Chapter 4

Discussion

This study set out to resolve the structure-activity relationship of MAs from *M. tuberculosis* in terms of both their antigenicity, i.e. their capacity to be recognised by TB patient serum antibodies, and the ability of MAs to attract cholesterol, because it may play a critical role in the entry of the bacillus into the host macrophage and its mechanism to survive in that normally hostile environment.

The first indication that MAs are antigenic was obtained indirectly by the production of antibodies against cord factor in mice and rabbits by a Japanese research group. They injected rabbits with methylated bovine serum albumin complex of cord factor to obtain antiserum. The anti-cord factor IgM antibodies were detected by a precipitin reaction. It was suggested that the antigenic epitope may be the trehalose moiety (82). In 2005, Fujita *et al.* (64) published evidence that suggested that the MA rather than the sugar component of trehalose di- and monomycolates, was the antigenic determinant that was recognized by anti-cord factor antibodies from tuberculosis patients. This same notion was more directly demonstrated by Pan *et al.* (112) who coated ELISA plate wells with the pure, separated MA methyl esters from hexane solutions and showed that the methoxy-mycolates were the predominant antigens, while little TB patient antibody binding could be demonstrated with the α- or keto-mycolates. Fujiwara *et al.* (65) showed that anti-cord factor IgG in rabbits recognized MA subclasses of *M. tuberculosis* and *M. avium.* All this work on the antigenicity of cord factor stemmed from Japanese laboratories that focused on bringing a commercial kit for serodiagnosis of TB on the market. This kit is currently on clinical trial.

Yano *et al.* (157) found that antibodies in TB patients recognized not only MA esterified to a mono- or disaccharide (cord factor), but also free MAs with a carbon number of 14 or more as antigens. The MAs were extracted from *Mycobacterium, Nocardia, Rhodococcus* and *Corynebacterium* species and were esterified to trehalose. The trehalose MA esters comprised of trehalose bound to 1 to 4 MAs. These antigens were then brought into contact with an antibody-containing sample and analyzed by ELISA. They also showed that *M. tuberculosis*

derived antibodies that react with cord factor were present in the serum of TB^+ patients, but not in healthy controls.

The preferred antigenicity of cord factor in TB patients was confirmed in this study by showing that MA bound to trehalose was recognised by TB^+ patient serum, but not by the TB^- patient serum when analysed by ELISA. In chapter 2 the issue around the antibodies responsible for recognition of cord factor and MA was discussed. It is in fact not that simple to determine. Although cord factor was not recognised by TB^- patient serum, free MA was recognised. It seems that there are different types of antibodies, one type that is only found in TB^+ patients and that can recognise MA bound to trehalose as cord factor, and another type that can only recognise free MA, but not the MA bound to trehalose. TB^+ patients have both types of antibodies in their serum. The epitope on cord factor that is important for recognition by TB^+ patient serum is not the trehalose, but a combination of trehalose with MA. Our results therefore confirms that of Fujiwara *et al.* (65). They found that the different types of MA from different *Mycobacterium* species have unique antibody specificity in TB patients. Therefore, trehalose is not a public cross-reactive epitope, but forms a unique complex epitope that depends on the MA it associates with. The results show a possible epitope that exists on MA and which disappears when bound to trehalose. The MA epitope is a natural epitope that is recognised by antibodies from both TB^+ patients and healthy people.

To determine the fine specifity of interaction of MA with antibodies, the three subclasses of MA from *M. tb* were separated and the antigenicity of two of the subclasses determined. The antigenicity of the MAs initially appeared not to reside in only one of the subclasses, but this finding was not entirely convincing, because of possible contamination of the α-MA with methoxy-MA and the lack of sufficient amounts of keto-MA to be tested. This finding therefore does not yet support my hypothesis i.e. that the recognition of MAs by TB patient serum antibodies resides in only one of the subclasses. However, the statement could still hold true if contamination of one preparation of subclass with other subclasses can be totally ruled out. This may be easier to achieve by chemical synthesis of each of the various subclasses, rather than their isolation from a natural extract.

Both the mycolic acid motif and the mero chain components of natural MA are important epitopes for antigenicity (Chapter 2 and 3). It may be possible that this epitope on MA is

hidden when MA is bound to arabinogalactan in the cell wall, or that this epitope can only be recognised as free MA that might be found in lipoproteins in TB patients. It might well be that this is the immunogen that induces the formation of anti-MA antibodies in TB patients.

In recent years free MAs were used as antigens for the serodiagnosis of tuberculosis. They had good potential because HIV-TB co-infected patients maintained high antibody levels to MAs (127). It has been shown with ELISA that free MAs were not adequate for serodiagnosis of tuberculosis; it was only 57% accurate. An association between MAs and cholesterol was hypothesized (134) that might explain the low accuracy.

AmB accumulates to a cholesterol coated surface and also to a natural MA coated surface in a waveguide resonant mirror biosensor, but not to a synthetic protected α-MAs coated surface (24). Similarly, I demonstrated that cholesterol accumulated on a natural MA surface, but not on the synthetic protected α-MAs surface or a surface of methylated natural MA. The association of both AmB and cholesterol to MA depended on the formation of hydrogen bonds that also affect the structural rigidity of the molecules. The attraction between MA and cholesterol was specific and depended on a particular conformation that the free MAs assume in the phospholipid bilayer of liposomes. This will probably also apply to biological membranes.

Cholesterol may be attracted to MAs through hydrophobic Van der Waals interactions or by a more specific interaction between certain similar features present in both molecules. Free MAs assume a ‘W’ conformation with all the four alkyl chains folded to each other (145, 146). The condensed conformation of MAs was proposed by several groups and is based on Langmuir studies. The ‘W’ conformation could resemble the shape of cholesterol. In its extended form, the structure of MA can hardly relate to that of cholesterol or be able to attract cholesterol to any degree of specific association. According to Alving *et al.* (9, 10) naturally occuring autoantibodies against cholesterol reacting with the 3β-hydroxy group of cholesterol are present in the serum of almost every healthy individual. This was confirmed by Horváth *et al.* (79) and Bíró *et al.* (26). If MAs are able to attract cholesterol, it might be possible that cholesterol antibodies from TB^- patients recognise these MAs.

Retzinger *et al*. (118) and Villeneuve *et al.* (145, 146) proposed that the long MA mero chain is kinked and folded in the lipid bilayer to form three tightly packed bends of hydrocarbon. Similar folded conformations of oxygenated MA was proposed to explain experimental observations obtained in different studies (70, 75, 76). The alignment of the acyl chains due to folding may allow hydrophobic interactions by intra-molecular stacking that is enabled by the hairpin bend induced by the proximal *cis*-cyclopropane group. Methoxy-MA may relate better to cholesterol than keto-MA by retaining a tetrahedral structure around the oxygenated carbon, homologous to the structural architecture around the 3-hydroxy group of cholesterol. This all supports the folded conformation proposed by Siko (134).

The stereochemically controlled synthesis of MAs was first achieved by Al Dulayymi and co-workers (3-8, 38, 90), but to clarify the absolute stereochemistry of the functional groups in the mero chain as it occurs in nature, these compounds must be tested for biological activity and antigenicity.

Antibodies that are present in TB^+ patient serum recognise a number of synthetic MAs. All of these were also recognised by serum from TB^- patients. The α-MA, the negative control antigen, and the different keto- and hydroxy-MAs were not recognised by TB^+ patient antibodies. This demonstrates that the antigenicity of MA is not only dependent on the mycolic motif, but that the mero chain is critical in the manifestation of biological activity. In terms of the oxygenated groups in the mero chain, the methoxy seems to be more active.

One diastereomer of synthetic methoxy-MA was more avidly recognised by TB^+ serum than the other, and weaker by TB- serum compared to the rest of the MAs. It also is the one that most closely approximates the signal strength of antibody binding to natural MA by TB^+ patient sera. It can therefore be concluded that, of these synthetic compounds, *SS*-*SR*-methoxy-MA would be the most appropriate antigen to use in a serodiagnostic assay for tuberculosis. It may well represent one of the antigenically active components that occurs in natural MA and that elicits specific antibody production in patients with TB.

There are other questions. Can the mycolic acid motif retain its activity if it is esterified with some other groups, for instance arabinogalactan or 5-BMF. In these cases, a hydroxyl group would still be in the vicinity of the carboxyl group to form hydrogen bonds with the methoxy-

group in the folded mero chain? What is the role of the C_{24} chain? Is the C_{24} chain important for biological activity? It would be worthwhile to investigate in order to manipulate MAs for its exploitation as biologically active compound, eg. to find a minimum antigenic structure.

Proving that these synthetic MAs are recognized by antibodies in TB^+ patient serum when analyzed by ELISA, is only the beginning. It would be worthwhile to investigate other biological properties of the different subtypes of these synthetic MAs. MAs elicit an immune response, stimulate double negative T-cells (67) and stimulates mainly the macrophages following intraperitoneal administration of MAs into mice. This is achieved by converting the MAs into cholesterol-rich foam cells (89).

This thesis reports a stereocontrolled chemical synthesis of a biologically active MA. The chemical synthesis of MA and its derivatives can be used to control TB and other lung diseases in a variety of ways. In diagnostics it now can allow the determination of the minimum antigenic structure for recognition by antibodies in TB^+ patient serum as well as for other mycobacterial diseases like leprosy, Crohn's disease, Buruli ulcer and *M. avium* complex diseases. In biosensor technology the synthetic MA can be directly coupled to the gold disc surface of the cuvettes, eliminating additional washing and coating steps. In therapy, a synthetic medicine based on MA structure can be designed against asthma and arthritis, as well as in TB, eg. as an inhibitor of MA biosynthesis or the inhibition of the TACO-proteins. Synthetic MA can be included into nanoparticles for immunomodulation to exploit its anti-inflammatory effect.