CHAPTER 4

Combination of Chemotherapy with Mycolic Acids During Treatment of *M. tuberculosis* Infected Balb/c Mice

4.1 Introduction

Tuberculosis therapy has in the past three decades improved due to a better understanding of the mechanism of interaction between the host, *Mycobacterium tuberculosis* and the drugs (Stead and Dutt, 1988; Grosset, 1995). The bacterial load in the lung cavities is high because *M. tuberculosis* is an obligate aerobe growing in proportion to the oxygen concentration of the environment. For most drugs to be effective, the bacteria they are targeting must be actively growing. Because *M. tuberculosis* grows slowly and exhibits a remarkably high mutation rate, the duration of daily chemotherapy usually needs to exceed six months (Mitchison, 1969; Chopra and Brennan, 1997; Mitchison, 1998; Cynamon *et al.*, 1999).

4.1.1 Anti-tuberculosis drugs

The first antibiotic that proved to be effective towards *M. tuberculosis* was streptomycin (SM), which became available 61 years after the discovery of *M. tuberculosis*. More drugs have since been discovered, some of which are more effective than streptomycin. The standard therapy for tuberculosis was defined a number of years ago, and includes the usage of isoniazid (INH) and ethambutol (EMB), with or without SM. These drugs target different populations of growing *M. tuberculosis* (Fig 4.1).

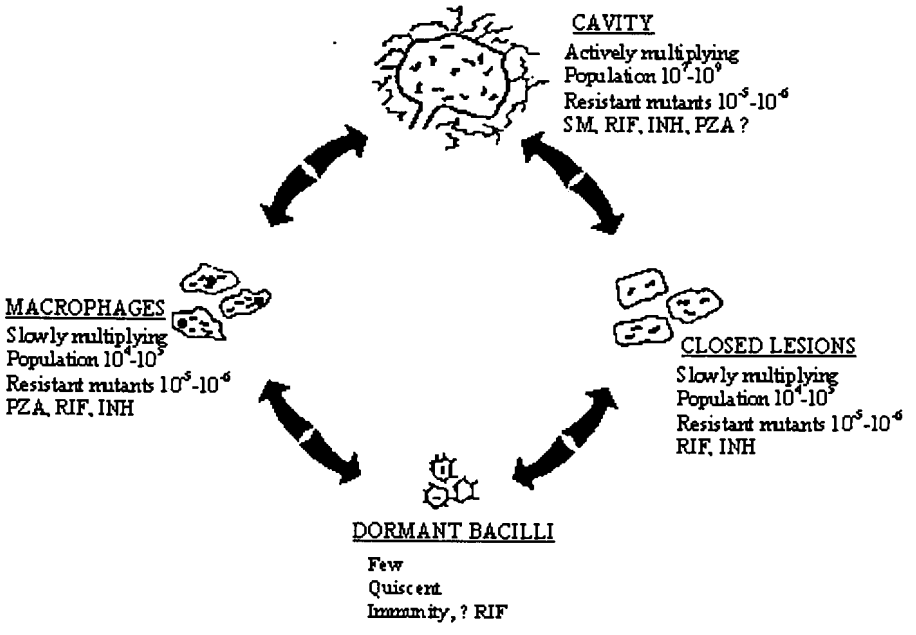
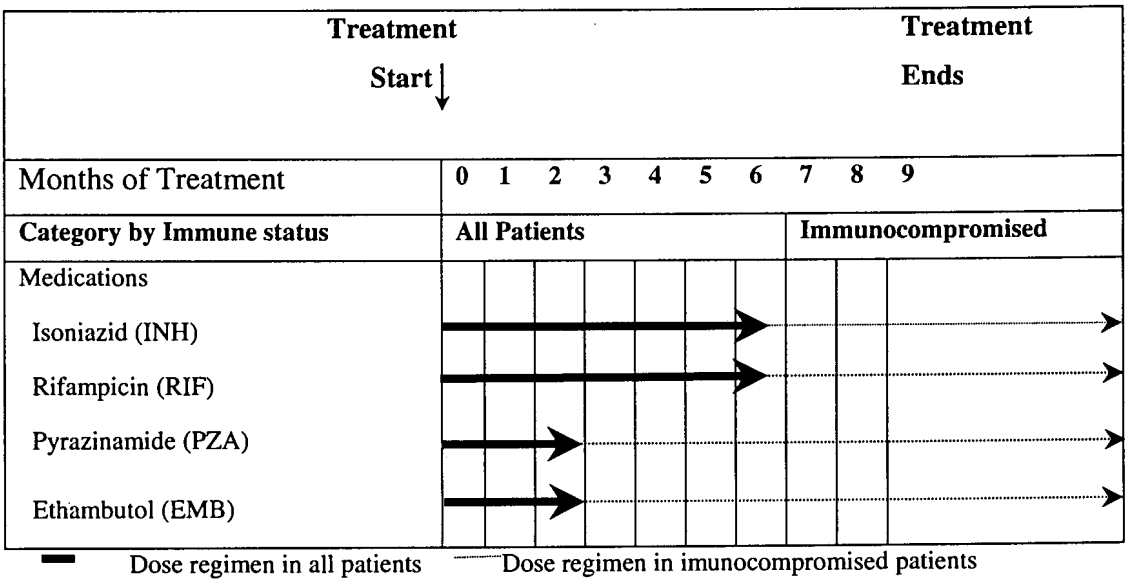


Figure 4.1: Bacterial populations during progression of tuberculosis. (Stead and Dutt, 1988)

This therapy was found to be effective on condition that it is continued for up to 24 months (Stead and Dutt, 1988; Harding and Bailey, 1994). Lack of compliance with the 24-month therapy created complications such as relapse of the disease and mutations of *M. tuberculosis*. The discovery of rifampicin (RIF) and a new understanding of the mechanism of action of an old drug, pyrazinamide (PZA), resulted in the reduction of the treatment to 9 or even 6 months. Today the World Health Organization (WHO) strongly recommends the use of a regime of a combination of four drugs (Table 4.1). These are administered to the patient in the presence of a trained health care worker in a process referred to as the directly observed treatment short course (DOTS).

Table 4.1: Drugs recommended for tuberculosis short course treatment



4.1.2 T-cell mediated immunity and tuberculosis chemotherapy

There is plenty of evidence implicating T-cell mediated immune responses in the resistance to mycobacteria (Orme *et al.*, 1993; Porcelli *et al.*, 1995; Tsukaguchi et *al.*, 1995). Tentori *et al.* (1998) and Giuliani *et al.* (1998) went a step further and described the involvement of a cytokine-induced expression of CD1b in antigen presenting cells (APC). They also implied that double negative (DN) T-cell response might be involved in chemotherapy and resistance to tuberculosis. DN T-cells react specifically to mycolic acids derived from the cell-wall of *M. tuberculosis* and presented by professional antigen presenting cells (APC) (Beckman *et al.*, 1994).

Various rifamycins (chemical derivatives of RIF), such as rifampetine (RFP) and rifalazil (RZL), are drugs that are widely used in chemotherapy against *M. tuberculosis* infection (Lenaerts *et al.*, 1999; and Lenaerts *et al.*, 2000). Administration of chemotherapeutic

drugs against *M. tuberculosis* infection has been found to reduce B and T cell-dependent responses, including chemotaxis and DTH (Van Vlem *et al.*, 1996). The use of chemotherapy alone in the absence of an effective immune system may not be effective. This could be partly responsible for the relatively large rate of relapse and the lengthy period required to treat *M. tuberculosis* infection. Combining chemotherapy and immunotherapy has demonstrated the potential to curb this problem (Liakopoulou, 1989; Van Vlem *et al.*, 1996; Lowrie *et al.*, 1999). These observations prompted Tentori *et al.* (1998) to further investigate the effects of RIF on macrophages (MΦs), with regard to antigen presentation by CD1b molecules. Their investigations were performed on cytokine-activated monocytes (CAM) [adherent mononuclear cells (AMNC) activated with either IL-4 or GM-CSF or both] and they found that administration of RIF to these cells increased expression of CD1b (Tentori *et al.*, 1998; and Giuliani *et al.*, 1998). This suggested that the administration of RIF could be beneficial in improving mycobacterial antigen presentation, but detrimental in other forms of cell mediated immunity. From this arose the idea that the efficiency of chemotherapy may be enhanced by concomitant immunotherapy (Hernandez-Pando *et al.*, 2000; Rook and Hernandez-Pando, 1994).

Studies in animal models have suggested that immunotherapy in conjunction with chemotherapy could serve as a valuable approach to curb the spread of tuberculosis. A number of studies have shown that immunotherapy with DNA vaccination combined with chemotherapy shift the immune system from that which renders bacteriostasis to that which is bactericidal (Huygen *et al.*, 1996; Lowrie *et al.*, 1999). This has been achieved by establishment of cellular immunity that ultimately resulted in the production of cytokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12) to support the chemotherapeutic effect on tuberculosis with the appropriate bactericidal immune response. The use of IL-12 DNA "vaccine" in combination with chemotherapy has been shown to clear mycobacteria from the lungs (Lowrie *et al.*, 1999). The effects observed with DNA vaccines could also be attributed to the immunostimulatory 'adjuvant' effect of the plasmid itself (Krieg *et al.*, 1998).

4.2 Aims

The objective of this study was to assess the effect of combining chemotherapy (antibiotic treatment) and mycolic acids administration on the clearance of *M. tuberculosis*. This approach has been motivated by previous studies in which mycolic acids were observed to have the ability to prolong the survival of *M. tuberculosis*-infected mice (Chapter 2 and Siko, 1999). Pretorius (1999) indicated that mycolic acids also have the ability to induce expression of IL-12 and IFN-γ in mice infected with *M. tuberculosis*, the cytokines known to protect against tuberculosis.

The chemotherapy experiment was designed in such a way as to determine whether mycolic acids administered as a homogenous mouse serum conjugate to intranasally infected mice could shorten the duration of the antibiotic treatment to which the experimental animals were subjected.

The effect of antibiotic treatment, and of a combined treatment comprising antibiotics and mycolic acids, on the spread of *M. tuberculosis* in experimental animals was studied. The animals from the experimental groups were randomly sacrificed 4, 16, 37 and 54 weeks after the start of the *M. tuberculosis* infection, and their lungs and spleens were analysed for the number of viable mycobacteria present.

4.3 Materials

4.3.1 Cultures

Mycobacterium tuberculosis H37Rv ATCC 27294 - a virulent strain, originally isolated from an infected human lung.

The cultures were purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA.

4.3.2 Growth medium used for the cultivation of *M. tuberculosis*

Middlebrook 7H-10 agar medium (in plates) was used as growth medium both for the cultivation of *M. tuberculosis*, prior to the extraction of mycolic acids and for the enumeration of *M. tuberculosis* in animal organs.

A detailed composition of the ingredients necessary for the preparation of these media as well as the conditions recommended for their sterilization, are given in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (1980, Chapter 6, pp 83-105; Second Edition, revised by E E Nel, H H Kleeberg and E M S Gatner). Media were prepared by staff of the National Tuberculosis Institute of the Medical Research Council of South Africa, in Pretoria.

4.3.3 Reagents

Reagents used for: the extraction, saponification and derivatization of mycolic acids; the purification of mycolic acids; and for the HPLC analysis of mycolic acids are described in detail by Siko (1999) and Goodrum, Siko *et al.* (2001)

4.3.4 Washing and dilution of *M. tuberculosis*

The harvested mycobacteria used for infecting the experimental animals were washed in sterile 0,9% m/v NaCl (Chemically Pure, Saarchem, RSA) and adjusted to the appropriate concentration.

Diluent used for the preparation of serial dilutions, preceding the determination of viable counts of *M. tuberculosis*, was prepared by dissolving Tween 80 (Chemically Pure, Merck) in 0,9% m/v NaCl (Chemically Pure, Saarchem) to a concentration of 0,01% v/v and distributing and autoclaving it in 9,0 ml aliquots into test-tubes. The autoclaved tubes with diluent were stored at 4^0C.

4.3.5 Antibiotics

Isoniazid (Noristan-Isoniazid) - manufactured by Noristan Ltd, Waltloo, Pretoria, South Africa;
Rifampicin (Rimactane) - manufactured by Ciba-Geigy Ltd, Basel, Switzerland;
Pyrazinamide (Rolab-Pyrazinamide) - manufactured by Spartan, Kempton Park, South Africa.

4.3.6 Preparation of organ homogenates

Sterile saline (0,9% m/v NaCl, Saarchem, Chemically Pure, RSA) was used for the preparation of the organ homogenates.

Diluent for the preparation of serial dilutions of the organ homogenates, preceding the determination of the concentration *M. tuberculosis* in various mouse organs was the same as that described for dilution of *M. tuberculosis* (see 4.3.4).

4.3.6.1 Blood decontamination

N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH):

i) NaOH pellets 4 g in 100 ml distilled water;

ii) Sodium citrate 2,9 g in 100 ml distilled water.

After autoclaving at 121°C for 15 minutes, both solutions were mixed together with 1,0 g of N-acetyl-L-cysteine powder.

Phosphate buffer 0,15 M, pH 6,8:

iii) Anhydrous Na_2HPO_4 9.47 g in 1000 ml distilled water;

iv) Anhydrous K_2HPO_4 9.07 g in 1000 ml distilled water.

Solutions iii and iv (50 ml) were mixed and the pH checked and adjusted to pH 6,8 with either of the solutions.

4.3.7 Experimental animals

Eight to twelve weeks old female Balb/c (a tuberculosis-susceptible strain) mice were used in the chemotherapy experiment. The mice were inbred for at least 11 generations at the Animal Centre at the South African Institute for Medical Research in Johannesburg. Male mice of corresponding age were used for the collection of sera necessary for the preparation of mycolic acids/mouse serum conjugates. Mice cubes, manufactured by EPOL and autoclaved tap water were provided *ad libitum.*

Sanitation-Bronocide, manufactured by Essential Medicines (Pty) Ltd, was used for sanitation purposes.

4.3.8 Plasticware

The following plasticware was used: Disposable Petri dishes (Promex, RSA); disposable tissue culture dishes (Promex, RSA); Sterile, disposable, 50 ml centrifuge tubes (Corning, USA) and disposable sterile tips (Elkay, Denmark)

4.3.9 Preparation of organ homogenates

Instruments and materials used for preparation of homogenates were as follows: Sterile scissors; sterile tweezers; sterile teflon "homogenisers"; sterile tubes with heparin; sterile glass rods (used for the spreading of mycobacterial suspensions on the surface of agar plates) and crushed ice.

4.4 Methods

4.4.1 Cultivation of bacteria

The mycobacteria for the extraction of mycolic acids were cultivated at 37°C using Middlebrook 7H-10 agar medium plates.

The sterility of the media was confirmed visually before use in the experiments by incubating them at 37^0C for 24 h.

For routine extraction of mycolic acids approximately 3-4-week old *M. tuberculosis,* grown on Middlebrook 7H-10 agar medium plates were used.

For the preparation of bacterial suspensions used for the experimental induction of tuberculosis, approximately 2-week old cultures of *M. tuberculosis*, grown on Middlebrook 7H-10 agar medium plates, were used.

4.4.2 Viable bacterial counts

For the viable count determination, serial suspensions of the harvested bacteria were prepared in the diluent medium (as specified under 4.3.4) to a density corresponding to a McFarland standard 4 (approximate optical density (OD) of 1,0; using a Beckman DU 65 spectrophotometer, at 486 nm). Tenfold serial dilutions were prepared using 9 ml aliquots of the diluent medium. From the last three dilutions corresponding to 10^{-3}, 10^{-4} and 10^{-5} of the original suspension, aliquots of 0,1 ml (100 µl) were withdrawn and spread over the surface of Middlebrook 7H-10 plates. The plates were incubated at 37^0C and the developed colonies counted after two to three weeks.

Statistical analysis of the bacterial counts included the mean values of bacterial counts and standard deviations.

4.4.3 Preparation of mycolic acids from bacterial samples

Mycolic acids were prepared according to a method described in chapter 2.

4.4.4 Preparation of conjugates

The required mass of mycolic acids (2,5 mg) was dissolved in 200 µl chloroform and added to 10,0 ml of mouse serum, previously filtered through a 0,22 µm filter. Thus, the volume of dissolved mycolic acids constituted 2% of the volume of mouse serum.

The sample was sonicated using a Branson Sonifier B 30 Cell Disruptor, (at 20% duty cycle, output control of 2, for 50 pulses, at room temperature). The sample was maintained for 1 hour at room temperature, to allow air bubbles formed during sonication to escape. In order to remove chloroform, nitrogen was bubbled through the conjugate until the chloroform odour was removed. The conjugate was prepared immediately before administration to the experimental animals.

4.4.5 Preparation of bacterial suspensions

The cells of *M. tuberculosis* H37 Rv, harvested from Middlebrook 7H-10 agar medium plates, were suspended in the diluting buffer (0,01% v/v Tween 80 in 0,9% m/v NaCl) and homogenized. After centrifugation in a Beckman J-6 centrifuge for 20 min at 1 580 g, the cells were washed with a sterile solution of 0,9% m/v of NaCl and adjusted to a concentration corresponding to a McFarland standard No.4. After the confirmation of the total direct bacterial count, carried out on an autoclaved suspension in a Neubauer counting chamber, the suspension was further diluted in the sterile solution of 0,9% NaCl to obtain concentrations of *M. tuberculosis* corresponding to 10^3, 10^4 and 10^5 cells/ml.

The viable counts of the mycobacteria in the suspensions were confirmed by plating 100 µl aliquotes of the relevant dilutions onto Middlebrook 7H-10 agar medium, incubating the plates at 37^0C for two weeks and counting the number of colony forming units (CFU).

The suspensions were introduced into the experimental animals in aliquots of 100 µl or 60 µl per animal.

4.4.6 Experimental animals

Eight to twelve weeks old female Balb/c mice were accommodated in cages with a floor area of 450 cm^2, with 8 mice per cage.

Environmental conditions: Temperature and humidity in the animal facility were set at 20^0C (+/- 1^0C) and 40% (+/- 10%), respectively. Lighting was provided by means of fluorescent tubes. A light-darkness cycle of alternating 12 hour periods was set up.

Mice were housed in transparent polypropylene cages with tight fitting stainless steel lids. Wooden shavings, after autoclaving, were provided as nestling material. Animal rooms, mice cages and glass bottles were cleaned and decontaminated once a week using Bronocide. Water bottles, after washing, were autoclaved once a week.

Mice were maintained and caged in Techniplast animal isolators marketed by Labotec, South Africa. A positive pressure of 4 atm inflated the isolator. It was equipped with an air inlet pre-filter (with the pore size of 0,6 μm) through which the incoming air was filtered and an outlet HEPA (High Efficiency Particulate Air) filter (with a pore size of 0,22 μm) through which the outgoing air was filtered before leaving the isolator. The airflow rate was regulated at 7 exchanges per hour. Animal rooms, the animal cage

isolators and water bottles were cleaned and decontaminated once a week using Bronocide. Individual identification of mice was accomplished by making ear marks.

4.4.7 Inoculation with *M. tuberculosis* H37 Rv suspensions

The introduction of the *M. tuberculosis* suspensions was performed in a biosafety cabinet class III in the PIII facilities at the Tuberculosis Institute of the Medical Research Council in Pretoria. The introduction of the bacterial suspensions *via* intranasal route was carried out as follows:

Mice were anaesthetized with 5% diethylether. The bacterial suspensions were introduced into the nostrils of mice in aliquots of 60 μl per animal. The suspensions were released drop-wise into the nostrils using autoclaved pipette tips, while the animals were in dorsal recumbence. Control animals received an equivalent volume of sterile saline, *i.e.*, 60 μl introduced intranasally.

4.4.8 Introduction of MA-serum conjugate and mouse serum

The administration of the mycolic acids conjugates was carried out *via* the intravenous route, after mice were heated for 5 min in a heating box to effect vasodilation of the tail veins. The mycolic acids-mouse serum conjugate was administered by introducing 5 μg or 25 μg mycolic acids in 100 μl mouse serum per mouse. Control animals received 100 μl of mouse serum introduced in the same manner.

4.4.9 Administration of antibiotics

Three antibiotics were selected for the experiment, namely: isoniazid, rimactane (rifampicin) and pyrazinamide. Although isoniazid and pyrazinamide are water-soluble and could be administered to the experimental animals with drinking water, these

compounds are chemically unstable and their potency would vary throughout the experiment. For that reason it was decided to introduce the appropriate dosages of antibiotics mixed with crushed mouse pellets. The dosages administered to mice were calculated on the basis of the daily dosages recommended for humans, expressed in terms of the mouse metabolic weight.

Isoniazid and pyrazinamide tablets were crushed using a pepper mill, mixed with the contents of rifampicin capsules and introduced into the mouse pellets crushed to powder in such a way that each 5 g portion of mouse feed contained: 1 mg isoniazid, 1,4 mg of rifampicin and 1,3 mg of pyrazinamide. Assuming that a mouse consumes 5 g of food per day, it should consume appropriate quantities of the respective antibiotics to sterilize the lungs of infected mice. Food required for the mice in each cage was then transferred using a spatula to the compartments of the special trays prepared specially for this purpose.

4.4.10 Preparation of organ homogenates

The experimental animals to be sacrificed were selected from each experimental group at random at 4, 16, 37 and 54 weeks after the infection with *M. tuberculosis* H37Rv. All the steps were carried out in a sterile manner. The specimens for further investigations were prepared as follows:

i. The animals were sacrificed by neck dislocation. The spleens and the lungs were removed using sterile scissors and tweezers and placed into individual, sterile small tissue culture dishes, suitably marked with the mouse number and the type of the organ removed. Each organ was weighed and the mass recorded.

ii. The spleens and the lungs were transferred into sterile homogenizers into which aliquotes of 300 μl sterile saline were introduced. After satisfactory homogenates

were prepared, the suspensions were diluted with the sterile saline to reach the concentration of approximately 1 mg/ml.

iii. The homogenates were decontaminated according to the method specified in section 4.4.11 and the volume of the homogenates adjusted to 1,5 ml. Serial dilutions were prepared in the diluting medium.

iv. In the course of these experiments the following dilutions of the lungs' and spleen's decontaminated homogenates were prepared: undiluted, 10^{-1}; 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}.

v. Duplicate or triplicate plates were prepared for each dilution by evenly spreading the introduced aliquots with the sterile glass rods. Each tube with a respective dilution was vortexed prior to plating.

vi. The inoculated plates were placed in an incubator at 37°C and regularly checked for growth and the presence of contaminations.

4.4.11 Decontamination of organ homogenates

Decontamination of spleen and lungs homogenates was carried out according to the method recommended by Kubica *et al.*, (1963, 1964) and was performed as follows:

Aliquots of the spleen or lungs homogenates were introduced into 10 ml centrifuge tubes. Into each tube an equal volume of N-acetyl-L-cysteine-sodium hydroxide (NACL-NaOH) was added. Caps on the test tubes were tightened securely and the content was mixed using a test tube vortex until completely liquefied, for approximately 10-20 seconds. The mixtures were allowed to stand for 15 minutes at room temperature.

The tubes were then filled up with sterile phosphate buffer pH 6,8 and centrifuged at 2000 g for 15 minutes using a Labofuge GL centrifuge. The supernatants were discarded and 1,5 ml of sterile phosphate buffer pH 6,8 was introduced into each tube. The sediments were resuspended and were considered to be undiluted, decontaminated sample homogenates.

4.4.12 Plating of decontaminated homogenates

Appropriate dilutions of the decontaminated homogenates were prepared in the diluting medium (0,01 % Tween 80 in saline) and aliquots of 100 μl of the respective dilutions were plated onto the Middlebrook H-10 agar plates.

Duplicate or triplicate plates were prepared for each dilution by evenly spreading the introduced aliquots with the sterile glass rods. Each tube with a respective dilution was vortexed prior to plating. The inoculated plates were placed in an incubator at 37°C and regularly checked for growth and the presence of contaminants.

4.4.13 Experimental set-up

The set up of the study is indicated in Table 4.2.

Table 4.2: Experimental setup of the the chemotherapy experiment groups.

Group No	Treatment and infection with *M. tuberculosis*	Administration of antibiotics	Number of mice per group
1	100 µl saline *i.n.*. No infection with *M. tuberculosis*	Yes	10
2	100 µl saline *i.n.*. No infection with *M. tuberculosis*	No	6
3	25 µg MA *i.v.* *M. tuberculosis i.n.*	Yes	12
4	25 µg MA *i.v.* *M. tuberculosis in*	No	12
5	100 µl serum *i.v.* *M. tuberculosis in*	Yes	12
6	100 µl serum *i.v.* *M. tuberculosis i.n.*	No	12
7	*M. tuberculosis i.n.*.	Yes	6
8	*M. tuberculosis i.n.*.	No	5

1) *i.v.* - intravenous inoculation and *i.n.* – intranasal inoculation

4.5 Results

4.5.1 Confirmation of doses of *M. tuberculosis* and mycolic acids

By determining the colony forming units (cfu) of *M. tuberculosis*, it was established that the sub-culture of *M. tuberculosis* used in this study was diluted to a concentration of 2,5 x 10^6 cfu/ml. The dose of *M. tuberculosis* introduced intranasally into the animals was therefore confirmed to be 2,5 x 10^5 cfu/mouse/60µl saline.

By analysing the mycolic acids sample used to treat the animals by means of HPLC, it was established that the mycolic acids/mouse serum conjugate used in study comprised 25 µg mycolic acids per animal.

4.5.2 Comparison of the masses of the removed organs

The animals in this experiment were randomly selected and sacrificed 4, 16, 37 and 54 weeks after the infection with *M. tuberculosis* H37Rv as indicated in fig. 4.2.

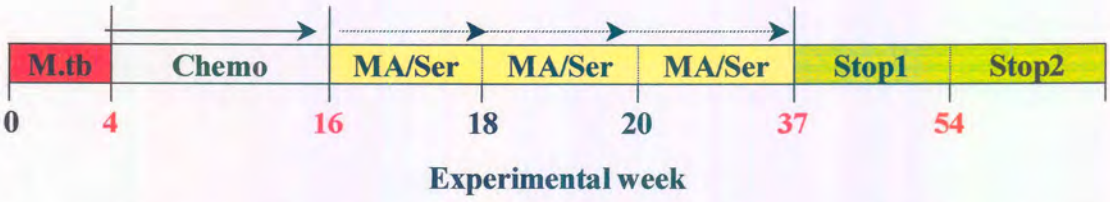


Figure 4.2: A time line display of the combined chemotherapy and MA pre-treatment experiment. The solid line indicates every day ad lib administration of antibiotics ending where the arrow ends and the dashed line indicates weekly administration of MA. The experimental weeks range from week 0 to 54 with weeks numbered in red depicting weeks of organ extraction. M.tb = intranasal *M. tuberculosis* infection; Chemo = administration of INH, PZA and RIF; MA/Ser = administration of mycolic acids or serum; Stop1 and Stop2 = organ bacterial counts to determine *M. tuberculosis* sterility.

The recorded masses of the removed organs were subsequently used for calculating the total number of bacteria present in each respective organ, assuming that the homogenates comprised a uniformly distributed suspension of mycobacteria present in the organs.

The organ masses were compared between various groups of mice. The masses of the spleens and lungs removed from the sacrificed *M. tuberculosis* infected and uninfected animals are presented as organ indices (Fig. 4.3). The masses and indices calculated of the spleens and particularly of the lungs of the infected and control mice at week 4 after *M. tuberculosis* infection appeared to be different. The mean value of the lungs' mass of the mice that had been infected with *M. tuberculosis* was found to be twice as high as that of the control group, that is: 0,4 g ± 0,1 compared to 0,2 g ± 0,1. The lung/body ratios were likewise affected with 1,77 ± 0,153 recorded for the infected group compared to 0,87 ± 0,5 for the control group. The results imply that intranasal infection was causing the expected disease. The viable mycobacterial cell counts from the infected and non-infected animals also confirmed this effect. The counts recorded for the mouse lungs (on average $5.2 \times 10^6 \pm 2{,}17 \times 10^5$) were two orders of magnitude higher than those obtained for the mouse spleens ($6{,}11 \times 10^4 \pm 4{,}71 \times 10^3$).

The organ mass determinations and concomitant viable mycobacterial cell counts at week 16 were done to establish whether the infection with mycobacteria was controlled by the antibiotics treatment and to what a degree the infection was spreading within the organs of mice. The organ indices of the spleens and lungs removed from the sacrificed *M. tuberculosis*-infected and uninfected animals at week 16 are presented in Fig. 4.3.

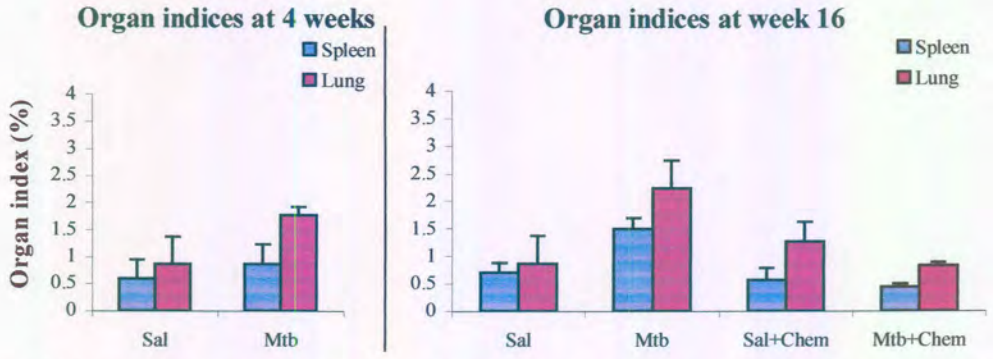


Figure 4.3: Measured organ indices of the spleens and lungs removed from Balb/c mice (4 weeks after infection and 16 weeks after infection). The organ index is expressed as a percentage of organ per body mass. The organs were removed four and sixteen weeks after infection with *M. tuberculosis* or saline treatment. The bars represent an average of three samples. "M.tb" on the x-axis of the graph represents infection with *M. tuberculosis*, "Sal" represents saline treatment, and Chem represents chemotherapy.

The mass of the spleens and the lungs isolated from the uninfected, control animals undergoing or not undergoing chemotherapy as well as from those comprising infected mice undergoing chemotherapy, were similar and considerably smaller than those isolated from the infected mice not undergoing chemotherapy. The spleens isolated from the animals of the first three groups (Sal+Chem, M.tb +Chem and Sal) were on average twice as small (0,133 g ± 0,05) as the corresponding organs isolated from infected and untreated animals (0,367 g ± 0,057). The lungs isolated from uninfected mice and those that received chemotherapy (Sal, Sal+Chem, and M.tb+Chem) were on average 2,5 times smaller than those isolated from the infected and untreated mice (M.tb), eg. Sal = 0,233 g ± 0,087 compared to M.tb = 0,533 g ± 0,115. The lung/body ratios accordingly registered 0,978 ± 0,363 for uninfected (Sal) compared to 2,23 ± 0,503 for the *M tuberculosis* infected (M.tb) mice.

The number of colonies (expressed as colony forming units, cfu) grown on agar plates, recovered from the organs of the control and experimental mice after a 16-week incubation period is presented in Table 4.3.

Table 4.3: Average number of colony forming units recovered from the suspensions of the lungs and of the spleens 16-weeks.after infection

Treatment Group	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
Saline (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2 x 10^5 H37Rv (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
Saline (in)		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2 x 10^5 H37Rv (in)		
Mouse 1	2,4 x10^5 ± 6,7x10^4	1,4 x10^6 ± 1,1 x10^5
Mouse 2	1,1 x10^5 ±3,2x10^4	3,7 x10^6 ± 9,5 x10^5
Mouse 3	5,8 x10^5 ± 4,5 x10^3	Contamination

The results in Table 4.3 clearly illustrate the bactericidal properties of the antibiotics used for the treatment of the infected mice. The results recorded for the mice not undergoing chemotherapeutic treatment, might also indicate that the infection with *M. tuberculosis* H37 Rv in the lungs spread further in the spleens of the infected mice.

On analysing the results presented in Figures 4.4 the protective effect of the antibiotics is confirmed, showing that no macroscopic evidence for relapse was observed, 21 weeks after discontinuation of chemotherapy. In the remaining cases of infected animals not receiving chemotherapy it was evident that no protective effect was visible by mycolic acids treatment (Mtb+MA, compared to Mtb+Ser and Mtb alone)

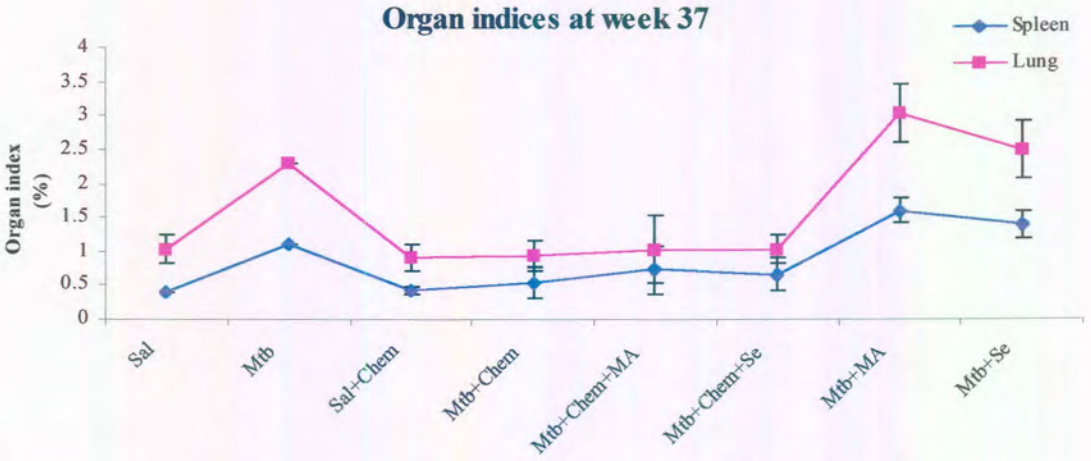


Figure 4.4: Organ indices of the spleens and lungs removed from Balb/c mice and expressed as a percentage of total body mass. The organs were removed 37 weeks after infection with *M. tuberculosis* or saline treatment. The bars represent an average of three samples. "Mtb" on the x-axis of the graph represents infection with *M. tuberculosis*, "Sal" saline treatment, "Chem" chemotherapy, "MA" treatment with mycolic acids conjugated to serum and "Ser" treatment with serum.

The mycobacterial counts from the lungs and spleens from the experimental mice sacrificed at week 37 confirmed the observations made by comparing the organ masses and indices (Table 4.4).

Table 4.4: Average number of colony forming units recovered from the suspension of the lungs and of the spleens 37 weeks after infection

Treatment groups	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
Saline (in) and Chemotherapy		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2×10^5 H37Rv (in) + Chemotherapy +25 μg MA		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	$1,2 \times 10^3 \pm 6,4 \times 10^2$	Neg
2×10^5 H37Rv (in) + Chemotherapy + Serum		
Mouse 1	Neg	Neg
Mouse 2	$2,4 \times 10^2 \pm 1,1 \times 10^2$	60
Mouse 3	Neg	$8,4 \times 10^3 \pm 3,9 \times 10^2$
2×10^5 H37Rv (in) + Chemotherapy		
Mouse 1	30 ± 10	$4,6 \times 10^4 \pm 2,2 \times 10^4$
Mouse 2	Neg	70 ± 14
Mouse 3	Neg	Neg
Saline (in)		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
2×10^5 H37Rv (in) + 25 μg MA		
Mouse 1	$1,3 \times 10^3 \pm 1, \times 10^3$	$5,9 \times 10^6 \pm 1,5 \times 10^6$
Mouse 2	$1,7 \times 10^3 \pm 4,2 \times 10^2$	$7,3 \times 10^5 \pm 6,4 \times 10^4$
Mouse 3	$1,2 \times 10^5 \pm 9,9 \times 10^2$	$6,7 \times 10^6 \pm 1,3 \times 10^6$
2×10^5 H37Rv (in) + Serum		
Mouse 1	$1,4 \times 10^4 \pm 4,1 \times 10^3$	$3,8 \times 10^5 \pm 1,1 \times 10^5$
Mouse 2	$1,1 \times 10^5 \pm 7,8 \times 10^4$	$2,2 \times 10^6 \pm 1,3 \times 10^6$
Mouse 3	$7,9 \times 10^5 \pm 1,9 \times 10^{24}$	$3,6 \times 10^5 \pm \times 10^2$
2×10^5 H37Rv (in)		
Mouse 1	$3,2 \times 10^4 \pm 5,6 \times 10^4$	$3,17 \times 10^2 \pm 4,2 \times 10^2$

No growth was found in the noninfected animals as expected. However, the cell counts show that organs were not sterilized from mycobacteria at week 37, with the exception of those that received combined chemotherapy and mycolic acids treatment. Table 4.5 summarizes the avarages of the groups that had a positive count.

Table 4.5: The calculated average colony forming unitsfrom groups with positive mycobacterial counts, 37 weeks after *M. tuberculosis* infection

Treatment group	**Spleens**	**Lungs**
Mtb + Chemotherapy + 25 μg MA	$1,2 \times 10^3 \pm 6,4 \times 10^2$ (1)*	Not detected
Mtb + 25 μg MA	$4,1 \times 10^4 \pm 6,8 \times 10^4$ (3)	$4,4 \times 10^6 \pm 3,2 \times 10^6$(3)
Mtb + Chemotherapy + Serum	$2,4 \times 10^2 \pm 1,1 \times 10^2$ (1)	$8,4 \times 10^3 \pm 3,9 \times 10^2$ (1)
Mtb + Serum	$3,05 \times 10^5 \pm 4,23 \times 10^5$ (3)	$9,8 \times 10^5 \pm 10,6 \times 10^5$ (3)
Mtb + Chemotherapy	30 ± 10 (1)	$3,3 \times 10^4 \pm 3,25 \times 10^4$ (2)
Mtb	$8,8 \times 10^4 \pm 3,5 \times 10^4$ (1)	$5,05 \times 10^5 \pm 3,6510^5$ (1)

Figures in brackets indicate the number of determinations

The significance of the sterilizing effect of MA and chemotherapy could not yet be determined, and was thus further investigated at week 54.

4.5.3 Mycobacterial cell counts at week 54

This was to establish the effectiveness of the antibiotic therapy 54 weeks since the start of the experiment and a potential effect of the simultaneous administration of mycolic acids on the number of mycobacteria detected in the spleens and lungs of the experimental animals. The results obtained using the organs isolated from 15 mice are presented in Table 4.6.

Table 4.6: Average number of colony forming units recovered from the suspension of the lungs and of the spleens 54 weeks after infection

Mouse No	SPLEENS *M. tuberculosis* count (Mean cfu/organ +/- SD)	LUNGS *M. tuberculosis* count (Mean cfu/organ +/- SD)
2 x 10^5 H37Rv (in) + Chemotherapy + 25 µg MA		
Mouse 1	Neg	Neg
Mouse 2	Neg	Neg
Mouse 3	Neg	Neg
Mouse 4	Neg	Neg
Mouse 5	Neg	Neg
Mouse 6	Not determined	$3{,}2x10^4 \pm 5{,}6\ x10^4$
2 x 10^5 H37Rv (in) + Chemotherapy + Serum		
Mouse 1	Neg	Neg
Mouse 2	Not determined	$3{,}17x10^2 \pm 4{,}2\ x10^2$
Mouse 3	Neg	Neg
Mouse 4	Neg	Neg
Mouse 5	Neg	Neg
Mouse 6	Neg	Neg
Mouse 7	Neg	Neg
Mouse 8	Neg	Neg
Mouse 9	Neg	Neg

Mycobacteria were not detected in any of the examined spleens and only in two out of 15 examined mouse lungs. This result confirms again that the antibiotic therapy appeared to be successful in controlling the infection with *M. tuberculosis* in Balb/c mice.

Mycobacteria were detected in the lungs of both the groups of mice that were placebo treated and treated with mycolic acids. This implied that no significance could be given to the observation at week 37, that mycolic acids appeared to exert additional protection over and above chemotherapy to combat progression of tuberculosis. It is also noteworthy that at week 37, the viable mycobacterial cell count of the spleens of mycolic acids plus

chemotherapy treated mice were higher than that obtained from infected mice receiving chemotherapy only.

The general observations made on these animals were that there was no cross-infection observed between the experimental animals assigned to various groups and maintained in the same isolator. Organs from groups of mice that were infected with *M. tuberculosis*, but did not received chemotherapy, showed high numbers of viable mycobacteria. The tested spleens showed approximately the same level of infection (approximately 1 x 10^4 cfu/plate) whereas the lungs appeared to harbour higher numbers of mycobacteria, ranging from 3,3 x 10^6cfu/plate (for the group treated with mycolic acids-mouse serum conjugate) to 1 x 10^6cfu/plate and 5 x 10^5cfu/plate (for the groups treated with the mouse serum only and not treated at all, respectively).

The results observed so far suggest that administration of mycolic acids had no effect in complementing chemotherapy by sterilizing infection where chemotherapy could not, as infection could be detected after week 54.

4.6 Discussion

This study envisaged emulating studies with DNA "vaccines" in combination with chemotherapy to effect clearance of the mycobacteria in a shorter chemotherapy. Mycolic acids were used to substitute for the immunotherapy to boost the immune system. This was motivated by previous observations that mycolic acids pre-treatment could slightly prolong survival of Balb/c mice that were intravenously infected with *M. tuberculosis* (Siko, 1999) and also induced the expression of IL-12 and IFN-γ in infected and uninfected Balb/c mice (Pretorius, 1999). As well as a later study (Chapter 2) where mycolic acids administration was shown to have a strong protective effect against intranasal infection with *M. tuberculosis*. The approach used in this study was to infect the mice with a minimal lethal concentration of *M. tuberculosis*, apply short treatment course with chemotherapeutic drugs to control the *M. tuberculosis* infection, later to be followed by mycolic acids treatment to boost the immune system.

In all the infected groups, more mycobacteria were detected in the lungs than in the spleen, primarily because in the intranasal infection route the lungs are the port of entry for the mycobacteria, simulating normal infection in humans. After 16 weeks of infection and 12 weeks of chemotherapy all the animals that received chemotherapy were found to be macroscopically clear of infection as compared to the *M. tuberculosis* infected animals that did not receive chemotherapeutic treatment. After termination of chemotherapy, mycolic acids were administered to animals three times from week 16 to week 20 and they were found to have no effect in containing the infection in either the lungs or the spleen after 37 weeks and 54 weeks of infection.

In the experiment performed here, no convincing evidence could be found of complementary benefit provided by mycolic acids administration in addition to the treatment of *M. tuberculosis* infection with chemotherapy. In previous studies (Siko, 1999; Pretorius, 1999) post-infection treatment of animals with mycolic acids were found to be ineffective in controlling tuberculosis. In contrast, pre-treatment with mycolic acids

was found to be effective to curtail subsequent *M. tuberculosis* infection (see Chapter 2). Pre-treatment of Balb/c mice with mycolic acids apparently primed the immune system by means of cytokine expression (Pretorius, 1999) to handle *M. tuberculosis* infection, whereas post-infection treatment with mycolic acids was ineffective against the spread of *M. tuberculosis* infection. In this study however, it was hoped that post-infection chemotherapy prior to mycolic acids administration would restore the responsiveness of the mice to mycolic acids. This appeared not to be the case.

In conclusion, mycolic acids were found to be inefficient as immunotherapeutic supplement to shorten the chemotherapy course to treat tuberculosis. Mycolic acids appeared to be efficient at priming the immune response to a protective mode before an infection, but found no application to be of benefit once the infection has already taken effect.

CHAPTER 5

Development of a biosensor antibody assay for *Mycobacterium tuberculosis* mycolic acids

5.1 Introduction

Scientists have discovered that the lipid rich mycobacterial cell wall (Minnikin and Goodfellow, 1980; Brennan and Nikano, 1995), contributes towards antigenicity of mycobacteria (Porcelli *et al.*, 1992; Sieling *et al.*, 1995). These observations led to the further discovery that mycolic acids, which are abundant mycobacterial cell wall lipids (Minnikin, 1982; Minnikin *et al.*, 1984), are specifically presented in an MHC independent mechanism on CD1 molecules (Beckman *et al.*, 1994; Sugita *et al.*, 1998).

Mycolic acids were found to induce an enhanced antibody response in Sprague Dawley rats that peaked at about 91 days (Siko, 1999). This evidence of the existence of antibodies against mycolic acids confirmed the observation in human tuberculosis patients of antibodies that recognise mycolic acids (Pretorius, 1999). Pan *et al.* (1999) independently corroborated these observations when they reported that the specificity of antibodies that were previously classified as anti-cord factor actually recognized a mycolic acids subclass.

5.1.1 Modern advances in immunoassays

The current rapid technology-driven advances in the genome and proteome research demanded an equal advancement in the techniques to analyse biomolecular interactions to keep pace. Since the antibody-antigen interactions involve reversible molecular

recognition, their interactions can be measured through association and dissociation in an immunoassay.

An immunoassay is a technique that measures the presence of a substance using an immunological reaction such as antibody-antigen interaction. In the 1950s, after the introduction of the technique of radiolabelling of proteins, antibody and antigen dissociation and association could be measured by using radiolabelled antigens or antibodies. The biggest limitation of radioimmunoassay (RIA) was the safety risk involved. Coupling of enzymes to proteins revolutionised immunoassays. The enzyme linked immunosorbent assay (ELISA) introduced in the late 60's (Avrameas, 1969; Avrameas, 1971) is today still extensively used to measure antibody-antigen interactions. The disadvantage of ELISA is that it detects only high affinity antibodies and the interactions are detected at the endpoint. Measurement of real-time binding kinetics of antibody-antigen interaction was possible already since the early 70's by means of techniques such as temperature dependent fluorescent polarization and fluorescent emission isotropy (Weltman and Davis, 1970; Portmann *et al.*, 1971; Dimitropoulos *et al.*, 1986) which required high purity of reagents and was limited by the unknown effect of covalent modification of the antibodies during labelling with fluorophores. The association and dissociation can now be quantified in impure samples of unlabelled antibodies by measuring the rate constants with optical biosensor technology (Badley *et al.*, 1987).

5.1.2 The Resonant mirror biosensor

Biosensors are analytical devices, which can convert a biomolecular interaction signal on a sensing surface into a quantifiable response signal without using any labels. There are two main configurations used for analysing a sample on the sensor surface: equillibrium analysis in the micro cuvette and steady state analysis in the micro flow cell (Fig. 5.1) (Freaney *et al.*, 1997). In both systems a wave of total internally reflected incident light is

generated at the interface of a substance with high refractive index and a substance with low refractive index.

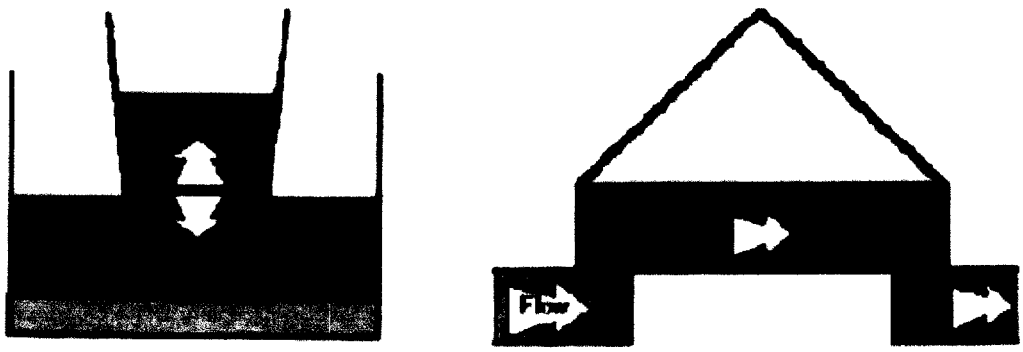


Figure 5.1: Target molecule delivery to the biosensor surface in either **A)** a micro cuvette or **B)** a micro flow cell.

The flow cell system is based on surface plasmon resonance (SPR). The SPR occurs when the sensing surface frees electrons in a film of metal, such as gold, oscillate and absorb energy from an incident light beam at a specific angle (SPR angle). The absorbance is then measured as a sharp decrease in the intensity as measured by a diode detector. In this system binding analysis is determined where one of the interacting partners is immobilised on a gold chip, which forms a wall of the flow cell (Fig. 5.2) (Myszka and Rich, 2000).

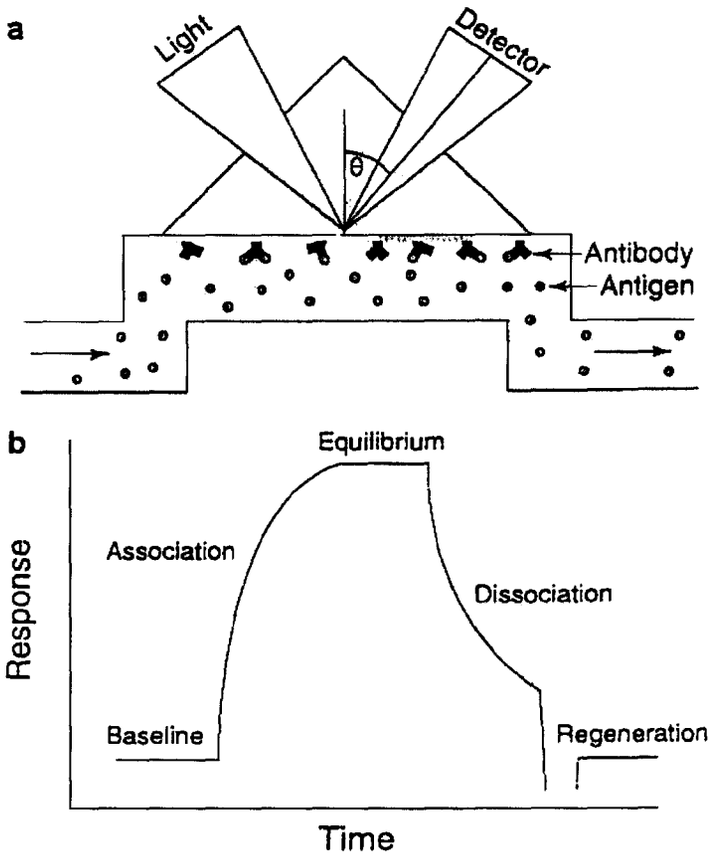


Figure 5.2: Flow system biosensor with the direction of the flow relative to the angle of the incident light depicted (a) and the resulting sensorgram (b) (Myszka and Rich, 2000).

Unlike the SPR flow system, the cuvette system employs a resonant mirror wave guiding technique. In this system, the resonant mirror internally reflects light beamed from one side of the cuvette through a prism to be measured the other side by a detector (Fig. 5.3). In this system, the changes in the evanescent field determines the angle that effects total reflection in the high refractive index layer that makes up the wave guide.

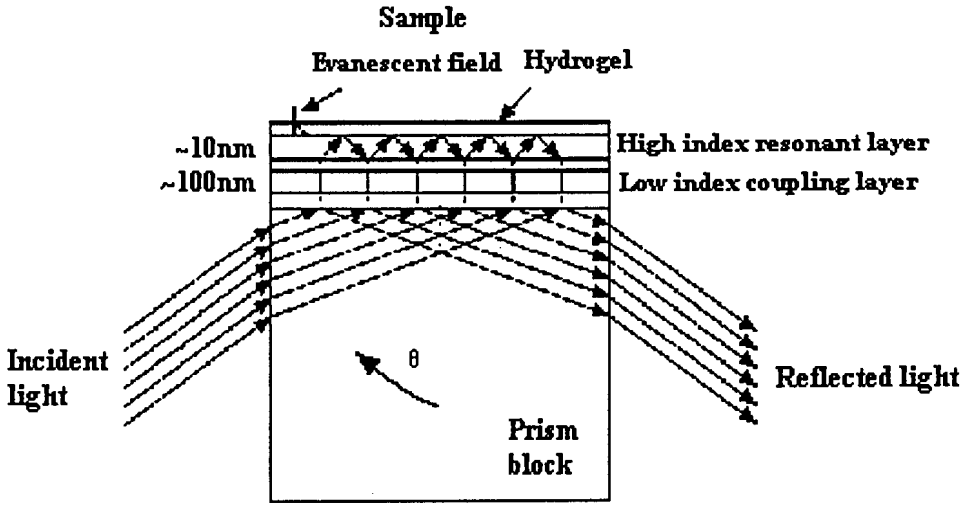


Figure 5.3: Representation of the IAsys resonant mirror-sensing device built in a cuvette (Pathak, 1995).

The region in the resonant layer where the path of the light is interrupted by the events that take place at the surface inside the cuvette is the evanescent field. The intensity decreases exponentially away from the resonant layer, which means that only reactions that occur close to this field can be monitored. When a ligand (such as an antibody or antigen) associates with the immobilised substance (antigen or antibody), the resonance angle changes and is registered with the instrument software in real-time. The change is measured in arc seconds units. An arc second is defined as 1/3600 of a degree with 3600 arc seconds making up a degree. Ligands immobilized onto the sensor surface by chemical means generate a sensorgram that can be characterised with available software (Fig. 5.2b).

In the equillibrium analysis configuration it is essential that the cuvette cell contents are stirred to ensure that mass-transfer is limited.

The IAsys affinity biosensor system uses a removable stirred cuvette system that is believed to have an advantage over the flow system in that the sample consumption is minimal, with no time constraints in terms of how long the sample is left in contact with its binding partner (Fig 5.4). This system is also available with two compartments (cells) suitable for measurements against a standard or direct comparison of sample binding properties.

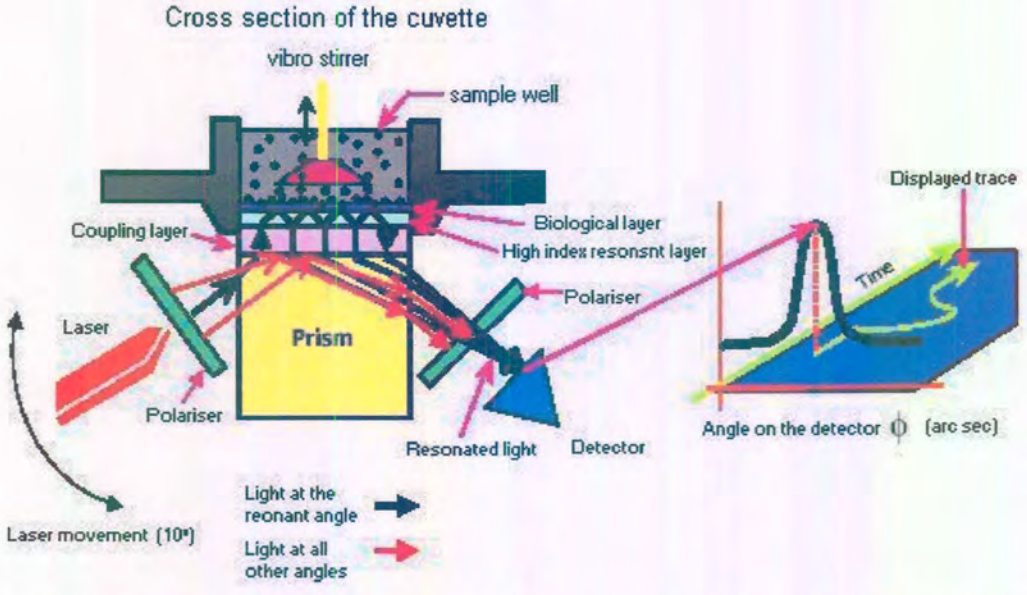


Figure 5.4: Schematic representation of the IAsys cuvette system with the vibro stirrers and oscillating laser light. (IAsys technical manual)

The sensor contains internal aspirators to remove solutions from the cells without removing the cuvette, making addition of other solutions fast and easy. The cuvettes are available with a choice of different derivatised sensor surfaces for different chemical immobilization reactions: carboxymethyl dextran for general protein immobilization, hydrophobic surfaces for creating lipid bilayers, carboxyl, and aminosilane surfaces for

specific immobilization of molecules, biotinylated surfaces for interacting with streptavidin labelled molecules and a non-derivatised surface. In this study the resonant mirror biosensor is used to develop a system by which anti-mycolic acids antibodies can be assesed to determine whether they can be regarded as surrogate markers for *M. tuberculosis* infection in sero-diagnosis.

5.2 Aims

This study aimed at developing a biosensor surface suitable for characterisation of the antibody-mycolic acids interaction using a resonant mirror biosensor.

5.3 Materials

5.3.1 Mycolic acids

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

5.3.2 Reagents

PBS-Azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per 1 *l* ultrapure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4

Guanidinium thiocyanate (GSCN, for synthesis; Merck, Munich, BRD; Cat No 820613) 3.5 mole per *l*

Cetylpyridinium chloride (CPC; Sigma, St. Louis, MO; Cat No C-9002) 0.02 mg per ml

Cholesterol (5-cholesten-3β-ol) (Sigma; Cat No C-8667): stock solution 100 mg per ml in chloroform (Merck, Darmstadt, BRD)

Phosphatidyl choline (pure) (PC-99; Sigma; Cat No P-3556): stock solution 100 mg per ml in chloroform

Phosphatidyl choline (from dried egg yolk) (PC-60; Sigma; Cat No P-5394): stock solution 100 mg per ml in chloroform

HCl 0.1 M

NaOH 50 mM

Ethanol 96% (v/v) in demineralized water

Saponin (Sigma; Cat No S-1252), 1 mg/ml in PBS-EDTA buffer

5.3.3 Resonant Mirror Biosensor apparatus

IAsys *plus* Resonant Mirror Biosensor (IAsys Affinity Sensors, Saxon Way, Bar Hill, Cambridge, UK)

5.3.4 Human sera

Human sera from three different sources were tested:

- Negative control sera from people who had never suffered from tuberculosis were obtained from healthy students and staff of the Department of Biochemistry, University of Pretoria, Pretoria, RSA and from healthy students from the Department of Medicine, University of Witwatersrand, Johannesburg, RSA.
- Tuberculosis patient sera obtained in the year 1994 at the King George V Hospital, Domerton, RSA, were a gift from the Medical Research Council National Tuberculosis Institute in Pretoria, RSA.
- Patient sera obtained in the year 2000 were kindly provided by Dr. A.C. Stoltz, Pretoria Academic Hospital, Pretoria, RSA, and Dr. G. Schleicher, Helen Joseph Hospital, Auckland Park, RSA.

5.3.5 Liposomes

- Phosphatidyl choline (PC) – 100mg/ml in chloroform
- Mycolic acids (MA) – 1mg
- Cholesterol (Chol) – 100mg/ml in chloroform
- PBS Azide EDTA (PBS/AE)
- Saline

5.3.6 ELISA

Goat anti-human IgG (H + L chains) antibody conjugated to peroxidase was obtained from Sigma (St Louis, MO, USA)

o-Phenylenediamine (Sigma, St Louis, MO, USA)

Hydrogen peroxide (Merck, Darmstadt, BRD).

Sterile, disposable 50 ml centrifuge tubes (Bibby Sterilin Ltd, Stone, UK)

Disposable pipettes (Bibby Sterilin Ltd, Stone, UK)

Disposable pipette tips (Bibby Sterilin, Serowell; Bibby Sterilin Ltd, Stone, UK)

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

Buffers

- **Acidification buffer:** Glycine HCL (0,2 M, pH 2.8)
- **Neutralisation buffer:** K_2HPO_4 (1 M in dddH_2O). The pH was adjusted to 9.0 with H_2KPO_4 (1 M) if necessary.
- **PBS buffer**: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 (anhydrous) and 1.05 g Na_2HPO_4 (anhydrous) per 1 *l* distilled water, adjusted to pH 7.4.
- **Diluting buffer:** 0.5% (m/v) carbohydrate- and fatty acid free casein (Calbiochem, La Jolla, CA) in PBS buffer adjusted to pH 7.4 was used for diluting of the sera and the immunoreagents.

5.4 Methods

5.4.1 Mycobacterial culture

Mycobacterium tuberculosis H37Rv (ATCC 27294) was cultured on Löwenstein-Jensen (LJ)-slants at 37°C for 3 to 6 weeks. Harvested cells with a total count varying between 10^4 and 10^6 per ml were suspended in 0.89 % sterile saline, washed twice by centrifugation (2000 x g for 15 min) and re-suspended in 0.89 % saline. Bacterial titres (viable counts) of samples were determined by counting colony forming units (CFU) in 1:10 to 1:1 000 000 dilutions on Middlebrook 7H10 agar after incubation at 37 °C for 4 to 6 weeks.

Total count was determined by means of a direct count using a Neubauer counting chamber and various dilutions of bacterial suspensions. Staff from the National Tuberculosis Institute of the Medical Research Council of South Africa, Pretoria, prepared the media. The sterility of all the media was confirmed before use in the experiments by incubating them at 37°C for 24 h to detect any turbidity.

The harvested bacteria were washed with sterile 0,9% m/v NaCl (Saarchem, Chemically Pure, RSA). Medium used for the preparation of serial dilutions, preceding the determination of viable counts of *M. tuberculosis* was prepared by dissolving Tween 80 (Merck, Chemically Pure) in 0,9% m/v NaCl (Saarchem, Chemically Pure) to a concentration of 0,01% v/v and distributing it in 9,0 ml aliquots into test-tubes. The autoclaved media were stored at 4°C.

5.4.2 Mycolic acids preparation

Mycolic acids were prepared from *M. tuberculosis* following a method described in Chapter 2.

5.4.3 Liposomes

For the preparation of mycolic acids only-containing liposomes, 90 µl of the phosphatidyl choline (PC-99) stock solution was added to an amber glass vial containing 1 mg of mycolic acids, mixed well to dissolve the mycolic acids, dried at 80°C under a stream of N_2, and then sonified in 2 ml saline for 5 min at room temperature. The "empty" phosphatidyl choline liposomes were made in the same manner with omission of the mycolic acids. For the cholesterol-containing liposomes, 60 µl of the phospatidyl choline (PC-60 or PC-99 as applicable) stock and 30 µl of the cholesterol stock were added to an amber glass vial with or without mycolic acids, mixed well, and then dried, suspended in saline and sonified as above.

Liposomes were divided into 200 µl aliquots, freeze-dried and stored at -20°C until use. Before use the liposomes were reconstituted with 2 ml PBS/AE buffer (PBS-EDTA buffer: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per 1*l* ultrapure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4), heated at 80°C for 15 min and then sonified as above. The final liposome concentration therefore came to 50 µg/ml.

5.4.4 ELISA

Mycolic acids originating from *Mycobacterium tuberculosis*, isolated as described above, and cholesterol (Sigma, St. Louis, MO; Cat No C-8667) were used at final concentrations of 60 µg/ml and 75 µg/ml, respectively. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85°C. The hot solutions were sonicated at 20% duty cycles and optimal output level for 1 min. The solutions were kept at 85°C and loaded into the ELISA plates. The respective antigens were dissolved in hot PBS and then sonicated, as described above. The wells were coated overnight at 4°C with 50 µl/well of antigen solution. The final antigen load was approximately 3 µg/well for mycolic acids or approximately 3.75 µg/well for the cholesterol.

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

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

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

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

The substrate solution comprising 10.0 mg *o*-phenylenediamine and 8.0 mg hydrogen peroxide in 10 ml of 0.1 M citrate buffer pH 4.5, was prepared immediately before use and introduced in 50 µl aliquots per well. The plates were incubated at room temperature

and the colour development was monitored at 5, 30 and 60 min after addition of the substrate using an SLT 340 ATC photometer (Thermo-Labsystems, Finland) at a wavelength of 450 nm.

5.4.5 Coating of biosensor cuvettes

Each cuvette was first allowed to equilibrate to room temperature before insertion in the IAsys apparatus. Binding of each component was monitored in real-time.

5.4.5.1 Coating of the hydrophobic cuvette

The following steps were followed for coating a hydrophobic cuvette:

- The inserted cuvette was washed 5 times with 50 µl 2-propanol followed by data collection for 1 minute and then washing 7 times with 60 µl PBS/AE with 10 minutes data collection.
- This was then followed by washing 7 times with 50 µl 2-propanol and 1 minute data collection after that. With 50µl remaining in the cuvette, 22µl of MA-cholesterol mixture dissolved in chloroform was added in one cell and in the reference cell, 20 µl of the control solution was added and coating data was collected for 1 minute followed by washing 7 times with 60µl PBS/AE and 5 minutes data collection.
- The cuvette cells were then washed 5 times with 50µl HCl and data collected for 1 minute, and then washed 7 times with 60µl PBS/AE with 5 minutes data collection. This was followed by 5 washes with 50µl NaOH for 1 minute and the cells were washed 7 times with 60µl PBS/AE followed by data collection for 5 minutes.
- Blocking was performed by adding negative sera to a final concentration of 10%, washed 5 times with 60µl PBS/AE and followed by 5 minutes data collection.

5.4.5.2 Coating of underivatised cuvettes with liposomes

The following steps were followed for liposome coating of the underivatised cuvette:

- The cuvette was washed 10 times with 60 µl PBS/AE. The buffer was replaced with 50 µl cetylpyridinium chloride solution. After 10 min the cuvette was washed 5 times with 60 µl PBS/AE.
- After the final wash, 25 µl of PBS/AE was added to the cuvette, allowing 5 min for the baseline to be established. Then 25 µl of liposomes (containing either phosphatidyl choline plus mycolic acids, phosphatidyl choline plus cholesterol, or phosphatidyl choline only) were added and data collected for 20 min.
- The cuvette was again washed 5 times with 60 µl PBS/AE and then aspirated. In order to stabilize the lipid complex on the cuvette surface and to reduce non-specific binding, 50 µl of saponin solution was added to the cuvette. After 20 min of data collection the cuvette was washed 5 times with 60 µl PBS/AE.
- After 10 min of data collection base line was established by aspirating and replacing with 25 µl of PBS/AE. At least 5 min was allowed for a stable baseline to be established. In the first experiment, saponin (1 mg/ml) was included in the final washing and base-line solutions.

5.4.6 Binding of human antibodies in the biosensor

Serum dilutions were prepared in PBS/AE, except for the first experiment where saponin (1 mg/ml in PBS/AE) was used. An appropriate amount of serum dilution was introduced into the cuvette to give a final dilution as indicated in the result graphs. Data for the antibody binding were accumulated for 10 min after each serum addition. Washing the cuvette 3 times with 60 µl PBS/AE, after the final serum addition, effected the dissociation. Dissociation data were accumulated for 5 min.

5.4.7 Inhibition of antibody binding by pre-incubation with liposomes

Serum was diluted in the appropriate liposome suspension (made with PC-99) and incubated for 20 min at room temperature. A 10 µl aliquot of the mixture was then added to the cuvette and binding data were accumulated for 10 min. Dissociation was effected by washing the cuvette 5 times with 60 µl PBS/AE. Data on the dissociation were accumulated for 5 min.

5.4.8 Washing and regeneration of biosensor cuvettes

In order to regenerate the cuvette surface, the cuvette was washed five times with 50 µl 0.1 M HCl, seven times with 60 µl PBS/AE, five times with 50 µl 100 mM NaOH, seven times with 60 µl PBS/AE, three times with 50 µl 96% ethanol and finally 7 times with 60 µl PBS/AE. In the first experiment, the cuvette was regenerated after the dissociation event by washing 5 times with guanidinium thiocyanate (3.5 M), followed by extensive washing with saponin (1 mg/ml) in PBS/AE, before the second incubation with antibody at a higher concentration was introduced.

5.5 Results

IAsys biosensor cuvettes are supplied in either underivatised, or in various pre-derivatised forms. Of these, only the hydrophobic and underivatised cuvettes appeared to be suitable for coating with mycolic acids.

5.5.1 Anti-mycolic acids antibodies on a hydrophobic cuvette

The hydrophobic cuvette was considered to be suitable over the other types, based on the applicable IAsys protocol provided. The protocol supplied by the instrument manufacturer was first adapted for the purpose of this study. In order to determine the feasibility of testing anti-mycolic acids antibodies on the biosensor, the ability to differentiate between anti-mycolic acids antibodies in a tuberculosis patient serum and a negative control serum was tested.

The hydrophobic cuvette was coated as described in 5.4.5.1. The binding of antibodies to a mycolic acids-cholesterol phosphatidyl choline-60 (MA-Chol-PC60) coated surface differed between tuberculosis positive patient and tuberculosis negative control sera in accordance with their respective ELISA signal (Figure 5.5). ELISA signal to background values of patient 1799 was 6 times (with an absorbance reading of 1577) compared to 1.5 times (with an absorbance reading of 679) for the negative control serum GS8. The ELISA signal was obtained from mycolic acids coated plate and the background signal from an un coated plate.

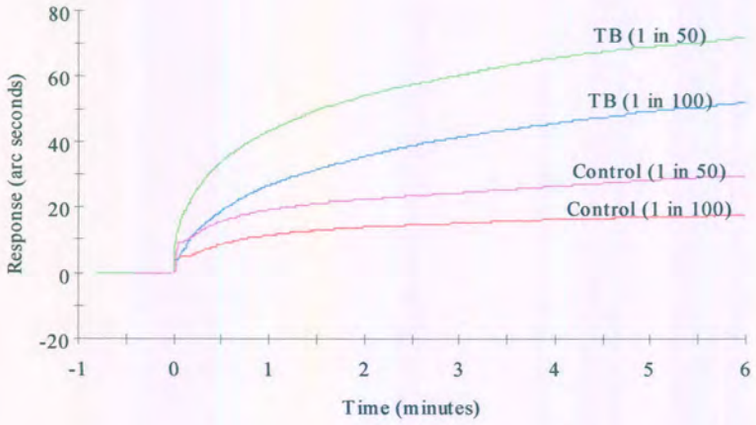


Figure 5.5: Binding profile of tuberculosis positive patient serum (TB) and negative control serum (Control) on a hydrophobic cuvette coated with mycolic acids-cholesterol phosphatidyl choline. Sera analysed were diluted 1in 50 and 1 in 100 in PBS/AE.

In spite of the difference between tuberculosis patient and negative control serum binding, it remained important to indicate the specificity of the interaction with the mycolic acids surface. This was attained through comparing the anti-mycolic acids antibody binding profiles of patient serum on a MA-Chol-PC60 and Chol-PC60 coated surfaces.

The results are indicated in Fig. 5.6. There were no obvious differences in binding of antibodies from the tuberculosis positive patient (diluted 1 in 800 PBS/AE) on either mycolic acids-cholesterol (MA-Chol-PC60), or cholesterol phosphatidyl choline 60 (Chol-PC60). There are two possible explanations for this observation: The patient serum binds non-specifically to the coated biosensor surface, or there is a cross-reactivity of binding of antibodies or serum components to cholesterol and mycolic acids.

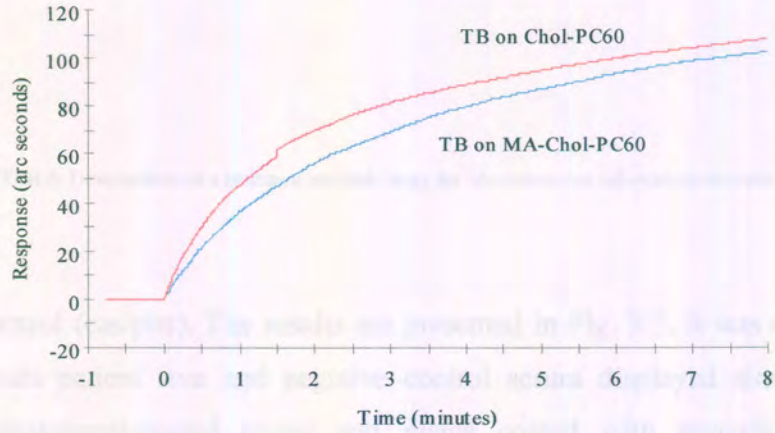


Figure 5.6: Binding profile of tuberculosis patient 1799 serum (TB) on a hydrophobic cuvette coated with mycolic acids-cholesterol phosphatidyl choline 60 (MA-Chol-PC60) or cholesterol phosphatidyl choline 60 (Chol-PC60). Sera analysed were diluted 1 in 800 PBS/AE.

5.5.2 ELISA confirmation of the cholesterol and mycolic acids cross-reactivity

Enzyme-linked immunosorbent assay (ELISA) was used to investigate a possible cross-reactivity of antibody binding between cholesterol and mycolic acids. Whereas the biosensor cannot distinguish between binding of antibodies or of other serum components, ELISA specifically reports the binding of antibodies. As patients who suffer from tuberculosis produce antibodies directed against the mycolic acids in the cell wall of *M. tuberculosis*, ELISA can detect these antibodies, as well as antibodies directed against cholesterol. The response in antigen-coated wells was compared to the response signal in control wells that had not been coated with antigen.

In this experiment, the antibody recognition of cholesterol was compared with that of mycobacterial mycolic acids within the same ELISA plate, using tuberculosis patient serum (PC se), negative control (NC se), high cholesterol patient serum (+CP se) and

casein-PBS control (cas/pbs). The results are presented in Fig. 5.7. It was observed that both tuberculosis patient sera and negative control serum displayed similar antibody binding on cholesterol-coated plates and plates coated with mycolic acids. The tuberculosis patient serum (patient 1799) was the strongest antibody signal generating serum from a group of 214 tuberculosis patients tested on ELISA plate coated with mycolic acids. This strong signal was again observed on the cholesterol-coated plates but was not observed with serum from a patient suffering from high blood cholesterol levels. This confirmed the observations made on the biosensor that there could be cross-reactivity of binding of tuberculosis patient antibodies to cholesterol and mycolic acids.

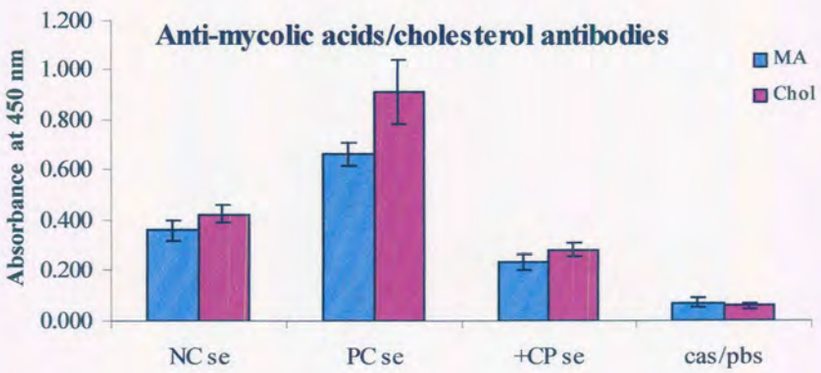


Figure 5.7: Antibodies binding profile on ELISA plates coated with either cholesterol (Chol) or mycolic acids (MA). Sera from different samples were analysed. NC se = healthy control serum, PC se = tuberculosis patient 1799 serum, CP se = cholesterol patient serum, cas/pbs = casein-PBS.

In this study, where mycolic acids were used together with cholesterol as antigens in the ELISA using tuberculosis patient as test serum, almost equal signals were produced in wells coated with cholesterol or mycolic acids alone. It was thus appropriate to look into possible ways in which mycolic acids could theoretically mimic the chemical structure of cholesterol.

5.5.3 Modeling of the possible molecular structural mimicry between cholesterol and mycobacterial mycolic acids

Based on the observations made on the biosensor and ELISA on the possible existence of molecular mimicry between mycolic acids and cholesterol, different mycolic acids folding structures were investigated. This generated a possible folded structure of the two oxygenated mycolic acids species (i.e. keto mycolic acids and methoxy mycolic acids, Figure 5.8). Only oxygenated methoxy-mycolic acids could assume a folded structure in which mimicry of cholesterol could be envisaged, with the methoxy-group of mycolic acids corresponding to the hydroxyl position of cholesterol. In its folded structure with all the oxygenated groups clustered on one side of the molecule and a hairpin bend induced by the cyclo-propane moiety of the long hydrocarbon chain of mycolic acids, a mimicry to the structure of cholesterol certainly appeared feasible. Keto mycolic acids do not mimic cholesterol as the double bonds between oxygen and carbon (red circle on Fig 5.8) prevent formation of such a structure.

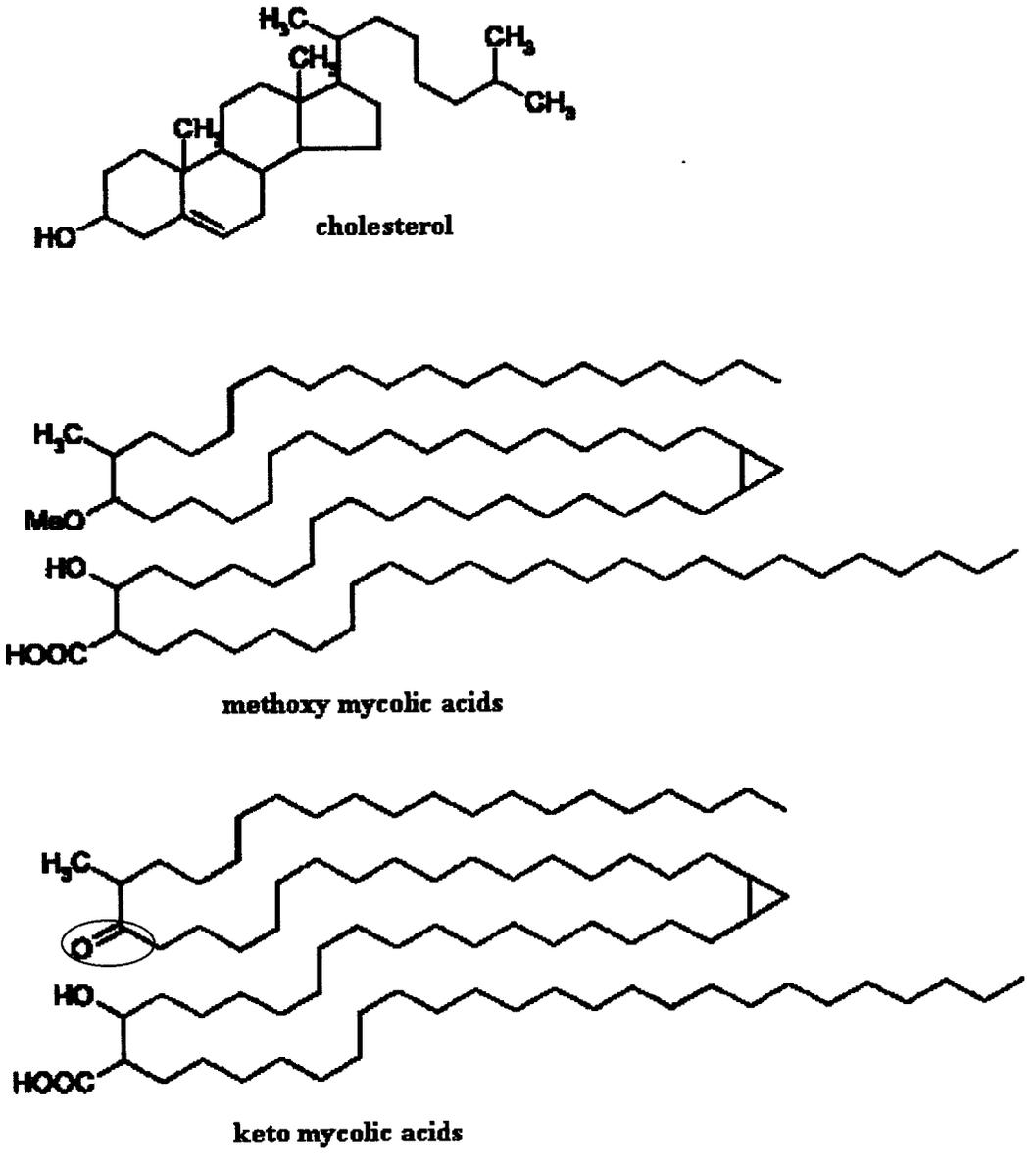


Figure 5.8: The structures representing the proposed possible molecular mimicry between the methoxy- mycolic acids and keto-mycolic acids and cholesterol

With evidence gathered so far, it became important that this cross-reactivity between mycolic acids and cholesterol be further characterised on the biosensor. The limiting factor in this regard was the instability of the available hydrophobic cuvette. The hydrophobic cuvette proved to be difficult to work with, as experiments could not be reproduced on the same cuvette after regeneration steps. Experiments could only be reproduced on new cuvettes, which are very expensive.

5.5.4 Coating the underivatised cuvettes with mycolic acids

A new approach was needed to be implemented, that would allow the repeated use of non-derivatised cuvettes coated with mycolic acids or cholesterol. The problem was solved by using a novel approach that involved the activation of the surface with a cationic detergent. The hydrophilic surface of the non-derivatised cuvette was made hydrophobic by activation with cetylpyridinium chloride (CPC), and could subsequently be coated stably with mycolic acids or cholesterol containing liposomes.

The optimum concentration of CPC was found to be around 0.02 mg/ml. The results indicated that addition of MA-liposomes before CPC addition produced inadequate binding, while addition of mycolic acids-liposomes after CPC activation showed an increase in binding of about 500% (Fig 5.9).

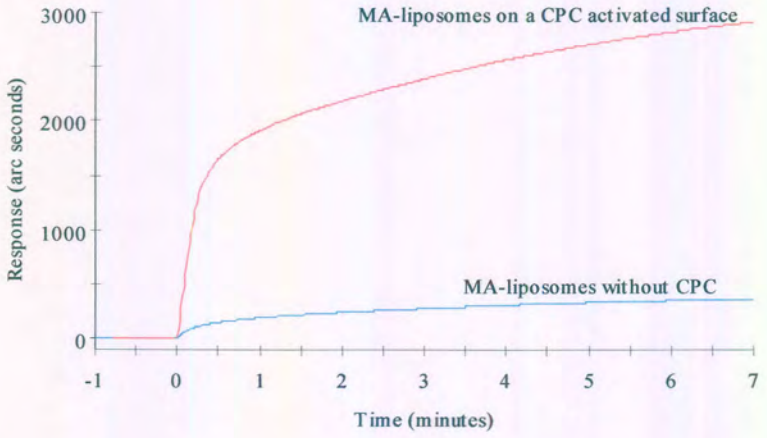


Figure 5.9: Mycolic acids-cholesterol liposomes (PC 60) coated on a non-derivatised cuvette activated with CPC (red line) or left in PBS/AE (blue line).

With the liposomes immobilised on the surface, the next step was to find the optimum concentration of a neutral surfactant (saponin) to be used to stabilise the surface and also to block. Saponin was used as it also binds cholesterol. The results indicate that titration with aliquots of a 1 mg/ml saponin solution produced saturation at around 2000 arc seconds. Titration with a higher (10 mg/ml) concentration of saponin did not have any further beneficial effects (Fig. 5.10). From this data it was concluded that an effective amount of saponin was 25 µl of a 1mg/ml saponin solution to effect optimal blocking of cholesterol containing liposome coats in underivatised cuvettes. It is expected that this optimisation needs to be done for every batch of saponin that is acquired for biosensor applications.

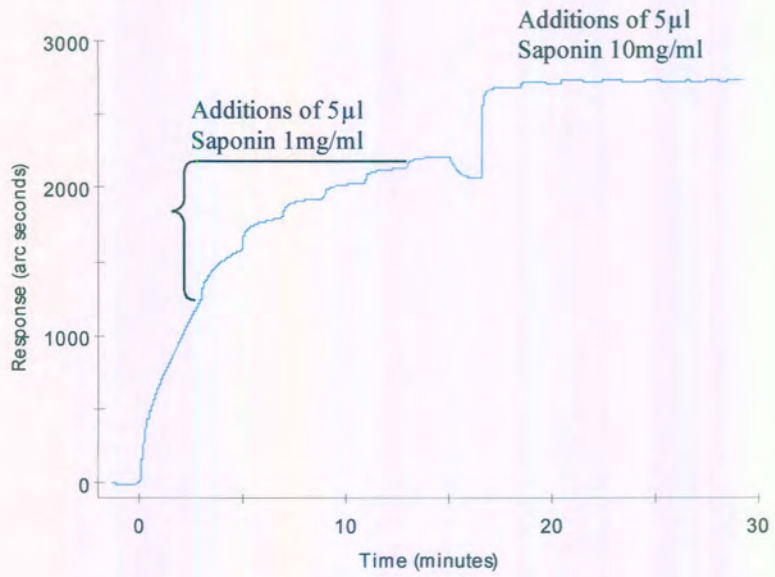


Figure 5.10: Titration of saponin on a non-equilibrated mycolic acids-cholesterol PC60 liposome surface.

With the non-derivatised cuvette coated with MA-chol liposomes, it was necessary to determine if this surface will be stable towards repeated binding cycles. The results indicated that the baseline remained stable and unchanged after repeated cycles, but repeated loading of tuberculosis patient serum at 1:200 dilution brought about decreased binding with every cycle without inflicting a drop in the baseline. Fig. 5.11 shows the decline in signal of the same serum concentrations after each regeneration step that removed the bound antibodies. It was as if repeated binding of tuberculosis patient serum of the same concentration caused the gradual depletion of mycolic acids from the surface.

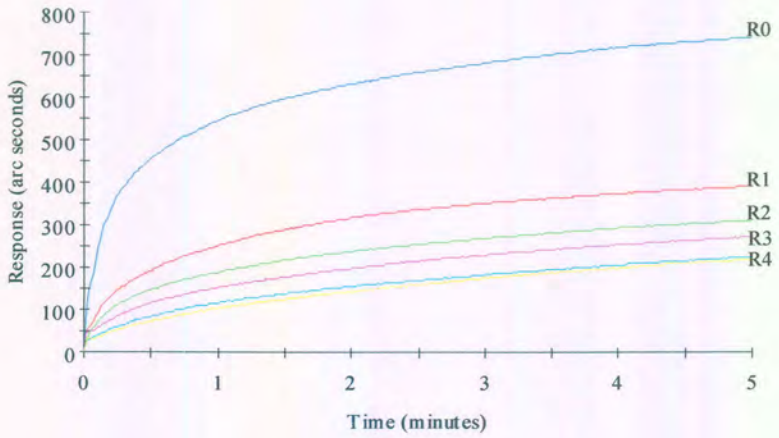


Figure 5.11: Apparent depletion of mycolic acids on the liposome-coated surface due to continued regeneration. After the first binding event (R0) depletion was measured by the declining binding signal after each acid-base regeneration cycle (R0 to R4).

The decrease in signal suggested that the affinity of the antibodies towards mycolic acids may be strong enough to effect removal of mycolic acids from the liposome coat during antibody removal at each regeneration. From these results it appeared to be essential that the surface be totally stripped after each binding and dissociation cycle and then recoated to be able to measure different samples.

Stripping of a cuvette surfaces was achieved by using washing steps that contained higher concentrations of acid (HCL 0.1M), base (NaOH 50mM), ethanol (96%, capable of removing surfactants from glass surfaces) and guanidinium thiocynate (GSCN, 3.5M) to restore the evanescent layer. It was then imperative to once again compare tuberculosis patient serum with negative control serum in this new approach towards coating the biosensor with mycolic acids containing liposomes. In these experiments the cuvette was coated with liposomes comprising phosphatidyl choline (PC-60), cholesterol and mycobacterial mycolic acids. Dilutions of tuberculosis patient 1799 (TB 1:200 and

1:800) serum displayed a stronger binding curve on this surface than similar dilutions of healthy control serum (Control 1:200 and 1:800) as shown in Fig. 5.12. This result was comparable to what was found with the hydrophobic cuvette coated with phosphatidyl choline (PC-60), cholesterol and mycobacterial mycolic acids, although the signal was here enhanced. These results were also comparable to those obtained with ELISA previously, implying that the binding to the coated biosensor surface was probably due to the binding of antibodies from the serum samples.

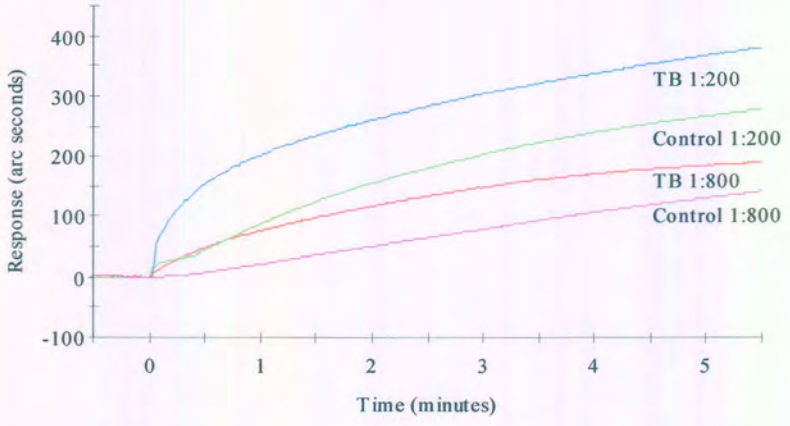


Figure 5.12: Resonant mirror biosensor binding profiles of tuberculosis patient (TB) and healthy control (Control) sera on underivatised CPC activated cuvettes coated with liposomes consisting of PC-60, cholesterol and mycolic acids. Serum dilutions are indicated on each curve.

5.5.5 Inhibition tests

Up to this stage the liposomes that were used contained cholesterol for stability. With the observations made on the apparent cross-reactivity between cholesterol and mycolic acids, the question remained as to whether anti-cholesterol antibodies can also bind to mycolic acids, i.e. whether the anti-cholesterol antibodies could be the same as those directed against mycolic acids. Inhibition tests were employed to assess the degree of cross-reactivity between cholesterol and mycolic acids. This required the use of lipomes that were free of cholesterol and that comprised of high purity of phosphatidyl choline (PC99). In order to determine the specificity of binding of antibodies to the mycolic acids coated biosensor surface, cholesterol-free liposomes were constructed for coating. These contained phosphatidyl choline (PC-99) and mycolic acids only. Tuberculosis patient 1799 serum was added in both cells of the mycolic acids coated cuvette in 1:1000 dilution to ensure that the response of the two cells were identical. Subsequently 1:100 dilution of the tuberculosis patient serum was added in both cells, the first pre-incubated with PC-99 liposomes and the second with PC-99/mycolic acids liposomes.

Pre-incubation of the patient serum with PC-99/mycolic acids liposomes resulted in an inhibition of binding to the surface, as shown in Fig. 5.13. No inhibition was seen when pre-incubation was done with PC-99 liposomes. These results provide evidence that the sera of tuberculosis patients contain antibodies that specifically recognize mycolic acids. The results also show that pre-incubation of tuberculosis patient serum with PC-99/cholesterol liposomes could not inhibit the antibody binding response on a mycolic acids-containing biosensor surface. This suggested that even though the tuberculosis patient may have produced antibodies that recognised cholesterol they have an even higher affinity for mycolic acids. Alternatively, these results may imply that the antibodies to mycolic acids are not identical to those against cholesterol.

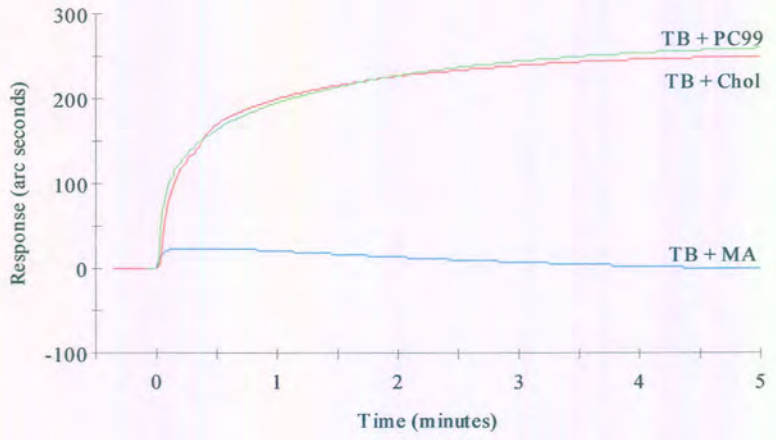


Figure 5.13: Inhibition assay of TB patient sera with mycolic acids on a mycolic acids coated surface. TB patient serum of the highest titre on ELISA was compared to the inhibitory effects of cholesterol and empty liposomes (PC99). TB + MA = TB patient serum pre-incubated with mycolic acids liposomes/PC99, TB + Chol = TB patient serum pre-incubated with cholesterol liposomes/PC99, and TB + PC99 = TB patient serum pre-incubated with empty liposomes/PC99

The specificity of the antibody-antigen interaction was tested with tuberculosis negative sera as negative control, to establish that the mycolic acids inhibition that was observed was only due to the presence of antibodies to mycolic acids in tuberculosis patients. The results indicated that when the cuvette cell was coated with mycolic acids and the negative serum was also incubated with mycolic acids, there appeared to be no inhibition (Fig.5.14). The strong binding of negative control serum to the mycolic acids coated surface can possibly be due to antibodies against cholesterol that also bind mycolic acids. The interaction between these antibodies and mycolic acids could not be inhibited by

cholesterol, arguing against such reasoning. The binding of negative control serum to mycolic acids therefore could simply be due to non-specific binding

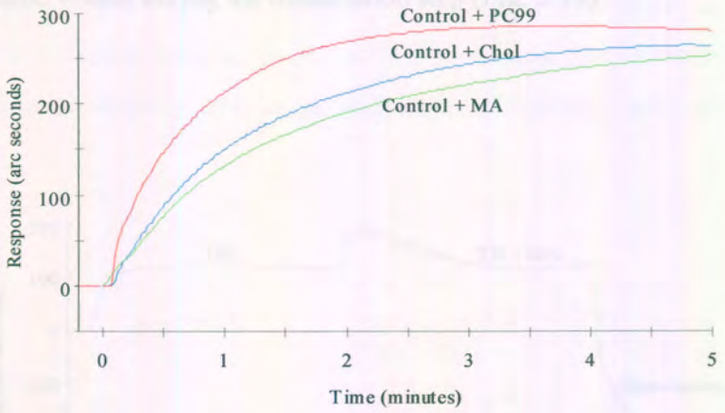


Figure 5.14: Inhibition assay of negative control sera with mycolic acids on a mycolic acids coated non-derivatised cuvette surface. The negative control serum was also pre-incubated with cholesterol and empty liposomes (PC99). Control + MA = control serum pre-incubated with mycolic acids PC99 liposomes, control + Chol = control serum pre-incubated with cholesterol PC99 liposomes, and control + PC99 = control serum pre-incubated with empty PC99 liposomes.

As the results from ELISA suggested that antibodies that recognise mycolic acids may also recognise cholesterol, the cuvette cells were also coated with cholesterol and the inhibition experiments repeated as it was done with mycolic acids coated cells. Immobilisation of cholesterol/PC99 liposomes followed by binding of tuberculosis patient sera that had been pre-incubated with mycolic acids, resulted in the inhibition of the binding. This however, did not only result in the inhibition of the binding, but also

resulted in the mycolic acids damaging the coat surface. This made it especially difficult to establish if the inhibition was specifically due to the interaction of the mycolic acids with the antibody or whether it also involved the destabilisation of the cholesterol coated surface that became visible during the dissociation step (Fig. 5.15).

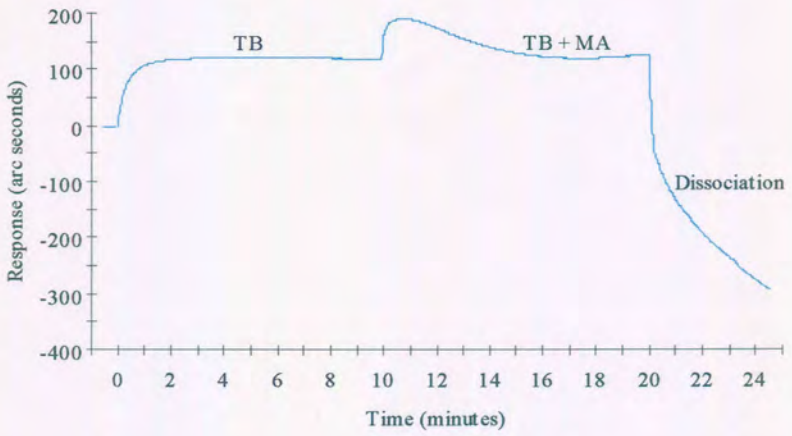


Figure 5.15: Inhibition of binding assay of tuberculosis patient 1799 serum with mycolic acids/PC99 liposomes on a cholesterol coated cuvette surface. TB + MA = TB patient serum pre-incubated with mycolic acids liposomes/PC99 and Dissociation = three times PBS/AE wash. Note the decay of the cholesterol coat by the response falling below the base-line during dissociation.

The effect that mycolic acids had on the cholesterol surface suggested that even though there was inhibition of binding, it would be difficult to interpret the result. It thus became important to investigate the possibilities of mycolic acids interacting with the liposomes bound on the biosensor surface.

5.5.6 Cholesterol-mycolic acids interactions

Addition of soluble mycolic acids/PC99 to a cholesterol/PC99 coated surface in the biosensor, revealed that there might be strong interactions between mycolic acids and cholesterol on the surface (Fig. 5.16). Addition of soluble PC99 liposomes had no such effect. The mycolic acids may have folded into structures that resemble the stacked cholesterol molecules, thereby interacting with coated cholesterol and removing it from the surface.

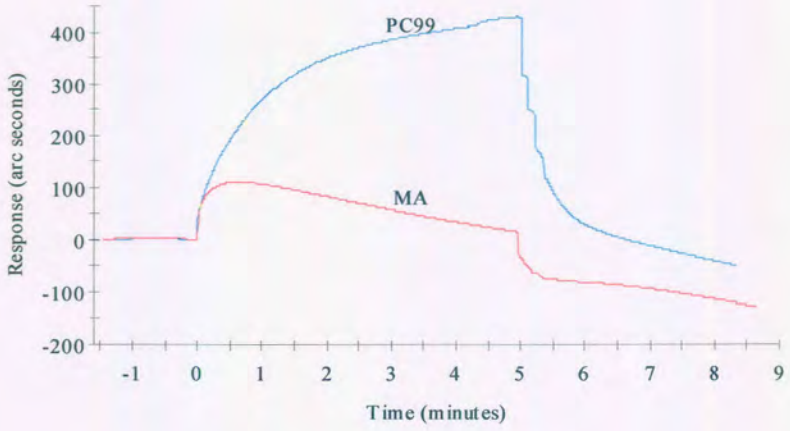


Figure 5.16: Binding assay of mycolic acids on a cholesterol-coated surface. MA= mycolic acids / phosaphatidyl choline (99 % pure) liposomes and PC99 = phosaphatidyl choline (99 % pure) liposomes.

An experiment was done to investigate if cholesterol will be able to bind to a mycolic acids surface. The results in Fig. 5.16 indicated that cholesterol goes after the mycolic acids, thereby escaping from the cholesterol coat when incubated with soluble mycolic acids, but accumulating into the mycolic acids coated surface when incubated with soluble cholesterol (Fig.5.17). This implies that pre-incubation of patient serum with

cholesterol/PC99 liposomes that may be expected to show inhibition of binding to a mycolic acids surface, if cross-reactivity between cholesterol and mycolic acids binding to the antibodies existed, may result in an artefactual positive binding curve due to cholesterol accumulation into the mycolic acids layer. The results taken together therefore argue in favour of a direct cross-reactivity of binding of antibodies to cholesterol and mycolic acids.

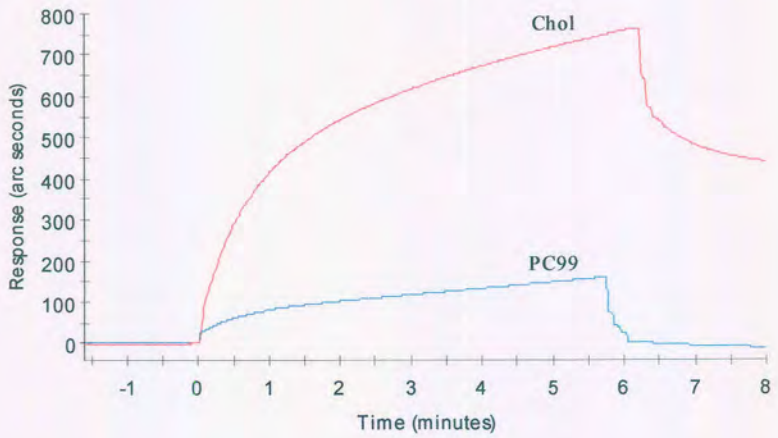


Figure 5.17: Biosensor binding profile of cholesterol on a mycolic acids -coated surface. Chol = binding profile of after addition of cholesterol/PC99 liposomes; PC99 = binding profile after adding PC99 liposomes

Data presented so far indicated that a tuberculosis patient serum that gave a high antibody binding signal to mycolic acids on ELISA could be inhibited by pre-incubation with mycolic acids. It was also indicated that there might be cross-reactivity between antibodies binding to cholesterol and mycolic acids. The question that arose from these observations was whether this could be extended to other tuberculosis patient sera that had low signal on ELISA. The results indicated that the binding of the antibodies could

still be inhibited with mycolic acids although it was not as pronounced as displayed by that of the high titre patient serum (Fig. 5.18).

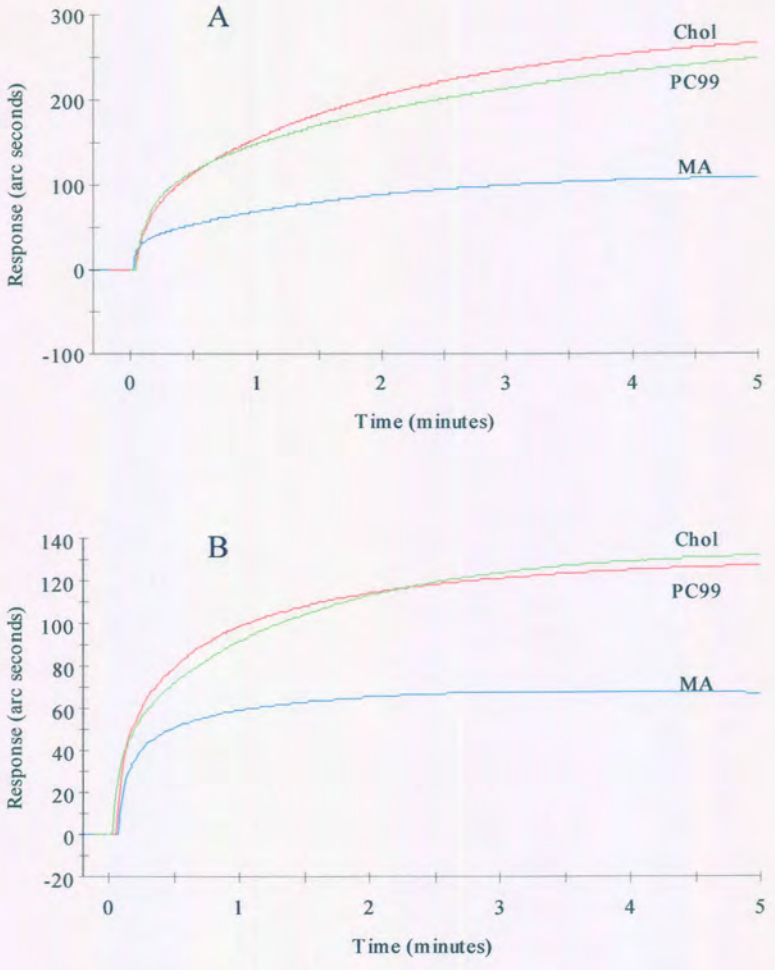


Figure 5.18: Biosensor binding profile of an inhibition assay of TB and HIV positive patient sera with mycolic acids on a mycolic acids coated surface. TB patient sera of intermediate titre on ELISA was compared with the inhibitory effects of cholesterol and empty liposomes (PC99). MA = TB patient serum pre-incubated with mycolic acids PC99 liposomes, Chol = TB patient serum pre-incubated with cholesterol PC99 liposomes, and PC99 = TB patient serum pre-incubated with empty PC99 liposomes. A= Medium titre tuberculosis patient P50 serum (relative ELISA signal = 1.23), B= lower titre tuberculosis patient P48 serum (relative ELISA signal = 0.56).

5.6 Discussion

Tuberculosis patients produce antibodies directed against *Mycobacterium tuberculosis* mycolic acids (Pretorius, 1999; Pan *et al.*, 1999). Mycolic acids constitute the most abundant lipids in the cell wall of *Mycobacterium tuberculosis*. These antibodies were detected by an enzyme-linked immunosorbent assay (ELISA). The response in antigen-coated wells was compared to the response signal in control wells that had not been coated with antigen. The ability to detect antibodies directed against mycolic acids using ELISA prompted further characterization of the binding of these antibodies. Because the biochemical reactions between antigen and antibody are normally reversible, the association and dissociation could then be determined in real-time using a resonant mirror biosensor. Because of the hydrophobic nature of the antigen, the first challenge was to find and develop a suitable surface on the biosensor cuvette. After several attempts on the hydrophobic biosensor surface using standard protocols, the underivatised surface was found to be suitable for innovating a homogenous mycolic acids coat after activating the surface with a cationic detergent and coating with mycolic acids containing liposomes. Both cholesterol/PC60 and mycolic acids/cholesterol/PC60 containing liposomes coated cuvette surfaces produced similar binding profiles with tuberculosis patient serum. This corroborated the results found with ELISA that mycolic acids specific antibodies were present in tuberculosis patients.

In this study, it was discovered by serendipity that there could be a relationship between mycolic acid and cholesterol structure, as antibodies binding to mycolic acids were found to also bind cholesterol. ELISA determination confirmed that these binding results wer due to antibodies specifically binding to mycolic acids and cholesterol thereby pointing to a possible molecular mimicry between mycolic acids and cholesterol. Theoretical folding of mycolic acid structure indicated that methoxy mycolic acids could take on a form that resembled cholesterol. This agrees with the observations made by Pan *et al.* (1999) that tuberculosis patients have antibodies that specifically have affinity for methoxy-, but not for keto- oxygenated mycolic acids, nor non-oxygenated α-mycolic acids.

On the basis of these results it is proposed that mycobacterial mycolic acids may show a structural mimicry with cholesterol, such that antibodies directed against mycolic acids can also recognize cholesterol. In support of this, immobilized mycolic acids were found to capture cholesterol from dissolved cholesterol liposomes in the biosensor, while cholesterol was withdrawn from the cholesterol coated surface when exposed to dissolved mycolic acids liposomes thereby demonstrating the attraction of cholesterol to bind to mycolic acids. This opens the possibility that mycobacteria may accumulate cholesterol from the host body fluids and that the mycolic acids cholesterol sandwich may present itself for binding to cell-receptors and antibodies. Interestingly, although cholesterol could not inhibit the binding of tuberculosis patient antibodies to a mycolic acids coated biosensor surface, the reaction could actually have remained invisible due to the concomitant accumulation of cholesterol into the mycolic acids-liposome coated cuvette surface.

Altogether, the results obtained with the biosensor experiments come up in support of a structural mimicry between mycolic acids and cholesterol. This conclusion relies on the observation that cholesterol serves well as an immobilised binding ligand for anti-MA antibodies. The binding of antibodies to mycolic acids can be prevented by pre-incubation of the patient serum with mycolic acids-liposomes, confirming the specificity of the antibodies. Additional experiments may be required to provide absolute proof of specificity of binding of antibodies to mycolic acids. Lipoproteins complexed with antibodies may hydrophobically associate with mycolic acids or cholesterol coat, thereby giving only apparent specificity of binding. Although unlikely, this possibility cannot yet be totally excluded.

The results obtained in this study open up new insight into a possible way of infection by *M. tuberculosis*. A number of recent studies have indicated that cholesterol is important for mycobacterial entry into the macrophages (Av-Gay and Sobouti, 2000; Gatfield and Pieters, 2000; Pieters and Gatfield, 2002). Pathogenic *M. tuberculosis* was found to

require cholesterol not only as a carbon source but also for mycobacteria to be able to infect cells. In this study cholesterol has been shown to interact with mycolic acids, suggesting a possible mechanism involved in mycobactrial entry into macrophages.

Based on these data we hypothesise that mycobacteria accumulate cholesterol by a molecular attraction between mycobacterial mycolic acids and cholesterol, thereby mimicking the structure of oxidised LDL. In this way, *M. tuberculosis* may facilitate their uptake into the host macrophage via attachment to type A scavenger receptors. The proposed molecular attraction between the mycobacterial mycolic acids and the cholesterol in the host cell membrane may aid in the engulfment of the mycobacterium via a "lipid zipper"-like mode of action (Gatfield and Pieters, 2000). The molecular association between mycobacterial mycolic acids and membrane cholesterol may also explain why the membrane of the phagosome appears to be tightly associated with the engulfed mycobacterium (de Chastellier *et al.*, 1995; de Chastellier and Thilo, 1997). Because the mechanism of cholesterol involvement has not been fully elucidated, further investigations into the interactions between cholesterol and mycolic acids may shed new light in the development of new anti-tuberculosis drugs.

CHAPTER 6

General Discussion

Tuberculosis has been the scourge of mankind since the beginning of human history. This is mainly because *M. tuberculosis* can persist inside the human body without causing disease. The major reasons for the unabated spread of tuberculosis can be identified as insufficient protection offered by the world-wide vaccination programme based on the use of BCG, problems associated with the detection of tuberculosis, problems associated with treatment of tuberculosis and the occurrence of multi-drug resistant strains of *M. tuberculosis*, interaction with HIV infection, and socio-economic aspects. These problems call for a fresh approach to control the tuberculosis problem.

Raffel *et al.*, had in 1949 for the first time showed that the immunogenicity of mycobacterial cell wall can be extended to mycobacterial lipids. Most mycobacteria are endowed with specific, highly antigenic glycolipids that are immunogenic like the phenolic lipids of *M. leprae* and *M. bovis*, the peptidolipids of the *M. avium*, and the acylated trehalose-containing lipooligosaccharides of species such as *M. kansasii*, *M. szulgai*, and *M. malmoense*. A search for analogous structures in *M. tuberculosis* has revealed antigenicity in diglycosyl-diacylglycerol and the immunogenic phosphomannoinositides. The dominant carbohydrate-containing antigen of *M. tuberculosis* (responsible for the high-titer anti-arabinofuranosyl activity in tuberculous sera) is lipoarabinomannan, which has been purified in the native state from *M. tuberculosis* and shown to contain both phosphatidylinositol and phosphoinositol side-branches (Brennan, 1989; Barry *et al.*, 1998; Barry, 2001). Trehalose dimycolate (cord factor from *M. tuberculosis* cell wall) was found to produce extensive granuloma formation in mice (Bekierkunst *et al.*, 1969). Tuberculosis patients were also later found to produce antibodies to cord factor (Kato, 1972). This confirmed that the complex *M. tuberculosis* cell wall lipids contribute to the outcome of the dialogue between the pathogen and the host.

The cell wall of *Mycobacterium* is a very complex structure that accounts for the spore-like properties of the bacterium (Brennan and Nikaido, 1995; Daffe and Draper, 1998; Barry, 2001). In particular mycolic acids exist as the most abundant cell wall lipid in *M. tuberculosis* with many biological functioning other than simply providing a protective layer. In 1994, the group of Michael Brenner in Boston (USA), found that mycolic acids evoked an immune response through presentation on human CD1b (Beckman *et al.*, 1994). Since then the group in Boston has focussed their attention on the further characterisation of antigen presentation on CD1 molecules.

In this laboratory the focus fell on various applications of mycolic acids in fields including immunotherapy and tuberculosis diagnosis. The first challenge was to obtain purified mycolic acids in large quantities. This hurdle was crossed using a liquid-liquid extraction process on a counter current distribution system (Siko, 1999; Goodrum *et al.*, 2001). Experiments preformed on cell cultures, mice, rats, and tuberculosis patient serum suggested that:

- Mycolic acids pre-treatment of *M. tuberculosis* infected mice enhanced survival (Lombard, 2002)
- Mycolic acids pre-treatment of *M. tuberculosis* infected mice induced expression of IL-12 and IFN-γ in the lungs (Pretorius, 1999)
- Administration of mycolic acids to uninfected mice induced expression of IL-12 and IFN-γ in the lungs (Pretorius, 1999)
- Administration of mycolic acids to Sprague Dawley rats elicited anti-mycolic antibodies (Siko, 1999; Ten Bokum *et al.*, 2002 submitted)
- Mycolic acids pre-treatment prevented the onset of heat-killed *M. tuberculosis* induced adjuvant arthritis in Lewis rats (Siko, 1999; Ten Bokum *et al.*, 2002 submitted)
- Treatment with mycolic acids induced proliferation of CD4/CD8 double negative T cells (Goodrum *et al.*, 2001)

- Tuberculosis patients express antibodies against mycolic acids (Pretorius, 1999; Schleicher *et al.*, 2002)

With such overwhelming evidence on the potential of mycolic acids to be of benefit in the medical field and to elicit immune responses, the question remained about the mechanism of mycolic acids action.

6.1 Mycolic acids in murine tuberculosis

Previous studies indicated that protection against tuberculosis provided by mycolic acids could be due to production of pro-inflammatory cytokines such as IFN-γ and IL-12 in the lungs and not in the spleen (Pretorius, 1999). Induction of these cytokines is normally associated with a shift of the immune system towards a Th1 mode (Mossmann and Coffman, 1989). In these studies, an unequivocal shift in the Th1/Th2 balance was not detected in either the spleen or the lungs (Siko, 1999). The animals for these studies were infected intravenously, leading to systemic infection and cytokines were determined by means of end-point PCR. Because a response to mycolic acids administration was prominent in the lungs, the experiment was repeated, but this time expression of cytokines were analysed after the intranasal route of infection to introduce *M. tuberculosis* directly into the lungs of mycolic acids pre-treated animals. In addition, real-time quantitative PCR was compared with semi-quantitative endpoint PCR. The results indicated that expression of pro-inflammatory cytokines IL-12, IFN-γ, and TNF-α in mice that were pre-treated with a sub-optimal dosage of mycolic acids (5μg) was inhibited. This was not directly in agreement with the survival data observed. The optimum dose of mycolic acids (25μg/mouse) maintained the expression of pro-inflammatory cytokines to the same level as in *M. tuberculosis* infected mice that did not receive mycolic acids, even though these levels were generally higher than those of the animals receiving 5 μg/mouse. Compared to other studies where systemic *M. tuberculosis* infection was established via intravenous infection, mycolic acids pre-treatment did not enhance IL-12 or IFN-γ expression.

The pre-treatment of mice with 25 μg mycolic acids/mouse correlated with an enhanced (90%) protection of mice against subsequent disease progression over the 40 weeks following *M. tuberculosis* infection. Although, a significant expression of IL-12 in mycolic acids treated, uninfected mice were detected compared to untreated-uninfected mice the IL-4/IL-12 and IL-4/IFN-γ ratios clearly excluded any shift towards Th1. This indicates that mycolic acids may contribute to the expression of pro-inflammatory cytokines, but the protective mechanism involved is not simply a shift towards Th1 orientation. Flynn *et al.* (1995) indicated that with IL-12 administration, enhanced survival could be attributed to a shift towards a Th1 mode in the lungs. Although mycolic acids were also able to elicit an IL-12 expression response, the mechanism of induced protection was clearly different and did not rely on subsequent IFN-γ production in the lungs. Rather, an effect of mycolic acids on macrophages appeared to contribute to protection. Stoltz (2002) explored the possible mechanism of mycolic acids action and found that in the peritoneal macrophages, mycolic acids appeared to have interesting effects. The observations made were that peritoneal macrophages were not activated according to the classical pathway, which only involved the Th1 cytokines but rather were activated via the alternative pathway which also involved Th2 cytokines. In these studies mycolic acids were shown to induce IL-10. An alternative macrophage activation response to mycolic acids in the peritoneum was in contrast with what was previously observed in the lungs (Pretorius, 1999) and requires further investigation with alveolar macrophages.

6.2 Mycolic acids in immunocompromised mice

C57Bl/6 mice are more resistant to *M. tuberculosis* infection than Balb/c mice. Previous studies indicated that mycolic acids pre-treatment of Balb/c mice intravenously infected with *M. tuberculosis* brings them to the level anti-tuberculosis resistance of C57Bl/6 mice (Siko, 1999). Mycolic acids administration were however shown to have no significance effect on the survival of C57Bl/6 mice. C57Bl/6 mice were shown to have a high base

level of IL-12 in the lungs (Pretorius, 1999). The observation that mycolic acids brought the resistance of Balb/c mice to that of the level of C57Bl/6 mice, inferred the possibility that if C57Bl/6 mice could become susceptibile to *M. tuberculosis* infection then mycolic acid pre-treatment may restore resistance. This aims at the possibility of enhancing the innate immune response to control tuberculosis in HIV co-infected individuals. Like C57Bl/6 mice, humans are significantly resistant to *M. tuberculosis* infection. Of persons infected with *M. tuberculosis*, only 10% develop tuberculosis. Infection with HIV renders them much more susceptible to disease progression upon infection with *M. tuberculosis.*

Infection of C57Bl/6 mice with LP-BM5 murine leukaemia virus renders them immunodeficient, with symptoms similar to those observed in humans that have developed AIDS. For this reason this model of C57Bl/6 mice infected with LP-BM5 MuLV has been termed murine AIDS (MAIDS) and has been extensively used in anti-retroviral drug research. Ian Orme and co-workers (1992) indicated that *M. avium* infection is increased when MAIDS developed in C57Bl/6 mice. The MAIDS model was therefore chosen to investigate the potential benefits of mycolic acids pre-treatment in LP-BM5 infected C57Bl/6 mice before infection with *M. tuberculosis.*

In this study, co-infection of C57Bl/6 mice with LP-BM5 MuLV and *M. tuberculosis* manifested itself as a realistic model as there was increased susceptibility to tuberculosis disease progression compared to animals that were mono-infected. This suggested that the model could be useful as a valuable tool in studying tuberculosis in an AIDS environment, aiming at a better control of tuberculosis in sub-Saharan Africa.

This study provided an unexpected outcome: Unlike with Balb/c mice, the effects of mycolic acids administration in C57Bl/6 mice were not observed in the lungs but were now observed in the spleens. Mycolic acids induced expression of IL-12 in the spleens of mice that were co-infected with LP-BM5 MuLV and *M. tuberculosis*. This induction was however maintained to the level of uninfected mice. There was no observable effect on the level of IFN-γ expression. There was also a down-regulation of IL-10 expression in these animals suggesting that mycolic acids induced a shift towards pro-inflammatory

cytokine expression as opposed to anti-inflammatory cytokine expression. Anti-inflammatory/pro-inflammatory cytokine ratios emphasised that mycolic acids did enhance the expression of pro-inflammatory cytokines. This trend was apparent in both IL-10/IL-12 and IL-10/IFNγ ratios in the spleens and suggested that mycolic acids induce a pro-inflammatory cytokine response in C57Bl/6 mice, which was, however, not enough to uphold innate protection in immunocompromised animals.

6.3 Immunotherapy with mycolic acids

The major problem with tuberculosis chemotherapy is that it should be maintained without interruption for a period of 6 to 9 months. Lack of compliance results in multi-drug resistant strains. Studies have focused on combining chemotherapy with immunotherapy to combat this problem. Because mycolic acids have been shown to induce expression of IL-12, this study focused on applying this property of mycolic acids as a potential immunotherapeutic support treatment in combination with chemotherapy. Studies have indicated that administration of IL-12 alone is not sufficient to control *M. tuberculosis* infection, but IL-12 in combination with chemotherapy was successful (Flynn *et al.*, 1995; Lowrie *et al.*, 1999). Similarly, mycolic acids as such could not be used independently to cure tuberculosis. In Balb/c and C57Bl/6 mice already infected with *M. tuberculosis*, treatment with mycolic acids had no observable effects (Siko, 1999).

When Balb/c mice were infected with *M. tuberculosis* and the animals were subsequently treated with chemotherapeutic drugs as well as mycolic acids, no improved sterilisation of the lungs in terms of growth of *M.tuberculosis* could be achieved, in comparison to mice that only received chemotherapy with isoniazid, pyrazinamide and rifampicin. There could be any, or a combination of several explanations for the negative outcome of this experiment:

- Mycolic acids may not have induced IL-12 at sufficient levels of expression to effect the same support for chemotherapy as was obtained with the IL-12 gene therapy repoted by Lowrie *et al.* (1999).
- Mycolic acids administration may have induced biological effects in addition to IL-12 expression that could have antagonised the effect of IL-12, such as IL-10 secretion or the activation of a type of macrophage that could antagonise the effect of IL-12 (Stoltz, 2002).

Taken together, the potential of mycolic acids to be applied as an immunotherapeutic support for chemotherapy against tuberculosis found no support in the mouse experiment performed here.

6.4 Mycolic acids in a biosensor based serodiagnostic assay for tuberculosis

The tuberculosis epidemic is aggravated by lack of fast and reliable methods for diagnosing *M. tuberculosis* infection. In 1999, Pan *et al.* indicated that oxygenated mycolic acids are recognised by antibodies in tuberculosis patients in an ELISA assay, and that such antibodies may act as surrogate markers for infection with *M. tuberculosis*. Schleicher *et al.* (2002) from our group, reported however that the predictive value of such an assay was poor, when tested among South African patients. It thus became important to understand the mechanisms of interactions between specific patient antibodies and mycolic acids. The light evanescent biosensor appeared well suited to offer fast and reliable information on this as it measures the interaction of molecules in real time. Mycolic acids are highly insoluble in a number of solvents and this provided a challenge in first finding a suitable surface for analysing the interaction of mycolic acids and their antibodies. This study envisaged developing such a surface to measure interactions between antibodies and the mycolic acids immobilised in the cells of a biosensor cuvette.

A non-derivatised biosensor cuvette was selected to immobilise mycolic acids, after activation of the surface with a cationic surfactant. Mycolic acids were deposited on the

surface using cholesterol-containing liposomes. The coat was blocked with a neutral surfactant. A binding profile was established from a tuberculosis patient serum that differed from negative control serum on a mycolic acids coated surface. The anti-mycolic acids antibodies could be inhibited by pre-incubation with mycolic acids indicating specificity of these antibodies. Interestingly a similar binding profile was established on a control surface coated with cholesterol liposomes that contained no mycolic acids. Binding of patient antibodies to cholesterol was confirmed with ELISA to be an antibody antigen interaction. This suggested that both mycolic acids and cholesterol may act as ligands for antibodies in patient and control sera, and that there may even be a degree of cross-reactivity between mycolic acids and cholesterol towards antibody recognition.

Cross-reactivity between cholesterol and mycolic acids appeared not to be far-fetched according to theoretical modelling of folded mycolic acids and cholesterol structures. The insight obtained by modelling could even explain why Pan *et al.* (1999) had found oxygenated mycolic acids to be the specific ligand for anti-mycolic acids antibodies. The cross-reactivity between cholesterol and mycolic acids as ligands for anti-mycolic acids antibodies require more rigorous testing with antibody binding assays other than ELISA and the biosensor, which rely on ligand-coated surfaces of a hydrophobic nature. Final proof of specificity and cross-reactivity will require determination of the affinity of these ligands with purified immunoglobulins from patient sera. This will be addressed by other researchers on this project.

6.5 Conclusion

The cholesterol-mycolic acids mimicry observed with biosensor analysis provides some tantalizing possibilities and explanations of recent observations: During the year 2000 a number of reports appeared in the scientific literature implicating cholesterol in the survival of pathogenic *M. tuberculosis* in macrophages *in vitro*. Av-Gay and Sobouti (2000) in their observations suggested that cholesterol might have a role in mycobacterial infection other than as a carbon source. Gatfield and Pieters (2000) reported that

cholesterol depletion of macrophages blocked mycobacterial entry. These observations suggested a crucial role played by cholesterol during infection.

In previous studies it was shown that mycolic acids could not protect mice against tuberculosis if administered after infection but was only effective when administered as a pre-treatment before *M. tuberculosis* infection (Siko, 1999). Protection rendered by mycolic acids could be due to their blocking of the scavenger receptor (SR). Macrophages express SRs that are able to bind oxidised low-density lipoproteins (LDL). Low-density lipoproteins are rich in cholesterol. Scavenger receptors derive their names from the fact that they bind a variety of unrelated ligands. The SRs were reported to be the most important cell surface receptors on the macrophage involved in the binding and subsequent entry of *M. tuberculosis* (Zimmerli *et al.*, 1996). The possible role played by mycolic acids in the protection against tuberculosis could be through binding the SRs and thereby preventing the adherence of the bacterium. Mycolic acids may also have biological effects that may explain why there is a narrow window of dosage of mycolic acids where they are protective. Concentrations of mycolic acids higher or lower than 25μg were previously observed to have no protective effect (Siko, 1999).

A different perspective on the possible mechanism of mycolic acids protection is provided by the observations that mycolic acids pre-treatment induced pro-inflammatory cytokine expression in the lungs of *M. tuberculosis* mice, without changing the T-heper bias. This protection could be through activation of macrophages. Lower concentrations of mycolic acids resulted in suppression of these cytokines and rendered the animals more susceptible to disease progression than untreated *M. tuberculosis* infected mice. One possible explanation for the narrow window of dosage affectivity was provided by Stolz (2002): He found that mycolic acids may render the macrophages super-targets by inducing a cholesterol hunger that prepared the macrophages for *M. tuberculosis* infection. Higher doses were however enough to evoke protective cytokine responses and thus induce the bactericidal activity of macrophages, while overdoses may block the bactericidal activity similar to high dose antigen tolerance in activation of B-cells.

Another view could be that mycolic acids are involved in determining the critical macrophage membrane lipid structural transition by association with cholesterol. In this case the lower dose of mycolic acids may render the macrophage super-targets of *M. tuberculosis*, the optimal dose of mycolic acids could then be protective, and a higher dose could induce a stage where the macrophage becomes paralysed to respond to infection.

In more practical terms, a molecular mimicry between mycolic acids and cholesterol as derived from the results with the biosensor may create the possibility of synthesising a more effective drug that targets the cholesterol, by binding to the mycolic acids surrounding the invading bacterium. This study opens the possibility of designing a compound comprising known organic compounds that bind to cholesterol, cholesterol-containing molecular complexes or chemical entities with cholesterol structural mimicry, linked to organic compounds with anti-mycobacterial or anti-inflammatory or anticoagulant properties via a weak covalent bond or a strong non-covalent bond, which should slowly dissociate in an aqueous environment. The cholesterol-binding organic compound can also be linked to a reporter molecule for the purpose of selectively binding to the infectious agent, thereby allowing its detection. These options may provide a worthwhile approach to future research that may contribute to controlling tuberculosis in the world and specifically in sub-Saharan Africa, where the disease is so dramatically aggravated by AIDS.