vlakke kan moontlik toegeskryf word aan die opregulering van Th1 response in pasïente na voltooing van suksesvolle behandeling van tuberkulose.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED IN THIS TEXT

APC	antigen presenting cells
BSA	bovine serum albumin
CMI	cell mediated immunity
CPM	counts per minute
dATP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
DHEA	dehydropepiandrosterone
dGTP	deoxyguanidine triphosphate
dNTP	deoxynucleotide triphosphate
DTH	delayed type hypersensitivity
dTTP	deoxythymidine triphosphate
ELISA	enzyme-linked immunosorbent assay
ESD	enumerator sub-districts
EtBr	ethidium bromide
FCS	foetal calf serum
GIS	geographical information system
HDM	house dust mite
IFN-γ	interferon-gamma
Ig	immunoglobulin
IgG	immunoglobulin G
IgE	immunoglobulin E
IL-2	interleukin-2

IL-4	interleukin-4
IL-5	interleukin-5
IL-10	interleukin-10
IL-12	interleukin-12
kU/l	kilo-units per liter
Log	logarithm
Ln	natural logarithm
MHC	major histocompatibility complex
m/v	mass per volume
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
SD	standard deviation
SI	stimulation index
RT-PCR	reverse transcriptase polymer chain reaction
RPMI	Roswell Park Memorial Institute
Taq	*Thermus aquaticus*
TB	tuberculosis
TBE	tris-borate-EDTA buffer
TCR	T cell receptor
TGF-β	transforming growth factor βeta
TNF-α	tumour necrosis factor-alpha

Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
v/v	volume per volume ratio

CHAPTER 1

INTRODUCTION

1.1 BIOLOGY OF THE IMMUNE SYSTEM

To enable an organism to survive in an unfriendly environment, the immune system must be able to mount a destructive immune response against non-self without destroying the host itself. The immune system must therefore be able to distinguish self from non-self. Healthy individuals protect themselves against microbes by means of innate and adaptive immunity. The adaptive or specific immune response can be classified into two types, namely humoral and cell mediated immunity. Humoral immunity is mediated by B cell derived antibodies responsible for specific recognition and elimination of antigens, while helper and cytotoxic T cells regulate cell mediated responses.

1.1.1 Innate and acquired immunity

The non-specific or innate immune system is not affected by previous contact with an infectious agent (Abbas *et al.*, 1991). The immediate line of defence is the skin, which when intact, is impenetrable to most infectious agents. Mucus, secreted by the membranes lining the inner surfaces of the body, acts as a protective barrier to block the adherence of bacteria to epithelial cells. Microbial and other foreign particles held within the adhesive mucus are eliminated by mechanical designs such as ciliary movement, coughing and sneezing (Abbas *et al.*, 1991). If micro-organisms do penetrate the body, two main defensive operations come into play, namely the destructive effect of bactericidal enzymes and the mechanism of phagocytosis. The main phagocytic cells are polymorphonuclear neutrophils and macrophages. Organisms attach to their surface, activate the engulfment process and are then taken up by these cells where they fuse with cytoplasmic granules. A formidable array of oxygen-dependent and oxygen-independent microbicidal mechanisms, within the cell come into play. The complement system, a multi-component enzyme cascade, is used to attract phagocytic cells to the microbes and to enhance phagocytosis.

Micro-organisms which overwhelm or elude the innate non-specific immune mechanisms, come up against the host's second line of defence. Antigens coming into contact with the cells of this form of immunity initiate the adaptive immune response.

Leukocytes specialised for processing and presenting antigen for the acquired immune response include monocytes, macrophages, Langerhans cells, Kupffer cells and dendritic cells (Huston, 1997). All these cell populations express both class I and class II major histocompatability complex (MHC) molecules, which present peptides processed through alternative mechanisms for recognition by T cell receptors (TCR). Macrophages and monocytes participate in inflammatory and often granulomatous immune responses throughout the body, while Langerhans cells and Kupffer cells process antigen encountered in the skin and liver, respectively. Dendritic cells abide mainly within the lymphoid tissues and are the most effective antigen presenting cells. B cells also have the potential to process and present antigen in the context of MHC class II molecules, following internalisation of antigen engaged by their membrane immunoglobulins (Ig). Table 1.1 depicts the barriers, circulating molecules and cells involved in innate and acquired immunity.

Table 1.1

Cells involved in innate and acquired immunity.

	Innate	Acquired
Physiochemical barriers	Skin, mucous membranes	Cutaneous and mucosal immune systems; antibody in mucosal secretions.
Circulating molecules	Complement, collectins	Antibodies
Cells	Phagocytes (neutrophils and macrophages), NK cells, eosinophils and basophils	T and B Lymphocytes

(Taken from Abbas *et al.*, 1991)

1.1.2 Cells of the immune system

Lymphocytes consist of three major populations: T cells, B cells and natural killer (NK) cells. T cells are phenotypically defined by their cell surface expression of a T cell

receptor (TCR) which binds antigen presented by antigen presenting cells (APCs). B cells on the other hand, are phenotypically defined by their cell surface expression of trans-membrane Ig that can bind unprocessed antigen independent of APCs. NK cells are morphologically large granular lymphocytes and are phenotypically defined by the absence of either trans-membrane cell surface TCR or Ig as well as by the presence of the cell surface molecules CD16 and CD56.

1.1.2.1 T lymphocytes

More than 90 % of all peripheral T cells in mouse and man express a T cell receptor which is composed of an α and β chain. These T cells express either the CD4 or CD8 accessory molecule (αβ T cells). $CD4^+$ T cells provide help for antibody production, mediate delayed hypersensitivity responses and interact with peptide:MHC class II complexes. $CD8^+$ T cells have cytotoxic or suppressor functions and interact with peptide:MHC class I complexes.

1.1.2.2 B lymphocytes

The hallmark of B cells is their production of Ig. The major Ig classes in humans are IgA, IgD, IgE, IgG, IgM (Abbas *et al.*, 1991).

1.1.3 Cytokines

Cytokines are low molecular weight proteins usually in the order of 15-25 kDa (Nicod, 1993). These proteins are involved in cell growth and differentiation, inflammation, immunity and repair. Cytokines play a key role in facilitating communication between immune cells and orchestrating both the activation and damping of the immune system in response to infection or injury. Cytokines are produced upon stimulation and are effective in the pg/ml to ng/ml range. Once released, their half-life is short. Unlike hormones that are transported via the blood stream to their target organs, cytokines normally act in the local milieu in a paracrine or autocrine manner. Multiple cytokines are produced by T cells, B cells, NK cells, macrophages and mast cells following stimulation and exert an array of functions (Table 1.2). Cytokines are grouped into 2 classes depending on their function. Type 1 cytokines activate macrophages, cytotoxic T cells and NK cells and play

Table 1.2

Cytokines with origin and function.

Cytokine	Source	Function
IL-1	Macrophages, fibroblasts	Proliferation of B- & T cells Induces fever Induces acute phase proteins Induces IL-6
IL-2	T cells	Growth factor: B- & T cells Activation of NK cells
IL-4	Mast cells, T cells	Inhibition of Th1 activation Proliferation of B- & mast cells Isotype switch to IgG4 and IgE
IL-5	Mast cells	Production of IgE & IgM Production of activated B cells
IL-10	T- & B cells	Inhibition of Th1 activation
IL-12	T cells, macrophages	Differentiation of T cells Inducer of IFN-γ production Activation of NK cells
TNF-α	Macrophages, T cells	Tumour cytotoxicity Cachexia
TNF-β	CD 4 T cells	Anti-viral activity Activation of phagocytic cells Endotoxic shock
IFN-γ	NK cells, T cells	Inhibition of Th2 activation Anti-viral activity Anti-microbial activity Macrophage activation Antagonism of IL-4 action
TGF-β	T- & B cells	Wound repair Immuno-suppression

a crucial role in the generation of the cell mediated immune response to intracellular organisms such as mycobacteria, viruses and protozoa. Type 2 cytokines lead to the stimulation and differentiation of B cells, eosinophils and mast cells, which are involved in the humoral response.

1.1.4 Development of Th1 and Th2 subsets of $CD4^+$ Th cells

1.1.4.1 Discovery of discrete T helper (Th) subsets

In 1986, Mosmann *et al.* first demonstrated that mouse $CD4^+$ T helper cells could be classified into distinct populations based on their cytokine secretion patterns. These two populations were termed Th1 and Th2. Th1 cells produce IL-2, IFN-γ and TNF-β (Figure 1.1). Th2 cells produce IL-4, IL-5, IL-6 and IL-13 (Figure 1.1). It was originally accepted that IL-10 is a product of Th2 cells (Fiorentino *et al.*, 1989), but it has become clear that IL-10 is also secreted by Th1 cells (Somasse *et al.*, 1996) and by activated macrophages. IL-12, produced by activated macrophages and dendritic cells, is the principal Th1-inducing cytokine. The early presence of IL-4 at the time of antigen presentation and recognition is the most potent stimulus for Th2 differentiation. T cells that produce a mixture of Th2 and Th1 cytokines have been termed Th0.

1.1.4.2 Heterogeneity and interaction of the Th1 and Th2 subsets

Th1 cells, by their production of IFN-γ, are responsible for directing cell-mediated immune responses leading to the elimination of intracellular pathogens (Sher and Coffman 1992; O'Garra, 1998). Functionally, Th1 cytokines are required for delayed type hypersensitivity (DTH), macrophage activation and the production of IgG2a in mice and IgG3 in humans. Th2 cytokines regulate IgE production, induce eosinophilia and enhance the production of IgG1 (mice) and IgG4 (humans) (Street *et al.*, 1991; Abbas *et al.*, 1996). Cytokines produced by each subset, inhibit the development and activity of the other subset (Figure 1.1). IFN-γ produced by Th1 cells amplifies Th1 development and inhibits proliferation of Th2 cells, whereas IL-4 produced by Th2 cells blocks activation of Th1 cells. The net result of cytokine-mediated self-amplification and cross-regulation

is that once a T cell immune response begins to develop along one pathway, it tends to become progressively polarised in that direction (Abbas *et al.*, 1996).

Furthermore, Abbas *et al.* (1996) proposed that the two subsets should be categorised on the basis of their function (*i.e.* type 1/type 2) rather than on the cell type that produces them (Th1/Th2). This is necessary as some of the Th1 and Th2 cytokines have been shown to be produced by non-$CD4^+$ cells and by non-T cells (Clerici and Shearer, 1994).

1.1.4.3 Cytokine induced Th development

IL-12 is the main Th1-inducing cytokine. This cytokine is produced by macrophages upon their encounter with microbial products such as components from viruses and intracellular bacteria such as mycobacteria. Th1 development is also IFN-γ dependent (Belosevic *et al.*, 1989; O'Garra, 1998). The effects of IFN-γ on Th1 development may be mediated via action on the macrophage to up-regulate IL-12 production (Trinchieri, 1995) or by direct effects on the T cell.

The development of Th2 cells has been attributed to the exposure of naïve $CD4^+$ T cells to IL-4 at the initiation of an immune response (Seder and Paul 1994; O'Garra, 1998). The effects of IL-4 in inducing Th2 development are dominant over Th1 polarising cytokines, so that if IL-4 levels reach a certain threshold at the beginning of an immune response, Th2 cells will differentiate, leading to a progressive increase in IL-4 levels. This may explain why chronic stimulation, particularly in the absence of inflammatory signals delivered by the innate immune response, as well as the magnitude of an immune response, may drive Th2 responses (Abbas *et al.*, 1996).

1.1.4.4 Other factors influencing Th subset development

Mycobacterial antigens such as purified protein derivative (PPD) induce a Th1 response, whereas parasite antigens such as Toxocara excretory substance induce a Th2 response (Del Prete *et al.*, 1991; reviewed by Constant and Bottomly, 1997). Low antigen concentration and low-dose infections tend to induce Th1 responses, whereas high doses induce Th2 development (reviewed by Constant and Bottomly, 1997) (Figure 1.2). It is

possible that at low doses of antigen, the principal antigen-presenting cells (APCs) are dendritic cells or macrophages (Abbas *et al.*, 1996) (Figure 1.2). Both dendritic cells and macrophages produce IL-12, tilting the balance of the specific T cell response towards Th1 differentiation. When the antigen concentration is high, it may be presented by APCs that do not secrete IL-12, thus favouring Th2 development. It is also possible that high concentrations of antigen lead to repeated T cell stimulation, thus increasing IL-4 production and Th2 development, or induce a state of immunological tolerance, which inhibits Th1 cells. Different concentrations of antigens may also trigger different signals from T cell antigen receptors, which may influence the pattern of differentiation. The amount of pathogens can also influence the type of host response. Immunising mice with 10^7 *M. vaccae* induces a Th1 response, whereas 10^9 organisms induce a mixed response (Hernandez-Pando and Rook, 1994).

Another factor known to influence T cell development is co-stimulation, which refers to signals provided by APCs that work together with antigen to enhance specific T cell responses. The best defined co-stimulators are two structurally related proteins, B7-1 and B7-2 (CD80 and CD86 respectively), both of which activate T cells by interacting with the CD28 receptor on their cell surface (Lenschaw *et al.*, 1996). The development of both Th1 and Th2 cells is dependent on co-stimulation. High levels of co-stimulation promote Th2 responses, probably because increasing the magnitude of initial T cell activation increases IL-4 production. Enhanced IL-4 production promotes the IL-4 dependent autocrine Th2 differentiation. Th1 development on the other hand, depends on IL-12 produced by APCs and appears to require lower levels of stimulation via the TCR and CD28. Fully developed Th2 cells respond to antigen by secreting IL-4 without B-7 co-stimulation, whereas Th1 cells continue to require the CD28/B-7 interaction for activation and the ability to produce IL-2 and IFN-γ, which are important for proliferation. Once a Th2 cell clone has developed, it will continue to secrete IL-4 and hence progressively proliferate independently of the type of APC and the presence of B-7/CD28 interaction.

Glucocorticoids enhance Th2 activity and synergise with IL-4 (Daynes *et al.*, 1991). Araneo *et al.* (1989) tested whether glucocorticoids affected the capacity of T cells to produce IL-4, IL-2 and IFN-γ. They found evidence both *in vitro* and *in vivo* that physiological doses of glucocorticoids enhanced the production of IL-4. Further *in vivo* studies showed an enhancement of IL-2 and IFN-γ production upon T cell activation, following treatment with metyrapone, a glucocorticoid inhibitor. Since treatment with metyrapone causes an increase in the production of dehydroepiandrosterone (DHEA) by T cells, the effect of DHEA on normal cells stimulated with anti-CD3 *in vitro* was also tested. DHEA enhanced the secretion of IL-2, but not IL-4 (Daynes *et al.*, 1990). These results suggest that elevating concentrations of glucocorticoids may profoundly affect the patterns of cytokines produced in a subsequent immune response. Glucocorticoids impede T cell proliferation by inhibiting IFN-γ and IL-2 production, but enhance IL-4 production and B cell differentiation.

1.1.4.5 Signalling pathways

Initial events in signal transduction via antigen and cytokine receptors, generally involve the phosphorylation of tyrosine, serine and threonine residues within the cytoplasmic domains of trans-membrane proteins and in soluble cytoplasmic proteins. IL-12 and IL-4 have been implicated as the cytokines regulating the polarising of naïve T cells to produce type 1 or type 2 cytokines. The IL-12 and IL-4 receptors associate with members of the JAK family. Ligand binding activates the kinases, which phosphorylate the cytoplasmic domains of the cytokine receptors on tyrosine residues. Cytoplasmic Stat (signal transducer and activator of transcription) molecules bind to the phosphorylated receptors, become phosphorylated on tyrosine residues by JAK kinases and subsequently dimerise via their SH2 domains. The binding of IL-4 and IL-12 by their receptors typically results in rapid tyrosine phosphorylation of the Stats. Signalling by IL-4 occurs

through activation of the Stat6. Knockout of the *Stat6* gene results in deficient Th2 responses (Abbas *et al.*, 1996).

IL-12 activates Stat1, Stat3, and Stat4 (Jacobson *et al.*, 1995). Of these, Stat4 appears to be selectively activated by IL-12. Knocking out either IL-12 or Stat4 results in markedly reduced Th1 responses. In animal models (O'Shea, 1997), a deficiency of Stat1 impairs IFN-γ-induced signal transduction, thereby increasing susceptibility to viral infections.

1.1.4.6 Th1/Th2 balance in animal models

The specific role that Th1 and Th2 cells play in determining the outcome of parasitic infection is illustrated dramatically in a mouse model of *Leishmania* infection. *Leishmania major* is an obligate intracellular parasite which causes death when injected into susceptible mice, *i.e.* Balb/c, but only causes a limited disease that is cured in resistant mice, *i.e.* C57BL/6. Balb/c mice exhibit a Th2-like response that is associated with severe, generalised disease and these animals are characterised by high IL-4, IgE, low IFN-γ levels and weak DTH reactions. On the other hand, C57BL/6 mice produce a Th1-like response that is associated with a contained, local infection and cure and these mice are characterised by low IL-4, IgE, high IFN-γ levels and strong DTH reactions. The susceptibility of Balb/c mice has been shown to be dependent on the production of IL-4 early in infection, while control of infection and resistance to re-infection in other mouse strain is dependent on IFN-γ.

1.2 ONTOGENY OF THE IMMUNE SYSTEM

The ontogeny of the host defence system begins during the first month of gestation with hematopoietic stem cells located in the yolk sac (Huston, 1997). By the third month of gestation, hematopoiesis occurs predominantly in the liver until the skeletal elements are formed. Thereafter, bone marrow is the major site of hematopoiesis. The hematopoietic stem cells differentiate into granulocytes, monocytes and lymphocytes as well as erythrocytes. Beginning two months into gestation, lymphocytes destined to become T cells migrate from the bone marrow into the developing thymus for their further maturation. The maturation of B lymphocytes occurs in bone marrow under the influence of the stromal reticular cells. By birth, the immune system has developed into a sophisticated network, connecting central locations of immune cell production with peripheral tissues for immune surveillance.

1.2.1 Age–related risk of infectious diseases

The progressive maturation of the immune system during infancy and early childhood, results in distinct periods of susceptibility to infection. The first month of life is characterised by susceptibility to gram-negative and group B streptococcal infections. Between one and six months of age, viral infections are frequent. During the next 12 months, viruses and bacteria expressing polysaccharide, particularly acute inflammatory cocci, are the major causative agents. There is progressive formation of immunologic memory and full immunologic functions from the age of two years (Pabst *et al.*, 1980). Imbalances in the increasingly sophisticated immune system cause other forms of immunologic diseases to manifest in children over two years of age.

Pabst *et al.* (1980) proposed that a peak of resistance to a number of infections occurs around 10 years of age. In their study, overall mortality in man followed a parabolic curve, with the nadir at 12 years of age, the time when total lymphoid tissue mass in the body has reached its peak. It is suggested that after this age, overall mortality due to

infectious diseases rises again, while lymphoid tissue mass declines and the numbers of immunoglobulin producing cells wane (Pabst *et al.*, 1980).

The rate of development of progressive tuberculosis also changes with age (Smith *et al.*, 1997). In New York, P. W. Beavan *et al.* (1950) found that the highest estimated rate of progressive tuberculosis was in children younger than 14 years of age (78.7 cases/1000 people infected). Similarly, in Norway the highest incidence of disease (73%) was the group from birth to children 6 years of age (Gedde-Dahl, 1952; Smith *et al.*, 1997) even though the total number of children in the cohort was low. Meningitis and miliary tuberculosis occur more frequently in young children than in older children and adults. In the United States, meningitis developed in 15-20% (Miller *et al.*, 1963, reviewed by Smith *et al.*, 1997) of children younger than one year with reactive tuberculin tests (Smith *et al.*, 1997). The number of infected individuals in whom disease developed, decreased with increasing patient age, with the lowest rate (25%) usually in individuals between the ages of 25 and 29 years.

1.2.2 Observations on the developmental stages of the immune system

1.2.2.1 Macrophages

There are indications that macrophages gradually maturate during childhood (Pabst *et al.*, 1980), with alveolar macrophages showing qualitative differences between adults and neonates (Smith *et al.*, 1997). A rapid postnatal rise in the number of alveolar macrophages, from 24 – 48 hours of age, to adult levels has been demonstrated in various species, including human neonates (Holt 1995; reviewed by Smith *et al.*, 1997). Many of the effector functions of alveolar macrophages are decreased in neonates relative to adults (Weiss *et al.*, 1986; Kurland *et al.*, 1988). Animal data have consistently demonstrated reduced microbial killing by alveolar macrophages and decreased microbial clearance from the lungs, in the neonatal period (Smith *et al.*, 1997). Whether deficits in alveolar macrophage function could impede the control of mycobacterial infections in the lungs of neonates, is unknown.

1.2.2.2 Antigen presentation

Certain aspects of antigen presentation appear to be less efficient in young infants compared with adults. Monocytes from human neonates are able to present antigens to maternal T cells as efficiently as maternal monocytes, implying intact MHC class II-restricted presentation of peptide antigens (Smith *et al.*, 1997). The dendritic cell population is uniquely specialised for the presentation of antigen to naïve T cells (Steinman *et al.*, 1991). Human cord blood dendritic cells, however, are less efficient APCs and stimulators of allogeneic responses than their adult counterparts (Hunt *et al.*, 1994). These findings may reflect diminished expression of MHC and certain co-stimulatory molecules. Thus the ability of dendritic cells from infants to present antigen to naïve, neonatal T cells appears reduced and does not attain adult competence until after 1 year of age (Clerici *et al.*, 1993). This view is strongly supported by recent studies in mice (Ridge *et al.*, 1996). These authors show that presentation of antigen by primed dendritic cells to naïve, neonatal T cells induces protection, whereas tolerance may result from presentation by inefficient APCs especially when antigen dose is high. Whether the same paradigm applies to human beings has yet to be tested.

These data imply that limitations in the function and perhaps the number of dendritic cells in the neonate may have adverse effects on the efficiency of antigen presentation. Available data suggests that peripheral tissues are seeded with bone marrow derived dendritic cell precursors late in gestation. At birth, however, dendritic cell population densities are generally much lower than in adults (Holt, 1995). This may contribute to a delay in initiation of an appropriate antigen-specific response, particularly in the draining lymph nodes of the infant respiratory tract (Holt, 1995; Nelson, 1995) and thereby contribute to the development of disease.

1.2.2.3 Lymphocytes

It has been known for more than half a century that the immune system is functionally less mature at birth (Kato *et al.*, 1935). Despite the newborn baby having strong immunologic armour, they need an additional few years to become fully capable of effectively handling severe pathogens. Within the first years of life, the immune system

undergoes a process of sequential development that is both programmed genetically and stimulated externally by antigen exposure. It is therefore expected that the absolute size of lymphocyte sub-populations would vary with age, because of the progressive expansion and maturation of the immune system.

During intra-thymic ontogeny, most developing T cells progress through stages in which the cells are sequentially $CD4^-CD8^-$, then $CD4^+CD8^+$ and then either $CD4^+CD8^-$ or $CD4^-CD8^+$. During B cell ontogeny, the stages of B cell maturation can be identified by progressive rearrangement of the Ig heavy and light chain genes and by distinct changes in cell surface phenotype (Fearon and Locksley, 1996). Comparisons among age groups from neonates through adults, revealed continuous declines in the absolute numbers of leukocytes, total lymphocytes, T- and B cells as well as NK cells (Erkeller-Yuksel *et al.*, 1992; Comans-Bitter *et al.*, 1997). However, the percentage of T cells within the total lymphocyte population increases with age (Erkeller-Yuksel *et al.*, 1992). These findings confirmed results obtained by Wiener *et al.* (1990) suggesting an increase in T cell percentages with age, but with no significant changes in the CD4/CD8 ratio across age groups. Alternatively, other studies support the idea of an elevated CD4/CD8 ratio in cord blood (Solinger *et al.*, 1985) or in the first year of life (Yanase *et al.*, 1986). Comans-Bitter *et al.* (1997) found limited fluctuations in $CD4^+$ and $CD8^+$ T cell subsets, but age related changes in the CD4/CD8 ratio with higher median values during the first 2 years of life and lower values at birth, older children and in adults.

NK cells are defined as CD3-negative lymphocytes that express CD16, CD56 or both. A variable proportion of these cells also co-express CD8 and CD57. The highest NK absolute count and percentage of NK cells are found in cord blood (Erkeller-Yuksel *et al.*, 1991; Comans-Bitter *et al.*, 1997). However, there are progressive increases in the percentages of NK cells from early childhood (2 to 5 years) onwards, in contrast with the absolute numbers that stay stable after the first 2 months of life. NK cells in cord blood are reported to be functionally less active than those in adults, perhaps as a reflection of

Table 1.3

Differences in the developmental stages of lymphocytes.

Compartment	Differences
T cells	Strongly decreased production of IL-4, IFN-γ and other cytokines by neonatal cell (Wilson *et al.*, 1986; Wakasugi *et al.*, 1985; Taylor *et al.*, 1985) Age related switch from $CD45RA^+$ to $CD45RO^-$ phenotype (Hannet *et al.*, 1992)
B cells	Strongly diminished immunoglobulin secretion by neonatal B cells (Gathings *et al.*, 1981; Splawsi *et al.*, 1991) Doubling of the proportion of B cells expressing CD23 between infancy and childhood (Hannet *et al.*, 1992)
Macrophages	Neonatal macrophages are functionally immature; phagocytosis and chemotaxis are impaired (Wilson, 1986; Taylor and Bryson, 1985)
NK cells	NK cells in cord blood are less active and have reduced IFN-γ production (Hannet *et al.*, 1992; Abo *et al.*, 1983)

their immaturity or because of a delay in the development of other cellular elements that may generate cytokines necessary for full NK activation (Table 1.3).

In humans, γδ T cells account for 1 to 5% of adult peripheral blood T cells, whereas negligible numbers are detected in the cord blood of new born infants (Smith *et al.*, 1990). The number of γδ T cells is reported to increase progressively with age (Pechhold

et al., 1994). The subset of γδ T cells that recognises isoprenyl pyrophosphates of mycobacteria (the vγ2vδ2 subset) is abundant in adults, but rare in neonates (Tsuyuguchi *et al.*, 1991; Smith *et al.*, 1997).

The percentage and absolute count of $CD19^+$ B lymphocytes are reported to increase during the first 5 months of life, remaining stable until approximately 5 years, followed by a gradual decrease at adult age (Comans-Bitter *et al.*, 1997).

1.2.2.4 Cell surface markers in naïve versus memory T cells

CD45 T cells. Differential expression of CD45 iso-forms has been used to classify functionally heterogeneous $CD4^+$ T cell subsets. "Memory" T cells which are responsive to recall antigens are generally $CD45RA^-RO^+$, whereas their "naïve" precursors are $CD45RA^+RO^-$ (Hassan *et al.*, 1993). $CD45RA^+$ cells are gradually replaced by $CD45RO^+$, presumably as a result of repeated antigen exposure. A plateau phase of $CD45RO^+$ cells is commonly reached between 15 – 20 years (Hannet *et al.*, 1992). The switch from $CD45RA^+$ to the $CD45RO^+$ phenotype appears to reflect an age-related accumulation of memory cells in the circulation. These phenotypic changes are associated with functional changes, such an increased proliferative response to IL-2 and an increased production of IFN-γ (Hannet *et al.*, 1992). *In vitro* $CD45RO^+$ T lymphocytes show enhanced help for IgE synthesis (Akbar *et al.*, 1991). These two populations are considered to represent different maturation stages of cells from the same lineage. As determined by the surface expression of the CD45 molecule, 90% of circulating neonatal T cells correspond with adult naïve T cells (Akbar *et al.*, 1988). Ageing leads to the replacement of virgin T cells by memory T cells, resulting in adult blood consisting of ~40% memory T cells ($CD45RA^-RO^+$) and ~60% naïve T cells ($CD45RA^+RO^-$) (Sanders *et al.*, 1988).

$CD25^+$ T cells. The IL-2 receptor (CD25) is expressed on activated T cells and to a lesser degree on B cells, NK cells, monocytes and macrophages. The IL-2 receptor is useful as a marker of activation, but not for discrimination between virgin and memory cells as the expression is lost when cells return to the resting state.

CD40/CD40L. Recently, two comparable studies were published, both suggesting an association between ineffective expression of CD40L on cord blood T cells and the low Ig production in the new born (Brunoni *et al.*, 1994; Fuleihan *et al.*, 1994). Cord blood mononuclear cells (MNC) have no (Brunoni *et al.*, 1994) or decreased (Fuleihan *et al.*, 1994) expression of CD40L compared with adult MNC. This defect can be largely attributed to the lack of antigenic exposure in the neonate, because *in vivo* primed cord blood T lymphocytes could express substantial amounts of CD40L upon appropriate stimulation (priming and re-stimulation with PMA and ionomycin). This ineffective CD40L expression by cord blood $CD4^+$ T cells together with their failure to produce a number of co-stimulatory cytokines might be regarded as the primary cause for the poor Ig production in the neonate.

$CD23^+$ B cells. CD23, the low affinity receptor for the Fc portion of IgE, is also a presumed activation antigen. The number of B cells that express CD23 nearly doubles between infancy and adulthood (Hannet *et al.*, 1992).

1.2.2.5 Cytokines

There is evidence for progressive maturation of T cell cytokine production with increasing age. Mechanisms responsible for these modifications may involve differences in naïve and memory sub-populations of T cells in the various age-groups, as it has been found that naïve and memory T cells differ in the profile of cytokines they produce (Lewis *et al.*, 1988). Table 1.4 shows contradictory observations of changes in T lymphocyte development and function as well as cytokine production.

It is widely accepted that the potential of neonatal T cells to produce IFN-γ is markedly reduced (Miyawaki *et al.*, 1985; Wilson *et al.*, 1986; Holt 1995; reviewed by Smith *et al.*, 997). IFN-γ production by neonatal T cells is decreased to as low as 10% of the adult IFN-γ production (Wilson *et al.*, 1986). Several reasons for this low IFN-γ production have been suggested: an intrinsic deficiency in the capacity of neonatal T cells to produce IFN-γ (Wilson *et al.*, 1986), an increased sensitivity of neonatal T cells to the inhibitory effect of PGE_2 (Wakasugi and Virelizier, 1985), or an inefficient accessory cell activity

of neonatal macrophages (Taylor and Bryson, 1985). The reduced capacity to produce IFN-γ coincides with the time when the infant's gastrointestinal and respiratory tracts are first exposed to environmental antigens and this may be important in the development of specific allergic disease (Warner *et al.*, 1991; reviewed by Koning *et al.*, 1996). The human fetus and neonate are unusually susceptible to infection with intracellular pathogens. Resistance to these infections is mediated in part by T lymphocyte-dependent macrophage activation, particularly by IFN-γ. Wilson *et al.* (1986) proposed that the inability of neonates to produce adult levels of IFN-γ might contribute to the neonate's enhanced susceptibility to intracellular pathogens. Children acquire adult-equivalent levels 3-5 years after birth (Miyawaki *et al.*, 1985; Frenkel *et al.*, 1987; Holt, 1995).

IL-2 production by neonatal T cells has been reported to be similar to that produced by adult T cells (Miyawaki *et al.*, 1985; Wilson, 1986; Fairfax *et al.*, 1988), or to be significantly reduced (Watson *et al.*, 1991). The difference may be due to the use of different stimulating agents, which potentially elicit distinct patterns of cytokine production (Miyawaki *et al.*, 1985). The expression of IL-2 receptor is comparable to those in adults (Wilson *et al.*, 1986).

Neonatal monocytes exhibit diminished capacity to produce IL-6 and TNF-α, which may also contribute to their defective inflammatory reactivity. The diminished presence of IL-6 and TNF-α may also be an important factor in the reduced febrile response of neonates. TNF-α secretion by neonatal monocytes is also less sensitive to IFN-γ stimulation than adult monocytes (Holt, 1995).

IL-4 production appears to be strongly decreased in neonatal T cells (Table 1.4). In adults, IL-4 and IFN-γ are mainly produced by memory ($CD45RO^+$) T cells, whereas IL-2 is produced by both memory ($CD45RO^+$) and naïve ($CD45RA^+$) T cells. Therefore, the acquisition of memory T cells may be a determinant of maturation for IL-4 and IFN-γ production. The fact that naïve T cells produce IL-2, but low levels of IFN-γ and IL-4 (Lewis *et al.*, 1988; Salmon *et al.*, 1989) could explain the reduced IL-4 levels observed in neonates and children younger than 10 years (Tang and Kemp, 1995).

IL-4 induces class switch in B cells to IgE production. This effect is inhibited by IFN-γ. Reports of increased IL-4 production and reduced IFN-γ production in children and adults with atopic disease suggest possible roles for IL-4 and IFN-γ in the pathogenesis of atopic disease (Koning *et al.*, 1996). Atopic disease is more dominant in infants and children, with 85% of cases commencing in the first 5 years of life. Increased IL-4 and/or reduced production of IFN-γ in early childhood could predispose this age group to develop atopic disease. In addition, neonates with a positive family history of atopic disease who are at risk of developing atopic disease have reduced secretion of IFN-γ in mitogen-stimulated in cord blood mononuclear cell cultures compared with neonates without a family history (Tang and Kemp, 1993). Increased IL-4 production might also be expected in these groups (Table 1.4).

An inhibitory modulator of IFN-γ production (such as IL-10) could exert a greater effect on neonatal than on adult IFN-γ secretion. This could happen either through higher levels of neonatal IL-10 production by accessory cells or greater sensitivity of IFN-γ producing cells to the inhibitory effects of IL-10. In a study done by Scott *et al.*, (1997), the neutralisation of IL-10 in culture increased IFN-γ production. Chedda *et al.* (1996) however, argues against a role for higher IL-10 production in neonates. They reported a decreased production of IL-10 by neonatal T cells and monocytes in response to various stimulating agents (Table 1.4).

Table 1.4

Controversial observations of changes in T lymphocyte development and function as well as in cytokine production.

Observation	Seen in	Reference
CD4/CD8 ratio increases with age	Human PBMC	Utsuyama *et al.*, 1992
CD4/CD8 ratio decreases with age	Human PBMC	Ceuppens *et al.*, 1982 Solinger *et al.*, 1985
IL-4 production increases with age	Mouse spleen	Ernst *et al.*, 1990
IL-4 production decreases with age	Human PBMC	Al-Rayes *et al.*, 1992
IL-10 production increased at birth	Human PBMC	Scott *et al.*, 1997
IL-10 production decreased at birth	Human PBMC	Chedda *et al.*, 1996
IFN-γ production increases with age	Human PBMC; mRNA & protein	Chopra *et al.*, 1989
IFN-γ production decreases with age	Human PBMC; mRNA	Gauchat *et al.*, 1988
NK activity declines with age	Mice, internal lymphoid tissue	Albright *et al.*, 1983
NK activity unchanged with age	Human PBMC	Murasko *et al.*, 1986

1.2.3 Implications of the immature immune system

1.2.3.1 Enhanced susceptibility to certain infectious diseases

Although many of the maturational differences in the host response in young children may not be intense, the attained effect could result in a delayed and less effective host response. The neonatal period as well as early life is marked by an increased susceptibility to certain infections. This is seen by the T cell-mediated host response only beginning to improve by 4 to 8 weeks of age and most of the critical components appear to reach adult competence by 1 to 2 years of age. Immunising infants might therefore reduce the susceptibility, but severe infections such as Group B Streptococci, may occur too early in life to allow sufficient time for infant immunisation. Infants however, do not respond to certain bacterial capsular polysaccharides before the age of two years, which creates a window of susceptibility to infections at the time of disappearance of protective antibodies.

Immunising pregnant women in order to transfer protective antibodies transplacentally to infants would have several advantages: immune responses in pregnant women are normal, some inactivated vaccines (*e.g.* tetanus toxoid) already have a long safety track record in pregnant women and the transplacental transfer of IgG is efficient (Kovarik and Siegrist, 1998). Group B Streptococci cause bacteremia and pneumonia within the first 5 days of life in infants born to mothers infected with group B Streptococci. It has been suggested that many cases of group B meningitis could be prevented by immunising pregnant mothers to the type III polysaccharide, thus providing their new borns with protective antibodies. After testing this theory, Kovarik and Siegrist (1998) found that 80 % of the infants born to these mothers still had protective antibodies to type III group B Streptococci at 1 month of age.

The application of maternal immunisation might be limited by the relatively short persistence of transferred antibodies, leading to a requirement for infant immunisation at a time of potential interference by maternal antibodies. Maternally derived antibodies can inhibit infant responses to an extent that depends on a variety of factors: levels of maternal antibodies, age of infant at immunisation, dose of vaccine, type of antigen and

route of administration (Kovarik and Siegrist 1998). The immunogenicity of vaccines in new-borns and young infants emerges as an important factor for early-life protection.

It is widely accepted that the neonatal T cells are immunocompetent, but that their differentiation is biased towards a Th2 profile under neutral conditions. The Th2 biased cytokine milieu could also contribute to the enhanced susceptibility to certain infectious diseases in early life. It is not clear whether this Th2 bias can be attributed to the Th2 cytokine milieu of pregnancy, to altered interactions of APC with T cells, or to both of these factors.

1.2.3.2 Differences in the clinical manifestation of certain diseases

The progressively maturing immune system could also result in differences in the clinical manifestation of diseases. This is seen in the manifestation of TB with adults presenting with more severe immunopathology than children (discussed later). Differences in the manifestation of other diseases such as measles and mumps are also observed. A possible reason could be the fact that the cells of the immune system in early life are less sensitive to specific Ag resulting in insufficient cytokine production .

1.3 IMMUNOPATHOGENESIS OF TUBERCULOSIS

1.3.1 The disease

Tuberculosis (TB) is typically a disease of the lungs as these organs serve as the major port of entry. A long incubation period, dormant infection and a chronic course are characteristic of the disease. It is estimated that one third of the total world population harbour the organism without any clinical manifestations of the disease. In these cases, a long-lasting but labile balance between host immune response and the mycobacteria develop. Infection transforms into disease in only a small number of infected individuals when the balance tips in favour of the infection. Still, the vast majority of infected individuals remain at risk of developing the disease as the immune system generally fails to fully eradicate the mycobacteria. Thus, a potent immune response is required to control mycobacterial growth effectively. However, inadequate healthcare, poverty, malnutrition and war increase the rate of reactivation (reviewed by Rook, 1996). Moreover, TB is one of the first secondary infections to be activated in HIV-positive subjects.

Among all infectious agents, *M. tuberculosis* is considered to give rise to the highest morbidity rates and is, therefore, responsible for more deaths than any other single pathogen (McDonought *et al.*, 1993). The upheaval of significant levels of multiple-drug-resistant *M. tuberculosis* isolates further complicates treatment and control, emphasising the need to develop new strategies for prevention and treatment of tuberculosis beyond currently available anti-microbial therapies (Bloom and Murray, 1992). A report by the World Health Organisation predicts that by the year 2005, TB will have killed 4 million people annually (Kaufmann, 1993). Africa has the highest TB incidence in the world with 272 new cases per 100 000 population per year. The overall TB incidence in South Africa is 366 new cases per 100 000 population per year with the highest (718/100 000) in the Western Cape (Beyers *et al.*, 1996).

1.3.2 Macrophages as the first line of defence

After inhalation of aerosolised *M. tuberculosis*, fewer than 10 % of *M. tuberculosis* will reach the respiratory bronchioles and alveoli. Most of the organisms will settle in the upper respiratory epithelium, from which where they are expelled by the mucociliary escalator (Fenton 1996). Bacteria arriving in the deep lung are phagocytosed by alveolar macrophages and are either killed or else survive intracellularly to initiate an infection. The role of macrophages in intracellular bacterial infection is twofold, representing an essential habitat for growth of the organism, but also an effector of defence. Alveolar macrophages are equipped with multiple microbicidal mechanisms to rid the host of infecting micro-organisms (McDonough *et al.*, 1993). If the bacteria survive their encounter with the alveolar macrophages, infection is ultimately established with the bacteria gaining access to the lymphatics or the bloodstream.

Killing of ingested *M. tuberculosis* takes place within the macrophages. A fierce array of oxygen-dependent microbicidal mechanisms come into play, which include lysosomal hydrolases, reactive oxygen intermediates (ROI), such as H_2O_2 and O_2^-, and reactive nitrogen intermediates (RNI) such as NO and NO_2^- (Kaufmann 1993). The role of ROI in anti-mycobacterial defence is controversial as tubercle bacilli are remarkably resistant to killing by oxygen radicals and hydrogen peroxide *in vitro* (Chan *et al.*, 1992) and they may deploy mechanisms to evade ROI *in vivo*. ROI combined with RNI can significantly enhance mycobacterial killing. The capacities of resident alveolar macrophages and recruited monocytes to destroy mycobacteria differ significantly and progressively throughout the course of the infection. Some bacilli may persist and replicate within the alveolar macrophage. After the processing of the mycobacterial proteins into peptides, the peptides are presented to the T cells by macrophages in association with major histocompatability complex (MHC) II. The transition from resting macrophages to activated macrophages is induced by cytokines.

1.3.3 T lymphocytes

T lymphocytes are vital for the acquisition of protection against tuberculosis. All three known T-cell populations – $CD4^+$ $\alpha\beta$ T cells, $CD8^+$ $\alpha\beta$ T cells and $\gamma\delta$ T cells – seem to

participate in the acquisition of optimum protection (Kaufmann, 1993). Early in the course of TB, prior to the development of a specific T cell response, IFN-γ may be provided by NK cells. Release of IFN-γ is stimulated by IL-12, which is secreted by the infected macrophages (Fulton *et al.*, 1996). IFN-γ and TNF-α stimulate IL-12 secretion by infected macrophages, creating a positive feedback loop for macrophage activation. In a study done by Barnes *et al.* (1993), a strong positive correlation between expansion of γδ T cells and manifestations of human TB was found, suggesting that γδ T cells contribute to immune resistance against tuberculosis. Furthermore, human γδ T cells are activated by *in vitro* stimulation with mycobacterial components, independent of previous infection or immunisation. *M. tuberculosis*-reactive γδ T cells produced IFN-γ, GM-CSF, IL-3 and TNF-α with negligible amounts of IL-4 and IL-5 (Rook and Bloom, 1994; Fenton *et al.*, 1996). This cytokine profile is consistent with a role in anti-mycobacterial defences. IFN-γ and GM-CSF are potent macrophage-activating factors (Kaufmann, 1993) and TNF-α enhances mycobacterial killing (Denis, 1991). To define the contribution of γδ T cells to anti-mycobacterial defences, it will be important to characterise the antigens recognised by γδ T cells. It is proposed that γδ T cells are involved in a transient and auxiliary fashion in the generation of an immune response to *M. tuberculosis* which is ultimately sustained by αβ T cells.

$CD4^+$ αβ T cells have traditionally been considered to be the major T cell subset in mice and humans to regulate protective immunity to mycobacterial infection (McDonough *et al.*, 1993) and mycobacterium-specific T cells isolated from mice and humans immune to *M. tuberculosis* consistently express the $CD4^+$ phenotype (Orme *et al.*, 1993). It appears that $CD8^+$ T cells contribute to the control of mycobacterial infection. This is suggested by the increased growth of bacteria in tissues, including the lungs, of mice depleted of or lacking $CD8^+$ T cells (reviewed by Rook and Hernandez-Pando 1996). The mice, consequently devoid of functionally active $CD8^+$ T cells, suffer severely from experimental tuberculosis. In humans, the only evidence supporting a role of $CD8^+$ T cells in controlling mycobacterial infections, is the large numbers of these cells in the periphery of well-formed granulomas, which are indicative of an effective host response

(reviewed by Kaufmann, 1993). It is suggested that cytotoxic activity assists in control of infection by the lysis of cells unable to kill mycobacteria, which would allow them to be engulfed by more microbicidal monocytes and activated macrophages (Orme *et al.*, 1993).

Depressed Th1 responses have been reported for patients with active TB. Production of Th1 cytokines by *M. tuberculosis*-stimulated peripheral blood mononuclear cells (PBMC) was lower for PBMC from TB patients than from healthy tuberculin reactors (Surcel *et al.*, 1994; Zang *et al.*, 1995). Whether the depressed Th1 responsiveness of PBMC from TB patients is accompanied by increased numbers of Th2 cells, is not known. Within pleural, lymph node and pulmonary TB lesions, Th1 cells predominate, suggesting that reduced Th1 responses of PBMC may not reflect events within the lesions and the possibility has been raised that sequestration of Th1 cells at the site of disease may deplete their numbers in blood (Barnes *et al.*, 1990; Lin *et al.*, 1996).

1.3.4 Cytokines in protection and immunopathology

The immune response eliminates microbial pathogens through an inflammatory response that may be harmful to host tissue. Tissue necrosis and fibrosis are characteristic manifestations that are thought to be the result of cytokines produced during the inflammatory response (reviewed by Rook and Hernandez-Pando, 1996). Activated alveolar macrophages produce TNF-α, IL-12 and 1,25-dihydroxyvitamin D (Barnes *et al.*, 1989). Many symptoms of TB such as fever, weight loss and tissue damage, resemble the pathological effects of TNF-α. Evidence that these symptoms may indeed depend on TNF-α in human tuberculosis has come from experiments using thalidomide, which decreases the half-life of the mRNA for this cytokine (Moreira *et al.*, 1993). Patients treated with thalidomide show rapid relief and weight gain (Kaplan, 1994). TNF-α is essential for immunity, but may also be responsible for pathology.

IFN-γ appears to be a key effector cytokine in control of mycobacterial infection via macrophage activation (Barnes *et al.*, 1993). IFN-γ, along with TNF-α, is critical for the effective abating of mycobacterial growth and development of long-term immunity

(reviewed by Smith *et al.*, 1997). IFN-γ, TNF-α and 1,25-dihydroxyvitamin D may further induce inhibition or killing of intracellular *M. tuberculosis* by macrophages.

In TB patients, TGF-β production has been shown to be present in blood monocytes, giant cells and epithelioid cells within pulmonary lesions (Toossi *et al.*, 1995). TGF-β displays several potent immunosuppressive activities that may be relevant to the pathogenesis of TB. T cell responses to Ag, including IL-2 dependent proliferation and secretion of Th1 cytokines, are inhibited by TGF-β, as are the effector functions of cytotoxic T cells and NK cells. TGF-β also interferes with macrophage functions including the generation of RNI and ROI in response to IFN-γ and TNF-α. Replication of *M. tuberculosis* within human macrophages is enhanced in the presence of TGF-β, suggesting that induction of this immunosuppressive cytokine may be a strategy by which the tubercle bacillus manipulates the host response to its own advantage (Hirsch *et al.*, 1994). TGF-β production may benefit the infected host by limiting the extent of tissue damage caused by excessive cell mediated immune (CMI) responses and may help contain infection by promoting fibrosis and healing of tuberculous lesions.

Although immunity to tuberculosis requires a type 1 response, in tuberculous mice, type 2 cytokines are also expressed (Orme *et al.*, 1993). The same is true in humans, though less strikingly evident and is most apparent when peripheral responses are considered. Studies of peripheral blood mononuclear cells pre-cultured with mycobacterial antigen *in vitro* are unreliable, because the rapid production of IFN-γ from NK cells and from Th1 cells that are almost always present tends to inhibit full expression of Th2 cytokines. If peripheral blood lymphocytes are examined without pre-culture, it becomes apparent that the IL-4 gene is indeed expressed in patient's peripheral blood mononuclear cells (Schauf *et al.*, 1993).

1.3.5 Tuberculosis in children and adults

1.3.5.1 Clinical differences in manifestation of TB

Noticeable clinical differences are observed between children and adults with tuberculosis. In children the disease is usually the result of first contact with *M. tuberculosis*. On first contact, children's immunological apparatus is still naïve and unprimed (Hannet *et al.*, 1992). Adulthood disease ("secondary" TB) however, is most often attributable to the reactivation (the Koch phenomenon) of a previously dormant infection. Children with tuberculosis usually present with lymphadenopathy, the complications thereof or with systemic spread of the organism. Adults usually have more pronounced systemic effects such as severe weight loss, high fever and more severe immunopathology that includes cavitation and fibrosis.

Progressive tuberculosis develops more often in young children than in adults. This may be largely due to the naïve nature of the immature host.

1.3.5.2 Immunological differences in manifestation of TB

Alveolar macrophages are the first cells to be infected with mycobacteria. Studies have shown that alveolar macrophages are qualitatively different in neonates compared to adults (Smith *et al.*, 1997). Animal data have consistently demonstrated reduced microbial killing by alveolar macrophages and decreased microbial clearance from the lungs in early life (Martin *et al.*, 1987; Geertma *et al.*, 1993; Holt, 1995). Defective microbial killing may be related, at least in part, to the high surfactant present in the first 1 – 2 weeks of neonatal life (Jackson *et al.*, 1988; reviewed by Smith *et al.*, 1997).

Recruitment of monocytes contributes to local control of infection, because these cells generally have greater anti-microbial activity than alveolar macrophages. The absolute numbers and anti-microbial activity of neonatal and adult monocytes are similar, although their activity against mycobacteria has not been compared thus far (Smith *et al.*, 1997). However, chemotaxis of neonatal monocytes is diminished *in vitro* (Rahunathan *et al.*, 1982) and recruitment of monocytes into tissues is also diminished (Bullock *et al.*, 1969; reviewed by Smith *et al.*, 1997). Monocyte chemotaxis attains adult levels by

6 – 10 years of age. The capacity of neonatal monocytes and macrophages to produce and respond to several cytokines that may contribute to protection against *M. tuberculosis*, is similar or reduced compared to adults (discussed in Section 1.2). Also, the ability of neonatal monocytes and macrophages to support IFN-γ production by NK cells or T lymphocytes after LPS or mitogen stimulation, is less than half of adult cells (Taylor *et al.*, 1985).

Age-related differences also exist in certain aspects of the antigen-specific T cell-mediated immune response, which likely contributes to the greater susceptibility of infants and young children to infection with *M. tuberculosis*. Dendritic cells are uniquely specialised to present Ag to naïve T cells. Human cord blood dendritic cells, however are less efficient APCs. This may reflect diminished expression of MHC and other co-stimulatory molecules. The ability of dendritic cells to present Ag to naïve T cells seems reduced and only attains adult competence after 1 year of age.

Limited clinical data suggest that an antigen-specific T cell mediated response to mycobacterial antigens may develop less efficiently *in vivo* in the neonate and young infants than in the older child or adult (Figure 1.3).

FIGURE 1.3

The primary pre-immune pulmonary response to mycobacteria

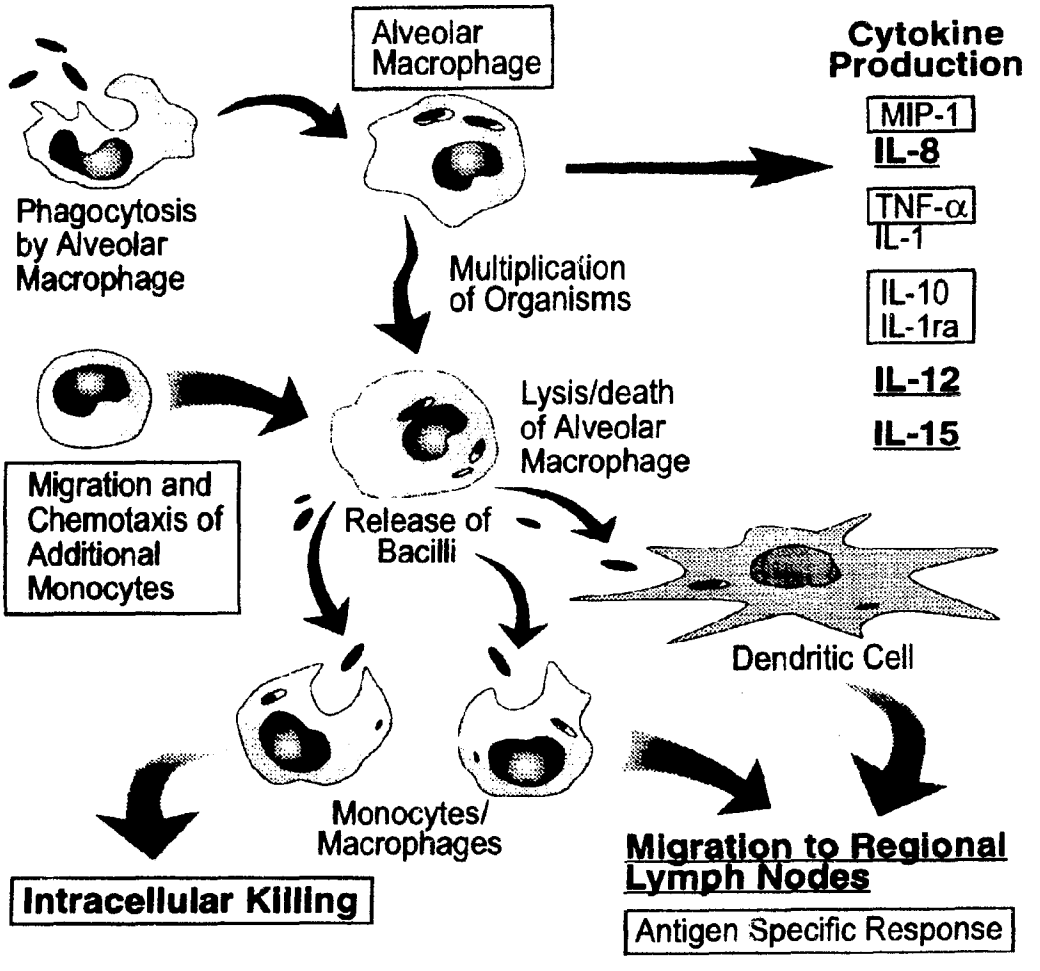


Figure 1.3 The cells, functions or mediators shown in boxes are those for which deficiencies compared with those in adults have been found in neonates and in some cases also in infants and young children. Underlined text indicates that insufficient or conflicting data exist. For those shown in regular type, there appear to be no differences (discussed in Section 1.2) (Taken from Smith *et al.*, 1997).

1.4 REGULATION OF IgE LEVELS

Th2 lymphocyte development, primed by IL-4, is associated with IL-5 and IL-13 cytokine production and promotes the secretion of IgE and the recruitment of eosinophils as part of the natural immune response to large extra-cellular parasites, such as helminths. The capacity of common allergens to stimulate IgE responses and thus to produce allergic diseases, has tended to overshadow the fact that helminthic parasites are possibly the most potent inducers of IgE responses (Lynch *et al.*, 1998). Parasitic helminths cause enormous morbidity among humans especially in the developing countries.

1.4.1 Cytokine regulation of IgE responses

IgE synthesis is dependent on at least two signals provided by T cells (Romagnani, 1990). One signal is delivered by T cells recognising MHC II antigens on B cells and the other by T-cell-derived IL-4 (Vercelli *et al.*, 1989). These two signals are likely to synergise in the activation of B cells into IgE production. IL-4 stimulation of B cells which bear IL-4 receptors even in their resting state, increases MHC class II molecule density by 5- to 10-fold. An increased density of MHC class II antigens on B cells may enhance their capacity to activate a subset of T cells recognising autologous MHC class II antigens and self peptides or plus foreign peptides. Thus, IL-4 would, on the one hand, enhance T/B cognate recognition and on the other hand, drive the B cell response toward the IgE isotype, acting as an IgE-switching factor.

Cytokines play a crucial role in the regulation of IgE synthesis. It is well established that IL-4 and IFN-γ are the main regulatory cytokines of IgE production. King *et al.* (1993) demonstrated a reciprocal relationship between IL-4 and IFN-γ production in the IgE response. The mechanism by which IFN-γ inhibits IgE production has previously been shown to be a block of the effect of IL-4 on B cells (Nutman *et al.*, 1984 and de Vries *et al.*, 1991) as well as suppression of the ongoing IgE production by already switched cells. Soluble CD23 (which is specifically induced by IL-4) was found to be significantly elevated in sera of patients with atopic disorders (Rousset *et al.*, 1991) and helminth-

infected individuals (Yanagihara *et al.*, 1990) compared with controls. Binding of IgE to mucosal mast cells occurs via the high affinity IgE receptor $Fc_{\varepsilon}R$. Following contact with allergen and the subsequent cross-linking of the $Fc_{\varepsilon}RI$-bound IgE molecules, mast cell activation occurs through $Fc_{\varepsilon}RI$ and this leads to the release of a complex array of biological mediators which orchestrate local mucosal inflammation.

1.4.2 Factors regulating IgE synthesis

IgE is the hallmark for Th2 activation. Genetic factors, environmental factors and helminth infections can induce Th2 responses with a subsequent increase in IgE levels.

1.4.2.1 Genetic factors

A positive family history has been shown to be an important risk factor for the development of atopic disease. In a study of Swedish children, Bjorksten *et al.* (1994) found that if a child has one allergic parent, the risk of becoming allergic is approximately 20 %. This is increased to 60 % with two allergic parents, whereas without allergic parents the risk is around 10 % (Bjorksten *et al.*, 1994). Allergic diseases are presently considered as at least a two-locus inheritance, with expression being influenced by environmental variables, resulting in heterogeneous expression. Moreover, an individual's likelihood of developing allergic disease is not constant over time, even if the general propensity is genetically determined.

Evidence of linkage of high levels of total serum IgE to loci on chromosome 5q31.1, including the cytokine gene cluster (5q31), has been reported (Marsh *et al.*, 1994). It was also demonstrated that elevated serum IgE is co-inherited with a trait for bronchial hyper-responsiveness and that a gene governing bronchial hyper-responsiveness is located near a major locus that regulates serum IgE levels on chromosome 5q. Further support for genetic influences comes from several studies reporting significantly higher cord blood IgE concentrations in male than in female subjects (Halononen *et al.*, 1991; Hansen *et al.*, 1992), although conflicting information exists (Ownby *et al.*, 1991). Cookson *et al.* (1989) found a strong linkage of a marker on chromosome 11q13 with asthma and atopy in large pedigrees.

1.4.2.2 Environmental factors

The principal feature so far that distinguishes atopic from non-atopic individuals is their predisposition to develop antigen-specific IgE antibodies upon repeated exposure to low doses of foreign proteins (*i.e.* pollen, animal products, house dust mite and food). This results in higher serum IgE levels in atopic than in non-atopic subjects.

Outdoor pollution, by combustion by-products (CO_2, NO_x, SO_2, CO), solid particles and ozone, is able to enhance allergic sensitisation. The actual contribution of pollutants acting as stimulants in allergy development is, however controversial. The prevalence of allergic diseases in the more polluted former East Germany was found to be less than that in West Germany, against expectations. Epidemiology studies therefore show that atopy is more common in westernised communities (Williams *et al.*, 1994).

Air pollutants can act as irritants and precipitate respiratory symptoms in established asthma. It is notable that nitrogen dioxide levels, a valuable index of traffic-related pollution that is relevant to West Germany, were the same in both East and West Germany. The reunification of Germany provided a unique opportunity to study two genetically similar populations who over the past 45 years have been exposed to different living conditions and levels of environmental pollution. In particular, the type of air pollution differed greatly between Western and Eastern Germany (Mutius *et al.*, 1992) with Western Germany having an increased exposure to house dust mite and other environmental toxins. This resulted in a rise in the incidence of atopic disorders in Western Europe compared to Eastern Europe with high incidences of infectious diseases.

An increase in the levels of indoor allergens has been considered as one potential mechanism – in particular, the case for likely increases in house dust mites in more "luxurious" modern homes with more furnishings and higher relative humidities which suit the mite. Though the level of mites can be related to the risk of sensitisation and wheezy illness, it is not believed that rising house dust mite levels account for the general rise in atopy. Asthma has risen in American regions, such as the extreme South West and North East, where relative humidity is low and where sensitisation to the cockroach is

more prominent; mite levels cannot account for the different rates of grass sensitisation noted between Western and Eastern Europe for 1990 (Von Mutius *et al.*, 1992).

Insects have been recognised as inhalant allergens long before mites came to the foreground. In urban dwellings, particularly in lower socio-economic communities, cockroach debris can be a major component of house dust allergen (Garcia *et al.*, 1993). Although cockroach infestation is ubiquitous, it is greatest in urban areas and numerous studies have confirmed the fact that cockroach allergens play a significant role as sensitising agents for the induction and exacerbation of urban asthma (Kang, 1976).

It is likely that a set of specific infections that strongly promote Th1 immunity has the potential to inhibit atopic disorder by the repression of Th2 immunity. It is believed that the role of such an infection in repressing atopy depends on a number of factors, including its timing, anatomical site, dose and delay; exposure to other infections; and host characteristics such as genetic variables and nutritional status. Prospective and experimental studies are needed to investigate the action of *M. tuberculosis* and other micro-organisms, through natural infection or immunisation schedules, in deviating immunity away from atopy.

1.4.2.3 Helminth infestations

Gastrointestinal roundworm parasites, including those within the genera Necator, Ancylostoma, Ascaris, Trichuris and Strongyloides, infect approximately one billion people world wide and are believed to cause approximately one million deaths annually (Finkelman *et al.*, 1997). Children in developing countries are particularly likely to be infected by gastrointestinal nematodes; in some endemic areas the prevalence by age 10 approaches 100 % (Finkelman *et al.*, 1997). Fincham *et al.* (1996) reported that in rural areas of the Western Cape, infection was frequent in children with a prevalence of up to 90 %. These children are mainly infected by *Trichurius trichuria*, *Ascaris lumbricoides*, *Enterobius vermicularis* and *Hymenolepis nana*. Risk of infection by intestinal parasites is proportional to human population density in a contaminated environment and to ignorance of how to prevent infection on the part of communities and individuals.

Infection with helminths leads to a dramatic induction of Th2 responses, well characterised in rodent models by induction of IL-4 and down-regulation of IFN-γ. Street *et al.*, (1989) found that spleen, lymph node and lung cells isolated from *Nippostrongylus brasiliens* infected mice secrete increased levels of IL-4, IL-5 and IL-10, but reduced levels of IL-2 and IFN-γ after *in vitro* mitogen stimulation. These effects all suggest that the selective activation of a strong Th2 response is occurring in these animals.

Infection by helminths is generally associated with high levels of IgE, eosinophilia and mastocytosis: all responses that are associated with the presence of Th2-type cytokines. Demonstrations that eosinophils and IgE can kill parasites *in vitro* has led to the wide-spread belief that these Th2-dependent responses are primarily responsible for the destruction of large extra-cellular parasites (Lynch *et al.*, 1992). However, a striking contradiction occurs *in vivo*. In most helminth infections, heavy parasite burdens occur despite Th2 responses and direct *in vivo* evidence for the role of eosinophils, IgE or mast cells controlling helminth infections remains scarce. IL-4 plays a critical role in the control of intestinal helminth infections. *In vivo* experiments with neutralising antibody to IL-4 have shown that worm expulsion is dependent on IL-4 during infection with *Trichuris muris* (Finkelman *et al.*, 1988). IL-4 is not required for worm expulsion in several other intestinal helminth infections, but does have a major impact on the intensity of infection and the production of eggs (Finkelman *et al.*, 1988).

The relationship between socio-economic level, conditions of hygiene and parasitic infection in developing countries is well established (Anderson and May 1982; Hagel *et al.*, 1993). Also well studied is the dual effect of helminth infection on the synthesis of IgE. These parasites can stimulate specific IgE antibody responses (Lynch *et al.*, 1998) which may be involved in protective mechanisms against the infection (Miller *et al.*, 1981; Capron *et al.*, 1987; Kay *et al.*, 1985; Hagel *et al.*, 1993). These parasites can also cause non-specific polyclonal stimulation of IgE synthesis (Turner *et al.*, 1978; Hussain *et al.*, 1980; Finkelman *et al.*, 1988; Hagel *et al.*, 1993; Lynch *et al.*, 1998). It has been suggested that this polyclonal synthesis of IgE can suppress the expression of allergic reactivity via saturation of mast cell Fc_ε receptors (Godfrey and Gradidge 1976; Lynch *et*

al., 1987; Lynch *et al.*, 1998) or inhibition of specific antibody responses (Lynch *et al.*, 1992; Hagel *et al.*, 1993). Therefore, despite high levels of IgE and other features of Th2 cell activation, allergic responses are rarely observed in helminth-infected individuals as a result of the Fc_{ε} receptor saturation.

1.4.2.4 Microbial exposure

The pattern of microbial exposure in childhood has changed considerably with westernisation as a result of cleaner food, water and less crowded accommodation. The changing patterns of microbial exposure in recent decades in westernised communities could represent an important mechanism for the rise of atopy.

A historical cohort study in Guinea-Bissau, West Africa, examined the relationship between a history of measles in childhood and the results of allergen skin prick testing to a range of aero-allergen preparations, including those from the mites *Dermatophagoides pteronyssimus* and *Dermatophagoides farinae* and mixed grass pollens, in young adulthood (Shaheen *et al.*, 1996). They found, however that infection with measles in early childhood may prevent the development of atopy. Declining family size has probably contributed to this fall as severe infections in early childhood are less likely with fewer older siblings in the family.

In a population of Japanese school children a strong inverse association between DTH to *M. tuberculosis* and atopy was found (Shirakawa *et al.*, 1997). Positive tuberculin responses predicted a lower incidence of asthma, lower serum IgE levels and cytokines biased toward Th1 type (Shirakawa *et al.*, 1997). Therefore, exposure to intracellular infections early in life predicted less atopy. Such intracellular infections typically induce Th1 immunity which might down-regulate Th2 mechanisms that are implicated in atopy.

Single or repeated exposure to mycobacterial Ag results in a Th1 response. When animals are sensitised to filarial and PPD Ag simultaneously, no deviation of Th responses to either Ag occurs. In contrast, establishment of a filarial-driven Th2 response before

induction of the PPD T cell response results in the deviation of the latter to the Th2 subset by an IL-4 dependent mechanism (Pearlman *et al.*, 1993).

1.5 Aims of the present study

There were three aims to the present study:

1. To determine if cytokine responses of peripheral blood mononuclear cells (PBMC) from children differ from those of adults
2. To investigate if chronic Th2 stimulation, indicated by high serum IgE levels, was associated with a high incidence of TB
3. To determine if prominent Th2 responses, manifested by high total serum IgE levels, would be down regulated during the course of TB treatment

[Detailed aims are provided in Chapters 3 – 7.]

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tissue Culture Reagents

RPMI 1640 medium was purchased from ICN Biochemical Research products, Ohio, USA. Foetal Calf Serum (FCS) was obtained from Delta Bioproducts, Kempton Park, Johannesburg. Penicillin Streptomycin (Pen.Strep.) and L-Glutamine were purchased from Highveld Biologicals, Kelvin, Johannesburg.

Human AB serum was obtained from Grootte Schuur Hospital, Cape Town.

KSLMS medium was supplied by Highveld Biologicals, Kelvin, Johannesburg.

Phytohaemagglutinin-P (PHA) extracted from *Phaseolus vulgaris*, was obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Purified Protein Derivative (PPD) was a gift from Dr. S. Ress (Clinical Immunology, University of Cape Town, South Africa).

2.1.2 Radiochemicals

[methyl-^{3}H] Thymidine (specific activity 25 Ci/mmol) was purchased from Amersham Life Sciences, Buckinghamshire, England.

2.1.3 Polymerase chain reaction (PCR) and Enzyme-linked immunosorbent assay (ELISA) kits

PCR kits containing *Taq* polymerase, PCR buffer and $MgCl_2$ solution were purchased from Boehringer Mannheim, GmbH, West Germany.

ELISA kits for all cytokines measured, were obtained from Ridge Diagnostics, Genzyme Corporation, USA.

2.1.4 Radioimmuno- and Fluoroimmunoassay kits

Radioimmunoassay (RIA) kits for serum IgE determination, were purchased from Pharmacia AB, Uppsala, Sweden.

Specific IgE Fluoroimmunoassay kits were obtained from Pharmacia AB, Uppsala, Sweden.

2.1.5 Primers

Primers were as described by Yamamura *et al.* (1992) and were obtained from Genosys, Cambridge. The sequences of the cytokine specific primer pairs, 5' and 3', are as follow:

β-actin

5' GTGGGGCGCCCCAGGCACCA

3' CTCCTTAATGTCACGCACGATTTC

Interleukin-2

5' ACTCACCAGGATGCTCACAT

3' AGGTAATCCATCTGTTCAGA

Interleukin-4

5' CTTCCCCCTCTGTTCTTCCT

3' TTCCTGTCGAGCCGTTTCAG

Interleukin-10

5' ATGCCCCAAGCTGAGAACCAAGACCCA

3' TCTCAAGGGGCTGGGTC[illegible]GCTATCCCA

Interferon-γ

5' AGTTATATCTTGGCTTTTCA

3' ACCGAATAATTAGTCAGCTT

Tumor necrosis factor-α

5' TCTCGAACCCCGAGTGACAA

3' TATCTCTCAGCTCCACGCCA

2.1.6 Analytical grade reagents were obtained as follows:

2.1.6.1 BDH Chemicals (Poole, England)

Chloroform, Hydrochloric acid (HCl), Potassium chloride (KCl), Sodium chloride (NaCl).

2.1.6.2 Boehringer Mannheim (GmbH, West Germany)

Deoxyadenosine triphosphate (dATP), Deoxycytosine triphosphate (dCTP), Deoxyquanidine triphosphate (dGTP), Deoxythymidine triphosphate (dTTP).

2.1.6.3 Merck (Darmstadt, Germany)

Boric acid, β-mercaptoethanol (BME), Propanol, Tris-(hydroxylmethyl) amino methane (Tris).

2.1.6.4 Sigma (St. Louis, Missouri, USA)

Diethyl pyrocarbonate (DEPC), Ethylene diamine tetra acetic acid (EDTA), Ethidium bromide (EtBr), Ficol/Histopaque, Tri-Reagent (for RNA extraction).

2.1.6.5 Other suppliers

Agarose was supplied by FMC Bioproducts, Rocklands, Maine, USA.

Bovine Serum Albumin (BSA) was supplied by Delta Bioproducts, Kempton Park, Johannesburg.

Insta-Gel Plus Scintillation Liquid was obtained from Packard, Instrument Company, Groningen, The Netherlands.

2.1.7 Media and buffers

DEPC

0.1 % DEPC (v/v)

Eppendorfs and tips were covered with DEPC overnight and before autovlaving.

DNA loading buffer

30 % Glycerol, 0.15 % (v/v) Zylene Cyanol

PBS/BSA

8 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4

The solution was autoclaved before the addition of filter sterilized BSA, final concentration of 0.25 % (w/v).

RPMI (Tissue Culture)

10.4 g RPMI 1640 powder was dissolved in 900 ml ddH_2O and stirred for 30 minutes. The pH was changed to 4.0 with HCl. 2.1 g $NaHCO_3$ and 10 ml of L-glutamine were added to the medium to recover a pH of 7.0. The solution was stirred for 30 minutes, before the pH was adjusted to 7.33. After the addition of 10 ml Pen.Strep and 0.03 mM BME, the medium was filter sterilised using an autoclaved filter unit.

Tris-Borate (TBE)

89 mM Tris, 89 mM Boric acid, 20 mM EDTA

2.2 METHODS

2.2.1 Peripheral blood mononuclear cell (PBMC) isolation and culture

PBMC from adults and children were isolated from 10 ml heparinized venous blood. The blood was diluted 1:1 with PBS/BSA (as defined in Section 2.1.7), layered over a Ficoll/Histopaque (Sigma) gradient and centrifuged at room temperature for 25 minutes at 1 800 rpm. Using a sterile 10 ml pipette, the mononuclear fraction was removed and resuspended in 10 ml PBS/BSA. The cells were then washed twice in 10 ml PBS/BSA by centrifuging for 7 minutes at 1 200 rpm. The pellet was resuspended in 1 ml PBS/BSA before the cells were counted. The cells were cultured in serum free medium (KSLMS) at a concentration of 10^6 /ml in 96-well plates (1.8 x 10^5 cells per well), unstimulated and polyclonally stimulated with 10 µg/ml Phytohaemagglutinin (PHA). Cells were cultured for a maximum of 72 hours with mitogen stimulation, depending on the optimum time of cytokine expression and production. Culturing was performed in 5% CO_2, 95% air at 37°C in a humidified incubator.

2.2.2 Harvesting of cells

Supernatants and cells were harvested after an 18-hour and 72-hour incubation period following PHA stimulation. Supernatants harvested after the 18-hour incubation, were used for the measurement of IL-2 and TNF-α production. A 72-hour incubation was required for optimal IFN-γ, IL-10 and IL-4 measurement. The supernatants were stored at -20°C until further assay. RNA was extracted from the cells after an 18-hour incubation period as described in Section 2.2.6.

2.2.3 Proliferation study

Cellular proliferation was assessed at 72 hours following PHA stimulation. The assessment was preceded by radioactive labeling of the cultured cells with 0.5 µCi [^{3}H] thymidine for 20 hours. This method is the most common assay for mitogenesis which measures the incorporation of tritiated thymidine into the DNA of dividing cells (Virella, 1993). The cells were harvested on glass fiber filters using a Titertek Cell Harvester (Flow Laboratories). [^{3}H] Thymidine incorporation by the dividing cells was measured

by liquid scintillation counting and expressed as counts per minute (CPM) of triplicate cultures. A stimulation index (SI) was then calculated as follows:

$$SI = \frac{\text{CPM in mitogen-stimulated lymphocytes}}{\text{CPM in unstimulated control lymphocytes}}$$

2.2.4 Dialysis of foetal calf serum and determination of steroid levels

Dialysis tubing was prepared by boiling in 0.01 M EDTA for an hour. The tubes were rinsed three times with ddH_2O and stored in 1 mM EDTA, 3 mM $NaNa_3$ at room temperature. One end of the tube was clamped tightly. Using a sterile pipette, 10 ml FCS was aliquoted into the tubes. The other end of the tube was closed, where after it was stirred for 18 hours in 1 liter of Tris/Saline, 3 mM NaN_3 at 4 °C. During this time, dialysis buffer was changed twice.

Samples of FCS and dialysed FCS were sent to the Dept. Chemical Pathology, Tygerberg Hospital for the determination of the hormonal composition of the two samples.

2.2.5 Enzyme-linked immunosorbent assays (ELISA)

The production of IFN-γ, TNF-α, IL-2, IL-4 and IL-10 were measured by the ELISA technique as described by the manufacturers (Ridge Diagnostics, Genzyme Corporation). In principle, an ELISA kit consists of a 96-well microtiter plate pre-coated with immobilized mouse monoclonal antibody to the human cytokine measured. The kit also provides all reagents needed for the assay. Serial dilutions were made of the standards as stipulated in the protocol. Optimal dilution of the tissue culture supernatants for measurement of each cytokine was determined (Table 3.5). One hundred µl of the diluted samples and standards were added to each test well and incubated at 37°C for 1 hour to allow the specific cytokine, if present to be bound by the antibodies on the microtiter plate. The wells were washed prior to the addition of 100 µl of biotinylated antibody. The plates were incubated at 37°C for 1 hour and washed again.

After washing, 100 µl peroxidase-labelled streptavidin reagent was added, which bound to biotin in the immune complex on the plate. Following incubation at 37°C for 15 ± 5 minutes, the wells were washed after which 100 µl substrate solution was added to the wells, producing a blue colour in the presence of peroxidase. The colour reaction was stopped by the addition of 100 µl of stop solution, changing the blue colour to yellow.

The intensity of the yellow colour is proportional to the amount of cytokine present in the samples or standards. The absorbance of each well was read at 450 nm and a standard curve was constructed to quantify the cytokine concentrations in the samples. Figure 2.1 depicts a typical standard curve used for the calculation of the concentrations of the samples assayed.

2.2.6 RNA extraction

1.8×10^5 PBMC were plated per well as described in Section 2.2.1. Cells were lysed in 200 µl TRI Reagent after which lysates were transferred to diethyl pyrocarbonate (DEPC)-treated eppendorfs. Sample volumes did not exceed 10 % of the volume of TRI Reagent used for extraction (GIBCO BRL, Life Technologies RNA isolation protocol). The cells in the TRI Reagent were kept at -20°C until further analysis.

2.2.6.1 Phase separation

Stored lysates were thawed and incubated for 5 minutes at room temperature. This allowed the complete dissociation of nucleoprotein complexes. 180 µl of chloroform was added to the lysates, where after the eppendorfs were vigorously shaken by hand for 15 seconds and then incubated at room temperature for 5 minutes. Following the incubation, samples were centrifuged at 12 000 *g* at 4°C for 15 minutes after which the aqueous phase (containing the RNA) was removed. The volume of the aqueous phase is approximately 60 % of the volume TRI Reagent used for initial extraction (Section 2.2.6).

FIGURE 2.1

Example of a typical standard curve used in the ELISA

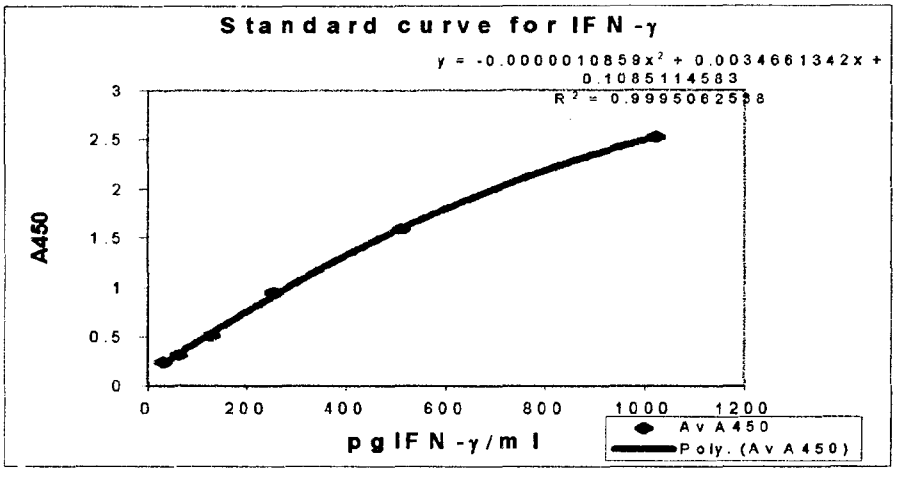


Figure 2.1 A typical standard curve used to determine the concentrations of the unknown samples. A standard curve was constructed each time an assay was performed and a different curve was drawn for each cytokine.

2.2.6.2 RNA precipitation

The aqueous phase was transferred to a fresh eppendorf with 2 µl of glycogen. The RNA was precipitated from the aqueous phase by mixing with 500 µl isopropyl alcohol, incubated at room temperature for 10 minutes and subsequently pelleted by centrifugation for 10 minutes at 12 000 *g* at 4°C. The RNA precipitate, often not visible before centrifugation, formed a gel-like pellet on the side of the tube.

2.2.6.3 RNA wash and resuspension

After the supernatant was removed, the RNA pellet was washed with 750 µl 75 % ethanol by centrifugation at 7 500 *g* for 10 minutes at 4°C. The ethanol was removed, the pellets dried under vacuum for 5 minutes followed by resuspension in 10 µl DEPC-treated water. To ensure complete resuspension of the pellets, samples were heated at 55°C for 10 minutes. RNA was quantitated spectrophotometrically of which
2 µg was electrophoresed on a 1 % agarose gel containing 1 x TBE (as described in Section 2.2.9) to assess the quality of the RNA.

2.2.7 Generation of cDNA

Ten µl of RNA was transferred to DEPC-treated PCR tubes to which 2 µl of oligo dT was added. The samples were incubated at 70°C for 10 minutes. Four µl of 5 x First Strand Buffer, 2 µl of 100 mM DTT and 1.6 µl (2.5 mM) dNTP were added after which the tubes were incubated at 42°C for 2 minutes in a PCR machine (Hybaid). After the addition of 1 µl Reverse transcriptase (MMLuV), the samples were incubated at 42°C for 50 minutes, followed by an incubation period of 15 minutes at 70°C to inactivate the reverse transcriptase.

2.2.8 Amplification of cDNA

The cDNA was amplified by means of the polymerase chain reaction (PCR). To 2 µl of cDNA, 4 µl dNTPs of 2.5 mM, 5 µl 1 x PCR buffer, 3 µl $MgCl_2$ of 2.5 mM, 50 pmoles of each primer (5' and 3') and 2.5 units of Taq polymerase (Boehringer Mannheim) was

added. The reaction mix was made up to 50 µl with water. Mineral oil was added to prevent evaporation during high temperature incubations. A positive control (RNA extracted from PHA stimulated PBMC from a volunteer) as well as a negative control consisting of no template was included in every PCR reaction.

PCR cycles for IL-2, IL-4 IFN-γ and β-actin were as follows:
94°C for 30 seconds (denaturation step)
55°C for 30 seconds (annealing step)
72°C for 1 minute and 30 seconds (elongation step).

PCR cycles for TNF-α and IL-10 were as follows:
94°C for 30 seconds (denaturation step)
60°C for 30 seconds (annealing step)
72°C for 1 minute and 30 seconds (elongation step).

The above three steps were repeated for 35 cycles.

2.2.9 Agarose gel electrophoresis

The DNA fragments obtained by PCR were analyzed by electrophoresis using a 1.5% agarose gel. The agarose was dissolved in 1 x TBE by boiling in a microwave oven. Ethidium bromide was added to a final concentration of 0.5 mg/ml. 5 µl of DNA loading buffer was added to 20 µl PCR product. The samples were heated for 5 minutes at 55°C, electrophoresed for 1 hour on a 1.5% agarose gel and visualized by UV-light.

2.2.10 IgE radioimmunoassay

This work was done by Mr M. Voget from the Dept. Chemical Pathology, Tygerberg Hospital. The quantitative determination of total serum IgE, was performed according to the protocol supplied by the manufacturers (Pharmacia AB). Assays on standards and sera were performed in duplicate. In principle, standard or serum samples were incubated with ^{125}iodinated IgE and with a specific amount of anti-IgE. IgE in the standard or serum

competed with the labelled IgE for binding to anti-IgE. The amount of iodinated IgE binding to anti-IgE was measured in a gamma counter and is indirectly proportional to the amount of IgE present in the samples. IgE levels were expressed as kU/l. Samples containing > 1 000 kU/l IgE/I were diluted and quanitified in a second run. The detection limit of this test is < 2 kU/l with no cross reactivity of IgA, IgD, IgM and IgG at physiological concentrations.

2.2.11 Statistical Analysis

Results are presented either as individual means or median group values with standard deviations (SD). Statistical significance was calculated using the Wilcoxin Rank test. Correlation analysis was conducted usinf the Spearman rank correlation coefficient test.

CHAPTER 3 – 7

RESULTS

CHAPTER 3

ESTABLISHING CELL CULTURE CONDITIONS

3.1 Background and aims of the present study

There are marked differences in the manifestation of TB between children and adults (Section 1.3.6). The aim of the present study was to determine whether differences in cytokine production exists between peripheral blood mononuclear cells (PBMC) from children and adults, which may contribute to the pathology seen in children and adults.

Before cytokine responses between children and adults could be compared, the effects of various culture media and stimulating agents on proliferation and cytokine secretion of PBMC from adults were studied. For *in vitro* analyses of cytokine responses by human PBMC, while most investigators use RPMI supplemented with human AB serum (Reiner *et al.*, 1990; Boom *et al.*, 1991; Barnes *et al.*, 1993), others prefer RPMI supplemented with foetal calf serum (FCS) (Surcel *et al* 1994; Zhang *et al.*, 1994). Foetal calf- and AB serum however, contain high levels of steroid hormones (Section 3.1.2) that might have an effect on proliferation and cytokine responses (Ramirez *et al.*, 1996). The ideal therefore would be to use a medium that is serum free. One such serum-free medium is KSLMS (Shirakoto *et al.*, 1983). The production of cytokines and proliferation of PBMC in the presence and absence of stimulants were therefore compared in KSLMS and in RPMI supplemented with 10 % FCS or in 5 % human AB serum, respectively.

Human lymphocytes can be stimulated *in vitro* by specific antigens or by mitogenic substances. In principle, when studying the function of lymphocytes the preferred approach would be to stimulate the cultures with a specific antigen (Balkwill, 1991). However, very few T cells and even fewer B cells in the peripheral blood will carry specific receptors for any antigen, even if the individual has already developed a memory response to that particular antigen (Virella, 1993). In contrast, polyclonal mitogens bind to carbohydrate determinants present in T cell membranes, inducing greater proliferative

and cytokine responses. The most widely used mitogens are plant glycoproteins (lectins) such as phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PMW) (Balkwill, 1991). In this chapter, we compared the proliferation and cytokine secretion, following stimulation by PBMC by PHA and purified protein derivative (PPD). Conflicting results from other workers, concerning the time points for cell and supernatant harvesting were observed (Barnes *et al.*, 1993; Zhang *et al.*, 1994). The kinetics of proliferation and cytokine production in different media was therefore determined.

3.2 Examination of the hormonal composition of foetal calf serum

Several steroid hormones modulate the balance between the Th1 and Th2 responses. Dehydroepiandrosterone (DHEA) is known to promote a Th1 response (Rook and Zumla 1997), whereas glucocorticoids induce the reverse effect (Ramirez *et al.*, 1996). Testosterone levels are known to be low during a Th2 response (Rook *et al.*, 1996) whereas estradiol and progesterone levels are high during a Th1 response. The levels of estradiol, progesterone, DHEAS and testosterone were determined in both FCS and in dialysed FCS (Table 3.1). FCS contained low levels of DHEAS and testosterone, but high levels of estradiol and progesterone. Extensive dialysis did not reduce the levels of estradiol and progesterone. It is difficult to explain the apparent increase in estradiol and progesterone levels following dialysis. Removal of an inhibitor might explain this finding, but a possible inhibitor has not been described in the literature. It is therefore possible that these hormones may have had an influence on the *in vitro* immune responses of the populations studied.

Table 3.1
The composition and concentration of hormones in FCS before and after dialysis.

Hormones	FCS	Dialysed FCS	Physiol. Conc.
Estradiol	249.2 pmol/l	320.4 pmol/l	110-370 pM (female)
Progesterone	1.5 nmol/l	2.8 nmol/l	0.3-3.8 nM (female)
DHEAS	0.14 µmol/l	< 0.14 µmol/l	0.95-11.7 µM (male)
			2.17-15.2 µM (female)
Testosterone	0.6 nmol/l	< 0.4 nmol/l	9.4-37 nM (male)

TABLE 3.2 and FIGURE 3.2

Thymidine incorporation and stimulation indices for PBMC following PHA stimulation.

Mean CPM values obtained for unstimulated and stimulated PBMCs following PHA stimulation. Proliferation was assessed for a 5 day period and is expressed as a SI.

Medium	Days	Unstimulated	Stimulated	SI
KSLMS	1	153	191	1
	2	241	2 669	11
	3	783	65 822	84
	4	2 196	48 897	22
	5	2 607	86 016	33
RPMI/FCS	1	57	399	7
	2	72	4 000	55
	3	298	89 513	300
	4	404	62 654	155
	5	968	53 278	55
RPMI/AB	1	38	382	9
	2	252	15 120	60
	3	468	75 164	160
	4	497	49 222	99
	5	1 774	56 796	32

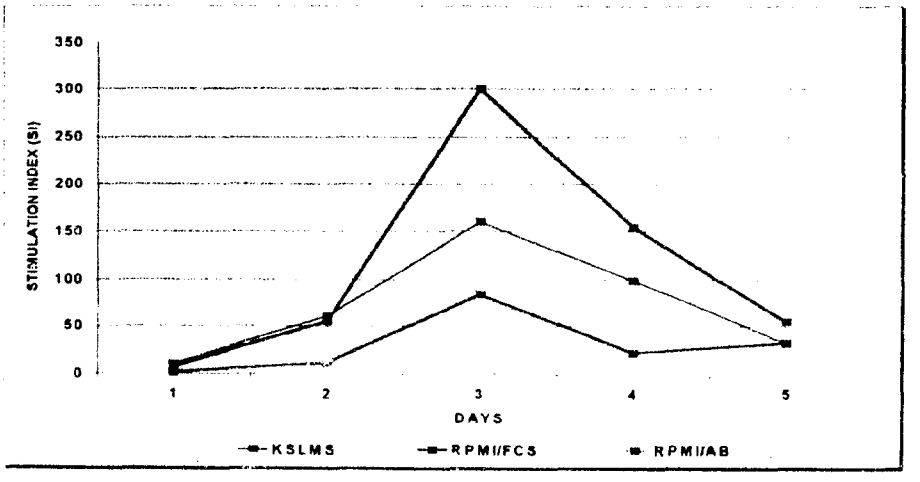
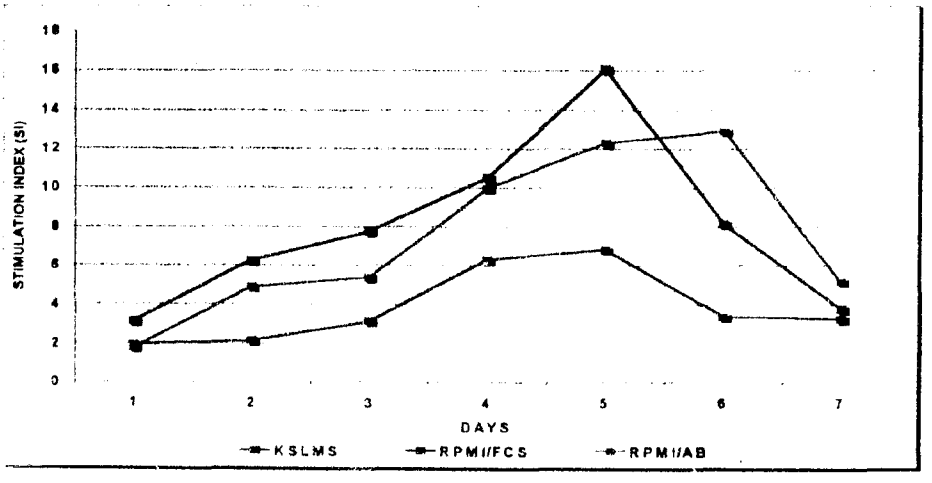


TABLE 3.3 and FIGURE 3.3

Thymidine incorporation and stimulation indices for PBMC following PPD stimulation.

Mean CPM values obtained for unstimulated and stimulated PBMCs following PPD stimulation. Proliferation was assessed for a 7 day period and is expressed as a SI.

Medium	Days	Unstimulated	Stimulated	SI
KSLMS	1	69.0	136.3	1.9
	2	117.6	256.6	2.2
	3	94.3	297.3	3.2
	4	861.0	5 427.6	6.3
	5	404.0	2 768.0	6.8
	6	673.3	2 289.3	3.4
	7	1 109.6	3 648.3	3.3
RPMI/FCS	1	120.0	379.0	3.2
	2	83.0	522.0	6.3
	3	111.0	865.8	7.8
	4	289.0	3 034.5	10.5
	5	404.2	6 499.0	16.1
	6	369.0	2988.9	8.1
	7	4 375.0	16 650.0	3.8
RPMI/AB	1	33.0	329.0	9.9
	2	282.0	503.0	1.8
	3	428.0	2 314.0	5.4
	4	468.0	2 269.0	4.9
	5	392.0	4 865.3	12.4
	6	462.0	5 979.0	12.9
	7	284.0	3 506.0	12.3



3.4 Evaluation of cytokine production by PBMC following PHA stimulation in KSLMS medium

The supernatants of PHA stimulated cells were harvested at various times (18, 24, 48 and 72 hrs). This was performed so as to determine the time for optimal production of each cytokine to be assayed. ELISAs were done on supernatants diluted 1:10 and 1:25 as described in Section 2.2.5. Absolute values are depicted in Table 3.4. As seen in Figure 3.4 (A), IFN-γ production in culture supernatants is already evident at 18 hours, increasing with time. At a 1: 10 dilution, IFN-γ production reaches a peak value of 1 070 pg/ml, after 72 hours in culture. At a 1: 25 dilution, a peak value of 800 pg/ml was obtained 48 after PHA stimulation. For both the 1: 10 and 1: 25 dilutions, TNF-α gave peak values after 48 hours. As seen in Figure 3.4 (B), at 1: 10 an increased production of TNF-α can already be detected at 18 hours (2 500 pg/ml) and 24 hours (2 560 pg/ml). Although low background values were obtained in these experiments, in Section 4.5 high background values were found in the populations studied.

Although IL-2 could be detected at 18 hours, maximum IL-2 production was detected 48 hours after PHA stimulation, which previously has been documented (Miyawaki *et al.*, 1985). At the 1: 10 dilution, IL-4 peaked at 18 hours with a concentration of 1000 pg/ml, declining to 510 pg/ml after 72 hours. There was however, variation in the production of IL-4 at the 1: 25 dilution, but with a peak value of 1550 pg/ml after 72 hours. In a study done by Hernandez *et al.* (1994), the supernatants for IL-4 assay were harvested at 24 and 48 hours (Figure 3.4 D). Maximum IL-10 production occurred after 18 hours for the 1: 10 diluted supernatant, declining from a concentration of 220 pg ml to 145 pg/ml at 72 hours (Figure 3.4 E). There was however no change with time in the concentration of the supernatant diluted 1:25. Higher background values were however found for experiments presented in Section 4.5 compared to values obtained in for these experiments. These higher background levels were unexpected. Technical reasons such as inadequate washing may have caused artefactually high levels in these initial ELISA assays.

Table 3.4

ELISA results on time courses done identifying the time for harvesting of supernatants for optimal production of IFN-γ, TNF-α, IL-2, IL-4 and IL-10 with PHA stimulation.

Cytokine	Time (hr)	Control		PHA-stimulation	
		1: 10	1: 25	1: 10	1: 25
IFN-γ (pg/ml)	18	0	25	120	102
	24	0	25	440	512
	48	0	25	1 002	800
	72	0	25	1 070	762
TNF-α (pg/ml)	18	200	0	2 250	1 250
	24	212	0	2 560	1 275
	48	215	89	3 300	2 725
	72	88	0	3 110	2 200
IL-2 (pg/ml)	18	1 080	2 800	2 100	400
	24	1 210	25	3 320	2 825
	48	0	8 950	4 430	7 725
	72	180	0	5 510	2 850
IL-4 (pg/ml)	18	450	0	1 000	1 250
	24	1 100	375	630	200
	48	460	2 425	630	1 175
	72	950	25	510	1 550
IL-10 (pg/ml)	18	140	0	220	25
	24	140	0	220	25
	48	25	0	200	25
	72	60	0	145	25

After ascertaining the optimum time and dilution for each cytokine, it was decided to use the supernatants as listed in Table 3.5.

Table 3.5

Optimum time for harvesting and dilution of supernatants for ELISA.

Cytokine	Dilution	Time Point
IFN-γ	1: 10	72 hours
TNF-α	1: 10	18 hours
IL-2	1: 25	72 hours
IL-4	1: 25	72 hours
IL-10	1: 5	18 hours

3.5 DISCUSSION

3.5.1 Establishing PBMC culturing conditions

3.5.1.1 Reasons and implications for using KSLMS as culture medium

For the present study, it was decided to use KSLMS as culture medium, because of its serum-free property. Hormonal levels of FCS and dialysed FCS were determined and it was found that FCS did exhibit high to normal levels of estradiol and progesterone and low levels of DHEAS and testosterone. Little to no effect was observed in the concentrations of the hormones of interest after dialyses. Since the aim of the present study was to determine cytokine production by PMBC in children and adults, it was important to minimise hormonal interference as they have been implicated to have an effect on the Th1/Th2 balance.

KSLMS, being serum-free, lacks fibronectin required for effective growth of antigen presenting cells. Therefore, cells cultured in KSLMS showed poor antigen specific responses, as was observed for PPD stimulation. Subsequently, only a non-specific stimulant such as PHA was used in this study. In future studies, whole blood assays (Elsasser-Baile *et al.*, 1991; Weir *et al.*, 1994; Petrovsky *et al.*, 1995) will be implemented whereby cells will be cultured in autologous serum. For this method the minimum amount of blood is needed as the blood is diluted 1: 10 with RPMI without foetal calf serum.

3.5.1.2 Cytokine production by PBMC in different media

As mentioned before, most studies used RPMI/AB or RPMI/FCS as culture media for the analysis of cytokine production by PBMC. It was therefore difficult to compare optimum times for cytokine production obtained in this study with that found in the literature. Time courses were consequently set up whereby PBMC were cultured in KSLMS, unstimulated and stimulated with (10 μg/ml) PHA. The supernatants were harvested daily, followed by analysis by ELISA, measuring the production of IFN-γ, TNF-α, IL-2, IL-4 and IL-10.

In the present study it was found that IFN-γ production peaked after 72 hours in culture using a dilution of 1: 10. For optimal IFN-γ production, it was agreed to harvest supernatants after a 72 hour PHA stimulation. This was consistent with results obtained by Miyawaki *et al.* (1985), Boom *et al.* (1991) and Pirenne-Ansart *et al.* (1995) when they determined the optimal time point for IFN-γ production ranginf from 48 to 72 hours. For IL-2 and IL-4 measurements, supernatants were harvested at 72 hours with a dilution factor of 1: 25 for the ELISA. Supernatants for TNF-α and IL-10 determination were harvested at 18 hours, with supernatants diluted 1: 10 and 1: 5 for TNF-α and IL-2, respectively.

CHAPTER 4

CYTOKINE PRODUCTION BY PBMC FROM CHILDREN AND ADULTS

4.1 Hypothesis

There is a difference between children and adults in the manifestation of tuberculosis and certain other infections as previously described. We hypothesise that these contrasts could be due to differences in the immune response between children and adults, with adults presenting with more severe immunopathology than children. The severe immunopathology observed in adults may possibly be attributed to a more vigorous immune response in adults.

4.2 Aim of the present study

The aim of the present study was to measure differences, if any, in the cytokine production by PBMC from children and adults. Cytokine production has been repeatedly evaluated in neonates and in young children with various diseases, but few comparative studies of cytokine production in healthy children and adults have been performed. Subsequently, in this initial study, cytokine production was measured in healthy individuals.

4.3 Sample population

Blood samples were collected from nine healthy, Mantoux positive adults (20-50 years of age) and nine children (2-5 years of age). All individuals investigated, had normal chest X-rays. Subjects came from two well-defined adjacent suburbs with a high incidence of tuberculosis. Ravensmead and Uitsig as well as from health workers at the Tygerberg hospital.

4.4 Specific methods

PBMC from children and adults were isolated as described in Section 2.2.1. The cells were cultured in KSLMS medium, unstimulated and stimulated with PHA (10 μg/ml). As described in Section 2.2.2, supernatants were harvested at times specified in Table

3.5 and stored at -20°C until assayed. ELISAs for IFN-γ, TNF-α, IL-2, IL-4 and IL-10 were performed using the supernatants at the dilutions specified in Table 3.5.

Cellular proliferation was assessed at 72 hours, after the cells were pulsed with [^{3}H] thymidine for 20 hours (Section 2.2.3). The cells were harvested onto glass fibre filters, whereafter thymidine incorporation was measured by liquid scintillation counting. Proliferation was expressed as CPM values and as a stimulation index (SI). Results are shown in Tables 4.1 and 4.2 respectively. Total RNA was extracted from cell pellets (Section 2.2.6) for cDNA synthesis and amplification of cDNA by PCR (Section 2.2.7).

4.5 Cytokine production by PBMC from children and adults

The concentrations of cytokines (pg/ml ± SD) produced by PBMCs by the adults and children of the sample population, are shown in Tables 4.1 and 4.2 respectively.

PBMC from adults when stimulated with PHA produced significantly higher levels of IFN-γ (1 104.11 ± 2 075.83 pg/ml) than children (12.8 ± 10.69 pg/ml, $p < 0.005$) (Figure 4.1). The production of IL-10 by PBMC from children (2 081 ± 1 125) and adults (1 650 ± 1 256.16 pg/ml) were similar, showing no significant difference (Figure 4.1). IL-10 values were high even in the unstimulated supernatants for both adults and children. After correcting for the high background levels, the children showed an IL-10 yield of 1 621.27 ± 959.79 pg/ml compared with adults 1 212.88 ± 984.41 pg/ml. IL-10 levels showed a clustering around the mean for both children and adults. In this study, no significant difference was found between children (1 952.29 ± 1 453.92 pg/ml) and adults (1 032.22 ± 1 148.85 pg/ml) for the production of IL-2 (Figure 4.1).

No significant difference was found for IL-4 production between PBMC isolated from children (486.66 ± 577.58 pg/ml) and adults (211.42 ± 187.89 pg/ml) (Figure 4.1). As shown in Figure 4.1, no significant difference was found between children and adults in the production of TNF-α. PBMC from children produced 2 500.56 ± 1 710.5 pg/ml TNF-α compared with 3 751.66 ± 3 743.32 pg/ml in adults. The TNF-α background levels were high for children and

adults. The corrected values for TNF-α production in children and adults were 877.22 ± 1 499.87 pg/ml and 2 303.88 ± 3 024.2 pg/ml, respectively.

4.6 Analysis of cytokine mRNA levels in children and adults

cDNA from the PBMC obtained from children and adults were diluted neat, 1: 10 and 1: 100 prior to PCR. The PCR fragments were subsequently electrophoresed on 1.5 % agarose gels. On average, in children IFN-γ mRNA levels could be detected at a dilution of 1: 10 and in adults at a dilution of 1: 100. In children, IL-2 mRNA was detected at neat and in adults at a dilution of 1: 10. The same trend was observed for TNF-α mRNA levels in children and adults. For children and adults, IL-4 and IL-10 mRNA could only be detected at neat. As shown in Table 4.3 and Figure 4.2, children and adults produced similar levels of cytokine message.

Table 4.3

A summary of mRNA cytokine expression in 9 children and 9 adults.

mRNA detected at neat is represented by 1 +, detected at 1: 10 by 2 + and detected at 1: 100 by 3 +.

Cytokine	Children	Adults
IFN-γ	2 +	3 +
IL-2	1 +	2 +
TNF-α	1 +	2 +
IL-4	1 +	1 +
IL-10	1 +	1+

Figure 4.2

An example of mRNA cytokine profiles in a child and an adult

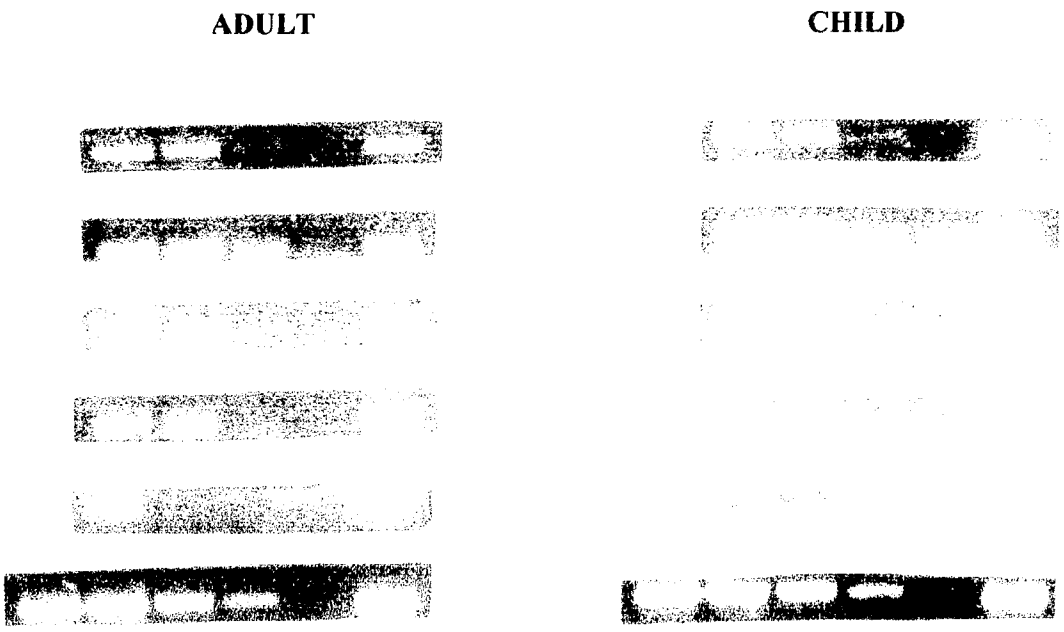


Figure 4.2 Two gels representing examples of mRNA cytokine profiles in a child and an adult. Similar trends were seen in all 9 children and 9 adults. Prior to PCR, cDNA was diluted neat, 1: 10 and 1: 100. For amplification of β-actin cDNA, the sample was further diluted to 1: 1 000. In each run, a positive and negative control was included. PCR products were run on 1.5 % agarose gels and visualised under UV-light.

Row 1: **IFN-γ**
Row 2: **IL-2**
Row 3: **TNF-α**
Row 4: **IL-4**
Row 5: **IL-10**
Row 6: **β-actin**

4.7 DISCUSSION

4.7.1 Proliferative responses of PBMC from children and adults

Following stimulation with PHA, cellular proliferation was measured at 72 hours and expressed as a SI. The low SI obtained can be ascribed to the absence of serum in the culture medium, KSLMS. KSLMS medium lacks fibronectin which is essential for the adherence of APCs. PBMC isolated from children showed a mean SI value of 24.16 ± 29.82 compared with adults attaining a mean value of 26.63 ± 29.84. This difference in SI values was shown not to be significant.

4.7.2 Lower IFN-γ production in children than in adults

The results demonstrate that healthy children differed markedly from adults in IFN-γ production after stimulation with PHA. Adult IFN-γ levels reached a mean of 3 058 ± 3 842 pg/ml compared to childrens' mean level of 12.8 ± 10.69 pg/ml. IFN-γ production has frequently been measured and evaluated in neonates where it is reduced compared to adults (Miyawaki *et al.*, 1985; Wilson *et al.*, 1986; Holt 1995; reviewed by Smith *et al.*, 1997).

4.7.2.1 Possible explanations for the decreased IFN-γ production in children

IFN-γ is mainly produced by memory T cells (Miller, 1996). Neonates and young children have a higher naïve to memory T cell ratio, therefore IFN-γ production is expected to be lower in early life. NK cells are also a major source of IFN-γ. The highest absolute count and percentage of NK cells are found in early life (Erkeller-Yuksel *et al.*, 1991; Comans-Bitter *et al.*, 1997), although these cells are reported to be functionally less active at a young age.

Taylor and Bryson (1985) implied that macrophage immaturity is responsible for the neonate's decreased ability to produce PHA-induced IFN-γ. A deficiency in IL-12 production by macrophages of young children may contribute to low IFN-γ production by T cells, as IL-12 is required for IFN-γ production by T cells. It is widely accepted that neonatal T cells are immunocompetent, but that their

differentiation is biased towards a Th2 profile under neutral conditions (Kovarik and Siegrist, 1998). IL-4 at the time of priming has been shown to be sufficient to inhibit the IL-12Rβ2 chain expression on naïve T cells and, therefore, to direct the cells towards Th2 responses even in the presence of IL-12 (Kovarik and Siegrist, 1998). In the present study, it was found that children and adults both produced high levels of IL-10. IL-10 is known to inhibit the development of Th1 cells and subsequent production of IFN-γ. It can therefore be argued that the reduced IFN-γ production in children is a result of the inhibitory effect of IL-10, although this does not hold true for adults showing high IL-10 and IFN-γ production. Scott *et al.* (1997) however, supports the inhibitory effect of IL-10 on early life IFN-γ production, arguing that T cells in early childhood exert a greater sensitivity to IL-10 inhibition than T cells of adults. Of the five adults producing less than a 100 pg/ml of IFN-γ, only two adults produced IL-10 levels greater than the mean, arguing against inhibitory effect of IL-10 on IFN-γ.

4.7.2.2 Implications of low IFN-γ production in early life

IFN-γ is important as it enhances the ability of macrophages to phagocytose and destroy invading organisms. Deficient production of this cytokine could subsequently lead to an enhanced susceptibility to certain infectious diseases in early life.

4.7.3 IFN-γ levels in adults

A wide distribution in the production of IFN-γ was found in adults. Although we only studied 9 adults, the distribution of IFN-γ levels appear to be trimodal. One group of adults produced levels similar to that found in children; another group showed clustering around the median and the last group producing levels higher than the mean. Pirenne-Ansart *et al.* (1995) previously described a bimodal distribution with the one group producing levels similar to that found in children and the other group producing very high levels.

Due to the unusual distribution of IFN-γ production in adults (as seen in the present study and in work done by Pirenne-Ansart *et al.* (1995)), it can be assumed that in a population or community a certain population of adults will show a child-like IFN-γ

profile. From this, it can be speculated that these adults exhibiting a child-like profile, may have enhanced susceptibility to infections with intracellular organisms.

4.7.4 Production of IL-2, TNF-α, IL-4 and IL-10 by children and adults

As IL-4 is normally produced by memory cells it is expected that IL-4 production in children would be lower than in adults. It has previously been reported that IL-4 production in neonates is reduced (Lewis *et al.*, 1988; Lewis *et al.*, 1991; Kurtzhals *et al.*, 1992; Lilic *et al.*, 1997). These results are contradictory to those found in the present study where children attained a mean level of 534.88 ± 536.5 pg/ml compared with 236.6 ± 189.1 pg/ml produced by adults. It is important to note that the children were recruited from a community with a high intestinal parasite infestation rate. It is reported that up to 90 % of the children in the Western Cape are infested with parasites such as *Ascaris lumbricoides* and *Trichurius trichuria* (Fincham *et al.*, 1996). The high IL-4 production in the children could be the result of the immune response elicited against these intestinal parasites. Three of the nine children (participating in the present study) had serum IgE levels > 1 000 kU/l (data not shown).

No significant differences were found between children (1 952.29 ± 1 453.92 pg/ml) and adults (881 ± 1 083.34 pg/ml) for the production of IL-2. Dissociation between IL-2 and IFN-γ for neonates has been reported, confirming the results obtained in this study. Previous studies carried out in neonates, but not in older children and only assessing mitogen stimulation demonstrated either normal (Hayward *et al.*, 1981) or decreased IL-2 production (Hassan *et al.*, 1996). In the present study, the production of IL-10 in children (2 080 ± 1 125 pg/ml) was comparable with that found in adults (2 081.16 ± 1 125.46 pg/ml).

4.7.5 Analysis of cytokine mRNA levels in children and adults

In most cases, mRNA cytokine expression was comparable with cytokine production in both children and adults. However, there was a discrepancy with IFN-γ message expression and protein production in children. Several groups have previously

reported that healthy neonates have a reduced expression of IFN-γ message with subsequent reduced protein production (Lewis *et al.*, 1986). The presence of IFN-γ message, but low product could be due to the short half-life of IFN-γ mRNA or it could be ascribed to the presence of an inhibitor preventing the translation of the protein. These reasons are speculative, as this phenomenon has only previously been described in children suffering from atopic dermatitis (Tang *et al.*, 1994).

CHAPTER 5

IgE LEVELS IN A COMMUNITY WITH A HIGH INCIDENCE OF TUBERCULOSIS

5.1 Setting

The geographical areas from where participating individuals resided, comprised of two adjacent Cape Town suburbs, Ravensmead and Uitsig. These areas have a total surface area of 2.42 km^2, a population of 34 294/100 000 population, a birth rate of 29.3/1 000 population and an infant mortality rate of 38/1 000 live births. The TB notification rate in these two communities is more than 1 000/100 000 (Beyers *et al.*, 1996).

5.2 Hypothesis

We hypothesised that individuals or communities with prominent Th2 immune responses may have an increased susceptibility to *M. tuberculosis* infection. The high TB incidence reported in Ravensmead and Uitsig could be ascribed to a combination of factors:

- Environmental factors such as poverty and the consequences thereof
- Overactive Th2 response down regulating the Th1 response
- Inherent insufficient Th1 response

5.3 Aims of the present study

The aims of the present study were threefold:

- To correlate TB incidence with education, economical and social variables in the community (all variables were obtained from the 1991 Census report).
- To measure serum IgE levels in individuals recruited from the community.
- To correlate serum IgE levels with TB incidence, education, economical and social variables in the community.

5.4 Sample populations

For the determination of IgE levels in healthy individuals from the Ravensmead/Uitsig communities, blood samples were obtained from healthy adults (> 15 years of age) visiting the Ravensmead and Uitsig clinics. Most of these individuals were healthy mothers bringing their children for routine visits to the clinics for evaluations or immunisations. This resulted in a preponderance of females in this cohort. These individuals were Mantoux skin test positive or negative, with no prior or present clinical manifestation of disease. Individuals with a history of TB, asthma, diabetes, hypertension or auto-immune or with acute infections were excluded. HIV status was not determined routinely, but it is known that the incidence of HIV infection in the Western Cape is low, being about 2 %. The ideal was to sample blood from individuals from each of the 39 enumerator sub-districts (ESDs), but for this initial study, blood samples could only be obtained from individuals living in 30 ESDs. The number of samples acquired from the 30 ESDs are described in Tables 5.1 A and 5.1 B.

5.5 Specific methods

5.5.1 Geographical information system

According to the 1991 population census data, the two areas of Ravensmead and Uitsig, were divided into 39 census blocks or enumerator sub-districts (ESDs) with an average population of 900 (ranging from a minimum of 627 to a maximum of 1267). Property boundaries were obtained from the Western Cape Regional Services Council (WCRSC) and Parow Municipality for both Uitsig and Ravensmead. The boundary files were subsequently imported into a computer-based (ARC-INFO) geographical information system (GIS) and integrated with clinical and notification data from the two residential areas for the 10-year period 1985-1994. To calculate the notification incidence of TB per ESD, the boundaries used for the 1991 population census report were superimposed on the property boundary coverage. The official records of the Western Cape Regional Services Council (WCRSC) (now the Cape Metropolitan Council) (CMC) provided data on all notified cases of TB in the area. From these records the name, address, type of TB and age of each notified case from 1 January 1985 to 31 December 1994 were collected.

The TB incidences per ESD (for 1991) was subsequently calculated for adults (> 15 years of age).

For each ESD, the census report yielded demographic variables such as age and sex; social variables such as number of households, education, type of housing and employment and economic variables such as annual income per capita. From these data Dr. A. van Rie (Dept. Pediatrics and Child Health, University of Stellenbosch Medical School, Tygerberg) and Prof. L. Zietszman (Dept. Geography, University of Stellenbosch) determined one value per ESD for crowding, economic status, maternal and paternal education level and socio-economic status. By using the GIS, the TB incidence could be related to demographic, social and economic variables in each ESD.

- Crowding was calculated as the average number of adults (> 15 years of age) per room (sleeping rooms and living rooms).
- Economic status was defined as the average annual income of a household.
- Maternal and paternal education status, were defined as the percentage of female and male adults (> 15 years of age) having completed primary education.
- The economic index was equal to the average income per adult.
- The educational index was determined by the median adult education level by yearly standard.
- A socio-economic index for each ESD could be calculated as follows:

$$\frac{\text{Economic index} + \text{education index}}{2}$$

5.6 Geographical distribution of TB incidence in the community

The geographical spread of TB incidence per ESD is portrayed in Figure 5.1. A notable variation in TB incidence is observed. The TB incidence per ESD varied from < 500 to 3 074/100 000 population. The ESDs depicted in red to dark red are those areas with a TB incidence of > 3 000/100 000 population. The ESDs shown in lighter colours indicate those areas with low TB incidence. It is noteworthy that the areas with the highest reported TB incidence are lower Uitsig and middle Ravensmead.

FIGURE 5.1

Geographical distribution of TB incidence per ESD

Table 5.1 A

TB incidence, socio-economic index, crowding and female literacy in the 30 ESDs studied. Also shown is the mean serum IgE levels (kU/l) per ESD and the number of healthy individuals sampled per ESD. Indicated are the 30 ESDs from which the 141 individuals were recruited.

ESD	TB Inc.	SE- index	Crowd.	Literacy (F)	IgE	No.
31540	1 070	53.05	1.00	79.2	49	2
31544	955	41.77	1.10	61.2	120	3
31545	1 367	48.22	1.10	79.8	29	3
31547	2 268	52.65	1.32	81.9	373	1
31549	185	56.99	0.98	85.2	124	7
31551	977	58.32	1.30	88.4	184	5
31552	562	51.10	1.44	75.1	619	1
31553	366	51.93	1.66	81.5	73	6
31554	1 310	53.0	1.21	77.4	160	4
31555	285	52.46	1.52	79.0	4	1
31556	821	58.61	1.16	83.8	87	3
31557	734	50.70	0.89	77.3	114	2
31559	683	47.21	1.67	70.5	172	8
31560	293	46.74	1.65	77.1	106	6
31561	1 217	48.66	1.23	67.0	53	11
31562	500	45.97	1.65	65.1	140	4
31563	1 218	53.47	1.10	80.6	281	2
31565	0	63.28	1.16	88.3	29	2
41622	2 847	34.97	1.86	54.8	622	6
41623	1 046	39.96	1.49	56.3	485	22
41624	2 435	41.54	1.68	61.3	633	4
41625	2 091	41.70	1.55	58.1	830	5
41626	1 587	41.99	1.41	61.6	958	7
41627	1 825	40.12	2.01	66.9	884	4
41628	3 074	35.38	1.74	50.7	602	5
41629	676	48.25	1.53	83.5	45	3
41630	920	45.46	1.71	74.7	50	5
41631	1 456	48.15	1.60	65.3	161	5
41632	165	61.69	1.09	91.1	161	1
41634	555	56.21	1.07	77.6	116	3

KEY:

ESD	Enumerator sub-district
TB Inc.	TB incidence
SE-index	Socio-economic index
Crowd.	Crowding
Literacy (F)	Female literacy
No.	Number of samples per ESD

Table 5.1 B

TB incidence, socio-economic index, crowding and female literacy for the 9 remaining ESDs from which no individuals could be recruited.

ESD	TB Inc.	SE- index	Crowd.	Literacy (F)	IgE	No.
31541	1 070	53.05	1.00	79.2	-	-
31542	1 664	50.72	1.14	73.7	-	-
31543	1 541	40.43	0.95	67.8	-	-
31546	1 176	45.52	1.30	75.2	-	-
31548	580	59.92	1.04	86.8	-	-
31550	510	59.28	1.09	88.1	-	-
31558	692	54.13	1.04	86.1	-	-
31564	592	60.20	0.95	90.5	-	-
41633	295	59.84	1.11	88.9	-	-

5.7 TB incidence related to social and economic variables

After establishing the TB incidence per ESD, it was possible to relate it to other parameters. The socio-economic index was subsequently correlated with the TB incidence per ESD. As expected, a negative correlation was found between socio-economic index and the incidence of TB per ESD ($r = -0.57$ $p < 0.001$) (Figure 5.2 A). The ESD with the lowest index (34.97) also had one of the highest notification rates of TB, 2 847/100 000 population. The highest index 63.28 was found in an ESD with no notified TB cases. A negative correlation was found between female literacy and TB incidence with a correlation coefficient of –0.58, $p < 0.001$ (Figure 5.2 B). No significant correlation was found with crowding ($r = 0.34$, $p = 0.06$) (Figure 5.2 C).

5.8 Serum IgE levels in the community

5.8.1 Serum IgE levels measured

In an initial study, serum IgE levels were measured on healthy and diseased individuals (227 adults and 33 children). Subsequently, the mean serum IgE levels attained for the healthy and diseased adults and children, were 481 and 421 kU/l, respectively. (Normal IgE levels in Caucasians are < 100 kU/l and < 200 kU/l in the coloured population of the Western Cape). From this pool of individuals, the mean and median serum IgE levels

were calculated for the 140 healthy males and females (> 15 years of age). Mean serum IgE levels for the healthy individuals attained levels of 275 kU/l compared to the 206 kU/l obtained for the diseased adults and children.

5.8.2 Geographical distribution of serum IgE levels in the community

As portrayed in Figure 5.3, serum IgE levels are shown as median LogIgE levels per ESD. A remarkable variation was observed in the spread of serum IgE levels per ESD. Those ESDs marked in red are indicative of the areas with the high serum IgE levels. Areas with the lower serum IgE levels are marked in lighter colours.

5.9 TB incidence related to serum IgE

The median IgE per ESD was calculated as well as the Ln function thereof and subsequently correlated to TB incidence per ESD. Values for TB incidence per ESD are depicted in Table 5.1 A. A significant correlation was found to exist between Ln serum IgE levels and TB incidence with $r = 0.57$, $p < 0.001$ (Figure 5.4 A).

5.10 The association between serum IgE, social and economic variables

As the median LnIgE was determined for each ESD, it was subsequently correlated with the socio-economic index, crowding and female literacy. These values are shown in Table 5.1.

A significant correlation ($p < 0.05$)was found between Ln serum IgE levels and socio-economic index with a correlation coefficient of –0.47 (Figure 5.4 B). Likewise, a significant correlation was found between IgE levels and female literacy with $r = -0.51$, $p < 0.01$ (Figure 5.4 C) On the other hand, no significant correlation was found between IgE and crowding ($r = 0.35$) (Figure 5.4 D).

FIGURE 5.2 (A)

The correlation between TB incidence and socio-economic index per ESD

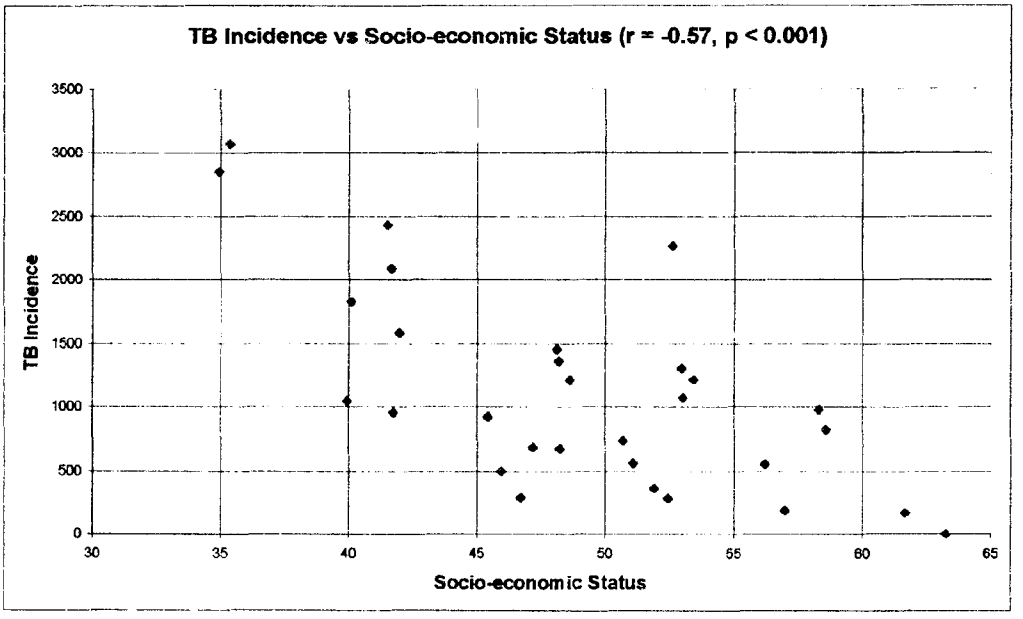


Figure 5.2 (A) The association between TB incidence and the socio-economic index per ESD. A negative correlation was found between these two parameters with $r = -0.57$, $p < 0.001$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.2 (B)

The correlation between TB incidence and female literacy per ESD

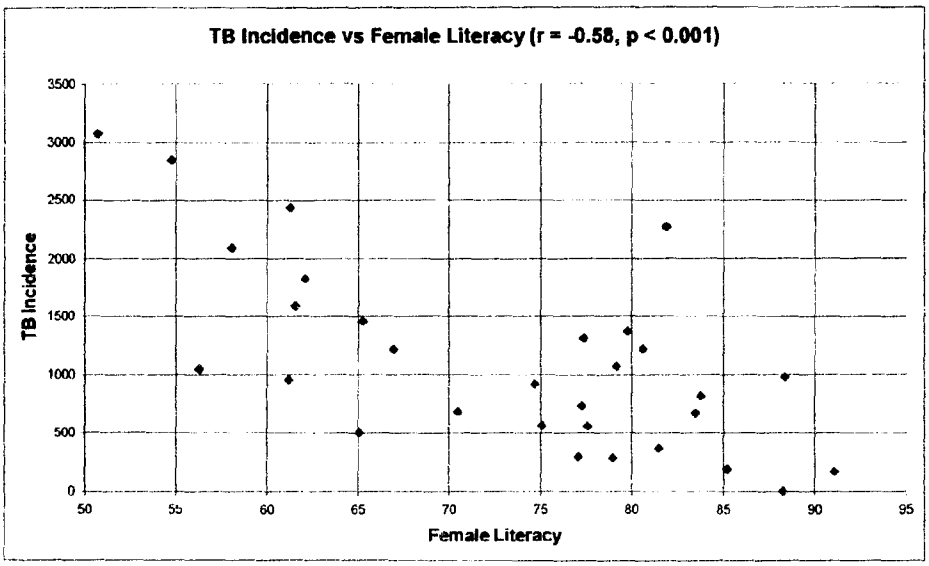


Figure 5.2 (B) The association between TB incidence and female literacy per ESD. A negative correlation exists between these two parameters with $r = -0.58$, $p < 0.001$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.2 (C)

The correlation between TB incidence and crowding per ESD

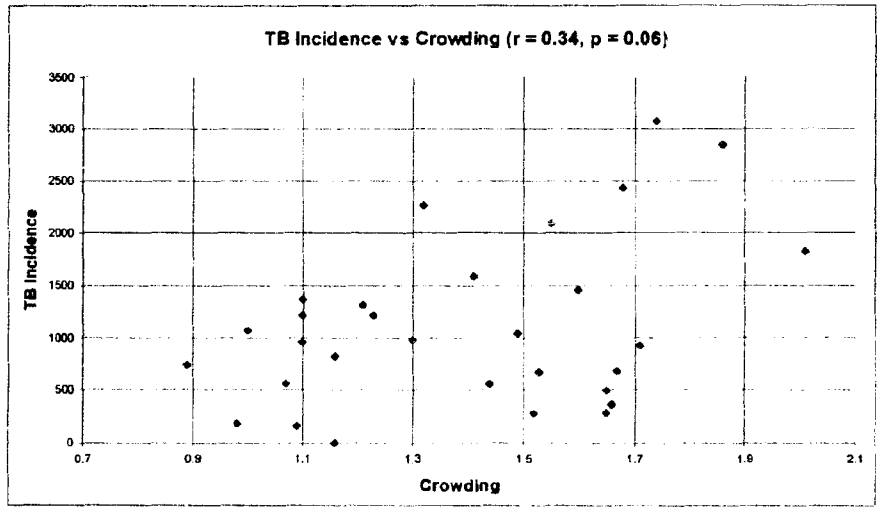


Figure 5.2 (C) The association between TB incidence and crowding per ESD. The correlation between these parameters was not significant with $r = 0.34$, $p = 0.06$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.3

Geographical distribution of serum LnIgE per ESD

FIGURE 5.4 (A)

The correlation between TB incidence and median LnIgE per ESD

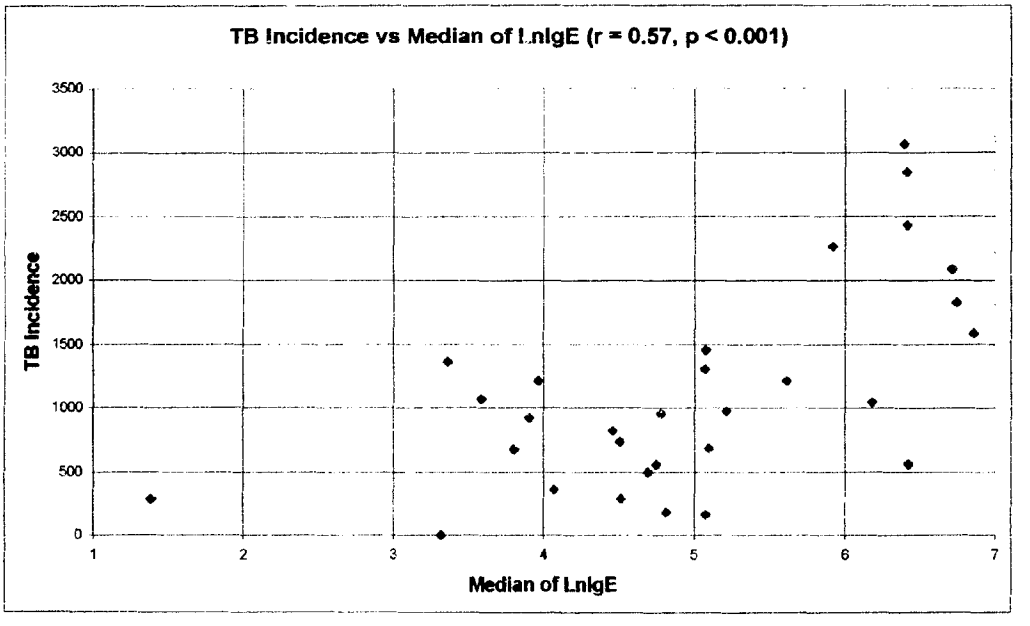


Figure 5.4 (A) The association between TB incidence and median LnIgE per ESD. A positive correlation exists between these two parameters with $r = 0.57$, $p < 0.001$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.4 (B)

The correlation between LnIgE and socio-economic index per ESD

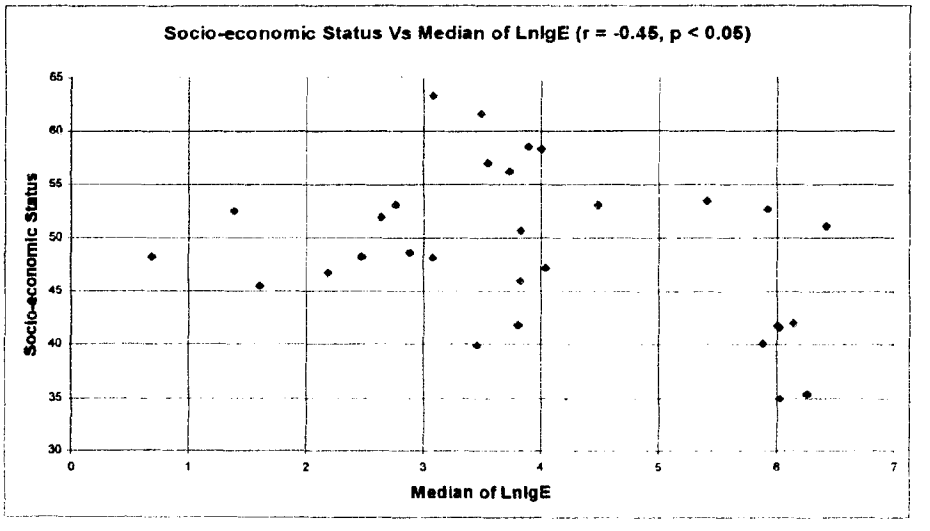


Figure 5.4 (B) The association between socio-economic index and the median LnIgE per ESD. A negative correlation exists between these two parameters with $r = -0.47$, $p < 0.05$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.4 (C)

The correlation between median LnIgE and female literacy per ESD

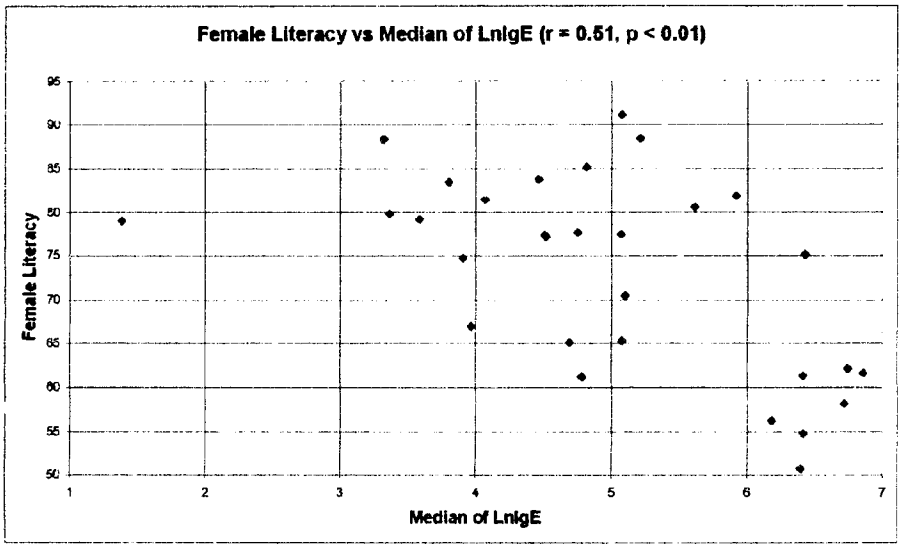


Figure 5.4 (C) The association between female literacy and median LnIgE per ESD. A negative correlation was found between these two parameters with $r = -0.51$, $p < 0.01$. The correlation coefficients were determined using the Spearman rank test.

FIGURE 5.4 (D)

The correlation between median LnIgE and crowding per ESD

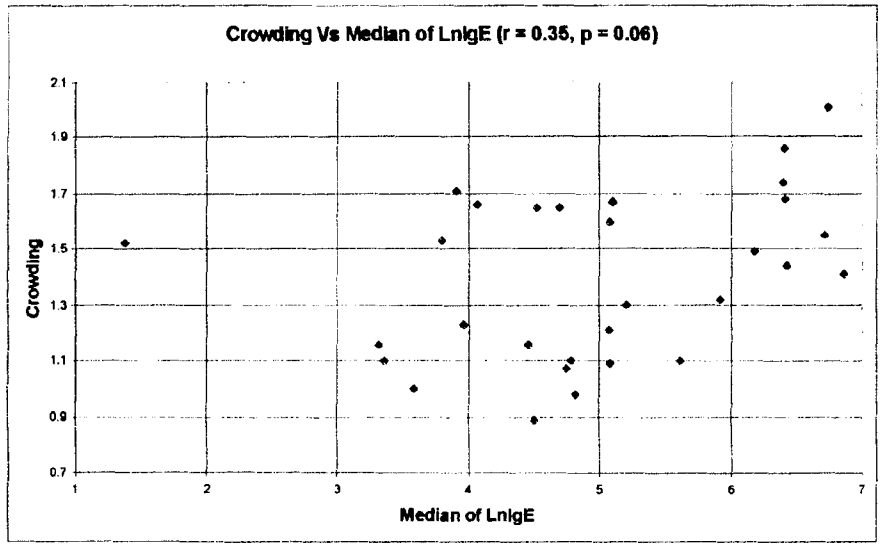


Figure 5.4 (D) The association between crowding and median LnIgE per ESD. No significant correlation was found between these two parameters, with r = 0.35, p = 0.06. The correlation coefficients were determined using the Spearman rank test.

5.11 DISCUSSION

5.11.1 Factors contributing to the high TB incidence in the community

5.11.1.1 Ethnicity and the incidence of TB

Stead *et al.* (1993) made a strong argument for a genetic cause for the apparent racial differences to susceptibility to TB. After performing repeat skin testing of 25 398 initially tuberculin negative nursing home residents, these authors found that 13.8 % of black residents had evidence of new infection compared to 7.2 % of whites. Among those infected, no racial differences were found in those developing clinical TB later. The difference in susceptibility, according to race, was also reported by Long and Jablon, (1955). In their study reviewing medical records after World War II, a significantly higher incidence of apparently new infection was found among non-whites (4.2 %) than among whites (2.8 %, $p < 0.05$). These findings are contradictory to those of Torchia *et al.*, (1977), who found no evidence of a difference in susceptibility between non-whites and whites.

The mechanism of a racial difference in susceptibility is, however only speculative. Davies (1985) has suggested a deficiency in vitamin D as a factor in the higher susceptibility to TB of darker skinned individuals, as calcitriol is essential for the phagocytosis of the bacilli by the macrophages. Studies in animals have shown that variation in susceptibility to *M. tuberculosis* is due to genetic differences in the ability of unstimulated macrophages to ingest and lyse the bacilli (Lurie *et al.*, 1952, Skamene 1989). Considerable variation is, however, found in the vigour with which human macrophages ingest and kill *M. tuberculosis*. Crowle and Elkins (1989) found that macrophages from non-whites permit significantly more replication of *M. tuberculosis* than those from whites ($p < 0.001$) This appears to provide an explanation at the cellular level as to the difference found in susceptibility. Well-recognised racial differences in the defence against malaria also exist.

In the present study, 99.7 % of the sample population were coloured *i. e.* of mixed ethnic origin. If race does play a role in susceptibility to TB, it might contribute to the high TB

incidence in the study area. This should however, be further substantiated by genetic studies in this community. Squatter camps in the Western Cape, with a predominantly black population, appear to have lower TB notification rates than Ravensmead and Uitsig. It is therefore possible that ethnicity could play a role in the susceptibility to TB.

5.11.1.2 Social and economic factors

The relationship between the socio-economic level and incidence or spread of infectious diseases is well recognised. Spence *et al.*, (1993) and Hagel *et al.*, (1993) reported that the level of poverty correlated with the incidence of TB and Ascaris infection, respectively. Poor socio-economic conditions are generally due to poverty resulting in poor nutrition, poor hygiene, inadequate sanitation, *etc*. Taken together, these factors or combinations thereof, could contribute to an enhanced susceptibility to TB in the community. It is therefore not surprising to find a significant correlation between the socio-economic level and TB incidence per ESD. It was expected that crowding should correlate with TB incidence as close confinement would favour the spread of disease. It was therefore surprising to observe that crowding did not correlate with the TB incidence per ESD.

5.11.1.3 Education

Education is crucial as it plays a role in determining the economic status of a person. A sound education and good economic status would enable individuals to establish and afford a healthy and clean environment. The more educated the parent is, the more likely he or she is to seek help at a clinic or health institution when they experience symptoms. With a higher education level, the parents are more likely to understand the importance of screening their children for possible infection with not only *M. tuberculosis*, but other diseases as well. The educated parent will be more aware of the preventative measures of the disease. In our study, female literacy was correlated with TB incidence in the community as in most cases the mothers are responsible for monitoring the health of the children. The significant correlation between TB incidence and female literacy reflects the importance of education in the community.

5.11.2 Possible reasons for the high serum IgE levels in the community

The causal agent or agents giving rise to the high IgE levels in the community, is not known. It has previously been described that 90 % of the children in the Western Cape are infested with intestinal parasites (Fincham *et al.*, 1996) leading to chronic Th2 responses. High rates of parasite infestation are probable in Ravensmead and Uitsig. Parasite loads, however, have not been determined in the study groups. Another factor proposed to shift the Th1 response to a Th2 response is stress (Rook *et al.*, 1994)). Stress in the community could mainly be derived from poverty. Unemployment, violence, poor housing low or no income *etc.* are all factors that are prevalent in the community. Environmental factors such as industrial pollution and common allergens (cockroaches, and house dust mite) lead to the manifestation of atopic disorders being the result of an active Th2 response. Any combination of these factors could lead to the overwhelming activation of the Th2 response in the community. As the Th1 and Th2 immune responses are mutually antagonistic, persistent activation of the Th2 response could predispose the community to an enhanced susceptibility to infections, in this case TB.

5.11.3 Correlation between serum IgE levels and TB incidence

A remarkable variation was observed in the TB incidence as well as serum IgE levels in the different ESDs. Possible reasons for the high TB incidence and serum IgE levels have been discussed previously. In this study the reason for the high serum IgE levels have not been investigated, but it is known that infestation with intestinal parasites such as *Ascaris lumbricoides* and *Trichurius trichuria*, is rampant in the Western Cape, where up to 90 % of children are infested with *Ascaris lumbricoides*. IgE is an important component of host-protective immune responses against the helminthic parasites. These infections not only stimulate the production of specific IgE, but also can non-specifically induce polyclonal IgE synthesis that may result in highly elevated total serum IgE levels.

Similarly, in an Ethiopian study, Bentwich *et al.* (1995) showed that intestinal parasites aggravate the course of HIV, which like TB requires a cell-mediated immune response. High serum IgE levels, a surrogate marker for Th2 activation induced by intestinal

parasites, could render the community more susceptible, resulting in the positive correlation between serum IgE levels and TB incidence.

CHAPTER 6

IgE LEVELS ON PRESENTATION OF TUBERCULOSIS AND AFTER COMPLETION OF TREATMENT

6.1 Hypothesis

TB induces a potent Th1 response at the site of disease. Given that Th1 cytokines can down regulate Th2 responses, we hypothesised that Th2 responses and total serum IgE levels (a surrogate marker of Th2 responses) as well as IgE levels against specific antigens will be down regulated in patients with TB.

6.2 Aims of the present study

The aims of the present study were twofold:

1. To compare total serum IgE levels in TB patients on presentation with IgE levels following completion of treatment.
2. To compare IgE against Ascaris and common allergens in TB patients on presentation and after completion of treatment with specific IgE levels of healthy controls from the same community.

6.3 Sample population

Serum was collected from 33 healthy adolescents, being Mantoux positive and showing normal chest X-rays. The age, gender and serum IgE levels of the control group are shown in Table 6.1. Serum was also obtained from 30 adolescents on presentation with TB and two months following completion of treatment. Table 6.2 depicts the age, gender and IgE levels measured on presentation of disease and after treatment.

After determining the total serum IgE levels on the 30 TB patients, sufficient serum was available from 23 patients for the measurement of Ascaris- and common allergen specific IgE (on presentation of disease and after completion of treatment).

6.4 Serum IgE levels at onset and following completion of TB treatment

For the 30 TB patients, serum IgE levels were significantly lower at two months following completion of treatment (mean = 164 ± 30 kU/l, $p < 0.0001$) (Table 6.2) than on presentation with disease (mean = 441 ± 84 kU/l) (Table 6.2). Although the decline in serum IgE levels is apparent for both sexes, that seen in females was steeper and reached lower levels than observed for males (Figure 6.1). On presentation, the 17 females had mean serum IgE levels of 477 ± 83 kU/l declining significantly to 174 ± 26 kU/l, $p = 0.00061$ after completion of treatment. The 13 males had mean serum IgE levels of 393 ± 164 kU/l on presentation decreasing significantly to 150 ± 61 kU/l, $p = 0.00083$ after completion of treatment.

There was no significant difference between the serum IgE levels found in the 16 control females (335.7 ± 92.3 kU/l) (Table 6.1) compared with the 17 diseased females on presentation (477 ± 83 kU/l) (Table 6.2). Similarly, no significant difference was seen between the 17 control males (216.6 ± 74.5 kU/l) (Table 6.1) and the 13 diseased males on presentation (393 ± 164 kU/l) (Table 6.2). The changes in the serum IgE levels observed in males and females are shown in Figures 6.2 A and 6.2 B.

Table 6.1

Serum IgE levels measured on 33 healthy controls including 16 females (F) and 17 males (M). The ages of the controls are also indicated. Mean serum IgE levels (kU/l) per gender as well as for the total group are shown.

Control	Age	Gender (F/M)	Serum IgE
C1	16	F	35
C2	14	F	557
C3	17	F	352
C4	11	F	220
C5	15	F	533
C6	13	F	30
C7	16	F	237
C8	16	F	253
C9	19	F	1 047
C10	16	F	1 251
C11	13	F	40
C12	15	F	68
C13	15	F	163
C14	14	F	52
C15	15	F	510
C16	15	F	23
Mean ± SD			**335 ± 92.3**
C17	11	M	589
C18	17	M	32
C19	18	M	171
C20	15	M	77
C21	19	M	65
C22	18	M	4
C23	17	M	117
C24	13	M	116
C25	16	M	83
C26	17	M	588
C27	15	M	96
C28	16	M	39
C29	15	M	250
C30	18	M	1 202
C31	14	M	71
C32	16	M	66
C33	16	M	166
Mean ± SD			**216.6 ± 74.5**
Mean ± SD		**33 controls**	**275.8 ± 338**

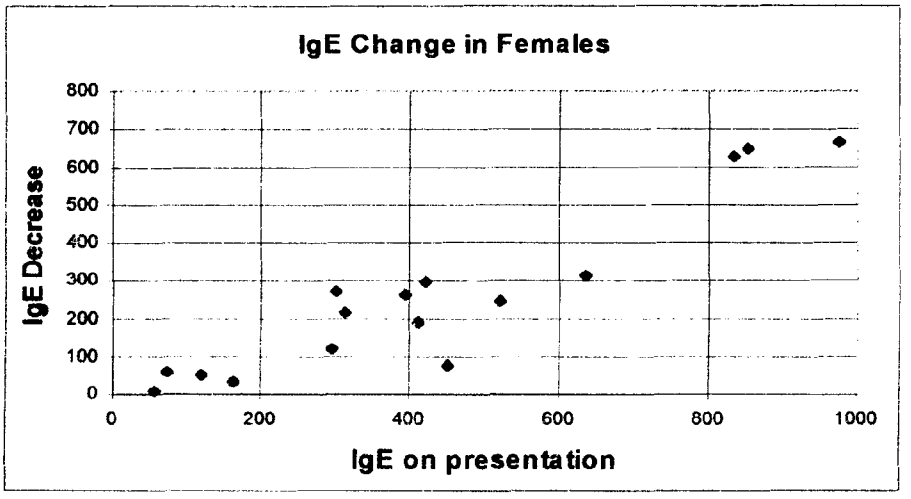
Table 6.2

Serum IgE levels (kU/l) of 30 adolescents on presentation of disease and two months following completion of treatment. Mean ± SD for both sexes are shown.

Patient	Age	Gender	IgE (before)	IgE (after)
TB 1	17	F	411	222
TB 2	17	F	854	203
TB 3	19	F	420	125
TB 4	17	F	975	310
TB 5	17	F	1 291	226
TB 6	18	F	312	99
TB 7	13	F	120	68
TB 8	15	F	394	131
TB 9	17	F	295	176
TB10	19	F	637	324
TB11	19	F	301	30
TB12	18	F	833	204
TB13	14	F	521	275
TB14	18	F	75	15
TB15	16	F	451	374
TB16	16	F	164	129
TB17	12	F	57	48
Mean ± SD			**477 ± 83**	**174 ± 26**
TB18	19	M	75	22
TB19	16	M	87	62
TB20	14	M	35	19
TB21	12	M	641	167
TB22	18	M	89	40
TB23	14	M	8	4
TB24	15	M	14	8
TB25	19	M	559	280
TB26	18	M	135	121
TB27	19	M	627	313
TB28	18	M	502	45
TB29	18	M	146	68
TB30	13	M	2 194	801
Mean ± SD			**393 ± 164**	**150 ± 61**
Mean ± SD	30 patients		**441 ± 84**	**164 ± 30**

FIGURE 6.2 (A)

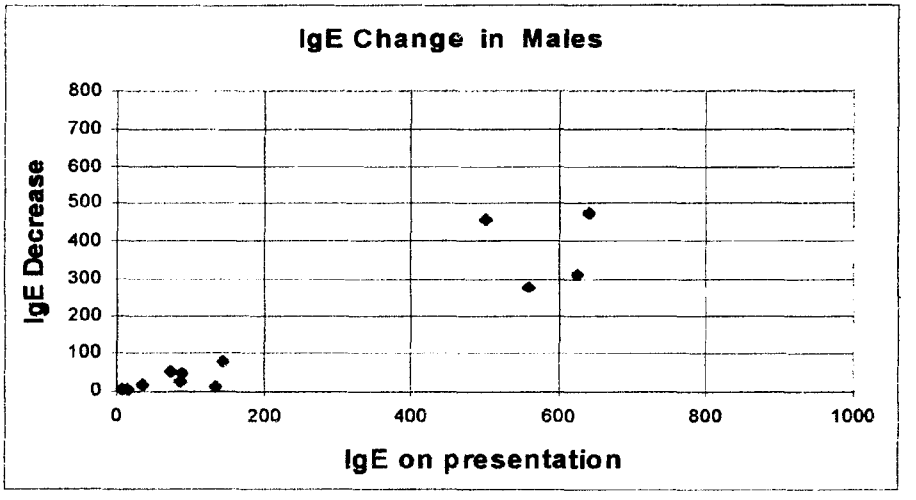
The change in mean serum IgE levels in females on presentation and after treatment



Figures 6.2 (A) Serum IgE levels were determined on 17 females on presentation of disease and after completion of treatment.

FIGURE 6.2 (B)

The change in serum IgE levels measured in males on presentation of and after treatment



Figures 6.2 (B) Serum IgE levels were determined on 13 males on presentation of disease and after completion of treatment.

6.5 Specific IgE on presentation of disease and after treatment

Specific IgE levels against Ascaris, cockroaches and house dust mite were determined on the 33 healthy controls and a subset of the TB patients on presentation of disease and after successful completion of treatment. Specific IgE levels shown in Table 6.3 are discussed below.

6.5.1 Specific IgE levels of control adolescents

Specific IgE levels in the 33 control adolescents were as follow for Ascaris, house dust mite and cockroach:

- Asacris specific IgE levels were 1.933 ± 4.05 kU/l.
- House dust mite specific IgE levels were 3.356 ± 16.17 kU/l.
- Cockroach specific IgE levels were 0.44 ± 0.6 kU/l.

6.5.2 Ascaris specific IgE on presentation and after treatment

Ascaris specific IgE levels were determined on the 33 healthy controls and 23 TB patients on presentation and after treatment (Figure 6.3 A). Of the 33 controls, 20 individuals had specific IgE levels equal to 0.3 kU/l whereas the other 13 individuals had levels > 0.3 kU/l.

Of the 23 TB patients

- 7 individuals were non-responders, with specific IgE levels of ≤ 0.3 kU/l before and after treatment
- 13 individuals had specific IgE > 0.3 kU/l on presentation decreasing after treatment
- 3 individuals showed a increase in specific IgE levels after treatment

6.5.3 Cockroach specific IgE on presentation and after treatment

As shown in Figure 6.3 C, cockroach specific IgE levels were measured on the 33 controls with 29 individuals having levels equal to 0.3 kU/l and the other 4 individuals attaining levels higher than 0.3 kU/l.

Of the 23 TB patients

- 18 individuals were non-responders, with specific IgE levels of 0.3 kU/l before and after treatment
- 1 individual had specific IgE ≥ 0.3 kU/l on presentation increasing after treatment
- 4 individuals showed a decrease in specific IgE levels after treatment

6.5.4 House dust mite specific IgE on presentation and after treatment

When determining house dust mite specific IgE on the 33 healthy controls, 25 individuals attained levels equal to 0.3 kU/l while the other 8 had levels higher than 0.4 kU/l (Figure 6.3 B).

Of the 23 TB patients

- 17 individuals were non-responders, with specific IgE levels of 0.3 kU/l before and after treatment
- 1 individual had specific IgE ≥ 0.3 kU/l on presentation increasing after treatment
- 5 individuals showed a decrease in specific IgE levels after treatment

Table 6.3

Ascaris, house dust mite (HDM) and Asacris specific IgE levels measured on 23 TB patients on presentation of disease and again after successful completion of treatment. Values are presented in kU/l.

TB patients	IgE		Ascaris		HDM		Cockroach	
	Before	After	Before	After	Before	After	Before	After
TB 1	411	222	3.0	2.2	0.3	0.3	0.3	0.3
TB 2	854	203	5.1	2.6	0.3	0.3	0.6	0.4
TB 4	975	310	4.8	1.9	0.3	0.3	0.7	0.3
TB 5	1 291	226	0.8	0.3	11.1	3.4	6.0	1.7
TB 6	312	99	0.3	0.3	19.3	39.6	0.3	0.3
TB 8	394	131	2.2	2.5	0.3	0.3	0.3	0.3
TB 9	295	176	14.4	0.3	0.7	0.3	0.3	0.3
TB 10	637	324	0.4	0.3	0.3	0.3	0.3	0.3
TB 12	833	204	0.5	0.3	1.8	0.6	0.3	0.3
TB 13	521	275	2.2	0.5	0.3	0.3	0.3	0.3
TB 14	75	15	0.3	0.3	0.3	0.3	0.3	0.3
TB 15	451	374	51.4	24.6	0.3	0.3	0.3	0.3
TB 16	164	129	1.7	0.7	0.3	0.3	0.3	0.3
TB 18	75	22	0.4	0.3	0.3	0.3	0.3	0.3
TB 19	87	62	0.3	0.3	0.3	0.3	0.4	0.3
TB 21	641	167	1.5	0.4	0.3	0.3	0.3	0.3
TB 22	89	40	0.3	0.3	0.3	0.3	0.3	0.3
TB 23	8	4	0.3	0.3	0.3	0.3	0.3	0.3
TB 24	14	8	0.3	0.3	0.3	0.3	0.3	0.3
TB 25	559	280	1.4	0.3	0.7	0.3	0.3	0.3
TB 26	135	121	0.5	0.6	0.3	0.3	0.3	0.3
TB 29	146	68	0.3	0.3	0.3	0.3	0.3	0.3
TB 30	2 194	801	13.2	14.8	71.6	68.9	1.1	1.1
Mean ± SD			**3.8±9.7**	**2.3±5.8**	**3.9±13.5**	**4.9±15.8**	**0.5±0.1**	**0.9±0.32**

6.6 DISCUSSION

6.6.1 Mean serum IgE levels in controls

The normal IgE level for Caucasians is < 100 kU/l and for the coloured population of the Western Cape it is < 200 kU/l. For the control group the mean serum IgE levels was 275.8 ± 338 kU/l (Table 6.1). This levels was not significantly different to levels obtained on presentation of disease 441 ± 84 kU/l (Table 6.2). The high serum IgE levels in controls and patients on presentation of TB may be ascribed to intestinal parasite infestation (Fincham *et al.*, 1996).

6.6.2 Decline in serum IgE levels after completion of TB treatment

In every patient, a decline in serum IgE levels was observed after completion of successful treatment. This significant decline can most probably be ascribed to chronic Th1 stimulation induced by *M. tuberculosis*. *M. tuberculosis* elicits a Th1 response characterised by IFN-γ production. IFN-γ promotes Th1 development thereby suppressing further Th2 progression with subsequent inhibition of IL-4 induced B-cell switching to IgE synthesis.

Other reasons responsible for the decline are possible, but unlikely. As yet, no reports implicating TB treatment in the down regulation of serum IgE levels, has been documented. Conversely, rifampicin binds to and activates glucocorticoid receptors. Recent evidence reveals that glucocorticoids enhance a Th2 rather than a Th1 response (Ramirez *et al.*, 1996). Furthermore, reduction in parasite burden resulting in decreased IgE levels has not been described for TB drugs. It is unlikely that patients received anti-helminthics during their TB treatment.

Shirakawa *et al.* (1997) and Shaheen *et al.* (1996) have previously reported down regulation of Th2 responses due to chronic Th1 stimulation. Shirakawa *et al.* (1997) found an inverse relation between IgE levels and delayed type hypersensitivity responses induced by repetitive BCG vaccinations and/or environmental mycobacteria. Although

Shirakawa's study was performed on healthy children as opposed to the TB patients used in our study, they were able to show that chronic Th1 stimulation down regulates IgE responses. Similarly, Shaheen *et al.* (1996) showed that natural measles infection inducing a Th1 response suppresses Th2 responses, manifested by a decline in serum IgE or specific IgE levels.

6.6.2.1 Proposed methods of down regulating pre-existing Th2 responses

In Section 6.6.2, we proposed that chronic Th1 stimulation induced by environmental mycobacteria or repetitive BCG vaccinations could effectively down regulate Th2 responses. This was also further demonstrated by Wang and Rook, (1997) who showed that when immunising ovalbumin preimmunised Balb/c mice with a single injection of *M. vaccae* (a killed preparation of *M. vaccae*), serum IgE and allergen specific IgE levels were suppressed.

Down regulation of Th2 responses can also be accomplished in an allergen-specific manner with DNA vaccinations whereby differentiation of Th1 cells are promoted, with subsequent inhibition of Th2 cell differentiation. The use of Ag-IL-12 fusion proteins show great potential for down regulating Th2 responses. The fusion protein converted a Th2 dominant response characterised by high IL-4 and IgE levels, into a Th1-dominated immune response in an Ag-specific manner. These methods, however, use a single Ag, whereas individuals in the community are exposed to various Ag from *M. tuberculosis* and *Ascaris lumbricoides*. In a community with a high incidence of intestinal parasite infestations as well as TB, it would be more advantageous to down regulate pre-existing Th2 responses than chronically stimulate Th1 responses. This can ultimately be achieved by introducing de-worming programmes reducing the parasite loads. Eradication of intestinal parasites will down regulate Th2 responses, ultimately improving individual's ability to cope with *M. tuberculosis* infection.

6.6.2.1.1 Plasmid DNA vaccinations

In a study performed by Raz *et al.* (1996), it was demonstrated that gene immunisation induces a Th1 response that dominates over an ongoing protein-induced Th2 response in

an antigen-specific manner. The mechanism by which IgE production is down regulated is not clear, but they propose it may be due to the induction of new antigen-specific Th1 cells that are able to down-regulate the differentiation of new Th2 cells. Furthermore, IgE has a short serum half-life (Haba *et al.*, 1985), which could explain the rapid decline in the IgE titer once IgE synthesis is suppressed by the secondary plasmid DNA (pDNA) immunisation. Immunisations with pDNA might be beneficial to down-regulate Th2 immune responses induced by allergic disorders and parasitic infections.

6.6.2.1.2 Ag-IL-12 fusion proteins

IL-12 secreted by macrophages is potent in driving the differentiation of naïve $CD4^+$ T cells toward the Th1 phenotype, by enhancing the production of IFN-γ. Studies have shown that IL-12 has potential as an immunomodulator and adjuvant in models of allergy and of infectious diseases (Wynn *et al.*, 1995). In a study performed by Kim *et al.*, (1997), they found that IL-12 covalently linked to the Ag OVA (an Ag that is responsible for allergy in humans), reduced allergen-specific production of IL-4 and enhanced allergen specific IFN-γ. The OVA-IL-12 fusion protein was much more effective than free rIL-12 in reducing Ag-specific IL-4 synthesis and in reducing IgE synthesis. Furthermore, in a setting of pre-existing high IL-4 and IgE synthesis, the OVA-IL-12 fusion protein greatly reduced OVA-specific IgE production while increasing OVA-specific IgG2a production and greatly enhanced Th1 dominant cytokine production in an Ag-specific fashion. These results suggest that Ag-IL-12 fusion proteins may be beneficial in the treatment Th2-dominated responses.

6.6.3 Specific IgE levels on presentation and after treatment

The decline in total serum IgE levels during treatment was highly significant. A significant decline in specific IgE levels against Ascaris was observed ($p = 0.04$, McNemer's test). Although stool specimens were not obtained in order to quantify the rates and severity of parasite infestation in these patients, more patients has Ascaris-specific IgE antibodies than the controls. However, the patients on presentation had similar Ascaris-specific IgE levels than the controls. Parasite infestation and TB are both associated with poverty (Spence *et al.*, 1993; Hagel *et al.*, 1993) and social deprivation.

In this study, controls and patients were from the same communities and the increased frequency of Ascaris-specific antibodies in patients raises the question of whether infestation with *Ascaris lumbricoides* contributes to susceptibility to TB. It is noteworthy that *Ascaris suum* impairs T cell function in mice (Ferreira *et al.*, 1994) and that Ascaris spends a stage of its life cycle in the lungs, where it may induce a local type 2 immune response. A limited number of studies in mice and humans indicate that parasite-induced type 2 dominance may suppress cell-mediated immunity.

No significant decline in common allergen specific IgE levels were observed during the course of disease. A limitation in this study was the small sample population.

CHAPTER 7

Specific allergen and Ascaris specific IgE levels in ESDs with a high and low TB incidence

7.1 Hypothesis

The TB incidence per ESD ranged from < 500 to > 3 125/100 000 (Section 5.1). We hypothesised that individuals in those ESDs with the highest TB incidence could have the highest specific IgE levels.

7.2 Aim of the present study

Ascaris, house dust mite and cockroach specific IgE levels were determined in 15 individuals residing in 5 ESDs with low TB incidence, as well as on 15 individuals residing in 2 ESDs with high TB incidence.

7.3 Sample population

The sample population used in the present study was the same as that used in Section 5.2.3. A subsection of the individuals was taken from 5 ESDs with the lowest and 2 from ESDs with high a TB incidence, respectively.

7.4 Specific IgE levels in ESDs with high and low TB incidence

As shown in Tables 7.1 and 7.2, the only significant difference between high and low TB incidence ESDs was found in Ascaris specific IgE ($p < 0.05$). For the 15 individuals in the low incidence ESDs, a mean of 6.08 ± 0.54 kU/l was obtained. In the ESDs with the high TB incidence, a mean of 11.08 ± 8.16 kU/l was measured. In most cases, individuals were non-responders, having very low specific IgE levels for Ascaris, house dust mite and cockroach allergens. Specific IgE levels against house dust mite, attained levels of 8.51 ± 8.42 kU/l and 13.11 ± 25.63 kU/l in the high and low TB incidence ESDs,

respectively. Specific IgE levels against cockroaches, came to 6.18 ± 0.68 kU/l and 5.94 ± 0.13 kU/l in the high and low TB incidence ESDs, respectively.

7.5 Discussion

It is difficult to make a strong argument for high Ascaris infestation rates in ESDs with a high TB incidence due to the small sample populations. Parasite loads in stool samples need to be determined in the community in order to determine if the high TB incidence in these areas is associated with intestinal parasite infestations.

Table 7.1

Specific allergen (house dust mite (HDM) and cockroach) and Ascaris specific IgE levels determined in 5 ESDs with a low TB incidence.

ESD (Low TB incidence)	Ascaris	HDM	Cockroach
31549	0.3	0.9	0.3
31549	0.3	0.3	0.3
31549	0.4	4.6	0.3
31549	1.1	0.3	0.3
31553	0.3	0.3	0.3
31553	0.3	0.3	0.3
31553	0.3	0.3	0.3
31560	0.3	1.1	0.8
31560	0.3	0.3	0.3
31560	0.3	0.3	0.3
31560	0.3	0.3	0.3
31560	2.3	33.1	0.5
31565	0.3	0.7	0.3
31565	0.3	0.3	0.3
41632	0.3	0.6	0.3
Mean ± SD	**6.08 ± 0.54**	**8.51 ± 8.42**	**5.94 ± 0.13**

Table 7.2

Specific allergen (house dust mite (HDM) and cockroach) and Ascaris specific IgE levels determined in 2 ESDs with a high TB incidence.

ESD (High TB incidence)	Ascaris	HDM	Cockroach
41622	4.7	1.7	2.9
41622	2.8	0.7	1.1
41622	1.2	0.3	0.3
41622	0.4	0.4	0.3
41622	0.3	0.6	0.3
41622	2.0	0.4	0.3
41622	2.5	0.4	0.3
41628	0.3	0.3	0.3
41628	25.0	6.5	0.3
41628	3.5	0.3	0.3
41628	24.1	100.0	0.8
41628	0.3	0.3	0.3
41628	0.3	0.3	0.3
41628	6.4	0.3	0.3
41628	10.1	0.3	0.3
Mean ± SD	**11.18 ± 8.16**	**13.11 ± 25.63**	**6.18 ± 0.68**

CHAPTER 8

CONCLUSION

CHAPTER 8

CONCLUSION

8.1 Cytokine responses in children and adults

For this initial study, serum-free medium was used as opposed to medium supplemented with either human AB - or foetal calf serum. However, as was shown in this thesis, estrogen and progesterone levels in foetal calf serum were high. These high levels could ultimately alter the cytokine production by PBMC from children and adults. Consequently, a serum free medium, KSLMS was chosen as culture medium for this study. The choice of culture medium would also determine the type of stimulating agent used. Serum free medium lacks fibronectin which is needed for the spreading and adherence of APCs after specific antigen stimulation. Therefore, we decided to culture the isolated PBMCs from children and adults in KSLMS medium, unstimulated or stimulated with a non-specific stimulant, PHA.

We hypothesised that the differences seen in the clinical manifestation of TB between children and adults might be ascribed to a more vigorous immune response in adults compared to children. PBMC were subsequently isolated from whole blood obtained from 9 healthy children (2 – 5 years of age) and 9 healthy adults (20 – 50 years of age). The children produced significantly less IFN-γ than adults. No further differences was, however, observed in the production of TNF-α, IL-2, IL-4 and IL-10. The low IFN-γ levels produced in children might support our hypothesis. IFN-γ along with TNF-α, is critical for the effective elimination for mycobacterial growth (Smith *et al.*, 1997). Orchestration of these two cytokines might be responsible for the more severe immunopathlogy observed in adults compared to adults.

One limitation of this study was the use of only a non-specific stimulating agent (PHA). However, in future studies, cytokine responses in children and adults will be assessed by culturing whole blood diluted 1: 10 with RPMI (Elsasser-Beile *et al.*, 1991; Petrovsky *et al.*, 1995). Therefore, the presence of autologous serum would allow the use of a specific antigen such as purified protein derivative (PPD) and possibly live *M. tuberculosis* as stimulating agents. Possible reasons for the presence

of IFN-γ mRNA, but low IFN-γ protein levels in children will also be pursued. Although only 9 children (2 –5 years of age) and 9 adults were in used in this thesis, future studies will aim to investigate cytokine responses in healthy individuals over a broader age spectrum. These responses will subsequently be compared to cytokine responses measured in individuals with TB recruited from a community with a high incidence of TB.

8.2 Immune responses in a community with a high TB incidence

Our second hypothesis stated that the high TB incidence of > 1 000/100 000 population (Beyers *et al.*, 1996) in the Ravensmead and Uitsig, might be due to a Th2 dominance and/or to the contributory consequences of environmental factors. For this reason, serum IgE levels, surrogate markers for Th2 activation, were determined on 140 healthy males and females in the community. High serum IgE levels were found ranging from 4 kU/l to 958 kU/l. (Normal IgE levels in the coloured population of the Western Cape is < 200 kU/l and for Caucasians < 100 kU/l). Mean serum IgE levels were determined per ESD and subsequently correlated to the TB incidence per ESD. A significant positive correlation was found, confirming our hypothesis that the high TB incidence in the community might be due to an over active Th2 response. The positive correlation stressed the importance of identifying the causative agents for the high serum IgE levels. Even though parasite loads have not been determined in the community, it is commonly accepted that low socio-economic levels are associated with parasite infestation (Anderson and May, 1982; Hagel *et al.*, 1993). Further studies are, however, needed to determine if intestinal parasite infestation may contribute to the development of TB by driving Th2 responses. This can only be achieved by an interventional study, *i.e.* by studying if regular de-worming reduces the incidence of TB. On the level of public health and preventative measures, a greater emphasis should be placed on eradication of intestinal parasite infections with subsequent down regulation of serum IgE responses. Such eradication may modulate the baseline immune responses and lead to an improved ability of especially individuals in the community to cope with infections.

The 1991 Census report provided social and economic variables for Ravensmead and Uitsig. From the report, socio-economic levels as an index, female literacy, and

crowding were calculated and subsequently correlated to the TB incidence per ESD. These variables demonstrated significant negative correlation with TB incidence except for crowding that showed no significant correlation. These findings established that education as well as socio-economic levels in this community might contribute to the high TB incidence. As TB is such a prevalent disease in the community, TB education should be initiated at primary school level. In this way, the child will grow up knowing the symptoms of the disease, the treatment thereof and ultimately, the preventative measures involved. Furthermore, improvement of existing TB education programs compensating for the illiterate parent should also be addressed. In addition to education, the improvement of living conditions in the community could ultimately reduce the risk of developing TB. It can therefore be argued that although environmental factors are not directly responsible for the high TB incidence in the community, they might contribute to susceptibility to TB.

In this study, serum IgE levels were determined on 140 individuals recruited from 30 ESDs. Future studies will involve the recruitment of at least 5 individuals per 39 ESDs. Further correlation will be made between serum IgE levels, TB incidence and the social and economic variables in the community. These variables will be obtained from the 1996 Census report. Furthermore, stool samples will be collected from children in the community, whereby quantitative parasite egg counts will be determined.

8.3 Down regulation of Th2 responses after treatment for TB

It was further hypothesised in this thesis that dominant Th2 responses might be down regulated by chronic Th1 stimulation induced by *M. tuberculosis* infection. For this reason, serum IgE levels were determined on TB patients on presentation and again following completion of successful treatment. A significant decline in serum IgE levels was observed after completion of successful TB treatment. Females had higher serum IgE levels on presentation and they showed a steeper decline in serum IgE levels than males. These findings confirmed our hypothesis that Th2 responses are down regulated after treatment for TB. Further support of this hypothesis was given by Shirakawa *et al.* (1997) and Shaheen *et al.* (1996). Both groups found that chronic Th1 stimulation, whether induced by repetitive BCG vaccinations (Shirakawa *et al.*,

1997) or natural measles infection as opposed to measles immunization (Shaheen *et al.*, 1996), down regulated Th2 responses manifested by a reduction in IgE responses.

Two important implications of these findings are highlighted:

1) Chronic Th1 stimulation can down regulate the Th2 responses enabling individuals in the community to cope with *M. tuberculosis* infection. This can be achieved by repetitive BCG vaccinations or ideally, the administration of a new, efficient anti-TB vaccine. These implications apply mostly to third world communities such as Ravensmead and Uitsig.
2) In addition, down regulation of Th2 responses is also important in the treatment of atopic disorders that are more prevalent in first world countries.

After measuring serum IgE levels on presentation of TB and after treatment, specific IgE levels against Ascaris and common allergens were measured on a subset of the above mentioned TB patients. A significant decline in Ascaris specific IgE levels was found after completion of successful treatment. No significant differences was, however, observed in the specific IgE levels against cockroaches and house dust mite. A limitation of this study was the small sample number. Future studies will involve measuring serum IgE levels and cytokines on presentation of TB, at regular intervals during treatment and after completion of successful treatment.

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