PROPERTIES OF ANTI-MYCOLIC ACID ANTIBODIES IN HUMAN TUBERCULOSIS PATIENTS

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LIST OF CONTENTS

CHAPTER 1: ANTIBODY PRODUCTION TO MYCOBACTERIAL LIPID ANTIGENS

CHAPTER 2: ANTI-MYCOLIC ACID ANTIBODIES IN PULMONARY TUBERCULOSIS PATIENTS TREATED OR CO-INFECTED WITH HIV

CHAPTER 3: PROPERTIES OF ANTI-MYCOLIC ACID ANTIBODIES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-GalCer	α-galactocylceramide
3β-OH	3β-hydroxyl group
5-BMF	5-Bromofluorescein
ACHA	anti-cholesterol antibodies
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cells
Apo B-100	apoproteinB-100
ApoAI	lipoprotein AI
ATCC	American Type Culture Collection
BCG	Bacillus Calmette Guerin
BIA	biomolecular interaction analysis
BSA	bovine serum albumin
CD	cluster of differentiation molecules
CE	cholesterol ester
CEases	cholesterol esterases
CETP	cholesteryl ester transfer protein
CF	cord-factor
CIC	circulating immune complexes
CM	chylomicrons
CPC	cetyl pyridium chloride
CR	complement receptor
CTL	cytotoxic T-lymphocytes
DAT	diacyl trehaloses
DC	dendritic cells
DCA	dicarboxylic fatty acid
$dddH_2O$	double distilled, de-ionised water
DMPC	dimyristoyl-phosphatidylcholine
DMPG	dimyristoyl-phosphatidylglycerol
DN	double negative

EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FA/s	fatty acid/s
Fc	fragment crystalline
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
GMM	glucose monomycolate
GPI	glycosyl phosphatidylinositol
HCl	hydrochloric acid
HDL	high density lipoproteins
HIV	human immune deficiency virus
HNTN	HIV negative, TB negative
HNTP	HIV negative, TB positive
HPTN	HIV positive, TB negative
HPTP	HIV positive, TB positive
IAsys	interaction analysis system
IC	immune complex
IDL	intermediate density lipoprotein
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
LAM	lipoarabinomannan
LDL	low density lipoproteins
LE	late endosomes
LM	lipomannan
LP	lipoproteins
Lyso-PC	lyso phosphatidylcholine

M. tb	*Mycobacterium tuberculosis*
MA	mycolic acid
mAG	mycolyl arabinogalactan
MHC class I/II	major histocompatibility complex class I/II
MIIC	MHC class II antigen loading compartment
MR	mannose receptor
MRC	Medical Research Council
MZB	marginal zone B cells
NAA	natural autoantibodies
NaCl	sodium chloride
NaOH	sodium hydroxide
NKT cells	natural killer T cell
NRF	National Research Foundation
OPD	ortho-phenylenediamine
OxLDL	oxidized LDL
P	probability
P57	patient 57
PAT	penta-acyl trehalose
PBS	phosphate buffered saline
PBS/ AE	phosphate buffered saline containing azide and EDTA
PC	L-α-phosphatidylcholine
PDIM	phthiocerol dimycocerosate
PEG	polyethylene glycol
PG	peptidoglycan
PGL	phenolglycolipids
Phos Ag	phospho antigens
PIM	phosphatidylinositol mannoside
PL	phospholipid
r	correlation
r^2	strength of the relationship

rHDLs	reconstituted discoidal high-density lipoproteins
SAT	sulphated tetra-acyl trehalose
SD	standard deviation
Seph-A	Sepharose protein - A
SL	sulpholipids
SM	sphingomyelin
Sp-A	surfactant protein A
SPSS	statistical package for social sciences
SR	scavenger receptors
TAG	triacylglycerol
TAP	transporters associated with antigen processing
TAT	triacyl trehalose
TB	tuberculosis
TBGL	tuberculosis glycolipid antigen
TCR	T-cell antigen receptor
TDM	trehalose dimycolate / cord factor
TH cells	T helper cells
TMM	trehalose monomycolates
TRIS	tris (hydroxymethyl) aminomethane
TST	tuberculin skin test
t-test	independent samples t-test
UC	unesterified cholesterol
VLDL	very low density lipoproteins

SUMMARY

Tuberculosis has re-emerged as a global health threat today. Current tuberculosis diagnosis is too slow in general and unsensitive in HIV burdened populations. Exposure to mycobacterial antigens in a country with a high prevalence of tuberculosis leads to false positive test results. Serodiagnosis would have been ideal, but was hitherto not successful.

Mycolic acid (MA) is the major lipid cell wall constituent of *Mycobacterium tuberculosis*, the etiological agent of this disease. In this study an antibody response to the MA molecules are investigated as a possible surrogate marker for tuberculosis. In previous studies, IgG antibodies to MA in TB infected, HIV seronegative patients were detected in human sera, with promising results. In this study the ELISA results detecting anti-IgG antibodies to MA in TB and HIV co-infected patients showed a low sensitivity and specificity. The study, however, showed that antibodies to MA are prevalent in HIV seropositive patients. The presentation of MA on the CD1 molecule to T cells might explain why anti-MA antibodies are detected in HIV seropositive patients.

The properties of anti-mycolic acid antibodies were investigated to explain the low sensitivities and specificities of the ELISA test. An ELISA was done comparing signals to MA and cholesterol as coating antigen. A degree of cross-reactivity of anti-MA antibodies to cholesterol was obtained. In using the IAsys biosensor it was shown that anti-MA antibodies were inhibited with MA and cholesterol as antigens in liposomes with cholesterol as the weaker antigen.

An antibody response to MA might prove to be a good surrogate marker for tuberculosis when measured in an IAsys biosensor based serodiagnostic test, where the serodiagnosis does not depend on the detection of high affinity anti-MA antibodies only.

OPSOMMING

Tuberkulose het herrys as ‘n wêreldwye gesondheidsrisiko vandag. Die huidige diagnose daarvan is te stadig en oor die algemeen te onsensitief in HIV-belaste populasies. Die algemene voorkoms van tuberkulose lei tot vals positiewe diagnose deur blootstelling aan tuberkulose antigene. Serodiagnose sou ideaal kon wees, maar was tot nou toe onsuksesvol.

Mikoolsuur (MS) is die oorwegende selwandlipied van *Mycobacterium tuberculosis*, die etiologiese agent van tuberkulose. In hierdie studie word ‘n teenliggaamreaksie teen MS ondersoek as ‘n surrogaatmerker vir tuberkulose. Vorige studies het die teenwoordigheid van IgG teenliggame teen MS in tuberkulose besmette MIV sero-negatiewe pasiënte aangetoon met belowende resultate. In hierdie studie word die teenliggame teen MS in TB- en MIV-dubbel-geïnfekteerde pasiënte ondersoek en ‘n lae sensitiwiteit en spesifisiteit van binding word aangetoon. Die studie toon egter aan dat die teenliggame teen MS algemeen in MIV positiewe pasiënte voorkom. Die presentering van MS deur CD1 molekules aan T selle, mag verduidelik hoekom hierdie teenliggame steeds teenwoordig is in MIV positiewe pasiënte.

Die eienskappe van die teenliggame teen MS is ondersoek om te verklaar waarom ‘n lae sensitiwiteit en spesifisiteit vir die ELISA-toets bekom word. ‘n ELISA is uitgevoer om teenliggaamseine teen geïmmobiliseerde MS en cholesterol as antigene te ondersoek. ‘n Mate van kruisreaktiwiteit van die teenliggame teen MS met cholesterol is gevind. Deur gebruik te maak van die IAsys biosensor is gewys dat teenliggame teen MS geïnhibeer is met MS en cholesterol as antigene in liposome. Cholesterol was die swakker antigeen.

‘n Teenliggaamreaksie teen MS kan dalk as ‘n goeie surrogaat-merker vir tuberkulose serodiagnose bewys word met ‘n IAsys biosensor gebasseerde tegniek, waar die serodiagnose nie slegs afhanklik is van hoë affiniteit teenliggame teen mikoolsuur nie.

CHAPTER 1: ANTIBODY PRODUCTION TO MYCOBACTERIAL LIPID ANTIGENS

1.1 INTRODUCTION

Today the single most fatal infection of the human race is tuberculosis (TB). It is caused by the rod-shaped *Mycobacterium tuberculosis* (*M.tb*) pathogen. Concomitant infection of HIV and the appearance of multi-drug resistant mycobacterium strains have led to the re-emergence of this disease (Sirakova *et al*., 2001; Anderson *et al*., 2000; Bennett *et al*., 2000). It is estimated that a third of the world's population is infected with latent *M. tb* (Boom *et al*., 2003). *M. tb,* as one of the most successful human pathogens, has evolved to escape the host immune system. The main attribute to the Mycobacterium's survival is its characteristic cell wall composition, which aids in resistance to some anti-microbial drugs. The cell wall is very high in lipid composition (50-60%), thus impermeable and resistant to hydrolytic molecules, enzymes and even small hydrophobic and amphiphatic molecules (Sirakova *et al*., 2001; Anderson *et al*., 2000; Liu *et al*., 1996). Although scientific investigation into lipids dates back to 1779 (Mead *et al*., 1986), it was only in the 1900s that lipids were identified as being immunogenic. This chapter investigates possible mechanisms for the production of antibodies to lipid antigens of *M. tb.*

1.2 *Mycobacterium tuberculosis*

TB is mainly contracted by the respiratory route. The mycobacterium enters the host organism and is engulfed by macrophages, neutrophils or dendritic cells (DC). As little as 1 to 3 tubercle bacilli might cause infection as they are suspended in the air stream directly entering the alveolar spaces of the human lung. Heavier bacillary particles with more bacilli and bits of caseous material fall on mucosal surfaces of the bronchial tree and the nasopharynx. Cillia then move these bacilli up the bronchial tree and are eventually swallowed (Schlossberg, 1999). Mycobacteria are facultative intracellular parasites, which can survive in alveolar macrophages.

M.tb mostly gains entry to mononuclear phagocytes through many receptors that include complement receptor types 1, 3, and 4, the mannose receptor (MR), the surfactant protein A (Sp-A) receptors, the Fc receptors, CD14 and the scavenger receptor A (Zimmerli *et al*., 1996). Receptor mediated entry into these cells results in the uptake of bacilli into phagolysosomes.

Phagosomes containing dead bacteria are taken up by the endocytic pathway to fuse with acidic lysosmes for digestion. Live *M. tb*, however, avoids fusion of the phagolysosome with late endosomes (LE) and lysosomes. The mycobacteria now persist within a non-acidified intracellular compartment, showing early endocytic properties see Figure 1.1. (Barker *et al*., 1997).

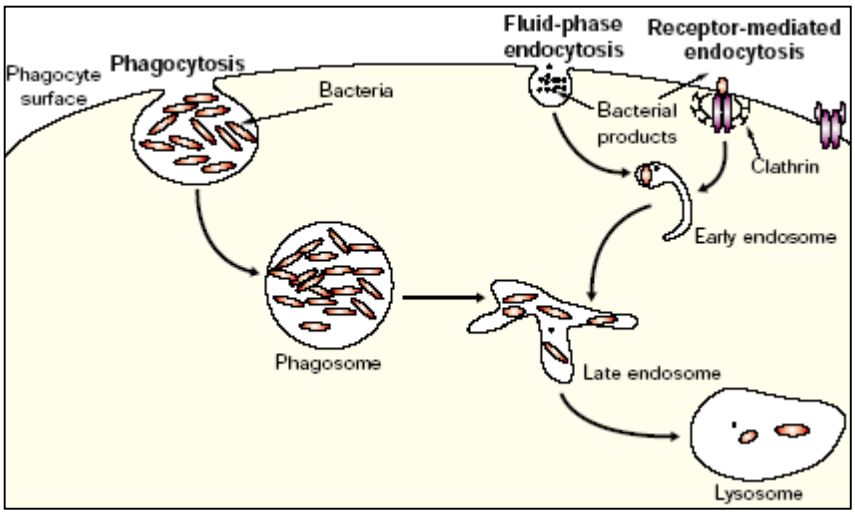


Figure 1.1: *Ingestion of bacteria by phagocytes*
Bacteria can be phagocytosed into phagosomes, whilst bacterial products can be taken up by fluid-phase endocytosis or receptor mediated endocytosis into early endososomes (clathrin coated pits). Bacteria and bacterial products are degraded in endosomal compartments fused with lysosomes (Pieters, 2001).

Local innate immunity (mostly through alveolar macrophages) fails to control the slowly replicating bacilli. The intracellular digestion of mycobacterial cells release macromolecular compounds that activate macrophages and migrating lymphocytes at the inflammation site to form a tubercle with a caseous centre (Nuzzo *et al*., 2002 & Richards *et al*., 2002). The slow growth of *M. tb* and chronic nature of *M. tb* infection result in prolonged exposure of the host to a large diversity of antigens derived from *M. tb*, resulting in the development of adaptive immunity. T cells then control, but do not eradicate persistent *M. tb* bacilli in infection (Boom *et al*., 2003).

When *M.tb* infection reaches the advanced stage, the solid caseous center of the tubercle liquefies. When such liquefied lesions discharge their contents a cavity is formed and the tubercle bacilli can now grow extracellularly. The released material enters the bronchial tree where the bacilli spread to other parts of the lung and to the environment. Bacilli in the primary lesions enter the bloodstream directly when the capillaries and venules within these lesions are eroded by the caseous necrotic process. Such bacilli are carried by the pulmonary veins to the left side of the heart, enter the aorta, and then are distributed throughout the body to every organ (Dannenberg and Collins, 2001).

1.3 DIAGNOSIS OF TUBERCULOSIS

It is essential that a fast, effective and inexpensive diagnostic test for TB is developed as early diagnosis stands central to the control of the disease (Van Rie *et al*., 2000; Anderson *et al*., 2000). Early diagnosis of TB would not only limit the spread of the disease, but would lead to early and more successful treatment of patients.

A problem associated with diagnosis is to distinguish between latent and active infection. The gold standard of active TB diagnosis today involves clinical examination and direct microscopic examination of sputum and culture of bacteria. Sputum smear tests for acid fast bacilli have a 50% sensitivity whilst the sputum culture on solid media has a long turnaround time in the laboratory (6-8 weeks). The use of liquid media shortens the time period required to +/- 10 days but the media and hardware are relatively expensive.

Bacillus Calmette-Guerin (BCG) vaccination to prevent TB disease leads to the loss of many diagnostic applications as memory immune responses can lead to cross-reactivity and false positive test results. Until the year 2001, the only way to detect latent TB was through the tuberculin skin test (TST). It relies on an immune response directed against proteins from heat killed *M. tb* cultures. The TST has poor specificity as both BCG vaccination and infection with other mycobacterium strains may lead to false positive diagnosis (Anderson *et al*., 2000). Immuno-suppressive conditions (e.g HIV) could further limit this cell-mediated response by decreasing sensitivity. Recently developed test involves the detection and quantification of the cytokine, interferon-γ, after the introduction of purified protein derivative (PPD) to blood T lymphocytes. One such assay, the QuantiFeron assay, was found to detect active TB more

accurately but it responded more to BCG vaccination, suggesting that it could not replace the TST skin test for the detection of latent TB infection (Fietta *et al*., 2003).

Rapid molecular methods have been developed to diagnose new cases of TB. These tests can also determine drug resistance and identify the mycobacterial strain of infection. Most of these molecular tests involve the polymerase chain reaction, a molecular amplification technique that is powerful and sensitive. Low levels of contamination can lead to false positive test results. A high degree of training of laboratory personnel (technicians) is therefore required (Anderson *et al*., 2000; Hugget *et al*., 2003). Most of these tests are associated with relatively high costs, which can be viewed as a problem in developing countries (Chan *et al*., 2000).

Diagnosis is more difficult in certain groups of individuals like the elderly, children and HIV infected patients. Serologic assays to detect antibodies to protein antigens are unreliable in HIV burdened populations because of the associated immune suppression. No single, suitable immunodominant antigen has emerged to date, but a number of antigens have been identified that are recognized by a majority of healthy, tuberculin skin-test positive persons (Boom *et al*., 2003).

Serodiagnostic tests are considered fast and only one intravenous specimen is needed.
One example is ELISA (enzyme linked immunosorbent assay), a method used for the detection of antibodies directed against immunodominant antigens. The test is complicated by the fact that *M. tb* is an intracellular pathogen. The wide antibody range of specificities exclude any that contribute to protection, while the polymorphic nature of MHC molecules underlies the errant reproducibility of ELISA–results from individual to individual, depending on the antigen used and even with the use of whole cells as antigenic determinants (Harrington III *et al*., 2000; Mauch *et al*., 1988).

This dissertation aims at the development of a serodiagnostic assay based on antibodies to mycolic acids as surrogate markers for infection.

1.4 HIV CO-INFECTION

The HIV and TB infection combo has led to an increase in active tuberculosis disease. HIV infection leads to increased rates of latent tuberculosis reactivation and greatly enhances the progression into active tuberculosis following new infection. Extrapulmonary tuberculosis is also much more common (Zumla *et al*., 2000; Godfrey-Faussett & Ayles, 2003). TB might also worsen the course of HIV associated immunodepression (Van Loenhout-Rooyackers & Veen, 1998; Kirschner, 1999; Ranjbar *et al*., 2004). Cavitatory tissue damage is reduced in the lungs of HIV-1 TB patients, where the immune system cannot successfully respond to *M. tb*. HIV infected individuals show abnormalities in fat distribution and hypocholesterolaemia is found (Grunfeld *et al*., 1989; Lucas *et al*., 1994; Crook & Mir, 1999; Rodwell *et al*., 2000).

$CD4^+$ T cells are important in peptide recognition of presented antigens, and are involved in Th1 and Th2 immune reactions. The HIV virus targets cells expressing the CD4 molecule, especially $CD4^+$ T cells, through the HIV protein gp120. The $CD4^+$ T cell and the virus fuse together, infection occurs and the abundance of the $CD4^+$ T cell subset decreases (from between 800 - 1200 in healthy individuals to below 200 $CD4^+$ T cells per cubic millimeter of blood in AIDS patients). Opportunistic infections such as TB then sets in, resulting in progressive primary TB infection, reactivation of endogenous mycobacteria or enhanced susceptibility to infection by *M. tb*.

In the serodiagnosis of tuberculosis, false negative results are obtained as the production of antibodies to peptide antigens decrease with the degree of immune suppression associated with HIV infection (Van der Werf *et al*., 1992; Roitt *et al*., 1998). This may be avoided if serodiagnosis is based on the detection of anti-lipid antigen antibodies as will be explained below.

1.5 HUMAN IMMUNE RESPONSE TO MYCOBACTERIA

Macrophages are the primary effector cells for the control of *M. tb* and are essential for processing and presentation of antigens to T cells. After an initial innate phase, acute T cell mediated adaptive immunity develops to control the proliferating bacilli. This is followed by a chronic memory immune phase necessary for control of persistent bacilli and surveillance for

possible remission of infection. Immune failure during acute or chronic adaptive immunity results in clinical TB, resulting in *M. tb* spreading to other organs in the body. The balance of the host–pathogen interaction in *M. tb* infection is determined by the interaction of T cells and infected macrophages. The relative importance of different T cell subsets and mechanisms employed by *M. tb* to interfere with macrophage and T cell function probably depends on the phase of the infection (Boom *et al*., 2003).

The ability of *M. tb* to modulate antigen presenting and microbicidal function of macrophages is essential for its survival (Boom *et al*., 2003). To survive (and thrive) in macrophages, *M. tb* has evolved mechanisms to block immune responses that include modulation of phagosomes, neutralization of macrophage effector molecules, inducing the secretion of inhibitory cytokines and interfering with antigen processing for presenting to T cells (Boom *et al*., 2003). Mycobacteria remain in the phagosomal compartment and are capable of blocking phagosome maturation to avoid the aggressive conditions within phagolysosomes (Schaible and Kaufmann, 2000).

1.6 ANTIGEN PRESENTATION IN TUBERCULOSIS

In lymphoid aggregates associated with the respiratory tracts, macrophages function as antigen-presenting cells (APC), initiating specific T cell mediated immune responses leading to increased T cell proliferative responses in the draining lymph nodes and ultimately the spleen (Gheorghiu, 1994). Mycobacteria with their peptide and lipid antigens are phagocytosed through receptor mediated recognition. Typical receptors for the uptake of *M. tb* include the mannose receptor (MR), complement receptor 3 (CR3), Fc and scavenger receptors (SRs). CD1b is endocytosed together with mycobacteria at the plasma membrane in clathrin coated pits. This leads to the formation of coated vesicle structures, which then transits to early endosomes (Figure 1.2). MHC class II is sorted at the trans Golgi network and is directed to endosomes in association with the invariant chain (Ii).

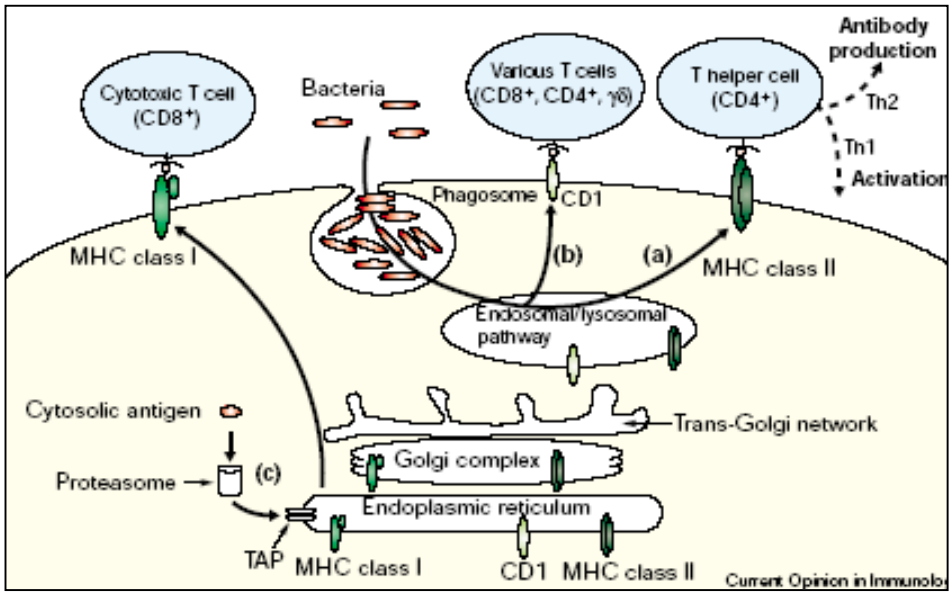


Figure 1.2: *Antigen presentation to T cells*

a. *Degraded protein antigens from the endosomal/lysosomal pathways are presented through the MHC class II molecules to T cells. T cells recognize the class II MHC molecule and the peptide antigen complex. The stimulation of Th1 or Th2 T helper cells are now effected.*
b. *Lipid moieties derived from microbes are presented to various T cell subsets including $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells by CD1 molecules.*
c. *MHC class I molecules present peptide antigens obtained from the cytosol to $CD8^+$ cytotoxic T cells, leading to rapid lysis of the infected presenting cell. These proteins are processed by the proteasome and transferred to the endoplasmic reticulum through transporters of antigenic peptides (Pieters, 2001).*

CD1b (lipid presentation) and MHC II molecules (peptide presentation) are then transported to the MHC class II antigen loading compartment (MIIC). Here they bind their respective antigens and are expressed on the cell surface for recognition by T cells through T cell receptors (TCR). CD1b endocytosis and trafficking to this MIIC were shown to be directed by an amino acid signal in the cytoplasmic tail of the CD1b molecule. Trafficking through these lysosomal compartments for antigen loading onto CD1b is only seen with dead mycobacteria (Barker *et al.*, 1997).

The interaction between T cells and infected mϕs is central to the protective immunity against *M. tb* (Figure 1.3). $CD4^+$ T cells have an essential role, while other T cell subsets such as $CD8^+$, γδ TCR^+ Tcells (γδ Tcells) and CD1 restricted T cells fulfill important supportive roles (Gheorghiu,1994).

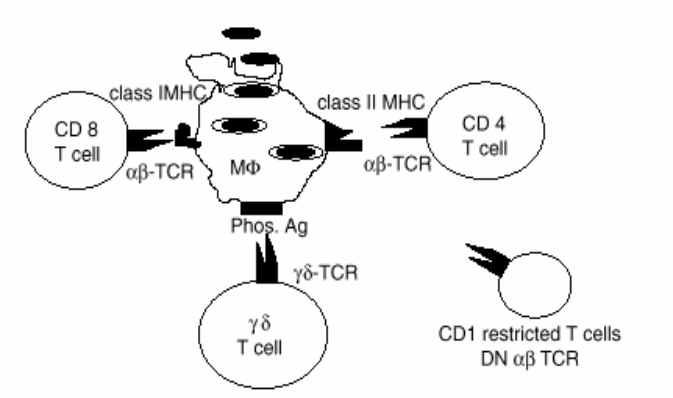


Figure 1.3: *A schematic presentation of functional T cell subsets in M. tuberculosis infected macrophages (Boom et al., 2003). Phos Ag- phospho antigens; mϕ - macrophage, DN - Double negative αβ TCR; TCR - T cell receptor, MHC- major histocompatibility complex.*

1.6.1 PRESENTATION OF PEPTIDE TUBERCULOSIS ANTIGENS

Mycobacterium peptide antigens are presented to the immune system through the major histocompatibilty complex molecules I and II (MHC I and II). The MHC family of molecules has a high polymorphism and function by presenting peptide antigens after processing in antigen presenting cells (APC) to T cells for further immune responses.

Class I MHC molecules present peptides derived from antigens (like viral antigens) that are present or synthesized in the cytosol. Peptides may also derive from microbial origin, from bacteria that are capable of migrating into the cytoplasm (Boom *et al*., 2003). Once in the cytosol, antigens are cleaved by proteasomes. Peptide fragments are delivered to the endoplasmic reticulum (ER) through specialized transport molecules, the transporters associated with antigen processing (TAP). In the ER the peptide fragments are further trimmed prior to association with the newly synthesized class I MHC molecules. The peptides are loaded into a cleft formed by the distal first and second domains of the MHC class I heavy chain. The complex is transported to the cell surface where it presents peptides to $CD8^+$ T cells. MHC-class-I-restricted $CD8^+$ T cells then primarily act by killing infected host cells (Schaible and Kaufmann, 2000).

Peptides presented by MHC class II molecules are generated in the endosomal–lysosomal system by proteases (Figure 1.2). The MHC class II molecules are composed of two heavy chains, the α chain and the β chain, with the distal domains of both chains forming a cleft into

which peptides are loaded. MHC-class-II-restricted $CD4^+$ T helper cells function through cytokine secretion to activate macrophages and/or B cells and play an important role in protective immunity against bacteria and protozoa (Schaible and Kaufmann, 2000).

Owing to the high polymorphism of MHC molecules different antigenic peptides are selected for presentation by different individuals (Schaible and Kaufmann, 2000).

1.6.2 LIPID ANTIGEN PRESENTATION

Many 'signature' bacterial lipids exist that are not encountered in vertebrate organisms. These foreign lipids are useful targets for various components of the immune system. Glycolipids are products of complex biosynthetic pathways, have a structural function in bacterial cell walls and are not susceptible to alterations by gene mutations. Their presentation to T cells therefore do not require polymorphic host ligand proteins. For this reason, glycolipid antigens presented by non-polymorphic CD1 molecules should induce an immune response in most or all individuals of an infected population (Schaible and Kaufmann, 2000).

CD1 molecules are lipid antigen presenting molecules. The overall structure of CD1 resembles that of MHC class I molecules, consisting of an extracellular β-2 microglobulin bound to an α chain with three extracellular domains (α1, α2, and α3), a transmembrane region, and a short cytoplasmic tail (Figure 1.4). The antigen binding site comprising the α1 and α2 domains of CD1 is very hydrophobic in comparison to that of the MHC molecules.

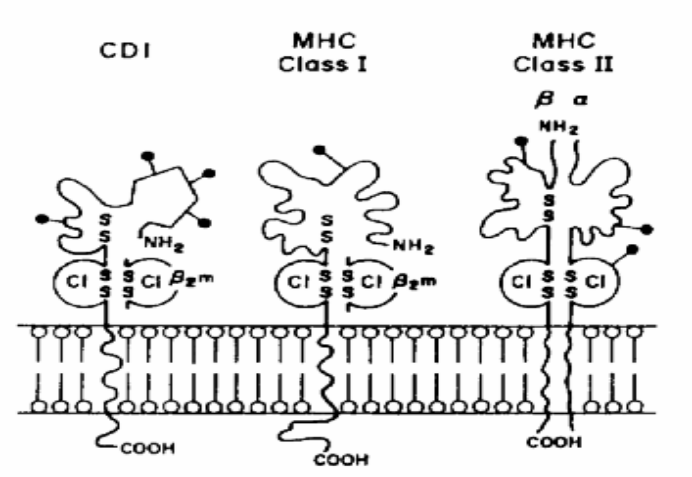


Figure 1.4: *Comparative structures of the antigen presenting molecules: CD1 and MHC I,II.*
CD1 and MHC I have a cytoplasmic tail, a transmembrane domain and 3 extracellular domains. MHC II, a heterodimer, has an additional cytoplasmic tail (Moody et al., 1996).

CD1 is conserved throughout much of the mammalian order (Tangri *et al*., 1998) with the C1-domain having nearly equal levels of homology with that of both class I and class II MHC molecules (Schaible and Kaufmann, 2000). CD1 molecules comprise several isotypes designated CD1 a-e, divided into two families; Group I (CD1a,b,c,e) and Group II (CD1d) (Wang *et al.,* 2000). CD1 molecules are expressed on APC (Sugita *et al*., 1998). In Table 1.1 the antigen presenting molecules CD1 and MHC I, II are compared in relation to their expression, T cell activation and function.

Table 1.1: *Comparison of expression, function and T cell recognition of CD1 and MHC molecules*

	MHC I	**MHC II**	**CD1a,b,c (group 1)**	**CD1d (group 2)**	**CD1d (group 2)**
Species	Human, mouse	Human, mouse	Human	Human (CD1d)	Mouse (CD1.1)
Genomic location	MHC	MHC	Non-MHC	non-MHC	non-MHC
Major cellular expression	Nucleated cells, hematopoietic cells*	B cells, DC (mature)*	APC, B cells (CD1c), Langerhans cells (CD1a,c), Cortical thymocytes, DC (immature)*	Cortical thymocytes, B cells, Intestinal epithelial cells, nonlymphoid tissues	B cells, monocytes, DCs, mϕ, Cortical thymocytes, Intestinal epithelial cells, Liver cells
Antigens presented	Endogenous peptides	Exogenous peptides	Endogenous/Exo-genous glycolipids, phospholipids & lipids.	Endogenous/Exo-genous glycolipids, phospholipids & lipids, glycosylated ceramides	Endogenous/Exo-genous glycolipids, phospholipids & lipids, glycosylated ceramides
T cell subsets stimulated	$CD8^+TCR\alpha\beta$	$CD4^+TCR\alpha\beta$	DN, $CD8^+$, $CD4^{+a,b}$, $TCR\alpha\beta$, $TCR\gamma\delta$	DN, $CD4^+$, $TCR\alpha$ (NKT), diverse $TCR\alpha\beta$	DN, $CD4^+$, $TCR\alpha$ (NKT), diverse $TCR\alpha\beta$
TCR diversity	High	High	Moderate high	Low	Low
Surface coreceptor	CD8	CD4	CD4, DN, CD8	CD4, DN, CD8	CD4, DN, CD8
T cell functions	CTL	Th1/Th2	CTL, Th1, Th2 anti-microbial immunity	Th1, Th2, regulation of anti-self responses	Th1, Th2, regulation of anti-self responses

Table constructed from Gumperz and Brenner, 2001; Sieling, 2000 and Park *et al*., 1998a.
a, Cardell *et al*., 1995. b, Sieling *et al*., 2000a.
* Indicates elevated cell surface expression. CTL – cytotoxic T lymphocyte; DC – Dendritic cell; DN – double negative ($CD4^-CD8^-$); NKT – Natural killer T cell

1.6.2.1 CD1 AND MYCOBACTERIA

Group 1, glycolipid-specific, CD1a-, b- and c-dependent cytotoxic T cells have been shown to be involved in the host response against TB (Schaible and Kaufmann, 2000). CD1 expression was restricted to a mature population of DC occurring in the tuberculoid lesions that was found efficient in presenting lipid antigens to CD1b restricted T cells (Sieling *et al*., 1999).

Group 2 CD1-restricted T cells are not involved in protection to TB infection (Sieling, 2000a). Although effects of Group 2 CD1-controlled immune responses in TB are conditional rather than essential (Szalay *et al*., 1999), mycobacterial infection *in vitro* increased CD1d surface expression on murine DCs and mϕ (Schaible and Kaufmann, 2000).

Unstimulated human peripheral blood-derived macrophages do not typically express group 1 CD1 proteins and cannot present mycobacterial lipid antigens. Group 1 CD1 proteins are rapidly upregulated on monocytes after exposure to cytokines such as GM-CSF and IL4 *in vitro.* The cytokine induction is related to the *in vivo* immune response, accounting for the expression of CD1 in pathology samples (Moody *et al*., 1996).

The upregulation of CD1a, b and c molecules on immature DCs suggests that group 1 CD1 restricted T cells may function at an earlier phase in immune responses than MHC-restricted T cells. Since responses that are restricted by MHC class I or II may be suppressed in mycobacterial infections, CD1-based antigen-presentation and associated T cell responses afford an independent opportunity to detect microbial infection (Gumperz and Brenner, 2001).

Studies on cellular level showed that the antigen presentation of mycolic acids (MA) to the host may avoid the use of MHC-class I and II molecules, by being presented on group I CD1 molecules. Expression of CD1 molecules was found to correlate with an increased T cell immunity to *Mycobacterium leprae* (Sieling *et al*., 1999). CD1-restricted T cells against mycobacteria that promote cell mediated immune responses was derived from healthy individuals, TB patients and HIV infected TB patients (Gong *et al*., 1998; Sieling, 2000a).

1.6.2.2 PRESENTATION OF MYCOBACTERIAL LIPIDS TO T CELLS

Porcelli *et al*., (1992) showed evidence for the first time implicating CD1 as an antigen presenting molecule restricting the recognition of antigens by T cells. They isolated a human T cell line that proliferated in response to *M. tb* – derived antigens in a CD1b-restricted fashion. This $CD4^{-}CD8^{-}$ T cell line (DN1) showed a dose dependent proliferation response when $CD1^{+}$ activated monocytes were used as APC. This proliferation was successfully inhibited by antibodies to CD1b but not with antibodies to CD1a,c or MHC molecules. Only CD1b expressing cells that were pulsed with mycobacteria were recognized and killed by the DN1 T cell line when transfectant cells expressing CD1a,b or c were used in cytolytic assays. Human CD1b and c molecules elicit cytolytic and IFNγ responses by presenting mycobacterial glycolipids to $CD8^{+}$ or DN T cells (Schofield *et al.,* 1999). Trehalose dimycolate (TDM), but not sulfolipid (SL, 2,3,6,6'-tetraacyl trehalose 2'-sulphate - as a structurally similar glycolipid without mycolic acid), strengthened protective cellular immune

responses. TDM effects included the down regulation of IL-4-producing NK T cells whilst expanding a population of IFN-γ secreting NK T cells. IL-4 production could favour humoral immunity (Ryll *et al.*, 2001).

Lipids of internalised mycobacteria are not restricted to phagosomes but are released into the endocytic network of infected macrophages. CD1 molecules 'sample' mycobacterial glycolipids from different intracellular sites in the infected cell (Figure 1.5). Cell to cell transfer of mycobacterial lipids was suggested, as non-infected macrophages were also observed to take up these lipids - possibly in the form of exosomes. Exported glycolipids were shown to colocalize with members of the CD1 family, possibly to become targets for immune recognition by CD-1 restricted T Cells (Neyrolles *et al*., 2001).

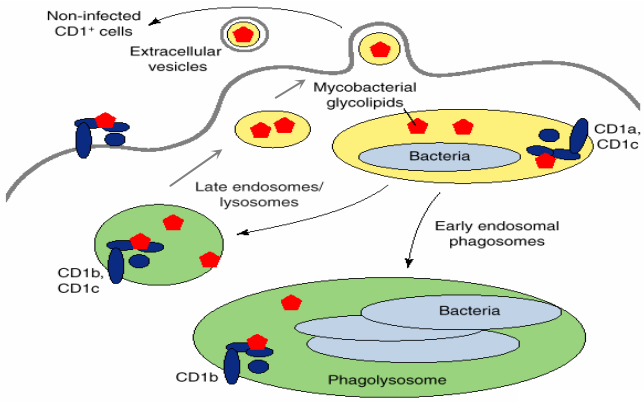


Figure 1.5: *Intersection of CD1 molecules with glycolipid antigens. CD1a and CD1c interact with mycobacteria in the phagosome at an early stage of infection. CD1b is present in mycobacterial phagolysosomes. CD1b and CD1c also converge with glycolipids transported from phagosomes into late endosomes or lysosomes (Schaible et al., 2000).*

APCs (including DC) expressing CD1b, mediate the specific T cell recognition of mycobacterial lipid antigens. These lipids include free mycolates, glycosylated mycolates and diacylglycerol based PL such as phosphatidylinositol mannoside (PIM) or lipoarabinomannan (LAM) and are presented by different CD1 epitopes as shown in Appendix 1 (Matsuda & Kronenberg, 2001). Mycolate and glucose monomycolate (GMM) exist within the mycobacterial cell wall both as free compounds and covalently linked to larger glyco-conjugates such as trehalose dimycolate (TDM, cord factor) or arabinogalactan. Cord factor has amphiphatic character allowing it to interact strongly with cell membranes thereby activating immunocytes (Barry III *et al*., 1998).

1.6.2.3 LIPID PROCESSING FOR ANTIGEN PRESENTATION

Lipid antigens require internalization by APCs prior to presentation for antibody production (Moody *et al*., 1999). Capturing of extracellular glycolipid antigen may be mediated by surface receptors such as the MR, as suggested for LAM, and subsequently be delivered to a low pH environment within endosomes (Moody *et al*., 1999). The presentation of both LAM and MA is dependent on the acidification of endosomes. At a pH below 5 the properties of CD1b and c are changed to allow the interaction of CD1 and non peptide ligands. There is unfolding of the α-helical antigen binding part, and the groove becomes deeper and narrower. CD1 is targeted to this endosome through a tyrosine-based motif encoded in the short intra-cytoplasmic tail. Antigens delivered to the MIIC then intersects with the CD1 pathway on the cell surface (Park *et al.,* 1998a). The CD1 molecule thus links the innate immune system (recognition of common pathogen-associated molecular patterns by pattern recognition receptors) and the adaptive immune system (presentation to T cells).

1.7 TCR SPECIFICITY

Structural considerations as well as functional assays using variants of antigenic lipids suggest that the two acyl chains of the lipids bind the hydrophobic groove of CD1 in an extended conformation. The hydrophilic head, usually the carbohydrate moiety, contacts the TCR loops. The lipid antigens can thus interact with T cells in an aqueous environment (Moody *et al*., 1996). The fine specificity of recognition was found to depend on the type of carbohydrate rather than on the length of the lipid moieties (Park *et al.,* 1998a). Glucose monomycolate shows a 2R,3R conformation. Both the orientation of the lipid branches and the stereo configuration of the 3-hydroxyl group in mycobacterial glucose monomycolate were required for recognition by the CD1 molecule. The carbohydrate and adjacent elements of the lipid that form the functional unit are referred to as the hydrophilic cap.

The molecular mechanism of TCR interaction specificity occurs by a trimolecular interaction of the TCR with the hydrophilic cap of the antigen protruding from the CD1 groove (Figure 1.6, Moody *et al*., 2000a).

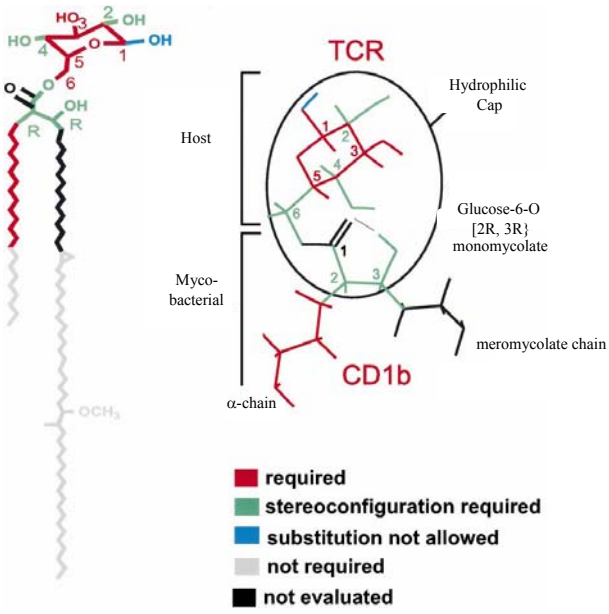


Figure 1.6: *Molecular specificity of the CD1-TCR complex with glucose monomycolate (Moody et al., 2000a).*

CD1b presents free MA to several T cell lines, indicating that the MA structure is sufficient for the uptake and presentation by APC and recognition by certain T cells (Jullien *et al*., 1997; Niazi *et al*., 2001).

It was postulated by Grant *et al*. (1999), that MA specific TCRs had positively charged residues in the TCR binding regions. These residues were positioned to interact with lipid antigen head groups protruding from the CD1b hydrophobic pockets, between the CD1 α helices. The most polar region of the mycolic acid residue consists of a carboxylic acid group and a hydroxyl group that might interact with positive charges in the CD1 TCR complex as depicted in Figure 1.7 a and b (Grant *et al*., 1999; Phyu *et al*., 1999).

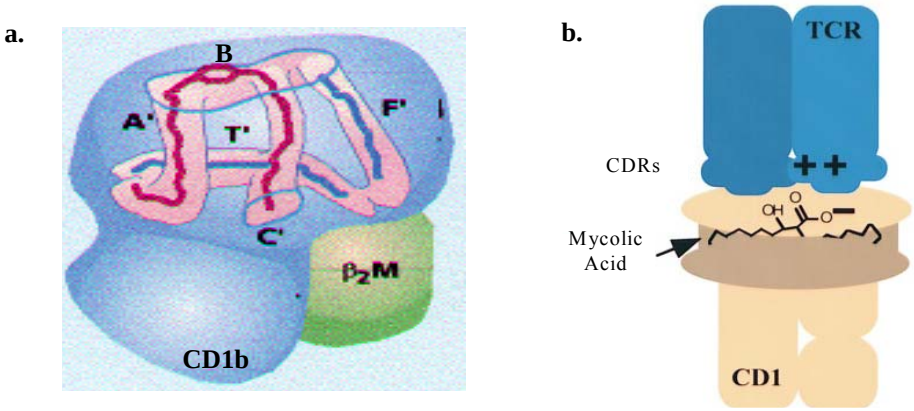


Figure 1.7a: *Proposed fit of glycolipids into the CD1b antigen binding groove for adaptive immune responses. The antigenic short chain fits into C', the longer chain fits into A', T' and F'. The polar regions (B) are then available to interact with the TCR (Niazi et al., 2002).*
Figure 1.7b: *Interaction of MA polar regions with the CD1-TCR hydrophobic pocket (Grant et al., 1999).*

The different hydrophilic caps of phosphatidylinositol mannoside (PIM), glucose monomycolate (GMM), and free mycolate on the α-helical face of CD1b are of appropriate sizes to interact with the TCR antigen binding site without steric interference (Moody *et al.,* 1999).

Different T cell lines responded to free mycolate or GMM without cross-reactivity. The ability of DN T cells to discriminate between MA mixtures from different species is probably due to the fine structural properties of the antigen, such as functional groups in the meromycolic chain (R groups), chain length, degree of unsaturaton, isomerism or glycosylation (Moody *et al.,* 1996). DN1 T cells responded in a CD1b-restricted fashion to free mycolic acids from *M. tb*. Another CD1-b restricted cell line responded to glycosylated analogues. A CD1b restricted T cell line, DN1, only responded to MA with certain oxygen containing meromycolic chains. The differential response of the two T cell lines was not considered a property of the APC-antigen interaction, but rather due to the antigen/T cell interaction, suggesting T cell specificity for their hydrophilic caps (Moody *et al.,* 1996).

APCs were unable to interconvert lipid antigens by enzymatic or chemical deglycosylation or glycosylation. APCs were also unable to cleave mycobacterial TDM at its most chemically labile linkages to yield antigenic free mycolates or GMM for presentation by CD1b (Moody *et al*., 1999; Vinay *et al*., 1996; Davis, 2002; Daffé & Etienne, 1999).

1.8 CD1 RESTRICTED T CELLS

Human T cells that are restricted by group 1 CD1 molecules have features in common with classical MHC-restricted T cells: They express diversely rearranged TCRs that specifically recognize distinct foreign antigens and they are clonally expanded in response to microbial infections (Moody *et al*., 2000b).

The first human CD1-restricted T cells to be cloned were DN ($CD4^-CD8^-$) T cells expressing αβ TCR, which represent only a small fraction of peripheral blood T cells. More recently derived clones include both CD8/TCR$\alpha\beta^+$ and $CD4^+$ T cells. This indicates that T cells that are restricted by group 1 CD1 are phenotypically heterogeneous and may comprise a significant fraction of the total T cell population (Rosat *et al*., 1999; Gumperz and Brenner, 2001 & Moody *et al*., 1996).

All mycobacteria specific group-1 CD1 restricted T cell lines that have been examined to date secrete type 1 cytokines (cytotoxic immune responses) such as IFN-γ. They have not been found to secrete type 2 cytokines (humoral immune responses) such as IL-4 (Moody *et al*., 1996; Boom *et al*., 2003). CD1 restricted T cells were found to lyse activated macrophages and B lymphoblastoid targets efficiently (Moody *et al*., 1996). Lysis of highly infected macrophages lead to direct killing of the bacteria or the uptake of these organisms by other macrophages which could then effectively dispose of the bacteria.

DN CD1 restricted T cells participate in the elimination of infected macrophages via an interaction between Fas and Fas ligand whereas the CD8 T cells use a granule dependent mechanism to kill the mycobacteria (Braud *et al*., 1999). $CD1^+$ DC were found to present lipid antigens to $CD4^+$ T cells at the site of disease, leading to a Th1 response and increased CD1 expression (Sieling *et al*., 2000a). $CD4^+$ CD1 restricted T cells were found to produce TH1 cytokines similar to DN and $CD8^+$ T cells on stimulation.

DN T cells might exert a protective effect in reducing the local inflammatory responses and thus reducing tissue damage (Jullien *et al*., 1997). They may also be involved in the regulation of autoimmune diseases (Niehues *et al*., 1994; Hammond *et al*., 1993).

A correlation exists between the presence of γδ T cells in the blood and lungs of tuberculosis patients and the extent of tuberculosis disease (Phyu, 1999; Boom *et al*, 2003). TCR γδ dependent recognition of CD1a and CD1c isoforms was observed in the absence of an exogenous antigen, suggesting that the T cells recognized an endogenous antigen-CD1 complex or the CD1 molecules alone (Gumperz and Brenner., 2001).

MA are presented to αβ TCR^+ T and γδ TCR^+ T cells through the CD1b molecule (Beckman *et al*., 1994 & Porcelli *et al*., 1992), but no primary or even secondary CD1-restricted responses to mycobacterial lipids have been obtained. The published mycobacteria-specific CD1-restricted lines were all observed after multiple rounds of selection by *in vitro* stimulation.

1.9 AUTOIMMUNE DISEASE OF CD1 RESTRICTED T CELLS

A regulatory role for CD1-restricted T cells in autoimmune disease is becoming increasingly clear. IL-4 production from CD1d restricted NK T cells protects against self-recognition, whereas IFN-γ production is associated with disease (Sieling, 2000). Apoptotic fragments of cells (resulting from autoimmune disease) can be taken up and presented to T cells by $CD1^+$ DCs. These apoptotic fragments express cellular phospholipids (PL) normally separated from the immune system. T cells might thus be activated that recognize self-PL to set an autoimmune response in motion. Group 1 CD1-restricted T cells, with their more diverse TCR repertoire, recognize foreign lipid antigens. When self-tolerance is broken, group 1 CD1-restricted T cells can become effector cells in autoimmune destruction (Sieling, 2000). The limited TCR diversity of group 2 CD1-restricted T cells may serve to recognize a limited number of self-antigens and regulate autoimmune responses. Some murine CD1d-restricted NKT cell hybridomas responded specifically to the presentation of certain abundant cellular lipids —such as phosphatidylinositol and phosphatidylethanolamine (Gumperz *et al*., 2000).

A significant population of T cells residing in peripheral tissue may be constitutively triggered through their CD1-autoreactive TCRs. Tissue specific autoreactive T cells distinguish between different types of murine CD1-expressing cells. This suggests that a

diverse set of different self ligands might be responsible for this variability (Park *et al*., 1998a; Park *et al*., 1998b; Brossay *et al*., 1998).

1.10 CD1 RESTRICTED T CELLS AND ANTIBODY PRODUCTION

CD1d-restricted T cells may regulate the production of antibodies that recognize lipid-containing antigens, a possibility that correlates with the expression of CD1d on certain human and murine B cells. Activation of CD1d-restricted NKT cells by α-galactocylceramide (α-GalCer) rapidly lead to polyclonal stimulation of lymphocytes including NK-, T- and B-cells (Gumperz and Brenner, 2001). When α-GalCer was introduced into mice together with a protein antigen the cytokine response was of a Th-2 type. α-GalCer stimulated antigen specific T cell responses and IgE production to the protein antigen (Singh *et al.,* 1999).

CD1-restricted T cells in mice promote IgG antibodies against malarial glycosyl phosphatidylinositol (GPI)-anchored proteins. The specificity of both the antibody and T cells was against the glycolipid (Schofield *et al*., 1999; Sieling, 2000).

The lymphatic system plays a major role in antibody production as it is here where B cells and T cells exchange information. The existing paradigm to explain T cell help for antibody production to peptide antigens hold that B cells bind and internalize antigens via surface immunoglobulin, process the antigen, and then present peptide fragments on a MHC II molecule to the TCR (Sieling, 2000). Two molecular mechanisms for the generation of specific T cell responses to non-protein microbial antigens have been proposed (Moody *et al*., 1996). First, APC expressing CD1 can present various mycobacterial cell wall lipids and glycolipids to T cells. Second, γδ T cells bearing the VγVδ2 T cell receptor recognize monoalkyl phosphates from mycobacteria in the absence of antigen presenting molecules (Moody *et al.,* 1996). DCs survey the peripheral tissues and return to the lymphoid system displaying foreign antigens and co-stimulatory molecules (Roark *et* al., 1998). $CD1^+$ B cells have the unique function of recognizing (through immunoglobulin receptor) and presenting (through CD1 molecules) nonpeptide antigens to CD1-restricted T cells in order to recruit help for antibody production (Sieling, 2000). The recognition of nonpeptide antigens by CD1-

restricted T cells and the expression of CD1 on B-cells suggest that these T cells might promote antibody production for nonpeptide, highly repetitive structures, such as polysaccharides.

Any attempt to implicate CD1-restricted T cells in antibody responses necessarily requires that B cells express CD1. Unlike cytokine-activated monocytes, which express CD1a, CD1b and CD1c, B-cell populations in peripheral blood, tonsil, spleen and lymph nodes express only CD1c. A CD1c B-cell population has been identified in human intestinal and colonic lamina propria. CD1 expression is high (particularly CD1c) among marginal zone B cells (MZB). The marginal zone in the spleen is an important microanatomic site for the generation of antipolysaccharide antibodies (Fairhurst *et al*., 1998; Roark *et al*., 1998). The marginal zone B cells are stationed in the spleen at the point where blood enters the marginal sinuses. These cells are well situated to capture blood-borne antigens.

The MR delivers lipoglycan antigens to endosomes for T cell presentation by CD1b. B cells expressing CD1 can trap non-protein antigens for presentation. T cells that recognize non-protein antigens in association with CD1 may interact with the CD1/CD21 expressing marginal zone B-cells to augment antibody responses to non-protein antigens (Amano *et al*., 1998 & Roark *et al*., 1998).

CD1-restricted $CD4^+$ T cells are the major source of IL-4 production, while non-CD1-restricted $CD4^+$ T cells play a substantial role in IFN-γ production (Wang *et al.,* 2000). IL-4 is very important for the differentiation into Th2 cells.

Upon mycobacterial infection CD1 expression is upregulated. Although the natural ligand for CD1d is not known this epitope expression is also increased. CD1d-restricted T cells were proven to be potent stimulators of humoral immunity (Singh *et al*., 1999; Schofield *et al*., 1999; Sieling, 2000). A major CD1d restricted T cell line is the NK T cells (Molano *et al*., 2000; Matsuda & Kronenberg, 2001). These T cells release high amounts of IL-4 upon TCR stimulation. The IL-4 produced in this manner might now assist B cells to produce antibodies. IL-4 up-regulates the expression of CD1 a, b and c (Porcelli *et al*., 1992).

1.11 ANTIBODY PRODUCTION TO MYCOLIC ACIDS

Human tuberculosis patients produce IgG antibodies to MA (Pan *et al*., 1999). How this comes about may be envisioned as follows:

Mycobacteria infect the human host to activate a host of immune responses (Figure 1.8).

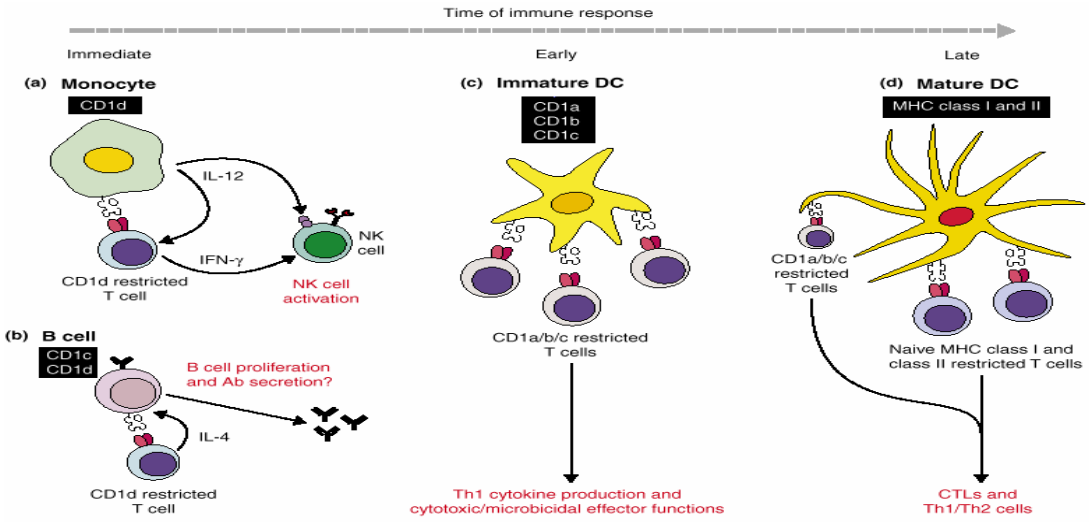


Figure 1.8: *Course of the immune response upon Mycobacterium infection.*
CD1 restricted T cells may become activated prior to classical MHC-restricted T cells. (a) In the immediate phase after infection CD1d restricted T cells interact with monocytes leading to NK cell activation through cytokines. (b) CD1d and CD1c restricted T cells also interact with B cells and secrete cytokines to lead to B cell activation and antibody secretion. (c) As the immune responses continue monocytes differentiate into immature DC expressing CD1a,b and c. Microbial lipid antigens are now presented to CD1-restricted T cells.(d) Later in the immune response, MHC class I and II restricted T cells are also activated by mature DC (Gumperz & Brenner, 2001).

Mycobacteria are delivered to the alveolar spaces of the lung. Some bacilli might be swallowed to be delivered to the digestive tract. Macrophages acting as APC in the lung take up these bacilli. The lipid antigenic material might now be presented through the CD1 molecule to T and B cells which might lead to antibody formation.

Mycobacteria that are swallowed are delivered to the digestive tract. Peyer's patches have special capabilities for sampling intestinal contents. Dr Kenzaburo Kumagai in 1923 fed viable *M. tb* to rabbits showing their uptake by Peyers patches (Owen, 1999). The antigenic lipid material is ingested and might then be presented to DC functioning as the main APC in the digestive tract.

Alternatively the antigenic lipid material might be seen as nutritional lipids. Low density lipoproteins (LDL) are produced to transfer lipids through the systemic circulation to tissues. Lipoproteins (LPs) are also involved in the binding and subsequent transport of a number of water insoluble compounds. Cholesteryl ester transfer protein (CETP) facilitates the transport of lipid soluble drugs between different lipoprotein subclasses (Kwong and Wasan, 2002). The antigenic mycobacterial lipids might be incorporated into these LPs. The antigenic material might now circulate the periphery in the LP. In the lymphatic system the antigens might come into contact with APC, B and T cells.

CD1b is responsible for the presentation of MA to T cells. In APC (DC) the CD1b molecule is expressed in late endosomes or lysosomes. Dead mycobacteria containing MA will come into contact with CD1 in these organelles. Glycolipid material, however, can traffic through the phagosomal system and was shown to be transferred to non-infected bystander cells (Schaible *et al*., 2000). Lipid material shed from live mycobacteria can thus still progress through the phagosomal system to come into contact with the CD1b molecules.

In Figure 1.9 the so-called palmitate hypothesis is explained that provides a mechanism for T cell dependent Antibody responses to non-protein antigens. The palmitate molecule anchors diverse covalently attached capsular polysaccharides into the non-polymorphic, hydrophobic groove of CD1. B cells then internalise these palmitate bearing capsular polysaccharides through surface IgM. The antigen is then processed and the lipid-substituted terminal fragment is then presented through CD1c. This theory provides the possibility that B cells can directly elicit T cell help in the production of anti-polysaccharide antibodies (Fairhurst *et al*., 1998).

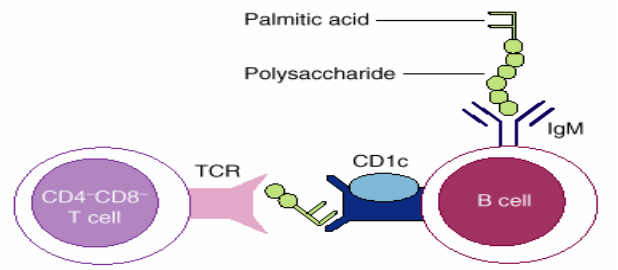


Figure 1.9: *The palmitate hypothesis where B cells directly elicit T cell help for antibody production (Fairhurst et al., 1998).*

IgM acting as antigen receptors on the B cells may thus bind and internalise MA (free or as part of TDM). Antibodies to MA might then be produced with the help of CD1b restricted T cells. B cells, however, do not express CD1b. Another mechanism for antibody production needs to be suggested. The possibility exists that antibodies to MA might be selectively produced although the mechanism remains unclear.

What is clear, however, is that IgG antibodies to MA need not come about by CD4 T cell intervention, and may therefore be better surrogate markers for infection with *M. tb* in HIV burdened populations than anti-protein antigen antibodies. The strength of antibody binding signals in serodiagnosis may, however, be influenced by the presence of MA antigen in the blood. Being such a large lipid, the most likely carrier for MA are the lipoproteins.

1.12 MYCOLIC ACID ANTIGEN ACCUMULATION IN BLOOD

Two possible ways can be envisaged whereby MA can accumulate in the blood, i.e. absorption from the digestive tract and or shedding from infected and phagocytic cells. In both cases it is necessary to have a basic understanding of how lipids are generally absorbed and transported in the body.

1.12.1 DIETARY FAT

Exogenous diet fat and cholesterol are absorbed through the intestine and transported in plasma as chylomicrons (CMs) containing Apoprotein B-48. A smaller CM remnant (with apolipoprotein C-II) is produced upon triacylglycerol (TAG) hydrolysis into free fatty acids (FA) and glycerol. These remnants are taken up by the liver and TAG rich very low density lipoproteins (VLDL) is synthesized with Apo B-100. Lipids delivered to the liver are processed for excretion into bile or are re-assembled into lipoprotein particles, mostly VLDL. The liver synthesizes fat and cholesterol, which are assembled into these VLDL particles before secretion into circulation for lipid distribution to peripheral tissues. In plasma, the VLDL TAG is broken down to free FAs and glycerol with the formation of smaller VLDL remnants and IDL. Hydrolysis (hepatic lipase) of VLDL TAGs convert the lipoproteins to cholesterol-rich LDL particles which distribute cholesterol to extrahepatic tissues or return to

the liver in an LDL receptor mediated process (Hui, 2003). ApoproteinB-100 (ApoB-100) is a non-exchangeable apoprotein adjusting to the diameter change from 80-200 nm for a VLDL particle to ± 22 nm of a LDL particle in the metabolic processes (Hevonoja *et al*., 2000).

1.12.2 LOW DENSITY LIPOPROTEIN

LDL vary in density, size, structure and composition. LDL is a large, spherical, compositionally complex lipoprotein particle containing a single copy of an apoprotein B-100 (apoB-100) protein (4536 amino acid residues) and ± 3000 lipid molecules (Figure 1. 10).

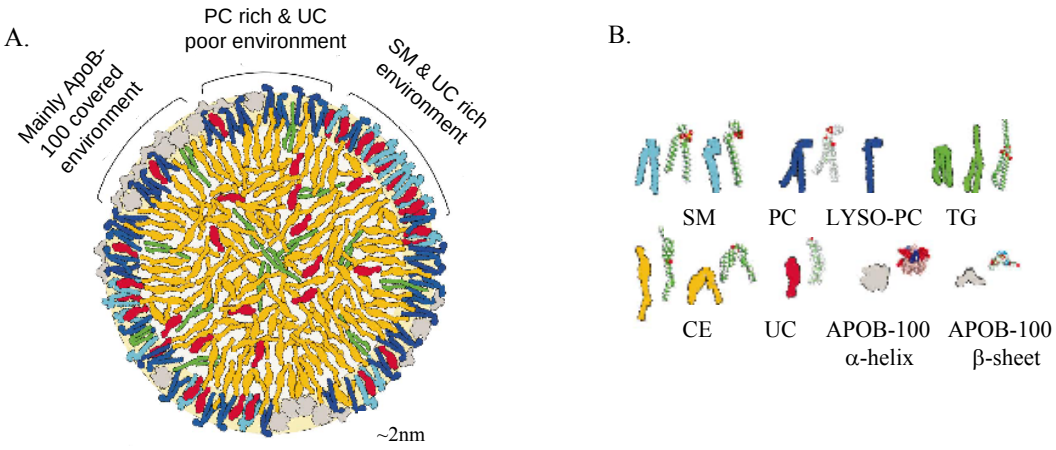


Figure 1.10: *Schematic molecular model of a LDL particle at around body temperature.*

A. *The depicted LDL particle shows a surface monolayer of 2 nm, and an average composition of 20% protein, 20% phospholipids, 40% CEs, 10% UC, and 5% TGs. The molecular components are drawn at the correct percentages and size ratios. Different domains are illustrated at the particle surface with interpenetration of core and surface lipids.*

B. *Key to the molecular molecules (Hevonoja et al., 2000).*
SM - sphingomyelin, PC - phosphatidylcholine, Lyso-PC – lyso phosphatidylcholine, TG - triglyceride, CE – cholesterol ester, UC – unesterified cholesterol, ApoB-100 – apoproteinB-100.

ApoB-100 is a big, insoluble protein that encircles LDL in kinks with a ribbon and bow conformation coming into contact with the lipids in all three concentric layers of the LDL particle. The ApoB fraction is necessary for binding to LDL receptors on fibroblasts whilst the lipid fraction is important for binding to LDL receptors (e.g. CD36) on platelets (Chappey *et al*., 1995; Pedrenõ *et al*., 2000).

The outer amphipathic monolayer of PL headgroups surround an interfacial layer consisting of an interpenetrating core and surface lipids. The hydrophobic core consists of approximately 170 triglyceride (TAG) and 1600 cholesteryl ester (CE) molecules. The surface monolayer comprise about 700 phospholipid (PL) molecules. The main PL components are phosphatidylcholine (PC, ± 450 molecules/LDL particle) and sphingomyelin (SM, ± 185 molecules/LDL particle). Of the 600 molecules of unesterified cholesterol (UC), ±33.3% reside in the core and ± 66.6% in the surface layer (Hevonoja *et al*., 2000).

In LDL, cholesterol esterases (CEases) hydrolyses CEs with an increase in core hydrophilicity as the UC concentration increases. The hydrophobic FA chains of the surface PLs are now in an energetically unfavorable environment. UC molecules then solubilize in membrane-like structures leading to the generation of liposomes and complex multilamellar structures (Hevonoja *et al* ., 2000).

Choline containing PC and SM constitute most of the outer leaflet in resting plasma membranes. The membrane's capacity to solubilize cholesterol is a function of the SM content. When the cell surface SM is depleted, the efflux of UC to an extracellular UC acceptor (e.g. LDL) increases (Slotte., 1999; Hevonoja *et al*., 2000; Wolf & Chachaty, 2000; Wolf *et al*., 2001).

Cholesterol is insoluble in an aqueous medium and its critical aggregation concentration is 30nM (Slotte, 1999). UC molecules do not form a tight complex with the PL polar headgroups. The OH group of UC resides with the ester carbonyl oxygen of the PC molecules in a membrane. UC molecules in LDL and high density lipoproteins (HDL) particles are readily susceptible to oxidation by cholesterol oxidase, because the molecular packing of lipids in the LDL or HDL monolayer (spherical curved membranous surface of the particles), is looser than in the biological bilayer membranes (Hevonoja *et al*., 2000). Oxidized LDL (oxLDL) is taken up from circulation by macrophages through scavenger receptors (SR) like SR-A and CD36 to produce foam cells in atherosclerotic plaques (Yamaguchi *et al*., 2002).

1.12.3 HIGH DENSITY LIPOPROTEINS

High density lipoproteins (HDL) are central in the reverse cholesterol transport pathway stimulating cholesterol efflux from extrahepatic cells to the liver for excretion from the body (Toledo *et al*., 2000). HDL is formed in the circulation from lipid free lipoprotein AI (ApoAI) and PLs. The lipid free apoprotein AI requires five PC molecules to become a lipoprotein-like particle that is a good acceptor of cellular cholesterol molecules because of SM's high affinity for cholesterol (Slotte, 1999). High density lipoproteins associate with the HDL receptor, scavenger receptor BI (SRB1), on the liver and 50% of the CE in HDL is selectively taken up. The remaining 50% will be transferred to ApoB containing lipoproteins (VLDL, IDL and LDL). Nascent HDL is made primarily in the liver and intestine to contain mostly PL and apolipoprotein A-I with very little cholesterol. Cholesterol ester poor HDLs are circulated back to the plasma to pick up more cholesterol (Kwiterovich, 2000).

Reconstituted discoidal high-density lipoproteins (rHDLs) contain two apo-AI molecules (243 amino acid residues each) and ± 200 lipid molecules (Hevonoja *et al*., 2000). Tricerri *et al*. (1998) showed that discoidal rHDL interact with lipid vesicles and induce a very rapid and transient leakage of the internal aqueous content of the vesicles. Leakage was dependent on rHDL size and cholesterol content of both rHDL and vesicles. Rothblat *et al.* (1992) proposed that some apoAI domains of discoidal rHDL could interact with lipid bilayers of cholesterol donor membranes and facilitate cholesterol efflux. Initially the cholesterol acceptors are small cholesterol-poor discs containing PL and ApoAI in a conformation that allows a high affinity for binding to cholesterol rich membranes (vesicles/lipid bilayer). They are present in low concentrations in plasma and in higher concentrations in the lymph.

Cholesterol enrichment along with a size increase leads to a change in the ApoAI conformation decreasing the rHDL affinity for the membranes along with cholesterol adsorption and desorption rates. Large cholesterol-rich pre-β HDL particles are released. Cholesterol accumulates at the central region of the rHDL disc until the concentration is high enough to force net flux toward vesicles. Bidirectional transfer of cholesterol between cell membranes and the primary lipoprotein acceptors can be mediated through diffusion or a collision model. In the activation collision model cholesterol becomes activated in the donor particle and is then transferred during collision with the acceptor particle. The acceptor lipoprotein interacts with the cell membrane through an apoprotein domain which triggers

intracellular cholesterol mobilization toward the cell surface, facilitating cholesterol transfer (Toledo *et al*., 2000).

Cholesterol transfer between HDL and LDL occur between lipoproteins of different sizes and compositions. The rate constant for cholesterol transfer between rHDL and LDL was dependent on the donor/acceptor ratio. With little rHDL, the rate constant for cholesterol transfer from LDL was higher to cholesterol free than cholesterol containing rHDL. The opposite was true with excess rHDL present (Toledo *et al*., 2000). This implies that molecules can be transferred between the lipoproteins as a function of the size, concentration and phospholipid composition of the lipoproteins.

1.12.4 FATE OF MYCOLIC ACIDS

If MA is swallowed as sputum after coughing it follows the route of dietary fat, to be incorporated by the liver into LDL particles. MA-containing LDL particles will presumably follow the route of normal LDL, up to the stage where they become oxidized and taken up from the circulation by macrophages.

After *Mycobacterium tuberculosis* ingestion by macrophages, lipid containing moieties of the mycobacterial cell wall were trafficked out of the mycobacterial containing macrophages. These lipids were found in extracellular vesicles isolated in culture medium and shown to be taken up by uninfected macrophage bystander cells. These vesicles are thought to have emerged from acidic vesicles and resemble the MIIC compartments of B lymphocytes. These exosomes have been released from various cell types and may function in several immuno-regulatory actions (Beatty *et al.,* 2000).

1.13 AIM OF STUDY

The aim of this study was to investigate the use of anti-MA antibodies in TB patients as surrogate markers for TB infection with reference to HIV endemic areas. The dynamics of the interaction between anti-MA antibodies and MA-antigens in human sera is investigated, where it is expected that MA antigens are presented on lipoprotein complexes to their antibodies.

CHAPTER 2: ANTI-MYCOLIC ACID ANTIBODIES IN PULMONARY TUBERCULOSIS PATIENTS

2.1 INTRODUCTION

Immunological mechanisms against *M. tb* have mainly been investigated in murine models. Often a low dose of bacilli are introduced through an aerosol, to resemble human infection. The immune resistance to *M. tb* relies mainly on cellular immunity, which is stimulated by proteins secreted by the bacilli. However, an appreciable number of B cells have been found in granulomas of TB infected mice. Not much is known about the natural evolution of antibodies against *M. tb* in mouse models. Mycobacteria produce a wide variety of glycolipids that are located in the outermost layers of the cell. Several of these compounds [cord-factor (CF), diacyltrehaloses (DAT), phenolglycolipids (PGL), sulpholipids I (SL-I) and lipoarabinomannan (LAM)] have been studied as target molecules for serology in diagnosis of TB (Figure 2.1). These molecules also interact with host immune cells and contribute to pathogenesis i.e. they are potential virulence factors. Their structures are based on very characteristic methyl-branched long-chain acids and alcohols (Minnikin *et al*., 2002).

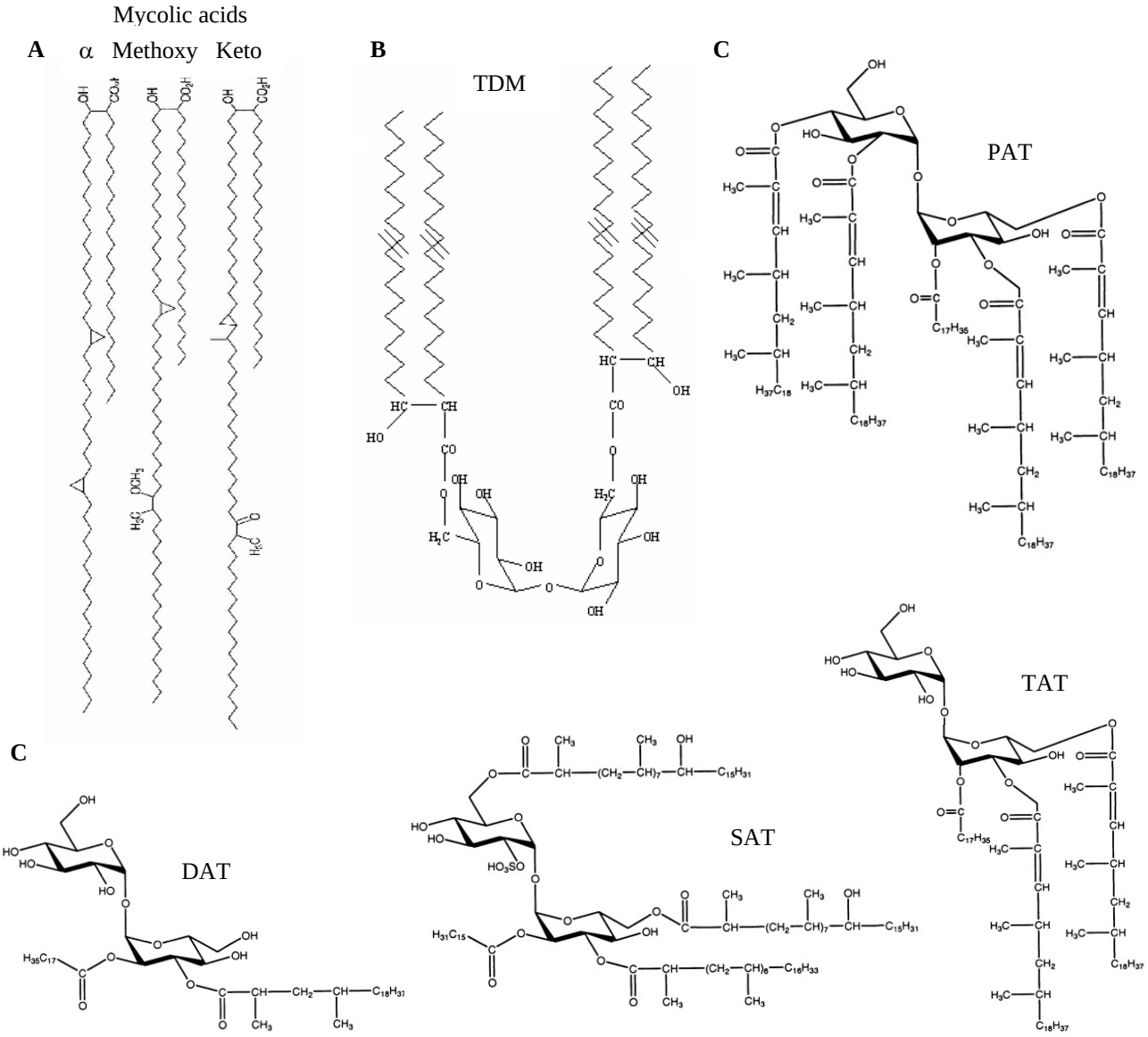


Figure 2.1: *Structures of M. tuberculosis mycolic acids and glycolipids based on trehalose.*

A. *Schematic representation of the α, methoxy-, and keto-mycolic acids.*

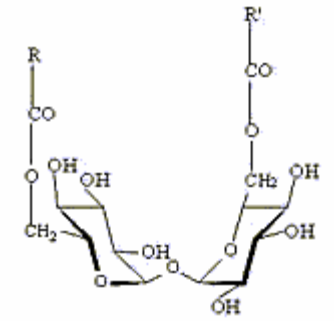
B. *Trehalose dimycolate (TDM)*

C. *Complex glycolipids. Pentaacyl trehalose (PAT) based on mycolipenic acids, diacyl trehaloses (DAT1) based on mycosanoic acids, sulphated tetra-acyl trehalose (SAT) based on phthioceranic and hydroxyphtioceranic acids and triacyl trehalose (TAT). (Figure from Vergne & Daffé, 1998; Minnikin et al., 2002; Joyce & Van Kaer, 2003; Rousseau et al., 2003).*

Masahiko Kato, in 1973, showed that rabbit antibodies to TDM were predominantly of the IgM isotype. Antibodies to TDM was only detected after immunization of TDM conjugated to methylated BSA and not with BCG infection (Kato, 1973). Cardona *et al.* (2002) ascribed the

immunoglobulin M (IgM) dominated response to these antigens to the IFN-γ that is elicited upon immunization. IFN-γ generally supports IgM production *in vivo* by activated human B-lymphocytes.

The existence of naturally occurring human immunoglobulin G (IgG) antibodies to lipids was then showed by several investigators (Verschoor *& Bye*, patent application *PCT/GB 95/00856,* 1995; Verschoor *et al.*, patent application *PCT/GB 98/00681,* 1998; Pan *et al.*, 1999; Harrington III *et al.*, 2000; Horváth *et al*., 2001b). MA was identified as an antibody inducing antigen by Verschoor *et al.* (patent application *PCT/GB 98/00681,* 1998). Fujiwara *et al.* (1999) reported that the hydrophobic moiety rather than the carbohydrate moiety in cordfactor was detected by IgG antibodies in rabbits. Pan *et al.* (1999) reported that the detection of anti-cord factor IgG antibodies in active (smear or culture positive) and inactive (smear and culture negative) TB patients was useful in early diagnosis of the disease. They showed differential responses of antibodies to TDM from *M. tb* (higher response) and *M. avium* (lower response). The structure of cord factor is shown in Figure 2.2 indicating the positions where MA is linked to the trehalose.



6,6'-dimycoloyl-alpha-D-trehalose
R-CO- and R'-CO. = mycoloyl residues

Figure 2.2: *Structure of cord factor (TDM), showing positions for mycolic acid (Asselineau and Laneelle, 1998).*

Pan *et al*. (1999) tested the specificity of anti-cord factor IgG antibody for its ability to distinguish the three subclasses of mycolic acids. In *M. tb* it was shown that antigenicity manifested predominantly with the methoxy mycolic acids. Antibodies directed against these wax-like structures discriminated between MA subclasses on the basis of branched chains, chain length and polar groups. The anti-cord factor IgG showed a higher affinity for binding to methoxy MA than to the α- and keto mycolic acid subclasses.

Mycolic acids are presented to αβ TCR^+ T and γδ TCR^+ T cell cells through the CD1b molecule (Beckman *et al*., 1994). The CD1 molecules are unique antigen presenting molecules resembling the MHC family of antigen presenting molecules. The CD1 family is able to present lipid antigens from their hydrophobic ligand binding grooves to T cells. Naidenko *et al*. (2000) showed that the β-hydroxyl group of MA alone is the minimal requirement for the hydrophilic part of the structure to be recognized by CD1b-restricted mycolic acid specific T cells. The hydroxyl group on the second (β) carbon of mycolic acid was required for glucose monomycolate recognition. The free MA fraction prepared from 6,6'– trehalose dimycolate could stimulate the DN1 T cell line, whereas the fatty acid containing fraction prepared from 6,6'– trehalose dibehenate was inactive. The authors concluded that MA rather than trehalose or other fatty acid components determined the CD1b restricted antigen.

This dissertation aimed to shed more light on the possibility that human antibodies to MA might function as surrogate markers for TB infection. For this purpose a short term immune antibody memory would be ideal, as well as an ability to maintain the production of such antibodies even under conditions of HIV infection.

2.2 MYCOLIC ACIDS

The first MA was isolated from the tubercle bacilli in 1929 by Anderson R.J. (Anderson, 1929). The name mycolic acid was proposed in 1935 for a portion of the lipid fraction from *M. tb* by F.H. Stodola, Alex Lusuk and R.J Anderson (Stodola *et al*., 1938). Structural characterization followed in 1950 by Asselineau (Asselineau & Lederer, 1950).

MA are described as a group of long chain α-alkyl, β-hydroxy fatty acids. MA has a largely asymmetric structure around the hydrophilic headgroup. They have a general structure of R1-CH(OH)-CH(R2)-COOH. R1 is a meromycolate chain (50-60 carbons) and R2 is a shorter α-branch (22-26 carbons). They are the major constituents of the inner leaflet of the lipid bilayer of the mycobacterial cell wall, where they form an effective impermeable barrier to protect the mycobacteria from antimicrobial agents (Liu & Nikado, 1999). In mycobacterial cells, MA are

predominantly covalently bound to the arabinogalactan polysaccharide. Some are found as trehalose monomycolates (TMM) and dimycolates (TDM) (Kremer *et al*., 2000; Chatterjee, 1997; Kawamura *et al*., 1997). TMM is involved in the transfer of MA into the cell wall.

M. tb is characterised by a range of complex glycolipids based on trehalose. Lipids containing multi-branched fatty acids are proposed to interact with a covalently bound monolayer of mycolic acids to provide the characteristic mycobacterial outer membrane cell wall structure. The essence of the mycobacterial cell enveloped is presented in Figure 2.3.

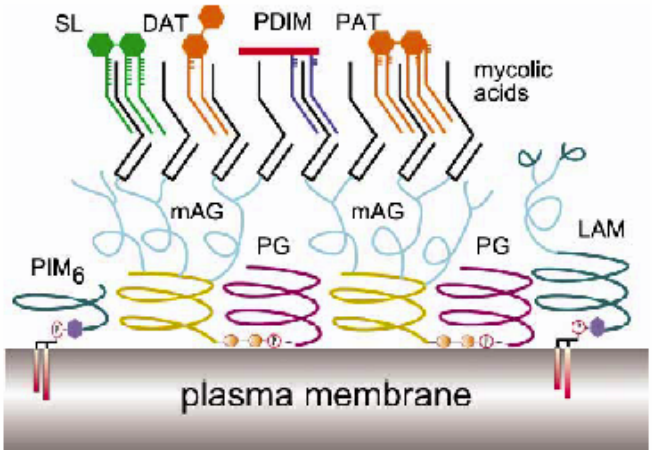


Figure 2.3: *The arrangement of structural components in the cell envelope of M. tuberculosis. Possible interactions of methyl branched long chain components with a MA matrix is shown.*
A relatively conventional plasma membrane and peptidoglycan layer is connected by a linker unit to an arabinogalactan, which bears MA on some of its arabinose termini.
Mycolyl arabinogalactan (mAG) is connected to peptidoglycan (PG) by a phosphoryl linker unit. Complex free lipids (Phthiocerol dimycocerosate-PDIM, DAT, SL) interact with mAG.
LAM and PIM are anchored in the plasmamembrane. LAM and lipomannan (LM) are lipopolysaccharides based on a phosphatidyl mannoside which may locate into the plasma membrane (Minnikin et al., 2002).

MA are classified on the basis of their oxygen groups within the mero moiety as shown in Figure 2.4.

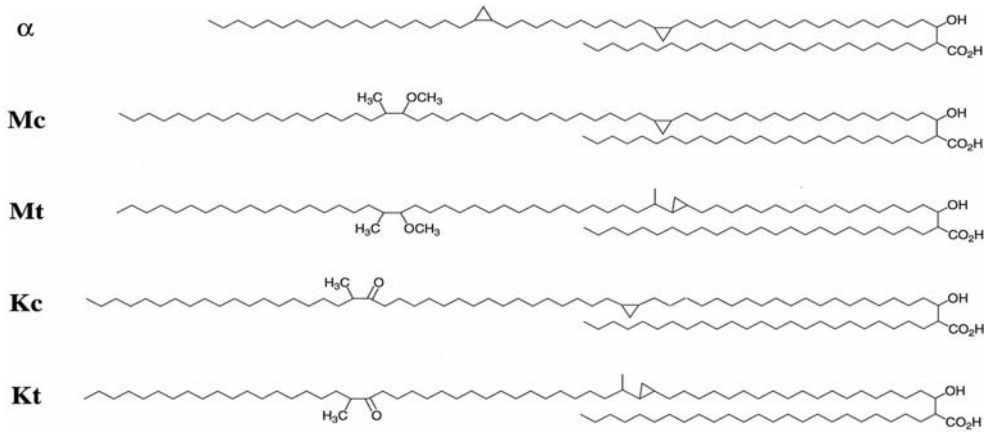


Figure 2.4: *Mycolic acid types, arranged from top to bottom in order of decreasing concentrations in M. tuberculosis (Yuan et al., 1997).*
α: α-mycolates: Contain no oxygen functionality in the mero (long chain) moiety
Kc/Kt: Keto/oxo mycolates (cis/trans): play an essential role in the intracellular growth of this pathogen in its host.
Mc/Mt: Methoxy mycolates (cis/trans): play a role in humoral responses.

Retzinger *et al.* (1981) have proposed that the disaccharide residue of the trehalose mycolates resides in the water phase, whilst the long mycolic acids are kinked and folded in the lipid bilayer as six tightly packed acyl chains.

α-Branched MA as used in adjuvant emulsions may expose extensive hydrophobic domains onto which proteins might adsorb. Wedge shaped hydrophobic domains are formed because of the large ratio of the areas between the hydrophobic chains (folded up) and the hydrophilic head group. This folding up of the MA prevents the formation of cylindrical micelles, which are unable to adsorb proteins. Hasegawa and Leblanc (2003) suggested that, through the measurement of limiting molecular areas, MA can be presented as double chain or triple-chain molecules. Surface pressure was applied to α-MA monolayer films on water by a Teflon-coated moving barrier. α-MA from different mycobacterium species including *M. tuberculosis* were investigated. The length of the MA short chain was found to be important. As little as 2 additional carbons showed increased aggregation among the MA resulting from stronger molecular interaction.

α - MA is thought to be a double or triple chain structure (Figure 2.5). At a low surface pressure the mero group in all MA are expected to be doubly folded (triple hydrocarbon chain, Figure 2.5a), but with increased surface pressure the mero chains unfold. The mero group is then singly folded (Figure 2.5b) and is structurally supported by the short chain through hydrophobic interaction only at the proximal part of the mero chain.

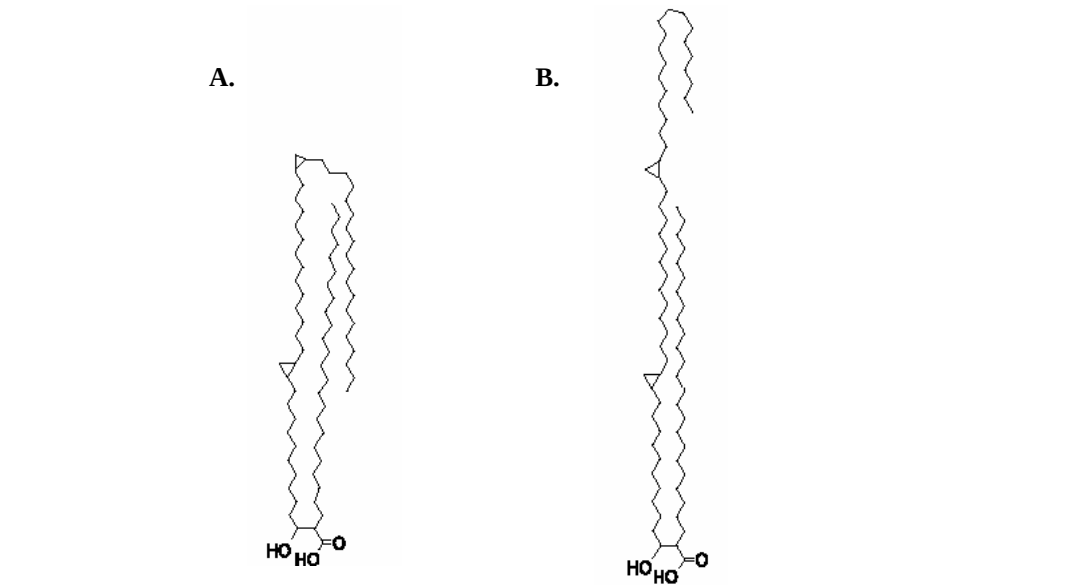


Figure 2.5: *Scheme representing the triple chain and double chain structures of α - mycolic acids..*
A. *The triple-chain molecule in which the short chain part is partly interacting with the mero group.*
B. *The mero group is extending by pressure and the short chain part is not interacted with the linear part of the mero group (Hasegawa & Leblanc., 2003).*

The molecular area for the keto MA could not be explained by the double chain model and the authors suggested a triple chain model. The unfolding of the keto MA would be largely inhibited by the strong molecular interactive forces. The molecular area for methoxy MA was intermediate to that of the alpha and keto MA (Hasegawa & Leblanc, 2003).

The folding of the MA might be important for recognition by CD1 molecules. The hydrophilic headgroup is then presented to T cells to result in various immune functions including antibody formation to MA.

2.3 PROBLEM STATEMENT

Detection of mycobacterial infection in its active state requires a diagnostic test that can exclude false positive and false negative results. A method of diagnosis is needed where a positive diagnosis reflects the disease and is not related to the vaccinated status of the individual against TB or exposure to non-pathogenic *Mycobacterium sp* (Raqib *et al*., 2003). Thus in an immunological approach, a short term immune memory (as in the innate immune system) might present less false positive diagnosis then the longer term immune memory (typical of the adaptive immune) system. HIV infection and other immune diseases also lead to the formation of auto-antibodies and immune complexes. It is thus also important to see if the dissociation of these complexes might improve the sensitivity of the ELISA method to be used (Chia *et al*., 1979).

Many studies investigated the use of non-protein antigens for serological diagnostic tests in tuberculosis. The same non-protein antigens in different studies often result in different sensitivities and specificities. Harrington III *et al.* (2000) used a multi-antigen based enzyme immunoassay to improve test specificity and sensitivity over the use of a single antigen. Most of these studies appear promising for diagnosis, but very few studies investigated diagnosis in HIV co-infected populations.

This chapter reports results obtained in the investigation of the application of mycolic acids, a lipid antigen from the *M. tb* cell wall, as antigen in ELISA. The study investigated the use of the anti-MA antibody as a surrogate marker for TB infection with special reference to HIV co-infection. The effect of TB chemotherapy on the prevalence of anti-MA antibodies in TB patients is also investigated.

2.4 HYPOTHESIS

Humans actively infected with *M. tuberculosis* will express antibodies to the lipid antigen, MA, that can be used in a serodiagnosis assay for active tuberculosis infection in an environment where HIV infection is common.

2.5 AIMS

Antibody levels to MA were determined in TB negative and TB positive patients through ELISA. The specificity, sensitivity, positive- and negative- predictive values for the assay were determined against results obtained from patients diagnosed for TB on sputum microscopy and culture. A comparison is also made of the anti-MA antibody levels in TB positive, HIV seropositive patients to those in TB positive, HIV seronegative patients.

2.6 MATERIALS AND METHODS

TB patients produce antibodies against the MA in the cell wall of *M. tuberculosis*. To investigate the possibility of using the prevalence of antibodies to MA as surrogate marker for infection in an ELISA based serodiagnostic test, screening of 118 patients from the year 2000 and 214 patients from 1994 were carried out. The mycolic acid antigen was purified from the *M. tuberculosis* cell wall and immobilized on the ELISA plate surface. ELISA plates were from Sero-Well®, Bibby Sterilin Ltd, Sterilab, Stone, UK. After serum, conjugate and substrate incubation the presence of antibodies directed against MA could be detected in an indirect ELISA.

2.6.1 REAGENTS AND BUFFERS

Di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$); dihydrogen potassium phosphate (KH_2PO_4); potassium chloride (KCl); sodium chloride (NaCl) all of analytical grade, were obtained from Merck (Darmstadt, BRD).

Casein from bovine milk (carbohydrate/ fatty acid free) was obtained from Calbiochem (La Jolla, CA). Goat anti-Human IgG (whole molecule) peroxidase conjugate, O-phenylenediamine (OPD) and polyethylene glycol (PEG) 8000 were obtained from Sigma, St Louis, MO, USA. Hydrogen peroxide was from Merck (Darmstadt, BRD).

Ultra-pure double distilled, de-ionized water ($dddH_2O$) was autoclaved prior to use.
The Mettler AE 163 (Protea Laboratory, Sandton, SA), a semimicro balance with 0.01mg resolution was used. It has two weighing ranges 30.00000g and 160.0000g with readability's of 0.01mg and 0.1mg respectively. Another toploading balance used was the Sartorius portable, PT 1200, (Microsep, Göttingham, Germany) with a balance capacity of 1200g and a readability of 0.1g.

20x PBS (phosphate buffered saline) stock solution was prepared by dissolving 160g NaCl, 4g KCl, 4g KH_2PO_4, 23g Na_2HPO_4 in a total of 900ml- $dddH_2O$, with the use of a magnetic stirrer. The solution was brought to a final volume of 1000ml with $dddH_2O$ and was filtered through a 0.22µm filterpaper. The solution was stored in 50ml aliquots at room temperature. PBS (0.154M) was prepared by diluting 1ml 20x PBS with 19ml sterile $dddH_2O$. A normal working solution at a pH of 7.4 was obtained.

Glycine-HCl (1M) with a pH of 2.8 was prepared as follows. Glycine (1.50g) was weighed out and dissolved in 80ml $dddH_2O$. The pH was then adjusted with diluted HCl. The final volume of 100ml was obtained through addition of $dddH_2O$. The solution was stored at 4^oC.

K_2HPO_4 (50ml, 1M) with a pH of 9 was prepared as follows. K_2HPO_4 (8.80g) was dissolved in 40ml dddH_2O. The final volume of 50ml was obtained through addition of dddH_2O. The solution was stored at 4^oC.

The substrate solution was prepared just before use and was kept in the dark. OPD (10 mg) and of H_2O_2 (8 mg) were weighed into 10 ml of 0,1M citrate Tri-sodium buffer, pH 4,5.
The citrate buffer was prepared as follows: citrate Tri-sodium (0.1M, 450 ml) and citric acid (0.1M, 450 ml) solutions were prepared by weighing out 14.71g and 10.5g respectively. The citric acid solution was then added to the tri sodium citrate solution until a pH of 4.5 was obtained. The solution was then brought to a final volume of 1000 ml with the addition of dddH_2O. The solution was aliquoted into 100ml portions and autoclaved.

2.6.2 STUDY POPULATION

2.6.2.1 STUDY WITH SERA COLLECTED IN 2000

Patient serum samples from the year 2000 were kindly provided by: Dr. G.K. Schleicher, Helen Joseph Hospital, Auckland Park, Johannesburg). Patients were adults between the ages of 18 and 65. TB positive patients (59) were matched for age, gender and race with TB negative (59) patients as closely as possible. These patients could be divided into the following subgroups:

- 19 HIV negative, TB negative (HNTN)
- 20 HIV negative, TB positive (HNTP)
- 40 HIV positive, TB negative (HPTN)
- 39 HIV positive, TB positive (HPTP)

TB infection was diagnosed with smear positive tests. Appropriate sputum samples were stained with the Ziel-Neelsen stain and analyzed microscopically for acid fast bacilli. All sputum samples considered positive for acid fast bacilli were submitted for mycobacterial culture and analyzed radiometrically (Bactec-460 TB Hood, Becton Dickinson and Co, USA). None of the TB positive patients were treated for TB at the time of sampling. The TB negative patients consisted of

control subjects from the general medical wards with medical conditions other than tuberculosis, but showing no evidence of active tuberculosis. All patients had routine sputum analysis, haematological, biochemical and serological blood tests (including tests for HIV) as part of the usual clinical practice. $CD4^+$ T cell counts were also provided. Demographic, clinical and laboratory data were recorded (Schleicher *et al*., 2002).

For the determination of sensitivity, specificity, positive and negative predictive values sera from 10 healthy individuals were obtained. The individuals were healthy by all outward appearances and showed no clinical signs or symptoms of any illness.

The study was approved by the Institutional Review Board for Human Research. Informed consent was obtained from all patients prior to enrolment.

Blood was withdrawn from patients in sterile Vacutainer tubes (with brown lids, Aquila Health Care, Pinegowrie, SA). These tubes were silicone coated and without any anticoagulants (Helen Joseph hospital, Johannesburg). Samples were stored and transported to the laboratory at 3-5ºC. After the blood clotted, serum was removed from the blood clot with plastic pipettes. The serum samples were then centrifuged in an Eppendorf centrifuge (326g,, 5 min, 4°C). This was done to remove any red blood cells that were still in the serum. All the serum samples from one patient were then combined in a 10 ml tube and after gentle mixing samples were aliquoted in 500µl portions into 1.8ml cryo tubes (NUNC™ Brand products, Nalge Nunc international, Denmark) and stored at -70°C until use.

Samples were thawed and then γ-irradiated (30 Grates, Pretoria Academic hospital) as an additional safety precaution. Both γ-irradiated and non-irradiated sera were analyzed in ELISA to confirm that antibody binding activity was not affected by γ-irradiation (data not shown).

2.6.2.2 STUDY WITH SERA COLLECTED IN 1994

Serum samples from tuberculosis patients from the year 1994 were obtained (already separated from the blood clot) from the Medical Research Council (MRC), Clinical and Biomedical TB Research Unit: King George V Hospital, Durban. These samples could be divided into the following subgroups, depending on whether they were already undergoing antibiotic treatment or not:

- 34 TB infected but not treated, HIV- (Rf-HIV-)
- 28 TB infected but not treated, HIV+ (Rf-HIV+)
- 116 TB infected and TB treated, HIV- (Rf+HIV-)
- 36 TB infected and TB treated, HIV+ (Rf-HIV+).

Experiments were carried out in the year 2000.

2.6.3 ELISA METHOD FOR ANTI - MYCOLIC ACID ANTIBODY DETECTION

The MA batches used were obtained in aliquots from S. van Wyngaardt (Department of Biochemistry, University of Pretoria). MA were derived from *M. tuberculosis* H37Rv, ATCC 27294. This virulent strain was isolated from human lung tissue and purchased from the American Type Culture Collection (ATCC), Maryland, USA. The TB culture was maintained at the National Tuberculosis Research Programme of the MRC of South Africa, Pretoria. The MA were then purified from the mycobacterial cell wall as described by Goodrum *et al.* (2001). The purified MA obtained were used as antigen in ELISA.

Weighed MA (±240 µg) was dissolved in 4 ml of 0.154M PBS (pH=7.4) as follows:
The vials containing the MA and PBS were transferred to a heatblock for 20 minutes at 85°C. The MA-PBS (antigen) and PBS (control) were then sonicated at 20% duty cycles and output of 2, for one minute. The vials were kept in the heatblock whilst pipetting 50µl into the wells of the ELISA plate. The final antigen load was approximately 3µg per well. The plates were left on the

bench to reach room temperature after which the plates were inspected with a light microscope. The presence of oily drops were indicative of successful MA coating. To determine background (non-specific) binding, PBS alone was coated as control. The plates were transferred in plastic bags to a 4°C cold-room and left overnight. The coating solution was flicked out. As blocking buffer 400µl 0.5% (m/v) carbohydrate and fatty acid free casein/PBS was added per well for 2 hours. One litre 0.5% casein/PBS buffer (blocking and washing buffer for the ELISA) was prepared by weighing out 5g casein and mixing it into a paste with 50ml 20xPBS. This paste was then diluted with the addition of 900ml dddH_2O and incubated in a 37°C waterbath for 2 hours to dissolve completely. The solution was left overnight at 4°C. The pH was then determined and adjusted to 7.44 with the addition of 1M NaOH. A final volume of 1000ml was obtained with the addition of dddH_2O.

The wells were aspirated before loading the serum samples. Serum samples were tested as 1:20 serum dilution by diluting 25µl of serum in 475µl casein/PBS. For serum precipitates, 8% PEG was prepared to precipitate the immune complexes and IgG in patient serum. The 8% PEG solution was prepared by weighing out 0,8g PEG 8000 and dissolving this in 10 ml 0.01M PBS buffer. PBS (approximately 0.01M) was prepared by diluting 1 ml 0.154M PBS with 9ml sterile dddH_2O. Patient serum (100 µl) was added to 100µl PEG 8000 and left overnight at 4^oC. The following day the tubes were centrifuged at 1600g, for 30 minutes at 4^oC. The supernatant was discarded. The pellet was washed two times with 4% PEG (1:1 dilution of 8% PEG in 0.01M PBS) and subsequent centrifugation at 1600g for 30 minutes, at 4^oC in a Sorvall® RMC 4 centrifuge (Du Pont®). After the pellet was washed, it was dissolved in 100 µl 0.154M PBS. Glycine-HCl (50 µl) was added to the samples on ice and left to stand for 15 minutes to release the antibodies from complexes. KH_2PO_4 (1M, 25 µl) was added to neutralize the acid environment. This prevented the antibodies that were just dissociated to be damaged by the lower pH (Chia *et al*., 1979 & Yamashita *et al*., 1992). DddH_2O (25 µl) and Casein/PBS (1800 µl) was added to the samples to obtain a final volume of 2 ml and a 1:20 dilution equivalent of the original serum. All samples were kept on ice through out the whole experiment.

Dilute patient sera (50 µl) or patient precipitate (50 µl) was pipetted into ELISA plate wells. Each sample was gently mixed just before loading it in quadruplicate into the antigen coated wells. The plates containing samples were then incubated for 60 minutes at room temperature. The serum samples were removed and disposed of into a suitable container to be autoclaved. The wells were then washed three times with Casein/PBS and aspirated before the addition of the reporter antibody. An original Well Wash4, purchased from labsystems (Finland), was used for all the wash steps of the ELISA. Goat anti-human IgG peroxidase conjugate was used in a 1:1000 dilution (diluted in casein/PBS). Conjugate (50µl) was pipetted into each well and the plates were incubated for 30 minutes at room temperature. The contents were aspirated and disposed into a suitable container to be autoclaved. The wells were then washed three times with casein/PBS and aspirated before the addition of substrate solution (50 µl) to each well.

Colour development was measured after 5, 30 and 60 minutes of incubation. Absorbancies were determined with a SLT 340 ATC photometer, using a 450nm measuring filter and a 690nm reference filter. Absorbancy values obtained after 30 minutes were transferred into a spread sheet and box and whisker diagrams were constructed. Corrected ELISA signals were obtained by subtracting the background values on PBS coated plates from the signals obtained on the MA coated plates.

Statistical analysis:

Microsoft Excel was used for box and whisker diagrams and the Statistical package for social sciences (SPSS), at the University of KwaZulu-Natal, Pietermaritzburg campus, was used for the Mann-Whitney test and the independent samples t-test for comparing means (equal variances not assumed). The independent samples test is a parametric t-test to compare the means from two independent samples, testing whether it is likely that the samples are from populations having different mean values. A normal distribution is assumed (Altman, 1994; http://www.amherst.edu/; http://www.wellesley.edu; http://www.une.edu.au). P values smaller than 0.05 indicates a significant difference between the two groups tested.

The Mann-Whitney test is a non-parametric test analogue of the t-test used for two independent samples. A normal distribution is not assumed, as this test is independent of distribution

assumptions. The data is ranked on scores. A difference in tendency is obtained that is not influenced by one or a few extreme values. P values smaller than 0.05 indicates a significant difference between the two groups tested (Altman, 1994; Howell, 1999).

For the determination of sensitivity, specificity, positive and negative predictive values the serum of 10 healthy control subjects were used. The cut-off value defining patient positive and negative test signals were determined at 2 standard deviations from the mean of the healthy control subjects values.

2.7 RESULTS

2.7.1 STUDY WITH SERA COLLECTED IN 2000

All of the serum samples collected in the year 2000 were from not treated TB patients. The samples were carefully matched for age, gender and race. No statistically significant differences for these characteristics were obtained between the TB positive patients and the TB negative controls (Schleicher *et al*., 2002). The TB negative control patients were recruited from the general medical ward and had medical conditions other than tuberculosis infection. These samples were tested as serum preparations and immune complex (IC) precipitate preparations. The corrected ELISA signals are indicated in box and whisker diagrams (Figure 2.6a and b). This style of presentation of results not only allows comparison of the mean signals determined for each study group, but also gives a visual representation of the distribution of samples within a population.

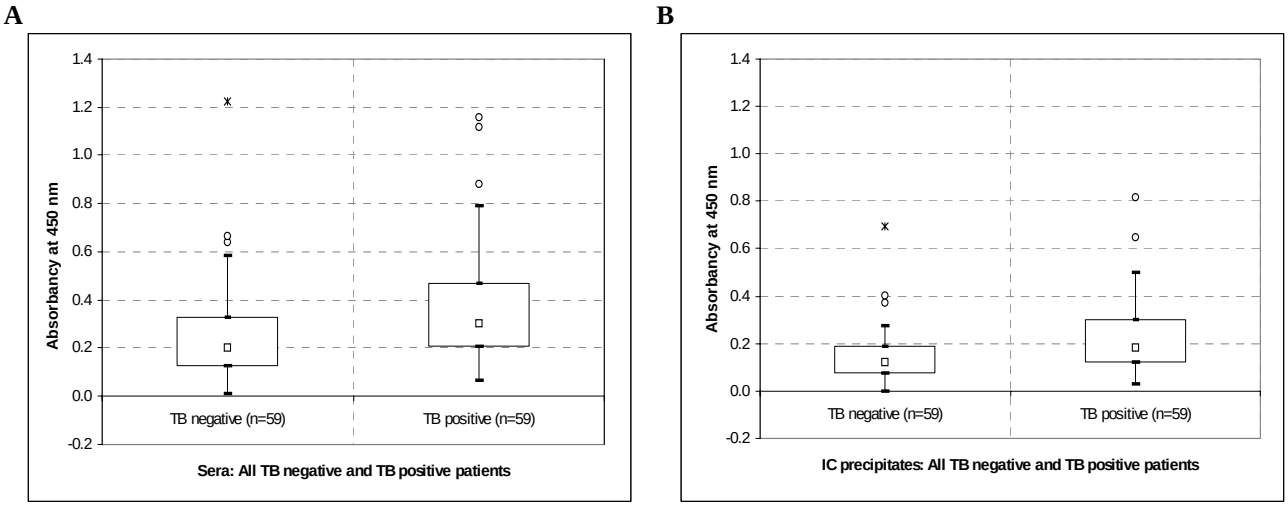


Figure 2.6: *ELISA results of the year 2000 collection of human sera tested against mycolic acids to compare TB negative and TB positive sub-divisions for significance of difference.*
A. *Sera*
B. *IC precipitates*
*Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ indicates non-outlier maximum; ⊥ indicates non-outlier minimum; ° Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

The medians of the two groups tested differ as indicated by the small square inside the large square (box). The height of the boxes indicate the absorbancies where 25-75% of the population was found, and was found to be different for the subgroups tested. Although the distribution of patient results in each of the groups differed (box), a large degree of overlap was seen between the TB negative and TB positive patients.

When comparing the corrected signal intensities (mean ± SD) in these two graphs the TB positive patient group had statistically significant higher anti-mycolic acid antibody levels than the TB negative group in both the sera and the IC precipitates. The independent samples t-test (equal variances not assumed) for comparing means was used to determine the statistical significance of the results obtained. In TB positive patients the mean corrected signal intensity (mean ± SD) of sera was 0.364 ± 0.234 and in IC precipitates it was 0.226 ± 0.150, this was higher than the corrected signal intensities in sera 0.249 ± 0.200 ($p=0.0053$) and IC precipitates 0.143 ± 0.110 ($p = 0.0009$) of TB negative patients. The Mann Whitney Z test was done to confirm results obtained by the independent samples t-test. For the sera and the IC precipitates the P values were determined at 0.001 and 0.0004.

When the same samples were re-grouped and presented as HIV seronegative versus HIV seropositive individuals the results in Figure 2.7 were obtained.

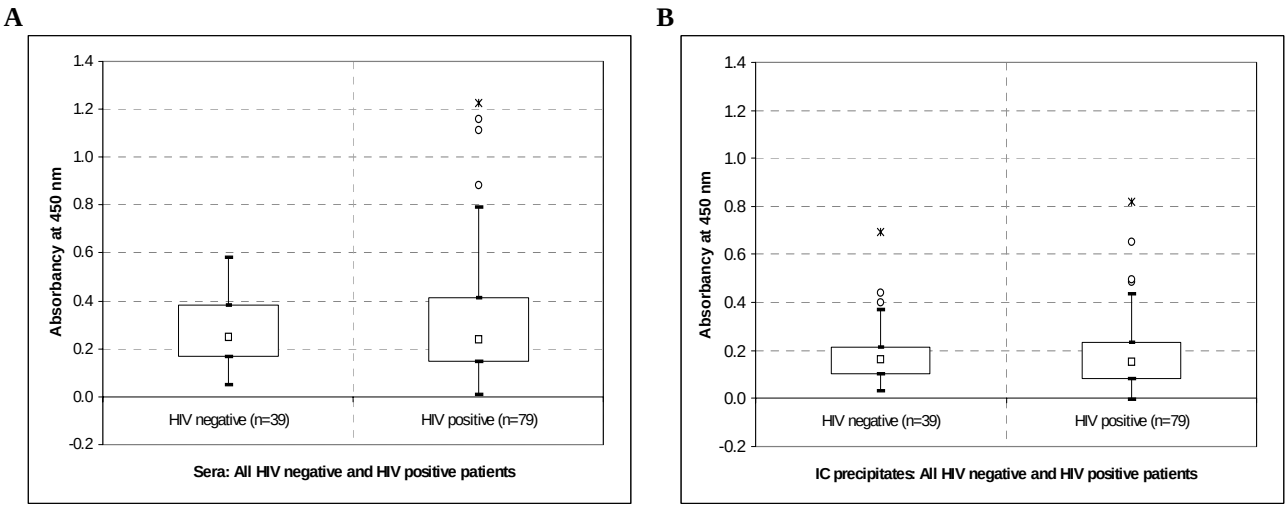


Figure 2.7: *ELISA results of the year 2000 collection of human sera tested against mycolic acids to compare HIV negative and HIV positive sub-divisions for significance of difference.*
A. *Sera*
B. *IC precipitates*
*Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ indicates non-outlier maximum; ⊥ indicates non-outlier minimum; ° Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

Comparing the signals of HIV negative and HIV positive individuals to MA as lipid antigen, no significant differences were observed. In HIV negative patients the mean corrected signal intensity (mean ± SD) in sera was 0.276 ± 0.149 and in IC precipitates it was 0.184 ± 0.127 in comparison to the corrected signal intensities in sera 0.321 ± 0.253 (p=0.207) and IC precipitates 0.184 ± 0.144 (p = 0.993) of HIV positive patients. In these groups the HIV negative and HIV positive groups contained equal numbers of both TB positive and TB negative patients.

The Mann Whitney Z test confirmed the results obtained by the independent samples t-test (equal variances not assumed). For the sera and the IC precipitates respectively the P values were determined at 0.858 and 0.667. These results imply that the ELISA test for detecting the prevalence of anti-MA antibodies is not affected by the HIV-infection status of the patients.

The sensitivity of the test refers to the number of TB positive patients correctly testing positive, whilst the specificity refers to the number of TB negative patients correctly testing negative. Positive and negative test results were defined to be higher and lower respectively, than the mean plus 2 SD (0.137 + 0.094) of ten healthy controls tested. The positive and negative predictive values refer to the degree of certainty that can be associated with a positive or negative result respectively, obtained from the diagnostic test. The scores of these four criteria, for the assessment of the ELISA test, were notably low. For the IC precipitates (n=118) the sensitivity of the ELISA was 37% and the specificity was 85%. The positive predictive value was 71% and the negative predictive value was 57%. These values indicate that the ELISA will not be effective in diagnosis. The discovery of the imperviousness of the ELISA signals to HIV-infection status (Figure 2.7) is, however, important.

A positive aspect of the test became apparent when matched control graphs were drawn for HIV seropositive individuals comparing TB positive and TB negative patients (Figure 2.8).

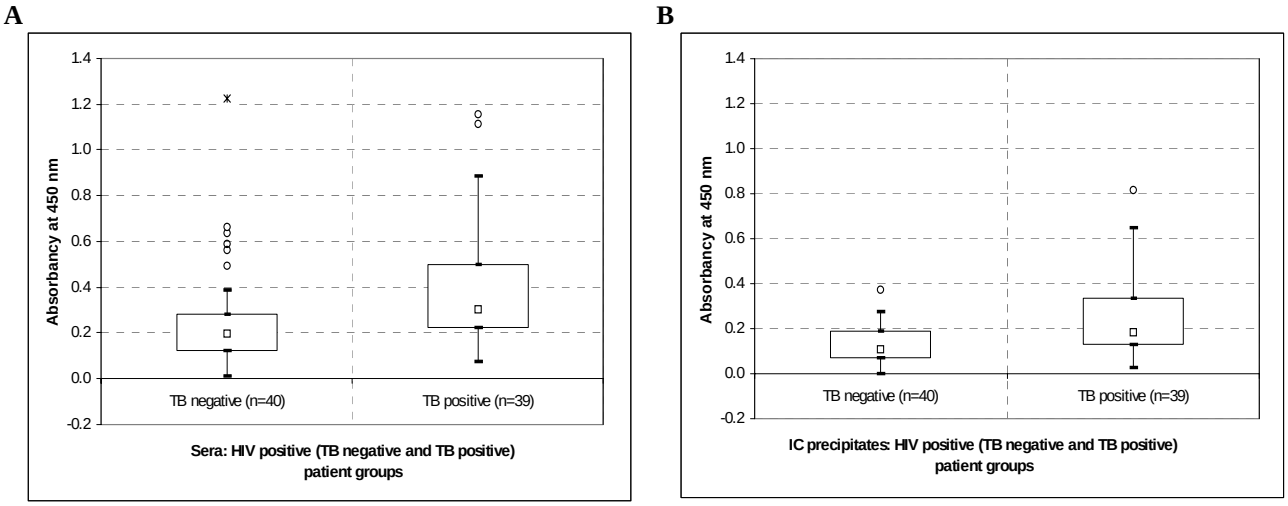


Figure 2.8: *ELISA results of the year 2000 collection of human sera tested against mycolic acids to compare TB negative and TB positive sub-divisions for significance of difference in HIV positive samples.*
A. *Sera*
B. *IC precipitates*
Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ *indicates non-outlier maximum;* ⊥ *indicates non-outlier minimum;* ° *Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

In the HIV positive patient group the mean corrected signal intensity (mean ± SD) in sera for TB negative patients was 0.252 ± 0.225 and in IC precipitates it was 0.128 ± 0.083 in comparison to the corrected signal intensities in TB positive sera 0.394 ± 0.263 (p=0.013) and IC precipitates 0.243 ± 0.169 (p = 0.0003). The difference between TB positive and TB negative groups were significant. The Mann Whitney Z test confirmed the results obtained by the independent samples t-test (equal variances not assumed). For the serum dilution and the IC precipitates respectively the P values were determined at 0.001 and 0.001.

In this group the surprising observation is made that HIV positive TB positive individuals respond to MA as lipid antigen with a significantly higher mean signal intensity than the TB negative HIV positive patients. With HIV infection the CD4 T cells in the HIV infected humans decrease. This would expectedly lead to a reduction in the production of antibodies by the depletion of T cell help to antibody producing B cells.

In this study the HIV-seropositive patients in the TB positive group had a significantly lower mean CD4 T-lymphocyte count than the HIV sero-negative TB positive group (138 x 10^6 cells/l +/- 140 versus 289 x 10^6 cells/l; p=0.0013).

To test whether a decrease in CD4 T cells correlated to a decrease in antibody signal to the MA as lipid antigen, the corrected ELISA signals obtained were plotted against the relevant CD4 counts of the patients in a correlation graph (Figure 2.9).

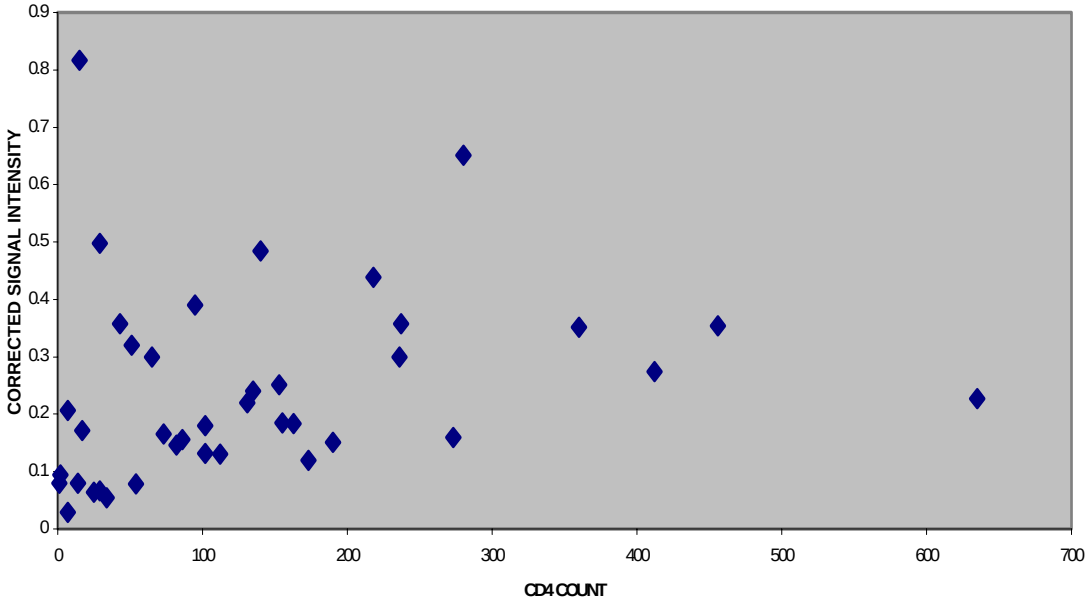


Figure 2.9: *A comparison of the anti-mycolic acid antibody levels in IC precipitates of sera with the relevant CD4 T-lymphocyte count (x10^6 cells/l) in HIV-seropositive patients with tuberculosis (n=39) (Schleicher et al., 2002).*

No correlation (*correlation coefficient,* r=0.225, *strength of the relationship,* r^2 = 0.05 and p=0.168) was found between the CD4 count and the anti-MA ELISA signal obtained. These results suggest that antibodies to MA might still be a good surrogate marker for the detection of tuberculosis infection in HIV seropositive individuals provided that an adequate technology could be developed to detect the antibodies.

2.7.2 STUDY WITH SERA COLLECTED IN 1994

All the sera from 1994 were TB positive and could be divided into TB treated and TB not treated subgroups. Box and whisker diagrams for the ELISA results to determine the prevalence of anti-mycolic acid antibodies in these patients are shown in Figure 2.10 a and b.

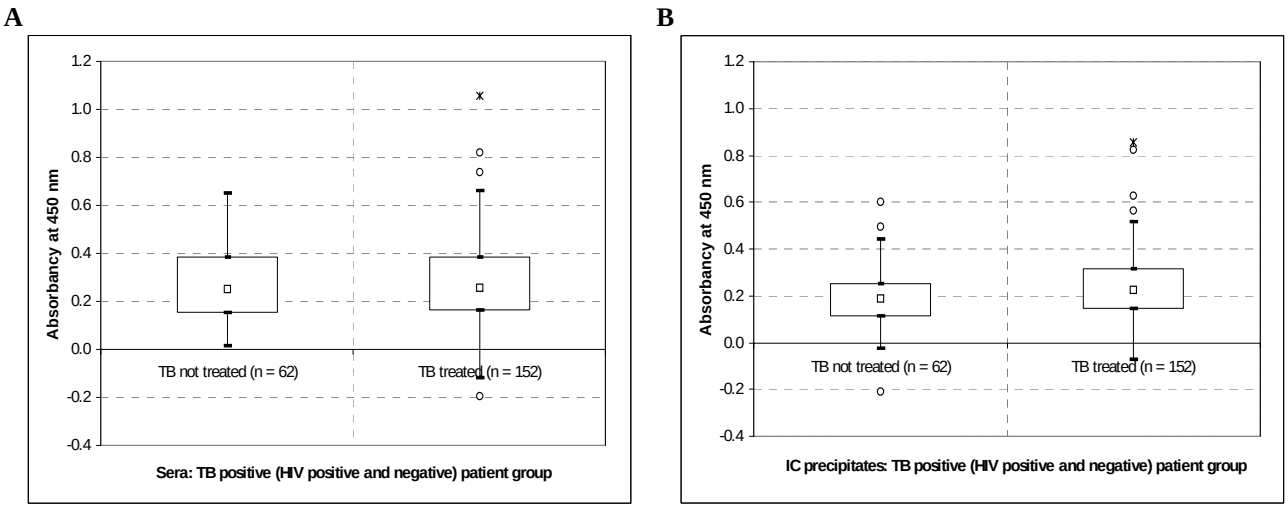


Figure 2.10: *ELISA results of the year 1994 collection of human TB patient sera tested against mycolic acids to compare TB not treated and TB treated sub-divisions for significance of difference.*
A. *Sera*
B. *IC precipitates*
*Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ indicates non-outlier maximum; ⊥ indicates non-outlier minimum; ° Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

From the serum dilution of TB positive patients no difference was found in the ELISA signal of anti MA antibodies with TB non treatment 0.277 ± 0.159 as compared to TB treatment 0.285 ± 0.182 (p=0.722). This might indicate that TB treatment has no apparent effect on the free circulating antibodies to MA. However, for the IC precipitates (Figure 2.10b) the mean for the TB not treated group 0.191 ± 0.127 is significantly lower than the mean for the TB treated group 0.237 ± 0.155 (p=0.027). The precipitated immune complexes contain the lipid antigen and its associated antibodies. The Mann Whitney Z test confirmed the results obtained by the independent samples t-test (equal variances not assumed). For the serum dilution and the IC precipitates respectively the P values was determined at 0.846 and 0.048.

When the HIV positive population is divided into TB not treated and TB treated subgroups the results presented in Figure 2.11 were obtained.

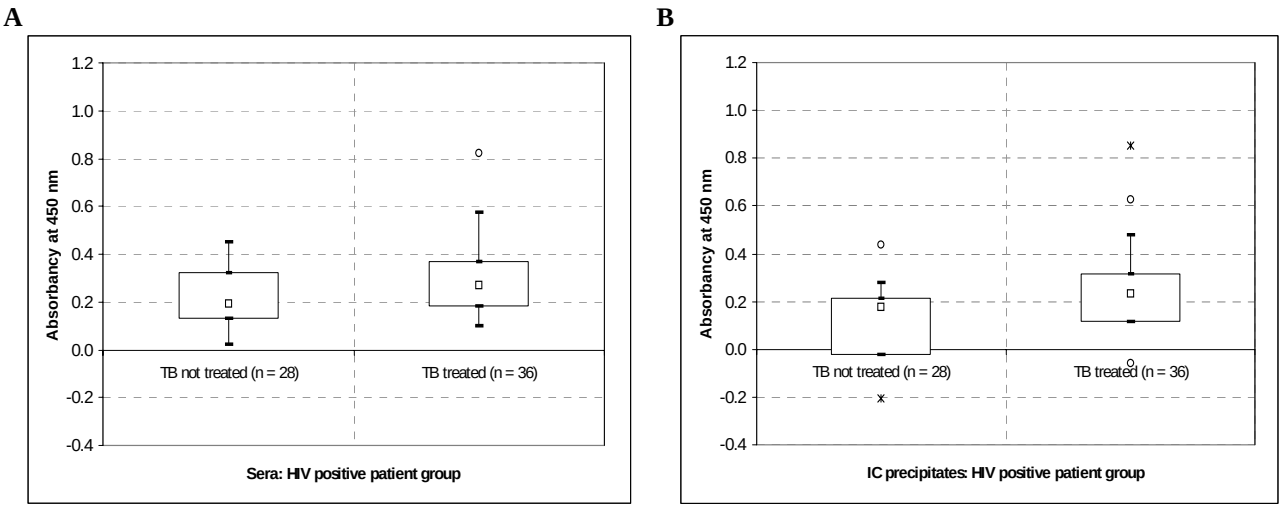


Figure 2.11: *ELISA results of the year 1994 collection of human sera tested against mycolic acids to compare TB not treated and TB treated sub-divisions for significance of difference in HIV positive samples.*
A. *Sera*
B. *IC precipitates*
*Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ indicates non-outlier maximum; ⊥ indicates non-outlier minimum; ° Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

In the HIV positive subgroup a lower average corrected sera ELISA signal intensity (mean ± SD) was determined in the TB not treated 0.223 ± 0.125 than the TB treated 0.301 ± 0.155 (p =0.030) samples (Figure 2.11a). The IC precipitates (Figure 2.11b) for the TB not treated patients 0.162 ± 0.111 gave a significantly lower corrected ELISA signal than the TB treated samples 0.274 ± 0.156 (p = 0.001). This indicated that TB treatment increased the anti-MA antibody signal detected by ELISA in HIV positive individuals. The Mann Whitney Z test confirmed the results obtained by the independent samples t-test (equal variances not assumed) for the IC precipitates by recording a P value of 0.001. For the sera however the Mann-Whitney test determined a P value of 0.063 indicating no statistical difference between the TB not treated and TB treated group. This differs from the independent samples t-test which measures possible differences between the means of two groups tested. The Mann-Whitney test measures differences in the central tendencies of the two groups indicating that extreme values should not influence this test. This could indicate that a single value in the study group influences the mean in the independent samples t-test such that significant differences can be obtained.

When the HIV negative population is divided into TB not treated and TB treated subgroups the results presented in Figure 2.12 were obtained.

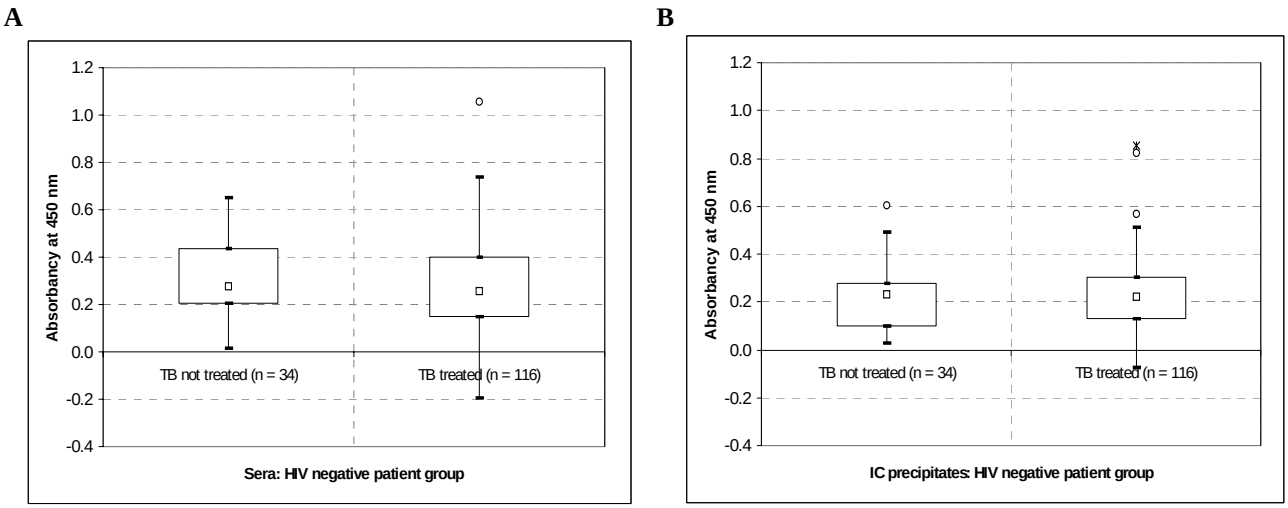


Figure 2.12: *ELISA results of the year 1994 collection of human sera tested against mycolic acids to compare TB not treated and TB treated sub-divisions for significance of difference in HIV negative samples.*
A. *Sera*
B. *IC precipitates*
*Large boxes indicate absorbancies where 25% to 75% of the population lie; Small squares indicate median values; ⊤ indicates non-outlier maximum; ⊥ indicates non-outlier minimum; ° Indicates outlier values and * indicates extreme values. (n=number of patients in each group).*

In the TB positive HIV negative group, the anti-tuberculosis treatment (n=116) did not increase ELISA signals significantly in either the serum diluted or precipitated preparations as no statistically significant differences to the TB not treated group (n=34) were obtained. This might indicate that TB treatment has no apparent effect on the free circulating antibodies to MA in HIV negative TB positive patients. For the sera (Figure 2.12a) the mean for the TB not treated group 0.321 ± 0.171 is similar to the mean for the TB treated group 0.281 ± 0.190 (p=0.249). For the IC precipitates (Figure 2.12b) the mean for the TB not treated group 0.215 ± 0.136 is similar to the mean for the TB treated group 0.226 ± 0.153 (p=0.710). The Mann Whitney Z test confirmed the results obtained by the independent samples t-test (equal variances not assumed). For the sera and the IC precipitates respectively the P values was determined at 0.202 and 0.753.

Although no data was available on the course of TB treatment for the patients tested here, it appeared that the antibodies associated in the circulating immune complexes (CIC) were

increased. Upon treatment with anti-tuberculosis drugs, *M. tb* are killed and the lipid antigens might be shed. The antigenic load at the infection site and peripheral blood is then increased. The amount of MA taken up in the immune complexes might now be higher with a simultaneous increase in the amount of anti-MA antibodies associated. This might have led to an increase in the ELISA signal in the IC precipitates of the TB treated patients. As was found with the year 2000 collection, this observation was especially pronounced in the HIV-positive population.

2.8 DISCUSSION

Humans infected with *M. tb* can produce IgG antibodies to MA (Verschoor *et al.*, patent application *PCT/GB 98/00681,* 1998; Pan *et al.*, 1999, Alving & Wassef, 1999; Harrington III *et al.*, 2000). In this study an attempt was made to show that anti-MA antibodies can be used as a surrogate marker for the diagnosis of active tuberculosis infection, especially in HIV endemic areas. It was deemed of importance that the negative control patients were not healthy controls, to resemble field conditions more closely.

With the 2000 serum sample collection, isolated MA antigen from the *M. tb* cell wall was used in an ELISA based serological test to determine the application of the assay in a population of HIV seronegative and seropositive hospitalized individuals. The study revealed a low sensitivity and specificity. This is contradictory to optimistic results published by Pan *et al.* (1999) for a similar ELISA test where TDM was used as antigen. The difference in performance of their test compared to this study may be ascribed to the fact that the samples used here were obtained from a population where there is a higher combined TB and HIV prevalence.

Precipitation of the immunecomplexes in this study led to better data to demonstrate the difference between TB positive and TB negative patients than with the diluted serum. Precipitation of the circulating immune-complexes was shown in previous studies to increase the sensitivity of ELISA tests for anti-lipid antibodies. Antibodies associated in these complexes in unprecipitated sera might not be available to bind to the MA antigen surface and might be washed away during the washing cycles of the ELISA. By precipitating these immune-complexes

and then breaking them up with pH extremes, the anti-lipid antibodies were found to bind better to the antigen captured on the ELISA plate surface (Sousa *et al*., 2000).

A positive aspect of the use of anti-MA antibodies is the fact that the prevalence of anti-MA antibodies remained unaffected in HIV co-infected patients. In the TB positive population the HIV positive individuals responded with higher anti-MA ELISA signals than the HIV-seronegative patients. This would be a significant contribution to current TB diagnostic tests as antibodies to protein tuberculosis antigens are generally suppressed in HIV positive individuals (Diagbouga *et al*., 1997; Devi and Ramalingam, 2003; Talbot *et al*., 2004). This study also shows that there is no correlation between $CD4^+$ T cell counts and the anti-MA ELISA signals. This could be ascribed to the recently discovered function of the CD1 molecule to present lipid antigens to $CD4^-$, $CD8^-$ T cells. This might further indicate that CD4 T lymphocytes are not required for the CD1–MA restricted pathway of antigen presentation. This finding motivates further investigation into the possible use of anti-MA antibodies as surrogate markers for active tuberculosis disease.

Although the 1994 study was conducted in an uncontrolled manner the results seem to indicate an increase of ELISA signals to MA in TB treated patients relative to TB not treated patients. A previous study by Maekura *et al*. (2001) showed a decrease in antibody levels to glycolipids in TB positive patients treated effectively with chemotherapy. Patients were divided into two groups according to their initial antibody ELISA signal to a tuberculosis glycolipid (TBGL) antigen, a tuberculosis glycolipid mixture containing TDM and more hydrophilic TB glycolipids. When patients were subsequently subjected to anti-tuberculosis chemotherapy, it was observed that patients with an initial high signal on ELISA showed decreasing signals, while patients with an initial low signal showed an increase in the signal in the first month of anti-tuberculosis treatment.

In this study treatment appeared to increase the antibody levels to MA. The difference in these studies might be explained by the period of treatment. It is expected that with initial treatment the *M. tuberculosis* bacilli will be killed leading to a release of antigenic material at the infection site.

An initial increase in antibody levels is expected with the increase in concentration of the antigenic lipids being shed. As treatment continues the lipid antigens will be removed from the host and will therefore not stimulate the production of antibodies to MA. With a longer period of treatment the antigenic load will decrease. The amount of free circulating antibodies is then expected to increase whilst the antibodies in CIC (circulating immune complexes) should decrease (Sousa *et al*. 2000).

Maekura *et al*. (2001) showed that after six months of anti-tuberculosis treatment the antibody titres to glycolipids decreased significantly. Although the authors suggest that a 2-3 year period is necessary to return the antibody levels to normal, this significant decrease after 6 months of treatment could indicate that the CD1 restricted immune reaction might be of short term immune memory. This could decrease the number of false positive test results obtained in serodiagnosis to lipid antigens due to vaccination to TB (Maekura *et al*., 2001, Maekura *et al*., 1993). The ELISA assay might then also be employed in the monitoring of the compliance with anti-mycobacterial therapy by the TB positive patients.

2.9 ASSESSMENT OF ELISA FOR ANTI-MA ANTIBODY DETECTION

Although the sample population was relatively small and the standard deviations in the study groups relatively high, this study makes a contribution as it was set up to simulate true field conditions in a country with not only a high prevalence of tuberculosis disease but HIV co-infection as well.

From the results obtained it is observed that relatively high signals to MA are obtained in the TB negative control group as is reflected by the low specificity obtained. This is expected as the negative controls are from a population with a high incidence of *M. tb* infection. Maekura *et al.* (2001) suggest that the cutoff point used, should differ in countries with lower and higher disease prevalence. Although patients were screened for active TB and selected on negative test results, these patients might have come into contact with mycobacterial antigens and therefore show high antibody responses to MA. Furthermore, as these patients are selected from a general ward in the

hospital where patients were admitted for a variety of reasons, it is possible that other non-tuberculous infections or non-infectious inflammatory responses could result in the formation of non-specific lipid antigens. This could result in false positive test results.

Another possible problem arises from MA being a hydrophobic (lipid) antigen. It is possible that this lipid could interact hydrophobically with other lipid complexes (e.g. the lipoproteins) in circulation. When antibodies associated with MA in these complexes now bind to MA on the ELISA plate other antibodies associated with their various antigens will now also be detected. This might lead to an increase in ELISA signals due to non-specific association with MA. Another possibility explaining false positive test results might be cross-reaction of the anti-MA antibody with other lipids present in the host.

False negative ELISA diagnosis was also common in this study. In the TB positive patient group relatively low signals are obtained as indicated by the low sensitivity of the assay. The low signals obtained might be as a result of the affinity of the antibodies for MA as captured ELISA antigen. The antibody generally appears to be of low affinity. This would implicate that although antibodies to MA are present in large amounts, it would not be detected by ELISA. The ELISA assay involves various wash steps that could dissociate low affinity antibody associations to MA. It might also be possible that some patients present high affinity antibodies to MA. In this case anti-MA antibodies would associate with MA in the patient serum with a stronger affinity than in ELISA, as the antibody is not available to associate with the inferior presentation of the MA antigen on the ELISA plate.

2.10 CONCLUSION

Gong *et al.* (1998) reported that *M. tb*–reactive CD1-restricted T cells could be expanded from HIV-infected patients. The CD1-restricted T cell lines were αβ TCR^+ $CD4^-$ and could therefore not be infected with HIV. Another feature of CD1 molecules is the fact that they are nonpolymorphic so that CD1-restricted antigens are likely to be recognized by most individuals (Gong *et al*., 1998). This novel pathway (CD1 molecules) of lipid antigen presentation might be the reason why antibodies to MA remain detectable in TB positive HIV seropositive patients.

The fact that anti-MA antibodies still recognize MA in HIV seropositive patients with active pulmonary tuberculosis warrants further investigation into the use of anti-MA antibodies in a possible serodiagnostic test for TB.

The next chapter will investigate some of the properties of the anti-mycolic acid antibodies in an attempt to explain the low sensitivity and specificity of the ELISA assay obtained here.

CHAPTER 3: PROPERTIES OF ANTI-MYCOLIC ACID ANTIBODIES

3.1 INTRODUCTION

In the previous chapter it was observed that a number of hospitalized HIV negative, TB negative patients also had antibodies that could recognize MA, just like the tuberculosis patients. Other infections and diseases, other than *M. tb* and tuberculosis, may therefore elicit antibodies recognizing MA, explaining the false positive results obtained in the year 2000 collection of sera. Alternatively, antibodies to other hydrophobic antigens present in blood may associate with the lipoproteins, which in turn may hydrophobically adsorb to the MA coated ELISA plate wells. The ELISA would then also detect these antibodies that are not against MA, leading to false positive test results.

The classical function of the immune system is the protection of the host against foreign antigens. Surprisingly, however, antibodies exist that recognize self-antigens (autoantigens) like phosphorylcholine and cholesterol. These immunoglobulins, or natural autoantibodies (NAA), do not need B cell stimulation for their synthesis. They are present in the normal antibody pool of all vertebrates and do not require deliberate immunization with the target antigen for their production (Cabiedes *et al*., 2002). These autoantibodies are immunoglobulins that react with at least one self-antigen and originate in healthy individuals or in patients with autoimmune disease. Autoreactive antibodies, B- and T-cells are present in healthy individuals and are selected in foetal life. NAA are produced when B cells leave the bonemarrow to become naturally activated by an autoantigen or by an idiotypic complementary V region of an antigen receptor. Most of the NAA in adult serum are of IgG class, although IgM and IgA also exist. NAA recognize both self and foreign antigens (Lacroix-Desmazes *et al*., 1998, Poletaev & Osipenko, 2003).

NAA (IgG) in adult serum could also arise from natural autoreactive B cells encountering epitopes on the surface of foreign antigens that are cross-reactive with self-epitopes. These NAA might be polyreactive but recognize specific epitopes. Some of the cells producing polyreactive NAA could undergo affinity maturation into high affinity antibodies to foreign antigens or pathogenic high-affinity antibodies to self antigens. Antibodies are thought to require high affinity of binding to antigens to be efficient against pathogens. NAA might be of

low affinity where networks of antibodies result in novel biological properties aiming at the regulation of the antibody response (Lacroix-Desmazes *et al*., 1998).

Most disease-associated autoantibodies arise from naturally occurring unmutated autoantibody templates through a process of somatic diversification and self-antigen driven selection, similar to that involved in the affinity maturation of foreign antigen specific antibodies. Antigenic pressure on a polyreactive NAA-producing B cell clone may lead to somatic mutation and the selection processes that result in higher affinity for antigen. This is accompanied by a loss in polyreactiveness. The encountering of foreign antigen, cross-reactive with self epitopes in the first years of life, stimulates the maturation of the self-reactive antibody repertoire. Pathogenic autoantibodies (IgG) show high affinity binding to self antigens (Lacroix-Desmazes *et al*., 1998).

Autoreactive B cells constitute a considerable part of the B-cell repertoire. The NAA secreted are self-reactive but not self-specific (Opezzo & Dighiero, 2003). In AIDS patients an increased level of many natural autoantibodies are found, explaining the description of AIDS as an autoimmune disease (Quan *et al*., 2001).

3.1.1 FATTY ACID ANTIGENS

Fatty acids (FAs) are relatively small, hydrophobic and highly mobile molecular structures with vital biological functions and a ubiquitous distribution. One would not expect to find antibodies to FA. The immune system, furthermore, rarely has the opportunity to interact with these structures as FA hydrophobicity makes it virtually impossible for them to stay in aqueous solution, except as a lipid bilayer. Surprisingly, natural anti-FA antibodies have been discovered in several situations. It has been suggested that antibodies to FAs can be generated in response to the exposed edges of disrupted host cell membranes, damaged by viral infection or through autoimmune cell destruction (Maneta-Peyret *et al*., 1987).

Although an increasing amount of evidence is found in literature that antibodies recognize lipid antigens, still little information is available on the affinity and possible cross-reaction of antibodies to fatty acids per sé. The mycolic acids are one group of naturally occurring FAs

that promote an immune response probably because of their rigidity and dimensions (Fujiwara *et al*., 1999; Pan *et al*., 1999).

Harris *et al.* (2003) reported that FA could be rendered immunogenic: Linoleic acid (C18:2) was modified by substituting the distal methyl group with a carboxyl group to yield a dicarboxylic fatty acid (DCA). The antibodies showed cross reactivity between FA variants differing in the number, position and configuration of their double bonds. They concluded that the anti-DCA antibody paratope (binding site) was structured to accommodate the hapten in a way that depended on the precise shape of the acyl chain. This FA epitope is determined by the particular double bond pattern of the unsaturated acyl chain, as well as the polar head group. They suggested that FAs become much more effective as B-cell epitopes when presented with their hydrophilic carboxyl group exposed on the surface of immunogenic conjugates (Harris *et al*., 2003).

3.1.2 CHOLESTEROL AS AUTOANTIGEN

Horváth *et al*. (2001b) showed that sera of every healthy human subject contained various amounts of naturally occurring IgM and IgG anti-cholesterol antibodies (ACHA). Alving and Wassef (1999) proposed that endotoxin, found ubiquitously in the environment, could act as a potent adjuvant for the induction of antibodies to cholesterol. The cholesterol source of antibody induction could come from localised sites of infection and inflammation where cellular debris is gathered. Infection or colonalization of bacteria could lead to a high amount of free cholesterol. Gram-negative bacteria, bacterial endotoxin or certain other microorganisms could stimulate the production of naturally occurring ACHA. They demonstrated their tenet by injecting Mycoplasma into rabbits, which led to production of IgG to cholesterol.

Dijkstra *et al.* (1996) showed that polyclonal and monoclonal mouse antibodies reacted with the 3β-hydroxyl group of cholesterol and structurally similar sterols (oxidized sterols) retaining the 3β-OH group. In the cell membrane the tiny 3β-OH group of cholesterol (recognised by ACHA) is located at a protected site (lowest point) between other phospholipids at the surface of the lipid bilayer. When the cholesterol concentration increases

in the membrane or the lipoproteins, the antibodies gain easier access to cholesterol that then becomes more exposed on the surface (Dijkstra *et al*., 1996; Agirre *et al*., 2000).

In the body ACHA (mainly IgG and IgM) bind to LDL, VLDL and intermediate density lipoprotein (IDL), but not to HDL. LDL dose-dependently inhibited human ACHA (IgG) binding to solid phase cholesterol (Horváth *et al*., 2001b). HDL has a higher protein to cholesterol ratio at the surface of the lipoprotein. This higher protein presence may inhibit antibody binding through steric hindrance, charge effects or hydrophobic blocking of the small 3β-OH group of cholesterol. ACHA did, however, bind to the lipid fraction extracted from HDL (Dijkstra *et al*., 1996; Agirre *et al*., 2000).

Alving and Wassef proposed in 1999 that ACHA bind reversibly to cholesterol in circulating lipoproteins (LDL, VLDL, IDL but not HDL), and opsonize the latter for removal by scavenger macrophages. These circulating lipoprotein-ACHA immune-complexes are taken up by cells expressing anti-cholesterol antibody receptors. Free ACHA were however still detected in human serum samples (Alving and Wassef, 1999). It could be that oxidation of the lipids in LDL provides the trigger for antibody recognition of LDL. CIC-LDL with autoantibodies showed elevated levels of oxysterol (Tertov *et al*., 1996).

Interestingly, Horváth *et al.* (2001a) showed that ACHA are higher in HIV positive individuals than in HIV negative individuals. The ACHA level correlated negatively with the cholesterol concentration in the blood. They inferred that the presentation of concentrated cholesterol on HIV particles acted as immunogen to elicit ACHA (Horváth *et al*., 2001a). This could then possibly also explain the higher signal obtained to MA in the HIV positive, TB positive group shown in the previous chapter.

3.1.3 CHOLESTEROL AND *Mycobacterium tuberculosis*

Prof. J.A. Verschoor and Dr. D.G.R. Siko of our research team used the resonant mirror biosensor in an attempt to determine the affinity of the anti-MA antibody for MA. As MA was highly insoluble in water, the research team used cholesterol containing liposomes as MA carriers. Liposomes with MA were used as antigen in the biosensor experiments, whereas liposomes without MA were used as control. After a series of experiments it was concluded

that cholesterol complicated the interpretation of the biosensor antibody-MA binding results obtained against the MA antigen. It was suggested that the anti-MA antibody might cross-react with cholesterol to yield false positive test results (Siko, 2002).

Cholesterol is a major sterol constituent of eukaryotic membranes. It is involved in the stabilisation of membranes and plays a key role in hormonal and signalling pathways. Pathogenic *M. tb* take up cholesterol, but do not use it as a carbon source like their non–pathogenic counterparts. It was then proposed that *M. tb* accumulate cholesterol in their membranes to mimic eukariotic cell membranes (Av-Gay and Sobouti, 2000). It was shown by Gatfield & Pieters, (2000) that cholesterol was essential for the successful uptake of *M. tb* by the host immune cells. In addition, the cholesterol and cholesterol ester concentrations in macrophages of *M. tb* infected patients were higher than in healthy individuals. Cholesterol also played a protective role as it helped to transform the macrophage into the perfect biotype for the pathogen. Moreover, cholesterol appeared to prevent lysosomal degradation of *M. tb* (Gatfield & Pieters, 2000).

M. tb has a characteristic cell wall, which is very rich in lipids. The bacilli are preferentially taken up by macrophage SR-A (Zimmerli *et al*., 1996). The precise ligand on the mycobacterial cell wall for this uptake is unknown, but may well be related to cholesterol. Scavenger receptor A (SR-A) molecules are integral membrane glycoproteins expressed in tissue macrophages and foam cells. They recognise polyanionic macromolecules including modified LDL like the naturally occurring oxidised LDL (Hsu *et al*., 1995; Febbraio *et al*., 1999). The recognition of oxLDL by SR-A results in the formation of foam cells that could lead to the formation of atherosclerotic plaques. It was shown in our laboratory that the uptake of MA by macrophagess resulted in the formation of foam cells (Stoltz, 2002). It was postulated that MA might be the mycobacterial ligand recognised by SR-A. MA, by its presumed mimicry with cholesterol, may stack with the latter in hydrophobic association. *M. tb* may therefore approach the macrophages in a cholesterol coat with particular affinity for SR-A.

Here, the hypothesis that MA mimics cholesterol in the body is further tested. As ELISA was shown not to be an ideal test for anti-MA antibody detection (Chapter 2) other more efficient antibody assays may be required to find reliable evidence to test the hypothesis. Thanyani (2003) convincingly showed that the resonant mirror biosensor was a far more reliable

technique to demonstrate anti-MA antibody activity in human serum samples. The reason for this appeared to be the omission of a washing step after contacting the antigen with antibody and the suitability of this technology to confirm specificity by inhibition of binding experiments.

3.1.4 HYPOTHESIS

Antibodies recognize MA specifically, but are cross-reactive with cholesterol.

3.1.5 AIM

To test the possible cross-reactivity between the anti-MA antibody to MA and cholesterol, by means of a correlation study using ELISA and inhibition experiments on the biosensor.

3.2 MATERIALS AND METHODS

3.2.1 SEPHAROSE PROTEIN A ASSAY

To determine if antibodies to MA recognize MA specifically and not simply through hydrophobic association of lipids onto the hydrophobic surface of the MA-coated ELISA plate, patient serum samples were tested in a fluorescence assay. Total serum IgG of the patient was captured onto Sepharose-protein A (Seph-A). Fluorescently labeled MA was then added in liposome carriers. The amount of labeled MA retained after extensive washing was determined as a measure of direct binding of MA to its antibodies. The values obtained were correlated with the ELISA signals obtained in Chapter 2.

3.2.1.1 REAGENTS AND BUFFERS

Cholesterol (5-Cholesten-3β-ol) was obtained from Sigma (St. Louis, MO, USA). L-α-Phosphatidylcholine (L-α-Lecithin) Type XVI-E, isolated from fresh egg yolk was purchased as a lyophilized powder from Sigma-Aldrich (St. Louis, MO, USA). Fluorescently labeled MA was obtained from S. van Wyngaardt (Department of Biochemistry, University of Pretoria). Already isolated aliquoted MA (see chapter 2) was used for fluorescence labelling with 5-bromofluorescein (5-BMF). 5-BMF was purchased from Molecular Probes (Leiden, the Netherlands). Chemically pure chloroform was from Saarchem (Halfwayhouse, Gauteng, SA). Protein A immobilised on Sepharose® 6MB for affinity chromatography was obtained from Fluka (Steinham, Switzerland). The Sepharose Protein-A had a binding capacity of 6 mg human IgG/ml gel. Sodium azide for Sepharose – Protein A (Seph-A) storage was obtained from Sigma (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) was obtained from Merck (Fedsure park, Midrand, SA). Tris-HCl (10mM, 100mM and 1M) was prepared by dissolving 0.121g, 0.606g and 3.029g Tris in 0.1l, 0.05l and 0.025l dddH_2O respectively. The pH was adjusted to eight with 1N HCL (10mM Tris-HCl) and 10N HCl (100mM and 1M Tris-HCl) before making up to the final volume with dddH_2O. HCl was from Saarchem, Johannesburg, SA. Glycine (50mM, from MERCK, Fedsure park, Midrand, SA) was prepared by dissolving 0.188g glycine in 0.05l dddH_2O, and adjusting the pH to three with 1N HCl before final volume adjustment. Nunc™ dark plates (Black microwell SH plates) were from Nulge Nunc International, Denmark. Balances used were the Mettler AE 163 (Protea Laboratory, Sandton, SA) and the Sartorius portable (Microsep, Göttinggen, Germany) as in chapter 2. For quick centrifugation of the Seph-A suspensions the Eppendorf microfuge 5414S (Eppendorf, Hamburg, Germany) was used at 15000 rpm at room temperature. Buffers were autoclaved and aliquoted before storage at 4ºC.

3.2.1.2 METHODS

3.2.1.2.1 LIPOSOME PREPARATION

Liposomes as carriers for lipids were prepared from stock solutions containing 100mg phosphatidylcholine in 1000µl chloroform and 100mg cholesterol in 1000µl chloroform. All lipids were dissolved in cold chloroform by vortexing and were kept on ice to decrease evaporation of chloroform. Fluorescently labeled MA (approximately 500µg/vial) was obtained from Mrs S. van Wyngaardt (University of Pretoria). All work involving labeled MA was done in darkness. The required amounts of lipids (see Table 3.1) were transferred in chloroform to clean glass vials, after which the chloroform was evaporated by placing the vials in a heatblock at 85°C under nitrogen gas for 10 minutes. Phosphate buffered saline (0.154M, 1ml) was then added to all the liposome vials. The final liposome composition is indicated in Table 3.1.

Table 3.1: *Amount of lipids in liposome preparations used in the Sepharose protein A assay.*

No	Liposome type	PC (ul stock)	PC (mg)	Cholesterol (ul stock)	Cholesterol (mg)	MA (ul stock)	MA (ug)	0.154M PBS (ml)
1	PC	90	9	0	0	0	0	1
2	Cholesterol	90	9	45	4.5	0	0	1
3	MA	90	9	0	0	0	240	1

All the vials were transferred to the heatblock at 85 °C for 30minutes, after which liposomes were vortexed for 1 minute. Liposomes were then pulse sonified at output 3 and 30% duty cycle for 1 minute.

The efficiency of MA labeling was tested in Nunc dark plates by performing a serial dilution (1:1) in 0.154M PBS. Fluorescence was measured in a Fluoroscan Fluorometer (Thermo Labsystems, Oy, Helsinki, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Liposomes were stored overnight at 4 °C and vortexed for 1 minute before use.

3.2.1.2.2 SEPHAROSE-PROTEIN A PROTOCOL

Sepharose-protein-A (Seph-A) was used to isolate IgG from patient sera. The captured IgG was then exposed to 5-bromofluorescein (5-BMF)-labeled MA in liposomes. The general principle is presented in Figure 3.1.

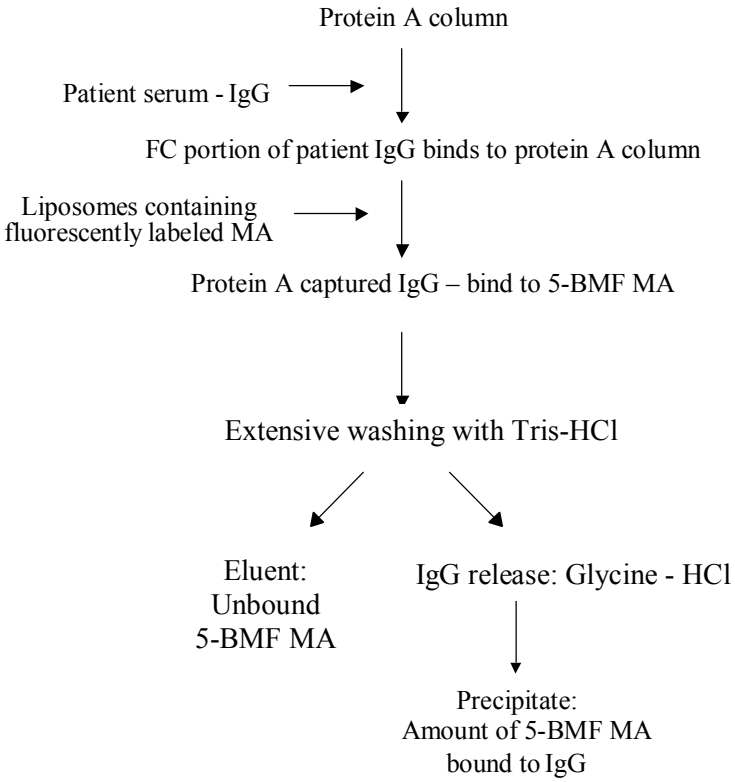


Figure 3.1: *An outline of the basic approach used in the Sepharose protein A assay.*

Empty Eppendorf tubes were weighed for each sample to be tested. Sepharose beads in storage buffer were added to the weighed Eppendorf tubes and centrifuged for 3 seconds. Buffer at the surface of the Seph-A was removed by pipette aspiration. The Eppendorf tubes were weighed again and the mass of the wet Seph-A determined. This process was repeated until all the Eppendorf tubes contained approximately 0.060g Seph-A. The Seph-A was then washed three times with 1000ul 10mM Tris HCl buffer (pH = 8) to remove all azide containing storage buffer. First, 1200ul 10mM Tris HCl buffer was added to the Eppendorf tubes before vortexing for 30 seconds. A volume of 1000ul buffer was then replaced with 1000ul new washing buffer. This was repeated another two times. After all buffer was aspirated, the Eppendorf tubes were centrifuged for 7 seconds and stored overnight at 4°C in

100ul 10mM Tris HCl buffer. Before use, the Tris HCl buffer was again aspirated and 950ul 10mM Tris HCl buffer were added. In clean Eppendorf tubes 5ul 1M Tris HCl buffer (pH = 8) was added to 50ul patient serum. Of this solution, 50ul was then transferred to the Seph-A tubes to obtain an effective 1:20 dilution of serum.

Samples were incubated for 1 hour at room temperature with mechanical rotation to allow association of all the IgG present in patient serum to protein A. As negative control, patient serum was replaced with 50ul 10mM Tris HCl buffer. Samples were centrifuged for 7 seconds and 800ul of the supernatant removed. To wash off all the unbound proteins, 1000ul 100mM Tris HCl buffer (pH = 8) were added and again discarded after vortexing and centrifugation. This was repeated twice. Following these washes, 10mM Tris HCl buffer was used in the same way to repeat the washes an additional two times. All supernatant was discarded before the addition of 900ul 1x PBS.

Fluorescently labeled MA liposomes were vortexed for 1 minute before addition of 100ul to Seph-A in the tubes. The tubes were rotated at room temperature in darkness for 1 hour. This step allowed an interaction between the captured patient IgG and the liposomes containing the fluorescently labeled MA. Samples were vortexed for 30 seconds and centrifuged for 7 seconds. Of the supernatants 100ul were transferred to Nunc dark plates. Of the remaining 900ul supernatant, 600ul was discarded and substituted with 1000ul 10mM Tris. Samples were vortexed, centrifuged and 100ul supernatant transferred to the dark plates. From the remaining supernatant 900ul were discarded. This process was repeated up to 15 times until no more fluorescence was removed from the samples. The fluorescent readings of the serial washing supernatants were taken at the excitation wavelength of 485nm and the emission wavelength of 538nm to determine when baseline fluorescent readings were obtained to indicate adequate removal of excess non-bound fluorescently labeled MA. When no more fluorescence was detected in the supernatant, captured IgG was released by the addition of 500ul Glycine (0.1M, pH=2.7). Samples were rotated for 2 minutes, vortexed, centrifuged and 435ul supernatant transferred to Eppendorf tubes containing 65ul 1M Tris to neutralise the acid used in the IgG releasing step. Of the neutralised solution, 100ul were transferred into the Nunc dark plates in quadruplicate. The release process was repeated once more to determine the efficiency of IgG release, although only the first release was used in data analysis. The amount of fluorescence detected at the excitation wavelength of 485nm and the emission wavelength of 538nm was then considered as an indication of the amount of MA that bound

specifically to anti-MA antibodies. The experiments were repeated four times to obtain quadruplicate values for each patient serum tested. The method was adapted from Harlow and Lane (1998).

A correlation graph indicating the 95% confidence intervals between the corrected fluorescent signals of released IgG-MA complexes and the corrected ELISA results of each individual serum tested was constructed in the FigP software programme (version 2.98, Durham, NC, USA; at the University of Pretoria).

An ELISA was conducted to ascertain that the amounts of IgG captured from the patient samples were similar (data not shown). Lower fluorescent signals could thus not be ascribed to lower IgG binding. Of the IgG released and neutralised in 1M Tris HCl buffer, 225ul were diluted by the addition of 1025ul 10mM Tris HCl buffer. Of this dilution, 200ul were then used in ELISA in duplicate to create a serial 1:1 dilution in 10mM Tris HCl buffer. These sample-IgG coated ELISA plates were incubated overnight at 4°C. Plates were flicked out, aspirated and then blocked with casein-PBS for 1 hour. Goat anti-human IgG (whole molecule) peroxidase (100ul) was used as detection antibody in a 1:1000 dilution in casein PBS for 30 minutes. A 30-minute substrate (OPD and H_2O_2 in citrate buffer, 100ul) incubation followed. Absorbancies were determined at 450nm.

3.2.2 CHOLESTEROL AND MYCOLIC ACID ELISA

The ELISA experiments were carried out as described in Chapter 2. The ELISA plate was divided into three sections. One section was coated with approximately 3ug MA/well, the second section with approximately 3ug cholesterol/well and the last section coated with PBS. The ELISA signals to MA and cholesterol were then compared with each other after plate normalization relative to a negative control sample included on all ELISA plates. The normalized values were obtained by subtracting the average patient background signal on PBS coated plates from both the patient average signal on MA and cholesterol coated plates. This was done for the negative control patient sample as well, which was the same on all the plates.

The correlation coefficient between the response signals for cholesterol and MA was then determined. For the construction of graphs indicating the 95% confidence bands the patient sera results were normalized to the same healthy control TB negative serum result determined on every plate. The average and standard deviations of the quadruplicate signals for MA, cholesterol and PBS were obtained for both patient and the healthy TB negative control sera tested. The average signal obtained in PBS coated wells was then subtracted from the average signal obtained in MA or cholesterol coated wells. The patient values in MA or cholesterol coated plates were then divided by the healthy TB negative control sera values on MA or cholesterol coated plates. The final standard deviation was calculated. For the corrected signal (subtracting signals on PBS) the square root of the sum of the standard deviations for the signal on MA and PBS coated plates were determined. For normalization when dividing with the negative control patient the process was repeated. The square root of the sum of the standard deviations for the corrected patient and the corrected negative control sera for MA and cholesterol coated plates were determined. These points were then used to construct linear regression graphs in FigP.

3.2.3 BIOSENSOR EXPERIMENTS

The Iasys plus Resonant Mirror Biosensor (Iasys Affinity Sensors, Saxon Way, Bar Hill, Cambridge, UK) was used as an additional method to measure the binding of MA to anti – MA antibodies in a TB positive, HIV positive patient. Two non-derivatized cuvettes were used. The possible cross- reactivity with cholesterol was also investigated in this way using a cholesterol concentration range to inhibit antibody binding to MA.

3.2.3.1 REAGENTS AND BUFFERS

Saline was prepared by dissolving 0.9g NaCl (MERCK NT laboratories, SA) in 100ml dddH_2O. PBS (1x) was prepared by diluting 25ml 20x PBS stock (Section 2.6.1) with 475ml dddH2O. This buffer was then used to prepare PBS/AE. Ethylene diamine tetra-acetic acid (EDTA, 1mM) and 0.025% (m/v) sodium azide in PBS/AE was obtained by dissolving 0.186g EDTA (Titriplex ® III from MERCK, Fedsure Park, Midrand, SA) and 0.125g sodium azide (Sigma, St. Louis, MO, USA) in 500ml 1x PBS. A Stock solution of cetyl-pyridium

chloride (CPC, Sigma, St Louis, MO, 0.2mg/ml in PBS/AE) was diluted to a concentration of 0.02mg CPC/ml PBS/AE (200ul stock CPC + 1800ul PBS/AE). Saponin (Sigma, St Louis, MO) was used at a concentration of 0.2mg saponin per ml in PBS/AE. Potassium hydroxide (KOH, 12.5 M) obtained from Saarchem (Fedsure Park, Midrand, SA) was used for cuvette regeneration. KOH (30.068 g) was dissolved in 50ml dddH_2O. Ethanol (95%), for cuvette regeneration was from BDH, MERCK (Halfway House, Gauteng, SA).

3.2.3.2 METHODS

3.2.3.2.1 LIPOSOMES

Liposomes as carriers for lipids were prepared from stock solutions containing: 100mg phosphatidylcholine (PC) in 1000µl chloroform and 100mg cholesterol in 1000µl chloroform. Phosphatidylcholine (L-α-Phosphatidylcholine ,L-α-Lecithin, Type XVI-E), isolated from fresh egg yolk was purchased as a lyophilized powder from Sigma-Aldrich (St. Louis, MO, USA)]. Cholesterol (5-Cholesten-3β-ol), was obtained from Sigma (St. Louis, MO, USA)]. Chloroform was obtained from Saarchem (Halfwayhouse, Gauteng, SA). All lipids were dissolved in cold chloroform by vortexing and then kept on ice to decrease evaporation of chloroform. MA was obtained from Mrs S. van Wyngaardt already aliquoted in amounts of approximately 1 mg/vial. The required amounts of lipids in chloroform were transferred to clean glass vials.

Liposomes were prepared to contain the amounts of lipids as shown in Table 3.2.

Table 3.2: *Amount of lipids used in stock preparations of liposomes used in the biosensor experiments*

No	Liposome Description	PC (ul stock)	PC (mg)	Cholesterol (ul stock)	Cholesterol (mg)	MA (ul stock)	MA (mg)	Saline (ml)
1	PC	90	9	0	0	0	0	2
2	Cholesterol-10	60	6	10	1	0	0	2
3	Cholesterol-20	60	6	20	2	0	0	2
4	Cholesterol-30 *	60	6	30	3	0	0	2
5	Cholesterol-40	60	6	40	4	0	0	2
6	Cholesterol-50	60	6	50	5	0	0	2
7	Cholesterol-60	60	6	60	6	0	0	2
8	MA	90	9	0	0	100	1	2

** Liposomes normally used by TB team in biosensor experiments*

After evaporation of chloroform on the heatblock (85°C) under a nitrogen stream for 10 minutes, 2ml saline was added to each vial and vortexed for 1 minute. Vials were transferred to the heatblock (85°C) for 5 minutes and vortexed. After another 25 minutes on the heatblock vials were vortexed for 1 minute. Liposomes were pulse- sonified at output 3 and 30% duty cycle for 1 minute, aliquoted by pipetting 200ul into new clean glass vials and transferred to -70°C for 2 hours. Samples were then freezedried overnight. Dry liposome preparations were stored at –70 °C until used.

Before use, 2ml PBS/AE were added, samples heated for 30 minutes at 85°C on the heatblock and each vial pulse-sonicated (output 3 and 30% duty cycle) for 1 minute. Liposomes were stored at 4°C and could be used for 1 week. The liposomes now contained the amounts of lipids presented in Table 3.3.

Table 3.3: *Amount of lipids present in final liposomes as used in the biosensor experiments.*

No	Liposome Description	PC (mg/ 200ul aliquot)	**PC Final (ug/ml)**	Cholesterol (mg/200ul aliquot)	**Cholesterol Final (ug/ml)**	MA (mg/ 200ul aliquot)	**MA Final (ug/ml)**	**Total ug lipid**
1	PC	0.9	**450**	0	-	0	-	**450**
2	Cholesterol-10	0.6	**300**	0.1	**50**	0	-	**350**
3	Cholesterol-20	0.6	**300**	0.2	**100**	0	-	**400**
4	Cholesterol-30 *	0.6	**300**	0.3	**150**	0	-	**450**
5	Cholesterol-40	0.6	**300**	0.4	**200**	0	-	**500**
6	Cholesterol-50	0.6	**300**	0.5	**250**	0	-	**550**
7	Cholesterol-60	0.6	**300**	0.6	**300**	0	-	**600**
8	MA	0.9	**450**	0	-	0.1	**50**	**500**

** Liposomes normally used by TB team in biosensor experiments*

3.2.3.2.2 BIOSENSOR PROTOCOL

Patient 57, a HIV seropositive patient of the year 2000 collection of serum obtained by Dr. G.K. Schleicher, was used as a proven TB^{+}-serum. Both cells of the non-derivatized cuvettes were used. A biosensor stirrer speed of 75% was maintained. Resonance scans were performed to confirm cuvette surface integrity throughout the experiment. The cuvette was washed 3x with 60ul PBS/AE, aspirated and 60ul PBS/AE added. After a stable baseline was obtained for 1 minute, the cells were aspirated and 50ul CPC (0.02mg/ml PBS/AE) added. Surface activation was allowed for 10 minutes to create a hydrophobic surface for liposome interactions. The cuvette was washed 5x with 60ul PBS/AE and left for 1 minute before aspiration. PBS/AE (25ul) was then added. After 5 minutes, the MA liposomes to be used for

the coating of the cuvette surface was vortexed for 1 minute and 25ul added to the 25ul PBS/AE already in each cell. Liposome binding (association) was monitored for 20 minutes. After washing 5x with 60ul PBS/AE and allowing 1 minute to achieve a baseline, 50ul saponin (1mg/ml in PBS/AE) was added as a blocking agent and left for 10 minutes. Cells were washed 5x with 60ul PBS/AE and left for 1 minute before aspiration. PBS/AE (25ul) was added and left for 5 minutes. Serum (10ul) diluted in PBS/AE (1:500 dilution) was added and incubated for 10 minutes to ensure synchrony of the cells. In Eppendorf tubes 1ul of a patient serum (P57) was pre-incubated in 180ul inhibition liposomes, containing competing antigens, for 20 minutes. Here the experiment differed for the two cells. For example, one cell received 10ul of the P57 serum (1ul) diluted in 180ul PC liposomes, whilst the other cell received 10ul of the P57 serum (1ul) diluted in 180ul cholesterol containing liposomes. Cells were washed 5x with 60ul PBS/AE and 5 minutes were allowed for monitoring dissociation. The monitoring was then paused for regeneration as follows: Cells were washed 3x with 50ul 95% ethanol and left for 30 seconds. Cells were then washed 10x with 100ul PBS/AE, rinsed 5x with 50ul 12.5M KOH and left for 1 minute. Cells were finally washed with 10x 100ul PBS/AE. Cuvettes were stored in 100ul PBS/AE at 4 °C wrapped in parafilm. The biosensor stirrers were washed with dddH_2O in a wash cuvette. The stirrer was then switched off and cells were washed 10x with 100ul dddH_2O. DddH_2O (120ul) was used to wash the stirrers and the cuvette surfaces for an additional 5 minutes at 100% stirrer speed, after which the cuvette was washed 3x with 120ul dddH_2O and stored at 4°C.

3.3 RESULTS

3.3.1 CORRELATION OF ELISA AND FLUORIMETRIC SEPHAROSE PROTEIN A DETERMINATION OF ANTI-MYCOLIC ACID ANTIBODIES

When using ELISA to detect antibodies to lipids rather than proteins several complications have to be considered. The use of detergents as blocking agents (e.g. Tween) must be avoided as lipids can be washed off the ELISA plate surface. Fatty acid free casein-PBS is therefore preferred as blocking and washing buffer. The hydrophobicity of the ELISA plate surface is another complication. If the plate is not properly blocked antibodies might associate through hydrophobic interaction to the plate surface and then be falsely detected as antibodies recognizing the lipid antigen.

Another effect expected with the use of MA as antigen is that antibodies associated with lipids in immune complexes might accumulate on the MA coated surface. The antibodies detected might thus not be necessarily directed to MA only.

To avoid the use of a hydrophobic surface for specific detection of anti-MA antibodies, various patient IgG antibodies were separately captured by their Fc portion onto protein A particles in suspension. The open antigen binding sites were then exposed to a solution of fluorescent MA in liposomes. After extensive washing, the amount of retained fluorescence was measured as an indication of how much MA bound. These data were then correlated to the relevant patient sera's ELISA signal generated on MA coated ELISA plates. Eight patients were randomly chosen and the correlation graph indicating 95% confidence bands is shown in Figure 3.2.

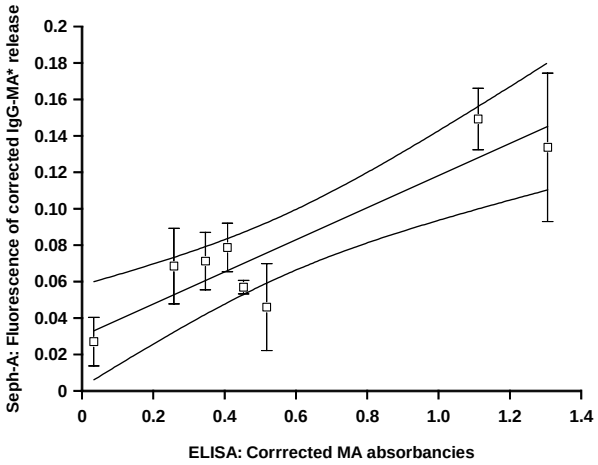


Figure 3.2: *Linear regression analysis between ELISA signals on MA coated plates and fluorescent MA captured on Protein A Sepharose immobilized antibodies. A random collection of eight human sera was used that consisted of 1 HNTP, 1 HPTN, 1 Untyped, 2 HNTN and 3 HPTP patients. The 95% confidence bands are indicated. The correlation coefficient was determined (r = 0.907) and the strength of the relationship (r^2 = 0.823).*

From Figure 3.2 it can be concluded that the signals for antibody binding to MA obtained from ELISA and the fluorescent MA release from patient antibody on protein A correlate (r = 0.907). This indicates that the antibodies recognize MA specifically. The antibodies detected in ELISA are thus not ascribed solely to the non-specific adsorption of antibody-lipid complexes on the hydrophobic surface created on the ELISA plate.

3.3.2 CHOLESTEROL ELISA

The putative cross-reactivity of patient antibodies to MA and cholesterol was investigated with ELISA on plates coated with either MA or cholesterol (Figure 3.3).

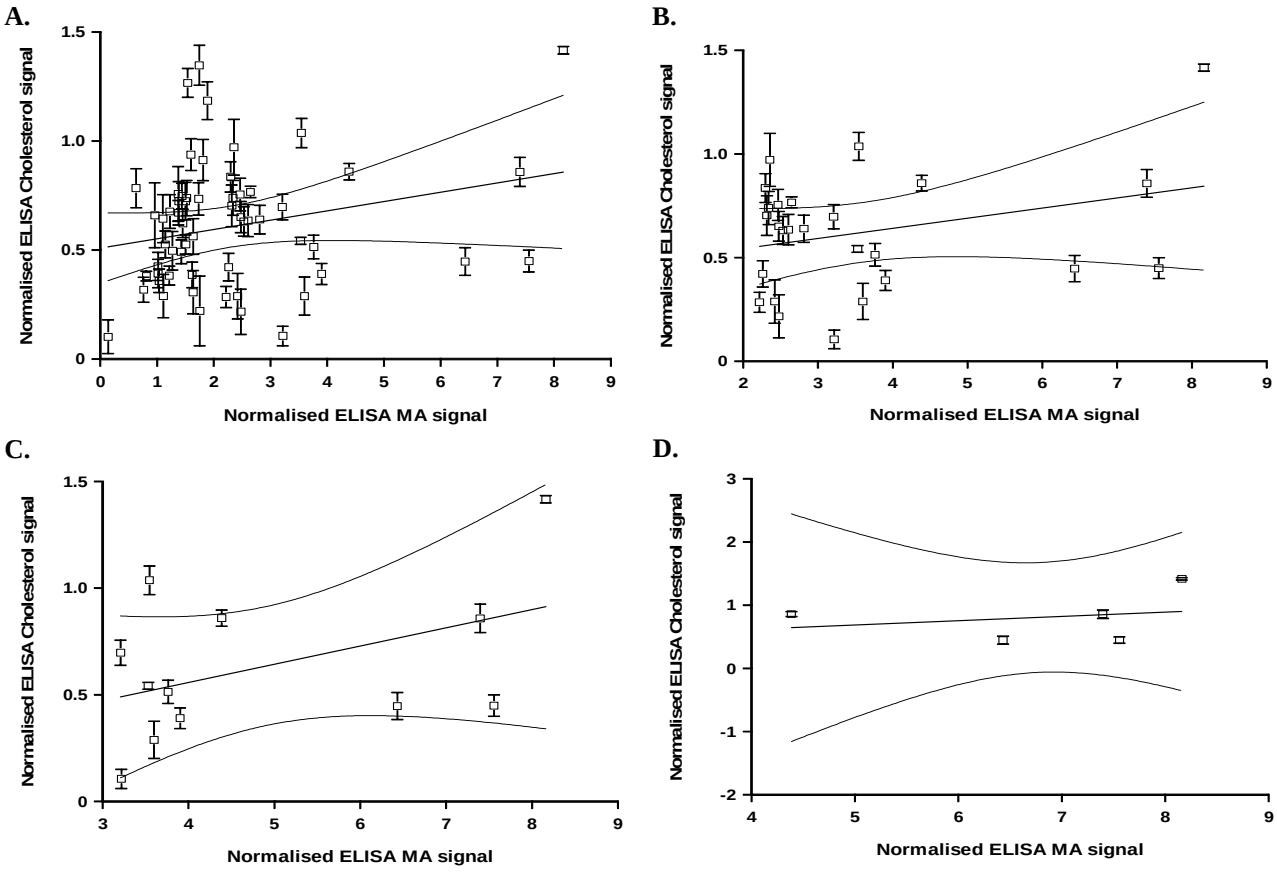


Figure 3.3: *Linear regression analysis of the ELISA results obtained against MA and cholesterol of patient sera tested on MA and cholesterol coated ELISA plates. The 95% confidence bands are indicated.*

- ***A.*** *All randomly chosen patients (HNTP = 11, HNTN = 10, HPTN = 6, HPTP = 33). Correlation coefficient r = 0.238, strength of the relationship r^2 = 0.057.*
- ***B.*** *Those patients with a normalized value to MA greater than 2 (HNTP = 3, HNTN = 3, HPTN = 4, HPTP = 16). Correlation coefficient r = 0.300, strength of the relationship r^2 = 0.090.*
- ***C.*** *Those patients with a normalized value to MA greater than 3 (HNTP = 2, HNTN = 1, HPTN = 3, HPTP = 6). Correlation coefficient r = 0.452, strength of the relationship r^2 = 0.204.*
- ***D.*** *Those patients with a normalized value to MA greater than 4 (HNTP = 0, HNTN = 0, HPTN = 2, HPTP = 3). Correlation coefficient r = 0.253, strength of the relationship r^2 = 0.064.*

On analysis of 60 patients tested (HNTP = 11, HNTN = 10, HPTN = 6, HPTP = 33) it was observed that a low correlation existed between the signals to cholesterol and MA (strength of the relationship = 0.057). As expected, an increase in correlation coefficients was observed with an increase in normalized anti-MA signal, as antigen binding of negative sera is not expected to correlate with binding to any antigen. When normalized ELISA signal ratios to

MA above 2 was used, the remaining 26 patients gave a correlation coefficient of 0.300. The remaining 12 patients that gave a normalized ELISA signal ratio of 3 or more gave a correlation coefficient of 0.452. The strongest strength of the relationship was obtained with the highest correlation coefficient at normalised anti-MA ELISA signals above 3 (Figure 3.4). At a normalized anti-MA ELISA ratio above 4, the strength of the relationship was lost (r^2=0.064), probably due to the low number of data points (n=5).

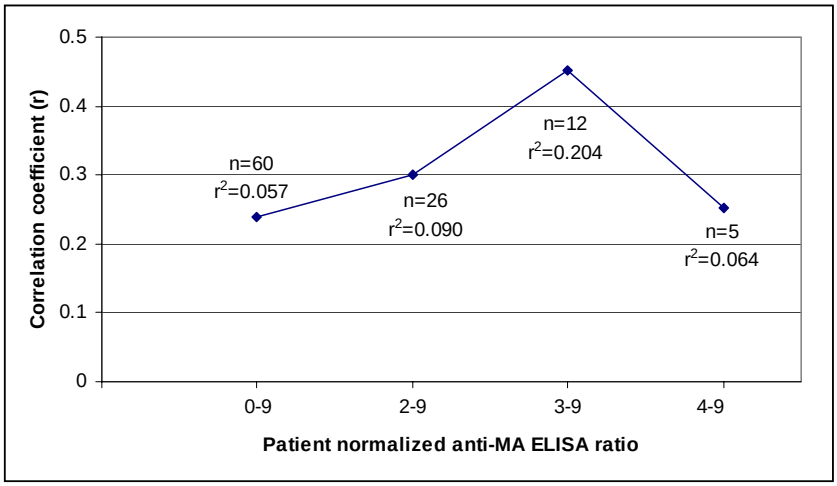


Figure 3.4: *Relating the correlation coefficients of the ELISA results obtained against MA and cholesterol of patient sera tested on MA and cholesterol coated ELISA plates to normalized signal categories.*

These same samples were then analysed in their respective groups according to TB and HIV infection status of the patients (Figures 3.5 -3.8).

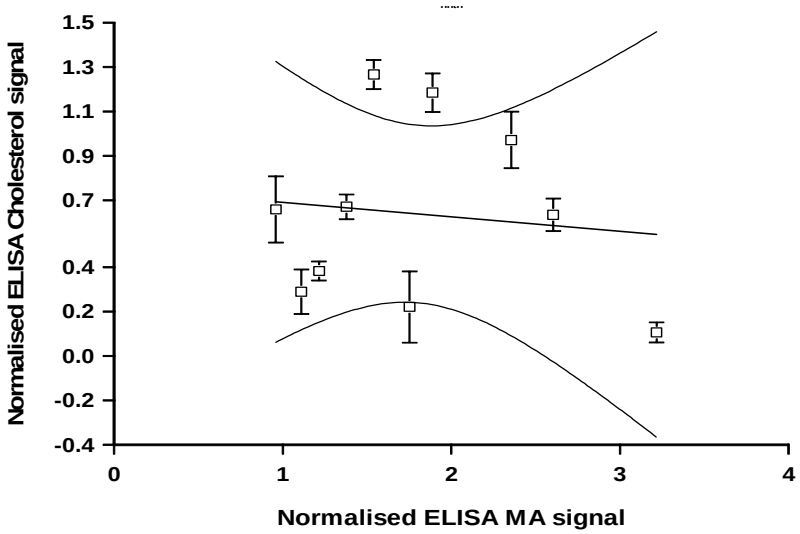


Figure 3.5: *Linear regression analysis of the ELISA results obtained against MA and cholesterol of HNTN (n=10) patient sera tested on MA and cholesterol coated ELISA plates. The 95% confidence bands are indicated. The correlation coefficient of the normalized MA and cholesterol signals was r = -0.118 with a strength of the relationship of r^2 = 0.014.*

For the HNTN group (Figure 3.5) no correlation (r = -0.118) was found between the signals to cholesterol and MA. This was expected, as the patients were not naturally exposed to MA. Positive ELISA signals detected in this group might be due to antibodies directed against cholesterol elicited by the particular diseases for which the patients in this population group were hospitalized.

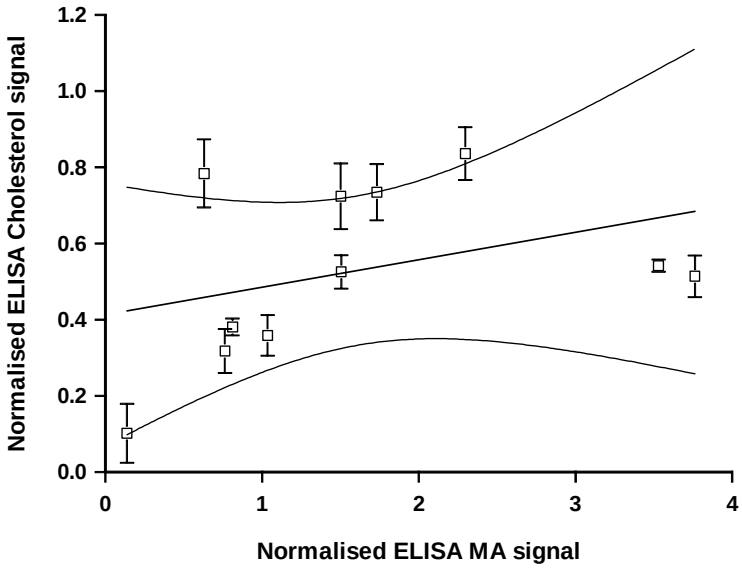


Figure 3.6: *Linear regression analysis of the ELISA results obtained against MA and cholesterol of HNTP (n=11) patient sera tested on MA and cholesterol coated ELISA plates. The 95% confidence bands are indicated. The correlation coefficient of the normalized MA and cholesterol signals was r = 0.370 with a strength of the relationship of r^2 = 0.137.*

In the HNTP patient study group (Figure 3.6) the patients are HIV negative and the immune system should be functioning effectively. A positive correlation coefficient between MA and cholesterol binding of antibodies (r = 0.370) was obtained. Although the strength of the relationship was not good (r^2=0.137) the correlation in this patient subgroup is better than that obtained in the HNTN group (Figure 3.5) as expected. A degree of cross-reactivity of patient antibodies between cholesterol and MA may therefore be observed among these data with a low level of confidence.

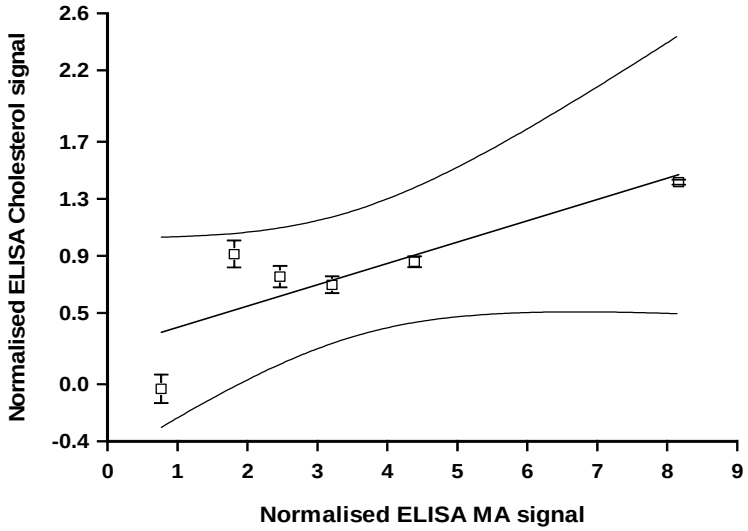


Figure 3.7: *Linear regression analysis of the ELISA results obtained against MA and cholesterol of HPTN (n=6) patient sera tested on MA and cholesterol coated ELISA plates. The 95% confidence bands are indicated. The correlation coefficient of the normalized MA and cholesterol signals was* $r = 0.815$ *with a strength of the relationship of* $r^2 = 0.663$.

Among the HPTN patients (Figure 3.7) the correlation between antibodies to cholesterol and MA is unexpectedly the highest (r = 0.815) to a reasonable degree of reliability (r^2 = 0.663). These patients have not been exposed to MA as antigen, but may have elevated anti-cholesterol antibodies that cross-react with MA. If these samples were representative of the study population and if antibodies to cholesterol cross-react with MA, then one may conclude that antibodies directed to cholesterol are elevated under HIV positive conditions. This group may also contain patients that were actually TB positive, but not identified as such with the state of the art technology.

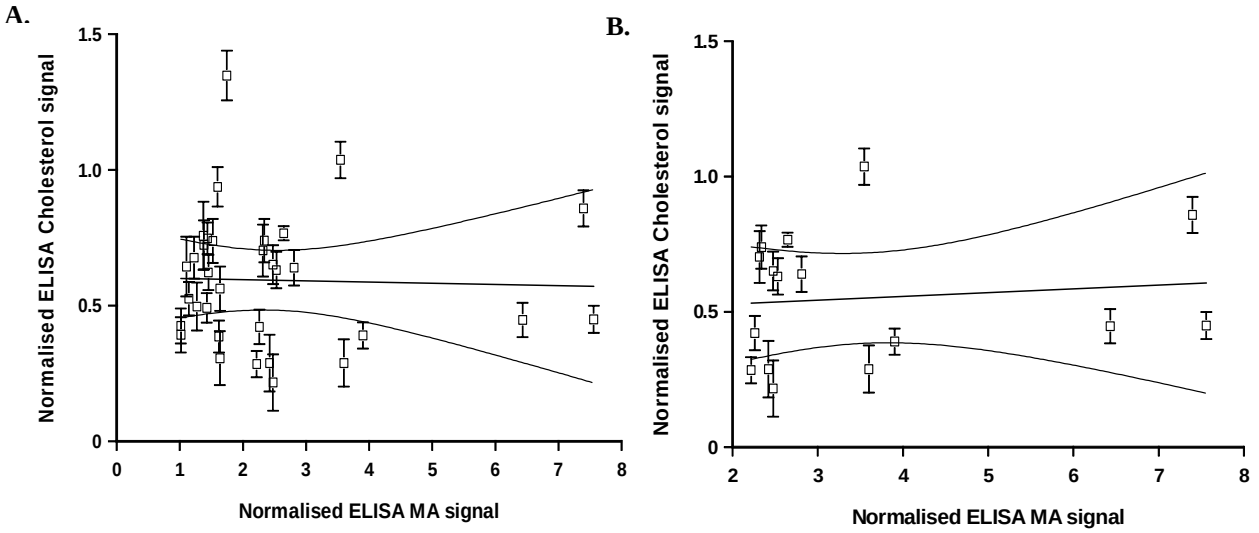


Figure 3.8: *Linear regression analysis of the ELISA results obtained against MA and cholesterol of HPTP patient sera tested on MA and cholesterol coated ELISA plates. The 95% confidence bands are indicated.*

A. *All HPTP patients (n=33). The correlation coefficient of the normalized MA and cholesterol signals was r = -0.030 with a strength of the relationship of r^2 = 0.001.*

B. *HPTP patients giving a normalized value to MA higher as 2 (n=16). The correlation coefficient of the normalized MA and cholesterol signals was r = 0.108 with a strength of the relationship of r^2 = 0.012.*

In the HPTP group (Figure 3.8a) no correlation was observed between antibody binding to MA and cholesterol (r = -0.030). These patients were expected to produce antibodies to MA due to TB infection, but the signals to MA did not correlate with those against cholesterol. An indication exists that the correlation between antibodies to MA and cholesterol will increase with an increase in MA signal in the HPTP group. When the same graph is drawn but now only considering patients with a normalized signal to MA higher than two the correlation coefficient increased to 0.108, but with poor strength of the relationship r^2 = 0.012 (Figure 3.8b).

It is clear that a definite answer to the correlation between antibody binding to MA and cholesterol is not to be found with the ELISA technique. This is evident from the large number of false positive and false negative signals that were obtained for patients in all the different groups.

3.3.3 BIOSENSOR INHIBITION EXPERIMENTS

To establish whether a possible cross-reactivity between antibodies to cholesterol and MA exists, even in the HPTP patient group, a real-time antigen-antibody binding experiment was conducted on a resonant mirror biosensor.

The biosensor cuvette was coated with liposomes containing MA in both cells. Diluted patient serum was added to allow binding to the MA antigen and to ensure the synchronized response in both cells. Patient serum was then pre-incubated in Eppendorf tubes containing MA liposomes as test sample, PC liposomes as control sample or cholesterol liposomes to investigate possible cross-reactivity.

Figure 3.9 shows a typical biosensor profile obtained for this application of the Iasys biosensor. The cuvette surface was activated with CPC (b), coated with MA (c), blocked with saponin (e), and calibrated with patient serum diluted in PBS/AE (g). The next step involved the monitoring of the amount of antibody binding after serum had been inhibited with liposomes containing MA, cholesterol or phosphatidylcholine (h). The specificity of the antibody binding to the MA antigen coated on the cuvette surface was thus established. Monitoring of dissociation of antibody binding was effected with PBS/AE washes (i). Only the association profiles of the patient serum with MA are of interest to test the hypothesis.

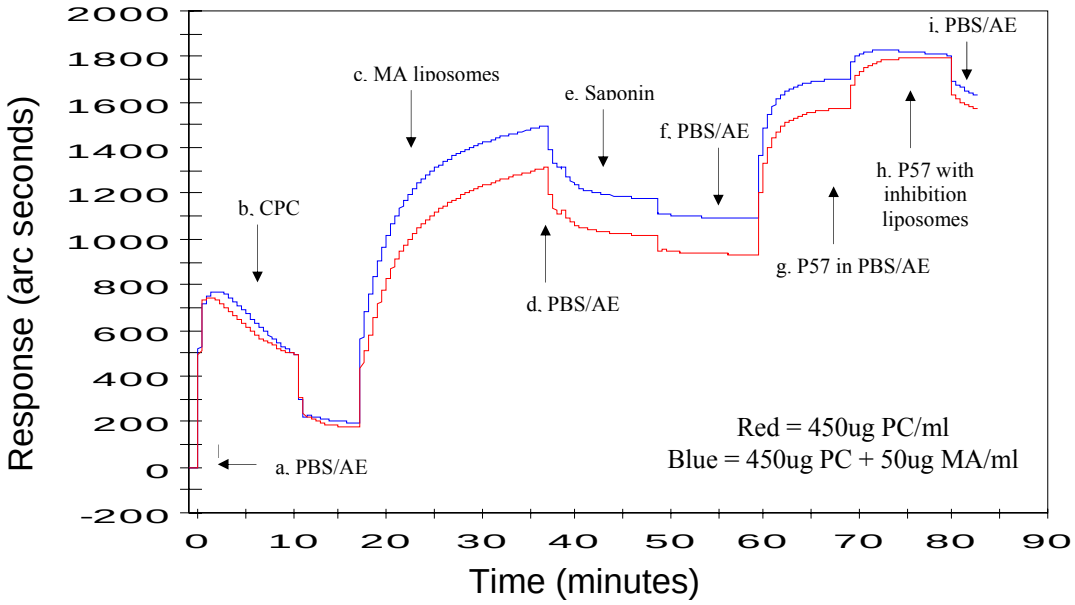


Figure 3.9: *A typical IAsys biosensor binding profile to measure the prevalence and specificity of antibody to MA in a patient serum sample.*
PBS/AE baseline (a), surface activation by CPC (b), coating with MA liposomes (c), PBS wash step (d), saponin blocking (e) and PBS wash steps (f). Diluted patient serum (P57) in PBS (g) serum (P57) response after pre-incubation in two different inhibition liposomes (h) and dissociation by PBS/AE washing steps (i).

Patient 57, a TB positive/HIV positive patient who gave a high normalized ELISA signal (7.557) to MA and an intermediate normalized signal (0.450) to cholesterol on ELISA, was used. In Figure 3.10 the association of the patient serum with the MA coated surface is shown after pre-incubation with either MA-liposomes or empty liposomes.

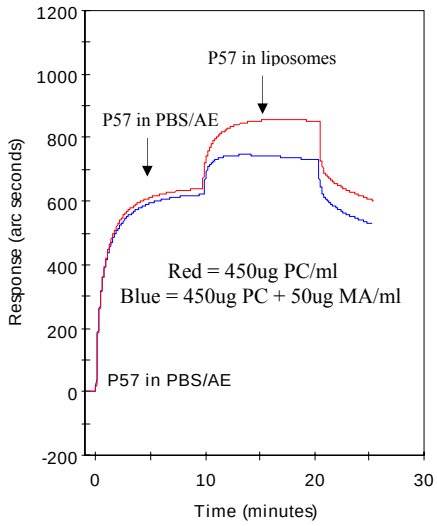


Figure 3.10: *Biosensor profiles obtained for determining the prevalence and specificity of patient serum to MA. The red line represents P57 serum preincubated in PC liposomes as control. The blue line represents P57 serum preincubated in MA liposomes.*

Pre-incubation of the patient serum with MA liposomes inhibited the binding to MA relative to pre-incubation with PC-only liposomes, thereby confirming the specificity of binding of antibodies to MA.

When this same experiment was repeated using inhibition-liposomes containing 150ug/ml cholesterol as pre-incubation ligand with the serum (Figure 3.11), the serum pre-incubated with cholesterol liposomes exhibited a better binding than that obtained with empty liposomes. In this particular experiment the binding event consisted of the accumulation of cholesterol as well as the binding of antibody remaining after inhibition of the serum with cholesterol (See discussion).

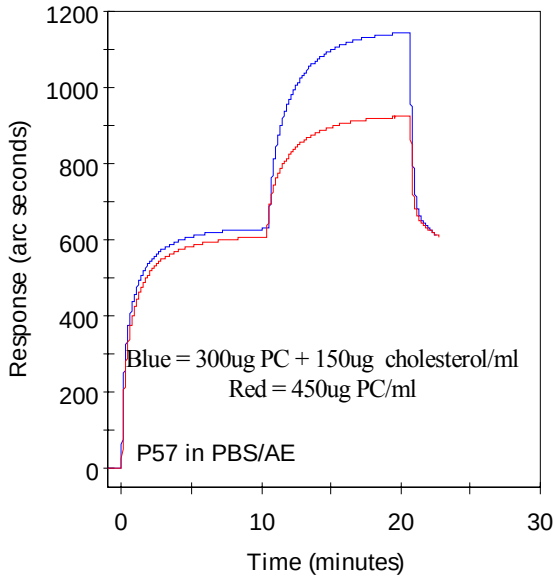


Figure 3.11: *Biosensor profiles obtained for determining the cross-reactivity between cholesterol and MA towards binding of anti-MA antibodies in patient serum. The red line represents P57 serum preincubated in PC liposomes and binding to a MA surface. The blue line represents P57 serum preincubated in cholesterol liposomes and binding to a MA surface.*

The relative influence of the cholesterol concentration in the liposomes and the inhibition of binding of antibodies to MA was then titrated. The inhibiting cholesterol containing liposomes were prepared as a concentration range to quantitate its inhibitory effect on antibody binding against MA as antigen.

The initial cholesterol concentration of 150µg/ml (Figure 3.9) was used in all experiments as a reference for comparison. Figure 3.12 shows the results obtained when the serum of patient P57 was pre-incubated with the concentration range of cholesterol as inhibitor prior to biosensor monitoring of binding to immobilized MA.

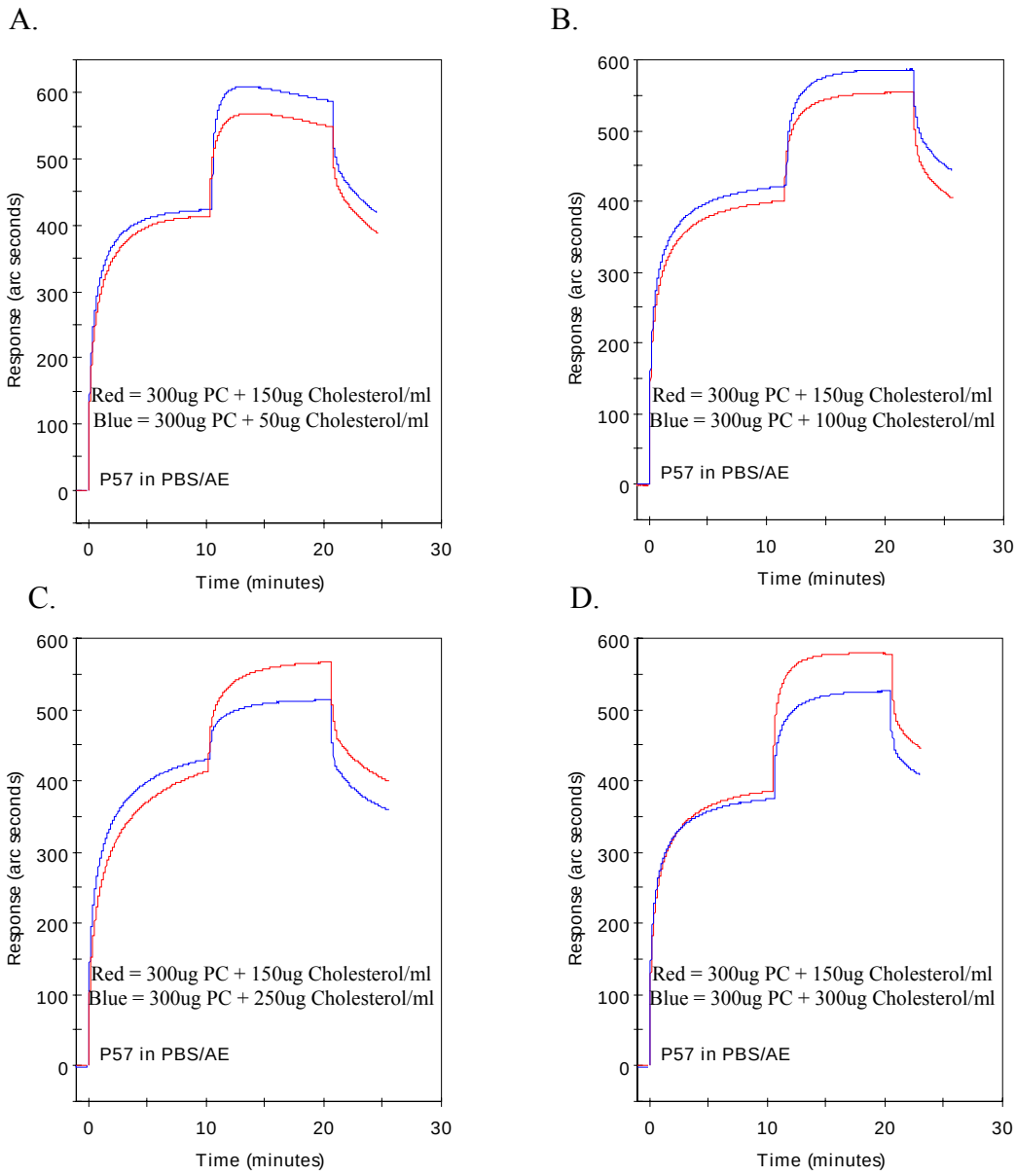


Figure 3.12 A-D: *Biosensor profiles obtained for determining the strength of interaction of antibodies in patient serum to MA after pre-incubation with liposomes of a cholesterol concentration range. In all graphs the red lines represent the sensorgrams after pre-incubating the serum with 150ug cholesterol/ml liposomes. The blue line shows the binding profile of patient serum pre-incubated with the other cholesterol concentrations used in the range, i.e. A, 50ug/ml; B, 100ug/ml; C, 250ug/ml; and D, 300ug/ml.*

From Figure 3.12 it is evident that serum pre-incubation with lower cholesterol concentrations do not show a net inhibition of the binding to MA, but that the cholesterol accumulation onto the cuvette surface is overtaken by the inhibition of antibody binding effected at higher cholesterol concentrations. These results confirm that the cholesterol concentration in the pre-incubation inhibition experiments is important. Antibodies to MA appear to be inhibitable with cholesterol only when the cholesterol concentration is sufficiently high. To confirm this, the experiment was repeated in another cuvette at the highest cholesterol concentration of inhibitory liposomes (300ug cholesterol/ml). The sensorgrams in Figure 3.13 are of patient

serum pre-incubated with PC liposomes (red line) and the high concentration cholesterol liposomes (blue line).

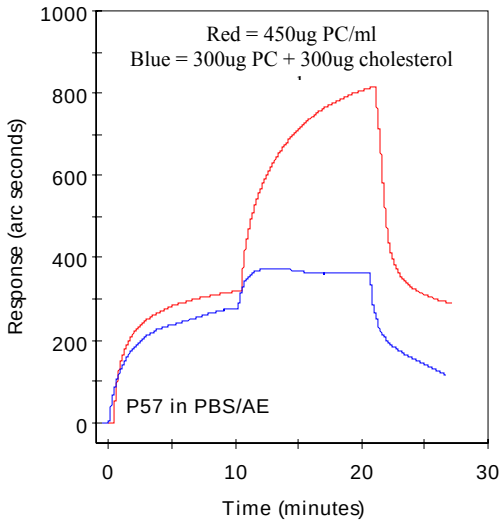


Figure 3.13: *Biosensor profiles to demonstrate the antigenic cross-reactivity of antibodies in patient serum to MA and cholesterol. The red line represents binding of antibodies in P57 serum preincubated with PC liposomes and the blue cell contains P57 serum preincubated with 300ug Cholesterol/ml liposomes.*

Here the results of Figure 3.12 were confirmed, in that the higher cholesterol concentration during pre-incubation effected a significant decrease in the binding curve. These data suggest that the antibodies recognising MA are inhibited by high cholesterol concentrations, in support of the hypothesis that cross-reactivity exist for antibody binding to MA and cholesterol, with MA being the stronger antigen.

3.4 DISCUSSION

In an attempt to prove with ELISA, that there is a cross-reactivity of binding of anti-MA antibodies in TB patients between MA and cholesterol, the specificity of IgG for binding MA was first confirmed by capturing IgG by their Fc portions on Seph-A, the degree of binding to 5-BMF labeled MA was measured by fluorimetry. The results were compared with those obtained by ELISA for several patient and control sera and suggested that the antibodies recognise MA specifically. The ELISA results are therefore not generally due to a non-specific hydrophobic adsorption of lipid-antigen complexes to immobilized antigen.

ELISA was subsequently done to compare antibody binding signals to MA and cholesterol as coating antigens. Some correlation was found in the ELISA signals to MA and cholesterol in the total study group of patient and control sera. The correlation was lost where antibodies bound with a lower affinity (low ELISA signal) to MA, probably due to the washing procedures of ELISA that removes bound antibody of low affinity.

Positive correlation coefficients were obtained for the HNTP and HPTN patient groups, but not the HNTN group. The HPTP patient group showed a weakly positive correlation. The positive correlation between anti-MA and anti-cholesterol antibodies in HPTN patients was particularly interesting. This patient group does not have active tuberculosis disease and are therefore not exposed to MA like the HNTP and HPTP patient groups. Horváth *et al.* (2001a) reported that ACHA levels were significantly higher in HIV positive individuals than in HIV seronegative controls. The higher levels of ACHA in HIV positive individuals could contribute to the decrease of cholesterol levels (hypocholesterolaemia) observed in HIV patients normally associated with altered lipid metabolism (Lucas *et al*., 1994; Horváth *et al.,* 2001a). The increased levels of ACHA in HIV positive individuals was explained as follows.

- Serum concentrations of several natural antibodies increase with HIV infection due to polyclonal B cell activation associated with high serum immunoglobulin concentrations (Horváth *et al*., 2001a).
- Antibodies to cholesterol are produced in the HIV infected patient upon exposure to certain micro-organisms, due to the release of phospholipids and cholesterol from cells or cellular debris. The antigenicity of the cholesterol is thus increased due to the higher propensity of the HIV infected patient towards infectious diseases (Alving & Wassef, 1999, quoted in Horváth *et al*., 2001a).

- An increased immunogenicity of cholesterol because of its abundance in the envelope of the HIV virion and its presentation to the immune system. HIV antigen was able to inhibit the ELISA signal of IgG to cholesterol (Horváth *et al*., 2001a).

The higher levels of ACHA in HPTN patient sera appears to be responsible for the anti-MA ELISA signals obtained, supporting the notion of anti-cholesterol antibodies that cross-react with MA.

No anti-MA antibodies were expected in the HNTN patient group as these patients were not infected with m*ycobacteria*. Although low levels of ACHA were predicted in all humans (Horváth and Bíró, 2003), cross-reactivity with MA at such low ACHA concentrations may not be detectable. This could indicate that antibody production to cholesterol need a stimulus to achieve concentrations that can be detected in an anti-MA ELISA.

Sera from the HNTP patient group showed a significant degree of cross-reaction between cholesterol and MA. The antibodies present here are directed to MA, as the patients were TB infected and these same antibodies appeared to cross-react with cholesterol. As HNTP patients were exposed to MA, it might be possible that the mycobacteria acted as stimulus to assist the affinity maturation of ACHA into antibodies able to detect MA as well. This antibody might show a stronger affinity towards MA than to cholesterol, developed through the process of affinity maturation. Alternatively, two populations of antibodies could be present in TB patient sera of which one binds MA and the other cholesterol. This becomes an unlikely scenario in the face of the results obtained in the HPTN population. In this population, anti-cholesterol antibodies are elicited that cross-react with MA, whereas in the HNTP population anti-MA antibodies are elicited that cross-react with cholesterol. One can therefore conclude that ELISA analysis of HNTP and HPTN sera gave reasonable indication that anti-MA antibodies cross-react with cholesterol.

From the above, one would have expected that HPTP sera analysed by ELISA to provide even stronger data in support of the cross-reactive binding specificity of serum antibodies to MA and cholesterol. This was found, however, not to be the case. Only a weak positive correlation was found between anti-cholesterol and anti-MA ELISA data in this population. HPTP patients are usually very sick with various kinds of infections. In a study by Harries *et al*. (1998) Malawian, HIV positive TB positive patients were treated with chemotherapy

consisting of streptomycin, rifampicin, isoniazid and pyrazinamide. One third of the HIV positive patients and one half of HIV positive smear negative patients died during treatment. One half of these deaths occurred in the first month of treatment. A huge amount of antibody-antigen complexes may build up in the blood that effectively neutralizes anti-lipid antibodies with lipid antigens, hindering their detection with ELISA. The fact that one out of sixteen sera was found with a notably high ELISA signal to cholesterol, but not to MA, as well as two that gave high signals to MA, but not to cholesterol argued against the hypothesis of cross-reactive antibodies to cholesterol and MA. It is, of course, possible that under fatally sick conditions, the control over the maturation of the antibody response becomes so disturbed that extreme affinities of binding arise to auto-antigens, such that the cross-reactivity becomes hard to detect (Lake *et al*., 1994; Nardi and Karpatkin, 2000).

As the reliability of the ELISA test to detect anti-cholesterol and anti-MA activity is not ideal (Schleicher *et al*., 1999) one HPTP serum was analysed on a resonant mirror biosensor to get a more accurate and quantifiable measure of the degree of cross-reactivity of binding of antibodies to MA and cholesterol. It was found that both MA liposomes and cholesterol liposomes preincubated with patient serum could inhibit the signal to MA, but the two sets of results were not directly comparable. Pre-incubation of serum with cholesterol liposomes caused both an inhibition of antibody binding to MA and an opposite effect due to accumulation of cholesterol onto the MA coated solid phase. A relatively low concentration of cholesterol (150ug/ml) preincubated with patient serum caused an increase in the binding profile relative to serum preincubated with empty liposomes. Previous work done in our laboratories by D.G.R. Siko and S.T. Thanyani indicated that cholesterol is accumulated into the MA coat of the biosensor cuvette (Siko, 2002). MA, by its apparent molecular mimicry of cholesterol, was shown to attract cholesterol, explaining the increase in mass accumulation.

Dijkstra *et al*. (1996) and Agirre *et al.* (2000) stated that the concentration of cholesterol in lipoproteins is important for antibody recognition of cholesterol. The cholesterol threshold differed with the PL composition in vesicles used. In 2000, Agirre *et al.* reported that the molar cholesterol ratio in small unilamellar vesicles containing dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylglycerol (DMPG) had to be over 50% for ACHA (IgM) to induce aggregation of the vesicles. The work done on the biosensor could not, unfortunately, quantitatively determine the cholesterol concentration at which the signal to MA can be inhibited. Egg phosphatidylcholine was used of which the exact

composition and therefore molar mass was uncertain, but qualitatively cholesterol was shown to indeed influence the signal obtained to MA in a concentration dependent manner. At the highest amount of cholesterol used in this study the signal to MA as surface antigen was inhibited. This concentration dependent effect may be explained by the existence of a minimum cholesterol concentration in membranes or liposomes above which antibodies are able to interact with cholesterol (Dijkstra *et al*., 1996; Agirre *et al*., 2000).

Taken together, the evidence presented in this chapter generally supports the hypothesis that antibodies recognising either cholesterol or MA, cross-react with the other antigen, although the complexity of measuring this requires care in the interpretation of the data. The accumulation of cholesterol from free liposomes in the MA coat on the biosensor is in itself circumstantial evidence of a molecular attraction between hydrophobic components that may suggest a degree of mimicry between cholesterol and MA (Siko, 2002). It nevertheless complicated the quantitative interpretation of the biosensor results of cholesterol-inhibited HPTP patient serum on MA coated cuvettes.

If cross-reactivity of antibody binding to cholesterol and MA is assumed based on the evidence above, then the small number of false positive ELISA results that was obtained (Chapter 2) can now conveniently be explained by the prevalence of antibodies directed to cholesterol in non-TB patients. False negative ELISA results may also be explained: antibodies present in sera may be difficult to detect in ELISA under certain circumstances. The antibodies removed from circulation as CICs in patients with advanced tuberculosis disease would be those of higher affinity, as the lower affinity antibodies might not be as readily associated with cholesterol or MA for clearance by scavenger macrophages. These lower affinity antibodies appear to be washed away during the ELISA washing between steps. These basic concepts have already been successfully applied in the design of a biosensor based TB serodiagnostic assay (Thanyani, 2003).

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

TB remains a global health threat. A fast, reliable diagnostic test for TB is essential to reduce the threat of TB. Early diagnosis of TB will lead to early treatment of infected individuals decreasing the disease infection rate. In countries with a low tuberculosis incidence, false positive reactions are observed, mainly due to environmental mycobacteria. In countries with a high tuberculosis prevalence, false positive test reactions result mainly from exposure to mycobacterial antigens (Arias-Bouda *et al*. 2003). TB diagnosis today involves acid fast bacilli microscopy with less than 50% sensitivity. The golden standard for TB diagnosis involves the growth of sputum cultures which is much more sensitive, but can take up to 6 weeks to produce test results. A fast, effective and affordable diagnostic method is needed of adequate sensitivity and specificity. TB serodiagnosis has the potential to fit these requirements allowing the further benefit of easy sample collection.

Eradication of the TB epidemic involves addressing TB and HIV co-infection as well as multidrug resistant TB (Raviglione, 2003). Not only does the dangerous liaison between HIV and *M. tb* lead to more deaths, but TB diagnosis is also severely complicated. HIV targets $CD4^+$ T cells and impairs the host's immune defense, allowing TB infection to progress into active tuberculosis (Kaufmann & Schaible, 2003). The production of antibodies to protein antigens is impaired concomitantly with a loss in $CD4^+$ T cells. Several serodiagnosis antigens including proteins, polysaccharides, peptidoglycans, glycolipids and phospholipids (Chan *et al*., 2000) indicate promising results for serodiagnosis. Most of these tests lost sensitivity and specificity when applied in HIV burdened populations such as in sub-Saharan Africa (Grange, 1984). For example Lawn et al. (1997) reported that the sensitivity of the Mycodot assay, a serodiagnostic assay based on the LAM antigen was 56% in HIV negative patients and only 25% in HIV positive patients. This trial was performed in Ghana, with a high HIV-seroprevalence in TB positive patients. TB serodiagnosis thus remains problematic, especially in HIV burdened populations.

In 1999 it was suggested for the first time that mycobacterial cell-wall lipids could be used to solve the problem in tuberculosis serodiagnosis in HIV burdened populations. This is because some mycobacterial lipid antigens are presented to human T cells (mainly double negative T cells) through CD1 molecules, a lipid antigen presenting molecule. These antigens were predicted to be detectable by antibodies in HIV co-infected patients, as $CD4^+$ T cells were not exclusively required for antibody formation (Verschoor & Onyebujoh, 1999).

Several glycolipid antigens were used in serodiagnosis for tuberculosis. Kawamura *et al*. (1997) investigated the use of an anti-TBGL antigen consisting of TDM and a specific glycolipid fraction. Harrington III *et al*. (2000) used a lipid antigen cocktail containing TDA, DAT, SL-1 and PIM showing that the disruption of immune complexes after precipitation increased the test sensitivity. Maekura *et al*. (2001) used a tuberculous glycolipid antigen (TBGL) consisting of hydrophilic glycolipids and TDM in an enzyme immunoassay. The sensitivities determined were 89.6% and 73% for smear and culture positive pulmonary tuberculosis patients and smear and culture negative pulmonary tuberculosis patients respectively. The controls used were representative of a population with a low incidence of tuberculosis disease (Maekura *et al*., 2001). Cardona *et al*. (2002) used a tuberculosis aerosol infection model in mice relating to the route of tuberculosis infection in humans. They showed that antibodies against *M. tb* cell wall glycolipids (DAT and SL) were produced in higher concentrations than antibodies directed to several protein antigens.

In this study anti-MA antibodies were investigated as a possible surrogate marker for tuberculosis infection. MA is the most abundant lipid in the mycobacterial cell wall. MA are presented to T cells through human CD1 b molecules. This could allow the production of anti-MA antibodies in HIV positive individuals in contrast to anti-peptide antibodies which are present at decreased levels because of the destruction of $CD4^+$ T cells. MHC class I and II molecules show extensive polymorphism that allows the presentation of a wide range of protein antigens to $CD4^+$ T cells, but all individuals of a species do not present the same antigen with equal efficiency (Calabi & Milstein, 2000). By circumventing the use of MHC molecules, MA might be an immunodominant antigen detected by all individuals of a species by its presentation on the CD1 molecule that shows a very limited polymorphism. Antigens presented by these molecules are expected to be less diverse than protein antigens and therefore more ubiquitously active in TB patients (Calabi & Milstein, 2000).

IgG antibodies to MA in mice were not detected (Goodrum, 1998), but IgG antibodies to MA in humans were detected in a study by Pretorius (1999). To avoid false positive results due to tuberculosis vaccination, a short term immune memory is required for the analyte antibodies in serodiagnosis. Maekura *et al*. (2001) showed a decrease in antibody titres to TBGL after 6 months of anti-tuberculosis treatment. A short term immune memory to MA might exist, which will further the use of MA in a serodiagnostic test to detect the prevalence of anti-MA antibodies in TB patients. This system might then allow the diagnosis of tuberculosis disease even in HIV seropositive individuals.

Results obtained by Pan *et al*. (1999) using TDM, a MA acid containing glycolipid, showed promising diagnostic potential. The current study, however, is the first to investigate the diagnostic potential of anti-MA antibodies in a population with a high prevalence of tuberculosis infection including HIV-seropositive and HIV-seronegative patients. The TB negative patient sera used as control samples were obtained from hospitalized non-TB patients to resemble field conditions more closely and were well matched with TB positive patient sera for age, gender and race (Schleicher *et al*., 1999). Although significant differences between TB negative and TB positive patients were obtained in the sera and IC precipitates, a high degree of overlap between the two populations disqualified the test as a viable alternative to current TB diagnostics.

A significant contribution is made in the current study by showing that the IgG response to mycobacterial MA remains conserved in HIV sero-positive individuals. This finding contradicts Simmoney *et al*. (1997), where lower IgG antibody responses to glycolipid antigens were obtained in HIV co-infected patients. The current study suggests that antibody production to MA is independent of CD4 cell counts (Schleicher *et al*., 1999).

The possibility of TB diagnosis in HIV seropositive patients warranted the further investigation into the anti-MA antibody test. False negative test results may be explained by the relative affinities detectable of anti-MA antibodies in the presence of a high antigenic load in circulation. Higher affinity antibodies might be in association with circulating immune complexes and thus unable to associate with the immobilized MA as coating antigen. Precipitation of these immune complexes followed by acid dissociation increased the diagnostic potential of the ELISA method used. False positive test results could be due to the detection of non specific antibodies by means of the hydrophobic interaction of the MA

containing immune complexes with the hydrophobic MA coat on the ELISA plate surface. An experiment was designed to avoid the use of a hydrophobic plate surface. Fluorescently labeled MA was captured on antibodies captured by their Fc-domains onto Prot-A sepharose and measured by release of fluorescence at low pH. The correlation obtained in this study with the ELISA results indicated that the antibody binding in ELISA was not due to non-specific hydrophobic surface association. The ELISA method however could still not detect low affinity antibodies to MA. These antibodies could be washed off during standard ELISA procedures and could explain the false negative test results obtained.

Another source of false positive test results could stem from a possible autoimmune reaction. Sieling *et al.* (2000b) proposed that nonpeptide self-antigens are presented to DN T cells. Double negative T cells were implicated in autoimmune diseases (Niehues *et al.*, 1999). Although the production of antibodies to self-lipid antigens seem unlikely, several such auto-antigens have been identified. $CD1c^+$ B cells have evolved to recognize lipid antigens through surface Ig and present them to DN T cells to recruit help for IgG production. Mouse $CD1^+$ B cells produce IgM antibodies spontaneously and are deficient in IgG production unless they receive T cell help. $CD1^+$ B cells might thus be prone to anti-self responses if inappropriate T cell help is provided. $CD1^+$ B cells are located in the marginal zones of lymphoid tissues, where B cells are more responsive to nonpeptide antigens. DN T cells from systemic lupus erythematosus patients produced IL-4, which induced DN Th cell mediated isotype switching from IgM to IgG in lupus patients (Sieling *et al*., 2000b).

In a study by Petrovas *et al*. (1999) a possible cross-reactivity of antibody binding was indicated between cardiolipin and phosphatidyl serine. The negative charges on these phospholipids were suggested as epitopes, as the antibody might be charge dependent. These antibodies had a low binding avidity.

Verschoor and Siko found that anti-MA antibodies were autoreactive to cholesterol. Biosensor studies showed that MA attracted cholesterol, implicating a possible structural resemblance between MA and cholesterol (Siko, 2002). It was deemed necessary to investigate a possible cross-reaction of anti-MA antibodies to cholesterol in an attempt to explain the false positive tests results observed in the ELISA. An ELISA was done to compare antibody binding to MA and cholesterol. A degree of correlation was obtained for the total study population, indicating that a possible cross-reaction of anti-MA antibodies to

cholesterol might be present. This correlation was found in the HNTP and, surprisingly, also in the HPTN patient groups. Horváth *et al.* (2001a) showed that anti-cholesterol antibodies (ACHA) were present at significantly higher levels in HIV sero-positive individuals than in HIV sero-negative individuals. The antibody production to cholesterol was reported to be independent of CD4 counts. This could indicate that the anti-MA antibodies in the HNTP patient group cross-reacted with cholesterol, whilst ACHA in the HPTN patient group cross-reacted with MA. This suggested that the same antibody might be binding to both MA and cholesterol and supports the hypothesis that anti-MA antibodies cross-react with cholesterol.

The positive correlation expected between MA and cholesterol ELISA signals in the HPTP group was not observed. The biosensor method was then applied to one HPTP patient serum. The specificity of binding to the MA antigen was confirmed by pre-incubating the patient serum with MA containing liposomes. A decrease in the binding profile was observed, indicating inhibition of the binding of antibodies to MA. Cholesterol was then used in the biosensor inhibition experiments with MA as coated antigen. It was shown that the anti-MA antibody could be inhibited with increasing cholesterol concentrations. The observation that cholesterol only inhibited antibody binding at high concentrations might be ascribed to the liposome carriers used. As the cholesterol concentration increases in membranes or lipoproteins it becomes more exposed on the surface and ACHA gain easier access to the 3β-hydroxyl group of cholesterol (Dijkstra *et al*., 1996; Agirre *et al*., 2000). A possible cross-reaction between anti-MA antibodies to cholesterol were thus confirmed with the biosensor technique.

It was expected that antibodies to cholesterol are increased in HIV seropositive patients. Alving and Wassef proposed in 1999 that cholesterol antibodies might contribute to a reduction in cholesterol concentration in the human body. Hypocholesterolaemia was found in HIV seropositive patients (Grunfeld *et al*., 1989; Crook and Mir, 1999). In the HPTP group, antibodies associated with cholesterol or MA could thus be removed from the circulation by opsonization. It is reasonable to suggest that the higher affinity antibodies will be removed from circulation first. This could explain why the cross-reactivity between MA and cholesterol could better be confirmed with the biosensor application that detects lower affinity antibodies, than the ELISA, that only reports high affinity antibodies that can resist extensive washing of the plate.

Two different antibody populations might recognize MA and cholesterol. These antibodies are likely to cross-react with the other antigen albeit at a lower affinity. HIV infection also leads to the abnormal functioning of B cells where elevated numbers of cells spontaneously secrete antibodies and lapse to T cell independent B cell activation (Petrovas *et al*., 1999). In the very sick HIV and TB co-infected patients, more of these non-specific antibodies could then be prevalent and may include low affinity anti-cholesterol and/or anti-MA antibodies.

The cross-reactivity between MA and cholesterol can be explained by a possible functional similarity between MA and oxLDL. Both of these entities are taken up by macrophages to produce cholesterol rich foam cells (Stoltz, 2002). Mycobacteria bind to macrophages in cholesterol-rich domains of the host cell plasma membranes through receptors including MR, CR and scavenger receptors (SR) (Tailleux *et al*., 2003). OxLDL also gains entry into macrophages through SR (Yamaguchi *et al*., 2002). Antibodies to cholesterol are directed to the 3β-hydroxyl group (Dijkstra *et al*., 1996; Agirre *et al*., 2000). Fujiwara *et al*. (1999) showed that anti-cord factor antibodies recognized the methoxy MA subclass in TDM, whilst Pan *et al*. (1999) were the first to publish an IgG humoral immune response to this MA subclass. A structural similarity between cholesterol and methoxy MA can thus be suspected. Although ketomycolates are the most abundant oxy-mycolic acids, the methoxymycolates are found mostly in slow growing mycobacteria species (Yuan *et al*., 1998).

In 2002, Grant *et al*. proposed a hypothetical model to explain the recognition of a combinatorial epitope of MA by the TCR of DN T cells. They determined that the oxygen groups (keto or methoxy) of the MA main alkyl chain and a free carboxylic acid was necessary for the CD1b-MA complex to be recognized by DN1 TCR. α-MA were not recognized by DN T cells whilst keto and methoxy MA were. Methylation of the carboxylic acid abolished this TCR recognition. Natural structural modifications of this carboxylic acid such as in TDM and glucose monomycolate were also not recognized by DN1 T cells in contrast to the free MA which were recognized. A hypothetical model (Appendix 2) was suggested in which MA would fold up in order to present an epitope recognized by the TCR. CD1b binds the hydrophobic alkyl chains of MA allowing the polar functions of MA to group together. For keto MA, a combinatorial epitope for TCR recognition appears to be formed consisting of a keto function, a hydroxy group and a free carboxylic acid. In keto methylester-MA the carboxylic acid group is modified and TCR recognition is inhibited. In the α-MA the

keto function is absent and TCR recognition is inhibited (Grant *et al*., 2002). The methoxy MA folded on this theoretical model, might mimic cholesterol and explain the cross-reactivity of anti-MA antibodies to cholesterol.

The biosensor technique measures binding association in real time and does not require extensive washing between steps. The detection of low affinity antibodies are thus possible. Thanyani (2003) demonstrated that the number of false negative and false positive scores was reduced to insignificance when an IAsys biosensor was applied to determine anti-MA antibodies as surrogate marker for TB infection in 17 sera. Future investigations may include the measurement of the duration of the antibody response to MA by the biosensor application. A short term immune memory to MA would be ideal for the monitoring of compliance to anti-tuberculosis therapy with the biosensor, but destroy the vision that MA may be used in a vaccine against tuberculosis. A hypothesis by Beckman *et al*. (1994) imply the possibility that MA may be used as a vaccine subject, but has hitherto not been proven convincingly, despite the claims by Dascher *et al*. (2003). These authors reported a degree of short term protection against TB that was obtained by MA administration, but failed to show long term immunity.

4.2 CONCLUSION

This study investigated the use of anti-MA antibodies as a surrogate marker for tuberculosis disease. The standard ELISA assay allowed the detection of anti-MA antibodies in tuberculosis patients, but at low sensitivity and specificity. A significant finding was the prevalence of these anti-MA antibodies in TB and HIV co-infected patients. It was indicated that these anti-MA antibodies cross-reacted with cholesterol. The anti-MA antibodies showed a wide range of affinities to MA among various patients, with a bias towards lower affinity antibodies. The use of anti-MA antibodies as surrogate markers of TB infection would show potential if a technique could be employed that was suitable to detect low affinity antibodies. The IAsys biosensor proved suitable for this purpose. Another significant contribution of the biosensor application was the observation that, although anti-MA antibodies cross-react with cholesterol, the affinity for cholesterol was too low to affect the diagnostic potential of the test. This prepares the way to a brand new approach in TB serodiagnosis that may well uproot many of the current imperfect applications for TB serodiagnosis.

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APPENDIXES

APPENDIX 1: STRUCTURES OF CD1 RESTRICTED ANTIGENS

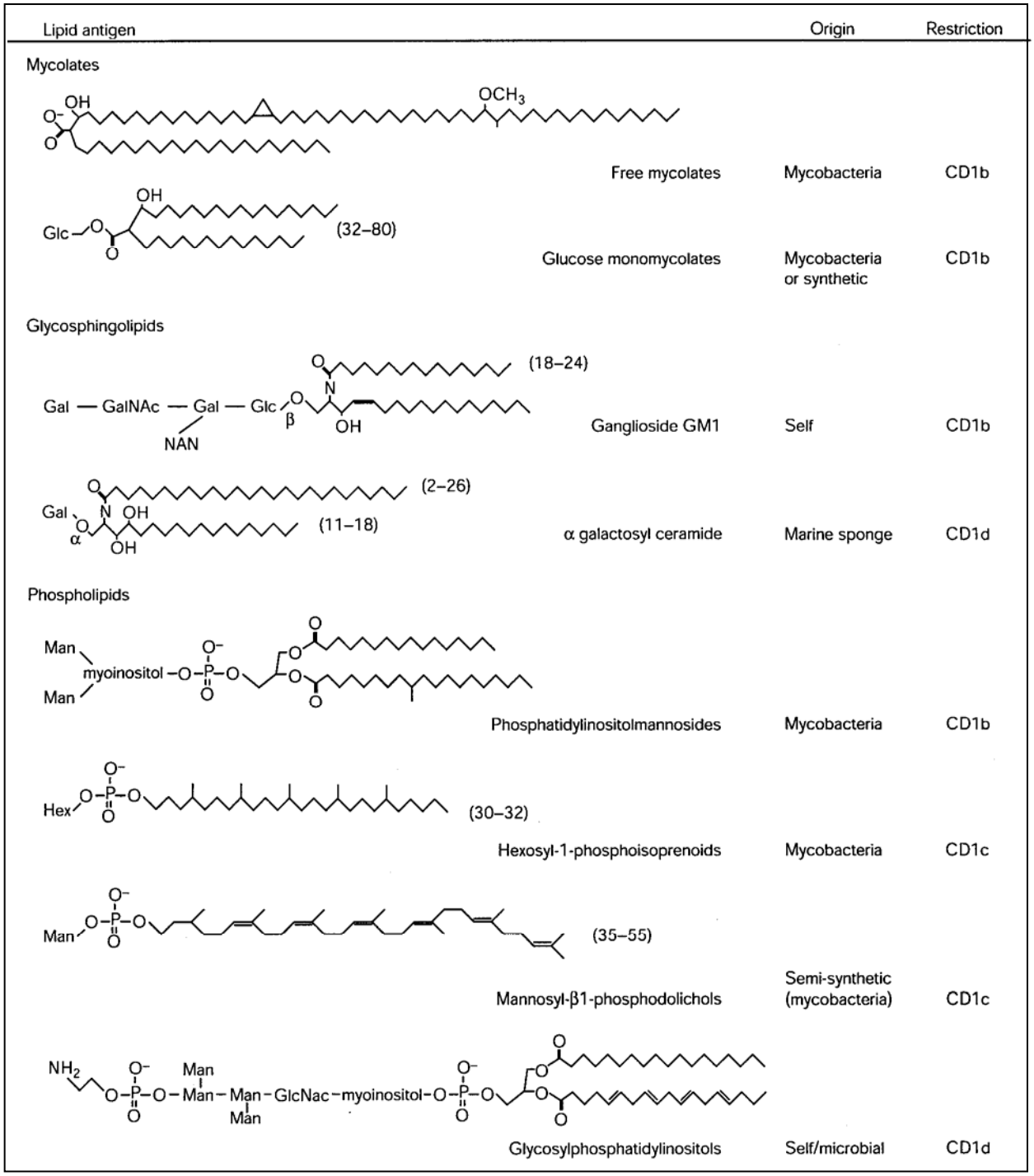

Figure A: *The structures of lipid containing antigens with their origin and CD1 epitopes. Figure from Matsuda & Kronenberg., 2001.*

APPENDIX 2: A HYPOTHETICAL MODEL FOR THE FOLDING OF MA

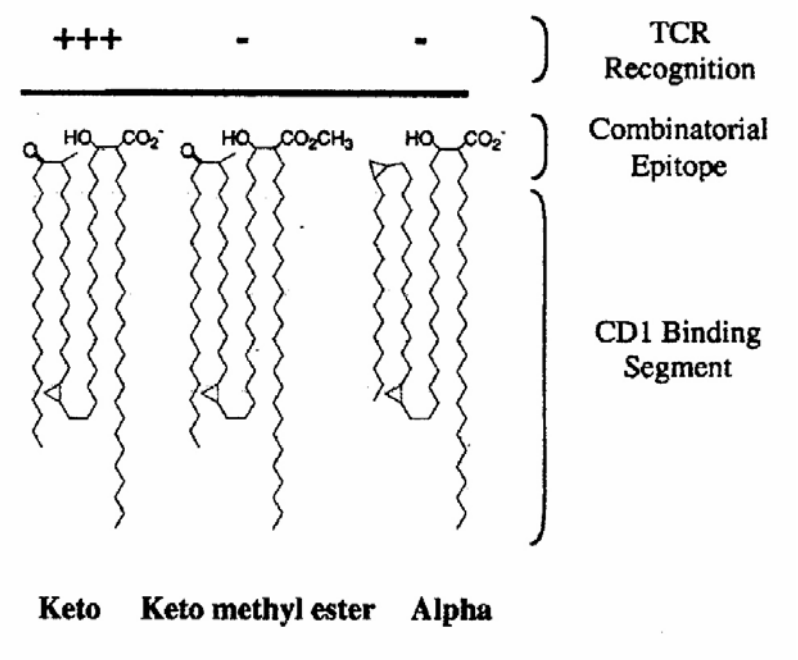

Figure B: *A hypothetical model in which the polar functions of MA are brought in proximity. A combinatorial epitope is produced in keto MA that is comprised of a keto function, a hydroxy group and a free carboxylic acid. If this carboxylic acid is covalently modified (keto methyl ester) TCR recognition is inhibited. Similarly if the keto function is absent like in α-MA TCR recognition of the antigen is also inhibited. (Figure from Grant et al., 2002).*