A DESCRIPTIVE STUDY OF IgG SUBCLASSES AND ALLOTYPES IN CHILDREN WITH PULMONARY TUBERCULOSIS IN THE WESTERN CAPE

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CONTENTS

ABSTRACT:

IgG SUBCLASSES AND IMMUNOGLOBULIN ALLOTYPES IN CHILDREN WITH PULMONARY TUBERCULOSIS IN THE WESTERN CAPE.

STUDY OBJECTIVES:
An analysis of IgG subclasses and allotypes in children with pulmonary Tuberculosis (PTB) in the Western Cape.

DESIGN:
Consecutive children under 15 years of age with microbiological proven PTB over an 8 month period- November 1993 to July 1994.

SETTING:
Teaching Hospitals in Cape Town.

PATIENTS:
Thirty-five cases were selected from 99 consecutive cases that were *Mycobacterium tuberculosis* positive, 15 of which were of Mixed and 20 were of Black Ancestry.

CONTROL GROUP:
Sera were selected from 224 Black Ancestry (59 children and 165 adults) and 211 Mixed Ancestry (67 children and 144 adults) that had no evidence for active tuberculosis or a history of previous TB diagnosis.

MEASUREMENTS AND RESULTS:
IgG subclasses, total IgG,and five allotypes: G1m(a), G1m(f), G2m(n), G3m(b1), G3m(g1) were determined by ELISA techniques. In the Mixed ancestry group the G1m(f) ($p= 0.01$), G2m(n) ($p= 0.04$) and G3m(g1) ($p=0.001$) allotypes were less frequently found in children with proven PTB. In the Black Ancestry subjects the G3m(g1) allotype was significantly less common than in the control group ($p < 0.001$).

CONCLUSIONS:
Because allotypes are inherited in a Mendelian fashion, the observed association of childhood PTB and certain allotypes strengthens the hypothesis that a genetic susceptibility exists to acquiring TB.

DECLARATION

I, **Stephanus Theron Potgieter** hereby declare that the work on which this thesis is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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11 June 1997

DATE

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CHAPTER 1

IgG SUBCLASSES AND ALLOTYPES

1.1 GOAL OF THIS STUDY

To determine if a relationship exists between IgG allotype variability, the IgG subclass levels and infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) in Black and Mixed Ancestry children of the Western Cape. If such a relationship can be established it may be possible to target specific groups of individuals for preventative, screening or intervention strategies. Such an association would also support the existence of a genetic predisposition to acquiring *M. tuberculosis*. High IgG subclass levels and certain Allotypes have been associated with protection against infection from encapsulated organisms. The levels of IgG Subclasses in *M. tuberculosis* disease were therefor investigated.

Specific objective

The presence of five allotypes: G1m(a), G1m(f), G2m(n), G3m(g1) and G3m(b1) were determined and correlated with IgG subclasses levels in children with pulmonary tuberculosis. Allotypic frequency and IgG subclass levels were compared with a similar race and age matched control population.

Hypothesis

There is a significant association between the IgG allotypic expression and IgG subclass levels in Mixed Ancestry and Black children with pulmonary tuberculosis in the Western Cape.

1.2 IgG SUBCLASSES

1.2.1 Introduction

The human Immunoglobulin system has five classes: IgG, M D, E and A. These classes differ in their antigenic determinants and physiological and biological activities.

The basic molecular structure of the immunoglobulin molecule consists of 2 heavy and 2 light chains each with variable and constant regions. The IgG structure consists of one pair of light chains (Lamda or Kappa) linked to one of 4 different heavy gamma chains. Each heavy and light chain has variable and constant regions of approximately 110 amino acid residues. The constant regions of the different gamma chains exhibit significant sequence homology.

Within IgG and IgA subclasses of immunoglobulin are recognized by the antigenic uniqueness of their heavy chains. These proteins are encoded separately within the heavy chain genome located on chromosome 14 at band 32 (Croce et al 1979). The IgG subclasses also differ in their biological activities (see Table 1.1).

TABLE 1.1: IgG SUBCLASS BIOLOGICAL ACTIVITY

	IgG1	IgG2	IgG3	IgG4
Proportion of total IgG	60-65%	20-25%	5-10%	3-4%
Placental transfer	++	±	++	++
Staphylococcus protein A binding	+	+	-	+
Fc Receptor binding:				
Monocytes	+++	-	+++	++
Neutrophils	+	-	+	-
Lymphocytes	-	-	-	-
t ½ (days)	21-33	20-33	7-8	21-23
Complement fixation	+++	++	++++	±

References:
R Jefferis and D Kumararatne (1990)
J Gibson (1987)

1.2.2 Factors influencing subclass levels

- **Age**

The four subclasses differ in proportion and absolute levels according to age. IgG1 and IgG3 attain adult levels at an earlier age than IgG2 and IgG4. Normal values should therefore always be interpreted according to age adjusted ranges (Lee et al 1986).

- **Antigenic stimulation**

Responses to protein antigens are produced predominantly by IgG1 and IgG3. Polysaccharide antigens evoke a heterogeneous response which is predominantly IgG2 (Jefferis et al 1990). During prolonged antigenic stimulation, such as with pulmonary tuberculosis, increased levels of IgG (total) and IgG4 occurs. Subtle differences and deficiencies of subclasses are difficult to interpret and may be masked if only total IgG is measured.

- **Race and Sex**

Differences between White and Black American children have been documented (Shackelford et al 1985). There are also differences among the Mixed Ancestry and Black populations in the Western Cape (Goddard et al 1992).

- **Genetic factors**

Gm allotypes influence IgG subclass levels (Oxelius 1993a) and since IgG allotypes differ among population groups (see 1.3), where possible normal ranges, specific for each grouping, should be used.

Deficiencies in IgG subclasses (mainly IgG2) have been associated with disease, particularly due to encapsulated organisms (Umetsu et al 1985). Pulmonary TB has not been associated with any subclass deficiency.

1.3 IgG ALLOTYPES

1.3.1 Introduction

Allotypes are specific genetic variants of light and heavy chains of the Immunoglobulins. They are due to alleles on the Ig constant region genes that differ mostly in single amino acid sequence changes. Allotypes are inherited in a Mendelian fashion. The functional value of these variants is not known but a number of associations that relate to immunoglobulin production and disease have been found. There are multiple allotype alleles of IgG1 and IgG3 subclasses, one recognised allele for IgG2 subclass and two for IgA. On the kappa light chain 3 alleles are recognized (Table 1.2).

TABLE 1.2: HUMAN IMMUNOGLOBULIN ALLOTYPES

	Domains	Allotypes Alphabetical	Numerical
Heavy Chains			
IgG1	CH3	G1m(a)	G1m(1)
	CH3	G1m(x)	G1m(2)
	CH1	G1m(f)	G1m(3)
	CH1	G1m(z)	G1m(17)
IgG2	CH2	G2m(n)	G2m(23)
IgG3	CH3	G3m(b0)	G3m(11)
	CH2	G3m(b1)	G3m(5)
	CH3	G3m(b3)	G3m(13)
	CH2	G3m(b4)	G3m(14)
	CH3	G3m(b5)	G3m(10)
	CH2	G3m(g1)	G3m(21)
	CH3	G3m(g5)	G3m(28)
	CH2	G3m(u)	G3m(26)
	CH3	G3m(v)	G3m(27)
	CH3	G3m(s)	G3m(15)
	CH3	G3m(t)	G3m(16)
	CH3	G3m(c3)	G3m(6)
	CH3	G3m(c5)	G3m(24)
IgA2	CH2		Am(1)
	CH3		Am(2)
IgE			Em(1)
Light chains			
Kappa			Km(1)
			Km(2)
			Km(3)

Reference: WHO Notation for genetic factors of human immunoglobulins. Bull Wld Hlth Org 1965; 33:721.
Lefranc and Lefranc: 1990

The nonmenculature has evolved in an alphabetical or a numerical system. In this thesis the alphabetical system is used.

Combinations of allotypes, called haplotypes, are characteristic for population groups (Lefranc and Lefranc 1990, Steinberg 1973, Steinberg 1969 Johnson et al 1977).

Haplotypes found commonly among:

- Caucasians:
  - Gm b, f, n, u, v
  - Gm b, fu, v
  - Gm g, a, z, u, v
  - Gm g, a, x, z, u, v
- Negroid population haplotypes
  - Gm b, a, z, v
  - Gm b, c, a, z, u, v
- Khoisan haplotypes
  - Gm a, z, g
  - Gm a, b, z
- Mongolian haplotypes
  - Gm b, v, a, z
  - Gm b, u, v, a, f, n

1.3.2 Specific allotypes

Gm allotypes

G1m(a) consist of 2 point mutations on the CH3 homologous region of IgG1 heavy chain. G1m(f) and G1m(z) are genetically linked and are due to different point mutations at the same position in the CH1 homologous, region and thus would be expressed reciprocally.G2m(n) is the only allotype recognised for IgG2 and is found on the CH2 domain. The G3m(b^1) allotype is carried on the CH2 domain as is G3m(g^1). All the allotypes have iso-allotypes found on the other IgG subclasses (Lefranc and Lefranc 1990). This has relevance when quantitation is done using monoclonal specific antibodies in the ELISA system. The presence (and detection) of iso-allotypes can give spurious results if the relevant IgG subclass is not selected by capture ELISA techniques. G3m(b^1) allotype is carried in close proximity to a number of other allotypes. Thus G3m(b^1), (b^o), (b^5) and (b^3) markers are collectively referred to as G3m(b).

Km allotypes

The three alleles are all on the constant region of the kappa light chain: Km(1), Km(1,2) and Km(3). The differences amount to single amino acid substitutions. Km(1) is rare in Caucasians (8%) and present in about 50% of Negroids (Lefranc and Lefranc 1990).

Factors influencing allotypic expression

Conformational changes in vitro which could be responsible for epitope masking may influence allotype detection. Heterozygosity for the specific allotypes is known.(

Oxelius et al 1993b) Factors such as age, sex and infection have not been found to alter expression of allotypes (Goddard 1994).

1.3.3 Allotypes IgG Subclasses and disease associations

i. IgG subclass levels
Certain allotypes in some population groups have been associated with decreased levels of a particular subclass (IgG2 and G2m(negative) (Oxelius 1993a) or increased levels, IgG3 and G3m(b) (Morrel et al 1972, Jefferis et al 1990). The influence of G1m(a) and G1m(f) on subclass levels has not been well defined. Goddard (1994) found that G1m(f) marker had a negative effect on IgG (total) and IgG1 levels in the Mixed Ancestry children studied in Cape Town. This is the opposite of the findings in the Finish population (Sarvas et al 1991). The G2m(n) marker has also been associated with altered IgG subclass levels (Granoff et al 1992, Jefferis et al 1990).

ii. Antibody production to vaccines
Sarvas et al (1989) found higher IgG2 subclass antibodies against pneumococcal polysaccharides in Finish homozygotic individuals whom expressed G2m(n). In black (Negroid) children the IgG antibody response to *H. influenzae* polysaccharide vaccine was found to higher in those who expressed Km(1) and G2m(n) (Granoff 1986).

iii. Immune mediated diseases
A number of autoimmune diseases including multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosis have been associated with various allotypes (Dogoryan JM, Guitard E, Seregas MT 1992). Recently an association with Kawasaki disease has also been reported (Shulman 1993).

iv. Bacterial infection
Granoff and others (1984) reported a decreased susceptibility among black USA children who expressed Km(1) allotype to infection with *H. influenzae* meningitis.
In Mixed Ancestry children with invasive *H. influenzae* infection G2m(n) and Km(3) were less frequent, whereas in Black children only Km(3) was decreased (Goddard 1994).

It therefor appears that allotypes do influence a number of disease related factors but not in a consistent manner.

1.3.4 Literature review of allotypes and *M. tuberculosis*

Publications on the association between allotypes and *M. tuberculosis* are limited to three articles; one in an adult Indonesian population, the other in a Russian group of adult patients and in a Tuvinian childhood population.

Gibson and others (1987) reported on a study group of 121 smear-positive pulmonary tuberculosis patients and 33 healthy controls from the same town in East Java. Their important findings were: The Km(1) allotype occurred significantly less in tuberculosis patients than among controls and the phenotypes which lacked G1m(z), G3m(g1) and Km(1) were more common amongst the TB group. Anti-mycobacterial antibody levels were also influenced by allotypic expression in that IgG2 specific antibody was decreased in those without G1m(z) or G3m(g1). IgG4 specific antibody levels were

elevated if the Km(1) allotype was present. G2m(n) was not tested. In this study a large panel covering G1m, G3m and kappa allotypes were tested. The populations genetic diversity is well illustrated by the excess of 10 phenotypes found. A very small control group (33) could have influenced their results. Of all those having tuberculosis in the study group only a third had a phenotype that differed from the control group. The strength of the allotypic association was therefore only modest.

Chukanova and others (1994) studied 214 adults with pulmonary tuberculosis and 203 controls. They looked at HLA typing, erythrocyte enzymes and IgG allotypes. The most significant finding was that HLA DR7, Hp 1-7 and the absence of G1m(a) and G1m(x) in the presence of G1m(f) was associated with a favourable outcome. HLA DR2, Hp 2-2 and the absence of G1m(x) but the presence of G1m(a) and (f) led to chronicity, judged by chest X-rays at the end of a course of standard therapy. The authors used a limited phenotypic panel and could not demonstrate a difference between controls and patients but only a difference amongst patient outcome at termination of therapy.

Matrakshin and others (1993) found among a number of polymorphic systems, including HLA-typing and red blood cell enzymes, that there was a positive association in the 73 children with G1m(x) and tuberculosis in comparison with the 273 child controls. This was so for the group that expressed G1m(x,a,f).

Conclusion:
Some evidence, albeit inconclusive, exists that IgG allotypic expression influences and/or modifies susceptibility to *M. tuberculosis* infection.

1.3.5 Selection of phenotypic panel in this study

To determine the influence of allotypic expression in *M. tuberculosis* the following factors were considered in selecting allotypes for the study:

a. Allotypes identified from previous work with *M. tuberculosis*: Km(1) and G3m(g1) (Gibson 1987) and G1m(a); G1m(f) (Chukanova 1994).

b. Allotypes associated with IgG subclass expression: G3m(g^1) and G3m(b^1) (Jefferis and Kumararatne 1990), G2m(n) (Oxelius 1993a).

c. Allotypes that are prevalent in the Western Cape population under study:

	Blacks	Mixed Ancestry
Km1	60%	?
G1m(a)	99%	58%
G1m(f)	<3%	30%
G2m(n)	3%	44%
G3m(b1)	64%	53%

Reference: Jenkins 1970, Goddard 1994.

1.4 *MYCOBACTERIUM TUBERCULOSIS*

1.4.1 Introduction

The World Health Organisation (WHO) estimates that one third of the world's population is infected with *M. tuberculosis*. This figure might be as high as 40% in

sub-Saharan Africa. In South Africa the overall incidence rate for 1992 was 366:100 000 (Eggers et al 1994). In the Western Cape there is an alarming incidence rate of 718:100 000 among the Mixed Ancestry population group. It is calculated that this group is experiencing a bimodic epidemic curve: one part of the community having a decreasing and the other (younger) section an increasing incidence (Kustner 1995).

The high incidence rate among the Mixed Ancestry population group in the Western Cape has not been adequately explained. Socio-economic factors that promote *M. tuberculosis* infection are an obvious factor. Poor living conditions and overcrowding, the extent of case finding and of contact screening are important in determining the incidence of infection but it is possible that other factors such as genetic influences may also play a role.

1.4.2 Bacteriology

M. tuberculosis was first described by Robert Koch in 1882 and has since been studied intensively, but, there is still more to unravel.

M. tuberculosis is a facultative intracellular pathogen and resembles other pathogens such as Leishmania, Toxoplasma gondii and Legionella pneumophilia (Kaufman 1993). These organisms enter humans' cellular milieu via the macrophage and escape intracellular killing for some time, giving rise to their progressive local and systemic effects. Furthermore they have the ability to remain dormant in the human body and to multiply many years later. Within the human body, under certain conditions, organisms multiply extracellularly an example being pulmonary cavities where *M. tuberculosis* bacilli lie free within the caseous material.

M. tuberculosis is also characterised by its slow dividing time (doubling time ± 12-20 hours) (Wheeler 1988) and its ability to survive outside the human host for long periods (Nyka 1974). The nutrients needed for in vitro culture is nothing more than the usual organic carbons, nitrogen and trace elements (Wheeler 1988).

The cell wall consists of a plasma membrane, the cell wall proper and an outer attached capsule (Draper 1982). All three structures consist mainly of lipids giving it it's waxiness and characteristic acid fast staining property. The skeleton or supporting framework is made up from peptidoglycans (polysaccharides), arabinogalactan and mycolic acids. Also attached to the skeleton are glycolipids and proteins. Lipoarabinomannan (LAM) is one such glycolipid that produces a strong immunological response although not unique to *M. tuberculosis* (McNeil and Brennan 1991). Other glycolipids that are important immunologically are the sulphatides.

The characteristics of the cell wall and the slow adaptive metabolism is most likely responsible for the innate ability of the organism to resist the human host defence mechanisms (Ehlers 1993).

1.4.3 Host immune responses to *M. tuberculosis*

Recent reviews on this topic by Dunlop and Briles (1993); Buschman and Skamene (1988); and Edwards and Kirkpatrick (1986) have been published.

Droplets containing live *M. tuberculosis* bacilli are inhaled and ingested by alveolar macrophages. Multiplication progresses until a threshold is reached (4-8 weeks later), when bacilli are transmitted via lymphatics to regional lymphnodes or via the bloodstream to distant sites.

M. tuberculosis enters macrophages by a process of phagocytosis. The probable binding sites - called ligands - of *M. tuberculosis* are complement receptors three and four (also known as MACI, part of the ß2 family) (Schlesinger et al 1990). This binding enables the bacillus to avoid the respiratory burst and also interferes with the phagolysome-lysosome fusion process. The other factors that aid evasion of intracellular killing are the waxy coat, the sulfatides (high molecular weight glycolipid sulfates), lipoarabinomannan and increased adenosine monophosphate production by the bacillus (Dunlop and Briles 1993).

The Cellular immune response is initiated by clonal expansion of primed T-helper cells that activate peripheral macrophages by gamma interferon production. Delayed hypersensitivity (DTH) develops which is characterised by skin reactions to intradermally injected PPD (purified protein derivative). The DTH response is probaly T-cell dependant and leads to macrophage killing and tissue damage by liquefaction. The T-helper subsets Th1 and Th2 and cytotoxic T-cells (CD8+) each produce a typical cytokine pattern: Th1 cells mainly interferon gamma (IFNγ),interleukin-2 (IL-2) and tumour necrosis factor beta (TNFβ) and Th2 cells IL-4 and IL-10. Once activated through INFγ, macrophages can then kill off the bacilli. Natural killer cells lyse other cells to liberate *M. tuberculosis* bacilli which can then be phagocytosed effectively by activated macrophages and this leads to an abated initial infection. If the DTH response predominates, liquefaction and caseation causes extensive tissue damage. (Kaufman 1993, Dunlop 1993).

The humoral immune response to *M. tuberculosis* infection has been mostly regarded as non-protective and in many instances detrimental. An association between disseminated disease and high antibody titres has also been described (Lenzine et al 1977). It is tempting to speculate that the high IL-4 levels, causing high antibody levels in patients with *M. tuberculosis*, could be determental to the host defence due to down regulating the Th1 response (Surcel et al 1994)

Antibodies may however be **protective**. The following evidence from the literature supports this:

a. A study of childhood tuberculosis found the highest specific *M. tuberculosis* antibody titres in circulating immune complexes from children with healed pulmonary tuberculosis and calcified chest lesions, but lower levels in children with miliary tuberculosis (Ashtekar et al 1992).
b. Specific antibodies to certain cell membrane antigens of *M. tuberculosis* i.e. lipoarabinomannon (Costello et al 1992) and the 38-kDa protein (Harboe 1992) might have protective value since they are associated with localised disease and not with dissemination.
c. In animal models it is reported that specific anti-mycobacterial antibodies impaired delayed hypersensitivity reaction in rats skin tested with PPD (Drexhage et al 1980).

d. Other evidence comes from a study on macrophages of mice infected by mycobacterium bovis (Forget et al 1976). When the sera of mice that have overcome the disease were added to in vitro cultures this resulted in enhanced killing of *M. tuberculosis* by macrophages.
e. Specific antibodies when present in autologous serum, increases the degree of lysosomal fusion with the phagosomal membrane in human macrophage cultures infected with M.bovis (Armstrong et al 1975).

One can therefore conclude that antibodies against mycobacterial species may play a **immune modulating** role.
As a diagnostic tool antibody measurement has not yielded promising results, mainly because of the inability to find discriminating antibody levels between dormant and active *M. tuberculosis* infection.

1.4.4 Genetic susceptibility

Genetic susceptibility to mycobacterium species is a much more vexing question. To support this theory the following is quoted:
Epidemiological evidence:
a. Twin studies: concordance for tuberculosis was higher among monozygotic than dizygotic twins (Canstock 1978).
b. Studies on USA racial groups in similar environments indicated a higher susceptibility to *M. tuberculosis*.(Stead et al 1990).

Laboratory investigations:
a. In vitro studies on macrophage killing ability that differs between Negroid and Caucasian (Crowle 1990).
b. Murine models in which the 'BCG' gene mapped on chromosome 1 rendered the animal resistant to M.bovis (Skamene 1989). Recently linkage studies have shown that in the human genome homology exist on Chr 2q with the Bcg gene in the mouse. (Skamene E 1994).
c. Decreased production of tumour necrosis factor (a cytokine responsible for macrophage activation) in four genetically related children from Malta with atypical mycobacterial disease (Newport and Levin 1994).

Genetic determined markers:
A worse outcome in pulmonary tuberculosis in patients with HLA DR2 (Chukanova et al 1994) and MHC class III C4A component (Santos et al 1994) has been described. Associations between tuberculosis and immunoglobulin allotypes have also been described (Gibson et al 1987; Chukanova et al 1994; Matrakshin et al 1993).
There are fierce opponents of the genetic susceptibility hypothesis who claim that environmental factors such as overcrowding and other health issues including nutrition and poor case and contact tracing plays the most important role in rendering the host susceptible (Kushigemachi et al 1984).

1.5 Rationale for the study

From the literature review it is clear that *M. tuberculosis* is still highly prevalent and will possibly become the most important infectious disease in sub-Saharan Africa. Although cellular immune mechanisms play a dominant role, humoral responses could at the very least modify the disease process. There is some evidence that genetic susceptibility to *M. tuberculosis* exists. Allotypic markers of IgG are genetically determined and thus provides a way of studying the genetic determined responses to mycobacterial infection in humans.

CHAPTER 2

STUDY DESIGN

2.1 STUDY DESIGN AND POPULATION DEFINITIONS

The study was undertaken in conjunction with a parallel study 'The immunological aspects of pulmonary tuberculosis in association with Vitamin A supplementation in children of the Western Cape' (principal author W Hanekom) between August 1993 and June 1994. Approval by the Ethics and Research Committee of the University of Cape Town was obtained prior to embarking on this study.

2.1.1 Population definitions

Black population: In accordance with accepted usage 'Black' is employed for describing persons of indigenous African lineage. The population in the Western Cape are largely Xhosa speaking.

Mixed Ancestry population: The term 'mixed ancestry' is used to describe this group rather than Coloured or Cape Coloured. The population has a mixed lineage in origin. (see section 2.3.2.)

2.2 PATIENT COHORT

2.2.1 Criteria for selection

The patient cohort comprised of 99 consecutive children with suspected *M. tuberculosis* disease who presented themselves or were referred for in-patient treatment at three paediatric training hospitals in Cape Town. Their ages ranged from 3 months to 14 years and 3 months. The World Health Organisation criteria (WHO) were used to make the diagnosis of active *M. tuberculosis* disease (Table 2.1). Only the 35 patients with microbiological confirmation for *M. tuberculosis* were selected for further analysis of the IgG subclasses and allotypes.

Exclusion criteria were the following:

i. Recent (within 3 months prior) use of high dose Vitamin A therapy since the immunological effects of Vitamin A supplementation was studied as well.
ii. The use of steroids. Most of the children with Tuberculous meningitis (TBM) received oral prednisone for prolonged periods (28 days) and were excluded due to the effect of steroids on immunological measurements. Only 1 TBM child was included in the study.
iii. Children with skeletal manifestation of tuberculosis as a presenting feature were generally not available for selection.
iv. Refusal by parent or caretaker for blood sampling.

Thirty five children (female 15; male 20) were included of whom 20 were Black and 15 of Mixed Ancestry.

TABLE 2.1: WHO CRITERIA for the diagnosis of Pulmonary Tuberculosis in children under five years of age.

Suspected case:

- An ill child with a positive contact
- Any child who doesn't return to normal after measles or whooping cough.
- Any child with loss of weight, cough and wheeze who doesn't respond to antibiotic therapy.
- Any child with abdominal swelling or a hard painless mass and free fluid.
- Any child with painless firm swelling in a group of superficial lymph nodes.
- Any bone or joint lesion of slow onset.
- Any child with signs suggesting of meningitis.

Probable Tuberculosis:

- A suspect case with:
  >10 mm induration on tuberculin reaction;
  Suggestive radiological appearances on chest, bone or joint films;
  Suggestive histological findings in biopsy material;
  Favourable response to specific antituberculosis therapy.

Confirmed Tuberculosis.

- A probable case with:
  Microscopic detection of tubercle bacilli from secretions or tissues;
  The identification of the tubercle bacilli as *Mycobacterium tuberculosis* by culture characteristics.

2.2.2 Data collected

Demographic data

The age, sex, race and permanent address were collected. Factors relating to social status and housing conditions, as well as the caretaker and disease contacts were recorded. Immunisation status, birth history and birth place were also recorded.

Clinical symptoms and signs

Symptoms pertaining to anatomical localisation, hypersensitivity phenomena and malnutrition were illicited. Each child was fully examined and the findings recorded.

Skin testing

Mantoux responses were recorded 72 hours after administration of PPD intradermally. Not all the patients could be personally seen after injecting the PPD and the 72 hour recordings were therefore done by multiple health professionals.

Chest X-rays

Chest X-rays were obtained at diagnosis, at 6 weeks and at 12 weeks and were reported on by one radiologist in the radiology department at the Red Cross Children's Hospital.

The X-ray appearances were recorded and classified into pathological groupings:

Primary pulmonary TB	-	Lymphadenopathy only and/or
	-	Single parenchymal lesion with lymphadenopathy
Progressive pulmonary TB	-	Lymphobronchial disease with or without airway impingement and/or
	-	Haematogenous dissemination and/or
	-	Pericardial disease and/or
	-	Pleural disease
Post primary/secondary TB	-	Cavitation or fibrosis with or without calcifications.

2.2.3 Disease severity score

To compare the extent of pulmonary TB among subgroups several authors have used different criteria. Some include symptoms that relate to the disease, specific signs of the disease; special investigations such as X-rays or bacteriological results and response to therapy (Tidjani et al 1986). Allotypes could be associated with disease modulation and therefore a severity score was developed. This score system uitilises age, nutritional status, X-ray appearances, haemoglobin and albumin values. (Table 2.2)

TABLE 2.2: SEVERITY SCORE

	0	1	2
Nutritional status: (weight for height mean)	≥ 95% of mean	87-94% of mean	< 87% of mean
Albumin	≥ 32 g/dl	25-31.9 g/dl	< 25 g/dl
Haemoglobin	≥ 10.5 g/dl	8.5-10.4 g/dl	< 8.5 g/dl
Chest X-ray appearances	Primary	Progressive	Secondary
Age:	> 5 years	2-5 years	< 2 years

The following statements are made to motivate the selection of age, nutritional status, X-ray appearances and two laboratory parameters:

M. tuberculosis induces acute malnutrition as is clear from children with extensive disease (Hussey et al 1991). Weight for height was used to eliminate the effect of chronic malnutrition that is associated with patients from poor socio-economic backgrounds as was demonstrated in a group similar to this study population (Hugo-Hamman 1987).

The common finding of disseminated *M. tuberculosis* occurring in younger children (Seth et al 1993; Sinder et al 1988; Jacobs et al 1993) warrants the inclusion of age in the severity score.

Chest X-ray appearances correlate with the known pathophysiology of the disease progressing from the Gohn complex to lymphogenic or bronchogenic spread and later

tissue necrosis and cavitations (Dunlop et al 1993). Miliary (pulmonary) tuberculosis was grouped under progressive primary disease due to this pathophysiological concept. Since age was used as a denominator and most miliary *M. tuberculosis* occurs in the under 2 age group (Hussey et al 1991) this specific chest X-ray appearance was not weighted separately.

The haemoglobin and serum albumin values were included due to the known effect of *M. tuberculosis* on bone marrow suppression and its effect on general nutrition (DesPrez et al 1990).

2.3 CONTROL GROUP

2.3.1 Selection

The control group consisted of four separately collected cohorts:

i. Children of Mixed Ancestry (67): Aged 6 m - 13 years. Sampling was done in conjunction with the National Nutrition and Vitamin A Survey (1994). All the children's growth parameters were checked and examined clinically to exclude lung pathology. Blood was obtained with written consent.
Mixed Ancestry adults (144): Ages were not recorded. Volunteer blood donors were asked to donate an extra sample. Each donor was weighed, haemoglobin checked and a health questionnaire completed. Only samples from individuals that were not ill by these criteria were selected.
Total Mixed Ancestry group: (211)

ii. Black children (59): Aged 6 to 18 months. Samples were obtained as part of measles vaccination intervention study (Hussey G personal communication 1993). All patients were examined and growth parameters recorded. Only healthy children were selected.
Black adults (25): Volunteer blood donors were screened in the same way as the blood donors of the Mixed Ancestry group (see (i) above).
Black adults (149): A cohort screened for sexually transmitted diseases. All patients were examined and weighed and patients without chest symptoms and who were HIV negative were selected
Total Black race group: (233).

2.3.2 Genetic composition

Mixed Ancestry population
The Western Cape Mixed Ancestry population has its origins routed in an admixture of a number of different race groups.

The first inhabitants of the Western Cape are by all accounts (verbal, written and archaeological) a group called Khoi or also known as Hottentots (±AD 1500). The Khoi and another closely related group San (Bushmen) shared geographical areas with the Khoi being pastorlists and the San the hunters. With increasing pressure on both groups by other occupants (European and African) the two groups became intermingled and are now referred to as the Khoisan.

From historical facts the groups contributing to the Mixed Ancestry population group in the Cape were: Hottentots (Khoi), Caucasian (Western Europe), Bushmen (San) and freed slaves (Negroid and Indonesian from Madagascar, Java and Sumatra) (Marais 1957). But also from data based on genetic markers that included immunoglobulins, blood groups, red blood cell enzymes and serum proteins the contributing groups are the same namely Khoi, San, European, Negroid and Indonesian (Nurse et al 1985).

The relative contribution of each of the race groups to Cape Mixed Ancestry population group is virtually impossible to determine. No important founder-gene abnormalities identify this group and due to the many different factors involved in gene drift each race group's contribution cannot be accurately established.

This group can be expected to share some common genetic inherited markers with its neighbours such as the Nguni. The Nguni who some time later became the Black Xhosa speaking group has been included in this study.

The present day Mixed Ancestry population group was formed in the late 1700's and has developed it's own socio-cultural identity in many aspects, especially as a result of Apartheid laws. Although further racial admixture would have continued, but on a much smaller scale, it can at present be considered as a fairly homogenous group.

Black population

The Black Xhosa speaking population group in the Western Cape is closely related to the other Nguni Bantu groups: Pedi and Zulu. The Nguni pastoralists are grouped together and separated from the Sotho-Tswana Bantu by linguistic and social characteristics. The Sotho of the early Iron Age inhabited mainly the interior plateau regions and came into close contact with the San - assimilating some of the language characteristics, whereas the Nguni inhabited the coastal regions migrating gradually southwards from present day Swaziland (Oliver and Fagan 1975).

Clear distinction between the present day Nguni groups evolved during the late 16th and early 17th centuries after the Mfecane - an event of large scale upheaval involving most of the Negroid groupings of Southern Africa. Pressures relating to European occupation of the Southern Cape regions, population growth and the need for increasing land usage common to the pastoral life style, and dominating groups such as the Zulu under Chief Chaka, are all factors that contributed to the Mfecane.

There is evidence that the Xhosa group has assimilated some of the Khoi members when judged by allotypic expression (Steinberg et al 1975), linguistic characteristics (Maingard 1934) and historical evidence. This is also supported by archaeological findings which dates early Bantu and Koisan inhabitations during the early Iron Age to the same geographical areas covering most of the eastern Southern Africa coastline (Oliver et al 1975).

The important episodes of population upheaval and possible genetic related changes are listed below (Nurse et al 1985):

Date	Source	People group	Effect
1713	Small pox epidemic	Khoi	Movement away from Southern Cape towards Namibia and Northern Cape
Early 1800's	Mfecane	Nguni	Northward migration towards Malawi

Changes brought on by present day economic factors (such as urbanisation) and apartheid laws (migrant labourers) have probably not changed the genetic composition significantly although this has not been formally studied.

This suggests that since the mid 19th hundred century no major factors involving genetic admixture has occurred in the two population groups under study

CHAPTER 3

METHODS

3.1 CLINICAL DATA CAPTURE FORM

All the data collected was recorded on a specially devised questionnaire and later transferred to an analytic programme (EpiInfo, Version 6). See Appendix 6.

3.2 ELISA TECHNIQUE

All allotypes were tested by ELISA techniques. Of the five ELISA tests used, three were newly established tests for this laboratory The IgG subclasses were measured by techniques established in the Immunology Laboratory at the Red Cross Children's Hospital (Goddard et al 1992).

Written consent was obtained from the parent(s) or accompanying caretaker before blood was taken from the child. The sera were separated and aliquoted on the day of collection and stored at -70°C until analyzed.

3.2.1 Introduction to the ELISA technique

The enzyme linked immunosorbent assay, was developed from the older radioimmuno assay where a solid support was used to bind antibody. Radio labelled antigen was then captured and analyzed. In 1972 Engwall and Perlmann described a refined method by which antibodies were linked to an enzyme (alkaline phosphatase) for detecting antigen on a solid phase. With the addition of a suitable substrate and a chromogen, measurement by spectrometry could be undertaken.

Allotypes were measured previously by haemagglutination methods and this has remained the gold standard (De Lange et al 1989). As newer and more specific antibodies and reliable reference sera became available the ELISA technique has been used more extensively. The ELISA technique has some major advantages over haemagglutination (Table 3.1).

TABLE 3.1: HAEMAGGLUTINATION VERSUS ELISA TECHNIQUE

	Haemagglutination	ELISA
Methodology		
Ease	+	++
Time	+++	++
Reproducibility	++	++
Costs	++	+
Moab* availability	+	+++
Quantitation	Not possible	Possible
Samples done per run	10-20	> 40

* Moab = Monoclonal antibody

The greatest limitation of the ELISA technique is the lack of specific and suitable antibodies for a few remaining allotypes (Nelson et al 1990).

3.2.2 Description of methods used: (Detailed description included in Appendices)

Indirect ELISA: (Allotyping - G1m(a), Gm(f), G2m(n), G3m(g^1))
The test sera and a positive and negative control sera are absorbed onto a plastic surface (Nunc microtitre plate). After blocking with a protein (casein 1%) to prevent non-specific binding a detector monoclonal antibody (Moab) is added. Thereafter a conjugated species-specific antibody is used to detect the amount of Moab that adhered to the test and control sera antigens. Antibodies used are animal derived: mouse detector monoclonal antibody and goat conjugated antibodies. Horseradish peroxidase is used as the conjugating enzyme with orthophenylene diamine (OPD) as chromogen and hydrogen peroxidase as substrate. The amount of antibody captured by the coated antigen (test serum) is then determined spectrometrically.

Inhibition ELISA: (Allotyping G3m(b^1)
No monoclonal antibody that is specific for G3m(b^1) is available. The only Moab that includes G3m(b^1) also reacts with u and u allotypes. The procedure was therefore adapted in that a known serum that contained the allotype G3m(b^1) but not u/v was used for coating the microtitre plates. The test serum is then mixed with the monoclonal antibody and allowed to react before plating. If the G3m(b^1) allotype was present in the test serum, the Moab is prevented from reacting with the coating serum. By adding a negative and positive control, inhibition could be confirmed.

Capture ELISA: (IgG1, 2, 3 and 4)
Commercially available monoclonal antibodies are absorbed onto the solid phase (microtitre wells). The positive control (in standard dilutions), negative control and test sera are then added. A conjugated goat anti-human antibody is then used for subclasses IgG2, IgG3 and IgG4. For IgG1 a biotinylated human derived Moab is used with streptavidin. Ornythine phenyladianime and horseradish peroxidase is again used as chromogen substrate to obtain a spectrometric reading. Quantitation is then done via the standard curve, constructed with each run.

Km(1) Allotype
The lack of a suitable monoclonal antibody against Km1 meant that this assay could not be adapted to be discriminatory and used for analysis in this study. The only Km1 monoclonal antibody (Moab) available is human derived. The other more commonly available goat derived anti-human Moab can be detected by an enzyme labelled mouse anti-goat antibody. We attempted to biotinylate the human derived antibody with a commercially available kit. This would allow streptavidin to be used as a conjugate. Three preparations were done but none performed adequately, with a high signal to background ratios due to non-specific binding. As a last resort immune-precipitation in agarose gel was attempted for the Km1 antibody, but no precipitation lines were observed in four different dilutions.

3.2.3 Quantification:

Quantification is achieved by equating a known standard serum concentration with a specific optic density.

Because of the availability of commercial and/or WHO standards exact quantitation could be achieved for G2m(n), G1m(f) and the four IgG subclasses. The method employed is through heterologous inter-polation from standard curves generated with each run. Parallelism of the test serum and standards were obtained (Goddard 1992).

Semi quantification:
The lack of available standards for the allotypes G1m(a), G3m(b^1) and G3m(g^1) necessitated that these assays were semi-quantitative.

Reference sera containing the allotypes in question and negative sera were obtained commercially. Test serum results could therefore be grouped into equal or greater than the positive sera, lesser or equal to negative sera, or had optic density levels between the positive and negative reference sera.

These intermediate results represent individuals that are most likely heterozygous for the particular allotype. With the availability of myeloma proteins that are allotypic specific, accurate quantification can be done in certain research laboratories (Ota et al 1991, Oxelius 1993a). Using the technique of immunoprecipitation (Rautonen N et al 1989) has established that heterozygosity gives quantitative differences in optical density. This study did not attempt to identify homo and heterozygocity for the allotypes tested.

3.2.4 ELISA technique standardisation and control

The following standardisation and method controls were undertaken according to McLaren et al (1981):

a. Inter-assay variation and sensitivity:
The inter-assay coefficient of variation was determined for the allotypes that could be accurately quantified (G1m(f) and G2m(n)). Inter-assay coefficient of variation never exceeded 15% (G1m(f) 14.1% and G2m(n) 14.0%).

In the semi quantitative ELISA test (G1m(a); G3m(g^1) and G3m(b^1) the use of positive and negative control sera on each plate confirmed the result as positive, negative or indeterminant (less than positive control, more than negative control). The indeterminant group was categorised further by adding control sera mixtures to each run. The positive control serum was mixed with the negative control serums in dilutions of 1:2, 1:4, and 1:20. This was done for G1m(a) and G3m(g1). All optical densities (OD) that were two standard deviations above the negative control sera were considered negative, those between the positive control and two standard deviations below the 1:4 dilution was considered positive and the group that fell in between was reanalysed. If on a second evaluation the OD remained above the 1:20 dilution of the positive sera the result was accepted as positive.

b. Specificity and optimisation of test conditions:
Checkerboard analysis were used in preliminary runs of each allotype to determine optimal dilutions, coating times and temperatures. Only non-specific backgrounds (NSB) of less than 10% of the signal obtained were accepted. Runs were repeated if NSB was more than 10%. As far as possible the same batch of enzyme labelled antibodies were used to minimise the variability of enzyme-substrate reactions.

c. Standardisation between assays was achieved by using the same batch of reference sera for each allotype and the same procedure under the same working conditions. Most reactions took place at room temperature which was controlled between 18° and 22°C.

Validation of allotyping by haemagglutination:
The ELISA determination of G1m(f) and G2m(n) allotypes were validated earlier (Goddard 1994) by a WHO recognized reference laboratory: The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

3.2.5 Statistical Analysis:

Data was entered on a statistical program distributed by the Centre of Disease Control, Atlanta, USA(EpiInfo version 6) Analysis was performed by comparing 2 x2 tables by means of the Chi- square or Fisher exact tests. Non parametric data were calculated by applying Barlett's test for homogeneity and the Kruskal-Wallis H test. Due to the ever changing reference ranges (according to sex, age and race) for IgG (total) and subclasses the mean values were used. Multiples of the Mean Age Norm were then calculated and compared. Thus each ancestry cohort could be grouped together for comparisons.(see Table 4.5)

CHAPTER 4

RESULTS

4.1 CLINICAL DATA OF PATIENT COHORT

In this section the proven TB cohort is compared with probable TB group to investigate the simalarities and important factors predisposing children to aquire pulmonary tuberculosis.

4.1.1 Demographic data:

Thirty five children's serum samples with definite *M. tuberculosis* either on direct microscopy and/or culture were used for allotype determination. The demographic data of these patients and 64 probable cases (organism negative) are depicted in Table 4.1.

TABLE 4.1: DEMOGRAPHIC DATA (Rounded off percentages in brackets)

	TB Confirmed (n = 35)		**TB Probable (n = 64)**		**Total (n = 99)**
Race					
Mixed Ancestry	15	(43)	20	(31)	35
Black	20	(57)	44	(69)	64
Sex					
Male	20	(57)	32	(50)	52
Female	15	(43)	32	(50)	47
Age (months)					
Median	40		38		39
Range	3-171 mths		6-164 mths		3-171 mths
Immunisations					
Complete	23	(65)	54	(84)	77
BCG received	25	(71)	56	(88)	81
Skin testing					
Tine > Grade I	7	(20)	21	(33)	28
Mantoux > 10 mm	28	(80)	49	(77)	77
TB contact positive	20	(57)	23	(36)	
Housing					
Shack	14	(40)	28	(44)	42
Brick house	15	(43)	20	(31)	35
Room/flat	3	(8.5)	10	(16)	13
Other	3	(8.5)	5	(8)	7
Unknown	-		1	(1)	2

Table 4.1 continued:

	TB Confirmed		TB Probable		Total
Overcrowding					
> 4 persons/room	9	(27)	12	(18)	21
Income					
One parent employed	17	(49)	33	(52)	50
Both parents employed	3	(8)	5	(8)	8
Both unemployed	15	(43)	26	(40)	41
Caretaker					
Mother	18	(51)	41	(64)	59
Family member	14	(40)	8	(12.5)*	22
Creché/school	2	(6)	11	(17)	13
Unknown	1	(3)	4	(6.5)	5

- p: (Mantel Haenszel) 0.0017

As expected the children came from an impoverished background with overcrowded housing conditions. The TB proven group had less contact with medical services judged by completed immunisations (65%), than the TB probable group (84%).

There were more male than female patients (ratio 1:1.3) and more Black than Mixed Ancestry children. The median age was 40 months (range 3-171 months). Most were in the care of their mother (51%) but a larger number of proven TB group were in the care of a family member (40%), than the probable TB group (12.5%). The reason for this is not clear. Fifty seven percent of the proven TB group had a positive TB contact compared to only 36% of the probable TB group.

There were however differances between the Black and Mixed Ancestry cohorts. Important differences concerning housing (61% shacks versus only 9% in the Mixed Ancestry group) and contact with health services, as judged by immunisation coverage. Only 60% of the Black children were fully immunised where as 94% of the Mixed Ancestry children were fully immunised. Employment figures were about the same for the two race groups. One could therefore conclude that the Mixed Ancestry group is less socially deprived and has the opportunity to present themselves earlier for diagnosis and therapy (Table 4.2).

TABLE 4.2: DEMOGRAPHIC DATA: BLACK VERSUS MIXED ANCESTRY
(Rounded off percentages in brackets)

	Black (n = 64)	Mixed Ancestry (n = 35)	p Value*
Sex:			
Male	36 (56)	16 (46)	
Female	28 (44)	19 (54)	
Age (months)			
Median	42	67	
Range	3-164	5-171	
Immunisations			
Complete	44 (69)	33 (94)	p<0.01
BCG received	47 (73)	34 (97)	p<0.01
Skin testing			
Tine > Grade I	14 (22)	5 (14)	
Mantoux > 10mm	59 (92)	2 (97)	NS
Housing			
Shack	39 (61)	3 (9)	p<0.001
Brick house	11 (17)	24 (69)	P<0.001
Room/flat	6 (9)	7 (20)	NS
Other	6 (9)	0 (0)	NS
Unknown	2 (3)	1 (3)	
Overcrowding			
> 4 persons/room	15 (23)	6 (17)	
Income			
One parent employed	35 (55)	20 (57)	
Both parents employed	3 (5)	4 (11)	
Both unemployed	26 (41)	11 (31)	NS
Caretaker			
Mother	43 (67)	16 (46)	
Family member	13 (20)	9 (26)	
Creché/School	5 (8)	3 (6)	
Unknown	3 (5)	2 (6)	

* Yates corrected

4.1.2 Disease Profile:

The following symptoms were recorded on enrollment: coughing, loss of weight, loss of appetite, night sweats, shortness of breath, wheezing and fever. Of those with pulmonary tuberculosis nearly 85% presented with coughing, loss of appetite and fever.

Physical signs recorded included: Height and weight measurements, signs of hypersensitivity, lymphadenopathy detailed chest findings, central nervous system and

abdominal findings. Hypersensitivity attributed to *M. tuberculosis* was found in 5% and lymphadenopathy in 24%.

Those with definite proven *M. tuberculosis* did not differ in clinical signs at presentation from the probable TB group although chest X-ray findings were significantly different with the proven group showing more advanced disease as could be expected from the higher yield in microbiological positive results.(Table 4.3).

TABLE 4.3 CHEST X-RAY FINDINGS:

	TB Positive (n = 33)		Probable TB (n = 64)		p Values (Fischer exact)
Primary TB					
Primary complex	3	(9)	18	(28)	**0.037**
Lymphnodes	2	(6)	6	(9)	0.603
Progressive primary	23	(70)	35	(35)	
Miliary	7	(21)	1	(2)	**0.001**
Pleural	8	(24)	22	(34)	0.308
Post primary	4	(12)	0		**0.011**

Percentages in brackets

In four children other systems apart from the respiratory were also affected by tuberculosis:

Meningitis	=	1
Abdominal TB	=	1
Glandular TB	=	1
Bone TB	=	0
Pericardial effusions	=	1

4.1.3 Severity Score

The mean severity scores for children with proven TB and probable TB were 4.94 and 3.80 respectively. Due to the large standard deviation in each group this was not a significant finding (Table 4.4).

TABLE 4.4: SEVERITY SCORE MEAN VALUES

	PROVEN TB			PROBABLE TB		
	Mean	Median	SD	Mean	Median	SD
Age	1	1	-	1	1	-
Hb	0.74	1	0.74	0.66	1	0.73
CXR	1	1	0.48	0.59	1	0.53
Albumin	0.94	1	0.75	0.69	1	0.65
Height for weight	1.29	1	0.75	0.84	1	0.77
Mean (total score)	4.94	4	1.79	3.80	3	1.99

SD= standard deviation

4.2 PATIENT COHORT

4.2.1 IgG (total) and IgG subclasses

The normal values used for comparison were obtained from a study done among the same population (Goddard et al 1992). Only proven cases were analysed. Due to ever changing normal ranges (according to sex, race and age), the data obtained for this study is expressed as multiples of the mean value for the specific race and age categories (called multiples of the mean age norm or MMN). The mean values according to the specific race and age category (called mean age norm or MAN) thus provided the basis for comparing the individual values. (Table 4.5).

TABLE 4.5: SUBCLASSES: PROVEN TUBERCULOSIS PATIENTS

(gram/litre)	Mean	Maximum	Normal Range*	Mean Age Norm	Multiples Of Mean Age Norms
IgG Total					
All	22.875	45.730		14.288	1.634
Black	23.521	31.720	7.121-28.387	14.778	1.644
Mixed Ancestry	22.013	45.730	5.179-26.046	13.682	1.533
IgG1					
All	19.913	40.930		12.342	1.625
Black	20.770	29.590	6.997-27.019	13.347	1.570
Mixed Ancestry:					
Male	20.691	40.930	5.411-19.564	11.047	1.834
Female	17.089	22.850	5.241-24.560	10.964	1.581
IgG2					
All	1.707	3.300		0.923	2.026
Black	1.651	3.300	10.219-2.119	0.772	2.234
Mixed Ancestry:					
Male	1.930	2.740	0.200-3.212	0.987	2.032
Female	1.654	2.840	0.200-3.212	1.243	1.501
IgG3					
All	0.938	2.440		0.772	1.645
Black	0.835	1.7860	0.184-2.315	0.622	1.417
Mixed Ancestry:					
Male	0.986	1.890	0.176-2.355	0.622	1.736
Female	1.155	2.440	0.189-2.032	0.593	2.135
IgG4					
All	0.322	0.920		0.174	2.001
Black	0.291	0.920	0.004-0.840	0.173	2.046
Mixed Ancestry:					
Male	0.343	0.740	0.002-0.830	0.196	1.757
Female	0.369	0.760	0.006-0.809	0.156	2.101

* Reference: Goddard et al 1992

The mean values for both race groups were significantly higher than the reference ranges for IgG total and all four subclasses. This is best illustrated by the Multiples of Mean Age Norm (MMN), where the average MMN for IgG total was 1.634 (more than one and a half times elevated when compared to the reference mean value).

The subclasses IgG2 and IgG4 showed highest elevation for the patient group as a whole. The IgG2 (Blacks) and the IgG3 (Mixed Ancestry females) subclasses were the most raised in each racial group. Black patients' mean MMN as a group was 1.817 and the Mixed Ancestry patient group was 1.688 (no significant difference).

There was no correlation between age and MMN values (data not depicted).

There were too few numbers to analyse the effect of each allotype on the subclass values since each age group, race and sex group had it's own reference values.

4.2.2 Allotypes

When the two population groups with culture proven tuberculosis were compared, no significant differences could be found in the 5 allotypes measured (Table 4.6). Apparent differences between the two groups did not reach significance due to the small numbers analysed.

TABLE 4.6: ALLOTYPES IN CHILDREN WITH PROVEN *M. TUBERCULOSIS* (percentages positive in brackets)

Allotype	All (n = 35)		Black (n = 20)		Mixed Ancestry (n = 15)	
G1m(a)	35	(100)	20	(100)	15	(100)
G1m(f	3	(9)	0	(0)	3	(20)
G2m(n)	2	(6)	0	(0)	2	(13)
G3m(g1)	23	(66)	13	(65)	10	(67)
G3m(b1)	31	(86)	17	(85)	14	(93)

4.3 CONTROL GROUP

4.3.1 Allotypes

The Black and Mixed Ancestry control groups differed significantly in that more of Mixed Ancestry persons were G1m(f) and G2m(n) positive. Adults and children did not differ in allotype frequency (Table 4.7).

Only 51 Mixed Ancestry adults and 80 Black adults were tested for G3m(b1). This was because the method used, inhibition ELISA, needed dilutions of 1:50 and thus consumed reagents. Further analysis was not undertaken when it appeared that no significant differences were apparent between racial groups or the control and tuberculosis cohort.

TABLE 4.7: CONTROL GROUP: ALLOTYPES (percentages positive in brackets)

Allotype	Black	Mixed Ancestry	Black		Mixed Ancestry	
			Adult	Child	Adult	Child
	(n = 232)	(n = 211)	(n = 173)	(n =59)	(n = 145)	(n = 67)
G1m(a)	232 (100)	196 (92)	173 (100)	59 (100)	132 (91)	64 (96)
G1m(f)	14 (6)	120 (57)*	8 (5)	6 (10)	84 (58)	36 (54)
G2m(n)	11 (5)	92 (44)**	9 (5)	2 (3)	65 (45)	27 (41)
G3m(g^1)	188 (97)	197 (93)	173 (100)	15 (71)	135 (93)	62 (93)
G3m(b^1)	47 (92)	68 (85)	43 (92)	- -	74 (84)	21 (88)
	(n=51)	(n=80)	(n=47)		(n=56)	(n=24)

* p = 0.0018, OR 3.30

** p = 0.003, OR 3.74

4.4 PATIENT VERSUS CONTROL GROUP: ALLOTYPES

The most striking difference was found in the G1m(f), G2m(n) and G3m(g1) allotypes in the Mixed Ancestry population (Table 4.8). Children with TB had a significantly lower frequency of G1m(f), G2m(n) and G3m(g1) than the control group. This association was even stronger when the proven and probable TB children were analysed together. In the Black population the G3m(g1) allotype was also found to be of a lower frequency in the children with proven TB (Table 4.8).

TABLE 4.8: TB VERSUS CONTROL: ALLOTYPES (percentages positive)

Allotype	Black Population Proven TB (n=20)		Control (n=233)		p Value
G1m(a)	20	(100)	233	(100)	NS
G1m(f)	0	(0)	15	(6)	NS
G2m(n)	0	(0)	11	(5)	NS
G3m(g1)	13	(65)	189	(97)	**< 0.001** *
G3m(b1)	17	(85)	47	(92)	NS
				(n=51)	

* OR 20.35

Allotype	Mixed Ancestry Population Proven TB (n=15)		Control (n=211)		p value
G1m(a)	15	(100)	203	(93)	NS
G1m(f)	3	(20)	120	(57)	**0.01**
G2m(n)	2	(13)	92	(44)	**0.04**
G3m(g1)	10	(67)	202	(96)	**0.001**
G3m(b1)	14	(93)	68	(87)	NS
			(n=80)		

* Yates corrected

NS = non significant

4.5 HAPLOTYPES IN TB GROUP

The five allotypes tested for in the proven TB group consisted of the following haplotypes:

Haplotype 1:	G1m(a), G3m(b1), G3m(g1)	(n=17)
Haplotype 2:	G1m(a), G1m(f), G3m(b1), G3m(g1)	(n=1)
Haplotype 3:	G1m(a), G3m(b1)	(n=11)
Haplotype 4:	G1m(a), G3m(g1)	(n=4)
Haplotype 5:	G1m(a), G1m(f), G2m(n), G3m(b1)	(n=1)
Haplotype 6:	G1m(a), G1m(f), G2m(n), G3m(b1), G3m(g1)	(n=1)

The IgG subclass values were not distributed normally in each of the haplotypes studied, and therefore non-parametric statistics were used.

A weak significant difference between the mean values of IgG2 and IgG3 were found when those with versus those without haplotype 1 were compared (p = 0.04, see Table 4.9). On comparing the two race groups with each other for haplotype 1, there was no differences in the mean values of any of the subclasses.

There was no association between haplotypes and disease severity score (data not shown).

TABLE 4.9: HAPLOTYPE VERSUS MEAN SUBCLASS VALUES

Haplotype		Subclass (mean values)			
		IgG1	G2	G3	G4
1	Pos	12.086	0.753*	0.557*	0.139
	Neg	12.584	1.083	0.671	0.207
3	Pos	13.019	0.937	0.691	0.224
	Neg	12.032	0.916	0.581	0.151
4	Pos	13.342	0.847	0.631	0.172
	Neg	12.213	0.932	0.163	0.174

(Haplotypes 2, 5 and 6 excluded, one patient only in each haplotype)
* Kruskal-Wallis H (non-parametric statistics used) p = 0.04

In the Black Race group with proven TB the absence of the haplotype 1 was a significant finding (p = 0.006, OR 4.67). This was not found in the Mixed Ancestry Race group. In none of the other haplotypes were any significant differences between the race groups or those with and without proven TB.

4.6 DISEASE SEVERITY SCORE

When the allotypes were compared with the disease severity score attained, no influence could be demonstrated (Table 4.10).

TABLE 4.10: ALLOTYPES AND DISEASE SEVERITY

Allotype		Median Severity Score*							
		Proven TB		All TB		Proven TB cases			
						Black Ancestry		Mixed	
G1m(a)	Pos	5	(4.7)	5	(4.7)	5	(5.1)	5	(4.6)
	Neg	none		4	(3.8)	none		none	
G1m(f)	Pos	4	(4.0)	4	(4.2)	none		3	(3.7)
	Neg	5	(4.9)	4	(4.2)	5	(5.0)	5	(4.8)
G2m(n)	Pos	3	(3.0)	4	(4.0)	none		5	4.8)
	Neg	5	(4.9)	5	(4.2)	5	(4.8)	3	(3.0)
G3m(b1)	Pos	5	(5.6)	5	(4.7)	5	(4.9)	5	(4.7)
	Neg	4	(4.8)	4	(4.2)	6	(6.5)	4	(4.0)
G3m(g1)	Pos	5	(5.4)	4	(4.2)	6	(5.7)	5	(5.1)
	Neg	4	(3.9)	3	(3.8)	4	(4.0)	4	(3.7)

* Mean score in brackets

4.7 SUMMARY OF IMPORTANT FINDINGS

1. Mixed Ancestry children with proven tuberculosis expressed the G1m(f), G2m(n) and G3m(g1) allotypes less frequently. In Black children with proven tuberculosis, only G3m(g1) showed a similar association. This suggests that the absence of these Ig allotypes is associated with an increased susceptibility to *M. tuberculosis* infection.
2. Within the allotypes tested no effect on IgG subclass levels in children with pulmonary tuberculosis could be shown due to small numbers per catogory.
3. Disease severity was not associated with any change in allotype frequency.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 PATIENT SELECTION AND CHARACTERISTICS

The patient selection in this study suffers from some bias in that only children presenting themselves to hospitals in the Cape Peninsula were considered. These children would therefore differ from a non-hospitalised pulmonary TB paediatric patient group in the following ways:

i. More acute co-infections with viral or bacterial organisms
ii. More severe or complicated pulmonary TB
iii. Possibly better social status with sufficient resources to reach hospital.
iv. Selection criteria which virtually excluded bone TB and TB meningitis.

The data presented suggests that the Mixed Ancestry population group is a more socially privileged group with greater access to health services when compared to the Black Ancestry group. (table 4.2). The much higher incidence (718: 100 000 see page 16) is thus not easily explained on socio-economic factors alone. More research is needed to unravel this phenomena, but it suggests that other factors could be at play i.e. genetical influences.

To confirm the similarities in under-privileged communities such as our patient cohort, two other studies of paediatric TB patients were considered (Table 5.1). The one study was purely community based (Ravensmead, RSA) and the Uganda study that was a hospital based study.

The important differences between our study and those quoted are:
i. the higher percentage of cavitating x-ray appearances. This may reflect that the more ill children present to hospital when compared to community treated tuberculosis and that possible differences in interpretation (medical officer versus radiologist) could influence the X-ray assessments.
ii. the high percentage of negative intradermal tuberculin tests in the community studies and the large number of suspected TB cases in the Uganda study illustrate the difficulties experienced in reaching a definite diagnosis in childhood tuberculosis.

TABLE 5.1: COMPARISON OF DEMOGRAPHIC DATA

	Present Study (All TB cases) n = 99)	**Ravensmead* (Cape Town) (n - 124)**	**Uganda# (n = 210)**
Age			
< 2	32%	6%	All < 5 years
2-5	44%	34%	
> 5 years	23%	20%	

TABLE 5.1:COMPARISON OF DEMOGRAPHIC DATA (continued)

	Present Study (All TB cases) n = 99)	Ravensmead* (Cape Town) (n - 124)	Uganda# (n = 210)
Male:female	1.11	1.03	-
Over crowding	21%	-	-
Unemployment	42%	-	-
CXR findings:			
Lymphadenopathy (only)	8%	74%	17%
Pulm infiltrates	49%	26%	19%
Pleural/cavitations	30%	-	11%
Weight for age < 3rd %	36%	40%	-
TB contact	43%	53%	53%
Skin testing	Mantoux	Heaf	Mantoux
Positive	77%	16%	37%
Negative	11%	34%	63%
Not done	12%	27%	Nil
TB category (WHO)			
Suspected	-	20%	74%
Probable	64%	72%	10%
Proven	35%	18%	15%

References:
* Stoltz AP et al. 1990.
\# Miglion GB et al. 1992.

All three studies underline the fact that young children in poverty related settings are particularly prone to develop tuberculosis. Since our study's cohort may have differences in social and economic background (Table 4.1) it is possible than the case selection (of self-presenting hospital admissions) provided bias by selecting a more under-privileged group with a higher incidence of the disease. The majority of our cohort is of Black Ancestry which is due to the predominance of this culture group in the suburbs providing most of the admissions to the three teaching hospitals. The child adult ratio where roughly the same for each of the population groups (1:3 see table 5.2) The slightly more males in the proven and probable patient cohort is also found in the Ravensmead study but has not been adequatedly explained.

5.2 CONTROL GROUP SELECTION

Due to the difficulties of obtaining consent and case matched controls in a paediatric population, controls were selected from other readily available serum samples. This could lead to selection bias with genetic subgroups disproportionally represented. As pointed out earlier (Chapter 2.3.2) the population genetic drift has most likely remained constant over the last 40 years. There is no data to substantiate this but demographic patterns and population ratios have not changed significantly, except for an increased proportion of Xhosa speaking Blacks migrating to the Western Cape

since 1980. The origin of the Black population in the Western Cape has remained the same (Eastern Cape region, formerly Transkei and Ciskei) (Table 5.2).

The prevalence rate for pulmonary TB among those of Mixed Ancestry in the Western Cape region was estimated in 1992 to be 704:100 000 (Eggers et al 1994). There is thus a possibility that some of the control group were infected with pulmonary tuberculosis. The absence of significant weight loss and persistent coughing on direct questioning (all adult and child controls) supplemented with a normal clinical examination (in all children and Black Ancestry adult controls) makes this less likely.

TABLE 5.2: POPULATION STATISTICS - WESTERN CAPE REGION

		Total Population		TB Incidence			
	Year	Black	Mixed Ancestry	Black		Mixed Ancestry	
		(Rounded off to thousands)		(per 100000)			
	1950	60	297	200	(202)*	445	(414)
	1960	75	417	410	(428)	522	(526)
	1970	108	606	288	(334)	290	(305)
	1980	183	775	212	(215)	315	(316)
	1991	539	1991	198	(191)	709	(652)
	1995	640	2215	no data available			
<14yrs	1991	146 (26%)	645(32%)	no data available			

* Values in brackets are the National average figures

References: Tuberculosis Control Programme - 1992
Epidemiological Comments Vol 21(1); 1994
Population Census 1950, 1970, 1980
CSSD Report: Provincial Statistics 1994 (Part I), Printed by: The Government Printer, Pretoria

5.3 IgG AND IgG SUBCLASSES

The mean values of total IgG and the four subclasses were grossly elevated. This is in keeping with other chronic infections/inflammatory conditions such as histoplasmosis and leprae and has previously been described in tuberculosis (Lenzini et al 1997; Kaplan et al 1980).

The subclasses with the highest elevation were IgG2 and IgG4 (and IgG3 in the Mixed Ancestry female group). Specific IgG subclass stimulation in a disease like *M. tuberculosis* is due to multiple protein and polysaccharide antigens. There is evidence that certain protein antigens are more specific (ea: 32-kDa protein) than non-protein antigens (Harboe et al 1992). A number of other factors influence IgG subclass production including age, sex and allotype expression. Also it has been claimed that protein antigens influence IgG1 production most (Jefferis et al 1990). We could not find any influence of age, sex or allotype expression on these subclass values. Because of these multiple factors no firm conclusion can be drawn from the finding of IgG2 and IgG4 predominance in our study.

Further studies utilizing mycobacterium specific antigen detection and possibly isolated B-cells from disease patients might elucidate the significance of IgG subclass production.

5.4 SEVERITY SCORE

A disease severity score is a useful tool for monitoring disease response to therapy. Most scoring systems focus on one anatomical system and its' related symptoms and signs. In the severity score used in this study a multisystem approach was selected. Nutrition, as evaluated by height for body mass, respiratory involvement as reflected on chest x-ray, liver metabolism (and nutrition) as reflected by albumin values and haemopoiesis as depicted by haemoglobin concentration.

One other study on children with tuberculosis utilizing a severity score could be found (Tidjani et al 1986). This scoring system included symptoms and signs of lung disease, chest x-ray findings, sputum analysis for *M. tuberculosis* and response to therapy. In the above mentioned study the scoring system proved useful retrospectively in aiding TB diagnosis. Our scoring system could not differentiate between bacterial proven and non-proven cases. Age and allotype expression and subclass levels did not influence the severity score either. Since the study did not follow patients through till x-ray resolution, the score could not be validated against outcome.

5.5 ALLOTYPES

A comparison of allotypes frequency in the control groups with other studies in the same populations is shown in Table 5.3.

TABLE 5.3: COMPARISON OF ALLOTYPES IN SIMILAR SOUTH AFRICAN POPULATIONS

	Mixed Ancestry				Blacks		
Allotype	**Present Study**	**Goddard 1994**	**Jenkins 1970**	**Steinberg 1975/81**	**Present Study**	**Goddard 1994**	**Jenkins 1970**
G1m(a)	99.5%	-	95%	100%	100%	-	100%
G1m(f)	57%	62%	55%	8%	6%	2%	1.4%
G2m(n)	44%	34%	-	-	5%	3%	-
G3m(g^1)	95%	-	-	63%	97%	-	-
G3m(b^1)	87%	-	87%	61%	92%	-	86%

There is a good correlation between the studies quoted which strengthens the claim of representibility of the control groups used.

5.6 ALLOTYPES IN *M. TUBERCULOSIS*

Within the Black Ancestry cohort only G3m(g1) allotype showed an association with pulmonary tuberculosis. In the TB proven group of 20 children, 13 (65%) had

G3m(g1) allotype. In the control group 97% had the allotype. If however only children of the Black Ancestry group (cohort and controls) are compared this difference disappears. These child controls were all of one age group (8-12 months), and had (presumedly) a much lower IgG3 level than the adults. (Goddard 1994) It is thus possible that due to the high backgrounds in the ELISA used (two SD above negative gave a OD of 1.402), the test was not sensitive enough for decreased epitope frequency in the low IgG3 subclass concentrations in the child sera. In the Mixed Ancestry child controls the mean age was 35 months and here the significant differences remained if only children were compared. (Data not shown) Another possibility is that the small Black Ancestry child control group (only 21controls) showed heterozygosity in their expression of the G3m(g1) allotype. This could also lead to spureous negative results. **In conclusion**: the absence of the G3m(g1) allotype was associated with pulmonary tuberculosis in the Mixed and Black Ancestry groups (with some reservations). Among the Mixed Ancestry group there was a strong association (p = 0.01 and 0.04) of pulmonary tuberculosis with the absence of G2m(n) and G1m(f).

The findings from this study differs from the three other studies on TB and Allotypes. (Table 5.4)

TABLE 5.4: ALLOTYPES IN *M. TUBERCULOSIS*

	Population Studied			
Allotype	Indonesia*	Moscow#	Tuvinia+	Cape Town
G1m(a)	No difference	**Increased**	**Increased**	No difference
G1m(f)	No difference	**Increased**	**Increased**	***Decreased***
G1m(x)	***Decreased***	*Decreased*	**Increased**	Not done
G2m(n)	Not done	Not done	Not done	***Decreased***
G3m(g^1)	***Decreased***	Not done	Not done	***Decreased***
G3m(b^1)	No difference	Not done	Not done	No difference
Km(1)	***Decreased***	Not done	Not done	Not done

References: * Gibson et al 1987 # Chukanova et al 1994
+ Matrakshin 1993

Some obvious differences can be noted: Increased G1m(f) in the Russian and Tuvinian patients and decreased in the present study. Also the G1m(x) gave similar discordant findings. All four studies clamed to have selected cultural (and thus genetically) similar cohorts as their patient group. We could state that no allotype (or haplotype) is consistently associated with pulmonary tuberculosis or for that matter any other disease in different populations groups as is depicted in table 5.5. Genetic susceptibility associated with a allotypic phenotype is then rather dependend on other mechanisms / modulation. Consideration should be given whether allotypes are merely genetic markers for some more fundamental abnormality (?receptor gene expression) in handling infectious agents. Recently Saha et al (1994) showed that defective T cell mediated responses to mycobacterial antigens in the murine model was due to an inability of the macrophage cells to deliver appropriate co-stimulatory signals to the T-helper cells. Could it be that allotypic expression is associated with macrophage

innate resistance? Evidence that antibodies influence macrophage function is scanty but reported by Forget (1976) in murine *in vitro* models. However we could not demonstrate any allotypes influences on antibody levels in PTB in our cohort of children.

TABLE 5.5: DISEASE ASSOCIATIONS WITH ALLOTYPES G1m(f) AND G2m(n)

Reference	Age	Race	Disease	Allotype G2m(n)	G1m(f)
Goddard	Child	Mixed Ancestry	*H. influenzae*	Absent	No difference
1994		Black	Meningitis	No difference	No difference
Granoff et al 1984	Child	Black	*H. influenzae*	No difference	Not done
		White	Meningitis	No difference	Not done
Demaine et al 1984	Adult	White	Idiopathic Membranous Nephropathy	Not done	Decreased
Ambrosino et al 1985	Child	White	*H. influenzae*	Absent	Not done
Takala et al 1991	Child	White	*H. influenzae*	No difference	Not done

5.7 FURTHER RESEARCH AVENUES

The finding that a genetic difference in immunoglobulin allotypic markers is associated with *M. tuberculosis* disease in Mixed Ancestry and Black children indicates that further research into the genetic related pathogenic mechanism(s) in tuberculosis could be profitable. A study of lymphocyte proliferation, macrophage killing ability and other functional studies, correlated with immunoglobulin allotype expression could provide useful information in this regard.

Allotype expression should be correlated with levels of subclass specific *M. tuberculosis* antibody production to elucidate possible mechanisms and changes in the humoral response to *M. tuberculosis* antigens which may indicate a role for antibody production in recovery or protection from *M. tuberculosis*. If cytokine patterns could be determined from in vitro stimulated T- and B-cells in the examined group, the effect of T-helper cell (Th1 or Th2) activity could be better documented.

5.8 FINAL CONCLUSION

There is a clear difference in G2m(n), G1m(f) and G3m(g^1) allotype (Mixed Ancestry) and less so in G3m(g^1) (Black race group) expression between controls and children with active tuberculosis. This support the hypothesis of genetic

susceptibility in tuberculosis. The very high incidence of PTB in the Mixed ancestry population group might well be related to a genetic determined increased susceptibility to *Mycobacterium tuberculosis*. No influence of Allotypes on IgG Subclasses levels in PTB could be shown due to the small numbers in each group.

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APPENDIX 1

G1m(a) ALLOTYPE ELISA

Reagents:

1. a. Stock glycine buffer: 0.05 M
   Adjust pH to 2.5 with HCl.
   b. Working solution:
   Glycine/Tris Buffer (pH 7.0 - 7.5)
   Add solid Tris to stock glycine buffer until pH is between 7.0 - 7.5
2. Tris Buffered Saline (TBS): 0.2 M Tris, 0.15 M NaCl
   NaCl 9.0 gm
   Tris 2.42 gm
   Add distilled H_20 750 ml. Adjust pH to 7.5 using 1N HCl. Make up to 1 litre with distilled H_20.
3. Blocking solution: 1% Casein in 0.01 M Tris, 0.15 M NaCl
   Dissolve 9 g NaCl, 1.211 g Tris and 10 g Casein into 1 litre of distilled water. Stir until well dissolved.
4. Monoclonal antibody
   R2102 (1 ml lyphilized serum), CLB (Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service) diluted 1/1000 in 1/10 blocker in TBS.
5. Conjugate:
   Cappel HRPO goat anti-mouse (gamma chain specific) diluted 1/1000 in 1/10 Blocker in TBS.
6. OPD:
   Dilute powder with citrate buffer $\rightarrow$ 2.5 mg/ml. For two plates use at least 8 mg powder.
   8 mg $\rightarrow$20 ml citrate buffer. Immediately before use add H_2O_2 1 µl/ml (e.g Total volume = 20 mls, then add 20 µl H_2O_2)
7. Citrate Buffer (0.1 M)
   Solution A: Citric acid = 4.2 g/200 ml
   Solution B: Sodium citrate = 5.888 g/200 ml
   Add 20.5 ml solution A to 29.5 ml solution B
   Adjust pH to 5.0 with 4 M NaOH. Make up to 100 ml with distilled H_20.
8. 2N H_2SO_4
   53.3 ml/litre = 2N

Method:
Reference sera:
R1113 (+ve control diluted 1/10000 in glycine/Tris buffer)
R1117 (-ve control diluted 1/10000 glycine/Tris buffer)
Mixed sera: R1113 into R1117 to contain 50%; 20% and 5% of R1113.

Patient sera:
1/10000 dilutions with glycine/Tris buffer

Serum blanks:
Glycine/Tris buffer.

1. Coat flat bottomed microtitre plates with 100 µl of reference sera, patient sera and glycine/Tris buffer respectively for test and blank. Incubate for 30 minutes at room temperature.
2. Wash with TBS/0.01% Tween 20.
3. Block overnight at 4°C with 1% casein.
4. Wash 2x TBS/0.01% Tween.
5. Add 100 µl/well Moab diluted at 1/1000 in 1/10 blocker. Leave two rows as blanks (1/10 Blocker).
6. Wash 3x TBS/0.01% Tween.
7. Add 100 µl/well conjugate (1/1000). Incubate 30 minutes at room temperature.
8. Wash 5x TBS/Tween 0.01%.
9. Add 100 µl/well OPD/H_2O_2.
10. Stop reaction 50 µl/well 2N H_2SO_4.
11. Read at 492 nm.

APPENDIX 2

G1m(f) ALLOTYPE ELISA

Reagents:

1. a. Stock glycine buffer: 0.05 M
   3.75 g glycine/litre
   Adjust pH to 2.5 with HCl. Make up to 1 litre with distilled H20.
   b. Working solution:
   Glycine/Tris Buffer (pH 7.0 - 7.5)
   Add solid Tris to stock glycine buffer until pH is between 7.0 - 7.5
2. Tris Buffered Saline (TBS): 0.02 M Tris, 0.15 M NaCl
   NaCl 9.0 gm
   Tris 2.42 gm
   Add distilled H_20 750 ml. Adjust pH to 7.5 using 1N HCl. Make up to 1 litre with distilled H20.
3. Blocker: 2% BSA/0.05% Tween 20
   For 2 plates: 1.2 gm BSA. Add to 60 ml TBS. Add 30 µl Tween 20 and stir for 5 mins or until BSA has dissolved.
4. Monoclonal Antibody:
   Sigma anti IgG1 m(f)
   For two plates: Dilute 1/1500 i.e. 11 µl anti G1m(f) in 16.5 ml 1/10 blocker*
5. Conjugate: Cappel goat anti mouse IgG (gamma-chain specific)
   Dilute 1/1500 with 1/10 blocker
6. OPD: Dilute powder with citrate buffer → 2.5 mg/ml. For two plates use at least 8 mg powder.
   8 mg → 20 ml citrate buffer. Immediately before use add H_20_2 1 µl/ml (i.e. Total volume = 20 mls, then add 20 µl H_20_2)
7. Citrate Buffer (0.1 M)
   Solution A: Citric acid = 4.2 g/200 ml
   Solution B: Sodium citrate = 5.888 g/200 ml
   Add 20.5 ml solution A to 29.5 ml solution B
   Adjust pH to 5.0 with 4 M NaOH. Make up to 100 ml with distilled H20.
8. 2N H_2SO_4
   53.3 ml/litre = 2N

*1/10 blocker: Dilute 2% BSA/0.05% Tween, 1/10 with TBS

Method:
Standard curve: Dilute reference serum R1110 in glycine/Tris buffer - 1/500; 1/1000; 1/2500; 1/5000; 1/7500; 1/10000; 1/15000; 1/25000 and 1/50000.

Ref sera: R1117 (+ ve control) dilute 1/10000 in glycine/Tris buffer
R1113 (- ve control) dilute 1/10000 in glycine/Tris buffer

Patient sera: Dilute 1/10000 in Glycine/Tris buffer

Serum Blank: Glycine/Tris buffer.

1. Add 100 µl standard curve dilutions, serum blank, reference sera and patient sera to plate. Incubate 30' at room temperature.
2. Wash plates 1x with TBS/0.01% Tween 20.
3. Add 250 µl/well 2% BSA/0.05% Tween (Blocker). Incubate 30' at room temperature.
4. Wash plates 2x with TBS/0.01% Tween.
5. Add 100 µl/well. MoAb - anti G1m(f) 1/1500 to rows A-C and E-G. Add 100 µl/well 1/10 blocker to rows D and H. Incubate 30' at room temperature.
6. Wash plates 4x with TBS/0.01% Tween.
7. Add conjugate 100 µl/well - anti mouse GAM conjugate 1/1500. Incubate 30' at room temperature.
8. Wash plates 5x with TBS/0.01% Tween.
9. Add 100 µl/well OPD/H_2O_2. Incubate 15' at room temperature.
10. Stop reaction by adding 50 µl/well 2N H_2SO_4.
11. Read at 492 nm.

APPENDIX 3

G2m(n) ALLOTYPE ELISA

Reagents:

1. a. Stock glycine buffer: 0.05 M
   3.75 g glycine/litre
   Adjust pH to 2.5 with HCl. Make up to 1 litre with distilled H_20.
   b. Working solution: Glycine/Tris Buffer (pH 7.0 - 7.5)
   Add solid Tris to stock glycine buffer until pH is between 7.0 - 7.5
2. Tris Buffered Saline (TBS): 0.01 M Tris, 0.15 M NaCl .
   NaCl 9.0 gm
   Tris 2.42 gm
   Add distilled H_20 →750 ml. Adjust pH to 7.5 using 1N HCl. Make up to 1 litre with distilled H_20.
3. Blocker: 2% BSA/0.05% Tween 20
   For 2 plates: 1.2 gm BSA. Add to 60 ml TBS.
   Add 30 µl Tween 20 and stir for 5 mins or until BSA has dissolved.
4. Monoclonal Antibody:
   Sigma anti IgG2m(n)
   For two plates: Dilute 1/10000
   5 µl + 495 µl 1/10 blocker* (1/100) then take 160 µl of 1/100 and add to 14.4 mls 1/10 blocker.
5. Conjugate: Cappel goat anti mouse IgG (gamma-chain specific)
   Dilute 1/1500 with 1/10 blocker
6. OPD: Dilute powder with citrate buffer → 2.5 mg/ml.
   For two plates use at least 8 mg powder.
   8 mg → 20 ml citrate buffer. Just before use add H_20_2 1 µl/ml (i.e. Total volume = 20 mls, then add 20 µl H_20_2)
7. Citrate Buffer (0.1 M)
   Solution A: Citric acid = 4.2 g/200 ml
   Solution B: Sodium citrate = 5.888 g/200 ml
   Add 20.5 ml solution A to 29.5 ml solution B
   pH to 5.0 with 4 M NaOH. Make up to 100 ml with distilled H_20.
8. 2N H_2SO_4
   53.3 ml/litre = 2N
9. Standard curve: Use pooled AB serum

*1/10 Blocker: Dilute 2% BSA (0.05% Tween 20)

Method:
Standard curve: Dilute pooled AB serum in glycine/Tris buffer - 1/2000; 1/4000; 1/6000; 1/8000; 1/10000; 1/15000 1/20000; 1/30000; 1/50000.

Ref sera: R1110 (+ ve control) dilute 1/15000 in glycine/Tris buffer
R1113 (- ve control) dilute 1/15000 in glycine/Tris buffer
Patient sera: Dilute 1/15000 in Glycine/Tris buffer
Serum Blank: Glycine/Tris buffer

1. Add 100 µl standard curve dilutions, serum blank, reference sera and patient sera to plate. Incubate 30' at room temperature.
2. Wash plates 1x with TBS/0.01% Tween 20.
3. Add 250 µl/well 2% BSA/0.05% Tween (Blocker). Incubate 30' at room temperature.
4. Wash plates 2x with TBS/0.01% Tween.
5. Add 100 µl/well. Moab - anti G2m(n) 1/10000 to rows A-C and E-G. Add 100 µl/well 1/10 blocker to rows D and H. Incubate 30' at room temperature.
6. Wash plates 4x with TBS/0.01% Tween.
7. Add conjugate 100 µl/well - Cappel goat anti mouse IgG (gamma-chain specific) 1/1500. Incubate 30' at room temperature.
8. Wash plates 5x with TBS/0.01% Tween.
9. Add 100 µl/well OPD/H_2O_2. Incubate 15' at room temperature.
10. Stop reaction by adding 50 µl/well 2N H_2SO_4.
11. Read at 492 nm

APPENDIX 4

G3m(g1) ALLOTYPE ELISA

Reagents:

1. a. Stock glycine buffer: 0.05 M
   3.75 g glycine/litre
   Add distilled H_20 (900 ml)
   Adjust pH to 2.5 with HCl. Make up to 1 litre with distilled H20.

   b. Working solution:
   Glycine/Tris Buffer (pH 7.0 - 7.5)
   Add solid Tris to stock glycine buffer until pH is between 7.0 - 7.5

2. Tris Buffered Saline (TBS), 0.02 M Tris, 0.15 M NaCl
   NaCl 9.0 gm
   Tris 2.42 gm
   Add distilled H20 →750 ml. Adjust pH to 7.5 using 1N HCl. Make up to 1 litre with distilled H20.

3 Blocker: 1% casein/0.01% Tween 20 in 001 M Tris, 0.15 M NaCl
   Dissolve 9 g NaCl, 1.211 g Tris and 10 g Casein into 1 litre of distilled water. Stir until completely dissolved.

4. Monoclonal antibody:
   R2005 diluted 1/250 with 1/10 blocker**

5. Conjugate: Cappel goat anti mouse IgG (gamma chain specific), for use dilute 1/1000 in 1/10 blocker.

6. OPD: Dilute powder with citrate buffer 2.5 ml/mg. For two plates use at least 8 mg powder.
   8 mg → 20 ml citrate buffer. Immediately before use add H_20_2 1 µl/ml (i.e. Total volume = 20 mls, then add 20 µl H_20_2)

7. Citrate Buffer (0.1 M)
   Solution A: Citric acid = 4.2 g/200 ml
   Solution B: Sodium citrate = 5.888 g/200 ml
   Add 20.5 ml solution A to 29.5 ml solution B
   Adjust pH to 5.0 with 4 M NaOH. Make up to 100 ml with distilled H_20.

8. 2N H_2SO_4
   53.3 ml/litre = 2N

**1/10 Blocker: Dilute 1% Casein with TBS

Method:
Reference Sera:
R1113 (+ve control) dilute 1/30 in glycine/Tris buffer
R1117 (-ve control) dilute 1/30 glycine/Tris buffer
Mixtures of R1113 and R1117: to contain 50%; 20% and 5% of R1113.
Patient serum diluted 1/30 in glycine/tris buffer
Serum Blank: glycine/tris buffer

1. Coat a flat bottomed microtitre plate. Add 100 µl of reference sera and test sera and incubate for 90 min at 37°C. Add 10 µl of glycine/Tris to blank wells. Add mixtures of reference sera.
2. Wash 1x with TBS/0.01% Tween 20.
3. Block with 1% casein overnight at 4°C.
4. Wash 3x with TBS/0.01% Tween 20.
5. Add 100 µl/well of Moab. Incubate for 90 min at room temperature.
6. Wash 4x with TBS/0.01% Tween 20.
7. Add conjugate 100 µl/well. Incubate 30 min at room temperature.
8. Wash 5x with TBS/0.01% Tween 20.
9. Add 100 µl/well OPD/H_2O_2. Incubate 15 min at room temperature.
10. Stop reaction with 50 µl/well 2N H_2SO_4.
11. Read at 492 nm.

APPENDIX 5

G3m(b1) ALLOTYPE ELISA

Reagents:

1. a. Stock glycine buffer: 0.05 m
   3.75 g glycine/litre
   Adjust pH to 2.5 with HCl. Make up to 1 litre with distilled H20.
   b. Working solution:
   Glycine/Tris Buffer (pH 7.0 - 7.5)
   Add solid Tris to stock glycine buffer until pH is between 7.0 - 7.5
2. Tris Buffered Saline (TBS), 0.02 M Tris, 0.15 M NaCl
   NaCl 9.0 gm
   Tris 2.42 gm
   Add distilled H20 → 750 ml. Adjust pH to 7.5 using 1N HCl. Make up to 1 litre with distilled H20.
3. Blocker: 1% casein/0.01% Tween 20, 0.01 M Tris, 0.15 M NaCl
   Dissolve 9 g NaCl with 1.211 g Tris and 10 g casein into 1 litre of distilled water until dissolved.
4. Coating serum:
   R1110 (1ml lypohilized serum). Central Laboratory, Netherlands Blood Transfusion Service. Serum contains G1m(a,f); G3m(b0, b1, b3, b4, b5, u, v)
5. Monoclonal antibody:
   R2007 (1 ml lyophilized serum)
   Antiserum against: G3m(b1/u) dilute 1/2500 in 1/10 blocker
6. Conjugate: Cappel goat anti mouse IgG conjugated to horse radish peroxidase. Dilute 1/1000 with 1/10 blocker.
7. OPD: Dilute powder with citrate buffer → 2.5 ml/mg. For two plates use at least 8 mg powder.
   8 mg → 20 ml citrate buffer. Just before use add H_2O_2 1 µl/ml (i.e. Total volume = 20 mls, then add 20 µl H_2O_2)
8. Citrate Buffer (0.1 M)
   Solution A: Citric acid = 4.2 g/200 ml
   Solution B: Sodium citrate = 5.888 g/200 ml
   Add 20.5 ml solution A to 29.5 ml solution B
   Adjust pH to 5.0 with 4 M NaOH. Make up to 100 ml with distilled H_2O.
9. 2N H_2SO_4
   53.3 ml/litre = 2N

Method:
Patient sera: diluted 1/2500 in glycine/Tris
Reference sera:
R1110 (coating serum) dilute 1/1000 in glycine/Tris buffer
R1117 (+ve control) dilute 1/2500 in 1/10 blocker. Mix with equal volumes of Moab
R1113 (-ve control) dilute 1/2500 in 1/10 blocker. Mix with equal volumes of Moab.

1. Coat microtitre (flat bottomed wells) plates with 100 µl of R1110. Blank wells are filled with Glycine/Tris buffer. Incubate at 37°C for 30 min. Duplicates for each sera and one blank well is thus prepared.
2. Wash plates 1x with TBS/0.01% Tween 20.
3. Prepare dilutions of reference, patient and Moab sera mixtures. Add equal volumes and allow to react in shaking waterbath at 37°C for 45 minutes.
4. Coat wells with 100 µl of sera mixtures. The two reference sera wells are prepared with duplicate sera and Moab mixture, duplicate sera alone and duplicate Moab alone. Incubate for 30 min at room temperature.
5. Wash 3x with TBS/ 0.01% Tween.
6. Add 100 µl of conjugate and incubate 30 min at room temperature.
7. Wash 5x with TBS/Tween (0.01%).
8. Add 100 µl/well of OPD/H_2O_2. Incubate 15 min at room temperature in the dark.
9. Stop reaction by adding 50 µl/well 2N H_2SO_4.
10. Read at 492 nm.

APPENDIX 6

VITAMIN A IN TB STUDY
FIRST QUESTIONNAIRE

identification

No ___ Name _____ Race _ Sex _
Hosp _ Hospspec _____ Ward ___
HospNorxh _____ HospNonsh _____ HospNobch _____
DOB _____ Age ___ DAdmtostudy _____
Dafteradm __ DofTBRx __ DofADrec __
Random _ CatTBDx _ InclDstudy _
Ifnottbwhatwasitthen? _____

housing

Type _ Rooms _ Adults __ Child __
Elec _ Water _ Transkei _

family

Mmarried _ Mempl _ Fempl _
Sincome _ Income ____
Dcare _
Sibs __ PosPt _

immunizations

Complete _ BCGnodos _ Measdurtudy _ Specmeasles_____

nutrition

BWT ____ Size _
BFED _ BFPer __
ADFF _

tbhistory

ContAd _ ContCh _ Screened _
Proph _
LOA _ LOAPer __
LOW _ LOWPer __
RWT _
NSweats _ NSPer __
Cough _ CPer __
Wheeze _ WhPer __
SOB _ SOBPer __
Fever _ FPer __
Rill _
RRTI _
PITB _
RANTIB _
TBHxadd_____
PrevHx_____
Developm _

SECOND QUESTIONNAIRE

Study number ____
Name _____

clinical examination

WeeksRx6w __	WeeksRx3m __	WeeksRx6m __	
WTPr _____	WT6w _____	WT3m _____	WT6m_____
HTPr _____	HT6w _____	HT3m _____	HT6m_____
HCPr _____	HC6w _____	HC3m _____	HC6m_____
TnorPr _	T6w _	T3m _	T6m _
PalPr _	Pal6w _	Pal3m _	Pal6m_
OedPr _	Oed6w _	Oed3m _	Oed6m_
MalPr _	Mal6w _	Mal3m _	Mal6m_
ECPr _	EC6w _	EC3m _	EC6m_
RicPr _	Ric6w _	Ric3m _	Ric6m_
Ricadd_____			
FPr _	F6w _	F3m _	F6m _
ENPr _	EN6w _	EN3m _	EN6m_
LnPr _	Ln6w _	Ln3m _	Ln6m_
LaPr _	La6w _	La3m _	La6m_
LiPr _	Li6w _	Li3m _	Li6m_
CLPr _	CL6w _	CL3m _	CL6m_
CYPr _	CY6w _	CY3m _	CY6m_
BCGscPr _	BCGsc6w _	BCGsc3m _	BCGsc6m_

resp

RnorPr _	Rnor6w _	Rnor3m _	Rnor6m_
RrPr _	Rr6w _	Rr3m _	Rr6m_
RECPr _	REC6w _	REC3m _	REC6m_
DecMMPr _	DecMM6w _	DecMM3m _	DecMM6m_
DulLUPr _	DulLU6w _	DulLU3m _	DulLU6m_
DulLMPr _	DulLM6w _	DulLM3m _	DulLM6m_
DulLLPr _	DulLL6w _	DulLL3m _	DulLL6m_
DulRUPr _	DulRU6w _	DulRU3m _	DulRU6m_
DulRMPr _	DulRM6w _	DulRM3m _	DulRM6m_
DulRLPr _	DulRL6w _	DulRL3m _	DulRL6m_
DecAELUPr _	DecAELU6w _	DecAELU3m _	DecAELU6m _
DecAELMPr _	DecAELM6w _	DecAELM3m _	DecAELM6m _
DecAELLPr _	DecAELL6w _	DecAELL3m _	DecAELL6m _
DecAERUPr _	DecAERU6w _	DecAERU3m _	DecAERU6m _
DecAERMPr _	DecAERM6w _	DecAERM3m _	DecAERM6m _
DecAERLPr _	DecAERL6w _	DecAERL3m _	DecAERL6m _
BrbrLUPr _	BrbrLU6w _	BrbrLU3m _	BrbrLU6m _
BrbrLMPr _	BrbrLM6w _	BrbrLM3m _	BrbrLM6m _
BrbrLLPr _	BrbrLL6w _	BrbrLL3m _	BrbrLL6m _
BrbrRUPr _	BrbrRU6w _	BrbrRU3m _	BrbrRU6m _
BrbrRMPr _	BrbrRM6w _	BrbrRM3m _	BrbrRM6m _
BrbrRLPr _	BrbrRL6w _	BrbrRL3m _	BrbrRL6m _
CrLUPr _	CrLU6w _	CrLU3m _	CrLU6m_
CrLMPr _	CrLM6w _	CrLM3m _	CrLM6m_

CrLLPr _ CrLL6w _ CrLL3m _ CrLL6m_
CrRUPr _ CrRU6w _ CrRU3m _ CrRU6m_
CrRMPr _ CrRM6w _ CrRM3m _ CrRM6m_
CrRLPr _ CrRL6w _ CrRL3m _ CrRL6m_
IStPR _ ISt6W _ ISt3M _ ISt6M_
EStPr _ ESt6w _ ESt3m _ ESt6m_
EWhlocPr _ EWhloc6w _ EWhloc3m _ EWhloc6m _
IWHPr _ IWH6w _ IWH3m _ IWH6m_
ChDescribe
_TrPr _ Tr6w _ Tr3m _ Tr6m_
NEBPr _ NEB6w _ NEB3m _ NEB6m_
TOLPr _ TOL6w _ TOL3m _ TOL6m3
Rch6w _ Rch3m _ Rch6m_
PATHPr _ PATH6w _ PATH3m _ PATH6m_
SBIPr _
NECC6w _ NECC3m _ NECC6m_
Radd

abd

AnorPr _ Anor6w _ Anor3m _ Anor6m_
HepPr _ Hep6w _ Hep3m _ Hep6m_
SplPr _ Spl6w _ Spl3m _ Spl6m_
PLnPr _ PLn6w _ PLn3m _ PLn6m_
AscPr _ Asc6w _ Asc3m _ Asc6m_
PLEPr _ PLE6w _ PLE3m _ PLE6m_
GOR _ GORRx _ GORSx _
Ach6w _ Ach3m _ Ach6m_
Abdadd

cvs

CVnorPr _ CVnor6w _ CVnor3m _ CVnor6m_
CVch6w _ CVch3m _ CVch6m_
CVadd

cns

CNnorPr _ CNnor6w _ CNnor3m _ CNnor6m_
TBMPr _ TBM6w _ TBM3m _ TBM6m_
HcefPr _ Hcef6w _ Hcef3m _ Hcef6m_
TbclPr _ Tbcl6w _ Tbcl3m _ Tbcl6m_
HlossPr _ Hloss6w _ Hloss3m _ Hloss6m_
VlossPr _ Vloss6w _ Vloss3m _ Vloss6m_
LTSxPr _ LTSx6w _ LTSx3m _ LTSx6m_
CNabnPr _ CNabn6w _ CNabn3m _ CNabn6m_
SeizPr _ Seiz6w _ Seiz3m _ Seiz6m_
DevrPr _ Devr6w _ Devr3m _ Devr6m
CNch6w _ CNch3m _ CNch6m_
CNadd

other systems

ENTnorPr _ ENTnor6w _ ENTnor3m _ ENTnor6m _
ENTch6w _ ENTch3m _ ENTch6m_
ENTadd_____
SknorPr _ Sknor6w _ Sknor3m _ Sknor6m_
Skch6w _ Skch3m _ Skch6m_
Skadd_____
SpnorPr _ Spnor6w _ Spnor3m _ Spnor6m_
RMSSnorPr _ RMSSnor6w _ RMSSnor3m _ RMSSnor6m _
MSSch6w _ MSSch3m _ MSSch6m_
MSSadd_____
Other1_____
Other2_____
Other3_____
Other4_____
Other5_____
Ievent6w _ Ievent3m _ Ievent6m _
Ieventadd_____

skin tests

TinePr _ Tine6w _ Tine3m _ Tine6m_
MantPr __ Mant6w __ Mant3m __ Mant6m__
CreacPr _ Creac6w _ Creac3m _ Creac6m_

treatment
INH _ Rif _ PZA _ Etham _ Ethion _ Strep _
Other_____
Dose _ Compl _ Complspec_____
Sterdur _ Stercourses _ Sterindic_____
MVTA&D __ MVTother __ Fe __ Fedose __ Folate __
Antib _ Respsup _

chemistry/micro: tb diagnosis only

Satype1 _ Satype2 _ Satype3 _ Satype4_
Date1 _____ Date2 _____ Date3 _____ Date4_____
Prot1 _____ Prot2 _____ Prot3 _____ Prot4_____
sprot1 _____ sprot2 _____ sprot3 _____ sprot_____
ADA1 _____ ADA2 _____ ADA3 _____ ADA4_____
Glob1 _____ Glob2 _____ Glob3 _____ Glob4_____
Cl1 ___ Cl2 ___ Cl3 ___ Cl4 ___
sCl1 ___ sCl2 ___ sCl3 ___ sCl4___
Gluc1 _____ Gluc2 _____ Gluc3 _____ Gluc4_____
GlucL1 _ GlucL2 _ GlucL3 _ GlucL4_
sgluc1 _____ sgluc2 _____ sgluc3 _____ sgluc4_____
Lymph1 _____ Lymph2 _____ Lymph3 _____ Lymph4_____
Poly1 _____ Poly2 _____ Poly3 _____ Poly4_____
MNC1 _____ MNC2 _____ MNC3 _____ MNC4_____
LDH1 ___ LDH2 ___ LDH3 ___ LDH4___
sLDH1 ___ sLDH2 ___ sLDH3 ___ sLDH4___

histology

Satype4 _ Satype5 _
Date4 _____ Date5 _____

SugTB4 _ SugTB5 _
Inconcl4 _ Inconcl5 _
AFB+4 _ AFB+5 _

mycobacterial culture

Satype6 _	Satype7 _	Satype8 _	Satype9 _	Satype10 _
Date6 _____	Date7 _____	Date8 _____	Date9_____	Date10 <dd/
mCult6 _	Cult7 _	Cult8 _	Cult9 _	Cult10 _
Mtb _	Mtb7 _	Mtb8 _	Mtb9 _	Mtb10 _
Sens6 _	Sens7 _	Sens8 _	Sens9 _	Sens10 _
Other6 _	Other7 _	Other8 _	Other9 _	Other10 _

cultadd_____