

CHAPTER 1

Tuberculosis: the disease and the immune response to it

1.1 Introduction

The most frequent and important causative agent of human tuberculosis is *Mycobacterium tuberculosis*. Occasionally tuberculosis is caused by the closely related organisms *Mycobacterium bovis* (transmitted by unpasteurized milk) and *Mycobacterium africanum* (seen in small proportions in West and Central Africa). Another important disease caused by a pathogen of the genus *Mycobacterium* is leprosy, for which the agent is *M. leprae*. Recently, a number of other species of *Mycobacterium* have become important opportunistic pathogens in immunocompromised individuals, mainly AIDS-sufferers, where they can cause tuberculous or non-tuberculous symptoms of infection (Kaufmann and Andersen, 1998).

1.2 Properties of the etiological agent

M. tuberculosis is an aerobic, rodshaped, non-spore-forming bacillus of about 0.5 x 3 μm , classified as acid-fast due to its ability to retain dyes when treated with acid. The reason for the acid-fastness can be found in the chemical wax that surrounds the mycobacteria and which consists mainly of mycolic acids (McNeil, *et al.*, 1991). In the mycobacterial cell wall, mycolic acids (MA) are linked to underlying arabinogalactan and peptidoglycan. Lipoarabinomannan, another component of the cell wall, is involved in the pathogen-host interaction and facilitates the survival of the bacilli in the host macrophages. Cord factor (trehalose-dimycolate) seems to play a role in the virulence of the bacteria by mediation of cytokines and is responsible for the growth of the organism in rope like arrangements (Saita *et al.*, 2000). During infection and disease, cord factor is also partly responsible for the formation of granulomas.

Pathogenic bacteria belonging to the *Mycobacterium tuberculosis* complex cause tuberculosis. In two-thirds of cases, the lungs are affected, although any other organ in the body can be involved (Antonucci *et al.*, 1995). Mycobacteria grow

very slowly and normally take up to 24 hours to double in number in cell culture media (Antonucci *et al.*, 1995).

1.3 Epidemiology of Tuberculosis

According to the WHO report on the *M. tuberculosis* epidemic and anti-tuberculosis drug resistance in 1997, the projected figures for the next decade are alarming. In the 216 reporting member countries of the WHO, an estimated 7.4 million tuberculosis cases occurred in 1995 with nearly 3 million deaths. The eleven countries in the Southern African sub-region contribute about 275 000 new cases every year.

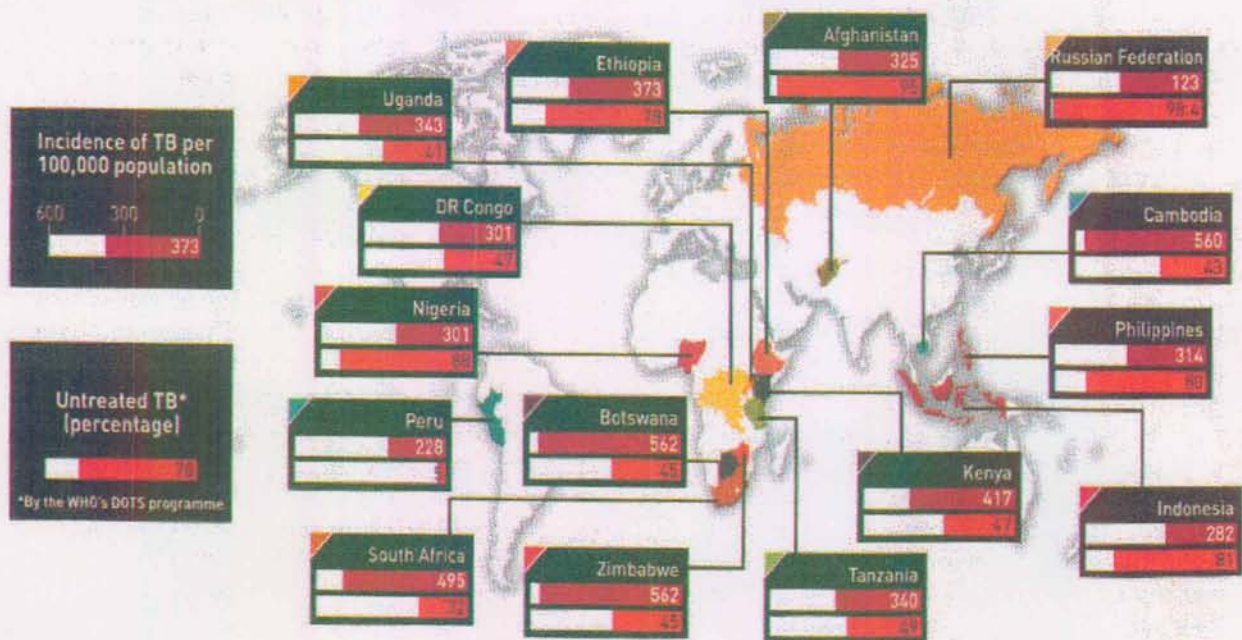


Figure 1.1 Incidence of TB cases per 100 000 of the population for developing countries worldwide and the number of untreated cases as percentage of occurrence. Taken from Coglán and Concar, 2001.

Compared to other developing countries, South Africa has the fourth highest incidence of TB cases per 100 000 of the population. This represents a TB frequency that is dramatically higher, compared to the USA and Western Europe.

In April 2000, South Africa had an estimated 103 700 new cases, representing 495 cases per 100 000 of the total population (Coglan and Concar, 2001).

HIV infection appears to play an important role in the alarming rate of TB infection in Southern Africa (Verschoor and Onyebujoh, 1999, Corbett and De Cock, 2001). If the rate of increase of TB stays the same in South Africa, the prediction for the year 2006 will be 1117 cases per 100 000 of the population, with 848 per 100 000 cases directly attributable to HIV infection. This means that at least 3.5 million new cases and 90 000 deaths due to tuberculosis are expected in the next decade. Already, hospitalizations for tuberculosis increased 350% from 1991 to 1998 (WHO, Fact sheet no 104, 2000), implying a pending disaster for the next decade.

1.4 Infection

M. tuberculosis is most commonly transmitted from a patient with infectious pulmonary disease to another by droplet nuclei, which are aerosolized by coughing, sneezing or speaking. As many as 3000 infectious nuclei remain suspended in the air for several hours after one cough, from where they may gain access to the terminal air passages by inhalation (Fenton and Vermeulen, 1996). Less than 10% of the inhaled bacilli reach the respiratory bronchioles and the alveoli. The external innate immune systems, e.g. the mucus and the epithelium of the upper respiratory tract, filter out and remove most of the bacteria.

Other routes of infection, such as trans-dermal or trans-placental, are uncommon and of no epidemiological significance. Patients with cavitary pulmonary disease or tuberculosis of the respiratory tract (endobronchial or laryngeal), produce sputum with as much as 100 000 bacilli per millilitre.

Up to 75-80% of people exposed to *M. tuberculosis* bacilli do not develop active disease. The risk of acquiring *M. tuberculosis* infection is determined mainly by exogenous factors as explained in a later section.

In contrast to the risk of acquiring TB infection, the risk of developing the disease after being infected depends largely on endogenous factors, including innate susceptibility to the disease and the level of functioning of cell-mediated immunity (Fenton and Vermeulen, 1996). Innate susceptibility is determined by genetic factors which, although not related to immune function, does confer differing degrees of susceptibility in different populations of experimental animals (McMurray, 2001).

1.5 Clinical stages of tuberculosis

Mycobacteria reaching the lung are phagocytosed by alveolar macrophages. Some of the bacilli are destroyed within the phago-lysosomes of the macrophages and the rest remain inside the macrophage vacuoles for an undetermined period of time. Inside these vacuoles the bacilli can replicate and then escape by destroying the macrophage after 2 to 3 weeks, to infect other macrophages (Fenton and Vermeulen, 1996).

Granulomas, formed at the sites of infection by the attraction of monocytes, lymphocytes and neutrophils during the inflammation reaction, contain macrophage-derived giant cells and epithelioid cells. The formation of granulomas is a result of an interaction between macrophages and T lymphocytes and the secretion of cytokines by stimulated cells of both types. The degree of granuloma formation depends upon the number of mycobacteria present in the tissue and on the number of macrophages present at the infection area.

In the cytokine/chemokine cascades that develop around the tuberculosis infection, the relationship between the tissue-destructive delayed-type of

hypersensitivity (DTH) and the T cell-enhanced specific bactericidal killing of mycobacteria by macrophages usually determines the outcome of the disease (Kaplan and Freedman, 1996). How this balance can be manipulated chemically, is currently not fully understood.

1.6 Primary tuberculosis

The majority of primary infections are asymptomatic. The development of a positive tuberculin test between 3-6 weeks after infection is usually the only evidence. A peripheral lung lesion (Ghon focus) accompanied by ipsilateral hilar lymphadenopathy can sometimes be seen on röntgen images (Fenton and Vermeulen, 1996). The pulmonary complex is about 10 mm in diameter and consists of a central zone of caseous necrosis surrounded by palisaded epithelioid histiocytes, Langerhans cells and lymphocytes (Fenton and Vermeulen, 1996). In almost all the cases, a primary lesion will organize. Clinical signs at this stage are unusual except if a lung collapse, caused by enlarged lymph glands, develops. Live tuberculous bacilli may still be present in these scarred foci and may persist for years (Bloom and McKinney, 1999). Complications of primary infection include epituberculosis (pneumonitis and collapse), obstructive emphysema, bronchiectasis, pleural effusion, and associated hypersensitivity phenomena (like Erythema nodosum).

1.7 Secondary tuberculosis

After primary infection, the pathogens may lie dormant and the disease quiescent without any symptoms for decades. Secondary tuberculosis could either start from direct progression of primary disease, inhalation of additional bacilli (exogenous re-infection), reactivation of quiescent primary disease or haematogenous spread of bacilli to the lung. The subsequent lesions nearly always develop in the apices of the lungs because of the high oxygen concentration.

Post-primary tuberculosis progresses despite the existing immunity acquired during the primary exposure, and is considered the most prevalent form of the disease (Fenton and Vermeulen, 1996).

If the healing process is impaired, the lesions do not calcify. They may erode and rupture into adjacent bronchi to form cavities in which *M. tuberculosis* and/or other opportunistic fungi can multiply freely (Nardell, 1993). Leakage of living bacilli into adjacent bronchi will spread the disease in the host and in sputum, discharging it into the air.

Early in the disease, symptoms may be non-specific, such as loss of weight, night sweats, persistent cough, fatigue and loss of appetite (Fenton and Vermeulen, 1996). Systemic features of tuberculosis include anaemia, fever, weight-loss and clubbing of the fingers. In acute pulmonary tuberculosis, lung symptoms become prominent. Upper zone crackles may be heard and haemoptysis may be seen as a result of consolidation. Other complications include pleurisy, pleural effusion, empyema, laringitis, Poncet's arthropathy, airflow obstruction, cor pulmonale, aspergilloma, amyloid, carcinoma of the lung, Adult Respiratory Distress Syndrome (ARDS) and eventually death.

Tuberculosis is a multi-system disease and involvement of liver, urogenital system, adrenal glands, spine, joints, larynx and central nervous system has been reported. Haematogenous spread of postprimary tuberculosis starts when infected lesions erode and rupture into vessels leading to infection of any organ of the body (Raviglione *et al.*, 1995).

1.8 Miliary tuberculosis

Haematogenous spread of bacilli in patients with poor immunity, resulting in disseminated disease, is known as miliary tuberculosis.

Miliary tuberculosis can be a complication of either primary or secondary tuberculosis. In high prevalence areas, the majority of cases occur shortly after

primary infection. In comparison, mostly elderly people develop military tuberculosis in low prevalence areas, representing reactivation.

The name is derived from the small seed-like appearance of the lesions. The lungs are always involved and any other organ variably so. Two forms exist: acute and chronic “cryptic” military tuberculosis.

Complications include meningitis and ARDS. This is an acute medical emergency necessitating prompt treatment to avoid a fatal outcome (Swaminathan, 2000).

1.9 Laboratory detection of tuberculosis

Major disadvantages exist in the traditional laboratory detecting methods. A positive result culture *i.e.* a culture of *Mycobacterium tuberculosis*, may take up to eight weeks for completion but remains a very reliable means of detecting the presence of the bacteria. This in turn leads to further spread of the disease due to the delay in treatment. The normal culture of bacilli can be shortened to 10 -12 days using radiometric determination of bacterial growth in liquid medium, known as the BACTEC technique. This relatively high-tech approach requires a sophisticated infrastructure (Piersimoni *et al.*, 2001)

Tuberculosis is also diagnosed by detection of the whole organism in sputum samples, using the microscope and acid fast stain. The microscope has a sensitivity of detection of at least 5000 – 10 000 bacilli per milliliter of sputum.

Molecular approaches, which were recently introduced, result in more rapid and sensitive detection and drug sensitivity assays, but are expensive and require trained personnel and sophisticated infrastructure. DNA probes are specific, but need 10 000 to 100 000 organisms for a positive signal. At present, the main interest is in the PCR or DNA amplification RT-PCR of parts of the genome of the mycobacteria (Kambashi *et al.*, 2001).

1.10 Tuberculosis Vaccine Research

The only tuberculosis vaccines available for use in humans are attenuated strains of *M. bovis*, i.e. Bacille Calmette Guerin (BCG). This vaccine was introduced in France more than 70 years ago. BCG is still widely used, but controversy surrounds the efficacy of the vaccine (Fine *et al.*, 1994).

New or improved vaccine developments currently investigated are as follows:

- Genetically improved protective antigen BCG or auxotrophic vaccines: improvements relate to genetically engineered attenuation and introduction of cytokines to ensure a Th1 response (Guleria *et al.*, 1996).
- Protein subunit vaccines: this approach involves use of low molecular weight proteins. Typically secreted mycobacterial proteins, are used as vaccines (Wiker *et al.*, 1992).
- DNA subunit vaccines: administration of plasmid DNA expression vectors has been shown to result in protein expression *in vivo*. The translated proteins are immunologically active and elicit humoral as well as cell-mediated immune responses. The approach allows inclusion of cytokine genes to solicit the desired immune response (Donnelly *et al.*, 1994, Lowrie *et al.*, 1994, Lowrie, 1999).

Except for the DNA vaccination method tested in mice, above methods have hitherto met with little success.

1.11 Interaction with HIV infection

Various diseases predispose humans to become vulnerable to the development of tuberculosis. The most important and potent risk factor is HIV infection, which suppresses cellular immunity (Bhatia, 2000).

The risk of developing active disease from latent *M. tuberculosis* is directly related to the degree of immunosuppression in the individual. For non-HIV

infected people, the *lifetime* risk of developing tuberculosis is 10 %. In HIV infected patients it may be 10 to 15 % *per year* (Bhatia, 2000). Other conditions known to increase the risk of developing active tuberculosis among persons infected with the tubercle bacilli include:

1. lymphoma, leukemia and other malignant neoplasm's
2. silicosis
3. haemophilia
4. chronic renal failure and hemodialysis
5. insulin dependent diabetes mellitus
6. immunosuppressive treatment and associative nutritional conditions (Bloom *et al.*, 1994).

The presence of healed, fibrotic tuberculosis lesions constitutes a serious risk of active disease in patients with above conditions.

In patients with HIV infections, endogenous reactivation of pre-existing infection, progression from infection to tuberculosis and transmission of mycobacteria bacilli to non-infected people occurs (Rieder, 1994). In non-African countries, all forms of tuberculosis are now considered an AIDS-defining disease. Tuberculosis is the most common infection in HIV infected patients. Doubling of the current numbers of co-infection is expected in the next decade as a consequence of the impact of the HIV epidemic (Bhatia, 2000).

The pathology and clinical features of tuberculosis are similar in an HIV infected patient prior to acquiring AIDS as in patients without HIV infection. While the CD4⁺ count declines, the clinical and pathological nature of tuberculosis infection changes and tuberculosis becomes atypical (decreased cavitations, increased extra pulmonary involvement, false PPD test) and disseminated. During this stage, the giant cell and epithelioid granulomas with scanty tubercle bacilli become infrequent.

During the advanced stages of AIDS, the macrophage response is lowered, granulomas become rare and contain abundant mycobacterial bacilli (Bhatia, 2000).

Tuberculosis infected HIV patients are more likely to have extrapulmonary tuberculosis, and greater frequency of other manifestation of HIV disease such as wasting, oral candidiasis and lymphadenopathy (Bhatia, 2000).

1.12 The immune response to tuberculosis

M. tuberculosis is an intracellular pathogen infecting several mammalian cells *in vitro*, but is almost exclusively found in macrophages *in vivo*. Cells can internalize particles and solutes via different strategies including pinocytosis, receptor-mediated endocytosis, pinocytosis and phagocytosis. The ingestion of bacilli by macrophages is generally regarded as part of a non-antigen-specific process, broadly known as the innate immune response, which then orchestrates an antigen-specific response, broadly known as the adaptive immune response (Kruth *et al.*, 1999).

1.12.1 Innate immunity

Innate immunity comprises four types of defense barriers, namely anatomical, physiological, phagocytic and inflammatory. Tuberculosis elicits unique responses in the latter three:

Anatomic barriers consist of the skin and mucous membranes. Mechanical, pH and mucus entrapment prevents uptake and entry of the pathogen in the first line of defense (Kuby, 1997).

Physiological barriers make use of temperature, pH, oxygen tension as well as soluble factors to combat spreading of pathogens. Soluble factors such as lysozyme, interferons and complement work together to bring about damage to pathogens, either by destroying them or facilitating their clearance (Kuby, 1997).

Interferon gamma (IFN γ) seems to be the crucial effector molecule in mice and humans against a variety of intracellular pathogen infections, including *Mycobacterium tuberculosis* (Flynn *et al.*, 1993).

By inducing reactive nitrogen intermediates (RNI), IFN γ mediates its protective effect, killing intracellular mycobacteria. In humans, the role of RNI is not as clear as in mice. RNI appears to link the innate and adaptive immunity in tuberculosis. Nitric oxide (NO), generated by infected macrophages, determines the life span and function of specific lymphocytes at the infection site (Sciorati *et al.*, 1999).

Phagocytic barriers comprise the ingestion of extracellular macromolecules via endocytosis and particulate matter via phagocytosis. Endocytosis occurs via either pinocytosis or receptor mediated endocytosis (Kuby, 1997). Macromolecules dissolved in extracellular tissue fluid are internalised by inward folding (invagination) and sealing of small regions of plasma membrane to form endocytic vesicles. These are approximately 0.1 μ m in diameter. The endocytic vesicles fuse with each other and are delivered to endosomes. Endosomes are intracellular acidic compartments and are involved in sorting. Endosomes fuse with primary lysosomes to form secondary lysosomes. Primary lysosomes contain degradative enzymes that break down the ingested materials into small products.

With phagocytosis the material is 10 to 20 times larger and the plasma membrane expands around the particle to form phagosomes. Once the material is ingested, phagosomes fuse with lysosomes and digestion is via the same route as endocytosis (Kuby, 1997). Phagocytosis of either *Mycobacterium tuberculosis*, or large latex beads induced interleukin 12 (IL12) secretion by the macrophage, whereas tumour necrosis factor (TNF) was produced only in response to *M. tuberculosis* and not to latex beads (Ladel *et al.*, 1997).

Patocytosis is a process whereby aggregated low density lipoproteins, microcrystalline cholesterol and small (<0.5 μ m) polystyrene microspheres enter a labyrinth of membrane-bound compartments that remain

connected to the surface. It seems to be triggered by the hydrophobic nature of the particle entering. Mycolic acids and other bio-lipids from the cell wall of mycobacteria could enter the macrophage in this way (Kruth, *et al.*, 1999).

Inflammatory barriers are created by tissue damage caused by a wound or invasion by pathogenic micro-organisms. The cardinal signs of inflammation, *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain) and *functio laese* (loss of function), are reflected in three events during inflammation:

1. Vasodilatation
2. Increase in capillary permeability
3. Influx of phagocytes into damaged tissue

Among the chemical mediators of inflammation released into the area, acute phase proteins, histamine, kinins and chemokines abound. The hypothesis proposed for *M. tuberculosis* infection is that erosion of a bacterium into the interstitium of the lungs causes inflammation. Increased capillary permeability promotes influx of macrophages, but also allows haematogenous spread of bacteria. Important to this hypothesis is that bacilli must escape the local environment very early during infection, to explain the kinetics of generation of T cells (Orme and Cooper, 1999). Granulomas start forming from the interstitial pneumonitis within 15-20 days. Interferon gamma secretion in the tissue activates macrophages to stop the further progress of infection. Dendritic cells in lung tissue migrate to lymphoid tissue after activation. Without infection the activation of dendritic cells doesn't occur. Alveolar macrophages control this by IL10, transforming growth factor β (TGF β) and prostaglandin secretion. If infection is established in the interstitium, macrophages ingest bacilli and release IL12. This drives the maturation of dendritic cells to a Th1 phenotype (Orme and Cooper, 1999).

In mice the Th1 response is followed by a Th2 response, which may be essential to limit the inflammation and minimize tissue damage. The Th2 may also contribute to the immunosuppression that is frequently seen in advanced tuberculosis. In HIV infected tuberculosis patients, this is more apparent, as the immunosuppression affects the Th1 response. Once again the macrophages contribute by producing suppressor cytokines such as IL10 and TGF β . Th2 cells activate B cells and initiate antibody production in TB infection (Grange and Stanford, 1994, Fine *et al.*, 1994). No protective role for these various antibodies have been found. Th2 cells activate B cells and initiate antibody production - this is also known to occur in TB infection (Grange and Stanford, 1994, Fine *et al.*, 1994).

1.12.2 Acquired Immunity

Acquired or specific immunity reflects a functional system capable of recognizing specific antigens by antibodies and the antigen receptors on T cells, leading to the elimination of the antigens. The acquired immunity is adaptive and displays four attributes:

- 1) Antigen specificity
- 2) Practically unlimited diversity
- 3) Immunological memory
- 4) Discrimination between self and non-self.

Immunity to tuberculosis consists of a variety of complex interactions between different cell populations and secreted cytokines to combat infection through inflammation.

T cells, rather than antibodies cause protection against tuberculosis. This was already observed in the 1940's. Later, a number of authors confirmed that the presence of antibodies to different antigens of *Mycobacterium* in infected individuals did not correlate to susceptibility or resistance against disease. (Andersen, 1997).

1.12.2.1 T cells involved in *Mycobacterium tuberculosis* infections.

T cells contributing to protection in tuberculosis include the CD4⁺, CD8⁺ single positive and CD4⁻CD8⁻double-negative types, expressing either the $\alpha\beta$ or $\gamma\delta$ antigen receptors. Among these subsets, an array of different mechanisms of antigen recognition and specific effector functions are available to combat disease.

CD4⁺ T cells

The importance of T cells in the defense against tuberculosis was demonstrated by aggravation of disease in CD4⁺ depleted mice (Orme, 1987). Moreover, transfer of CD4⁺ T cells from immunised animals, protected non-immunised TB infected mice. The dramatic decrease of resistance to tuberculosis in AIDS patients, shows that CD4⁺ T cells also have an important protective effect in humans (Barnes and Modlin 1996). Protection against *Mycobacterium tuberculosis* infection by T cells seems to be antigen-specific and mediated by secreted cytokines. The cytokines activate macrophages, which kill the bacteria. Among these cytokines, interferon γ appears to be the crucial one. The cytolytic activity of CD4⁺ T cells against mycobacterially infected monocytes has been described, but how this manifests *in vivo* is not known (Kaufmann and Andersen, 1998). CD4⁺ cytotoxic T cells (CTLs) isolated from broncho-alveolar lavage, indicated that they play a role in local immune responses against *M. tuberculosis*. The MHC class II molecule restricted lysis of target cells. It was noted that alveolar macrophages are more resistant to lysis than blood monocytes. It was further observed that CTL clones, specific for *M. tuberculosis*, lyse a number of targets, using the granule exocytosis as well as the Fas - Fas ligand (FasL) pathway (Oddo *et al.*, 1998, Manfredi *et al.*, 1998).

CD8⁺ T cells

β 2-Microglobulin forms part of the structure of MHC class I proteins, the ligand for CD8⁺. The importance of CD8⁺ T cells is demonstrated in β 2-microglobulin deficient mice. These animals lack mature CD8⁺ T cells and are highly

susceptible to mycobacterial infection. The susceptibility could also derive from defects in the CD1 presenting system, which also contains β 2-microglobulin (Porcelli and Modlin, 1999, Moody *et al.*, 1999). Vaccination of mice with a DNA vaccine encoding the mycobacterial heat shock protein 65, revealed that protection was predominantly mediated by CD8⁺ T cells (Tascon *et al.*, 1996). These cells were detected in high frequency, secreted IFN γ , and were highly cytolytic. In *M. tuberculosis* infected humans, Tan *et al.* (1997) detected CTLs against tuberculosis in the lungs of patients. Using ELISPOT, Lalvani and Hill (1998) demonstrated IFN γ producing CD8⁺ T cells in the peripheral blood of TB infected patients.

Until recently, it was shown that all CD8⁺ cell-lines recognize antigens from *M. tuberculosis* in the context of classic MHC class I molecules. It is now realized that non-classical class I (MHC class Ib and CD1) presenting molecules are also recognized. A subset of CD8⁺ cells, which recognize CD1 presented non-peptide tuberculosis molecules, were able to recognize and lyse TB infected macrophages irrespective of whether they were derived from healthy donors, TB or HIV/TB co-infected persons (Stenger and Modlin, 1998).

IFN γ , produced by CD8⁺ T cells, contributes to protection by complementing macrophages and CD4⁺ T cells: Tascon *et al.* (1996), demonstrated that *M. tuberculosis* infection could be controlled by transferring CD8⁺ cells to MHC class II deficient mice. However, the protection was lost if the donor cells were taken from IFN γ deficient mice. This suggests that IFN γ , rather than cytotoxicity, provides the mechanism of protection.

Double-negative $\alpha\beta$ CD1 restricted T cells

Double negative (CD4⁻CD8⁻) T cells with the $\alpha\beta$ antigen receptor, recognize non-protein antigens presented on the CD1 molecules (Beckman *et al.*, 1994). These CD1 restricted double negative (DN) T cells, contribute to the response of the

host against mycobacterial infection. As with CTLs, DN T cells lyse macrophages infected with *M. tuberculosis*, and produce IFN γ (Tascon *et al.*, 1998). It was also demonstrated that DN CD1-restricted T cells recognize antigen on the surface of *M. tuberculosis* infected cells (Sieling *et al.*, 1995). Fas-FasL interaction mediated the cytotoxicity of DN CD1-restricted T cells, but no effect was seen on the viability of the bacteria. This is in contrast to CD8⁺ T cells, which lysed the infected macrophages via a Fas independent, granule-dependent mechanism, killing both the host cell and the bacteria. Thus, the CD8⁺ and DN phenotypically distinct subsets of human CTLs use different mechanisms to react to the infected cells with distinctly different outcomes.

T cells with the $\gamma\delta$ antigen receptor

T cells with the $\gamma\delta$ antigen receptor ($\gamma\delta$ -T cells), are important in the resistance against tuberculosis: they accumulate in human lesions caused by *M. tuberculosis* infection and respond *in vitro* to non-protein mycobacterial antigens by proliferation (Stenger and Modlin, 1999). Moreover, their ability to produce IFN γ in response to *M. tuberculosis* infected macrophages supports this hypothesis (Tsukaguchi *et al.*, 1995). *In vivo*, protection mediated by $\gamma\delta$ -T cells seems to be dependent on the size of the mycobacterial inoculum. $\gamma\delta$ -TCR deficient mice can control *M. tuberculosis* infection if the amount of bacteria is not too high. The infection was found to be more pyogenic than granulomatous, hinting at a role of $\gamma\delta$ -T cells to regulate cellular traffick at the site of infection, rather than directly combatting the infection or infected host cells. $\gamma\delta$ -T cells migrate into the mycobacterial lesions to contain infection. FasL is upregulated on activated $\gamma\delta$ -T cells induced by mycobacterial antigens during chronic disease. If the infection turns chronic, the *M. tuberculosis* reactive $\gamma\delta$ -T cells are cleared via apoptosis through Fas ligandation. This in turn contains the tissue damage, due to limitation of inflammation, but allows a chronic tuberculosis infection (Li *et al.*, 1998).

1.13 Cytokines in tuberculosis

Cytokines are soluble proteins produced by a range of cells. They work at nano- to picomolar concentrations on cell membrane receptors to regulate and modulate signal transduction, cell proliferation, activation and differentiation. They induce proliferation and effector function of a variety of cells that are involved in the elicitation of both the innate and adaptive immune responses.

Interleukin 1

IL1 is produced upon stimulation of human monocytes with lipoarabinomannan (LAM) from the cell walls of *M. tuberculosis* (Zhang and Rom, 1993, Wallis *et al.*, 1990). IL1 may contribute to the fever that is characteristic of TB infection, as it is an endogenous pyrogen (Dinarello, 1984). It may also enhance the inflammatory response by inducing the macrophages to produce IL6 and tumour necrosis factor α (TNF α). IL1 produced by macrophages, drives IL2 receptor expression by T cells. If peripheral blood monocytes secrete too much IL1, the upregulation of IL2 receptors can have a suppressive effect on T cell proliferation through consumption of IL2 (Barnes and Modlin, 1996).

Interleukin 2

IL2 determines whether a T cell will proliferate and become a functional effector cell. The interaction of the IL2 receptor and its agonist enhances clonal selection and proliferation of CD4 memory T cells to change into IFN γ secreting cells. T cell proliferation can be induced through upregulation of IL2 receptors and IL12 production (Platanias and Vogelzang, 1990).

Interleukin 6

IL6 is a potent T cell proliferation and B cell maturation factor, leading to increased production of immunoglobulins by activated B cells (Hirano *et al.*, 1990). It may therefore mediate the hyperglobulinemia that is characteristic of tuberculosis. IL6 gene-disrupted mice cannot control acute intravenous infection (Ladel *et al.*, 1997). IL6 is increased 10 000 times in cultures of infected macrophages, compared to uninfected controls (VanHeyningen *et al.*, 1997). In

humans, the IL6 from lung lavages of patients with active pulmonary tuberculosis was also found to be high, indicating that IL6 may play a key role in *M. tuberculosis* infection. Lipoarabinomannan (LAM) as well as muramyl dipeptide, both components of mycobacterial cell walls, have been shown to stimulate IL6 production (Zhang *et al.*, 1994a). Moreover, mycobacteria had to be metabolically active to induce this IL6 induction. This data suggests that the organism must synthesize or process an effector molecule responsible for IL6 production. At this stage it is not clear what role IL6 plays in the mycobacterial infection. It seems that low levels of IL6 are necessary for differentiation and growth of infected macrophages, whereas high concentrations of IL6 suppress the macrophages' ability to stimulate T cells (VanHeyningen *et al.*, 1997)

Interleukin 12

Secretion of IL12 by the infected macrophages and the production of IFN γ by natural killer (NK) and other cells, lead to Th1 cell development. Th1 is usually associated with effective immunity leading to containment of tuberculosis. Anti-IL12 antibodies inhibit the proliferation of T cells against *M. tuberculosis* (Zhang *et al.*, 1994b).

Both IL2 and IL12 have profound influence on the adaptive immunity. IL12 induces the development of Th1 cells, which increase immunity to intracellular pathogens (Flynn *et al.*, 1995a). The production of IFN γ , and proliferation of T- and NK cells are also enhanced by IL12 (D'Andrea *et al.*, 1992, Kobayashi, 1989). IL12 binds to high-affinity $\beta 1/\beta 2$ interleukin receptor complexes on the T- and natural killer cells. Mutations of these receptors lead to severe mycobacterial infections. Both IFN γ and IL12 receptor deficiencies predispose to mycobacterial infections and impair the formations of mature granulomas (Altare *et al.*, 1998). IL12 mRNA and -protein are high in response to *M. tuberculosis* (Zhang *et al.*, 1994b). Thus, IL12 participates in a resistant immune response against *M. tuberculosis*. IL12 has the capacity to induce proliferation of cytolytic T cells only upon co-stimulation of the T cell receptor with antigen or anti-CD3 (Bertagnolli *et al.*, 1992). IL12 might be a candidate to control the cytolytic part of the initial

immune response against microbial pathogens by T cell proliferation, but only in the presence of antigen. The cytolytic activity of CD8⁺ T cells and NK cells are also enhanced by IL12 (Flynn *et al.*, 1995b, Gately *et al.*, 1994). IL10 and TGFβ in contrast, inhibit IL12 synthesis and suppress Th1 responses (D'Andrea *et al.*, 1992).

Interleukin 10

IL10 has suppressive functions by down-regulation of the MHC molecules and inhibition of the production of monokines. Antibodies against IL10 in TB patients enhanced the production of IFNγ (Gong *et al.*, 1996). This suggests that IL10 may inhibit the immune response against *M. tuberculosis* in humans. Mycobacteria induce IL10, which inhibits the Th1 response to mycobacteria, probably by inhibiting the synthesis of IFNγ by NK cells and macrophages. If the production of IL10 by macrophages and IL4 by CD4⁺ NK cells takes place at an early stage of the infection when innate immunity plays a critical role, the differentiation of T cells will be biased towards Th2 cell development. This may worsen the prognosis of the disease (Kaufmann, 1995).

IL10 was originally described as a Th2 cytokine, but is also secreted by alternatively activated macrophages and human Th1 cells (Abbas *et al.*, 1996). Production of IL10 may be part of an alternatively activated macrophage system controlling the Th1 effect to protect the lungs from disseminated necrotic damage.

Tumour necrosis factor α (TNFα)

TNFα exhibits both protective and pathologic effects in *M. tuberculosis* infection. Physiological concentrations seem to be important to anti-mycobacterial immune defenses. It has been speculated that physiological concentrations of TNF at the site of infection contribute to granuloma formation, control of the disease and mycobacterial elimination. Orme and Cooper (1999) and Rhoades *et al.* (1995), looking into the relationship between these mechanisms, proposed that protection is cytokine- and DTH driven. Protective immunity is an attempt by the

host to prevent further spread of the infection. This is accomplished by the innate response acting via $IFN\gamma$ and the adaptive system characterized by clonal expansion of specific T cells. These T cells secrete IL12 and then $IFN\gamma$. In granuloma formation, influx of macrophages is regulated via chemokines, mostly produced by leukocytes and local tissue cells. Large amounts of macrophages and monocytes move into the infection site long after protective immunity has been activated. Because DTH and protective immunity occur at the same time, it was always seen as one reaction. According to Orme and Cooper (1999), the key element in both protection and DTH reactions is $TNF\alpha$. Classically activated macrophages are a good source of this cytokine. $TNF\alpha$ drives the DTH reaction by chemokine secretion, recruiting monocytes into the region. However, TNF is a double edged sword, on one side necessary for granuloma formation and protection, but on the other side leading to continued influx of monocytes and tissue damage (Orme and Cooper, 1999).

If increased $TNF\alpha$ is found in the blood, systemic manifestations like fever and cachexia is found (Barnes and Modlin, 1996, Tracey and Cerami, 1994). Disruption of the gene coding for the $TNF\alpha$ receptor in infected mice, leads to decreased survival (Flynn *et al.*, 1995). Patients with tuberculous pleuritis, who mount a resistant immune response to infection, have concentrated $TNF\alpha$ at the site of infection (Barnes *et al.*, 1990). Human alveolar macrophages and mononuclear cells produce $TNF\alpha$ in large quantities in response to *M. tuberculosis* (Valone *et al.*, 1988).

Transforming growth factor β (TGF β)

TGF β is produced constitutively by monocytes from TB patients and inhibits IL2-dependent T cell proliferation and IL2 receptor expression (Ortaldo *et al.*, 1991). TGF β down-regulates MHC class II expression on macrophages and inhibits cytokine production by macrophages (Oswald *et al.*, 1992, Toossi *et al.*, 1995). Macrophages are only activated by $IFN\gamma$ if TGF β is neutralized, indicating that TGF β inhibits anti-mycobacterial immune defenses. In macrophages, as in

lymphocytes, TGF β 's level of control is not regulated by mRNA expression, but in secretion and activation of latent forms. Its function may be to contain the extent of inflammation following infection, in order to limit damage to non-infected surrounding tissues (Toossi *et al.*, 1997, Toossi and Ellner, 1998).

Interferon γ (IFN γ)

Interferon gamma is a pleiotropic cytokine, playing a central, but complex role in resistance of the host to pathogens. IFN γ exerts its activities on macrophages and lymphocytes, resulting in classically activated macrophages and differentiated T cells to deliver a Th1 type of immune response. Except for regulation of several aspects of the immune system, IFN γ also:

1. Stimulates bactericidal activity of phagocytes,
2. Stimulates antigen presenting through MHC class I and II molecules,
3. Facilitates leukocyte-endothelium interactions,
4. Influences cell proliferation and apoptosis,
5. Control the expression of a variety of genes whose function is unknown at this time (Boehm *et al.*, 1997).

Such a variety of effects by a single cytokine is coordinated by complex patterns of cell specific gene regulation. It is known that over 200 genes are regulated by IFN γ (Boehm *et al.*, 1997).

For a long time it was thought that only T cells and NK cells express and secrete IFN γ . As a general rule, both these cell types require cooperation from accessory cells being in some form of activated state. For T cells to produce optimal amounts of IFN γ , the following signals are needed:

1. a ligand for the antigen receptor (specific or aspecific)
2. a balanced cytokine combination (TNF α , IL12, IFN γ)
3. contact of accessory cells through adhesion molecules.

Macrophages are intimately related to the IFN system, so much so that an "IFN-macrophage alliance" has been postulated (Mogensen and Virelizier, 1987). All attention is currently given to the effects of IFN γ on macrophages, assuming that

other cells are the source of the IFN γ . The biological effects of IFN γ are summarized in Table 1.1.

Table 1.1. Biological effects of IFN γ on macrophages. (Gessani and Belardelli, 1998)

Function	Effect
Classical macrophage activation	↑
Alternatively activated macrophages	↓
Cytokines and chemokines production	↑ ↓
MHC class II antigen expression	↑
Fc γ R1 expression	↑
Nitric oxide production	↑
Differentiation	↑
Antiviral activity	↑

Until a few years ago, it was not believed that macrophages could produce IFN γ . Recently, Fultz *et al.* (1993) and Fultz and Vogel (1998), as well as Gessani and Belardelli (1998) provided proof that the IFN γ gene is constitutively expressed in unstimulated, resting peritoneal macrophages. In addition, if these macrophages were stimulated by LPS treatment, increased levels of IFN γ mRNA, as well as cell associated immunoreactive IFN γ was found. Moreover, it was demonstrated that IFN γ can up-regulate its own gene expression, by accumulation of both its mRNA in the cytoplasm and protein in the culture medium.

Convincing proof is now available that macrophages can indeed produce IFN γ under certain physiological as well as pathological conditions. The functional role of this is not yet understood, but it seems that the regulatory mechanisms activating transcription of this gene differ in macrophages in comparison to lymphocytes. Only a single signal will activate transcription of IFN γ in macrophages as opposed to at least two signals in lymphocytes. This emphasizes the importance of macrophages as early players in the immune

response, giving a direct response to infection. It has been well demonstrated that shortly after infection, IL12 is secreted in huge amounts, giving the major signal for local release of IFN γ . This in turn may act as the auto-stimulatory signal for further cytokine production. In peritoneal macrophages, IFN γ is spontaneously secreted, implicating that under physiological conditions certain macrophages are in a classical activated state (Gessani and Belardelli, 1998).

The finding that macrophages can respond to IL12 by producing IFN γ , adds a new component to the role of macrophages in the relationship between innate and acquired immunity.

It appears that the activated macrophages and the Th1 CD4⁺ T cells play the major role in TB immunology, leading to an acute phase inflammatory response. Monocytes attracted by many chemotactic factors, ingest the bacilli released by the lysed macrophages. The tissue-damaging stage is a result of a delayed hypersensitivity (DTH) reaction, destroying nonactivated TB infected macrophages.

1.13.1 The role of Th1 and Th2 cytokine responses in *M. tuberculosis* infections

Mycobacterium tuberculosis is a facultative intracellular organism. Eradication of infection is only possible through cellular immunity.

In the Th1 responses, macrophages loaded with *M. tuberculosis* secrete IL12 which in turn activates lymphocytes to secrete IL2 and IFN γ , both being Th1 cytokines. From various sources, it seems that IFN γ alone is not enough for eradication of disease (Flynn, 1999).

In Th2 responses, elevated levels of IL4, IL5, IL6 and IL13 are found together with IgE secretion and activation of eosinophils and mast cells. IL4, possibly derived from basophils, mast cells or NK1.1 T cells, induce the Th2 response.

IL10 is found in both Th1 and Th2 responses and in alternatively activated macrophages. In murine mycobacterial infection, the Th1 response found in the first three weeks after infection, changed to a mixed response after day 50. Abbas *et al.* (1996) proposed that the Th2 mostly present in chronic infections, down-regulate the tissue damage found in pure Th1 responses.

The type, dose and route of antigen, can also influence the polarization toward a Th1 or Th2 response (Table 1.2). Mycobacterial antigen such as purified protein derivative (PPD) induces a strong Th1 response, whereas *Toxocara* excretory antigen induces a Th2 response. Immunization with 10^7 *Mycobacteria vaccae*, induces a Th1 response, compared to 10^9 bacilli which leads to a mixed Th1/Th2 response (Hernandez-Pando and Rook, 1994). Low or high dose soluble protein with uptake via pinocytosis induces a Th2 response (Constant and Bottomly, 1997). As will be discussed later, phagocytosis via the different receptors tends to polarize the immune response to Th1 and IL12 production.

Table 1.2 Factors that regulate the polarization of Th1 or Th2 immune responses.

Factors involved	Th1	Th2
APC	Dendritic cell, Macrophage	B cell
Cytokines	IL12, IFN γ	IL4
Ag dose	Low	Very low or very high
Co-stimulation	B7-1	B7-2
Steroid	DHEA	Glucocorticosteroid

1.14 Factors that determine resistance or susceptibility to tuberculosis

The search for an effective immunotherapy against tuberculosis requires that cognisance is taken of genetic factors that influence resistance or susceptibility to disease. The following is known to critically determine the outcome of tuberculosis infection in humans:

IFN γ receptor (IFN γ -R)

Levin and Newport (1999) described 6 children having disseminated infection with atypical *Mycobacterium* species in 1996. All were living on Malta. Four were from the same village, of which three were from the same family. This suggested a common immunological genetic disorder. Comprehensive testing revealed impairment in IFN γ production in response to mycobacterial infection. All the children lacked an IFN γ receptor on their leucocytes. When children with impaired IFN γ -R were vaccinated with BCG, they developed disseminated infection. The frequency of occurrence of this phenomenon was found at 0.59 cases per million vaccinated children, making IFN γ -R deficiency very rare.

Natural resistance-associated macrophage protein (*Nramp*) –

In the mouse model, resistance/susceptibility to infection could be related to a single autosomal gene. This gene, *bcg*, is located on chromosome 1 and is not mycobacterium specific. The gene product determined by cloning and sequencing, was called natural resistance-associated macrophage protein, abbreviated *Nramp*. This protein, expressed by macrophages and other APC's, was found to modulate the susceptibility to mycobacterial infection. Human *Nramp* has also been cloned, and the cell distribution is the same as in mice. It is present on chromosome 2q35. It leads to 4 times less tuberculosis infections compared to the *Nramp* defective-genotype. This demonstrates that *Nramp* is an important gene in the variability in susceptibility to tuberculosis (Arias *et al.*, 1997). *Nramp* has two related actions:

1. activation of macrophages to kill intracellular organisms without the help of T cells, through activation of nitric oxide (NO) (Arias *et al.*, 1997).
2. influencing the antigen presentation to CD4⁺ T cells to modulate the body into a Th1 response (Arias *et al.*, 1997).

The method by which the *Nramp* induces the Th1 response is not yet known, but IL12 could be a mediator.

IL12 receptor –

IL12 binds to high-affinity $\beta 1/ \beta 2$ IL12 receptor complexes on the T- and NK cells. Mutations of these receptors lead to severe mycobacterial infections. Both IFN γ and IL12 receptor deficiencies predispose to mycobacterial infections and impair the formations of mature granulomas (Altare *et al.*, 1998). IL12 mRNA and protein are high at the infection site in patients with TB pleuritis. The pleural fluid cells produce IL12 in response to *M. tuberculosis* challenge (Zhang *et al.*, 1994b). Thus, IL12 participates in a resistant immune response against *M. tuberculosis*. IL12 has the capacity to induce proliferation of cytolytic T cells only upon co-stimulation of the T cell receptor with antigen or anti-CD3 (Bertagnolli *et al.*, 1992).

Antigen presenting molecules – The susceptibility to disease is often genetically determined by alleles or genes that code for membrane proteins involved in presenting antigens to the immune cells. The most important of these are coded for by the Major Histocompatibility Complex (MHC) of genes.

MHC class 1 molecules consist of one α -globulin subunit in association with $\beta 2$ microglobulin. It binds endogenous peptides and present them to CD8⁺ T cells. Cytosol derived peptides are from endogenous intracellularly digested proteins. Endogenous proteins are known to be degraded within the cytoplasm by the large (26S) LMP-containing proteasome complex. These peptides are transported into the endoplasmic reticulum by an ATP binding transporter, TAP. Here they interact with calnexin associated MHC class 1 molecules and calnexin is released. Finally the MHC class 1 molecule-peptide complex is transported from the ER through the Golgi complex to the plasma membrane.

A substantial role for MHC1 molecules in mycobacterial infection is demonstrated by $\beta 2$ -microglobulin deficient mice. These mice rapidly die from *M. tuberculosis*, but not BCG infection (Kaufmann and Ladel, 1994).

However, it must be kept in mind that β 2-microglobulin deficiency not only leads to absence of active CD8⁺ cells. The observed effect can be due to other effects, such as deficient CD1 presentation (Kaufmann and Ladel, 1994).

MHC class II (MHCII) molecules on macrophages and B cells present antigenic peptides to CD4⁺ T helper (Th) cells. Most of these peptides are exogenous proteolytic degradation products loaded onto newly synthesized MHCII molecules. MHCII molecules are synthesized in the endoplasmic reticulum (ER) as a transmembrane glycoprotein consisting of homodimerized α and β -chains, of which the C luminal domains comprise the peptide binding groove. In the ER, the α and β -homodimers associate with the invariant chain (Ii), a non-MHC-encoded integral membrane glycoprotein. Interaction between a luminal domain of Ii known as class II associated Ii peptide (CLIP), and the putative peptide-binding groove of MHC II, prevents peptide binding in the ER. As soon as the MHC-Ii complex is formed, it is transported to the Golgi complex. After glycosylation, the MHC-Ii is transported to the trans-Golgi (TGN) network and targeted to the endocytic network.

Using immunoelectron micrography, it was shown that a majority of intracellular MHC class II are found in late endosome structures, with numerous internal membrane vesicles and sheets typical for late endosomes (LE) and lysosomes [MHC class II compartments (MIICs)]. These MIICs structures also contain lysosomal membrane proteins and enzymes and little or no transferrin receptor. In macrophages, MIICs represent the primary site for peptide loading. Some class II molecules that bind peptide are recycled from the surface of the cell.

Mutant mice with MHCII deficiency, and thus non-functional CD4⁺ T cells, die of *M. tuberculosis* and BCG infection. This points to an essential role of CD4⁺ and MHC II molecules (Kuby, 1997).

1.15 Mycobacterial mycolic acids (MA) in resistance or susceptibility to tuberculosis

Whereas much is known on the role of protein antigens from *Mycobacterium tuberculosis* in prototype vaccines against tuberculosis, much less is known on the effect that biolipid antigens have. The most abundant cell wall biolipid of *M. tuberculosis* is mycolic acids (MA). Mycolic acids (MAs) are a class of long-chain fatty acids present in mycobacteria and some other related genera, i.e. *Nocardia*, *Rhodococcus* and *Corynebacterium* (Minnikin and Goodfellow, 1980). MAs are located within the bacterial cell wall, covalently esterified to peptido-glycan-linked arabinogalactan polysaccharides, and also non-covalently, hydrophobically associated in the form of 6,6'-trehalose dimycolate (Brennan and Nikaido, 1995). Although uniform in their α -branched, β -hydroxy structure, mycolic acids are heterogeneous with regard to chain length, number of double bonds, cyclopropane groups and side groups (keto-and methoxy-groups) (Minnikin and Goodfellow, 1980, Brennan and Nikaido, 1995). The C₆₀-C₉₀ MAs are characteristic for mycobacteria (McNeil *et al.*, 1991). The first evidence for immunoregulatory properties of MA was obtained by the observation that presentation of MA to T cells occurred by professional antigen-presenting cells (APC) in a MHC-independent manner through CD1b molecules (Beckman *et al.*, 1994). These experiments revealed a unique role for MA in stimulating the unconventional subset of CD4⁺CD8⁻ T cells.

1.15.1 CD1 as MA presenting membrane protein

CD1 molecules, unlike the MHC molecules, are capable of presenting non-peptide foreign lipids and glycolipid-microbial antigens to specific T- cells. CD1 molecules are surface glycoproteins (50 000 dalton) expressed as glycosylated heavy chains, non-covalently associated to β 2 microglobulin. CD1 is related to MHC, but equally distant from MHC class I and II. It probably originated from a primordial antigen presenting molecule at an equal evolutionary time as the MHC class I and II molecules. CD1 glycoproteins differ from MHC class molecules by being:

- encoded by genes outside the MHC
- non-polymorphic
- independent of the transporter associated with antigen-presentation (TAP) or the invariant chain, required for trafficking to MHC class I and II respectively (Behar *et al.*, 1999)

There are five non-polymorphic CD1 genes in the human: CD1a, -b, -c, -d and e, compared to the rodents' CD1d1 and CD1d2. The β 2-microglobulin-associated protein products from these genes are grouped in:

Group 1 CD1 (consisting of human CD1 a, -b and -c) and

Group 2 CD1 (consisting of human CD1d and -e and murine CD1d1 and CD1d2 (Brossay *et al.*, 1997).

The group 1 molecules are abundantly expressed on professional antigen presenting cells such as dermal- and lymph node dendritic cells, Langerhans cells and mantle zone B cells, whereas group 2 CD1 proteins are expressed on the epithelium of the intestine. CD1 homologues are found in all mammalian species examined to date (Porcelli and Modlin, 1999). There are several hints that the unique functions of the CD1 may be related to the special properties of the species of T cells that interact with the molecule. Table 1.3 shows the CD1 molecules found in various mammals.

Table 1.3 Size and complexity of the CD1 gene in various mammals (Porcelli and Modlin, 1999).

Species	Total CD1 genes	Number of genes for indicated isoform				
		CD1a	CD1b	CD1c	CD1d	CD1e
Human	5	1	1	1	1	1
Mouse	2	0	0	0	2	0
Rat	1	0	0	0	1	0
Guinea Pig	$\cong 10$?	>5	>3	?	>1
Rabbit	$\cong 8$?	>1	?	>1	?
Sheep	$\cong 7$?	>3	?	?	?

In addition to mycolic acids and phosphoglycolipids, glucose monomycolate (GMM) is also presented on hCD1b. hCD1a, b and c present acyl-chain-containing polar antigens. hCD1a and hCD1c present lipids recognized by CD8⁺ cells. CD1 molecules have also been shown to present *Haemophilus influenza* type b glycolipids (Fairhurst *et al.*, 1998). In table 1.4 the major differences between Group 1 CD1 (humans only) and CD1d (mouse and human) are listed.

Table 1.4 Comparison of antigen recognition mediated by group1 CD1 and CD1d molecules (Burdin *et al.*, 2000).

Property	Group1(CD1)	CD1d
Stimulation of autoreactive T cells	Yes	Yes
Stimulation of NK T cells	No	Yes
Presentation of α -GalCer	No	Yes
Presentation of LAM	Yes	No
Presentation of GMM	Yes	?
Reactivity with microbes	<i>M. tuberculosis</i> <i>H. influenza</i>	<i>P. falciparum</i> <i>T. brucei</i>
Requirement of acidic pH for binding to lipid antigen	Yes	No
TCR usage	Variable	Invariant or restricted

In mice, two types of T lymphocytes reactive to CD1d have been described. The CD8⁺TCR $\alpha\beta$ ⁺ cytotoxic T lymphocytes are specific for foreign, hydrophobic peptides, whereas the CD4 single positive or CD4⁺/CD8⁻ double negative T cells recognise the CD1d without foreign antigen, *i.e.* they are auto-reactive. It is currently not clear whether a self ligand should be bound to mCD1 in order to effect recognition by T cells. The auto-reactive anti-mCD1d cells can be subdivided depending on their expression of an invariant TCR α -chain and the cell surface phenotype for NK1.1. If both are present, these cells are called natural

killer T cells or NK T cells. The heterogeneity of the mCD1d auto-reactive T cells is seen by the number of reactivity patterns (almost the same as the amount of T cells tested), and is not related to the level of expression of mCD1d on APC. It is speculated that the heterogeneity might reflect the requirement of the mCD1d T cells to be able to recognize members of a diverse set of autologous ligands such as lipoglycans. It is still undefined what the nature of CD1d bound lipoglycans, required to stimulate CD1d auto-reactive T cells, must or should be.

Brossay *et al.* (1998) demonstrated that some of the CD1 auto-reactive T cells require wild type CD1 that trafficks to endosomes. This suggests that CD1 might bind to a self-ligand derived from the endosome. Metabolic radioactive labelling and mass spectrometry helped to identify glycosylphosphatidylinositol as a natural ligand of mCD1d (Joyce *et al.*, 1998). Using a synthetic glycolipid α -galactosylceramide (α -GalCer), it was shown that both hCD1d and mCD1d can bind this ligand and that either can stimulate NK T cells in both mice and humans. This emphasizes the conservation of the CD1 system.

Crystal structure analysis of the CD1d molecule (Figure 1.2) showed an MHC class 1-like fold with a narrow, deep electrostatically neutral and highly hydrophobic antigen-binding groove (Zeng *et al.*, 1997). CD1d binding models place the hydrophobic acyl portion of the lipid molecule into two large hydrophobic pockets. Hydrophilic or polar groups are on the top of the groove. Compared to CD1b, CD1d does not need acidic conditions to induce the conformational changes required for loading the antigen into the hydrophobic pocket of the groove. The nature and the length of the acyl chains buried in the antigen-binding groove do not significantly affect the presentation capability of CD1d (Porcelli *et al.*, 1998). It seems that the CD1d binding groove has less demanding structural requirements in presentation to NK T cells than Group I CD1 (CD1b).

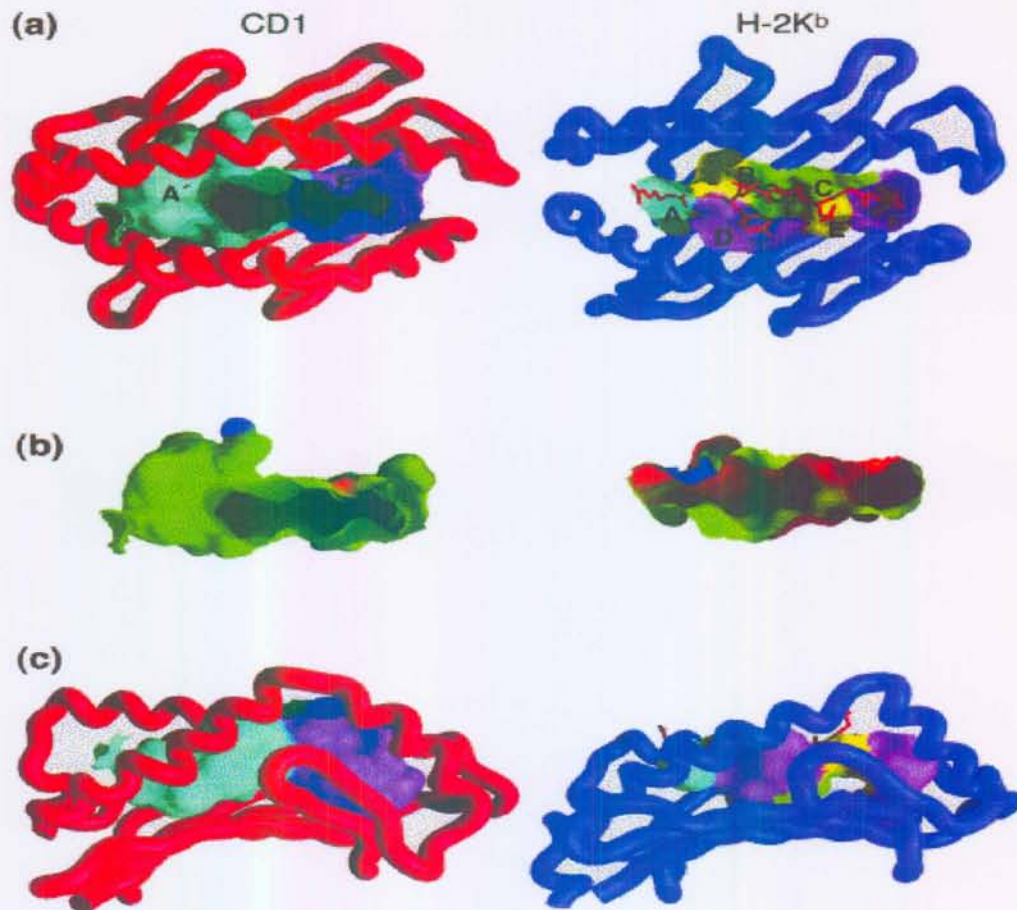


Figure 1.2 Comparison of the ligand binding grooves of CD1 and MHC class I(a) The molecular surface of the groove is shown superimposed on a worm diagram of the backbone of the $\alpha 1$ and $\alpha 2$ domains for CD1d (left) and H-2k^b(right). The groove pockets (A' and F') for CD1d and A-F for H-2k^b are differentiated by colour. (b) Electrostatic potential of the groove surface of CD1d and H-2k^b. Areas with a negative charge are coloured red and areas with a positive charge are blue. Green represents various areas of neutral charge. (c) Side view of the $\alpha 1$ and $\alpha 2$ domains with the groove surface superimposed on the backbone as in (a) and (b). (Zeng *et al.*, 1997).

A critical aspect that still has to be resolved, is the loading of the CD1 groove. Are the lipids processed before loading, and how are they inserted into the groove of the CD1 protein? The human CD1b protein is found in a variety of endosomal compartments, also in those in which the MHC class II is found to load peptides. These MIICs are lipid rich late endosomes. The lipid rich composition of the MIICs is an ideal place to concentrate the foreign lipid antigens, such as mycolic acid. In addition, MIIC contains a wide array of degradative enzymes, which may be involved in the trimming of large glycan components of some CD1 presented antigens, such as those found in LAM.

Sieling *et al.* (1999), showed that CD1 is abundantly expressed in the tuberculoid form of *Mycobacteria leprae* infection compared to lepromatous leprosy. In the tuberculoid or self-healing form of leprosy, INF γ secreting Th1 cells are recruited, in comparison to the lepromatous form where Th2 immunity prevails. This suggests that the CD1 molecule expression may determine the predominance of Th1 or Th2 response.

CHAPTER 2

Aims of this study

The appropriate immune response to bacterial invasion is related to the cell wall structure of the invading organism. There are four main bacterial cell wall types:

- Gram-positive
- Gram-negative
- Mycobacteria
- Spirochaetes

Different immunological mechanisms have evolved to destroy and remove the different groups of bacteria. One example is demonstrated by LPS micelles from gram-negative bacteria that modulate the immune system. By deploying a complex pathway, LPS is neutralized by binding to lipoprotein particles or binding to soluble CD14 (sCD14), thereby passing it on to membrane receptors. The above mechanism is accomplished by a lipid binding protein that assist in the transfer, starting a cascade of reactions leading to cytokine release and immune modulation (Roitt *et al.*, 2000). Another example is provided by isolated *Chlamydia pneumoniae* lipopolysaccharide (cLPS), which induces a cholesterol ester accumulation and foam cell formation in macrophages (Kalayoglu and Byrne, 1998).

Thus, macrophages that are chronically exposed to cLPS may accumulate cholesterol and contribute to atheroma development in pursuit of destroying the microbial pathogen.

As MA comprises 40% of the dry weight of *Mycobacteria tuberculosis*, it may be anticipated to have at least some effect on the invaded cell and host. The complexity of the mycobacterial cell wall makes it difficult to relate single components to the effect they have on the immune system. It may be argued that the effect of the cell wall as a unit may be quite different than the sum of the effects of each component.

In Chapter 1, the importance of tuberculosis as a disease, the mechanics of the mycobacterial infection and the response of the immune system to the infection were discussed, giving special reference to the newly discovered importance of mycolic acids as immunoregulatory substance. Previous work at the University of Pretoria indicated that some important immunoregulatory cytokines were secreted when MA was introduced in mice, providing increased survival upon challenge with living *Mycobacterium tuberculosis*. This dissertation concerns itself with shedding new light on the role that mycolic acids may play in the progression of tuberculosis or immune resistance to it.

Giving the chemical and physical nature of MA, the first obstacle was to get MA into a soluble form to be introduced into the body. The isolated MA was tested for the effects it may have on mouse peritoneal macrophages. The mouse model was used, as the immune system of the mouse is well known and frequently used in mycobacterial research. Macrophages from the mouse peritoneal cavity were chosen, due to their abundance and ease of isolation. Also, immune markers are readily available.

Mycobacterium tuberculosis escapes the innate immune system and evades destruction inside the macrophage after phagocytosis by various systems. Some of these systems prevent the phagosome to mature into late stage endosomes. In chapter 3, the MA and MA-carriers were studied to learn how they were phagocytosed, and how the phagosomes subsequently developed into late stage endosomes by using fluorescently labelled MA.

The effects of MA on mouse macrophages were found to involve differences in activation status as well as morphology. Chapter 4 deals with the activation and survival of macrophages and characterization of the phenotype of the cells that develops after loading with MA.

In Chapter 5, the effect of MA on the immune system and the killing of *Mycobacterium tuberculosis* are examined. Evidence is presented for a prominent role for MA in the innate immune system. The role of reactive oxygen and nitrogen intermediates are examined in the activation of macrophages with subsequent foam cell formation and killing of the bacteria.

Chapter 6 summarizes the evidence to support a role for MA on the innate immune system. As with the LPS in *Chlamydia pneumonia*, this isolated part of the mycobacterial cell wall seems able to activate macrophages into foam cell formation. The activation status of these foam like cells are discussed.

The complete genomic sequence of *M. tuberculosis* suggests that it contains sterol biosynthetic enzymes as well as two putative cholesterol degrading enzymes (Cole and Barrell, 1998, Cole, 1998, Bellamine *et al.*, 1999). This gives us ground to speculate on *M. tuberculosis* induced foam cells as a possible carbon and energy source for latent intracellular Mycobacteria.

In addition, a possible effect that *Mycobacterium tuberculosis* infection might have on cardiovascular diseases through immune modulation by MA, is proposed.

CHAPTER 3

Evaluation of carriers for mycolic acids

3.1 Introduction

3.1.1 Mycolic acids: properties and solubility

Mycolic acids are high molecular weight, α -branched, β -hydroxy fatty acids, present in the bacterial genera *Mycobacterium*, *Rhodococcus*, *Nocardia* and *Corynebacterium*. Mycobacterial mycolic acids are covalently bound to arabinogalactan via ester bonds (McNeil *et al.*, 1991). Mycolic acids also occur as components of soluble trehalose dimycolate (cord factor) and trehalose monomycolate in the cellular matrix (Besra *et al.*, 1994).

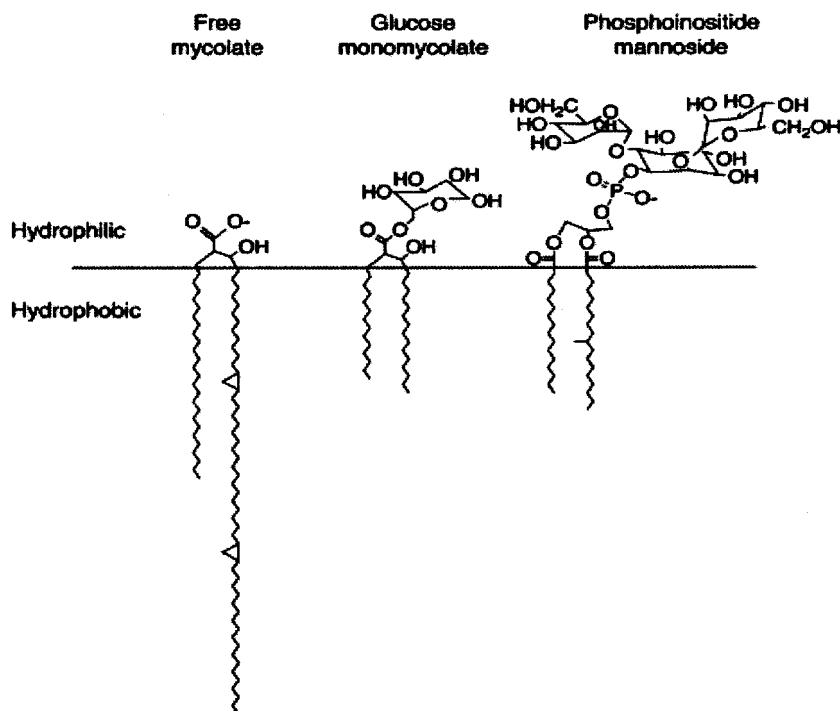


Figure 3.1 Structure of mycolic acid, glucose monomycolate and phosphoinositide mannoside.

Mycobacterial mycolic acids can be distinguished from those of other genera by

- being the largest mycolic acids (C60-C90)
- having the longest side chains (\geq C20-C24)
- containing one or two unsaturated groups, existing as double bonds or cyclopropane rings
- containing functional groups with oxygen in addition to the β -hydroxy group
- being methyl branched in the main carbon backbone (Liu *et al.*, 1996).

Mycolic acids can be divided in three subclasses namely α -mycolates, ketomycolates and methoxymycolates, according to the chemical groups associated with the carbon backbones.

The extracted and purified mixture of mycolic acids appears white and waxy with a melting point of approximately 40°C. It is insoluble in water, but soluble in non-polar organic substances such as chloroform, dichloromethane and tetrahydrofuran.

3.1.2 Dissolving hydrophobic substances in water

To enhance the water solubility of a drug, organic solvents are used which are miscible with water. Alternatively, surface-active agents (surfactants) are used which orientate into concentrated polar and non-polar layers (micelles) in solution. This has been used to solubilize substances such as vitamins, oils and resins (Florence and Rogers, 1971).

For a substance to dissolve, the solvent must overcome the forces of intermolecular attraction of the solute. According to the principle of like dissolves like, ionic compounds dissolve more readily in polar water by virtue of ion-dipole interactions, whereas non-polar substances dissolve more easily in organic solvents as a result of dipole or induced dipole interactions (Van der Waals, London or Debye forces). The solubility of a drug substance is due mainly to the polarity of the solvent, often expressed as the dipole moment, referred to as the

dielectric constant. Solvents with high dielectric constants dissolve ionic substances and are water soluble, whereas solvents with low dielectric constants do not dissolve ionic substances. The former are polar solvents such as water and methanol, while the latter are non-polar substances such as chloroform and oils (Lawrence, 1989).

3.1.2.1 Emulsions

Emulsification is the heterogenous dispersion of one immiscible liquid in another. An emulsion is formed when two immiscible liquids are agitated together and become dispersed in one another. Water coalesces much faster than oil and two phases are formed if the emulsion is not stable. If a stabilising agent (emulsifier) is added to the two mixed, immiscible liquids, one phase may become translucent, but the other will remain in emulsified form. Emulsifiers are simple additives that usually consist of a non-polar hydrocarbon chain with a polar group at the end. The hydrocarbon chain is soluble in oil and the polar head in the water. Thus, emulsifiers selectively locate at the interface between aqueous and oil phases or at the surface defined by the aqueous and air boundaries (Lawrence, 1989). Their presence cause a reduction in the surface tension of water at the interface with the oil, the degree of which determines the stability of an emulsion when the phases are mixed.

3.1.2.2 Micelles

Micelles come about when amphiphilic molecules in water segregate their hydrophobic portions from the solvent by self-aggregation. Micelles can be spheres, discs, oblate or prolate ellipsoids, or long cylinders and can be ionic or non-ionic. Amphiphiles must have a certain concentration to form or produce a structure to shield the tails from the water. This concentration is called the critical micellar concentration (CMC).

At ultra-low interfacial tension, a micro-emulsion forms spontaneously, which has the appearance of a clear solution. By increasing the concentrations of the

emulsifying agent, a change is induced in the micro-emulsion at the so-called critical micellar concentration (CMC), when mostly spherical structures are formed with the hydrocarbon chains projected to the inner part and the polar groups to the surface contacting the aqueous phase (Rosoff *et al.*, 1988). These are called micelles and have a diameter of approximately 5 nm. Inverse micelles can also be produced by amphiphiles in non-aqueous solutions (Lawrence, 1989).

Micelles cannot be detected optically and appear as translucent solutions. Solutes with poor water solubility, such as fatty acids, will dissolve in the inner part of these micelles and thus become solubilized. In general, micellar solubilisation is saturable at approximately 10 % (m/v) solute concentration, depending on parameters such as the chemical properties of the solute and the type of emulsifying agent used. The maximum amount of solute that can be incorporated into a given emulsion system at a fixed emulsification agent concentration, is termed the maximum additive concentration (MAC). Higher solubilisation is achieved when the micellar solution is changed to a micro-emulsion. Micro-emulsions are also called swelled micelles, and consist of a homogenous transparent system of low viscosity and contains a high percentage of both oil and water as well as emulsifier. Micro-emulsions form spontaneously when the components are mixed in the appropriate ratios, and are thermodynamically stable.

The difference between micro- and macro-emulsions is the size of the particles. In the case of macro-emulsions, the size is usually above 0.15 μm and the solution is not transparent (Attwood *et al.*, 1989).

3.1.3 Carriers to solubilize mycolic acids

The carrier used to mobilise MA in aqueous solution or suspension for administration to cells or animals, can influence the absorption, processing and presentation by the macrophages or antigen presenting cells (APC). The carrier

must therefore be chosen in a way that takes into account the effects on the immune system.

3.1.3.1 Cyclodextrins

Cyclodextrins (CD) are a family of cyclic oligosaccharides produced by enzymatic modification of starches that can be applied as alternatives to micellar solubilisation. They are crystalline, homogenous, non-hygroscopic substances which are of a torus-like macro-ring shape, built up of glycopyranose units (Szejtli, 1988). Szejtli (1988) discussed the physical and chemical properties of cyclodextrins in depth. The most important characteristics of the α -, β - and γ -cyclodextrins used in this investigation, are summarised in Table 3.1.

Table 3.1 Properties of cyclodextrins

	α	β	γ
Molecular weight	973	1135	1297
Glucose monomers	6	7	8
Internal cavity diameter (Å)	5	6	8
Water solubility (g/100 cm ³)	14.2	1.85	23.2
Melting range (°C)	255-260	255-260	240-245

Cyclodextrins are consumed by humans or animals as oral drug-carriers or as food-additives. On entering the gastric area, they undergo rapid dissolution and dissociation-association equilibrium is reached. Only insignificant amounts of CD are absorbed from the intestinal tract into the circulation. Because the bulk of the cyclodextrins is metabolized in the colon by the microflora, their effect on passive diffusion (Attwood *et al.*, 1989) need not be considered.

According to Szejtli, the acute toxicity of cyclodextrins, defined as LD 50 values, could not be accurately determined because high doses given orally did not result in any deaths of the animals. Similarly, sub-acute toxicity studies did not reveal any abnormalities in the experimental animals (Szejtli, 1988). Intravenous injections at a dose of 100 mg/kg for α -cyclodextrin and 788 mg/kg for β -cyclodextrin, showed signs of nephrotoxicity characterized by proximal tubule

alterations accompanied by cytoplasmic vacuolation, cell disintegration and amorphous mineralisation (Szejtli, 1988). Subcutaneous administration of approximately 400 mg/kg of β -cyclodextrin to male and female rats, produced signs of intoxication consisting of sleepiness and respiratory problems, which generally disappeared after 24 hours. Intramuscular administration led to the ulceration of the injection site after 32 daily injections into the same area with a dose corresponding to 80 mg/kg, but no mortality was recorded. No kidney damage could be seen after 12 days injecting a daily dose corresponding to 20 mg/kg. At a dose of 50 mg/kg, alteration was recorded in the kidneys and irreversible nephrotoxicity resulted. No mutagenic or teratogenic changes were recorded (Szejtli, 1988).

Fatty acids are generally easily soluble in cyclodextrins (Szejtli, 1988). The solubility of members of the homologous series of aliphatic fatty acids in an aqueous cyclodextrin solution, increases from caproic acid onwards. On average, five CH groups are co-ordinated to one cyclodextrin molecule. [The available chain length is almost identical to the actual length of the fatty acid molecule (Attwood *et al.*, 1989).]

3.1.3.2 Polyethylene glycol (PEG)

Polyethylene glycols are molecules of the general formula: $\text{HO}-(\text{-CH}_2\text{CH}_2\text{O})_n\text{-H}$. The type nomenclature *i.e.* 300, 400, 6000 *etc.*, indicates the molecular mass (MW) of a particular compound.

The PEGs with low molecular mass such as 300 MW and 400 MW are clear liquids, almost tasteless and odourless. PEGs with higher molecular mass have a soft and waxy appearance at room temperature. A solution of 50% m/v PEG in water is a clear liquid with low viscosity. PEGs are soluble in water, chloroform and acetone. They are insoluble in ethers, fats, fatty oils and paraffins (Blume *et al.*, 1993).

PEGs 1500 to 6000 are used as carriers for active molecules, which are insoluble or sparingly soluble in water. They also act as lubricants and binders for the production of tablets (Attwood *et al.*, 1989). PEGs are substances with very low toxicity and good compatibility with the skin.

PEGs with low molecular weight, such as PEG 300 and 400, are good solvents for numerous substances that are not soluble in water. The solvent power is ascribed to a complex formation between the active substance and PEG. Some of these complexes are loose and reversible, but sometimes PEG complexes can inactivate a molecule by binding too strongly, eg penicillin G. Consequently, the influence of the PEG on the efficiency and uptake of the active substance needs to be tested in each particular case.

3.1.3.3 Sesame seed oil

Sesame oil is also known as benne or teel oil from the seeds of the cultivated *Sesamum indicum*. The oil consists of olein, stearin, palmitin, myristin, linolein, sesamin and sesamol. It is a pale yellow oil and odourless with a bland taste. It solidifies at -5°C . Sesame oil is soluble in chloroform, ether, petroleum ether and carbon disulphide, while only slightly soluble in ethanol and insoluble in water (Ten Wolde *et al.*, 1997).

Sesame oil is a non-toxic, edible oil, which finds frequent application in the food and cosmetic industries. Sesame oil was also found not to be toxic when in an injectable form. It has been used as a carrier for gold for injections in patients with rheumatoid arthritis (Ten Wolde *et al.*, 1997).

3.1.3.4 Solutol HS 15

Solutol HS 15 is also known as polyethylene glycol 660 hydroxystearate, and is known as a non-ionic solubilizer for injection solutions. It is a white paste at room temperature and becomes liquid at 30°C .

Solutol HS 15 consists of a polyglycolester of 12-hydroxystearic acid (70%) as hydrophobic component and polyethylene glycol 660 (30%) as hydrophilic component. The main fatty acid component is 12-hydroxystearic acid, but stearic and palmitic acid are also present in detectable amounts (Fromming *et al.*, 1990).

Solutol HS 15 is very stable. Prolonged exposure of the Solutol/water mixture to heat, may induce physical separation into two phases which can be reversed by homogenisation. Aqueous solutions of Solutol can be sterilised by autoclaving at 120°C. The solubility of Solutol decreases with increasing temperature. Solutol is soluble in chloroform, water, ethanol and 2-propanol. In these solvents it forms clear solutions (Information sheath, Pharmaceutical specialities, BASF, 1997).

The following LD₅₀ figures are based on the Technical Information for Solutol HS 15 (BASF, 1997).

LD ₅₀	mouse intraperitoneally	> 8,5 g/kg
LD ₅₀	mouse intravenously	> 3,16 g/kg
LD ₅₀	rabbit intravenously	> 1,0 < 1,4 g/kg

3.1.3.5 Microbeads

In the development of single-shot vaccines, biodegradable, non-toxic microspheres have been used to introduce immunogens to elicit a protective immune response. Polymeric microbeads of poly (lactic-co-glycolic) acid (PLGA) and other polymer types, were being used which released the immunogen structurally and chemically intact (Cleland *et al.*, 1994).

Fluorescent microspheres are currently used in a variety of diagnostic tests and blood flow experiments. The microspheres used in these assays are hydrophobic particles that will passively absorb a number of molecules (Powell *et al.*, 1996).

Macrophages and other cells take up particles and micro-organisms via phagocytosis. By loading immunogen on the non-toxic, non-degradable microsphere vehicle, the phagocytosis of these microspheres by macrophages and the effect of the immunogen on the immune system can be studied. By using non-degradable microspheres, the secondary immunological influence of the antigen carrier can be minimized. This system would allow the study of the MA as immunogen mainly to pave the way to a more complex biodegradable vehicle in the future (Eldridge *et al.*, 1991).

3.1.3.6 Liposomes

Charged lipids and neutral lipids doped with a charged amphiphile, spontaneously form dispersions of unilamellar vesicles with diameters ranging from 0.1 to 2 μ m. Phospholipids, a main constituent of biological membranes, have a high tendency to aggregate in bilayers. Upon sonication, these transform into closed, self-sealing, solvent-filled vesicles called liposomes. These liquid crystals, when first formed, consist of several bimolecular lipid lamellae, separated by aqueous layers. Sonication causes the transformation of these units into unilamellar liposomes. Water-soluble drugs can be entrapped in liposomes in the aqueous layers, while lipid-soluble drugs can be intercalated within the hydrocarbon layers of the lipid interior (Farhood *et al.*, 1995, Bangham, 1983).

Important considerations in the preparation of lipid vesicles are lipid surface charge, vesicle size and aqueous volume of the liposomes. Large uni- or multilamellar liposomes are cleared rapidly from the circulation on intravenous injection, and are targeted to mainly the reticulo-endothelial system. An addition of cholesterol to the bilayer is beneficial for maintaining the physical structure (Deamer and Gavino, 1983, Bakouche and Gerlier, 1986). The net charge of liposomes can be varied by incorporation of negatively or positively charged molecules such as diacetylphosphate or stearylamine respectively. Sending the

liposome solution through a polycarbonate sieve of precise pore diameter, can control the homogeneity of size of the liposomes.

A disadvantage of liposomes is that phospholipids are susceptible to oxidation. This requires special care during storage, such as maintaining them in a nitrogen atmosphere (Florence and Rogers, 1971).

Liposomes can be administered intravenously, orally or intramuscularly. Direct administration to the nose or the lungs as an aerosol of liposomes looks promising as a mode of delivery.

3.1.4 The route of administration

The nature of the product to be administered and the desired pharmacological actions are factors determining the route of administration to be employed. Each route of administration is associated with certain limitations and provisos. One of the most important considerations is the volume of the drug which must be administered. In humans, large volumes (*i.e.* greater than 10 ml), can only be introduced via the intravenous route, although the rate of infusion is critical. Volumes up to 10 ml can be introduced intraspinaly, while the intramuscular route is normally limited to 3 ml, subcutaneous to 2 ml and intradermal to 0.2 ml.

The choice of the solvent is critical to the intended route of administration. Intravenous and intraspinal injections are restricted to dilute aqueous solutions, whereas oily preparations, solutions, suspensions and emulsions can be injected intramuscularly and subcutaneously (Florence and Rogers, 1971).

As most infections of TB is found in the lung, an inhalant route seems appropriate. This will place the drug directly into the biosphere where it elicits its function.

3.1.5 Isotonic/iso-osmotic nature of injectables

A solution is iso-osmotic to host tissue fluid into which it is introduced, when the amount of dissolved solutes is equal in both systems. Injectable fluids are

formulated to be iso-osmotic to reduce the irritation that can result from osmotic incompatibility with the body tissues or, if introduced intravenously, to reduce the damage to erythrocytes. If an iso-osmotic preparation is injected into the body, there is no net fluid flux from or to the injection site, and hence no hindrance on passive diffusion (Duma and Akers, 1984, Florence and Rogers, 1971).

Isotonic and iso-osmotic are only synonymous if the dissolved solute cannot pass through membranes. If membranes are permeable to solutes (as is the case with aqueous solutions of glucose, urea and alcohol), the solution acts like pure water and both solute and solvent pass through the membrane into cells, causing them to swell and burst (Duma and Akers, 1984).

3.2 Aims and objectives

The experiments carried out in the course of this investigation were aimed at exploring the suitability of the following carriers for administration of mycolic acids:

1. Cyclodextrins α , β and γ ;
2. Polyethylene glycols 300 and 6000;
3. Sesame seed oil;
4. Solutol HS 15;
5. Polystyrene microspheres and
6. Liposomes using phosphatidylcholine and cholesterol.

3.3 Experimental

3.3.1 Materials

3.3.1.1 General reagents

Cyclodextrin α was obtained from Sigma Chemical Co (St Louis, MO).

Cyclodextrins β and γ were a gift from the Department of Chemistry at the University of Pretoria.

Chloroform was from Saarchem (Analytical Grade, RSA).

5-Bromomethylfluorescein (5-BMF) and propidium iodide were purchased from Molecular Probes (Leiden, The Netherlands).

Potassium carbonate and 18-crown-6 ether were obtained from Sigma Chemical Co (St Louis, MO).

Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany).

Silica gel GHL plates were from Merck.

PEG 300 and PEG 6000 were obtained from Merck (Darmstadt, Germany).

Sesame seed oil at 100% purity was obtained from Pick & Pay, a South African general retail store.

Saline (0,9% NaCl) in infusion bags was purchased from Adcock Ingram Critical Care, Johannesburg, South Africa.

Fluorescent microspheres (1 micron diameter) were obtained from Molecular Probes (Leiden, The Netherlands).

Solutol HS 15 was a gift from BASF Pharmaceuticals (Ludwigshafen, Germany).

Phosphatidylcholine and cholesterol were from Sigma Chemical Co (St Louis, MO).

Macrophage cell lines from C57BL/J6 (4.4) and Balb/C (2C11/12) mice were gifts from Prof. Johan Grooten (University of Gent) and Prof. Patrick de Baetselier (University of Brussels) respectively.

3.3.1.2 Reagents for HPLC

For the preparation of reagents used in mycolic acid separation on HPLC, double distilled deionized water and HPLC grade methanol (BDH, Poole, UK) were used.

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

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

Reagent C: Potassium bicarbonate (2%) Analytical Grade (BDH, Poole, UK) dissolved in methanol-water (1:1). Potassium bicarbonate (10 g) 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., Rockford, IL, Cat. No 48891) were dispensed in 500 μ l quantities into small amber-coloured screw cap vials with Teflon-coated septa. The caps were tightened and the vials were wrapped with ParafilmTM. Reagent D was stored at 4°C.

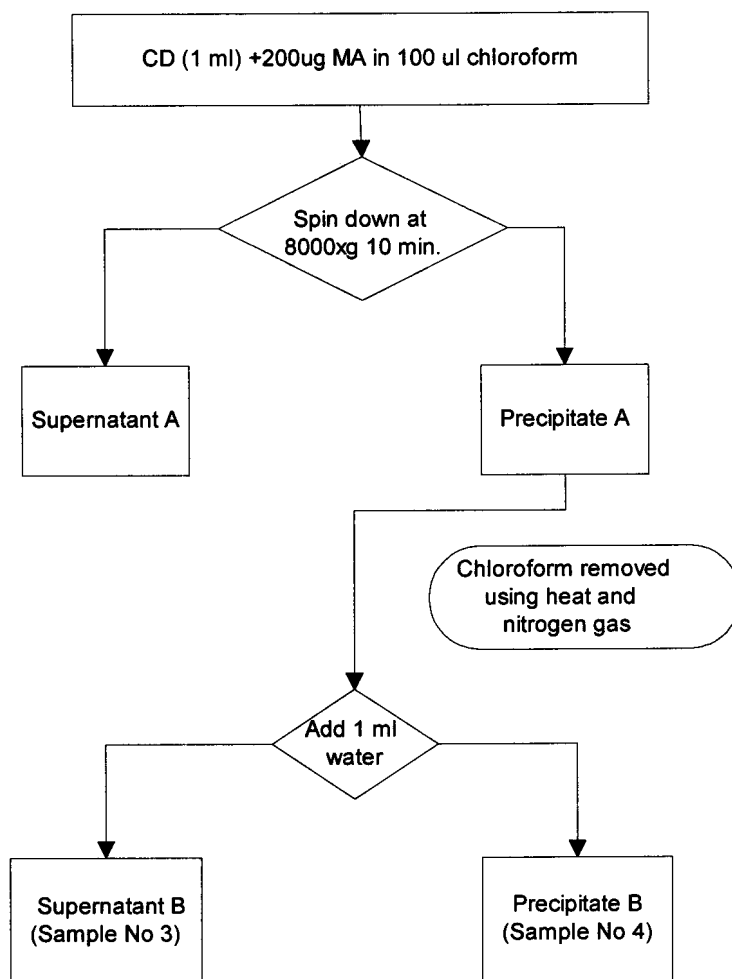
Reagent E: Reagent E was prepared by mixing reagent B 1:1 with methanol.

HPLC Standard: High molecular weight internal standard (C-100) from Ribi ImmunoChem Research Company (Hamilton, MT, Cat No R-50). The standard, 1 mg, was dissolved in 20 ml chloroform HPLC Grade (BDH, Poole, UK) at 4°C and aliquots of 100 μ l were dispensed into 4 ml amber WISP vials, dried, capped with Teflon-coated septa and stored at 4°C.

3.4 Methods

3.4.1 Preparation of cyclodextrins α , β and γ - mycolic acid complexes

Saturated solutions of α , β and γ cyclodextrins were prepared in double distilled water. The solutions were heated to 80°C and cooled down, which resulted in the formation of a clear solution. In each vial of a set of three, 1,0 ml of saturated solutions of cyclodextrins (CD) α , β and γ were introduced. Mycolic acids were added as chloroform solutions according to the scheme in Fig. 3.2. As high temperature does not influence the structure of cyclodextrins, chloroform was removed by heating the samples at 80°C under a stream of nitrogen. Samples from all the preparations were extracted using chloroform, weighed and analysed by HPLC for quality control.



Negative control: saturated CD solution 1 ml + 100 μ l chloroform
Sample 1 and 2 – equivalent to sample 3 and 4

Figure 3.2. Lay-out of the procedure to prepare cyclodextrin-mycolic acid complexes and sampling for quality control.

3.4.2 Solubility of mycolic acids in aqueous solutions of PEG 300 and 6000

3.4. 2.1 PEG 300

In a first vial containing 100 µg MA, 200 µl of a 100% solution of PEG 300 was added. In a second and third vial containing 200 µg MA each, 200 µl 50% (v/v) solution of PEG 300 in water were added. The solutions were heated to 80°C for 30 min to melt the MA into the PEG 300. During this time, the vial was vortexed and heated again. After the mixtures were allowed to cool down to 20°C, 50 µl samples were withdrawn from each vial and analysed for the presence of MA by HPLC. The empty vials were also rinsed with chloroform and sampled for MA determination by HPLC.

3.4. 2.2 PEG 6000

Into a vial containing 490 µg MA, 200 µl of a 20% (v/v) solution PEG 6000 in water was added. The solution was heated to 80°C, vortexed for 5 minutes and allowed to cool down to 20°C. Between each step the solubility was evaluated visually. The volume of the solution was then increased to 1 ml by adding 200 µl aliquots of a 20% solution of PEG 6000. After the introduction of each aliquot, the content of the vial was evaluated visually. The sample was heated and vortexed as described for the first step. The concentration of PEG 6000 in the solution was then increased to 40% by introducing solid PEG 6000. Heating and vortexing of the sample was done in between. The stability of the resulting homogenous suspension was then determined by leaving it at room temperature for 3 days, taking care not to disturb the suspension. Undissolved MA was observed floating on the surface.

In order to establish the concentration of dissolved MA, a 200 µl sample was carefully withdrawn from the bottom of the vial, the solution shaken and a second sample of 200 µl, containing the suspended MA, was also withdrawn. This allowed the determination of the non-soluble MA fraction. The remaining suspension was then removed, the vial extracted three times with 500 µl chloroform and these combined rinses analysed by HPLC.

3.4.3 Sesame seed oil as carrier for mycolic acids

A sample of 430 μg mycolic acid was dissolved in 200 μl chloroform and 200 μl of sesame oil added. The vial was vortexed and heated to 80°C. Nitrogen gas was blown over the oil surface to remove chloroform. After the removal of chloroform, the remaining 200 μl of the MA mixture in sesame oil was divided into two identical parts:

- A) Duplicate samples of 50 μl of the sesame oil/MA preparation were transferred to vials containing 200 μl chloroform and the HPLC standard.
- B) To the remaining part of the sesame oil and MA preparation, chloroform (100 μl) was added and vortexed. Duplicate samples of 100 μl were withdrawn and analysed by HPLC.

The first set of duplicate samples were to represent the solubilized MA in sesame oil, while the second set were to represent the solubilized MA as well as the insoluble MA left in the vial. The control consisted of 50 μl sesame oil and 200 μl chloroform.

3.4.4 Solutol HS 15 as carrier for mycolic acids

3.4.4.1 MA introduced from a chloroform solution

A stock solution of MA in chloroform was prepared at a concentration of 5 mg/ml. Three concentrations (5%, 10% and 25% v/v) of Solutol HS 15 were prepared in water. To 200 μl aliquots MA stock solution, aliquots of 200 μl of the different concentrations of Solutol HS 15 were added. Control solutions of MA in chloroform and Solutol HS 15 alone were used as positive and negative controls respectively.

3.4.4.2 MA introduced as a dried solid

To prepare an unsaturated MA solution, 10% Solutol in 0,9% saline (1 ml) was added to 250 μg dried MA in a sterile Eppendorf tube. The sample was heated

for 10 min at 80°C, vortexed for 10 seconds and left to cool down to room temperature. A 100 µl sample was used for HPLC analysis.

To prepare a saturated MA solution, 10% Solutol in 0,9% saline (200 µl) was added to 3 mg dried MA in a sterile Eppendorf tube. The sample was heated for 10 min at 80°C, vortexed for 10 seconds and left to cool down to room temperature. Three 50 µl samples, taken from the bottom of the vial, were prepared for HPLC.

3.4.4.3 Toxicity testing of Solutol HS 15

3.4.4.3.1 *In vitro* toxicity testing

Three concentrations of Solutol HS 15 (100 µl each of 1, 0,5 and 0.1% v/v in PBS) were added to C57BL/J6 macrophage cultures (1ml) containing 2×10^5 cells and incubated for 10, 30 and 60 minutes. The macrophage cultures contained either 10% or 20% (v/v) foetal calf serum. The Solutol HS 15 was removed by washing four times with complete medium (2ml) before the cells were put back in the incubator. After 24 hours the cells were dissociated from the flask and tested for survival by staining with 5 µl propidium iodide per 1ml cell suspension and analysing on the FACS.

3.4.4.3.2 *In vivo* toxicity testing

Ten mice were injected intravenously with 100 µl Solutol HS 15 (10% v/v saline) with and without mycolic acids (25 µg). Daily weighing and examination was done for 30 days to record any signs of toxicity.

Two mice were injected intraperitoneally with 100 µl Solutol HS 15 (10% v/v) with and without MA (25 µg). After 2 hours the peritoneal macrophages were removed, one half tested for viability using propidium iodide on the FACS and the other put into culture and assessed for viability after 24 hours using the trypan blue exclusion test. As negative control, peritoneal exudate macrophage cells obtained from non-injected mice were used.

3.4.4.4 Effect of Solutol HS 15 on haemolysis

The turbidity of fresh human erythrocyte suspensions in different concentrations of Solutol HS 15 was measured at 690 nm to determine the latter's isotonicity. The decrease in turbidity is proportional to the degree of lysis of the erythrocytes.

Human red blood cells were suspended in Iso M after collection in EDTA tubes. **Iso M** was made up by taking 200 ml of Tris/NaCl buffer pH 7.4 (Tris 24.2 g, NaCl 163.0g made up to one litre, adjusted with HCl and diluted to 2 litres), adding 6.7 ml of 0,15 M MgSO₄ and 3 ml of 0,1 M CaCl₂ and diluting to 2 litres.

Iso BSA was made up by dissolving BSA (1 g) in 1 litre of Iso M.

All cells were washed in Iso BSA and used as the standard in which no lysis occurs.

Haemolysis of erythrocytes was tested by exposing the cells to the following samples listed below:

Experimental samples:

1. Solutol-saline (10% v/v) containing MA introduced as a dry solid (250 µg/ml) and autoclaved for 20 minutes at 121°C
2. Solutol-saline (10% v/v)
3. Saline (positive control)
4. Water (negative control)
5. Solutol-water 10% v/v MA 250 µg/ml

Solutions of MA were prepared by placing an amount of Solutol into a vial with MA, heating it up to 80°C for 5 min and vortexing it for one minute. These solutions were then left to cool down to 20°C.

A standard curve indicating the percentage haemolysis expressed as change in turbidity at 690 nm was prepared as follows: EDTA-blood (2 ml) suspended in Iso M (5 ml) was centrifuged at 750g and the buffy coat removed. An aliquot consisting of 2% erythrocytes in Iso M (named as suspension A) was used as the sample comprising 100% intact erythrocytes, *i.e.* 0% haemolysis. From this 1.5, 1.0 and 0.5 % erythrocyte suspensions were prepared in triplicate.

All test suspensions (1-5) were added to a 96-well flat-bottomed plate (90 μ l) and a background value was obtained on a Titertek Multiscan colourimeter. The 96 well plate was then cooled on ice. After 10 min on ice, 10 μ l of uniformly suspended erythrocytes (suspension A) was added to all the wells. The 96-well plate was then incubated at 37°C. After 15 min the erythrocytes were resuspended and the turbidity recorded as absorbance at 690 nm in a Titertek Multiscan colourimeter.

3.4.4.5 Determination of the particle size of Solutol HS 15 / MA

Different concentrations of Solutol HS 15 (10% v/v) were prepared in water or saline. Mycolic acids (250 μ g), saponified or not saponified, were placed into 4 ml glass vials. To each of these, 1ml of the prepared concentrations of Solutol HS 15 was added. The samples were heated for 10min at 80°C and vortexed for 10 seconds. Solutions of water alone, Solutol alone and saline alone were also used. Autoclaving of some samples was done at 121°C for 20 minutes. The solutions were left to cool down to room temperature and particle sizes were evaluated by light scattering in a flow cytometer. Effects of autoclaving on the micelle size, size distribution and pH of the suspensions were also evaluated.

3.4.5 Microsphere carriers for mycolic acids

3.4.5.1 Preparation of the microspheres

Insoluble polystyrene microspheres were used as a transport vehicle to load MA into the macrophages. Uptake via phagocytosis was tested using these fluorescent microspheres with MA melted onto the surface. This was never intended as an injectable, but only as a method of testing the immunological presentation by APC.

Saponified MA (125 μ g) in chloroform was put into an Eppendorf tube that was silanated and dried at 100°C under a stream of nitrogen gas. Microspheres were diluted in PBS to 2×10^7 per ml, heated to 100°C on a heat block and 500 μ l hot

suspension added to the melted MA at 100 °C. The suspension was then sonicated with a Branson B30 sonifier (at 50% power output for 1 minute), allowed to cool down and again sonicated at room temperature for 10 seconds. The microsphere-mycolic acids suspension was prepared just prior to use.

3.4.5.2 Toxicity testing of microspheres

Uncoated or MA-coated microspheres were added to a C57BL/J6 macrophage cell line culture for phagocytosis. After 24 hours the macrophages were dissociated from the flasks and tested for cell death using trypan blue.

3.4.6 Liposomes as carriers for MA

The original methods of Bangham, (1983) for the preparation of liposomes were followed. This involved the deposition of a thin lipid film from an organic solvent medium on the walls of a container, followed by agitation with an aqueous solution of the material to be encapsulated.

3.4.6.1 Solubility of MA in liposomes

Phosphatidylcholine and cholesterol were both made up to 100mg/ml in chloroform. A mixture of phosphatidylcholine (161 μ l) and cholesterol (97 μ l) was put into a 5ml new sterile glass vial. MA (500 μ g) was added and the chloroform removed by heat (80°C) under a stream of nitrogen gas. PBS (2 ml) was added. A liposome suspension was obtained by sonicating the heated (80°C) phosphatidylcholine / cholesterol / MA mixture. An MA-free liposome suspension was made and used as negative control. Suspensions of 200 μ l were sampled for analysis by HPLC.

3.4.6.2 Toxicity of liposomes

To C57BL/6J macrophage cultures of 2×10^5 cells in 3ml medium, 100 μ l of the liposome suspensions with and without MA were added. After 24 hours cell survival was recorded by using the trypan blue exclusion method.

3.4.6.3 Effect of liposomes on haemolysis

To one millilitre of a 1% packed cell volume per volume (pcv/v) erythrocyte suspension in RPMI 1640 medium, the following volumes of liposomes were added:

1. 0 μ l
2. 200 μ l liposomes only
3. 200 μ l liposomes containing 50 μ g MA

The suspensions were incubated at 37°C for 1 hour before the supernatants were tested for the presence of lactate dehydrogenase on an auto-analyzer at Dept of Chemical Pathology at University of Pretoria. The positive control consisted of a (1% v/v) erythrocyte suspension in which all cells were lysed by sonication.

3.4.7 Macrophage cultures

Macrophage cultures C57BL/J6 (4.4) and BALB/C (2C11/12) were maintained in RPMI 1640 (Life Technologies, Paisley, U.K.), supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100U/ml), sodium pyrovalate (1 mM) and β mercaptoethanol (5×10^{-5} M).

3.5 Results

3.5.1 Cyclodextrin as carrier for mycolic acids

When adding chloroform aliquots with or without mycolic acids to the saturated solutions of cyclodextrins, precipitates were observed in all the CD preparations tested. Testing the supernatant fractions after centrifuging down the precipitates (Sample 3 in Fig 3.2) for the presence of mycolic acids, gave negative results, similar to the negative controls (Samples 1 and 2). Upon attempting to redissolve the precipitated CD in water, only those precipitates that did not contain MA dissolved. This confirmed that a specific interaction occurred between the dextrins and the MA. When analysing the precipitate fractions (Sample 4, Fig 3.3) very little mycolic acids were recovered. Fig 3.3 shows the yields of mycolic acids recovered in chloroform extracts of the CD precipitates, indicating that mycolic

acids were only recovered from the γ -CD precipitate complexes. The low yields recovered probably reflect poor extractability of MA from CD-MA complexes using chloroform. This serves as circumstantial evidence that the MA were indeed complexed to, rather than adsorbed onto the cyclodextrins, since adsorbed mycolic acids would have been recovered equally well from all three types of CD. Moreover, the probability that the mycolic acids were adsorbed onto the surface of the CD molecules are small, as the outer parts of the molecules are hydrophilic and the inner parts hydrophobic. The difficulty of extracting MA from the CD using chloroform may also pose a problem for the release of mycolic acids from the MA-CD complexes in the gastro-intestinal tract when taken orally. As the majority of cyclodextrins can be toxic in the injectable form, and the mycolic acids absorption from the gastric tract may pose a problem, it is unlikely that this method of mycolic acid administration will be suitable.

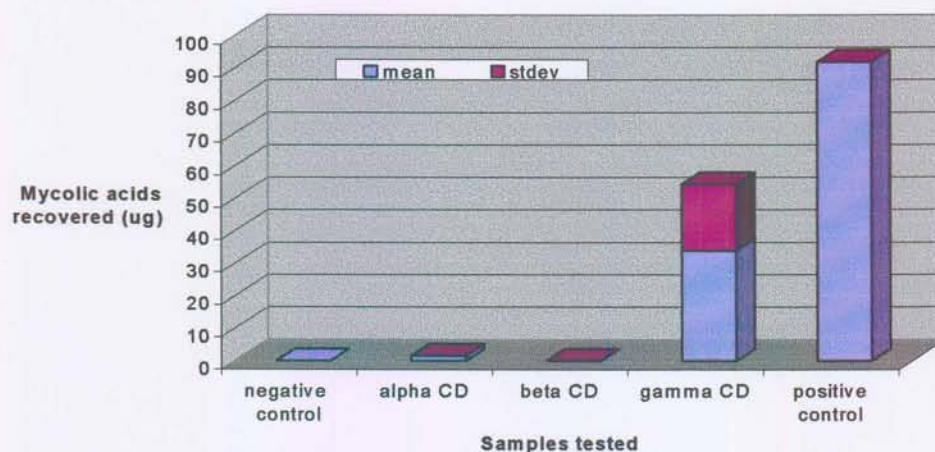


Figure 3.3 Recovery of mycolic acids from the three types of cyclodextrins used. Blue bars represent the average of triplicate values.

3.5.2 PEG as carrier of mycolic acids

When testing the solubility of MA in PEG as such or in aqueous PEG solutions, it was almost impossible to assess the degree of solubilisation by eye. The results of HPLC in Table 3.2 indicate that PEG 300 (undiluted or 50% v/v in water) does not solubilize mycolic acids. Upon using aqueous PEG 6000 solutions for the

solubilization of MA, a suspension was obtained which could not be dissolved by adding more of the PEG solution or by adding solid PEG. When 40% (m/v) PEG 6000 in water was used to solubilize MA, a suspension was obtained. Although 77% of the expected mycolic acids were recovered in the suspension (Table 3.3), the mycolic acids were not in solution. After leaving the mixture undisturbed for 3 days, the mycolic acids could be observed floating on the surface, while the clear solution drawn from the bottom of the tube did not contain any significant amount of dissolved mycolic acids.

Although PEG solutions heated to a temperature of 80°C appeared to solubilize MA, an unstable suspension resulted upon leaving the mixtures undisturbed to cool to room temperature. This cooling resulted in oil droplets floating on the surface of the solution. PEG was therefore considered to be an unsuitable carrier for mycolic acids.

Table 3.2 HPLC analysis of samples from attempted MA solubilisation in PEG 300.

SAMPLES	VOLUME WITHDRAWN	AMOUNT MA EXPECTED	AMOUNT MA RECOVERED	PERCENTAGE RECOVERY
Undiluted PEG 300/MA	200 µl	100 µg	4,1 µg	4,1%
50% PEG 300/MA Sample 1	50 µl	50 µg	0.00 µg	0%
50% PEG 300/MA Sample 2	50 µl	50 µg	1.01 µg	2.0%
MA (control)	200 µl	100 µg	92,0 µg	92,0%
PEG 300 alone	50 µl	0 µg	0.00 µg	-
MA recovered from the vial	200 µl	0 µg	0.00 µg	-

Table 3.3 HPLC analysis of samples from attempted MA solubilization with a 40% (m/v) aqueous PEG 6000 solution.

Samples	Volume taken	MA expected	MA recovered	Percentage recovery
PEG 600/MA *	200 μ l	98 μ g	0 μ g	0%
PEG 600/ MA#	200 μ l	98 μ g	75,5 μ g	77.0%

* This sample was not shaken and 200 μ l was taken from the bottom of the vial.

This sample was shaken and the concentration determined on 200 μ l of the suspension.

3.5.3 Sesame oil as carrier for mycolic acids

Mixing the oil with chloroform solutions of mycolic acids tested the solubility of mycolic acids in sesame oil. After the mycolic acids were brought into solution in a vial containing the mycolic acids/chloroform and sesame seed oil, the chloroform was evaporated off, leaving the mycolic acids to either remain in oil solution, or to stick to the sides of the tube. The first duplicate sample that was taken (Experiment A), was for determining the amount of MA dissolved in the sesame oil and the second set (Experiment B), to determine both the solubilized MA, and insoluble MA left in the vial.

From the results it appears as if mycolic acids are soluble in sesame oil. About 60% mycolic acids could be recovered from the amount of oil that was spiked. The high degree of variation and the low yields could have been due to erroneous pipetting of the viscous oil. It is clear from Table 3.4 however that the mycolic acids were dissolved in the oils and were not left sticking to the wall of the tube.

Table 3.4 Concentration of MA dissolved in Sesame oil, as detected by HPLC

Samples	Volume taken	MA recovered	MA expected	Percentage recovery
Experiment A				
1.MA /Sesame oil	50 µl	48,0 µg	112,5 µg	42,6%
2.MA /Sesame oil	50 µl	77,0 µg	112,5 µg	68,4%
Total amount	100 µl	125,0 µg	225 µg	58,1%
Experiment B				
1.Sesame oil/vial	50 µl	88,0 µg	112,5 µg	78,2%
2.Sesame oil/vial	50 µl	28,0 µg	112,5 µg	24,8%
Total amount	100 µl	125,0 µg	225 µg	53,9%
Sesame oil alone	100 µl	0 µg	0 µg	-
Positive control	100 µl	100 µl	92 µl	92%

3.5.4 Solutol as carrier for MA

3.5.4.1 Solubility of mycolic acids in aqueous Solutol

To determine the optimal concentration of Solutol to solubilize mycolic acids for administration into animals, mycolic acids were aliquoted in 1 mg quantities and 200 µl of Solutol/water solutions added in concentrations of 5, 10 and 25% (v/v). Dissolution was effected by heating to 80°C. After cooling, the amount of mycolic acids dissolved in each sample was measured with HPLC. The results are presented in Fig. 3.4.

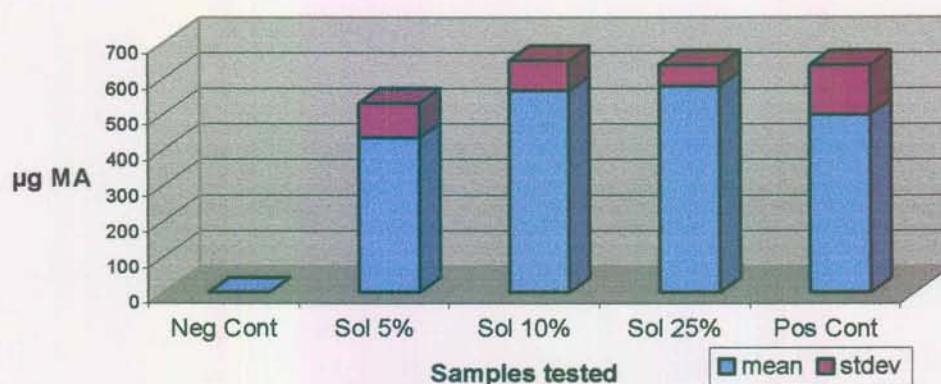


Figure 3.4 Concentration mycolic acids recovered from micelles using different concentrations of Solutol HS 15. The positive control consists of mycolic acids in chloroform. The negative control is Solutol alone. These solutions were obtained by using mycolic acids in chloroform and then removing the chloroform with heat and nitrogen gas. All determinations were done in triplicates.

Saturation of the solution of mycolic acids in Solutol/water was achieved between 5 and 10% (m/v) Solutol. As nothing was gained by increasing the concentration of Solutol to 25%, a 10% Solutol suspension was taken to be optimal.

Using 10% v/v Solutol HS 15 in saline (200 µl), adding it to 3 mg dried MA in an Eppendorf tube, heating it to 80°C, cooling it down to room temperature and releasing a sample for HPLC, it was shown that up to 9.7 mg/ml mycolic acids could be accommodated in 10% Solutol. This solution was not clear and consisted of a macro emulsion. No improvement could be gained by increasing the Solutol concentration to 25%. Based on this observation, Solutol at 10% was taken to be the optimal concentration for this carrier. It has a capacity to carry in excess of 9 mg/ml of mycolic acids.

3.5.4.2 *In vitro* testing of toxicity of Solutol HS 15

To determine the toxicity of Solutol, *in vitro* cultures of a C57BL/J6 derived cell line of macrophages were exposed to three concentrations of Solutol at three different times as indicated in Fig 3.5.

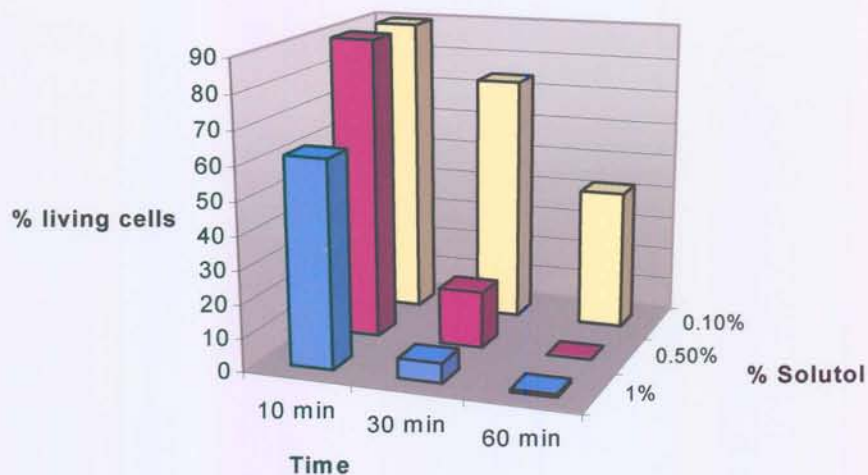


Figure 3.5 *In vitro* toxicity of Solutol tested on a mouse macrophage cell line using variable time and concentrations of exposure. Surviving cells were counted via flow cytometry using propidium iodide. Each bar represents the average of triplicate cell cultures.

The results from Fig 3.5 show that Solutol is toxic to the cells at concentrations as low as 0.1%. Exposure to Solutol concentrations higher than 0.1% practically killed all the cells after 30 to 60 minutes. Increasing the serum in the *in vitro* cell growth medium from 10 to 20%, decreased the toxicity somewhat (results not shown).

3.5.4.3 *In vivo* toxicity of Solutol HS 15 and mycolic acids

To determine whether Solutol HS 15 at 10% is toxic to animals, ten mice were injected intravenously with 100 μ l of Solutol solution with and without MA. No weight change or other macroscopic change were observed which could imply toxicity. In addition, no cell death could be detected among peritoneal macrophages two hours after Solutol HS 15 injection. In Figure 3.6 the number of living macrophages obtained from mouse peritoneal exudate after intraperitoneal injection of 10% Solutol (100 μ l) could be seen after 24 hours in culture.

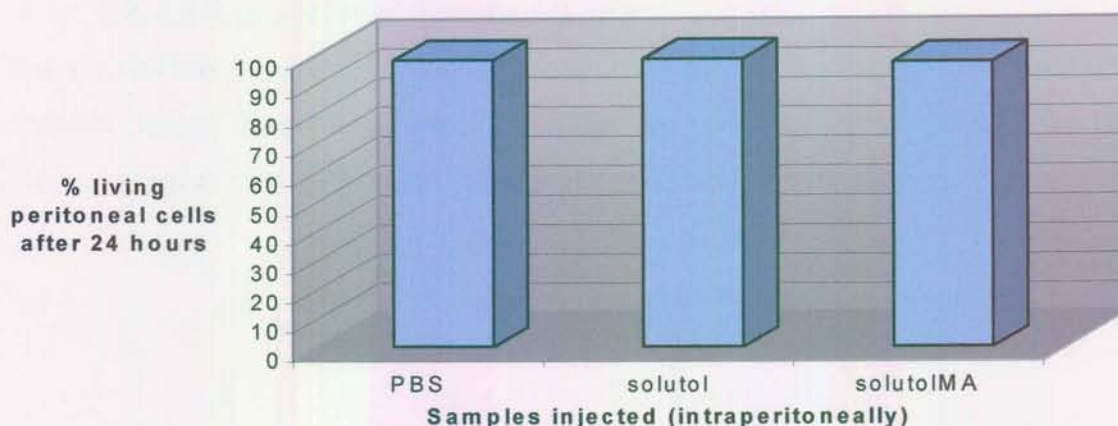


Figure 3.6 *In vivo* toxicity of solutol after two hours of intraperitoneal exposure. Percentage living macrophages were measured after 24 hours in culture. Solutol with or without mycolic acids (25 μg) was injected IP and after 2 hours peritoneal macrophages were removed and put into culture for 24 hours before survival was assessed by trypan blue exclusion.

Having established that Solutol appeared non-toxic to mice when injected intraperitoneally, mycolic acids (25 μg in 100 μl of 10% Solutol) was injected intraperitoneally and the macrophages obtained two hours later from the peritoneal exudate. After 24 hours in culture, the cells appeared healthy and unaffected by the exposure to Solutol/mycolic acids (Fig 3.6).

3.5.4.4 Haemolytic activity of Solutol HS 15

In order to assess the potential risk of intravenous injection of mycolic acids/Solutol, the haemolytic activity of several preparations were tested in a quantitative haemolysis assay. The effects of MA saponification and water/saline medium were tested on the pH and haemolytic properties of the various Solutol preparations. In Table 3.5 the haemolysis results are shown and the pH values listed for the samples treated in various ways.

Table 3.5 Haemolytic activity and pH of Solutol and Solutol/Mycolic acids preparations.

SAMPLE	%HAEMOLYSIS	PH
Solutol-saline 10% v/v MA 250 µg/ml	6 %	6.02
Solutol-saline 10% v/v	6 %	5.50
Saline	0 %	5.58
Water	100 %	6.21
Solutol water 10% v/v	50 %	5.52
Solutol water 10 % v/v MA 250 µg/ml	50%	5.62

As expected, saline alone and water alone gave 0 and 100% lysis, respectively. If Solutol (10% v/v water) was used with and without mycolic acids, there was still 50% haemolysis of the erythrocytes added. If saline and Solutol were combined, then 6% haemolysis was obtained, indicating that Solutol micelles can haemolyse small amounts of erythrocytes. By adding mycolic acids to the Solutol saline, no change in the haemolysis was seen. From these results it seems that micelles of mycolic acids do not increase haemolysis of erythrocytes. The pH of the 10% v/v Solutol/saline is affected only moderately by adding 250µg/ml mycolic acids. Moreover, it appears not to be necessary to buffer the solution, as a pH of 6 is acceptable for an injectable.

3.5.4.5 Results from light scattering

The size of the micelles is of significance, as it will be injected intravenously into animals. Using 1 μ m beads as size reference, micelles were analysed by light scattering on a flow cytometer. In Figure 3.7 the results are shown.

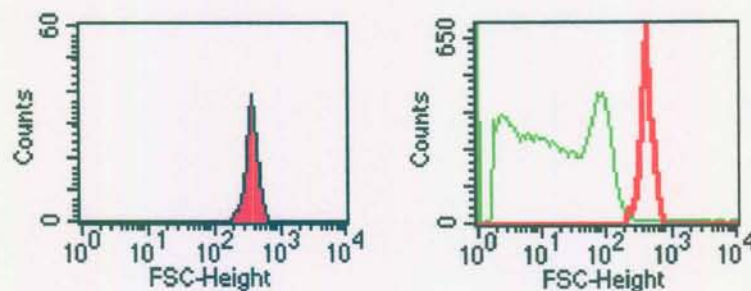


Figure 3.7 Histogram plots of micelles using 10% Solutol and mycolic acids at 250 μ g/ml. Beads that are 1 μ m in diameter were used as a size reference. Liposomes were all smaller than 1 μ m in size. The beads (size 1 μ m) are shown in red.

It was concluded that the final product (Solutol 10% v/v saline and 250 μ g mycolic acids sterilised by autoclaving) consists of micelles smaller than 1 μ m in diameter.

3.5.5 Microspheres as carriers for MA

3.5.5.1 Loading of the microspheres

The method of coating the microspheres by melting the mycolic acids onto the microspheres was successful, as seen on the high resolution scanning electron microscope. Recovery of mycolic acids in organic solvents (chloroform or tetrahydrofuran), from the polystyrene microspheres, was hampered due to the incompatibility of chloroform and polystyrene. The solubilized polystyrene mixture could not be separated on an HPLC column. The ideal microsphere system would comprise of biodegradable polymer microspheres, that are compatible with organic solvents. In Figure 3.8 the uncoated and coated microspheres are shown.

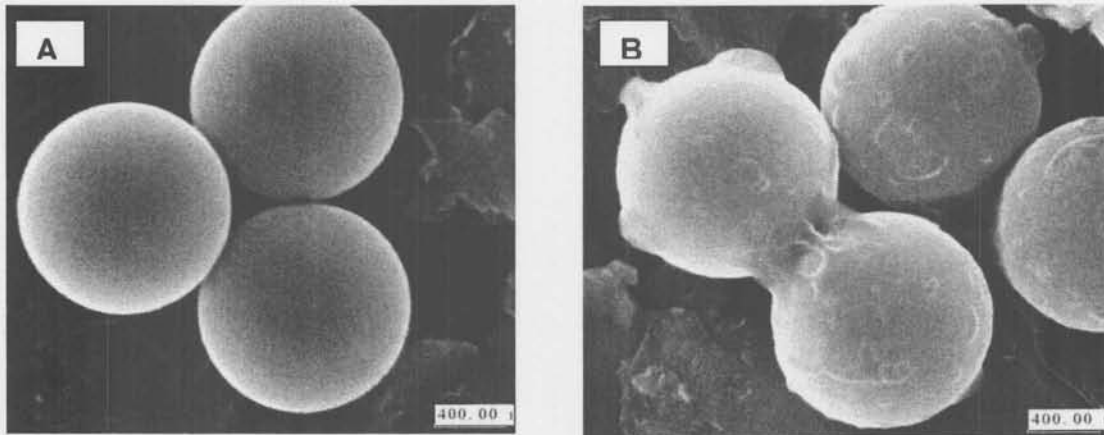


Figure 3.8 Polystyrene microspheres. (A) Microspheres without MA and (B) MA melted onto the microspheres .

3.5.5.2 Toxicity testing of beads

No macrophage cell death occurred with any concentration microspheres (beads) or microspheres-MA that was given to the macrophage cultures. Overloading the macrophages with more than 20 microspheres per cell didn't seem to have an effect on the growth of the macrophages.

Microspheres have previously been successfully applied as carrier for protein vaccines and here microspheres were used as a delivery system for mycolic acids to peritoneal macrophages *in vivo* and *in vitro*. In both cases the need for biodegradable microspheres is not important. Using non-degradable microspheres, the effects of the product can be isolated from the effects of the microspheres. Moreover, polystyrene microspheres have hydrophobic surfaces and mycolic acids stick well to these surfaces. If this delivery system has good immunomodulating effects, biodegradable microspheres can in principle be considered for delivery of mycolic acids into humans.

3.5.6 Liposomes as carriers for mycolic acids

3.5.6.1 Solubility of mycolic acids in liposomes.

Liposomes made with phosphatidyl choline and cholesterol accommodate the mycolic acids into solution. The amount of mycolic acids recovered from liposomes ranged between 70 and 110 % of the expected value.

3.5.6.2 Toxicity of liposomes and mycolic acids

The pH of the liposome solution was buffered at pH 7.4 by the PBS in preparing the liposomes for toxicity determination. No toxicity could be detected in adding liposomes or the liposomes loaded with mycolic acids to macrophage cultures (results not shown).

3.5.6.3 Haemolysis of erythrocytes using liposomes

The presence of lactate dehydrogenase released when erythrocytes lyse gives an indication of the ability of the liposomes to haemolyse cells.

Haemolysis of erythrocytes, tested by measuring lactate dehydrogenase activity amounted to none in all liposome-added samples. In Figure 3.9 the effect of liposomes on haemolysis is seen.

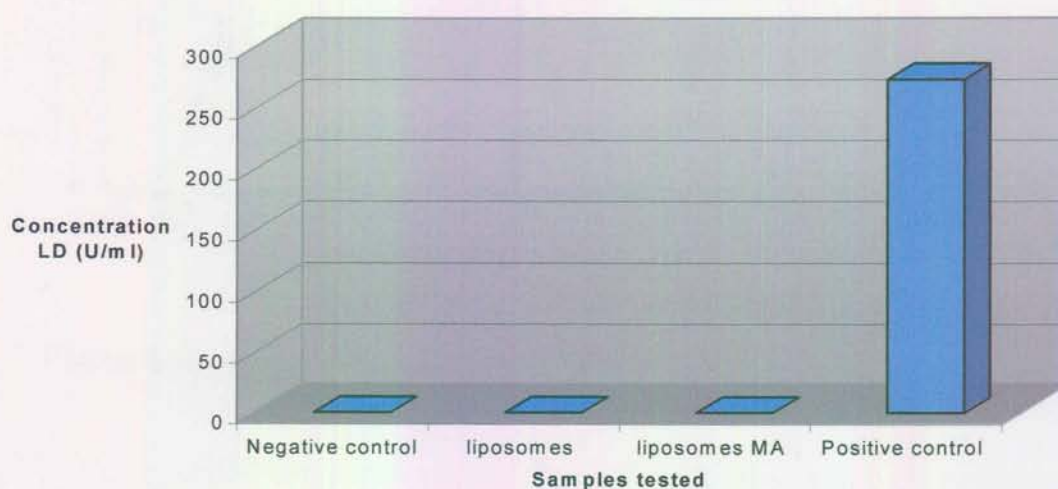


Figure 3.9 Haemolysis of erythrocytes using lactate dehydrogenase release as indicator. The negative control is 1% erythrocytes without liposomes. The positive control is 1% erythrocytes lysed by sonication. Samples were incubated at 37°C and 4°C, with no difference in haemolysis rate.

3.5.6.4 Sizing of liposomes

Using the flow cytometer, the size of the liposomes were determined in comparison to a 1 μm polystyrene bead (Figure 3.10).

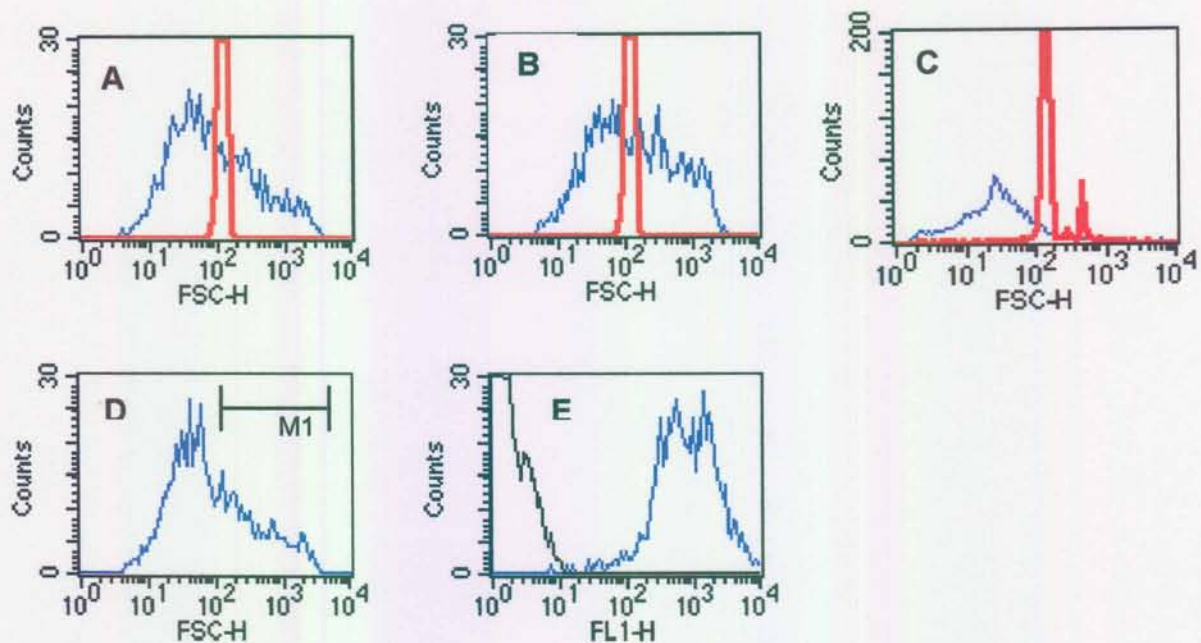


Figure 3.10. Sizing of liposomes with and without MA in the flow cytometer. Red represents beads (1 μm in size) and blue the liposomes with and without MA (A) liposomes alone (B) liposomes-MA (C) liposomes-MA sent through a 0.8 μm polycarbonate sieve (D) liposomes-MA in the sediment after centrifugation at 13 000g for 5 minutes (E) difference in fluorescence of liposomes after labelling with 5-bromo-methyl fluorescein.

Liposomes varied in size from around 3 μm to very small. The average size is around 0,8 μm . In liposomes alone, 37% of the particles are bigger than 1 μm and in MA-containing liposomes 47% are bigger than 1 μm . Liposomes were then sent through a polycarbonate sieve to obtain a more homogenous liposome suspension. This excluded the larger population, leaving liposomes of mainly 0,8 μm size. If liposomes were centrifuged at high speed, less of the bigger

liposomes were found in the precipitate. The size profile after nebulizing the liposomes-MA showed that the liposomes withstand the process well.

3.6 Discussion

Pharmaceutical formulations should display high availability, ease of administration and chemical and biological stability. The carrier must be immunologically inactive, or should augment the effects of immunity towards the antigen.

Mycolic acids form a solid wax at room temperature and need to be forced into a dispersion of acceptable size using heat and/or organic solvents. The extremely hydrophobic, large sized properties of mycolic acids demand their introduction in an aqueous environment on an inert carrier, while still enabling release and effector activity. Such preparations may consist of emulsions, micelles or liposomes. These are rarely used for parenteral administration, because lipid particles must usually be smaller than 1 μm to prevent emboli in blood vessels (Duma and Akers, 1984). The size range of the particles are of importance, as this may determine the method of uptake (pinocytosis or phagocytosis), which can influence the intracellular trafficking leading to presentation of the antigen. In liposomes, the size of the vesicles influences the Th1 or Th2 outcome of the immune response to the protein antigen content (Brewer *et al.*, 1998). The different carriers for MA were first evaluated for their ability to solubilize MA and then for the effect they had on the tissues and cells. Functionality of the solubilized MA was assessed separately.

Inclusion of a solute into a cyclodextrin (CD) cavity, substitutes its water content. This may provide the driving force to complexation of CD and the solute. Fatty acid solubility can increase up to 30 fold by complexing with cyclodextrins, depending on the aliphatic chain length. On average, five CH_2 groups are co-ordinated to one cyclodextrin molecule. If αCD is used, it seems that long chain fatty acids have to be stretched to be accommodated into the available cavity. Thus, depending on the

type of CD, formation of complexes brings about differences in the stretching of the aliphatic chain of various fatty acids. As the mycolic acids are not soluble in a water solution, the effect of using chloroform as solubilizer may influence the availability of the CD cavity. Chloroform enters the cyclodextrin cavity and must be released before the mycolic acid can enter. The dissolution of mycolic acids in chloroform and added to a preheated cyclodextrin solution, did not negatively affect the incorporation of mycolic acids. Although the α -, β - and γ -cyclodextrins appeared to interact with mycolic acids in water, the complexes precipitated out of solution. This implies that the complexes are not water soluble and that a big part of the mycolic acid molecule is still in contact with the water. Extraction of mycolic acids from these complexes appeared to be problematic, especially with the smaller α - and β -CD types. If stretching of the mycolic acids occurred, it might have influenced the release, biological activity and solubility of the complexes. Cyclodextrins were therefore not considered to be likely candidates as carriers for mycolic acids in pharmaceutical preparations destined for oral administration or intramuscular injection.

Various sparingly water-soluble substances have been accommodated in solution by using polyethylene glycol. The power to dissolve the active substance is ascribed to a complex formation with PEG. PEG has been used in formulas to make submicron lipid emulsions (Lindberg, 1999), liposomes (Ng *et al.*, 2000), and other forms of injectables (Lee *et al.*, 1999). PEG is a suitable gellant for simple aliphatic hydrocarbon liquids but lack compatibility with many oils. PEG is often derivatized to contain groups that enable solubilization of the different oils and fats that are usually not soluble in standard PEG (Miller *et al.*, 1998). PEG polymers as the low molecular weight liquids or as aqueous solutions of the higher molecular weight solids, were unable to solubilize any significant amount of mycolic acids. The results showed clearly that mycolic acids cannot be solubilized by PEG as such.

In contrast to PEG, sesame oil was able to solubilize mycolic acids, although the recovery by HPLC showed a high degree of variability. Sesame oil has been used as carrier in injectables using gold as the product. No anti-inflammatory effects were seen in supplementing the diet of volunteers with high levels of sesame oil (Ten Wolde *et al.*, 1997). Its complexity as a mixture of natural oils, however, detracts from its solubilization properties, especially with mycolic acids. As the effect of mycolic acid as an immunomodulating agent is to be determined, complex mixtures of this kind are not desirable.

Solutol HS 15 was developed as carrier system for non-watersoluble products. It contains PEG linked to a mixture of mainly 12- hydroxystearic acid as mono- and di-esters, and thirty percent pure poly-ethylene glycol (660 MW). Non-water soluble substances such as vitamin A, D, E and K are solubilized in these systems making use of its micellar nature. When using Solutol as a drug carrier, the reversal of the multi-drug resistance (MDR) of tumour cells to anti-cancer drugs was found to be a side-effect (Coon *et al.*, 1991). Solutol easily accommodated mycolic acids in an aqueous environment in the form of micelles.

The Solutol micelles appeared to be toxic to mouse macrophage cell cultures at very low concentrations. This toxic effect was hardly noticeable after *in vivo* injection and could be decreased *in vitro* by working in 20% foetal calf serum (FCS). Because the immune cells *in vivo* were not affected by the Solutol, this can still be a good option for a carrier of mycolic acids.

In contrast to Solutol, no toxicity could be detected in using polystyrene microspheres in macrophage cultures or by *in vivo* loading of peritoneal macrophages. High uptake of beads by both cultured and peritoneal derived macrophages was observed. Using the beads, it was found that their consumption by macrophages was erratic. Some macrophages were crammed with beads and others had none. Moreover, it was also observed that a high amount of mycolic acids melted onto the polystyrene beads, made them float to

the surface if suspended in aqueous medium. The highest MA-containing beads, were thus not taken up if added to *in vitro* macrophage cultures.

The process of phagocytosis of beads tends to release IL12 depending on the size of the particle (Fulton *et al.*, 1996). That might influence the ensuing immune response. MA on biodegradable beads might therefore provide a useful method of MA-induced immunomodulation. The inconsistent phagocytosis of beads and the fact that beads are not biodegradable, necessitated investigation into alternative methods of loading MA into macrophages.

Solubilization of MA by using liposomes was investigated. The liposome is a biodegradable system that can carry hydrophilic drugs in its enclosed aqueous environment and the hydrophobic drugs in the lipid bilayers. Liposomes were first characterised by Bangham *et al.* (1965). Although liposomes were initially designed as models for biological membranes, their potential for drug delivery was soon realised.

There are several reasons why liposomes are preferred as carriers for drugs: Liposomes obtained from natural phospholipids are biocompatible, immunologically inert and biodegradable. A large variety of molecules can be incorporated into the liquid or the lipid phase. Drugs are shielded from the external environment, thereby limiting the side effects. Drugs can be delivered by fusion and endo- or phagocytosis. Antibodies can confer active targeting of liposomes.

Liposomes injected intramuscularly or subcutaneously are released from the injection site and are taken up by the reticulo-endothelial system (RES) at a rate determined mostly by the size of the liposomes. The bigger the size, the slower the release from the injection site. Retention of liposomes in the draining lymph nodes, is both size- and charge dependent. Very small liposomes rapidly appear in the circulation after subcutaneous injection (Dams *et al.*, 2000, Oussoren *et al.*, 1999). Clearance of liposomes after intravenous injection also depends on size and

surface charge of the liposomes (Gabizon and Papahadjopoulos, 1992). Rigid, small size (100-200 nm) liposomes tend to be retained in the blood without degradation. These RES-avoiding, long-circulating liposomes passively accumulate in tumours due to the permeable vasculature in these tissues. Therefore these long-circulating liposomes are useful tools in tumour imaging and therapy. By adding PEG or glucuronide derivatives into the liposomes, the circulation time could be manipulated. (Dams *et al.*, 2000). PEG containing liposomes are sterically stabilised and are thus considered promising tools for delivery of therapeutic agents. Repeated injection of PEG liposomes dramatically enhanced the clearance of subsequently injected PEG liposomes and should be taken into consideration when repeated administration of PEG liposome-carried therapeutic drugs is required (Dams *et al.*, 2000).

The formulation of liposomes requires careful consideration of procedures and materials to ensure liposome stability. For example, phosphatidylcholine-cholesterol liposomes carrying the drug carboplatin, showed no degradation or size change during six months of refrigerated storage, but hydrolysis of the phosphatidylcholine was increased when an antioxidant, ascorbyl palmitate, was added (Pietzyk and Henschke, 2000). Free radical peroxidative damage against phospholipid membranes *in vivo* can be reduced by adding reducing agents such as alpha carotene to the liposomes (Farombi and Britton, 1999). Surfactants should preferably be avoided, but up to 1% can be tolerated.

Immunogenicity of a protein antigen, encapsulated inside liposomes, could be manipulated by alternating the neutral lipids/cholesterol/negatively charged phospholipids ratio. Other factors included the PC acyl chain length. Increasing fatty acid unsaturation decreased the immunogenicity of the liposome encapsulated antigen (Bakouche and Gerlier, 1986). In contrast, Yasuda *et al.* (1979) showed that immunogenicity in mice of a liposomal membrane-embedded hapten, was not influenced by cholesterol concentration or degree of saturation of the fatty acids. Membrane bound antigens on liposomes with phospholipids that have a high

transition temperature, such as synthetic distearoyl PC, were more immunogenic than low transition temperature lipids (*i.e.* PC) (Dancey *et al.*, 1978).

The cell wall of *Mycobacterium* spp is composed of an asymmetric lipid bilayer according to model proposed by Minnikin and Goodfellow (1980). The inner leaflet contains MA linked to arabinogalactan, and the outer leaflet contains extractable lipids such as trehalose-containing glycolipids, phenolic glycolipids or glycopeptidolipids. X-ray diffraction studies showed that MA hydrocarbon chains arrange in a tightly packed parallel, quasi-crystalline array, mainly organised in a direction perpendicular to the cell surface. Most of the extractable lipids underwent major thermal transitions between 30°C and 60°C. In contrast to this, MA exhibited a transition phase at a temperature of between 60°C and 70°C. The mycobacterial cell wall therefore appears to consist of a bilayer with an outer leaflet of moderate fluidity and an inner mycolic acid layer of extremely low fluidity (Liu *et al.*, 1996). Here the MA-containing liposomes have a mixture of low and high transitional temperature lipids, as well as cholesterol. Liposomes-MA thus constitute a novel form of liposome, probably of low fluidity and low permeability. Immunogenicity is at this stage not predictable as the antigen is also part of the lipid membrane and the immunity mechanism towards MA is unknown.

Liposomes as drug vehicles can be used to deliver drugs into various compartments of the body. Liposomal carriers have been successful in enhancing the clinical efficacy of a number of drugs from inhalation into the lungs, to topical application on the skin (Farhood *et al.*, 1995). Pulmonary delivery of glucocorticosteroids and cyclosporine (in liposomes) has been done by liquid aerosol generated nebulizers. The size of the droplets was in the range of 1µm, thereby allowing deep penetration into the lung.

Tuberculosis is mainly a pulmonary disease and the transmission of the bacilli is almost exclusively in and from the lungs. Drug delivery directly into the biophase (the lungs), would perhaps improve efficacy of MA as it is not necessary for the

liposomes to circulate the blood system to reach the target area. Dilution or elimination of the drug will then also not be such a problem.

From the results seen with incorporation of mycolic acids into liposomes, the preparation of a liposome carrier system seems feasible. The low toxicity seen in this system towards cells was encouraging. The liposomes-MA could also be nebulized in an aerosol.