IMMUNE RESPONSES 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 dissertation 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

It is estimated that about one third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). Of those infected, only 10 % will develop disease of which 3-5 % will relapse after completion of treatment. Susceptibility to *M. tuberculosis* or relapse following treatment, may be due to environmental influences such as poverty-related factors including intestinal parasites (helminths), and/or genetic factors, all of which can influence the immune response to *M. tuberculosis*. In the current study, the epidemiology of mycobacterial infection and helminths was studied in two adjacent suburbs of Cape Town, South Africa. These communities had a tuberculosis notification rate of over 1 000/100 000 population with rampant infestations by helminths such as *Ascaris lumbricoides* and *Trichuris trichiura*. *M. tuberculosis* infection and Bacille Calmette Guerin (BCG) vaccination induce a Th1 (type 1) immune response, while a Th2 (type 2) immune response is required for expulsion of intestinal parasites. Type 1 and type 2 responses negatively cross regulate each other *in vitro* and in experimental models. The interaction of these two immune responses in the study community, were investigated. It was hypothesised that susceptibility to *M. tuberculosis* and progression to disease may be increased in individuals mounting prominent type 2 immune responses, manifested by high serum IgE levels. Furthermore it is proposed that that poverty-related factors and intestinal parasites, specifically those trafficking through the lungs, could further augment the type 2 dominance in the study community.

Results presented show that serum IgE concentrations, surrogate marker for type 2 activation, were high among healthy adults, confirming the dominance of type 2 responses. When characterised in census blocks or enumerator sub-districts (ESDs), IgE levels correlated with the tuberculosis notification rate per ESD. The notification rate of tuberculosis also correlated with the socio-economic status, female literacy and population density of the study population. Although these correlations do not necessarily imply a causal relationship, these factors are associated with susceptibility to *M. tuberculosis*. It was also shown that IgE concentrations decreased significantly after successful treatment of tuberculosis, showing that IgE concentrations in humans can be down-regulated under these circumstances, presumably due to enhancement of a type 1

response. Furthermore, as a reason for the high serum IgE concentrations in the study population, the helminth burden was subsequently measured in all primary school children in the study community. Results show that more than 50 % of the children recruited were infected with *A. lumbricoides* and/or *T. trichiura*. Schools situated in the poorest areas with the highest tuberculosis notification rates, presented with the highest prevalences of helminths. All the children, irrespective of their helminth status or their participation in the study, subsequently received ant-helminthic treatment.

The BCG vaccination scar status and Mantoux skin test responses were available on a sub-sample of the above-mentioned school children. Although it is assumed that most children receive BCG vaccination in the neonatal period, only two thirds of the children had evidence of a BCG scar. The results show that the prevalence of BCG scar positivity, while independent of age, was lower in children around 11 years of age. In contrast to the broad constancy of BCG scar expression, the percentage of children showing Mantoux reactivity increased with age, from 13 % at 6 years to 65 % at mid teenage. The time course of Mantoux conversion with age indicated that any tuberculin sensitivity, induced by the BCG, waned within the first few years of life and that PPD responsiveness thereafter was induced by environmental exposure to *M. tuberculosis*. Contrary to the Th1/Th2 paradigm, the prevalence of helminth infection in children with a BCG scar was marginally lower than in those without one. A relatively weak positive correlation was found between tuberculin responsiveness and helminth infection and this correlation was most marked in children without a BCG scar. In this subgroup, children who were infected with helminths were more likely to be PPD responsive than those who were not infected. The data showed that conversion to PPD sensitivity predisposed to helminth infection. The results suggest that the effect of helminth infection on the development of clinical tuberculosis is such that those with large worm burdens and who make good PPD responses are likely to be resistant whereas those who deal very effectively with these parasites and who make weaker PPD responses are more likely to be susceptible. The data also indicate that the BCG vaccine used in this study does not give rise to a latent infection whereas the pathogenic *M. tuberculosis* does so and repeatedly stimulates an immune response to it.

In a separate study, it was demonstrated how the host response to *M. tuberculosis* differs in patients at risk for developing tuberculosis after successful completion of treatment, compared to those who have protective immunity. Individuals participating in the study were also interviewed to understand their social and economic background and how it relates to the disease. Purified protein derivative (PPD) and *M. tuberculosis*-induced cytokine responses were determined in the study groups. The results show that single immunological marker of susceptibility could not be distinguished, but rather immunological patterns of susceptibility were observed. Individuals who have had tuberculosis once before and who had been cured, presented with an immuno-suppressive profile, which included high concentrations of IL-10, TGF-β as well as high IgE levels. This type of profile suggests that although these individuals have had tuberculosis once before, they have not acquired protective immunity and would be susceptible to re-infection and progression to disease. Furthermore, the interviews conducted showed that most of the people included in this study were poor, unemployed, undernourished and lived in overcrowded conditions. It seems inevitable that those individuals with the immuno-suppressed profile living in poverty would present with a second episode of tuberculosis in the near future.

We conclude that in the study community, which has a typical third world setting, poverty-related factors including helminths, could contribute to a dominant type 2 immune response which in turn, would down-regulate the protective type 1 response, resulting in an enhanced susceptibility to *M. tuberculosis* and progression to disease.

OPSOMMING

Dit word beraam dat ongeveer een derde van die wêreld se populasie geïnfekteer is met *Mycobacterium tuberculosis* (*M. tuberculosis*). Van diegene wat wel geïnfekteer is, sal slegs 10 % siekte ontwikkel met 3-5 % wat 'n relaps episode sal ervaar na voltooiing van behandeling. Vatbaarheid vir *M. tuberculosis* of 'n relaps episode gevolg na behandeling, mag toegeken word aan armoede-verwante faktore wat intestinale parasiete (helminte) asook genetiese faktore, insluit. Hierdie faktore het die vermoë om die immuun respons teen *M. tuberculosis* te beïnvloed. In die huidige studie, is die epidemiologie van die mikobakteriele infeksie en helminte bestudeer in twee aangrensende voorstede van Kaapstad, Suid Afrika. Hierdie gemeenskappe het 'n tuberkulose aanmeldings koers van 1 000/100 000 populasie met verpreide infestasies met helminte soos *Ascaris lumbricoides* and *Trichuris trichiura*. Infeksie met *M. tuberculosis* en vaksinasie met Bacille Calmette Guerin (BCG), induseer 'n Th1 (tipe 1) immuun respons, terwyl 'n Th2 immuun respons benodig word vir die eliminasie van intestinale parasiete. Die interaksie tussen die twee immuun response was in die huidige studie populasie bestudeer. Dit word gepostuleer dat persone met 'n sterk tipe 2 immuun respons, gemanifesteer deur hoë serum IgE vlakke, vatbaar is vir infeksie met *M. tuberculosis* en progressie tot siekte. Verder was dit voorgestel dat armoede-verwante faktore en intestinal parasiete, veral parasiete wat deur die longe beweeg, 'n dominante tipe 2 respons verder kan versterk.

Die resultate voorgestel, wys daarop dat serum IgE konsentrasies, 'n surrogaat merker vir tipe 2 aktivering, hoog was in gesonde volwassenenes. Dit het die siening van 'n dominante tipe 2 respons bevestig. IgE vlakke was bereken vir elke sensus blok of enumerator sub-distrik (ESD) en het gekorreleer met die tuberkulose annmeldings koers per ESD. Die aanmeldings koers het ook gekorreleer met die sosio-ekonomiese status, vroulike geletterdheid en populasie digtheid. Alhoewel hierdie korrelasies nie noodwending dui op 'n oorsaak en gevolg verhouding nie, is dit duidelik dat hierdie faktore kan bydra tot vatbaarheid vir *M. tuberculosis*. Dit was ook getoon dat IgE konsentrasies beduidend afneem na suksesvolle behandeling van tuberkulose. Dit wys daarop dat IgE konsentrasies in mense afgeruleer kan an waarskynlik dui op 'n verhoogde tipe 1 respons. Verder, as 'n rede vir die hoë IgE konsentrasies in die studie

populasie, is die helmint ladings gevolglik in alle primere skool kinders in die studie populasie, gemeet. Die resultate dui daarop dat meer as 50 % van die kinders ingesluit, geïnfekteer was met *A. lumbricoides* en/of *T. trichiura*. Skole in areas met die hoogste armoede syfer en tuberkulose annmeldings koers, het ook die hoogste prevalensie van helminte gehad. Alle kinders, ongeag hulle helmint status of hulle deelname in die studie, het gevolglik anti-helmintiese behandeling ontvang.

BCG vaksinasie littekens en Mantoux vel toets response was beskikbaar op 'n sub-populasie van die bogenoemde skool kinders. Alhoewel dit aanvaar word dat die meerderheid van kinders BCG vaksinasie in die neonatale periode ontvang het, het slegs twee derdes van die kinders 'n BCG litteken getoon. Die resulatate dui daarop dat die prevalensie van BCG litteken positiwiteit, onafhanklik van ouderdom, laer was in kinders rondom die ouderdom van 11 jaar. In kontras met die konstante uitdrukking van BCG littekens, het die persentasie van Mantoux reaktiwiteit verhoog met ouderdom vanaf 13 % by 6 jaar tot 65 % teen 15 jarige ouderdom. Die tyd koers van Mantoux omskakeling met ouderdom dui daarop dat tuberkulin sensitiwiteit, geïnduseer deur BCG, afneem binne die eerste paar jaar van lewe en dat PPD responsiwiteit daarna deinduseer word deur omgewings blootstelling aan *M. tuberculosis*. In kontras met die Th1/Th2 paradigma, was die prevalensie van helmint infeksies in kinders met 'n BCG litteken marginaal laer teenoor hulle sonder 'n litteken. 'n Relatiewe swak posititiewe korrelasie was gevind tussen tuberkulin responsiwiteit en helmint infeksie. Hierdie korrelasie was meer beduidend in kinders sonder 'n litteken. In hierdie sub-groep, was die helmint-geïnfekteerde kinders meer geneig om PPD responsief te wees teenoor hulle wat nie geïnfekteer was nie. Die data wys daarop dat omskakeling na PPD sensiwiteit kan lei tot infeksie met helminte. Die resultate stel voor dat die effek van helmint infeksie op die ontwikkeling van kliniese tuberkulose van so 'n aard is dat diegene met groot wurm ladings en wat goeie PPD response toon, meer geneig sal wees om weerstandig te wees. Diegene egter wat die parasiete beter kan beheer and wat goeie PPD response toon, sal meer geneig wees om vatbaar te wees vir tuberkulose. Die data dui ook daarop dat die BCG vaksien wat in die studie gebruik was, nie lei tot latente infeksie nie, terwyl patogene *M. tuberculosis* dit wel doen en herhaardelik die immuun respons sal stimuleer.

In 'n aparte studie, was dit gedemonstreer dat die gasheer-respons teen *M. tuberculosis* in pasiënte wat die gevaar loop om na suksesvolle voltooiing van behandeling, weer tuberkulose te ontwikkel, verskil van diegene wat beskermende immuniteit het. Onderhoude was ook gevoer met indiwidue wat deelgeneem het aan die studie, om ten einde hul sosiale en ekonomiese agtergrond te verstaan en hoe dit gekoppel is aan die siekte. Purified protein derivative (PPD) en *M. tuberculosis*-geinduseerde sitokien response was in die studie groepe bepaal. Die resultate wys daarop dat alhoewel 'n enkele immunologiese merker nie geidentifiseer kon word nie, was immunologiese patrone vir vatbaarheid wel opgemerk. Indiwidue wat reeds een episode van tuberkulose gehad het en suksesvolle behandeling ontvang het, het 'n onderdrukte immuun profiel getoon. Dit het ingesluit hoë vlakke van die sitokiene, IL-10 en TGF-β asook hoë vlakke van serum IgE. Hierdie tipe profiel stel voor dat ten spyte van die vorige tuberkulose episode, hierdie persone nie beskermende immunitiet ontwikkel nie en dus vatbaar is vir her-infeksie en progressie tot siekte. Die onderhoude het getoon dat die meerderheid van mense in die studie populasie onder armoedige oorbevolkte omstandighede lewe, wat werkloosheid en ondervoeding insluit. Die studie het verder getoon dat hierdie indiwidue met die onderdrukte immuun profiel en wat in armoede lewe, in die nabye toekoms vatbaar is vir 'n tweede episode van tuberkulose.

In die studie gemeenskap, met 'n tipiese derde wêreld opset, is daar gewys dat armoede-verwante faktore en helminte, mag bydra tot 'n dominante tipe 2 immuun respons wat op sy beurt, die beskermende tipe 1 response sal af-reguleer. Dit sal uiteindelik lei tot verhoogde vatbaarheid vir *M. tuberculosis* en uiteindelik progressie tot siekte (tuberkulose).

ABBREVIATIONS USED IN THIS TEXT

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ARTI	Annual risk of tuberculosis infection
BCG	Bacille Calmette-Guerin
CCR	Chemokine
CD	Cluster determinant
CFP	Culture filtrate proteins
CMI	Cell mediated immunity
CR	Complement receptor
CTL	Cytotoxic T-lymphocytes
DC	Dendritic cells
DHEA	Dehydroepiandrosterone
DNAse I HS	DNAse I hypersensitivity site
DTH	Delayed type hypersensitivity
epg	eggs per gram
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target to 6 kDa
ESD	Enumerator sub-district
FACS	Fluorescence activated cell scanning
GIS	Geographical information system
Hb	Hemoglobin
HDM	House dust mite
HIV	Human immunodeficiency virus
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAM	Lipoarabinomannan
Ln	Natural logarithm

LPS	Lipopolysaccharide
MBP	Myelin basic protein
MDR	Multi-drug resistant
MHC	Major histocompatability complex
MR	Mannose receptors
NFAT	Nuclear factor of activated cells
NK	Natural killer
NIP45	NFAT interacting protein 45
Nramp	Natural resistance associated macrophage protein
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
pDNA	plasmid DNA
Pl	Platelets
PFA	Paraformaldehyde
PHA	Phytoheamagglutinin
PPR	Pattern recognition receptor
PPD	Purified protein derivative
RBC	Red blood cells
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SP-A	Surfactant protein A
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCCR	T-cell cytokine receptor
TB	Tuberculosis
TCR	T-cell receptor
TGF-β1	Transforming growth factor β1
Th	T helper
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptor
Tr	Regulatory T-cell

TST	Tuberculin skin test
WBA	Whole blood assay
WBC	Whole blood count
WHO	World health organization

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CHAPTER ONE
INTRODUCTION

Host resistance against infectious agents comprises two major defence systems, namely the innate and adaptive responses. Interactions between these two branches of host defence are regulated by cells of the immune response and soluble factors, including cytokines. Generally speaking, the immune response to a pathogen can develop toward a cellular type or toward a more allergic type of response. When well developed, these gear the immune response for the effective elimination of different types of pathogens, with a type 1 response being more effective against intracellular pathogens, such as *Mycobacterium tuberculosis* (*M. tuberculosis*). Type 2 responses, in contrast, favour the elimination of parasites such as helminths. Immune responses are not only induced by infectious agents, but also by vaccines. In this thesis, the influence and interaction of *M. tuberculosis*, helminth infections and vaccination of the human immune response, were investigated and will be introduced in the following pages.

1.1 Cells of the immune system

Immune responses are mediated by leukocytes and by the soluble molecules which they secrete. One important group of leukocytes and the phagocytic cells, such as the monocytes, macrophages and polymorphonuclear neutrophils. These cells bind to microorganisms, internalise them and then kill them. Another important set of leukocytes is the lymphocytes. Lymphocytes consist of three major populations: T-cells, B-cells and Natural killer (NK) cells. T-cells phenotypically defined by their cell surface expression of a T-cell receptor (TCR) that 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 immunoglobulin (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 transmembrane cell surface TCR or Ig and by the presence of the cell surface molecules, CD16 and CD56.

1.1.1 $CD4^+$ and $CD8^+$ T-lymphocytes

Most T-cells, express a T cell receptor (TCR) composed of an α and β chain. These cells co-express either the CD4 or CD8 accessory molecule (αβ T-cells). $CD4^+$ T-lymphocytes are capable of recognising small peptides (about 15 amino acids) that are presented by the MHC II surface protein of antigen presenting cells (APCs). APCs could be monocytes, dendritic cells or activated B-cells. Naïve $CD4^+$ T-cells can be subdivided into two groups, namely $CD4^+$ T-helper 1 (Th1) and –2 (Th2) cells (Figure 1.1). The functional roles of Th1 and Th2 cells can largely be defined based on the cytokines they produce and would mediate cell-mediated and humoral immunity (Abbas *et al.,* 1996). $CD8^+$ T-lymphocytes recognise peptides containing about 9 amino acids that are presented on the MHC I molecule which is found on the surface of most nucleated cells. $CD8^+$ T-cells form the largest part of cytotoxic lymphocytes (CTL). Effector function of $CD4^+$ and $CD8^+$ T-lymphocytes would involve neutrophils, macrophages, basophils and eosinophils (Kopf *et al.*, 1995; reviewed by Chtanova and Mackay, 2000).

1.1.2 NK1 T-lymphocytes

These T-cells can secrete large amounts of cytokines upon primary stimulation. Most NK1 T-cells are $CD4^+$ or $CD4^-CD8^-$ cells and express mostly an invariant TCR (Lantz and Bendelac, 1994). This T-cell subset is likely to play an immunu-regulatory role as they are capable to secreting IL-4 within minutes of primary activation (Yoshimoto *et al.*, 1995). Thus, NK T-cells may regulate the Th1/Th2 balance, but their precise role remains unclear.

1.1.3 γδ T-lymphocytes

With the alternative γδ-receptor, γδ-T-lymphocytes constitute the third population of T-lymphocytes. In peripheral blood their presence is limited to less than 10 % of all T-cells

(Kaufmann, 1996). Present on some epithelia, they form part of the first barrier for the entrance of pathogens. These cells are large granular lymphocytes that can develop a dendritic morphology in lymphoid tissues. In general γδ T-cells are MHC-restricted and they function largely as cytotoxic T-cells (Abbas *et al.*, 1996).

1.1.4 B-lymphocytes

Each B cell is genetically programmed to encode a surface receptor specific for a particular antigen. Having recognised its specific antigen, the B-cells multiply and differentiate into plasma cells, which produce large amounts of antibodies. The hallmark of B-cells is their production of immunoglobulins (Ig) (Abbas *et al.*, 1996).

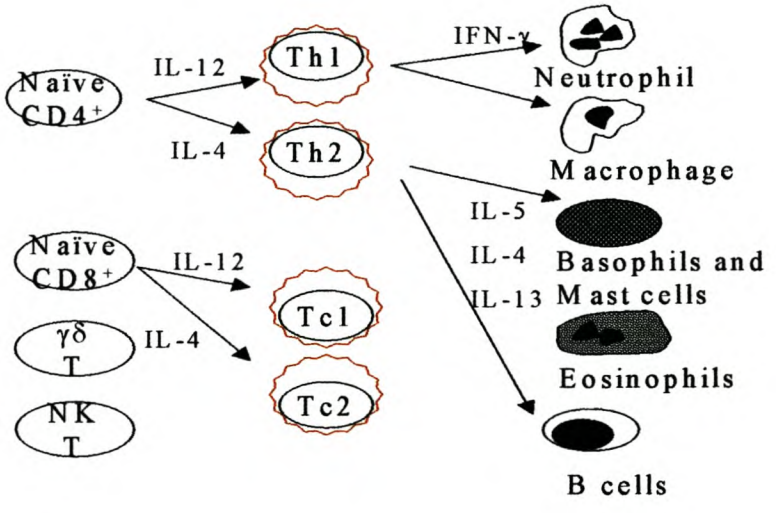


Figure 1.1 Effector T-cell subsets and their cytokine production profiles. (Adapted from Chtanova and Mackay, 2000)

1.2 Th1 and Th2 cell biology

1.2.1 Definition

In 1986, Mosmann and co-workers first suggested that mouse $CD4^+$ T-helper cells could be classified into distinct populations based on their cytokine secretion patterns. Cytokines are low molecular weight proteins usually in the order of 15-25 kDa mass range (Nicod, 1993). The two major populations were termed T-helper 1 (Th1) and T-helper 2 cells (Th2) (Figure 1.2). The basis for this selective action resides in the cytokines made by the $CD4^+$ T-cells. Th1 cells produce interleukin-2 (IL-2), interferon γ (IFN-γ) and lymphotoxin preferentially, whereas Th2 cells are prone to produce IL-4, IL-5, IL-6, IL-10 and IL-13. In turn, IL-2 and IFN-γ promote the development of strong cell-mediated immunity, whereas the Th2-cytokines promote allergic responses. However, it has been suggested 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 (*i.e.* Th1/Th2). This was 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). In this context, Th1 or Th2 would be referring to the type of helper cell involved, whereas type 1 and type 2 would be referring to the immune response induced. T-cells producing both IFN-γ and IL-4 are known as Th0 cells. The Th0 cells are lymphocytes that did not polarize during maturation and thus took on attributes of both Th1 and Th2 cells (Firestein *et al.,* 1989). A type 1 immune response is responsible for the effective elimination of intracellular pathogens such as *M. tuberculosis* and *Listeria monocytogenes*, whereas a type 2 immune response favours the elimination of parasites such as helminths (Figure 1.2).

Functionally, Th1 cells participate in cell-mediated responses, including delayed Type hypersensitivity (DTH) and provide moderate help for B-cells. IFN-γ stimulates phagocytosis and also upregulates expression of class I and class II major histocompatibility complex (MHC) molecules (Volk *et al.,* 1986; reviewed by Spellberg and Edwards, 2001), thereby stimulating antigen presentation to T-cells. Th2 cells, on the other hand, stimulate high titres of antibody production (Lundgren *et al.*, 1989; Punnonen and de Vries, 1994). In particular, IL-4 and IL-10 and IL-13 activate B cell proliferation,

antibody production and class switching. In fact, class-switching from IgG to IgE cannot occur without the presence of IL-4 and IL-13 (Kuhn *et al.*, 1991; Snapper *et al.*, 1991), making the production of IgE a perfect bio-assay for the presence of Th2 cells *in vivo*. IL-4, IL-5, IL-9 and IL-13 have all been strongly implicated in allergic and atopic reactions. In addition to their stimulatory effects, Th1 and Th2 cells cross-regulate one another. IFN-γ, produced by Th1 cells, directly suppresses IL-4 secretion and thus inhibits differentiation of naïve Th0 cells into Th2 cells (Li *et al.*, 1999). Conversely, IL-4 and IL-10 inhibit the secretion of IL-12 and IFN-γ, blocking the ability of Th0 cells to polarise into Th1 cells (D'Andrea *et al.*, 1993).

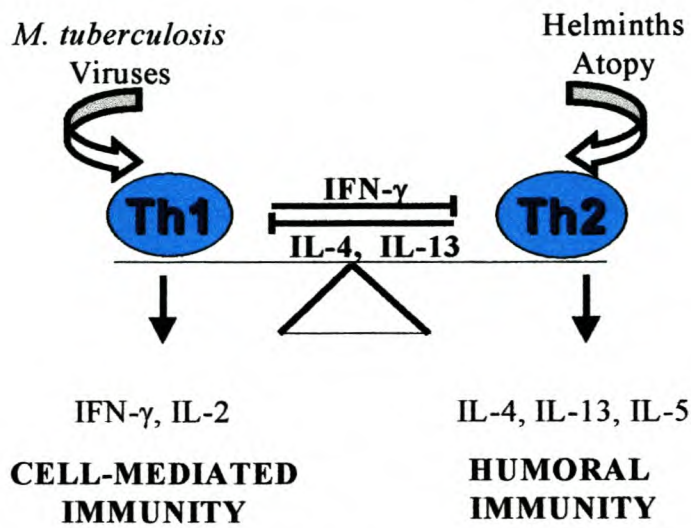


Figure 1.2 The two distinct $CD4^+$ T-helper subsets, Th1- and Th2 cells. Th1 cells produce IFN-γ and IL-2, while Th2 cells mainly produce IL-4, IL-5 and IL-13

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. Additional populations of $CD4^+$ T-helper lymphocytes called Th3 and T-regulatory 1 (Tr1) cells have been described. The Th3 subset, produces high levels of transforming growth factor β (TGF-β) with varying amounts of IL-4 and IL-10 (Chen *et al.*, 1994) and has an immunosuppressive phenotype

in experimental models of oral tolerance and auto-immunity (Miller *et al.*, 1992). Tr1 cells, which appear to be similar to Th3 cells, secrete unusually high levels of IL-10 and lower levels of TGF-β and they have also been implicated in the general suppression of immunity (Groux *et al.*, 1997; Buer *et al.*, 1998).

Table 1.1 Different cytokines and their functions (Abbas *et al.*, 1996; O'Garra, 1998)

Cytokine	Source	Function
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	Tumour cytotoxicity Cachexia
TNF-β	CD4 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	Immuno-suppression

1.2.2 Factors influencing T-helper commitment

Many factors influence the maturation of an early stage T-cell. The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 and (STAT4) and 6 (STAT6) respectively, are key determinants of the outcome (Murphy and Reiner, 2002). It has also been proposed that antigen, dose, co-stimulators, genetic modifiers and other non-cytokine factors have crucial roles in determining the dominance of a Th-cell response.

1.2.2.1 Commitment to a Th1 phenotype

1.2.2.1.1 Pattern recognition receptor signalling

Th1 cells control resistance to many pathogens such as bacteria, protozoa, fungi and viruses. However, prior to the induction of specific acquired responses, these pathogens first activate the innate immune system (Abbas *et al.*, 1996). In contrast to the clonotypic receptors characteristic of B- and T-lymphocytes, APCs express non-clonal sets of recognition molecules called pattern recognition receptors (PPRs), which bind conserved molecular structures shared by large groups of pathogens (Murphy and Reiner, 2002). An important PPR family includes Toll-like receptors (TLRs), homologous to the Toll molecule in *Drosophila* (Hemmi *et al.*, 2000). Characterised members of the TLR family display a certain degree of specificity for pathogen ligands: TLR2 binds to components of Gram-positive bacteria and mycobacteria; TLR4 is a part of lipopolysaccharide (LPS) receptor and is essential for the recognition of both Gram negative bacteria and respiratory syncytial virus; TLR5 recognises bacterial flagellin (from Gram positive and – negative bacteria) and TLR9 is a receptor for unmethylated DNA (Fickenscher *et al.*, 2002). They all use similar intracellular signalling pathways resulting in the expression of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-6.

1.2.2.1.2 Role of dendritic cells

Immature DCs are bone-marrow derived cells present in most non-lymphoid tissues, where they exhibit a potent capability to capture and process antigen. Inflammatory mediators or microbial agents promote the migration of DCs into secondary lymphoid organs (Abbas *et al.*, 1996). As they migrate, DCs mature, losing their Ag-capture ability

and gaining an increased capacity to prime other cells (Moser and Murphy, 2000). This migratory capacity makes the DC the most relevant APC, clearly distinguishing it from tissue-resident macrophages. In addition to pathogen-derived peptide complexed with MHC class II molecules, DCs carry signals that bias the choice of Th effector cell appropriate to the specific infectious agent they have encountered in the tissue (Gagliardi *et al.*, 2000). Based on *in vitro* studies with human blood DCs, the DC1 and DC2 dichotomy was proposed to describe the capacity of monocyte-derived myeloid DCs to stimulate allogeneic T-cells to secrete IFN-γ and plasmacytoid DCs to prime T-cells to provide IL-4 (Sallusto *et al.*, 1999; Rissoan *et al.*, 1999). However, subsequent reports have shown that the induction of a distinct Th-phenotype is not dependent on a particular DC lineage but instead, depends on the state of activation and maturation of the DC (Moser and Murphy, 2000). Studies with the fungus *Candida albicans* support the hypothesis that the Th-cell response depends on the nature of the pathogen and not the type of DC; yeast-pulsed DCs stimulate a protective Th1 response, whereas hyphae-pulsed DCs induce a non-protective Th2 response (d'Ostiani *et al.*, 2000).

1.2.2.1.3 Cytokine-induced Th1-cell development

IL-12

The Th1-promoting ability of DCs correlate with their ability to produce IL-12. This heterodimeric cytokine, composed of p40 and p35 subunits, is a crucial mediator between innate and adaptive immunity (Dorman and Holland, 2000). Mice deficient in IL-12, IL-12 receptor B1 or the IL-12-specific transcription factor, STAT4, displayed greatly impaired secretion of IFN-γ when infected with such pathogens (reviewed by Murphy and Reiner, 2002). Cells of the innate immune system can produce a positive feedback mechanism that increases the secretion of IL-12 by IL-12. IL-12 directly stimulates NK cells to produce IFN-γ, which augments the synthesis of IL-12 by DCs. As IL-12 plays such as selective role in driving Th1 responses, the priming of DCs for the secretion of IL-12 is likely to be determined exclusively at the time of exogenous microbial stimulation; subsequent interactions of DCs with primed T-lymphocytes in lymphoid organs, although known to augment the production of IL-12 p70 by DCs are not necessarily Th1-specific (Dorman and Holland, 2000). Activated macrophages, as well as

DCs, can secrete IL-12 together with other pro-inflammatory cytokines. Their ability to migrate to the lymph nodes and spleen makes tissue-resident macrophages unsuitable APCs for priming $CD4^+$ lymphocytes, but they play a significant role in providing the micro-environment required for optimal induction of Th1 effectors at the site of infection. Although IL-12 plays a dominant role in the commitment to a Th1 phenotype, residual Th1 responses can develop in its absence *in vivo*. Patients who lack a functional IL-12R are susceptible to mycobacterial and *Salmonella* infections (De Jong *et al.*, 1998). Nevertheless, T-cells from these patients still secrete low levels of IFN-γ. Such observations indicate that additional factors might be implicated in Th1-development, but that IL-12 is required for the optimal secretion of IFN-γ by Th1-cells. This notion is supported by the observation that the expression of functional IL-12R is maintained throughout the lifetime of Th1 effectors (reviewed by Dorman and Holland, 2000).

IL-18

IL-18 augments the production of IFN-γ by differentiated Th1 cells, despite its inability to drive the development of Th1 on its own (Robinson *et al.*, 1997). The combination of IL-18 and IL-12 can induce the production of IFN-γ by differentiated Th1 cells in the absence of signalling through the TCR (Robinson *et al.*, 1997; Yang *et al.*, 1999). The IFN-γ production is more prolonged than that induced by TCR cross linking (Yang *et al.*, 1999), which indicates that cytokine-driven IFN-γ production is not coupled to the NFAT pathway. *In vivo* it seems that IL-12 and IL-18 act synergistically to induce maximum IFN-γ production (Takeda *et al.*, 1998; Neighbors *et al.*, 2001). Although the administration of exogenous IL-18 augments protective immunity against intracellular pathogens, the role of endogenous IL-18 appears to be subordinate to endogenous IL-12. Moreover, in the absence of IL-12, IL-18 promotes Th2-cell development.

IFN- γ

Specific changes occur at the IFN-γ locus in the course of Th1 commitment. DNAse I hypersensitivity site (DNAse I HS) have been observed in the first and third introns of the IFN-γ gene (Rengarajan *et al.*, 2000). In addition, the locus is hypomethylated in Th1, but

not Th2 cells (Young *et al.*, 1994). The transcription factors IRF-1 and Stat4 have been implicated in Th1 differentiation because mice deficient for each of these factors lack Th1 cells (Wurster *et al.*, 2000). However, the exact roles that these factors play in Th1 cytokine generation is not clear. Thus, although these proteins are clearly important in the transcriptional regulation of the IFN-γ gene, none has shed light on the Th1-restricted expression of IFN-γ.

A substantial advance in elucidating Th1 lineage commitment and IFN-γ expression has recently been provided with the isolation of a novel protein, T-bet (T-box expressed in T-cells). T-bet is a member of the T-box family of transcription factors that regulate several developmental processes and seems to be expressed in developing and committed Th1 cells (Rengarajan *et al.*, 2000). T-bet expression strongly correlates with IFN-γ expression; it is specifically upregulated in primary Th cells differentiated along the Th1 but not the Th2 pathway (Szabo *et al.*, 2000). Szabo and co-workers (2000) have also shown that transduction of T-bet into fully polarised Th2 cells converts them into IFN-γ secreting Th1 cells and simultaneously represses the Th2 cytokines IL-4 and IL-5. T-bet thus appears to function by initiating a Th1 differentiation program while repressing Th2 differentiation.

In addition, T-bet seems to be involved in remodelling of the chromatin structure of the gene that encodes IFN-γ (Mullen *et al.*, 2001) as well as in the induction of expression of the IL-12Rβ2 (Mullen *et al.*, 2001; Afkarian *et al.*, 2002) either through an autolytic loop or the autocrine effects of IFN-γ signalling (Mullen *et al.*, 2001; Afkarian *et al.*, 2002). Both $CD4^+$ and $CD8^+$ T-cells, as well as NK cells express T-bet, but appears that there is less dependence on T-bet for high level expression of IFN-γ in $CD8^+$ T-cells than in $CD4^+$ T-cells, or NK cells (Szabo *et al.*, 2002). Rengarajan and co-workers (2000) proposed a model for Th1/Th2 polarization that involves a balance between the Th1-specific T-bet and the Th2-specific GATA3. GATA3 is a zinc-finger transcription factor that seems to be crucial for inducing transcriptional competence of the Th2 cytokine cluster, which includes the genes encoding IL-13, IL-4 and IL-5 (reviewed by Murphy and Reiner, 2002). The naïve Th-cell would receive signals through the TCR and co-

stimulatory molecules. If the conditions at the time of activation favour the initiation of Th1 differentiation, T-bet is induced and promotes Th1 lineage commitment. However, if GATA3 is induced via Stat6 activation, the Th cell is driven down a Th2 pathway followed by up-regulation of c-Maf and subsequent Th2 polarisation (Ho *et al.*, 1998). Factors like NFAT are present in both subsets and inducibly bind to specific sites (Ranger *et al.,* 1998). This mutually exclusive expression pattern of T-bet and GATA3 during Th1 and Th2 differentiation respectively, lends support to this model. Furthermore, the two transcription factors may antagonise the development of the opposite subset by directly regulating each other's expression as well as by remodelling cytokine loci. Thus, the relative abundance of T-bet and GATA3 may determine the Th1/Th2 fate of a Th0 cell (Rengarajan *et al.*, 2000).

IL-23

IL-23, a recently discovered cytokine, is composed of the p40 sub-unit of IL-12 paired with a unique chain, p10 (IL-23α), that is distantly related to IL-12 p35 (Oppmann *et al.*, 2000). IL-23 binds IL-12Rβ1 and not IL-12Rβ2; as it interacts instead with a unique receptor sub-unit, IL-23R (Parham *et al.*, 2002). It s reported that IL-23 activates STAT4 (Oppmann *et al.*, 2000) and it might act during the induction of Th1-cell development in co-operation with IL-18 (Seder *et al.*, 1992). However, a role for IL-23 in initiating early Th1-cell development has not been established yet. In addition, Il-23 might also enhance the stimulatory capacity of DCs (Belladonna *et al.*, 2002).

IL-27

Another heterodimeric cytokine, IL-27, was recently reported by Pflanz and co-workers (2002). This cytokine is composed of EB13 (Epstein-Barr virus-induced gene) (Devergne *et al.*, 1996) and p28, a subunit related to IL-12 p35. IL-27 is produced by APCs, inducing the proliferation of naïve T-cells selectively by acting with IL-12 to promote IFN-γ production and its ligand for TCCR, a receptor on T-cells that is involved in early Th1-cell development (Chen *et al.*, 2000; reviewed by Murphy and Reiner, 2002). The exact mechanism by which IL-27 and TCCR act in early Th1-cell development are not

clear, but it seems that IL-27 interacts synergistically with IL-12 at an early point in Th1-cell commitment (Robinson *et al.*, 2002).

1.2.2.1.4 Chemokines

Chemokines are also known to promote Th1 responses. The microbial induction of CC-chemokine receptor-5 (CCR5) – binding chemokines might trigger the secretion of IL-12 by DCs (Lalani *et al.*, 2000). In addition, DCs can promote the Th1 response through secretion of B-chemokines, which bind CCR5 on $CD4^+$ lymphocytes. IL-23, a complex of p19 and IL-12 p40, binds to the IL-12Rβ1 chain and an additional unidentified receptor sub-unit (Rengarajan *et al.,* 2000). A possible candidate is a recently described T-cell cytokine receptor (TCCR) homologous to IL-12Rβ2; mice deficient in this receptor display impaired Th1 responses and an increased susceptibility to infection with *Listeria monocytogenes*, despite apparently unaffected endogenous production of IL-12.

1.2.2.1.5 Transcriptional factors

The genes encoding cytokines are in an inactive form (Avice *et al.*, 2000) in resting $CD4^+$ T-cells. During the differentiation of Th1 cells, specific remodelling of chromatin occurs at the locus encoding IFN-γ. Hypomethylation and DNAse I-hypersensitive sites (DNAse I HS) in the first and third introns of the gene have been observed in Th1 but not Th2 cells (Young *et al.*, 1994; Agarwal and Rao, 1998). The respective contributions of TCR and cytokine-receptor signalling to the induction of expression of the transcriptional factors required for the activation of the gene encoding IFN-γ are poorly understood. The translocation of nuclear factor of activated cells (NFAT), a positive regulator of the expression of IFN-γ, is TCR-specific but not restricted to the Th1 lineage (Peng *et al.*, 2001). It is probable that TCR triggering promotes the up-regulated expression of cytokine and/or chemokine receptors and the subsequent receptor-ligand interactions then result in the induction of more specific transcription factors. Although signalling through IL-12R activates STAT1, STAT3 and STAT4, only STAT4 has been implicated in Th1-cell development, and even its precise role in the activation of the gene encoding IFN-γ is unclear at present (Ma and Trinchieri, 2001). Some of the transcription factors associated with the development of Th2 cells act as negative regulatory elements for Th1 cytokines

and vice versa. IL-12-independent pathways of Th1-cell commitment are supported by evidence that STAT4/STAT6$^{-/-}$ animals develop equivalent numbers of IFN-γ secreting T-cells as wild-type mice and mount an *in vivo* Th1 cell-mediated delayed type hypersensitivity response (Ma and Trinchieri, 2001). Conversely, the transcriptional factor T-bet, which can induce transactivation of an IFN-γ reporter gene and is specifically expressed in Th1 cells generated in the presence of IL-12, suppress the expression of genes encoding IL-4 and IL-5 and induces the synthesis of IFN-γ when ectopically expressed in Th2 cells (Afkarian *et al.,* 2002). Evidence for the cross-regulation of expression of IL-4 and IFN-γ by transcriptional factors provides a molecular basis for Th1-Th2 cross-regulation, in which cytokines that augment the development of cells of one subset suppress the other. This mutual antagonism provides an economic process to polarise the immune response towards an effector type most suitable for the resolution of each pathogenic challenge.

1.2.2.1.6 Transcription of the gene encoding IFN-γ

IL-12 and IL-18-induced IFN-γ production correlates with induction of expression of the GADD-family proteins, GADD45β and GADD45γ (reviewed by Murphy and Reiner, 2002). Yang and co-workers (2001) have shown that over-expression of GADD45β augments IFN-γ production and the absence of GADD45γ decreases IFN-γ production, thereby inhibiting Th1-cell development. Furthermore, Afkarian and colleagues (2002) have demonstrated that IL-12 and IL-10 induced IFN-γ production strongly depends on STAT4, whereas TCR signalling can induce IFN-γ production, although at a reduced level, in *STAT*$^{-/-}$ Th1 cells.

1.2.2.2 Commitment to a Th2 phenotype

1.2.2.2.1 The default Th2 response

T-helper events leading to the differentiation of Th2 cytokine-producing CD4^{+} cells during disease might not require the activation of DCs by microbial adjuvants, as characterises the Th1 response. The injection of soluble Ag without adjuvant is sufficient to trigger a Th2 response, but not a Th1 response (reviewed by Spellberg and Edwards,

2000). Indeed, sensitisation to ovalbumin without adjuvant followed by local lung challenge triggers a strong B7-1 and/or B7-2 dependent Th2 response (Lenschow *et al.*, 1995; Kuchroo *et al.*, 1995). Previous studies have shown that IL-4, produced by a non-T-cell or an unconventional T-cell, provided the initial signal for the differentiation of Th2 cells (reviewed by Spellberg and Edwards, 2000). In this model, ligation of the IL-4R expressed by $CD4^+$ T-cells triggered the activation of STAT6, driving the differentiation of naïve T-cells to Th2 cells. IL-4 is perhaps unique among the Th2 cytokines in that it can stimulate the development of Th2 cells from naïve T-cells. However, although the activation of STAT6 is required for the amplification and stabilisation of Th2 cells, more recent in vitro and in vivo findings indicate that conventional $CD4^+$ T-cells can initially differentiate to produce IL-4 and other Th2 cytokines in the absence of IL-4R and STAT6 signalling. There is some evidence that microbial structures associated with certain parasites, including *Schistosoma mansoni*, *Acanthoceilonema vitae* and *Nippostrongylus brasiliensis*, might promote the Th2 response, but it is also increasingly clear that in other cases, such microbial adjuvants are not required for the Th2 response to develop (Erb *et al.,* 2002). Taken together, these studies suggest that, the Th2 response might develop through a default pathway, when microbial constituents are not available to stimulate DCs to produce IL-12 and other Th1-inducing cytokines.

1.2.2.2.2 Cytokine-induced Th2-cell development

IL-4

IL-4 was recognised early on to promote the development of the Th2-cell subset (Swain *et al.*, 1990; Le Gros *et al.*, 1990; Seder *et al.*, 1992). It was shown that 87 bp of sequence upstream of the transcription initiation site was sufficient for TCR-inducible and Th2-specific expression, and sites (P0-P4), located within the 5' flanking region were shown to be critical for inducible expression of IL-4 (Szabo *et al.,* 1997). Proteins of the nuclear factor of activated T-cells (NFAT) family bind specifically to these sites and co-operate with activator protein (AP-1) factors like Fra and Jun at sites P1 and P4 to induce IL-4 transcription (Rengarajan *et al.,* 2000). Studies have shown that mice lacking one of the three known NFAT familiy members, NFATc1 (NFATc) have impaired IL-4 production,

whereas mice lacking NFATc2 and NFATc3, (NFATp and NFAT4) displayed extreme overproduction of IL-4 and other Th2 cytokines, demonstrating a negative regulatory role for NFAT in IL-4 production (Ranger *et al.*, 1998a; Ranger *et al.*, 1998b). Although NFAT and AP-1 proteins are important for IL-4 transcription, they are present in both Th1 and Th2 subsets and, thus, do not explain the Th2-restricted expression of IL-4. A major advance towards elucidating this came with the confirmation that the proto-oncogene c-Maf (a member of the basic-region leucine-zipper family) is specifically expressed in Th2 but not Th1 cells (Ho *et al.,* 1996). c-Maf promotes skewing towards Th2-cell development when it is expressed in transgenic mice (Ho *et al.*, 1998), and c-Maf-deficient mice have a selective defect in IL-4-production (Kim *et al.,* 1999; reviewed by Murphy and Reiner, 2002). Thus, c-Maf is specific for IL-4 and is critical for high levels of IL-4 production but is not sufficient for the initiation of IL-4 transcription. The search for additional genes that are preferentially expressed in Th2 cells led to the isolation of GATA3. The expression of GATA3 is induced rapidly by IL-4, through STAT6, increasing from a low level in naïve T-cells to a high level in Th2 cells (Ouyang *et al.*, 1998). Whereas the requirement for STAT6 and c-maf in Th2-cell development has been confirmed using gene-targeting, this has not been reported so far for GATA3, because this seems also to be an essential factor for normal thymocyte development and embryonic survival (reviewed by Murphy and Reiner, 2002). In addition, GATA3 has been shown to directly regulate IL-5 and IL-13 expression and thus appears to play a more global role in regulating Th2 cytokines (reviewed by Murphy and Reiner, 2002).

IL-13

Studies have shown that although IL-4 deficient mice showed impaired Th2 responses (Takeda *et al.*, 1998), Th2 responses were not completely abrogated in these mice (Kopf *et al.*, 1993; Brewer *et al.*, 1996). Evidence for this had emerged from comparative studies performed by Barner and co-workers (1998) using IL-4 and IL-4Rα-deficient mice with the same genetic background, which were infected with the nematode *Nippostrongylus brasiliensis*, a strong inducer of primary Th2 responses in mice. Results from their study showed that Th2 cell differentiation was more severely affected in IL-4Rα-deficient mice than in IL-4 deficient mice, the latter still being able to respond to IL-

13. This led to the conclusion that the development of Th2 cells can be regulated by IL-4 or IL-13 (Barner *et al.*, 1998). Consistent with this finding, several studies have shown that STAT6, IL-13 and IL-4/IL-13-deficient mice also showed impaired *in vitro* and *in vivo* Th2-cell development (Takeda *et al.*, 1996; Satoskar *et al.*, 1997; McKenzie *et al.*, 1999). However, the mechanism whereby IL-13 effects Th2 polarization remains unclear, since, *in vitro*, IL-13 cannot directly promote growth and differentiation of naïve $CD4^+$ T-cells (Somasse *et al.*, 1996; reviewed by Brombacher, 2000). Directly, IL-13 has an immunoregulatory interaction with various Th differentiation factors, including IL-4, IL-6, IL-12 or IL-18. Indirectly, IL-13 may also play a role in B-cell switching to the production of IgE antibodies. Emson and co-workers (1998) have shown that IL-13 transgenic mice express elevated levels of IgE even in the complete absence of IL-4. Moreover, in response to *Schistosoma* eggs, the blocking of endogenous IL-13 reduced total serum IgE levels (Chiaramonte *et al.*, 1999; reviewed by Brombacher, 2000).

1.2.2.3 Factors influencing the Th1/Th2 balance

1.2.2.3.1 Dose of antigen

Low antigen concentration and low-dose infections tend to induce Th1 responses, whereas high doses induce Th2 development (reviewed by Constant and Bottomly, 1997). Immunising mice with 10^7 *M. vaccae* induces a Th1 response, whereas 10^9 organisms induce a mixed response (Hernandez-Pando and Rook, 1994). This could be explained by the fact that at low doses of antigen, dendritic cells or macrophages might be serving as APCs. Both dendritic cells and macrophages produce IL-12, tilting the balance of the specific T-cell response towards Th1 differentiation (Abbas *et al.*, 1996). 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 (reviewed by Spellberg and Edwards, 2001). Different concentrations of antigens may also trigger different signals from T-cell antigen receptors, which may influence the pattern of differentiation.

1.2.2.3.2 Antigen presenting cells (APCs)

The type of APC stimulating the T-cell also plays a role in the polarization of the lymphocyte to a Th1 or Th2 phenotype (Kalinski *et al.*, 1997; Liu *et al.*, 1998). Studies have shown that stimulation by B-cells are prone to inducing a type 2 outcome, which is consistent with their need to drive a type 2 response to Ab production (Liano and Abbas, 1987). Macrophages, on the other hand, stimulate a type 1 outcome (Schmitz *et al.*, 1993). Dendritic cells, being the most potent APCs, are capable of driving both type 1 and 2 outcomes (Croft *et al.*, 1992; Masten and Lipscomb, 1999), depending on the local cytokine milieu.

1.2.2.3.3 Co-stimulation

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 (Abbas *et al.*, 1996). The best defined co-stimulators are two structurally related proteins, B7-1 and B7-2 (CD80 and CD86), both of which activate T-cells by interacting with the CD28 receptor on their cell surface (Lenschow *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 (Abbas *et al.*, 1996). 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 (Lenschow *et al.*, 1996). Fully developed Th2 cells respond to Ag 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 (Tao *et al.*, 1997). Once a Th2 cell clone has developed, it will continue to secrete IL-4 and hence progressively proliferate independent of the type of APC and the presence of B-7/CD28 interaction.

1.2.2.3.4 Glucocorticoids

Glucocorticoids are powerful inducers of the type 2 immune response and powerful inhibitors of the type 1 outcome, directly inducing IL-4 and IL-10 production from lymphocytes and APCs (Daynes *et al.*, 1990; Ramirez *et al.*, 1996) while suppressing the secretion and effects of IFN-γ, IL-2 (Boumpas *et al.*, 1991) and IL-12 (Vieira *et al.*, 1998). However, in order to suppress lymphocyte proliferation and type 2 cytokine production, glucocorticoids must be present at least 10^{-7} *M* concentrations (Moynihan *et al.*, 1998).

Araneo and co-workers (1989) 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 activation following treatment with metyrapone, a glucocorticoid inhibitor. Since treatment with metyrapone causes an increase in the production of dehydroepiandrosterone (DHEA), 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 (reviewed by Spellberg and Edwards, 2001).

1.2.2.3.5 Strength of signal

The affinity of the TCR for the MHC/stimulating-antigen complex, the presence of certain co-stimulatory molecules on the APC, and the timing of TCR ligation by the MHC/antigen complex have each been shown to modify type 1 and type 2 outcomes (Lenschow *et al.*, 1996; reviewed by Spellberg and Edwards, 2001). However, there is much confusion in this area, as evidenced by an abundance of conflicting reports on whether high or low affinity, present or absent co-stimulation, and short or long ligation, mediate Th1 or Th2 polarisation (reviewed by Spellberg and Edwards, 2001). This lack of concordance likely reflects study-to-study variation in the complex interactions between these difficult-to-measure variables.

1.2.3 Regulatory T-cells

Current dogma suggests that immunity to infection is controlled by either type 1 or type 2 responses, discriminating on the basis of cytokine secretion and function. However, a further subtype of T-cells, with immunosupressive functions and cytokine profiles distinct from either Th1 or Th2 cells, termed regulatory T-cells (Tr-cells) has recently been described (Groux *et al.*, 1997; Doetze *et al.*, 2000; Cavani *et al.*, 2000). Tr-cells can be subdivided into different sub-populations, namely, type 1 Tr-cells, which secrete high levels of IL-10 and moderate levels of TGF-β and type 3 Tr-cells (Th3) cells (Chen *et al.*, 1994; Fukaura *et al.*, 1996), which primarily secrete TGF-β and $CD4^+CD25^+$ T-cells, which inhibit immune responses through cell-cell contact (Shevach *et al.*, 2000). In addition to the various $CD4^+$ Tr-populations, $CD8^+$ Tr-cells, which secrete either IL-10 or TGF-β (Giliet *et al.*, 2000; Garba *et al.*, 2002) have also been identified. It has been reported that Tr-cells can be induced against bacterial, viral and parasite antigens *in vivo* (McGuirk and Mills, 2002).

1.2.3.1 Phenotype and functions of Tr-cells

1.2.3.1.1 Tr1-cells

Tr1-cells, with a unique cytokine profile distinct from that of Th0, Th1 or Th2 cells, produce IL-10, IL-5 with or without TGF-β, but with little or no IL-2, IL-4 or IFN-γ. (Groux *et al.*, 1997). Functional studies have shown that Tr1-cells have immuno-suppressive properties and have been shown to prevent the development of Th1-mediated autoimmune diseases (Groux *et al.*,1997; reviewed by McGuirk and Mills, 2002). However, the suppressive effects of Tr1-cell clones are reversed by neutralising IL-10 (Groux *et al.*, 1997; McGuirk *et al.*, 2002), suggesting that, regardless of their antigen specificities, Tr1-cell suppression is a bystander effect mediated through the production of IL-10.

1.2.3.1.2 Th3-cells

Another subset of $CD4^+$ Tr-cells, namely Th3-cells, was identified in studies of oral tolerance (reviewed by McGuirk and Mills, 2002). The *in vivo* significance of Th3-cells and a role for TGF-β in their induction, was suggested by the recent finding that certain

tumour cell lines actively produce TGF-β (Seo *et al.*, 2001). This induction of TGF-β by regulatory Th3 cells, might represent a novel strategy engaged by tumours to suppress the induction of protective tumour-specific cytotoxic T-lymphocyte (CTL) responses (Seo *et al.*, 2001). Furthermore, because TGF-β is expressed and acts on multiple cell types, TGF-β-secreting Th3-cells might play a role in immune regulation and T-cell homeostasis (Gorelik *et al.*, 2002).

1.2.3.1.3 $CD4^+CD25^+$ Tr-cells

Another regulatory T-cell population, $CD4^+CD25^+$ T-cells, comprise approximately 5-10 % of the peripheral T-cell pool and exhibit potent immuno-suppressive abilities both *in vivo* and *in vitro* (Shevach *et al.*, 2000, reviewed by McGuirk and Mills, 2002). The regulatory effect of this population was thought to be mediated by cytokine production, but appeared to be more related to their ability to inhibit IL-2 production, by a mechanism dependent on cell-cell contact and expression of the inhibitory co-stimulatory molecule CTLA-4 (Thornton *et al.*, 2000; Read *et al.*, 2000).

1.2.5 Th1 and Th1 type responses and association with disease

As previously mentioned, a Type 1 immune response is needed to effectively cope with *M. tuberculosis* infection whereas a Type 2 response is associated with atopic disorders and the host's response against parasite infection. In the light of this, the three diseases and the influence of environmental factors on susceptibility to these infectious agents, will be discussed in greater detail in the following sections of this dissertation:

1) Tuberculosis (Type 1 response) (Section 1.3)
2) Infection with parasites (Type 2 response) (Section 1.4)
3) Atopy and atopic disorders (Type 2 response) (Section 1.6)

1.3 TUBERCULOSIS

Tuberculosis is one of the common recognised infectious diseases world-wide, contributing to significant morbidity and mortality (Snider *et al.,* 1994). It has been estimated that one third of the global population harbours the organism, although only 5 - 10 % of infected adults develop pulmonary disease in the absence of immunosuppression (Israel *et al.,* 1941; Mohan *et al.*, 2003). A compromised immune system often related to poverty-related factors and HIV/AIDS, are risk factors for developing reactivation disease in later life.

A report by the World Health Organisation predicts that by the year 2005, *M. tuberculosis* will kill 4 million people annually (Kaufmann, 1993). In 1995, the overall TB notification rate in South Africa was 366 new cases per 100 000 population per year with the highest (718/100 000) in the Western Cape (Department of Health, Republic of South Africa, 1996).

1.3.1 Pathogenesis of *M. tuberculosis* infection

Infection by *M. tuberculosis* is associated with a wide range of outcomes: from containment of infection and possible development of protective immunity, to the development of disease as a consequence of rapid initial mycobacterial replication or reactivation of latent infection after a period of mycobacterial dormancy. The ability of *M. tuberculosis* to be associated with a pathogenesis allowing such diverse outcomes is dependent on the following factors, amongst others: a) the ability of *M. tuberculosis* to survive and replicate within host mononuclear phagocytes; b) dormancy, allowing the organism to survive for prolonged periods of time within the tissues of the host; and c) the interaction of the organism with the host which contributes both to pathogenesis and protective immunity.

1.3.1.1 Infection with *M. tuberculosis*

Subsequent to an aerosol inhalation of a few (up to 5) bacilli (Toossi *et al.,* 1998), it is thought that fewer than 10 % of *M. tuberculosis* bacilli will reach the respiratory bronchioles and alveoli. Most of the organisms (90 %) would have settled in the upper

respiratory epithelium. The respiratory bronchial epithelium is remarkably resistant to infection by *M. tuberculosis*. Although direct evidence of anti-mycobacterial action is lacking, the bronchial epithelium can produce anti-microbial peptides with a wide spectrum of activity (Diamond *et al.,* 1991).

According to Dannenberg and co-workers (1994) bacteria arriving in the lung, have four potential fates:

1) By killing all bacilli, the host response can be so effective, ensuring that the infected individual has no chance of developing tuberculosis at any time in the future or;
2) The organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary tuberculosis or;
3) The bacilli may become dormant and never cause disease, such that the infected individual develop latent infection, manifested only by a positive PPD skin test (Mantoux response) or;
4) The latent organisms can eventually reactivate resulting in clinical disease.

1.3.1.2 Interaction between *M. tuberculosis* and macrophages

The initial defence against infection with *M. tuberculosis* is alveolar macrophages. Macrophages are capable of inhibiting growth of the bacillus through phagocytosis and subsequent antigen presentation to recruited T-lymphocytes (reviewed by Schluger and Rom, 1998).

Phagocytosis normally begins with the phagocytic cell engulfing the invading microbe in a membrane-bound tight vacuole (Figure 1.3). Experimental reports have shown that in the *M. tuberculosis*/mononuclear phagocyte interaction, the creation of this vacuole is accompanied by binding of the organism to the phagocyte through complement receptors CR1, CR3 and CR4 as well as mannose receptors (MR) and other cell surface receptor molecules (Schlesinger *et al.,* 1996). These authors have also demonstrated that there may be differences between specific binding mechanisms for virulent and avirulent strains of mycobacteria, as blocking complement receptors with monoclonal antibodies inhibits phagocytosis of *M. tuberculosis* strain H37Ra and the strains, Erdman and

H37Rv, but down regulation of MRs is associated with decreased binding of only the virulent strains (Schlesinger *et al.*, 1998). Furthermore, it was shown that the interaction between MRs on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial glycoprotein lipoarabinomannan (LAM), which is present on the cell wall of the mycobacteria, including virulent strains of *M. tuberculosis* (Sclesinger *et al.*, 1994; reviewed by Schuler and Rom, 1998).

1.3.1.3 Fate of *M. tuberculosis* after phagocytosis

Once *M. tuberculosis* is engulfed into phagosomes, the organism is subjected to killing via a variety of mechanisms, including: a) phagosome-lysosome fusion; b) generation of reactive oxygen intermediates and c) generation of reactive nitrogen intermediates, particularly nitric oxide.

Macrophages have the ability to produce 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). Studies have shown that RNI is more important than ROI in human mycobacterial defence (Walker *et al.*, 1981; Chan *et al.*, 1992).

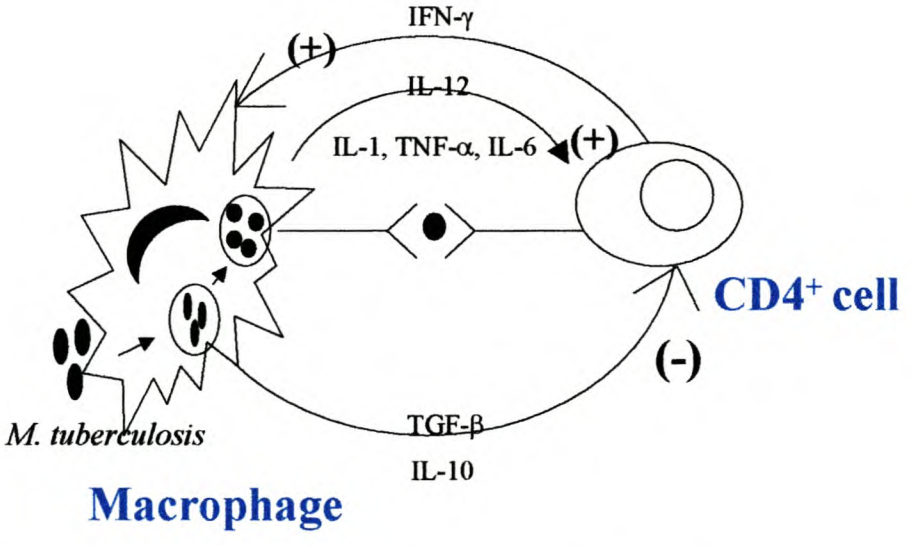


Figure 1.3 Interaction between *M. tuberculosis*, macrophages and $CD4^+$ T-cells

Chan and colleagues (1992) demonstrated that murine macrophages stimulated by either IFN-γ and either LPS or TNF-α are capable of inhibiting growth of *M. tuberculosis*. This inhibition is independent on the macrophage's ability to produce reactive ROI as the inhibition could be attained using the macrophage cell line D9, which is ROI-deficient, supporting the notion that ROI have a small role in defence against mycobacteria. Chan and co-workers (1992) have also shown that that the anti-mycobacterial activity of macrophages correlated with the induction of L-arginine-dependent production of toxic nitrogen species, including NO, NO_2 and HNO_2. Furthermore, Macmicking and co-workers (1997) have shown that in genetically modified mice lacking the ability to produce inducible nitric oxide synthase (iNOS -/- knockout mice), *M. tuberculosis* replicated faster than in wild type animals, suggesting a significant role for nitric oxide in mycobacterial host defence. Nicholson and co-workers (1996) have further examined freshly obtained human alveolar macrophages from patients with tuberculosis and could demonstrate that on average, 65 % of macrophages from every patient studied, reacted with a specific human antibody against iNOS, whereas only 10 % of macrophages from normal subjects stained positively. In addition, BAL samples also contained iNOS mRNA. Tubercle bacilli seemed to be less resistant to killing by oxygen radicals and hydrogen peroxide *in vitro* (Chan *et al.,* 1992) and may also deploy the following mechanisms to evade ROI *in vivo*:

a) mycobacteria can enter macrophages by engaging cell-surface mannose receptors or the complement receptors CR1 and CR3, ligation of which does not trigger the macrophage's oxidative burst,
b) sulfatides secreted by tubercle bacilli can down-regulate ROI,
c) LAM inhibits protein kinase C activity and transcriptional induction by IFN-γ, both of which are important for ROI generation,
d) LAM is itself an efficient scavenger of ROI. ROI combined with RNI significantly enhances mycobacterial killing.

Another possible mechanism involved in macrophage defence, is apoptosis, or programmed cell death. Apoptosis was induced in a dose-dependent manner in BAL cells recovered from patients with tuberculosis, particularly in macrophages from HIV-

infected individuals (Placido *et al.,* 1997). Klinger and colleagues (1997) have shown that apoptosis associated with tuberculosis is mediated through a down-regulation of bcl-2, an inhibitor of programmed cell death. Mooloy and co-workers (1994) have further demonstrated that apoptosis of macrophages results in reduced viability of mycobacteria contained within.

Natural resistance associated macrophage protein (Nramp) is crucial in transporting nitrite from intracellular compartments such as the cytosol to more acidic environments such as the phagolysosome where it can be converted to NO (Blackwell *et al.,* 1996). Defects in Nramp production or function might therefore be expected to lead to defective production of nitric oxide and increased susceptibility to intracellular pathogens such as mycobacteria. Blackwell and co-workers (1994) have further demonstrated that susceptibility can be mapped to defects in the *nramp1* gene (originally called the *bcg* gene). However, the relevance of Nramp to tuberculosis is controversial as Medina and co-workers (1996) have shown that although *nramp1* may indeed control resistance to *M. bovis* in mice, resistance to infection with *M. tuberculosis* may be unrelated to mutations at this locus in mice (Medina *et al.,* 1996a; Medina *et al.,* 1996b).

Nevertheless, through intracellular trafficking and a combination of events which may include alkalinization of the phagosome (Godon *et al.,* 1980), prevention of phagosome-lysosome fusion (Armstrong *et al.,* 1975), escape from the phagosome (McDonough *et al.,* 1993) or evasion of killing by either ROI or RNI, *M. tuberculosis* may survive in the macrophage which may be a crucial step in the establishment of disease or the latent state of infection.

1.3.1.4 Role of T-lymphocytes in host defence against *M. tuberculosis*

T-lymphocytes are vital for the acquisition of protection against tuberculosis. All known T-cell populations – $CD4^+$ $\alpha\beta$ T-cells, $CD8^+$ $\alpha\beta$ T-cells, $\gamma\delta$ T-cells, cytotoxic T-cells – seem to participate in the acquisition of optimum protection. However, $CD4^+$ T-cells are undoubtedly the major effector cells in cell-mediated immunity in tuberculosis (Boom 1996).

1.3.1.4.1 $CD4^+$ T-lymphocytes

Active immune surveillance is required to maintain the quiescence of *M. tuberculosis* foci, and $CD4^+$ T-cells are critical to cell-mediated anti-tuberculosis immunity, presumably because they are the primary source of the protective cytokine IFN-γ. The importance of IFN-γ responses in mycobacterial infections was highlighted by the observation that children with hereditary IFN-γ receptor 1 deficiency, were prone to infection with ubiquitous mycobacteria or dissemination of BCG after vaccination (Newport *et al.,* 1996; Jouanguy *et al.,* 1996; Jouanguy *et al.,* 1997). Furthermore, IFN-γ was successfully used as adjunctive therapy in patients with refractory non-tuberculosis mycobacterial infection (Holland *et al.,* 1994) and has shown promising results in patients with multidrug-resistant pulmonary tuberculosis (Condos *et al.,* 1997). Surcel and co-workers (1994) studied proliferative responses and cytokine production in peripheral blood mononuclear cells taken from patients with tuberculosis and from normal control subjects, in response to stimulation with mycobacterial antigens *in vitro*. They found that patients with tuberculosis had increased proliferation of cells secreting IL-4 but not IFN-γ, in response to stimulation and in comparison with cells from healthy subjects. Sanchez and co-workers (1994) studied 45 patients with pulmonary TB and 16 tuberculin skin test positive control subjects and found that patients had less IFN-γ and more IL-4 production than the control subjects. They concluded that tuberculosis patients had a Th2-type response in their peripheral blood, whereas tuberculin skin test positive patients had a Th1-type response. It is suggested that there might in fact be a compartmentalisation of the cellular response in patients with active TB. Reports have shown that *M. tuberculosis* Ag-specific IFN-γ producing cells (Schwander *et al.,* 1998) and spontaneous IFN-γ production (Condos *et al.,* 1998) in patients with pulmonary tuberculosis are compartmentalised to the site of infection despite ongoing disease.

It has been suggested that IL-12 production may be an important regulator of T-cell phenotypes in tuberculosis. Zhang and co-workers (1994) found that cytokine production in pleural fluid from patients with tuberculosis pleurisy had high levels of IL-12 after stimulation of pleural fluid cells with *M. tuberculosis*. As IL-12 is known to induce a Th1-type immune response in undifferentiated $CD4^+$ cells, a Th1 response was suggested

at the site of disease. Further confirmation of the role of IL-12 as a regulator of the T-cell phenotype response came from a study comparing patients with MDR tuberculosis to tuberculin-negative and tuberculin-positive subjects. McDyer and co-workers (1997) found that stimulated PBMCs from MDR-TB patients had less proliferation and secretion of IL-2 and IFN-γ than did cells taken from healthy PPD-positive or negative control subjects. In this study, IFN-γ production was restored when PBMCs were supplemented with IL-12 prior to stimulation.

1.3.1.4.2 $CD8^+$ Cell phenotypes in tuberculosis

$CD8^+$ T-lymphocytes recognize peptide fragments of antigens processed in the cytosol and presented in the context of MHC Class I molecules, which are found on the surface of all nucleated cells. These cells participate directly in lysis of infected cells and induction of apoptosis of their target-cells. Several groups have attempted to characterise the role of $CD8^+$ cells in the blood and lungs of patients with pulmonary tuberculosis and the data have been conflicting at times. Bose and colleagues (1995) showed that peripheral blood CD4/CD8 ratios were depressed in patients with newly diagnosed disease or in chronically non-responding patients, but ratios normalised if and when patients responsed to therapy. This reduced ratio was felt to be due to depletion of $CD4^+$ cells rather than to proliferation of $CD8^+$ cells. An increase in $CD8^+$ cells in the BAL of patients with active tuberculosis along with marked increases in the number of BAL cells expressing IFN-γ and IL-12mRNA was reported by Taha and colleagues (1997). The above-mentioned studies point to a potential role for $CD8^+$ T-cells in the immune response to tuberculosis. The exact function of these cells in tuberculosis, is however unclear. $CD8^+$ T-cells could be involved in cell lysis and apoptosis, but evidence also exist that they are capable of secreting cytokines such as IFN-γ and IL-4 and thus may play a role in regulating the balance of Th1- and Th2-cells in the lungs of patients with pulmonary tuberculosis.

1.3.1.4.3 γδ T-cells in tuberculosis

In a study done by Barnes and colleagues (1993), a strong 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. Upon mycobacterial stimulation *in vivo,* these cells proliferate rapidly with a cell number increasing from 1-2% to 40% of the total circulating T-cells. *M. tuberculosis*-reactive γδ T-cells can be found in the peripheral blood of tuberculin-positive healthy subjects and these cells are cytotoxic for monocytes pulsed with mycobacterial antigens. Ueta and co-workers (1994) studied healthy contacts and compared them with persons who had not had contact with patients with tuberculosis. They found that tuberculin-positive persons in frequent contact with active cases had had a greater percentage of γδ T-cells in their peripheral blood than those without contact with active cases. Patients with active tuberculosis also had no increase in γδ cells as a percentage of total circulating T-lymphocytes (Ueta *et al.*, 1994). Taken together, these studies indicate that γδ T-cells may indeed play a role in early immune responses against *M. tuberculosis* infection and may be an important part of the establishment of protective immunity in those patients with latent infection (Ladel *et al.*, 1995). 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, (Ellner, 1997) GM-CSF, IL-3 and TNF-α with negligible amounts of IL-4 and IL-5 (Barnes *et al.*, 1996), a profile consistent with a role in anti-mycobacterial defences. However, in order to define the contribution of γδ T-cells to anti-mycobacterial defences, it will be important to characterise the antigens recognised by these particular cells.

1.3.1.5 Induction of cytokines by *M. tuberculosis*

The cytokine profile, at any stage of *M. tuberculosis* infection or even earlier, determines the success of the host in containment of mycobacterial growth. The importance of IFN-γ, TNF-α, IL-12 and IL-10 in the host immune response against *M. tuberculosis*, have been highlighted in aforementioned sections of this chapter. In the following section, the role of the immuno-suppressive cytokines such as TGF-β and IL-10 will be discussed.

Active tuberculosis is also associated with suppression of T-cell responses (Ellner, 1997) and enhanced production and/or activity of immuno-suppressive molecules such as TGF-

β1 and IL-10. TGF-β1 and IL-10 overlap with each other in many of their biological effects, including T-cell suppression, macrophage deactivation and interference with APC function (de Waal-Malefyt *et al.,* 1992; Wahl, 1992). In a study of tuberculosis patients from Karachi, Pakistan, neutralising antibody to TGF-β1, normalised lymphocyte proliferation in response to PPD and significantly increased PPD-induced production of IFN-γ in the PBMC of healthy PPD-reactive household contacts of patients (Hirsch *et al.,* 1996) were detected. In addition, co-culture with neutralising antibody to IL-10 augmented T-cell-proliferation to PPD in blood from TB patients but not their household contacts. Furthermore, Jacobs and colleagues (2000) have shown that cell-mediated immunity is enhanced in the absence of IL-10, resulting in a robust granuloma formation, which accelerates the clearance of mycobacteria. In contrast, stimulation of PBMC with PPD and the mycobacterial 30 kDa alpha antigen induced greater secretion of TGF-β1, but not IL-10, in patients than household contacts. In recent studies of patients with active TB in Uganda, Othieno and co-workers (1999) have observed enhanced production of both IL-10 and TGF-β1 in PPD-activated PBMC culture supernatants from TB patients compared to healthy PPD skin test-reactive control subjects. Furthermore, co-culture of PBMC from TB patients with neutralising antibodies to either TGF-β1 or IL-10 significantly increased PPD-induced production of IFN-γ in TB patients. Whether TGF-β1 and IL-10 synergise with one another or function independently to enhance the suppression of the IFN-γ response in TB patients, however, is not known. Maeda and co-workers (1995) showed that TGF-β1 enhanced production of IL-10 by peritoneal macrophages in both normal and tumour-bearing mice. Recently, Othieno and co-workers (1999) found that together, TGF-β1 and IL-10 potentiate the down-regulatory effect on *M. tuberculosis*-induced T-cell production of IFN-γ. TGF-β1 alone enhances IL-10 production. As TGF-β has been detected in granulomas during active tuberculosis in humans (Wahl, 1992), the IL-10/TGF-β1 interactions may result in the suppression of mononuclear cell functions at the site of infection. In addition, TGF-β renders T-cell hyporesponsive to antigen stimulation and impairs mycobacteriocidal activity of *M. tuberculosis*-infected monocytes (Comstock 1992; Hirsch *et al.*, 1994).

In a study performed by Hirsch and co-workers (1999), they found that at the time of diagnosis, PPD-stimulated production of IFN-γ by PBMCs from TB patients were depressed, compared with that of healthy control subjects, whereas TGF-β1 and IL-10 were increased. PPD induced production of IL-10 and TGF-β1 returned to baseline by 3 months, whereas IFN-γ production remained depressed for at least 12 months. This indicates that the immuno-suppressive cytokines are present early during the course of *M. tuberculosis* infection but are also long lasting, presumably relating to a primary abnormality in T-cell function.

1.3.1.6 Immunogenetics of tuberculosis

Genetic differences in individuals could also contribute to susceptibility to tuberculosis. This hypothesis is supported by the observations that pulmonary tuberculosis concordance was higher among monozygotic twins than dizygotic twins and the finding that the *nramp1* genotype correlated with disease among West Africans (Comstock, 1978; Bellamy *et al.,* 1998; Hill, 1998). Other polymorphic human genes have been identified, some of which affect the cellular immune response to *M. tuberculosis* and may influence the outcome of the host response to mycobacterial infection (Bellamy *et al.,* 1999; Wilkinson *et al.,* 1999). Similarly, differences have been observed in the survival of various inbred mouse strains after inoculation with virulent *M. tuberculosis*, indicating that the genetic background can have a profound effect on the control of infection (Medina and North, 1998). Recently, Chackerian and colleagues (2001) investigated that immunological differences between susceptible C3H/HeJ (C3H) mice and resistant C57BL/6 (B6) mice. They found a correlation between the appearance of IFN-γ-producing $CD4^+$ T-cells in the lungs and resistance to *M. tuberculosis*. Susceptibility in the C3H mice was manifested by an increased mycobacterial burden and reduced survival, which was associated with delayed appearance of cytokine-producing pulmonary T-cells suggesting an important role for the early appearance of IFN-γ-producing lymphocytes in the lung in resistance to infection with *M. tuberculosis*. Other genes reportedly associated with resistance or susceptibility to *M. tuberculosis*, include vitamin D receptor (Delgado *et al.*, 2002), mannose binding protein (Hoal van Helden *et al.*, 1999) and surfactant protein (Floros *et al.*, 2001).

Studies by Altare and co-workers (1998), Jouanguy and co-wokers (1997) and Newport and co-workers (1998) have shown that defects in the genes encoding IL-12 and the receptors for IL-12 and IFN-γ in certain patients with severe atypical mycobacterial infections. More recently, a case control and family study has shown a clear association with IFN-γ and susceptibility in a general population with high incidence (Rossouw *et al.*, 2003). Given the strong correlation between control of tuberculosis and cell-mediated immunity, the genetic differences between susceptible and resistant individuals are likely to be immunological.

1.3.1.7 Recurrence or re-infection

First exposure to tuberculosis frequently results in a clinically silent infection. Often the only sign is a positive PPD skin test . Most clinically relevant TB is thought to be a post-primary infection caused by the reactivation of a previously dormant primary (endogenous) infection. However, recurrent tuberculosis might be due to either relapse or exogenous re-infection.

There have been some reports of exogenous re-infection with multiple drug-resistant strains of *M. tuberculosis*, but these were restricted to HIV-positive patients who were immuno-compromised (Fine and Small, 1999). However, findings from a study conducted in two suburban communities in Cape Town, South Africa, suggest that re-infection may be more common than previously thought (Van Rie *et al.*, 1999). Recent models based on estimates of the annual risk of infection and the incidence of tuberculosis have suggested that the relative contribution to exogenous re-infection increases in parallel with the incidence of the disease (Vynnycky *et al.*, 1997). In countries with a low incidence of tuberculosis, recurrence seems to be attributable to relapse in most areas (Sudre *et al.*, 1999; de Boer *et al.*, 2000). However, in such countries it has been generally been accepted that the combination of host immunity and limited exposure to *M. tuberculosis* made exogenous reactivation a more plausible explanation of recrudescence of disease (Vynnycky *et al.*, 1997). Studies from areas with a high incidence have reported re-infection cases to vary from 12 % to 75 % (Das *et al.*, 1993; Das *et al.*, 1995; Sahadevan *et al.*, 1995; van Rie *et al.*, 1999). The varied estimates

of the relative risk of recurrence could be ascribed to differences in the risk of new *M. tuberculosis* infection and possibly the influence of HIV-1 on relapse and re-infection (Sonnenberg *et al.*, 2001).

1.3.2 VACCINATION AGAINST TUBERCULOSIS

1.3.2.1 Bacille Calmette Guerin (BCG) vaccine

BCG vaccines have been given to hundreds of millions people since 1921, more than any other vaccine. The capacity of this vaccine to protect against tuberculosis, is still debated, since randomised clinical trials have provided estimates ranging from 80 % protection to no benefit (Comstock, 1988; Rodrigues *et al.*, 1993; Colditz *et al.*, 1995; reviewed by Fine, 1995). Reasons for this widely varying degree of protection in efficacy are discussed in Section 1.3.2.5 of this thesis.

1.3.2.2 Historic records on BCG generation

The history of BCG begins with experiments by Calmette and Guerin in the first decade of the 20th century. It was developed by attenuation of a wild type isolate of *M. bovis*, which is closely related to *M. tuberculosis* (> 90 % DNA homology).

To study tuberculosis in cattle, Calmette and Guerin used cultures of bovine tubercle bacilli (*M. bovis*). For optimum oral infection of cattle and guineapigs, bile was added to the culture medium in order to prevent the bacteria from clumping (reviewed by Behr 2002). Within a few months, a colony type was observed which was less virulent to guinea-pigs. For 13 years, the bacteria were grown in the presence of bile. A total of 230 passages (by changing the culture medium every 2 weeks), were achieved by 1921 (reviewed by Behr, 2002). During that time, the bacteria had a progressive decrease in virulence as measured in several different animals, and eventually an unidentified human being was injected with 44 000 bacteria intravenously to show that attenuation had successfully been accomplished (reviewed by Behr, 2002).

By 1921, BCG was administered to individuals as a preventative measure against tuberculosis. However, as it was impossible to lyophilise BCG at that time or put away a

stock of the strain at –80 ^{0}C, BCG continued to be grown in much the same conditions that had resulted in its original attenuation until lyophilisation of BCG-Pasteur after 1173 passages in 1961 (reviewed by Behr, 2002). Presently, all BCG strains are prepared as lypophilised stocks that are resuspended before inoculation. In 1924, BCG lots were distributed to different countries for local preparations of vaccine. BCG was propagated in these laboratories, namely to prevent BCG from reverting to virulence while preserving an acceptable degree of what was called "potency". Until the 1960's and 1970's, each of these BCG vaccine laboratories developed its own daughter strain of BCG, named after the laboratory director, city or country (reviewed by Osborn, 1983; reviewed by Behr, 2002).

Recent data indicates that a large number of mutations have taken place during the long *in vitro* propagation of *M. bovis* BCG (Behr *et al.*, 1999; Gordon *et al.*, 1999). It is known that these mutations have resulted in the deletion of many open reading frames, encoding several important T-cell antigens such as the ESAT-6 (early secretory antigenic target of 6 kDa) and CFP10 (Andersen *et al.*, 1995; Skjot *et al.*, 2000). This BCG provides relatively exclusive protection in all animal models of TB infection. Paradoxically, the efficacy of BCG in several of the more recent field trials has been low or non-detectable (Fine, 1995). Generally speaking, trials conducted in the 1940's and 1950's demonstrated the vaccine to be highly effective in developed countries such as the UK imparting 88 % protection, (Springett, 1969; reviewed by Fine, 1995), 94 % in Denmark (Hyge, 1956; reviewed by Fine, 1995) and 72 % in North America (Levine *et al.*, 1946; reviewed by Fine, 1995). More-recent trials in developing countries (Ponnighaus *et al.*, 1992; Tuberculosis Prevention Trial, 1980; reviewed by Fine, 1995) demonstrated no detectable protection against pulmonary TB in Malawi and South India.

However, Behr (2002) argues that much of the evidence of differences between BCG strains comes not from evolution of the same strain being used in a population, but rather when one BCG strain is replaced by another. These changes could be followed by observations of an increase or decrease in rates of tuberculosis or vaccine-associated events. These have been well-described when BCG-Glaxo and BCG-Pasteur are

interchanged, the Pasteur strain causing greater numbers of lesions (Milstein and Gibson, 1990). In a case-control study done in Columbia, the BCG strain was changed during the study period, suggesting that the benefit was reduced when a Danish strain replaced a form of BCG-Tokyo (Shapiro *et al.*, 1985).

1.3.2.3 Evaluation of BCG vaccination in South Africa

Since 1970, BCG vaccination has been administered percutaneously in South Africa using the Heaf gun with 20 needles. About ten years later, the Heaf gun was replaced by a Japanese disposable tool with 9 needles (Glatthaar and Kleeberg, 1977; reviewed by Kibel *et al.*, 1995). According to the Department of National Health and Population Development (1990) and other limited published information, neonatal BCG vaccination coverage rates should be above 80 % in most areas in South Africa. Until recently, BCG-Tokyo 172 was used percutaneously in South Africa. (The vaccine was prepared in South Africa at the Vaccine Institute in Cape Town). Recently, in South Africa, a locally made form of BCG-Tokyo 172 administered percutaneously has been replaced by intradermal BCG-Danish 1331. The use of BCG-Tokyo will be reviewed in Chapter 6 of this thesis.

In the Western Cape Province of South Africa however, which has particularly high incidence rates of tuberculosis, there are serious concerns as to the efficacy of this method of vaccination. Despite having been adequately vaccinated, many infants and young children still develop miliary, meningitic and other forms of invasive forms of tuberculosis (Hussey *et al.*, 1991). These concerns are difficult to substantiate, given the current high TB incidence and the high rates of BCG vaccination (96-99 %), as it is possible that the incidence of such complications might have been even higher in the absence of vaccination.

Percutaneous BCG stimulates skin test hypersensitivity to tuberculin, but this does not appear to equate to clinical protection against the invasive forms of the disease. Skin reactivity only persists for about 2 years (Kibel *et al.*, 1995). BCG scars produced by this method are also very superficial or totally absent and ulceration or lymphadenitis is encountered very rarely (Kibel *et al.*, 1998). It is reported that the majority of immunised

infants show no mark or scar on the arm when seen at 3 months in child health clinics (Kibel *et al*, 1995). This apparent lack of protection could be related to 1) poor immunogenicity of the strain of BCG used (Tokyo), or 2) the use of a percutaneous technique rather than the internationally acceptable intradermal method. There may be a simpler explanation that the tools used are ineffective in producing deep enough skin penetration, either because of problems with design or constant re-use. Technical factors as well as the strain of vaccine used, may well be the reason for the poor efficacy of BCG vaccination in South Africa. The Japanese tool consists of a hollow plastic cylinder with 9 fine stainless steel needles implanted in a disc near one end and a rectangular flange which fits around the cylinder at the needle end. The flange is used to spread the vaccine and according to the original description, to control the depth of penetration of the needles. The rim measures 2.0 cm in diameter. The technique of administration is as follows: The right upper arm is cleaned with spirits and allowed to dry. One drop of vaccine is applied on the skin over the deltoid; the vaccine is spread with the flange over an area equal to a diameter of 2 cm and the needles of the tool are pressed through the vaccine into the skin until the sides of the flange touch the skin. The tool is withdrawn and rotated and the procedure is repeated once in new-borns (18 punctures) and twice in older subjects (27 punctures). In a successful vaccination, it should therefore be possible to see 18 puncture marks immediately after the procedure. Where no BCG scar is visible, vaccination is repeated at the 3-month visit. Vaccination of older children has been discontinued.

1.3.2.4 Characteristics of acquired cellular immunity to tuberculosis

For a live attenuated vaccine to provoke an immunological response, the vaccine has to be provided in a living state, survive long enough after inoculation to provoke the desired immune response and express the antigenic components necessary to trigger the appropriate response (reviewed by Behr, 2002). These factors are known to vary among BCG strains, while indirect evidence suggests that survival in the host could also vary. The proportion of BCG vaccine that is dead versus live varies greatly after reconstitution. Most lyophilised BCG vaccines are 90-95 % dead bacteria, with BCG-Tokyo as the exception, estimated to contain perhaps 25 % live bacteria at vaccination. After

inoculation, the bacteria have to survive long enough to induce an immune response. Vaccine properties that enable survival in the host are important for their ability to present an immunising stimulus to the host, while at the same time, these same properties could also predict whether the vaccine can itself cause disease. Evidence suggests that perturbations in the mycolic acid repertoire of BCG strains could induce a pathway whereby survival in the host in compromised. Mycolic acids are the major constituent of the bacterial cell wall and therefore influence the interaction between the bacterium and its environment. BCG strains after 1931 do not produce these acids. Altered production of methoxymycolates result in reduced bacterial survival in macrophage cell cultures.

Currently, a point of particular concern in the development of vaccines that require cell-mediated immune responses, is the induction of long-lived immunological memory (Seder and Hill, 2000). In this regard, it is striking that BCG generally protects against childhood manifestations of the disease, but as the children reach adolescence, the incidence of TB increases and protection by BCG is no longer observed (Stern *et al.*, 1998). It seems therefore that immunity induced by BCG is only efficient in early life (Anderson, 2001). $CD4^+$ T-cells are essential for immunological memory (Orme, 1988; Anderson, 1996), but there is strong evidence from various experimental models suggesting that $CD4^+$ memory T-cells revert to the naïve phenotype over time (Bell *et al.*, 1998). A possible solution to this problem would be to boost a waning memory immune response just before adolescence, thereby returning the resting memory cells to the activated state (Andersen, 2001).

1.3.2.5 Variation in protection by BCG

Formal evaluation of BCG vaccines began in the 1930's but it was not until the late 1950's that scientists became aware of discordance between various results. Estimates of protection imparted by BCG against pulmonary tuberculosis vary from zero to 80 %. This variability has been attributed to strain variation in BCG preparations, to genetic and nutritional differences in populations, to environmental influences such as sunlight exposure, poor cold-chain maintenance, or exposure to environmental mycobacterial infections (reviewed by Fine, 1995). Evidence accumulated to date suggests that regional

differences in environmental mycobacteria may be responsible for much of the variation observed between populations in the efficacy of BCG against pulmonary tuberculosis. Apart from poor quality or improper handling of the vaccine, various other factors can be expected to influence the protective effect of BCG. These other factors will be discussed briefly:

- Geographical evidence
- Immunological background of new-borns when immunised
- Differences in strains, doses and vaccination schedules
- Exposure to environmental mycobacteria
- Strain variations in BCG preparations

1.3.2.5.1 Geographical evidence

It appeared that BCG provided better protection at higher latitudes which led to the notion that exposure to certain mycobacteria common in warmer regions might explain the loss of protection offered by BCG in these areas (reviewed by Fine, 1995). As there are many species of environmental mycobacteria, individuals exposed to them could be sensitised and impart protection against tuberculosis and that BCG could do little to improve upon such naturally acquired protection.

1.3.2.5.2 Immunological background of new-borns when immunised

Early immunization is required to protect infants from pathogens to which they may be exposed during early childhood, but is limited by the immaturity of the immune system of the neonatal immune system (Marchant and Newport, 2000; Siegrist, 2001). Furthermore, data obtained from animal models indicates that the neonatal immune response is biased toward Th2. These animals preferentially develop Th2-type responses following immunization and are generally deficient in Th1 responses (Barrios *et al.*, 1996; Beverly, 1997; Donckier *et al.*, 1998). Also, *in vitro* studies on cord blood mononuclear cells have shown that lymphocytes from new-borns have a deficient IFN-γ production in response to mitogens (Wilson *et al.*, 1986; Roncarolo *et al.*, 1994). Recent data have also shown that human cord blood-derived dendritic cells have a profound defect in the production of IL-12, suggesting that type 1 responses could also be defective

in human new-borns (Goriely *et al.*, 2001). As a result of this biased response, a new-born's response to a vaccine could be impaired. However, a recent study by Marchant and co-workers (1999) demonstrated for the first time that *M. bovis* BCG vaccination induced potent Th1 responses to mycobacterial Ags in new-borns. They also found that the Th1 response induced by BCG was of similar magnitude to infants immunized at 2 or 4 months of age. The Th1 response persisted one year after vaccination and indicated that BCG vaccination at birth activates memory T-cells. However, in a recent South African study (Hussey *et al.*, 2002), it was found that cord blood mononuclear cells differentially elevated the production of IL-5 and IL-10 in response to BCG.

Furthermore, it has been established that maternal infection or prenatal exposure to soluble Ags in humans such as mumps (Aasa *et al.*, 1972), serogroup A and C meningcoccal polysaccharide vaccines (McCormick *et al.* 1980), tetanus (Gill *et al.*, 1983), *Toxoplasma gondii* (Hara *et al.*, 1996), environmental allergens (Prescott *et al.*, 1998) and filariasis and schistosomiasis (Malhotra *et al.*, 1999) leads to immunologic sensitization of the developing foetus (King *et al.*, 1998). These studies confirmed the notion that these infections may biase immunity and could alter the efficacy of vaccination. Finally, it has also been shown that apart from the immunological background of the new-born, tuberculin sensitivity diminishes very rapidly in newborns after vaccination (Beskow *et al.*, 1980).

1.3.2.5.3 Exposure to environmental mycobacteria

Studies in humans and animals have shown that exposure to environmental mycobacteria can protect against subsequent challenge with *M. tuberculosis*. Cohort studies have shown that individuals with low or intermediate tuberculin sensitivity, indicative of prior exposure to environmental mycobacteria, are at lower risk to tuberculosis than are individuals with either no tuberculin skin sensitivity or with very strong sensitivity at the start of follow-up (reviewed by Fine, 1995). Two of the three BCG trials with the most stringent criteria for excluding individuals with any prior tuberculin sensitivity found high efficacy of the vaccination (Hart and Sutherland, 1977; Aronson, 1948; reviewed by Fine, 1995) This is consistent with the environmental mycobacteria hypothesis because

the absence of prior sensitivity indicates that the trial participants were less likely to have had exposure to cross-reacting infections. Furthermore, a decline in protection is observed in several BCG vaccine trials. Such declines are predictable as a result of continued exposure of the trial population to environmental mycobacteria. Studies by Cheng and co-workers (1993) found that although peripheral blood monocytes from unvaccinated teenagers in the Chingleput trial area in South India were more efficient at inhibiting *M. microti* growth *in vitro* than were monocytes from unvaccinated teenagers in England, BCG vaccination enhanced this capacity to a greater extent in the English vaccinees. They interpreted their observations as evidence that the South Indian vaccinees had greater immunity against mycobacteria due to their greater previous exposure to environmental mycobacteria and that BCG had less scope to increase the bacteriostatic potential in the South Indian children because of this higher baseline due to prior heterologous immunity.

Classic experiments by Palmer and Long (1966) demonstrated that prior exposure of guinea pigs to *M. fortuitum*, *M. avium*, or *M. kansasii* imparted 15 %, 50 % or 85 % as much protection respectively, against *M. tuberculosis* as did BCG. However, the combination of BCG and an environmental mycobacterium provided no more protection than did BCG alone. More recently, in a study conducted in Malawi, Black and co-workers (2001) presented strong evidence that exposure to various environmental mycobacteria is highly prevalent in an area where BCG fails to protect against pulmonary tuberculosis, suggesting that prior exposure to such mycobacteria, can influence the immune response to BCG vaccination. Recently, Brandt and colleagues (2002) demonstrated that mycobacteria of the *M. avium* complex isolated from a Malawian population, sensitised mice to block the replication of BCG, thus giving a possible explanation as to how environmental mycobacteria could interfere with BCG vaccination.

The evidence for environmental mycobacterial involvement in naturally derived immunity to TB, in particular in the tropical countries, may appear at odds with the fact that TB is now most prevalent in just those areas where this natural immunity should be widespread. However, environmental mycobacteria are by no means the only influence

on TB epidemiology. Socio-economic conditions, mediated through crowding and contact or through nutritional or other influences on general health status, are also important factors in all populations (Fine, 1995; Bentwich *et al.*, 2001).

1.3.2.5.4 Strain variation in BCG preparations

Hart and Sutherland (1977) and Behr (2002), argued that the strain differences between BCG preparations were responsible for most of the observed variation in efficacy. The fact that BCG vaccines produced by different manufacturers were known to differ, lent credence to his hypothesis. Though one cannot prove that strain differences are not necessarily responsible for the observed differences in efficacy, the fact that similar vaccines perform very differently in different settings indicates that this cannot be the entire explanation. Prominent examples are provided by the Glaxo-freeze dried BCG, which gave good protection against tuberculosis in England (Sutherland and Springett, 1983; reviewed by Fine, 1995), but not in Malawi (Ponnighaus *et al.*, 1992; reviewed by Fine, 1995), and by the Danish BCG, which performed well in the original British trial (Hart and Sutherland, 1977; reviewed by Fine, 1995), but provided very little protection in the South India/Chingleput trial (Tuberculosis Prevention Trial Madras, 1980; reviewed by Fine, 1995).

1.3.2.6 Strategies for TB vaccine development

Ideally, an effective vaccine against tuberculosis, should be : a) safe and inexpensive, b) provide worldwide protection against infection and disease irrespective of geographical location, c) protect after a single administration, d) induce long-lasting immunological memory, e) can be combined with other childhood vaccines and f) would not compromise the tuberculin skin test (Mantoux skin test). As BCG fails to prevent pulmonary TB in adults, the most prevalent form of this disease (Kaufmann, 2000; Andersen, 2001), the design of novel vaccines against tuberculosis is urgently required.

1.3.2.6.1 Live mycobacterial vaccines

Live bacterial vaccines have the theoretical advantage that many antigens can act together to induce optimal protection. Furthermore, live vaccines would be expected to remain viable in the tissues for a prolonged period of time, thereby ensuring that efficient memory is induced and maintained. With progress in the development of molecular tools for manipulation of mycobacteria, the number of reports on successful attenuation of mycobacterial strains has increased rapidly (Doherty *et al.*, 2002; D'Souza *et al.*, 2002). For example, Jackson and colleagues (1999) and McAdam and co-workers (1995), by using selectable markers, such as antibiotic resistance genes and both transposon mutagenesis and allelic exchange, have shown that auxotrophic mutants could be generated that have reduced virulence owing to a lack of functional genes for the synthesis of nutritional growth factors. The latest development in this field however, is signature-tagged mutagenesis, which has been used in several reports to generate panels of hybrids with varying levels of attenuation (Cox *et al.*, 1999; Camacho *et al.*, 1999). Many of these attenuated mutants have mutations in the genes that control synthesis of lipids in the outer cell wall, which is important for the survival of the bacteria in the lung (Cox *et al.*, 1999; Camacho *et al.*, 1999). Although several of these constructs are protective against subsequent infection with tuberculosis in mice, there seems to be a correlation between virulence and protection and none of the mutants tested so far has given higher levels of protection than BCG in the animal models (Anderson, 2001). Attempts have also been made to improve the immunogenicity of BCG, either by enhancing its $CD8^+$ T-cell stimulating capacity through induction of listeriolysin, which targets the Ags for MHC class I presentation (Hess *et al.*, 1998; Hess and Kaufmann, 2001), or by the expression of Th1 cytokines (Murray *et al.*, 1996). However, these different approaches are always complicated by the potential risk of increasing the virulence of the vaccine strain.

1.3.2.6.2 Subunit and DNA vaccines

The subunit and DNA approaches are based on the assumption that a few antigens are sufficient to induce and maintain a protective immune response. These approaches have the advantage of being safe and stable even in immuno-compromised individuals.

In the development of sub-unit vaccines, the major focus has been on vaccines based on culture filtrates (Pal and Horwitz, 1992; Anderson, 1994), or secreted proteins purified from culture filtrates such as components of the 30-33 kDa Ag85 complex (Ag85A, Ag85B and Ag85C) (Horwitz *et al.*, 1995), the 38 kDa phosphate-binding protein (Falero-Diaz *et al.*, 2000) and the low molecular mass antigen, ESAT-6 (Brandt *et al.*, 2000).

The Ag85 complex is a promising vaccine candidate as it sensitizes the immune system for strong T-cell proliferative responses and IFN-γ production in most healthy individuals infected with *M. tuberculosis* or *M. leprae* (Launois *et al.*, 1994) and in BCG-vaccinated mice (Huygen *et al.*, 1992), but not in tuberculosis or lepromatous leprosy patients (Huygen *et al.*, 1988; Launois *et al.*, 1991). Antigen 85 belongs to a family of proteins with fibronectin-binding capacity and mycolyl-transferase activity, which are involved in the final stages of mycobacterial cell wall assembly (Hess *et al.*, 2000a; Hess *et al.*, 2000b). Furthermore, Ag85B of BCG shows 99 % homology with its cognate of *M. tuberculosis*. It has been reported that immunization with naked plasmid DNA (pDNA) encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated responses and confer significant protection to C57BL/6 mice challenged by the aerosol or intravenous route with *live M. tuberculosis* H37Rv (Huygen *et al.*, 1996; Baldwin *et al.*, 1999; Kamath *et al.*, 1999). Recently, priming with Ag85 DNA was shown to augment the protective efficacy of *M. bovis* BCG (Feng *et al.*, 2001) and recombinant BCG overexpressing Ag85 was found to have increased immunogenicity and efficacy in guinea pigs (Horwitz *et al.*, 2000). Furthermore, Weinreich and colleagues (2001) have shown that a fusion protein consisting of Ag85B and ESAT-6 is a very promising protein-subunit vaccine candidate for tuberculosis.

Another promising tuberculosis DNA vaccine consists of DNA encoding the 40-kDA protein PstS-3 (Tanghe *et al.*, 1999). PstS-3, PstS-1 and PstS-3 are surface-exposed lipoproteins that are putative mycobacterial phosphate transport proteins, homologous to phosphate-binding protein PstS of *Escherichia coli* (Lefevre *et al.*, 1997).

Although the immunogenicity of DNA vaccines in humans is promising, increasing the potency of DNA vaccines would be advantageous. Increased pDNA-induced antibody responses can be obtained by complexes with conventional adjuvants, such as monophosphoryl lipid A (Sasaki *et al.*, 1998a), alum (Ulmer *et al.*, 1999) and QS-21 saponin (Sasaki *et al.*, 1998b). Priming with DNA followed by boosting with either purified protein (Tanghe *et al.*, 2001) or recombinant modified virus Ankara (McShane *et al.*, 2002) was also shown to increase the immunogenicity and protective efficacy of DNA vaccines consisting of DNA encoding Ag85A. D'Souza and co-workers (2002) have also shown that cationic lipids can be used as adjuvants for DNA-based vaccination with Ag85 and PstS-3 from *M. tuberculosis*, resulting in significant increased antibody titers and levels of Th1-type cytokine production in the spleen and lungs, and showing more sustained cytolytic CTL responses as well as increased protective efficacy against an intravenous challenge with bioluminescent *M. tuberculosis* H37Rv.

1.3.2.6.2 Future prospects

Most research today in focused on the development of the ideal vaccine that could be given at birth and provide lifelong protection. As already mentioned, the majority of the population that would be targeted with a novel vaccine is already sensitised either by previous infection, exposure to other mycobacterial or by BCG vaccination. It will therefore be of crucial importance to define the vaccination requirements under these circumstances.

1.3.3 Tuberculin skin test (TST) reactivity

For years, the TST has been the only screening tool available for the detection of latent *M. tuberculosis* infection. Despite dependence on the TST and its wide-spread use, the test is subject to significant variations and limitations as it does not reliably differentiate between *M. tuberculosis* infection, BCG vaccination and exposure to mycobacteria other than *M. tuberculosis* (Huebner *et al.*, 1993).

Tuberculin, a blend of antigens produced from killed tubercle bacilli, was first introduced by Robert Koch in 1890 who recognised it as a diagnostic tool for tuberculosis (Rieder,

1999). Attempts to identify a better tool to determine infection more accurately and to distinguish infection from disease with such a test have so far been unsuccessful.

At the beginning of this century, von Pirquet introduced a cutaneous tuberculin skin test (Rieder, 1999). In this technique, tuberculin was dropped on the cleaned skin and the skin was subsequently scratched. He found the test's sensitivity to be less than 100 % in serious forms of the disease. Shortly afterwards, Moro (1909) (reviewed by Rieder, 1999) introduced a simplified cutaneous test, using tuberculin cream rubbed onto the skin (Rieder, 1999) with results comparable with the von Pirquet method. Mendel and Mantoux (reviewed by Rieder, 1999) almost simultaneously introduced the intradermal technique, allowing the administration of an exact dose of tuberculin with needle and syringe. Today, world wide preference is given to the intradermal technique, thus called the Mantoux skin test.

In 1955, the Statens Serum Institute in Copenhagen, Denmark produced a large batch of tuberculin for international use and called it Tuberculin PPD RT 23 (Magnusson and Bentzon, 1958; Guld *et al.,*1958; reviewed by Rieder, 1999). Tween 80 was added to the tuberculin resulting in a stronger potency compared to the previously used PPD-S. Currently, a standardised technique (Mantoux: intradermal) at a standardised dose (2 TU PPD RT 23 or 5 TU PPD-S) is used.

1.3.3.1 Post vaccination scars and vaccine efficacy

The second most common measure of the effect of the BCG vaccination is ascertainment and measurement of scar formation. In studies of vaccine prevalence and/or efficacy, the presence or absence of a BCG vaccine scar is routinely used as a surrogate marker of vaccination status, in place of or in addition to vaccination records (Liard *et al.*, 1996; Zodpey *et al.*, 1996; Dhadwal *et al.*, 1997; Surekha *et al.*, 1998; Awasthi and Boin 1999). Although 20-50 % of BCG vaccine recipients will not develop a scar (Friedland *et al.*, Grindulus, 1984; Lockman *et al.*, 1999; reviewed by Menzies 2000; Floyd *et al.*, 2000), scarring is a stable phenomenon (Fine *et al.*, 1989). Scarring generally is thought to at

least represent "effective" vaccination, since it is associated with subsequent TST reactivity (Collas *et al.*, 1968; Chhatwal *et al.*, 1994).

In a recent study performed by Jason and co-workers (2002), BCG-scarred individuals with mycobacteremia had proportionally more memory T-cells and more activated $CD8^+$ T-cells than did unscarred persons, explained by scarred persons being previously exposed and responsive to BCG antigens. Furthermore, the BCG-scarred people also had a relatively greater anti-inflammatory cellular cytokine response, possibly as a regulatory, dampening response to cellular activation. More importantly, these people had a type 2 dominance, although mycobacterial infection is optimally handled with a type 1 response. In case-control studies that classified the status of the BCG vaccine on the basis of post vaccination scars, the range of efficacy of the BCG vaccine was 50-60 % (Coldits *et al.*, 1994). However, it is possible that individuals without scars had in fact been vaccinated but failed to manifest an adequate local reaction. In a large survey conducted by Fine and colleagues (1994) in sub-Saharan Africa, the incidence of tuberculosis was virtually identical among individuals with and without scars after BCG vaccination.

1.3.3.2 The effect of BCG vaccination and environmental mycobacteria on tuberculin reactivity

Numerous studies involving different vaccines, age groups and populations have consistently shown that > 90 % of BCG vaccine recipients develop tuberculin reactions ≥ 10 mm in diameter within 8-12 weeks after vaccination (reviewed by Menzies, 2000). However, it has also been shown that tuberculin reactivity after BCG vaccination in infancy wanes rapidly in all individuals (Karalliede *et al.*, 1985; Menzies and Vissandjee, 1987; Great Britain Medical Journal, 1994; reviewed by Menzies, 2000), suggesting that tuberculin reactivity after BCG vaccination is primarily affected by the age at vaccination. Several studies have shown that the frequency distribution of tuberculin reactions among individuals vaccinated in infancy (Lifschitz *et al.*, 1965; Joncas *et al.*, 1975; Menzies and Vissandjee, 1987), corresponded with that seen among people who had been sensitised to non-tuberculous mycobacteria.

The protective efficacy for BCG vaccine is good for infants but less so for older children and adults. The results of the above-mentioned studies of tuberculin reactivity suggest that the immune response to the BCG vaccine is different in infancy than in childhood. Post-vaccination tuberculin reactivity is not an indicator of protective efficacy of BCG vaccination as it is not an indicator of immunity to *M. tuberculosis*. The ideal BCG vaccine would stimulate the development of effective immunity yet would have no effect on tuberculin reactivity.

The specificity of the tuberculin skin test varies in different areas as there as large variations in the presence of environmental mycobacteria, which induce cross-reactions (Huebner, 1993; reviewed by Rieder, 1999). The ability to distinguish infected from non-infected persons is largely dependent on the ration of the prevalence of infection with environmental mycobacetria, usually causing smaller reactions (< 10 mm in induration size) to the prevalence of infection with *M. tuberculosis*, usually causing larger reactions.

1.4 PARASITE IMMUNOLOGY

In 1947, in his article, "This Wormy World", Norman Stoll published estimates of the numbers of helminth infections in humans in different regions of the world (Stoll, 1947). These estimates were based on extrapolating from prevalence surveys of communities in which stool samples were examined for the presence of helminth eggs of various species. An estimated 644 million infections (30 % prevalence) with *Ascaris lumbricoides* (large roundworm), 355 million (16 %) with *Trichuris trichiura* (whipworm) and 457 million (21 %) with hookworms (*Necator americanus* and *Ancyclostoma duodenale*) were reported. Now, more than fifty years after Stoll's publication, estimates of numbers of infections remain virtually unchanged (Chan, 1997). Several estimates of global and regional prevalence and of numbers of infections have been made in recent years and all give a similar picture. It is estimated that a third of the world's population is infected with intestinal helminths (≈ 1.5 billion people) (Chan, 1997). Severity of disease depends upon the worm burden, but in children even moderate intensities of infection may be associated with stunted growth, slow mental development, aneamia and malnutrition (Bundy *et al.*,

1992). In developing countries, children born in areas where intestinal nematodes are endemic, harbour worms for the rest of their lives.

1.4.1 Age and infection

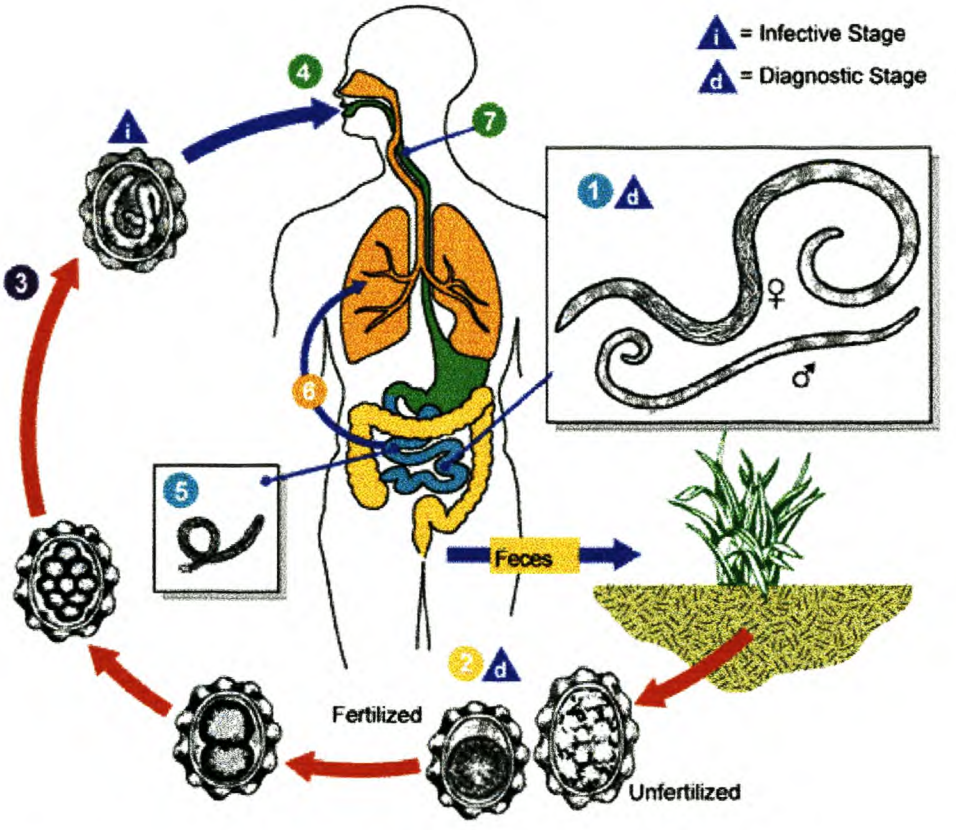
School children harbour the most intense infections with some of the most common worms. They are thus the age group most at risk of morbidity and, simultaneously, the major contributors to transmission (Wong *et al.,* 1988; Bundy *et al.,* 1991). Theory and practice indicate that targeting treatment at school children reduce infection levels in the community as a whole. The age-dependent pattern of infection prevalence is similar amongst the major intestinal helminths, exhibiting a rise in childhood to a relatively stable plateau in adulthood. Maximum prevalence is usually attained before 5 years of age with *A. lumbricoides* and *T. trichiura*. The reduction in worm burdens in adults has been attributed to two possible processes: 1) an age-dependent reduction in infection; or 2) an increase in host resistance with age (Warren 1973; Bradley *et al.,* 1974). Heavy infections may also be aggravated in families (Forrester *et al.,* 1988). Studies have shown that more families tend to contain individuals who are heavily infected with *T. trichiura* and *A. lumbicoides* than would be expected by chance, whereas families with a mixture of both heavy and light infected individuals are less common than would be predicted (Bundy *et al.,*1992).

For most helminthic species the initial rise in the intensity of infection with age closely mirrors the rise in prevalence, but the rise in intensity occurs at a slower rate. The age at which the highest mean intensity occurs, depends on the life span of the helminth species and is dependent of local rates of transmission (Bundy *et al.,* 1992). The most important differences in the relationship between age and the intensity of infections with these species become apparent after the peak

1.4.2 Particular helminths

The life cycle of two helminths, *Ascaris lumbricoides* and *Trichuris trichiura* will be discussed as these helminths are of particular importance in the context of this thesis.

1.4.2.1 Life cycle of *Ascaris lumbricoides*



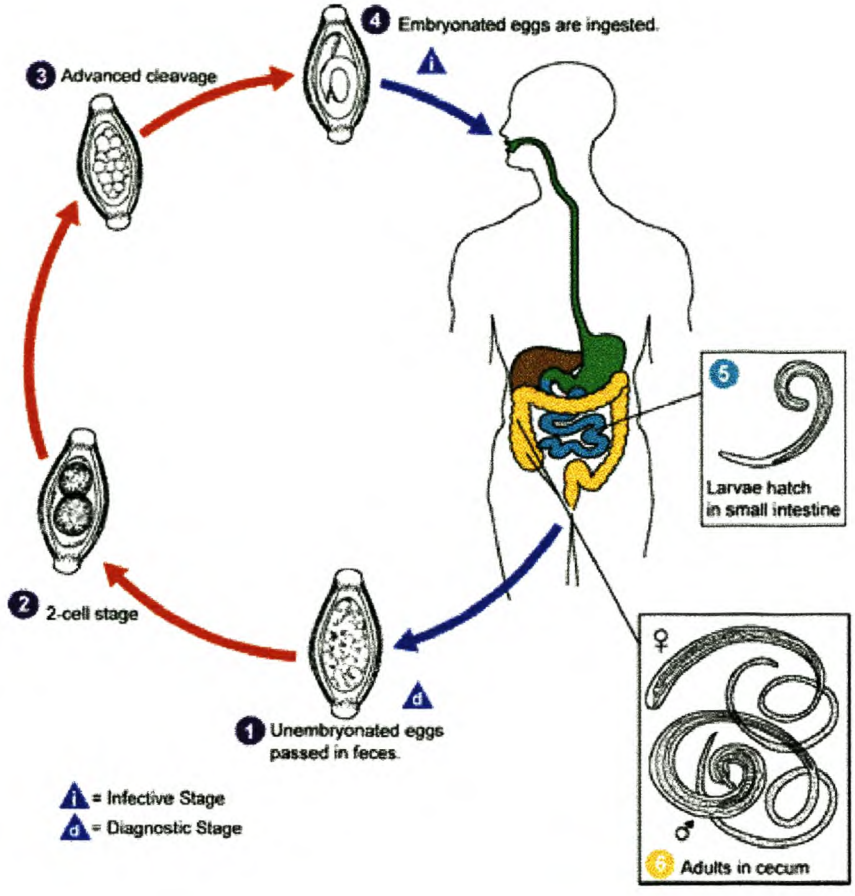
Life cycles obtained from the Centre for Disease Control (CDC) website: (http://www.dpd.cdc.gov/dpdx/default.htm)

An egg requires to be in a warm shady moist habitat for two to three weeks to develop into a first stage larva. If the eggs are picked up and swallowed, they pass to the duodenum where the larvae moult to infective second stage larvae and hatch. Adult worms live in the lumen of the small intestine. 1 A female may produce up to 240,000 eggs per day, which are passed with the feces 2. Fertile eggs embryonate and become infective after 18 days to several weeks 3, depending on the environmental conditions (optimum: moist, warm, shaded soil). After infective eggs are swallowed 4, the larvae hatch 5, invade the intestinal mucosa, and are carried via the portal, then systemic circulation to the lungs. The larvae mature further in the lungs (10 to 14 days), penetrate

the alveolar walls, ascend the bronchial tree to the throat, and are swallowed 7. Upon reaching the small intestine, they develop into adult worms. Between 2 and 3 months are required from ingestion of the infective eggs to oviposition by the adult female. Adult worms can live 1 to 2 years (Fripp, 1983).

Roundworms are the most prevalent of the intestinal helminthic infections, affecting one fourth of the world's population (Cox, 2002). Ascariasis is an ancient infection, and *A. lumbricoides* eggs have been found in human coprolites from Peru dating 2277 BC and Brazil from 1660 to 1420 BC. There are also records of *A. lumbricoides* in a Middle Kingdom Egyptian mummy dating from 1938 to 1600 BC (reviewed by Cox, 2002). Severe cases can cause serious morbidity and mortality due to intestinal blockage (Savioli *et al.,* 1992). Chronic ascariasis has been implicated in the development and persistence of malnutrition in children and may have long-term effects on anthropometric indicators of growth (Crompton, 1992). *A. lumbricoides* is the most common human helminthic infection and has a worldwide distribution. The highest prevalence of this helminth is normally in tropical and subtropical regions, and areas with inadequate sanitation.

1.4.2.2 Life cycle of *Trichuris trichiura*



The unembryonated eggs are passed with the stool ❶. In the soil, the eggs develop into a 2-cell stage ❷, an advanced cleavage stage ❸, and then they embryonate ❹; eggs become infective in 15 to 30 days. After ingestion (soil-contaminated hands or food), the eggs hatch in the small intestine, and release larvae ❺that mature and establish themselves as adults in the colon ❻. The adult worms (approximately 4 cm in length) live in the cecum and ascending colon. The adult worms are fixed in that location, with the anterior portions threaded into the mucosa. The females begin to oviposit 60 to 70 days after infection. Female worms in the cecum shed between 3,000 and 20,000 eggs per day. The life span of the adults is about 1 year.

1.4.3 Immune responses associated with expulsion of helminths

Children in developing countries are particularly likely to be infected with gastrointestinal nematodes; in some endemic areas the prevalence by age 10 approaches 100 % (Finkelman *et al.,* 1997). Fincham and co-workers (1996) reported that in rural areas of the Western Cape, infection was frequent in children with a prevalence of up to 90 %. These children were mainly infected by *T. trichuria*, *A. lumbricoides*, *E. vermicularis* and *Hymenolepis nana*. 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 (Urban *et al.*, 1991; Else et al., 1994; Finkelman *et al.*, 1997; Bancroft *et al.*, 1997). It is therefore generally accepted that helminthic infections induce a type 2 immune response. Type 2 responses correlate with diminished worm burdens, whereas type 1 responses allow chronic infection and scarring to develop.

However, there was a notion that type 2 immunity is a maladaptive response to helminth infection. This notion was directly challenged by a study performed by Bancroft and colleagues (1998). These investigators compared immune responses in wild-type IL-$4^{-/-}$, and IL-$13^{-/-}$ knock-out mice following infection with *T. muris*. Wild-type mice mounted strong type 2 responses that successfully cleared the helminth. IL-4 knockout mice failed to mount type 2 responses; they produced no IL-4 and had markedly diminished IL-5 and IL-13 levels, which resulted in a huge increase in worm burden. In contrast to IL-4 knockout mice, production of IL-4 and IL-5 by the IL-13 knockout mice was only mildly diminished in comparison to that in wild-type animals, indicating that they still mounted type 2 responses. Nevertheless, despite their apparent type 2 response, the IL-13 knockout mice suffered from twice the worm burden than the IL-4 knockout mice, thus showing that a type 2 immune response is protective against *Trichuris* only if IL-13 is present. The importance of IL-13 for helminth expulsion, has been confirmed by several other studies (Barner *et al.*, 1998; Mohrs *et al.*, 1999; Mohrs and Brombacher, 2000; reviewed by Brombacher, 2000).

Urban and co-workers (2000) showed that IFN-γ has an important and specific influence on worm expulsion following *T. muris* infection. In its absence, either IL-4 or IL-13 can induce expulsion, while both type 2 cytokines are required for expulsion in its presence. The marked IL-13 elevations observed in that particular study, following B7 and IFN-γ blockade may be required for the development of an effective protective response in the absence of IL-4. However, it is also possible that IFN-γ may inhibit type 1 or type 2 IL-4R signalling and/or expression; recent findings suggest that IFN-γ may directly inhibit IL-4 signalling by up-regulation of SOCS-1 (Losman *et al.,* 1999). Alternatively, IFN-γ may have a direct inhibitory effect on worm expulsion by interfering with IL-4 and IL-13 action on non-lymphoid target cell populations in the intestine that mediate worm expulsion. IL-4 and IFN-γ have previously been shown to have opposing effects on other target-cells, including MHC II expression by B-cells (Oliver *et al.,* 1987; Mond *et al.,* 1986). Direct effects of IFN-γ on such target cell populations may also explain the decreased worm burden found in IL-13 antagonist-treated IL-4/IFN-γ double-deficient mice, compared with IL-13 antagonist-treated IL-4-deficient mice.

Street and co-workers (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.

1.4.4 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 that helminthic parasites are possibly the most potent inducers of IgE responses (Lynch *et al.,* 1998).

1.4.4.1 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 (Lynch *et al.*, 1998). 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. King and co-workers (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; de Vries *et al.,* 1991) as well as suppression of the ongoing IgE production by switched cells.

1.4.4.2 The IgE blocking hypothesis

The "IgE blocking hypothesis" was the first scenario used to explain the inverse association between helminths and allergy. Clinical allergy requires efficient cross-linking of high affinity IgE receptors (FcεRI) on mast-cells and basophils (Holt *et al.,* 1999). At least two FcεRI-bound IgE molecules must capture a single allergen to induce mediator release. Helminth infections are often associated with highly polyclonal IgE, which is not specific for parasite antigen. If IgE saturates FcεRI on mast-cells and blocks binding of specific IgE directed either to parasite antigen or to environmental allergens, it could inhibit degranulation and immediate hypersensivity responses to allergens. In numerous studies, the protective effect of helminth infections on allergic reactivity was associated with high levels of total polyclonal IgE (Godfrey, 1975; Merrett *et al.,* 1976; Lynch *et al.,* 1987).

1.5 ATOPIC DISORDERS

The prevalence of atopic disorders, in particular asthma, is increasing in developed as opposed to developing countries, with 20-30 % of the population affected (Erb, 1999). Atopy, characterised by raised IgE levels, underlies allergic diseases such as asthma, rhinoconjunctivitis and eczema. The interaction of an environmental allergen with the innate immune system, its uptake by APCs and the subsequent T-cell priming leads to the stimulation of cytokines such as IL-4, IL-5 and IL-13 (Burrows et al., 1989; Abbas *et al.*, 1996). These cytokines interact with their receptors to stimulate IgE production and increased numbers of eosinophils and mast-cells; all these components are capable of precipitating inflammation in the respiratory tract.

Allergic responses to common environmental antigens, such as those derived from house dust mites, plant pollens or animal proteins, lead to clinical disorders such as asthma, hay fever, eczema and allergic rhinitis (Del Prete, 1992). It is argued that genetic and environmental factors interacting with each other, lead to the production of IL-4 through Stat6-mediated signalling and the activation of specific downstream transcription factors such as c-Maf, GATA-3, NIP45 and NFATc in naïve T-cells resulting in the development of allergen-specific Th2 $CD4^+$ T-cells (Erb, 1999). Once generated, Th2 cells activated by allergen secrete IL-4, IL-5 that in turn, stimulate the production of IgE and IgG1 by B-cells as well as stimulating the development of eosinophils in the bone marrow and their recruitment into inflamed tissues.

The most important factor governing the pathogenesis of atopic disorders, is the cross-linking of IgE Fcε receptors on mast-cells, after encountering allergen, which leads to mast cell degranulation. Histamine, heparin and proteases, released by mast-cells, mediate biological effects such as vasodilation, intestinal and or bronchial smooth muscle contraction, mucous secretion and local proteolysis.

1.6 IMMUNO-MODULATION BETWEEN DISEASES

1.6.1 Immuno-modulation by helminths

Concurrent helminths may alter the immune response to non-parasite antigens, as is suggested by the findings of experimental animal models of helminth infections (Kullberg *et al.,* 1992; Actor *et al.,* 1993; Pearlman *et al.,* 1993; Walzl *et al.*, 2003) and human studies of the impact of concurrent tissue helminth infections on post-vaccination and other immune responses (Sabin *et al.,* 1996; Cooper *et al.,* 1998; Cooper *et al.,* 1999).

1.6.1.1 Helminths and HIV and TB

Two of the worst current epidemics in the world are tuberculosis and HIV/AIDS. According to a recent WHO report, the global prevalence of *M. tuberculosis* infection in 1997 was 32 % (1.86 billion people). During that year new cases of TB totalled approximately 8 million. There were 16 million new cases of active disease and close to 2 million people died from it. Regarding HIV infection, according to the Joint United Nations programme on AIDS (UNAIDS), an estimated > 35 million people are presently infected with HIV-1 and > 100 million people will be carrying the virus in < 10 yrs. The highest incidences of both TB and HIV infections occur in the same countries, mainly in sub-Saharan Africa and Southeastern Asia. As HIV and TB infections are expanding in areas endemic for helminths, it is important to examine the effects of helminthic infections on the immune response of helminth-infected individuals towards HIV or TB.

1.6.1.1.1 Helminths and HIV

In addition to the importance of the disease because of the worldwide scale of infection, the significance of helminthic infections has dramatically increased with the advent of HIV infections (Bundy *et al.,* 2000). Bentwich and colleagues (1995 and 1999) have proposed that helminthic infections have detrimental effects on the ability to mount an effective immune response to other infections, particularly HIV and tuberculosis. Furthermore, they have suggested that the rapid progression to AIDS observed in developing countries may be attributed in part, to helminthic co-infections (Bentwich *et al.,* 1995; Bentwich *et al.,* 1999). Helminthic co-infections may also diminish the efficacy

of vaccines (Bentwich *et al.,* 1999; Bundy *et al.,* 2000). Clearly, helminthic infections can have a significant impact on epidemiological patterns of other diseases that have devastating health consequences.

The relationship between helminths and HIV was documented in a number of publications by Bentwich and co-workers (1996 to 1999) in particular. Studies on the immigration of Ethiopian Jews to Israel, some of them infected with HIV, provided the following conclusions:

a) The vast majority of the Ethiopian immigrants were infected with helminths and had dominant Type 2 immune profiles that returned to normal after eradication of helminths (Bentwich, 1996 and 1997).
b) Peripheral blood mononuclear cells obtained from Ethiopian immigrants were highly susceptible to infection with HIV (Shapiro-Nahor *et al.,* 1998)
c) The susceptibility was associated with marked immune activation and also with increased expression of HIV co-receptors and decreased secretion of *in vitro* of B-chemokines (Kalinkovich *et al.*, 1999)
d) The rate of progression of HIV infection, the plasma viral load, the immune activation profile and the response to highly active anti-retroviral treatment in the Ethiopian immigrants were similar to that of non-Ethiopian Israeli's once the helminth infections were eradicated when they were living in Israel (Galai *et al.,* 1997; Bentwich *et al.*, 1998; Weisman *et al.*, 1999)
e) Anti-helminthic treatment reduced HIV plasma viral load (Elias *et al.*, 2001).

1.6.1.1.2 Helminths and TB

The outcome of mycobacterial infection is dynamic in that it not only depends on factors associated with the pathogen, but also on the host and environment. In a recent study conducted by Lienhardt and co-workers (2002) in West Africa, they found that tuberculosis was associated with low Th1 and high Th2 reactivity *in vivo.* They found that current helminth infection was more frequent in tuberculosis patients than in household controls or community controls, confirming the notion that the high prevalence

of helminthiasis could impair protective immunity against mycobacteria (Bentwich *et al.*, 1999).

1.6.1.2 Helminths and atopy

Atopy and wheezing have been linked in both children and adults to elevated total serum IgE concentrations in more affluent societies (Nyan, 2001). However, intestinal helminth infections, endemic in many tropical populations, also cause polyclonal stimulation of the synthesis of IgE and this can modulate the expression of allergic reactivity in tropical populations with high helminth infection rates (Nyan, 2001). It is suggested that the high levels of non-specific IgE and downregulatory Th2 cytokines induced by worm infection offer some adaptive protection against pro-inflammatory diseases, and this has been cited as a reason for the supposedly low incidence of atopic disease in societies where worms are prevalent (Nelson, 1992). However, when one considers that people who are heavily infected with helminths world-wide are rarely affected by allergic diseases, then it is clear that a strong Th2 response is not the sole factor in precipitating an allergic attack. Meta-analysis of data from early surveys showed that, despite the variation in methodology and clinical assessment of allergy, the prevalence of parasitic infections was inversely associated with the prevalence of asthma (reviewed by Yazdanbakhsh *et al.*, 2002). More recently, studies have shown a consistent inverse association between helminth infections and either skin reactivity to environmental allergens or clinical scores, such as airway hyperresponsiveness, wheeze and asthma (Lynch *et al.*, 1987; van den Biggelaar *et al.*, 2000; Nyan *et al.*, 2001). In most of these studies, > 30 % of the study subjects carried substantial levels of house dust mite-specific IgE (HDM-IgE), suggesting that despite IgE sensitization to environmental allergens, helminth-infested subjects are somehow protected from mast cell degranulation and inflammatory responses in affected organs.

The burden and chronicity of helminth infections is an important variable that may determine whether helminths act as a risk factor for, or confer protection against allergic diseases. In Venezuela, the classification of helminth-infested populations into those with none, light or heavy worm burdens, showed that light helminth infections are associated with the amplification of allergen-specific IgE responses and a high skin reactivity,

whereas heavily parasitized subjects are protected from atopic skin reactivity despite a high degree of sensitization to mites (Lynch *et al.,* 1987). Clinical allergic symptoms in those with light helminth infections were alleviated after de-worming (Lynch *et al.,* 1997), but were exacerbated in individuals who had had heavy worm burdens (reviewed by Yazdanbakhsh *et al.,* 2002). This reinforces the notion that heavy helminth infections, protect against allergy. These observations might also explain the situation in industrialised countries where exposure to *Toxocara* spp. (reviewed by Yazdanbakhsh *et al.,* 2002), leading to seropositivity, is associated with an increased prevalence of airway symptoms. Such infections are presumably light and sporadic, allowing exposure to helminth antigens that potentiate Th2 responses without the inhibitory component that is associated with heavy and chronic conditions.

1.6.1.3 Atopy and mycobacteria

A putative link between exposure to mycobacteria and less atopy was illustrated in a study of Japanese children (Shirakawa *et al.,* 1997) in whom strongly positive tuberculin responses in early life were associated with less asthma, rhinoconjuctivitis and eczema in later childhood. Experimental data on the inverse link between mycobacteria and atopy come from experiments in mice which uniformly showed that exposure to mycobacteria had impressive Th2 limiting effects (Wang *et al.,* 1998; Erb *et al.,* 1998; Herz *et al.,* 1998). Thus, exposure of mice susceptible to the experimental induction of allergy with ovalbumin to different mycobacterial preparations resulted in a major limitation of systemic or pulmonary Th2 responses, pulmonary eosinophil infiltration and anaphylactic pulmonary reactions in response to allergens. Furthermore, Wang and colleagues (1998) have demonstrated that subcutaneous injection of heat killed *M. vaccae*, inhibited production of IgE and IL-5 by splenic cells. Erb and co-workers (1998) have shown that intranasal BCG inhibited pulmonary eosinophilia and IL-5 synthesis in response to ovalbumin and to the nematode worm *N. brasiliensis*, an effect dependent on IFN-γ. Intravenous BCG promoted IFN-γ synthesis and inhibited a range of Th2 phenomena including secretion of IL-4 and IL-5 and IgE/IgD1, airway eosinophilia and responsiveness to ovalbumin (Herz *et al.,* 1998). By confirming the notion that mycobacterial preparations can limit experimental allergy in mice, it is then possible that

immunisation with mycobacterial preparations could be developed to prevent atopy and asthma in humans.

1.6.1.4 Atopic disorders, parasites and the hygiene hypothesis

There has been a significant increase in the prevalence of allergic diseases in the past few years. There is a considerably lower prevalence of allergic diseases in developing countries. Furthermore, clear differences in the prevalence of allergies between rural and urban areas within one country has also been shown. For example, in Ethiopia, asthma is more prevalent in urban areas than in rural villages (Yemaneberhan *et al.,* 1997) and asthma is more common in residents of urban Germany than in farmers living in rural Bavaria (von Ehrenstein *et al.,* 2000). To explain these phenomena, environmental factors associated with more industrialised and urban living have been studied extensively, but there is little consistent evidence to suggest that obvious risk factors, such as increased exposure to indoor inhalants, pollution or changes in diet and breast feeding, could account for the rise in atopic diseases. However, another category of environmental factors such as childhood infections, has shown an overwhelming and consistent negative association with atopy and allergic diseases. Allergic sensitization is over-represented among first-born but is less frequent in children with large families (Strachan, 1989) and those attending day care (Kramer *et al.,* 1999), suggesting that a frequent exchange of infections may have a protective effect.

Exposure to food and orofecal pathogens such as hepatitis A, *Toxoplasma gondii* and *Helicobacter pylori*, reduces the risk of atopy by > 60 % (reviewed by Yazdanbakhsh *et al.,* 2002). Studies of gut commensals indicate differences in the rate of microbial colonization as well as the bacterial type involved in children with and without a predisposition to allergy (Sepp *et al.,* 1997). Therefore, it has been proposed that the lack of intense infections in industrialised countries owing to improved hygiene, vaccination and use of antibiotics may alter the human immune system such that it responds inappropriately to innocuous substances. This so-called "hygiene hypothesis" (Strachan *et al.,* 1989) has been given an immunological framework in which the balance between Type 1 and Type 2 immune responses are pivotal (Matricardi *et al.,* 2000). It has been

proposed that limited exposure to bacterial and viral pathogens during early childhood results in a insufficient stimulation of Th1 cells, which in turn, cannot counterbalance the expansion of Th2 cells and results in a predisposition to allergy.

The immunological explanation for the hygiene hypothesis has been very important in directing strategies to prevent allergic diseases. Induction of allergen-specific Th1 responses to BCG or DNA vaccination is being advocated (Wohlleben *et al.,* 2000) on the basis of the promising results obtained in experimental animals.

1.7 Aims of thesis

It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). Of those infected, 10 % will develop disease of which 3-5 % will relapse after completion of treatment. Susceptibility to *M. tuberculosis* or relapse following treatment, may be due to environmental, genetic and/or immunological factors. In 1995, two adjacent suburbs (community A and B) in Cape Town were reported to have a TB notification rate of 1000/100 000 (Beyers *et al.*, 1996). Furthermore, up to 90 % of children in the Western Cape province have been reported to be infected with intestinal parasites (Gunders *et al.*, 19993). Thus, in the current study, immunological and environmental reasons for the high TB notification rate or enhanced susceptibility to *M. tuberculosis* in the study communities will be investigated. As infection with *M. tuberculosis* and parasites induce different types of immune responses, immune responses of individuals in the communities will be characterised. The following aims will therefore be investigated in this thesis:

1. In order to characterise the immune profile of individuals in the communities, serum IgE levels will be determined. The measured IgE concentration will be calculated per census block or enumerator sub-district (ESD) and will then be correlated with the TB notification rate as well as with the socio-economic index, crowding and literacy in the ESDs of this community (Chapter 3).
2. To determine whether successful treatment for tuberculosis, would downregulate type 2 immune reponses (Chapter 4).
3. To determine the prevalence of helminths in children in all the primary schools in the communities and whether environmental factors would influence the prevalence of helminths on a community level (Chapter 5).
4. To determine the relation between BCG scar positivity, Mantoux skin test responses and helminth prevalence as markers of Th1 and Th2 immune responses (Chapter 6)
5. To determine how the host response to *M. tuberculosis* differ in patients at risk for being re-infected, compared to those who have protective immunity by identifying a common immunological marker or pattern (Chapter 7).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials and Methods

Materials and methods are listed and described following the order of the chapters in this thesis.

Determination of serum IgE concentrations (Chapters 3 and 4)

2.1.1. Solutions and reagents required for the IgE radio-immunoassay

IgE radio-immunoassays were obtained from Pharmacia, Uppsala, Sweden. Protocol and reagents were provided by the manufacturers.

2.1.1.1 IgE Radio-immunoassay

This work was performed at the Department of Chemical Pathology, Tygerberg Hospital. The quantitative determination of total serum IgE, was performed according to the protocol supplied by the manufacturers (Pharmacia, Uppsala, Sweden). 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, which is indirectly proportional to the amount of IgE present in the samples. IgE levels were expressed as KU/ml. Samples containing > 1 000 KU/ml IgE/I were diluted and qualified for a second run. The detection limit of this test was < 2 KU/ml with no cross reactivity of IgA, IgD, IgM and IgG at physiological concentrations.

Specific IgE against *Ascaris lumbricoides*, house dust mite, cockroach and Bermuda grass was measured using ImmunoCAP radio-allerg-sorbent (CAP RAST) tests (Pharmacia, Uppsala, Sweden).

Determination of helminth burden (Chapter 5)

2.1.2 Stool sample processing

2.1.2.1 Stool processing

Numbered specimen bottles (Sterilab) and a wooden spatula (Trust Hospital Supplies) with screw top lids were given to each child. Filled specimen jars were returned (decribed in Chapter 5). The consistency of each stool sample was recorded before 1 g of stool was weighed out with orange applicator sticks (Ysterplaat Medical Supplies) and the consistency (density) was graded (Table 2.1). This enabled a multiplication factor for the different consistencies to be applied to the egg counts making it comparable (Archer *et al.*, 1997). Each homogenised faecal sample was dispersed in 4 ml of 10 % aqueous formalin (Centrolab) and stored in a Bijou bottle (Sterilab). Fixed stool specimens were processed for light microscopy according to standard methods (Ash and Orihel, 1991; WHO, 1994). Eggs were counted under a 10 x objective of a light microscope and eggs per gram (epg) of stool was expressed per 1 g of faeces according to the following formula:

$$\text{number of eggs/g (epg)} = \frac{\text{(egg count)(consistency factor)}}{\text{weight of sample in grams}}$$

Table 2.1 Stool consistencies

Stool Consistency		**Multiplication factor**
Formed	(F)	Egg count x 1.0
Semi-formed	(SF)	Egg count x 1.5
Mushy	(M)	Egg count x 2.0
Mushy-diarrhoea	(MD)	Egg count x 3.0
Diarrhoea	(D)	Egg count x 4.0
Watery	(W)	Egg count x 4.5

Determination of a common immunological marker of susceptibility to *M. tuberculosis* (Chapter 7)

2.1.3 Enzyme-linked immunosorbent assays (ELISA)

2.1.3.1 Solutions and reagents required for ELISA

1) Phosphate Buffered Saline (PBS)

8 mM Nacl

2.6 mM KCL

8mM Na_2HPO_4

1.4 mM KH_2PO_4., pH 7.4

2) Tris-buffered Saline (TBS)

20 mM TRIS,

150 mM NaCl,

pH 7.3

0.2 μm filtered

3) Substrate Solution

1:1 mixture of Colour Reagent A (H_2O_2) and Colour Regeant B (Tetramethylbenzidine), (R&D Systems Cat # DY 999)

4) Stop Solution

2N H_2SO_4

5) De-lipidised bovine serum

DuoSet Diluent Concentrate 1, (R & D Systems Catalog # DY 999)

6) Microtiter plates

Costar EIA plates, (Cat. # 2592)

2.1.3.2 Whole blood assay (WBA)

The following protocol was performed in a Biosafety Level III facility: Blood was diluted 1: 10 with RPMI to a final volume of 25 ml after which 5 ml aliqouts of the diluted blood was made into 25 cm^2 tissue culture flasks. For each patient, blood was unstimulated and stimulated with 3.3 μg/ml PPD (PPD was a gift from Prof S. Ress, Clinical Immunology, Groote Schuur Hospital, Cape Town) and 1 x 10^5 colony forming units of *M. tuberculosis* H37Rv (ATCC 27294). In total, three flasks (*i.e.* unstimulated, PPD and *M. tuberculosis* stimulated) per patient were dedicated to cytokine measurement. After two and seven days in culture, supernatants were removed and stored at –20 °C for analysis by means of the ELISA technique.

2.1.3.3 Enzyme-linked immunosorbent assays (ELISA)

The production of IFN-γ, IL-12, IL-2, TNF-α, IL-10, and TGF-β were measured by the ELISA technique using kits obtained from R & D Systems. The ELISAs were performed according to instructions supplied by manufacturers. The kits contained a Capture Antibody (Ab) (anti-human-cytokine), Detection Ab (biotinylated anti-human cytokine), Standard (recombinant human cytokine) and Streptavidin conjugated to horseradish-peroxidase (HRP). Microtiter plates were obtained from Costar. The general ELISA protocol was the same for all six cytokines measured, except in isolated instances which will be highlighted. For the measurement of each cytokine, the optimal dilution of tissue culture supernatants, working concentrations of each Ab and standard, were previously determined in our laboratory (Table 2.2). All standards and samples were assayed in duplicate.

2.1.3.3.1 Plate preparation

The capture Ab was reconstituted and diluted to the working concentration (Table 2.2) after which 96-well micotiter plates were coated with 100 μl of the diluted antibody. The plates were sealed and incubated overnight at room temperature. The following day, plates were washed with Wash buffer (Table 2.3) repeating the process twice for a total of three washes. In our laboratory, a squirt bottle was used for the washing process. After the last wash, any remaining Wash Buffer was removed by inverting the plates and

blotting it against a clean paper towel. The plates were then blocked by adding 300 µl of Block Buffer (Table 2.2) to each well and incubated for one hour after which the plates were washed following the same above-mentioned washing process.

2.1.3.3.2 Assay procedure

A 100 µl of diluted sample (supernatant harvested from whole blood assay; described in Section 2.1.3.2) (Table 2.2) and a serially diluted standard was added to the plate in duplicate. The plates were covered and incubated for two hours at room temperature followed by a washing step. 100 µl of diluted Detection Ab was added to each test well and incubated for another two hours.

This was followed by another washing step after which, 100 µl peroxidase-labelled streptavidin reagent was added, which binds to biotin in the immune complex on the plate, and incubated at room temperature for 20 minutes. The wells were then 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.

Table 2.2 Concentrations at which samples and reagents were used in ELISA

	CTRL Dilution	**Sample** Dilution	**1ndAb** Dilution	**2ndAb** Dilution	**STD** Dilution
IFN-γ	1:2	1:11	4 µg/ml	100 ng/ml	1000 pg/ml
IL-12	1:2	1:2	4 µg/ml	150 ng/ml	1000 pg/ml
IL-2	1:2	1:2	2 µg/ml	50 µg/ml	1000 pg/ml
TNF-α	1:5	1:2	4 µg/ml	150 µg/ml	1000 pg/ml
IL-10	1:2	1:2	2 µg/ml	50 µg/ml	1000 pg/ml
TGF-β	1:15	1: 15	2 µg/ml	150 µg/ml	1000 pg/ml

CRTL: Unstimulated sample

1nd Ab: Primary antibody

2nd Ab: Secondary antibody

STD: Standard

The above-mentioned procedure was the same for cytokines measured, except in the case of TGF-β. As TGF-β1 is in a latent form, it had to be activated first by the addition of 1N HCl to acid activate the samples followed by the addition of 1.2 N NaOH/0.5 M HEPES for subsequent neutralisation. The following procedure was followed:

10 µl of 1N HCl was added to 50 µl of sample. The samples were mixed well and incubated for 10 minutes at room temperature. For the neutralisation of the samples, a further 10 µl of 1.2 N NaOH was added to the samples. Diluted sample and standard were added to plates and the general assay procedure was followed.

Table 2.3 Preparation and dilution of reagents during ELISA

Cytokine	Wash buffer	Block buffer	Reagent Diluent
IFN-γ	0.05 % Tween 20 in PBS, pH 7.4	1 % BSA, 5 % Sucrose in PBS with 0.05% NaN_3	0.1 % BSA, 0.05 % Tween 20 in TBS
IL-12	0.05 % Tween 20 in PBS, pH 7.4	1 % BSA, 5 % Sucrose in PBS with 0.05% NaN_3	0.1 % BSA, 0.05 % Tween 20 in TBS
IL-2	0.05 % Tween 20 in PBS, pH 7.2-7.4	1 % BSA, 5 % Sucrose in PBS with 0.05% NaN_3	0.1 % BSA, 0.05 % Tween 20 in TBS
TNF-α	0.05 % Tween 20 in PBS, pH 7.2-7.4	1 % BSA, 5 % Sucrose in PBS with 0.05% NaN_3	0.1 % BSA, 0.05 % Tween 20 in TBS
IL-10	0.05 % Tween 20 in PBS, pH 7.2-7.4	1 % BSA, 5 % Sucrose in PBS with 0.05 % NaN_3	0.1 % BSA, 0.05 % Tween 20 in TBS
TGF-β	0.05 % Tween 20 in PBS, pH 7.2-7.4	1 % BSA, 5 % Sucrose in PBS with 0.05% NaN_3	1.4 % de-lipidised bovine serum, 0.05 % Tween 20 in PBS

2.1.4 Fluorescence activated cell scanning (FACS) analysis

2.1.4.1 Solutions and reagents required for FACS

FACS Lysing solution were provided by the Manufacturers (Beckton Dickinson) (BD) The monoclonal antibodies used were obtained from Becton Dickinson.

2.1.4.1.2 Fluorescence activated cell scanning (FACS) analysis

The standard protocol provided by Becton and Dickenson for three colour staining was adjusted to the following protocol:

2.1.4.1.2.1 Preparation of cells for ex *vivo* analysis by FACS

Heparinised blood obtained from subjects, was diluted 1: 10 with RPMI (RPMI 1640 with glutamine, Gibco) to a final volume of 10 ml after which the diluted blood was centrifuged at 1 800 RPM for five minutes at room temperature. The supernatant was removed and the pellet resuspended in FACS buffer (PBS, 1 % Fetal Calf Serum) and washed twice, by centrifuging at 1 800 RPM for 5 minutes. After the second wash, the pellet was resuspended in 1 ml FACS buffer (PBS, 1 % Fetal Calf Serum) and aliquoted into FACS tubes. All antibodies used were diluted 1: 4 with FACS buffer, afterwhich 20 µl of diluted antibody was added to the FACS tube. For each assay (*i.e* for each patient), which included samples harvested on day 0 and day seven, the FACS tube would contain the following combination of pre-aliquoted antibodies:

Tube 1: α-CD45-PerCP, α-CD3-PE, α-CD4-FITC
Tube 2: α-CD45-PerCP, α-CD3-PE, α-CD8-FITC
Tube 3: α-CD45-PerCP, α-CD4-FITC, α-CD25-PE
Tube 4: α-CD45-PerCP, α-CD8-FITC, α-CD25-PE

It is important to note that the dye conjugated antibodies have the same excitation wavelength (488 nm), but different emission maxima: PerCP: > 650 nm (red); PE: 575 nm (orange-red) and FITC: 525 nm (green). The cell-antibody suspension was incubated at room temperature for 30 minutes. This was followed by a another washing step whereby the FACS buffer (PBS, 1 % Fetal Calf Serum) was added to the cell-antibody suspension and spun at 1 800 RPM for 5 minutes. The cells were then incubated for 10 minutes at room temperature with 2 ml of the FACS lysing buffer (1.5 % formaldehyde, 5 % diethyleneglycol; BD). After lysis, followed by a final washing step, the cells were fixed by the addition of 300 µl of 4 % paraformaldehyde (PBS and Formaldehyde). The fixed cells were kept at 4°C overnight for FACS analysis the following day.

2.1.4.1.2.2 Preparation of cells after seven days in culture

The following protocol was performed in a Biosafety Level III facility: Blood was diluted 1: 10 with RPMI to a final volume of 25 ml after which 5 ml aliqouts of the diluted blood was made into 25 cm^2 tissue culture flasks. For each patient, blood was stimulated with 1 x 10^5 colony forming units of *M. tuberculosis* H37Rv (ATCC 27294). After the 7 day incubation period, the same protocol was followed as mentioned above for the fresh blood. The only difference would be that directly after culture, the supernatants were removed and discarded after which the cells in the tissue culture flasks were resuspended in FACS buffer (PBS, 1 % Fetal Calf Serum) and transferred to a 50 ml tube, for washing.

2.1.4.1.2.3 Three colour staining

For immunofluorescence staining, cells were anaylsed by using a flow cytometer (Bection Dickinson). Analysis was performed using a software application (CELLQUEST, Becton Dickinson). In all cases, the CD45 antibody was used to gate the leukocyte population. For each assay, a total of 3 000 gated events were acquired per tube (discussed in Chapter 7).

2.1.5 Full blood count (FBC) measurement

The measurement of full blood counts (FBC) was performed in the Department of Heamatology, Tygerberg Hospital by means of routine laboratory methods using a Coulter Counter.

2.1.6 Statistics

2.1.6.1 Correlation by means of Spearman's rho (rank correlation)

Spearman rank correlation (SPSS) was used throughout this section to determine the association between parameters. Spearman's rho is used in situations where the numerical variables are not normally distributed. There are however several characteristics of the correlation that deserve mention. The value of the correlation coefficient (***r***) is independent of the particular units used to measure the variables. However, the value of ***r*** is influenced by extreme values and thus does not provide a good description of the

relationship between two variables when the distribution of the variables is skewed or contain outlying values.

2.1.6.2 Significance (One-way ANOVA)

The Kruskal-Wallis non-parametric test was used to compare three or more unpaired groups.

CHAPTER THREE

IMMUNOLOGICAL AND ENVIRONMENTAL FACTORS ASSOCIATED WITH A HIGH TB NOTIFICATION RATE IN A DEFINED COMMUNITY

3.1 Background

The average African human host is exposed to a huge number of infectious agents from early childhood. These agents would include bacteria, viruses and parasites (Annan *et al.*, 1986; Hodes *et al.*, 1988; Bentwich *et al.*, 1995). In the light of this, it would be expected that increased signs of immune activation could occur in such affected populations. This notion was confirmed by Bentwich and co-workers (1995) showing that such activation shifts the cytokine balance towards a type 2 response, which makes the host more susceptible to infectious agents.

The Western Cape region of South Africa has one of the highest reported incidences of tuberculosis in the world (Beyers *et al.,* 1996). Investigations in a suburb of Cape Town revealed marked variations in the incidence of TB within these suburbs. The poorest areas within this community have the highest TB incidence. However, the driving force behind the epidemic cannot be attributed solely to crowding, which facilitates rapid transmission of *M. tuberculosis*, poor socio-economic conditions or reactivation of latent infection (Warren *et al.,* 1996). As a result of people living in these communities being continually exposed to infectious agents, we suggest that environmental factors and biased immune activation could be responsible for enhanced susceptibility to the infectious agents. This notion is supported by Beyers and co-workers (1998)

demonstrating a marked correlation between total serum IgE levels and the notification rate of TB in the various sub-districts in Cape Town. Taken together, it is possible that immune activation, specifically a Th2 dominance (manifested by high serum IgE levels) could contribute to the high notification rate of TB in especially the above-mentioned communities.

3.2 Hypothesis

Changes in the host immune response may account for the high incidence of tuberculosis in the study community. Such changes would render the host more susceptible to *M. tuberculosis* infection and less capable of controlling the infection once it is acquired.

It is well recognised that an effective type 1 immune response is required for protection against *M. tuberculosis*. Given that such responses are downregulated by type 2 responses, we postulate that individuals with prominent type 2 immune responses may have an increased susceptibility to *M. tuberculosis*. As class-switching from IgG to IgE cannot occur without the presence of IL-4 and IL-13 (Kuhn *et al.,* 1991; Snapper *et al.,* 1991), the production of IgE serves as a perfect bio-assay for the presence of Th2 cells *in vivo*. We therefore hypothesise that in the study population, elevated serum IgE levels, the surrogate marker of type 2 activation, will correlate with the high notification rate of TB well as with environmental factors.

3.3 Aims of this study

1. To measure serum IgE levels in healthy individuals living in the community.
2. To characterise the serum IgE levels per census block or enumerator sub-district (ESD). For this reason, approximately five individuals per ESD were recruited.
3. To correlate the levels of total serum IgE of normal healthy adults in this community with the TB notification rate, as well as with the socio-economic index, crowding and female literacy in the ESDs of this community.

3.4 Materials and methods

3.4.1 Study area

The investigation was carried out in two adjacent suburbs of Cape Town, South Africa. These suburbs cover an area of 3.4 km^2, and have a population of 38 656 (1996 census). The total notified cases of TB in the area and in these suburbs were recorded in 1995 as 1 339/100 000 (Beyers *et al.,* 1996). The area is served by two local authority health clinics and a tertiary care referral hospital adjacent to the suburbs. BCG coverage is more than 90 % as it is routinely administered in the neonatal period (Kibel *et al.*, 1995).

3.4.2 Sample populations

Serum IgE levels were previously measured in this community, but only 140 individuals could be recruited (Beyers *et al.*, 1998). In the present study however, IgE levels were not only determined on 519 healthy individuals (≥ 15 years of age), but also on 48 healthy children (< 15 years of age). Most of the recruited individuals were mothers bringing their children for visits to the clinics for routine evaluations or immunisations. This resulted in a preponderance of females in this cohort. These individuals were either Mantoux skin test positive or negative, with no prior or present clinical manifestation of disease. The healthy individuals recruited had no history of TB, asthma, diabetes, hypertension, auto-immune diseases or presented with any acute infections. As HIV status is not determined routinely, the status of the recruited individuals is unknown. However, it is known that the prevalence of HIV infection in the Western Cape is low. Informed consent was obtained from each of the individuals included in this study. The study was also approved by the Ethics Committee of the Faculty of Medicine, Stellenbosch University.

3.4.3 Geographical information system (GIS)

According to the 1996 population census data, the two areas are divided into 39 census blocks or enumerator sub-districts (ESDs). 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).

To calculate the notification rate of TB per ESD, the boundaries used for the 1996 population census report were superimposed on the property boundary coverage. The official records of the Cape Metropolitan Council (CMC), provided data on all notified cases of TB in the area. From these records the address, type of TB and age of each notified case, were collected. The TB notification rate per ESD (for 1996) was subsequently calculated for adults (≥ 15 years of age).

3.4.4 Calculation of demographic variables

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, one value per ESD for crowding, economic status, maternal and paternal education level and socio-economic status, were calculated. The TB notification rate could be related to demographic, social and economic variables in each ESD. These variables were calculated as follow:

- Crowding was calculated as the average number of adults (> 15 years of age) per room (sleeping rooms and living rooms). (According to the census report, living rooms included the kitchen as well).
- 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 school education (7 years of schooling).
- The economic index was equal to the average income per adult.
- The educational index was determined by the average adult education level by yearly standard.
- A socio-economic index for each ESD was calculated as follows:

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

3.5 RESULTS

3.5.1 Serum IgE levels

Total serum IgE levels were determined on 567 healthy individuals from Ravensmead and Uitsig. These individuals had no previous history of TB and were either Mantoux (+) or Mantoux (-). The ages in this particular study population ranged from 1 to 83 years.

This group was further divided into two groups, namely children (< 15 years of age; n = 48) and adults (≥ 15 years of age; n = 519). In Table 3.1, descriptive statistics are given for the total population (1- 83 years of age), of children and adults defined by age. In order to determine whether adults had dominant type 2 responses, only serum IgE concentration in adults (*i.e.* ≥ 15 years of age) was considered. The normal mean serum IgE for the adult "coloured" population of the Western Cape has been described as being < 200 kU/l. Thus, in order to compare serum IgE levels described in this section with that of the normal levels, we would be referring to the mean serum IgE measured and not the median (unless otherwise mentioned).

A mean serum IgE level of 380.5 ± 664.7 (median = 125.4) kU/l was calculated for the total population of 567 individuals aged 1 – 83 years of age (mean age = 28.8 yrs). The highest recorded IgE concentration was 4 887.5 kU/l. In the adult population (≥ 15 years of age) (mean age = 31.0 yrs) a mean IgE concentration of 373.7 ± 667.8 (median = 122) kU/l was measured, which was similar to what we have previously recorded in 141 healthy individuals (Beyers *et al.,* 1998). Although not significantly different from the adults, a mean serum IgE concentration of 454.1 ± 631 (median = 212.0) kU/l was calculated for the 48 children included in the study. The highest IgE concentration measured in the children was 2 998.0 kU/l. Because of the comparable mean and median values, no statistically significant differences in serum IgE were found among the three groups (*i.e.* total population, adults and children).

Table 3.1 Serum IgE levels measured in the total population (1-83 years of age), adults (≥ 15 years of age) and children (< 15 years of age)

	Total population	**Adults**	**Children**
n	567	519	48
Mean age (yrs)	28.8	31.0	5.0
IgE (ku/l)			
Mean ± SD	380.5 ± 664.7	373.7 ± 667.8	454.1 ± 631.0
Median	125.4	122.0	212.5
Minimum	0	0	0
Maximum	4 887.5	4 887.5	2 998.0

3.5.2 Distribution of serum IgE levels in adults and children

As shown in Table 3.2, the majority of adults enrolled in the study (60 %), had normal serum IgE of < 200 kU/l, with the subsequent 40 % presenting with levels above 200 kU/l. Seven percent (7 %) of the adults had levels ≥ 1 000 kU/l.

Three (6.3 %) of the 48 children included in the study, had an IgE concentration above 1 000 kU/l with an overall 52.1 % (n = 25) of the children having levels above 200 kU/l (Table 4.2).

Table 3.2 Distribution of serum IgE concentration in children and in the adult population

Description	**Prevalence**	
	Adults	**Children**
% individuals with serum IgE = 0 kU/l	3/519 (0.6 %)	1/48 (2.1 %)
% individuals with serum IgE < 200 kU/l	309/519 (59.5 %)	22/48 (45.8 %)
% individuals with 1000 ≤ IgE ≥ 200 kU/l	169/519 (32.6 %)	22/48 (45.8 %)
% individuals with serum IgE > 1000 kU/l	39/519 (7.5 %)	3/48 (6.3 %)
% individuals with serum IgE ≥ 200 kU/l*	208/519 (40.1 %)	25/48 (52.1 %)

* Percentage of individuals with serum IgE levels above the normal level of 200 kU/l

3.5.3 Age-related differences in serum IgE concentration

After examining the data by age group the results showed that children between the ages 6 to 14 years (286.2 kU/l) and 1 to 5 years (212.1 kU/l) had the highest median serum IgE levels (Table 3.3.1), rendering them the most vulnerable in terms of susceptibility to *M. tuberculosis*.

Further analysis showed that of the 567 individuals, 232 (40.9 %) individuals aged between 1 and 80 years had an IgE concentration of > 200 kU/l (median = 528 kU/l and mean = 832.0 kU/l). In Table 3.3.2 showed that the IgE concentration was highest in the oldest (median = 677 kU/l) and youngest (median = 556.0 kU/l) age groups. The percentage of individuals in the other age groups with high serum IgE levels, ranged from 24 % (15-20 yrs) to 46 % (31-40 yrs). However, no statistical differences in the measured serum IgE levels were found among the age groups.

Table 3.3.1 Age-related differences in serum IgE concentration

Age groups (yrs)	**n**	**Prevalence**	**Mean ± SD**	**Median**
Total	567			
> 50 years	36	36/567 (63.4 %)	323.2 ± 645.9	81.4
41-50	49	49/567 (86.4 %)	359.7 ± 748.2	122.0
31-40	131	131/567 (23.1 %)	437.5 ± 737.1	163.5
21-30	222	222/567 (39.2 %)	393.5 ± 638.1	124.9
15-20	81	81/567 (14.3 %)	247.4 ± 579.0	84.8
6-14	10	10/567 (1.8 %)	415.4 ± 399.4	286.2
1-5	38	38/567 (6.7 %)	464.3 ± 683.9	212.5

Table 3.3.2 Age-related differences among individuals with serum IgE level > 200 kU/l

Age groups (yrs)	n	Prevalence (%)		Mean ± SD	Median
		X/232	Total population		
Total	232		232/567 (40.9 %)	831.6 ± 855.9	527.8
50 yrs	10	10/232 (4.3 %)	10/567 (1.7 %)	981.7 ± 973.4	677.0
41-50	18	18/232 (7.7 %)	18/567 (3.2 %)	857.0 ± 1079.1	410.0
31-40	61	61/232 (26.9 %)	61/567 (10.7 %)	859.1 ± 914.4	536.4
21-30	98	98/232 (42.2 %)	98/567 (17.3 %)	810.8 ± 780.8	525.0
15-20	20	20/232 (8.6 %)	20/567 (3.5 %)	777.0 ± 1006.1	525.0
1-14	25	25/232 (10.8 %)	25/567 (4.4 %)	813.4 ± 706.4	556.0

3.5.4 Gender-related age differences in serum IgE concentrations

As discussed in Section 3.4.2, there was a preponderance of females in this cohort resulting in 445 females (78 % of study population) and 122 males (22 % of study population) included in this study (Table 3.4). A median IgE value of 117.8 kU/l (mean: 358.9 kU/l ± 643.9) was obtained for the females compared with the median IgE value of 152.1 kU/l (mean: 459.6 ± 732.8) obtained for the males.

Although differences were observed in the median IgE concentrations between males and females in the different age groups, no statistical differences were calculated. However, the results showed that the IgE concentrations were much higher in the younger females (*i.e.* 1-20 years of age), whereas IgE levels in males were higher in the older age groups (*i.e.* 21-50 years of age). The difference between young males and females (*i.e.* 1-20 years of age) was however not significant ($p = 0.533$), whereas IgE levels differed significantly ($p = 0.02$) between males and females in the older age groups (*i.e.* 21-50 years of age).

Table 3.4 Gender-related age differences in serum IgE concentrations

Age groups (yrs)	Females			Males		
	n	Prevalence	Median	n	Prevalence	Median
Total	445			122		
> 50 years	30	30/445 (6.7 %)	88.7	6	6/122 (4.9 %)	75.2
41-50	44	43/445 (9.6 %)	119.5	5	5/122 (4.0 %)	133.2
31-40	106	105/445 (23.6 %)	151.5	25	25/122 (20.5 %)	283.0
21-30	180	180/445 (40.5 %)	115.9	42	42/122 (34.4 %)	224.4
15-20	61	60/445 (13.4 %)	90.3	20	20/122 (16.4 %)	80.0
6-14	5	5/445 (1.1 %)	710.0	5	5/122 (4.1 %)	116.0
1-5	19	18/445 (4.0 %)	216.0	19	19/122 (15.6 %)	135.0

3.6 Association studies

In order to establish the distribution of type 2 responses in the community, serum IgE levels were subsequently calculated for each ESD (Table 3.5.2). Similarly, the 1996 TB notification rate was obtained for in order to establish the distribution of the TB rates in the community (Table 3.5.2). Furthermore, from the Census Report, crowding, female literacy and a socio-economic index (Tables 3.5.2) were calculated on ESD level. In the following section, the association between serum IgE, TB notification rate and the demographic and socio-economic variables will be shown.

3.6.1.1 Geographical information system (GIS)

In Figures 3.5.1 and 3.5.2, the geographical distribution of the TB notification rate and median serum IgE levels per ESD is shown for the two communities. Those areas indicated in red to purple would be the ESDs with the highest median IgE concentration and highest TB notification rate, whereas the areas in yellow would indicate low levels of IgE and TB notification rates. The median serum IgE ranged from 20 – 525 kU/l (Table 3.5.1), while the TB notification ranged from 0 – 1 063/100 000. These distributions confirm the correlation between TB notification rate and serum IgE levels (Figures 3.1.1 and 3.1.2).

Table 3.5.1 Mean and median serum IgE levels measured in the individuals ≥ 15 years of age, with the number of healthy individuals per ESD.

ESD No.	n	Mean Age (yrs)	Serum IgE (kU/l) Mean	SD	Median
1010254	12	24.7	145	163	99
1010255	10	31.2	451	678	64
1010256	5	30.4	265	274	138
1010257	13	29.0	214	241	133
1010258	10	26.9	217	229	92
1010259	13	28.0	143	132	78
1010260	10	31.0	168	255	72
1010261	12	29.4	221	261	112
1010262	6	34.5	45	49	33
1010263	13	32.3	116	79	97
1010264	8	45.6	117	290	472
1010265	8	33.6	339	285	311
1010266	8	39.6	194	193	150
1010267	14	31.1	236	69	387
1010268	15	29.2	189	226	112
1010269	10	27.6	63	96	20
1010270	15	27.1	81	84	49
1010271	7	28.3	92	70	80
1010272	2	21.6	66	27	70
1010273	14	32.1	239	253	141
1010274	16	29.8	288	356	146
1010275	26	26.7	212	484	72
1010276	9	33.1	201	212	99
1010277	8	34.1	111	121	50
1010278	4	33.0	93	157	20
1010279	9	31.5	129	201	35
1020254	25	33.8	770	1 285	330
1020255	35	32.7	961	1 190	485
1020256	24	30.9	647	846	475
1020257	22	30.7	621	759	357
1020258	35	31.8	481	674	189
1020259	23	33.4	687	917	363
1020260	19	27.6	725	895	525
1020261	7	28.5	58	41	45
1020262	11	29.1	456	655	133
1020263	16	28.8	178	233	93
1020264	9	33.4	104	98	67
1020265	6	36.3	173	297	57
1020266	7	29.8	203	146	184

ESD. No. : Number of enumerator sub-district

Table 3.5.2 Ecological factors obtained from the 1996 Census Report

ESD	*Pop.	#TB case rate	Crowding	$SES	@Female Lit.
1010254	7476	625.6	1.3	0.62	76.97
1010255	10094	578.5	1.2	0.71	72.84
1010256	11075	322.2	1.3	-0.09	79.37
1010257	25201	250.6	1.1	0.21	78.33
1010258	91913	420.2	1.1	-1.06	72.16
1010259	16782	1 063.8	1.1	0.22	79.31
1010260	15182	581.4	1.2	-0.40	73.05
1010261	9850	573.6	1.2	0.67	83.96
1010262	12015	0	1.3	1.19	88.81
1010263	8802	377.4	1.3	1.07	85.37
1010264	12142	607.3	1.5	0.85	83.00
1010265	16858	300.8	1.2	0.73	86.44
1010266	14024	545.8	1.1	0.00	77.61
1010267	18786	91.4	1.1	0.45	77.97
1010268	15352	307.4	1.3	0.40	82.52
1010269	19189	183.9	1.1	0.33	75.29
1010270	9048	123.0	1.2	0.99	86.10
1010271	11222	99.3	1.3	1.09	91.33
1010272	11250	338.4	1.3	1.02	85.11
1010273	14938	715.7	1.1	-0.10	74.26
1010274	10898	526.3	1.2	0.29	72.34
1010275	8538	793.7	1.1	0.17	70.09
1010276	11796	253.2	1.1	0.48	73.56
1010277	10055	529.1	1.2	0.60	81.59
1010278	9448	155.9	1.5	1.69	89.31
1010279	8012	0	1.4	1.30	90.04
1020254	19485	714.3	0.8	-0.32	62.06
1020255	15266	846.3	0.9	-0.71	54.94
1020256	15577	536.5	0.9	-0.36	61.61
1020257	16047	921.2	0.9	-0.59	61.93
1020258	12781	864.2	0.9	-0.17	68.75
1020259	11265	580.3	0.8	-0.41	68.06
1020260	22273	443.1	0.9	-0.24	65.61
1020261	88513	123.0	1.0	-0.02	85.88
1020262	104893	0	1.1	-0.05	74.64
1020263	12981	184.7	1.2	0.20	77.98
1020264	11387	264.3	1.4	0.54	90.70
1020265	11037	0	1.4	1.33	86.64
1020266	16448	257.5	1.2	0.65	75.17

* Population: Population density, *i.e.* number of individuals per m^2 per ESD

$SES: Socio-economic index/conditions

@ Female Lit.: % Females (≥ 15 years of age) completing primary school education

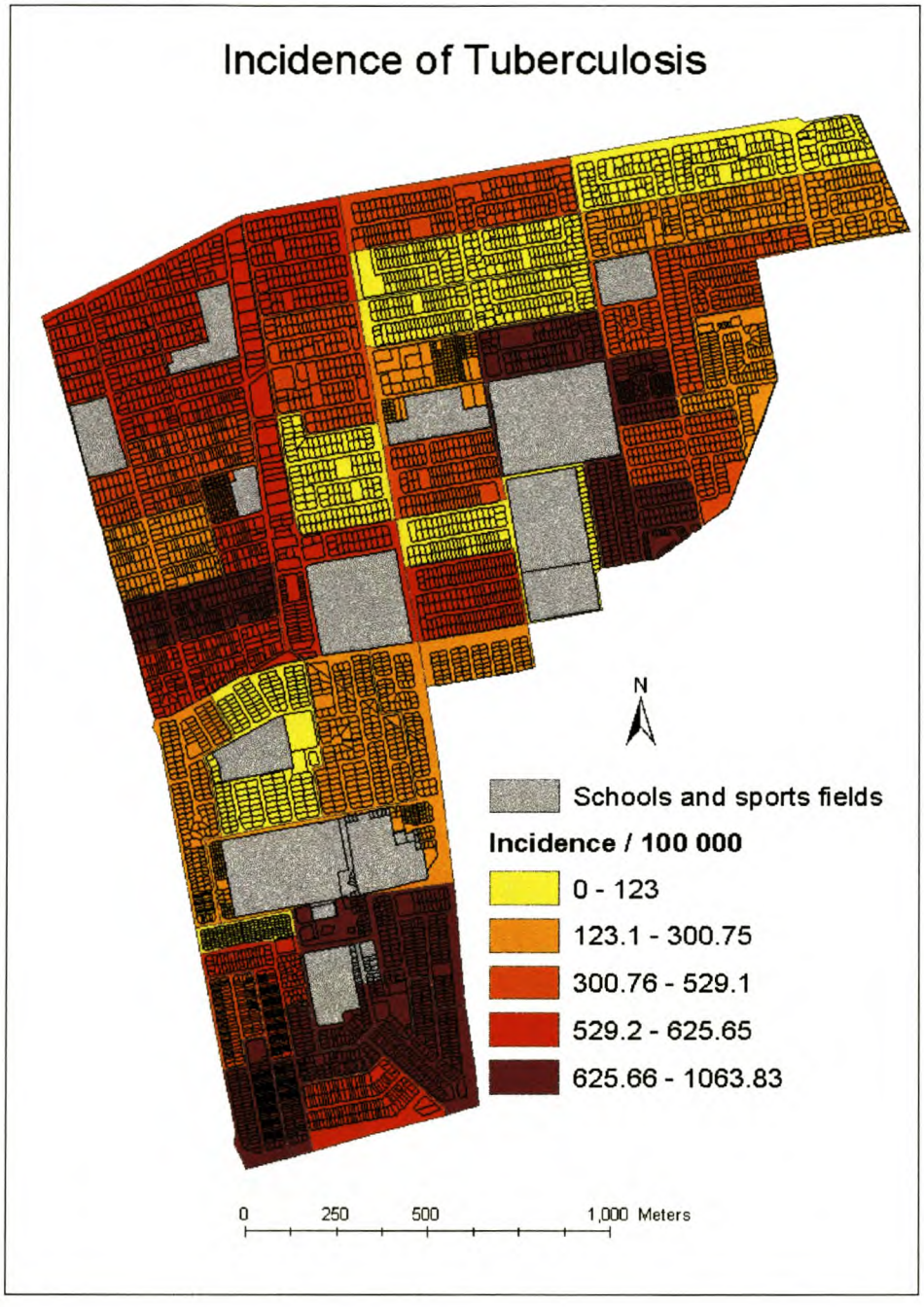


Figure 3.5.1 Geographical distribution of TB notification rates per ESD

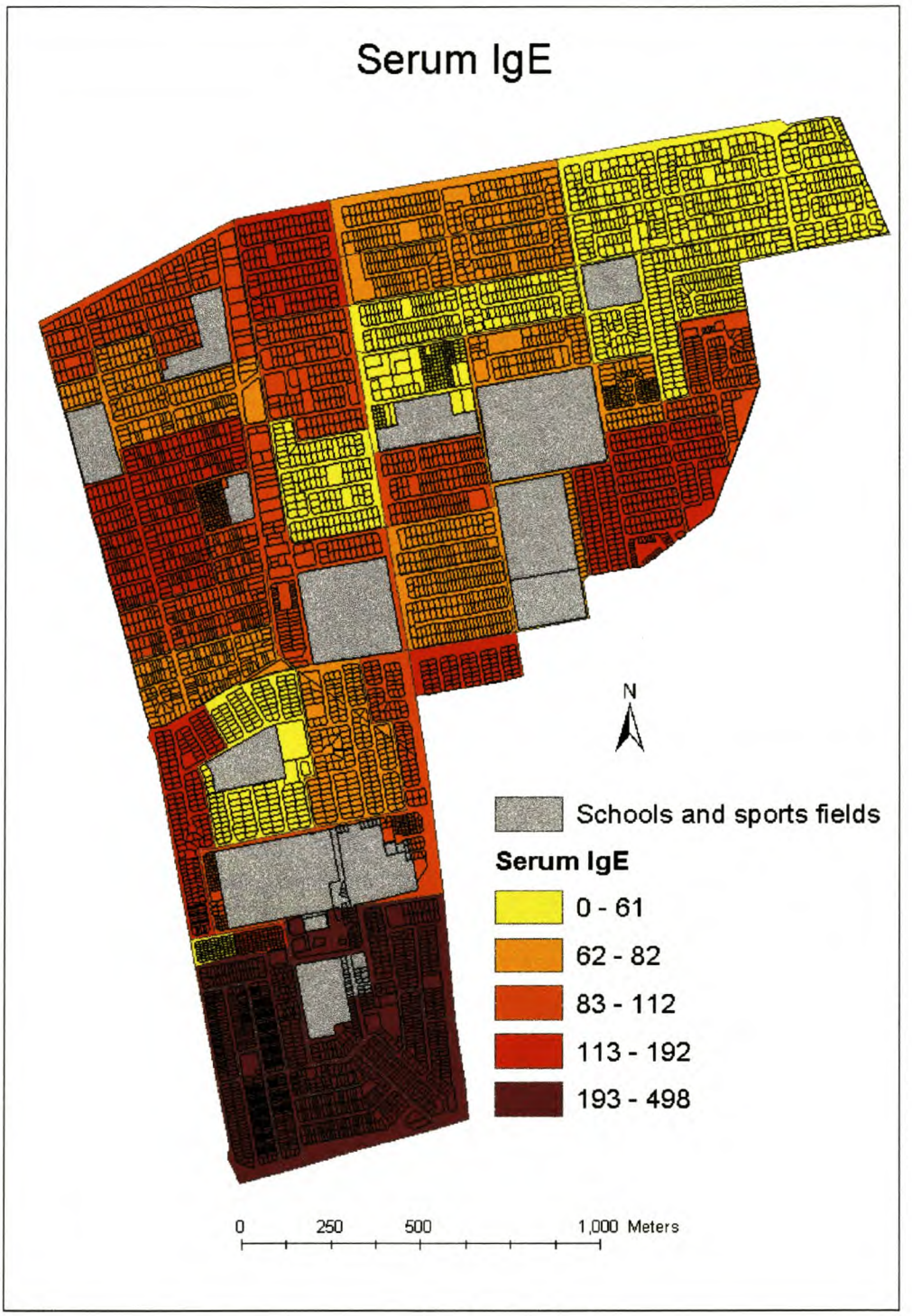


Figure 3.5.2 Geographical distribution of the median serum IgE concentrations per ESD

3.6.1.2 Correlation between serum IgE and TB notification rate

The mean ± standard deviation (SD) and median serum IgE per ESD are shown in Table 3.5.1. Due to the large distribution of IgE values, median LnIgE levels were used for the association studies. Figure 3.2 shows the scatter plot for the correlation between the median serum IgE levels and TB notification rate. A significant correlation was found between these two parameters ($r = 0.53$; $p = 0.005$). Special mention is made of those data points marked in red (■). These three marked ESDs: 1020254; 1020256; 1020260 are located in Uitsig and median serum IgE levels calculated were 330 kU/l, 475 kU/l and 525 kU/l, respectively. The corresponding TB notification rate for the three ESDs were 536\100 000; 714\100 000 and 443\100 000 respectively. These three ESDs will be marked throughout this section of this thesis.

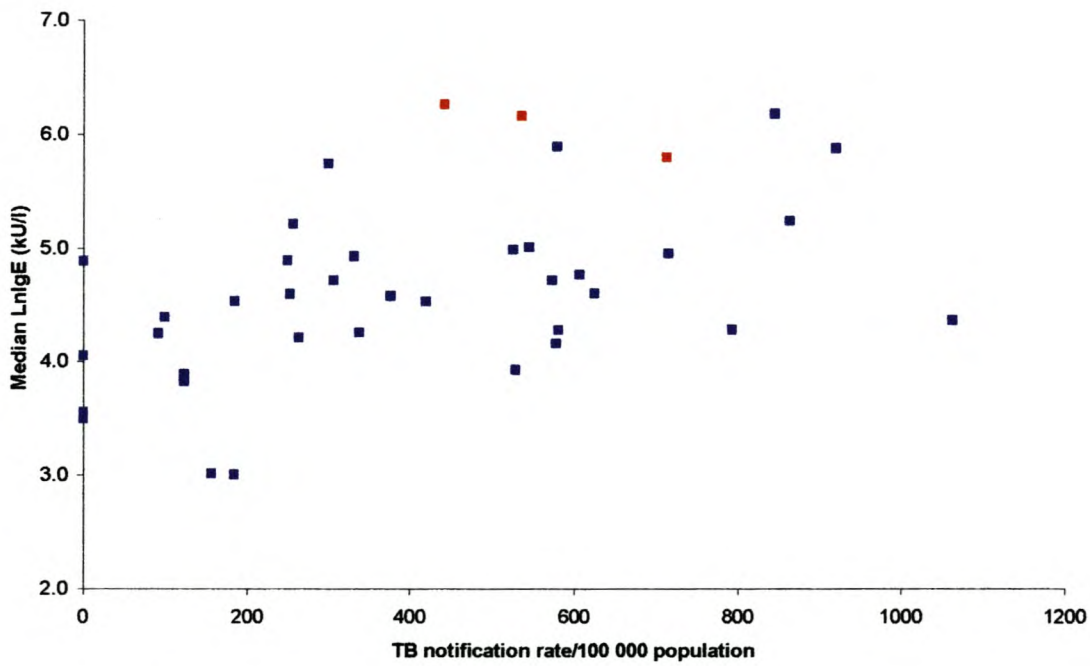


Figure 3.2 Scatter plot of median serum LnIgE levels correlated with TB notification rate

3.6.1.3 Association between the TB notification rate and socio-demographic factors

Crowding, socio-economic status and the percentage of females who have completed primary education (female literacy) could be calculated (formulas described in Section 3.4.4) for each ESD, by using data obtained from the 1996 Census report. The TB notification rate was subsequently correlated with the above-mentioned variables.

Scatter plots of the aforementioned correlations are shown in Figures 3.3.1 – 3.3.2. A significant correlation was found between the TB notification rate and socio-economic status ($r = -0.53$; $p = 0.005$) and TB notification rate vs. female literacy ($r = -0.6$; $p < 0.001$). However, contrary to previous work done by our group (Beyers *et al.*, 1998), a significant inverse association was found between the TB notification rate and crowding ($r = -0.42$; $p = 0.007$). In the three correlations, the three ESDs marked in red (■) had amongst the highest TB notification rates, the poorest socio-economic status, highest level of crowding and lowest percentage of females with primary school education.

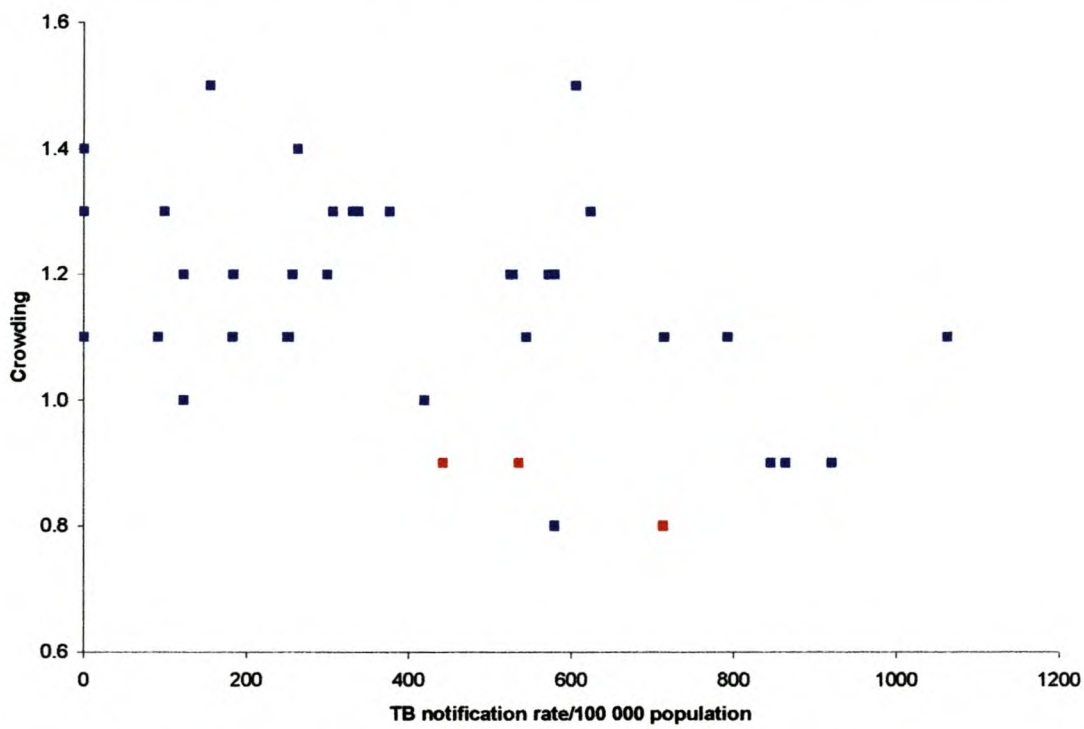


Figure 3.3.1 Scatter plot of the TB notification rate correlated with crowding

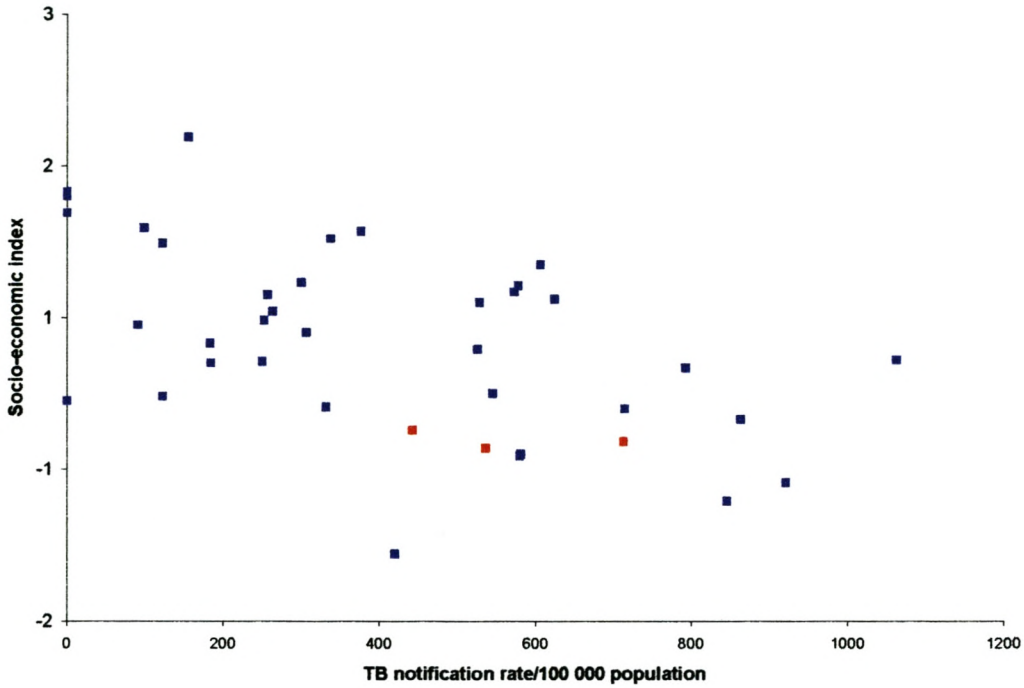


Figure 3.3.2 Scatter plot of TB notification rate correlated with the socio-economic index

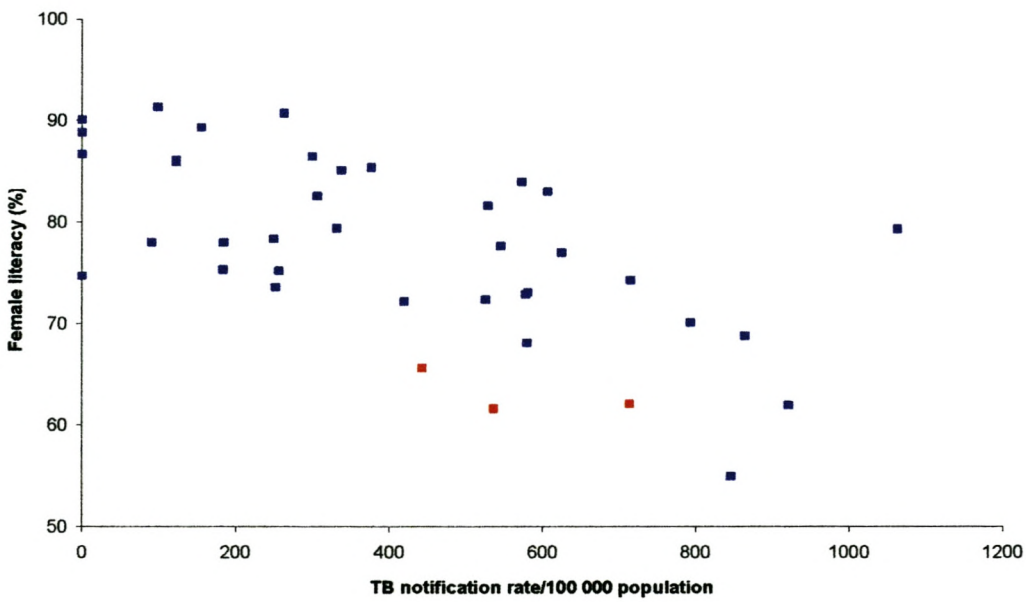


Figure 3.3.3 Scatter plot of the TB notification rate correlated with the % females that have completed primary school education

3.6.1.4 Association between the TB notification rate and population density

The population density for each ESD, is given in Table 3.5.2.

No significant correlation was found between the TB notification rate and population density ($r = 0.36$; $p = 0.13$). Two of the three ESDs marked in red were the most densely populated.

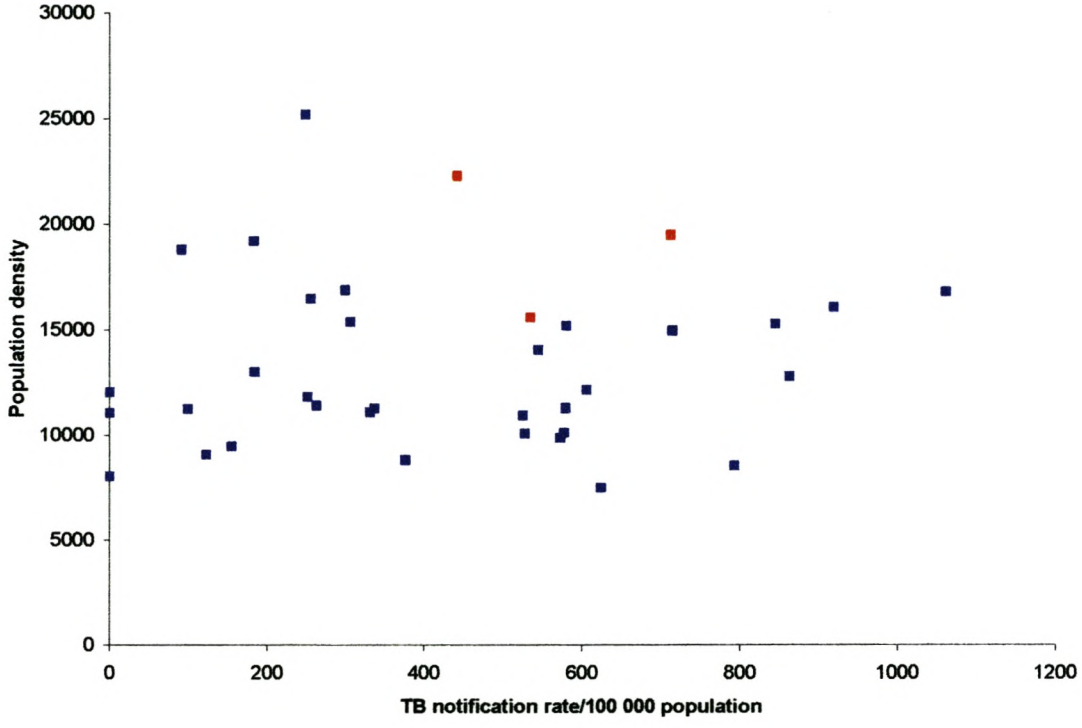


Figure 3.4 Scatter plot of correlation between TB notification rate and population density

3.6.1.5 Association between serum IgE levels and socio-demographic factors

The association between median LnIgE level and crowding, socio-economic status and female literacy were also determined.

As shown in Figures 3.5.1 – 3.5.3, significant inverse correlations were found between median LnIgE vs. socio-economic status ($r = -0.69$; $p = 0.01$) and median LnIgE vs. female literacy ($r = -0.69$; $p = 0.01$). A significant positive correlation was found between median IgE and crowding ($r = 0.52$; $p = 0.01$).

A significant correlation was also found between median serum IgE and population density ($r = 0.44$; $p = 0.01$).

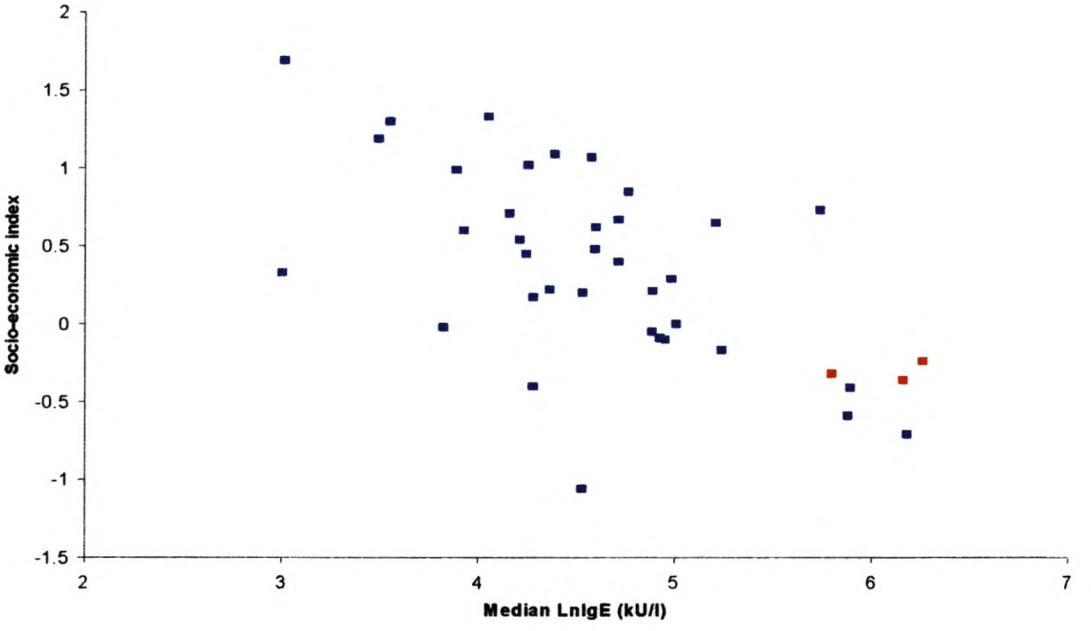


Figure 3.5.1 Scatter plot of correlation between median LnIgE and socio-economic index

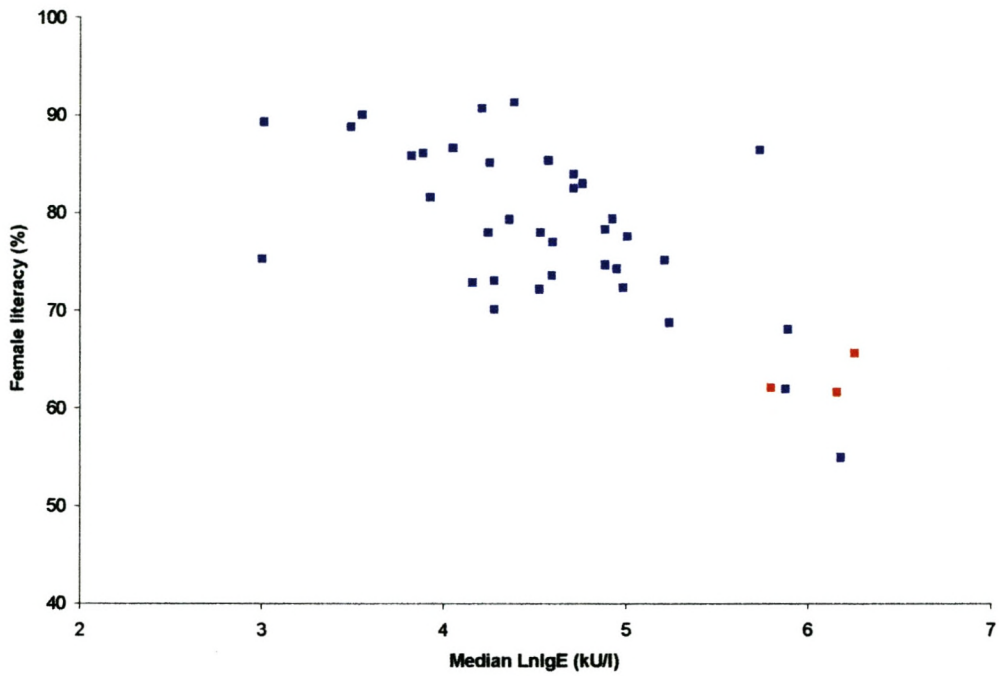


Figure 3.5.2 Scatter plot of correlation between median LnIgE and percentage females having completed primary school education

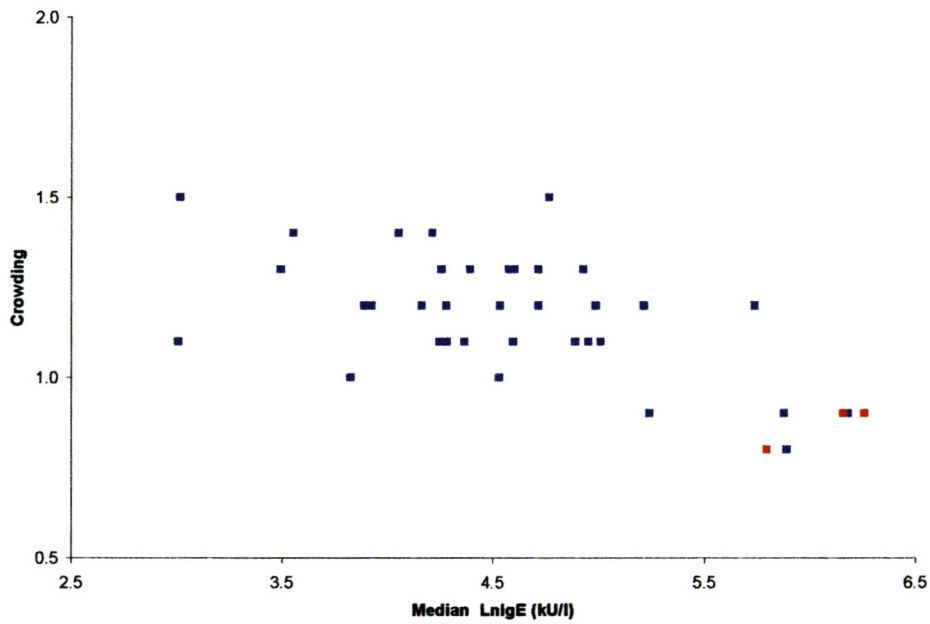


Figure 3.5.3 Scatterplot of correlation between crowding and median LnIgE

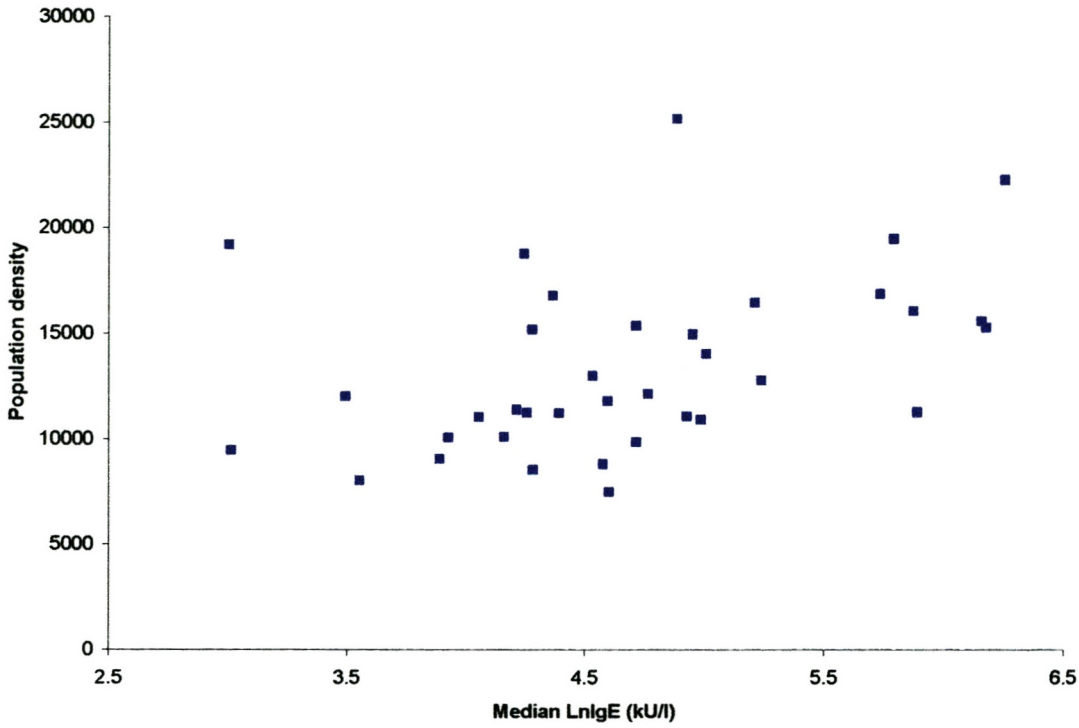


Figure 3.5.4 Scatter plot of correlation between median LnIgE and population density

3.7 Discussion

3.7.1 Serum IgE levels

The results presented in this study showed that 40 % of the adult population had high serum IgE levels. This is similar to levels described in control subjects in a previous study performed by our group (Beyers *et al.,* 1998). Elevated serum IgE levels was also reported in healthy individuals in developing countries such as Indonesia (Yong *et al.,* 1989) and in the Gambia (Nyan *et al.,* 2000). This would support the opinion that in developing countries, the host immune response is biased towards a Th2 immune profile (Bentwich *et al.,* 1999). Bentwich and colleagues (1999) further argued that this biased type 2 immune response, would influence the host's ability to mount a protective cellular response against infectious agents. The high serum IgE levels found in the study community could be the result of helminth infections, resulting in chronic Th2 activation. However, we have not determined the helminth status on these adults. In a pilot study performed in this community, the worm burden was determined on adults with active TB, but results have shown that the majority of these subjects (approximately 98 %) tested negative for helminths (data not shown). The high IgE concentration in these adults could suggest IgE-associated protection against helminths (Faulkner *et al.,* 2002) resulting in chronically activated type 2 immune responses effectively expelling helminths eggs, which in turn would explain the absence of helminth eggs in these adults. It is also possible that dominant type 2 immune responders are genetically and/or environmentally determined, resulting in their inability to mount type 1 responses, thus rendering them highly susceptible to infection with *M. tuberculosis* and progression to disease. However, as elevated IgE concentrations are also synonymous with asthma (Shirakawa *et al.,* 1996; Cookson and Moffat, 1997), atopy (ISAAC 1998; Strachan, *et al.*, 1999) and stress (Rook and Zumla, 1997; Kan and Fox, 2001), further studies on healthy adults in this community are required to determine the reason for their activated type 2 responses. The results have also shown that more than half of the children had the highest measured IgE levels compared to adults. This is not surprising, as high serum IgE levels in children would be ascribed to helminth infections, which in all likelihood would render them susceptible to infection with *M. tuberculosis*. Furthermore, it is interesting to note that

IgE levels in the young females (*i.e.* 1-20 years of age) were higher compared to the young males. In contrast, the older males (*i.e.* 21-50 years of age) presented with higher IgE levels compared to the older females. This findings suggest that young females might be more susceptible and males from the age of 20 years might be more susceptible to *M. tuberculosis* infection and progression to disease. This corroborates previous findings by Daynes and co-workers (1990 and 1995) as well as Fischer and colleagues (1991), suggesting that maturational and hormonal factors may influence the host's immune response and in this particular study, affect their susceptibility to *M. tuberculosis*.

3.7.2 Serum IgE levels and TB notification rate in enumerator sub-districts (ESDs)

The GIS maps clearly showed the marked variation in IgE concentration as well as TB notification rate among the ESDs. These variations could be attributed to the uneven distribution of socio-economic conditions in the community, resulting in different levels of exposure to infectious agents and subsequent burden of disease. The three ESDs that were highlighted, are of the poorest areas in the community and similar areas should be targeted for intervention.

3.7.3 Immunological and environmental factors contributing to the high TB notification rate in the study community

The association studies showed significant correlation between the TB notification rates and serum IgE levels as well as demographic and socio-economic factors. However, it is important to note that at this stage, these are not causal relationships, but only proves an association between the two variables measured. The results also confirmed the assumption that differences in life circumstances may confer different susceptibilities to *M. tuberculosis* (Spence *et al.*, 1993; Classen *et al.*, 1999; Head, 2000).

The significant correlation between IgE levels and the TB notification rates among the ESDs, would suggest that type 2 activation is associated with host susceptibility to *M. tuberculosis* infection and progression to disease. This is in concordance with findings by Pearlman and co-workers (1993), Bentwich and co-workers (1999) and Markus and Fincham (2001) suggesting that dominant type 2 responses could result in susceptibility

to not only *M. tuberculosis*, but also HIV. However, in this study community, case-control studies are required to confirm this notion.

Socio-economic conditions have traditionally been cited as risk factors for infection with *M. tuberculosis* (Dubos and Dubos, 1987; van Rie *et al.*, 1999), HIV and worms. As the socio-economic status of a community is determined by the level of education and economic environment (Section 3.4.4), it is proposed in this thesis that education is an important factor as it directly determines the economic status of a person (and thus the ability to afford a healthy environment). The significant association between the TB notification rate and percentage of adult females having completed primary school education, confirms the notion that the more educated the mother is, the more likely she is to seek help when experiencing symptoms, potentially resulting in a shorter exposure time so that prophylactic steps can be taken. The educated parent will also be more aware of the preventative measures of the disease and will most probably understand the importance of seeking help at a clinic or health institution. Level of education is normally highly correlated with socio-economic status and has been used in many studies as a marker for socio-economic status (Hargreaves and Glynn, 2002). The positive correlation between the TB case rates and the socio-economic index is therefore not surprising.

Surprisingly, an inverse association was found between the TB notification rate and crowding. This is unexpected as crowding has consistently been shown to be associated with tuberculosis (Dubos and Dubos, 1952; Gryzbowski, 1991; van Rie *et al.,* 1999a). Overcrowding facilitates transmission and spread of disease. It is important to note that crowding is calculated as the average number of adults per room, which according to the Census Report, included sleeping rooms, living rooms and kitchens. This obviously would have reduced the number of people per room, thus possibly explaining the inverse association with the TB notification rate. However, there is no doubt that overcrowding is a problem in the study community as Ellis and co-workers (1997) have shown that in some cases homes with three bedrooms (with the living room utilised as one) had to accommodate 16 to 18 people. Furthermore, population density, which refers to the number of people per m^2, did not correlate with the TB notification rates. This proves that

most areas in the study community are in fact densely populated and would therefore not show a correlation with the TB notification rate. Furthermore, it shows that overcrowding in the study community is the norm.

CHAPTER FOUR

DECLINE IN TOTAL SERUM IgE LEVELS AFTER TREATMENT FOR TUBERCULOSIS

The work presented in this chapter was published as:

Decline in total serum IgE after treatment for tuberculosis.

Adams, J.F.A., Scholvinck, E.H., Gie, R.P., Potter, P.C., Beyers, N. and Beyers, A.D.

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This work is an exact copy of the published article, except for the numbering and style that have been changed to conform to style of this thesis. All cited literature is compiled in the Reference list at the end of thesis

4.1 Introduction

It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) and that about 90 % of infected individuals do not develop clinical disease (Israel *et al.*, 1941). The outcome after infection with *M. tuberculosis* is determined by cell mediated immunity. Macrophages infected with *M. tuberculosis* secrete IL-12, which induces the development of type-1 lymphocytes secreting IL-2 and IFN-γ (Abbas *et al.*, 1996). IFN-γ, in turn, activates macrophages and enhances their microbicidal activity. The importance of an appropriate type-1 response for the successful elimination of mycobacterial infections is highlighted by recent reports of uncontrolled mycobacterial infection in patients with defective IFN-γ or IL-12 receptors (Newport *et al.*, 1996; Jouanguy *et al.*, 1996; Altare *et al.*, 1998; De Jong *et al.*, 1998). A type-2 response, on the other hand, is characterised by the secretion of IL-4, -5, -6, -10 and -13 and by the production of non-opsonic antibodies including IgG4 and IgE. This response is usually elicited by helminthic infections or an atopic predisposition.(Abbas *et al.*, 1996) Type-1 and type-2 cells negatively cross regulate each other *in vitro* and in experimental animals: IFN-γ inhibits a type-2 response, while IL-4 inhibits a type-1 response (Abbas *et al.*, 1996).

Delayed type hypersensitivity reactions such as tuberculin skin tests reflect a type-1 response while IgE production reflects a type-2 response. Several recent epidemiological studies indicate that mycobacterial or viral infection may reduce IgE levels and/or suppress atopy (Rook and Stanford, 1998). Japanese schoolchildren who were Mantoux skin test positive had lower IgE levels and type-2 cytokines than tuberculin negative individuals (Shirakawa *et al.*, 1997) and natural measles infection reduces the incidence of atopy to half of that seen in vaccinated children (Shaheen *et al.*, 1996). In mice allergic to ovalbumin, treatment with killed *M. vaccae* inhibits IgE and interleukin 5 responses (Wang and Rook, 1998), and infection with *M. bovis* BCG suppresses ovalbumin-induced airway eosinophilia (Erb *et al.*, 1998). These studies suggest that a type-2 response can be suppressed by a type-1 response *in vivo*. We hypothesised that *M. tuberculosis*-infected individuals, who successfully contained the organism and did not develop disease, would give prominent type-1 response and low IgE concentrations, whereas patients presenting

with tuberculosis would have a less efficient type-1 response and higher IgE concentrations. Furthermore, given that successful outcome after infection with *M. tuberculosis* is driven by a type-1 response, we hypothesised that successful treatment for tuberculosis would down regulate type-2 responses.

4.2 Methods

This study was done in the Western Cape Province of South Africa, where the incidence of TB was 682/100 000 in 1995 (Department of Health, Republic of South Africa, 1996) and where infestation with intestinal parasites, especially *Ascaris lumbricoides* and *Trichuris trichuria*, is common (Gunders *et al.*, 1993). Adolescents were studied, because the incidence of tuberculosis between the ages of 5 and 12 years is low (Starke *et al.*, 1992), because the diagnosis of tuberculosis in adolescents (and adulthood) is more accurate than in childhood (Kahn and Starke, 1995), and because IgE concentrations vary less than in early childhood (Bundy *et al.*, 1991; Needham *et al.*, 1992). Between 1995 and 1998, 50 adolescents with tuberculosis were referred to us from socioeconomically poor suburbs in the Cape Town metropolitan area, where BCG is routinely administered in the neonatal period and where BCG coverage is over 98 %. Controls were recruited by asking each patient to bring along a friend from the same peer and age group. Controls were randomly taken from this group. Of the 50 patients, four were excluded from the study because they did not complete their therapy and 13 were not analysed because serum was not collected for IgE determination at diagnosis and after therapy. The final sample consisted of 33 patients (19 females, 14 males; mean age 15.1 [SD 2.3] years) and 37 controls (18 females, 19 males; mean age 15.2 [SD 1.9] years).

All patients had clinical features consistent with tuberculosis, supported by typical chest radiograph findings or positive cultures for *M. tuberculosis* or both. Chest radiograph findings included cavities in the upper lobes (20 cases), hilar lymphadenopathy (two cases), pleural effusions (seven cases) and four smear or culture-positive cases where we could not classify the chest radiograph findings in any of these categories.

A Mantoux skin test (0.1 ml [5TU] Japanese freeze-dried tuberculin, induration measured after 48-72 hours) was done on 31 patients and 35 controls. All patients included in the study were successfully treated with directly observed short course combination chemotherapy. Venous blood was taken from control subjects and from patients before initiation of treatment and following successful completion of treatment (mean follow-up time 9.8 [1.24] months, after initiation of treatment). We did not do a Mantoux skin test on the patients after treatment.

Total serum IgE levels were measured with a radioimmune assay (Pharmacia, Uppsala, Sweden). On a random selection of sera (which included 23 patients and 33 controls) specific IgE against *A. lumbricoides*, house dust mite, cockroach and Bermuda grass was measured using ImmunoCAP radio-allergo-sorbent (CAP RAST) tests (Pharmacia, Uppsala, Sweden). HIV-1 and HIV-2 infection was excluded in all subjects with a microparticle enzyme immunoassay (AxSYM, Abbott, Wiesbaden-Delkenheim, Germany). All the subjects or legal guardians gave their written informed consent and had HIV-test counselling. The study was approved by the Ethics Committee of the University of Stellenbosch.

Non-parametric statistical tests were used and computed with the help of the SPSS programme. Analyses were done using the Wilcoxon signed-rank test, the Mann-Whitney test for comparison of independent groups and the Spearman rank test for correlations. The Fisher exact test was used to compare specific IgE levels between controls and TB patients. The McNemar exact test was used to compare IgE levels before and after treatment of patients. Individuals with measurable specific serum IgE concentrations (> 0.3 kU/ml) were considered to be responders and individuals with concentrations below 0.3 kU/ml were considered to be non-responders. Level of significance was set at the 95 % cut-off point.

4.3 Results

The mean serum IgE level was 313 kU/l (SD 352, median 171 kU/l) in control subjects and 457 kU/l (SD 455, median 394 kU/l) in patients before treatment (p = 0.085) (Figure

4.1). Mean IgE levels in patients after treatment was 175 kU/l (SD 173, median 129 kU/l). The post-treatment concentrations did not differ significantly from concentrations in controls. In patients the difference in IgE concentration before and after treatment was highly significant ($p < 0.0001$); in every patient the IgE level was higher before than after treatment. The decrease in IgE correlated with the level of IgE on presentation ($r = 0.93$, $p < 0.001$) (Figure 4.2).

Mean *Ascaris*-specific IgE was significantly lower in controls (1.73 kU/l, SD 3.94, median 0.30 kU/l) compared to patients before treatment (4.62kU/l, SD10.89, median 1.10 kU/l) ($p = 0.023$). Mean *Ascaris*-specific IgE in patients following treatment was 2.39 kU/l, SD 5.71, median 0.40 kU/l; $p = 0.0625$). The specific IgE levels for cockroach, house dust mite and Bermuda grass were not significantly different between controls and patients before and after treatment.

Eight controls and two patients did not have any Mantoux skin test induarion. After excluding individuals who showed no induration after Mantoux testing, the mean induration of controls (19.05mm, SD 6.52) did not differ from that of patients before treatment (21.8mm, SD 5.69). The size of Mantoux induration correlated inversely with serum IgE ($r = -0.406$; $p = 0.023$) in patients, but no statistical difference between these two correlation coefficients (Fischer z test) could be demonstrated.

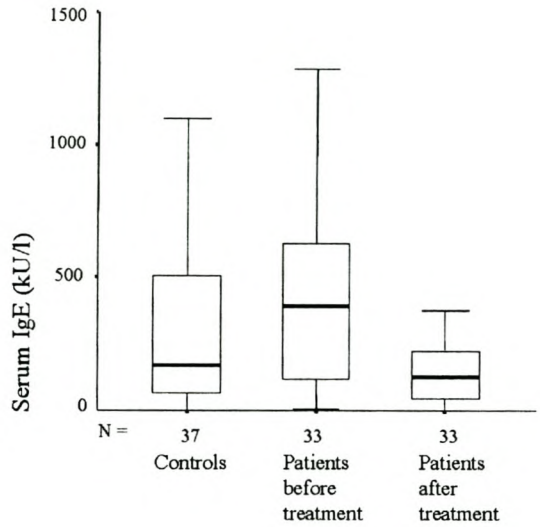


Figure 4.1 Serum IgE concentrations in controls, patients before treatment and patients after treatment for tuberculosis. Median IgE (171 kU/l), 394 kU/l and 129 kU/l in controls, patients before treatment, and patients after treatment, respectively) are indicated with horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles.

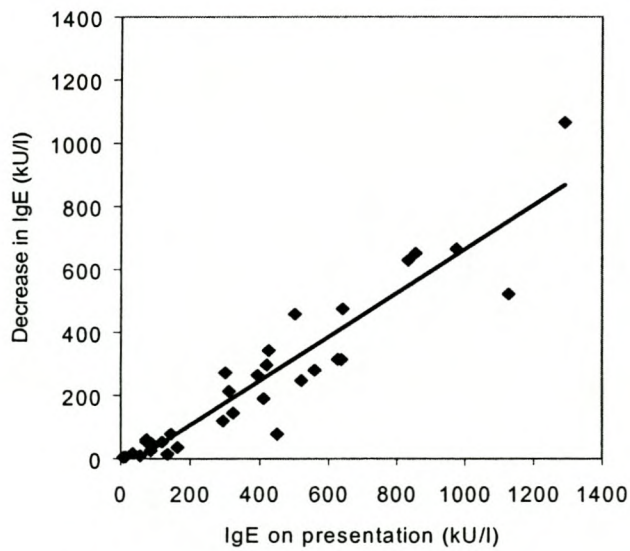


Figure 4.2 Correlation of serum IgE concentrations of patients on presentation with the decrease in serum IgE concentrations following treatment of tuberculosis

4.4 Discussion

This study was done in a community with a high incidence of tuberculosis (Beyers *et al.*, 1996) and a high infestation rate with the parasites *A. lumbricoides* and *T. trichuria* (Gunders *et al.*, 1993) a situation which is common in many developing countries. Most control individuals (28 of 36 tested) had Mantoux test indurations over 15 mm and the distribution of skin-test responses was similar to that of patients, indicating that most controls had been exposed to *M. tuberculosis* (Snider, 1982; Arnadottir *et al.*, 1996). Mean total serum IgE levels in both the controls and patients presenting with tuberculosis were higher than the normal range for individuals living in better socio-economic circumstances. This may reflect the high level of intestinal parasite infestation in the study community. At presentation with tuberculosis, patients had higher total IgE levels than controls, but the difference was not statistically significant. Therefore, the present study does not support our hypothesis that healthy individuals infected with *M. tuberculosis* have lower type-2 responses than patients with tuberculosis. One group, however, previously reported lower IgE concentrations in health workers exposed *to M. tuberculosis* than in a large cohort of Indonesian patients presenting with tuberculosis (Yong *et al.*, 1989).

The key finding from this study is a pronounced and consistent decline in IgE concentrations in patients after successful treatment for tuberculosis. Trivial reasons for the striking decrease in total IgE levels in tuberculosis patients are unlikely. First, reduction in IgE levels by antituberculosis drugs has not been documented. On the contrary, rifampicin binds to and activates the glucocorticoid receptor (Calleja *et al.*, 1998) and there is mounting evidence that glucocorticoids can enhance type-2 rather than type-1 responses (Ramirez *et al.*, 1996). Second, reduction in the parasite burden, which could decrease IgE concentrations, has not been described for antituberculosis drugs. Third, we could not document that any of the patients received antihelminthics during their tuberculosis treatment. Serum IgE was not repeated 9 months later in the control individuals, but IgE concentrations in worm-infested populations rapidly increase during childhood and thereafter stabilise in early adulthood, or gradually decline over many years (Bundy *et al.*, 1991; Needham *et al.*, 1992). Significant changes in the IgE

concentrations of the control individuals are therefore highly unlikely. Taken together, the decline in IgE levels in patients following successful treatment of tuberculosis supports the hypothesis that successful treatment of tuberculosis is associated with down regulation of type-2 responses. Previous publications that documented the reversal of skin-test anergy (Maher *et al.*, 1992) and an increase in T-cell responses (Wilkinson *et al.*, 1998) support the concept of enhanced type-1 responses after chemotherapy of tuberculosis.

We did not examine stool specimens to quantify the rates and severity of parasite infestation in our patients, but more patients with tuberculosis had ascaris-specific IgE antibodies than controls. Parasite infestation and tuberculosis are both associated with poverty and social deprivation (Maizels *et al.*, 1993; Spence *et al.*, 1993). 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 *A. lumbricoides* contributes to susceptibility to tuberculosis. It is noteworthy that *A. suum* impairs T cell function in mice (Ferreira *et al.*, 1995) and that ascaris spends a stage of its lifecycle in the lungs, where it may induce a local type-2 immune response. Studies in mice (Pearce *et al.*, 1991; Actor *et al.*, 1993; Pearlman *et al.*, 1993) and human being (Greene *et al.*, 1985; Sartono *et al.*, 1995) indicate that parasite-induced type-2 dominance may suppress cell mediated immunity. Bentwitch and colleagues (1995) hypothesised that intestinal parasites exacerbate the course of HIV infection (Bentwich *et al.*, 1995), which like tuberculosis is controlled by cell mediated immunity. In 1994, Bundy and co-workers calculated that 28 % of the world's population is infected with ascaris and 25 % with *T. trichuria*, causing significant morbidity (Chan *et al.*, 1994). If intestinal helminths indeed exacerbate HIV disease or tuberculosis, their burden on global morbidity may be even higher.

The inverse correlation between IgE concentrations and Mantoux size in patients may be due to cross regulation of type-1 and type-2 response. In this study the apparent lack of a similar correlation in controls may be due to small size of the cohort studied. Shirakawa and colleagues (1997) found lower IgE levels in a large cohort of Japanese

schoolchildren who were tuberculin skin-test positive and reasoned that mycobacterial infections may reduce type-2 responses including atopy.

In conclusion, this study and others (Shirakawa *et al.*, 1997; Rook and Stanford, 1998; Wang and Rook, 1998; Erb *et al.*, 1998) suggest that the immune response to *M. tuberculosis* and to nonpathogenic mycobacteria suppressses a type-2 response, which plays a key role in the induction and maintenance of high IgE concentrations in parasite infestation and in disorders such as atopy.

CHAPTER FIVE

PREVALENCE OF HELMINTHS IN A COMMUNITY WITH A HIGH TB NOTIFICATION RATE

5.1 Prevalence of helminths in the epidemiological field site

5.1.1 Introduction

In the previous section of this thesis, we found that polyclonal serum IgE levels were higher than normal in our community of research. The next aim therefore was to determine the reasons for the high serum IgE levels. Three possible explanations were identified namely, atopy, stress and helminth infection. In the current study however, it was decided to measure the helminth burden in children in the study community. Helminths are known to be potent inducers of the type 2 immune response. An overview of atopy, stress and helminths as inducers of the type 2 immuen response is given per introduction to this chapter.

Allergic reactions to common environmental antigens such as those derived from house dust mites, plant pollens or animal proteins, lead to clinical disorders such as asthma, hay fever, eczema and allergic rhinitis (Erb, 1999). These symptoms are associated with high levels of IgE and allergen-specific IgE (Saban *et al.,* 1994; Shakib *et al.,* 1994; Wand and Rook, 1998) and eosinophilia and are dependent on, IL-4 and IL-5. In fact, it is estimated that 10 – 30 % of the population in developed countries can be considered to suffer from clinical allergy (Yazdanbakhsh *et al.,* 2001). However, there is a considerably lower prevalence of allergic diseases in developing countries (The International Study of Asthma and Allergies in Childhood

(ISAAC) Steering committee 1998; Yazdanbakhsh *et al.,* 2002) compared with developed countries.

Psychological stressors have been shown to induce a wide range of immunological alterations in cell-mediated and humoral immunity (Glaser and Kiecolt-Glaser, 1994) mediated by changes in cytokine production (Kang and Fox, 2001). When animals were placed in a stressful environment, a marked increase in plasma IL-6 levels was noted, demonstrating an acute change in response to stress (Zhou *et al.,* 1996). The findings on stress and cytokine production in human studies are however, less clear. Some studies have found that stressful events lead to a significant decrease in IFN-γ production and an increase in IL-1 and IL-2 production (Glaser *et al.,* 1990; Dobbin *et al.,* 1991), whereas others reported no change in IL-2 and IL-6 levels following exposure to stressors (Zakowski *et al.,* 1992; Dugue *et al.,* 1993). Despite these later findings, it is hypothesised that stress exposure favours a shift towards a Th2 profile. It has been postulated by Rook and Zumla (1997) that Gulf War syndrome (Chronic Fatigue syndrome) in Gulf War veterans, was the result of a shift toward a predominant Th2-type profile. Similarly, HIV progression has been linked to a cytokine shift toward a Th2-type predominance (Shearer and Clerici 1991; Sher *et al.,* 1992) and that chronic stress experienced by HIV-infected individuals, could contribute in part towards this shift. Furthermore, in a study performed by Kang and Fox (2001) on the effect of academic stress on the immune response of students, they found a significant decrease in IL-2 and IFN-γ and a significant increase in IL-6 suggesting a down-regulation of Th1 and a selective up-regulation of Th2 cytokines during the stressful exposure.

Several steroid hormones modulate T-cell responses. Dehydroepiandrosterone (DHEA) tends to promote a Th1 pattern, with increased production of IFN-γ (Daynes *et al.,* 1990; Daynes *et al.,* 1995) and IL-2 (Suzuki *et al.,* 1991). Unlike DHEA, glucocorticoids such as cortisol increase Th2 activity and are synergistic with Th2 cytokines (Fischer *et al.,* 1991; Wu *et al.,* 1991). The effect of stress on the immune response is therefore an important consideration. Psychological and physical stress activates the hypothalamo-pituitary-adrenal axis and leads to various changes, including increased production of cortisol. Deprivation of food and sleep result in a falling ratio of DHEA to cortisol, which correlates with a fall in delayed type hypersensitivity

responsiveness and a simultaneous rise in serum IgE concentrations. Examples of the effect of stress on the response to immunisation have been shown in animals. Stress that arises from crowding or restraint can increase mycobacterial growth in mice (Tobach *et al.,* 1956; Brown *et al.,* 1993). There is also convincing evidence that depression can be associated with excessive cortisol-mediated effects (Raven *et al.,* 1996) and that stress can lead to depression. Thus, depression tends to be associated with Th2-mediated disorders (Holsboer *et al.,* 1984).

Bernton and co-workers (1995) have shown that under stressful conditions, war recruits had elevated cortisol levels with a subsequent reduction in the DHEA/cortisol ratio and more importantly, under these conditions, the serum IgE levels of these recruits were elevated. In our study community, the high rate of unemployment, gang-related violence and other local stressors, probably result in anxiety. Measuring stress would have required interviews with individuals in the community by drawing up proper questionnaires. Urine samples could have been collected for measurement of cortisol levels. However, these measurements were not possible, because we did not have the infrastructure, personnel financing or expertise for conducting such a study.

In the current study, it was decided to measure intestinal helminths in children, as they are the most efficient known inducers of the Th2 immune response. Intestinal nematodes are very common human parasites and according to the most conservative estimates, affect over a quarter of the world's population (Chan, 1997). These infections present a serious public health problem, in developing countries especially, where the high prevalence could result in morbidity and also affect growth and development (Chan *et al.,* 1994; Albonico *et al.,* 1998). Helminth infections are characterised by eosinophilia, elevated IgE levels and mastocytosis. These responses are known to be controlled by type 2 cytokines such as IL-4, IL-5 and IL-13. Evidence from murine experimental models indicates that concomitant with an increase in type 2 cytokine production, helminth infections also cause down-regulation of type 1 cytokines such as IL-2 and IFN-γ (Mosmann *et al.,* 1991; Actor *et al.,* 1993). In rural areas of the South-Western Cape, infection was frequent in children at five schools in 1994. The mean prevalence of *Ascaris* was 58.9 %, but peaked at 90 % in one school (Gunders *et al.*, 1993). The children in that particular study were infected by one or more of the helminths *Trichuris*, *Ascaris*,

hookworm, *E. vermicularis* and *H. nana*. Infection by the protozoan *Giardia* was also detected. The high serum IgE levels measured on the adults (Chapter 4), confirmed our hypothesis of a dominance of type 2 activation in the study community. Although the IgE levels were measured in adults, the helminth burden was to be determined in primary school children between 5-15 years of age. School children harbour the most intense infections with some of the most common worms. They are thus the age group most at risk of morbidity and, simultaneously, the major contributors to transmission (Wong *et al.,* 1988; Bundy *et al.,* 1991). The importance of this study is further highlighted as the prevalence and intensity of helminthiasis would also reflect the extent of environmental pollution by human faeces and the health risk for a range of enteropathogenic infections.

5.1.2 Aims

The aim of this study was to determine the prevalence of helminths in children attending all the primary schools in the study community. Differences in the prevalence of helminths in the two communities (*i.e.* community A and 2) will also be determined.

5.1.3 Materials and methods

5.1.3.1 Study area

The investigation was carried out in the two adjacent suburbs of Cape Town, South Africa as described in Chapters 3 and 4 of this thesis.

5.1.3.2 Study population

The study population was a survey of 5 766 primary school children, between the ages of 5-15 years from whom a stool sample was obtained.

5.1.3.3 Stool sample collection

For the purpose of this study, all nine primary schools in the study community were included. Three of these schools were situated in community B and six in community A (Figure 5.1 and Table 5.1). A total number of 5 766 children were enrolled in these schools for the year 1999. Prior to commencement of the study, class lists with the name, surname, sex, date of birth and

address of each child were obtained from the teachers, for the compilation of our database. A number was subsequently allocated to each child which was coupled to the name of the school. For the collection and processing of stool samples, these coded labels were used in order to maintain confidentiality.

One day prior to sample collection, a box with the consent forms, brown paper bags and labelled specimen jars containing a wooden spatula, were delivered to each class. On this day, instruction was given to children to collect a stool sample the night before collection or the morning before school. They were instructed to use the wooden spatula to collect a sample and to place the spatula with the sample in the specimen jar. Simultaneously, the children were also educated about good hygiene practices in order to avoid helminth infections. The following day, the box containing the paper bags with the filled specimen jars and signed consent forms were collected from each class.

However, as seen in Table 5.1, only 97 % (5 581) of the total number of children in these schools received an allocated specimen jar. The children who did not receive a specimen jar, were either absent (150/5 776 = 2.6 %) on the week of distribution or their parents did not give consent (23/5 776 = 0.4 %) or their names were not on the class lists, because of late school entry (22/5 776 = 0.38 %). Table 5.1 also shows the number of children per school who had received specimen jars. In community A, 98 % (3 583/3 660) and in community B, 95 % (1 998/2 106) of children received a specimen jar.

5.1.3.4 Recovery rate

Specimen bottles were distributed to a school on a Monday and Wednesday, respectively, thus two schools per week could be targeted. This ensured that samples could be collected for at least two consecutive days after specimen jars were delivered (*i.e.* Tuesday to Friday). Overall, 73 % (4 062/5 581) of the children returned a stool specimen (Table 5.1). The recovery rate ranged from 68 % to 77 % in community B and from 69 % to 79 % in community A. The overall recovery was higher in community B (75 %) compared to community A (72 %). After collection, stool samples were immediately transported to the Medical Research Council, Department of Nutrition and Intervention Studies, for parasitological examination. The study

population subsequently referred to in this thesis, would be referring to those children returning a filled specimen jar (n = 4 062).

Table 5.1 Description of schools and recovery rates

Schools	**No. children in school**	**Bottles ascribed n (%)**	**Stool sample returned n (%)**
School 1	248	248 (100 %)	192 (77 %)
School 2	849	789 (93 %)	535 (68 %)
School 3	520	509 (98 %)	382 (75 %)
School 4	647	647 (100 %)	498 (77 %)
School 5	751	745 (99 %)	518 (70 %)
School 6	645	645 (100 %)	438 (68 %)
Community A	3 660	3 583 (98 %)	2 563 (72 %)
School 7	672	672 (100 %)	528 (79 %)
School 8	972	864 (89 %)	651 (75 %)
School 9	462	462 (100 %)	320 (69 %)
Community B	2 106	1 998 (95 %)	1 499 (75 %)

5.1.3.5 Parasitological examinations

Numbered specimen bottles with screw top lids were given to each child. The consistency of each stool sample was recorded before 1 g of stool was weighed out and the consistency (density) was graded as described in Chapter 2 of this thesis. Each homogenised faecal sample was dispersed in 4 ml of 10 % aqueous formalin and stored in a Bijou bottle. Fixed stool specimens were processed for light microscopy according to standard methods (Ash and Orihel 1991, WHO, 1994). Eggs were counted under a 10 x objective of a light microscope and eggs per gram (epg) of stool was expressed per 1 g of faeces. Microscopy was done by a research team from the Medical Research Council, led by Dr. John Fincham.

The prevalence of the following helminths and protozoa were determined:

	Abbreviations used in text
a) Protozoa:	
Giardia lamblia/duodenalis	*Giardia*
Entamoeba coli	*E. coli*
Entamoeba hystolytica/dispar (2 species)	*E. hystolytica*
Endolimax nana	*E. nana*
Entamoeba hartmanni	*E. hartmanii*
Chilomastix mesnili	*C. mes*
Iodamoeba bütschilli	*I. butch*
Blastocystis hominis	*B. hominis*
b) Helminths:	
Ascaris lumbricoides (roundworm)	*Ascaris*
Trichuris trichiura (whipworm)	*Trichuris*
Hymenolepsis nana (dwarf tape worm)	*H. nana*
Enterobius vermicularis (pinworm)	*Enterobius*
Taenia (tapeworm of pork or beef)	*Taenia*
Trichylostrongylus Hookworms	Hookw
c) Yeasts	

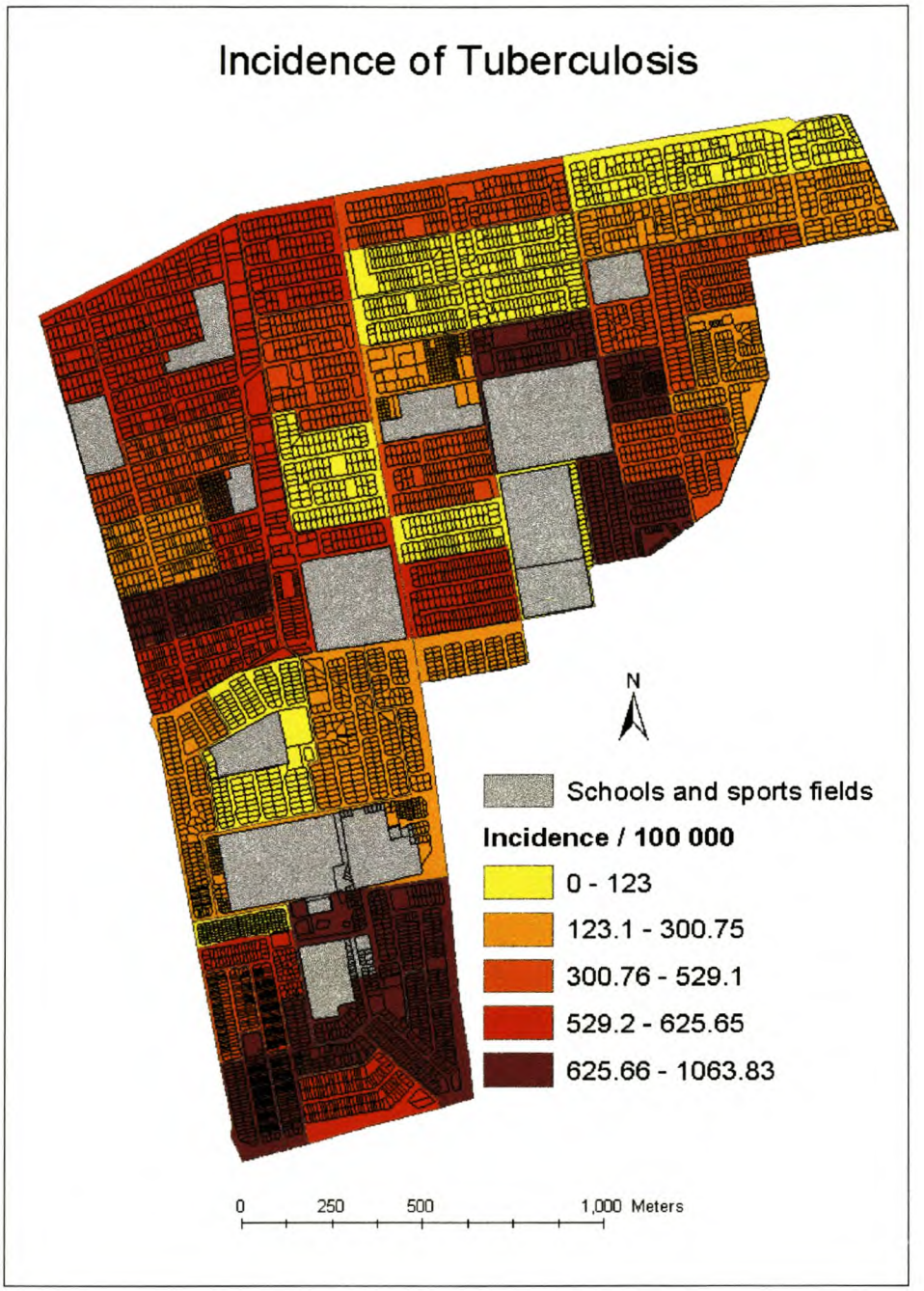
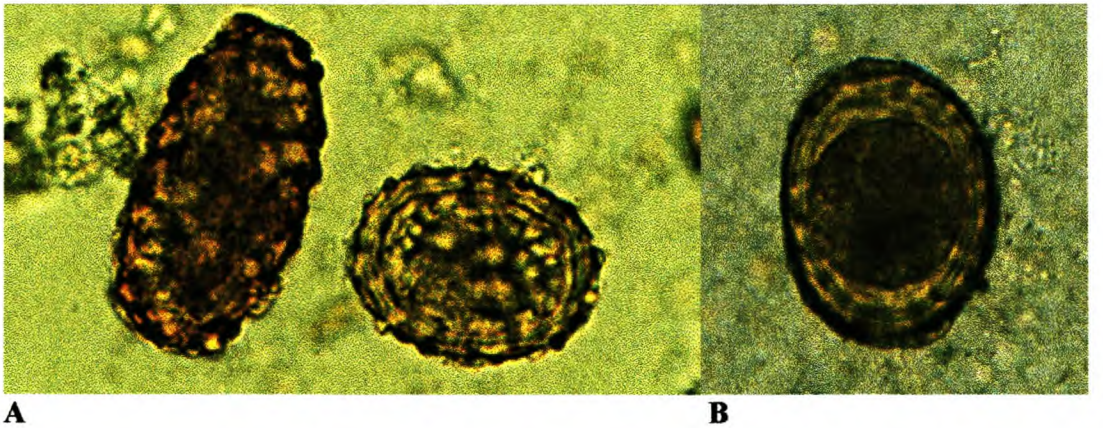


Figure 5.1 Geographical mapping of the nine primary schools in the communities

5.1.3.5.1 Microscopic analysis of stool samples

Photos of microscopic analyses (as seen under 10 x objective) of stool samples were obtained from the Centre for Disease Control (CDC) website: http://www.dpd.cdc.gov/dpdx/default.htm. Photos shown would reflect the parasites measured in this particular study.

Ascaris lumbricoides

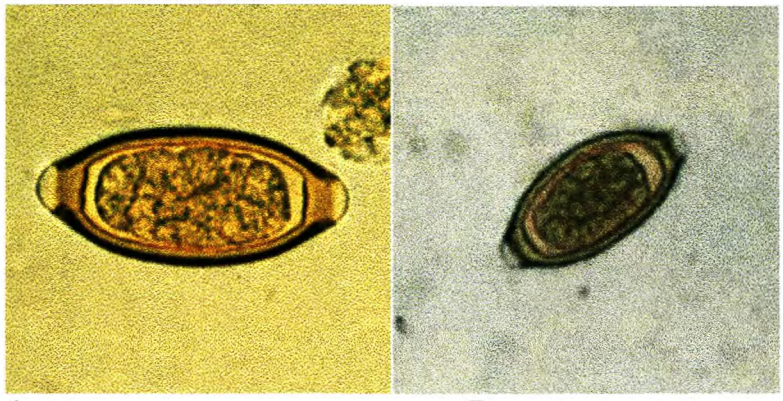


A **B**

A: Unfertilized and fertilized eggs (left and right, respectively).

B: Fertilized *Ascaris* egg, still at the unicellular stage. Eggs are normally at this stage when passed in the stool. Complete development of the larva requires 18 days under favorable conditions.

Trichuris trichiura

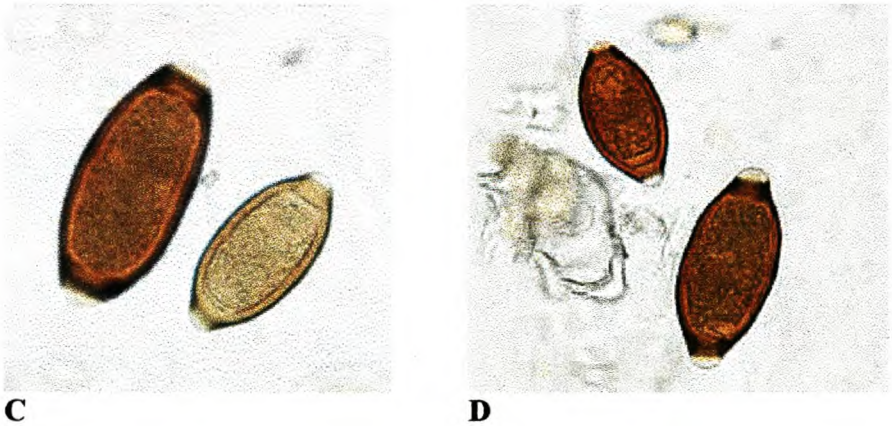


A **B**

A, B: *Trichuris trichiura* eggs (wet preparation). The diagnostic characteristics are:

- a typical barrel shape
- two polar plugs, that are unstained
- size: 50 to 54 µm by 22 to 23 µm

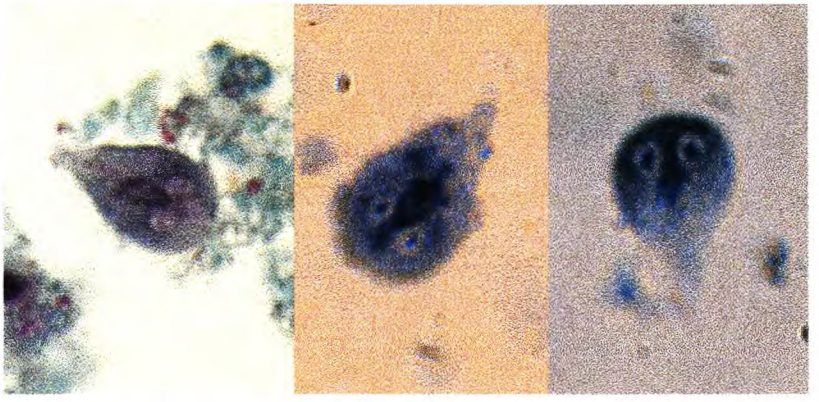
The external layer of the shell of the egg is yellow-brown (in contrast to the clear polar plugs). The egg is unembryonated, as eggs are when passed with the stool.



C **D**

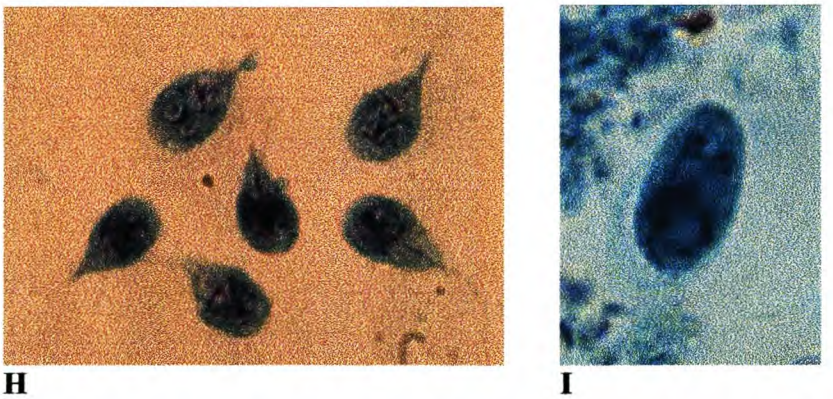
C, D: *Trichuris trichuria* eggs.

Giardia lamblia



E **F** **G**

E, F, G: Three trophozoites of *Giardia intestinalis*, stained with trichrome, (**E**), and stained with iron hematoxylin (**F** and **G**). Each cell has two nuclei with a large, central karyosome. Cell size: 9 to 21 µm in length.

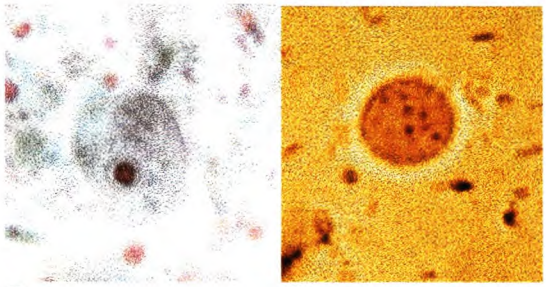


H **I**

H: *Giardia intestinalis* in culture. In these preparations, the flagellae (four pairs per cell) are clearly visible.

I: Cysts of *Giardia intestinalis*, stained with iron-hematoxylin

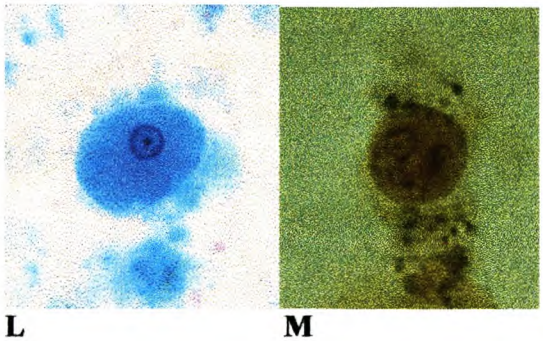
Amoebiasis



J **K**

J. *E. nana* trophosites

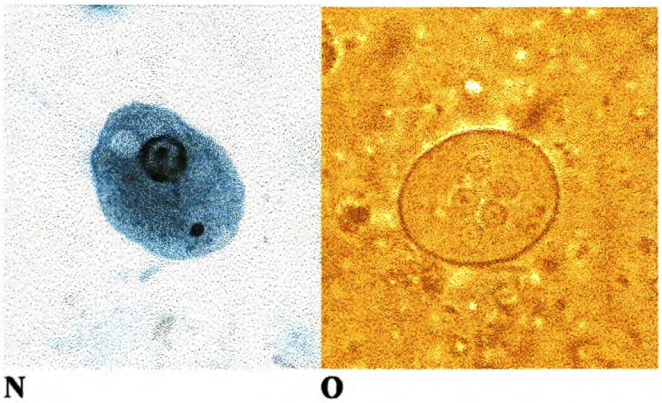
K. *E nana* cysts



L **M**

L. *Hartmani* trophozite

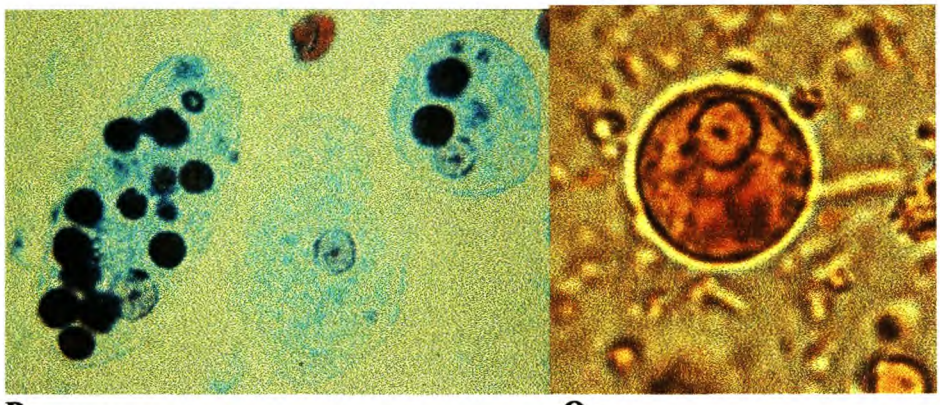
M. *Hartmani* cysts



N **O**

N. *E. coli* trophozite

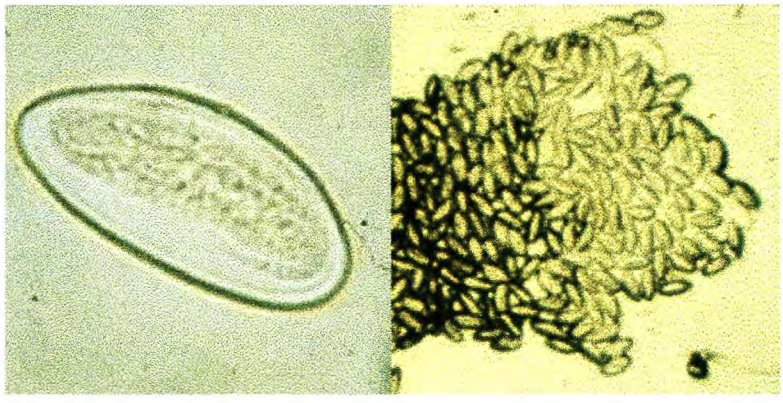
O. *E. coli* cysts



P **Q**

P. H*istolytica* trophozite

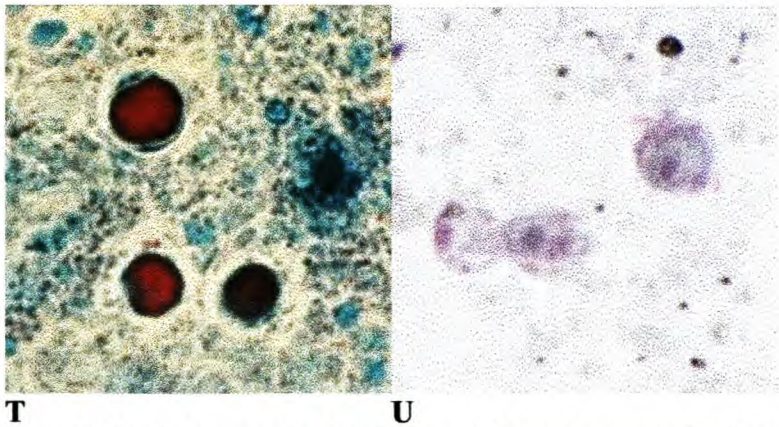
Q. *Hystolytica* cysts



R **S**

R. *Enterobius* egg

S. *Enterobius* eggs on cellulose tape prep



T **U**

T and U. *Blastocystis hominis* cyst-like forms stained in trichrome. The vacuoles stain variably from red to blue.

5.1.3.6 Anthropometric measurements

Anthropometric studies were carried out to determine the growth profile of the children. The height and weight of each child was monitored by standardised methods. Before weighing, shoes and heavy clothing were removed. Elevated hair arrangements of girls were adjusted. In order to assess growth, the height and weight of all the children were measured by the same method and the same health worker at all the schools. To assess growth in terms of a height-for-age-z-score (HAZ), children who were more than 12 years old were excluded resulting in a reduced sample size for this particular analysis. The SPSS statistical software was used to derive HAZ for each child. The score is the deviation from a reference value in a normal population (WHO, 1995), expressed as standard deviations of the WHO-reference population. HAZ was categorised into < -2 SD = stunted and ≥ -2 SD = normal.

5.1.3.7 Ethical considerations

Prior to enrolment, permission for conducting the study, was obtained from the Western Cape Education Department and school principals. Signed informed consent was obtained from parents or guardians. The study was approved by the Ethics Committee of the University of Stellenbosch. It was also decided that if estimated helminth prevalences should exceed the limit of 50 % set internationally by WHO, all the children in the nine schools, irrespective of their helminth burden or their participation in the study, will receive anti-helminthic treatment.

5.2 RESULTS

5.2.3 Gender and age profile

Information on gender and age of children who participated in the present study, is summarised in Table 5.2. Gender distribution was fairly equal for all schools, except in the case of School 6, where 62 % of the children were female. For the schools in community A, 49 % of the children were female. Gender distribution was the same in schools in both communities.

The mean and median ages per school were calculated to determine the age homogeneity of the children enrolled in the study. The age range (*i.e.* maximum and minimum age) per school is also shown. The means and medians were similar in all the schools, confirming the homogeneity of the study population.

Table 5.2 Gender and age of children at each school

			Age in years		
School	**Girls**	**Boys**	**Mean**	**Median**	**(Range)**
School 1	47 % (91/192)	53 % (101/192)	11.5	11.1	(5 - 17)
School 2	53 % (285/535)	47 % (250/535)	10.2	10.3	(5 - 16)
School 3	51 % (195/382)	49 % (187/382)	11.6	10.1	(6 - 16)
School 4	47 % (236/498)	53 % (262/498)	10.6	11.0	(5 - 16)
School 5	51 % (263/518)	49 % (255/518)	10.4	10.3	(4 - 16)
School 6	62 % (197/438)	38 % (241/438)	10.1	10.8	(6 - 17)
Community A	49 % (1267/2563)	51 % (1296/2563)	10.7	10.6	(4 - 17)
School 7	49 % (258/528)	51 % (270/528)	10.5	9.6	(5 - 16)
School 8	48 % (309/650)	52 % (341/650)	10.5	10.4	(5 - 16)
School 9	52 % (165/320)	48 % (155/320)	10.3	10.7	(6 - 16)
Community B	49 % (732/1498)	51 % (766/1498)	10.4	10.4	(5 - 17)

5.2.2 Sand in stool samples

Sand was present in 40.9 % of stool samples collected in community A. The highest prevalence of sand in stool was recorded in School 1 (71.9 %). In the rest of the schools in this area, the prevalence of sand ranged from 25.1 % (School 3) to 40.9 % (School 5) (Table 5.3).

In community B, 65.1 % of the stool samples analysed had evidence of sand. The highest prevalence was noted for School 8 (79.5 %) with equally high prevalence of sand in School 9 (75.0 %) and School 7 (60.2 %) (Table 5.3).

Table 5.3 The prevalence of sand in stool samples

School	Prevalence of sand in stool
School 1	36.9 % (71/192)
School 2	31.8 % (170/535)
School 3	25.1 % (96/382)
School 4	36.7 % (183/498)
School 5	40.9 % (212/518)
School 6	71.9 % (315/438)
Community A	40.9 % (1047/2563)
School 7	60.2 % (318/528)
School 8	79.2 % (418/650)
School 9	75.0 % (239/320)
Community B	65.1 % (975/1498)

5.2.3 Overall prevalence of helminths and protozoa

The prevalence (%) of all the parasites tested for the children (irrespective of their school of attendance), is listed in Table 5.4.1. Prevalence is firstly given for the two communities and secondly for the school with the highest prevalence for that particular parasite. Also, Table 5.4.2 shows the prevalences of worms (*i.e. Ascaris* and/or *Trichuris* infection) in the nine schools.

As seen in Table 5.4.1, the school with the highest prevalence of every helminth and protozoan tested, was School 8 situated in community B. In community A, the highest prevalence of helminths was observed in School 4 whereas Schools 4, 5 and 6 had the highest prevalence of protozoan infections. Overall, the prevalence of worms was highest in community B (66 %), with 77 % of children in School 8 testing positive for the presence of *Ascaris* and/or *Trichuris*. Descriptive statistics for prevalence and intensity of infection for *Ascaris* and *Trichuris* in the two communities are shown in Tables 5.4.3 and 5.4.4.

Table 5.4.1 Prevalence for each organism in community A (Com. A.) and community B (Com. B). The prevalences of the parasites are given for the two areas as well as for the school with the highest prevalence for that specific organism.

Area	Parasites	Prevalence (Area)	Highest Prevalence	*School
Com. A	*Ascaris*	16 % (398/2563)	22 % (109/498)	School 4
	Trichuris	38 % (972/2563)	55 % (237/498)	School 4
	H. nana	0.5 % (13/2563)	1.6 % (8/498)	School 4
	Taenia	0	0	
	E. vermicularis	1.0 % (26/2563)	1.5 % (85/535)	School 2
	Giardia	15 % (383/2563)	19 % (71/438)	School 6
	E. coli	14.4 % (370/2563)	26.0 % (112/438)	School 6
	E. hystolytica	0.5 % (268/2563)	21.6 % (112/518)	*School 5*
	E. nana	9.4 % (241/2563)	21.5 % (107/498)	School 4
	E. hartmani	1.8 % (45/2563)	3.0 % (13/438)	School 6
	C. mes	1.7 % (44/2563)	2.8 % (14/498)	School 4
	I. butch	1.4 % (37/2563)	3.1 % (16/518)	School 6
	B. hominis	6.6 % (170/2563)	15.0 % (79/535)	School 2
	Hookw	0.1 % (2/2563)	0.4 % (2/518)	School 6
	Yeasts	0.2 % (5/2563)	1.1 % (5/438)	School 6
Com. B	*Ascaris*	38 % (568/1498)	48 % (314/650)	School 8
	Trichuris	60 % (902/1498)	70 % (458/650)	School 8
	H. nana	3.9 % (58/1498)	4.6 % (30/650)	School 8
	Taenia	0	0	
	E. vermicularis	1.0 % (15/1498)	1.2 % (8/650)	School 8
	Giardia	12.4 % (186/1498)	22.2 % (117/528)	School 7
	E. coli	32.7 % (490/1498)	36.2 % (235/650)	School 8
	E. hystolytica	8.8 % (132/1498)	12.3 % (80/650)	School 8
	E. nana	27.2 % (408/1498)	30 % (193/650)	School 8
	E. hartmani	3.6 % (54/1498)	3.7 % (24/1498)	School 8
	C. mes	4.2 % (63/1498)	4.5 % (29/650)	School 8

I. butch	1.9 % (28/1498)	2.6 % (17/650)	School 8
B. hominis	18.4 % (276/1498)	17.7 % (115/650)	School 8
Hookw	0.1 % (2/1498)	0.3 % (2/650)	School 8
Yeasts	2.4 % (36/1498)	3.7 % (24/650)	School 8

*School - school with the highest prevalence of a specific helminth or protozoan.

Table 5.4.2 Prevalence of worms in the nine schools

School		***Trichuris***		***Ascaris***		**Worms**	
	n	**n**	**%**	**n**	**%**	**n**	**%**
School 1	192	71	37	33	17	79	41
School 2	535	172	32	48	9	188	35
School 3	382	134	35	38	10	141	37
School 4	498	237	55	109	22	249	50
School 5	518	212	41	114	22	260	50
School 6	438	146	39	56	15	165	44
Community A	2 563	972	38	398	16	1 085	42
School 7	528	322	61	200	38	354	67
School 8	651	458	70	314	48	504	77
School 9	320	122	38	54	17	128	40
Community B	1 498	902	60	568	38	986	66

Table 5.4.3 Descriptive statistics for prevalence and intensity of infection in community A

Descriptive measures	***Trichuris***	***Ascaris***
Prevalence of infection	37.9 %	15.5 %
Arithmetic mean epg of all children	198.1	80.6
Arithmetic mean epg of infected children	646.0	509.0
Geometric mean epg of all children	4.1	1.6
Geometric mean epg of infected children	101.6	17.2
Median epg of all children	0	0
Median epg of infected children	97.1	5.0
25^{th} percentileepg in infected children	21.4	2.0
50^{th} percentile epg in infected children	97.1	5.0
75^{th} percentile epg in infected children	400.0	165.5
90^{th} percentile epg in infected children	1 329.0	969.6
95^{th} percentile epg in infected children	3 144.3	1 605.20
Maximum epg per child	31 388.0	40 000.0

Table 5.4.4 Descriptive statistics for prevalence and intensity of infection in community B

Descriptive measures	***Trichuris***	***Ascaris***
Prevalence of infection	60.2 %	37.9 %
Arithmetic mean epg of all children	537.0	459.0
Arithmetic mean epg of infected children	896.0	1 222.0
Geometric mean epg of all children	20.2	7.1
Geometric mean epg of infected children	150.5	186.0
Median epg of all children	0	0
Median epg of infected children	178.0	194.0
25^{th} percentile epg in infected children	33.0	49.0
50^{th} percentile epg in infected children	178.0	193.5
75^{th} percentile epg in infected children	612.7	736.0
90^{th} percentile epg in infected children	2 175.8	2 426.4
95^{th} percentile epg in infected children	4 144.4	4 307.1
Maximum epg per child	52 560.0	28 800.0

5.2.4 Intensity of infection

In our study community, the overall prevalence of helminth infections was lower than in other endemic areas (Gunders *et al.*, 1993; Bradley and Buch, 1994; Fincham *et al.*, 1996), which led to the assumption that worm loads would be at an equally low level. Table 4.6.1 depicts the internationally defined criteria of intensity of infection for individuals categorised as being infected light, moderate or severe. The criteria depend on the species and have been set for *Trichuris*, *Ascaris*, hookworm and *schistosomes*. The number of children in each group as well as the percentages (of the total number of children tested) are shown in Table 5.5.1.

As seen in Table 5.5.2, the majority of children in community A tested negative for the presence of either *Ascaris* (69 %) or *Trichuris* (84 %). These proportions were somewhat similar in community B where 63 % of the children had no evidence of *Ascaris*, although only 40 % tested negative for *Trichuris*. In community A, most of the children infected had light *Ascaris* infections (11 %) and *Trichuris* infections (15 %). Only 0.2 % and 0.3 % of the children had epgs of > 10 000 for *Ascaris* and *Trichuris*, respectively.

In community B, the infections were also light, with most of the children falling into the 1 - 999 epg category (Table 5.5.2). The intensity of *Ascaris* infection ranged from 14 % of the children in the 1-99 epg; 15% in the 100 999 epg category and 6.7 % in the 1 000-9 999 epg category. One percentage and 0.3 % of the children in community B had epgs of > 10 000 for *Trichuris* and *Ascaris* respectively.

Table 5.5.1 Intensity of *Ascaris* and *Trichuris* infection in community A: Eggs per gram (epg) were categorised into the 5 groups ranging from 0 to > 10 000 epg. The number as well as the percentage (of the total number of children tested) of children in each category is shown

Category	**0**	**1-99**	**100-999**	**1000-9999**	**> 10 000**
Ascaris	2157	288	79	34	5
% of total	84.0	11.0	3.1	1.3	0.2
Trichuris	1777	393	293	92	8
% of total	69.0	15.0	11.0	4.0	0.31

Table 5.5.2 Intensity of *Ascaris* and *Trichuris* infection in community B: Eggs per gram (epg) were categorised into the 5 groups ranging from 0 to > 10 000 epg. The number as well as the percentage (of the total number of children tested) of children in each category is shown

Category	**0**	**1-99**	**100-999**	**1000-9999**	**> 10 000**
Ascaris	936	216	230	101	15
% of total	62.5	14.4	15.4	6.7	0.33
Trichuris	600	367	368	152	11
% of total	40.0	24.0	25.0	10.0	1.0

5.2.5 Age-related prevalence of *Ascaris* and *Trichuris*

Tables 5.6.1 and 5.6.2 and Figures 5.2.1 and 5.2.2 show the age-related prevalence of infection with *Trichuris* and *Ascaris* in community A and community B. Ages were categorised into the following groups: > 6 years; 6-8 years; 9-11 years; 12-14 years and +15 years of age.

In community B, the prevalence of trichuriasis increased from 21 % in the youngest age group to 38 % in the age group 15-17 years (Table 5.6.1). In contrast, the prevalence of ascariasis was 20 % in the youngest age group, declining steadily to 11 % in the age group 15-17 years. In contrast, the age-related prevalence of ascariasis and trichuriasis in community B followed a different pattern compared to that seen in community A (Table 5.6.2). In the children younger than 6 years of age, 35 % were infected with *Trichuris*. The prevalence increased, peaking at 62.6 % in the age group, 12-14 years, reaching a plateau. Prevalence of ascariasis increased from 28 % in the youngest age group of 40 % in the age group, 9-11 years, declining to 33 % in the age group, 15-17 years.

As shown in Figures 5.2.1 and 5.2.2, the prevalence of trichuriasis in both communities increased with age. The prevalence of ascariasis decreased in community A, whereas in community B, the prevalence of ascariasis stabilised at approximately 30 %.

Table 5.6.1 Age-related prevalence of *Ascaris* (*As*) and *Trichuris* (*Tt*) in community A

Age group	**< 6 yrs**	**6-8 yrs**	**9-11 yrs**	**12-14 yrs**	**+15 yrs**
# tested	64	758	896	713	101
# infected	14	206	284	236	39
% *Tt* infected	21.8	27.2	31.7	33.1	38.6
# tested	64	758	896	713	101
# infected	13	135	133	110	12
% *As* infected	20.3	17.8	14.8	15.4	11.8

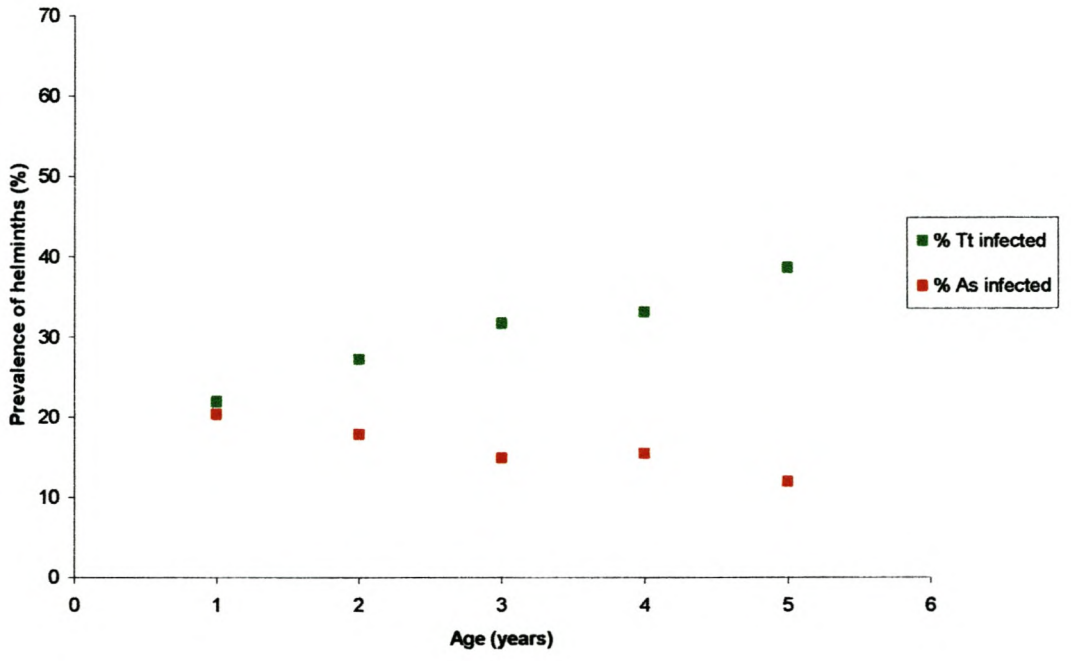


Figure 5.2.1 Age-related prevalence of *Ascaris* and *Trichuris* in community A

Key: 1 = < 6 years; 2 = 6-8 years; 3 = 9-11 years; 4 = 12-14 years and 5 = 15 years and older

Table 5.6.2 Age-related prevalence of *Ascaris* (*As*) and *Trichuris* (*Tt*) in community B

Age group	**< 6 yrs**	**6-8 yrs**	**9-11 yrs**	**12-14 yrs**	**+15 yrs**
# tested	14	521	552	348	24
# infected	5	293	344	218	15
% *Tt* infected	35.7	56.2	62.3	62.6	62.5
# tested	14	521	552	348	24
# infected	4	201	221	112	8
% *As* infected	28.6	38.6	40.0	32.2	33.3

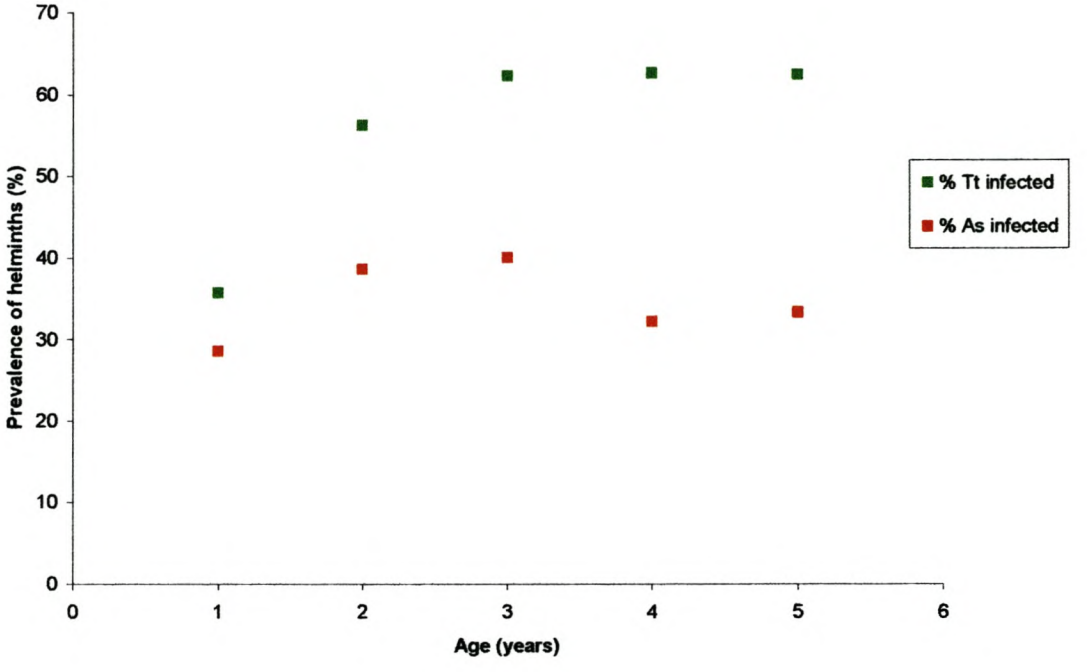


Figure 5.2.2 Age-related prevalence of *Ascaris* and *Trichuris* in community B

Key: 1 = < 6 years; 2 = 6-8 years; 3 = 9-11 years; 4 = 12-14 years and 5 = 15 years and older

5.2.6 Age-related prevalence by gender

The age-related prevalence of *Ascaris* and *Trichuris* in the two communities had similar distributions, thus no significant differences in prevalence of infection between the sexes were observed (Tables 5.7.1 to 5.7.3 and Figures 5.3.1 to 5.3.4).

Prevalence of *Trichuris* in community A appeared to be stable ranging from 25 – 30 % in boys and 15 – 21 % in girls. Apart from differences seen in prevalences between the youngest and oldest age groups, no apparent differences were observed between the sexes. The prevalence of *Trichuris* was lowest in girls younger than 6 years of age, increasing at 6-8 years of age and then from 9 to 15 years of age, as depicted in Figure 5.3.2. In contrast, the prevalence of boys infected with *Trichuris*, peaked in the age group 15 years and older. As shown in Figure 5.3.1, the prevalence of *Ascaris* in community A declined with age for both sexes from 21 – 15 % and 18 – 16 % in girls and boys respectively.

As depicted in Figure 5.3.3 in community B, the prevalence of *Ascaris* in girls with age ranged from 0 – 70 %. A sharp increase (from 0 – 52 %) in prevalence for girls in the youngest age group (< 6 years) and those aged 6-8 years was observed. A plateau is reached between the ages 6 to 14 years after which the prevalence of *Trichuris* in girls aged 15 years and older peaked at 70 %. *Trichuris* infection in boys however, was low in the youngest age group with 45 % infected cases and peaking at 60 % in the age group nine to ten years. The prevalence of ascariasis in boys was lowest at 18 % in the age group < 6 years, peaking in the age group, 6-8 years and then declined with age, as shown in Figure 5.3.4. The prevalence of ascariasis in girls was 62 % at age 5 years compared to the 15 % observed in the boys in this age group. Prevalence declined from 62 % to 40 % in the age group 6- 8 years declining steadily and reaching a plateau around 35 %. Overall, it seemed that the prevalence of ascariasis and trichuriasis in boys and girls in community A were stable with no significant differences between the sexes. In community B, although not significant, differences in prevalences between the sexes could be seen in the age groups, 5 years and 15-17 years.

Table 5.7.1 Age-related prevalence by gender of *Ascaris* infection in community A

	All	< 6	6-8	9-11	12-14	15+	Other
Males							
# tested	1275	36	369	435	359	59	17
# infected	207	6	63	69	59	8	2
% infected	16	17	17	16	16	14	12
Females							
# tested	1288	28	389	461	354	42	14
# infected	199	7	72	64	51	4	1
% infected	15	30	19	14	14	10	7
Total							
# tested	2563	64	758	896	713	101	31
# infected	406	13	135	133	110	12	3
% infected	16	20	18	15	15	12	10

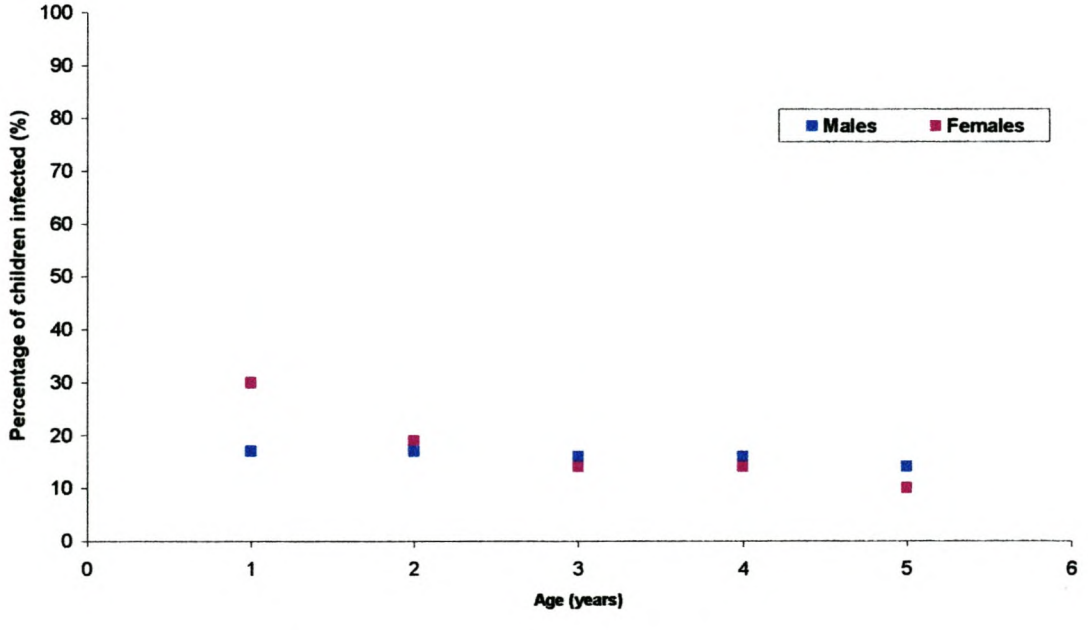


Figure 5.3.1 Graph of age-related prevalence by gender of *Ascaris* infection in Ravensmead.

Key: **1** = < 6 years; **2** = 6-8 years; **3** = 9-11 years; **4** = 12-14 years and **5** = 15 years and older

Table 5.7.2 Age-related prevalence by gender of *Trichuris* infection in community A

	All	< 6	6-8	9-11	12-14	15+	Other
Males							
# tested	1275	36	369	435	359	59	17
# infected	405	10	103	134	127	26	5
% infected	32	28	28	31	35	44	29
Females							
# tested	1288	28	389	461	354	42	14
# infected	381	4	103	150	109	13	2
% infected	30	14	26	33	31	31	14
Total							
# tested	2563	64	758	896	713	101	31
# infected	786	14	206	284	236	39	7
% infected	31	22	27	32	33	39	23

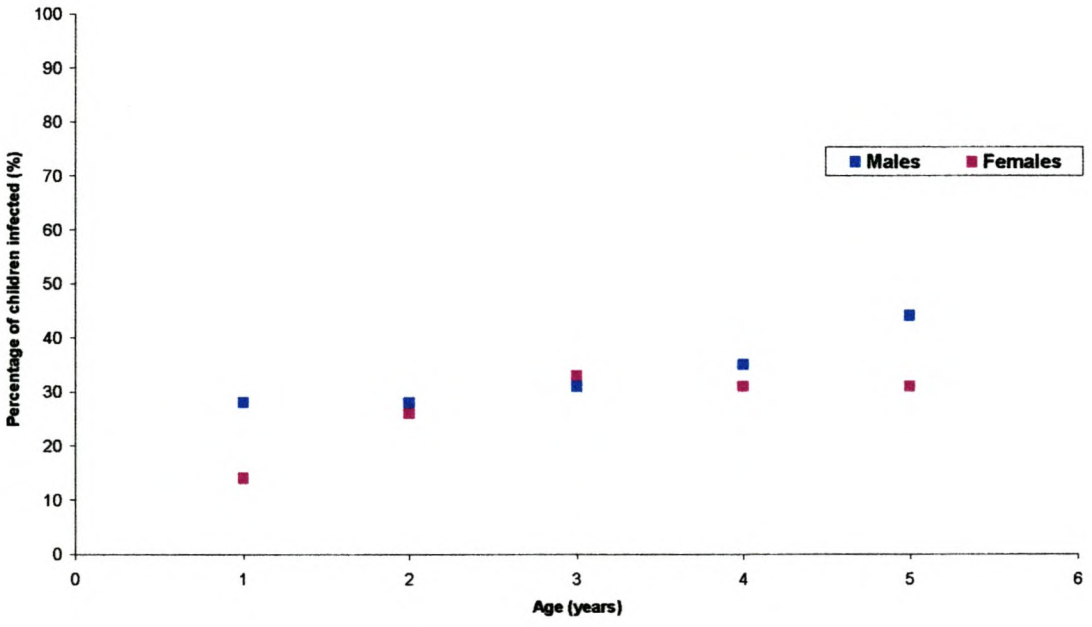


Figure 5.3.2 Graph of age-related prevalence by gender of *Trichuris* infection in community A

Key: 1 = < 6 years; **2** = 6-8 years; **3** = 9-11 years; **4** = 12-14 years and **5** = 15 years and older

Table 5.7.3 Age-related prevalence by gender of *Ascaris* infection in community B

	All	< 6	6-8	9-11	12-14	15+	Other
Males							
# tested	766	11	274	269	177	15	20
# infected	293	2	109	108	59	5	10
% infected	38	18	40	40	33	33	50
Females							
# tested	732	3	247	283	171	9	19
# infected	269	2	92	113	53	3	6
% infected	37	67	37	40	31	33	32
Total							
# tested	1498	14	521	552	348	24	39
# infected	562	4	201	221	112	8	16
% infected	38	29	39	40	32	33	41

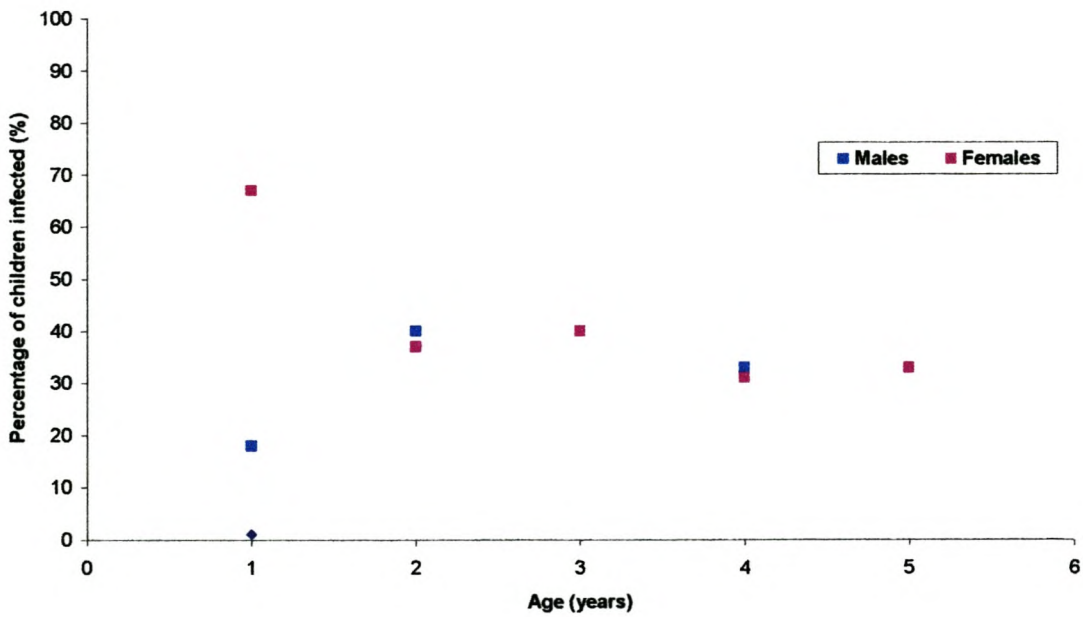


Figure 5.3.3 Graph of age-related prevalence by gender of *Ascaris* infection in community B

Key: **1** = < 6 years; **2** = 6-8 years; **3** = 9-11 years; **4** = 12-14 years and **5** = 15 years and older

Table 5.7.4 Age-related prevalence by gender of *Trichuris* infection in community B

	All	< 6	6-8	9-11	12-14	15+	Other
Males							
# tested	766	11	274	269	177	15	20
# infected	471	5	159	176	110	8	13
% infected	61	45	58	65	62	53	65
Females							
# tested	734	3	249	283	171	9	19
# infected	427	0	134	168	108	7	10
% infected	58	0	54	59	63	78	53
Total							
# tested	1230	14	523	552	348	24	39
# infected	898	5	293	344	218	15	23
% infected	73	36	56	62	63	63	59

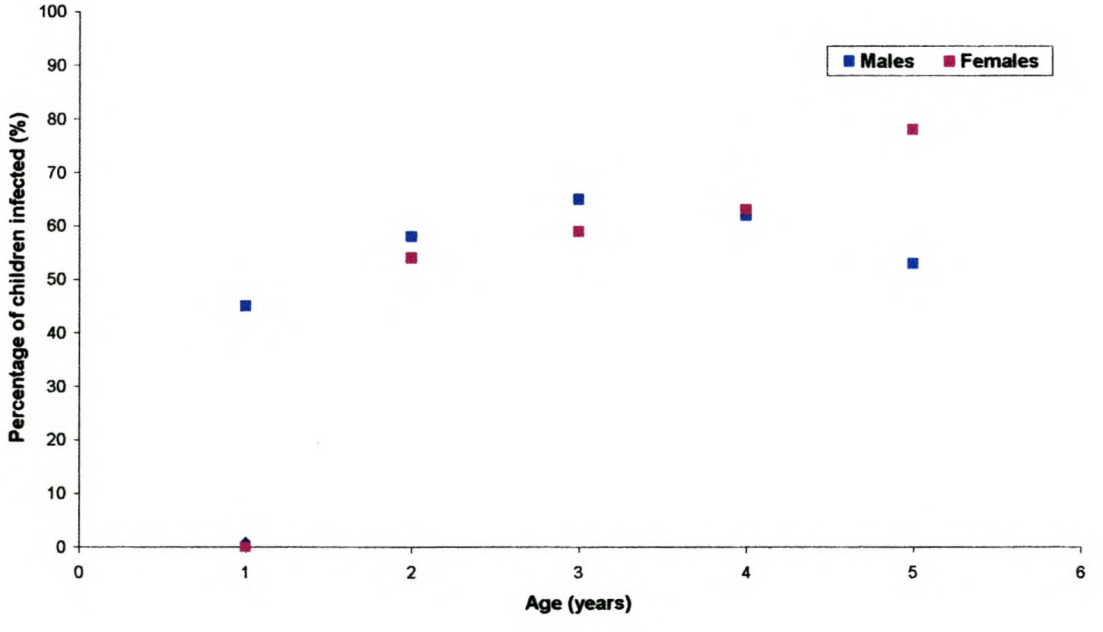


Figure 5.3.4 Graph of age-related prevalence by gender of *Trichuris* infection in community B

Key: 1 = < 6 years; **2** = 6-8 years; **3** = 9-11 years; **4** = 12-14 years and **5** = 15 years and older

5.2.7.1 Growth of children within the different schools

The height-for-age-z-score (HAZ) was only calculated on those children who participated in the Annual Risk of Tuberculosis Infection (ARTI) study (discussed in Chapter 7 of this thesis). This reduced the sample population from 5 766 to 1 667. The sample population was further reduced as the growth profile was restricted to children ≤ 12 years of age. This was done to avoid possible confounding by acceleration of growth due to puberty. HAZ was subsequently characterised into < -2 SD = stunted and ≥ - 2 SD = normal growth (WHO, 1995) and calculated for each child (Table 5.8.1).

Overall, the growth profile of the children seemed to be normal in community A, ranging from -0.52. to -1.20, for Schools 4 and 1, respectively. Although within the normal growth range, the growth profile in the three schools in community B, were skewed towards the negative. This corresponded with the higher prevalence of parasites in the schools in community B. A more descriptive analysis of those children with impaired growth is given in Table 5.8.2.

The prevalence of trichuriasis was significantly higher in those schools with the lowest mean z-score ($r = -0.80$; $p < 0.05$). Also, a significant association was found between the prevalence of ascariasis and the mean height for age z-scores ($r = -0.78$; $p < 0.05$).

Table 4.10.2 depicts the children in the schools with a z-score of < 2 SDs. With 23 % and 21 % respectively, Schools 7 and 8 had the highest prevalence of stunted growth.

Table 5.8.1 Growth of children at the different schools

School	No. of children	Mean age ± SD	Mean z score ± SD	% *Tt*	% *As*
School 1	103	10.5 (1.53)	-0.52 (0.97)	13 % (13/103)	13 % (13/103)
School 2	319	9.2 (1.87)	-0.53 (1.04)	9 (27/319)	16 (51/319)
School 3	209	9.5 (1.73)	-0.71 (1.12)	36 (76/209)	7 (18/209)
School 4	274	9.2 (1.81)	-0.52 (0.97)	50 (135/274)	21 (58/274)
School 5	286	8.9 (2.01)	-0.61 (1.01)	21 (61/286)	19 (54/286)
School 6	136	9.6 (1.28)	-0.89 (1.27)	40 (55/136)	18 (25/136)
School 7	211	9.4 (1.52)	-1.18 (1.28)	58 (122/211)	36 (75/211)
School 8	274	9.7 (1.46)	-1.20 (0.99)	72 (197/274)	47 (130/274)
School 9	157	9.9 (1.44)	-0.84 (0.98)	37 (58/157)	17 (26/157)

Tt* = *Trichuris ***As* = *Ascaris*** **z-score = height-for-age-z-score**

Table 5.8.2 Prevalence of stunted growth and worms. Also shown is the number of children with stunted growth in each school

School	No. of children	Mean HAZ	% Stunting	% *Tt*	% *As*
School 1	6	-2.41	5.8 % (6/103)	33.3 % (2/6)	33.3 % (2/6)
School 2	24	-2.51	7.5 (24/319)	12.5 (3/24)	29.1 (7/24)
School 3	26	-2.54	12.4 (26/209)	58.0 (15/26)	23.1 (6/26)
School 4	22	-2.53	8.1 (22/273)	63.6 (14/22)	27.3 (6/22)
School 5	24	-2.59	8.3 (24/286)	20.8 (5/24)	25.0 (6/24)
School 6	18	-2.50	13.2 (18/136)	61.1 (11/18)	27.7 (5/18)
School 7	34	-2.49	23.3 (49/210)	94.1 (32/34)	55.9 (19/34)
School 8	51	-2.62	21.5 (59/274)	86.3 (44/51)	70.6 (36/51)
School 9	19	-2.45	12.1 (19/157)	47.3 (9/19)	21.0 (4/19)

5.2.7.2 Growth of children in both communities and the prevalence of stunting

The distribution of HAZ in children in both communities, are shown in Figure 5.5. The distribution of z-scores for children was skewed towards negative values and differed from the normal reference distribution (WHO, 1995) in the mean value and prevalence of stunting. The sample mean of both communities was –0.5 vs. the normal reference value of zero. The children indicated under the curves to the left of the red line were stunted namely, 11.3 % for both communities vs. the normal rage of 2.5 % (WHO, 1995).

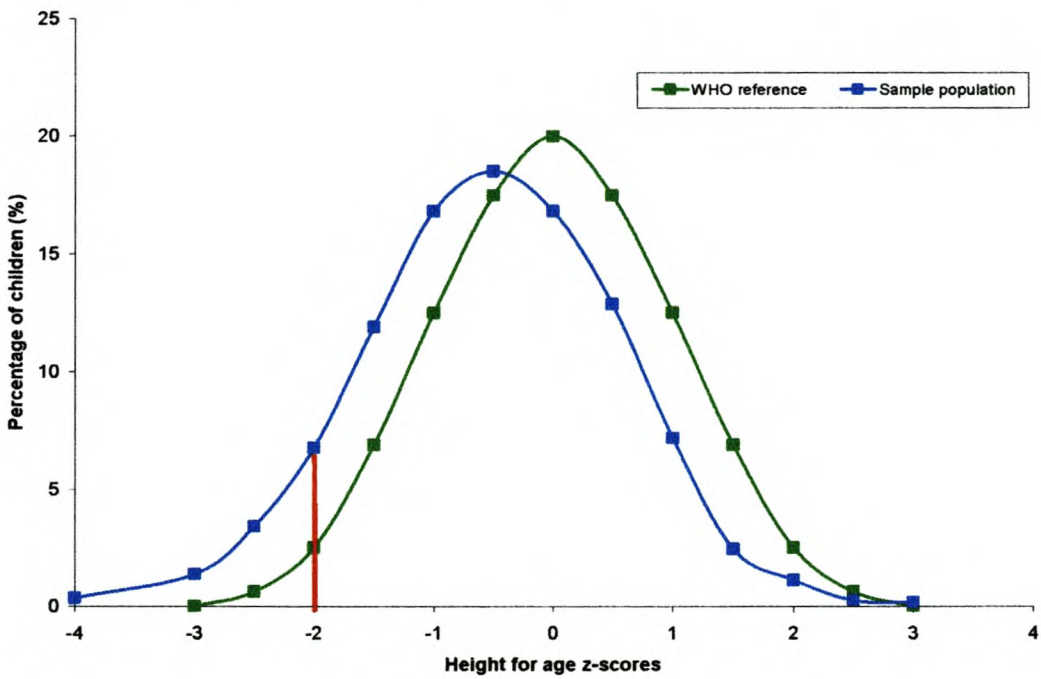


Figure 5.5 Distribution of height-for-age-z-scores of children in both communities (**blue**) and the reference population (**green**). The area under the curves to the left of the **red line** indicates the percentage of children who were stunted.

5.2.8 De-worming

As the prevalence of helminths was more than 50 % in the two communities, all the children in the nine schools studied, received ant-helminthic treatment. A total of 6 000 mebendazole (400 mg) (Vermox®) tablets were kindly donated by Janssen-Cilag Pharmaceuticals. Three and six months after collecting stool samples from the Uitsig and Ravensmead schools respectively, each child, irrespective of their helminth burden or whether they were tested or not, was given an mebendazole tablet. In cases where children were absent from school on the day the tablets were distributed, tablets were given to the teachers for distribution.

5.3 Influence of economic and environmental factors on the prevalence of helminths in the two communities

5.3.1 Background

Helminth infections are, in general, diseases of poverty and remain prevalent throughout the developing world where levels of sanitation, personal hygiene and maternal education are particularly low (Olsen *et al.,* 2001). Poor socio-economic conditions are among the key factors linked with higher prevalences of especially ascariasis, as are cultural differences relating to personal and food hygiene, housing style, social class and gender (O'Larcain *et al.,* 2000). Geohelminths are acquired through ingestion of fecally contaminated food or water or through contact with infected soil.

5.3.2 Aims

The purpose of this ecological study was to determine whether factors such as population density, crowding, socio-economic conditions, sanitation and female literacy would have an impact on the prevalence of worms in the community. The above-mentioned ecological factors were obtained and calculated from the 1996 Census Report as was discussed in Chapter 3 of this thesis. Also, a geographical information system will be used to determine the spatial distribution of the prevalence of *Ascaris* and *Trichuris* in the communities.

5.3.3.1 Materials and Methods

As was mentioned previously (Section 5.1.3.3), the addresses, age and sex of each child was obtained from the schools before commencement of the study. The addresses therefore, could be linked to an ESD (method described in Chapter 4). As a result only children living in Ravensmead and Uitsig and who had a known address were included in this ecological study.

5.3.3.1.2 Association studies

Correlation between factors were determined by means of Spearman Rank test using the SPSS statistical software.

5.3.3.1.2.1 Relevant factors calculated per ESD

In Tables 4.10.1 A and B, the relevant factors as calculated per ESD, are shown. The description of the factors is as follows:

5.3.3.1.2.2 Prevalence of worms

This column refers to the number of children infected with either *Ascaris* and/or *Trichuris*. Prevalences per ESD in Ravensmead ranged from 15-72 % (mean % per ESD = 35.70 %) compared with 21-86 % in Uitsig (mean % per ESD = 56.00 %).

5.3.3.1.2.3 Percentage non-flush toilets

Most of the households in the two communities had either flush or chemical toilets. The percentage non-flush toilets per ESD was calculated as follows:

$$\frac{\text{Number of non-flush toilets}}{\text{Number of households}} \times 100$$

As most of the households in the two communities had toilets, the % sanitation would be 100 %. In cases where this was not the case, pit or bucket latrines were indicated as being the type of toilet used.

5.3.3.1.2.4 Population density

Population density is calculated as the number of individuals per m^2.

5.4 Results

5.4.1 Spatial distribution of *Ascaris* prevalence and intensity of infection

As shown in Table 5.9 (Figure 5.6.1 and 5.6.2), the prevalence of *Ascaris* in community A ranged from 4.88 % to 64 % (measured in ESD no. **1020255**), compared with the range in community B from 5.88 % to 67 % (measured in ESD no. **1010255**).

In community A, the prevalence of *Ascaris* was highest in five neighbouring ESDs ranging from 41 % (**1010256**) to 67 % (**1010255**). Although Schools 1 and 5 are located in two of these neighbouring ESDs, the prevalence of *Ascaris* was low in both schools, 17 % and 22 %. A high prevalence (62 %) was also seen in ESD no. **1010279** which is located near School 2 having a prevalence of 9 %. Prevalences in the other ESDs were low. The intensity of *Ascaris* infection was low and in most cases a median of 3 epg was found. In ESD, no. **1010265,** median intensity was 320 epg, but this is due to the low numbers (3/32) in that particular ESD. In the remaining ESDs intensity levels ranged between 130 epg to 250 epg.

Similar to community A, prevalences were high in six adjacent ESDs in community B, ranging from 48 % (**1020257**) to 64 % (**1020255**). It is assumed that the majority of these children would either attend School 7 and 8, with prevalences of 38 % and 48 % respectively. The prevalences measured in the other ESDs were below 28 %. School 9 with a prevalence of 17 % is located in an ESD with a prevalence below 15 %. Five of the ESDs had levels below 70 epg. The majority of ESDs, had median intensity levels of > 100 epg, with the highest level recorded in ESD no. **1020264** (1927 epg). However, this high level is due to low numbers.

Table 5.9 *Ascaris* infection: Prevalence (%) and median intensity levels in the two communities

ESD	n	Prevalence	*Md* Intensity
1010254	9/16	56.26	2
1010255	21/31	67.74	2
1010256	18/38	47.37	3
1010257	16/39	41.03	3
1010258	6/36	16.67	2
1010259	26/50	52.00	52
1010260	17/65	26.15	46
1010261	8/43	18.60	8
1010262	2/34	5.88	188
1010263	2/15	13.33	321
1010264	3/14	21.43	1
1010265	3/32	9.38	320
1010266	17/78	21.79	240
1010267	17/84	20.24	250
1010268	7/80	8.75	13
1010269	5/68	7.35	2
1010270	8/28	28.57	3
1010271	11/22	50.00	3
1010272	5/23	21.77	2
1010273	48/129	37.21	130
1010274	13/81	16.05	10
1010275	28/74	37.84	3
1010276	12/30	40.00	3
1010277	14/27	51.85	2
1010278	4/12	33.33	2
1010279	5/8	62.5	3
1020254	52/96	54.17	178
1020255	54/84	64.29	358
1020256	50/94	53.19	227
1020257	60/125	48.00	126
1020258	40/141	28.37	165
1020259	44/82	53.66	445
1020260	48/88	54.55	286
1020261	8/55	14.55	46
1020262	8/41	19.51	65
1020263	23/94	24.47	45
1020264	2/41	4.88	1927
1020265	4/45	8.89	82
1020266	25/43	25.58	83

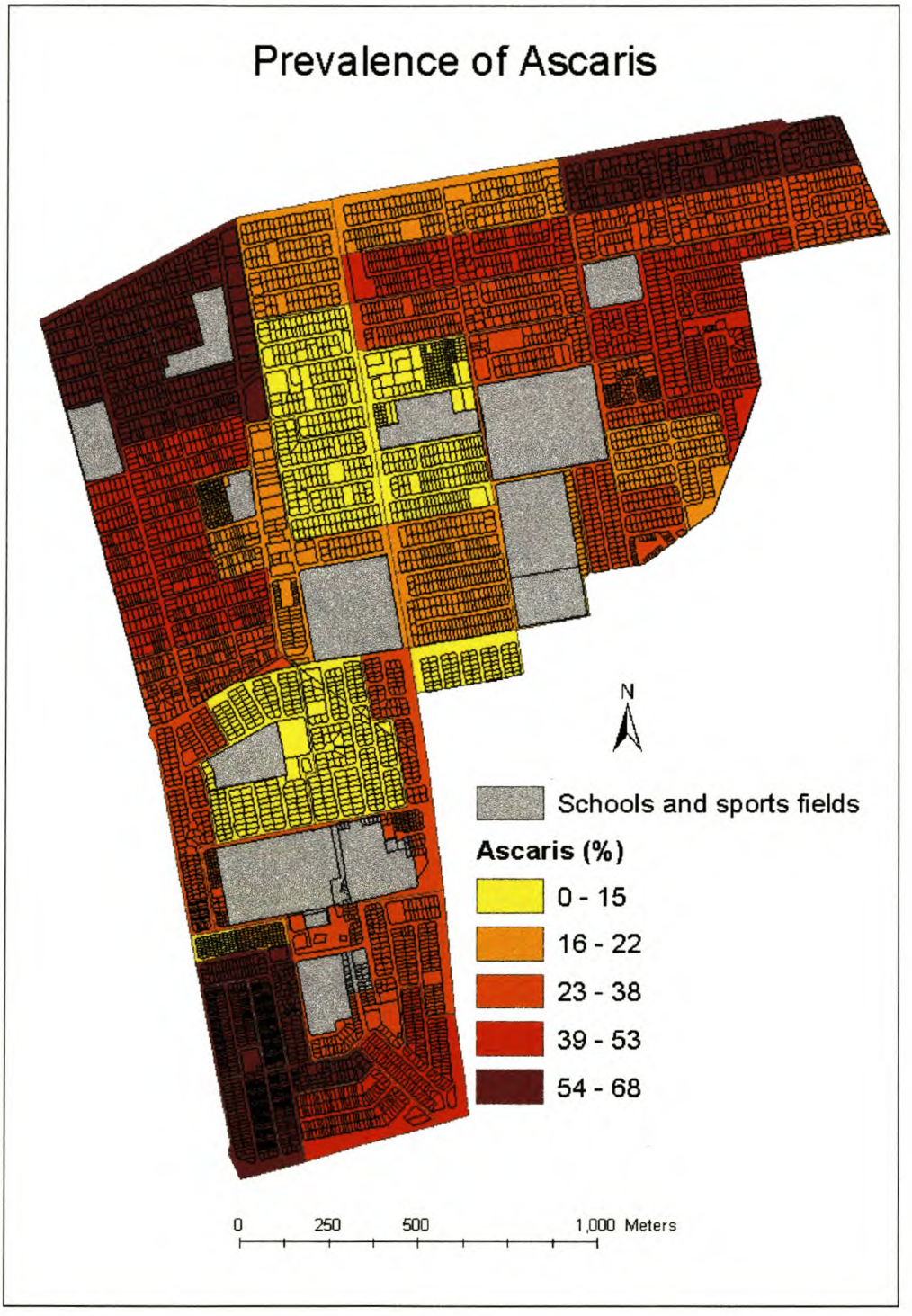


Figure 5.6.1 Geographical distribution of *Ascaris* prevalence (%) in the two communities

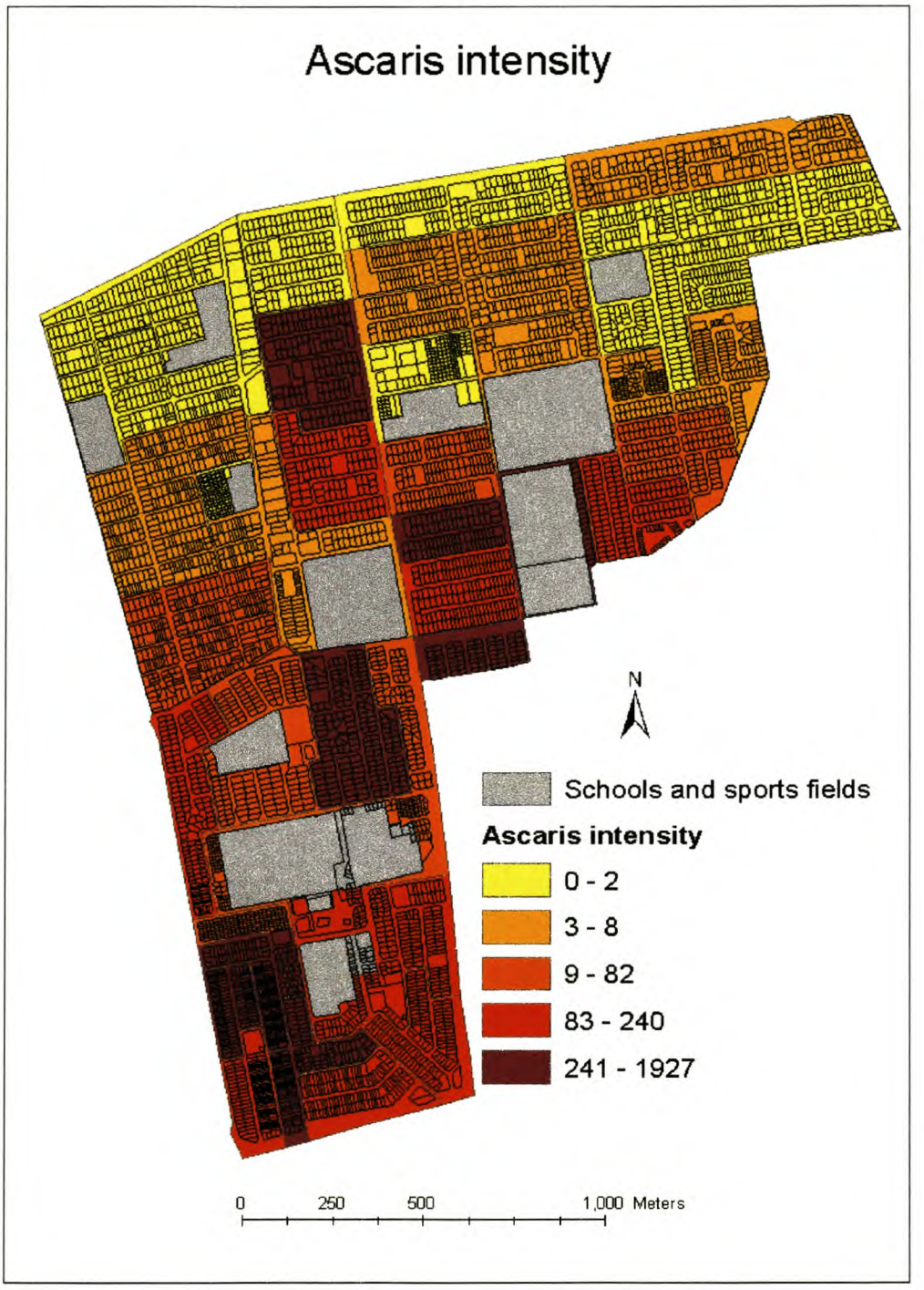


Figure 5.6.2 Geographical distribution of the intensity of *Ascaris* infection in the two communities

5.4.2 Spatial distribution of *Trichuris* prevalence and intensity of infection

In contrast to the spatial distribution of *Ascaris* in community A, the higher prevalences of *Trichuris* were found to be in ESDs adjacent to Schools 4 and 6, with prevalences of 39 % and 55 %, respectively. Two ESDs, **1010259** and **1010260,** had prevalences of 41 % and 53 % respectively, with equal high values for *Ascaris*, 41 % and 52 %. The highest prevalence (64.39 %) was recorded in ESD no. **1010273**. Higher intensity levels ranged from 137 epg to 343 epg. The distribution of prevalence and intensity was similar with the highest median intensity levels (343 epg) for *Trichuris* infection was recorded in ESD no. **1010273** (highest prevalence) (Table 5.10 and Figures 5.7.1 and 5.7.2). Intensity levels for the remaining ESDs ranged from 9 epg to 90 %.

With the exception of four ESDs in community B, prevalences in nine neighbouring ESDs ranged from 45 % to 82 % with the highest recorded in ESD no. **1020255**. Schools 7 and 8 primary, the schools situated in adjacent ESDs, had *Trichuris* prevalences of 61 % and 70 %, respectively. School 9, also located in the ESD with the lowest prevalence (17 %), **1020265**, had *Trichuris* prevalence of 38 %. The highest median ***Trichuris*** intensity level (450 epg) was recorded in ESD no. **1020255**, correlating with its high prevalence. Median intensity levels in community B ranged from 199 epg to 450 epg. The remaining ESDs had median levels below 90 epg.

Table 5.10 Prevalence (%) of *Trichuris* infection and median (*Md*) intensity levels in the two communities

ESD	n	Prevalence	*Md* Intensity
1010254	10/73	13.70	57
1010255	32/114	28.07	68
1010256	31/112	27.68	137
1010257	26/113	23.01	47
1010258	9/54	16.67	70
1010259	40/96	41.67	149
1010260	48/90	53.33	49
1010261	21/60	35.00	138
1010262	11/45	24.44	80
1010263	8/39	20.51	14
1010264	4/44	11.36	7
1010265	12/33	36.36	283
1010266	31/78	39.74	267
1010267	48/84	57.14	319
1010268	27/84	32.14	23
1010269	20/81	24.69	70
1010270	4/57	7.02	45
1010271	9/72	12.50	156
1010272	14/45	31.11	44
1010273	85/132	64.39	343
1010274	46/103	44.66	92
1010275	40/127	31.50	139
1010276	26/97	26.80	120
1010277	13/110	11.82	34
1010278	2/39	5.13	31
1010279	4/38	10.53	102
1020254	67/96	69.79	206
1020255	69/84	82.14	450
1020256	71/94	75.53	206
1020257	90/125	72.00	199
1020258	83/141	58.87	65
1020259	57/82	69.51	248
1020260	65/88	73.86	300
1020261	25/55	45.54	65
1020262	14/41	34.15	54
1020263	34/95	35.79	72
1020264	9/42	21.43	28
1020265	8/47	17.02	11
1020266	16/44	36.36	270

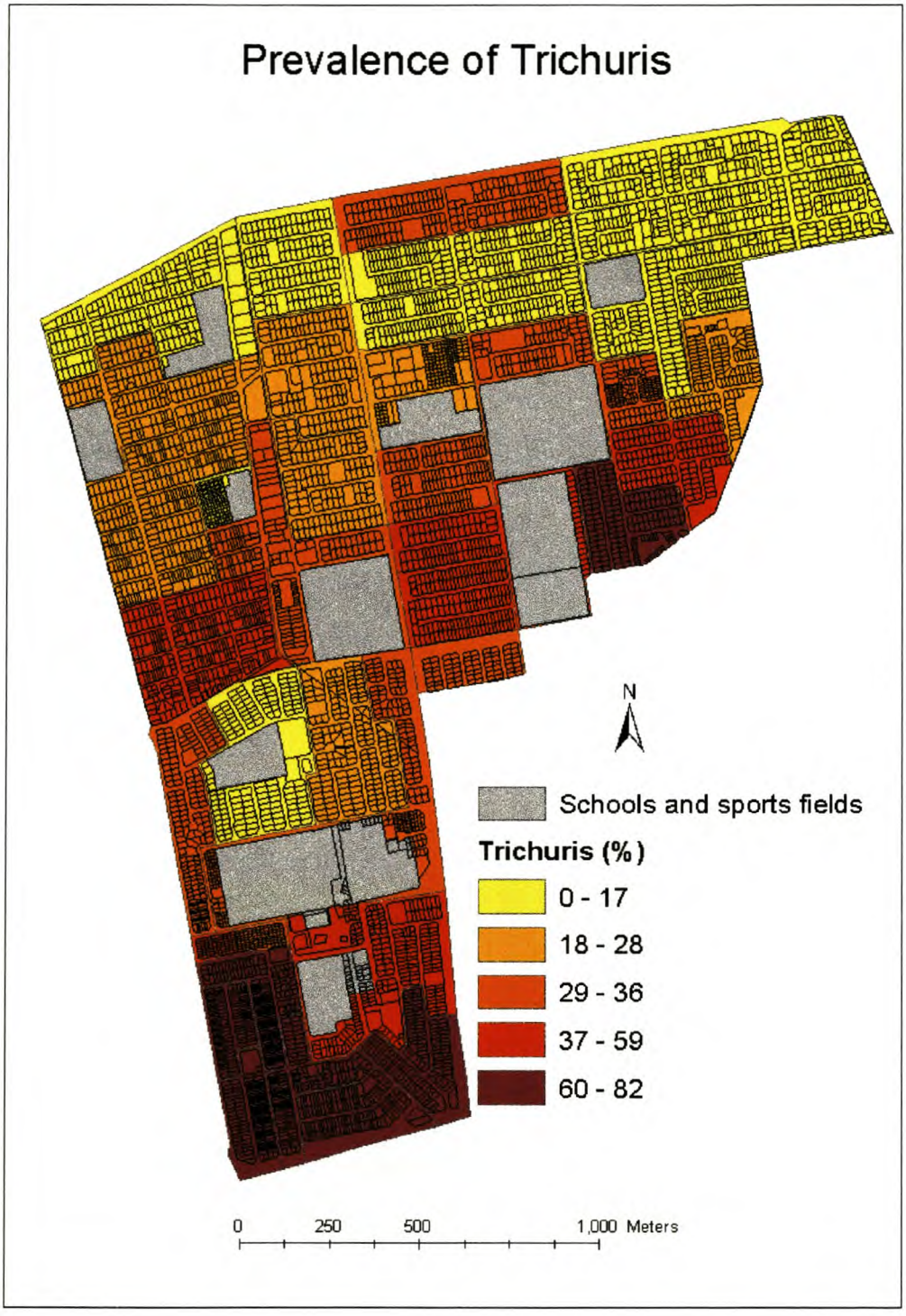


Figure 5.7.1 Geographical distribution of the prevalence of *Trichuris* infection in the two communities

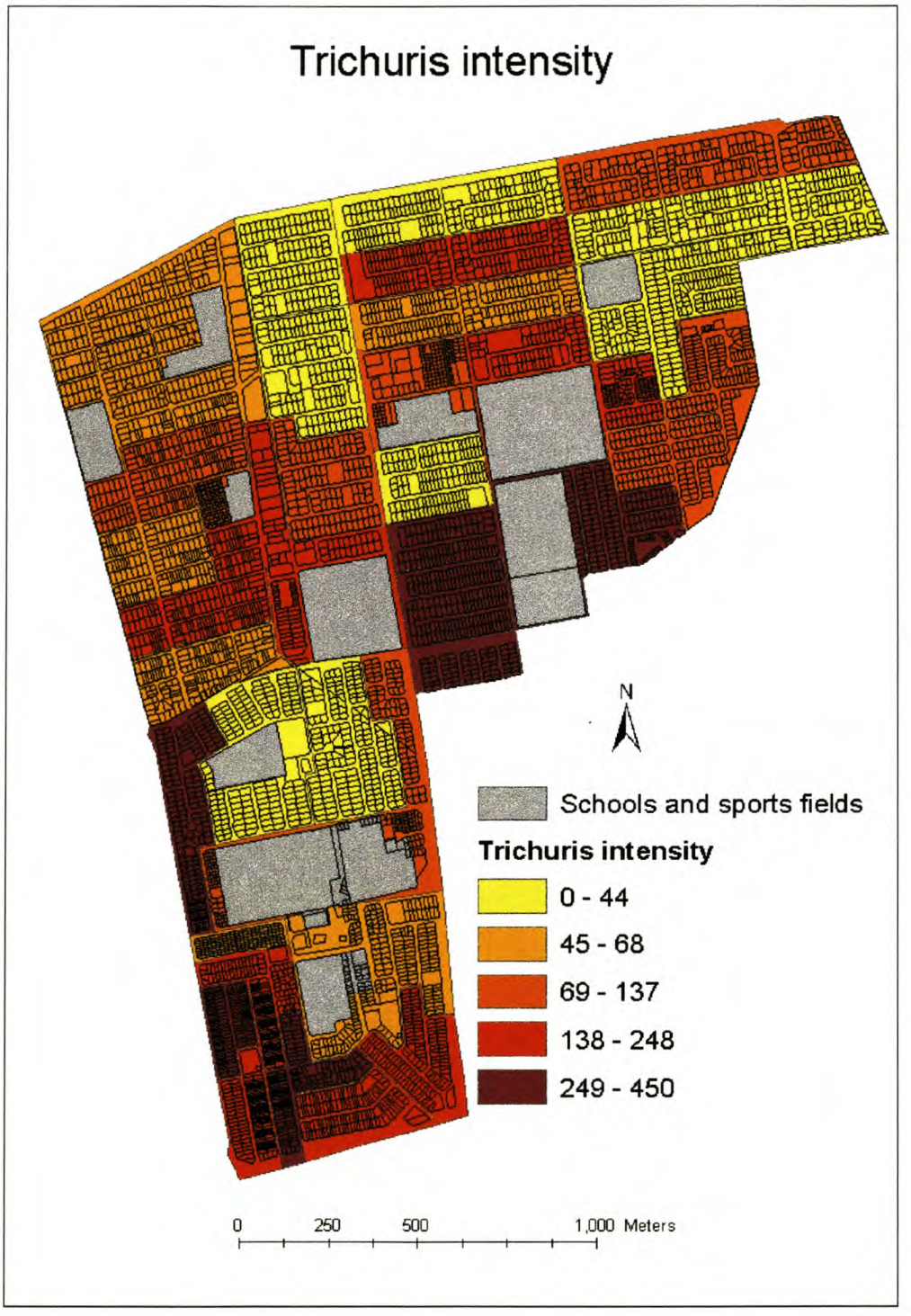


Figure 5.7.2 Geographical distribution of the intensity of *Trichuris* infection in the two communities

5.4.3 Spatial distribution of the prevalence of *Ascaris* and/or *Trichuris*

The prevalences of *Ascaris* and/or *Trichuris* is indicative of the number of children with worms (Table 5.11).

According to Figure 5.8, the majority of ESDs in community A appeared to have relatively low prevalences of worms ranging from 0 % to 34 %. Six ESDs however, had prevalences ranging from 40 % to 72 %. The ESD with the highest prevalence of worms (72 %) (**1020273**) is adjacent to School 4 with a prevalence of 50 %. The ESDs adjacent to **1020273** also had equally high prevalences of worms. In two ESDs adjacent to School 6 (prevalence of 44 %), 40 % and 60 % of the children, respectively, were infected with worms.

It is clear from Figure 5.8, that with the exception of three ESDs, most of the ESDs had high prevalences of worms ranging from 41 % to 86 %. The 86 % prevalence of worms was recorded in ESD, no. 1020255. Adjacent to this ESD, prevalences of 80 % (1020256 and 1020260), 79 % (1020257 and 1020254) and 78 % (1020259) were noted.

Table 5.11 Prevalence (%) of *Ascaris* and/or *Trichuris* infection in the two communities

Community A			**Community B**		
ESD	**n**	**Prevalence**	**ESD**	**n**	**Prevalence**
1010254	19/73	26.0	1020254	76/96	79.2
1010255	46/114	40.4	1020255	73/84	86.9
1010256	42/112	37.5	1020256	76/94	80.9
1010257	37/113	32.7	1020257	99/125	79.2
1010258	15/54	27.9	1020258	86/141	60.9
1010259	50/96	52.1	1020259	64/82	78.1
1010260	54/90	60.0	1020260	71/88	80.7
1010261	24/60	40.0	1020261	26/55	47.3
1010262	12/45	26.7	1020262	17/41	41.5
1010263	9/39	23.1	1020263	44/95	46.3
1010264	8/44	18.2	1020264	9/42	21.4
1010265	12/33	36.4	1020265	10/47	21.3
1010266	32/78	41.0	1020266	19/44	43.2
1010267	50/84	59.5			
1010268	29/84	34.5			
1010269	24/84	29.6			
1010270	11/57	19.3			
1010271	15/72	20.8			
1010272	18/45	40.0			
1010273	96/132	72.7			
1010274	54/103	52.4			
1010275	55/127	43.3			
1010276	33/97	34.0			
1010277	27/110	24.5			
1010278	6/39	15.4			
1010279	8/38	21.1			

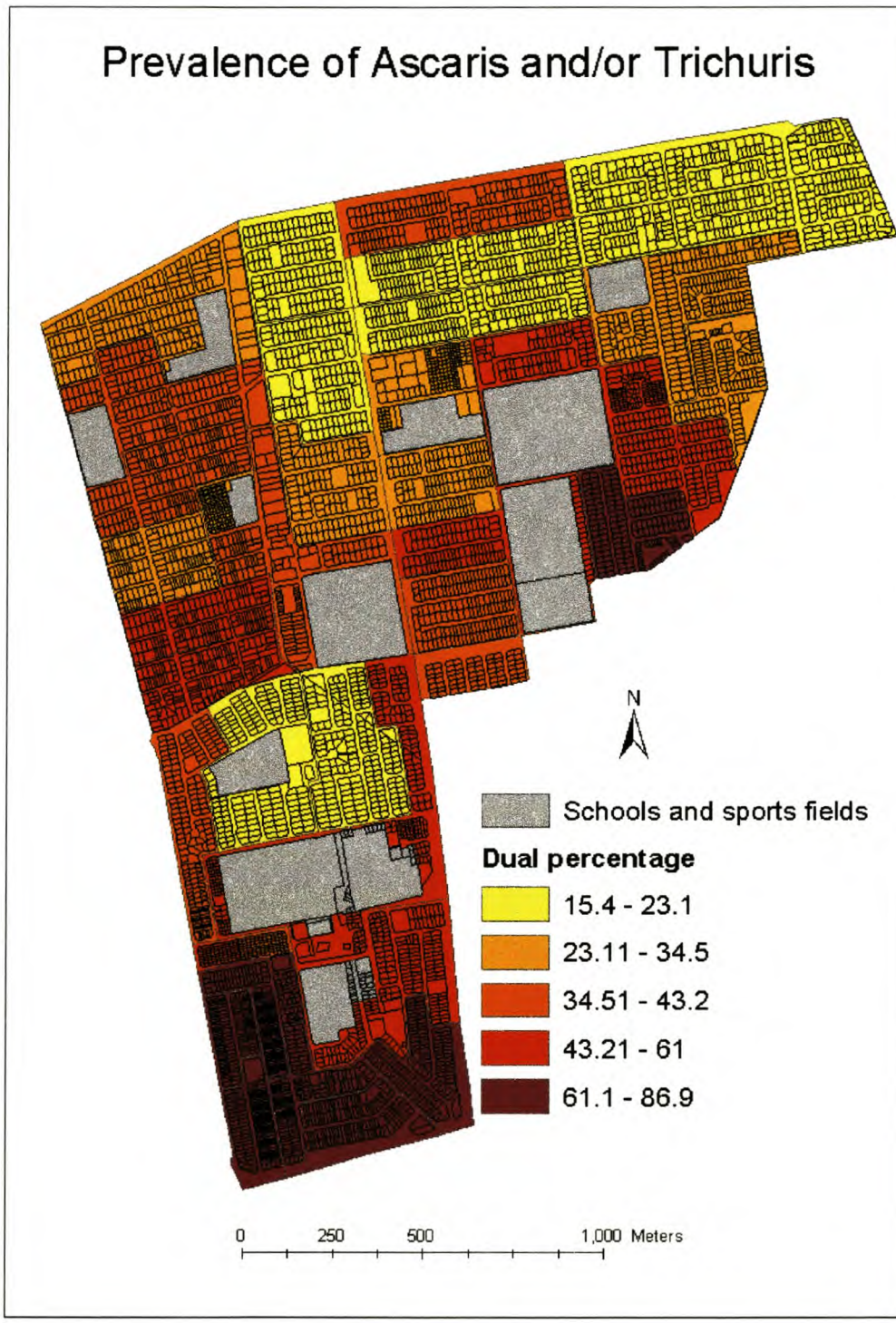


Figure 5.8 Geographical distribution of prevalence of *Ascaris* and /or *Trichuris* infection in the two communities

5.4.4 Association studies

5.4.4.1 Socio-economic conditions and prevalence of *Ascaris* and/or *Trichuris*

As expected, a strong negative association was found between socio-economic conditions and prevalence of worms ($r = -0.779$, $p < 0.01$) in the two communities.

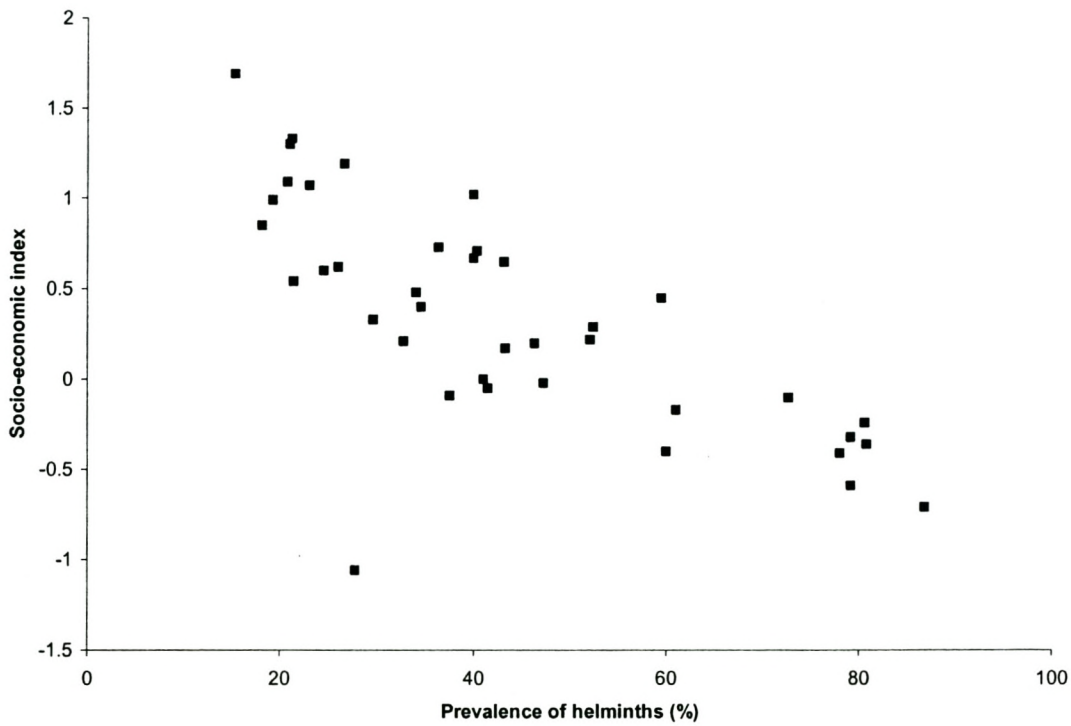


Figure 5.9 Scatterplot of the socio-economic index and the prevalence of helminths

Table 5.12 Environmental variables for the two communities

ESD	*Pop.	% worms	Sanitation	TB rate	Crowd.	SEI	F. Lit. (%)
1010254	7476	26.03	0.50	625.6	1.3	0.62	76.97
1010255	10094	40.35	3.62	578.5	1.2	0.71	72.84
1010256	11075	37.50	1.13	322.2	1.3	-0.09	79.37
1010257	25201	32.74	1.60	250.6	1.1	0.21	78.33
1010258	91913	27.78	0.00	420.2	1.1	-1.06	72.16
1010259	16782	52.08	0.32	1063.8	1.1	0.22	79.31
1010260	15182	60.00	0.00	581.4	1.2	-0.40	73.05
1010261	9850	40.00	0.26	573.6	1.2	0.67	83.96
1010262	12015	26.67	0.51	0	1.3	1.19	88.81
1010263	8802	23.08	0.54	377.4	1.3	1.07	85.37
1010264	12142	18.18	0.00	607.3	1.5	0.85	83.00
1010265	16858	36.36	0.00	300.8	1.2	0.73	86.44
1010266	14024	41.03	0.00	545.8	1.1	0.00	77.61
1010267	18786	59.52	1.53	91.4	1.1	0.45	77.97
1010268	15352	34.52	0.00	307.4	1.3	0.40	82.52
1010269	19189	29.63	0.00	183.9	1.1	0.33	75.29
1010270	9048	19.30	0.00	123.0	1.2	0.99	86.10
1010271	11222	20.83	0.00	99.3	1.3	1.09	91.33
1010272	11250	40.00	0.53	338.4	1.3	1.02	85.11
1010273	14938	72.73	0.00	715.7	1.1	-0.10	74.26
1010274	10898	52.43	0.00	526.3	1.2	0.29	72.34
1010275	8538	43.31	0.52	793.7	1.1	0.17	70.09
1010276	11796	34.02	0.00	253.2	1.1	0.48	73.56
1010277	10055	24.55	0.49	529.1	1.2	0.60	81.59
1010278	9448	15.38	0.41	155.9	1.5	1.69	89.31
1010279	8012	21.05	0.47	0	1.4	1.30	90.04
1020254	19485	79.17	0.10	714.3	0.8	-0.32	62.06
1020255	15266	86.90	0.00	846.3	0.9	-0.71	54.94
1020256	15577	80.85	0.29	536.5	0.9	-0.36	61.61
1020257	16047	79.20	0.00	921.2	0.9	-0.59	61.93
1020258	12781	60.99	0.25	864.2	0.9	-0.17	68.75
1020259	11265	78.05	0.00	580.3	0.8	-0.41	68.06
1020260	22273	80.68	2.47	443.1	0.9	-0.24	65.61
1020261	88513	47.27	0.00	123.0	1.0	-0.02	85.88
1020262	104893	41.46	0.46	0	1.1	-0.05	74.64
1020263	12981	46.32	0.52	184.7	1.2	0.20	77.98
1020264	11387	21.43	0.00	264.3	1.4	0.54	90.70
1020265	11037	21.28	0.00	0	1.4	1.33	86.64
1020266	16448	43.18	0.00	257.5	1.2	0.65	75.17

Variables as described in Section 4.3.3.2.1:
*Pop..: Total population
Crow.: Crowding
Sanitation: % non-flush toilets
TB rate.: TB notification rate
F. Lit.: Female literacy
SEI: Socio-economic Index

5.4.4.2 Crowding and the prevalence *of* Ascaris *and/or* Trichuris

An unexpected, inverse association was found between crowding and prevalence of worms ($r = -0.738$, $p < 0.01$) as it is known that crowding facilitates transmission and spread of disease. However, when correlated with the new TB cases reported, no association was found (Chapter 4).

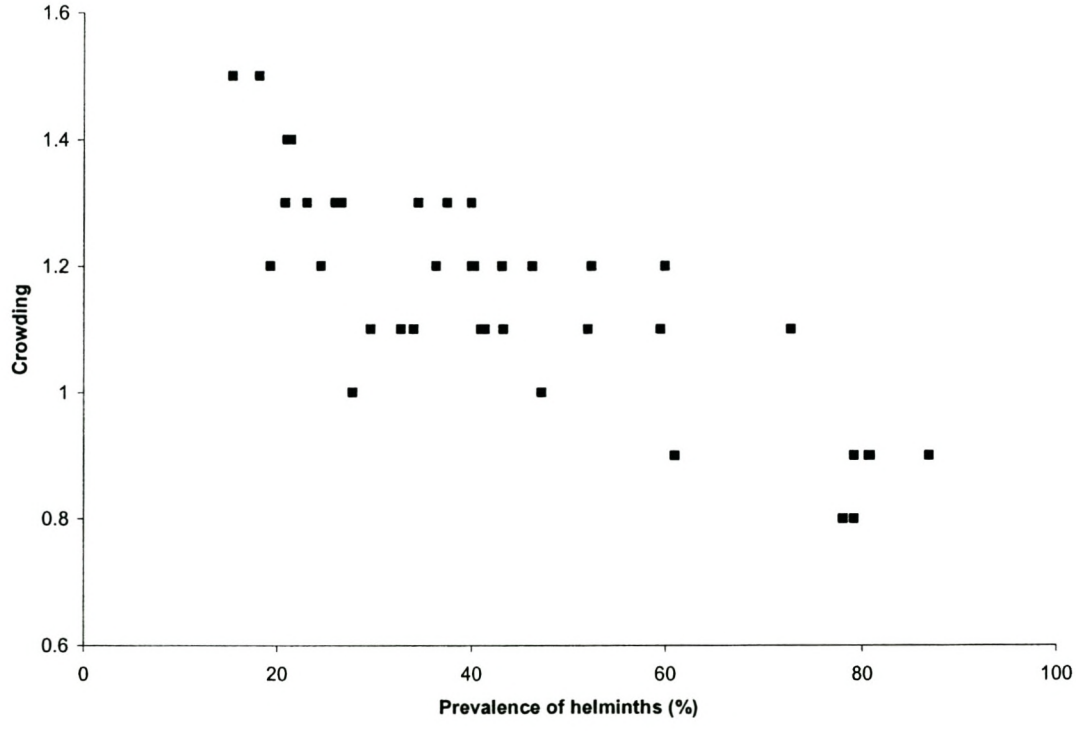


Figure 5.10 Scatterplot of crowding and prevalence of helminths

5.4.4.3 TB notification rate and prevalence of *Ascaris* and/or *Trichuris*

It is well known and accepted that tuberculosis and helminthiasis are both poverty-related diseases. The positive association found between the new TB cases reported and prevalence of worms ($r = 0.533$, $p < 0.01$) in the two communities, is therefore not surprising (Figure 5.11).

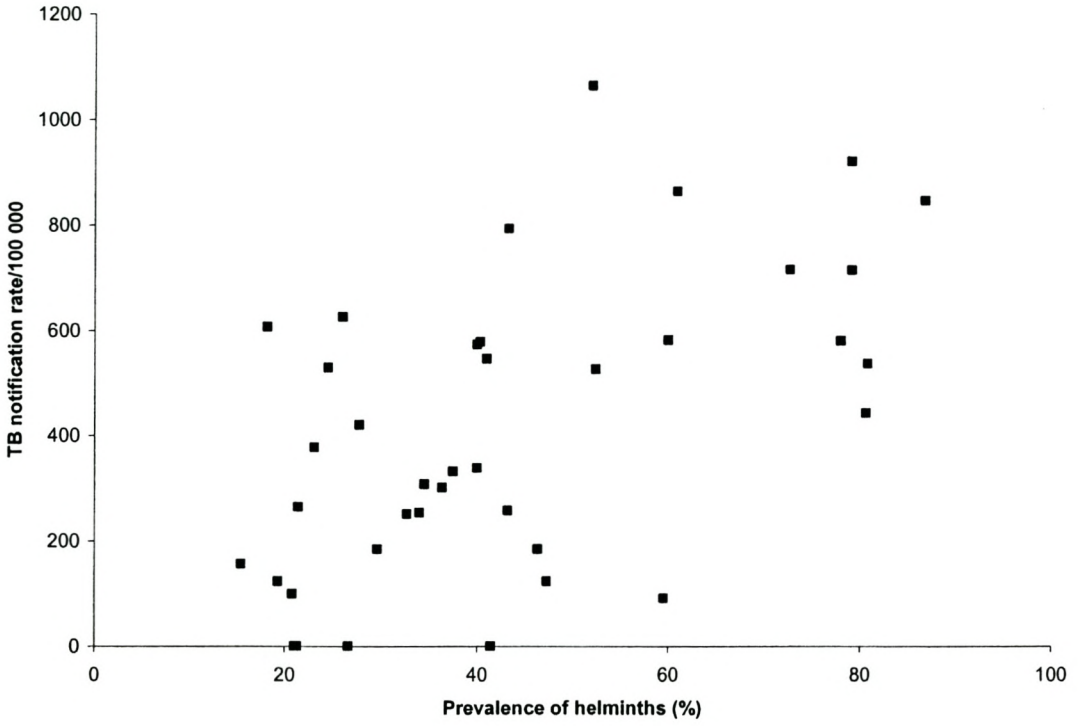


Figure 5.11 Scatterplot of TB notification rate and prevalence of helminths

5.4.4.4 Sanitation and prevalence of *Ascaris* and/or *Trichuris*

No association was found between sanitation and prevalence of worms (r = -0.011; NS) (Figure 5.12). However, as was shown in Section 5.2.2 of this chapter, most of the children enrolled in the study, had sand in their stools. It is therefore possible that although most households have flush/chemical toilets, the children, being the major contributors to transmission (Wong *et al.,* 1988 and Bundy *et al.,* 1991), do not necessarily always make use of the indoor toilet facilities.

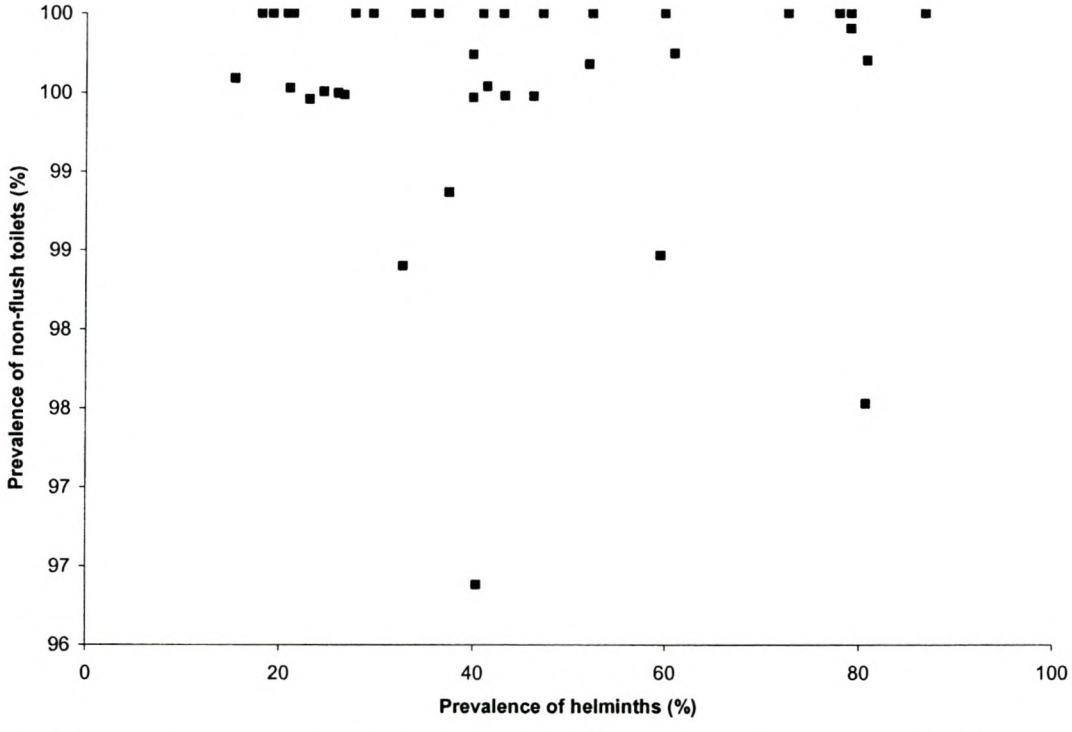


Figure 5.12 Scatterplot of percentage non-flush toilets and prevalence of helminths

5.4.4.5 Population density and prevalence of *Ascaris* and/or *Trichuris*

As expected a positive association was found between population density and the prevalence of worms ($r = 0.528$; $p < 0.01$) (Figure 5.13).

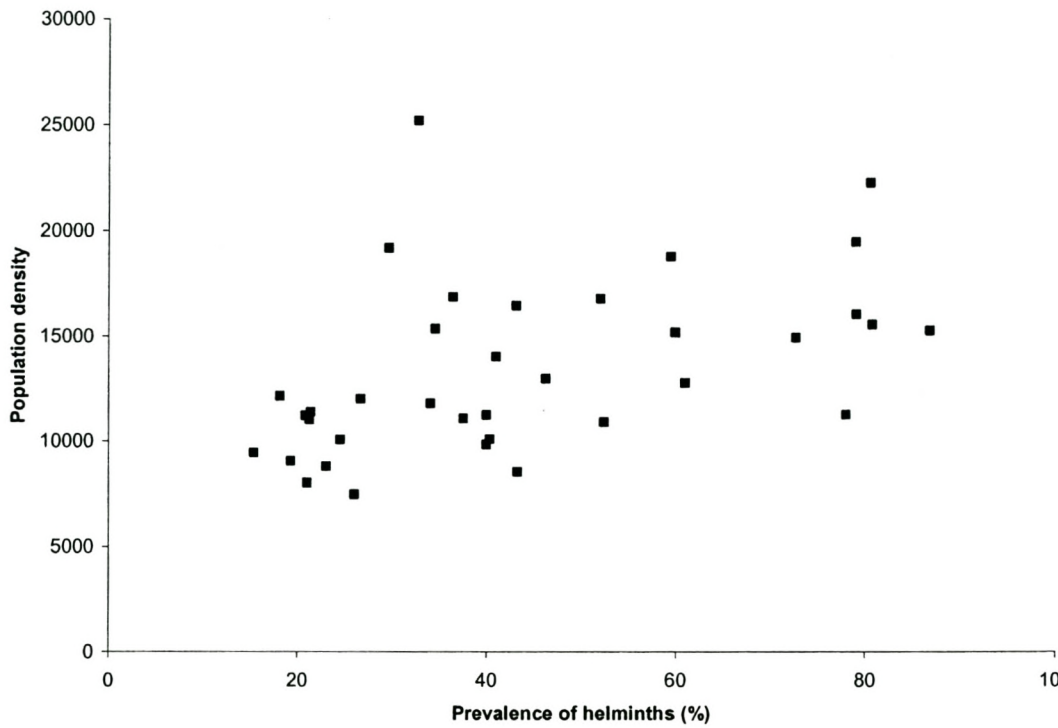


Figure 5.13 Scatterplot of population density and prevalence of helminths

5.4.4.6 Female literacy and prevalence of *Ascaris* and/or *Trichuris*

A strong negative correlation was found between the percentage of females completing primary education and the prevalence of helminths ($r = -0.704$; $p < 0.01$) (Figure 5.14).

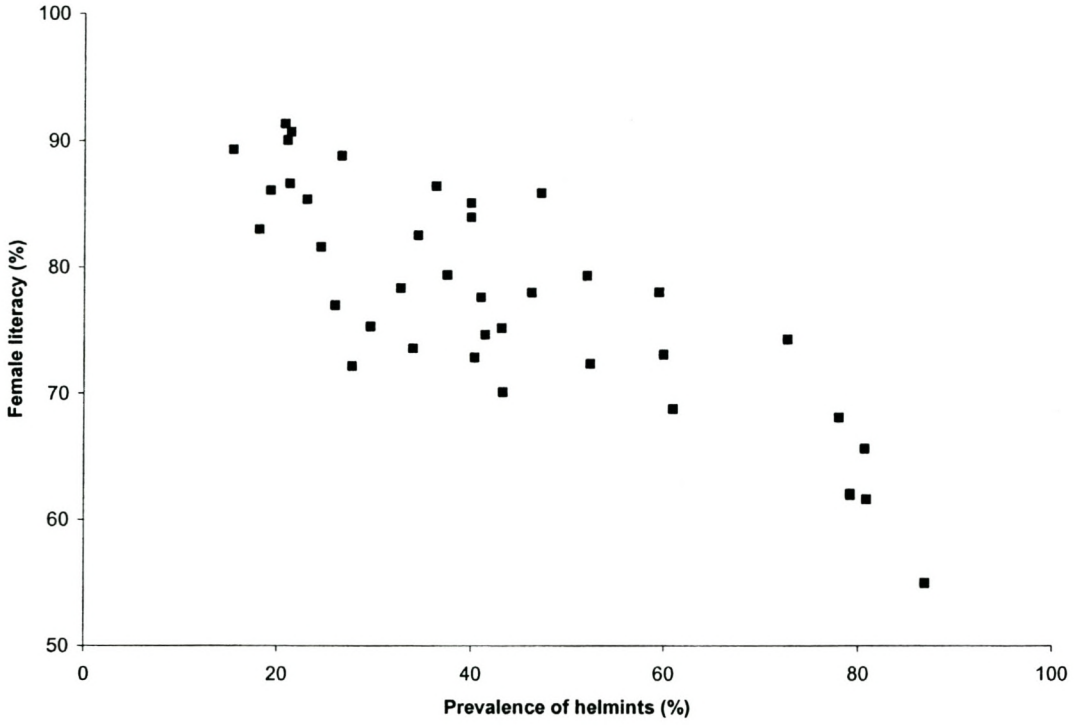


Figure 5.14 Scatterplot of percentage females completing primary school education and the prevalence of helminths

5.5 DISCUSSION

This study is the first of its kind in this particular community. The success of the study was largely attributed to the good communication between the health workers, school staff and learners.

5.4.1 Prevalence of helminths

In the present study, *Trichuris* was the most common parasite in both communities. Similar high prevalences of this helminth were previously reported in the Western Cape (Gunders et al., 1993; Fincham *et al.,* 1996; Arendse, 2000) and other parts of South Africa (Bradley and Buch, 1994; Jackson *et al.,* 1998). However, it is thought that some of the children in the two communities received anti-helminthic treatment. The treatment would result in expulsion of mainly male *Ascaris* adult worms (Van den Bossche 1982), which could explain why many of the *Ascaris* eggs found in the stool samples, were unfertilised (personal communication: Dr John Fincham, Medical Research Council) (data not shown). Also, anti-helminthics are less efficacious against *Trichuris* than against *Ascaris* (Bundy *et al.,* 1987; Bundy and Cooper, 1989; Chan *et al.,* 1994; Forrester *et al.,* 1998; Jackson *et al.,* 1998), which could have resulted in the higher prevalence of *Trichuris* in the study communities.

Furthermore, it is interesting to note that Schools 1, 2 and 3 in community A, had constantly lower prevalences compared to the other schools in this area. Although situated in community B, School 9 had equally low prevalences for all parasites tested.

5.4.2 Infection with parasitic protozoa

The protozoan *G. lamblia* causes infection of the intestinal tract, with diarrhoea as the most common symptom (Fraser *et al.,* 2000). In the present study, we found that cysts of *G. lamblia* were present in 15 % of children tested. However, because cysts are not shed at a constant rate (Fincham *et al.*, 1999) and may not be found in an isolated stool specimen from an infected child, prevalence of *Giardiasis* could have been higher if multiple feacal samples were tested over time. Transmission normally occurs through the ingestion of the infective cysts by faeces-to-hand-to-mouth or sewage-to-water-to-mouth (Fincham *et al.*, 1996). Because most of the

households have flush or chemical toilet facilities, the prevalence of *G. lamblia* (and any other parasite) can be attributed to children not washing hands after using the toilet.

It is estimated that all humans harbour nine species of intestinal amoebae, of which only one, *Entamoeba hystolytica*, is a pathogen (Cox, 2002). The life-cycle of amoebae is simple in that these organisms live and multiply in the gut and form cysts that are passed out in the faeces and infect new individuals when they are ingested in contaminated water or food. Apart from *Giardia*, the most common protozoa found in the present study, was *Entamoeba coli,* and *Endolimax nana*. The presence of these protozoa merely indicates that the infected individual ingested food or water, that was contaminated with stool containing the amoebae, thus indicating poor sanitation and or poor hygiene.

Two morphologically identical species and strains of *E. hystolytica* can be identified, namely, *E. hystolytica* which can cause disease and *E. dispar*, which cannot (Sargeaunt *et al.*, 1978). Distinction between the two species is only by serology and stool culture. The prevalence of 10.5 % in community A and 8.8 % in community B, certainly warrants the need for differential diagnosis as amoebiasis is a potential lethal disease. In the light of this fact, special mention is made of School 5 and 8 with prevalences of 21.6 % and 12.3 %, respectively. Tissue invasion by *E. hystolytica* has been associated with suppression of cell-mediated immunity; suggested to be responsible for the acquired protective immunity observed in recovering cases of extra-intestinal-amoebiasis (Ortiz-Ortiz *et al.*, 1975; Ghosh *et al.,* 1995) .

Blastocystis hominis was also one of the most common protozoa found in the present study. Although only 6.6 % of children in Ravensmead were infected, the prevalence of this protozoan in School 4 was 15 %. Prevalences were higher in community B with 18.4 % of children infected. *B. hominis* is probably the most common human gut protozoan in the world, with > 50 % prevalence in developing countries (Stenzel and Boreham, 1996). Within communities, groups from lower socio-economic levels who suffer from poor environmental hygiene, due largely to lack of water supply, sewerage and waste removal services are at greater risk of infection (Cirioni *et al.,* 1999; Tan *et al.,* 2002). *B. hominis* infections have been implicated in a variety of specific and non-specific intestinal disorders such as diarhoea, abdominal discomfort,

anorexia and flatulence (Boreham and Stenzel, 1993). It is not known how *B. hominis* is transmitted, although the number of people infected seems to increase in areas where sanitation and personal hygiene is inadequate. Very little is known about the host immune response to *B. hominis* except evidence from studies indicating that *B. hominis* can induce humoral immune responses (Chen *et al.,* 1987; Zierdt *et al.,* 1995; Hussain *et al.,* 1997).

5.4.3 School related prevalence

5.4.3.1 School 8 situated in community B

Special reference is made to School 8. This school is not only situated in an area (ESD) with the highest TB rate, but also in an area with the lowest household income, poorest socio-economic status, lowest female literacy level and the highest level of overcrowding. It is therefore not surprising that the highest prevalence of not only *Ascaris* and *Trichuris*, but every other helminth and protozoan tested in this study, were recorded at School 8. The intensity of infections with *Ascaris* and *Trichuris* were also highest at this school. Although stunting was not pronounced in community B, stunting and impaired growth were more prevalent in this school compared to the other schools. Since worm burden is associated with the severity of morbidity, these children are likely to suffer most at an age when they are both growing and learning (Nokes and Bundy, 1994). Geohelminth infection has been repeatedly shown to be associated with lower scores on tests of mental performance, educational achievements and school attendance in school-age children (Nokes *et al.,* 1991; Callender *et al.,* 1994; Nokes and Bundy, 1994; Simeon *et al.,* 1994; Sakti *et al.,* 1999).

Therefore, cognisance should be taken with regard to the possible negative effect of helminths on children in the schools in this area, especially School 8. In order to do so, attention should be focused on Schools 1 and 3 with minimal prevalences of worms and protozoa, in order to examine differences between schools with high prevalences. In this regard, teachers should be drawn into educational and environmental analyses and subsequent actions. Health promotion education, the adoption of hygienic practices and school-based deworming programmes, have shown to have long-term benefits and have proven to be effective (Holland *et al.*, 1996; Stolzfus, *et al.*, 1997; Mascle-Taylor *et al.*, 1999).

5.4.4 Growth profile of children

Although the population studied lives in areas that present several potential correlated risk factors for malnutrition and subsequent stunted growth, the prevalence of stunted growth was still relatively low. The growth of children in these communities were within the normal range with the exception of the three schools in community B with values skewed to impaired growth.

The present study has however, demonstrated a positive association between stunted growth and ascariasis ($r = -0.8$; $p < 0.05$) as well as trichuriasis ($r = -0.78$; $p < 0.05$). It is well recognised that helminth infections, by interfering with appetite and resulting in decreased food absorption through vomiting and diarrhoea (Stephenson *et al.,* 1987; reviewed by Sakti *et al.*, 1999), are associated with stunted growth, malnutrition and impairment of learning (Evans and Guyatt 1995; Savioli *et al.,* 1995). More specifically, Forrester and co-workers (1998) have shown that even symptomless trichuriasis impaired growth in children.

Taking into the account the importance of this subject for public health policies in especially developing countries, additional studies confirming the role of intestinal parasites in determining malnutrition are necessary.

5.4.5 Age-dependent prevalence and intensity of infection

The aggregation of worms in a few hosts has important implications: heavily infected hosts are of concern not only because they are most likely to experience disease, particularly if they are young and malnourished, but also because they may make a major contribution to the transmission of infections within their communities. Worms are normally not evenly distributed between hosts and it is typical to find that a large proportion of all worms occur in only a small proportion of all hosts (Elkins *et al.,* 1986; Holland *et al.,* 1989). Thus, the degree of aggregation of worms has important implications in our understanding of the epidemiology of infections and how to control disease.

The intensity of infection in the present study, is clearly age-dependent. Intensity of infection may however, also be influenced by the genetic, nutritional and physiological status of the host and by the age and the presence of other infections (Bundy *et al.*, 1987; Maizels *et al.*, 1993;

Booth *et al.*, 1998). It is well established that children are more heavily infected than adults and as such, harbour a disproportionate amount of worm burden within a community (Bundy *et al.*, 1987; Faulkner *et al.*, 2002).

In both communities we found that the prevalence of trichuriasis peaked in the children aged between 12 and 15 years of age. This confirms prevalences reported in other studies (Bundy *et al.*, 1987; Anderson *et al.*, 1993; Lon-Qi *et al.*, 1995; Smith *et al.*, 2001) showing that the peak intensity of *Trichuris* infection is normally between the ages 10 to 29 years, suggesting that *Trichuris* may be a more chronic infection than *Ascaris*. However, theoretical studies predict that acquired immunity can result in an early high peak in infection in areas of high transmission (Anderson and May, 1985; reviewed by Needham *et al.*, 1992). This would explain the peak in the 7 year old children obtained by Faulkner and co-workers (2002) in a community with a prevalence of 96.6 %.

In contrast to the prevalence of *Trichuris*, we found that the prevalence of *Ascaris* peaks at an early stage in community A (in children younger than 6 years of age) and in Uitsig the intensity of infection peaked between the ages 9 to 11 years old. There is evidence for age-dependency in the human immune response to *Ascaris* with intensity peaking in children aged 5-14 years old (Bundy *et al.*, 1987; Haswell-Elkins *et al.*, 1989; Turner *et al.*, 2002). The early peaks in *Ascaris* intensity obtained in communities A and B, could be due to the low prevalence of *Ascaris* in the community, specifically community A, or it could be ascribed to the development of protective immunity in infected individuals following sustained exposure and immune maturation (Warren *et al.*, 1973; Bundy *et al.*, 1991)

5.4.6 Age-prevalence by gender

Gender did not influence the likelihood of infection with either parasite in this community. Magambo and coworkers (1998) found that the infection rate of several intestinal parasites including *Trichuris* was higher in males compared to females in Sudan. An epidemiological study in Madagascar found that girls had a significantly higher prevalence and intensity of ascariasis (Kightlinger *et al.*, 1995), while another study from Guatemala did not find any gender differences with respect to parasitoses (Anderson *et al.*, 1993). These results may

indicate that gender may or may not play a role in parasitoses, depending on the region and other environmental or behavioral factors.

5.4.7 Geographical distribution of helminth prevalence and intensities

The value of using a geographical information system was highlighted in this particular study a enumerator sub-districts (ESDs) with "abnormal" immune responsiveness were identified due to th increased exposure to helminths. From the GIS in this study, we could see that individuals with th most severe helminth infections, were concentrated in the same ESDs. Thus, cognisance should b taken of children and adults residing in these areas as these ESDs also have the highest TB incidence lowest household income, poorest socio-economic status, lowest maternal education level and th highest level of overcrowding (Chapters 3 and 4).

The high helminth prevalence in these ESDs, confirms the notion that often a minority of a population will produce large amounts of eggs indicating heavy infections, while a majority will have light infections and produce few eggs (Palmer and Bundy, 1995). There is also a familial aggregation and predisposition to *Ascaris* and *Trichuris* infections (Chan *et al.*, 1994b) which does not appear to be genetically determined but may be more strongly determined by environmental and behavioural factors (Chan *et al.*, 1994c). Furthermore, geohelminth infection has been repeatedly shown to be associated with lower scores on tests of mental performance, educational achievements and school attendance in school-age children (Sakti *et al.,* 1999; Nokes *et al.,* 1991; Callender *et al.,* 1994; Nokes and Bundy, 1994; Simeon *et al.,* 1994). Therefore, as a long term engagement, routine health education for teachers (of schools situated in the mentioned areas), children and adults should be implemented with subsequent adoption of good hygienic practices and school-based deworming programmes.

5.4.8 Sanitation and hygiene

As helminth infections are normally associated with poor sanitation, Ravensmead and Uitsig proved to be the exception to the rule. All nine schools participating in the study, had flush toilets that are clean, as well as adequate facilities for washing hands. Almost all the households in this community have either flush or chemical toilets. In isolated cases, pit and bucket latrines are used as toilets. This would then explain the absence of any association between sanitation

and the prevalence of helminths. It is also possible that differences in prevalence between the two communities, may have been due to the degree of hygienic practices in each area. However, as children in most of the schools had high prevalences of sand in stool samples, it would indicate that the children might not necessarily make use of the toilet facilities at home.

5.4.9 Prevalence of infection and associated ecological variables

In 1994, Chan and co-workers estimated that worldwide there were 1 471 million and 1 048 million cases of *Ascaris* and *Trichuris* infections, respectively. Clinically, *Ascaris* can cause blockage of the intestine and *Trichuris* has been associated with dysentery (Cooper *et al.,* 1992). Both helminths have also been associated with stunted growth (Cooper and Bundy 1988, Adams *et al.,* 1994; Saldiva *et al.,* 1999) and impaired cognitive functions in children (Nokes *et al.,* 1992; Oberhelm *et al.,* 1998). Although many studies regarding intestinal parasites focus on reporting the prevalence and intensity of these infections, fewer studies have examined the socio-economic factors that affect transmission of intestinal helminths. Some studies have however, shown that the lack of latrines, occurrence of diarrhea, lower socio-economic status, inadequate disposal of human excreta and the level of sanitation in households are related to parasitoses (Cooper and bundy 1988; Holland *et al.,* 1988; Mahfouz *et al.,* 1997; Gamboa *et al.,* 1998).

We found that infection with *Ascaris* and or *Trichuris* occurred more frequently in the children who lived in an ESD where females had the least formal education. This was also reported by several other studies in other areas (Tshikuka *et al.,* 1995; Holland *et al.,* 1998; Smith *et al.,* 2001). Studies have shown that children living in homes without latrines have a higher prevalence of *Ascaris* and *Trichuris*, than those living in homes with latrines; the introduction of pit latrines can help lower the prevalence of diarrheal diseases (Hoque *et al.,* 1996). Although we did not compile information regarding the availability of water in these communities, the availability of water may also influence a household's sanitation level in general (VanDerslice and Briscoe 1995; Semenza *et al.,* 1998).

The results shown in this study indicate that there are certain factors observed in helminth transmission, which are common in developing countries, such as general contamination of the

living environment and overcrowded households. Low-socio-economic conditions and poor conditions of hygiene not only place children at higher risk of contracting infection, but also compromise their resistance to the parasites. Hence, knowledge of such factors may help reduce costs of studies for policy formulation and facilitate implementation of public health interventions designed to reduce the incidence of helminth infections in communities with similar environmental conditions and sanitary practices. Community-based mass chemotherapy on a regular basis can meet short term objectives of intestinal helminth reduction, and is the most cost-effective method. Health promotion education, the adoption of hygienic practices and school-based de-worming programmes can have long-term benefits and have already proven effective in other countries (Holland *et al.,* 1996; Mascle-Taylor *et al.,* 1999).

ADDENDUM TO CHAPTER FIVE

HYPERENDEMIC HELMINTHIASIS AND THE CONCEPT OF PREPUBERTAL VACCINATION AGAINST HIV/AIDS

Work presented in this addendum to Chapter 5, has been submitted for publication to *Acta Tropica* as:

Hyperendemic helminthiasis and the concept of prepubertal vaccination against HIV/AIDS

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Abstract

Prepubertal immunisation to reduce heterosexual spread of subtype C of HIV-1 is one of the objectives of the HIV/AIDS vaccine initiative in South Africa. A potential threat to this goal is the possibility that vaccine efficacy could be impaired by sustained or intermittent immune activation caused by endemic worm infestation. The results of surveys that were undertaken to determine the prevalence of helminthic infection in children and adolescents at schools in Ravensmead and Uitsig, which are adjacent residential areas in Cape Town, South Africa, are relevant in this context. The overall prevalence of ascariasis and/or trichuriasis, with the standard error bracketed, was 55.4 (0.8) % (n = 3 890). The range was from 43.1 (2.1) % in children who were 6 years old (n = 541) to 59.9 (3.3) % in adolescents aged 14 or more (n = 217). In addition to *Ascaris* and *Trichuris*, 2.2 (0.23) % of children had eggs of the dwarf tapeworm (*Hymenolepis nana*) in their faeces. These substantial infection rates were in an age group intended as a primary target for vaccination against HIV/AIDS in communities that are not the poorest of the poor, and which have been well-serviced for many years in terms of public health, medical, educational and other facilities. The socioeconomic and demographic conditions in the two communities have not changed over time and the population density is intermediate between that in slums and affluent suburbs. Where poverty is worse, such as in slums in Cape Town and Durban, the prevalence and intensity of worm infestation are known to increase, as does the incidence of HIV/AIDS as well as enteric and respiratory diseases. Development and testing of vaccines against HIV and the medical management of AIDS are costly and difficult for various reasons, whereas control of worm infestation is inexpensive, achievable and could yield spinoffs beyond currently-known benefits.

Keywords: vaccine; immunisation; poverty; *Ascaris*; *Trichuris*; *Hymenolepis*

5A.1 Introduction

In South Africa, HIV/AIDS has become the leading cause of death, especially in impoverished communities where helminthiasis is hyperendemic (Bentwich *et al.*, 1999; Ncayiyana, 2000; Arendse, 2001; Dorrington *et al.*, 2001; Mosala, 2001; Makgoba *et al.*, 2002; Fincham *et al.*, 2003a). The South African AIDS Vaccine Initiative (SAAVI) has been mandated to develop a vaccine against subtype C of HIV-1 that is safe, effective and affordable. To reduce the predominantly heterosexual spread of HIV, it is logical that immunisation should precede puberty (Makgoba *et al.*, 2002). However, worm infestation (especially by *Ascaris* and *Trichuris*) often peaks in children and can cause excessive skewing towards humoral immunity. This kind of immune profile might make some anti-HIV vaccines less effective because they are intended to work mainly via cellular immunity (McMichael *et al.*, 2002). Likewise, the efficacy of immunisation against tuberculosis (TB), cholera, diphtheria, tetanus and possibly other diseases, appears to be reduced when worms are highly endemic (Bentwich *et al.*, 1999; Bundy *et al.*, 2000; Cooper *et al.*, 2000a,b; Cooper *et al.*, 2001; Elias *et al.*, 2001; Wolday *et al.*, 2002; Fincham *et al.*, 2003a,b). Evidence that helminthiasis could be a factor in the infection dynamics of HIV and progression to AIDS has been published (Bentwich *et al.*, 1999; Bentwich, 2000; Bundy *et al.*, 2000; Markus and Fincham, 2000; Elias *et al.*, 2001; Markus and Fincham, 2001a,b,c; Fincham *et al.*, 2002; Wolday *et al.*, 2002; Fincham *et al.*, 2003a,b) and reviewed for the World Health Organization (Fincham *et al.*, 2003a). Geographically, there is substantial overlap of the global distributions of helminthiasis, HIV/AIDS, TB and poverty (Beyers *et al.*, 1996; Bentwich *et al.*, 1999; Bundy *et al.*, 2000). In the context of the possibility that ascariasis and trichuriasis could impair the efficacy of vaccination against HIV/AIDS, cognisance should be taken of the prevalence of infestation by soil-transmitted helminths in children and adolescents from the adjacent, relatively poor residential areas of Ravensmead and Uitsig in Cape Town. The incidence of HIV/AIDS is increasing in these communities, in which there is a high prevalence of active TB and where bacille Calmette-Guérin (BCG) vaccine administered soon after birth, does not appear to be effective (Beyers *et al.*, 1996; Adams *et al.*, 1999). Although the study areas are socioeconomically depressed, poverty and over-crowding are worse in other parts of the city.

5A.2 Methods

Permission to undertake the study was obtained from the Ethics Committee of the South African Medical Research Council. All the primary schools serving the community of Ravensmead (six schools designated A - F) and three of four schools serving Uitsig (designated G - I) were included in a helminthological survey. After informed consent had been obtained from parents or guardians, children were asked to provide a faecal sample and each child was supplied with a thread-topped sterile container in which to place the specimen. The formal-ether concentration technique was used to process the samples for microscopy (Ash and Oriel, 1987). Two experienced microscopists examined subsamples from each specimen for worm eggs and microscopic grains of silica as an indication of either deliberate (geophagia) or accidental ingestion of earth, both of which could result in eggs of *Ascaris* and *Trichuris* being swallowed. All children in the schools, *i.e.* including those who did not supply faeces, were dewormed with mebendazole (*Vermox*®, Janssen-Cilag) after sampling had been completed.

A logistic analysis of variance was used to assess relationships between the binary dependent variables in faecal samples (eggs of *Ascaris* or *Trichuris*, as proof of helminthic infection; and sand) and the independent variables (gender, age, school and residential area). Wald chi-square statistics were used to test for overall and individual effects. Age was treated as a variable with nine classes by number of birthdays elapsed, *i.e.* a child was taken as being six years old up to the seventh birthday, and so on.

To gain some perspective on poverty as a co-factor with the endemic diseases of interest, a comparison of population density was undertaken. The number of people per km^2 and living within each dwelling were calculated for the study areas as well as more affluent and poorer areas of Cape Town. A national census completed three years before the helminthological survey was the source of demographic data. A caveat with regard to the second variable, *i.e.* the number of people per house, is that the data did not include information on either the floor area of houses, which are bigger in the affluent suburbs, or on the number of houses (= shacks) in the slums.

5A.3 Results

There were 5 607 pupils in the nine schools, of whom 72.6 % (4 069/5 607) returned faecal samples (the school-based compliance range was 67.9 % to 78.3 %). The number of samples for analysis was 3 890 after 179 had been discarded because they were not labelled and/or the quantity of faeces was too small. The usable samples were supplied by 1 935 boys, 1 936 girls and 19 individuals for whom gender was not recorded (n = 3 890). Since the dependent variables (*i.e.* worm eggs and sand) were not influenced by sex, results are reported for the total sample (n = 3 890). More than half of these one-off samples contained eggs of *Ascaris* and/or *Trichuris* (Table 1). Worm infestation and ingestion of sand differed significantly by residential area, age and school. In Uitsig, more children had worm eggs and sand in their faeces (Table 1); and poverty as reflected by greater population density/km^2 (Table 2) and the appearance of houses, seemed worse. The number of people per dwelling in Ravensmead and Uitsig was similar, but in Ravensmead the houses are generally larger.

Children aged between 6 and 9 years old were more likely to have sand in their faeces, suggesting greater exposure to infection by *Ascaris* and *Trichuris*, which are soil-transmitted helminths. However, this was not reflected by the age-related prevalence of worm infestation because *Ascaris* was uniformly distributed; and children aged 11, 13 and 14+ had more *Trichuris*. Thus, ingestion of sand appeared to pertain more directly to poverty than to worm infestation. Children at some schools were more likely to have worms and/or sand in their faeces (Table 5A.1). This trend was consistently evident in the Uitsig schools, which is in line with the demographic (Table 5A.2) and probable socioeconomic differences between the areas.

The only other helminth eggs detected were those of the dwarf tapeworm (*Hymenolepis nana*). Prevalences were 2.2 (0.23) % overall, 1.4 (0.23) % in Ravensmead and 3.6 (0.5) % in Uitsig. The life cycle differs fundamentally from those of soil-transmitted helminths; and either humans or rats can be definitive hosts. The association with rats has implications for health as regards food hygiene and some infectious diseases.

Table 5A. Percentage of children having worm eggs or sand in an single faecal sample*, with standard errors bracketed

	Any egg	*As.* egg	*Tt.* egg	Sand	n
Overall	55.4 (0.8)	24.8 (0.69)	50.6 (0.8)	52.2 (0.8)	3890
Area					
Ravensmead	45.3 (0.99)	15.9 (0.73)	41.0 (0.98)	40.1 (0.97)	2528
Uitsig	74.2 (1.19)[b]	41.4 (1.34)[b]	68.4 (1.26)[b]	74.7 (1.18)[b]	1362
Age in years (by elapsed birthdays)					
6	43.1 (2.13)	22.4 (1.79)	36.0 (2.07)	54.2 (2.14)[e]	541
7	56.8 (2.27)	26.3 (2.02)	52.8 (2.29)	57.5 (2.27)[e]	475
8	58.7 (2.28)	27.4 (2.07)	52.9 (2.31)	59.5 (2.27)[e]	467
9	55.9 (2.28)	23.7 (1.95)	51.5 (2.29)	57.1 (2.27)[e]	476
10	57.4 (2.24)	26.8 (2.01)	50.4 (2.27)	52.5 (2.26)	488
11	59.5 (2.31)[c]	23.6 (1.99)	56.0 (2.33)[d]	45.2 (2.34)	454
12	54.1 (2.30)	24.2 (1.98)	49.3 (2.31)	49.3 (2.31)	471
13	59.1 (2.84)	25.3 (2.51)	57.1 (2.86)[d]	44.2 (2.87)	301
14	59.9 (3.33)	23.5 (2.89)	57.6 (3.36)[d]	41.0 (3.35)	217
Schools					
Florida (R)	40.2 (3.58)	16.9 (2.74)	36.5 (3.51)	32.3 (3.41)	189
Nebo (R)	59.2 (2.21)[f]	22.2 (1.87)	55.4 (2.24)[h]	39.2 (2.20)	495
Northway (R)	34.2 (2.09)	8.3 (1.22)	32.1 (2.06)	25.3 (1.91)	517
Pinedene (R)	37.6 (2.48)	9.9 (1.53)	35.5 (2.45)	25.1 (2.22)	383
Vorentoe (R)	46.1 (2.39)	15.4 (1.73)	41.5 (2.36)	71.8 (2.16)[i]	436
Webner (R)	50.0 (2.22)	22.1 (1.84)	41.3 (2.19)	42.9 (2.20)	508
Tygersig (U)	67.3 (2.05)[f]	38.2 (2.21)[g]	61.8 (2.21)[h]	60.1 (2.14)	526
Uitsig (U)	78.0 (1.83)[f]	47.2 (2.20)[g]	71.2 (1.99)[h]	74.1 (1.93)[i]	517
Wilmot (U)	79.6 (2.26)[f]	37.3 (2.71)[g]	74.6 (2.44)[h]	100 (0.00)[i]	319

R = Ravensmead; U = Uitsig

*Superscripts [b,c,d,ef,g,h,i] indicate significantly increased percentages within the categories of area, age, school, helminth and sand, respectively.

[a]Eggs of *Ascaris* and/or *Trichuris* were present in the sample, but not necessarily *Hymenolepsis nana*

Table 5A.2 Population density and occupation density of houses as indices of poverty in the study sites; as well as for different and poor areas in cape Town

Area	Tot	Area (km^2)	People/km^2 (range)	Number of homes	People/house[b]
Rav.	25 121[a]	2.45	10 246	4 819	5.21[b]
Uitsig	12 188[a]	0.96	12 720	2 487	4.90[b]
Affluent areas (n = 5)[c]	34 422[a]	45.57	755 (541-1 321)[e]	10 565	3.26[b]
Poor areas (n = 8)[d]	141 429[a]	5.47	25 855	no data	not known

[a]Adults and children; 1996 census

[b]These results are not a true reflection because affluent people live in bigger houses. In Ravensmead, the houses are usually larger than in Uitsig.

[c]Bishops Court, Constantia, Durbanville, Welgemoed and Plattekloof.

[d]Khayelitsha Sites A and C, Villikazi, Town 2, Taiwan, Section B, Green Point and G Mxenge. Many of the dwellings in these areas are small shacks crowded closely together.

[e]Population density over all the affluent and poor areas combined, with the range in brackets.

5A.4 Discussion

Recent surveys have confirmed that soil-transmitted worm infestation is widespread amongst South African children living in poverty (Fincham *et al.*, 1998 and 2003c; Appleton *et al.*, 1999; Arendse, 2001; Mosala, 2001). In studies such as the present one, where only a single faecal sample was obtained from each participant, the true prevalence will be higher than that recorded in the literature. The results reported here show that helminthiasis can occur frequently in adolescents and that it is not restricted to the poorest of the poor.

Attention has been drawn to options for immunising children against HIV/AIDS before they become sexually active in order to reduce the predominantly heterosexual spread of

infection in South Africa and elsewhere (Makgoba *et al.*, 2002). The helminthological results now described emphasise the need to research the immunological effects of co-endemic helminthiasis in terms of valid interpretation of the results of HIV vaccine trials and the effectiveness of mass immunisation; as well as the medical management of HIV/AIDS. In crowded, impoverished environments, even individuals (including adults) with no worm eggs in their faeces may intermittently be in a state of immune activation in response to repeated exposure to helminthic antigens (Bentwich *et al.*, 1999; Cooper *et al.*, 2000b; Elias *et al.*, 2001; Fincham *et al.*, 2003a). The question of whether immunological imbalance due to hyperendemic helminthiasis is frequent in children, and the possibility that sustained deworming programmes (preferably linked to poverty alleviation, effective sanitation and better housing) could rebalance the profile in a way that would be beneficial in respect of HIV/AIDS, should be high research priorities.

To conduct immunological and helminthological studies *per se* amongst children should not be difficult in general. However, inclusion of HIV in the equation creates complications. If research is carried out in poor communities where worms and HIV/AIDS are both hyperendemic (which it probably ought to be in order to capture the whole spectrum of confounding factors), it will be necessary to test for HIV infection and monitor "negative" status. A great fear of the disease and the stigma linked to positive status prevails in South Africa. Our experience has been that under these circumstances, very few families will give informed consent for participation in research. Furthermore, counselling about the implications of potential HIV positivity and seroconversion will be essential but difficult to manage.

The problems specified above appear to strengthen the case for immediate implementation of control and elimination of worm infestation in poorer South African communities *en masse*, as recommended internationally (Drake *et al.*, 2002; Savioli *et al.*, 2002). This kind of policy and practice would be relatively inexpensive; might help to combat HIV/AIDS; and would have other beneficial effects (Drake *et al.*, 2002; Savioli *et al.*, 2002; Fincham *et al.*, 2003a,b,c). The results obtained indicate a need to give schools, and probably creches, serving poverty-stricken communities higher priority for control of

helminthic infection. More than a decade could elapse before prepubertal vaccination against HIV/AIDS can be tested and become a reality. Therefore, control of worm infestation should not be delayed pending assessment of the extent to which co-endemic helminthiasis negatively influences either the effectiveness of vaccines against HIV/AIDS and other diseases or therapy for immunosuppression (Cooper *et al.*, 2000; Cooper *et al.*, 2001; Elias *et al.*, 2001; Markus and Fincham 2001a,b,c; Wolday *et al.*, 2002; Fincham *et al.*, 2003a,b,c).

CHAPTER SIX

THE ASSOCIATION BETWEEN BCG SCARRING, PPD SENSITIVITY AND INTESTINAL PARASITE INFECTION IN CHILDREN RESIDENT IN AN AREA WITH A HIGH TB NOTIFICATION RATE

The work presented in this chapter will be submitted to the *Journal of Clinical Immunology* as:

The association between BCG scarring, PPD sensitivity and intestinal parasite infection in children in an area with a high TB notification rate

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6.1.1 Introduction

Helminth infections are common in the developing world, with the main burden being borne by young children. These infections are marked by increasing levels of circulatory IgE, eosinophilia and mast cell hyperplasia, characteristic of T-helper 2 (Th2) driven effector mechanisms that involve the production of cytokines such as IL-4, IL-5 and IL-13. Cell-mediated immune responses on the other hand, associated with the activation of Th1 lymphocytes, are induced by infections such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *M. bovis* and are characterised by the production of IL-12 and IFN-γ, the predominant Th1 induced cytokines. There is good evidence that Th1 and Th2 responses have the capacity to inhibit each other and, as discussed below, immune responses to mycobacteria can be partly suppressed by infection with helminths and the converse is also demonstrable (Pearlman *et al.*, 1993). The genes that control Th1 and Th2 responses are polymorphic (Bentwich *et al.*, 1999) so that in an out-bred population there will be a great degree of variability in the relative vigour of these two responses. It may be anticipated that natural selection determines the optimum balance of alleles at the various immunological loci that control Th1 and Th2 responsiveness and this balance may be expected to depend on the prevalence of different types of pathogen in an environment. It follows that the outcome of an infectious disease will depend both on genetic factors and on environmental ones that influence the prevalence of other intercurrent infections that may modulate the response to the pathogen in question.

Although BCG is one of the world's most widely used vaccines, the protection that it imparts against tuberculosis is variable and inconsistent. Reasons for this inconsistency have been reviewed (Fine, 1995) but epidemiological studies have shown that the vaccine confers the least protection in areas where helminths are endemic (Bentwich *et al.*, 1999). There is evidence that the adverse effects of helminth infections on Th1 responses induced by BCG can be acquired in utero. New-borns whose mothers became infected by schistomiasis or bancroftian filariasis during pregnancy were frequently sensitised *in utero* to helminths (Malhotra *et al.*, 1997; King *et al.*, 1998; Malhotra *et al.*, 1999). These helminth-specific responses persisted into childhood and biased T cell immunity induced by BCG vaccination away from a protective Th1 response. Cellular immune responses to

Purified Protein Derivative (PPD) are also reduced in individuals with concurrent helminth infections (Pearlman *et al.*, 1993), suggesting that helminths may also influence tuberculin reactivity. Similarly, PPD skin negative onchocerciasis patients are less likely than uninfected individuals to convert to skin positivity following BCG vaccination (Stewart *et al.*, 1999). Paradoxically it has been reported that children with a BCG scar have a lower incidence, prevalence and intensity of infection with hookworm (Barreto *et al.*, 2000), however, a separate study failed to find such a negative correlation (Floyd *et al.*, 2001). Similarly, Elliot and colleagues (1999) found an inverse association between BCG scar positivity and intestinal nematode infestation among HIV-1 positive adults in Uganda.

6.1.2 Aims

In the Western Cape region of South Africa intestinal helminths, predominantly *Ascaris lumbrocoides* and *Trichuris trichuria* are endemic as is infection with *M. tuberculosis*. In response to the debate concerning whether the presence of a BCG scar is associated with a relative resistance to helminth infection we aimed to study a large number of children, immunised at birth with BCG, for the prevalence of *Ascaris* and *Trichuris*, tuberculin responsiveness and evidence of BCG scars in a community with a high TB notification rate.

6.2 Materials and Methods

6.2.1 Study area

The investigation was carried out in the two communities described in Chapter 4 of this thesis (Community A and B). The area is served by two local authority health clinics and a tertiary care referral hospital adjacent to the suburbs. BCG coverage is more than 90 % and is routinely administered in the neonatal period.

6.2.2 Study population

The study population was a sub-sample (n = 2 618) of a survey of 5 900 primary school children, 5-15 years of age for whom a stool sample was examined for helminth eggs (Chapter 5 of this thesis) and with a recorded BCG scar status and measured Mantoux

skin response. The age, sex, height, weight and address of each child were noted. Prior to enrolment permission was obtained from the Western Cape Education Department and the principals from each school. Signed informed consent was obtained from parents or guardians. The study was approved by the Ethics Committee of the University of Stellenbosch.

6.2.3 Parasitological examinations

Numbered specimen bottles with screw top lids were given to each child. The consistency of each sample was recorded before 1 g of stool was weighed and dispersed in 4 ml of 10 % aqueous formalin. Fixed stool specimens were processed for light microscopy by following the formalin-ether concentration technique. Helminth eggs were counted under a 10 x objective and eggs per gram (epg) of stool were calculated for the nematodes, *Ascaris* and *Trichuris* and the presence of the protozoan pathogen *Giardia lamblia/duodenalis* was also recorded.

6.2.4 Mantoux skin testing and BCG vaccination scar status

During an Annual Risk of TB Infection (ARTI) survey, the evidence of a BCG vaccination scar was recorded for all primary school children. For the Mantoux testing, a single batch of PPD RT 23 (Statens Seruminstitut, Copenhagen) was used. For each child, 0.1 ml (2U) RT 23 was injected intradermally into the volar aspect of the left forearm. The diameter of the area of induration was read after 72 hours using the "ball-point" method. The evidence of a BCG vaccination scar was noted on the day of reading of the Mantoux response. The administration of the PPD, reading of the Mantoux skin test and BCG scar status were performed by the same health worker. Anthropometric measures were also determined. Height was measured to the nearest millimetre and weight measured to the nearest 100 g.

Scores of standard deviation (z-scores) for height and age (*i.e.* height-for-age-z-score) (HAZ) were used to characterise the growth profile of children. HAZ was categorised into SD <-1.67 = stunted and SD ≥ -1.67 = normal.

6.2.5 Statistical analysis

The data were examined for possible association between pairs of variables by Cochran-Mantel-Haenszel statistics and the Breslow-Day test was used to test for homogeneity in Odds Ratios from data derived from different groups of children (see results for the selection criteria of these groups). The Mann Whitney test was used to determine the significance of differences between mean values of helminth eggs in stool samples from different groups. To examine simultaneously the data for the possible association of several variables, for example BCG scar status, Mantoux responsiveness and presence or absence of helminth infection, the observed frequency of the various subgroups was compared with the frequencies that would occur if all the variables were independent.

6.3 Results

6.3.1 BCG scar status, Mantoux skin test responses and helminth burden among the children studied

Table 6.1 shows the prevalence of the BCG scar status (*i.e.* -/+), Mantoux skin test responses and helminths in the 2 618 children included in this particular study. About one third (1 746/2618 = 66.2 %) of the children tested had evidence of a BCG scar with the majority of children (62.2 %) presenting with no Mantoux response (0 mm in induration size). Only 5.3 % of the children had a positive Mantoux skin test response (*i.e.* > 10 mm in induration size). The prevalence of *Trichuris* (39.1 %) and *Ascaris* (22.5 %) in this sub-sample were lower than reported in the previous chapter of this thesis.

Table 6.1 Prevalence of BCG scar status, Mantoux skin test responses and helminths

BCG scar status		Mantoux skin test (mm)			*Trichuris* (epg)		*Ascaris* (epg)	
% BCG^+ (of total)	% BCG^- (of total)	0 mm	<10 mm	>10 mm	0 epg	> 0 epg	0 epg	> 0 epg
66.6 % (1746)	33.3 % (872)	62.2 % (1617)	33.2 % (862)	5.3 % (139)	62.3 % (1592)	39.1 % (1026)	78 % (2028)	22.5 % (590)

In order to visualise the variation of these parameters with age, the first 2 600 children were divided in 26 group containing 100 children each (sorted according to ascending age). As shown in Figure 6.1 (Table 6.2) the prevalence of BCG scar positivity, while broadly independent of age, covering a range from 6-15 years, was lower in children around 11 years of age. In contrast, the percentage of children showing Mantoux reactivity increased with age, from 13 % at 6 years to 65 % at mid teenage (Table 6.2 and Figure 6.2). The percentage of children infected by *Trichuris* increased about twofold with age and most of this increase occurs in the first few years of this period (Table 6.2 and Figure 6.3). In contrast, the percentage of those infected by *Ascaris* showed virtually no change with age (Table 6.2 and Figure 6.4).

Table 6.2 Variation of BCG scar positivity and Mantoux responsiveness with age

Age groups (yrs)	% Mantoux positive	% BCG positive	% *Ascaris* positive	% *Trichuris* positive
6	12	78	19	21
7	18	76	17	20
7	30	78	25	37
8	22	66	19	36
8	22	71	18	42
8	19	72	18	36
8	26	74	36	39
9	32	64	23	37
9	27	67	31	43
9	32	60	25	45
10	26	57	20	34
10	41	52	17	37
10	32	62	21	41
11	32	60	23	37
11	32	53	21	47
11	27	56	23	45
11	43	64	18	43
12	42	59	28	47
12	41	61	27	38
12	32	69	14	33
13	44	75	18	37
13	40	77	17	38
13	35	75	18	32
14	49	68	22	51
14	52	73	21	48
15	57	77	22	45

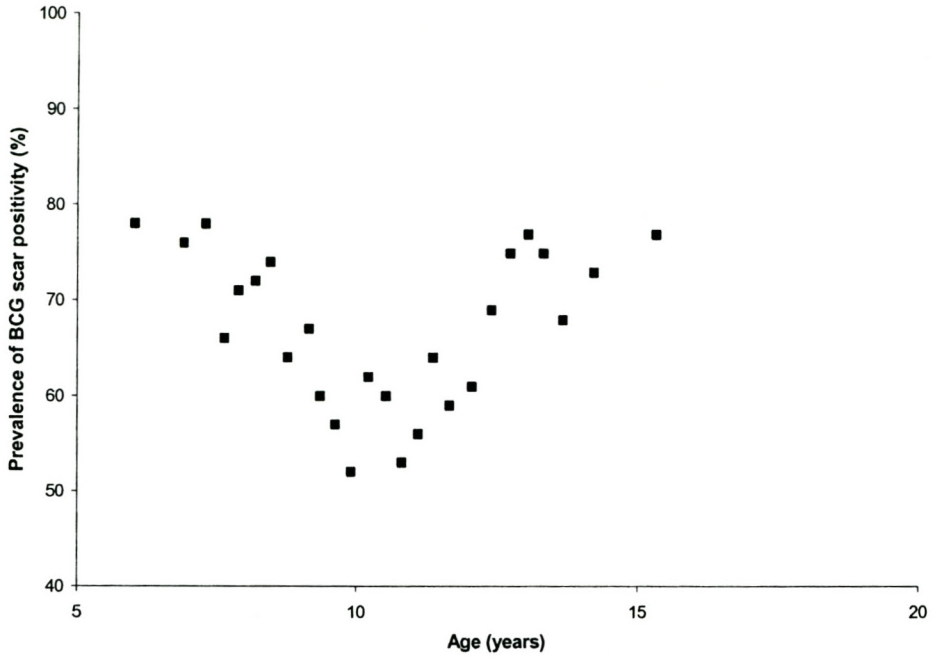

Figure 6.1 Variation of BCG scar positivity with age

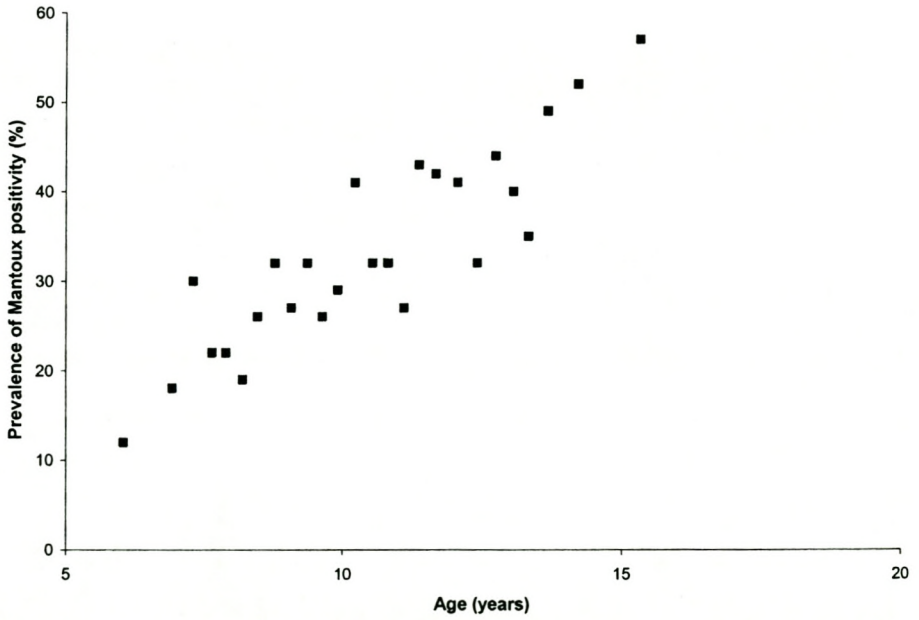

Figure 6.2 Variation of Mantoux skin test positivity with age

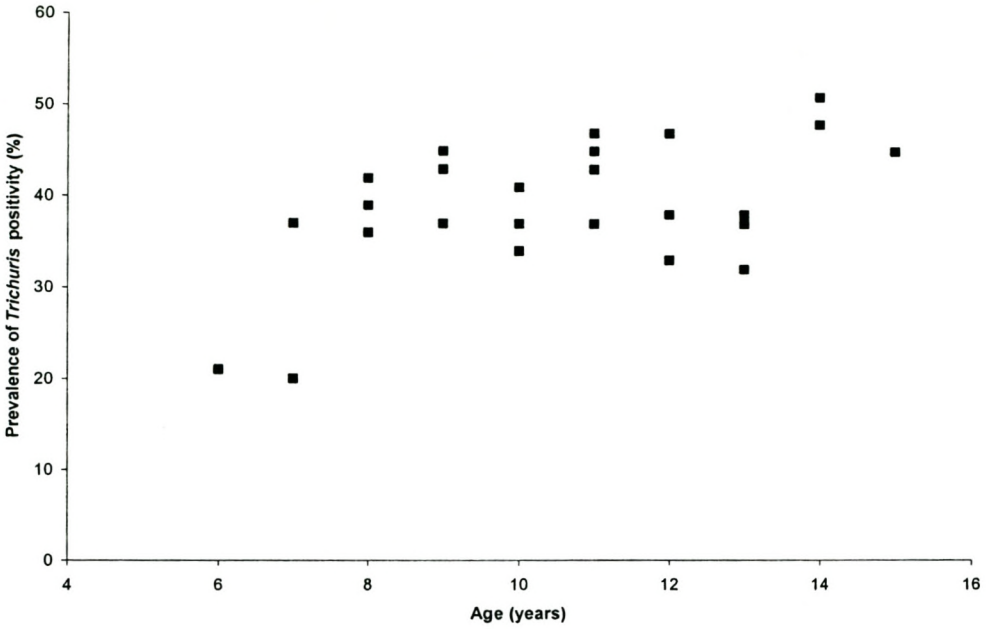

Figure 6.3 Variation of *Trichuris* positivity with age

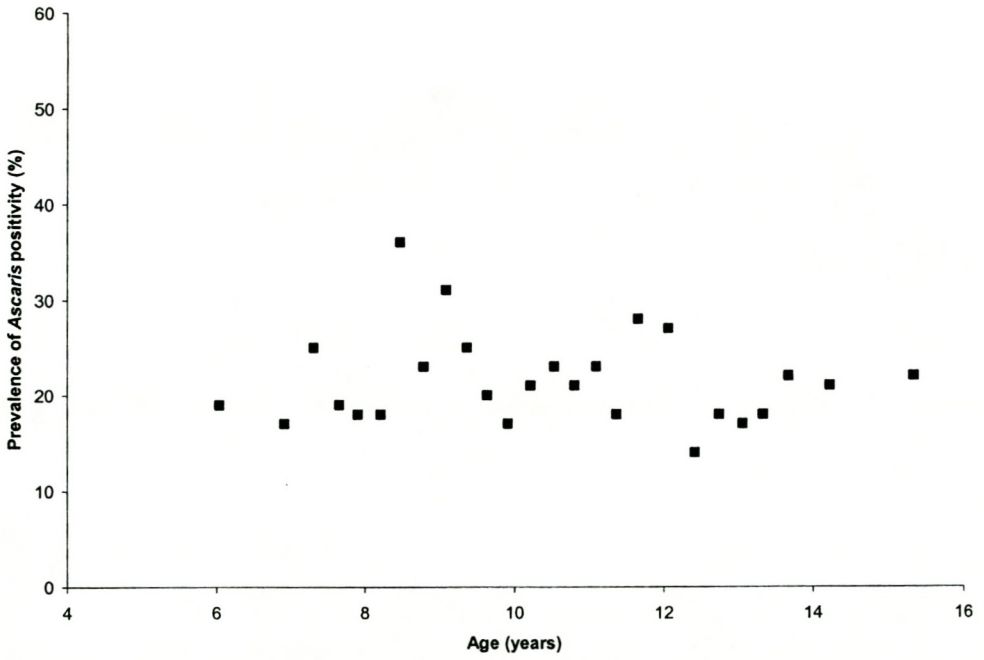

Figure 6.4 Variation of *Ascaris* positivity with age

6.3.2 BCG scarring and the presence of *Ascaris* and/or *Trichuris*

The possibility that genetic and/or environmental factors that influence BCG scarring might also affect resistance to helminth infection was examined by seeking an association between BCG scarring and the presence of an *Ascaris* and/or *Trichuris* infection (Table 6.3).

Table 6.3 Association between BCG scar status and helminths

Group	Subpopulation	Number in group	Percentage observed of total	Percentage expected
1	BCG^+ $helminth^+$	763	29.1	30.7
2	BCG^+ $helminth^-$	983	37.6	36.0
3	BCG^- $helminth^+$	443	16.9	15.3
4	BCG^- $helminth^-$	429	16.4	18.0

The numbers of individuals in each of the four groups was extracted from the data and the percentages observed (column 4) were compared with those calculated on the assumption that BCG scarring and helminth infection were independent variables (column 5). For example, the total number of children examined was 2 618 (groups 1, 2, 3, 4). Of these 1 746 (groups 1, 2) had a scar (*i.e.* 66.7 %) and 1 206 (groups 1, 3) were infected with helminths (46.0 %). Consequently, if scar formation and helminth infection are not associated the expected number of BCG^+ $helminth^+$ children is 2 618 x 0.667 x 0.460 = 803, which is 30.6 % of the total.

As the table shows there are fewer than expected numbers of individuals in Groups 1 and 4 and a correspondingly larger number in Groups 2 and 3. Consequently, a child who has a scar is less likely to have a helminth infection than does a child without one, (risk ratio 0.86). The effect, though small is statistically significant, even when *Ascaris* and *Trichuris* infections are analysed individually risk ratios for *Ascaris* and *Trichuris* infection are 0.95 ($p = 0.023$) and 0.92 ($p = 0.008$) respectively.

6.3.3 Association between BCG scar expression, Mantoux responses and helminths

When the whole population was examined for possible associations between BCG scar expression, Mantoux responses and helminth infection, no strong associations were found (Table 6.4). There was no correlation between BCG scar expression and Mantoux responsiveness (Table 6.4).

Table 6.4 Association between three parameters measured

Group	Subpopulation	Number in group	% of total	% BCG^+	% BCG^-	% calculated
1	$BCG^+ Mant^+ tt^+$	256	9.8	14.7		13.9
2	$BCG^+ Mant^+ tt^-$	406	15.5	23.3		24.1
3	$BCG^+ Mant^- tt^+$	382	14.6	21.9		22.7
4	$BCG^+ Mant^- tt^-$	702	26.8	40.2		39.3
5	$BCG^- Mant^+ tt^+$	183	7.0		21.0	16.7
6	$BCG^- Mant^+ tt^-$	147	5.6		16.8	21.1
7	$BCG^- Mant^- tt^+$	201	7.7		23.1	27.4
8	$BCG^- Mant^- tt^-$	341	13.0		39.0	34.7

In Table 6.4. the whole database, of 2 618 individuals was analysed in terms of the eight subpopulations in the table. The numbers in the 'Percent calculated' column were derived on the assumption that there were no associations between any of the variables (see legend to Table 6.1). However, because the frequency of *Trichuris* infection differed in the BCG positive and BCG negative subpopulations (groups 1-4 and 5-8) the 'percent calculated' values are given separately for these subpopulations.

It will be seen that for the BCG positive population there is quite close agreement between the observed and calculated percentages although there is a slight excess of Mantoux positive *Trichuris* positive individuals above chance (risk of being Mantoux positive if helminth infected or risk of Mantoux positive if not infected = 1.09). In the Mantoux negative populations the percentages of those infected by *Trichuris* are very

similar in both BCG positive and BCG negative subpopulations (35 % and 37 % respectively) so that about 36 ± 1 % of Mantoux negative children are infected regardless of their BCG status.

The only major departures from expectation occur in groups 5 and 6. These data show that in BCG negative subset a higher than predicted percentage of Mantoux positive individuals (group 5) are infected by *Trichuris*; a child who is infected is 1.58 times more likely to be Mantoux positive than a child who is not so infected. The result is statistically significant; the risk ratio for a child being Mantoux positive if infected by an intestinal parasite, regardless of its BCG scar status, is 1.303 ($p = 0.0002$). However, this result cannot be interpreted to imply that helminth infection predisposes to the acquisition of Mantoux sensitivity. From the data in Table 3 children who are BCG^- Mantoux positive are 1.48 times more likely to be infected by *Trichuris* than those who are BCG^- Mantoux negative.

The relative excess of those infected in the BCG negative Mantoux positive group largely accounts for the fact that the total percentage of those infected in the BCG negative population (44 %) is larger than that in the BCG positive one (36.6 %). In contrast, the frequency of Mantoux positive individuals is virtually the same in the BCG positive and negative subpopulations (compare the sum of groups 1 and 2 with those of groups 5 and 6). The most direct interpretation of these results is that the excess of *Trichuris*-infected individuals in group 5 arises because conversion to Mantoux positivity, in individuals without a BCG scar, is associated with an increase in susceptibility to helminth infection. An increase in susceptibility of similar magnitude was not found in the BCG positive subset suggesting that the immune response to BCG that leads to scar formation abrogates in some way the increased susceptibility to *Trichuris* seen in the scar-negative-Mantoux positive individuals (group 5). The percentage of those that are Mantoux positive increases with age, as does the percentage infected by *Trichuris*. However, numerical analysis of the data does not support the possibility that a higher than expected percentage of infected individuals in the Mantoux positive population arose simply as a consequence of age differences (Table 6.4). Further, this hypothesis does not account for

the absence of any excess of infected individuals in the BCG positive, Mantoux positive population (group 1). Finally, while the incidence of *Trichuris* infection in the BCG negative subpopulation was greater than in the BCG positive one this excess was not associated with an increase prevalence of Mantoux reactivity. Consequently, contrary to expectation, there is no evidence from the data that *Trichuris* infection predisposes to Mantoux conversion.

Table 6.5 shows a similar analysis of the data as that for Table 6.4 except that the results were sorted on the basis of *Ascaris*, rather than *Trichuris*, infection. Although the number of individuals infected with *Ascaris* is lower than that for *Trichuris* the general features of the two tables are similar. As in Table 6.4, the calculated percentages in the last column of Table 6.5 are derived on the basis of no association between the variables. In general the observed values depart from calculated ones only to a minor degree. However, as with Table 6.4 there is an indication that, in the BCG negative population helminth infection is more frequent in the Mantoux positive subset than chance would predict. Further, as comparison of rows 1,2,3 and 4 with rows 5,6,7 and 8 indicates, the percentage of BCG positives that are infected with helminth (20.3 %) is less than the percentage of BCG negatives that are so infected (25.4 %). Finally, the average epg for *Ascaris* and *Trichuris* in the BCG positive group is lower than it is in the BCG negative one (113 versus 284 and 263 versus 332 respectively).

Table 6.5 Population analysis of 2 618 children in terms of their BCG, Mantoux *Trichuris* (*Tt*) and *Ascaris* (*As*) phenotypes

	n	BCG (-/+)	Mean Mant. size (mm)	Mean *As* (epg)	Mean *Tt* (epg)	% of total	% of BCG^+	% of BCG^-	Percentage calculated
1	137	+	15.2	376	777	5.24	7.85		7.7
2	524	+	14.7	0.0	203	20.0	30.0		30.1
3	218	+	0.0	668	710	8.33	12.5		12.6
4	867	+	0.0	0.0	105	33.1	49.7		49.5
5	90	_	15.5	1 461	703	3.44		10.3	9.6
6	240	_	15.3	0.0	321	9.17		27.5	28.1
7	132	_	0.0	861	808	5.04		15.1	15.8
8	409	_	0.0	0.0	104	15.6		46.9	46.3

6.3.4 Value of the Mantoux response in predicting *Trichuris* infection

The data in Tables 6.4 and 6.5 indicate that there is a weak positive correlation between PPD responsiveness and helminth infection. Further evidence for such an association was sought by making a comparison of the mean number of *Trichuris* epg between those whose Mantoux response was > 10 mm and those whose response was < 10 mm. When BCG positive and BCG negative groups were analysed it was found that irrespective of the BCG status, the average number of *Trichuris* epg was significantly greater for those with the larger Mantoux reactivity; $p < 0.0001$ for the BCG negative group and 0.024 for the positive one by Mann Whitney two tailed test. The population results are shown in Table 6.6, for pooled BCG positive and negative groups.

Table 6.6 Number of individuals *Trichuris* infected individuals in two Mantoux size categories

Group	Mantoux size (mm)	n	Number infected	Number with more than 1 000 epg	Max. epg
1	< 10 mm	1751	627 (36 %)	85 (4.9 %)	22,100
2	> 10. mm	867	395 (46 %)	64 (7.4 %)	288,000

The table shows that individuals with a Mantoux response > 10 mm (group 2) are more likely to be heavily infected than those whose Mantoux response is < 10mm (group1). However, in numerical terms Group 1 contains more infected individuals and the magnitude of the Mantoux response is of very limited value in predicting a helminth infection.

6.3.5 Confounding variables in the association between Mantoux reactivity and parasite infection

The data from 2 618 individuals in the study were examined for possible differences in the prevalence of Mantoux reactivity in various subgroups. As shown in Table 6.7, when the data were examined for the possible influence on the frequency of Mantoux responsiveness of age, place of residence (community A or B), gender, or stunted growth, no correlations were observed (p values calculated by the Breslow-Day test). However, as reported above, significant association between Mantoux responsiveness and parasite infection was observed

Table 6.7 Confounding variables in the association between Mantoux responsiveness (Mantoux >10mm) and intestinal parasite infection (*Ascaris* and/ *Trichuris)*

Variable	Age			
	5-9 years		10 years and more	
	Risk ratio	p	Risk ratio	p
BCG	0.81	0.08	1.07	0.46
Area	0.92	0.49	0.89	0.18
Gender	1.11	0.40	1.05	0.60
Stunted	1.14	0.38	1.04	0.65
Parasites (*As/Tt)*	1.39	0.009	1.27	0.006

6.3.6 Mantoux responsiveness and helminth infection in children according to their growth profile

A comparison was made between stunted children and those showing normal growth with regard to the association between Mantoux reactivity and the presence of intestinal parasites (Table 6.8)

Table 6.8 Association between Mantoux responsiveness and presence of *Ascaris* and/or *Trichuris* in children based on their growth profile

	Normal growth		Stunted growth	
	Observed percentage	Expected percentage	Observed percentage	Expected percentage
Mant.$^{+}$ Parasite^{+}	18.9	16.4	24.7	24.5
Mant.$^{+}$ Parasite^{-}	13.1	15.5	11.8	12.0
Mant.$^{-}$ Parasite^{+}	32.5	35.5	42.5	42.7
Mant.$^{-}$ Parasite^{+}	35.6	33.1	21.0	20.8

The calculated percentages were derived on the assumption of no association between Mantoux reactivity and parasite infection. There were 1 998 children in the normal growth group and 600 in the stunted one.

The normal growth group show the positive association between Mantoux responsiveness and helminth infection seen in Tables 6.4 and 6.5. The corresponding risk ratios are 1.37 for being Mantoux positive if infected with parasites and 1.24 for being infected with parasites if Mantoux positive. In contrast, stunted children show no association between these variables (risk ratio = 1.0). The difference in the risk ratios for the two groups is significant ($p = 0.037$).

6.4 Discussion

6.4.1 Presence or absence of a BCG scar

The reason for this variation is unknown. However, at no age did the percentage of children with a BCG scar exceed 75 - 80 %. Whether the remainder fail to make a scarifying response to the vaccine or whether there are technological explanations (Kibel *et al.,* 1995; Kibel *et al.*, 1998) could not be answered by our data but the two suggested explanations are not mutually exclusive. There is evidence that maternal helminth infection can sensitise the foetus *in utero* and bias the immune response towards a type 2 immune response that might be expected to mitigate against a vigorous reaction to BCG (Malhotra *et al.,* 1999). Consequently, it may be that BCG scar development, while being determined before birth, is not solely a genetically determined variable. Studies on mothers are however required to establish or refute this possibility.

6.4.2 Variation of Mantoux reactivity with age and association with BCG scarring

From the results we could conclude that there is no Mantoux reactivity at birth. Given that virtually all children are immunised with BCG at birth the observed time course of Mantoux reactivity suggested that it is acquired from exposure to environmental mycobacteria, probably *M. tuberculosis* itself, rather than from the BCG vaccination. As this study did not include the determination of Mantoux responses in the first 5 years of life we cannot exclude the possibility that BCG vaccination led to a short-lived Mantoux

sensitivity that waned rapidly within this period. Such an effect has been previously reported (Menzies, 2000) and it has been concluded elsewhere (Stern *et al.*, 1996) that prior BCG vaccination does not negate the value of evaluating Mantoux responses in those at risk from tuberculosis.

The time course of PPD reactivity, is more likely to reflect post-natal exposure to *M. tuberculosis* than a delayed response to BCG immunisation at birth and, as mentioned above, the Mantoux response is probably useful in detecting those infected with *M. tuberculosis* regardless of whether they have been immunised in the neonatal period. It seems that BCG vaccination, at least when given very early in life, does not lead to sustained immunity to mycobacteria, (as judged by PPD responsiveness) in the same way that *M. tuberculosis* infection does in later childhood. It has been reported that BCG vaccination later in life does cause a more sustained, but not permanent, conversion to Mantoux reactivity (Menzies, 2000). With regard to the BCG vaccine used in this study the results are compatible with the concept that BCG, unlike *M. tuberculosis*, is unable to establish a latent infection. If BCG is rapidly eliminated in the neonatal period most of the lymphocytes in a young adult will never have been exposed to the vaccine.

6.4.3 Helminth infection

The percentage of children infected by *Trichuris* increased about twofold over the age range studied and most of this increase occurs in the first few years of this period. In contrast, the percentage of those infected by *Ascaris* showed virtually no change with age, indicating that the prevalence of this infection stabilises early in life, possibly as a consequence of anti-helminth treatment. There was no correlation between BCG scar expression and Mantoux responsiveness. The results suggested that one of these variables is largely genetically determined and the other by the environment this lack of association is, perhaps, not surprising. However, in principle the genes that determine BCG scar formation might have influenced responsiveness to PPD in those exposed to *M. tuberculosis*. The data do not support this possibility. Further, if PPD reactivity is a reliable sign of *M. tuberculosis* infection in these children, scar formation is not an indicator of resistance to infection. The average epg for *Ascaris* and *Trichuris* in the BCG

positive group was lower than in the BCG negative group leading to the conclusion that BCG scarring is associated with a relative reduction in the severity of helminth infection as well in its prevalence. The reason for this inverse relationship is not clear but it suggests that many individuals who make good Th1 responses, as judged by scarring after BCG vaccination, also make vigorous Th2 immune responses, evidenced by their ability to control helminth infections.

6.4.4 Mantoux responsiveness and helminth infection in children according to their growth profile

Stunting appeared to mimic the effect of helminth infection. The interpretation of this result is unclear. In principle infection with *M. tuberculosis* may cause stunting in a subgroup of children or that environmental factors that give rise to stunting may make stunted children more susceptible to *M. tuberculosis* infection or more likely to encounter infected individuals (Personal comminication: Dr. D.T. Loots, Department Paediatrics and Child Health, University of Stellenbosch). Given that poverty is associated with tuberculosis (Spence *et al.*, 1993; Davies, 1994) this latter possibility has some support.

6.4.5 Mantoux responsivess, BCG scarring and helminth infections

The three parameters, the presence of a BGC scar, the Mantoux reactivity and the presence of helminth infection are predominantly, but not totally independent. Compatible with the result of Elliot and colleagues (1999) and Barreto and colleagues (2000), there is a lower percentage of helminth-infected individuals in the BCG positive group. However, although the difference is highly significant it is not large. In a relatively large study like this one very weak correlations can be statistically highly significant. This significance does not detract from the conclusion that most of the variation seen in BCG scar formation and prevalence of helminth infection arises as a consequence of the impact of variables that were not identified in this study. Judging by the low level of Mantoux conversion in the younger children in this study, any modulating effect of BCG vaccination on susceptibility to helminth infection would be expected to have largely disappeared in infancy. Consequently, as suggested above, the presence of a BCG scar may be more indicative of the ability of an individual to make a

vigorous response to an immunological challenge than it is of a strong Th1 immune bias after the period of infancy.

The correlations between BCG scar expression, Mantoux responsiveness and helminth infection, while being weak, require some explanation. In Table 6.9 below it is shown that in qualitative terms an explanation can be provided if it is assumed that BCG scar expression is indicative of a vigorous immune response to both mycobacteria and helminths while Mantoux responsiveness is indicative of a mycobacteria–induced bias towards a Th1-type of immune reactivity. There is evidence for such a bias (Adams *et al.*, 1999).

Table 6.9 Immune responsiveness to helminths based on BCG scar status, Mantoux responses

Phenotype	Immune responsiveness	Susceptibility to helminth infection
BCG^{+} $Mantoux^{+}$	good responder but Th1 bias	intermediate
BCG^{+} $Mantoux^{-}$	good responder, no Th1 bias	resistant
BCG^{-} $Mantoux^{+}$	low responder and Th1 bias	susceptible
BCG^{-} $Mantoux^{-}$	low responder, no Th1 bias	intermediate

From Table 6.9, it shows that susceptibility to helminth infection is estimated on the assumption that the presence of a BCG scar is indicative of a vigorous immune response and that *M. tuberculosis* infection, as indicated by Mantoux responsiveness, diminishes immunity to helminths by biasing immunity away from a protective Th2 response. However, it is evident that the weakness of the causal relationships between the parameters measured implies that other factors, genetic and/or environmental are largely responsible for the variations seen. We have already discussed the evidence that susceptibility to BCG scarring is predominantly genetically determined and that Mantoux reactivity and helminth infection are acquired. However, the data do not exclude an environmental contribution to susceptibility to scarring after BCG vaccination nor to the

vigour of the Mantoux response. Similarly, as to be anticipated from the high degree of polymorphism in the immune system, genetic variation in the response to mycobacteria and to helminths is known to occur (Bentwich *et al.*, 1999).

Every possible combination of BCG scar expression, Mantoux responsiveness and helminth infection was represented in the children studied and it would be of value to know the relative susceptibility to tuberculosis in the different groups. Given that Mantoux responsiveness increase monotonically with age and reaches about 60 % at age 15 (Figure 6.2) the presence or absence of this reactivity in a child may depend only on whether he/she has been infected or not. There was no evidence from the graph that Mantoux prevalence versus age was beginning to plateau below 100 % and it appears that virtually all children in the study were susceptible to infection with *M. tuberculosis* and would become Mantoux positive as a result. Since the data support the view that Mantoux conversion is brought about by infection rather than vaccination, a child that is Mantoux positive but who does not have clinical disease, has a demonstrable degree of immunity.

The data in Table 6.3 also suggest that helminth infection does not predispose to infection with *M. tuberculosis* (as judged by acquired Mantoux reactivity). This conclusion receives support from the finding that the percentage of children that were Mantoux positive was essentially the same in Community B as it was in Community A although the former area had a significantly higher prevalence of helminth infection. However, this lack of a correlation cannot necessarily be taken to imply that helminth infection does not influence the development of clinical disease. In this context it is notable that patients with tuberculosis are not infected with helminths (private communication from Dr John Fincham) although it is known that they have very high serum IgE levels (Adams *et al.*, 1999) suggesting a strong Th2 bias. It may be that these individuals are able, for genetic reasons, to deal very effectively with helminth infections but, because the effector mechanisms associated with resistance to parasites are antagonistic to cell-mediated immunity, they are conversely less able to mount an effective Th1 type response to mycobacteria. In contrast, those with high parasite loads, indicative of inadequate Th2 responses may be relatively more resistant to tuberculosis. These individuals presumably

provide a reservoir of helminth infection that keeps the high Th2 responders polarised towards a TB-permissive Th2 response. The positive association between helminth infection and the vigour of the Mantoux response is compatible with this suggestion. In the present study 45% of all children were free of *Ascaris*, *Trichuris* and *Giardia*. It would be of value to know whether the subsequent development of tuberculosis is confined to members of this group. In this context it is notable that the prevalence of helminth infection declines in adulthood while susceptibility to tuberculosis increases (Bentwich *et al.*, 1999). If this decline in parasite infection with age is a reflection of an enhanced ability to mount a curative Th2 response the increase in tuberculosis may follow as a consequence.

CHAPTER SEVEN

CROSS-SECTIONAL STUDY IDENTIFYING IMMUNOLOGICAL MARKERS OF TUBERCULOSIS SUSCEPTIBILITY

7.1.1 Background

It is estimated that after a successful course of tuberculosis treatment, 3-5 % of these individuals will be develop tuberculosis again (Israel *et al.,* 1941). Active tuberculosis in patients with prior tuberculous disease can occur following endogenous reactivation or exogenous re-infection (Van Rie *et al.*, 1999; Bandera *et al.*, 2001). In the present study, recurrent tuberculosis was attributable to exogenous re-infection.

As already mentioned, a patient's ability to control TB is highly dependent on a type 1 immune response. We were interested in the peripheral immune response of patients who after a successful course of treatment, were diseased with TB again. One of the aims for the present study, was to determine a common immunological marker or pattern that can predict a patient's susceptibility towards TB. It has already been shown that the IFN-γR and IL-12R could serve as genetic markers in identifying susceptibility to *M. tuberculosis* as patients with non-functional receptors were highly susceptible to mycobacteria (Newport *et al.*, 1996; deJong *et al.*, 1998). Active tuberculosis is generally associated with suppression of T-cell responses (Ellner, 1997) and enhanced production and/or activity of immuno-suppressive molecules such as TGF-β1 and IL-10. TGF-β1 and IL-10 overlap with each other in many of their biological effects, including T-cell suppression, macrophage deactivation and interference with APC function (de Waal-Malefyt *et al.,* 1992; Wahl, 1992). In a study of tuberculosis patients from Karachi, Pakistan,

neutralising antibody to TGF-β1, normalised lymphocyte proliferation in response to PPD and significantly increased PPD-induced production of IFN-γ in the PBMC of healthy PPD-reactive household contacts of patients (Hirsch *et al.,* 1996) were detected. In addition, co-culture with neutralising antibody to IL-10 augmented T-cell-proliferation to PPD in blood from TB patients but not their household contacts. Furthermore, Jacobs and colleagues (2000) have shown that cell-mediated immunity is enhanced in the absence of IL-10, resulting in a robust granuloma formation, which accelerates the clearance of mycobacteria. In contrast, stimulation of PBMC with PPD and the mycobacterial 30 kDa alpha antigen induced greater secretion of TGF-β1, but not IL-10, in patients than household contacts. In recent studies of patients with active TB in Uganda, Othieno and co-workers (1999) have observed enhanced production of both IL-10 and TGF-β1 in PPD-activated PBMC culture supernatants from TB patients compared to healthy PPD skin test-reactive control subjects. Furthermore, co-culture of PBMC from TB patients with neutralising antibodies to either TGF-β1 or IL-10 significantly increased PPD-induced production of IFN-γ in TB patients. Whether TGF-β1 and IL-10 synergise with one another or function independently to enhance the suppression of the IFN-γ response in TB patients, however, is not known. Maeda and co-workers (1995) showed that TGF-β1 enhanced production of IL-10 by peritoneal macrophages in both normal and tumour-bearing mice. Recently, Othieno and co-workers (1999) found that together, TGF-β1 and IL-10 potentiate the down-regulatory effect on *M. tuberculosis*-induced T-cell production of IFN-γ. TGF-β1 alone enhances IL-10 production. As TGF-β has been detected in granulomas during active tuberculosis in humans (Wahl, 1992), the IL-10/TGF-β1 interactions may result in the suppression of mononuclear cell functions at the site of infection. In addition, TGF-β renders T-cell hyporesponsive to antigen stimulation and impairs mycobacteriocidal activity of *M. tuberculosis*-infected monocytes (Comstock 1992; Hirsch *et al.*, 1994).

Because of the wide BCG coverage and high TB notification rate in the study community as previously described in this thesis, even the healthy population is not immunologically naïve to mycobacterial antigens. Healthy controls show PPD skin test positivity, thus

making it an unreliable diagnostic marker for disease. It is therefore, important to carry out studies in TB endemic settings to identify additional immune discriminators predictive of early infection and progression of disease.

The need for the detection of immunological markers is needed as it could aid in identifying patients bearing the risk of higher susceptibility and to possibly apply a treatment different from that of "normal" individuals or alternatively, to be proactive in monitoring those individuals for repeat episodes of TB.

7.1.2 Aim

The aim of the study was to determine how the host response to TB differs in patients at risk for being re-infected, compared to those who have protective immunity. We aimed to determine whether people susceptible to a second infection and progression to disease reveal a common immunological marker. Also, we aimed to identify an immune parameter or pattern that could indicate susceptibility to *M. tuberculosis* progression to disease. All individuals included in this study will also be interviewed by a social anthropologist in order to understand their social and economic background and how it relates to the disease.

In the present study, we compared immunological markers of 1) adult subjects who have had more than two episodes of tuberculosis after cure, 2) adult subjects who had been cured after one episode of tuberculosis, 3) adult subjects with latent infection with *M. tuberculosis* (*i.e.* Mantoux positive) and 4) adult patients with newly diagnosed active TB.

7.1.3 Materials and methods

7.1.3.1 Sample population

7.1.3.1.1 Selection criteria for the subjects

Recurrence group (R)

- Individuals must have had at least two episodes of smear positive (or culture positive) tuberculosis
- All episodes must have been cured. Definition of cure: i) completion of course of treatment of the preceding tuberculosis, ii) must have been smear/culture positive at start of treatment and smear negative on completion of treatment of all episodes of tuberculosis
- They should not have been infected with multi-drug resistant *M. tuberculosis* strains during the initial or relapse episode
- The latest tuberculous disease must have been cured successfully at least six months prior to enrolment in study
- They should be HIV negative
- Sputum should be *M. tuberculosis* culture negative at time of enrolment

Cured (C)

- Diseased once before and cured
- Only one episode of TB in case history, which must have been successfully cured
- No multi-drug resistance
- Matching with recurrent group with last episode of TB for time-span between cure from TB and onset of the immunological studies
- Matched for enumerator sub-district (ESD) – controls should live in an ESD with similar or higher TB notification rate than recurrent group
- Matched for age and sex
- HIV negative and sputum should be culture negative at time of enrolment

Latent (L)

- Mantoux skin test positive with no previous history of having tuberculosis
- Skin test with 5 units of Japanese freeze dried PPD gives > 10 mm response
- No previous documented or suspected active TB disease
- Each individual should be matched for an individual in the "recurrent" and "cured" group for age (± 10 years) and gender

- Matching for ESD
- HIV negative and sputum should be culture negative at time of enrolment

Active tuberculosis (TB)

- Newly diagnosed tuberculosis (sputum should be culture positive at time of enrolment)
- No multi-drug resistance
- Match for age, sex and ESD
- HIV negative

7.1.3.2 Cellular assays

Methods used in this particular study include whole blood assays (WBA), fluorescence activated cell sorting (FACS), enzyme linked immunosorbent assays (ELISA) and radio immuno assays (RIA) all of which have been described in Chapter 2 of this dissertation. For this study, whole blood was used to perform the WBA and FACS analysis. A total volume of approximately 85 ml of blood was collected from each subject for the following tests:

1) Seven 10 ml Heparin vacutainer (BD 368480) for whole blood assays (WBA) and plasma
2) One 6 ml Na heparin vacutainer – for Fluorescence activated cell sorting (FACS)
3) One 4.5 ml EDTA vacutainer for full blood counts (FBC) and differential cell count (DIFF)
4) One 4.5 ml plain vacutainer for serum (HIV testing)

7.1.3.3 Interviews

A medical anthropologist interviewed subjects regarding contact with other people with tuberculosis, places of contact, any social problems and attitudes towards tuberculosis. In depth interviews were used to explore lifestyle aspects of the individuals in the sample population and their life histories that may have been relevant to their TB episodes. The interviews included gathering information which included biographical details and household composition, health history and history of TB.

7.1.3.4 Consent

Subjects were informed about the study and the type and volume of samples that would be requested. Written informed consent was obtained from all patients before enrolment into the study. Each patient also received pre- and post-test HIV counselling. The study was done according to the Helsinki declaration and ICH guidelines. Confidentiality was preserved by assigning subject study numbers. The identity of cases was only revealed to the clinical investigators of the project. Ethics permission was granted for a full detailed sociological interview (95/072).

7.2 Results

7.2.1 Description of patients

Table 7.1 gives a description of the cases studies which included ten groups consisting of four individuals each. These four individuals included a person with recurrent TB (R), a control subject who had been cured (C), an individual with latent TB (L) and a patient with newly diagnosed TB (TB).

Individuals enrolled in this study were adults between 25 and 55 years of age. The largest age difference between a re-infected individual and any of the other three in a group, was 10 years. As far as possible, the four individuals in a specific group were chosen from areas with a higher TB notification rate. If this was not possible, an individual residing in an area with a similar TB notification rate was chosen.

Table 7.1 Description of individuals within the ten study groups

Category		Sex	Age	End of TB Episode	*TB rate
Recurrent	(R1)	F	33	January 1999	1001
Cured	(C1)	F	29	November 1996	501
Latent	(L1)	F	22		2001
Active TB	(TB1)	F	29	November 1999	1501
Recurrent	(R2)	F	41	January 1999	#Outside
Cured Control	(C2)	F	38	March 1999	3501
Mantoux +	(M2)	F	43		#Outside
TB Active	(TB2)	F	45	January 2000	1501
Recurrent	(R3)	M	33	June 1997	#Outside
Cured Control	(C3)	M	28	October 1997	2001
Mantoux +	(M3)	M	28		2501
TB Active	(TB3)	M	34	November 1999	#Outside
Recurrent	(R4)	M	46	July 1995	1001
Cured Control	(C4)	M	46	June 1995	2001
Mantoux +	(M4)	M	44		1501
TB Active	(TB4)	M	39	May 2000	2001
Recurrent	(R5)	F	33	February 1999	3501
Cured Control	(C5)	F	34	March 1998	3501
Mantoux +	(M5)	F	37		2501
TB Active	(TB5)	F	36	March 2000	2501
Recurrent	(R6)	F	37	April 1998	Outside
Cured Control	(C6)	F	35	January 1998	95
Mantoux +	(M6)	F	39		Outside
TB Active	(TB6)	F	39	January 2000	95
Recurrent	(R7)	M	45	May 1998	95
Cured Control	(C7)	M	46	May 1998	95
Mantoux +	(M7)	M	53		3501
TB Active	(TB7)	M	45	September 2000	95
Recurrent	(R8)	M	51	April 1996	2001
Cured Control	(C8)	M	51	December 1998	Outside
Mantoux +	(M8)	M	59		2501
TB Active	(TB8)	M	57	March 2000	2501
Recurrent	(R9)	M	41	May 1996	501
Cured Control	(C9)	M	44	March 1998	3501
Mantoux +	(M9)	M	35		2501
TB Active	(TB9)	M	37	November 1999	1001
Recurrent	(R10)	F	29	November 1996	1001
Cured Control	(C10)	F	35	December 1998	1001
Mantoux +	(M10)	F	38		2501
TB Active	(TB10)	F	26	January 2000	1501

* TB notification rate per 100 000 population per ESD

Outside refers to individuals not living in Ravensmead or Uitsig

7.2.2 Composition of the blood

The results from the whole blood cell count and lymphocyte populations are summarised in Tables 7.2.1 (Recurrence group), 7.2.2 (Cured group) and 7.2.3 (Latent group). The composition of blood was only determined for individuals in these three groups as no sample was available for the newly diagnosed patients. For each individuals included, the concentration of white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), platelets (Pl), neutrophils, lymphocytes, monocytes, eosiniphils and basophils, were determined. The normal ranges for the aforementioned components are also shown. Values lying below or above the normal ranges are underlined.

7.2.2.1 Recurrent group

In this group, the concentration of WBC seemed to be within the normal range of 4.1 – 10.9 x 10^9/l. Only three patients; R3, R4 and R8 had lower than the normal range of red blood cells (Table 7.2.1). The low concentration of RBC in patients R3 and R4 could explain the correspondingly low levels of Hb measured in these two patients. Furthermore, the Pl concentration was within normal range for all the patients except R3. The platelet count for R3 was extraordinarily high (626.0 x 10^9/l), which is almost 1.6 x higher than the highest value that can be considered as normal (152.5 – 397.5 x 10^9/l). R3 presented with low numbers of RBC, decreased Hb levels and high Pl count which would lead to the diagnosis of iron deficiency. The differential white blood cell count however, did not show any striking abnormalities other than low lymphocyte counts in R2 and R7 and with R4 presenting with a low neutrophil count.

7.2.2.2 Cured group

As depicted in Table 7.2.2, the white blood cell count was normal in the cured group with only two males (C3 and C7) having low RBC counts. Only C3 had a correspondingly low concentration of Hb. Three individuals, namely C2, C3 and C6 had high levels of platelets in their blood. C7 was the only one presenting with a high concentration of neutrophils. C1 and C6 presented with high levels of eosinophils. In the cured group, C3 stands out presenting with a low RBC count, low Hb concentration and high concentration of platelets.

7.2.2.3 Latent group

The white blood cell count for all individuals in this group was within normal range (Table 7.2.3). Only one individual (M7) had a low RBC count and thus correspondingly low Pl count. The only other abnormalities within the latent group was M8 presenting with a low borderline lymphocyte count (1.4 $x10^9$/l) and M3 with a very high eosinopihil count (0.7 $x10^9$/l).

Table 7.2.1 Whole blood cell count on recurrent patients. Cell populations of lymphocytes (x 10^9g/l) are also shown. Abnormal values are underlined

Patient	Sex	WBC	RBC	HB	PL	Neutr.	Lymph.	Mono.	Eosin.	Baso.
R1	F	8.8	4.5	14.4	238.0	5.8	2.3	0.3	0.3	0.0
R2	F	4.5	5.4	13.6	223.0	2.6	1.4	0.2	0.1	0.1
R3	M	5.7	4.3	12.6	626.0	2.9	1.9	0.3	0.3	0.1
R4	M	5.2	4.3	12.8	200.0	1.9	2.7	0.5	0.1	0.0
R5	F	9.5	5.3	14.8	378.0	4.5	3.5	0.9	0.4	0.1
R6	F	8.4	4.0	13.2	143.0	5.6	2.2	0.3	0.1	0.1
R7	M	9.6	5.1	16.3	184.0	7.4	1.1	0.6	0.0	0.1
R8	M	5.7	4.4	13.4	252.0	2.8	2.3	0.3	0.1	0.0
R9	M	7.0	4.5	15.5	234.0	5.3	1.1	0.2	0.1	0.1
R10	M	7.3	4.5	14.5	320.0	4.8	1.7	0.3	0.1	0.1
Normal range		4.1-10.9	women 3.8-5.8 men 4.5-6.5	women 11.6-16.5 men 13.1-18.0	152.5-397.5	2.06-7.45	1.52-3.97	0.21-0.79	0.05-0.45	0.04-0.16

\# Not measured
* No sample available for particular patient

WBC white blood cells (x 10^9/l)
RBC red blood cells (x 10^{12}/l)
HB hemoglobin (g/dl)
Pl platelets (x 10^9/l)

Neutr.: Neutrophils
Lymph.: Lymphocytes
Baso.: Basophils
Eosin.: Eosinophils
Mono.: Monocytes

Table 7.2.2

Whole blood cell count of the cured group. Cell populations of lymphocytes (x 10^9g/l) are also shown. Abnormal values are underlined.

Patient	Sex	WBC	RBC	HB	PL	Neutr.	Lymph.	Mono.	Eosin.	Baso.
C1	F	7.8	5.4	15.1	288.0	3.8	2.8	0.4	0.8	0.1
C2	F	7.4	4.5	13.3	421.0	4.4	1.9	0.5	0.2	0.1
C3	M	7.4	4.0	9.7	544.0	3.7	2.6	0.5	0.3	0.0
C4	M	6.3	4.6	13.3	232.0	3.9	1.7	0.5	0.0	0.0
C5	F	6.8	4.3	13.8	355.0	4.2	2.0	0.3	0.1	0.0
C6	F	9.2	4.2	14.4	399.0	3.7	3.6	0.5	0.6	0.1
C7	M	10.5	4.1	13.5	247.0	7.6	1.9	0.3	0.3	0.0
C8	M	5.7	5.0	15.5	242.0	2.9	2.2	0.3	0.2	0.0
C9	M	7.0	4.0	13.9	224.0	#	#	#	#	#
C10	*									
Normal ranges		4.1-10.9	women 3.8-5.8 men 4.5-6.5	women 11.6-16.5 men 13.1-18.0	152-397	2.0-7.45	1.5-3.97	0.21-0.79	0.05-0.45	0.04-0.16

\# Not measured
* No sample available for particular patient

WBC white blood cells (x 10^9/l)
RBC red blood cells (x 10^{12}/l)
HB hemoglobin (g/dl)
Pl platelets (x 10^9/l)

Neutr.: Neutrophils
Lymph.: Lymphocytes
Baso.: Basophils
Eosin.: Eosinophils
Mono.: Monocytes

Table 7.2.3 Whole blood cell counts on latent group. Cell populations of lymphocytes (x 10^9g/l) are also shown. Abnormal values are underlined

Patient	Sex	WBC	RBC	HB	PL	Neutr.	Lymph.	Mono.	Eosin.	Baso.
L1	F	8.4	4.8	13.7	346.0	4.7	3.0	0.6	0.1	0.0
L2	F	10.2	4.8	13.7	284.0	6.1	3.4	0.2	0.1	0.1
L3	M	9.4	4.7	15.8	210.0	5.6	2.0	0.6	0.7	0.1
L4	M	5.6	4.7	13.5	245.0	3.2	1.6	0.3	0.4	0.0
L5	F	9.1	4.4	15.5	289.0	6.1	1.9	0.4	0.2	0.1
L6	*									
L7	M	8.4	4.0	12.2	348.0	5.4	2.1	0.4	0.1	0.0
L8	M	4.6	4.5	14.5	202.0	2.5	1.4	0.3	0.2	0.0
L9	M	3.8	4.8	12.6	183.0	1.4	1.9	0.2	0.1	0.1
L10	*									
Normal range		4.1-10.9	women 3.8-5.8 men 4.5-6.5	women 11.6-16.5 men 13.1-18.0	152.5-397.5	2.06-7.45	1.52-3.97	0.21-0.79	0.05-0.45	0.04-0.16

\# Not measured
* No sample available for particular patient

WBC white blood cells (x 10^9/l)
RBC red blood cells (x 10^{12}/l)
HB hemoglobin (g/dl)
Pl platelets (x 10^9/l)

Neutr.: Neutrophils
Lymph.: Lymphocytes
Baso.: Basophils
Eosin.: Eosinophils
Mono.: Monocytes

7.2.3 Serum IgE

7.2.3.1 Serum IgE concentrations among patient groups

The serum IgE concentration was measured on patients in all four patient groups (Table 7.3 and Figure 7.1). Normal IgE concentration for the study population would be 200 kU/l. The results are therefore presented with reference to the normal value of 200 kU/l.

7.2.3.1.1 Recurrent group

Serum IgE levels in the recurrent group was the lowest compared with the three other groups. A median level of 188.58 kU/l was measured for this group. Of the ten patients, five had levels above 200 kU/l with the highest two levels (R1 and R8) being 1000 kU/l. The lowest IgE level of 4.44 kU/l was measured for R5.

7.2.3.1.2 Cured group

With a median IgE level of 612.50 kU/l, the cured group had the highest levels compared with the other groups (range 52 – 1000 kU/l) with four of the ten individuals presenting with levels of 1000 kU/l. Only three individuals had levels within the normal IgE range.

7.2.3.1.3 Latent group

The latent group presented with a median IgE concentration of 245.84 kU/l (range 14 – 1000 kU/l) with six of the ten individuals presenting with levels of above 200 kU/l.

7.2.3.1.4 TB active group

The group with active TB had a median IgE concentration of 490.6 kU/l, the second highest level compared with the other groups. Six of the individuals presented with concentrations well above 200 kU/l with the highest being 1000 kU/l.

7.2.3.2 Differences in serum IgE levels between groups

It is quite clear that in all the above-mentioned groups, the measured IgE levels were mostly above the normal range of 200 kU/l. However, no significant differences in serum IgE levels were observed ($p = 0.64$) among the four groups. As seen in Figure 7.1, levels of IgE in the control, Mantoux + and active TB groups seemed to follow bimodal distributions.

Table 7.3 Serum IgE levels in the four study groups

Subject	Recurrent	Control	Latent	TB
1	1000.0	52.7	129.7	891.8
2	82.1	1000.0	41.7	14.3
3	105.6	1000.0	818.4	87.3
4	506.6	149.1	203.3	125.4
5	4.4	1000.0	288.4	578.3
6	85.7	228.4	1000.0	944.3
7	25.5	1000.0	1000.0	402.9
8	1000.0	904.6	14.7	55.9
9	271.6	71.0	126.4	1000.0
10	641.6	320.4	709.2	1000.0
Mean	372.3	572.6	433.2	490.6
Median	188.6	612.5	245.8	510.0
SD	391.8	437.7	402.2	422.1

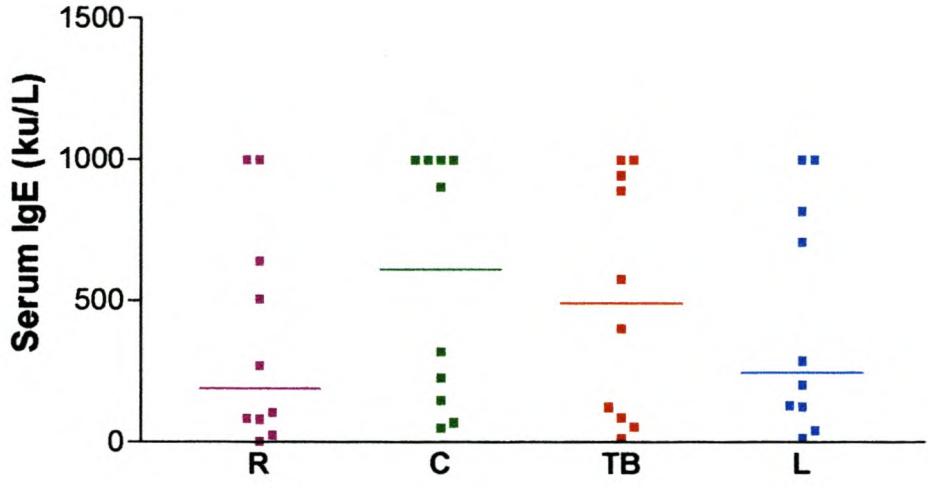

Figure 7.1 Serum IgE concentration among recurrence (R), cured (C), active TB (TB) and latent (L) groups. The median is indicated for each group

7.2.4 Immunophenotyping

As discussed in Chapter 2 (Materials and methods), whole blood from patients was cultured unstimulated (*ex vivo*) or stimulated with live *M. tuberculosis* for seven days. Cell were harvested according to the method outlined in Chapter 2 of this thesis. For this investigation, the following markers were chosen:

- CD3/CD4
- CD3/CD8
- CD4/CD25 (CD25 refers to the IL-2Rα which is an early activation marker)
- CD8/CD25

The method used for the analysis is described in Chapter 2 of this thesis. However, problems were encountered during this process. By using the method supplied by the manufacturer's, Becton Dickenson, we found that the red blood cells of individuals in the

cured and recurrence group would not lyse. For lysis of red blood cells, the FACS lysing solution supplied by the manufacturer's was used. As a result, it became impossible to accumulate 10 000 gated events during acquisition. In the current study a minimum of only 3 000 gated $CD45^+$ events could be acquired and will be presented in this thesis. In cases where less than 3 000 events were acquired, the result is not documented. Similar problems were also experienced by other members in our laboratory and the protocol supplied by the manufacturer was subsequently altered with the adoption of alternative methods of lysing red blood cells of tuberculosis patients. Examples of cases where 3 000 gated events were acquired as opposed to samples that had to be omitted due to low gated events are shown in Figures 7.2 A and B.

Figure 7.2 A An example of a case where less than 3 000 gated CD45 events were acquired

Figure 7.2 B An example of a case where 3 000 gated events were acquired

7.2.4.1 Cell surface marker expression as described for each patient group

Results from the immunophenotyping of individuals in the three groups are given in Tables 7.3.1 – 7.3.3. The TB active group was not included in this particular analysis.

7.2.3.1.1 CD3/CD4

7.2.3.1.1 Recurrent group

Due to the fact that the red blood cells of three individuals in this group did not lyse, only seven of the ten patients were included in this analysis (Table 7.3.1). The median percentage of cells expressing CD3/CD4 *ex vivo* on day 0 (R0) was 28.44 %, increasing significantly to 56.76 % ($p < 0.01$) after seven days (R7) in culture with *M. tuberculosis* (Figures 7.2.1 A and B).

7.2.3.1.2 Cured group

In this group ($n = 9$), the expression of CD3/CD4 cells also increased significantly from 32.28 % measured *ex vivo* (C0) to 55.57 % measured on day seven (C7) ($p < 0.01$) (Figures 7.2.1 A and B).

7.2.3.1.3 Latent group

The T-cells of the eight patients investigated in this patient group, expressed 36.26 % of the CD3/CD4 marker on day 0 (L0), increasing to 46.81 % on day seven (L7) ($p < 0.05$) (Figures 7.2.1 A and B).

7.2.3.1.2 Differences in CD3/CD4 expression among the patient groups

No significant differences were observed in CD3/CD4 expression among the three patient groups (Figure 7.2.1 C).

Table 7.3.1 Cell surface marker expression among the recurrence individuals ex vivo (Day 0) and after seven days in culture with *M. tuberculosis* (Day 7)

	CD3/CD4		CD3/CD8		CD4/CD25		CD8/CD25	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
R1	20.1	*	11.1	36.8	7.1	15.8	1.1	4.8
R2	*	22.2	*	11.2	*	6.2	*	0.8
R3	*	15.6	*	10.5	*	7.7	*	1.4
R4	36.9	56.8	15.3	14.8	2.6	25.3	0.1	1.5
R5	25.4	51.4	28.8	39.8	12.8	12.1	11.0	3.1
R6	28.4	62.1	2.8	18.2	3.8	35.2	0.4	7.1
R7	*	54.3	*	17.1	*	*	*	1.6
R8	39.1	59.3	3.4	2.8	1.4	8.3	0.0	0.2
R9	28.2	61.9	6.9	18.2	2.2	22.8	0.2	2.5
R10	58.8	55.7	20.5	27.7	7.8	5.9	4.3	0.3
Mean	33.3	48.5	12.7	19.7	5.4	16.7	2.1	2.6
Median	28.4	55.6	11.1	17.6	3.7	13.9	0.3	1.6
SD	12.7	18.1	9.5	11.7	4.0	10.3	4.4	2.2

Table 7.3.2 Cell surface marker expression among the cured individuals ex vivo (Day 0) and after seven days in culture with *M. tuberculosis* (Day 7)

	CD3/CD4		CD3/CD8		CD4/CD25		CD8/CD25	
	Day 0	**Day 7**	**Day 0**	**Day 7**	**Day 0**	**Day 7**	**Day 0**	**Day 7**
C1	27.8	54.9	11.0	15.0	3.4	20.3	3.8	3.6
C2	30.6	61.3	4.4	17.7	17.8	25.6	0.5	7.5
C3	*	44.8	*	17.9	*	19.8	*	2.6
C4	39.2	55.6	13.1	20.6	2.9	29.7	0.1	2.2
C5	51.3	55.1	*	19.1	7.1	11.0	0.1	10.0
C6	49.1	80.9	10.1	17.1	14.3	32.2	1.5	10.8
C7	*	57.6	*	3.1	*	19.5	*	0.5
C8	22.5	36.8	16.9	12.2	0.6	4.3	0.8	3.9
C9	34.1	*	15.2	*	3.5	*	0.1	*
C10	15.7	58.10	2.3	19.2	2.9	19.9	0.2	*
Mean	34.2	56.1	8.8	15.3	6.5	18.8	0.9	5.2
Median	33.8	55.6	10.2	17.4	3.2	19.9	0.3	3.8
SD	12.4	11.3	5.6	5.3	6.2	9.4	1.3	3.8

Table 7.3.3 Cell surface marker expression among the latent individuals ex vivo (Day 0) and after seven days in culture with *M. tuberculosis* (Day 7)

	CD3/CD4		CD3/CD8		CD4/CD25		CD8/CD25	
	Day 0	**Day 7**	**Day 0**	**Day 7**	**Day 0**	**Day 7**	**Day 0**	**Day 7**
L1	27.2	42.3	5.9	29.9	8.0	8.2	0.2	1.0
L2	35.1	61.3	18.6	26.2	4.2	24.0	0.8	3.0
L3	*	60.0	*	23.0	*	26.1	*	5.0
L4	37.3	74.7	5.8	9.4	*	47.6	0.1	2.2
L5	*	43.3	*	4.0	*	5.9	*	1.2
L6	37.6	48.0	35.9	49.4	6.2	5.9	7.1	3.0
L7	*	53.1	*	5.9	*	9.7	*	0.7
L8	12.2	45.6	21.1	31.5	0.3	9.9	0.8	0.5
L9	10.4	*	2.6	*	0.8	*	0.1	*
L10	50.3	90.6	21.6	8.4	4.3	80.6	0.0	1.3
Mean	40.8	50.9	16.1	20.5	7.3	20.6	0.5	1.3
Median	37.6	46.8	18.6	20.2	5.3	8.9	1.7	1.9
SD	17.7	21.8	11.7	14.5	8.7	24.9	2.6	1.5

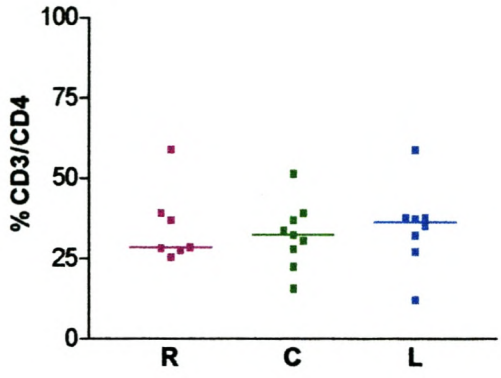

Figure 7.2.1 A CD3/CD4 expression in the groups on day 0 (*ex vivo*)

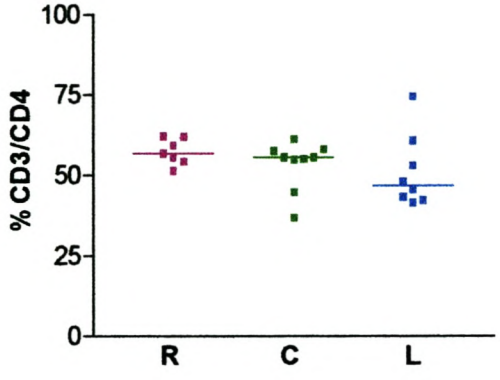

Figure 7.2.1 B CD3/CD4 expression in the groups after seven days in culture

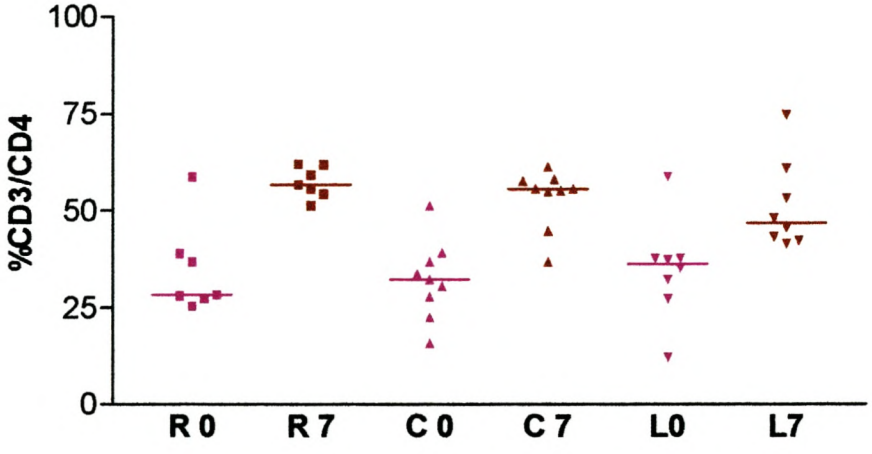

Figure 7.2.1 C CD3/CD4 expression in the groups on day 0 (*i.e.* R0, C0, L0) and after seven days in culture with live *M. tuberculosis* (*i.e.* R7, C7, L7)

7.2.3.2.1 CD3/CD8

7.2.3.2.1.1 Recurrent group

The median percentage cells expressing the marker CD3/CD8 was 11.10 % increasing to 18.16 % ($p > 0.05$) on day seven. Seven patients were included in this analysis (Figures 7.3.1 A and B).

7.2.3.2.1.2 Cured group

The median percentage of cells expressing the CD3/CD8 marker in this patient group was 10.10 % increasing significantly to 17.95 % ($p < 0.05$) on day seven.

7.2.3.2.1.3 Latent group

Although not significant, compared with the recurrence and controls groups, the percentage CD3/CD8 positive cells measured *ex vivo* was higher (16.28 %) in the latent group, increasing to 26.21 % ($p > 0.05$).

7.2.3.2.2 Differences in CD3/CD4 expression among the patient groups

No significant differences was observed in the percentage CD3/CD8 positive cells in the three groups (Figure 7.3.1 C).

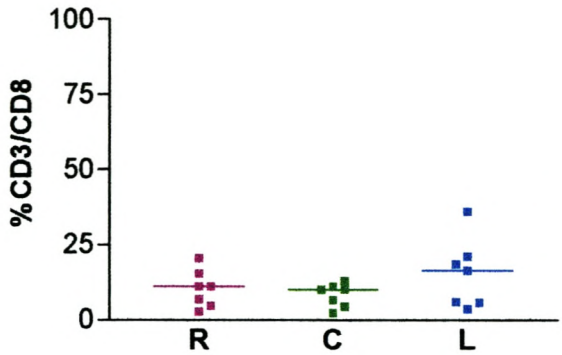

Figure 7.3.1 A CD3/CD4 expression in the groups on day 0 (*ex vivo*)

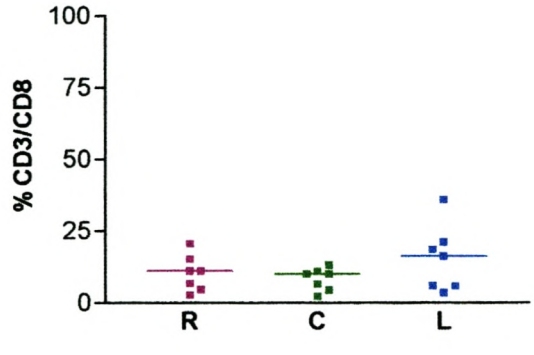

Figure 7.3.1 B CD3/CD8 expression in the groups after seven days in culture

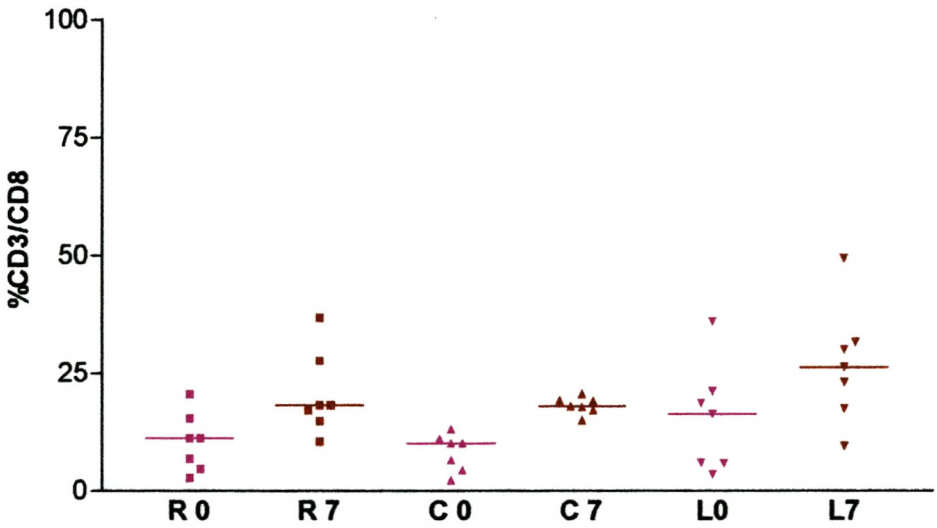

Figure 7.3.1 C CD3/CD8 expression in the groups on day 0 (*i.e.* R0, C0, L0) and after seven days in culture with live *M. tuberculosis* (*i.e.* R7, C7, L7)

7.2.3.3.1 CD4/CD25

7.2.3.3.1.1 Recurrent group

The median percentage activated T-cells increased significantly from 3.76 % measured *ex vivo* to 13.05 % measured on day seven ($p<0.05$) (Figures 7.4.1. A and B).

7.2.3.3.1.2 Cured group

Activated T-cells in the cured group showed the most dramatic increase from 4.26 % measured *ex vivo* to 19.87 % measured on day seven ($p> 0.05$).

7.2.3.3.1.3 Latent group

The median percentage CD4/CD25 positive cells was 4.25 % on day 0 increasing significantly to 9.17 % measured on day seven ($p< 0.05$).

7.2.3.3.2 Differences in CD4/CD25 expression among the patient groups

No significant differences was observed in the percentage CD4/CD25 positive cells in the three groups (Figure 7.4.1 C).

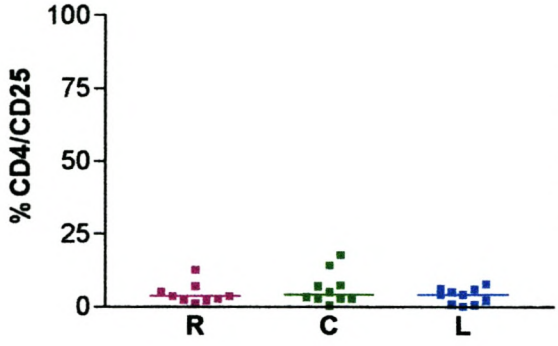

Figure 7.4.1 A CD4/CD25 expression in groups *ex vivo*

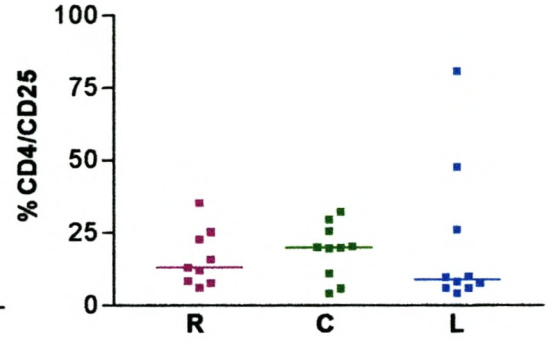

Figure 7.4.1 B CD4/CD25 expression in groups after seven days in culture with *M. tuberculosis*

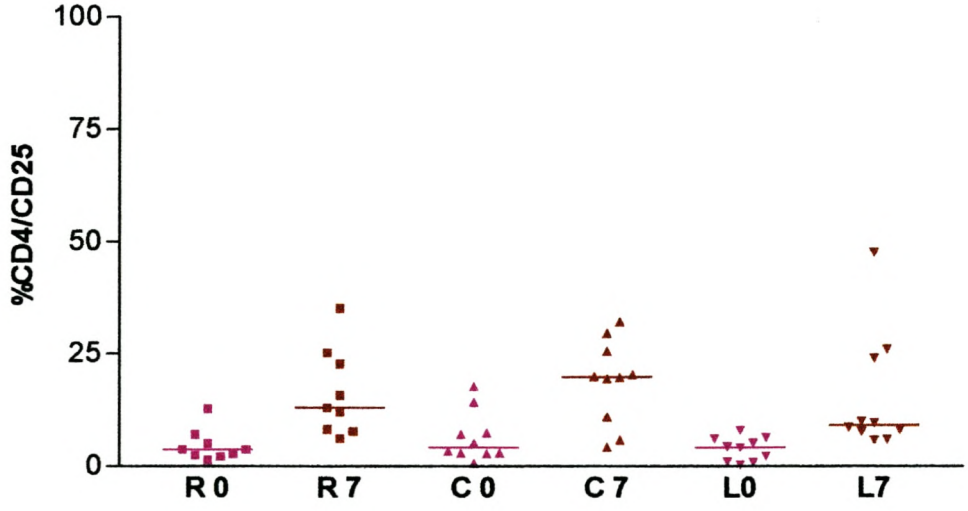

Figure 7.4.1 C CD4/CD25 expression in the groups on day 0 (*i.e.* R0, C0, L0) and after seven days in culture with live *M. tuberculosis* (*i.e.* R7, C7, L7)

7.2.3.4.1 CD8/CD25

7.2.3.4.1.1 Recurrent group

The number of positively stained CD8/CD25 cells were overall very low. The median percentage of activated CD8 T-cells in the recurrence group was 0.19 % increasing significantly to 1.48 % ($p < 0.05$) after seven days in culture (Figures 7.5.1 A and B).

7.2.3.4.1.2 Cured group

Although not significant, the cured group had the highest percentage of positively stained CD8/CD25 cells (0.41 %), increasing significantly to 3.8 % ($p < 0.05$) after seven days in culture.

7.2.3.4.1.3 Latent group

The median percentage of cells expressing CD8/CD25 in the latent group, was 0.21 % *ex vivo*, increasing significantly to 1.34 % ($p < 0.05$) after seven days in culture.

7.2.3.4.2 Differences in CD8/CD25 expression among the patient groups

No significant differences was observed in the percentage CD8/CD25 positive cells in the three groups (Figure 7.5.1 C).

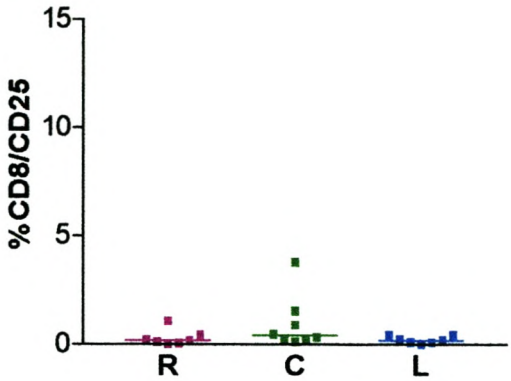

Figure 7.5.1 A CD8/CD25 expression in groups on day 0 (*ex vivo*)

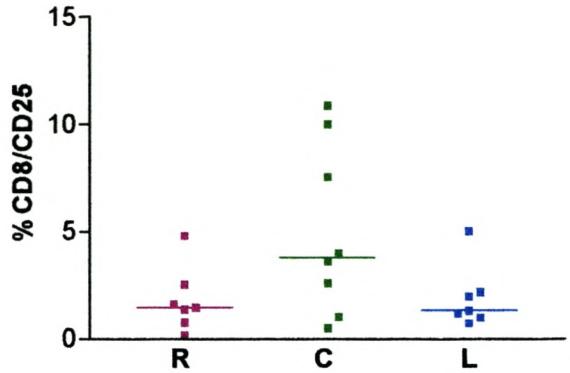

Figure 7.5.1 B CD8/CD25 expression in groups after seven days in culture with *M. tuberculosis*

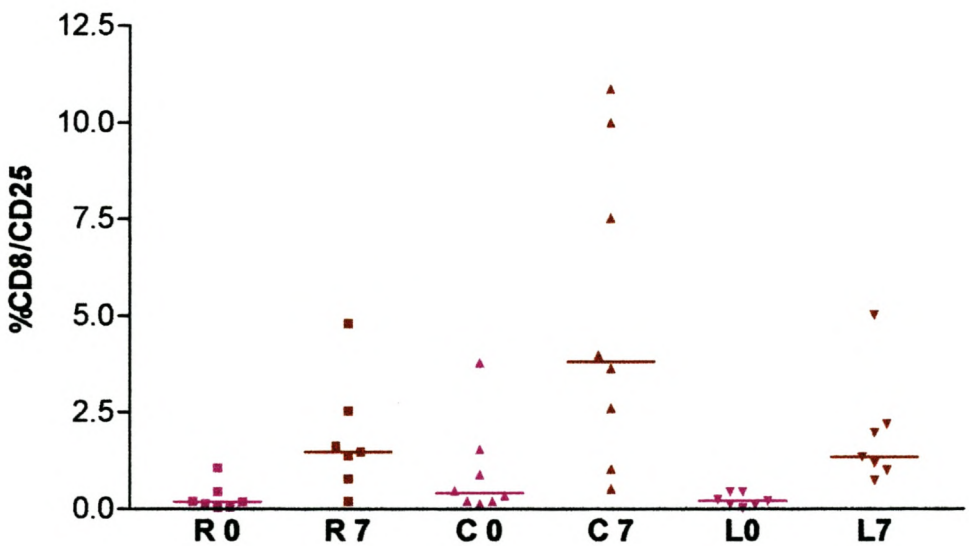

Figure 7.5.1 C CD8/CD25 expression in the groups on day 0 (i.e. R0, C0, L0) and after seven days in culture with live *M. tuberculosis* (i.e. R7, C7, L7)

7.2.4 Cytokine production

As outlined in Chapter 2, whole blood from patients was cultured unstimulated and stimulated with PPD and live *M. tuberculosis* for two and seven days, after which supernatants were harvested and stored at -20 °C until determination of cytokine profiles by means of the ELISA technique. The ELISA results for all subject groups (*i.e.* raw data) are shown in Tables 1 (A, B, C) – 4 (A, B, C) in the Appendix of this chapter. IL-10, TNF-α and TGF-β concentrations are expressed after the deduction of spontaneous secretion in the absence of any stimulus (unstimulated sample). These corrected median values as well as median values for IL-2, IL-12 and IFN-γ are given in Tables 7.4.1 A to D and will be quoted throughout the text. For the investigation, six cytokines were chosen for the following reasons:

- IFN-γ, as the signature Th1 cytokine as well as its importance in the immune response to *M. tuberculosis* (Jaffe *et al.*, 1991; Newport *et al.*, 1996; Jouanguy *et al.*, 1996)
- IL-12, as this cytokine induces and augments IFN-γ production (deJong *et al.*, 1998)
- IL-2, as growth factor (Abbas *et al.*, 1996)
- TNF-α, as this cytokine plays an important role in granuloma formation (Kindler *et al.*, 1989) and TNF-α also synergizes with IFN-γ to induce maximal macrophage activation (Britton *et al.*, 1993)
- IL-10, although not specifically a Th2 cytokine. It was chosen as a Th2 measure instead of IL-4, as the latter cytokine is rarely produced in cell culture and thus difficult to quantify (Petrovsky *et al.*, 1995). IL-10 inhibits the development, proliferation and cytokine production of Th1 cells (Moore *et al.*, 1993)
- TGF-β, an immuno-suppressive cytokine, suppresses monocytic activities (Toossi *et al* 1995).
- IL-13 was originally included in the study, but was abandoned after initial dose- and time-response ELISA results showed no detectable IL-13 levels.

Table 7.4.1 A Median PPD-stimulated cytokine responses after two days in culture

	IFN-γ	IL-2	IL-12	TNF-α	IL-10	TGF-β
R	104.7	17.19	186.9	124.6	337.6	513.6
C	170.7	0	362.0	197.2	243.2	1 165.2
L	597.1	12.17	168.5	353.8	31.5	200.1

Table 7.4.1 B Median PPD-stimulated cytokine responses after seven days in culture

	IFN-γ	IL-2	IL-12	TNF-α	IL-10	TGF-β
R	1 350.7	1.68	157.9	0	256.3	801.5
C	3 023.0	0	205.7	137.2	734.4	1 337.3
L	2 813.0	8.28	148.5	0	52.4	894.7

Table 7.4.1 C Median *M. tuberculosis*-induced cytokine responses after two days in culture

	IFN-γ	IL-2	IL-12	TNF-α	IL-10	TGF-β
R	7.02	0	41.73	0	0	0
C	0	0	105.5	6.25	29.64	162.45
L	26.23	0.57	312.9	38.85	0	210.2

Table 7.4.1 D Median *M. tuberculosis*-induced cytokine responses after seven days in culture

	IFN-γ	IL-2	IL-12	TNF-α	IL-10	TGF-β
R	1 183.4	4.26	133.81	113.6	45.72	265.2
C	1 947.0	0	88.18	47.45	36.22	541.15
L	1 572.0	11.79	145.8	0	52.4	285.8

7.2.4.1 Cytokine production as described for each patient group

7.2.4.1.1 IFN-γ

7.2.4.1.1.1 Recurrent group

As shown in Tables 7.4.1 A to D and Figures 7.7.1 A and B, PPD and *M. tuberculosis* induced IFN-γ production increased significantly from day two to day seven. Although IFN-γ production was lowest in the recurrent group, the increase in production from day two to day seven was steeper compared to the other two groups. For PPD stimulation, a median 104.7 pg/ml was measured on day two, increasing significantly ($p > 0.01$) to 1 350.7 pg/ml measured on day seven. Although lower compared to levels obtained after PPD stimulation, a median value of 7.02 pg/ml was measured on day two after *M. tuberculosis* infection and increased significantly ($p < 0.05$) to median 1 183.4 pg/ml measured on day seven.

7.2.4.1.1.2 Cured group

Although PPD and *M. tuberculosis* induced IFN-γ production also increased significantly from day two to day seven (Tables 7.4.2 A and B and Figures 7.7.1 and C and D), the incline was lowest in the cured group. A median IFN-γ value of 170.7 pg/ml was obtained on day two after PPD stimulation, increasing significantly ($p < 0.001$) to 3 023 pg/ml on day seven. A median value of 0 pg/ml) was measured on day two after *M. tuberculosis* infection, increasing significantly ($p < 0.001$) to 1 947.0 pg/ml on day seven.

7.2.4.1.1.3 Latent group

In this group, (Tables 7.4.3 A and B and Figures 7.7.1 E and F), PPD and *M. tuberculosis* induced IFN-γ production also increased significantly from day two to day seven. Of the three patient groups, IFN-γ production was highest in the latent group. After PPD stimulation, a median value of 597.1 pg/ml was obtained on day two, increasing ($p > 0.05$) to 2 813 pg/ml measured on day seven. A median of 26.23 pg/ml) was measured on day two after *M. tuberculosis* infection, increasing significantly ($p > 0.05$) to 1 572 pg/ml on day seven.

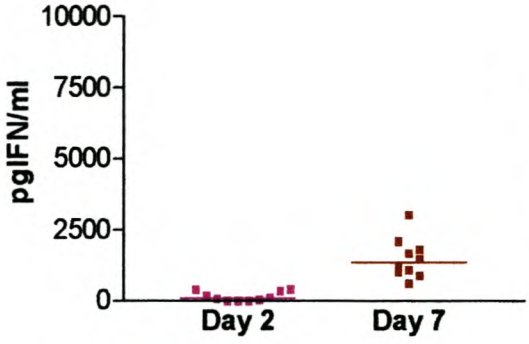

Figure 7.7.1 A PPD-induced production of IFN-γ in the recurrence group after two and seven days in culture

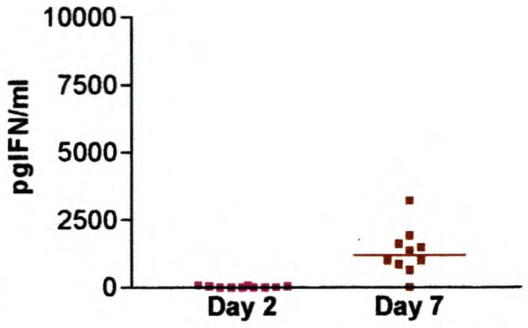

Figure 7.7.1 B *M. tuberculosis*-induced production of IFN-γ in the recurrence group after two and seven days in culture

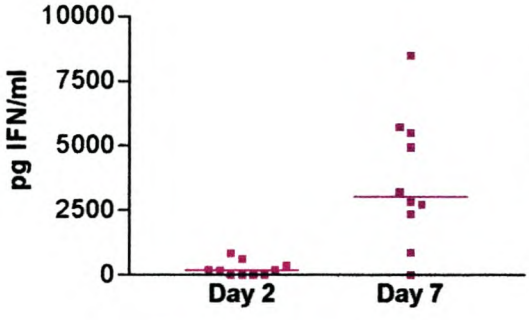

Figure 7.7.1 C PPD-induced production of IFN-γ in the cured group after two and seven days in culture

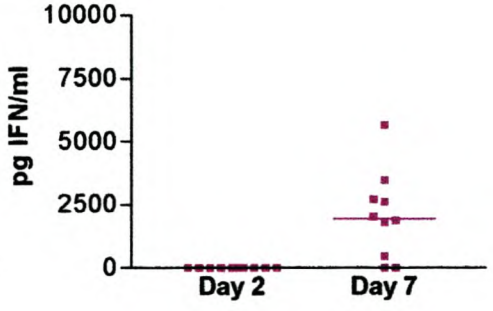

Figure 7.7.1.D *M. tuberculosis*-induced production of IFN-γ in the cured group after two and seven days in culture

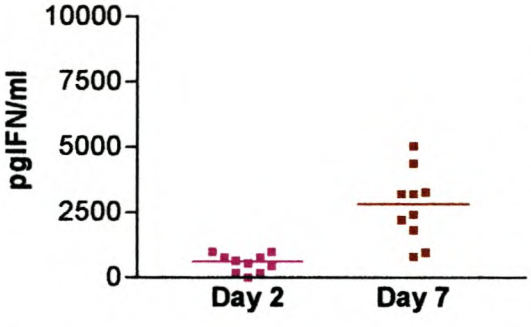

Figure 7.7.1 E PPD-induced production of IFN-γ in the latent group after two and seven days in culture

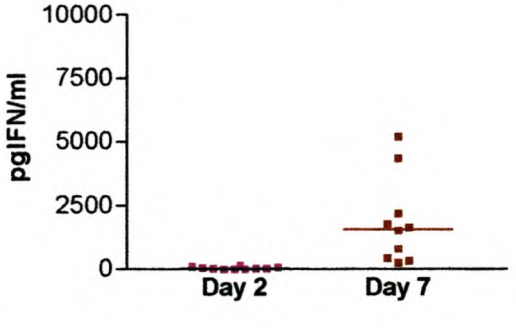

Figure 7.7.1 F *M. tuberculosis*-induced production of IFN-γ in the latent group after two and seven days in culture

7.2.4.1.1.4 Differences in IFN-γ production among subject groups

PPD stimulation As shown in Figure 7.7.2 A, after two days in culture with PPD, the only significant difference among the groups, was observed between the recurrent group (median 104.7 pg/ml) and the latent group (median 597.1 pg/ml) ($p < 0.05$). After seven days in culture, a significant difference in IFN-γ production was observed between the recurrent group (median 1 350.7 pg/ml) and the cured group (median 3 023 pg/ml) ($p < 0.05$) (Figures 7.7.2 C). Furthermore, a dramatic increase from day two to seven, was observed for both the recurrent and control groups, which could suggest that these two groups mounted more potent immune responses to PPD.

M. tuberculosis stimulation IFN-γ production after two days in culture with *M. tuberculosis*, was highest in the latent group (26.23 pg/ml) compared with the baseline levels measured in the two other groups (Figure 7.7.2 B). A significant difference in IFN-γ production was observed between the cured group (median 0 pg/ml) and latent group (median 26.23 pg/ml) ($p < 0.05$) on day two. However, no significant differences was found between the groups after seven days in culture (Figure 7.7.2 D).

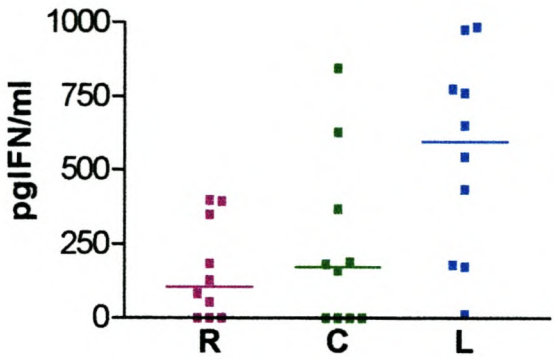

Figure 7.7.2 A PPD-induced IFN-γ production in the groups after two days in culture

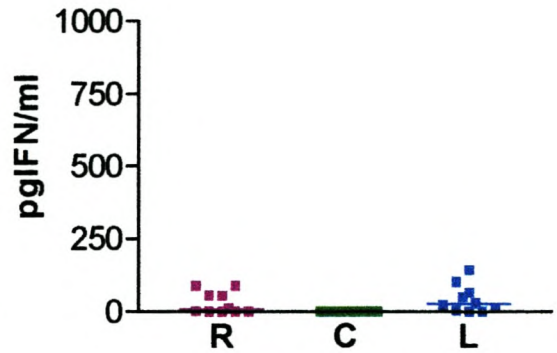

Figure 7.7.2 B *M. tuberculosis*-induced production of IFN-γ after two days in culture

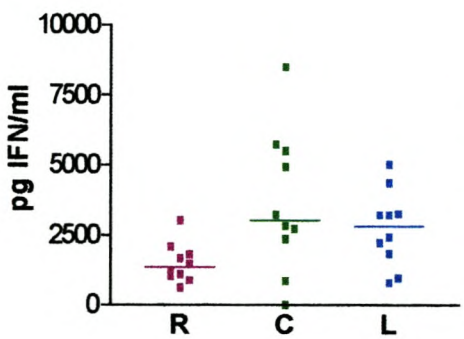

Figure 7.7.2 B PPD-induced production of IFN-γ after seven days in culture

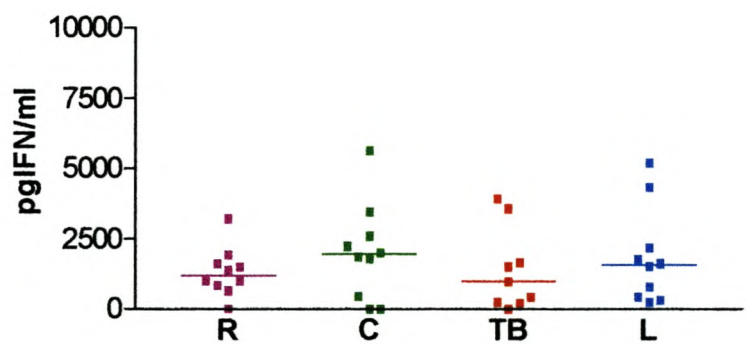

Figure 7.7.2 C *M. tuberculosis*-induced production of IFN-γ after seven days in culture

KEY: R = recurrence group
C = cured group
L = latent group
TB = active TB group

7.2.4.1.2 IL-2

7.2.4.1.2.1 Recurrent group

As expected, IL-2 production peaked early on day two with levels already low after two days in culture (Tables 7.4.1 A to D and Figures 7.7.3 A and B). The highest levels of IL-2 production was measured in the recurrent group. After PPD stimulation, a median IL-2 concentration of 17.19 pg/ml was measured on day two, declining significantly ($p < 0.05$) to 1.68 pg/ml on day seven. However, after *M. tuberculosis* infection, IL-2 levels increased from 0 pg/ml on day two to 4.26 pg/ml on day seven.

7.2.4.1.2.2 Cured group

As shown in Figures 7.7.3 C and D, there was no detectable levels of IL-2 in the cured group (Tables 7.4.1 A to D).

7.2.4.1.2.3 Latent group

As shown in Tables 7.4.1 A to D and Figures 7.7.3 E and F, IL-2 production in the latent group was low. No significant differences ($p < 0.05$) was found between IL-2 production on day two (median 12.17 pg/ml) and day seven (median 8.28 pg/ml) after PPD stimulation. Similar to that found in the recurrent group, after *M. tuberculosis* infection, a significant increase ($p > 0.05$) in IL-2 production was measured from day two (median 0.57 pg/ml) to 11.79 pg/ml measured on day seven.

7.2.4.1.2.4 Differences in IL-2 production among subject groups

Although a decline in IL-2 production was detected among the relapse group and latent group, no significant differences were observed for either PPD- or *M. tuberculosis*-induced IL-2 production.

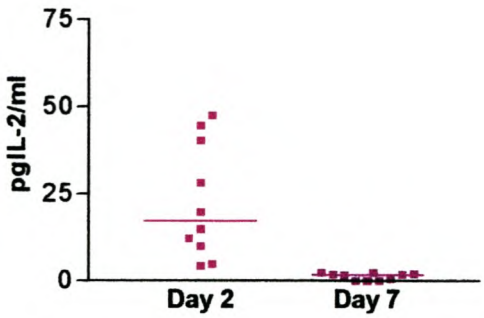

Figure 7.7.3 A PPD-induced production of IL-2 in the recurrence group after two and seven days in culture

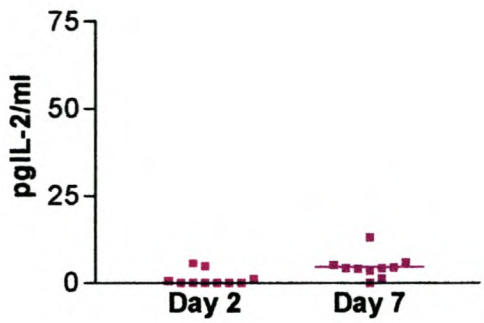

Figure 7.7.3 B *M. tuberculosis*-induced production of IL-2 in the recurrence group after two and seven days in culture

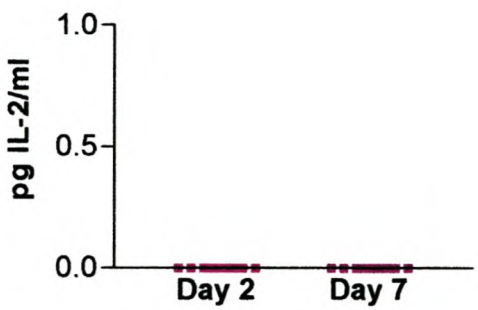

Figure 7.7.3 C PPD-induced production of IL-2 in the cured group after two and seven days in culture

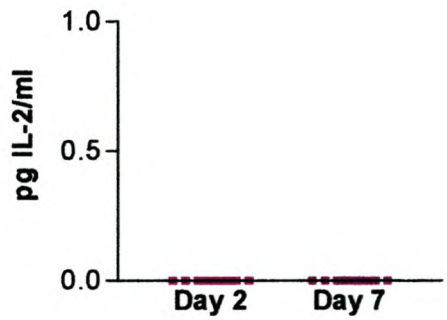

Figure 7.7.3 D *M. tuberulosis*-induced production of IL-2 in the cured group after two and seven days in culture

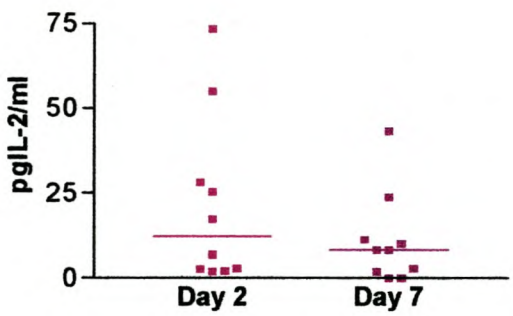

Figure 7.7.3 E PPD-induced production of IL-2 in the latent group after two and seven days in culture

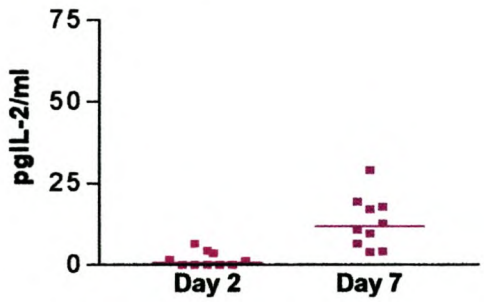

Figure 7.7.3 F *M. tuberculosis*-induced production of IL-2 in the latent group two and seven days in culture

7.2.4.1.1.3 IL-12

7.2.4.1.1.3.1 Recurrent group

Although IL-12 production decreased from day two (median 186.9 pg/ml) to 157.9 pg/ml measured on day seven, the difference was not significant ($p = 0.472$). After *M. tuberculosis* stimulation, a marginal increase ($p = 0.08$) from 41.73 pg/ml on day two to median 133.81 pg/ml measured on day seven was documented for IL-12 production (Tables 7.4.1 A to B and Figures 7.7.4 A and B).

7.2.4.1.1.3.2 Cured group

IL-12 production in the cured group was lowest compared with the other groups (Tables 7.4.1 A to B, and Figures 7.7.4 C and D). After PPD stimulation, a median value of 362.0 pg/ml was measured on day two declining ($p > 0.05$) to 205.7 pg/ml measured on day seven. IL-12 production after stimulation with *M. tuberculosis* decreased ($p = 0.129$) from 105.5 pg/ml measured on day two to 88.18 pg/ml measured on day seven.

7.2.4.1.1.3.3 Latent group

IL-12 production in the latent group was highest compared with the other groups and the decline in production from day two to day seven more significant and steep (Figures 7.7.4 E and F). After PPD stimulation, a median of 168.5 pg/ml was measured on day two declining ($p < 0.05$) to 148.5 pg/ml measured on day seven. IL-12 production after stimulation with *M. tuberculosis* decreased ($p > 0.05$) from 312.9 pg/ml measured on day two to 145.8 pg/ml measured on day seven.

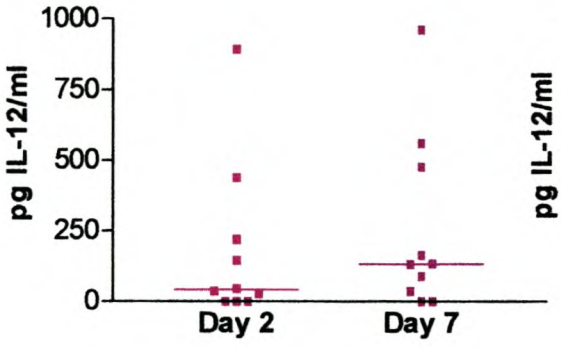

Figure 7.7.4 A PPD-induced production of IL-12 in the recurrence group after two and seven days in culture

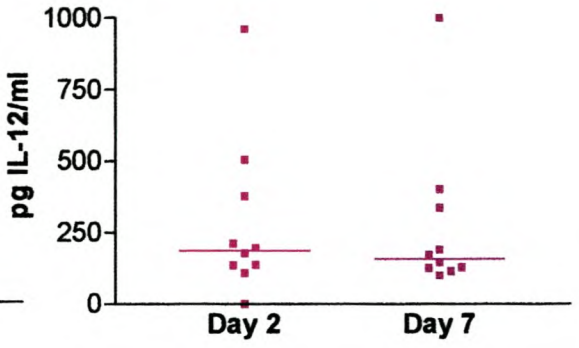

Figure 7.7.4 B *M. tuberculosis*-induced production of IL-12 in the recurrence group after two and seven days in culture

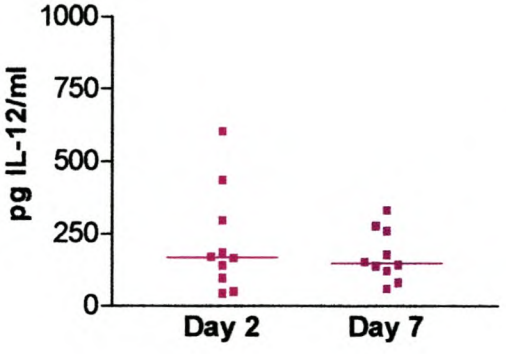

Figure 7.7.4 C PPD-induced production of IL-12 in the cured group after two and seven days in culture

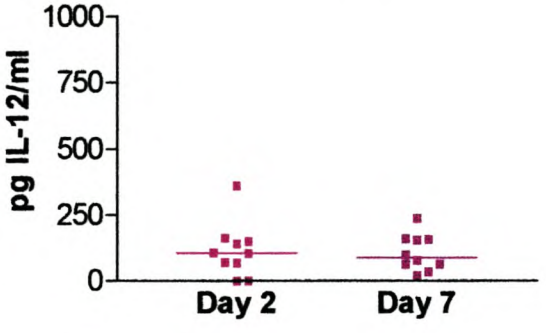

Figure 7.7.4 D *M. tuberculosis*-induced production of IL-12 after two and seven days in culture

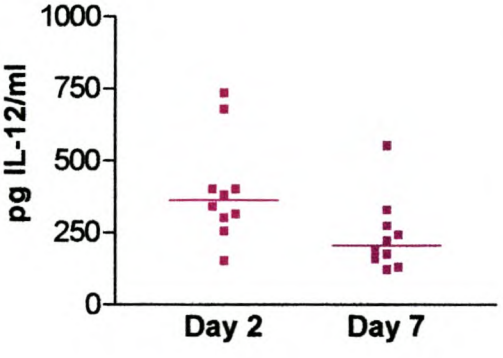

Figure 7.7.4 E PPD-induced production of IL-12 in the latent group after two and seven days in culture

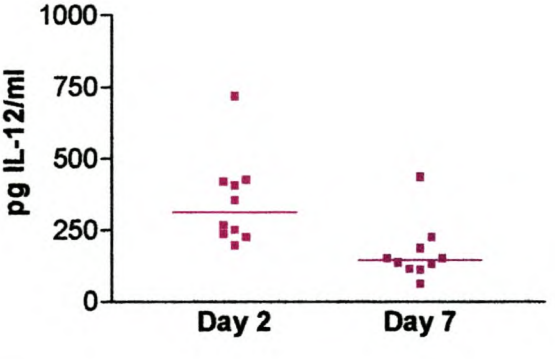

Figure 7.7.4 F *M. tuberculosis*-induced production of IL-12 after two and seven days in culture

7.2.4.1.1.3.4 Differences in IL-12 production among the subject groups

PPD stimulation IL-12 production after two days in culture was low in all three groups possibly due to the fact that IL-12 is the cytokine responsible for the early differentiation of naïve T-cells into Th 1 cells (Abbas *et al.*, 1996). After two days in culture, IL-12 production was lower among the recurrence (median 186.9 pg/ml) and control (median 362.0 pg/ml) individuals compared with the latent group (median 168.5 pg/ml). No significant differences was found in IL-12 production between the three groups (Figures 7.7.5 A and C). IL-12 production decreased in both the control (362.0 to 205.7 pg/ml) and latent groups (168.5 to 148.5 pg/ml) from day two to day seven.

M. tuberculosis stimulation IL-12 production after two days in culture with *M. tuberculosis* was highest in the latent group (median 312.9 pg/ml) (Figure 7.7.5 B). Significant differences in IL-12 production were found between the latent group and the cured group ($p < 0.05$) as well as between the latent and recurrence groups ($p < 0.05$). After seven days in culture the only increase in IL-12 production was seen among the recurrence individuals with the highest levels measured among the TB active group (median 145.7 pg/ml) (Figure 7.7.5 D). The low levels measured among the latent group differed significantly from levels measured among the recurrence subjects ($p < 0.05$) and TB active group ($p < 0.01$).

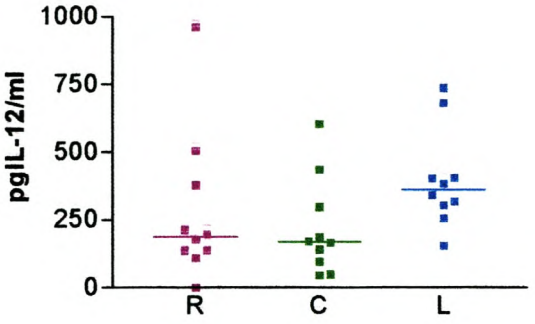

Figure 7.7.5 A PPD-induced production of IL-12 after two days in culture

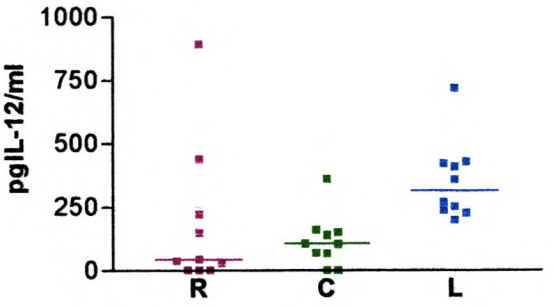

Figure 7.7.5. B *M. tuberculosis*-induced production of IL-12 in the groups after two days in culture

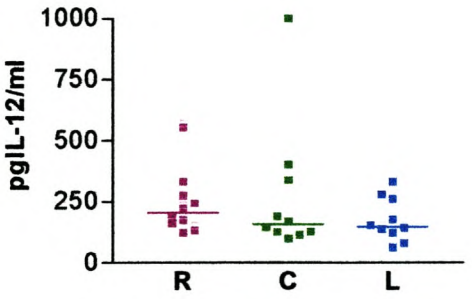

Figure 7.7.5 C PPD-induced production of IL-12 in the groups after seven days in culture

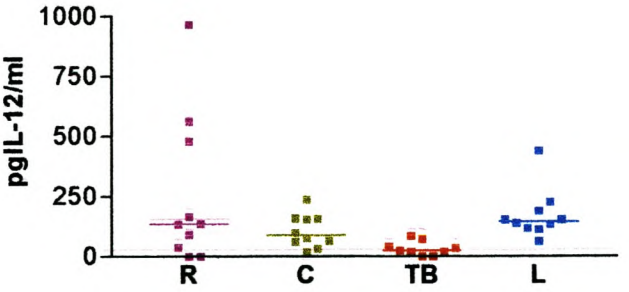

Figure 7.7.5 D *M. tuberculosis*-induced production of IL-12 in the groups after seven days in culture

7.2.4.1.1.4 TNF-α

7.2.4.1.1.4.1 Recurrent group

As shown in Tables 7.4.1 A and B and Figures 7.7.6 A and B, TNF-α production was lowest in this group compared with the cured and latent groups. For stimulation with PPD, a median of 124.6 pg/ml was measured on day two decreasing ($p < 0.05$) to 0 pg/ml on day seven. Although not significant, the only increase in TNF-α production increased from between day two to day seven after *M. tuberculosis* infection. A median value of 0 pg/ml was measured on day two increasing to 113.6 pg/ml measured on day seven.

7.2.4.1.1.4.2 Cured group

As shown in Tables 7.4.1 A to D and Figures 7.7.6 C and D, after stimulation with PPD, a median value of 197.2 pg/ml was measured on day two decreasing ($p > 0.05$) to 137.2 pg/ml) on day seven. For stimulation with *M. tuberculosis*, a median value of 6.25 pg/ml was obtained on day two increasing to 47.45 pg/ml measured on day seven.

7.2.4.1.1.4.3 Latent group

The highest level of TNF-α production was reported in the latent group for both PPD and *M. tuberculosis* stimulation (Tables 7.4.1 A to D and Figures 7.7.6 E and F). For stimulation with PPD, a median value of 353.8 pg/ml was measured on day two decreasing ($p < 0.001$) to 0 pg/ml on day seven. After stimulation with *M. tuberculosis*, a median value of 38.85 pg/ml was measured on day two decreasing to 0 pg/ml measured on day seven.

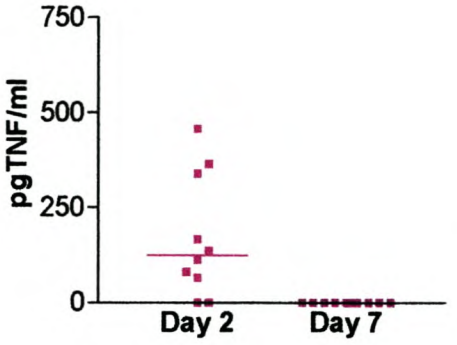

Figure 7.7.6 A PPD-induced production of TNF-α in the recurrence group after two and seven days in culture

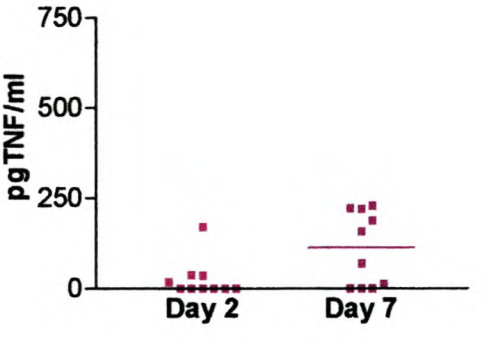

Figure 7.7.6 B *M. tuberculosis*-induced production of TNF-α in the recurrence group after two and seven days in culture

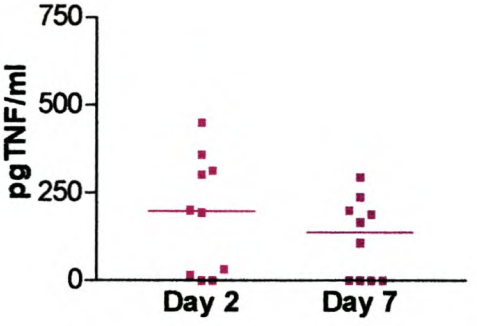

Figure 7.7.6 C PPD-induced production of TNF-α in the cured group after two and seven days in culture

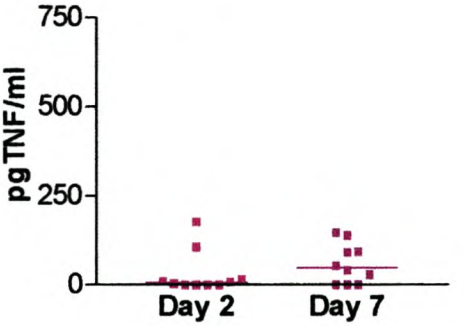

Figure 7.7.6 D *M. tuberculosis*-induced production of TNF-α in the cured group after two and seven days in culture

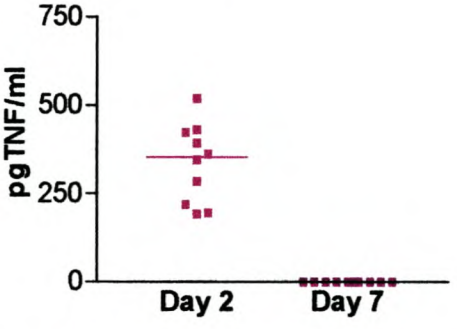

Figure 7.7.6 E PPD-induced production of TNF-α in the latent group after two and seven days in culture

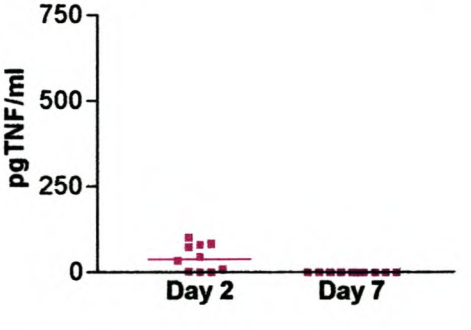

Figure 7.7.6 F *M. tuberculosis*-induced production of TNF-α in the latent group after two and seven days in culture

7.2.4.1.1.4.4 Differences in TNF-α production in the subject groups

On day two in culture with PPD, TNF-α production among recurrence group differed significantly from those individuals had been cured ($p < 0.01$) (Figure 7.7.7 A). After seven days in culture, the low levels of TNF-α produced by the recurrence individuals, differed significantly from levels obtained by the cured ($p < 0.01$) and latent groups ($p < 0.01$) (Figure 7.7.7 C).

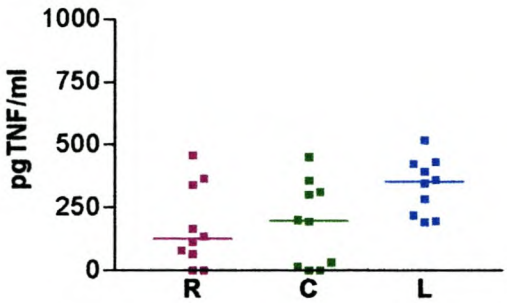

Figure 7.7.7 A PPD-induced production of TNF-α in the groups after two days in culture

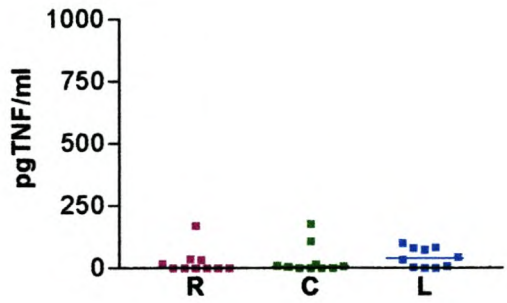

Figure 7.7.7 B *M. tuberculosis*-induced production of TNF-α in the groups after two days in culture

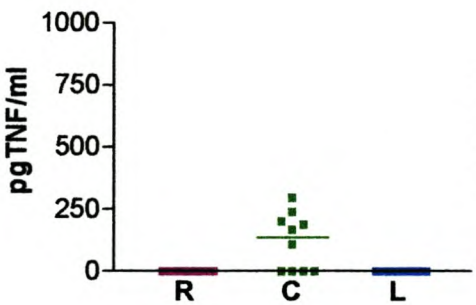

Figure 7.7.7 C PPD-induced production of TNF-α in the groups after seven days in culture

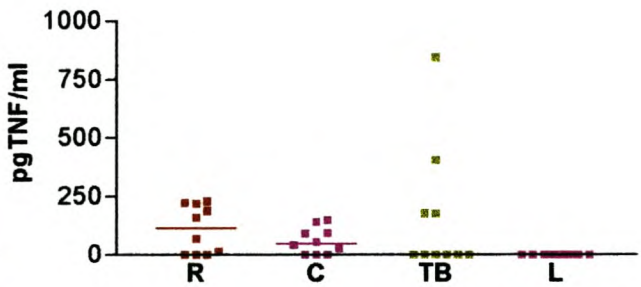

Figure 7.7.7 D *M. tuberculosis*-induced production of TNF-α in the groups after seven days in culture

7.2.4.1.1.5 IL-10

7.2.4.1.1.5.1 Recurrent group

As IL-10 is an immuno-suppressive cytokine (Moore *et al.*, 1993), it is important to note that not only was IL-10 production highest in the recurrent group, but levels were already elevated on day two (Tables 7.4.1 A to D and Figures 7.7.8 A and B). After stimulation with PPD, a median value of 337.65 pg/ml was obtained on day two decreasing ($p = 0.92$) to 256.95 pg/ml measured on day seven. The increase in production after *M. tuberculosis* infection, from day two to day seven, was marginal ($p = 0.054$) with a median value of 0 pg/ml measured on day two increasing to median 45.72 pg/ml obtained on day seven.

7.2.4.1.1.5.2 Cured group

The most significant increases in IL-10 production were observed in the cured group (Tables 7.4.2 A and B and Figures 7.7.8 C and D). After stimulation with PPD, a significant increase ($p = 0.002$) was measured from a median of 243.2 pg/ml on day two to 734.37 pg/ml measured on day seven. For stimulation with *M. tuberculosis*, an increase from a median value of 29.64 pg/ml measured on day two to a median value of 36.22 pg/ml measured on day seven.

7.2.4.1.1.5.3 Latent group

In the light of the immuno-suppressive properties of IL-10, it was not surprising to find that levels of this cytokine was lowest in the latent group (Figures 7.7.8 E and F). After stimulation with PPD, no significant increase ($p = 0.54$) was found between day two (median 31.5 pg/ml) and day seven (median 52.4 pg/ml). For stimulation with *M. tuberculosis*, a median value of 0 pg/ml was obtained on day two, increasing to 52.4 pg/ml ($p = 0.01$) on day seven.

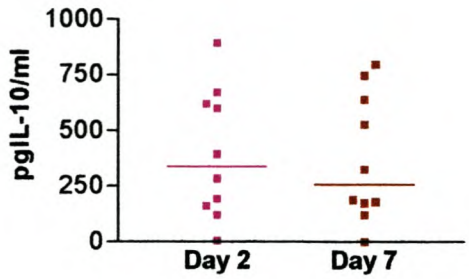

Figure 7.7.8 A PPD-induced production of IL-10 in the recurrence group after two and seven days in culture

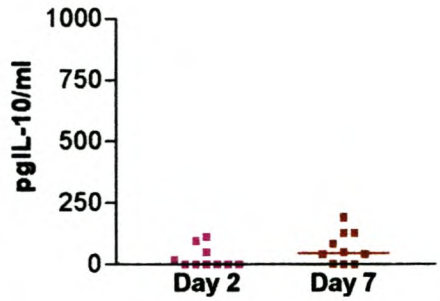

Figure 7.7.8 B *M. tuberculosis*-induced production of IL-10 in the recurrence group after two and seven days in culture

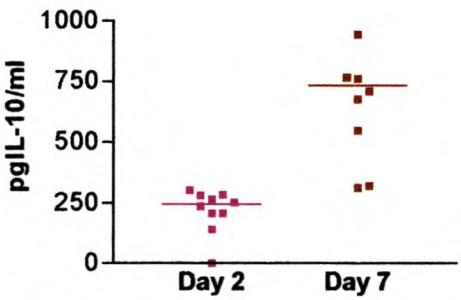

Figure 7.7.8 C PPD-induced production of IL-10 in the cured group after two and seven days in culture

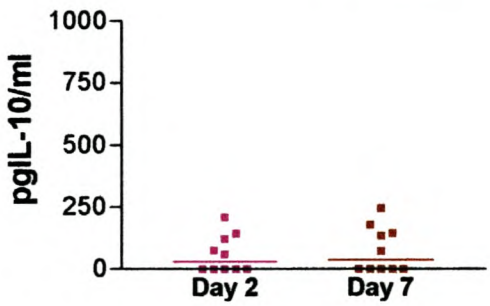

Figure 7.7.8 D *M. tuberculosis*-induced production of IL-10 in cured group after two and seven days in culture

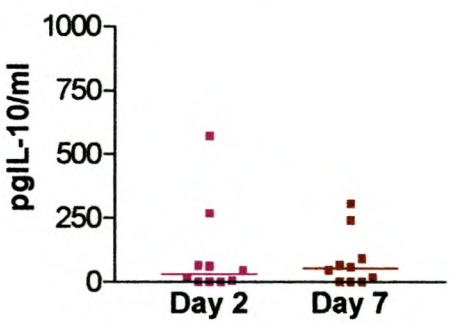

Figure 7.7.8 E PPD-induced production of IL-10 in the latent group after two and seven days in culture

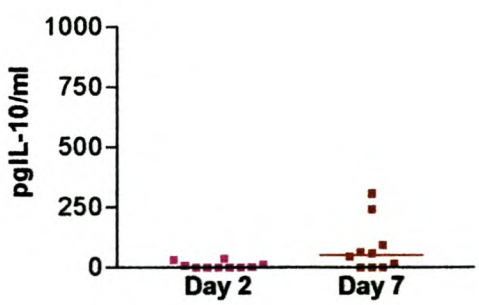

Figure 7.7.8 F *M. tuberculosis*-induced production of IL-10 in the latent group after two and seven days in culture

7.2.4.1.1.5.4 Differences in IL-10 production among subject groups

The only increase in IL-10 production was seen between the control and latent individuals after seven days in culture with PPD ($p < 0.001$) (Figure 7.7.9 C).

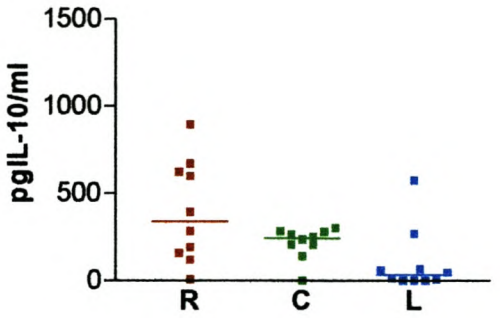

Figure 7.7.9 A PPD-induced production of IL-10 in the groups after two days in culture

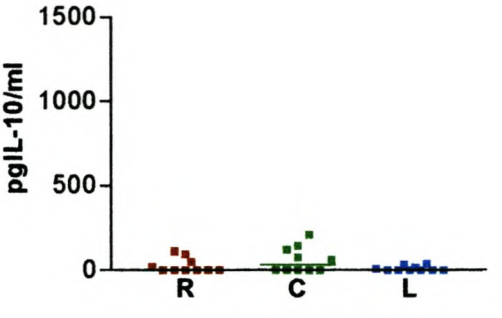

Figure 7.7.9 B. *M. tuberculosis*-induced production of IL-10 in the groups after two days in culture

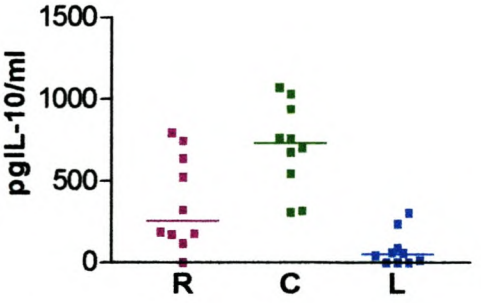

Figure 7.7.9 C PPD-induced production of IL-10 in the groups after seven days in culture

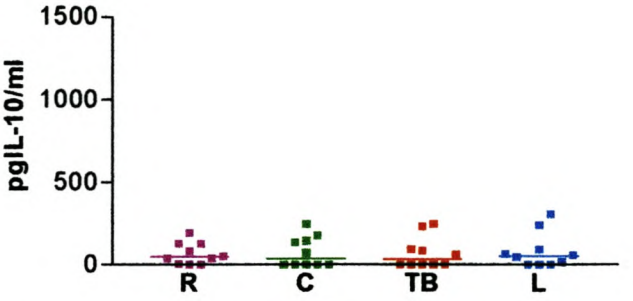

Figure 7.7.9 D *M. tuberculosis*-induced production of IL-10 in the groups after seven days in culture

7.2.4.1.1.6 TGF-β

7.2.4.1.1.6.1 Recurrent group

Similar to IL-10, TGF-β has immuno-suppressive properties with equally high levels of TGF-β were produced on day two (Tables 7.4.1 A to D and Figures 7.7.10 A and B). However, unlike IL-10, TGF-β was not the highest in the recurrent group. After PPD stimulation, TGF-β levels increased from a median value of 513.6 pg/ml to 801.5 pg/ml. For stimulation with *M. tuberculosis*, a median value of median 0 pg/ml was obtained on day two increasing ($p < 0.05$) to 265.2 pg/ml measured on day seven.

7.2.4.1.1.6.2 Cured group

The production of TGF-β was the highest in the cured group (Tables 7.4.1 A to D, and Figures 7.7.10 C and D). On day two a median value of 1 165.2 pg/ml was obtained after PPD stimulation, increasing to 1 377.3 pg/ml. After *M. tuberculosis* stimulation, TGF-β levels also increased from a median value of 162.4 pg/ml to 541.2 pg/ml.

7.2.4.1.1.6.3 Latent group

The production of TGF-β was the lowest in the latent group (Tables 7.4.3 A and B and Figures 7.7.10 E and F). A median value of 200.1 pg/ml was obtained on day two after PPD stimulation, increasing to 894.7 pg/ml. After *M. tuberculosis* stimulation, TGF-β levels increased from a median value of 210.2 pg/ml to 285.8 pg/ml.

7.2.4.1.1.6.4 Differences in TGF-β production among the subject groups

After two days in culture with PPD, TGF-β production was equally high among the recurrence (median 513.6 pg/ml) and cured groups (median 1 165.2 pg/ml), compared with the lower levels obtained in the latent group (median 200.1 pg/ml) (Figure 7.7.11 A). A significant difference was however only obtained between the cured and latent groups ($p < 0.01$) (Figure 7.10.6.2). As shown in Figures 7.7.11 C and 7.7.11 D, no differences were found among the groups after seven days in culture with PPD or *M. tuberculosis*.

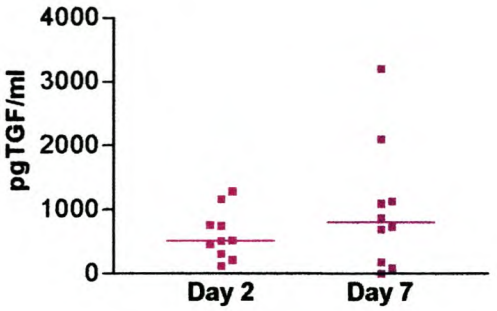

Figure 7.7.10 A PPD-induced production Of TGF-β in the recurrence group after two and seven days in culture

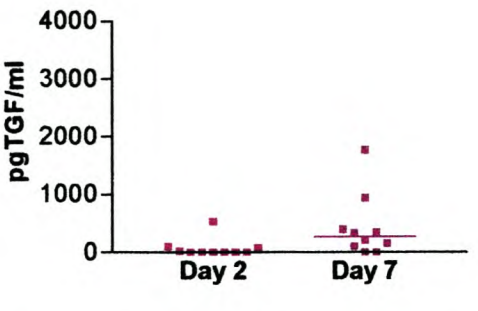

Figure 7.7.10 B *M. tuberculosis*-induced production of TGF-β in the recurrence group after two and seven days in culture

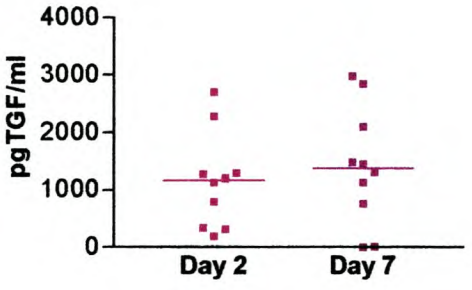

Figure 7.7.10 C PPD-induced production of TGF-β in the cured group after two and seven days in culture

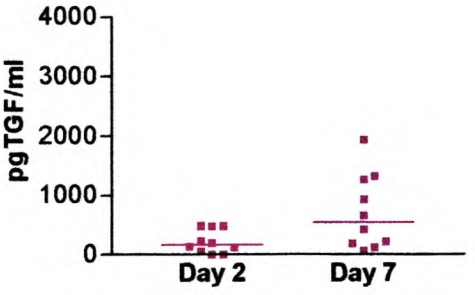

Figure 7.7.10 D *M. tuberculosis*-induced production of TGF-β in the cured group after two and seven days in culture

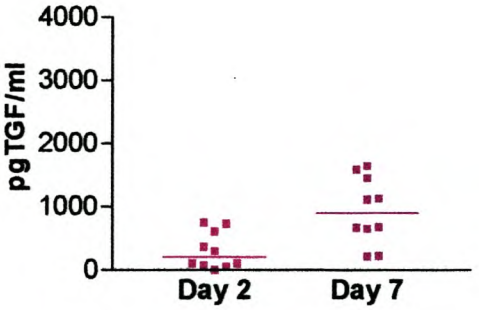

Figure 7.7.10 E PPD-induced production TGF-β in the latent group after two and seven days in culture

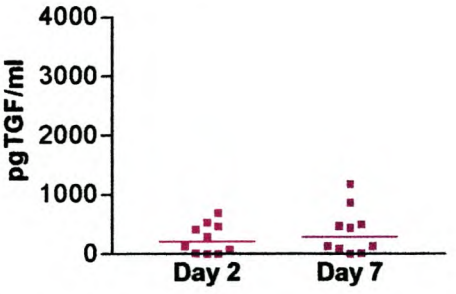

Figure 7.7.10 F *M. tuberculosis*-induced production of TGF-β in the latent group after two and seven days in culture

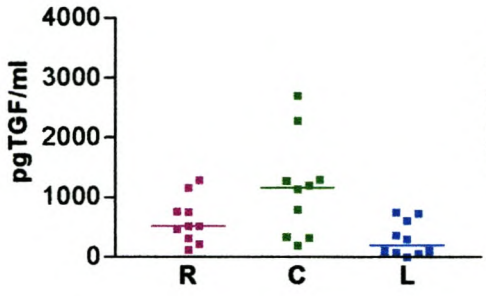

Figure 7.7.11 A PPD-induced production of TGF-β in the groups after two days in culture

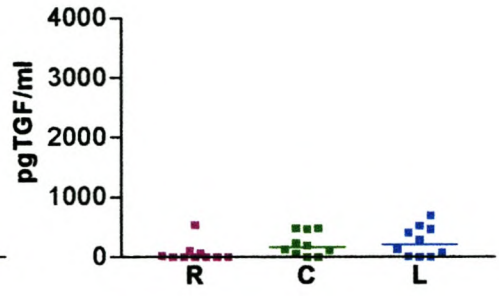

Figure 7.7.11 B *M. tuberculosis*-induced production of TGF-β in the groups after two days in culture

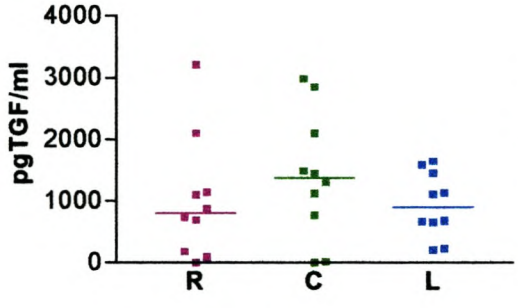

Figure 7.7.11 C PPD-induced production of TGF-β in the groups after seven days in culture

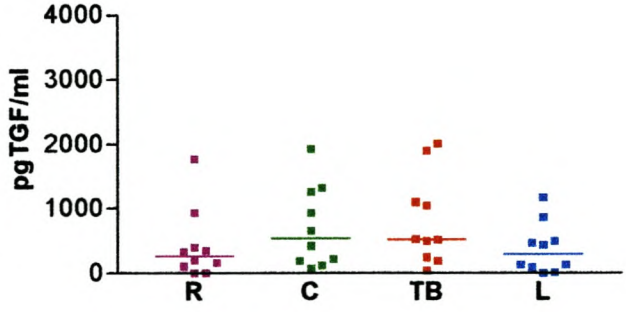

Figure 7.7.11 D *M. tuberculosis*-induced production of TGF-β in the groups after seven days in culture

7.2.4.1.7 TB active patient group

For this group of individuals, blood was only stimulated with live *M. tuberculosis* for seven days (Appendix). These results were shown with the above-mentioned results.

7.2.5 Social environment of subjects

Several striking themes emerged from the interviews of those who were prepared to be interviewed. Table 7.5 shows which individuals were included in the interviews as well as their source of income.

Most of the individuals interviewed basically lived from hand to mouth. Women would try to find work as chars while men would look for casual work in the building industry. Only one of the interviewed individuals was in full time employment and earned a regular wage. C6 had been working for 12 years as a machinist in a factory. R1 was retrenched and R10 stopped working as a live-in domestic after her third episode of tuberculosis. None of the other women interviewed was working. C2 had a chronic nervous condition, thus her sole source of income was a disability grant. Furthermore, almost everybody interviewed drank alcohol with at least one individual, R4 having a serious drinking problem. In addition to drinking almost everybody smoked cigarettes with most of them smoking at least two to three a day.

Table 7.5 Informants interviewed

Patient	Age	Income	Work
R1	33	none	none
R4	46	disability grant	N/A
R5	33	none	none
R7	45	none	none
R8	51	none	none
C1	29	none	none
C2	38	none	none
C6	35	wages	factory
C7	46	none	none
C8	51	none	none

Because of unemployment, most people would go hungry on a regular basis. In cases where these individuals had regular meals, it would be because his/her partner was in permanent employment. The individual's physical appearance confirmed their stories as

most of them were small and thin and some even emaciated. Furthermore, most of the households visited, were overcrowded.

Failure to recognize tuberculosis symptoms was a common occurrence among these individuals. Those who did have symptoms generally only had one and not the syndrome (a peristent cough, weight loss, night sweats, exhaustion, chest pain and backache), which they might have recognised. Because it is so cold in winter, people pile on blankets at night and then start to feel too hot. In summer, on the other hand, the houses are like ovens and people perspire at night. Sleeping in airless rooms with so many other people, it is normal to feel hot. Weight loss, particularly among women, was attributed to stress ad was not always unwelcome. Sleeping on the floor or sharing a bed with too many other people and hence constantly sleeping in awkward positions, explained backache (Head, 2001). Persistent coughs were explained as normal colds and flu until they became unbearable, or resulted in the expectoration of blood. Spitting blood was what finally prompted a number a people seeking medical advice.

7.3 Discussion

The aim of the present study was to identify an immune parameter or pattern that showed a difference between individuals resistant to *M. tuberculosis* infection and those who are susceptible. In most cases, overlapping cytokine production values were observed among the four groups studied, precluding the use of a specific cytokine for the discrimination between susceptible and resistant individuals.

7.3.1 Immune profiles of patient groups

Although we could not identify a specific marker that would predict susceptibility to *M. tuberculosis*, definite patterns among the four groups investigated were observed. These findings will be discussed based on the cytokine production as well as per group. The comparison of cytokine responses among the four groups following stimulation with PPD and *M. tuberculosis*, revealed only minor differences. Also, the values in the groups showed a large distribution, resulting in large standard deviations.

7.3.1.1 Composition of blood

Except for isolated cases within the three groups, the whole blood cell count and lymphocyte populations of individuals were mostly within the normal ranges. Although no striking abnormalities were found, the most common abnormality among the three groups, was a high concentration of platelets and low concentration of red blood cells (aneamia) with correspondingly low levels of hemoglobin. This would normally indicate iron deficiency, which in these cases would be attributed to helminth infection. However, other reasons for this type of profile are not excluded as the eosinophil counts, normally elevated during helminth infection, were within the normal range.

7.3.1.2 Serum IgE

The presence of serum IgE in the patient groups was used as a surrogate marker for Th2 activation, as class-switching from IgG to IgE cannot occur without the presence of IL-4 and IL-13 (Kuhn *et al.*, 1991; Snapper *et al.*, 1991).

Compared to the other groups, the recurrent group had the lowest IgE levels, negating the possibility of these individuals being Th2 dominant. The low serum IgE concentrations among these individuals could be the result of the potent *M. tuberculosis*-induced type 1 immune response downregulating the type 2 response (*i.e.* serum IgE) after successful completion of treatment (Adams *et al.*, 1999). We have previously shown that IgE decreased after successful completion of therapy, presumably due to enhancement of type 1 responses. This hypothesis would certainly hold true for those individuals who have recently completed therapy (*i.e.* R2, R5, R6 and R7) presenting with an IgE concentration ranging between 4 – 80 kU/l. However, for certain individuals in this particular group (R4, R8 and R10), having completed TB therapy more than three years ago and now presenting with high IgE levels (*i.e.* 300 – 1 000 kU/l), we predict that, due to the suppressive influence of a dominant type 2 response on a protective type 1 response, these individuals would be susceptible to re-infection with *M. tuberculosis* (Zhang *et al.*, 1994; Zhang *et al.*, 1995; Ellner *et al.*, 1997; Vanham *et al.*, 1997; Hussain *et al.*, 1997; Hernandez-Garay and Mendez-Sampiero, 2003).

A similar pattern was expected for the cured group, who have had TB once before and have successfully completed treatment. However, this group showed the highest median concentration of IgE (612.50 kU/l). This activated Th2 status would render them susceptible to re-infection with *M. tuberculosis*. A median IgE concentration of 245.84 kU/l was measured for the latent group which was close to the normal range of 200 kU/l.

It seems that the levels of serum IgE production could serve as a good immune indicator of susceptibility or resistance to a Th1-inducing pathogen. However, IgE levels should not be considered in isolation but should rather, in this case, be considered in conjunction with cytokine production.

7.3.1.3 Cytokine profiles

It is generally accepted that IFN-γ production is suppressed during tuberculous disease (Ellner *et al.*, 1996; reviewed in Kaplan and Freedman, 1996; Collins and Kaufmann, 2001, Hussain *et al.*, 2002). Ellner and co-workers (1996) have demonstrated that this

low IFN-γ secretion could be maintained for at least 12 months. This notion was confirmed by the fact that the recurrent individuals and the group with newly diagnosed tuberculosis, had the lowest measured IFN-γ concentration. The suppressed IFN-γ response among the recurrent individuals might also be attributed to the fact that they have had more than two episodes of TB, possibly resulting in chronic suppression of type 1 immune responses. It is also possible that these individuals could be genetically low IFN-γ producers. This was however not examined. Interestingly, the cured group showed the highest IFN-γ responsiveness which is indicative of immune activation. Moreover, the steep incline in IFN-γ production from day two to day seven among the recurrence and cured individuals, demonstrates that these individuals were able to mount stronger immune responses to mycobacterial antigens. The latent group showed low to intermediate levels of IFN-γ production. The question now asked is what should be the "normal" IFN-γ levels for individuals residing in Ravensmead and Uitsig.

IL-12, produced by activated macrophages, directly stimulates NK-cells and $CD4^+$ T-cells (Gately *et al.*, 1991; Bertagnolli *et al.*, 1992; Fulton *et al.,* 1996) to produce IFN-γ. It was therefore expected that levels of this cytokine would peak early (in this case, after two days in culture). The recurrence patients presenting with the lowest concentration of IFN-γ, intermediate amounts of IL-12 produced. Although IFN-γ production was highest in the cured group, their IL-12 production was approximately the same as for the recurrence individuals. Due to their suppressed immune response, individuals with active TB had the lowest IL-12 production. The latent group had the highest concentration of IL-12 produced, prompting the question as to whether immuno-competence should be measured by the presence of IL-12 and not IFN-γ.

As expected, IL-2 production was low among all the groups as this cytokine is an autocrine growth factor.

Flynn and Chan (2001) have demonstrated that without TNF-α, effective granuloma formation is diminished and bacterial numbers rapidly increase. Furthermore, in response

to *M. tuberculosis* infection, TNF-α synergises with IFN-γ to induce maximal activation of activated macrophages for production of IL-12, creating a positive feedback loop for macrophage activation (Collins *et al.*, 1995; Flynn *et al.*, 1995; Rook and Stanford, 1996; Britton *et al.*, 1998; Bean *et al.*, 1999). Recently, Mohan and co-workers (2001) have shown that TNF-α neutralisation resulted in fatal reactivation of persistent tuberculosis, suggesting that TNF-α is essential for the prevention of disease recrudescence. Our results showed that TNF-α levels were lowest among the recurrence individuals and those with active tuberculosis. However, the absence or presence of TNF-α would be a weak marker in predicting recurrence. Because of its early involvement and recruitment, TNF-α production was highest after two days in culture. Interestingly, the overall TNF-α production was highest among the latent group and also showed most dramatic increase from day two to day seven.

The suppression of T-cell responses during active tuberculosis (de Waal-Malefyt *et al.*, 1992), is generally associated with an enhanced production and/or activity of immuno-suppressive cytokines such as TGF-β and IL-10. These two cytokines overlap with each other in many of their biological effects, including T-cell suppression, macrophage deactivation, modulation of pro-inflammatory cytokines and interference with APC function (Ellner, 1997; Wahl, 1992). Suppressive effects of IL-10 on the pathophysiology of tuberculous infection have been well described (Bogdan *et al.*, 1991; Flesh *et al.*, 1994; de Vries, 1995; Murray *et al.*, 1997). *In vitro* studies examining the interactions between human peripheral mononuclear cells and the tubercle bacilli have shown that IL-10 has the ability to down regulate *M. tuberculosis*-induced production of IL-12 (Gong *et al.*, 1996; Fulton *et al.*, 1998). Furthermore, transgenic mice over-expressing IL-10 were less capable of clearing infection with BCG (Murray *et al.*, 1997), suggesting that IL-10 may impair elimination of mycobacteria from macrophages and thus contribute to the establishment of a persistent infection. In the present study, the enhanced production of these two cytokines among individuals in the recurrence and cured groups, is therefore not surprising. Othieno and co-workers (1999) have demonstrated that TGF-β specifically upregulates the production of IL-10 and that together these cytokines synergistically suppressed PPD-induced IFN-γ production. However, we have

demonstrated that by using association studies (data not shown), no significant correlation was found between TGF-β and IL-10 production. Compared to the other groups, the concentration of IL-10 and TGF-β were highest among the cured individuals who have had only one episode of tuberculosis. This is significant, as such an immuno-suppressive profile would imply susceptibility to re-infection among the cured individuals. Similar to the previous cytokines measured in the present study, IL-10 and TGF-β levels among the recurrence patients were intermediate. However, as it is well documented that IL-10 inhibits anti-mycobacterial immunity (Fiorentino *et al.*, 1991; de Waal-Malefyt *et al.*, 1992; Moore *et al.*, 1993; Othieno *et al.*, 1999; Jacobs *et al.*, 2001; Hussain *et al.*, 2002), the low IL-10 produced by newly diagnosed TB patients was surprising.

7.3.1.4 Immunophenotypic characterisation

It has been recognised that the cellular immune response, particularly by T-lymphocytes, plays a central role in controlling *M. tuberculosis* replication (Orme and Collins, 1983). T-cell-deficient mice have demonstrated increasing susceptibility to disease (Kauffman and Ladel, 1994), similar to the higher susceptibility to disease development observed in immuno-deficient patients (Vanham *et al.*, 1996). Furthermore, several groups (Singhal *et al.*, 1989; Rodrigues *et al.*, 2000; Swaminathan *et al.*, 2000) have demonstrated that $CD4^+$ and $CD8^+$ T-lymphocytes numbers are decreased in patients with active tuberculosis and that the $CD4^+$ T-lymphocyte counts were restored to normal levels after successful completion of therapy. In concordance with this notion, in the present study we found that the median percentage $CD4^+$ T-cells in the recurrent group was the same compared to the cured and latent groups . The same trend was observed after seven days in culture with *M. tuberculosis*. Although not significant, the increase in percentage $CD4^+$ T-cells to day seven was steeper among the recurrence individuals. This trend was corroborated by the steep increase in IFN-γ production from day two to day seven. The importance of $CD4^+$ T-cells in the immune response against *M. tuberculosis* has been well documented, specifically as these cells are the primary source of IFN-γ which in turn activates the effector responses of infected macrophages (Flynn *et al.*, 1993; Orme *et al.*, 1993; Lyadova *et al.*, 1998; Scanga *et al.*, 2000).

The importance of $CD8^+$ T-cells has been illustrated by studies in animal demonstrating that $CD8^+$ T-lymphocyte deficiency can result in susceptibility to tuberculosis (Flynn *et al.*, 1992) and that $CD8^+$ T-lymphocytes can contain the dissemination of *M. tuberculosis* (Feng and Britton, 2000). In the present study however, we found no differences in the expression of $CD8^+$ T-cells among the groups and no significant increases from day two to day seven.

In accordance with studies performed by Antas and co-workers (2002), $CD25^+$ cells, after stimulation with *M. tuberculosis*, were mostly $CD4^+$ T-cells and not $CD8^+$ T-cells. These results were in parallel with the early secretion of IL-2 by $CD4^+$ T-cells. Since every individual in the study population has been infected with *M. tuberculosis*, the *in vitro* activation of lymphocytes would be an indicator of immune sensitisation against the *M. tuberculosis* and is not necessarily related to resistance or susceptibility. No difference was found in the percentage activated $CD4^+$- and $CD8^+$ T-cells among the groups investigated.

7.3.2 Social factors contributing to susceptibility to *M. tuberculosis*

Results presented in this thesis suggest that susceptibility to *M. tuberculosis* is not only based on an immunological pre-disposition, but also depends on the environment and socio-ecnomic factors contributing to susceptibility to infection and progression to disease. Amongst those people interviewed, poverty-related factors such as poor nutrition, overcrowding and unemployment, seemed to be the norm. This again highlights the need for future strategy to relieve overcrowding, improve housing conditions and reduce poverty in order to reduce TB rates.

7.3.3 Immune profile of susceptibility to *M. tuberculosis*

Here special mention is made of individuals within the groups portraying a profile of susceptibility to re-infection with *M. tuberculosis*. As already mentioned, most of the individuals in the cured group showed profiles immune profiles of susceptibility to re-infection. From the results it can be predicted that individuals who have had TB once before and who had been cured will remain vulnerable to *M. tuberculosis* infection.

Furthermore, in an isolated case, R7 not only presented with a low lymphocyte count (1.1 x 10^9g/l), but also with a high concentration of type 1 cytokines, IL-12 and IFN-γ, as well as a high concentration of the immuno-suppressive cytokines, IL-10 and TGF-β. This type of immuno-suppressive profile would indicate that this patient would be vulnerable to infection. Recurrent TB is perceived as a result of patient non-compliance with treatment. It is therefore argued that better case finding and holding will improve the treatment rates. However, we have shown that even in cases where patients complete their treatment, they would still be immunologically and socially vulnerable to infection and progression to disease.

Table 1.A Cytokine production in the **Recurrence** group after a **2 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
IFN-γ										
Unstim	45.1	0.0	0.0	0.0	0.0	0.0	18.9	0.0	26.2	0.0
PPD	127.0	184.3	0.0	54.2	0.0	82.4	348.4	0.0	392.7	397.0
Mtb	88.5	0.0	0.0	2.0	12.0	88.5	0.0	56.2	0.0	0.0
TNF-α										
Unstim.	608.4	384.6	443.4	88.1	192.5	180.4	1 161.6	298.1	623.4	192.5
PPD	721.2	340.8	808.3	168.5	258.0	636.9	144.8	464.5	759.8	531.3
Diff. PPD	112.8	0.0	364.9	80.4	65.5	456.6	0.0	166.4	136.4	33.8
Mtb	55.3	336.1	431.1	65.4	180.3	350.3	1 180.1	295.8	660.9	227.4
Diff Mtb	0.0	0.0	0.0	0.0	0.0	169.9	18.5	0.0	37.5	349.0
IL-12										
Unstim.	0.0	23.6	29.5	0.0	82.6	69.1	909.0	0.0	0.0	535.2
PPD	377.8	108.2	135.8	0.0	196.8	177.1	960.2	212.5	137.7	505.7
Mtb	147.6	29.5	37.4	0.0	220.4	46.1	893.3	0.0	0.0	438.8
IL-2										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PPD	4.8	28.2	12.2	44.5	4.3	47.4	14.8	9.9	19.6	40.2
Mtb	0.0	0.0	0.0	5.7	0.0	4.8	0.6	0.0	0.0	1.1
IL-10										
Unstim.	966.2	266.2	297.8	20.0	154.4	157.3	597.5	395.6	672.8	300.7
PPD	1564.6	459.0.	1 191.1	180.2	274.8	777.1	1 267.4	678.6	678.6	693.0
Diff. PPD	598.4	192.8	893.3	160.2	120.4	619.8	669.9	283.0	5.8	392.3
Mtb	931.2	240.4	410.0	14.3	203.1	251.9	568.6	412.9	453.2	272.0
Diff. Mtb	0.0	0.0	112.2	0.0	48.7	94.6	0.0	17.3	0.0	0.0
TGF-β										
Unstim	941.8	674.9	522.7	467.2	400.3	340.9	645.2	589.7	455.7	789.1
PPD	1 690.0	1 960.0	1 284.0	978.6	1 560.0	459.0	860.0	1 050.0	765.0	1 305.0
Diff Mtb	748.8	1 285.1	961.3	511.3	1 159.7	118.1	214.8	460.3	309.3	515.9
Mtb	1 042.8	582.3	455.2	1 003.6	416.8	210.2	522.6	419.3	523.1	489.2
Diff Mtb	101.6	0.0	0.0	536.4	16.5	0.0	0.0	0.0	67.0	4.0

Unstim.: Unstimulated

Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*

Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 1.B Cytokine production in the **Recurrence** group after a **7 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
IFN-γ										
Unstim.	6.2	0.0	0.0	0.0	0.0	0.0	5. 8	0.0	10.4	0.0
PPD	886.8	1 678.3	1 100.1	1 225.9	620.1	1 815.8	3 022.8	1 475.5	2 093.5	1 027.5
Mtb	1 366.7	1 000.2	645.6	1 613.3	15.4	1 917.9	3 214.9	851.6	1 495.9	998.1
TNF-α										
Unstim.	324.1	243.7	266.6	50.3	143.5	185.7	575.8	158.7	558.6	703.4
PPD	136.2	103.4	168.9	39.6	81.0	108.6	379.5	62.0	177.5	269.0
Diff PPD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	303.4
Mtb	543.7	465.9	453.8	208.6	156.8	414.1	127.4	227.6	669.9	70.6
Diff. Mtb	219.6	222.2	187.2	158.3	13.3	228.4	0.0	68.9	0.0	0.0
IL-12										
Unstim.	55.1	59.0	33.4	60.0	96.4	200.7	629.6	0.0	330.8	114.9
PPD	114.9	128.7	145.4	337.3	100.1	190.6	170.3	1 000.0	401.7	126.2
Mtb	132.5	135.1	478.6	90.5	37.1	165.0	964.1	0.0	0.0	560.8
IL-2										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PPD	1.6	1.8	0.0	2.0	2.5	1.8	2.5	0.5	0.0	0.0
Mtb	4.5	4.3	3.6	6.1	1.4	5.2	13.0	4.3	0.0	4.0
IL-10										
Unstim.	454.8	230.3	147.6	0.0	121.1	156.5	375.0	375.0	227.4	147.6
PPD	1 092.6	419.3	673.3	174.2	242.1	953.8	1 122.1	351.4	407.5	472.5
Diff PPD	637.8	189.0	525.7	174.2	121.0	797.3	747.1	0.0	180.1	324.9
Mtb	581.7	233.3	274.6	41.3	162.4	348.4	425.2	197.8	206.7	230.3
Diff. Mtb	126.9	3.0	127.0	41.3	41.3	191.9	50.2	0.0	0.0	82.7
TGF-β										
Unstim.	1 226.3	1 141.8	987.1	1 241.8	1 230.1	411.2	805.7	766.9	672.6	1 000.4
Diff PPD	3 204.7	2 098.2	1 138.3	0.0	869.9	88.8	181.3	733.1	687.4	1 099.6
PPD	4 431.0	3 240.0	2 125.4	1 050.0	2 100.0	500.0	987.0	1 500.0	1 360.0	2 100.0
Mtb	2 991.4	1 471.5	1 089.7	2 177.8	924.2	261.8	1 006.4	924.7	1 020.0	1 395.4
Diff Mtb	1 765.1	329.7	102.6	936.0	0.0	0.0	200.7	1 578.0	347.0	395.0

Unstim.: Unstimulated

Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*

Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 2.A Cytokine production in the **Cured** group after a **2 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
IFN-γ										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	713.4	0.0
PPD	0.0	0.0	627.1	844.3	159.3	0.0	367.3	0.0	6 321.9	182.2
Mtb	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1 750.1	0.0
TNF-α										
Unstim.	532.4	346.5	335.8	300.1	214.3	383.2	164.2	481.9	641.3	575.2
PPD	547.7	569.4	648.9	607.9	415.3	576.6	523.6	513.9	943.5	1 025.7
Diff PPD	15.3	0.0	313.1	0.0	201.0	193.4	359.4	32.0	302.2	450.5
Mtb	542.9	350.4	299.9	314.3	391.8	376.8	271.2	412.9	617.6	583.8
Diff Mtb	10.5	3.9	0.0	14.2	177.5	0.0	107.0	0.0	0.0	8.6
IL-12										
Unstim.	119.1	60.5	175.5	72.3	161.6	123.9	25.2	46.8	692.4	349.3
PPD	165.8	96.2	297.1	186.8	435.5	171.2	44.3	49.4	812.2	603.6
Mtb	139.5	67.0	161.6	104.2	150.5	106.7	0.0	0.0	598.6	361.3
IL-2										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PPD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mtb	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-10										
Unstim.	0.0	131.2	98.1	142.0	210.0	250.0	241.0	111.0	222.0	159.0
PPD	0.0	412.3	400.6	348.3	350.5	485.9	524.7	361.5	486.7	365.5
Diff PPD	0.0	281.1	302.6	206.3	140.5	235.9	283.7	250.5	264.7	206.5
Mtb	210.3	253.4	241.5	0.0	0.0	163.2	190.9	186.6	222.2	218.3
Diff Mtb	210.3	122.2	143.4	0.0	0.0	0.0	0.0	75.6	0.0	59.3
TGF-β										
Unstim.	1 420.6	1 321.8	669.2	722.4	400.1	211.6	169.8	499.5	368.7	643.6
PPD	4 120.5	3 600.3	1 945.5	1 854.2	1 700.0	531.3	360.0	1 698.1	1 160.3	975.3
Diff PPD	2 699.9	2 278.5	1 276.3	1 131.8	1 299.9	319.7	190.2	1 198.6	791.6	331.7
Mtb	1 122.2	555.1	864.3	952.2	882.3	263.6	299.6	615.8	841.7	1 126.3
Diff Mtb	0.0	0.0	195.1	229.8	482.2	52.0	129.8	116.3	473.0	482.7

Unstim.: Unstimulated
Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*
Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 2.B Cytokine production in the **Cured** group after a **7 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
IFN-γ										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PPD	2 823.3	5 729.8	2 366.8	3 222.6	5 501.1	4 941.1	8 504.0	0.0	8 155.6	2 236.9
Mtb	2 605.7	1 800.1	1 877.8	3 470.8	2 016.6	0.0	5 650.5	0.0	733.7	2 726.8
TNF-α										
Unstim.	222.7	134.0	130.9	160.2	170.8	250.0	265.8	229.7	250.2	324.1
PPD	460.8	418.0	297.4	455.8	360.1	433.1	465.9	337.5	249.7	582.0
Diff. PPD	238.1	0.0	166.5	295.6	189.3	0.0	200.1	107.8	0.0	0.0
Mtb	370.2	227.2	222.2	299.8	212.1	244.7	294.8	152.1	187.1	377.7
Diff Mtb	147.5	93.2	91.3	139.6	41.3	0.0	29.0	0.0	0.0	53.6
IL-12										
Unstim.	134.0	50.1	110.9	72.3	175.5	109.3	6.3	62.3	482.3	206.9
PPD	153.2	143.6	260.1	138.1	331.9	121.2	80.4	60.9	560.4	277.6
Mtb	158.8	61.7	154.6	77.6	157.4	98.7	18.8	32.8	476.7	236.3
IL-2										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PPD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mtb	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-10										
Unstim.	352.0	421.3	544.1	397.4	548.7	613.4	483.0	210.0	357.2	376.8
PPD	672.3	1 364.3	1 577.3	1 075.1	1 309.7	1 321.1	1 555.4	521.6	1 156.5	924.2
Diff PPD	320.3	942.9	1 033.2	676.6	761.0	707.7	1 072.4	311.6	766.3	547.4
Mtb	424.4	566.1	498.2	211.0	260.5	309.1	478.3	456.3	534.6	510.3
Diff Mtb	72.4	144.8	0.0	0.0	0.0	0.0	0.0	0.0	177.4	133.5
TGF-β										
Unstim.	2 581.6	1 007.6	992.4	1 044.6	892.7	506.1	444.0	933.0	930.4	1 226.6
PPD	5 427.0	3 989.4	2 479.3	2 351.2	2 015.3	1 270.3	312.5	3 026.2	2 378.4	1 238.4
Diff PPD	2 845.4	2 981.8	933.3	1 306.6	1 122.6	764.2	0.0	2 093.2	1 448.0	11.8
Mtb	3 005.9	1 222.8	1 925.7	2 971.3	2 157.5	624.0	628.5	1 000.5	1 588.4	2.548.6
Diff Mtb	424.3	1 489.9	933.3	1 926.7	1 264.8	117.9	184.5	67.5	658.0	1 320.0

Unstim.: Unstimulated
Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*
Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 3.A Cytokine production in the **Latent** group after a **2 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
IFN-γ										
Unstim.	6.0	0.0	0.0	43.4	78.0	0.0	0.0	0.0	19.5	0.0
PPD	178.7	762.2	434.8	984.5	543.3	12.4	173.6	976.0	773.2	773.2
Mtb	14.9	0.0	0.0	102.7	143.1	5.0	49.9	22.4	52.4	29.9
TNF-α										
Unstim.	474.9	742.6	333.4	778.4	1 330.4	413.7	618.2	572.6	757.8	572.6
PPD	693.6	1 166.4	529.5	1 171.6	1 676.2	605.1	902.7	1 091.1	1 119.6	1 003.1
Diff PPD	218.7	423.8	196.1	393.2	345.8	191.4	284.5	518.5	361.8	430.0
Mtb	519.5	744.4	342.1	726.6	1 431.4	446.8	701.2	569.8	831.0	653.1
Diff Mtb	44.6	1.8	8.7	0.0	101.0	33.1	83.0	0.0	73.0	80.5
IL-12										
Unstim.	490.2	384.3	382.6	509.7	317.9	203.1	64.4	230.9	306.2	563.7
PPD	681.2	403.4	256.3	316.3	303.6	401.7	153.2	382.9	377.4	736.8
Mtb	251.8	428.4	720.1	237.8	340.2	356.8	226.6	269.0	660.9	408.2
IL-2										
Unstim.	8.5	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2
PPD	2.7	55.1	25.5	17.4	1.9	2.1	2.9	28.3	7.1	73.6
Mtb	18.4	4.2	0.0	0.0	0.0	0.0	1.2	1.5	0.0	3.5
IL-10										
Unstim.	37.9	25.2	167.9	235.1	37.2	36.4	104.4	72.6	48.4	92.2
PPD	610.3	12.6	437.2	159.9	43.6	36.4	169.2	134.9	65.3	138.3
Diff PPD	572.4	0.0	269.3	0.0	6.4	0.0	64.8	62.3	16.9	46.1
Mtb	69.7	0.0	205.5	110.1	45.2	38.0	118.4	73.4	41.2	84.8
Diff Mtb	31.8	0.0	37.6	0.0	8.0	1.6	14.0	0.8	0.0	0.0
TGF-β										
Unstim.	150.6	268.1	587.6	666.7	345.7	254.6	200.9	401.0	671.8	601.6
PPD	225.3	565.1	955.2	769.8	445.8	215.3	255.9	1 005.3	1 423.5	1 325.7
Diff PPD	74.7	297.0	103.1	100.1	0.0	55.0	604.3	751.1	724.1	200.1
Mtb	219.9	253.3	874.7	800.0	754.1	211.6	209.6	866.6	1 200.9	1 295.4
Diff Mtb	69.3	0.0	133.3	408.4	0.0	8.7	45.6	529.1	693.8	210.2

Unstim.: Unstimulated

Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*

Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 3.B Cytokine production in the **Latent** group after a **7 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis* (*Mtb*).

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
IFN-γ										
Unstim.	2.2	0.0	0.0	45.2	148.7	0.0	0.0	0.7	17.7	0.0
PPD	797.3	2 418.7	2 211.9	3 206.4	3 206.4	962.9	1 824.6	4 363.8	3 921.8	5 015.8
Mtb	323.3	789.6	1 623.7	1 768.2	2 183.1	255.9	428.5	4 359.2	2 377.1	5 209.1
TNF-α										
Unstim.	378.5	431.1	402.6	655.2	1 144.5	332.7	572.6	632.8	675.3	632.8
PPD	220.0	367.6	328.3	543.7	867.6	256.7	437.7	590.4	541.5	628.3
Diff PPD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mtb	245.1	358.9	265.4	911.9	185.7	282.7	316.1	452.2	323.4	628.1
Diff Mtb	0.0	0.0	0.0	0.0	256.7	0.0	0.0	0.0	0.0	0.0
IL-12										
Unstim.	402.2	154.1	161.3	144.5	176.1	258.9	83.0	112.4	172.4	251.8
PPD	553.4	274.8	243.4	176.1	222.9	188.7	131.7	161.3	289.6	329.8
Mtb	438.4	187.4	152.9	152.9	132.8	112.4	62.3	116.9	237.8	225.5
IL-2										
Unstim.	21.4	0.0	0.0	0.0	0.0	0.0	1.9	3.8	0.0	0.0
PPD	43.3	23.9	10.2	0.0	8.3	2.9	11.5	0.0	0.0	1.9
Mtb	28.9	17.8	12.7	6.4	9.6	3.8	19.4	16.9	22.6	10.8
IL-10										
Unstim.	65.6	38.2	196.7	232.2	85.7	102.2	309.5	172.5	118.8	1 512.9
PPD	111.4	12.7	503.7	296.3	144.7	87.5	550.6	266.1	135.4	284.9
Diff PPD	45.8	0.0	307.0	64.1	59.0	0.0	241.1	93.6	16.6	0.0
Mtb	65.5	7.3	198.6	245.4	122.5	435.8	393.4	204.1	128.1	266.1
Diff Mtb	0.0	0.0	1.9	13.2	36.8	333.6	83.9	31.6	9.3	0.0
TGF-β										
Unstim.	300.8	384.1	567.2	989.6	899.4	411.8	222.7	882.5	901.5	1 005.3
PPD	510.0	1 511.3	1 245.6	2 100.7	2 486.5	634.5	878.2	1 545.8	2 352.2	2 647.7
Diff PPD	209.2	1 127.2	678.4	1 114.0	1 587.1	222.7	655.5	663.3	1 450.7	1 642.4
Mtb	222.5	468.5	1 065.6	1 000.5	1 367.6	546.1	350.3	1 319.8	1 766.8	2 182.1
Diff Mtb	0.0	84.4	498.4	10.9	468.2	134.3	127.6	437.3	865.3	1 176.8

Unstim.: Unstimulated

Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*

Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

Table 4 Cytokine production in the **Active TB** group after a **7 day** incubation period, unstimulated and stimulated with PPD and live *M. tuberculosis*.

	TB1	**TB2**	**TB3**	**TB4**	**TB5**	**TB6**	**TB7**	**TB8**	**TB9**	**TB10**
IFN-γ										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mtb	253.2	1 504.7	975.6	825.2	225.5	0.0	3 572.8	1 660.6	433.6	3 930.0
TNF-α										
Unstim.	330.0	0.0	51.5	0.0	467.4	482.3	38.5	404.9	0.0	113.9
Mtb	299.3	844.4	455.6	0.0	426.7	657.6	214.9	402.9	0.0	75.0
IL-12										
Unstim.	74.6	15.1	62.1	34.6	86.1	57.9	0.0	37.4	19.1	16.1
Mtb	39.4	24.1	72.5	60.1	35.4	85.1	0.0	18.1	59.2	17.1
IL-2										
Unstim.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mtb	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-10										
Unstim.	0.0	0.0	0.0	0.0	84.2	208.6	0.0	0.0	0.0	0.0
Mtb	0.0	86.4	95.2	0.0	63.6	249.8	0.0	0.0	0.0	233.0
TGF-β										
Unstim.	400.0	692.5	529.9	600.0	283.7	192.5	1 086.4	902.2	852.8	1 159.5
Mtb	587.7	1 790.1	1 059.5	1 113.6	526.9	233.1	2 985.4	1 950.4	1 346.0	3 180.3
Diff Mtb	187.0	1 098.0	530.0	513.0	243.0	40.5	1 899.0	1 048.2	493.2	2 010.8

Unstim.: Unstimulated

Mtb. and PPD: Stimulated with PPD or live *M. tuberculosis*

Diff. Mtb and PPD: Stimulated concentrations subtracted from the unstimulated value

CHAPTER EIGHT

CONCLUSION

This study was conducted in a community with a high tuberculosis notification rate, a situation that is common in many developing countries.

It was argued in this thesis that the high TB notification rate in the study community could be due to dominant type 2 immune responses as it is known that type 1 and type 2 responses could down-regulate each other's development and function. A preponderance of dominant type 2 responses could then explain the high TB notification rate due to the suppressed type 1 immune response. Dominant type 2 responses in the study community were confirmed by the high serum IgE concentrations measured on healthy adults and children. Serum IgE concentrations were subsequently characterised per census block or enumerator sub-district (ESD) and correlated with the TB notification rate per ESD. A significant association was found between these two parameters. However, as this association does not necessarily imply a causal relationship, a longitudinal study is required to determine whether individuals with dominant type 2 responses do indeed develop tuberculosis. Significant associations were also found between the TB notification rate and socio-economic index, female literacy and crowding, suggesting that these factors could contribute to susceptibility to *M. tuberculosis*.

We were able to show a pronounced and consistent decline in IgE concentrations in patients after successful treatment for tuberculosis. This finding supported the hypothesis

that successful treatment of tuberculosis is associated with down-regulation of type 2 responses. Previous publications that documented the reversal of skin-test anergy (Maher *et al.*, 1992) and an increase in T-cell responses (Wilkinson *et al.*, 1998) support the concept of enhanced type 1 responses after chemotherapy of tuberculosis. In this study, controls and patients were form 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 tuberculosis. Bentwich and co-workers (1995) hypothesised that intestinal parasites exacerbate the course of HIV infection, which like tuberculosis is controlled by cell-mediated immunity. This study and others (Shirakawa *et al.*, 1997; Erb *et al.,* 1998; Rook and Stanford, 1998; Wang and Rook, 1998) suggest that the immune response to *M. tuberculosis* and to nonpathogenic mycobacteria suppresses a type 2 response, which plays a key role in the induction and maintenance of high IgE concentrations in parasite infestation and in disorders such as atopy.

As a possible reason for the high serum IgE concentrations, the helminth burden was measured on all primary school children in the study community. We have shown that more than half of the children tested, had worms (*i.e. Ascaris* and/or *Trichuris*). Prevalences of *Ascaris* and *Trichuris* were more than 70 % in schools situated in the poorest areas in the communities. These prevalences confirmed recent surveys showing that soil-transmitted worm infestation is rampant among South African children living in poor conditions (Fincham *et al.*, 1996; Arendse, 2001). All children in the nine primary schools, irrespective of their helminth burden or participation in the study, subsequently received ant-helminthic treatment. Furthermore, both helminths studied have also been associated with stunted growth (Cooper and Bundy, 1988, Adams *et al.,* 1994; Saldiva *et al.,* 1999) and impaired cognitive functions in children (Nokes *et al.,* 1992; Oberhelm *et al.,* 1998), which would ultimately influence the development of the children.

Helminth infection may also alter the immune response to non-parasite antigens through bystander effects. In the case of geohelminths infections, such as *A. lumbricoides*, with a pulmonary phase of larval migration, could affect responses in the pulmonary mucosa

(Cooper *et al.*, 2000). The pulmonary migrations of *A. lumbricoides* larvae would create a highly polarised type 2 immune environment in the lung mucosa, which will enhance type 2 cytokine production to non-parasite antigens such as *M. tuberculosis*. Observation by Cooper and co-workers (2000) lend support to this theory: significantly greater levels of IL-5 were secreted by the infection group in response to L3/L4 *Ascaris* antigens (*i.e.* lung stage parasites); and infected subjects secreted significantly greater amounts of IL-5 after stimulation with PPD. Thus, a type 2 immune response among individuals with significant exposure to *A. lumbricoides* or other hleminths might have an impact on susceptibility to *M. tuberculosis* and even HIV (Bentwich *et al.*, 1999) and warrants further investigation.

Although the helminth burden was not determined in adults, it is argued that because of the poor living conditions in the study community, the high serum IgE concentrations in adults could be due to helminth infections or it could be that these adults are in a state of intermittent immune activation in response to repeated exposure to worm antigens.

The Th1/Th2 paradigm was further examined by investigating the association between BCG scarring, Mantoux skin test responsiveness and helminth burden in children. The current study has shown that although most children in the study community were immunised with BCG in the neonatal period, only two thirds of the children had evidence of a scar. Furthermore, most of the children (60 %) were not Mantoux skin test responsive, with 33 % of the children having a Mantoux response smaller than 10 mm and only 4 % of the children presented with a positive Mantoux response (*i.e.* > 10 mm in induration size). The time course of Mantoux conversion with age indicated that any tuberculin sensitivity, induced by BCG, waned within the first few years of life and that PPD responsiveness thereafter was induced by environmental exposure to *M. tuberculosis*. Contrary to what one would anticipate from the Th1/Th2 paradigm, the prevalence of helminth infection in children with a BCG scar was marginally lower than in those without a scar. This supported previous findings by Elliot and co-workers (1999) as well as Barreto and colleagues (2000). Furthermore, children who were infected with helminths were 1.6 times more likely to be PPD responsive than those who were not

infected. However, taken together the data were consistent with the interpretation that conversion to PPD sensitivity predisposed to helminth infection, rather than the converse. The correlations found were relatively weak, but it indicated that factors not identified in this study are largely responsible for the variations seen. These factors are likely to be predominantly genetic although there was clear evidence that children with stunted growth were immunologically distinct from the main cohort of children studied. Also, as we did not have a measure of poverty, it was not considered as a confounder. Overall the results suggest that the effect of helminth infection on the development of clinical tuberculosis is such that those with large worm burdens and who make good PPD responses are likely to be resistant whereas those who deal very effectively with these parasites and who make weaker PPD responses are more likely to be susceptible. The data also indicate that the BCG vaccine used in this study does not give rise to a latent infection whereas the pathogenic *M. tuberculosis* does so and repeatedly stimulates an immune response to it.

In a separate study, it was demonstrated how the host response to *M. tuberculosis* differs in patients at risk for developing disease after successful completion of treatment, compared to those who have protective immunity. Individuals participating in the study were also interviewed by a social anthropologist in order to understand their social and economic background The aim therefore was to find a marker or trend that would indicate susceptibility to infection with *M. tuberculosis* and progression to disease. For this reason, PPD and *M. tuberculosis*-induced cytokine responses were determined in four study groups namely: 1) recurrence group (individuals who have had more than one episode of tuberculosis after successful completion of treatment), 2) cured group (individuals who have had only one episode of tuberculosis after cure), 3) latent group (individuals with latent *M. tuberculosis* infection) and 4) active TB group (patients diagnosed with tuberculosis (smear positive culture). From the experimental results, we could not distinguish a single immunological marker of susceptibility, but rather immunological patterns of susceptibility were observed. Individuals who have had tuberculosis once before and who had been cured, presented with an immuno-suppressive profile, which included high concentrations of IL-10, TGF-β as well as high IgE levels.

This type of profile suggests that although these individuals have had tuberculosis once before, they have not acquired protective immunity and would be susceptible to re-infection and progression to disease. Furthermore, the interviews conducted showed that most of the people included in this study were poor, unemployed, undernourished and lived in overcrowded conditions. These factors have consistently been associated with tuberculosis (Spence *et al.*, 1993; Davies, 1994; Head, 2001), further emphasising the need for social and economic programmes in this community to relieve the effects of unemployment and poverty in order to combat not only tuberculosis but also other infectious diseases in this community. Therefore it seems inevitable that those individuals with the immuno-suppressed profile living in poverty would present with a second episode of tuberculosis in the near future.

In this thesis it was shown that the risk of infection with *M. tuberculosis* in the study community, is related mainly to the background host immune response, educational attainment and socio-economic conditions. Furthermore, literature suggests and the current study supports the view that TB notification rates are highest in the areas of greatest poverty.

We conclude that in the study community, which has a typical third world setting, poverty-related factors such as crowding, illiteracy, poor socio-economic conditions and helminths, could contribute to a dominant type 2 immune response which in turn, would down-regulate the protective type 1 response, resulting in an enhanced susceptibility to *M. tuberculosis* and progression to disease with low levels of education and poor socio-economic conditions (Figure 8.1). The thesis also supports the notion that unless something is done to improve the social/living conditions of poor people in areas with high notification rates of tuberculosis, attempts to stem tuberculosis and through biomedical means will be limited.

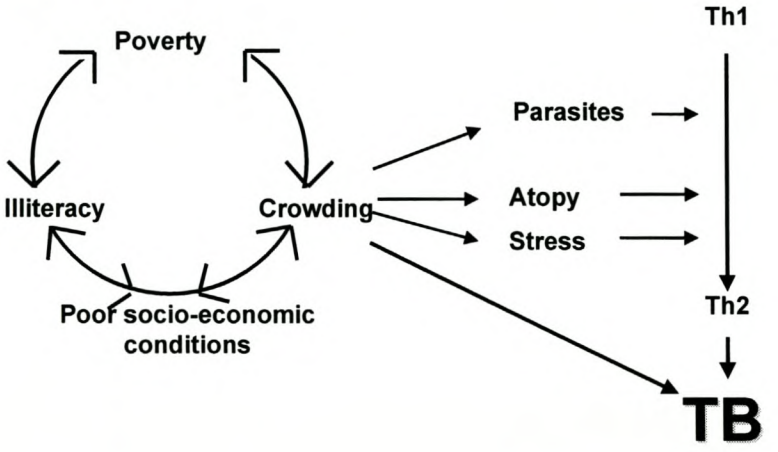

Figure 8.1 Poverty cycle

RECOMMENDATIONS

1) Although the majority of adults included in the study had IgE levels within the normal range, it would be important to characterise the immune profiles of those individuals with high serum IgE levels, by not only measuring cytokines, but also measuring parasite burdens as well as other markers of Th2 activation. These individuals would be most vulnerable to infection with *M. tuberculosis* and progression to disease.

2) Chemotherapy is currently the major tool used for the strategic control of ascariasis in particular as a short-term goal. Improvements in hygiene and sanitation are thought to aid long-term control. Environmental and economic conditions associated with worm infection in this community could mean that the population would remain at risk to helminth infection, even in the event of anti-helminthic drugs, decreasing infection rates only at a certain time. Public health programmes designed to decrease the prevalence of intestinal helminths should therefore focus on two areas, namely to decrease transmission through hygiene education and reducing human infections through regular anti-helminthic treatment. The potential advantage of drug treatment is a rapid reduction in infection. However, this would be costly and temporary as the children are rapidly re-infected (WHO, 1996). The need for medical intervention should therefore be minimised by means of education, socio-economic improvements, prevention of environmental contamination by sanitary disposal of sewage, provision of safe water and application of personal and community hygiene (Evans and Stephenson, 1995). However, as this will take many years, immediate practical objectives should be the determination of the prevalence, intensity and incidence of infections. In doing so risk factors for infection with helminths can be identified, targeted and addressed efficiently.

3) The results have shown that individuals who have had tuberculosis once before, do not have protective immunity against re-infection and progression to disease. It would be important to support these findings by the addition of more individuals to each

group and the inclusion of intracellular cytokine staining by means of FACS analysis, to the currently employed techniques.

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