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ANTIBODIES TO *MYCOBACTERIUM TUBERCULOSIS* MYCOLIC ACIDS IN PATIENTS WITH PULMONARY TUBERCULOSIS

GUNTER KLAUS SCHLEICHER

ANTIBODIES TO *MYCOBACTERIUM TUBERCULOSIS* MYCOLIC ACIDS IN PATIENTS WITH PULMONARY TUBERCULOSIS

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Medicine (Internal Medicine)

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ABSTRACT

ANTIBODIES TO *MYCOBACTERIUM TUBERCULOSIS* MYCOLIC ACIDS IN PATIENTS WITH PULMONARY TUBERCULOSIS.

Introduction and Aim: The waxy outer cell wall of mycobacteria consists mainly of mycolic acids (MA). The unique immuno-stimulatory properties of MA via the CD1-restricted antigen presentation pathway have been demonstrated in humans. Purification and isolation of *M.tuberculosis* (MTB) MA has allowed them to be applied as an antigen in an ELISA-based sero-diagnostic assay to detect specific antibodies in the sera of humans. The aim of the study was to measure the levels of antibody to MA in the sera of patients with culture proven pulmonary tuberculosis (PTB), and in control subjects without evidence of tuberculosis.

Methods: Sera from 118 human subjects were tested; 59 patients with proven PTB and 59 control subjects without evidence of tuberculosis. Each group consisted of HIV-seropositive and HIV-seronegative subjects. The endpoint was the detection of specific antibodies to MTB MA in the sera, before and after precipitation of immune complexes, by a newly developed ELISA using purified MA as an antigen and goat anti-human antibodies. The absorbancies were measured through a 450nm filter.

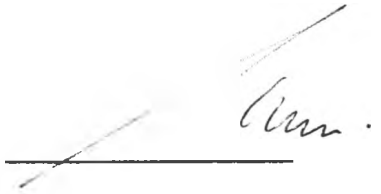
Results: The two groups of subjects were well matched for age, gender, race and HIV status. In patients with PTB compared to

subjects without tuberculosis, the corrected signal intensities (mean ± SD) in sera (0.3646 ± 0.2349 vs 0.2502 ± 0.2013, p=0.0053) and in precipitates (0.2261 ± 0.1509 vs 0.1432 ± 0.1102, p=0.0009) were significantly higher. In a subgroup analysis, patients with PTB who were HIV-seropositive had significantly higher antibody levels compared to HIV-seropositive patients without PTB, in both sera (0.393 ± 0.263 vs 0.253 ± 0.227, p=0.0132) and precipitates (0.242 ± 0.169 vs 0.128 ± 0.083, p=0.0003)

Conclusion: Infection with MTB in humans elicits a specific antibody response to mycobacterial MA. The antibody response is preserved in HIV-seropositive patients despite a declining CD4 T-lymphocyte count. This ELISA has the potential to be developed further to produce a rapid sero-diagnostic assay for the detection of tuberculosis.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Medicine (Internal Medicine) in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university.



Gunter Klaus Schleicher

11 September 2001

Date

DEDICATION

Dedicated to my wife, Claudia, and daughter, Gemma.

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TABLE OF CONTENTS

	PAGE
ABSTRACT	**i**
DECLARATION	**iii**
DEDICATION	**iv**
ACKNOWLEDGMENT	**v**
TABLE OF CONTENTS	**vi**
LIST OF TABLES	**xi**
LIST OF FIGURES	**xiii**

CHAPTER		PAGE
1.	**TUBERCULOSIS**	
1.1	INTRODUCTION	1
1.2	HISTORY	1
1.3	TUBERCULOSIS: THE GLOBAL EPIDEMIC	2
1.4	TUBERCULOSIS AND THE HIV EPIDEMIC	3
1.5	MICROBIOLOGY OF TUBERCULOSIS	5
1.6	MYCOBACTERIAL MYCOLIC ACIDS	6
1.7	IMMUNOPATHOGENESIS OF TUBERCULOSIS	
1.7.1	IMMUNOLOGY OF TUBERCULOSIS	7

1.7.2 CD1-RESTRICTED PRESENTATION OF MYCOBACTERIAL NONPEPTIDE ANTIGENS TO T-LYMPHOCYTES 10

1.7.3 THE IMMUNOGENICITY OF MYCOLIC ACIDS 12

2. THE DIAGNOSIS OF TUBERCULOSIS

2.1 INTRODUCTION 14

2.2 MICROSCOPY FOR ACID-FAST BACILLI 14

2.3 CULTURE OF MYCOBACTERIA 16

2.4 PHAGE SYSTEMS 17

2.5 CHEST RADIOGRAPHY 17

2.6 TUBERCULIN SKIN TESTING 18

2.7 BLOOD IMMUNE-BASED DIAGNOSIS 20

2.8 NUCLEIC ACID AMPLIFICATION ASSAYS 21

2.9 ADENOSINE DEAMINASE 22

2.10 SERODIAGNOSTIC ASSAYS FOR THE DETECTION OF TUBERCULOSIS 24

2.11 LIMITATIONS OF CURRENTLY AVAILABLE AGENTS OR TECHNIQUES FOR TUBERCULOSIS 27

2.12 AN ELISA FOR THE DETECTION OF ANTIBODIES AGAINST *M.TUBERCULOSIS* MYCOLIC ACIDS 28

3. THE STUDY

3.1 INTRODUCTION 30

3.2 HYPOTHESIS 30

3.3 STUDY OBJECTIVES 30

3.4 METHODS

3.4.1 GENERAL 31

3.4.2 PATIENTS 32

3.4.3 INCLUSION AND EXCLUSION CRITERIA 33

3.5 AN ELISA FOR THE DETECTION OF ANTIBODIES TO *M.TUBERCULOSIS* MYCOLIC ACIDS IN HUMAN SERA 34

3.6 DATA RECORDED 35

3.6.1 DEMOGRAPHIC AND CLINICAL DATA 35

3.6.2 LABORATORY DATA 36

3.6.2.1 HAEMATOLOGY 36

3.6.2.2 BIOCHEMISTRY 36

3.6.2.3 HIV AND SERUM T-CELL SUBSETS 36

3.6.2.4 SYPHILIS SEROLOGY 36

3.6.2.5 SPUTUM MICROSCOPY AND CULTURE 37

3.6.3 CHEST RADIOGRAPH 37

3.7 END POINT 37

3.8 DATA ANALYSIS 38

3.9 ETHICS 38

4.	**RESULTS**	
4.1	DEMOGRAPHIC DATA	39
4.2	PREVIOUS AND CURRENT MEDICAL CONDITIONS	39
4.3	CLINICAL DATA	41
4.4	LABORATORY DATA	
4.4.1	HAEMATOLOGICAL DATA	44
4.4.2	BIOCHEMISTRY DATA	44
4.4.3	CD4 T-LYMPHOCYTE DATA	46
4.5	MICROBIOLOGY DATA	46
4.6	CHEST RADIOGRAPHS	48
4.7	RESULTS OF THE ELISA TO DETECT ANTIBODIES TO *M.TUBERCULOSIS* MYCOLIC ACIDS IN SERA	
4.7.1	ALL TP AND TN PATIENTS	50
4.7.2	HIV-SEROPOSITIVE TP AND TN PATIENTS	50
4.7.3	HIV-SERONEGATIVE TP AND TN PATIENTS	55
4.8	THE RELATIONSHIP BETWEEN CD4 T-LYMPHOCYTE COUNT AND ANTIBODIES TO MYCOLIC ACIDS IN HIV-SEROPOSITIVE PATIENTS	57
4.9	SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE AND NEGATIVE PREDICTIVE VALUES FOR THE ELISA	57
5.	**DISCUSSION**	61

5. DISCUSSION 61

6. STRENGTHS AND WEAKNESSES OF THE STUDY 68

7. CONCLUSION 70

8. REFERENCES 72

9. APPENDIX

9.1 SUBJECT INFORMATION SHEETS 87

9.2 SUBJECT CONSENT FORM 91

9.3 ELISA METHOD 92

9.4 PATIENT DATA SHEET 97

LIST OF TABLES

TABLE		PAGE
4.1	DEMOGRAPHIC FEATURES OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS	40
4.2	PREVIOUS AND CURRENT MEDICAL CONDITIONS IN 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS	42
4.3	CLINICAL FEATURES OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS	43
4.4	HAEMATOLOGICAL AND BIOCHEMICAL LABORATORY DATA IN 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS	45
4.5	COMPARISON OF CD4 T-LYMPHOCYTE COUNTS IN 39 HIV-SEROPOSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 40 HIV-SEROPOSITIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS	47
4.6	COMPARISON OF RADIOGRAPHIC FEATURES ON CHEST RADIOGRAPH OF 20 HIV-SERONEGATIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 39 HIV-SEROPOSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS	49
4.7	RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 PATIENTS WITHOUT PULMONARY TUBERCULOSIS	51
4.8	RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 39 HIV-SEROPOSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 40 HIV-SEROPOSITIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS	53
4.9	RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 20 HIV-SERONEGATIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 19 HIV-SERONEGATIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS	56

4.10 SENSITIVITY, SPECIFICITY, POSITIVE AND NEGATIVE PREDICTIVE VALUES OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) IN 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 WITHOUT PULMONARY TUBERCULOSIS 60

5.1 COMPARISON OF THE SENSITIVITY AND SPECIFICITY OF DIFFERENT SERO-DIAGNOSTIC ASSAYS TO DETECT TUBERCULOSIS IN POPULATIONS WITH A HIGH PREVALENCE OF TUBERCULOSIS AND HIV INFECTION 65

LIST OF FIGURES

FIGURE		PAGE
4.1	ANTI-MYCOLIC ACID ANTIBODY LEVELS IN PRECIPITATED SERA OF 59 PATIENTS WITHOUT TUBERCULOSIS (TN) AND IN 59 PATIENTS WITH TUBERCULOSIS	52
4.2	ANTI-MYCOLIC ACID ANTIBODY LEVELS IN PRECIPITATED SERA OF 59 PATIENTS WITHOUT TUBERCULOSIS (TN) AND IN 59 PATIENTS WITH TUBERCULOSIS, GROUPED ACCORDING TO HIV STATUS	54
4.3	A COMPARISON OF THE ANTI-MYCOLIC ACID ANTIBODY LEVELS (PRECIPITATE) TO THE CD4 T-LYMPHOCYTE COUNT IN HIV-SEROPOSITIVE PATIENTS WITH TUBERCULOSIS	58
4.4	A COMPARISON OF THE ANTI-MYCOLIC ACID ANTIBODY LEVELS (PRECIPITATE) TO THE CD4 T-LYMPHOCYTE COUNT IN HIV-SEROPOSITIVE PATIENTS WITHOUT TUBERCULOSIS	59

1. TUBERCULOSIS

1.1 INTRODUCTION

More than a hundred years after the discovery of the tubercle bacillus, tuberculosis (TB) remains a major global health problem. Both the emergence of the human immunodeficiency virus (HIV) pandemic and multi-drug resistant strains of *Mycobacterium tuberculosis* have further exacerbated this already serious situation.

Despite major advances in the control of this disease, it continues to be a leading infectious cause of death, particularly amongst disadvantaged members of society (1).

1.2 HISTORY

Robert Koch first described the tubercle bacillus in 1882(2). Available evidence suggests that TB predates written records and has had a widespread occurrence for several millennia. The earliest evidence of TB in humans comes from the study of skeletal remains in Europe and Egypt, estimated to be over 5000 years old (3). Acid-fast bacilli and typical spinal deformities were found in a Peruvian mummy from around AD 700, providing evidence for pre-Columbian TB in South America (4).

The history of TB is linked to demographic and historical variations in social and environmental conditions. Population movements, urbanization, colonization, industrialization, and war have all led to changes in risk factor profiles in populations, and have affected the

occurrence and distribution of this disease (5). Through the ages the burden of TB has been borne by the most disadvantaged members of society, and is believed to be a social disease and a useful barometer of the standard of living in any society. Factors such as urbanization, inadequate housing, unemployment, poverty and the high prevalence of HIV have created large disparities between TB incidence and mortality rates amongst different racial groups in South Africa. Although the prevalence and mortality rates of TB declined steadily from 1900 to the mid-1980s, there has been a worldwide resurgence over the past 15 years, particularly in Africa (6).

1.3 TUBERCULOSIS: THE GLOBAL EPIDEMIC

In 1993 the WHO took the unique step of declaring TB to be a world health emergency (7). The resurgence of TB was observed in the mid-1980s and continued throughout the 1990s in both industrialized and developed countries.

In 1997, eight million new TB cases (95% in developing countries) and three million deaths from the disease (98% in developing countries) were estimated, with 32% of the world`s population (1,86 billion people) infected with *M.tuberculosis* (8). The global case fatality rate is currently 23%, but exceeds 50% in some African countries with high HIV prevalence rates (9). It is estimated that deaths from TB will increase from three million to five million people a year by the year 2015 (10).

In a recent report by WHO, South Africa was rated to have the eighth highest incidence of TB in the world, with 392 new cases

per 100 000 persons per year, and a prevalence of 604 cases per 100 000 persons (11). The percentage of the population infected with *M.tuberculosis* was estimated at 38%, whilst those co-infected with HIV and *M.tuberculosis* constituted 45% of all new cases of TB (11). The case fatality rate amongst all TB cases in South Africa has been estimated at 42% (11).

Over the past few years there have been major changes in the epidemiology of TB, with an increase in TB cases reported in both developed and developing countries (12). The principal reasons for this are world population increase, co-infection with HIV, poverty, TB control program decline, multi-drug resistant TB, and immigration (10). Parts of the world with the highest incidence of TB include sub-Saharan Africa, India, China, and Southeast Asia. In many of these areas the HIV epidemic is expected to dramatically increase the number of cases of TB over the next decade (13).

1.4 TUBERCULOSIS AND THE HIV EPIDEMIC

The HIV epidemic has played a major role in the resurgence of TB. HIV infection is the greatest risk factor for reactivating latent TB infection, with an estimated risk for developing TB in a tuberculin positive individual of seven to ten percent per year (14). HIV infection also markedly increases the susceptibility for new *M.tuberculosis* infection developing into active disease, which can be rapidly progressive (15). The increased susceptibility to TB is evident from an early stage of HIV infection, but becomes more pronounced as the degree of immunosuppression increases (16). The proportions of HIV-associated TB resulting from reactivation of

latent TB infection, and from progression of disease from recent infection vary according to the epidemiological circumstances (17). As a result, TB is particularly prevalent in populations likely to be co-infected with HIV and *M.tuberculosis*, especially in sub-Saharan Africa where 75% of these co-infected individuals live (17).

It is predicted that South Africa will become one of the countries most severely affected by HIV infection. The HIV prevalence for the whole country has increased from 0.76% in 1990 to 22.4% in 1999(18), with a parallel rise in the incidence of TB (19). Considering the concurrent spread of HIV in South Africa, it is predicted that the incidence of TB will rise by 10% - 20% every year.

The occurrence of TB in HIV-infected patients has been associated with immune activation and increased viral replication (20). *M.tuberculosis* increases HIV replication by inducing macrophages to produce tumour necrosis factor-alpha, interleukin-1, and interleukin-6 (21). Mortality rates of HIV-positive persons with TB are significantly higher than that of HIV-negative persons (6% vs 0.4%), despite comparable cure rates (93% vs 92%) using directly observed short-course rifampicin-containing regimens (22). The degree of immunosuppression is the most important predictor of survival in HIV-infected persons with TB, with prior opportunistic infections and CD4 T-lymphocyte counts of less than 200 cells per microlitre associated with increased mortality (23).

The clinical features of TB in patients with early HIV disease resemble those seen in HIV-seronegative patients. By contrast, HIV-seropositive individuals with advanced immunosuppression

are more likely to present with pulmonary TB with an atypical chest radiograph pattern, extra-pulmonary TB, or disseminated TB (24,25). In some studies HIV-seropositive patients with tuberculosis have a higher rate of smear-negative disease (26), with up to 43% of patients with culture-proven pulmonary tuberculosis being smear negative (27).

1.5 MICROBIOLOGY OF TUBERCULOSIS

M.tuberculosis belongs to the genus *Mycobacterium* that includes more than fifty other species, often collectively referred to as nontuberculous mycobacteria. TB is defined as a disease caused by members of the *M.tuberculosis* complex, which includes *M.tuberculosis, M.bovis, M. africanum*, and *M.microti.*

One distinguishing feature of the organisms belonging to the genus *Mycobacterium* is their cell envelope, which is characterized by a high lipid content (60% of the cell wall by weight)(28). The cell wall is composed of a core of three macromolecules covalently linked to each other (peptidoglycan, arabinogalactan, and mycolic acids), and a lipopolysaccharide (lipoarabinomannan), which is anchored to the plasma membrane (29). A variety of virulence factors of *M.tuberculosis* have been attributed to the cell wall structure of mycobacteria (30). Lipoarabinomannan resists the host cell oxidative response (31), and trehalose dimycolate and mycolic acids can elicit granuloma formation in animal tissue (32).

The cell wall components also give *Mycobacterium* its characteristic staining properties. The mycolic acid structure confers the ability to resist destaining by acid alcohol after being

stained by certain aniline dyes, leading to the term acid-fast bacillus (AFB)(33). *M.tuberculosis* has a slow rate of growth in culture, with a generation time of 20 to 24 hours on potato and egg based media (Middlebrook) or albumin in an agar based medium (Löwenstein-Jensen). In stained smears of clinical specimens, *M.tuberculosis* appears as 2-4 µm long rods, which may be evenly stained or beaded.

1.6 MYCOBACTERIAL MYCOLIC ACIDS

Mycolic acids are a complex group of long-chain alpha-alkyl, beta-hydroxy fatty acids produced by all mycobacteria. Mycolic acids in mycobacterial cell walls are present either as bound esters (arabinogalactan, a peptidoglycan-linked polysaccharide), or as lipids in trehalose dimycolate (also known as "cord factor", which consists of two mycolic acids linked to the disaccharide trehalose) (34). Similar mycolic acids are found in related taxa, for example, in *Corynebacterium* and *Nocardia* spp. (28).

Mycolic acids vary in the length of their fatty acid chains, and this variation in size is of value in defining the genera. The fatty acid component may also be modified by the presence of unsaturated bonds, cyclopropane rings, or methyl side chains. These are all termed alpha-mycolic acids. Others are more complex oxygenated mycolates, and are named after the group, i.e. keto-, methoxy-, epoxy- and wax-ester mycolic acids (28).

As well as being an important component of the mycobacterial cell wall, mycolic acids have been suggested as being virulence factors of *M.tuberculosis*. Mycolic acid glycolipids and cord factor have

been shown to elicit granuloma formation and induce prostaglandin-E2 and interleukin-1 synthesis in animal tissue (35).

Mycolic acid synthesis and metabolism is essential to the survival of *M.tuberculosis*. This has important therapeutic implications and has resulted in the use of drugs that inhibit mycolic acid biosynthesis, such as isoniazid and ethionamide, in the management of persons infected with tuberculous and non-tuberculous mycobacteria.

A method for the isolation and purification of mycobacterial lipid cell wall components elucidated in 1996 (36) has facilitated the investigation of the antigenic and immunostimulatory properties of mycolic acids.

1.7 IMMUNOPATHOGENESIS OF TUBERCULOSIS

1.7.1 IMMUNOLOGY OF TUBERCULOSIS

M. tuberculosis is transmitted from person to person via the respiratory route, by inhalation of droplet nuclei containing the tubercle bacilli. Entry into the lungs in a previously uninfected individual elicits a non-specific inflammatory response, which is usually asymptomatic (37). The inhaled cells of *M.tuberculosis* are ingested by alveolar macrophages and transmitted to the regional lymph nodes. The tubercle bacilli are either contained at this level, or reach the bloodstream and disseminate widely.

Most (90%-95%) primary and disseminated tuberculosis lesions heal in immunocompetent individuals, with tuberculosis – the clinical disease – developing in the minority who do not successfully contain the primary infection. The age of an individual,

general state of health, conditions of living, immunocompetence, nutrition, as well as ethnic and geographic differences affect the susceptibility to primary tuberculosis and its severity (38). Reactivation tuberculosis can develop following reactivation of a dormant primary or disseminated lesion in which bacilli survive within macrophages or granulomas in a dormant state. It usually occurs concomitantly with a period of waning of immune competence when the dormant bacilli are no longer held in check, and begin multiplying vigorously. The development of active tuberculosis in someone known to have been previously infected may be due to recrudescence of the initially infecting organism (endogenous reactivation), or re-infection with a new strain of *M.tuberculosis* (exogenous reinfection). A recent report from South Africa showed that re-infection was the cause of recurrent tuberculosis in 12 of 16 patients (39).

Two to six weeks after infection with *M.tuberculosis,* T-lymphocytes that specifically recognize *M.tuberculosis* antigens appear. This response can be demonstrated clinically by the development of a delayed-type hypersensitivity (DTH) response to intradermally injected tuberculin or PPD. T-lymphocyte activation is initiated by their contact with macrophages that have processed intracellular mycobacterial antigens and transported them to the cell surface, where they are presented in association with major histocompatability complex (MHC) proteins. This activation of T-lymphocytes by antigen-presenting cells (APC) results in their clonal proliferation, leading to the production of large numbers of antigen-specific lymphocytes. MHC class I molecules present to CD8-positive T-lymphocytes. MHC class II molecules present to

CD4-positive T-lymphocytes, which may either be Th1 or Th2 cells, depending on the cytokine profile they secrete upon activation.

Th1 cells secrete interleukin(IL)-2 and interferon gamma in response to IL-12 secreted by activated macrophages that are involved in processing and presenting ingested *M.tuberculosis* antigens. Activated macrophages and Th1 cells play important roles in DTH, containment of *M.tuberculosis* infection, and the formation of granulomas.

Th2 cells are also involved in the immune response to *M.tuberculosis*. Activated Th2 CD4 positive lymphocytes secrete IL-4 and IL-10, which activate B-lymphocytes and induce the production of antibodies (40). A protective role of the humoral immune response against *M.tuberculosis* has not been demonstrated. Th2 cells are considered to have a regulating function which allows them to terminate the defense reactions initiated by Th1 cells which, if left unchecked, could cause serious tissue damage (41).

Cytotoxic T-lymphocytes (CTL) are involved in protection against *M.tuberculosis*. CTL-mediated mycobacterial cell lysis involves a degranulation pathway that generates perforin and granzymes, and a Fas-Fas ligand dependent pathway that induces apoptosis of the target cell (42).

Antigen-presenting pathways other than those that are MHC class 1 or class 2 restricted can stimulate CTL responses. One such pathway, CD1-restricted CTL stimulation, will be discussed in more detail below.

Increased susceptibility to tuberculosis in HIV-infected persons is related to quantitative and qualitative defects of CD4-positive T-lymphocytes. On exposure to *M.tuberculosis*, T-lymphocytes from HIV-infected persons have an altered pattern of cytokine production. CD4 T-lymphocytes produce less interferon gamma but similar amounts of IL-4 and IL-10 as compared with T-lymphocytes from HIV-negative patients with tuberculosis (43). Thus, the reduced Th1 response and the enhanced Th2 response in HIV-infected patients contribute to their susceptibility to tuberculosis.

1.7.2 CD1-RESTRICTED PRESENTATION OF MYCOBACTERIAL NONPEPTIDE ANTIGENS TO T-LYMPHOCYTES

T-Lymphocytes are an essential component of efficient cell-mediated immunity against intracellular pathogens such as *M.tuberculosis*. The central paradigm is that T-lymphocytes recognize foreign antigens in the context of MHC class I and II molecules (44). T-lymphocyte recognition of antigen leads to their activation, lysis of infected macrophages, and the release of cytokines that activate macrophages to kill microbial organisms. Both CD4-negative-CD8-negative (double negative) and CD8-positive T-lymphocyte lineages have been shown to contain T - lymphocytes that recognize microbial lipid and glycolipid antigens in the context of human CD1 molecules (45). More recently the existence of CD4-positive CD1-restricted T-lymphocytes have been described in the lesions of leprosy patients (46).

The CD1 gene family consists of five non-polymorphic genes, CD1A, -B, -C, -D, and -E, mapped to a cluster on chromosome

1(47). Human CD1 are present on thymocytes and on professional antigen-presenting cells (APC), including dendritic cells, Langerhans cells, and mantle zone B-lymphocytes. All four of the known human CD1 proteins (CD1a, CD1b, CD1c, and CD1d) have been shown to mediate T-lymphocyte recognition of lipid or glycolipid antigens (48). Structural homology exists between CD1 and class I and II MHC molecules, suggesting an antigen presenting function. CD1 binds lipid antigen by accommodating the two lipid tails within the hydrophobic groove of its two distal membrane domains (48).

Human CD1a, CD1b and CD1c present lipid and glycolipid antigens of *M.tuberculosis* and *M.leprae* to T-lymphocytes (49). The two major antigens presented by CD1 are mycolic acids and lipoarabinomannan, both derived from the cell wall of mycobacteria (44). These antigens bind to APCs via pattern recognition molecules such as CD14 and the mannose receptor. The receptors then traffic such antigens through the endosomal pathway, where at acid pH, glycolipids bind via their lipid portions to CD1. The CD1-antigen complex then moves to the cell surface where it is recognized by the appropriate T-cell receptor (44). In this manner, the lipid and glycolipid antigens are first recognized by the innate immune system and then presented to the T-lymphocytes, which are part of the acquired immune response since their selection and expansion involves the development of memory. In this manner the CD1-restricted antigen presentation pathway links together the innate immune system (which includes macrophages, natural killer cells, and mast cells) and the acquired immune response.

CD4-positive and CD8-positive T-lymphocytes that are CD1 restricted produce high levels of interferon gamma, but little or no IL-4 upon stimulation with mycobacterial antigen (46, 50). This Th1 cytokine pattern directly contributes to cell-mediated immunity against intracellular infection by enhancing T-lymphocyte and macrophage activation. CD1-restricted T-lymphocytes also show a high degree of cytolytic activity and can lyse macrophages infected with virulent *M.tuberculosis*. Double negative CD1-restricted T-lymphocytes lyse targets through the Fas-Fas ligand pathway, while CD8 positive T-lymphocytes release granules containing perforin and granzymes (51). While double negative T-lymphocytes may have an immunoregulatory role by reducing the local cellular infiltrate, and thereby limiting tissue damage, the CD8-positive T-lymphocytes have the greatest impact in combating intracellular pathogens (42).

1.7.3 THE IMMUNOGENICITY OF MYCOLIC ACIDS

The immunogenicity of a molecule is its ability to induce an immune reaction. It is dependent on the chemical structure and properties of the molecule, and on the ability of the immune system to recognize it. Proteins, peptides, nucleic acids, and polysaccharides are naturally highly immunogenic, whilst lipids have until recently not been considered to be immunogenic.

In 1994 it was discovered that mycolic acid originating from *M.tuberculosis*, and presented via the CD1-restricted pathway, stimulated the proliferation of double negative T-lymphocytes (45). In the same year it was observed that *M.tuberculosis* mycolic acid conjugated to bovine serum albumin and administered to mice

elicited an antibody response that was specific to mycolic acid (52). On the basis of these observations it appears that mycolic acids are in fact immunogenic, and are capable of eliciting a cellular and humoral immune response.

In mice, antibodies to mycolic acids exist as soluble circulating IgM and IgG immune complexes that bind to their antigens with low affinity (52, 53). The omega-oxygenated fatty acid chains of mycolic acids appear to be the important antigenic determinants (54).

These specific antibodies to mycolic acids can be detected in human serum using a newly developed immunoassay device that will be described in greater detail in Chapter 2 (52, 53).

2. THE DIAGNOSIS OF TUBERCULOSIS

2.1 INTRODUCTION

The only confirmatory proof of infection with *M.tuberculosis* is the isolation of the organism from body secretions or tissue, either by direct microscopy of stained smears, or by culture. Although direct sputum smear examinations, combined with radiological examination of the lungs, still form the basis of detection of active pulmonary tuberculosis (PTB) (55), these techniques have many limitations which has resulted in the development of new techniques for the detection of *M.tuberculosis*. These include culturing of mycobacteria from secretions or tissue, detection of mycobacterial DNA in specimens, serological tests based on the detection of mycobacterial antigens or of human anti-mycobacterial antibodies, or immune-based tests.

2.2 MICROSCOPY FOR ACID-FAST BACILLI

A diagnosis of tuberculosis is commonly based on the finding of acid-fast bacilli (AFB) upon microscopic examination of a diagnostic specimen, such as a smear of expectorated sputum or of tissue (such as a lymph node biopsy) (56). Despite its limitations, such as a relatively low sensitivity and being highly operator dependent, microscopy is rapid, inexpensive, and easy to perform, with most modern laboratories using auramine-rhodamine staining and fluorescence microscopy.

Using routine Ziehl-Neelsen staining techniques, up to 83% of smear-positive cases of pulmonary tuberculosis are detected on

the first sputum specimen, 12% on the second, and 4% on the third (57). Evaluation of sputum requires that at least three good quality specimens of expectorated sputum should be collected, preferably in the morning, in all patients with suspected pulmonary tuberculosis. Smear from a non-processed sputum specimen can be positive if the specimen contains more than 100000 AFB per milliliter, but the sensitivity of this method can be increased to detect between 1000 and 10000 AFB per milliliter if the specimen is concentrated through appropriate processing and centrifugation, and the smear is examined by means of fluorescent microscopy (58). Sputum microscopy can be further improved by specimen homogenization using sodium hypochloride to liquefy the specimen and remove background debris (59).

A wide range of acid-fast smear positivity in expectorated sputum has been reported (31% to 81%) (60), but most studies find a 50% to 60% yield. False negative sputum microscopy may be due to inadequate specimen quality, inadequate number of specimens, poor staining technique, or inadequate technical expertise (58). If the patient cannot produce sputum spontaneously, specimens may be obtained by sputum induction with hypertonic saline or by fiberoptic bronchoscopy with broncho-alveolar lavage (61).

Although some studies have found no difference in the smear positivity rate between HIV-seropositive and HIV-seronegative patients (62), others have found that HIV-seropositive patients, particularly those with advanced disease, are more likely to present with smear negative PTB than are HIV-seronegative patients (63).

2.3 CULTURE OF MYCOBACTERIA

All clinical specimens suspected of containing mycobacteria may be inoculated onto egg- or agar-based media (e.g., Lowenstein-Jensen or Middlebrook 7H10) and incubated at 37°C under 5% CO_2 (56). Because most species of mycobacteria, including *M.tuberculosis*, are slow growing, four to eight weeks may be required before growth is detected.

A major improvement in mycobacteriology has been the development of commercial broth systems for mycobacterial growth detection. Automated culture systems, such as BACTEC 460 TB (Becton Dickinson and Co., Hood, USA), use added material for detection of mycobacterial growth by radiometric or colourimetric systems. These liquid systems allow for rapid growth and detection of mycobacteria within one to three weeks (64). Alternative non-radiometric growth detection methods using liquid culture (employing oxygen quenching and redox agents) have been developed (65), some of which have demonstrated performance comparable to BACTEC 460 TB (66).

Culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria per milliliter of material (67). Growth of the organism allows for precise species identification, drug susceptibility testing, and genotyping in epidemiological studies.

Culture improves the yield from sputum specimens to between 85% and 100% (68). Cultures of urine (69), stool (60), pleural fluid and biopsy (70), blood (71), and other body tissues generally have

a sensitivity of 80 to 85%, with a specificity of approximately 98% (72).

2.4 PHAGE SYSTEMS

Phage replication systems detect live mycobacteria in clinical specimens or in young liquid cultures using phages that infect and replicate in mycobacterial cells and act as indicators. These systems have been developed for both case-detection and drug susceptibility testing (73). A commercial product using this technology is now being developed for field testing.

2.5 CHEST RADIOGRAPHY

Although the classic picture of pulmonary tuberculosis (PTB) in adults is that of upper lobe disease with infiltrates and cavities, virtually any radiographic pattern may be seen (56). HIV infection alters the radiographic presentation of PTB, resulting in a higher prevalence of hilar adenopathy, pleural effusions, diffuse interstitial infiltrates, miliary pattern, lower zone consolidation, and less cavitation in comparison with HIV-seronegative patients with TB (74). Five to fourteen percent of HIV-infected patients with PTB have positive results on acid-fast staining of sputum, despite normal chest radiographs (75, 77).

In a mixed HIV-seropositive and HIV-seronegative population with PTB, the typical radiographic features of tuberculosis on chest radiographs were present in only 66% of cases when examined by a panel of experts (76), highlighting the limited sensitivity of diagnosing PTB on chest radiography alone.

2.6 TUBERCULIN SKIN TESTING

Tuberculin was first prepared by Robert Koch in 1890 from heat killed live cultures of *M.tuberculosis* (78). Old tuberculin is still made by a method almost identical to that first used by Koch, and is commonly used in veterinary medicine. Purified protein derivative (PPD) is prepared by precipitation with trichloroacetic acid, which removes some of the large carbohydrate antigens. PPD is more specific for the detection of *M.tuberculosis* infection and is the only material available for the standard intradermal Mantoux test (78).

A person infected with *M.tuberculosis* will react to intradermal injection of tuberculin with a delayed-type hypersensitivity (DTH) response mediated by T-lymphocytes. Cellular infiltration by T-lymphocytes in combination with other recruited inflammatory cells results in maximal induration at 48 to 72 hours after inoculation with intradermal antigen. A period of 4 to 6 weeks after primary infection is generally required for skin test conversion to occur (79).

A tuberculin skin test may be administered by multiple puncture devices, or by the intradermal injection of 0.1 milliliters of PPD (Mantoux skin test). Skin tests should be interpreted 48 to 72 hours after intradermal administration of antigen and the transverse diameter of induration measured in millimeters. Large-scale population studies have demonstrated a wide spectrum of results from PPD skin testing (78). The difference in size of the reaction depends in part upon the type of exposure to *M.tuberculosis*,

cross-reactions to other environmental mycobacteria, immunocompetence of the individual, and previous BCG vaccination.

The sensitivity of the test is approximately 95% in patients who have had tuberculosis that has been successfully treated with anti-tuberculosis therapy and do not have an underlying immunosuppressive disorder (78). Ten to 25 percent of patients with newly diagnosed PTB will have a negative skin test (80). These false negatives are due to a combination of factors, including an underlying immunosuppressive disorder (e.g., HIV infection, malnutrition), newborns or elderly patients with "waned" sensitivity, incorrect method of administration of tuberculin, error in recording skin reactions, and overwhelming infection with *M.tuberculosis* (78). HIV-infected patients with a CD4 T-lymphocyte count of less than $300x10^6$ cells per liter are more likely to have a false negative tuberculin skin testing (81).

The greatest drawback of tuberculin skin testing is the fact that most protein components in tuberculin or PPD are shared between mycobacterial species, or with unrelated species of bacteria (82). This greatly decreases the specificity of the skin test because individuals sensitized by prior exposure to non-tuberculous mycobacteria or vaccinated with BCG respond immunologically to tuberculin or PPD (83). False positive results also commonly occur in population groups with a high prevalence of tuberculosis where there is frequent contact with *M.tuberculosis*. In patient groups where the diagnosis of tuberculosis is often difficult to make, such as in HIV-seropositive individuals or children, the specificity of tuberculin skin testing may be less than 50% (80). Current PPD

skin testing is unable to reliably differentiate persons with asymptomatic *M.tuberculosis* infection from those with active tuberculosis, and is therefore not recommended as a diagnostic test for tuberculosis in adults in South Africa.

Cocktails of multiple purified antigens (such as ESAT-6 and MPT64) as skin test reagents have been developed in an attempt to improve the accuracy of skin testing. These cocktails have numerous advantages over traditional tuberculin testing, such as increased skin-test activity (84), improved sensitivity (85), and greater specificity (84). At present these skin test reagents are available in only a few specialized research laboratories and have not yet been commercially developed.

2.7 BLOOD IMMUNE-BASED DIAGNOSIS

PPD has been used for in-vitro detection of specific interferon gamma-secreting lymphocytes sensitized by mycobacterial antigens (86). The assay is done as a lymphocyte stimulation test with PPD in whole blood for 24 hours, followed by ELISA detection of interferon gamma produced after stimulation (87). This test has been developed commercially and results correlate well with tuberculin skin testing. It appears to be more sensitive than skin testing in immunocompromised patients (87). The blood test performs equally well in smear positive and smear negative patients, with a sensitivity range of 71% to 83% that was not statistically different in PTB versus extrapulmonary disease, or for smear-negative versus smear-positive cases (88).

2.8 NUCLEIC ACID AMPLIFICATION ASSAYS

Nucleic acid amplification assays (NAAs) using the polymerase chain reaction (PCR) have been developed to amplify the quantity of *M.tuberculosis* DNA or RNA in diagnostic specimens. These techniques are sensitive for the rapid detection of *M.tuberculosis* in a variety of specimens, including blood, sputum, urine, and biopsy specimens (89, 90, 91), usually in amounts too small to be seen by routine staining techniques.

NAAs can produce results within two to seven hours after specimen processing (92). However, technical expertise and skill are essential for reliable results, with false positives or false negatives occurring more frequently when technical proficiency is suboptimal. NAAs are highly specific for *M.tuberculosis* and are particularly helpful in the rapid differentiation of *M.tuberculosis* from non-tuberculous mycobacteria, such as *M.avium* complex (93). Several types of NAAs have been developed commercially, including Enhanced MTD (Gen-Probe, Inc., San Diego, CA), Amplicor (Roche Diagnostic Systems, Inc., Branchburg, NJ), and *M.tuberculosis* Direct Test (Gen-Probe, Inc., San Diego, CA).

Most clinical experience using NAAs has been gained using sputum specimens. Compared with culture, the sensitivity of NAAs is approximately 95% in patients with a positive AFB sputum smear, but only about 50% in sputum smear-negative cases (94, 95). Specificity is greater than 95% in either smear-positive or smear-negative samples (96). NAAs may remain positive for months despite appropriate antitubercular therapy and a good

clinical response (97). Thus, NAAs assist in diagnosis, but should not be used as a sole criterion for assessing infectivity or response to therapy. The United States Food and Drug Administration (FDA) guidelines recommend that NAAs should not be relied upon in the absence of smear or culture data (98). The FDA also recommends that NAAs should ideally be performed on all AFB smear-positive respiratory specimens; a positive result is diagnostic of infection with *M.tuberculosis* (99).

The use of NAAs has been investigated on non-respiratory specimens. The Amplicor assay on tissue and body fluid specimens was reported to have a sensitivity of 76% and a specificity of 99% (91, 99). NAAs do not perform well with pleural fluid samples as pleural fluid has many inhibitors which affect the test, resulting in poor sensitivity (92). NAAs have not been subjected to adequate testing on non-respiratory specimens to justify their routine use in this setting.

The limitations of NAAs are the requirement for highly skilled personnel and sophisticated equipment, poor sensitivity in smear-negative samples, and limited experience in non-research settings. NAA accuracy is highly dependent on the expertise and experience of the clinical laboratory (98). False positive results may be due to individual laboratory procedures, contamination, or might reflect amplification of DNA from non-viable mycobacteria (100).

2.9 ADENOSINE DEAMINASE (ADA)

ADA is a cellular enzyme that exists as two isoforms: ADA-1 is ubiquitous in all cells, including lymphocytes and monocytes, whereas ADA-2 is found only in monocytes (101). Elevated levels

of ADA are found in fluid exudates due to *M.tuberculosis* infection, but also in other diseases that result in activation of monocytes and lymphocytes.

Pleural fluid ADA concentrations are elevated in tuberculous effusions in both HIV-seropositive and HIV-seronegative patients. Using a cut-off level of 60U/L, the sensitivity is 95%, the positive predictive value is 96%, and the negative predictive value is 95% (102). False positives may occur in rheumatoid effusion, empyema, mesothelioma, lung cancer, parapneumonic effusion and haematological malignancies. Determination of the two isoenzymes may help to differentiate the causes of pleural effusion. ADA-2 is increased in tuberculous effusions, while ADA-1 rises in empyemas and parapneumonic effusions (103). Asians and immunocompromised patients may have lower levels of ADA, giving a false negative result (104).

ADA levels are elevated in the ascitic fluid of HIV-seropositive and HIV-seronegative patients with tuberculous peritonitis. Sensitivity ranges from 58% to 100% (105, 106), with decreased sensitivity in the presence of cirrhosis.

Patients with tuberculous pericarditis have elevated levels of ADA in pericardial fluid, with one study demonstrating a sensitivity of 93% and a specificity of 97% when pericardial ADA levels where above 40U/L (107). In another series, there was a positive correlation between high pericardial ADA levels and the subsequent development of constrictive pericarditis (108).

The level of ADA in cerebro-spinal fluid is elevated in patients with tuberculous meningitis, but has a sensitivity of only 44%-89% and a specificity of 75%-92% using a cut-off level of 5-8U/L (109, 110). False positives occur in patients with pyogenic meningitis, cerebral malaria, and brucellosis meningitis.

2.10 SERODIAGNOSTIC ASSAYS FOR THE DETECTION OF TUBERCULOSIS

Assays for the detection of immunologic responses to tuberculosis are based on the detection of specific antibodies in serum and other biological fluids of patients infected with *M.tuberculosis*. Arloing first recognized in 1898 that strong antibody responses are mounted during tuberculosis (111). Serodiagnostic assays have the potential for rapid and simple diagnosis, as well as distinguishing active tuberculosis from asymptomatic infection (112). The antibody response during infection with *M.tuberculosis* is directed against multiple mycobacterial antigens. Therefore, antigen recognition in tuberculosis is highly heterogeneous, and no single antigen or common set of antigens is recognized by serum antibodies from all patients (113). Various immunologically active components of *M.tuberculosis* have been studied. These include proteins, polysaccharides, peptidoglycans, phospholipids, and lipoarabinomannan (114, 115, 116, 117).

Serodiagnostic assays detecting serum antibodies to several mycobacterial antigens have been tested in clinical trails, with mixed results. Evaluation of a commercially available serodiagnostic kit for the detection of serum antibodies to the

M.tuberculosis 38-kDa antigen in a mixed HIV-seropositive and HIV-seronegative population with tuberculosis showed a sensitivity of 72,6% and a specificity of 94,9% (118). In Madagascar, an enzyme-linked immunosorbent assay (ELISA) to detect serum antibodies to the *M.tuberculosis* 45/47-kDa antigen in patients with both smear-negative and smear-positive PTB had a sensitivity of 76,9% and specificity of 73,2% (119). A Taiwanese study using an ELISA to detect serum antibodies to *M.tuberculosis* antigen A60 in a mixed population of patients with PTB and extra-pulmonary tuberculosis reported a sensitivity of 69,6% and a specificity of 92,1% (120).

One of the most extensively studied serodiagnostic tests for tuberculosis is the commercially available Mycodot kit (DynaGen, Inc., MA, USA) which detects IgG antibodies to mycobacterial lipoarabinomannan. When evaluated in developed countries in HIV-seronegative patients infected with *M.tuberculosis* the test had relatively good sensitivity (85%-93%) and specificity (89%-100%) (121, 122). However, when the same assay was evaluated in clinical trials in developing countries with a high prevalence of HIV-positivity, the sensitivity was only 16%-55%, with a specificity of 84%-97% (123, 124, 125). In particular, in HIV-seropositive patients with tuberculosis, especially if the CD4 count was less than 200 cells per milliliter, the sensitivity was below 40%.

In the process of selecting purified antigens for serodiagnostic purposes, it was repeatedly observed that single-antigen based assays never achieved satisfactory diagnostic performance (126). For this reason several multiantigen "cocktails" have been tested clinically. A combination of seven serological assays, tested in a

mixed population of patients with tuberculosis and negative controls, showed a maximum sensitivity of 84% and specificity of only 42%-55% (127). In a study of almost 600 Chinese patients with active tuberculosis and negative controls, a triple-ELISA test detecting antibodies against *M.tuberculosis* Antigen-60, 38-kDa antigen, and kp90 antigen, had a sensitivity and specificity of approximately 80%, making it a poor diagnostic tool for disease confirmation (128).

Antibodies against *M.tuberculosis* glycolipid antigens (DAT, PGLTb1, and LOS) circulate as immune complexes in sera of humans infected with *M.tuberculosis* (129). These antibodies can be detected by ELISA, the sensitivity of which can be improved by precipitation of the circulating immune complexes with polyethylene glycol (PEG) (129, 130). In clinical trials involving HIV-positive and HIV-negative patients infected with *M.tuberculosis*, the sensitivity ranged between 74% and 80%, with a specificity of 95% (130, 131). There was no significant difference in sensitivity between HIV-seropositive and HIV-seronegative patients, but sensitivity was lower for disseminated than for localized tuberculosis (131).

Cord factor (trehalose 6,6`-dimycolate) isolated from *M.tuberculosis* cell wall acts as an antigen to detect an antibody response in the serum of patients infected with *M.tuberculosis* (132). The exact antigenic epitope in cord factor has recently been established as methoxy mycolic acid (133). In HIV-seronegative patients with tuberculosis an ELISA detecting serum IgG antibodies to cord factor had a sensitivity of 81%-83%, with a specificity of 96%-100% (132, 134). Anti-cord factor antibody titres

have been shown to decline to normal levels after treatment with antituberculous chemotherapy (134).

Several ELISAs have been tested to detect *M.tuberculosis* antigens and antibodies in various secretions, such as sputum, bronchial washings, and other body fluids (135, 136, 137). Sensitivities ranged from 69% to 97%, but there are limited data in HIV-positive patients.

2.11 LIMITATIONS OF CURRENTLY AVAILABLE DIAGNOSTIC AGENTS OR TECHNIQUES FOR TUBERCULOSIS

Of the approximately 8 million people who will develop tuberculosis in 2001, only a minority will get a laboratory-supported diagnosis (138). Fast and accurate diagnosis of tuberculosis is an important element of global health measures to control the disease. The gold standard in diagnosis remains clinical examination, combined with direct microscopic examination of sputum and culture of mycobacteria. However, the sensitivity of sputum microscopy is low, and culture of *M.tuberculosis* takes two to eight weeks. Up to 50% of newly diagnosed tuberculosis patients may be smear- and culture-negative, and even the NAAs gives only an additional 15% positivity rate (133). In the era of HIV and *M.tuberculosis* co-infection, both radiography and tuberculin skin testing can no longer be relied upon for accurate diagnosis.

Serodiagnostic assays have the potential for providing a rapid, accurate, and inexpensive test for the diagnosis of tuberculosis. Unfortunately, such assays have frequently turned out to be disappointing in clinical practice after promising early reports (139).

(139). Currently available rapid serodiagnostic tests for tuberculosis suffer from four major limitations (140). Firstly, the majority have been developed in industrialized countries with lower disease prevalence than most developing countries. According to Bayes theorem, the positive and negative predictive values of a test vary according to the prevalence of a disease, making many of these tests unreliable when used in areas of high TB prevalence. A test with high sensitivity in a low prevalence environment may, therefore, have a poor positive predictive value in high-prevalence situations (140). Secondly, false positive results have occurred in patients with lung cancer, diabetes mellitus, bronchitis, autoimmune diseases, previous treated or untreated tuberculosis, exposure to environmental mycobacteria, and vaccination with BCG (140, 141). Thirdly, false negatives are common in immunocompromised and HIV-infected persons (123, 124, 125). Fourthly, errors in the design or reporting of TB diagnostic trials are common and include: inadequate statistical evaluation, inappropriate study design and sample size, inadequate gold standards for clinical and microbiological case-definition, and inappropriate population testing (139, 140). For these reasons, none of the currently available rapid serological field tests can be recommended for the diagnosis of tuberculosis in South Africa.

2.12 AN ELISA FOR THE DETECTION OF ANTIBODIES AGAINST *M.TUBERCULOSIS* MYCOLIC ACIDS

Work done by Verschoor *et al.* in South Africa, at the University of Pretoria, has led to the development of a method for the isolation and purification of mycobacterial mycolic acid (36). In experimental work, incorporation of *M.tuberculosis* mycolic acid into an

immunogenic conjugate elicited a specific antibody response. Anti-mycolic acid antibodies occurring spontaneously in human serum were found to be specific, but of low affinity (52, 53). An ELISA was developed by the same investigators for the detection of anti-mycolic antibodies in serum, or after precipitation with polyethyleneglycol (PEG).

Uncontrolled and unpublished early studies using stored serum from the Medical Research Council (MRC) tuberculosis serum bank had encouraging results. Using the new ELISA, sera from several groups of patients were tested (Verschoor JA, personal communication). In HIV-seronegative patients, the sensitivity of the ELISA was 89% (and 100% after precipitation of sera with PEG) in 35 newly diagnosed, untreated patients with TB, and 70%-86% in 122 patients with TB who were undergoing anti-tuberculous chemotherapy. In HIV-seropositive patients, the sensitivity was 58% in 24 patients with untreated TB and 72%-94% in 18 patients with TB who were undergoing anti-tuberculous chemotherapy. In another small study of 10 healthy health care workers with a high level of exposure to *M.tuberculosis*, but no history of clinical tuberculosis, all had low levels of anti-mycolic acid antibodies in their sera.

These results prompted the current study, in which sera were collected from patients (both HIV-seropositive and HIV-seronegative) with and without newly diagnosed pulmonary (smear-positive) tuberculosis. Anti-mycolic acid antibodies were then measured in the sera.

3. THE STUDY

3.1 INTRODUCTION

The waxy outer cell wall of *M.tuberculosis* consists mainly of mycolic acids. The unique immunostimulatory properties of mycolic acids via the CD-1 restricted antigen presentation pathway have been demonstrated in humans. Purification and isolation of these mycolic acids has allowed them to be applied as an antigen in an enzyme-linked immunosorbent assay (ELISA) sero-diagnostic test to detect specific antibodies in the sera of humans with active tuberculosis. This study was aimed at assessing the level of anti-mycolic acid antibodies in the sera of patients with active pulmonary tuberculosis, with the possibility of developing a much-needed rapid and accurate sero-diagnostic test for tuberculosis.

3.2 HYPOTHESIS TO BE TESTED

Humans who are actively infected with *M.tuberculosis* have elevated serum levels of specific antibodies to *M.tuberculosis* mycolic acids compared to humans who do not have active tuberculosis.

3.3 STUDY OBJECTIVES

The primary objective was to measure the level of specific anti-mycolic antibodies in the sera of patients with newly diagnosed, untreated, culture positive pulmonary tuberculosis. Adults with no evidence of active tuberculosis were used as a control group. The study investigated the diagnostic value of a recently developed

ELISA, based on the detection of specific antibodies to *M.tuberculosis* mycolic acids, as a possible surrogate marker of active pulmonary infection with *M.tuberculosis*. The results were compared with conventional diagnosis (based on sputum microscopy and culture) in patients with newly diagnosed active pulmonary tuberculosis and in adults with other medical conditions, but no evidence of active tuberculosis, to determine the sensitivity and specificity of the ELISA.

A secondary objective was to compare the level of specific anti-mycolic acid antibodies in the sera of HIV-positive and HIV-negative patients with active pulmonary tuberculosis relative to controls, in order to assess the accuracy of the ELISA in patients co-infected with HIV and *M.tuberculosis*.

3.4 METHODS

3.4.1 GENERAL

Sera from human subjects were tested for the presence of antibodies to *M.tuberculosis* mycolic acids using a newly developed ELISA. Patients were recruited from the general medical wards of the Helen Joseph Hospital, Auckland Park, Johannesburg, South Africa, between August and December, 2000. Subjects were interviewed and given prepared subject information sheets (appendix 1a and appendix 1b). Written consent (appendix 2) was obtained before enrollment and participation in the study. Demographic, clinical, and laboratory data were recorded. All recruitment, data capture and venesection was performed by the author personally. Ten milliliters of venous blood were collected in a red-topped blood collection tube (without anti-

coagulants) using a sterile needle and aseptic technique. The blood was stored at 3-5°C before being transported to the Department of Biochemistry, University of Pretoria, where the serum was separated, gamma irradiated, and stored at –70°C.

3.4.2 PATIENTS

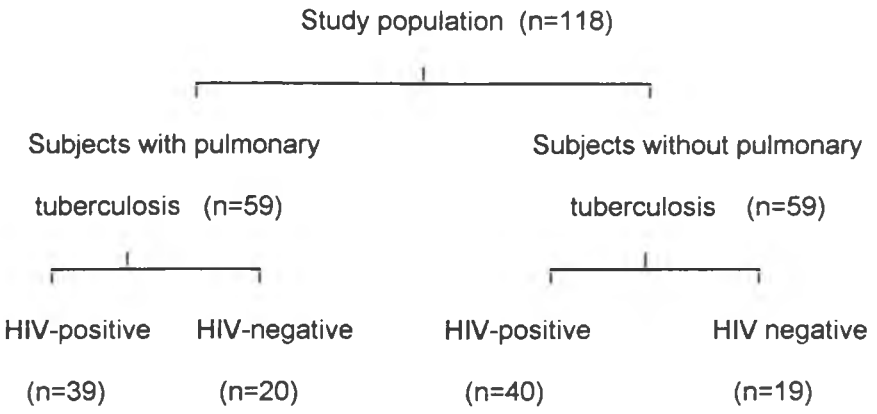
Two groups of patients were enrolled:

The **tuberculosis positive (TP)** group - patients with newly diagnosed, untreated, active (sputum smear-positive) pulmonary tuberculosis.

The **tuberculosis negative (TN)** group - control subjects with other medical conditions, but no clinical, radiological, or microbiological evidence of active infection with *M.tuberculosis*. These control subjects were matched (as best as possible) for gender, age and race.

Both groups of subjects included patients who were known to be infected with HIV and patients who were known to be HIV-seronegative.

After consultation with a biostatistician, a sample size of at least 100 subjects was estimated to be adequate for a pilot study of this nature. By the end of enrollment 118 eligible patients had been recruited for participation in the study.



3.4.3 INCLUSION AND EXCLUSION CRITERIA

Patients with newly diagnosed pulmonary tuberculosis:

Inclusion criteria - sputum positive for AFB on routine sputum staining with Ziel-Neelsen and microscopy
- sputum culture positive for *M.tuberculosis*
- clinical and radiographic features consistent with active pulmonary tuberculosis
- age between 18 and 65 years
- able to give informed consent

Exclusion criteria - a history of previously treated or untreated tuberculosis
- currently taking antibiotic treatment for *M.tuberculosis* infection

Control subjects without tuberculosis:

Inclusion criteria - no clinical, radiographic, or microbiological evidence of active infection with *M.tuberculosis*
- age between 18 and 65 years
- able to give informed consent

Exclusion criteria - a history of previous treated or untreated tuberculosis

- currently taking antibiotic treatment for *M.tuberculosis* infection

3.5 AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO *M.TUBERCULOSIS* MYCOLIC ACID IN HUMAN SERA

The ELISA was performed by preparing sera in a 1:20 dilution with 0.5% casein Phosphate Buffered Saline (PBS) and adding this to wells coated with mycolic acid antigen. After incubation for 60 minutes the wells were washed with 0.5% Casein PBS and bound antibody was detected with goat anti-human peroxidase conjugate. Absorbencies were measured at 5, 30 and 60 minutes using a photometer (Anthos SLT 340 ATC, Austria). The ELISA was repeated on sera that had been prepared with 8% Polyethylene Glycol (PEG) to precipitate circulating immune complexes. A detailed description of the method is given in appendix 3.

Each sample of precipitated and unprecipitated serum was tested by this method four times and an average signal intensity recorded. The corrected signal intensity was calculated by subtracting the signal value of the same serum on uncoated but blocked wells.

The author performed the ELISA several times to familiarize himself with the technique. However, the vast majority of the testing was performed by Miss Y.Vermaak from the Department of Biochemistry, University of Pretoria, South Africa.

3.6 DATA RECORDED

Patients were all admitted to the general medical wards of the Helen Joseph Hospital. All had routine sputum analysis, haematological, biochemical, and serological blood tests (including testing for HIV infection), as well as a chest radiograph, performed by the attending medical registrar and intern, as part of usual clinical practice.

All patients recruited into this study were interviewed and examined by the author. Demographic and clinical data, as well as the results from all investigations, were recorded on specially prepared Patient Data Sheets (appendix 4). Eligible patients were then enrolled into the study after written informed consent was obtained. Blood was then drawn for measurement of antibodies to *M.tuberculosis* mycolic acids.

3.6.1. DEMOGRAPHIC AND CLINICAL DATA

The demographic data recorded included age, gender and race of the patients. The clinical features documented included a history of the presenting symptoms, past medical history, drug history, smoking status, occupational dust exposure, and any allergies.

On examination of the patient the following findings were documented: blood pressure, pulse, temperature, respiratory rate, the presence or absence of cyanosis, pallor, lymphadenopathy, clubbing, wasting and oral candidiasis, as well as the clinical features on chest, cardiovascular, and abdominal examination.

3.6.2 LABORATORY DATA

3.6.2.1. HAEMATOLOGY

The haematological investigations included evaluation of haemoglobin levels, white cell, differential, and platelet counts (Beckman Coulter MAX-M, Beckman, USA). The erythrocyte sedimentation rate was measured by the Westergren method.

3.6.2.2. BIOCHEMISTRY

The initial serum urea, creatinine, electrolytes, C-reactive protein, albumin, and total protein were measured (H747 Automatic Analyzer, Hitachi, Japan).

3.6.2.3. HIV AND SERUM T-CELL SUBSETS

The HIV antibody was determined by a rapid screening ELISA (Determine Rapid HIV Antibody Test, Abbot Laboratories, USA). All positive results were confirmed by further third generation ELISA testing (Access, Pasteur Sanofi, France), as recommended by the World Health Organization. The results of the T-cell subsets determined by flow cytometry were noted and analyzed.

3.6.2.4. SYPHILIS SEROLOGY

Syphilis serology was determined with a non-treponemal screening test, the Rapid Plasma Reagin (RPR, Immunotrap Carbon Antigen, Omega Diagnostics, USA) Test, and a specific treponemal test, the *T.pallidum* Hemagglutination Assay (TPHA, Cellogost-Syphilis, Dade Behring, USA).

3.6.2.5. SPUTUM MICROSCOPY AND CULTURE

Expectorated sputum samples were stained with Ziel-Neelsen stain and microscopy performed for AFB. All sputum samples that were considered positive for AFB were submitted for mycobacterial culture by inoculation into appropriate broth culture media and analyzed radiometrically (BACTEC-460 TB Hood, Becton Dickinson and Co., USA). All positive mycobacterial culture specimens were further identified and confirmed as *M.tuberculosis*.

3.6.3. CHEST RADIOGRAPH

A routine postero-anterior and lateral chest radiograph was obtained on all patients. The chest radiograph was reported on by the author and the extent and nature of any lung infiltrates were noted. The presence or absence of hilar lymphadenopathy and/or pleural effusion were also noted.

3.7. END POINT OF THE STUDY

The end point was the detection of specific antibodies to *M.tuberculosis* mycolic acids in the sera of patients with active and untreated pulmonary tuberculosis before and after precipitation of immune complexes. Comparison of the accuracy of the results to conventional diagnostic testing (including sputum microscopy and culture) was done to assess the sensitivity of this potential sero-diagnostic assay. A group of subjects without any evidence of past or current active infection with *M.tuberculosis* acted as controls in order to assess the specificity of the test.

3.8. DATA ANALYSIS

The statistical analysis employed included various descriptive statistics, the paired Student`s t-test and the Mann Whitney U-test (2-tail) for the analysis of all continuous variables, and the Fisher`s exact (2-tail) test and the Chi-squared test for analysis of the categorical variables.

3.9 ETHICS

Clearance from the Committee of Research on Human Subjects of the University of the Witwatersrand was obtained for this research project (protocol number M00/06/19).

4. RESULTS

A total of 118 subjects were enrolled in the study to compare the levels of anti-mycolic acid antibodies between tuberculosis and non-tuberculosis patients. They consisted of 59 patients with sputum smear and culture positive, untreated pulmonary tuberculosis. Of these patients 39 were HIV-seropositive and 20 patients were HIV-seronegative. This group of patients will be referred to as the **tuberculosis positive (TP)** group. Another 59 patients from the general medical wards without any evidence of tuberculosis acted as controls. Of these, 40 patients were HIV-seropositive and 19 patients were HIV-seronegative. This group of patients will be referred to as the **tuberculosis negative (TN)** group. All the TP and TN patients met the inclusion and exclusion criteria for their respective groups.

4.1 DEMOGRAPHIC DATA

The TP group and TN group of patients were well matched in terms of age, gender, and race **(table 4.1)**. There were no significant differences between the two groups.

4.2 PREVIOUS AND CURRENT MEDICAL CONDITIONS

Various previous and current medical conditions were documented in the two groups of patients. There were no statistically significant differences between the two groups regarding HIV status, smoking status, occupational dust

TABLE 4.1

DEMOGRAPHIC FEATURES OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS

DEMOGRAPHIC FEATURES	TUBERCULOSIS POSITIVE (n=59)	TUBERCULOSIS NEGATIVE (n=59)	SIGNIFICANCE (p value)
Age, Years (mean ± SD)	36.2 ± 10.7	35.7 ± 9.9	NS
Males, no. (%)	30 (51%)	23 (39%)	NS
Females, no. (%)	29 (49%)	36 (61%)	NS
African, no. (%)	51 (86%)	47 (80%)	NS
Caucasian, no. (%)	1 (2%)	1 (2%)	NS
Mixed race, no. (%)	7 (12%)	11 (19%)	NS

SD = Standard Deviation

NS = Statistically Not Significant

Numbers denote number of patients and figures in brackets denote percentages

n = Total number of patients

exposure, non-tuberculous bacterial infections, the presence of neoplasms, auto-immune diseases, diabetes mellitus, syphilis infection, fungal infections, non-HIV viral infections, parasitic infections, and immunosuppressive therapy. The results are shown in **table 4.2.** The patients in the TN group were recruited from the general medical wards and thus had a significantly higher incidence of heart failure ($p=0.036$) and non-tuberculous bacterial infections such as community-acquired pneumonia, meningitis and soft-tissue infections ($p=0.0029$). The latter may have some significance on the study since it is currently unclear whether non-tuberculous infections stimulate an antibody response that may cross-react with anti-mycolic acid antibodies.

4.3 CLINICAL DATA

The clinical data are shown in **table 4.3**. The TP group of patients had a significantly higher mean temperature ($p<0.0001$), lower systolic blood pressure ($p<0.0001$), and higher respiratory rate ($p<0.0001$). Clinical cyanosis occurred significantly more frequently in the TP group ($p=0.012$). These features indicate that the patients in the TP group were significantly more ill than the TN control group of patients. This is an expected finding as symptomatic tuberculosis causes a significant systemic inflammatory response, often with features of sepsis. Also, many of the control patients had no evidence of infection since they were admitted for other medical conditions, such as heart failure.

TABLE 4.2

PREVIOUS AND CURRENT MEDICAL CONDITIONS IN 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS

CLINICAL CONDITION	TUBERCULOSIS POSITIVE (n=59)	TUBERCULOSIS NEGATIVE (n=59)	SIGNIFICANCE (p value)
HIV positive, no. (%)	39 (66%)	40 (68%)	NS
Previous or current smoker, no. (%)	27 (46%)	20 (34%)	NS
Previous or current occupational dust exposure, no. (%)	5 (8%)	3 (5%)	NS
Previous or current non-tuberculous chest infection, no. (%)	7 (12%)	6 (10%)	NS
Previous or current neoplasm, no. (%)	0 (0%)	5 (8%)	NS
Previous or current auto-immune disease, no. (%)	2 (3%)	2 (3%)	NS
Previous or current diabetes mellitus, no. (%)	2 (3%)	5 (8%)	NS
Previous or current asthma, no. (%)	1 (1.6%)	1 (1.6%)	NS
Previous or current syphilis infection, no. (%)	5 (8%)	7 (12%)	NS
Currently receiving immunosuppressive therapy, no. (%)	2 (3%)	1 (1.6%)	NS
Currently in heart failure, no. (%)	0 (0%)	6 (10%)	P=0.036
Current fungal infection, no. (%)	23 (39%)	16 (27%)	NS
Current non-HIV viral infection, no. (%)	0 (0%)	5 (8%)	NS
Current parasitic infection, no. (%)	0 (%)	2 (3%)	NS
Current non-tuberculous bacterial infection, no. (%)	0 (0%)	10 (17%)	P=0.00293

TABLE 4.3

CLINICAL FEATURES OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS

CLINICAL FEATURES	TUBERCULOSIS POSITIVE (n=59)	TUBERCULOSIS NEGATIVE (n=59)	SIGNIFICANCE (p value)
Temperature (°C mean ± SD)	38.09 ± 1.09	36.97 ± 0.6	P<0.0001
Systolic blood pressure (mmHg mean ± SD)	105.7 ± 14	124.3 ± 22.3	P<0.0001
Heart rate (beats per minute mean ± SD)	106.08 ± 18	88.5 ± 14.5	P<0.0001
Respiratory rate (breaths per minute mean ± SD)	24.7 ± 5.2	18.1 ± 4.2	P<0.0001
Cyanosis, no. (%)	15 (25%)	4 (7%)	P=0.0122
Generalized lymphadenopathy, no. (%)	37 (63%)	30 (51%)	NS

SD = Standard Deviation

NS = Statistically Not Significant

Numbers denote number of patients and figures in brackets denote percentages

n = Total number of patients

4.4 LABORATORY DATA

4.4.1 HAEMATOLOGICAL DATA

The results of the haematological investigations are shown in **table 4.4**. The mean haemoglobin level ($p<0.0001$) was significantly lower in the TP group. This may be the result of accelerated destruction or underproduction of haemoglobin and is frequently due to multifactorial causes in patients with tuberculosis. Ineffective haematopioesis may be due to nutritional deficiencies, advanced HIV infection, and an anaemia of chronic disorders that is frequently associated with symptomatic *M.tuberculosis* infection. The mean platelet count ($p=0.0165$) was significantly higher in the TP group, as was the mean erythrocyte sedimentation rate ($p<0.0001$). These laboratory investigations are frequently raised in systemic infections such as tuberculosis.

4.4.2 BIOCHEMISTRY DATA

The biochemistry results are shown in **table 4.4**. The mean C-reactive protein level was significantly higher in the TP group ($p<0.0001$), most likely as a non-specific marker of systemic inflammation. The mean serum sodium concentration was significantly lower ($p<0.0001$) in the TP group of patients. Hyponatraemia is frequently associated with gastro-intestinal losses, the syndrome of inappropriate anti-diuretic hormone secretion, and advanced HIV infection (142). The mean serum albumin level was significantly lower ($p<0.0001$) in the TP

TABLE 4.4

HAEMATOLOGICAL AND BIOCHEMICAL LABORATORY DATA IN 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 CONTROL SUBJECTS WITHOUT TUBERCULOSIS

LABORATORY DATA RECORDED	TUBERCULOSIS POSITIVE (n=59)	TUBERCULOSIS NEGATIVE (n=59)	SIGNIFICANCE (p value)
Haemoglobin (g/dL mean ± SD)	9.59 ± 2.48	11.86 ± 2.49	P<0.0001
White cell count (x 10^9 cells/L mean ± SD)	7.9 ± 3.28	8.8 ± 5.37	NS
Platelet count (x 10^9 platelets/L mean ± SD)	362.11 ± 192.23	287.42 ± 136.6	P=0.0165
Erythrocyte sedimentation rate (mm/hour mean ± SD)	89.93 ± 32.34	52.54 ± 39.55	P<0.0001
C-reactive protein (mg/L mean ± SD)	163.98 ± 86.36	62.05 ± 94.61	P<0.0001
Serum sodium (mmol/L mean ± SD)	132.57 ± 6.07	137.16 ± 6.53	P<0.0001
Serum potassium (mmol/L mean ± SD)	3.74 ± 0.66	3.58 ± 0.57	NS
Serum albumin (g/L mean ± SD)	24.79 ± 6.0	30.71 ± 7.79	P<0.0001
Serum total protein (g/L mean ± SD)	75.33 ± 12.44	76.27 ± 14.07	NS
Serum urea (mmol/L ± SD)	5.9 ± 6.4	6.96 ± 6.92	NS
Serum creatinine (µmol/L ± SD)	86.61 ± 52.15	114.0 ± 160.47	NS

SD = Standard deviation
NS = Statistically Not Significant
n = Total number of patients

group of patients. Low albumin levels may be a marker of malnutrition that frequently occurs during tuberculosis or may represent an acute phase catabolic response.

4.4.3 CD4 T-LYMPHOCYTE DATA

The CD4 T-lymphocyte count results for the HIV-seropositive patients in the two groups are shown in **table 4.5**. The HIV-seropositive patients in the TP group had a significantly lower mean CD4 T-lymphocyte count ($p=0.0013$). The number of HIV-seropositive patients with a CD4 T-lymphocyte count of less than 200 ($x10^6$ cells/L) was significantly higher in the TP group ($p=0.0005$). Although tuberculosis is found in HIV-seropositive patients during all phases of HIV infection, it is more common in the later stages of the disease associated with a low CD4 T-lymphocyte count (particularly when the CD4 T-lymphocyte count is less than 200 cells x 10^6/L).

4.5 MICROBIOLOGY DATA

All patients in the TP group had sputum that was reported as AFB positive on routine Ziel-Neelsen staining and microscopy. All smear positive sputum samples were submitted for mycobacterial culture with subsequent isolation of *M.tuberculosis*. All positive culture results were identified as *M.tuberculosis* and no cases of non-tuberculous mycobacteria were identified.

TABLE 4.5

COMPARISON OF CD4 T-LYMPHOCYTE COUNTS IN 39 HIV POSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 40 HIV POSITIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS

DATA RECORDED	HIV-SERO-POSITIVE WITH PULMONARY TUBERCUOSIS (n=39)	HIV-SERO-POSITIVE WITHOUT PULMONARY TUBERCULOSIS (n=40)	SIGNIFICANCE (p value)
CD4 T-lymphocyte count ($x10^6$ cells/L mean ± SD)	138.25 ± 140.49	289.55 ± 247.67	P=0.0013
CD4 T-lymphocyte count less than $200x10^6$ cells/L, no. (%)	30 (77%)	19 (47.5%)	P=0.0005

SD = Standard Deviation

NS = Statistically Not Significant

Numbers denote number of patients and figures in brackets denote percentages

n = Total number of patients

No patients in the TN group had positive sputum microscopy for AFB or *M.tuberculosis* isolated from any clinical specimens submitted for mycobacterial culture.

4.6 CHEST RADIOGRAPHS

All patients in both groups had a chest radiograph as part of standard medical care. A comparison of radiographic features on chest radiograph between HIV-seropositive and HIV-seronegative patients with pulmonary tuberculosis is shown in **table 4.6**. The HIV-seropositive patients had more features on the chest radiograph that were atypical of pulmonary tuberculosis than the HIV-seronegative TP patients, including a significantly higher incidence of hilar lymphadenopathy ($p<0.0001$) and pleural effusions ($p=0.042$). This is consistent with reports showing that HIV-seropositive patients with pulmonary tuberculosis rarely have classical upper lobe infiltrates with cavitation on chest radiographs, but rather have atypical features such as pleural effusions, hilar lymphadenopathy, diffuse bilateral air-space opacification, and pleural effusions (74).

TABLE 4.6

COMPARISON OF RADIOGRAPHIC FEATURES ON CHEST RADIOGRAPH OF 20 HIV-SERONEGATIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 39 HIV-SEROPOSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS

RADIOGRAPHIC FEATURES	HIV-SERO-NEGATIVE (n=20)	HIV-SERO-POSITIVE (n=39)	SIGNIFICANCE (p value)
Upper lobe infiltrates with cavitation, no. (%)	15 (75%)	19 (49%)	NS
Bilateral diffuse air-space opacification, no. (%)	13 (65%)	26 (66%)	NS
Hilar adenopathy, no. (%)	2 (10%)	30 (77%)	P<0.0001
Miliary pattern, no. (%)	0 (0%)	1 (2.5%)	NS
Pleural effusion, no. (%)	3 (15%)	11 (28%)	P=0.042

NS = Statistically Not Significant

Numbers denote number of patients and figures in brackets denote percentages

n = Total number of patients

4.7 ANTI-MYCOLIC ACID ANTIBODIES

4.7.1 ALL TP AND TN PATIENTS

The results of the ELISA to detect anti-mycolic acid antibodies performed on all TP patients and TN patients are shown in **table 4.7**. In TP patients, the corrected signal intensities (mean ± SD) in serum (0.364 ± 0.234, p=0.0053) and in precipitate (0.226 ± 0.150, p=0.0009) were significantly higher compared to corrected signal intensities in serum (0.250 ± 0.201) and precipitate (0.143 ± 0.110) in TN patients. A Box-and-whisker graph of these results is shown in **figure 4.1.**

4.7.2 HIV-SEROPOSITIVE TP AND HIV-SEROPOSITIVE TN PATIENTS

The results of the ELISA performed on HIV-seropositive TP and HIV-seropositive TN patients are shown in **table 4.8**. In HIV-seropositive TP patients, the corrected signal intensities (mean ± SD) in serum (0.393 ± 0.263, p=0.0132) and precipitate (0.242 ± 0.169, p=0.0003) were significantly higher compared to corrected signal intensities in serum (0.253 ± 0.227) and precipitate (0.128 ± 0.083) in HIV-seropositive TN patients. A Box-and-whisker graph of these results is shown in **figure 4.2.**

TABLE 4.7

RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 PATIENTS WITHOUT PULMONARY TUBERCULOSIS

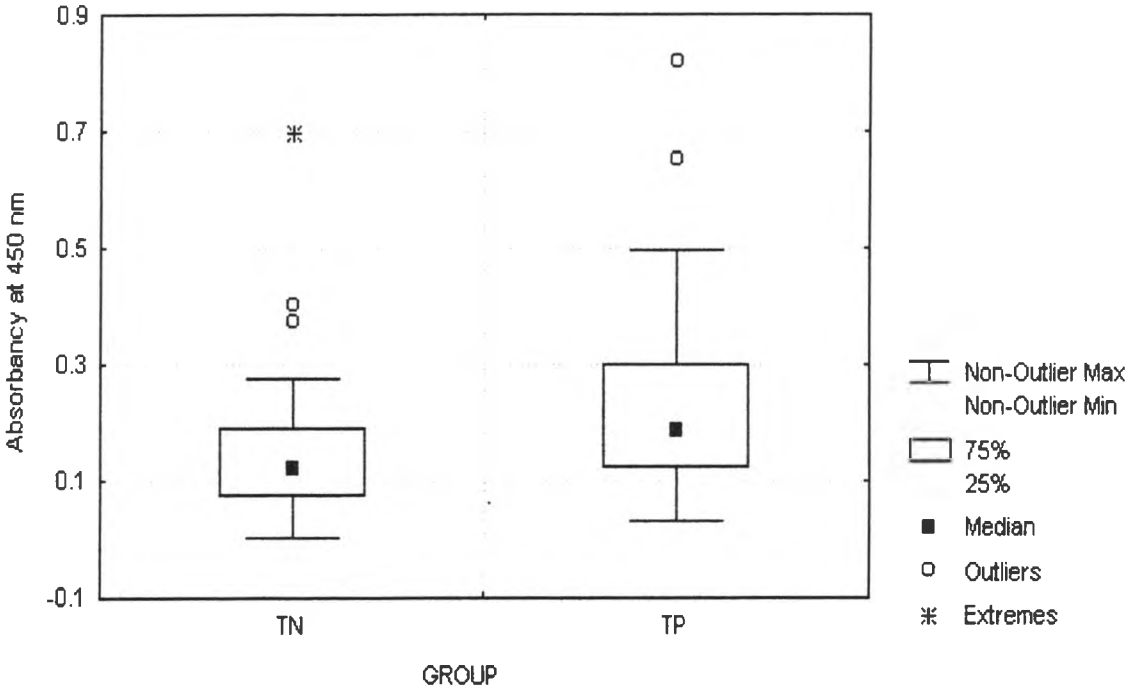
ELISA RESULTS	TUBERCULOSIS POSITIVE (n=59)	TUBERCULOSIS NEGATIVE (n=59)	SIGNIFICANCE (p value)
ELISA performed on serum absorbancy at 450nm (mean ± SD)	0.364 ± 0.234	0.250 ± 0.201	P=0.0053
ELISA performed on precipitate absorbancy at 450nm (mean ± SD)	0.226 ± 0.150	0.143 ± 0.110	P=0.0009

SD = Standard Deviation

NS = Statistically Not Significant

n = Total number of patients

FIGURE 4.1



ANTI-MYCOLIC ACID ANTIBODY LEVELS IN PRECIPITATED SERA OF 59 PATIENTS WITHOUT TUBERCULOSIS (TN) AND IN 59 PATIENTS WITH TUBERCULOSIS (TP)

TABLE 4.8

RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 39 HIV-SERO-POSITIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 40 HIV-SEROPOSITIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS

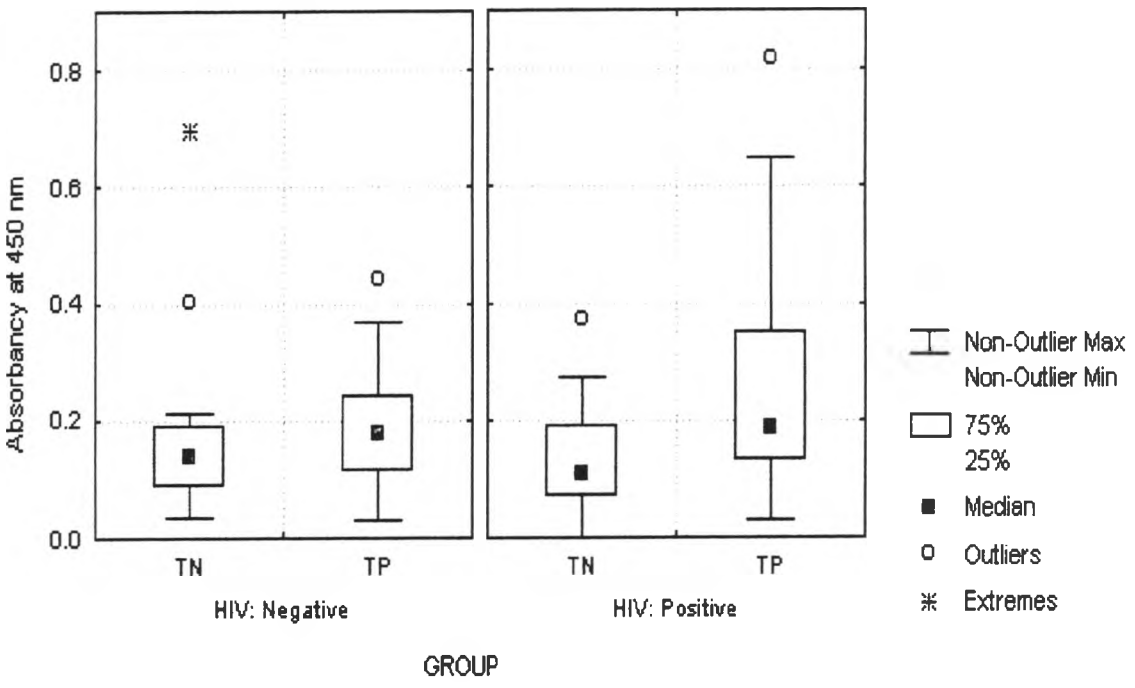
ELISA RESULTS	TUBERCULOSIS POSITIVE (n=39)	TUBERCULOSIS NEGATIVE (n=40)	SIGNIFICANCE (p value)
ELISA performed on serum absorbancy at 450nm (mean ± SD)	0.393 ± 0.263	0.253 ± 0.227	P=0.0132
ELISA performed on precipitate absorbancy at 450nm (mean ± SD)	0.242 ± 0.169	0.128 ± 0.083	P=0.0003

SD = Standard Deviation

NS = Statistically Not Significant

n = Total number of patients

FIGURE 4.2



ANTI-MYCOLIC ACID ANTIBODY LEVELS IN PRECIPITATED SERA OF 59 PATIENTS WITHOUT TUBERCULOSIS (TN) AND IN 59 PATIENTS WITH TUBERCULOSIS (TP), GROUPED ACCORDING TO HIV STATUS

4.7.3 HIV-SERONEGATIVE TP AND HIV-SERONEGATIVE TN PATIENTS

The results of the ELISA performed on HIV-seronegative TP and HIV-seronegative TN patients are shown in **table 4.9**. In HIV-seronegative TP patients, the corrected signal intensities (mean ± SD) in serum (0.307 ± 0.156, p=0.18) and in precipitate (0.193 ± 0.103, p=0.635) were higher compared to corrected signal intensities in serum (0.243 ± 0.136) and in precipitate (0.174 ± 0.143) in HIV-seronegative TN patients. These results did not reach statistical significance. A Box-and-whisker graph of these results is shown in **figure 4.2.**

TABLE 4.9

RESULTS OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 20 HIV-SERO-NEGATIVE PATIENTS WITH PULMONARY TUBERCULOSIS AND 19 HIV-SERONEGATIVE PATIENTS WITHOUT PULMONARY TUBERCULOSIS

ELISA RESULTS	TUBERCULOSIS POSITIVE (n=20)	TUBERCULOSIS NEGATIVE (n=19)	SIGNIFICANCE (p value)
ELISA performed on serum absorbancy at 450nm (mean ± SD)	0.307 ± 0.156	0.243 ± 0.136	P=0.18
ELISA performed on precipitate absorbancy at 450nm (mean ± SD)	0.193 ± 0.103	0.174 ± 0.143	P=0.635

SD = Standard Deviation

NS = Statistically Not Significant

n = Total number of patients

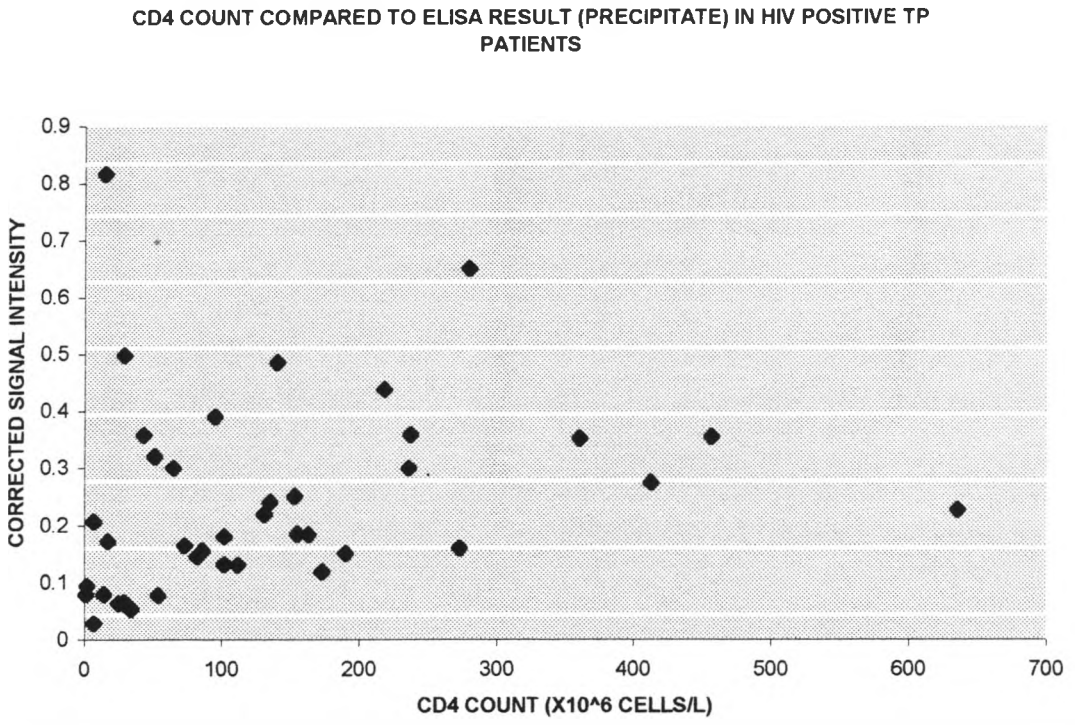
4.8 THE RELATIONSHIP BETWEEN CD4 T-LYMPHOCYTE COUNT AND ANTIBODIES TO MYCOLIC ACIDS IN HIV-SEROPOSITIVE PATIENTS

There was no significant decline in the ELISA signal intensity with a declining CD4 T-lymphocyte count in HIV-seropositive patients. The results of the comparison of the ELISA signal intensity to CD4 T-lymphocyte count in HIV-seropositive TP patients are shown in **figure 4.3** (correlation coefficient=0.225, r squared=0.05, p=0.168). The results of the comparison of the ELISA signal intensity to CD T-lymphocyte in HIV-seropositive TN patients are shown in **figure 4.4** (correlation coeffecient=0.119, r squared=0.014, p=0.462). These results argue against a significant correlation between anti-mycolic acid antibody levels and CD4 T-lymphocyte counts in HIV-seropositive patients with tuberculosis.

4.9 SENSITIVITY, SPECITIVITY, POSITIVE PREDICTIVE VALUE, AND NEGATIVE PREDICTIVE VALUES FOR THE ELISA

For serum, the sensitivity of the ELISA was 51% and the specificity was 63%. The positive predictive value was 58% and the negative predictive value was 56% **(table 4.10)**. For precipitate, the sensitivity was 37% and the specificity was 85%. The positive predictive value was 71% and the negative predictive value was 57% **(table 4.10)**.

FIGURE 4.3



A COMPARISON OF THE ANTI-MYCOLIC ACID ANTIBODY LEVELS (PRECIPITATE) TO THE CD4 T-LYMPHOCYTE COUNT IN HIV-SEROPOSITIVE PATIENTS WITH TUBERCULOSIS

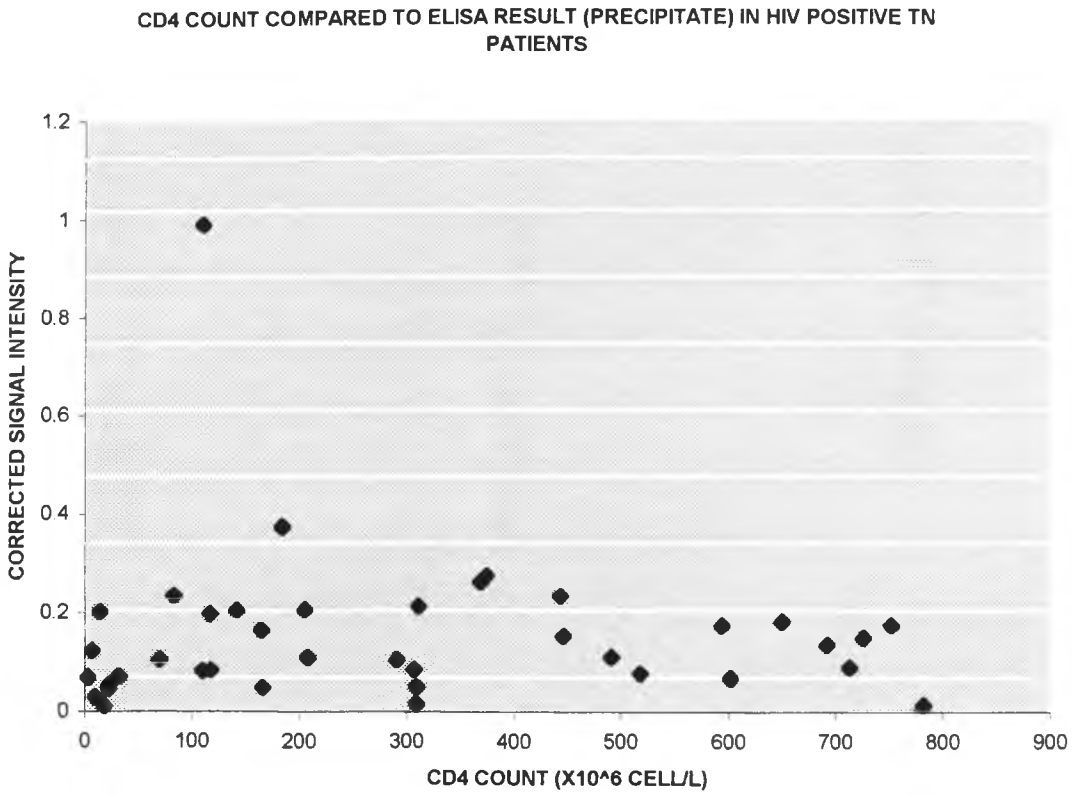
n = 39

correlation coefficient (r) = 0.225

r squared = 0.05

p = 0.168

FIGURE 4.4



A COMPARISON OF THE ANTI-MYCOLIC ACID ANTIBODY LEVELS (PRECIPITATE) TO THE CD4 T-LYMPHOCYTE COUNT IN HIV-SEROPOSITIVE PATIENTS WITHOUT TUBERCULOSIS

n = 40

correlation coefficient (r) = 0.119

r squared = 0.014

p = 0.462

TABLE 4.10

SENSITIVITY, SPECIFICITY, POSITIVE AND NEGATIVE PREDICTIVE VALUES OF THE ELISA TO DETECT ANTIBODIES TO MYCOLIC ACID IN THE SERA (AND PRECIPITATE THEREOF) OF 59 PATIENTS WITH PULMONARY TUBERCULOSIS AND 59 PATIENTS WITHOUT PULMONARY TUBERCULOSIS

ELISA RESULTS	SENSITIVITY	SPECIFICITY	POSITIVE PREDICTIVE VALUE	NEGATIVE PREDICTIVE VALUE
ELISA PERFORMED ON SERUM (n=118)	**51%**	**63%**	**58%**	**56%**
ELISA PERFORMED ON PRECIPITATE (n=118)	**37%**	**85%**	**71%**	**57%**

Values expressed as percentages

n = Total number of patients

5. DISCUSSION

At the beginning of the 21st century tuberculosis remains a major public health issue. In developing countries tuberculosis is a leading cause of morbidity and mortality with the spread of the HIV epidemic contributing significantly to the worsening situation. Co-infection with *M.tuberculosis* and HIV results in special diagnostic problems, especially in the resource-poor countries of sub-Saharan Africa where the majority of these patients are found.

Rapid and accurate diagnosis of patients with symptomatic tuberculosis is a cornerstone of global tuberculosis control strategies. The shortcomings of sputum microscopy (operator dependency, insensitivity, unavailability) seriously limit both the extent and quality of its application, and ultimately its impact on tuberculosis control (65). The rising incidence of smear-negative tuberculosis in countries where HIV infection is prevalent further limits the use of sputum microscopy. Less than 20% of the approximately 8 million annual cases of tuberculosis are currently identified as smear-positive (65).

A welcome new tool in the fight against tuberculosis would be a rapid screening serological test with high sensitivity (high negative predictive value) to exclude symptomatic tuberculosis-negative patients from tuberculosis diagnostic work-up in overburdened clinics in sub-Saharan Africa. Long the holy grail of tuberculosis diagnostics, serological tests have not yet demonstrated sufficient performance to warrant routine use in

national control programs. The quality of clinical trial data is too poor to make firm statements about the performance of most of the serological tests currently available. In cases of co-infection of HIV and *M.tuberculosis* most of these assays perform poorly when tested in field conditions in Africa, detecting less than a third of patients with active tuberculosis (65).

The characterization, purification and isolation of non-protein *M.tuberculosis* antigens, such as mycolic acids, has allowed the development of promising new serological tests which appear to have improved sensitivity in HIV-infected patients (65). The current study is the first one investigating the diagnostic potential of an ELISA based on detecting antibodies to *M.tuberculosis* mycolic acids in sera of HIV-seropositive and HIV-seronegative tuberculosis patients in a population with a high prevalence of tuberculosis. Control patients with various medical conditions and non-mycobacterial infections were recruited from the general wards, thereby simulating proper field conditions.

This is the first study to detect specific anti-mycolic acid antibodies in sera of HIV-seropositive and HIV-seronegative patients with active and untreated pulmonary tuberculosis. Studies by Maekura (132) and He (134) have shown elevated levels of antibodies to cord factor (trehalose-6,6`-dimycolate) in HIV-seronegative patients with tuberculosis. The antigenic epitope in cord factor has recently been shown by Pan (133)

and Fujiwara (143) to be the oxygenated mycolic acids, more specifically the *M.tuberculosis* methoxy mycolic acid. Simonney (129, 131) and Harrington (130) have shown that patients with tuberculosis have elevated levels of antibodies to several other *M.tuberculosis* lipid antigens, such as 2,3-diacyl trehalose (DAT), PGLTb1, LOS and SL-1. These studies also showed that precipitation of circulating immune complexes in the sera improved the sensitivity of the ELISA used. This was thought to be due to the recovery of anti-lipid antibodies from the immune complexes, which would otherwise have gone undetected by being washed away in the washing cycles of the ELISA.

The current study showed a relatively higher level of antibodies to *M.tuberculosis* mycolic acids in sera of HIV-seropositive patients with tuberculosis (0.393 ± 0.263) compared with HIV-seropositive patients without tuberculosis (0.253 ± 0.227). This difference was significant when the ELISA was performed on sera ($p=0.0132$), but even more so when performed on precipitated sera ($p=0.0003$). Furthermore, there was no significant relationship between declining CD4 T-lymphocyte counts and antibody levels in HIV-seropositive patients ($r=0.225$, $p=0.168$). As many HIV-seropositive patients in the TP group had advanced immunosuppression (mean CD4 T-lymphocyte count of 138.25 ± 140.49 $x10^6$ cells/L), this suggests that the antibody response to *M.tuberculosis* mycolic acid is well preserved despite a decline in CD4 T-lymphocyte count. This is in contrast to the study by Simonney (129) which showed lower IgG antibodies to other lipid *M.tuberculosis*

antigens (DAT, LOS) in HIV-infected tuberculosis patients compared with HIV-seronegative tuberculosis patients. Although CD4 T-lymphocytes have been shown to be involved in the CD1-restricted antigen presentation pathway in leprosy (46), they may not be clinically important in the antibody response to *M.tuberculosis* mycolic acids. Thus, the immune response to, and antibody production against *M.tuberculosis* mycolic acid may be preserved in HIV-infected patients with a declining CD4 T-lymphocyte count. This makes these antibodies an attractive reporter for serological diagnosis of tuberculosis in populations with a high prevalence of HIV infection.

The high levels of antibodies to mycolic acids in the TN control group and the wide standard deviations in both the TP and TN groups resulted in a poor sensitivity and specificity of the ELISA (51% and 63% for serum, and 37% and 85% for precipitate respectively). This is comparable to the results shown by Somi, Ratanasuwan, and Lawn (123, 124, 125) when the MycoDot sorodiagnostic assay was evaluated in African field conditions (sensitivity of 16%-55% and specificity of 84%-93%) (**table 5.1**). Studies by Simonney, Harrington, He and Maekura (129, 130, 132, 134) showed much lower levels of antibodies to *M.tuberculosis* lipid antigens in their control groups.

The reasons for the high levels of antibodies in the TN control group in this study are possibly two-fold. Firstly, there is a high

TABLE 5.1

SENSITIVITY AND SPECIFICITY OF DIFFERENT SERO-DIAGNOSTIC ASSAYS TO DETECT TUBERCULOSIS IN POPULATIONS WITH A HIGH PREVALENCE OF TUBERCULOSIS AND HIV INFECTION

SERO-DIANOSTIC ASSAY	SENSITIVITY	SPECIFICITY
ANTI-MYCOLIC ACID ANTIBODIES (SERUM) CURRENT STUDY (n = 118)	**51%**	**63%**
ANTI-MYCOLIC ACID ANTIBODIES (PRECIPITATE) CURRENT STUDY (n=118)	**37%**	**85%**
ANTI-LIPOARABINOMANNAN ANTIBODIES SOMI, et al. (n = 241)	**16%**	**84%**
ANTI-LIPOARABINOMANNAN ANTIBODIES RATANASUWAN, et al. (n = 594)	**56%**	**97%**
ANTI-LIPOARABINOMANNAN ANTIBODIES LAWN, et al. (n = 92)	**44%**	**97,5%**

Values expressed as percentages

n = Total number of patients

incidence of tuberculosis in the general population in South Africa with a high exposure level to *M.tuberculosis*. Although every effort was made to exclude patients with previous or current symptomatic tuberculosis from the control group, these patients may previously have had, or currently have, subclinical infection with *M.tuberculosis* with a measurable antibody response to mycolic acids. It is important to note that Maekura showed that, in patients with pulmonary tuberculosis, the anti-cord factor IgG antibody titres declined to `normal` levels after anti-tuberculosis chemotherapy (134). These results argue against long-lasting anti-mycolic acid antibodies in patients with effectively treated tuberculosis. One would therefore not expect that BCG immunization during childhood would cause a false positive result when measuring antibodies to *M.tuberculosis* mycolic acids.

Secondly, the control TN group of patients were recruited from the general medical wards of a busy secondary hospital. These patients were admitted for a variety of medical conditions, including heart failure, non-tuberculous infections and diabetes mellitus. It is possible that other non-tuberculous infections or non-infectious inflammatory responses can lead to the production of antibodies to non-specific lipid antigens, such as oxidized cholestrol complexes, which may cross-react with mycolic acids. This may cause false positive results in the mycolic acids based ELISA. For example, elevated levels of anti-cholesterol antibodies have been found in patients infected with *Trypanosoma* and *Mycoplasma* (144, 145).

When *M.tuberculosis* mycolic acid antibody levels were measured in the sera of 10 healthy controls, they were found to be much lower in sera (0.183 ± 0.059) and precipitate (0.142 ± 0.047) than in the TN group used in this study (Verschoor et al., unpublished data). In the studies by Simonney, Harrington, He and Maekura (129, 130, 132, 134) the control groups consisted of healthy volunteers and were not truly representative of patients found in actual clinical practice. This is an important reason why many serological tests for tuberculosis do not perform as well as expected when tested in the field in countries with a high prevalence of tuberculosis. In contrast to the studies performed in developed countries with a low prevalence of tuberculosis and HIV infection (121, 122), studies by Somi, Ratanasuwan, and Lawn (123, 124, 125) all showed poor results when the MycoDot test was evaluated in countries with a high prevalence of HIV infection, tuberculosis and non-tuberculous infections in the population.

6. STRENGTHS AND WEAKNESSES OF THE STUDY

The major strength of the study is that it was carried out under typical field conditions in which a serological test for tuberculosis would be a useful application i.e. in a developing country with a high prevalence of tuberculosis and HIV infection. The control group of TN subjects were not healthy volunteers, but patients with a variety of medical conditions and non-tuberculous infections, recruited from the general medical wards, again simulating field conditions. Each subject was evaluated individually by the author, fresh serum was reliably collected, and extensive demographic and laboratory data were recorded and analyzed. Pulmonary infection with *M.tuberculosis* was confirmed on culture of sputum, which is currently considered to be the gold standard for the diagnosis of pulmonary tuberculosis. Patients with HIV infection were found to have elevated levels of antibodies despite declining CD4 T-lymphocyte counts, thereby eliminating possible false negative results due to HIV-related immunosuppression.

The possible weaknesses of the study are the relatively small numbers of patients with tuberculosis (n=59) and control subjects (n=59) tested. Although strict adherence to the protocol was observed in order to exclude patients with tuberculosis from the control group, the high prevalence of tuberculosis and HIV infection in the population increases the likelihood that patients with subclinical or smear-negative tuberculosis may have inadvertently been included in the control group. This would lead to elevated levels of antibodies

to *M.tuberculosis* mycolic acids in the `tuberculosis negative` group of control subjects which would have been interpreted as false positives. Non-tuberculous infections, such as *Mycoplasma*, may have caused false positive results due to possible cross-reactivity with anti-cholesterol antibodies (145).

7. CONCLUSION

Sub-Saharan Africa is experiencing the devastating twin epidemics of HIV and tuberculosis. In many of these countries the time between onset of symptoms and diagnosis of smear-positive tuberculosis is 3-4 months (146, 147). This delay in diagnosis and treatment compromises the chances of cure and containing the epidemic. The diagnosis of tuberculosis in these resource-poor regions is fraught with difficulty because of a shortage of trained health personnel, poor diagnostic facilities, and an increasing percentage of patients with smear-negative tuberculosis.

An accurate, rapid and cheap sero-diagnostic test for tuberculosis would help to identify and treat symptomatic patients with tuberculosis. Such a strategy is a cornerstone of global tuberculosis control programs (148). Patients with tuberculosis, including those co-infected with HIV, demonstrate elevated levels of antibodies to *M.tuberculosis* mycolic acids. The ELISA to detect anti-mycolic acid antibodies evaluated in this study has poor sensitivity and specificity and is currently not suitable as a reliable sero-diagnostic assay for the diagnosis of pulmonary tuberculosis. Its use in the diagnosis of extra-pulmonary tuberculosis still needs to be investigated. The novel CD1-restricted lipid antigen presentation pathway, and the observation that the antibody response to mycolic acids is preserved in HIV-seropositive patients despite a declining CD4 T-lymphocyte count, makes this sero-diagnostic assay an

attractive potential test to diagnose tuberculosis in areas of high HIV infection prevalence.

Further work is needed in this field to develop a reliable diagnostic test to detect antibodies to lipid tuberculosis antigens, and to develop an affordable and accurate sero-diagnostic test for tuberculosis.

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APPENDIX 1a

SUBJECT INFORMATION SHEET

(for subjects without evidence of pulmonary TB)

Hello,

I am Doctor Gunter Schleicher from the University of the Witwatersrand Medical School and I am inviting you to participate in a research project to find a quick and reliable way to diagnose tuberculosis using a blood test.

Why are we doing this?

In South Africa many people suffer from TB.This is an infection that usually affects the lungs and can be transmitted from one person to another via respiratory secretions. The usual way of diagnosing TB is to test patients` sputum, but this is not always 100% effective and some patients may have TB with normal sputum tests.

We are trying to develop a blood test that will hopefully help us to diagnose TB quickly and accurately, especially in those patients with sputum tests that are not helpful.

What do we expect from you?

You do not have TB. In order to develop this blood test we need to test it on people without any evidence of TB infection to ensure that it is accurate. An experienced doctor or nurse will draw your blood once only. Your name will not appear on any of the samples or on any publications. Also, your blood will not be used for any other tests or experiments besides this TB project.

May I withdraw from this study?

Yes. You are free to withdraw at any time without providing any reasons and this will not affect your medical treatment in any way.

Why should I participate in this study?

Although you may not personally benefit from this study, the knowledge accumulated through testing your blood will prove advantageous to others.

You are encouraged to ask any questions that you may have in connection with this study at any time.

My staff or I will gladly answer your questions.

If you are happy to take part in this study, please read and sign the attached consent form.

Thank you

Dr Gunter Schleicher

(tel: 011 488 3334 bleep 3050)

APPENDIX 1b

SUBJECT INFORMATION SHEET

(for subjects with newly diagnosed pulmonary TB)

Hello,

I am Doctor Gunter Schleicher from the University of Witwatersrand Medical School and I am inviting you to participate in a reseach project to find a quick and reliable way to diagnose tuberculosis ("TB") using a blood test.

Why are we doing this?

In South Africa many people suffer from TB. This is an infection which usually infects the lungs and can be transmitted from one person to another via respiratory secretions. The usual way of diagnosing TB is to test patients' sputum, but this is not always 100% effective and some patients may have TB with normal sputum tests.

We are trying to develop a blood test that will hopefully help us to diagnose TB quickly and accurately, especially in those patients with sputum test that are not helpful.

What do we expect from you?

Your blood will be drawn once only by an experienced doctor or nurse. You and others will provide us with necessary samples to determine whether or not this test is suitable to be used in South Africa for TB testing. Your name will not appear on any of the

samples or on any publications. Therefore, your total anonimity is guaranteed. You can also be assured that your blood will not be used for any other experiment besides this TB project.

May I withdraw from this study?

Participation in the study is completely voluntary and you are free to withdraw at any time without providing any reasons an this will not affect your medical treatment in any way.

Why should I participate in this study?

Although you might not personally benefit from this study, the knowledge accumulated through testing your blood may prove advantageous to others. You are encouraged to ask any questions that you might have in connection with this study at any time. I or my staff will gladly answer your questions.

If you are happy to take part in the study, please read and sign the attached consent form.

Thank you

Dr Gunter Schleicher

(tel : 011 488 3334 Bleep 3050)

APPENDIX 2

PATIENT CONSENT FORM

I, _____the undersigned, understand and agree that a sample of my blood may be used in this study to measure antibodies to Mycobacterium Tuberculosis mycolic acids. The purpose of this study is to develop an ELISA-based sero-diagnostic assay for TB.

I am also assured that :

1. my blood will not be used for any other purpose other than these experiments;
2. my name, personal particulars or medical history will not be divulged to anyone outside the research team;
3. total confidentiality is guaranteed;
4. I may withdraw from this study at any time without providing reasons for doing so.

Patient signature ..... Date:

Doctor signature ..... Date:

Witness signature ..... Date:

APPENDIX 3

RECOGNITION OF CHOLESTEROL BY ANTI-MYCOLIC ACIDS ANTIBODIES IN SERUM FROM TUBERCULOSIS PATIENTS: ELISA ASSAYS

1 MATERIALS

1.1 Mycolic acids

Mycobacterial mycolic acids were isolated from a culture of *Mycobacterium tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum *et al.* (Goodrum, Siko et al. 2001).

1.2 ELISA reagents

PBS buffer: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 (anhydrous) and 1.05 g Na_2HPO_4 (anhydrous) per 1 *l* distilled water, adjusted to pH 7.4.

Diluting buffer: 0.5% (m/v) carbohydrate- and fatty acid free casein (Calbiochem, La Jolla, CA) in PBS buffer adjusted to pH 7.4 was used for diluting of the sera and the immunoreagents.

Blocking buffer: same as diluting buffer and used for blocking of ELISA plates.

Washing buffer: same as diluting buffer and used for washing of ELISA plates.

Coating antigens: Mycolic acids originating from *Mycobacterium tuberculosis*, isolated as described above, and cholesterol (Sigma, St. Louis, MO; Cat No C-8667) were used at final concentrations of 60 µg/ml and 75 µg/ml, respectively. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85°C. The hot solutions were sonicated at 20% duty cycles and optimal output level for 1 min. The solutions were kept at 85°C and loaded into the ELISA plates.

Conjugates: Goat anti-human IgG (H + L chains) antibody conjugated to peroxidase was obtained from Sigma (Cat No A-8667).

Substrate: *o*-Phenylenediamine (Sigma; Cat No P-1526) and hydrogen peroxide (Merck, Darmstadt, BRD; Cat No BDR 10366).

Substrate buffer: 0.1 M citrate buffer (0.1 M citric acid and 0.1 M tri-sodium citrate, adjusted to pH 4.5).

1.3 Human sera

Human sera were collected by Dr GK Schleicher from patients at Helen Joseph Hospital, Auckland Park, Johannesburg, RSA, between August and December 2000.

1.4Plasticware

The following plasticware was used:

ELISA plates: flat-bottom 96-well plates (Serowell; Bibby Sterilin Ltd, Stone, UK).

Sterile, disposable 50 ml centrifuge tubes (Bibby Sterilin).

Disposable pipettes (Bibby Sterilin).

Disposable pipette tips (Bibby Sterilin).

1.5 Reagents for precipitation of immune complexes

- **8 % PEG solution:** PEG 8000 (Sigma, Cat. no. P-2139), 1.6 g dissolved in 20 ml 0.01 M PBS.
- **Acidification buffer:** Glycine HCL (0,2 M, pH 2.8)
- **Neutralisation buffer:** K_2HPO_4 (1 M in dddH_2O). The pH was adjusted to 9.0 with H_2KPO_4 (1 M) if necessary.

2 METHODS

2.1 Antigen coating of ELISA plates

The respective antigens were dissolved in hot PBS and then sonicated, as described above. The wells of flat-bottom ELISA plates were coated overnight at 4°C with 50 µl/well of antigen solution. The final antigen load was approximately 3 µg/well for mycolic acids or approximately 3.75 µg/well for the cholesterol.

2.2 Blocking of ELISA plates

The coating solution was flicked out of the plates and replaced with 400 µl blocking buffer per well. Blocking was carried out for 2 hours at room temperature.

2.3 Precipitation of immune complexes

To undiluted serum (100 µl) in an Eppendorf tube, an equal volume of PEG 8000 in 0.01M PBS (pH = 7.4) was added, mixed and left overnight at 4°C. The precipitate was collected by centrifugation at 4°C for 30 min. The supernatant was discarded and the pellet washed two times with 4% PEG. After the pellet was washed, it was dissolved in 100 µl 0.154M PBS. Acidification buffer (50 µl) was added to the sample on ice to release the antibodies from the immune complexes. After standing for 15 minutes, neutralisation buffer (25 µl) was added. Double distilled water (25 µl) and ELISA diluting buffer (1800 µl) was added to the samples to obtain a final volume of 2 ml, representing a 1:20 dilution of serum.

2.4 Binding of human antibodies

The blocking solution was aspirated from the wells before loading of the serum or serum precipitate samples. Sera were diluted 20 times in diluting buffer. Aliquots of 50 µl were introduced into wells in quadruplicate. The plates were incubated at room temperature for 1 hour. The serum samples were removed from the wells, the wells washed three times with washing buffer using an Anthos Autowash automatic ELISA plate washer and then emptied by aspiration.

2.5 Quantitation of the bound antibodies

Peroxidase-conjugated anti-human IgG diluted 1:1000 in diluting buffer was introduced in aliquots of 50 µl per well and the plates were incubated for 30 min at room temperature. After removal of the conjugate, the wells were washed three times with the washing buffer and then emptied by aspiration.

The substrate solution comprising 10.0 mg *o*-phenylenediamine and 8.0 mg hydrogen peroxide in 10 ml of 0.1 M citrate buffer pH 4.5, was prepared immediately before use and introduced in 50 µl aliquots per well. The plates were incubated at room temperature and the colour development was monitored at 5, 30 and 60 min after addition of the substrate using an SLT 340 ATC photometer at a wavelength of 450 nm.

APPENDIX 4

PATIENT DATA SHEET

NAME:
STUDY NO:
AGE:
SEX:
RACE:

CLINICAL DETAILS

HISTORY:
PAST MEDICAL HISTORY:
DRUG HISTORY:
SMOKER:
OCCUPATIONAL DUST EXPOSURE:
ALLERGIES:

CLINICAL EXAMINATION

BP:
PULSE:
TEMPERATURE:
RESPIRATORY RATE:
CYANOSIS:
PALLOR:
LYMPHADENOPATHY:
CLUBBING:
WASTING:
ORAL CANDIDA:
CHEST:
CVS:
ABDOMEN:
OTHER:

LABORATORY RESULTS

SPUTUM AFB:
SPUTUM MYCOBACTERIAL CULTURE:

HEMOGLOBIN:
WHITE CELL COUNT:
DIFFERENTIAL COUNT:
PLATELET COUNT:

SODIUM:

POTASSIUM:
CHLORIDE:
CO2:
UREA:
CREATININE:
ALBUMIN:
TOTAL PROTEIN:

HIV SEROLGY (ELISA):
CD4 COUNT:
SYPHILIS SEROLOGY:

CRP:
ESR:

RADIOLOGY

CHEST RADIOGRAPH:

ANTI-MYCOLIC ACID ELISA

SERUM:
PRECIPITATE:

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Schleicher

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M00/06/19

PROJECT Antibodies To Mycobacterium Tuberculosis Mycolic Acids In Patients With Active Pulmonary Tuberculosis

INVESTIGATORS Dr GK Schleicher

DEPARTMENT Department of Medicine, Johannesburg Hospital

DATE CONSIDERED 00/06/30

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 00/07/10 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof C Feldman
Dept of Department of Medicine, Johannesburg Hospital

Works2\biol\015\HumEth97.wdb\M 00/06/19

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Dr G. K. Schleicher