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The Effects of Mycobacterial Mycolic Acids on Rodent Tuberculosis and Adjuvant Arthritis

by

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"Morena ke modisa wa ka, ga nkitla ke tlhoka sepe"

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List of abbreviations

AA	Adjuvant arthritis
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BCG	Bacillus Calmette Guerin
CA	Collagen induced arthritis
CCD	Countercurrent distribution
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CFU	Colony forming units
CI or II	Collagen type I or II
CMI	Cell mediated immunity
CTLs	Cytotoxic T lymphocytes
dddH_2O	Double distilled deionized water
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DN-T cell	Double negative T cell
DOTS	Directly observed treatment short-course
DTH	Delayed type hypersensitivity
ELISA	Enzyme linked immunosorbent assay
FA	Fatty acids
FIA	Freund's incomplete adjuvant
GC	Gas chromatography
GM-CSF	Granulocyte/monocyte colony stimulating factor
HIV	Human immune-deficiency virus
HPLC	High performance liquid chromatography
HSP	Heat-shock protein

Ig	Immunoglobulin
IFN	Interferon
IL	Interleuken
IRMA	Immunoradiometric assay
IS	Internal standard
KCl	Potassium chloride
LPS	Lipopolysaccharide
Mϕ	Macrophage
MA	Mycolic acids
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MOTTS	*Mycobacteria* other than tuberculosis
NaCl	Sodium chloride
NK	Natural killer
NOS	Nitric oxide synthetase
OA	Oil induced arthritis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
rRNA	Ribosomal RNA
RT	Reverse transcriptase
TB	Tuberculosis
T_H	T helper

TNF	Tumour necrosis factor
T-PBL	Peripheral blood T-lymphocyte
SCW-A	Streptococcal cell-wall induced arthritis
WHO	World Health Organization

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CHAPTER 1

Introduction

Tuberculosis - a disease

Records indicate that tuberculosis is of ancient origin. Skeletal remains of prehistoric humans dating back to 800 BC gave clear evidence of the disease. Egyptian skeletons dating back from 2500 to 1000 BC have shown that they had Pott's disease, a result of tuberculosis infection (Stead Dutt, 1994).

Although tuberculosis can affect any organ of the body, the lung is always the port of entry. The disease could result in fatigue, sweating and coughing, which in turn could lead to further transmission. Tuberculosis can thus be considered an airborne disease due to droplet-nuclei infection. Other methods of transmission are rare. Infection through handling contaminants is a common problem. Infection can occur through introduction of the bacilli through the skin. This was observed among laboratory workers who handled infected tissue and cultures. Books, clothes, bedding and eating utensils are not involved in the spread of tuberculosis.

Tuberculosis is still the major cause of death and accounts for about 25% of adult deaths in the developing world, a huge number compared to that caused by the combined contribution of diarrhoea, malaria and AIDS (Steyn, 1996). South Africa has one of the highest incidences of tuberculosis: more than 200 of every 100 000, hence about 35 people die of tuberculosis everyday in South Africa (Steyn, 1996).

The population dynamics of infection by tuberculosis takes the form of an "epidemic wave". Usually there is a sharp increase of infection running up to a peak followed by a gradual descent. This wave form of tuberculosis occurs by natural selection of susceptible persons' infections and has been established by following its course over 300 years, as illustrated by Grigg (Fig. 1)

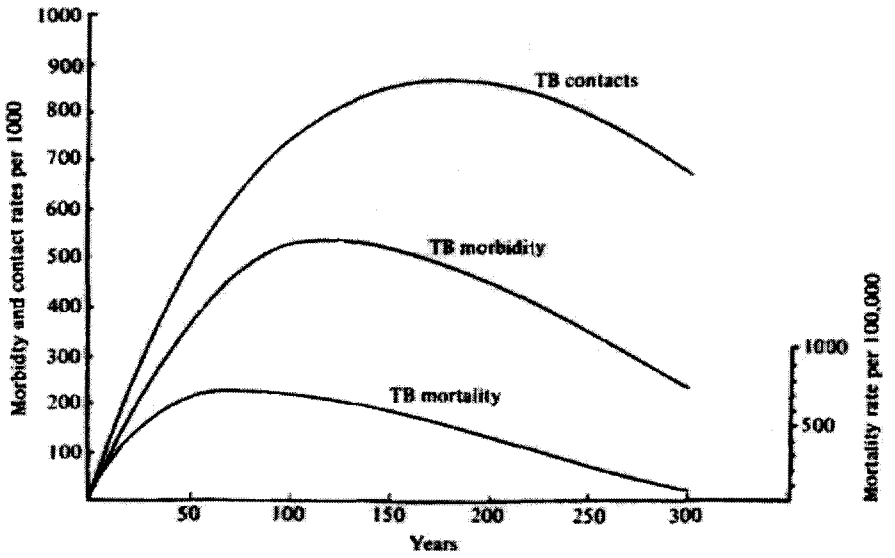

Fig.1: Theoretical concept of the development of a tuberculosis wave in a community (Grigg, 1958).

The monitoring and control of tuberculosis in developing countries is of great importance for the developed world, as the disease poses a threat due to the occurrence of multi-drug resistant *Mycobacteria* and additional spread through AIDS (Grange and McIntyre, 1979).

There is no simple diagnostic test for tuberculosis. Assessment of diagnostic tests requires careful consideration of their potential role and usefulness, as tuberculosis is easily mis-diagnosed. A simple, reliable, inexpensive test could be of great benefit. In poor countries the main priority is to block the transmission of the disease. Transmission can be reduced by finding the infectious cases timely and ensuring that there is sufficient medical care.

South Africa is burdened by one of the worst tuberculosis epidemics in the world, with rates more than double those observed in other developing countries. In 1998, tuberculosis cases in the country are expected to be between 154 000 (best scenario) and 195 800 (worst scenario), or between 351 and 446 per 100 000 of the total population. Of these 34% are expected to be also infected with HIV. Tuberculosis has been declared a top health priority by the South African Department of Health in November 1996 and the Minister of Health committed the Department to implement a new control programme based on the directly observed treatment short-course (DOTS) of the World Health Organisation (WHO) (Fourie and Onyebujoh, 1998).

Another important reason for the current failure to control tuberculosis is that even when the best available chemotherapy is used, treatment must be continued for at least six months. In countries like South Africa, this is not a practical proposition, because the patients feel well after a few weeks and, in the absence of proper health care, they stop taking the drugs. The solution is to implement the DOTS strategy, whereby the patient is supervised while taking every dose.

There is a good reason for the six months regimen. The chemotherapy kills the vast majority of bacteria in a few days, but resistant bacteria that are presumably not replicating are not killed by the drugs (Grange, 1992; Rook and Hernandez-Pando, 1996). Non-compliance with the six months treatment regimens leads to the emergence of multi-drug resistant (MDR) strains of tuberculosis causing organisms. This in turn requires extension of therapy with expensive drugs which will at best cure only half of them. Very few countries can afford this financial load.

The pathogen: *Mycobacterium tuberculosis* and related species

There are over 70 species of *Mycobacteria*. Of these, two are major pathogens: *Mycobacterium tuberculosis* discovered by Koch in the year 1882 and *Mycobacterium leprae* discovered by Hansen in the year 1874. The remaining *Mycobacteria* are environmental organisms, collectively known as MOTTS (*Mycobacteria* Other Than Tuberculosis) (Grange and McIntyre, 1979). *M. tuberculosis*, *M. avium*, *M. intracellulare*, and *M. kansasi* species account for about 90% of the potentially pathogenic *Mycobacteria* and 71% of the total *Mycobacteria* recovered in health laboratories in the US.

The *Mycobacterium tuberculosis* complex of organisms consists of the species *M. tuberculosis, Mycobacterium africanum, Mycobacterium bovis,* and *Mycobacterium necroti* (the last three are now considered to be sub-species of *M. tuberculosis*). *M. tuberculosis* can infect a wide variety of animals, of which man is the primary host. *Mycobacterium tuberculosis* is a slender, non-motile, non-encapsulated, straight or slightly curved bacillus, which does not form spores. *M. tuberculosis* is also an acid fast bacillus, aerobic, slow growing, sensitive to heat and resistant to drying and chemical disinfectants. The majority of species assigned to the genus *Mycobacterium* do not cause disease (Steyn, 1996).

All *Mycobacteria* are acid fast, *(i.e.* they do not decolour with acid and alcohol once stained with arylmethane dyes), aerobic, contain mycolic acids and have a 59-65% GC content in their genomes. The chromosome is not surrounded by a nuclear membrane but wrapped into a nuclear body, which justifies the classification of *Mycobacteria* as prokaryotes. The shape of

mycobacterial cells differ from species to species and even between individual strains depending on the growth conditions. Cells of *M. avium* may be coccoid while those of *M. kansasi* are often elongated (Grange, 1988).

Mycobacteria are different from other bacteria in that they have a complex cell wall that contains mycolic acids which proved to be resistant to heat break down due to their α-branched and β-hydroxylated chains. Anderson (1929) and Etemadi (1967) found that mycolic acids required 300 °C to be pyrolytically broken down to fatty acids. This contributes to the characteristic toughness of the cell wall of *Mycobacteria*.

Host-parasite interaction

M. tuberculosis elicits a series of battles between the host and the parasite. In this war the host's weapons are: the activated macrophage (a phagocyte powerful enough to kill or inhibit the tubercle bacilli that it ingests) and the ability to stop the intracellular growth of the parasite in the macrophage by killing the infected macrophage. The bacillus' weapons in this war are: logarithmic multiplication within the non-activated but infected macrophages and extracellular multiplication to reach tremendous numbers (Dannenberg,1994).

Cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) of the host are important processes in the pathogenesis of TB. CMI is important for the host response, as it is characterised by the increase in numbers of specific T lymphocytes. These lymphocytes produce

locally cytokines that attract monocytes or macrophages from the blood into the lesions and activate them. Interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) are major macrophage activators (Nathan *et al.*, 1988 ; Barnes *et al.*, 1989 and 1990; and Flesch and Kaufmann, 1987).

DTH is principally the same process as CMI but in this case DTH causes death of local macrophages and tissues. For about a century the relationship between DTH and CMI has been an issue of debate. Both inhibit the multiplication of *M. tuberculosis* equally well. Non-activated macrophages infected with many bacilli are killed by DTH. It is not known whether non-activated macrophages that are infected with fewer bacilli are recognisable targets for the activated lymphocytes to control the mycobacterial growth. The primary function of the CMI seems to be the activation of the surrounding uninfected macrophages (Dannenberg, 1989 and 1991). These macrophages now ingest and destroy the released bacilli.

Macrophages have a continual turnover in tuberculous lesions where many either die or penetrate deeper into the lesion. The accumulation of activated macrophages peak when CMI-DTH first develops (Dannenberg, 1994).

There are four stages of pulmonary tuberculosis. In stage one there is no bacillary growth, the bacillus is either destroyed or inhibited by the mature macrophage that ingests it. Failure to destroy the bacillus will lead to its multiplication and destroy the macrophage. In the second stage there is a logarithmic growth within non-activated macrophages of the developing tubercle when the macrophage fails to destroy the bacillus. In the third stage the immune system in the form of the CMI and DTH inhibits growth and results in decline in number of passive, viable bacilli. In the fourth stage the bacillus overcomes the host defence and the bacilli multiply for the

first time extra-cellularly (Dannenberg, 1994).

Tuberculosis is not a systemic disease. It is influenced by states of activation of local macrophages and lymphocytes around foci of infection causing lesions isolated from the rest of the organ or organism. In one area of the lung lesions may progress and in another area they may regress or stabilise. This can also be applied to parts of a single lesion, where one part may regress and another part may progress. The disease may even fluctuate between periods of exacerbation and remission (Dannenberg and Tomashefski 1988).

Gamma - delta T cells seem to be the major lymphocytes involved in the early primary response to tuberculosis infection (Kaufman, 1988; Born *et al.* 1990). Their cytotoxic effect limits the second stage of tuberculosis. Their influence on the CMI and DTH is still not clear.

Sub-populations of T-helper (T_H) cells(T_{H0}, T_{H1}, T_{H2}) can be differentiated by the cytokine-secretion pattern. T-helper (CD4+) lymphocytes are divided into two subsets due to the type of cytokines they produce. T_{H1} produces IL-2 and IFN-γ, while T_{H2} produces IL-4, IL-5, IL-6, and IL-10. IFN-γ inhibits proliferation of the T_{H2} and IL-4 and IL-10 inhibit the T_{H1} cells (Huygen *et al.*, 1992; Scott, 1993; Sypek *et al.*, 1993; and Heinzel *et al.*, 1993).

Evidence is increasing that the outcome of human disease may depend on the sub-populations of T-cells that predominate at the site of inflammation. Studies on reactive arthritis caused by *Chlamydia trachomatis*, indicated that of 58 T cell clones, 33 reacted towards *Chlamydia trachomatis* and 25 were not reactive. The cytokine patterns for IFN-γ and IL-4 were analysed and showed that 23 of the 33 antigen reactive clones secreted IFN-γ and not IL- 4 while the

remaining 10 exhibited T_{H0} pattern. Clones that were not reactive all expressed T_{H2} cytokine secretion (Simon *et al.*, 1992)

The role of TNF-α in tuberculosis is paradoxical. On the one hand there is enough evidence for its protective role, but on the other hand there is also evidence that TNF-α plays part in tissue damage that is characteristic of the human disease. Hernandez-Pando and Rook (1994) have reported that TNF-α frequently induces necrosis when injected into sites undergoing DTH responses to mycobacterial antigen. They suggest that in pure T_{H1} responses TNF-α may act as an additional macrophage activating factor. In mixed T_{H1} and T_{H2} or T_{H0} responses it may cause tissue damage. This mixed pattern is characteristic of tuberculosis.

The expression of protective immunity to *M. tuberculosis* in mice is mediated by T lymphocytes that secrete cytokines. These molecules then mediate a variety of functions including the activities of a parasite host macrophages and the recruitment of other mononuclear phagocytes to the site of infection in order to initiate granuloma formation. IFN-γ is thought to play a key role in these events. Studies on mice demonstrated that when the IFN-γ gene has been disrupted there was an inability to control or contain a normally sub-lethal dose of *M. tuberculosis* (Dannenberg, 1994).

In tuberculosis, the T_{H1} lymphocytes are important in activating the macrophages for CMI. T_{H2} cells are more involved in the production of antibodies. Much is still to be learned on the relation between tuberculosis and γδ-T cells, heat-shock proteins, $CD4^+$ and $CD8^+$ T cells and T-helper lymphocytes and their cytokines. In mice the difference in resistance to tuberculosis appears to be due to the differences in cytokines produced by the T_{H1} and the T_{H2} lymphocytes (Guery *et al.*,

1996).

Mycolic acids belong to a group of compounds which were generally considered to be non-immunogenic. Beckman *et al.* (1994) indicated that some of the T-lymphocytes in mice are able to recognise lipid antigens. They therefore predicted that mycolic acids could induce an immune response in animals that could possibly play an important role in the defence of the body against tuberculosis. Research into the role played by mycolic acids in the immunity against disease is hampered by the problems associated with purification of adequate amounts for *in vitro* and *in vivo* experimentation.

The aim of this study

In this study the immunoregulatory properties of mycobacterial mycolic acids are investigated with the aim to apply the compounds to shorten the chemotherapy regimen for tuberculosis patients, and possibly to avoid unwanted side effects of infection and/or therapy. A similar approach has been tested before: chemotherapy to kill rapidly metabolising extracellular bacilli and some intracellular organisms combined with a single injection of killed *M. vaccae* given early in chemotherapy, halved treatment failure rates and reduced deaths during treatment (Stanford and Grange, 1993; Stanford and Stanford, 1994). The mechanism by which *M. vaccae* might achieve this is still speculative. It has been reported that immunotherapy with autoclaved *M. vaccae* causes a rapid loss of agalactosyl IgG, detectable within 14-21 days, whereas chemotherapy alone causes agalactosyl IgG to rise further for up to 2 months.

In Chapter 2 the aim is to purify mycolic acids from mycobacterial cell walls in economic and sufficient quantities. The second part of the study (Chapter 3) investigates the effects of mycolic acids treatment on the immune response of mice before and after tuberculosis infection. In Chapter 4 the role of mycolic acids in adjuvant arthritis is investigated as a model for controlling the auto-immune side-reaction typically associated with tuberculosis.

CHAPTER 2

Purification of Mycolic Acids by Counter Current Distribution (CCD)

Introduction

Mycobacterial cells are enclosed by a complex, lipid-rich cell wall. It is considered to be the most complex of all cell walls in nature. This is due to its high lipid content that constitutes about 60% of the dry weight of the cell wall. Goren (1979) and Minnikin (1982) have intensively investigated mycobacterial lipids and found that these lipids contain long chain fatty acids. They include tuberculostearic, mycoserosic, phtheinoic, and mycolic acids and often contain unsaturated bonds, cyclopropane rings or methyl side chains. Mycolic acids are defined as a complex group of long-chain fatty α-alkyl, β-alkyl hydroxy fatty acids, which implies that there is an alkyl chain adjacent to the terminal carboxylic (-COOH) group. The general formula of mycolic acids is indicated in Fig. 2.1

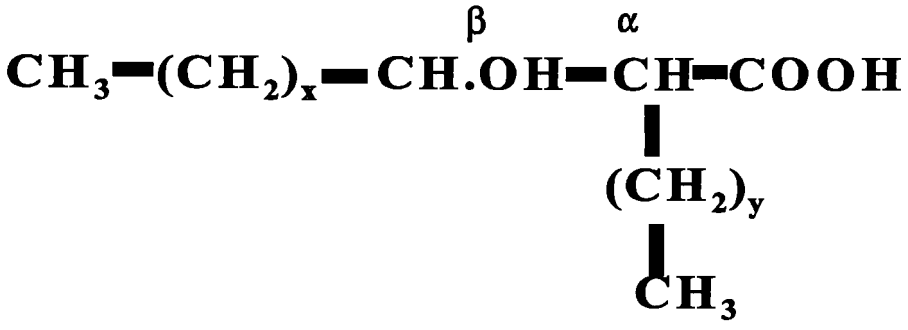

Fig.2.1 The general formula of mycolic acids (MA).

The number of carbon atoms in mycobacterial mycolic acids range from 60 to 90. *Mycobacteria* contain complex mixtures of mycolic acids which, when separated by two-dimensional chromatography of their methyl esters, fall into four main patterns represented by *M. tuberculosis, M. avium, M. fortuitum* and *M. chelonei* (Minnikin, 1982). *M. leprae* on the other hand has a simpler pattern of α- and keto-mycolic acids. Mycolic acids are usually covalently linked to sugars, as illustrated in Fig.2.2, where those forming the main cell wall are linked to the arabinose residues of the structural polysaccharide, arabinogalactan.

Mycolic acids occur in nature as mixtures of different types. They frequently form esters with carbohydrates, e.g. with arabinose forming the main cell-wall palisade and with trehalose forming dimycolyl trehalose, the cord factor which has been associated with the virulence of *M. tuberculosis.* All known mycolic acids have the basic structure $R^2CH(OH)CHR^1COOH$, (Fig.2.1) where R^1 is a C_{20} to C_{24} linear alkane and R^2 is more complex structure of 30 to 60 carbon atoms that may contain various numbers of carbon-carbon double bonds and/or cyclopropane rings, methyl branches or oxygen-functions such as C=O, $CH_3OCH=$ and COOH (The MERCK Index, 1989).

Mycobacterial cell-wall fractions are obtained by classical procedures which include disruption of the cells followed by differential centrifugation and repeated washing with buffers and water. Crude cell walls, obtained by disruption of intact bacterial cells, are rich in lipids. About 25% of their weight are composed of free lipids which can be removed by neutral solvents.

The main constituent of the purified cell wall is the covalent "skeleton" which consists of peptidoglycan to which molecules of arabinogalactan mycolate are covalently linked. Some

strains contain a glucan, while pathogenic and most vaccinating strains contain a poly-L-glutamic acid polymer (Petit and Lederer, 1984).

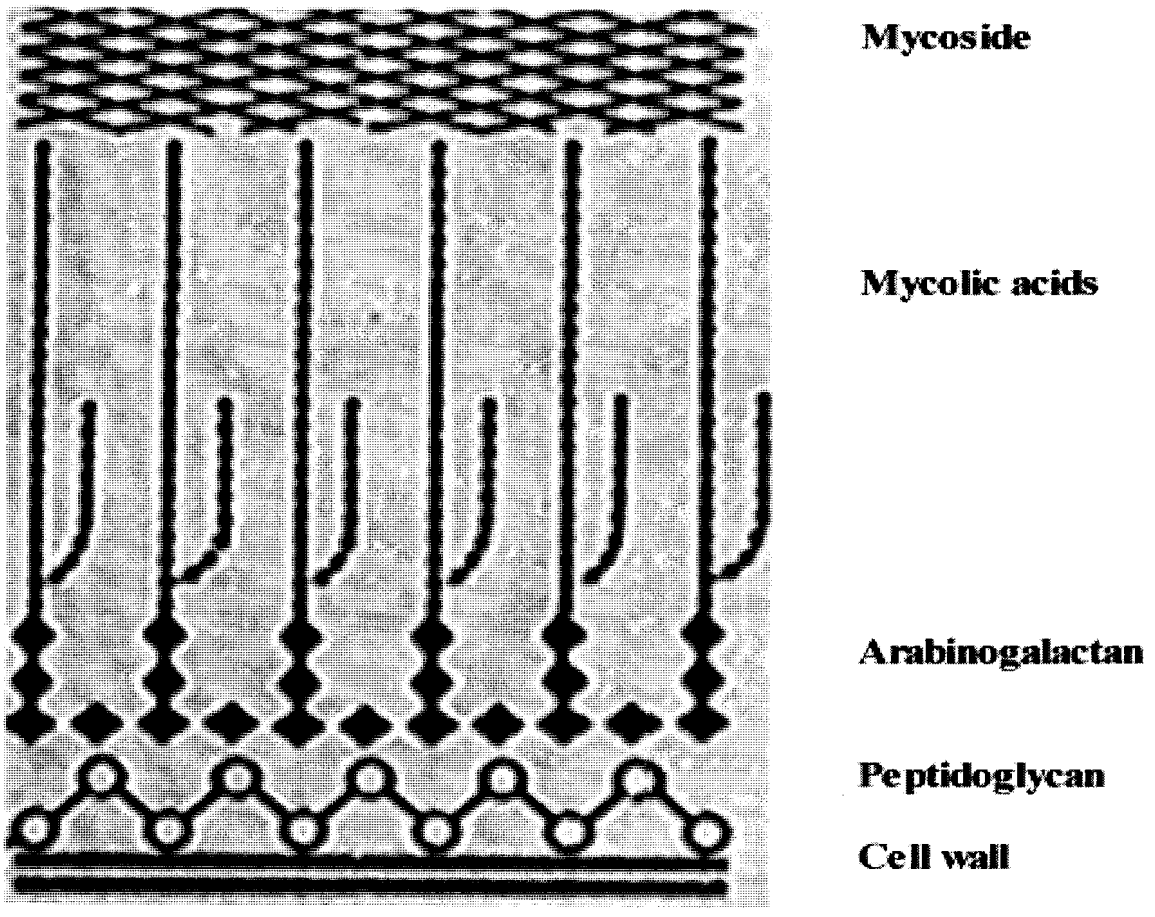

Fig. 2.2 Schematic representation of the mycobacterial cell wall.

Complex high molecular weight ß-hydroxy fatty acids with long alkyl chain at the α-position are characteristic of mycolic acids of the *Mycobacterium* species.

Due to the insolubility of mycolic acids in most organic solvents, purification of mycolic acids is not simple. Their solubility in chlorinated organic solvents such as chloroform and dichloromethane was exploited by Beckman *et al.* (1994) in their method to purify mycolic acids from the cell-wall extracts by HPLC in small preparative amounts.

In order to obtain enough purified mycolic acids to test our hypothesis concerning their immunological role in *M. tuberculosis*-infected animals, countercurrent distribution (CCD) was applied. In CCD, separation is effected by the flow of a liquid upper phase over a stationary lower phase thereby effecting differential transport of the various solutes in the two phases depending on their distribution constant between the two phases (King and Craig, 1962).

Hypothesis:

Mycolic acids (MA) can be isolated from mycobacterial cell-wall extracts and purified on a preparative scale using CCD.

Goals:

1. Purify *Mycobacterium tuberculosis* mycolic acids on a preparative scale in CCD.

2. Purify *Mycobacterium vaccae* mycolic acids to indicate that the CCD purification method is widely applicable.

Materials

Culture

Mycobacterium tuberculosis H37Rv ATCC 27294 - Type strain: A virulent strain, originally isolated from an infected human lung. The culture was purchased in lyophilized form from the American Type Culture Collection (ATCC), Maryland, USA.

Media

Growth media

The following media were used for the cultivation of *M. tuberculosis*:

Löwenstein-Jensen (LJ) medium (slants) and

Middlebrook 7H-10 agar medium (plates).

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).

The media were prepared by the National Tuberculosis Institute of the Medical Research Council of South Africa, Pretoria. The sterility of all the media was confirmed before they were used in the experiments by incubating them at 37°C for 24 h.

Media used for washing and diluting of *Mycobacteria*

The harvested bacteria were washed in 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.

Reagents

For the preparation of the reagents used for the extraction, derivatization and High-Performance Liquid Chromatography (HPLC) analysis of mycolic acids, HPLC Grade methanol (BDH) and double-distilled deionized water were used.

Reagent A: 25% potassium hydroxide (Saarchem, Analytical Grade) dissolved in methanol-water (1:1), *i.e.*, 62,5 g potassium hydroxide was dissolved in 125 ml water and 125 ml methanol (BDH, HPLC Grade) was added.

Reagent B: Concentrated hydrochloric acid (Saarchem, Analytical Grade) diluted 1:1 with water.

Reagent C: 2% potassium bicarbonate (BDH, Analytical Grade) dissolved in methanol-water

(1:1), 10 g potassium bicarbonate was dissolved in 250 ml water and 250 ml methanol was added.

Reagent D: para-bromophenacylbromide dissolved in acetonitrile and crown ether (Pierce Chemical Co, Illinois, USA) was dispensed in 500 ml quantities into small amber-coloured screw cap vials with Teflon-coated septa. The caps were tightened and the vials were wrapped with Parafilm. Reagent D was stored at 4°C.

Reagent E: Reagent E was prepared by mixing reagent B 1:1 with methanol.

HPLC Standard: High Molecular Weight Internal Standard (C-100) from Ribi ImmunoChem Research Company, Hamilton, MT. The standard, 1 mg, was dissolved in 20 ml chloroform (BDH, HPLC Grade) at 4°C and aliquots of 100 µl were dispensed into 4 ml amber WISP vials (using an Eppendorf dispenser), dried, capped with Teflon-coated septa and stored at 4°C.

Chloroform (Saarchem, Analytical Grade, RSA)

Methylene chloride (BDH, UK, HPLC-Grade)

Reagents A, B, C and E were prepared fresh prior to experiments, taking all the necessary safety precautions. The following reagents were used for the preliminary purification of crude bacterial extracts ("funnel extraction") and for the countercurrent purification of the extracted mycolic acids:

Chloroform (Saarchem, Chemically Pure)

Methanol (Saarchem, Chemically Pure)

Acetone (Saarchem, Chemically Pure)

Sodium chloride (Saarchem, Analytical Grade)

Double-distilled deionized water

Methods

Optimization of CCD separation of mycolic acids

Preliminary purification of crude mycobacterial extracts

In order to shorten the time required for the countercurrent purification of the crude mycobacterial extracts, an additional preliminary extraction step was introduced. This step had a dual purpose

(i) to remove unnecessary cellular components from the crude extract prior to the countercurrent purification and

(ii) to reduce the soap fraction in the crude bacterial extracts.

Gram quantities of the crude extracted material were suspended in a minimum volume of the lower phase solvent (usually 100 ml), transferred into a separation funnel and mixed with an equal volume of the upper phase solvent. The phases were allowed to separate and the upper phase was removed and stored at 4°C. Into the remaining lower phase, an equal volume of the upper phase solvent was again introduced and the process of the phase separation was repeated.

The second upper phase was removed and stored at 4°C and the second lower phase was dried in a Buchi Roto-evaporator RE 120, at 75 °C and its mass recorded.

Countercurrent purification of mycolic acids originating from *M. tuberculosis*

Countercurrent apparatus: A countercurrent apparatus produced by H O POST, Instrument Company Inc., Middle Village, New York was used during the investigations. The "trains" in this model consisted of 2 x 250 inter-connected tubes.

Solvent system used in the countercurrent apparatus

The solvent system used for the countercurrent separation consisted of: 42% v/v chloroform (Saarchem, Chemically Pure Reagent) 39% v/v methanol (Saarchem, Chemically Pure), 19% v/v ddd H_2O

The components were mixed, equilibrated and the upper and lower phases were collected using a separation funnel.

The composition of the upper phase was established to be:

15% v/v chloroform, 52% v/v methanol and 33% v/v ddd H_2O

The composition of the lower phase was established to be:

68% v/v chloroform, 27% v/v methanol and 5% v/v ddd H_2O

The countercurrent purification process was carried out under the following conditions:

A countercurrent distribution train comprising 50 tubes, numbered 0-49, was used in the experiments. The upper phase solvent, a volume of 600 ml, was introduced into a buffer reservoir. A sample of 235 mg of mycolic acids, after the preliminary purification, was dissolved in 20 ml of the lower phase solvent (divided into two aliquots of 180 mg in and 55 mg) and were introduced into first tubes, numbered "0" of each train respectively. Subsequently, 10 ml of the upper phase solvent was introduced into each of the first countercurrent tubes. Into the remaining 50 tubes aliquots of 10 ml of the lower phase were introduced. Upper phase, in volumes of 10 ml per cycle, was automatically dispensed into tube number 0, repeatedly over 50 cycles resulting in approximately 55-hour long operation. Thus, fifty five countercurrent cycles were performed, with each cycle consisting of 10 mixing pendula and 60 minutes phase separation time.

Initial load of crude extract after the funnel extraction:	125 mg
Number of cycles:	55
Equilibration time:	60 min

Removal of malachite green from the countercurrent-purified mycolic acids

To remove traces of malachite green derived from bacterial growth media, the countercurrent-purified material was selectively precipitated in the following manner. Countercurrent-purified mycolic acids (92 mg) were placed in a WISP vial into which 1,0 ml chloroform was introduced. The dissolved

mycolic acids were transferred into a pre-weighed round-bottom flask. The vial was rinsed twice with 1,0 ml chloroform and the two aliquots of chloroform were added to that already present in the round-bottom flask. Subsequently, acetone was introduced drop-wise in 500 µl aliquots. In total, a volume of 26 ml of acetone was introduced and the white flakes of the precipitated mycolic acids were washed twice with 20 ml acetone. The acetone supernatant with the dissolved malachite green was removed, the precipitate dissolved again in 12 ml cold (maintained at 4°C) acetone, transferred into Eppendorf's tubes and centrifuged at 8 000 g for 5 min. The regained pellets were washed twice with cold acetone. The final pellets were dissolved in chloroform, transferred into pre-weighed WISP vials, dried under a stream of nitrogen on a heat block at 80°C, capped and stored at 4 ℃ until required.

Determination of yield of the mycolic acids purified by countercurrent separation

In order to calculate the approximate yield of purification, the amount of the mycolic acids present in the samples obtained after the countercurrent separation/purification was compared to the amount of these compounds present in the crude cellular extract introduced into the countercurrent apparatus. The calculations were based on the results obtained by the HPLC analysis.

In order to increase the accuracy of the HPLC determination of mycolic acids, the High Molecular Weight Internal Standard (C-100) was introduced into the countercurrent-purified mycolic acids before the saponification. The main difference between the determination of mycolic acids after countercurrent purification and in the crude extract was the time of introduction of the Internal Standard. In the latter case, Internal Standard was added after the saponification.

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A sample of 0,5 mg of the countercurrent-purified mycolic acids was introduced into a WISP vial containing 5 mg of the High Molecular Weight Internal Standard (C-100). Saponification of mycolic acids was carried out with 2 ml of Reagent A at room temperature. The WISP vial was vortexed for 30 seconds. The extraction was carried out with 1,5 ml of Reagent B. After vortexing, the pH of the sample was checked and if necessary, adjusted to pH 1 with Reagent B.

Subsequently, 2,0 ml chloroform was added to each sample and vortexed for 30 seconds. The layers were allowed to separate. The bottom layers were removed with Pasteur pipettes, transferred to amber WISP vials and evaporated to dryness at 85°C in a heat block-evaporator under a stream of nitrogen. To neutralize traces of acid carried over, 100 µl of reagent C was added to each sample and the fluid evaporated to dryness at 85°C in a heat block-evaporator under a stream of nitrogen.

HPLC analysis and quantification of mycolic acids

Repeatability and accuracy of the pipette used for the distribution of the HPLC standard was determined. The precision was established to be +/- 1% and was confirmed prior to each aliquoting of the internal standard.

For the HPLC analysis 10 µl from each sample (maintained on ice during handling), was analysed. Control samples, *i.e.*, 10 µl of filtered methylene chloride, were run prior to each set of samples analysed. If a large number of samples was analysed, in order to validate the reliability of the HPLC apparatus, control samples were run after every three or four test samples.

The reverse-phase HPLC analyses were carried out using a Waters 600 E System Controller High Performance Liquid Chromatography apparatus consisting of:

- Microsep M741 Data Module;

Waters 712 WISP Autosampler;

Detector (Waters 486 Tunable Absorbance Detector);

Column: Nova-Pak C18 4 µm 3,9 x 150 mm and an end connector set for steel cartridge columns.

RKC Rex-C 4 Column Temperature regulator.

Running conditions were:

Mobile phase:

Solvent A: HPLC Grade methanol

Solvent B: HPLC Grade methylene chloride

Flow Rate: 2,5 ml/min

Column temperature: 30°C

The detector was set at 260 nm.

Prior to use, the solvents were sparged with Instrument Grade helium. High Purity Nitrogen was used to control hydraulics of the WISP vials autosampler.

The HPLC gradient initially comprised 98% (v/v) methanol (Solvent A) and 2% (v/v) methylene chloride (Solvent B). The gradient was increased linearly to 80% A and 20% B at one minute; 35% A and 65% B at ten minutes, held for 30 seconds and then decreased over 10 seconds back to 98% A and 2% B. This ratio was maintained for 4 minutes to allow for stabilization of the system prior to injection of the next sample.

Mathematical quantification of mycolic acids was carried out by comparing the combined peak areas of the tested samples to the peak area of the introduced quantity of the High Molecular Weight Internal HPLC Standard

It was essential for the calculation of the yield of the countercurrent separation that the mycolic acids determined by HPLC was within the tested linear range of the HPLC UV detector.

Solving the CCD emulsion problem with NaCl

Saline optimisation

The soap fractions from countercurrent purification were mixed with saline solutions to final concentrations of 0; 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; and 0.8 M. The emulsion breakage was measured by determination of the time for complete separation between the lower phase and the upper phase.

The minimum concentration of NaCl with the minimum duration of emulsion were chosen as the ones to be used for CCD. This concentration was used to titrate the phase diagram curve for the CCD bi-phasic solvent system according to the protocol described before and quoted from Patent Appl RSA 94/2575.

In the original system, water and chloroform formed the non-miscible components, while methanol served as the miscible integrator component. In this system saline was used to replace water.

Application of the saline-containing system in CCD purification of mycolic acids.

In this procedure the same protocol was followed as before (pages 18-19) but with saline replacing $dddH_2O$.

The solvent system used for the countercurrent separation consisted of:

42% v/v chloroform (Saarchem, Chemically Pure Reagent)

39% v/v methanol (Saarchem, Chemically Pure)

19% v/v 0,2 M NaCl (Saarchem, Chemically Pure).

The composition of the upper phase was established to be:

15% v/v chloroform, 52% v/v methanol and 33% v/v 0,2 M NaCl.

The composition of the lower phase was established to be:

68% v/v chloroform, 27% v/v methanol and 5% v/v 0,2 M NaCl.

The countercurrent purification process was carried out under the following conditions:

The conditions employed in this case were the same as before (pages 18-19), with the only difference being that 600 ml of the upper phase was introduced into a buffer reservoir. A sample of partially purified mycolic acids extract (187 mg) was dissolved in 50 ml of the lower phase solvent, divided into five aliquots and introduced into the first five tubes, numbered 0 to 4. Upper phase (10 ml per tube) was then introduced into the first five countercurrent tubes. CCD was then performed over 55 cycles resulting in an approximately 10-hour operation.

Initial load of crude extract after the funnel extraction:	187 mg
Number of cycles:	55
Equilibration time:	10-15 min

Removal of NaCl from the MA after CCD

After CCD, the dry, pooled MA were dissolved in chloroform and double distilled de-ionised water (ddd H_2O) (1:2) and vigorously mixed. After equilibration, the chloroform phase was separated from the water phase in the separation funnel, mixed with an equal volume of ddd H_2O and the extraction repeated. Finally, the chloroform and the second aqueous upper phase were dried (this was done to determine if all the NaCl had been removed by the first water extraction). MA was rinsed and precipitated with acetone to remove malachite green and stored at 4°C under acetone.

Purification of *Mycobacterium vaccae* mycolic acids

The purification method for *M. vaccae* MA followed the same principles as those of *Mycobacterium tuberculosis* MA. Crude MA extract (2.19 g) was extracted two times with non-saline upper and lower phases as described for *M. tuberculosis*. The only difference was that the funnel extraction was done with non-saline solvents to ease the gravimetric analysis of the extracts by excluding the step for removing the salts. This was followed by CCD with 50 cycles and 1 min equilibration time. The protocol followed is the same as that in pages 18 to 20.

Results

Countercurrent purification of mycolic acids originating from *M. tuberculosis*

A portion of the crude extract (approximately 3-4 g) was suspended in a minimum volume of the lower phase solvent (usually 100 ml), transferred into a separation funnel and mixed with an equal volume of the upper phase solvent and allowed to separate. The lower phase was then removed and an equal volume of the upper phase solvent was again introduced and the process of the phase separation was repeated.

From 2.7 g crude extract, 503 mg mycolic acids enriched material was obtained from the lower phase after funnel extraction. Of this, 235 mg was loaded on the CCD. In one tube train, 180 mg was loaded and in the other 55 mg. After 30 cycles with 45 min equilibrium time, separation was not complete.

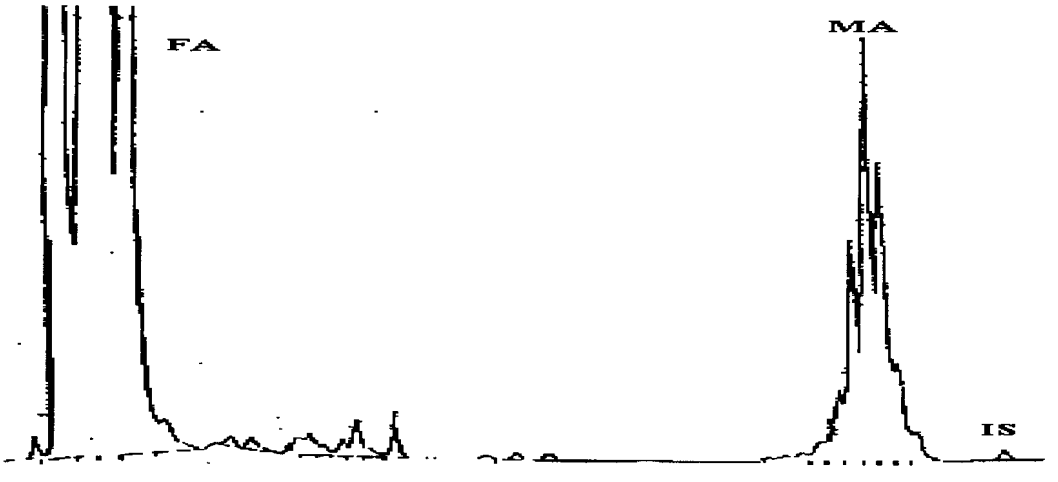

Fig.2.3: The HPLC profile of mycolic acids crude extract after funnel extraction. Crude extract was funnel-extracted twice with equal volumes of lower phase and the upper phase. FA represents the derivatised fatty acids peak, MA represents mycolic acids peak and IS represents the internal standard peak.

After a further 20 cycles, only the 55 mg sample loaded into the CCD train appeared to be successfully separated. The quality of the run was then determined using HPLC (Fig.2.4). In Fig.2.3 the HPLC profile of the funnel extract indicated that it consisted of about 55.56% of mycolic acids calculated using the internal standard peak.

In the first train loaded with 180 mg, there was no complete separation mainly because it was overloaded. This prompted a re-run of the combined and dried fractions that contained mycolic acids using the same conditions as before. A 79% recovery of pure mycolic acids was thus obtained (Fig 2.4). Mycolic acids from the second train loaded with 55 mg appeared to be pure as the yield was quantitative (measured as 120% based on HPLC analysis).

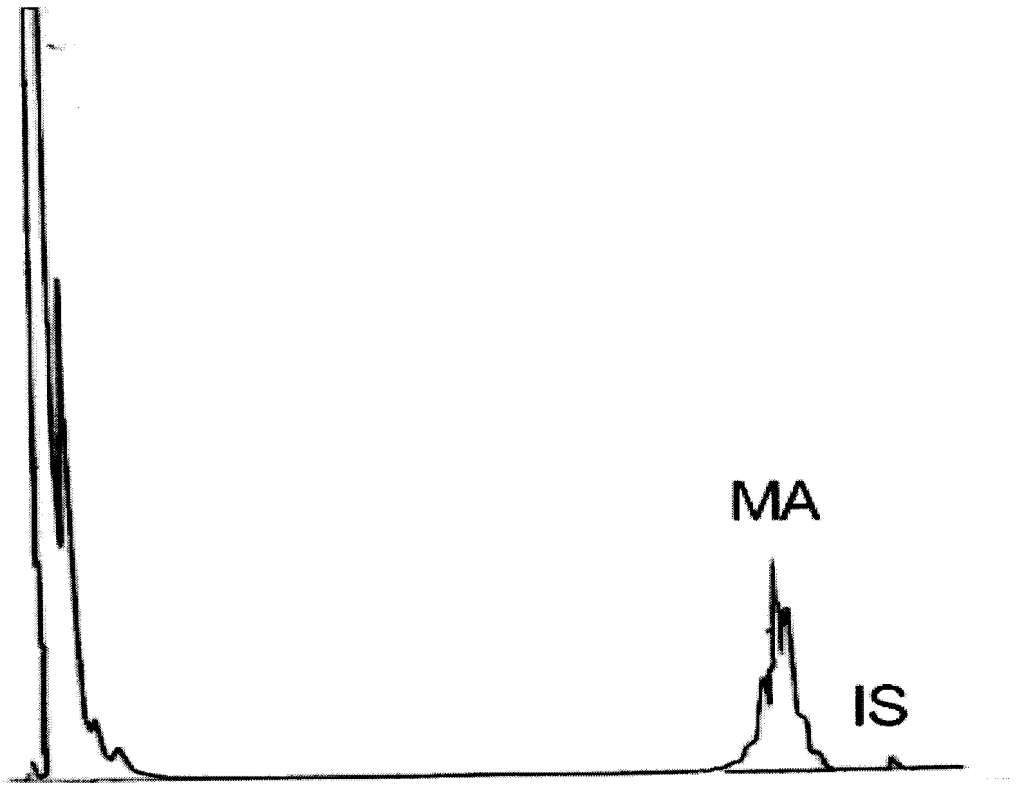

Fig.2.4: The HPLC profile of *M.tuberculosis* mycolic acids purified on CCD after two times funnel extraction of crude extract. Calculations of the amount of mycolic acids were based on the area of the internal standard peak. MA represents mycolic acids cluster of peak and IS the internal standard peak.

Optimisation of NaCl concentration

In this case the objective was to break emulsions formed that were thought to be responsible to limit the potential of CCD purification of mycolic acids.

Saponified fatty acids (soap) derived from the cell-wall extract after CCD and saline solution (in range of 0.1 - 0.8 M NaCl) were mixed to different concentrations and allowed to separate. It was found that addition of NaCl breaks the emulsions formed by the soap fractions and that a concentration of 0.2 M NaCl does this faster than 0.1 M NaCl. In the case where no NaCl was added the emulsion took a very long time to break.

Development of tri-component bi-phasic solvent system.

Different ratios of chloroform and 0.2 M NaCl were titrated with methanol to develop a phase diagram (Fig.2.5). Concentrations of chloroform in 0.2 M NaCl were prepared according to Table 2.1. The results obtained indicated that the titration curve is identical to the initial curve without NaCl in the system. This implicated that there will be no change in the phase composition of the CCD when NaCl is added to the system. The area under the curve represents solvent composition that separated into two phases. Such compositions can be chosen as points for the distribution of a particular solute between the two phases.

The point M on the phase diagram was obtained by drawing the line PQ pivoting over X such that PX:QX correlates with the volume ratio of lower phase:upper phase obtained by mixing the composition X. The point M is the mid-point of line PQ and when making up M, upper and lower phase of equal volumes are obtained, with the upper phase consisting of the composition P and the lower phase of composition Q, identical to that obtained when mixing composition X. Thus M consisted of 42% (v/v) chloroform, 39% (v/v) methanol and 19% (v/v) 0.2 M NaCl. This solvent system separated into two phases of equal volumes.

TABLE 2.1: Titration of chloroform and saline with methanol to develop a tri-component solvent system for saline CCD

TUBE	0.2M NaCl (ml)------(%)	Chloroform (ml)-------(%)	Methanol (ml)-------(%)	Total (ml)
1	1.0--------1.6	49.0-------80.4	11.0-------18.0	61
2	2.5--------2.8	47.5-------71.4	16.5-------24.8	66.5
3	5.0--------6.9	45.0-------62.0	22.5-------31.0	72.5
4	7.5--------9.67	42.5-------55.0	27.5-------35.4	77.5
5	10.0------12.0	40.0-------49.6	32.0-------38.4	82
6	12.5------14.6	37.5-------44.0	35.5-------41.5	85.5
7	15.0------17.0	35.0-------40.0	38.0-------43.0	88
8	17.5------19.0	32.5-------36.0	40.5-------45.0	90.5
9	20.0------21.4	30.0-------32.0	44.0-------46.4	94
10	22.5------23.0	27.5-------29.0	46.0-------48.0	96
11	25.0------25.5	25.0-------25.5	48.2-------49.0	98.2
12	27.5------26.2	22.5-------21.4	55.0-------52.2	105
13	30.0------28.0	20.0-------19.0	56.0-------52.0	106
14	32.5------31.0	17.5-------17.0	53.5-------51.7	103.5
15	35.0 -----33.5	15.0-------14.4	54.5-------52.2	104.5
16	37.5------35.5	12.5-------11.6	55.5-------52.6	105.5
17	40.0------38.5	10.0- -------9.6	54.0-------51.9	104
18	42.5------41.0	7.5- -------7.2	53.5-------51.7	103.5
19	45.0------46.2	5.0- -------5.1	47.5-------48.7	97.5
20	47.5------54.9	2.5- -------2.9	36.5-------42.2	86.5
21	49.0------68.6	1.0- -------1.4	21.5-------30.0	71.5

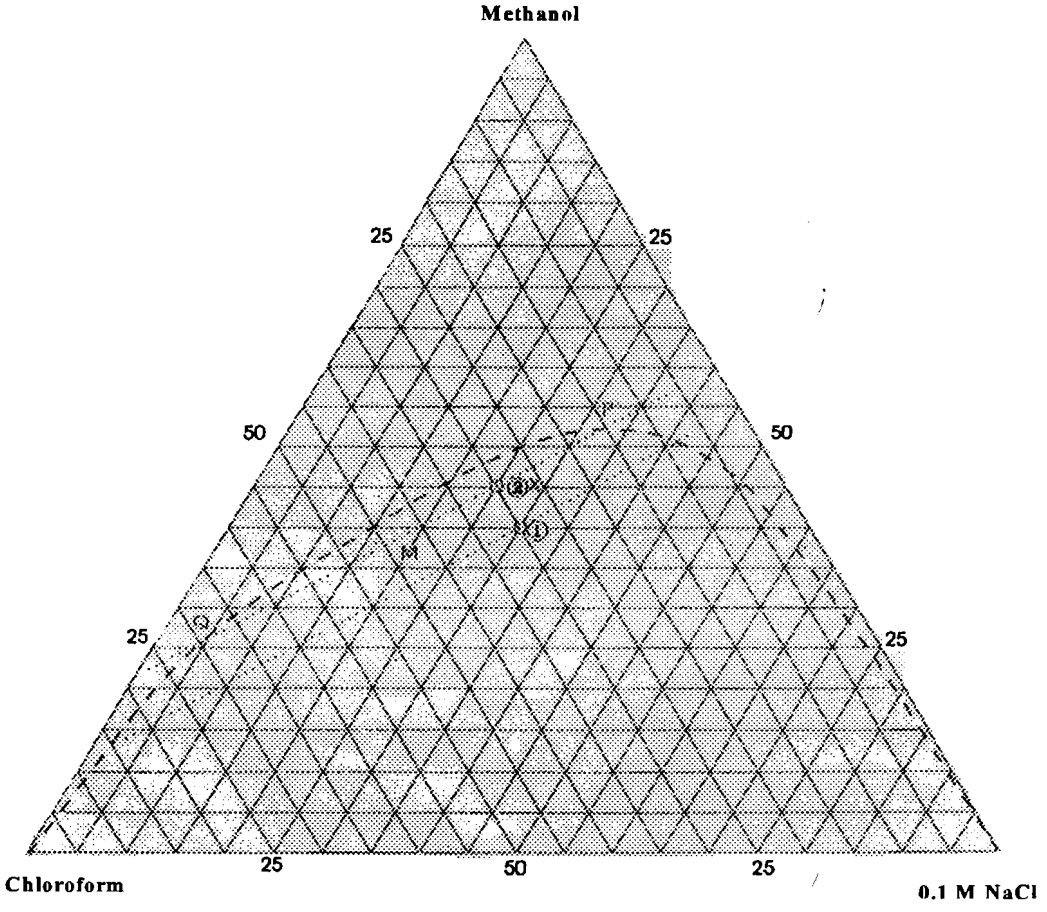

Fig. 2.5 Titration curve of CCD solvents using saline.

Use of saline in CCD

In this experiment 0.2 M NaCl was used to break the emulsions formed during CCD. The experiment was initially started by reducing the number of cycles from 50 to 30. Upon completion of the CCD run, tubes 0-3 were collected as pure mycolic acids and tubes 4 to 30 included in the determination of the gravimetric contents of each tube. After removal of salt it was found that the total amount of MA recovered was 51 mg free of malachite green (the latter comes from Löwenstein-Jensen agar slants on which *Mycobacteria* cells were cultured).

HPLC profiles obtained for each of the 4 tubes (Fig.2.6.1-2.6.2) revealed that separation was not complete. To achieve absolute separation of MA from the rest of the fatty acids and contaminants it was necessary to increase the number of cycles. The yield of MA was, however, high (Fig.2.6.1) and the amount of MA that was not completely separated from the rest of the impurities was negligible (Fig.2.6.2). The impurities could be due to the presence malachite green that was not removed from the sample as the amount of mycolic acids in that tube was negligibly low.

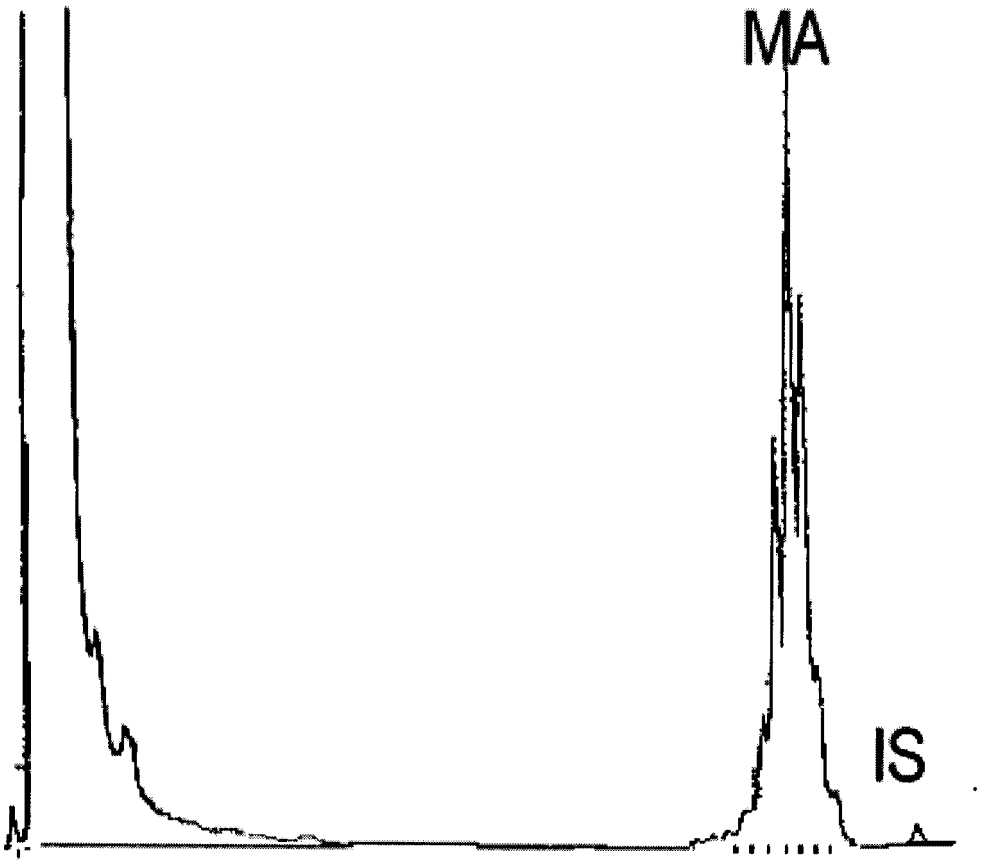

Fig.2.6.1: The HPLC profile of *M. tuberculosis* mycolic acids purified on saline CCD after two times funnel extraction of crude extract. Calculations of the amount of mycolic acids were based on the area of the internal standard peak. MA represents mycolic acids cluster of peaks and IS the internal standard peak.

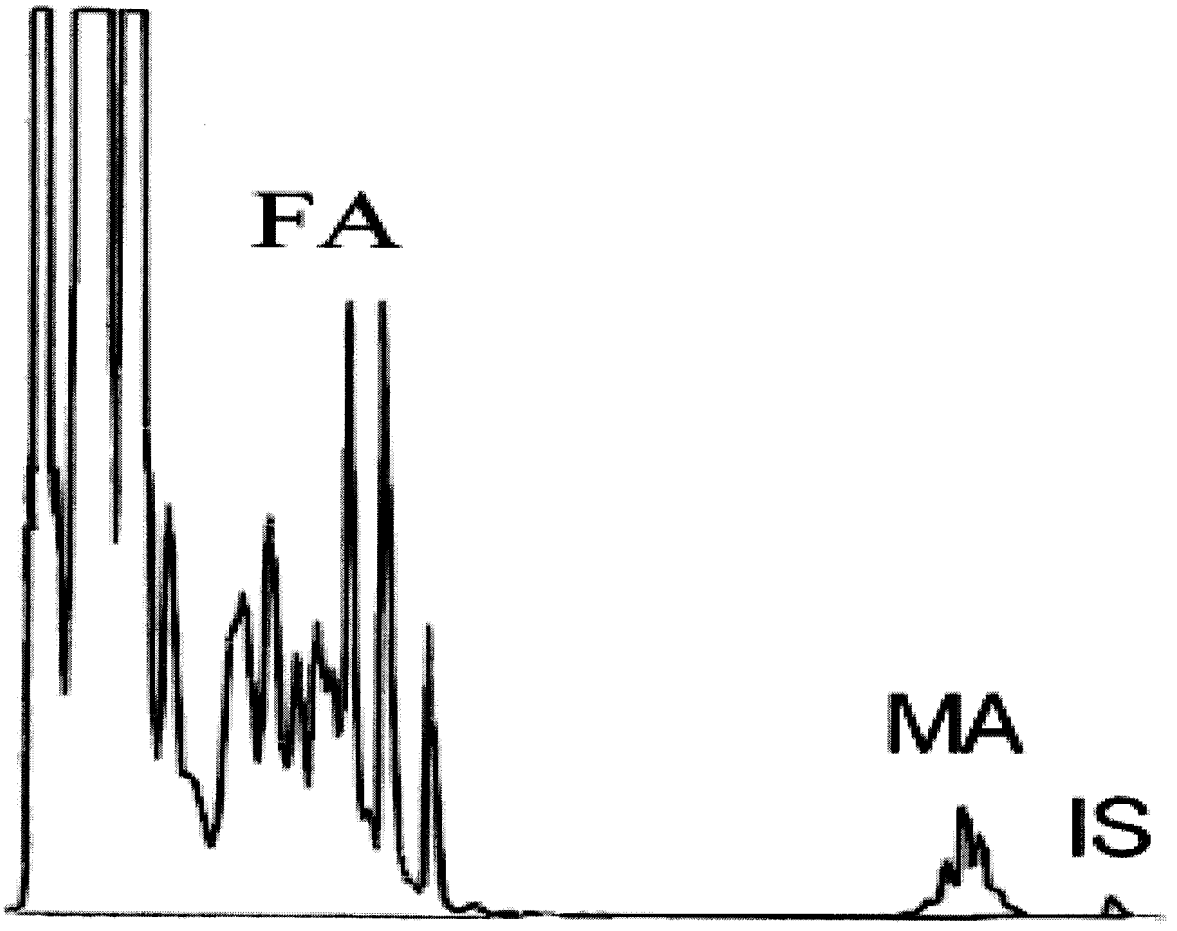

Fig.2.6.2: The HPLC profile of the trace amounts of *M.tuberculosis* mycolic acids found in the fourth tube of the saline CCD train. MA represents mycolic acids, IS the internal standard and FA fatty acids.

From the visual assessment of the solubility of the funnel-extracted mycolic acids in the two phases of the solvent system, more mycolic acids seemed to be dissolved in the lower phase than in the upper phase. This could be concluded from the observation that the lower phase remained yellow, while the upper phase became clear. This could be because the funnel extraction removed most of the impurities. Gravimetric analysis indicated that the amount of material that was initially dissolved in the lower phase before funnel extraction, decreased after funnel extraction. HPLC analysis indicated that mycolic acids predominantly remained in the lower phase, even during funnel extraction.

This method, wich uses NaCl in the CCD purification procedure, was then compared to the method of Goodrum (1998). In this case the use of NaCl in the CCD purification method poved to have more advantages over the previous method, which only uses $dddH_20$ (Table.2.2)

Table 2.2: Yield and purity of the mycolic acids originating from *M. tuberculosis*, purified using the improved method

Parameter	Original method (Goodrum, 1998)	Method with NaCl
Loaded mass of mycolic acids-crude extract	31,1 mg	3 760 mg
Mass of countercurrent-purified mycolic acids	3,5 mg	218 mg
Equilibration time	40 min	5 min
Number of cycles	24	30
Duration of the run	18 hours	3,5 hours
Yield	5,3%-10%	5,8%-7,8%

Purification of mycolic acids from *Mycobacterium vaccae*

Despite the fact that separation methods of MA from *M. vaccae* followed the same protocol as those of *M. tuberculosis*, it was found that the amount of MA extracted after funnel extraction was much lower than that obtained for *M. tuberculosis*. Only 89.5 mg was recovered from the 2.19 g of crude extract, compared to 503 mg from the 2.7 g of crude extract obtained from *M. tuberculosis*. This was then run on CCD, resulting in an even lower recovery of mycolic acids

(17% of funnel extract loaded, compared to 60% recovered from *M. tuberculosis.*). The HPLC pattern after CCD revealed that the purification was complete and successful, suggesting that this method of purification can be applied to purify mycolic acids from other *Mycobacteria*. This was deduced from comparing the HPLC pattern of mycolic acids from *M. tuberculosis* (Fig.2.6) and that from *M. vaccae* (Fig.2.7). It was shown by Minnikin *et al.*(1984) that *M. vaccae* has complex mycolic acids HPLC profiles, *ie.* three clusters of peaks against one cluster present in the HPLC profile of *M. tuberculosis.*

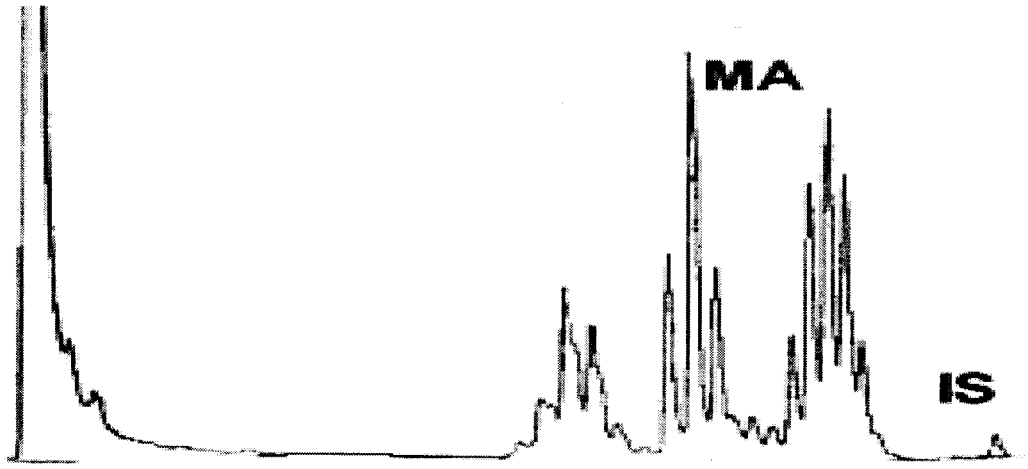

Fig.2.7: The HPLC profile of *M. vaccae* mycolic acids purified using saline CCD after two times funnel extraction of crude extract. Calculations of the amount of mycolic acids were based on the area of the internal standard peak. With MA representing mycolic acids cluster of peaks and IS the internal standard peak.

Discussion

Mycolic acids comprise the major constituent of the mycobacterial cell wall and provide the first line of defence against potentially lethal environmental conditions. This renders the assessment of the immunogenicity of mycolic acids indispensable for the investigations in the development of new vaccines for the prevention of tuberculosis and for other possible applications. The limiting factor in this case was that no purification protocols for mycolic acids extraction on a large scale existed. This study envisaged to obtain a fast, cost-effective, preparative and simple purification protocol for mycolic acids. This was obtained through the Counter Current Distribution (CCD) technique.

Mycobacterial cell envelopes contain high proportions of lipids whose diverse structures have obvious chemosystematic potential (Minnikin and Goodfellow, 1980; Minnikin 1982). Pathogenic and slow growing *Mycobacteria* such as *M. tuberculosis* modify their mycolic acids by cyclopropanation, while the fast growing saprophytic species such as *M. smegmatis* do not. This suggests that modification may be associated with an increase in oxidative stress experienced by the slow growing species (Yuan *et al.*, 1995). Although useful for classification purposes, this property of mycolic acids complicates the purification of single chemically pure members of this group of lipid molecules.

It should be noted that it is often difficult to isolate cell wall components from intact bacteria. Dobson *et al.* (1985) and Minnikin (1988 and 1982) described methods, based on those developed by Anderson (1929), whereby mycolic acids could be purified using tedious chemical methods which required numerous steps, making them useful only for small scale purification. The

introduction of the "new" spectroscopic and chromatographic techniques since the early 1960s has revolutionized the approaches to the analysis of these natural products. Thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) allowed rapid purification of mycobacterial cell-wall components (Dobson *et al.*, 1985). The method applied by Beckman *et al.* (1994) and Butler *et al.* (1991), where HPLC modification was applied to purify mycolic acids, required that mycolic acids be derivatized and afterwards be de-derivatized before using the purified MA for biological experiments. This again yielded mycolic acids at minute quantities. The use of CCD removed this limitation.

Initially, the application of CCD to purify MA (Goodrum, 1998) was lengthy and due to the formation of emulsions in the CCD tubes only 25 mg of pure MA could be obtained in a single run lasting 3 days. The emulsions were a result of saponified lipids from the mycobacterial cell-wall complex. In order to shorten the time required for the countercurrent purification of the crude mycobacterial extracts, an additional preliminary extraction step was introduced. This step had a dual purpose, i.e. (i) to remove the majority of cellular debris and (ii) to reduce emulsifying soaps in the crude bacterial extracts before the countercurrent separation step.

CCD purification of mycolic acids in this case only improved the ability to load and separate large amounts of pre-extracted mycolic acids in the purification system. Larger amounts of CCD pure mycolic acids could be obtained, but the time required to achieve this was still lengthy. This was because there were still emulsions formed during the mixing of the lower phase and the upper phase in the CCD purification system. The emulsion formation was identified as the limiting factor in the CCD purification system. This prompted the use of NaCl in the solvent system as an emulsion breaker. Occasionally emulsions are formed in the extraction of aqueous solution by

organic solvents, thus rendering a complete separation impossible. The emulsion may be broken by any of the following devices: mechanical means such as agitation with a glass rod or using a slow filtration process, an increase in the ionic species, addition of alcohol; or simply allowing the mixture to stand for some time (Furniss *et al.*, 1989). In this case, minimising the time of emulsion break-up during CCD was the main objective and the increase of ionic species in the bi-phasic separation system was best suited for this purpose. The reason for this was because the solubility of many organic substances in water is considerably decreased by the presence of dissolved inorganic salts (sodium chloride, calcium chloride, ammonium sulphate, etc.). This process is called "the salting-out effect". A further advantage is that the solubility of partially miscible organic solvents is considerably lower in salt solution.

The use of NaCl demonstrated that persistent emulsions could be avoided, thereby eliminating the time consuming factor in purifying mycolic acids. It is safe to say that the maximum amount of mycolic acids that can be purified with this method is determined by the maximum amount of mycolic acids that can be soluble in the lower phase solvent for loading into the CCD apparatus.

By applying modifications to the previous purification procedure (Goodrum, 1998), *i.e.*, by using NaCl, larger amounts of the extracted mycolic acids could be purified in a single run of CCD separation, without impairing the degree of their purity. This is illustrated by the results summarized in Table 2.2, for mycolic acids originating from *M. tuberculosis*.

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CHAPTER 3

Influence of Mycolic Acids on the T_{H1}/T_{H2} Cytokine Response

Introduction

Innate immunity in tuberculosis

Protection from infectious diseases such as tuberculosis could be achieved through innate (non-specific) immunity or through acquired (specific) immunity. Innate immunity refers to the basic non-specific resistance to a disease, which may involve anatomic, physiologic, endocytic/ phagocytic, and inflammatory mechanisms. Macrophages (MΦ), natural killer (NK) cells and neutrophils are the most likely cells to play an important role in the innate immune response to *M. tuberculosis* and may suffice to control tuberculosis infection before development of an acquired immune response (Vidal *et al.*, 1993). Interleukin 12 (IL-12) is produced by the macrophages, can affect a variety of immune processes and is thought to be a principal regulator of cytokines and T cell subsets (Flynn *et al.*, 1995). Interferon-γ (IFN-γ) produced by the NK cells and T helper (T_H) cells are induced by IL-12 (Chensue *et al.*, 1995b; D'Andrea *et al.* 1992; and Kobayashi *et al.*, 1989). Enhancement of proliferation of NK cells is also induced by IL-12

(Stern *et al.*, 1990; Kaufmann *et al.*, 1995; Wolf *et al.*, 1991; and Chan *et al.*, 1991).

IL-12 is therefore a potent immunoregulatory cytokine centrally involved in the protection against tuberculosis by its early secretion at the innate stage of the immune response to infection. IL-12 regulates the magnitude of the IFN-γ response at initiation of infection (Sieling *et al.*, 1994) and directs the acquired immunity to tuberculosis, mediated by T cells and executed by the macrophage (Ladel *et al.*, 1997b).

T-cells in tuberculosis

T cells are very important immunoregulatory cells, being involved in the activation of B cells and macrophages, in immune suppression and in cytotoxicity against certain targets (Londei *et al.*, 1991). T cells are antigen specific as opposed to their closely related members of the innate system, the NK cells.

T cells can be classified either as those that recognize an antigen in association with MHC class I molecule (CD8 T cells), or as those that recognize an antigen in the context of MHC class II (CD4 T cells) (Brown *et al.*, 1993; Kappes and Strominger, 1988; and Towsend *et al.*, 1989). Virtually all nucleated cells express class I MHC molecules. This ensures their detection and killing by the MHC class I restricted CD8 T cells upon becoming infected or mutated. Mononuclear cells express class II molecules for selective recognition by CD4 (helper) T cells.

Mycobacterium-specific CD4 T cells have been identified consistently in experimental and human tuberculosis (Kaufmann and Flesch, 1986; Otternhoff *et al.*, 1986; and Barnes *et al.*,1990; Barnes *et al.*, 1989). T cell depletion by specific monoclonal antibodies exacerbates the experimental infection of mice with *M.tuberculosis* and BCG (Orme, 1987).

Cytokine detection

Because of the wide spectrum of T-cell cytokine production, it is not an easy task to profile the T-cell cytokine response to disease. The detection of T-cell cytokines is problematic due to the very low concentration of cytokine produced, lower even by several orders of magnitude to those of other biological substances such as hormones and enzymes (Londei *et al.*, 1991; Londei *et al.*, 1989). This is because cytokines are expressed only transiently when they are required to assist in immuno-regulation (Buchan *et al.*, 1988).

Detection of cytokines *in vitro* is done either by immunoassay [radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA)] or by bioassay (Hamblin, 1993). Immunoassays rely on the interaction of a specific antibody and a particular cytokine. They are quick, sensitive and reliable, but have the disadvantage of detecting inactive, broken-down or denatured cytokines as well. Bioassays on the other hand may respond to more than one cytokine as they are often less specific (Contreras *et al.*, 1991).

In situ detection of cytokines is done mainly by RT-PCR. For T cells, a good correlation has been obtained between the mRNA level and the amount of cytokine protein produced. mRNA levels

can be detected using Northern blotting or slot blotting and hybridization, if the appropriate probe is available (Cherwinski *et al.*, 1987). Northern blots require a large amount of mRNA, making it impractical for determination of cytokines, which are expressed at such low levels. This requires highly sensitive methods to assess cytokine expression. The polymerase chain reaction (PCR) has proved useful in this regard, by the ability to amplify specific cDNAs, especially those present in low copy numbers (Taniguchi *et al.*, 1993).

T cells and cytokines

The discovery of different cell-surface markers among T cells allowed the separation of cytotoxic T lymphocytes (CTLs) from helper T cells (T_H cells) and to identify functional subpopulations among each group. The discovery of characteristic cytokine secretion by T cells provided a further base for classification of T-cell subpopulations: According to their secretion pattern, T_H segregate into T_{H1} cells which produce IL-2 and IFN-γ and T_{H2} cells which secrete IL-4, IL-5, IL-6, and IL-10 (Mossmann and Coffman, 1989; Mossmann, 1991). T_{H1} and T_{H2} antagonise one another by their respective cytokines, IFN-γ and IL-4 (Fig.3.1). Years ago it was thought that the CD4 T cell subpopulation was the major producer of cytokines (Mossmann and Coffman, 1989). Today it is established that CD8 T cells, activated killer cells and B cells, as well as other haemopoetic and non-haemopoetic cells can produce cytokines of a wide spectrum (Gordon and Galli, 1990).

IL-4 was initially referred to as a T-cell derived growth factor which induced resting B cells into

the S phase of the cell cycle after stimulation with anti-immunoglobulin antibodies (Hamblin, 1993). Due to this, the T_{H2} response towards infection is generally associated with an antibody response while the T_{H1} response is regarded as mainly cellular (Fig.3.1). This definition is not accurate, however, as characteristic isotypes of antibodies are associated with both T_{H1} and T_{H2} responses.

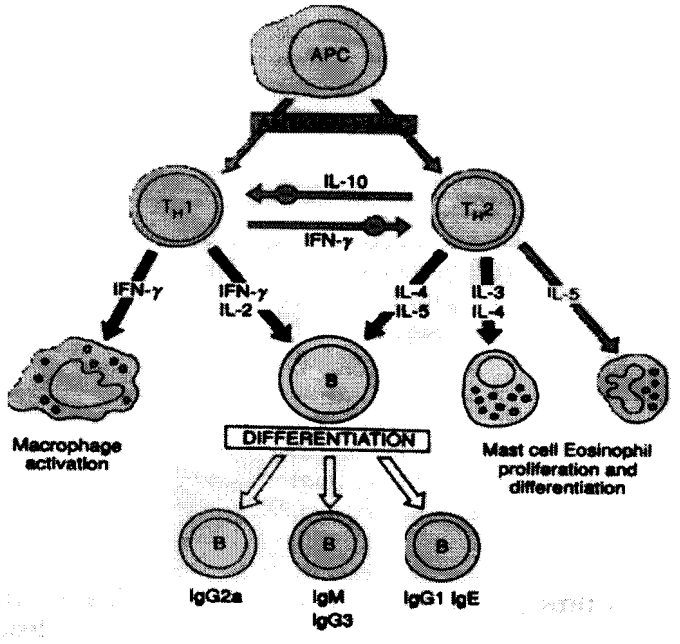

Fig.3.1: Immune responses mediated by T_{H1} and T_{H2} cells (Hamblin, 1993).

The murine T-cell response to tuberculosis is characterized by strong IL-2 and IFN-γ production (Kaufmann and Flesch, 1986). Various *in vitro* studies of murine and human systems have indicated that *Mycobacterium* reactive CD4 T-cells are potent IFN-γ producers (Toosi, 1996; and Kaufmann and Flesch, 1986), indicating the importance of T_{H1} immunity in combatting infection by tuberculosis bacteria. IFN-γ produced by the CD4 T cells, CD8 T cells and NK cells is probably the major macrophage-activating factor (Flesch and Kaufmann, 1993 and Andersen, 1997) (Table 3.1).

Table 3.1: Cytokines involved in Th1/ Th2 response

Cytokine	Major source	Likely mode(s) of action
IL-1	MΦ	Attraction of phagocytes
IL-4	T cell	Macrophage activation & control of phagocyte influx
IL-6	MΦ, T cell	Macrophage activation
IFN-γ	NK, T cell	Macrophage activation
TNF-α	MΦ	Macrophage activation & Granuloma formation
IL-10	T cell, MΦ	Inhibition of macrophage functions

Macrophage activation can be achieved *in vitro* by stimulation with IFN-γ, lipopolysaccharide (LPS) and TNF-α. Flesch and Kaufmann (1991) showed that resistance, through IFN-γ activation of MΦs, involves the action of reactive nitrogen intermediates (RNIs). Chan *et al.* (1991) indicated that inhibition of RNI production *in vivo* through nitric oxide synthethase (NOS) inhibitors, repealed the resistance to *M. tuberculosis* infection in mice.

In Balb/c mice, experimentally infected with *Listeria monocytogenes*, the important role played by IFN-γ produced by NK cells in early protection was demonstrated. It has become apparent that as long as IL-12 secretion is stimulated, IL-4 production will be diminished and the T_{H1} response will prevail (Kaufmann, 1995).

In the present study we investigated the role of MA, isolated from *M. tuberculosis*, in eliciting an immune response. MA biosynthesis has been a target of anti-tuberculosis drugs such as isoniazid (Goren, 1979; Takayama *et al.*, 1972). Evidence of immunoregulatory properties of MA was suggested by Beckman *et al.* (1994), through the observations that in humans, MA is

presented to the T cells by antigen presenting cells (APC) in a MHC-independent manner on CD1b, leading to the activation and proliferation of $CD4^-$,$CD8^-$ T cells. In this work, the study was performed on mice to see if there is any prevention or aggravation of tuberculosis due to MA administration in infected animals. The T_{H1} and T_{H2} cytokines in the spleen were also investigated to determine if MA induces any shift in the T_{H1}/T_{H2} balance.

Hypotheses:

1. Mycolic acids induce resistance to *M. tuberculosis* infection in Balb/c mice.

2. The degree of protection against tuberculosis provided by MA pre-treatment restores the more susceptible Balb/c mice to the status of the more resistant C57BL/6 mice.

3. MA are useful as therapy for *M. tuberculosis*-infected animals.

4. MA as such can induce T_{H1}/T_{H2} cytokines in the spleen of uninfected animals.

5. The survival results of infected animals correlate with the expression of T_{H1}/T_{H2} cytokines in the spleen.

6. MA treatment, before or after *M. tuberculosis* infection, changes the balance of T_{H1}/T_{H2} cytokines in tuberculosis susceptible Balb/c mice.

7. Saponified mycolic acids differ from the non-saponified mycolic acids in their biological effects on tuberculosis resistance.

Approach:

Optimisation of an infective dose of *M. tuberculosis* H37Rv that would be able to kill susceptible mice after a reasonably long incubation period, significantly shorter than that required for resistant mice, was a first priority. Secondly, the infection of Balb/c and C57BL/6 mice with the optimized dosage of *M. tuberculosis* H37Rv was performed, to test whether mycolic acids administration, both as pre-infection and post-infection treatment, could influence the onset and duration of tuberculosis. Finally, correlation of the survival results to the T_{H1}/T_{H2} cytokine balance in the spleen of *M. tuberculosis*-infected and uninfected Balb/c mice was done, using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Materials

Animals

Female C57BL/6 (resistant to tuberculosis) and Balb/c (susceptible to tuberculosis) mice between 8-11 weeks of age, inbred for 9 and 11 generations respectively by the South African Institute for Medical Research (SAIMR, Johannesburg) were used. Male mice from the same litter were used to collect serum for the adsorption of MA. Mice were kept in a glove isolator in a temperature and humidity controlled room.

Culture

M. tuberculosis H37Rv (ATCC 27294) was cultivated at 37 °C as described in the previous chapter (Chapter 2).

Reagents

Reagents used in the Semi-Quantitative Competitive Reverse Transcriptase Polymerase Chain Reaction (QC-RT-PCR):

Ethidium bromide (Boehringer Mannheim, Germany)

Formamide and formaldehyde (BDH, Poole UK)

Tris (Hydroxymethyl)-aminomethane (Merck, Darmstadt Germany)

EDTA (Ethylenediaminetetra-acetic acid) (Merck, Darmstadt Germany)

Sodium acetate (Merck, Darmstadt Germany)

TRI-reagent (Molecular Research Centre Inc, USA)

Formazol (Molecular Research Centre Inc, USA)

MOPS (3-(*N*-morpholino) propanesulfonic acid) (Sigma Chemicals, St Louis USA)

Diethyl pyrocarbonate (DEPC) (Sigma Chemicals, St Louis USA)

Oligo dT primers (Life Technologies Inc., Scotland)

Superscript RNase H Reverse Transcriptase (Life Technologies Inc., Scotland)

Recombinant RNasin (Promega Corporation, Woods USA)

Amplitaq Gold (Roche Molecular Systems, New Jersey USA)

Qiagen mini preparatory column Kit (Qiagen GmbH, Hilden Germany)

Tris EDTA buffer: Tris base 10 mM disodium ethylene diamine tetraacetate.$2H_2O$, pH adjusted to pH 8,3.

Methods

MA purification and preparation of MA-serum conjugate

Saponification, extraction and derivatization of MA were carried out as described by Butler *et al.* (1991), with modifications indicated in the previous chapter. MA was purified by counter current distribution described in Chapter 2.

MA-serum conjugates were prepared by analytically weighing out MA, dissolving it in chloroform and adding the solution to the mouse serum. The volume of the MA solution (10 µl containing 2.5 µg MA) constituted 2% of the volume of mouse serum. The mixture was then sonicated using a Branson Sonifier B-30 at output control 2 and 20% duty cycle for 50 pulses, at room temperature. Finally the chloroform was removed by bubbling nitrogen through the mixture. Serum without MA was treated in the same way and was used for the control experiment. Different dilutions of MA-serum conjugate were made by using chloroform-treated serum (prepared similarly to the MA adsorption process, but omitting the MA) as diluent.

Survival Studies

Mice were divided into groups of at least 8 mice per group. The mice were inoculated intravenously by injection of 4 X 10^4 colony forming units (CFU) of *M. tuberculosis*, in 100 µl 0.9% NaCl, via a lateral tail vein. One week prior to infection, mice from the pre-treatment groups were injected with the MA-serum conjugate (50 µg, 25 µg, or 12.5 µg MA) or with

chloroform-treated serum alone. Post-treatment mice were injected with MA-serum conjugate (8 µg or 16 µg MA) three times on a two day interval regime within the third week after infection. Three mice per group were sacrificed, by cervical dislocation, for cytokine determination 5 and 11 weeks after infection. Assessment of the degree of infection in various organs was carried out by comparing individual organs originating from various groups of experimental mice to the control organs, with the evaluators not aware which were treated mice and which were controls. The spleen, lungs, heart, liver, and kidneys were removed aseptically, immediately snap-frozen in liquid nitrogen and stored at -70°C. The 5-6 remaining mice were kept for the survival studies.

Organ indices assessment in Balb/c mice

Balb/c mice were used in this experiment to determine and compare the degree of infection in the lungs, spleens and livers after pre-treatment and treatment with MA. Mice were divided into seven groups of three mice per group. The groups comprised the following: those receiving no *M. tuberculosis* infection and no MA-serum, only (5 x 10^4 bacteria) infection; no infection but 25 µg MA-serum; 25 µg MA-serum pre-treatment and *M. tuberculosis* infection; 0 µg MA-serum pre-treatment and infection; *M. tuberculosis* infection followed by three times 16 µg MA-serum treatment; and infection followed by three times chloroform treated serum treatment. The organ indices of these mice were determined by weighing the individual mice, then dissecting the lungs, spleen and livers and determining their percentage mass per body mass. The individual organs were then photographed. These measurements were carried out using a Sartorius electronic scale

(with a range of 0,00-200,00 g and accuracy of 0,01 g) and a plastic beaker to contain the mice.

MA treatment of uninfected Balb/c mice

Balb/c mice were intravenous (i.v.) injected with 25 µg of the MA-serum conjugate or with serum alone as a control. Three mice each from both experimental and control groups were sacrificed by cervical dislocation after the MA (or serum only) treatment at the following time periods: 4 hr, 8 hr, 17 hr, 41 hr, 2 weeks and 4 weeks. The spleens were removed aseptically for cytokine profiling, immediately snap-frozen in liquid nitrogen and stored at -70 °C.

Semi-quantitative reverse transcription-PCR

Total RNA of the organs was extracted using TRI-reagent (Molecular Research Centre Inc, Cincinnati, USA), based on a method developed by Chomczynski and Sacchi (1987). Before the RT reaction, the RNA was co-precipitated with primer as described previously (Ausubel *et al.*, 1992). Total RNA (6 µg) was precipitated overnight at -20 °C in the presence of 3 pmol Oligo(dT)12-18 (Gibco BRL, Gaithersburg, MD, USA) with 0.1 M NaOAc, pH 5.5 and absolute ethanol (1:2). After centrifugation the pellet was washed with 70 % ethanol in DEPC-treated H_2O and allowed to dry briefly for about 10 to 15 minutes. Subsequently, the RNA was dissolved in a resuspension buffer [80 mM Tris-HCl pH 8.3, 90 mM KCl and 40 U RNasin (Promega, Madison, WI, USA)], heated to 70 °C for 10 minutes and followed by a 3 hour incubation at

37 °C. The RT reaction was performed with Superscript™ RNase H reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) as recommended by the manufacturer. Polymerase chain reaction (PCR) was carried out in a thermocycler [MJ Research Peltier Thermal Cycler PTC-200 (MJ Research Inc, Watertown, MA, USA)] in a 20 µl reaction volume containing 1.5 mM $MgCl_2$, 0.1 M dNTPs (Promega, Madison, WI, USA), 0.75 U of Amplitaq Gold Polymerase (Perkin Elmer Cetus, Norwolk, CT, USA), Amplitaq Gold PCR buffer and 70 pmol of sense and anti-sense primer (synthesized by Boehringer Mannheim, Indianapolis, USA). Primers for IL-4, IL-10, IFN-γ and their optimised annealing temperatures were as described by Reiner *et al.* (1993), for IL-12 by Bost and Clements (1995), and for ß-actin (Ma *et al.*, 1994). The PCR samples were electrophoresed through a 2% agarose gel containing 2 µg ethidium bromide per millilitre. The gels were scanned and analysed with the computer program NIH Image (version 1.61 on Apple Power Macintosh).

Optimization of PCR conditions

PCR conditions for effective analysis were first optimized for each cytokine using a multi-competitive plasmid, containing sequences for each cytokine to be determined. The PCR reaction mixture concentrations were varied to obtain maximum intensity and performance of the PCR reaction mixtures. Cytokine PCRs were optimised by using different plasmids containing DNA sequence fragments of the various cytokines to be evaluated / tested. These fragments of DNA are deletion mutations of fragments of the wild type cDNA for the individual cytokine. Both the mutated and the wild type cDNA can be amplified by using the same primers. The two different plasmids that were used for this purpose were:

i) a plasmid used for the determination of IL-12 [obtained from K Bost (University of Tulane, USA)]; and

ii) a plasmid used for the determination of IL-4, IL-10, IFN-γ [obtained from R M Locksley (University of California, San Francisco USA)].

Quantification of isolated RNA

Isolated RNA was first quantified by determining the absorbance readings on a spectrophotometer (Shimadzu, UV-visible recording spectrophotometer, UV-160 A) at 260 nm and 280 nm and the ratio determined to assess the purity of RNA. A ratio greater than 1.8 indicated pure RNA and a ratio smaller than 1.8 indicated contamination with proteins. DNA contamination of the RNA was assessed visibly on an electrophoresis gel, while simultaneously estimating the RNA concentration.

RNA concentration was determined electrophoretically by running the RNA through a 1% denaturing agarose gel containing formaldehyde (Sambrook *et al.*, 1989). These denaturing conditions prevent degradation of the RNA by RNases. The 1% agarose gel was prepared by melting 0.4 g agarose in 30 ml DEPC-treated H_2O in a microwave oven, allowing it to cool to 60 °C and then adding 4 ml of 10xMOPS buffer, 2.2 ml of 37% formaldehyde and adjusting the volume to 40 ml using DEPC treated H_2O.

RNA samples were prepared by adding 3 µg of RNA in DEPC-treated water (7 µl), 2 µl of 10xMOPS buffer, 3 µl of 37% formaldehyde and 10 µl of deionized formamide. The samples

were incubated at 55 °C for 15 minutes and then immediately placed in ice or at 4 °C. Ethidium bromide (0.2 µl), from a 10 mg/ml EtBr stock solution, was added to the RNA sample before it was loaded on the gel to enable visualization of the DNA with UV light. Pure, undegraded RNA giving the three rRNA bands (28S rRNA, 18S rRNA and 5S rRNA) on agarose gel electrophoresis, was used for the reverse transcriptase reaction (Sambrook and Maniatis, T. (1989). This was put up by adding EtBr in the tracking dye (15% Ficoll and 0.025% bromophenol blue in glycerol). The electrophoresis gel was ran at 69 volts for 1 hour in 1 x MOPS running buffer that contained DEPC-treated H_2O.

Results

Survival of infected mice treated with MA.

MA was administered to different mice that were to be, or had been infected with an optimized dose of *M. tuberculosis* to investigate if MA would influence the onset and duration of tuberculosis. As the lipid nature of MA allows dissolution only in chlorinated hydrocarbon solvents, MA was adsorbed on homologous mouse serum before being injecting into mice. Balb/c and C57BL/6 were injected with the MA-serum conjugate prior to (pre-treatment) or after (treatment) the infection with *M. tuberculosis*. Pre-treatment mice were injected with MA-serum conjugate one week prior to infection. Treatment mice were given MA-serum conjugate in three equal consecutive doses every other day, three weeks after infection. The effect of MA treatment and pretreatment on *M. tuberculosis* infected mice were then monitored.

Survival data of the susceptible Balb/c mice are shown in Fig.3.2.1. *M. tuberculosis* infected Balb/c mice pre-treated with 12.5 µg and 25 µg MA showed some protective effect while the 50 µg MA pretreatment appeared to make the mice more susceptible to tuberculosis than when left untreated, or treated with serum only. This suggests a protective effect of MA pre-treatment over a narrow dose range. In Fig.3.2.2. the survival data of the more resistant C57BL/6 mice showed no significant effect of pre-treatment with 0 µg, 12.5 µg, 25 µg and 50 µg MA on the prognosis of the disease. However, one can clearly see the difference in the susceptibility to tuberculosis of Balb/c and C57BL/6 mice by comparing Fig.3.2.1 and Fig.3.2.2 The onset of mortality in all groups, except the 25 µg MA pre-treatment groups, was considerably faster in the

Balb/c mice than in the C57Bl/6 mice. The 25 µg MA pre-treatment of the Balb/c mice conferred a resistance to tuberculosis on Balb/c mice similar to that of C57Bl/6 mice.

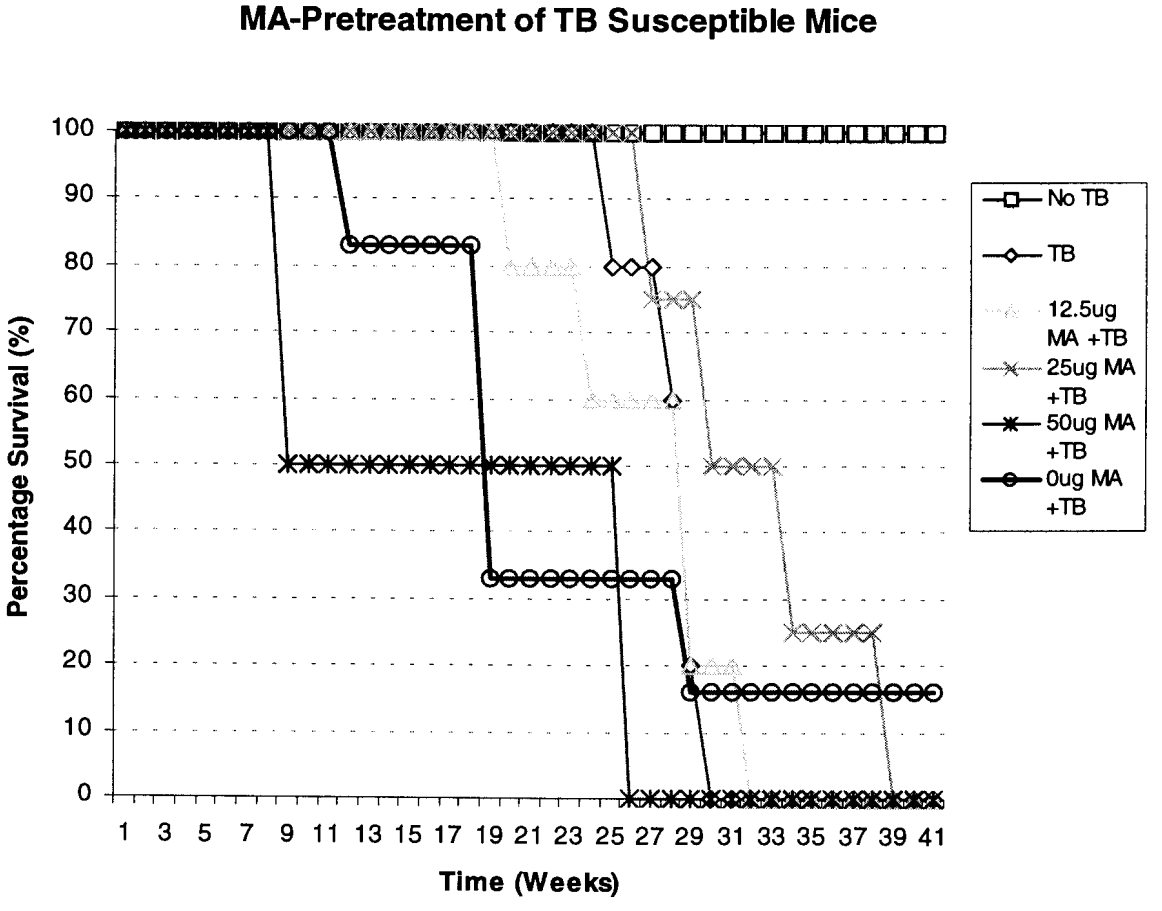

Fig.3.2.1: Survival of the tuberculosis-susceptible Balb/c mice, infected with *M. tuberculosis* and pre-treated with 0 µg, 12.5 µg, 25 µg and 50 µg MA conjugated to serum, before *M. tuberculosis* infection. Control mice were not given TB or MA treatment.

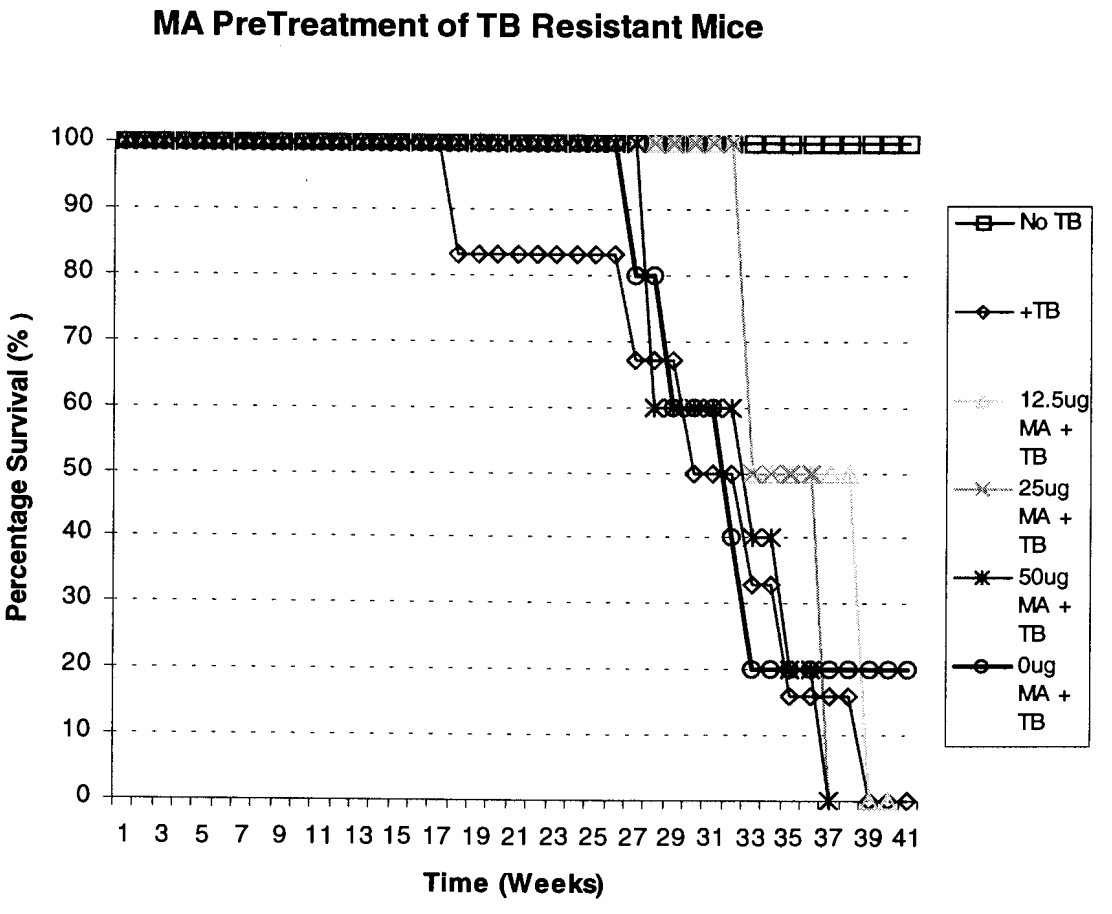

Fig.3.2.2: Survival of the tuberculosis-resistant C57Bl/6 mice, infected with *M. tuberculosis* and pre-treated with 0 µg, 12.5 µg, 25 µg and 50 µg MA conjugated to serum, before *M. tuberculosis* infection. Control mice were not given TB or MA treatment.

Fig.3.2.3 shows the survival of Balb/c mice after treatment with 0 µg, 24 µg and 48 µg MA three weeks after TB infection. Improved survival was only observed for the 48 µg MA treatment group. Fig.3.2.4 shows survival of C57BL/6 mice treated with MA after infection with TB. Again, C57Bl/6 showed no significant difference among the groups that were infected with TB and treated with MA afterwards. Although the difference between the susceptibility of Balb/c and C57Bl/6 mice could still be observed by comparing the time of onset of mortalities between Figs. 3.2.3 and 3.2.4, the differences were somewhat smaller.

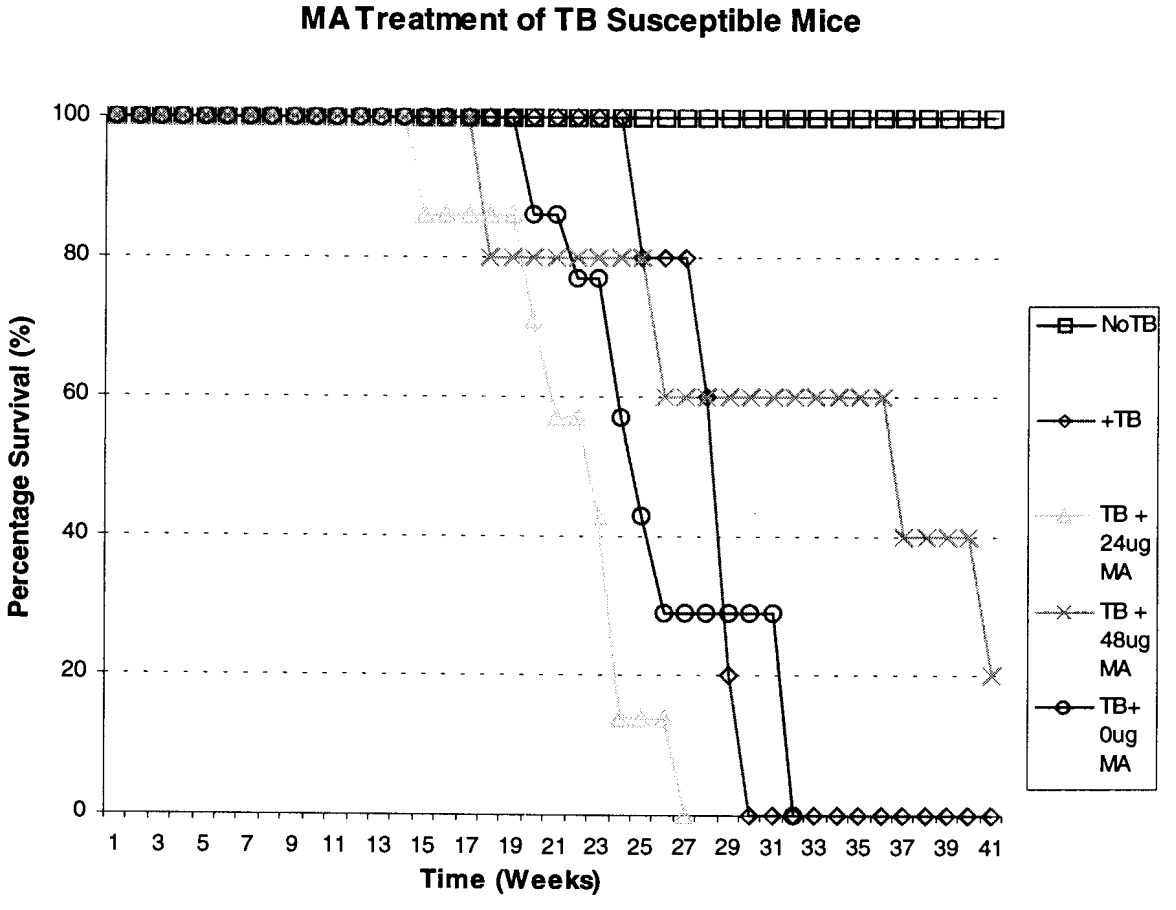

Fig.3.2.3: Survival of the tuberculosis-susceptible Balb/c mice, infected with *M. tuberculosis* and treated with 0 µg, repeated 3x8 µg (24 µg) and repeated 3x16 µg (48 µg) MA conjugated to serum, after *M. tuberculosis* infection. Control mice were not given TB or MA treatment.

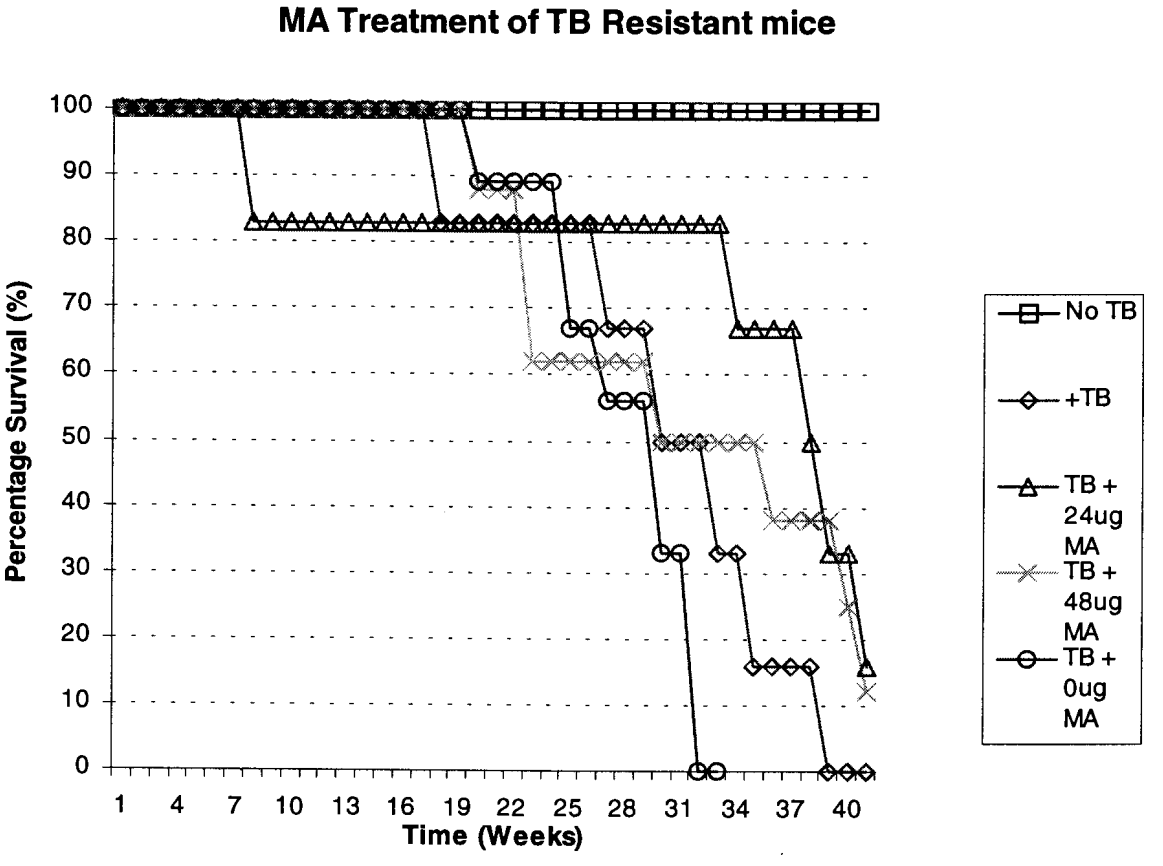

Fig.3.2.4: Survival of the tuberculosis-resistant C57Bl/6 mice, infected with *M. tuberculosis* and treated with 0 µg, repeated 3x8 µg (24 µg) and repeated 3x16 µg (48 µg) MA conjugated to serum, after *M. tuberculosis* infection. Control mice were not given TB or MA treatment.

Histopathology assessments

Table 3.2 gives an assessment of the macroscopic appearance of organs while they were dissected and removed for cytokine determination. At the first organ extraction, 5 weeks after the infection, the worst affected spleens were those from mice receiving serum only as a post-infection treatment. The best protected spleens appeared to come from groups that received MA treatment after infection. Mycolic acids pre-treatment and treatment also appeared to have some protective effect on the lungs when compared to serum post- and pre-treatment and no-treatment groups.

In the second organ extraction two weeks later, the spleens from all the groups appeared equally affected, but the lungs now appeared to be partially protected in all the groups of mice that received MA as treatment and pre-treatment. No significant differences could be observed in the appearances of the livers obtained from both the 5-week and the 7-week post-infection organ extractions.

These results gave the first indication that the lungs were the most responsive organs towards the MA-induced protection provided by MA.

Table 3.2 : Pathology studies of organs from Balb/c mice, observations noted immediately after organ extraction. Mice were treated with MA before and after infection with *M. tuberculosis* H37Rv with control groups receiving only H37Rv or nothing at all.

Group	Mass Loss per group after 60 days	Mortality 60 days after infection	1st Organ extraction (32 days after infection) Pathology				2nd Organ extraction (46 days after infection) Pathology			
			M	Spleen	Lungs	Liver	M	Spleen	Lungs	Liver
Uninfected	2	0	1	Normal	Normal	Normal	1	Normal	Normal	Normal
			2	"	"	"	2	"	"	"
			3	"	"	"	3	"	"	"
M.tb H37Rv Only	0	0	1	>>	+++	-	1	>>>	+++	Normal
			2	>>	+++	-	2	>>>	+++	"
			3	>>	+++	-	3	>>>	+++	"
12.5 µg single Pre-Treatment	1	1	1	>>	++	-	1	>>>	++	"
			2	>>	++	-	2	>>>	++	dark red
			3	>>	++	-	3	>>>	++	Normal
25 µg single Pre-Treatment	1	0	1	>>>	++		1	>>>	++	"
			2	>>	++	-	2	>>>	++	"
			3	>>>	++		3	>>>	++	"
50 µg single Pre-Treatment	2	0	1	>>>	++	-	1	>>>	++	dark red
			2	>>>	++	-	2	Normal	Normal	dark red
			3	>>	++	-	3	>>>	++	dark red
single Serum Pre-Treatment	0	0	1	>>>	+++	-	1	>>>	+++	"
			2	>>	+	-	2	>>>	+++	pale
			3	>>>	++	-	3	>>>	+++	Normal
8 µg Repeated Post Treatment	0	0	1	>	++	-	1	>>>	++	"
			2	>	++	-	2	>>>	++	"
			3	>	++	-	3	>>>	++	"
16 µg Repeat d Post Treatment	0	0	1	>	++	-	1	>>>	++	"
			2	>	+	-	2	>>	+++	"
			3	>	++	-	3	>>	+++	"
Repeated Serum Post Treatment	1	0	1	>>>	+++	-	1	>>>	+++	"
			2	>>>	+++	-	2	>>	+++	"
			3	>>>	+++	-	3	>>>	+++	"

Organ indices assessments in Balb/c mice

In a separate experiment aiming at assessing the infected state of the organs upon pre- and post-treatment with MA, the organ indices of Balb/c mice were determined as a percentage of body mass (Table 3.3).

Both the lungs and the spleens exhibited much larger organ indices for infected animals than for the non-infected, but the liver-indices remained constant. MA-serum treatment without *M. tuberculosis* infection did not affect the organ indices of lungs, livers and spleens in any way, boding well for the use of mycolic acids as an intravenous medication. *M. tuberculosis* infection appeared to dominate the change in organ indices, such that differences between mycolic acids-treated and untreated mice did not produce significant changes in respect of organ indices for the lungs, livers and spleens.

It therefore appears that organ index determination does not provide significant data to test the hypothesis that mycolic acids pre- or post-infection treatment protects lung or spleen from disease.

Table 3.3: Organ indices of Balb/c mice. Lungs, livers and spleens were dissected from individual mice and weighed. The organ indices were determined from their mass as a percentage of body mass.

		Organ Index		
Group	**Mouse No**	**Lungs**	**Liver**	**Spleen**
1	1.1	1.2	4.6	0.4
Uninfected & Untreated	1.2	0.7	5.5	0.4
	1.3	0.9	5.5	0.5
Average		0.9 ± 0.2	5.2 ± 0.5	0.4 ± 0.1
2	2.1	1.1	5.5	1.1
$5x10^4$ H37Rv	2.2	1.7	6.2	2.1
	2.3	1.4	5.6	1.3
Average		1.4 ± 0.3	5.8 ± 0.4	1.5 ± 0.5
3	3.1	0.8	4.5	0.3
25 µg MA Pretreatment only	3.2	0.8	4.7	0.5
	3.3	0.8	4.4	0.5
Average		0.8 ± 0	4.5 ± 0.2	0.4 ± 0.1
4	4.1	1.5	5.2	1.6
25 µg MA Pretreatment +TB	4.2	1.1	6.2	1.7
	4.3	1.1	5.6	1.2
Average		1.2 ± 0.2	5.6 ± 0.5	1.5 ± 0.3
5	5.2	1.1	5.2	1.4
Serum + TB	5.6	1.2	6.0	1.6
	5.7	1.1	5.6	2.1
Average		1.1 ± 0.1	5.6 ± 0.4	1.7 ± 0.4
6	6.2	1.0	5.7	1.4
TB + 3 x 16 µg MA Treatment	6.4	1.1	6.2	1.5
	6.7	1.2	5.3	1.5
Average		1.1 ± 0.1	5.7 ± 0.4	1.5 ± 0.1
7	7.1	1.6	5.8	1.5
TB + 3 x Serum Treatment	7.3	1.4	6.2	2.0
	7.4	1.5	6.5	2.2
Average		1.5 ± 0.1	6.1 ± 0.4	1.9 ± 0.4

Cytokine profile of non-infected MA treated mice

Short term (4 h, 8 h, 17 h, and 41 h) and long term (2 and 4 weeks) effects of MA treatment (25 µg) on the spleens of healthy Balb/c mice were investigated to find out if there will be any significant effects on the T_{H1}/T_{H2} cytokine bias. This was done to determine whether MA could influence the T_H cytokine secretion in any way when there is no infection present. For the purpose of determining the T_{H1}/T_{H2} cytokine profile of spleens of animals, IFN-γ was selected as a typical T_{H1} cytokine, while IL-4 and IL-10 were selected as typical T_{H2} cytokines.

Immunoregulatory effects of MA in Balb/c mice on the expression of IL-4 (Fig. 3.3.1) showed that, after 4 hours, the non-resaponified MA stimulated IL-4 expression stronger than resaponified MA, but this effect was not confirmed at 8, 17 and 41 hours after the administration of MA.

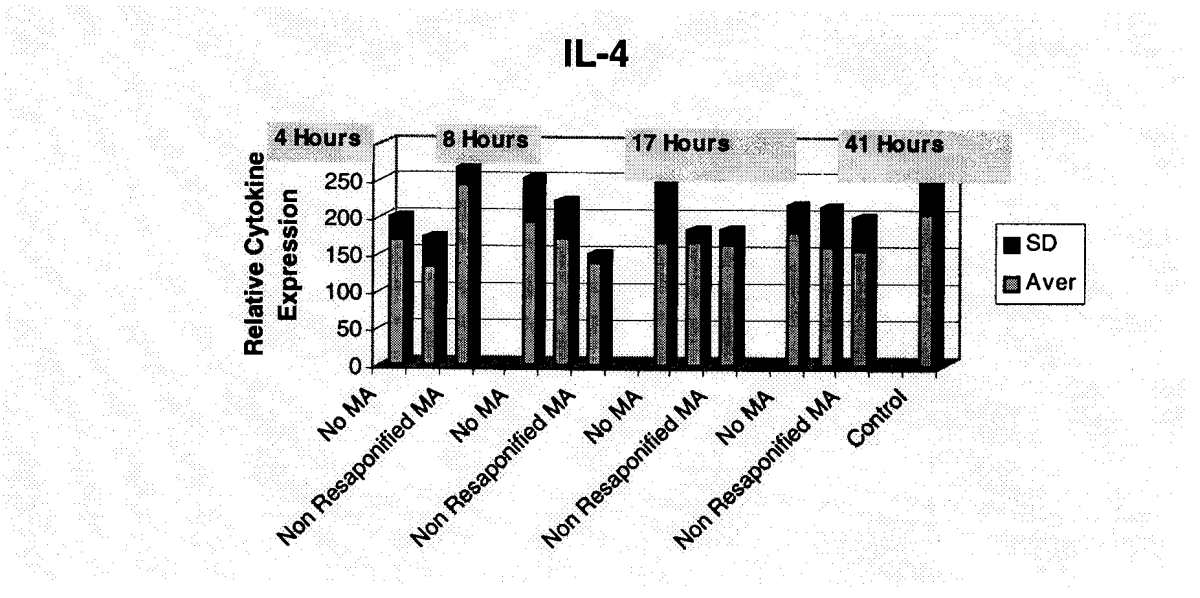

Fig. 3.3.1: Short term effects of treatment of Balb/c mice with 25 µg resaponified or non-resaponified MA and serum only on IL-4 expression in the spleen . Control mice were given no treatment. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

IFN-γ expression (Fig.3.3.2) appeared to be suppressed by the resaponified MA within 4 hours of administration of MA, when compared to the untreated, serum-only treated and non-treated controls, but this effect was not confirmed at 8, 17 and 41 hours after administration of MA. The levels of IL-10 expression (Fig.3.3.3) were not influenced by mycolic acids in any form (either resaponified or non-resaponified MA).

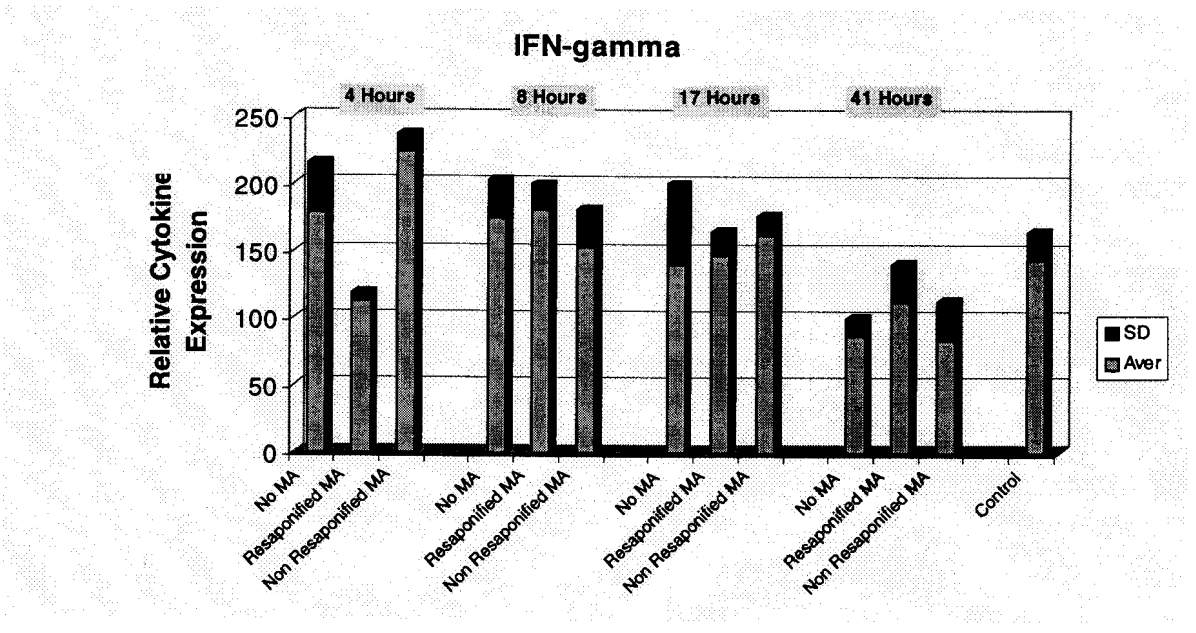

Fig. 3.3.2: Short term effects after treatment of Balb/c mice with 25 µg resaponified or non-resaponified MA and serum on IFN-γ expression in the spleen. Control mice were given no treatment. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

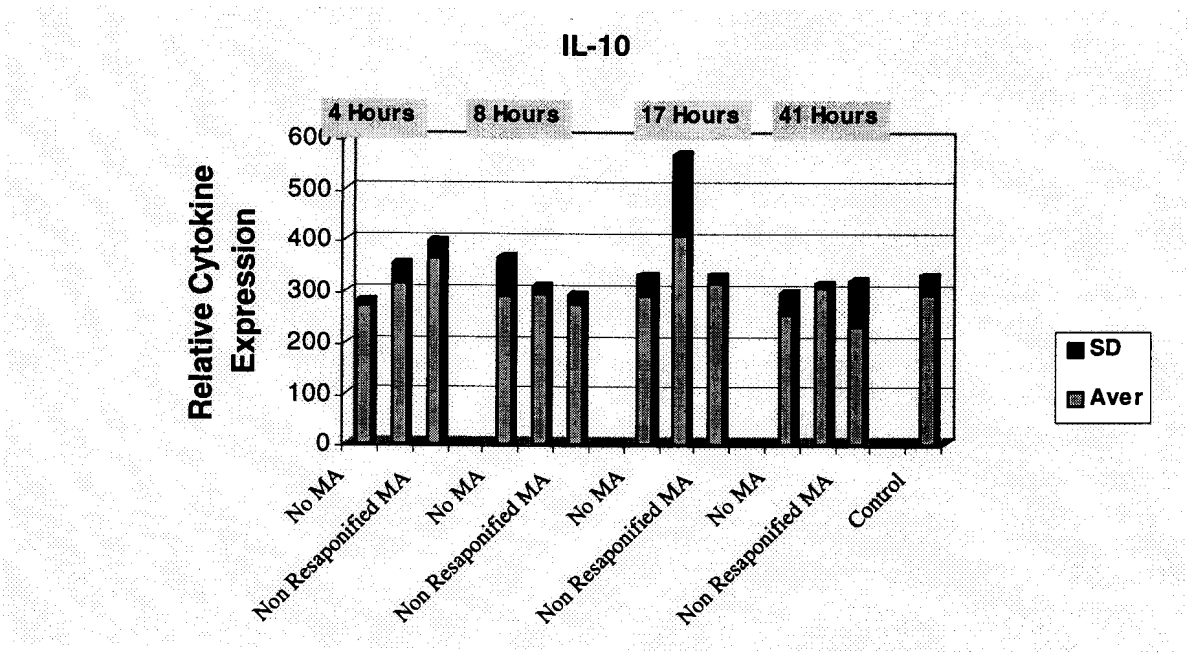

Fig. 3.3.3.: Short term effects of treatment of Balb/c mice with 25 µg resaponified or non-resaponified MA on IL-10 expression in the spleen. Control mice were given no MA and no serum. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

Longer term effects of MA on IL-4 (Fig.3.3.4) IFN-γ (Fig 3.3.5) and IL 10 (Fig 3.3.6) were also not observed, since expression of these cytokines after 2 weeks indicated that there was no pronounced difference between the non-resaponified MA, resaponified MA and the serum control groups. The effects were the same after 1 month, which were comparable to those of 2 weeks.

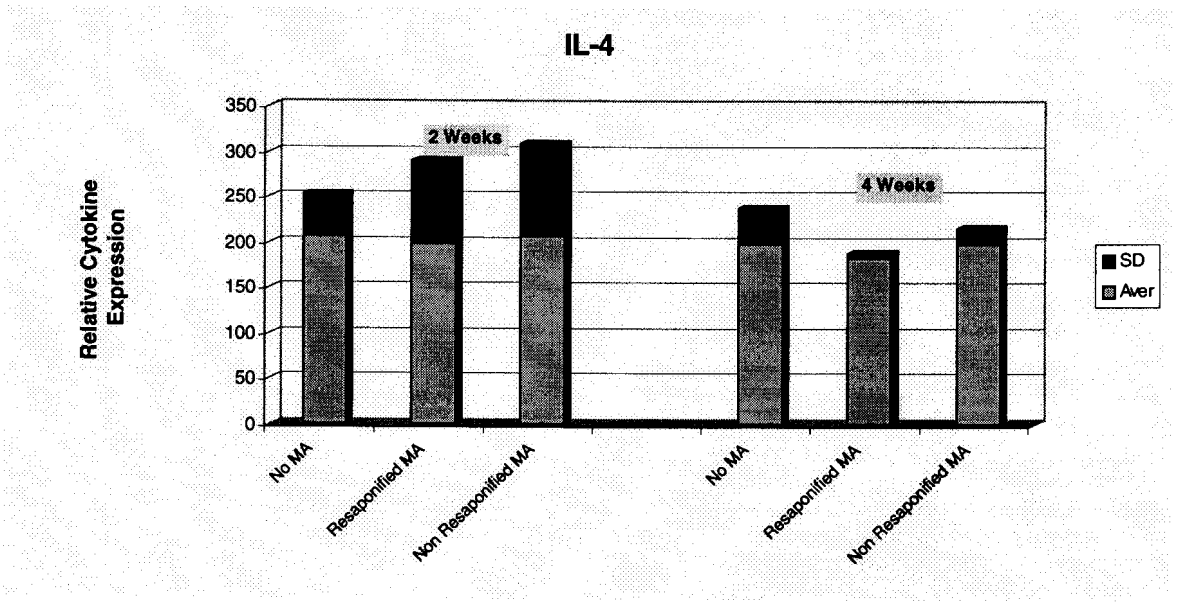

Fig. 3.3.4: Long term effects of treatment of Balb/c mice with 25 µg resaponified and non-resaponified MA on IL-4 expression in the spleen. Control mice were only given serum. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

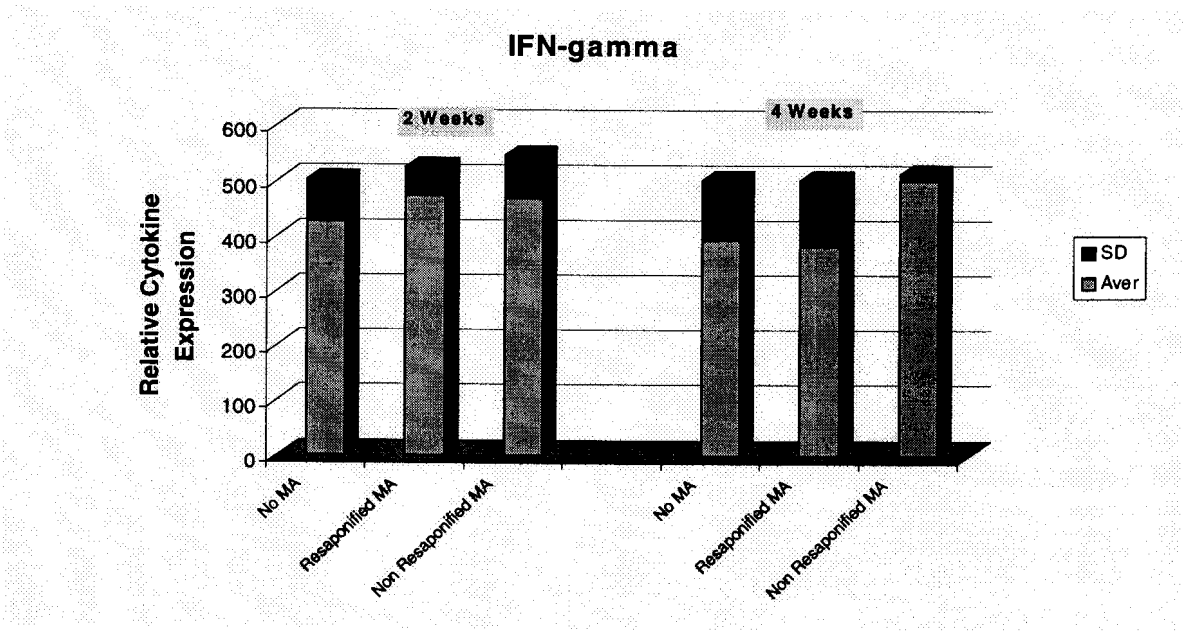

Fig. 3.3.5: Long term effects of treatment of Balb/c mice with 25 µg resaponified or non-resaponified MA on IFN-γ expression in the spleen. Control mice were only given serum. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

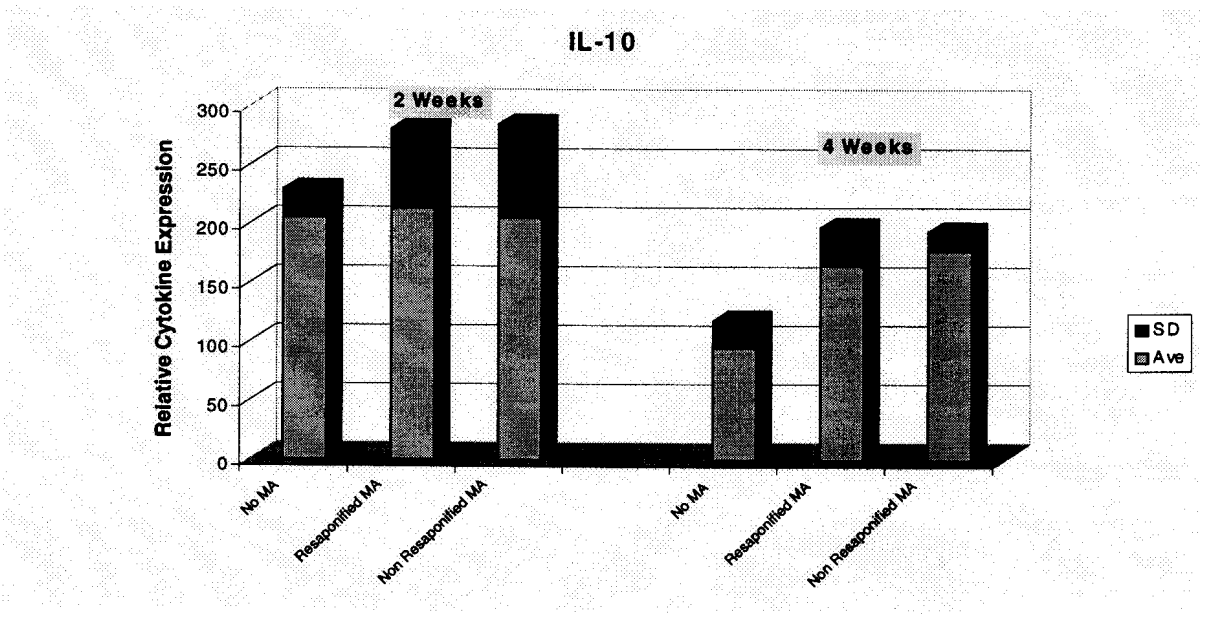

Fig. 3.3.6: Long term effects of treatment of Balb/c mice with 25 µg resaponified or non-resaponified MA effects on IL-10 expression in the spleen. Control mice were only given serum. "No MA" refers to treatment with only the homologous serum carrier. "Aver" refers to the average of three readings and "SD" refers to the standard deviation thereof.

In general, the levels of expression of the cytokines IL-4,IFN-γ and IL-10 within 41 hours of administration of MA, as indicated in Fig. 3.3.1, 3.3.2 and 3.3.3, remained stable. The expression levels of the three cytokines were already high in the untreated spleens and remained unchanged by mycolic acids or serum only treatment. No significant differences were observed between resaponified or non-resaponified mycolic acids-treatment over the short term (Fig. 3.3.1, 3.3.2 and 3.3.3) or the long term (Fig. 3.3.4, 3.3.5 and 3.3.6).

To establish whether immunoregulatory effects of mycolic acids could be demonstrated by administration and boosting with the MA-serum conjugates, healthy Balb/c mice were injected with 250 µg followed by two booster injections of 25 µg MA or serum only with two week intervals between administrations. IL-4, IL-10, IFN-γ and IL-12 were determined in the spleens at 24 hr and 48 hr after the last booster.

Fig.3.4 shows the data. Serum administration alone appeared to cause an increased expression of IL-4, IL-10 and IL-12, which peaked at or around 24 hours after the last administration, with decreased signals already apparent after 48 hours. Only IFN-γ remained undeterred by MA administration. The administration of MA adsorbed to serum had a profound effect on IL-4, IL-10 and IL-12: Although it did not significantly induce expression of these cytokines to higher levels than that induced by serum alone, it appeared to maintain the elevated expression of these cytokines over a longer period of time. The IFN-γ signals remained refractory, however. Each bar on Fig.3.4 represents data obtained after RNA of three samples from each treatment groups were quantified and equal concentrations pooled together before PCR quantification.

These results indicate that mycolic acids can be expected to exert immunoregulatory effects upon administration into mice, which may influence their susceptibility or resistance to tuberculosis. The findings that both IL-12 (leading to T_{H1}) and IL-4 (typical T_{H2}) expression is maintained by the administration of MA, does not allow a simple explanation of how MA provide protection against tuberculosis by the spleen cells. A simple bias towards a protective T_{H1} response was expected, but was not seen.

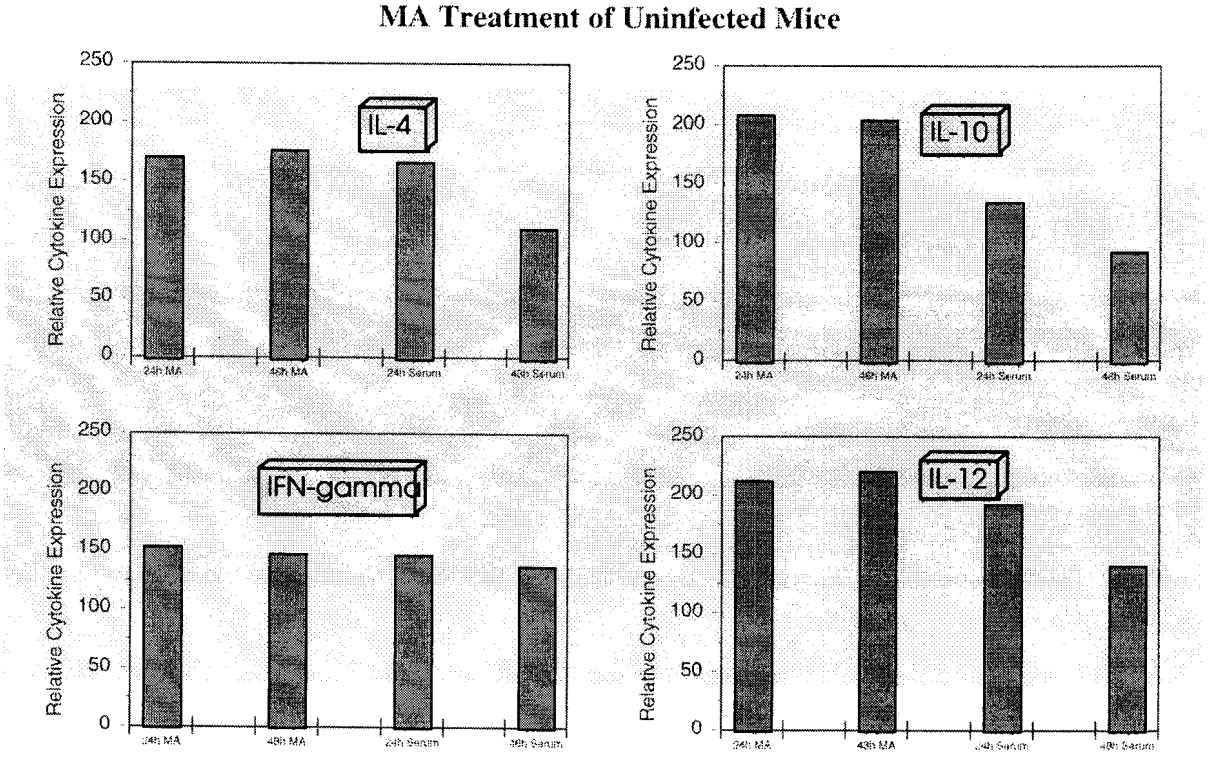

Fig.3.4.: Relative Cytokine Expression of Balb/c mice that were first immunized with 250 µg and then given 25 µg boosts of MA at different periods and the spleen removed 24 hours and 48 hours after the last boost.

Cytokine profile of infected MA-treated mice

Fig.3.5.1 presents the T_{H1}/T_{H2} cytokine profile of the spleens of MA pre-treated and *M. tuberculosis* infected mice analysed at 5 weeks after infection. For each cytokine determined, the range of expected expression levels of cytokines can be learned from the positive *M. tuberculosis* infected and negative non-infected controls. These are represented by the first two bars of each histogram in Fig.3.5.1. From this, reasonable variation can be expected for IL-10, IFN-γ and IL-12. IL-4 appeared to be expressed at high levels already in the non-infected control, whereas IL-

10, IFN-γ and IL-12 became induced more strongly by infection with *M. tuberculosis*. These results are corroborated by the week 7 data (Fig.3.5.2).

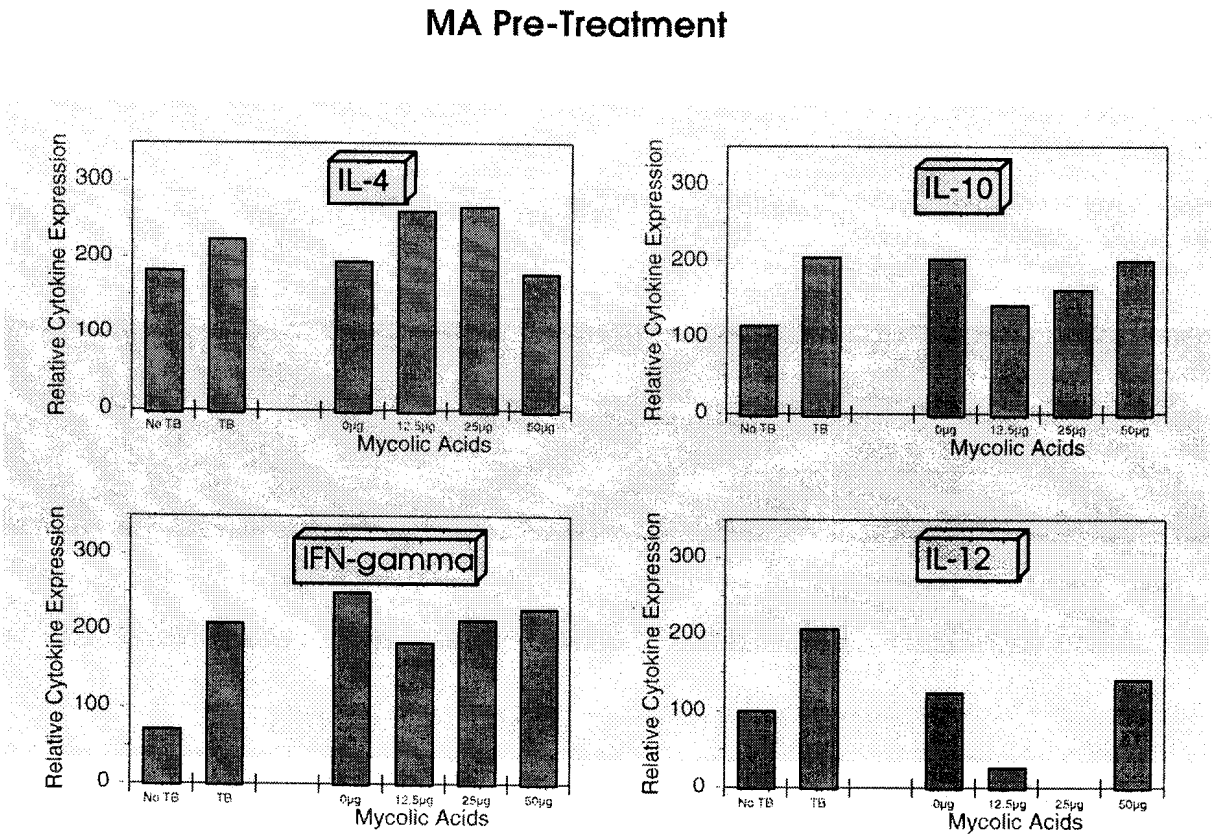

Fig. 3.5.1.: Cytokine expression after pre-treatment with MA. Spleens were dissected from Balb/c mice 5 weeks after infection with *M. tuberculosis* H37Rv and the levels of IL-4, IL-10, IFN-γ and IL-12 determined through RT-PCR. The animals were pre-treated with 12.5 µg, 25 µg, 50 µg or 0 µg MA conjugated to mouse serum

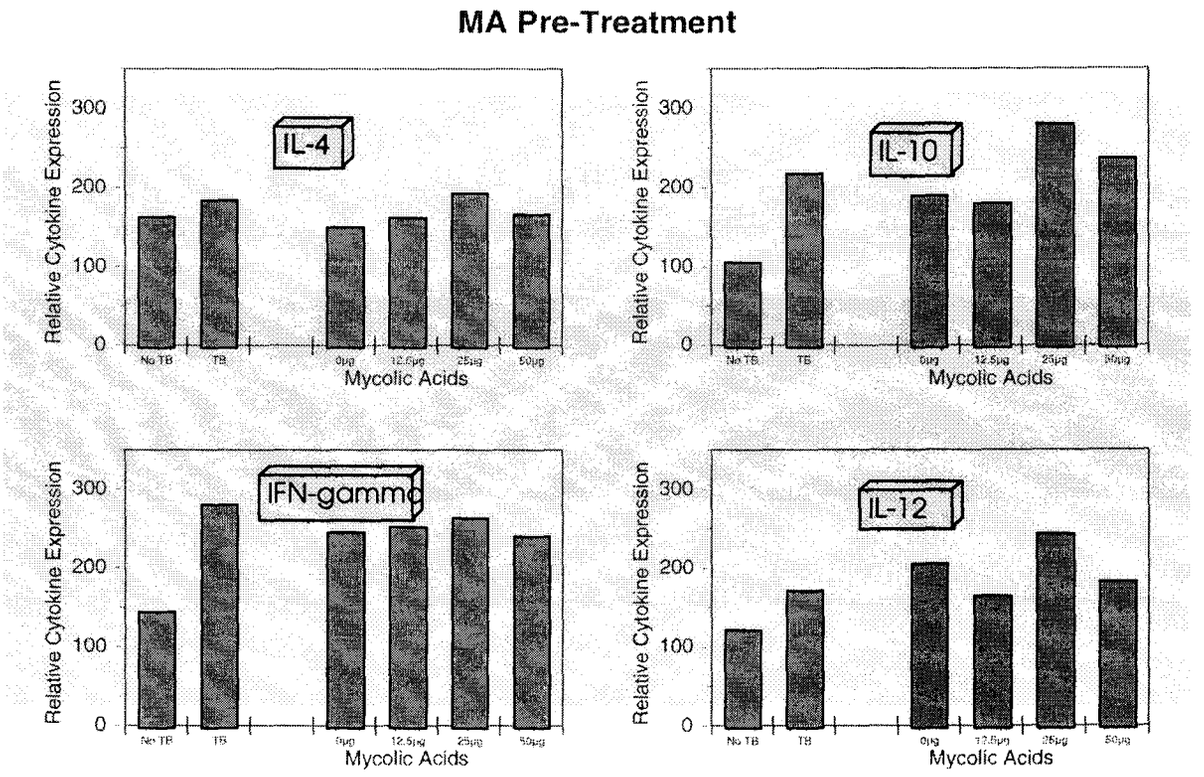

Fig. 3.5.2.: Cytokine expression after pre-treatment with MA. Spleens were dissected from Balb/c mice 7 weeks after infection with *M. tuberculosis* H37Rv and the levels of IL-4, IL-10, IFN-γ and IL-12 determined through RT-PCR. The animals were pre-treated with 12.5 µg, 25 µg, 50 µg or 0 µg MA conjugated to mouse serum.

Mycolic acids pre-treatment before infection of Balb/c mice provided protection against tuberculosis at a dose of 25 µg MA. This would suggest a stimulated IL-12/IFN-γ response (T_{H1}) at 25 µg MA pre-treatment. The 5-week and 7-week data do not strongly support this anticipated response. Although the IL-12 expression appears to be enhanced at 25 µg MA pre-treatment at 7 weeks (the sample for the 5 weeks 25 µg MA pretreatment was lost), so also were the signals for IL-10 and IL-4. At 5 weeks post-infection these differences were less pronounced. These data do not support a simple T_{H1} response in the spleen providing protection against TB at 25 µg MA administration one week before infection. The relationships between the four measured cytokines appear too complex at this stage to allow for a simple T_{H1}/T_{H2} ratio shift in the spleen as a mechanism of protection.

With post-infection MA treatment, peak levels of expression of IL-4, IFN-γ and IL-12 were observed for the 24 µg MA treatment groups at both 5 weeks (Fig.3.5.3) and 7 weeks (Fig.3.5.4) after infection. At 5 weeks, this observation was also made for IL-10, but was not convincing on the basis of the 5 week data. As in pre-treatment, these results did not correlate simplistically to survival, which peaked at 48 µg of MA treatment. Each bar represents data obtained after RNA of three samples from each treatment groups were quantified and equal concentrations pooled together before PCR quantification.

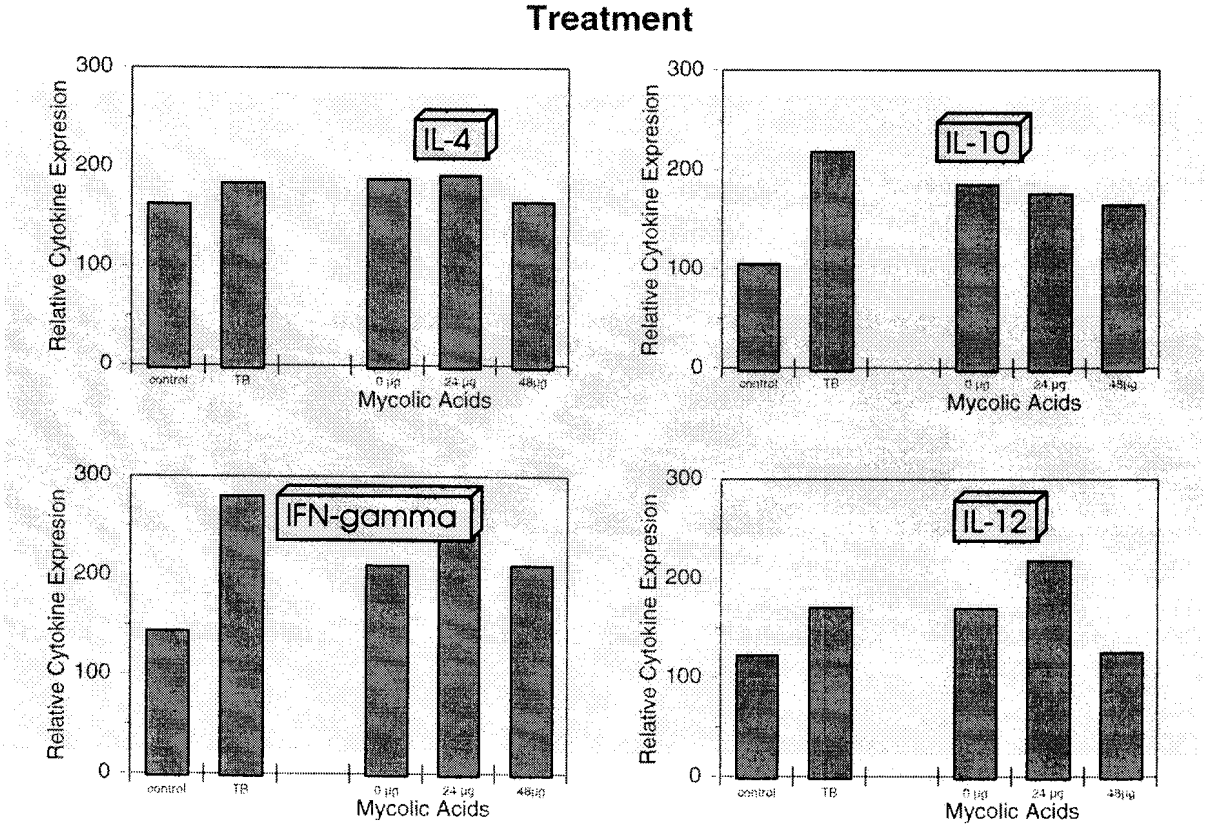

Fig. 3.5.3: Cytokine expression after treatment with MA. Spleens were dissected from Balb/c mice 5 weeks after infection with *M. tuberculosis* H37Rv and the levels of IL-4, IL-10, IFN-γ and IL-12 determined through RT-PCR. The animals were treated with 3 x 8 µg (24 µg), 3 x 16 µg (48 µg) or 0 µg MA conjugated to mouse serum.

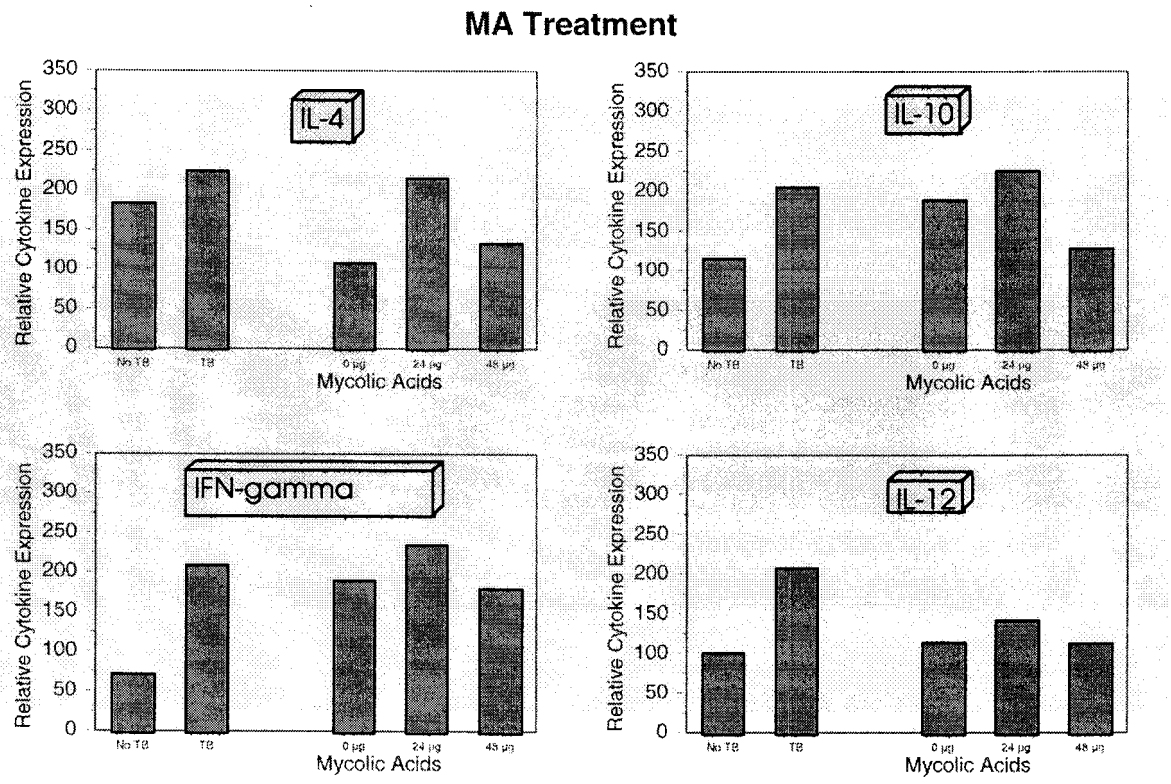

Fig. 3.5.4.: Cytokine expression after treatment with MA. Spleens were dissected from Balb/c mice 7 weeks after infection with *M. tuberculosis* H37Rv and the levels of IL-4, IL-10, IFN-γ and IL-12 determined through RT-PCR. The animals were treated with 3 x 8 µg (24 µg), 3 x 16 µg (48 µg) or 0 µg MA conjugated to mouse serum.

DISCUSSION

The discovery by Beckman *et al.* (1994) that mycolic acids exert immunoregulatory functions through stimulation of double negative T cells, prompted this study to determine the role played by mycolic acids (MA) in the enhancement of, or protection against *M. tuberculosis* infection. This was first tested in survival experiments, where the results suggested that there was some degree of protection rendered by MA administration. These experiments were done once it had been established what was the optimum dosage of *M. tuberculosis* that killed Balb/c mice but was ineffective in C57BL/6 mice. In this case the optimised dose of *M. tuberculosis* infection was found to be similar to that established by Flynn *et al.* (1995), i.e. 10^4 - 10^5 viable cells (CFU) per mouse.

In the survival studies the experiments were performed with the assumption that Balb/c mice are more susceptible to *M. tuberculosis* infection than C57BL/6 mice, which are considered to be more resistant (Flynn *et al.*, 1995 and Flynn and Bloom, 1996). The survival data obtained confirmed this. Flynn and Bloom (1996) have indicated that the increased resistance of C57Bl/6 mice was not due to their particular MHC alleles, but rather to the ability of these mice to launch an IFN-γ response of bigger intensity than the Balb/c mice. The IFN-γ response was directly linked to the IL-12 response that countered *M. tuberculosis* infection.

In this study it was found that treatment with MA does improve the resistance to *M. tuberculosis* infection. The resistance of Balb/c mice to tuberculosis was improved with 12.5 µg and increased with 25 µg MA pre-infection treatment, but in the case of 50 µg pre-treatment the animals were becoming more susceptible to infection as compared to the serum only pre-treated controls. With

the post-infection treatment group, protection was only observed at 48 µg MA while 24 µg MA showed no effect. Flynn *et al.* (1995) established that treatment of *M. tuberculosis*-infected resistant C57BL/6 mice with IL-12 did not have any significant positive or negative effects on the resistance of these mice to tuberculosis infection. In this study, it was established that C57BL/6 mice were refractory towards the effect of mycolic acids pre-treatment and treatment in respect of the enhancement or break-down of resistance to tuberculosis. This was observed even in the case of the 50 µg MA pre-treatment, a dose which was found to decrease resistance in Balb/c mice.

The data obtained from post-infection treatment studies suggest the possible application of mycolic acids in therapy of tuberculosis. This effect was evident only in Balb/c mice that received a high dose of MA (48 µg), a dose level which was found to be contra-indicative in pre-treatment studies. A lower dose of MA for post-infection treatment did not have any effect on the survival of these mice. In C57BL/6 mice, there was again no effect of post-infection treatment with MA on survival.

Flynn and Bloom (1996) provided evidence that both CD4 and CD8 T cells are important in the control of tuberculosis. They have investigated the role of T_{H1}/T_{H2} cytokines in response to tuberculosis and have established that IL-12 treatment increases resistance to *M.tuberculosis* infection in Balb/c mice. The resistance depended on the presence of IFN-γ, because when the latter was depleted, the resistance was removed. They have also established that treatment of infected Balb/c mice with IFN-γ had no significant effects. In their studies using the quantitative RT-PCR method, they have observed that levels of IFN-γ mRNA in the spleen cells are the same regardless of whether they have treated the animals with IL-12 or with PBS. The levels of IL-4

in the spleen were found to be four-to two-fold higher for PBS-treated mice as compared to IL-12-treated animals, but were still much lower than the IFN-γ responses, suggesting a strong T_{H1} response.

MA did not elicit any strong expression of IFN-γ mRNA nor any strong IL-4 mRNA expression according to the data obtained. The level of IL-4 mRNA expression after 24 µg post-infection treatment gave a two-fold increase over the serum-control and 48 µg treatment, but this did not correlate in any way with the survival data of the mice. There appeared to be no significant interdependent up- and down-regulation between the IL-4 and IFN-γ mRNA.

MA appeared not to elicit any significant immune response in the spleens of uninfected animals. This was not the case for the lungs, which were analysed in another study parallel to this one (Verschoor *et al.*, 1998). In that case, it was observed that in the lungs there was an increase of IL-12 mRNA expression after MA treatment and pre-treatment (Appendix A). Those findings indicated the importance of IL-12 expression in tuberculosis resistance. This was also observed in another parallel experiment (Appendix B), where IL-12 level in Balb/c and C57Bl/6 mice, infected with *M. tuberculosis*, were compared to non-infected control mice. In this experiment the various organs such as, the spleen, the liver, the kidney, the lungs and the heart were investigated for their immune cytokine response to *M. tuberculosis* infection. It was observed that infection stimulated the level of IL-12 expression in the liver and the kidney of both strains of infected mice. The lungs of the C57Bl/6 mice, on the other hand, showed a constitutively high level of IL-12 even before any infection. In the spleens, the IL-12 expression levels were the same for both strains of animals. The lungs of the Balb/c mice showed that infection with *M. tuberculosis* stimulated expression as compared to non-infection. These observations were in

agreement with Cooper *et al.* (1995) who discovered that upon impairment of IL-12 in C57BL/6 mice, resistance of C57BL/6 mice to *M.tuberculosis* is rescinded.

These studies do not support the suggestions by Cooper *et al.* (1993 and 1995) and Flynn *et al.* (1995), that secretion of IL-12 by the macrophages implies that the system will develop a T_{H1} response. The results obtained in the spleen, in conjunction with those obtained in the parallel study in the lungs, suggest nevertheless that MA induced protection is begotten in a similar way to that observed by Flynn *et al.* (1993 and 1995). It could also be due to innate immunity and not acquired immunity as suggested by Flynn *et al.* and Cooper *et al.* (1993 and 1995).

The effect of MA on cytokine expression was also investigated in uninfected Balb/c mice and there was no significant expression of IL-4, IL-10, IL-12, or IFN-γ in the spleen. Again in the parallel study it was found that, in the lungs, MA do have effects on the expression of IL-12 (Pretorius, 1999).

These studies complement one another and demonstrate that the MA-induced protective effect, observed in the lungs, does not always necessarily imply that the immune response is a T_{H1} or T_{H2} type. Post-infection treatment with MA clearly provides protection to tuberculosis in a different manner as when MA is injected before infection. Furthermore, no trace was found of a T_{H1} or T_{H2} bias induced in the spleen upon pre- or post-infection MA treatment.

In this case where the short term (4, 8, 17 and 41 hours) and long term (2 and 4 weeks) effects of MA were investigated, it was found that there was no effect on the immune response, regardless of whether MA was saponified or left as methyl ester form.

The results obtained thus far suggest that focus should be placed on the lungs as they are the ogan where an immune response is observed. In this case, direct infection of the lungs should be investigated as this is the normal route of infection in humans. In this way the direct effect of mycolic acids could be investigated and possibly the mechanism of protection could be elucidated.

CHAPTER 4

Effects of Mycolic Acids on Adjuvant Arthritis

Introduction

Tuberculosis has been a problem for humans since antiquity. Archeological evidence indicated that deformity of the bones and joints were associated with this disease. Today the level of pulmonary tuberculosis has declined in developed countries, but the extra pulmonary tuberculosis (including that of the bones and joints) has remained the same (Davidson and Fernandez, 1994).

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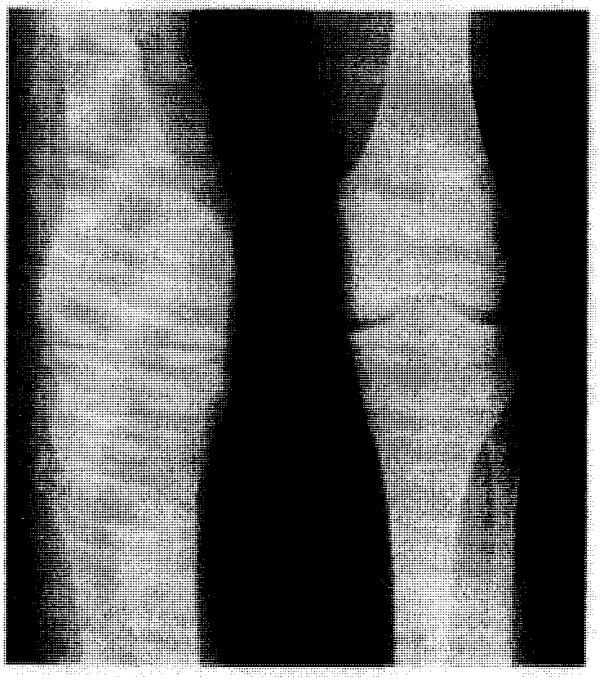

Fig.4.1: Tuberculosis of the knee characterised by lytic bone destruction and swelling. The radiograph shows the normal knee on the right (Davidson and Fernandez, 1994).

Tuberculous, mycotic, and parasitic infections are relatively rare causes of arthritis. A typical clinical presentation of arthritis due to tuberculosis is mono-articular disease of the large weight-bearing joints, also known as spondylitis (Messner, 1987). In the susciptible host, primary tuberculosis normally begins in the lungs. In contrast, in the immune person, tuberculosis is characterised by infection and inflammation of organs and tissues other than the lungs. (Wallace and Cohen, 1976; and Farer *et al.*, 1979). In the case of skeletal tuberculosis, destruction of bone occurs without stimulation of new bone formation. When the joint is involved, soft tissue swelling is an early sign of tuberculous arthritis. Fig. 4.1 shows the general X-ray appearance of a tuberculous arthritic joint in comparison to a healthy joint. Destruction in this case is not accompanied by osteophyte (bone growth) formation, but the shadow of a cold abscess may be seen. The appearance of the affected joint depends on the stage of the disease. The quickest and most reliable method of diagnosis is a biopsy (Messner, 1987).

Rheumatoid arthritis

Rheumaoid arthritis (RA) was first thought to be a disease of females. RA in women was originally described by AJ Landre-Beauviais (1772-1840), who suggested that it represented a variant of gout. The term "rheumatoid arthritis" was first applied in 1958 by AB Garrod, who thought that the disease was an arthritic or joint disease that manifested some of the external characteristics of rheumatism (Moutsopoulos and Vlachoyiannopoulos, 1992; Dugwoson *et al.*, 1989a).

Over recent decades many different criteria have been used to define rheumatoid arthritis such as those of the American Rheumatism Association (ARA) (Dugwoson *et al.*, 1989b). RA is a common autoimmune disorder, with the major symptom being chronic inflammation of the joints. RA, like tuberculosis, is also not a new disease and has been described in historical writings and portrayed in paintings for at least a thousand years. It was hypothesized years ago that RA, commonly agreed to be triggered by a virus, could behave like other major chronic infectious diseases, many of which showed temporal epidemicity (Silman, 1992).

Today, RA is considered to be an autoimmune disease caused by T cell mediated inflammation and damage to articular elements. The activation of the T cells involves the recognition of an antigen, presented by an MHC class II molecule to the T cell receptor (Fathman, 1992). In many cases of rheumatoid arthritis, rheumatoid factors bind to the Fc of circulating normal IgG, forming IgM-IgG complexes that are then deposited in the joints (Kuby, 1994).

Etiology of rheumatoid arthritis

The etiology of RA still remains obscure, but microbial involvement has been suspected for several years. Several microorganisms have been incriminated but no claim has stood the test of time (Bennett, 1978). Microorganisms that have been suggested include difteroids (Olhagen and Mansson, 1968; Ottenhof *et al.*, 1986 and Holoshitz *et al.*, 1986), *Clostridium perfringens* (Gullberg, 1978), *Mycobacteria* (Pearson, 1963) and several viruses such as parvo viruses (Phillips, 1988), but these claims for etiological involvement have not been proved convincingly.

Arguments for infectious etiology of RA include the following:

1. septic arthritis has clinical similarities with RA;
2. some forms of sterile or non-RA joint inflammation could be related to infection elsewhere in the body;
3. in several animal models arthritis can be induced by bacterial cell-wall antigens;
4. bacterial cell-wall components have potent inflammatory properties;
5. RA patients show a high level of antibodies against bacterial cell-wall components (Saag and Bennett, 1987)

Tuberculosis has been suspected to be involved in RA. T-cells that respond to mycobacterial 65 KDa heat-shock proteins (HSP) have been found in the affected joints of experimental animals with bacterially induced arthritis. *M. tuberculosis* heat-shock protein antibodies have been found to be elevated in RA and juvenile arthritis patients (van Eden *et al.*, 1991). Human patients with RA have also been shown to have *Mycobacterium*-reactive T-cells in the peripheral blood and the joints. It is however unclear whether this is specific to the rheumatoid arthritis (McFadden and Colsten, 1997), hence the involvement of *Mycobacteria* in RA remains unproven.

There is considerable difficulty in defining RA for epidemiological studies. Current criteria were derived from cases of chronic disease and do not reflect the many types of short-lived inflammatory poly-arthritis arising in the population by a variety of causative agents and conditions (Arnett *et al.*, 1988). TB-associated arthritis is typically a short-lived inflammatory response in the joints of patients.

Cellular composition of rheumatoid arthritis in the joints

Investigation of the cellular composition of RA joints revealed an extensive infiltrate of haemopoetic cells, chiefly T cells and macrophages. Many of these appear to be activated when judged according to morphological criteria and surface markers. One of the most important activation markers is the expression of the HLA class II antigens. These are essential for antigen presentation and are expressed in a variety of cell types consisting of the T lymphocytes, B lymphocytes, macrophages, dendritic cells, endothelial cells and fibroblasts (Feldmann *et al.*, 1992; and Feldmann, 1989).

T lymphocytes are the most abundant cells in active RA. Many studies have demonstrated that the $CD4^+$ cells tend to concentrate in the perivascular nodules, whereas the $CD8^+$ cells are more diffusely scattered. $CD4^+$ cells are subdivided into subsets, depending on their CD45 expression. In normal blood, half of the $CD4^+$ T cells are $CD45RA^+$, indicating a "virgin state". Essentially all the cells in the RA joint lack the CD45RA and express CD45RO/CD29, indicating a "primed state" (Pitzalis *et al.*, 1987). Niedhart *et al.* (1996), investigated whether, in RA, the CD45 isoform expression of the peripheral blood T-lymphocytes (T-PBL) is related to indicators of autoimmunity, eg. IgM rheumatoid factors (RF). Their investigations indicated that in RA, factors like drug therapy, age and gender did not have major influences on the CD45RA/RO patterns, whereas the state of autoimmunity, indicated by levels of circulating RF against human IgG Fc, did. The patients with high IgM-RF had elevated proportions of $CD45RO^+$ T-PBL and this correlated with clinical parameters of disease activity (tender joint count) and outcomes (radiographic scores).

Takworski *et al.* (1989) investigated the specificity of antibodies produced in RA and found that there was high production of IgM and IgG. In their experiments, production of antibodies to IgG and to type I and type II collagen (CI and CII) was determined by enzyme linked immunospot assay (ELISPOT) in patients with RA and those with other inflammatory joint disease. Anti-CII-Ig secreting cells were found among the cells eluted from inflamed synovial tissue in 12 out of 13 patients with seropositive RA and nine out of 14 patients with sero-negative RA or with undetermined serum RF levels. In contrast, no anti-CII-Ig producing B-cells were present in any of the four patients with other joint diseases. By enabling analysis of anti-CII-Ig secretion at the single cell level in the ELISPOT method, the scarcity of cells is not limiting. In addition, ELISPOT avoids the problems of ELISA where serum anti-CII-Ig may be low due to the rapid adsorption of secreted antibodies on destroyed cartilage surfaces *in vivo*, or complex formation by RFs.

Cytokines in rheumatoid arthritis

Cytokines are essential for many processes in RA, such as cell growth and HLA class II expression, it is however not clear which cytokines are of major importance for different processes. The activation and differentiation of B cells are mediated by cytokines such as IL-4 and IL-6. IL-4 level is however negligible in the synovial fluid, while high levels of IL-6 have been detected in the synovial fluid (Hirano *et al.*, 1988). These high levels of IL-6 may explain the infiltration of the synovium by the plasma cells and the production of auto-antibodies including rheumatoid factors. The presence of RF-containing immune complexes may contribute to the pathogenesis of RA by inducing the production of IL-1 (Chantry *et al.*, 1989).

Fibrosis is an important component and complication of RA. Arthritic fibrosis leads to the deformation of joints, while pulmonary fibrosis can cause systematic, progressive damage to the lungs. Which cytokine drives the fibrosis in RA joints is not yet known. There are many candidates such as IL-1α and -ß and TNF-α. These may act indirectly *via* platelet-derived growth factor (PDGF) induction (Raines *et al.*, 1989). Feldman *et al.* (1990) chiefly investigated the cytokine production in the joints, since RA is mostly manifested in the synovial joints. They found that rheumatoid joints contain a wide variety of cell types which produce many cytokines locally. Due to this situation it was not surprising to find that virtually all cytokines were detected.

Activation of macrophages and MHC class II expression are critical in the generation of the immune response or inflammation. IFN-γ is potentially the most effective cytokine at inducing MHC class II expression in the absence of other factors (Portillo *et al.*, 1989). Negligible amounts of IFN-γ are produced by rheumatoid synovial cells (Firenstein *et al.*, 1990). This suggests that other factors are involved instead of IFN-γ which would explain the inflammatory response in arthritic joints (Fig. 4.2). Chantry *et al.* (1990) suggested the involvement of granulocyte/monocyte colony stimulating factor (GM-CSF), which induces HLA-DR (MHC class II) expression on human monocytes. Alvaro-Garcia *et al.* (1989) have also suggested GM-CSF to be both an important macrophage activator and an inducer of MHC class II expression in RA joint.

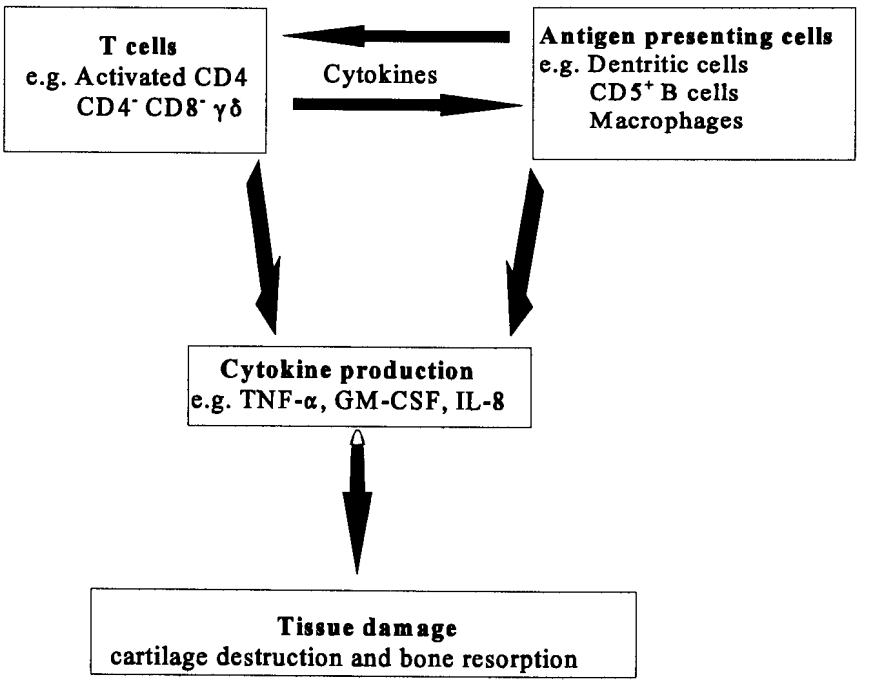

Fig. 4.2: Scheme of the cellular and cytokine interaction in rheumatoid arthritis.

Arthritis Models

Although the etiology of RA is unknown, microbial involvement is suspected, even though no particular microorganism has been incriminated (Sverijnen, 1990). Failure to pinpoint a single source of RA is demonstrated by the number and variety of animal models available to study arthritis. These include:

1. Antigen-Induced Arthritis
2. Collagen-Induced Arthritis (CA)
3. Streptococcal Cell Wall-Induced Arthritis (SCW-A)
4. Adjuvant-Induced Arthritis (AA)
5. Oil induced arthritis (OIA)

Susceptibility to arthritis varies greatly among rat strains, with Sprague Dawley and Lewis rats being highly susceptible and Buffalo and F344 rats being more resistant to chronic arthritis. The difference in susceptibility was found not to be MHC-related, as Lewis and F33 rats have the same MHC (Gill III *et al.*, 1987). Chronicity is an important feature of SCW-A, hence insight into the mechanisms which underlie chronicity might help to understand RA. The injection route is critical in determining the chronicity of the disease as indicated in Table 4.1 (Kool, 1992).

Table 4.1. Joint inflammation by bacterial cell-wall components administered via different ways.

Injection route	PG-PS	PG	PS
intra peritoneal	chronic	acute	oedema
intravenous	acute	acute	oedema
intra-articular	acute	acute	oedema

PG-PS: peptidoglycan-polysaccharide

Adjuvant Arthritis

AA is not a natural disease, but its study can bring the understanding of the role of the host-parasite interaction in induction of autoimmunity. It has been considered to be a model of RA because of its morphological features of synovitis, pannus formation and erosion of cartilage and bone progression to ankylosis, most prominently in the joints of the extremities. The disease was found to be influenced by the Ir gene of the MHC of rats (Batisto *et al.*, 1982). In this model, described by Pearson(1963) more than three decades ago, the connections between mycobacterial antigens, host genes and autoimmunity can be experimentally investigated. In this model, rats are administered complete Freund's adjuvant (CFA), made of killed *Mycobacterium tuberculosis* ground in mineral oil. After a period of about 12 days, rats develop a self limiting poly-arthritis

of limb joints leading to deformed paws (Pearson *et al.*, 1961). This requires that the rat strain that is to be used is not particularly susceptible to arthritis induction by oil alone, as was found for DA rats (Holmdahl *et al.*, 1992; Holmdah and Kvick, 1992). AA therefore provided the opportunity to explore experimentally the question of how the immune response to an environmental agent could trigger an immunological disease.

In the case of AA, arthritis could be explained by the inflammatory damage to the joints, which was an innocent bystander in the legitimate attack against mycobacterial antigens. It was conceivable that the joints were damaged by an autoimmune response against an endogenous, self-antigen. In this case *M. tuberculosis* could have triggered arthritis by expressing or modifying a self-antigen in the rat's connective tissues as a result of intra-cutaneous immunization. It was also possible that one of the *M. tuberculosis* antigens could be structurally similar to a joint antigen and that immunization with this cross-reactive epitope could lead to an attack against self-antigens in the joints.

This study investigates the effects that mycolic acids may have on adjuvant arthritis and aims to relate the observations to the protective immunoregulatory properties of mycolic acids in tuberculosis.

Hypothesis:

Mycolic acids, the major cell-wall components of *M. tuberculosis*, play a major role in the development of adjuvant arthritis.

Specific Aims:

To test whether:

1. administration of mycolic acids to arthritis-susceptible animals does not induce or enhance arthritis

2. mycolic acids provide resistance against development of *M. tuberculosis*-induced arthritis in susceptible rats, and

3. immunization of arthritis-susceptible rats with mycolic acids suspended in oil, induces mycolic acids-specific antibody production with long term memory.

Materials

Experimental Animals

Lewis rats (female), six weeks old, were obtained from Harlan (UK) through the South African Institute for Medical Research (SAIMR). Seventeen weeks old Sprague-Dawley female rats were purchased from the Animal Centre at the SAIMR in Johannesburg.

Bacteria

Lyophilized *Mycobacterium tuberculosis* H37Ra was purchased from Difco laboratories. Heat-killed *M. tuberculosis* H37Rv was prepared by autoclaving.

Reagents

Freshly saponified mycolic acids (prepared according to chapter 2) originating from *M. tuberculosis* H37Rv were suspended in FIA (10 mg/ml FIA) and diluted with FIA to required concentrations, *i.e.*:

0,1 mg MA/100 µl, 0,3 mg MA/100 µl and 1,0 mg MA/100µl.

ELISA reagents:

Basic buffer - PBS buffer: 8,0 g NaCl, 0,2 g Kcl (SAARCHEM, Krugersdorp SA), 0,2 g KH_2PO_4 and 1,05 g Na_2HPO_4 (MERK, Darmstadt Germany) per 1*l* distilled water, adjusted to pH 7,4.

Diluting buffer: 0,5% (m/v) casein (MERK, Darmstadt Germany) in PBS buffer adjusted to pH 7,4 was used for diluting of the experimental animals' sera (mixed with glycerol 1:1) and for the preparation of suitable dilutions of immunoreagents.

Blocking buffer: 0,5% (m/v) casein in PBS buffer adjusted to pH 7,4 was used for blocking of ELISA plates.

Washing buffer: 0,5% (m/v) casein in PBS buffer adjusted to pH 7,4 was used for washing of ELISA plates.

Coating antigen: unsaponified mycolic acids originating from *M. tuberculosis* H37Rv, at a final concentration of 0,067 µg/µl. To prepare the coating antigen, 1 mg mycolic acids was dissolved in 100 µl chloroform and the solution introduced into 15,0 ml PBS buffer adjusted to pH 7,4. The solution was autoclaved at 121°C for one hour.

Conjugates: Goat anti-rat antibody conjugated to peroxidase (H + L chains), (Cappel). Rabbit anti-human gamma chain specific peroxidase conjugate (Sigma,

St Louis USA).

Substrate: O-Phenylenediamine (Sigma, St Louis USA) and hydrogen peroxide (BDH, Poole UK).

Substrate buffer: 0,1 M citrate buffer (0,1 M citric acid [BDH, Poole UK] and 0,1 M Tri sodium citrate [Associated Chemical Enterprises, Reuven), adjusted to pH 4,5.

Methods

Preparation of bacteria

The dried *M. tuberculosis* H37Rv was suspended in Marcol 52 oil for immunising Sprague Dawley rats.

For immunising Lewis rats, lyophilised *M. tuberculosis* H37Ra was placed into a roughened mortar bowl and a few drops of Freund's incomplete adjuvant (FIA, purchased from Difco laboratories, Detroit USA) were added and the mixture was then ground to a paste over 120 seconds. Another few drops of FIA were then added and ground for 30 seconds. The final concentration of 100 mg bacteria per 10 ml of FIA was then reached.

Preparation of MA-oil suspension

Countercurrent purified mycolic acids prepared from *M. tuberculosis* H37Rv were dried under nitrogen (Chapter 2) and suspended in Marcol 52 oil to a final concentration of 1 mg/ml, by heating at 80 °C for three minutes and then vortexed. Dried mycolic acids were also added to FIA to give a final concentration of 2 mg per 100 µl and then diluted to give a final concentration of 1 mg per ml. This was done by heating them at 80 °C for 10 minutes in order to dissolve and the suspension was then vortexed.

Animal maintenance

Four Lewis rats were maintained in each cage, except for the animals of group 6, which were maintained three per cage, just as Sprague Dawley rats. The animals were maintained at the animal facilities of the Medical Research Council (MRC) in Pretoria. Rats were housed in transparent polypropylene cages with tightly fitting stainless steel lids. Wooden shavings, after autoclaving, were provided as nesting material. Rats were fed nutritionally balanced pellets, manufactured by Epol, South Africa. Autoclaved tap water was provided *ad libitum.*

Temperature and humidity in the animal facility were set at 20 °C (+/- 1 °C) and 40% (+/- 10%), respectively. Lighting was provided by means of fluorescent tubes. A light-darkness cycle of alternating 12 hour periods was set up. Animal rooms, rat cages and glass bottles were cleaned

and decontaminated once a week using Bronocide (manufactured by Essential Medicines (Pty.) Ltd) for sanitation purposes. Water bottles were autoclaved after washing once a week. Identification of the experimental animals was accomplished by making individual ear marks.

Mycolic acids in induction of arthritis

Sprague Dawley rats were divided into five groups of three: Those that received heat killed *M. tuberculosis* H37Rv in Marcol 52 (1 mg/ml), those that received Marcol 52 oil alone, those that received 0.01 mg/ml MA in Marcol 52, those that received 0.1 mg/ml MA in Marcol 52, and those that received 1 mg/ml MA in Marcol 52. Rats that received MA were immunized every second week to boost any MA response expected to be found. All the rats were bled every second week, starting from seven days after the first immunization. The joints of all the rats were measured every week to detect any development of swelling.

The effect of mycolic acids on adjuvant arthritis

Immunization of Lew rats was done according to Table 4.2, with Lewis rats receiving mycolic acids purified from *M.tuberculosis* H37Ra.

Table.4.2 Experimental set-up for determining the role of MA in adjuvant arthritis

Group number	Number of rats per group	Pre-treatment	Induction of adjuvant arthritis	Post-treatment
Day of the experiment		Day 0	Day 7	Day 11
Group 1	4	FIA only, 100 µl	1 mg H37Ra in 100 µl FIA	0
Group 2	4	0,1 mg MA in 100 µl FIA	1 mg H37Ra in 100 µl FIA	0
Group 3	4	0,3 mg MA in 100 µl FIA	1 mg H37Ra in 100 µl FIA	0
Group 4	4	1,0 mg MA in 100 µl FIA	1 mg H37Ra in 100 µl FIA	0
Group 5	4	0	100 µl FIA	0
Group 6	6	0	1 mg H37Ra in 100 µl FIA	0
Group 7	4	0	1 mg MA in 100 µl FIA	0
Group 8	4	0	1 mg H37Ra in 100 µl FIA	100 µl FIA
Group 9	4	0	1 mg H37Ra in 100 µl FIA	0,1 mg MA in 100 µl FIA
Group 10	4	0	1 mg H37Ra in 100 µl FIA	0,3 mg MA in 100 µl FIA
Group 11	4	0	1 mg H37Ra in 100 µl FIA	1,0 mg MA in 100 µl FIA
Group 12	4	0	0	0

ELISA determination of antibody response

Sterilin 96 well flat bottom plates (STERILIN, UK) were coated with 3 µg/well MA in hot PBS.

To MA (3,8 mg) was added 57 ml PBS and the suspension autoclaved for 20 minutes at 120 °C.

ELISA plates were transferred to the freezer for 1 hour and the hot MA solution was poured into a stainless steel container from where the solution was pipetted to coat each well with 50 µl MA. The plates were left at 4°C for 14 hours. PBS-MA solution was flicked out the following morning and the plates stored dry at 4°C. A casein buffer was used to dilute sera. The coated microtiter plates were blocked with 0,5% casein/PBS (200 µl) pH 7.4, for 2 hours at room temperature. The solution was flicked out and the wells aspirated. Serum dilutions were prepared while blocking the plates, keeping the sera solutions at 4°C until ready to continue with the ELISA.

Binding of animal antibodies: Rat sera (mixed with glycerol 1:1 v/v) were diluted further in the diluting buffer 1:10 v/v. The final dilution was therefore 1:20 v/v. Aliquots of 50 µl were introduced into wells in duplicate. The plates were incubated at room temperature for one hour. The sera were removed and the plates washed three times with the washing buffer using an Anthos Automatic Washer.

Quantification of the bound antibodies: A peroxidase anti-rat antibody conjugate diluted 1:1000 was introduced in aliquots of 50 µl per well and incubated at room temperature for 30 minutes. After the removal of the conjugate, the ELISA plates were washed three times with the washing buffer.

The substrate solution comprising 10,0 mg o-phenylene diamine 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 placed in a dark place and the colour development was monitored at 15, 30 and 60 minutes intervals using an SLT 340 ATC photometer at a wavelength of 450 nm.

Arthritis radiological assessment

Radiographs of the limbs originating from the control, arthritis and mycolic acids-treated rats were made using a Siemens Polymat 50 diagnostic X-ray machine. Fuji HRF film and Trimax T2 detail screens were used at a source-to-image distance of 109 cm. Exposure conditions were 42 kVp and 4mAs to optimise soft tissue visibility and bone detail.

Radiological examinations were carried out by Prof. R M Kirberger, Section of Pathology of the Veterinary Research Institute, Onderstepoort, Pretoria.

Results

Sprague Dawley rat arthritis induction

In order to determine whether mycolic acids of *M. tuberculosis* were arthritogenic, Sprague Dawley rats were immunized with 1 mg of dried *M. tuberculosis* H37Rv in Marcol 52 oil as arthritis positive control and this compared to 0.01, 0.1 and 1 mg MA in Marcol 52 oil (0.1 ml). A negative control, Marcol 52 oil only, was administered. No observable development of joint swelling and deformation was observed in this experiment. There was no development of arthritis related or other sickness in any of these rats. As such, no observation could be made in these rats to support any involvement of mycolic acids in arthritis. The experiment did show, however, that administration of mycolic acids up to 1 mg per animal did not have any observable adverse side-effects upon continual administration.

Mycolic acids-induced antibody production in the Sprague Dawley rats

Although the positive control in the experiment above did not show any arthritic symptoms, immunization of the remaining rats with MA was maintained at fortnightly intervals to determine any possible physiologic effect. Simultaneously the humoral immunogenicity of MA was measured by determining the induced antibody (Ab) response to mycolic acids. The Ab response was monitored and the ELISA results, after a treatment period of three months, are presented in Fig. 4.3.

A weak dose related response was observed for the induction of Abs specific for mycolic acids, immobilized on the ELISA plates. There was no observable development of Abs in those rats that only received oil and no boost.

Rats that obtained 1 mg MA treatment showed the development of Abs against MA that peaked at 91 days.

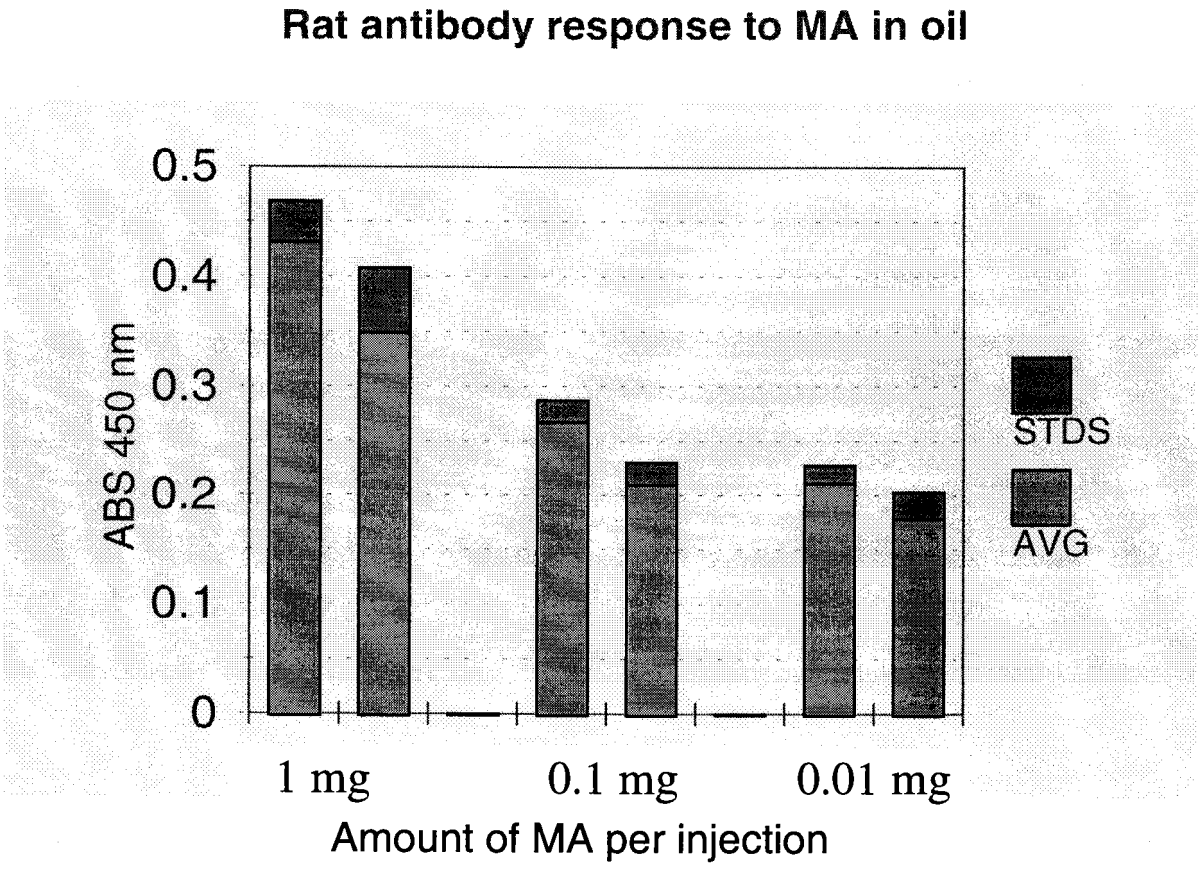

Fig.4.3: Antibody response against mycolic acids (MA) after 91 days. Rats were immunised every 14 days.

Mycolic acids prevent arthritis in Lewis rats

Lewis rats were used in this experiment as there was no development of arthritis in Sprague Dawley rats. Using the optimized protocol described by Wauben *et al.* (1994) for inducing arthritis in these rats, arthritis developed in the positive control group, which received only lyophilized *M. tuberculosis* H37Ra in FIA. The first arthritic symptom to be observed was necrosis at the site of injection (Fig 4.4), four days after the arthritic insult. This was followed by swelling of the knuckles and joints as well as nose bleeding by day 11. The symptoms peaked at days 16 to 21 and subsequently subsided, except for the necrosis. Complete recovery was observed within the next two weeks.

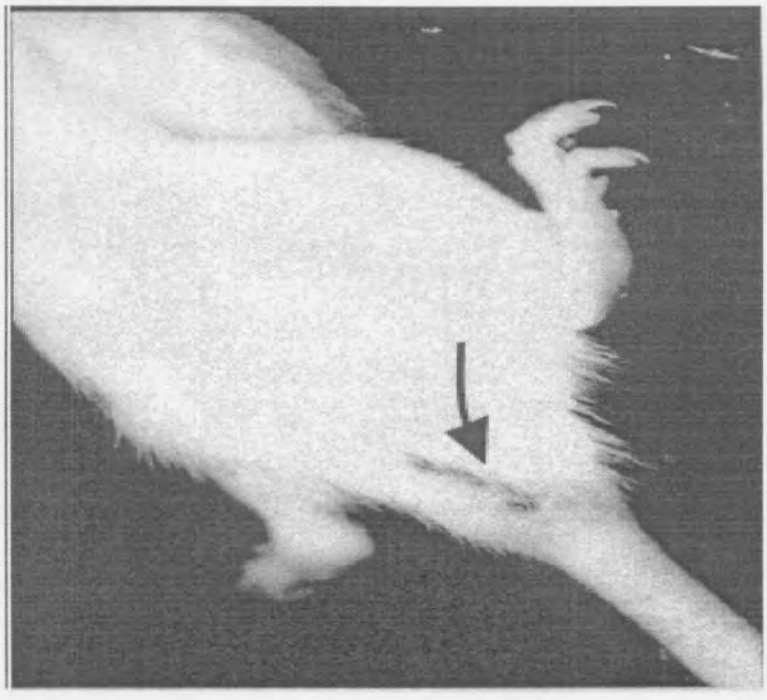

Fig. 4.4: Arthritic rat with necrotic development at the site base of injection in the Lewis rat tail. The rat is in the typical a swimming position. The arrow indicates the position of the necrosis.

The rats which were pre-treated with 0,1 mg and 0,3 mg mycolic acids developed less severe symptoms than those treated with FIA alone before arthritic insult. Three rats pre-treated with 1 mg MA did not develop any arthritic symptoms indicative of the presence of the disease. The rats which received injections of FIA only did not show any arthritic symptoms. In Fig.4.5, the typical swelling of the joints and knuckles in the front and hind paws of the experimental rats is shown by radiology. The results obtained in the experiment are shown in Fig 4.6. The swelling could not be attributed to the rats' mass gain, as it was observed that rats that were considered to be arthritic did not significantly gain mass as compared to those that received MA and those that did not receive arthritis induction (Fig.4.7).

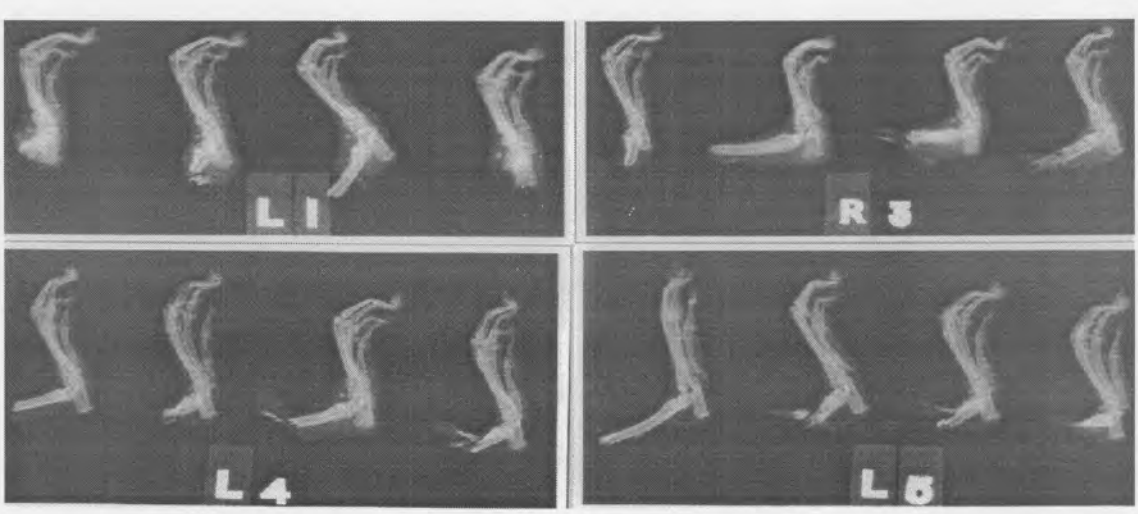

Fig. 4.5: X-ray photos of hind paws of Lewis rats after induction of arthritis. L represents the left hind paw, while R represents the right hind paw. L1: arthritis positive control; L5: arthritis negative control; R3: arthritis induced rats protected with 0.3 mg MA pretreatment; L4: arthritis induced rats protected with 1 mg MA pre-treatment. The paw third from the left is an arthritic limb of a rat in which MA pre-treatment was accidentally missed.

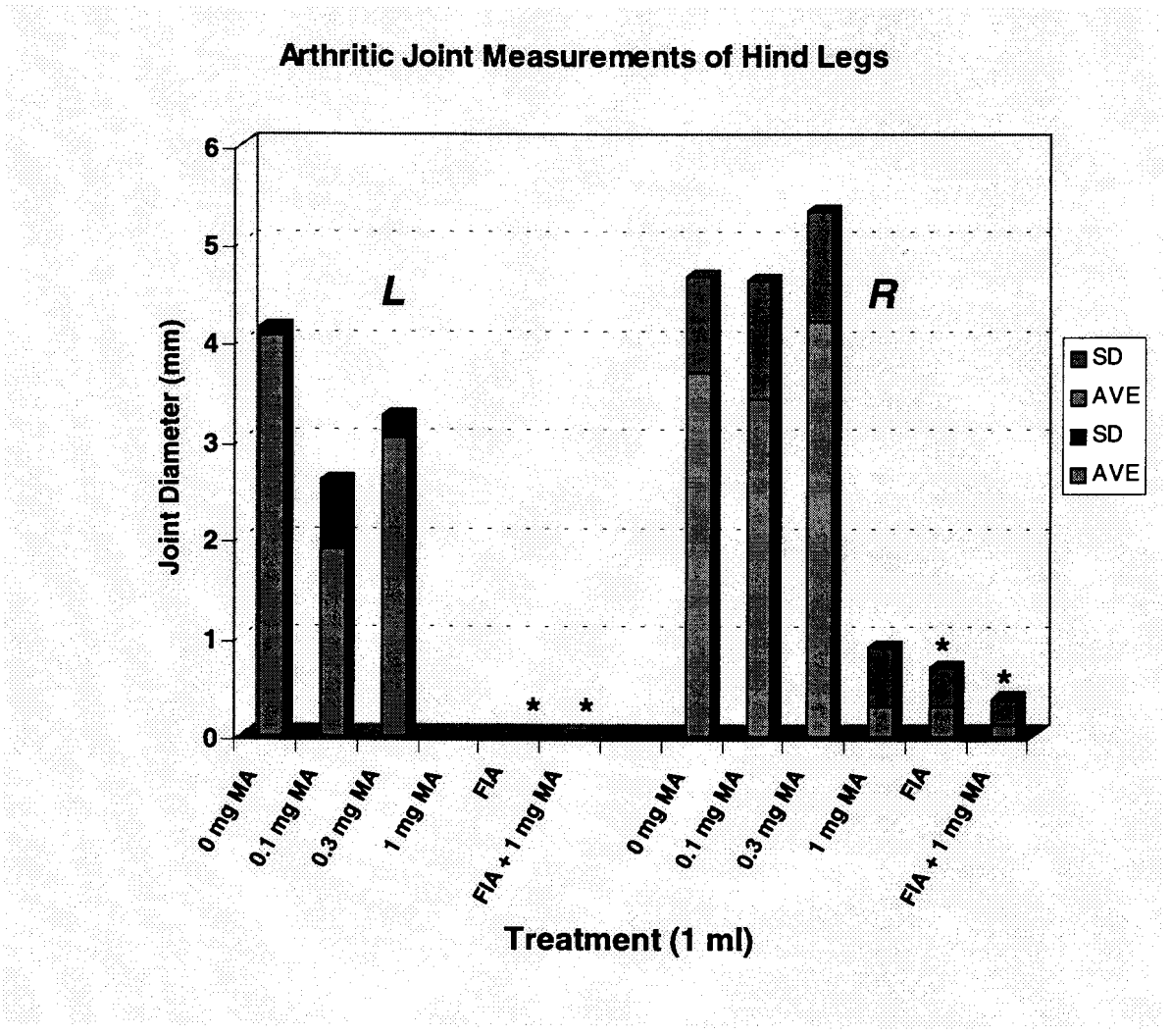

Fig.4.6: Arthritic joint measurements of rats treated with MA in Freund's incomplete adjuvant (FIA). These animals were given MA before arthritic insult. Controls given no MA lyophilized H37Ra in FIA (positive control-0 mg), only FIA (negative control), and FIA and 1 mg MA. **L** indicates the left hind limb and **R** the right hind limb, "AVE" the average and "SD" the standard deviation. (* indicate rats that did not receive lyophilized H37Ra in FIA but only FIA). The Diameter indicated is obtained by subtracting the diameter before arthritic insult from that obtained after arthritis induction.

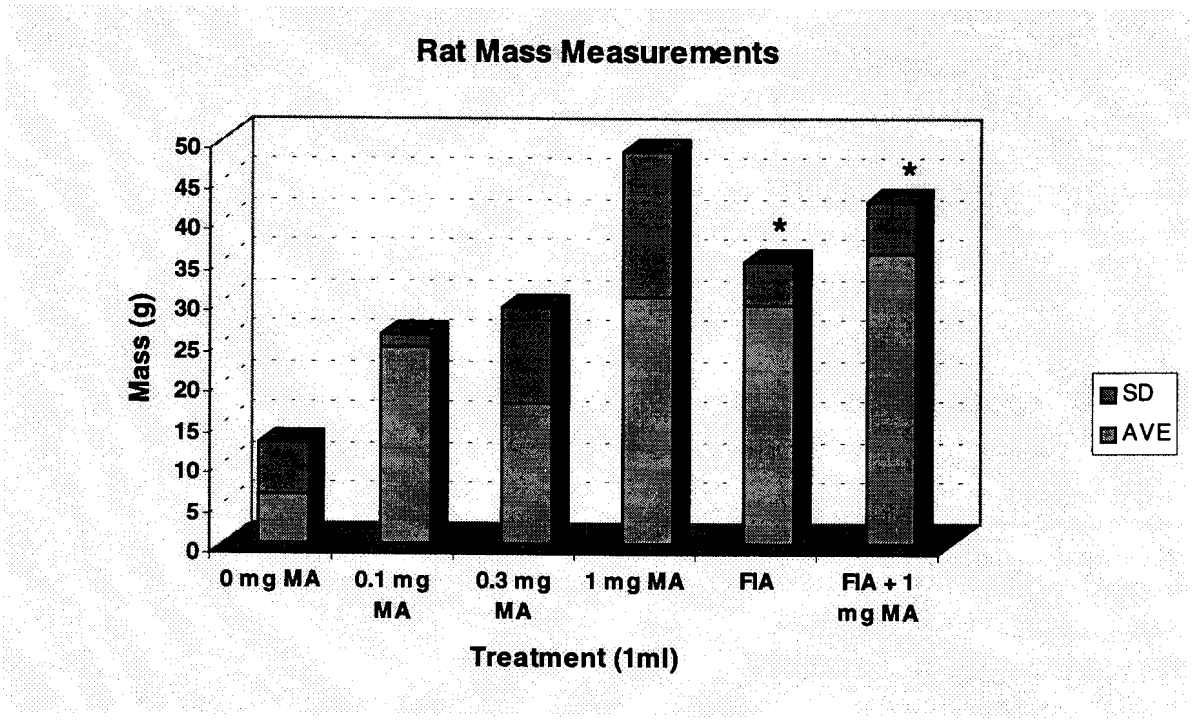

Fig.4.7: Mass measurements of rats given MA before arthritic insult. Controls given no MA lyophilized H37Ra in FIA (positive control-0 mg), only FIA (negative control), and FIA and 1 mg MA. "AVE" indcates the average and "SD" indicates the standard deviation. (* indicate rats that did not receive lyophilized H37Ra in FIA but only FIA)

The swelling and inflammation of hind leg joints of the rats in which AA was successfully induced, are clearly distinguished from those in rats that were protected with mycolic acids pre-treatment . These results indicate a protective influence of mycolic acids at a dose of 1 mg administered before priming with *M. tuberculosis* H37Ra in FIA.

Discussion

A number of studies have established that immunization of various susceptible animals with a non-arthritogenic dose of mycobacterial heat-shock protein (HSP) 65 in the skin before arthritic insult with *M. tuberculosis* H37Ra in FIA protects against arthritis (Van den Broek *et al.*, 1989). Our study indicates that MA provides protection against AA that is similar to that obtained through HSP 65. A strong HSP 65 response in rats was observed after immunization with *M. tuberculosis* (Thole *et al.*, 1987). The findings suggested that HSP 65 is associated with arthritis, irrespective of the arthritis inducing agent, as the effects were the same in a number of models. Administration of HSP 65 to arthritic animals, after arthritic insult, was found to exacerbate the disease (Van den Broek *et al.*, 1989). Induction of arthritis with *Mycobacteria* induced antibodies against HSP (Thole *et al.*, 1987 and van Eden *et al.*, 1988).

Mycolic acids biosynthesis has been a target for anti-tuberculosis drugs for years, but they were not suspected to be involved in eliciting an immune response until their immunoregulatory properties were suggested by Beckman *et al.* (1994). They determined that mycolic acids stimulate CD4/CD8 double negative (DN) T cells. In this study, it was observed that immunisation of Sprague Dawley rats with mycolic acids suspended in oil induced an antibody response. The antibody response to mycolic acids required high doses of MA, but was too weak to allow the confirmation of their specificity by ELISA inhibition assay. This observation emphasises the immunoregulatory properties of MA and opens a door to further investigations.

In the case of Lewis rats, dramatic immunoregulatory effects of MA could be demonstrated by the observation that MA pre-treatment before the arthritic insult, prevented the development of arthritis at 1 mg doses. This was comparable to the observation that 1 mg of MA elicited a weak Ab response against MA. MA administration after arthritis induction could not prevent arthritic development however, even at high doses of MA. The positive control animals that received *Mycobacteria,* but not mycolic acids treatment, developed severe but transient arthritis symptoms within two weeks after bacterial challenge, similar to that observed by Pearson *et al.* (1961).

No visible symptoms were apparent in mycolic acids pre-treated rats upon arthritic insult. Pearson (1963) could also induce protection against AA by challenging the young animals earlier with *Mycobacteria*, before the induction of AA. From these observations it can be speculated that MA induce proliferation of double negative T-cells, which are known to secrete IL-10. IL-10 is an immuno-suppressive cytokine that could suppress auto-immunity leading to adjuvant arthritis. These suggestions are in agreement with the evidence presented by Holoshitz *et al.* (1983) and Yoshino *et al.* (1991) demonstrating the T-cell nature of adjuvant arthritis. Our observations, however, did not agree with the findings by Koga *et al.* (1973) and Koga *et al.* (1976). In these studies it was found that wax isolated from *M. tuberculosis* H37Ra and H37Rv, emulsified in an adjuvant is arthritogenic. In our studies mycolic acids emulsified in FIA proved not to be arthritogenic. The rats also showed necrotic lesions at the site of inoculation (Fig.4.8). This was again in agreement with the observations of Wauben *et al.* (1994).

In conclusion, mycolic acids are shown in these experiments to be non-toxic to rats (chapter 4) as well as to mice (chapter 3). Moreover, they not only appear to partially protect against tuberculosis infection (chapter 3), but are also shown to prevent the onset of adjuvant arthritis. The persistence of antibodies to mycolic acids could provide an explanation for the protection against arthritis provided by MA. The antibodies were of low titre, however, making it unlikely that they could play a significant immunoregulatory role. The indications that mycolic acids could induce an innate immune response dictated by cytokines could provide a more probable mechanism of protection against adjuvant arthritis and tuberculosis.

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CHAPTER 5

Concluding Discussion

The World Health Organisation (WHO) has declared tuberculosis to be a global emergency, as one third of the world population is already infected (Rook and Hernandez-Pando, 1996). The breakdown of health care systems is leading to incomplete case and contact tracing, incomplete treatment and subsequently to increase in drug resistance. Moreover, tuberculosis is one of the most important secondary infections to be activated in HIV-positive individuals (Sturgill-Koszycki *et al.*, 1994).

An estimated 7.5 million TB incident cases occurred world-wide during the year 1990. An increase of 58% is predicted by the year 2005. It is estimated that 95% of HIV-associated TB cases are a result of HIV infection (Centres for Disease Control and Prevention, 1994). Problems that are still eminent in treating tuberculosis include poor patient compliance, multi-drug resistance (MDR) and altered immune responsiveness. Today acquired immune deficiency syndrome (AIDS) especially contributes to the latter.

The Centre for Disease Control (USA) recommended that all HIV patients, that are suspected to be infected with *Mycobacteria* should receive treatment against tuberculosis (Harding and Bailey, 1994). This is because during clinical presentation it is difficult to distinguish tuberculosis from the disease caused by *M. avium* complex, and in this case treatment of tuberculosis is more urgent than that of *M. avium.*

The problem with tuberculosis drug treatment is that many drug interactions may alter individual drug effectiveness, eg. they may increase the possibility of drug toxicity, such as when using isoniazid and phenytoin together. It is presumed that there are three subpopulations of tuberculosis organisms in active human tuberculosis infection, with the largest being bacilli that grow actively outside of the host cell and which may bring about drug resistance. The remaining two types bacilli would be the slow or intermittently growing types preferring an acid or neutral pH environment *in vitro*. The bactericidal drug rifampin is the only drug that is bactericidal to all three populations. Rifampin rarely displays side effects such as hepatitis, renal failure (at high doses) and/or skin irritations. Isoniazid is bactericidal to the extracellularly fast growing organisms and slow or intermittently growing organisms with acid pH preference. Isoniazid was found to be actively involved in the inhibition of the biosynthesis of mycolic acids (Takayama *et al.*, 1972).

The use of isoniazid alone generates drug resistance and as such isoniazid should not be used alone during treatment. The likelihood of organisms spontaneously developing resistance to two drugs is the product of their probabilities (1 in 10^6 in the case of isoniazid and 1 in 10^8 in the case of rifampin which results into 1 in 10^{14}) (Goble, 1986). The highest number of organisms found in human infection was up to 10^9 bacteria (Harding and Bailey, 1994). The common cause of drug resistance is non-compliance with therapy. The current increase in drug resistance is associated with areas where public health infrastructure is inadequate to ensure therapy compliance through direct supervision (Small *et al.*, 1993; Harding and Bailey, 1994). This is primarily caused by the duration of chemotherapy which requires a minimum of six months if isoniazid is used and up to 18 months if isoniazid cannot be used and ethambutol is used instead (Combs *et al.*, 1990; Cohn *et al.*, 1990). The combined drug preparation could again be toxic.

The problems of MDR in chemotherapy challenged researchers to engage in development of vaccines that are more potent than the currently used *M. bovis* BCG (bacillus Calmette Guerin). BCG is at present the only vaccine against tuberculosis. The first BCG vaccination was in 1921, but today still the immunology underlying its efficacy is largely unknown (Kaufmann and Andersen, 1998). BCG vaccination has not been able to prevent the rapid spread of tuberculosis (Ravn *et al.*, 1997). The development of new, improved, and preferably killed vaccines against tuberculosis has become a priority. Recent research outputs from several laboratories have encouraged the use of subunit vaccines (Andersen, 1994; Horwitz *et al.*, 1995).

The earlier attempts to construct tuberculosis subunit vaccines were performed in the sixties with different mycobacterial cell-wall-derived extracts (Ribi *et al.*, 1966). The problems that were observed demonstrated that mycobacterial cell walls contain large amounts of immuno-stimulatory components that mediate inflammatory reactions. In this light, recent research has focussed on the use of extracellular proteins from *M. tuberculosis* as a source of protective antigens (Andersen, 1994). The development of subunit vaccines has still not proven to be more efficient than BCG (Kaufmann and Andersen, 1998). This suggest that the whole organism is needed and not only a part of it.

From this it is clear that new methods and approaches for treatment and control of tuberculosis are required. Stanford and Grange (1993) suggested that the major step forward should come from immunology. Available evidence is gaining momentum to indicate or suggest that immunotherapy with *M. vaccae* (Stanford and Stanford, 1994) might be a powerful and affordable addition to the treatment of tuberculosis. The mechanism by which *M. vaccae* achieves this is still speculative.

Mycolic acids, the characteristic, abundant waxes of the cell wall of *Mycobacteria*, were investigated in this study. Mycolic acids, which occur as mixtures of different types, esterified to cell-wall carbohydrates, show immunoregulatory properties by stimulating CD4/CD8 double negative T cells. The first evidence for immunoregulatory properties of MA stemmed from the observation that in humans MA is presented to T cells by professional antigen presenting cells (APC) in a MHC-independent manner on CD1b (Beckman *et al.*, 1994).

The CD1 proteins are remotely homologous to MHC in their α1 and α2 domains. These non-MHC encoded CD1 molecules are non-polymorphic and have the ability to present antigens. The first remarkable discovery was that they seem to present lipid antigens instead of peptide derived antigens. Human CD1b presents cell-wall components of *Mycobacteria* including lipid mycolic acids and the lipoglycan lipoarabinomanan (LAM) to CD4/8 double negative T cells (Sugita *et al.*, 1998; Bendelac, 1995; Sieling *et al.*, 1995; Beckman *et al.*, 1994).

Porcelli *et al.* (1992) reported that proliferative and cytotoxic responses of human CD4/8 double negative T cells specific for *M. tuberculosis* can be restricted by CD1b and that these responses do not involve MHC. It was later found that the CD1b restricted antigen was mycolic acids associated with the mycobacterial cell wall (Beckman *et al.*, 1994). These observations were extended by the subsequent discovery that LAM is also presented by CD1b and CD1c molecules (Sieling *et al.*, 1995). This opened new possibilities in handling tuberculosis treatment, particularly AIDS-related tuberculosis cases. This again emphasised that the lipid components of mycobacterial cell walls are also necessary components of the subunit vaccines besides the proteins. Since the CD1 is not polymorphic, the system would not be under the threat of genetic resistance development.

The limiting factor of biological investigations based on the use of mycolic acids, is that purification of MA is complicated by their poor solubility in organic solvents, excluding chlorinated hydrocarbons. HPLC has been applied to purify the mycolic acids from various mycobacterial species, but required derivatization to be able to detect them, which had to be removed prior to their application in biological experiments. It could therefore only be used to produce small quantities of purified mycolic acids (Butler *et al.*, 1991).

During this investigation a novel, fast, cost-effective, preparative and simple purification of mycolic acids using the Counter Current Distribution (CCD) technique was developed. Crude mycobacterial cell-wall extracts were first made by saponification in potassium hydroxide methanol solution. Purification was then performed with CCD using a bi-phasic tri-component system, consisting of deionized distilled water, chloroform and methanol. CCD produced a high yield of pure mycolic acids, the quality of which was assessed using reversed-phase HPLC-analysis. The mycolic acids proved to be biologically active, as they could stimulate $CD4/8^-$ T cells *in vitro* (Goodrum, 1998).

To study the immunoregulatory properties of MA, the following aspects were investigated:

(1) The ability of mycolic acids to stimulate antibody production,

(2) the role of mycolic acids in immunomodulation during tuberculosis infection and

(3) the role of mycolic acids in the prevention of tuberculosis-associated arthritis.

(1) The ability of mycolic acids to stimulate antibody production was investigated in Balb/c mice (Goodrum, 1998) and in Sprague Dawley rats. In Sprague Dawley rats, the animals were immunised with three doses of mycolic acids (1 mg, 0.1 mg and 0.01 mg). A weak, dose-

dependent antibody response was observed which peaked at 91 days. These results confirm the observations made by Kato (1972), in which cord factor (trehalose dimycolate) was found to be immunogenic. In this work however, Kato (1972) reported that the α-D-trehalose moiety was the immunogenic part. This study indicates that mycolic acids are immunogenic on their own.

(2) The role of mycolic acids in immunomodulation during tuberculosis infection was investigated. CCD-purified mycolic acids were adsorbed on homologous serum and administered intravenously into Balb/c and C57Bl/6 mice either before, or after intravenous infection with *M. tuberculosis* H37Rv. The more resistant C57Bl/6 mice did not respond to these treatments. In Balb/c mice however, the survival studies indicated that there was a significant extension of lifespan when MA was injected prior to and after they were challenged with an optimised infectious dose of *M. tuberculosis.* This protection against tuberculosis was equivalent to that of the tuberculosis resistant C57Bl/6 mice, given an equal dose of *M. tuberculosis* infection.

The expression of protective immunity to *M. tuberculosis* in mice is mediated by T lymphocytes that secrete cytokines. These molecules then mediate a variety of functions including the activation of parasitised host macrophages and the recruitment of other mononuclear phagocytes to the site of infection in order to initiate granuloma formation. IFN-γ is thought to play a key role in these events. Studies on mice demonstrated that when the IFN-γ gene has been disrupted there was an inability to control or contain a normally sub-lethal dose of *M. tuberculosis*.

Sub-populations of T-cells (T_{H0}, T_{H1}, T_{H2}) can be distinguished by the cytokine-secretion pattern. Evidence is increasing that the outcome of human disease may depend on the subpopulations of T-cells that predominate at the site of inflammation. To understand the mechanism by which MA

induce protection, the T_{H1}/T_{H2} cytokine profiles in the spleen were analysed by semi-quantitative RT-PCR. There was no detectable influence of MA administration on the T_{H1}/T_{H2} bias that could be observed.

In a parallel experiment performed on the lungs of the same mice (Verschoor *et al.*, 1998, Pretorius, 1999), mycolic acids were found to induce elevated expression of IL-12 and IFN-γ. These observations supported the results obtained in survival studies. The mice were infected and their survival monitored and compared. Mycolic acids did not have any effect on C57Bl/6 mice, but in the case of Balb/c mice, mycolic acids rendered protection when these mice were pre-treated with a dose of 25 µg mycolic acids. Post mortem analysis indicated that protection occurred preferentially in the lungs. The mechanism of protection correlated with the stimulation of IL-12 and IFN-γ expression. These findings agreed with those of Flynn *et al.* (1995) and Cooper *et al.* (1995), on administration of IL-12 to Balb/c and C57Bl/6 mice infected with *M.tuberculosis*. The mechanism by which IL-12 increases resistance still needs to be explored. Many of the effects of IL-12 could be explained by the induction of IFN-γ (Flesch *et al.*, 1994). It is known that IL-12 induces NK and T cells to produce IFN-γ (Trinchieri , 1995; Trinchiery, and Gerosa, 1996).

(**3**) The role of mycolic acids in the prevention of tuberculosis-associated arthritis was investigated. Pure mycolic acids, suspended in mineral oil were administered intradermally into Lewis rats one week before the intradermal administration of an arthritis-inducing dose of lyophilized *M. tuberculosis* H37Ra. Positive control animals receiving *Mycobacteria,* but no mycolic acids treatment, developed severe but transient arthritis symptoms within two weeks after bacterial challenge. No visible symptoms were apparent in mycolic acids-treated rats. These

results suggested that mycolic acids administration before arthritic insult provides protection, while treatment after the arthritic insult could not alleviate or aggravate the symptoms of arthritis. These results shed new light on the observations made by Stanford *et al.* (1985), where *M. vaccae,* or fractions thereof, were shown to protect against arthritis development, or even alleviated the symptoms when administered after arthritis had already occurred. This study suggests that mycolic acids could be the biologically active part of the *M. vaccae* in the experiments of Stanford *et al.*, although mycolic acids could not provide remission from arthritis.

Another important aspect observed in all three of these approaches was that mycolic acids were non-toxic to the experimental animals, did not aggravate tuberculosis and did not induce any arthritic symptoms either. Mycolic acids did, however induce IL-12 and IFN-γ in the lungs when administered without any previous or subsequent infection (Pretorius, 1999).

The responsiveness of the lungs towards administration of mycolic acids allows for the prediction of a much enhanced protection against tuberculosis offered by mycolic acids against the disease, when the infection with *M. tuberculosis* occurs by the normal route, *i.e.* by inhalation into the lungs. It appears possible that the administration of mycolic acids could provide full protection against tuberculosis when infection with *M. tuberculosis* is introduced directly into the lungs. In addition, the administration of mycolic acids *via* inhaled vapours generated by a nebuliser could greatly improve the resistance towards infection with *M. tuberculosis* and also aid towards recovery from the disease.

At this stage it would be premature to extrapolate these findings to human beings, but it can be

expected that the response in humans towards the mycolic acids treatment should be similar or even better. This assumption is based on the observation that humans can present mycolic acids to the immune system on CD1b, the homologue of which has not yet been identified in mice.

Evidence thus far obtained demonstrated short term prevention or therapy of tuberculosis by mycolic acids. The experimental evidence currently available argues against a role for mycolic acids in vaccines, *i.e.* for using them to provide long term immune memory against infection with *M. tuberculosis*. The results obtained would suggest that T helper cells did not participate in the immune response upon challenge with mycolic acids either before or after the infection with *M. tuberculosis*. This was because there was no T_{H1}/T_{H2} bias observed in the spleen and because the antibodies that could be induced against mycolic acids in rodents in this study or in the work of Goodrum (1998), were of a low affinity and of the IgM isotype.

This study managed to provide a cost-effective method of purifying mycolic on a large scale so as to make biological studies with mycolic acids possible. The studies of Tentori *et al.* (1998) showed that rifampin (one of the drugs used in tuberculosis chemotherapy) induced expression of CD1b in human peripheral blood monocytes. This could be extrapolated to predict that there would be an increased presentation of mycolic acids if rifampin chemotherapy is combined with mycolic acids treatment. This could result in enhanced production of IL-12 and stimulation of CD4/8 double negative T cells when the combined therapy is administered.

The difficulties experienced by the current prolonged treatment regimen (six months) could be minimised in this way by the combined therapy/immunotherapy. This is based on the potential of mycolic acids to shorten the period of chemotherapy through its observed ability to induce

a protective immune response. This prediction is extrapolated from studies in rodents, in which the mode of mycolic acids presentation is unknown as they do not have the CD1b. In humans, with the proven ability to present mycolic acids on CD1b, the effect of mycolic acids could even be stronger than suggested.

Summary

Mycolic acids, the characteristic, abundant waxes of the cell wall of *Mycobacteria* were purified by Counter Current Distribution (CCD) from alkaline methanolytic crude extracts of bacteria, aiming at investigating their role in eliciting immune responses. Crude mycobacterial cell-wall extracts were first made by saponification in potassium hydroxide methanol solution. Purification was then performed with CCD using a bi-phasic tricomponent system, consisting of double distilled deionized water ($dddH_2O$), chloroform and methanol. Emulsions were formed in this system which in turn extended the purification time. The addition of a preliminary funnel extraction step, to reduce the saponified fatty acids in the crude extract, before CCD and the addition of NaCl as an emulsions breaker in the CCD solvent system, produced a high yield of pure mycolic acids. The purity of these mycolic acids were assessed using reversed-phase HPLC-analysis. This method proved not only to be applicable to purify mycolic acids from *M. tuberculosis* but was also applicable in purifying mycolic acids from other sources, such as *M. vaccae.*

The immunogenic properties of the purified mycolic acids were confirmed in experiments in which they induced the formation of antibodies in Sprague-Dawley rats when immunized in Marcol 52 oil. The antibody response was monitored by ELISA after 3 months of repeated immunization every second week. A dose-related response was observed for the induction of antibodies specific for mycolic acids, immobilized on the ELISA plates.

Mycolic acids also appeared to influence adjuvant arthritis. Pure mycolic acids, suspended in mineral oil were administered intradermally into Lewis rats one week before the intradermal administration of an arthritis-inducing dose of lyophilized *M. tuberculosis* H37Ra. Animals receiving *Mycobacteria,* but

no mycolic acids treatment, developed severe symptoms of arthritis within two weeks after bacterial challenge. No arthritis symptoms were apparent in mycolic acids treated rats. Mycolic acids treatment alone, did not produce arthritis.

Mycolic acids pre-treatment of *M. tuberculosis* H37Rv-infected mice, rendered tuberculosis susceptible Balb/c mice more resistant. This resistance was equivalent to that observed in tuberculosis resistant C57Bl/6 mice. Post-infection treatment of *M. tuberculosis* H37Rv-infected mice with MA had no effect. Resistance of C57Bl/6 mice is commonly associated with the expression of IL-12 and IFN-γ. The effect of mycolic acids in the spleens of *M. tuberculosis*-infected Balb/c mice was investigated. It was observed that there was no significant change on the T_{H1} and T_{H2} cytokines. The absence of mycolic acids-induced T_{H1}/T_{H2} cytokine bias implied that protection was not provided by the expression of IL-12 and IFN-γ in the spleen.

These results support the hypothesis that mycolic acids are immunogenic in respect of being able to induce specific antibodies, to provide resistance against tuberculosis and to prevent the development of adjuvant arthritis. The mechanism by which mycolic acids perform these tasks is unknown, particularly in these rodent models, which differ from humans, in that they do not have the CD1b that presents mycolic acids in humans. Unravelling this mechanism, can possibly aid the development of a pharmaceutical formulation that introduces MA into the body to enhance resistance to TB and prevent arthritis as an associated side-reaction.

Opsomming

Mikolsure, die kenmerkende wasagtige komponent van *Mycobacteria* was gesuiwer deur middel van teenstroomverdeling van alkaliese metanolitiese ru-ekstrakte van bacterië ten einde hul immunogeniese vermoëte toets. Ru-ekstrakte van mikrobakteriële selwande is vooraf berei deur saponifikasie van mikobakterië in 'n kaliumhidroksied metanoloplossing. Suiwering van mikolsure was uitgevoer met teenstroomverdeling in 'n bifasige trikomponentsisteem, saamgestel uit 'n mengsel van dubbelgedistilleerde-gedeioniseerde water (dddH_2O), chloroform en metanol. Hierdie sisteem het emulsievormings veroorsaak wat die skeidingstyd lank gemmak het. 'n Skeitregter-ekstrasiestap om gesaponifiseerde kortkitting vetsure uit die ru-ekstrakte te veminder, is voor teenstroomverdeling ingevoeg. Die toevoeging van NaCltot die fasesisteem het hoë opbrengste suiwer mikolsure gelewer in 'n verkorte skeidingstyd. Om die suiwerheid van die mikolsuurproduk te bepaal is omgekeerde fase HPLC gebruik. Hierdie suiweringsmetode is suksesvol aangewend, nie net vir suiwerings uit *M. tuberculosis* nie, maar ook vanuit *M. Vaccae.*

Die immunogeniese eienskappe van mikolsure is bevestig in eksperimente met Sprague-Dawley rotte waarin teenliggaamproduksie teen mikolsure waargeneem kan word, na immunisassie in Marcol 52 olie. Teenliggaamproduksie is deurlopend gemonitor en ELISA resultate verkry na 'n drie maande periode van immunisasie elke tweede week. Vir die induksie van mikolsuurspesifieke teenliggame is 'n dosis-afhanklike reaksie waargeneem op ELISA plate met gedek mikolsure op die oppervlak.

Dit het ook geblyk dat mikolsure adjuvant-artritis beïvloed. Een week voor die intradermale toediening 'n artritis induserende dosis van gevriesgroogde *M. tuberculosis* H37Ra, is gesuiwer mikolsure, gehersuspendeer in minerale olie, intradermaal aan Lewis rotte toegedien. Proefdiere wat alleenlik mikobakterieë, sonder voorafbehandeling met mikolsure ontvang het, het erge artritis simptome getoon,

binne twee weke na die bakteriële daging. Geen artritis simptome was waarneembaar in mikolsuurbehandelde rotte nie. Mikolsuurbehandeling in afwesigheid van bakterieë het geen artritis-simptome tot gevolg gehad nie.

Voorbehandeling met mikolsure het weerstandbiedendheid teen tuberkulose aan vatbare Balb/c muise verskaf wat met *M. tuberculosis* H37Rv-geïnfekteer was. Hierdie weerstandbiedendheid was gelykstaande aan die waargeneem in tuberkulose weerstandige C57Bl/6 muise. Mikolsuurbehandeling van muise nadat hulle met *M. tuberculosis* H37Rv-geïnfekteerde is, het geen beskermende effek gestoon nie. Weerstandbiedendheid van C57Bl/6 muise is geassoseer met die uitdrukking van IL-12 en IFN-γ. Voorafbehandeling met mikolsure is ook op *M. tuberculosis*-geïnfekteerde Balb/c muismilte ondersoek. Geen ooglopende effek was waarneembaar ten opsigte van die T_{H1}- en T_{H2}-sitokines nie. Aangesien daar geen waarneembare T_{H1}/T_{H2} sitokien-verandering opgemerk is nie, blyk dit dat die weerstandbiedendheid wat voorafbehandeling met mikolsureverleen, nie deur verhoogde uitdrukking van IL-12 en IFN-γ in die milt geskied nie.

Hierdie resultate ondersteun die hipotese dat mikolsure immunogenies van aard is ten opsigte van die indduksie van teenliggamproduksie, weerstanddiedendheid teen tuberkulose en die voorkoming van adjuvant artritis. Die meganisme waarvolgens mikolsure hierdie taak verrig is steeds onbekend, veral in die knaagdiermodel, aangesien hierdie diere nie die CD1b besit wat mikolsure presenteer aan die menslike immuunsisteem nie. Die ontrafeling van hierdie ontwikkeling van 'n farmasentiese formulering wat MA-toediening moontlik maak om weerstand teen tuberkulose te verhoog en om die ongewensde artritis newe-reaksie te vermy.

Appendix A

IL-12 expression in the lungs of Balb/c mice, pre-treated with mycolic acids before infection with *M. tuberculosis*. (Data and figure from Pretorius, 1999)

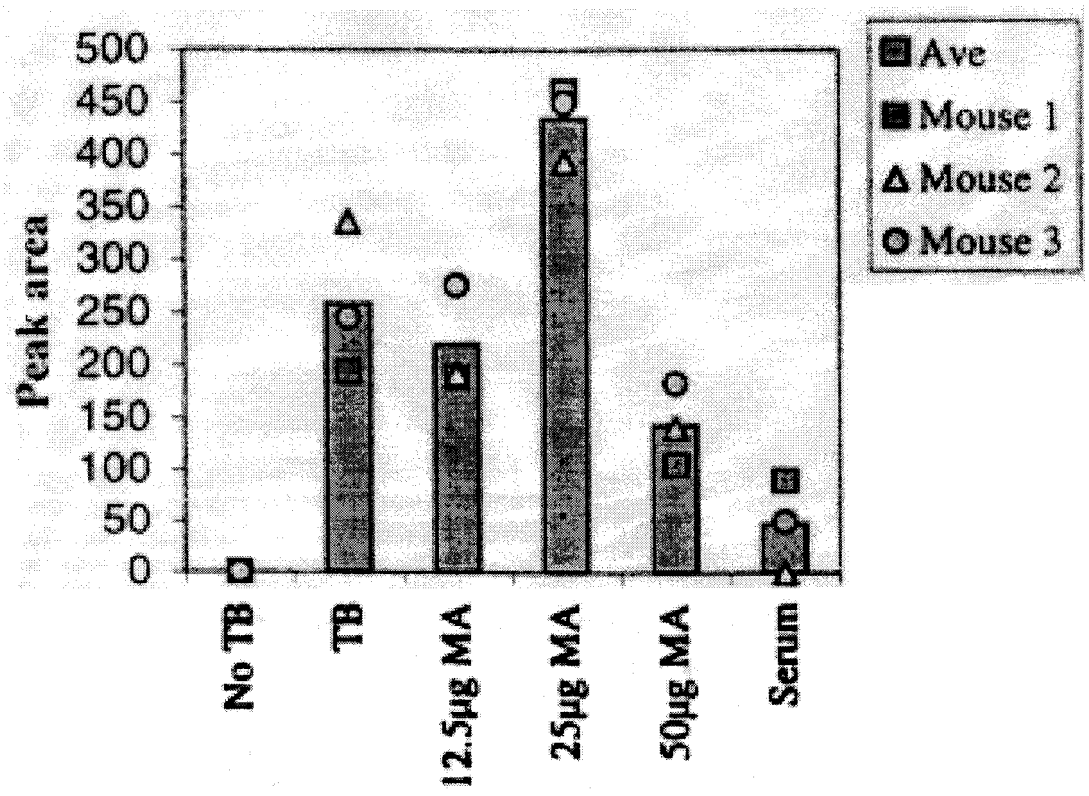

Fig.A: RT-PCR results of IL-12 expression in lungs of Balb/c mice pre-treated with mycolic acids before infection with *M . Tuberculosis*. There is a significant IL-12 response in mice that received 25 µg as compared to the serum control.

Appendix B

IL-12 expression in Balb/c and C57Bl/6 mice infected with *M. tuberculosis* compared with non-infected control mice. (Data and figure from Pretorius, 1999)

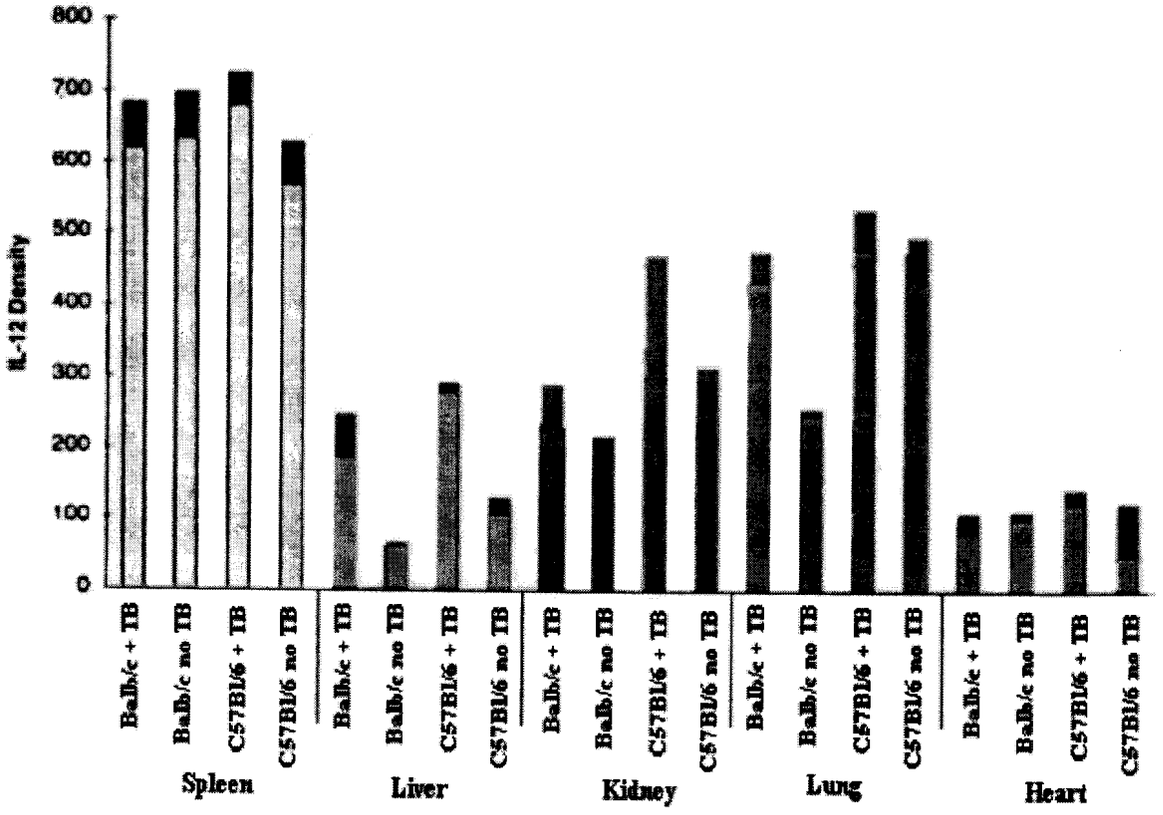

Fig.B1: RT-PCR results of IL-12 expression in lungs of Balb/c mice and C57Bl/6 mice infected with *Mycobacterium tuberculosis*. The spleen, liver, kidney, lungs, and heart, were removed two weeks after infection. The same procedure was performed in non-infected animals. The level of IL-12 expression in these organs were then investigated.

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