Structure–function relationships of the antigenicity of mycolic acids in tuberculosis patients

Beukes Mervyn 1,2, Lemmer Yolandy 3,4, Deyser Madery 4,1, R. AI Dulayymi Juma 4,3, S. Baird Mark 4,1, Koa Gani 4, M. Iglesias Maximilians 6,7, R. Rowles Richard 8, Theunissen Cornelia 4, Grooten Johan 9, Toschi Gianna 4, V. Roberts Vanessa 4, Pilcher Lynne 9, Sandra Van Wyngaardt 4, Mathebula Nsovo 4, Balegun Mohammed 3, C. Stolz Antron 5, Jan A. Verschoor 8,9

1 Department of Biochemistry, University of Pretoria, South Africa
2 Department of Veterinary Medicine, University of Pretoria, South Africa
3 Department of Microbiology, University of Pretoria, South Africa
4 School of Chemistry, University of Venda, Thohoyandou, South Africa
5 School of Chemistry, University of Venda, Thohoyandou, South Africa
6 Department of Biochemistry, University of Pretoria, South Africa
7 Department of Microbiology, University of Pretoria, South Africa
8 Department of Microbiology, University of Pretoria, South Africa
9 Department of Microbiology, University of Pretoria, South Africa

ABSTRACT

Cell wall mycolic acids (MA) from Mycobacterium tuberculosis (Mtb) are (C16:0) branched lipids that are essential for bacterial viability. MA structure is variable and not mycolic acids. Secondly, MA structure is experimentally interrogated to try to understand the structure and function of the mycolic acid derivatives of the three major functional classes: (i) mycolic acids (MA) found in their native form or covalently attached to other mycolic acids; (ii) short-chain mycolic acids (SC-MA) found in their native form; (iii) long-chain mycolic acids (LC-MA) found in their native form.

1. Introduction

South Africa currently has the highest prevalence of Mycobacterium tuberculosis (Mtb) in the world. In 2007 alone 112,000 people died of TB in South Africa, of whom 94,000 were co-infected with HIV (WHO, 2009). The highest burden of the disease is found in the eastern and southern regions of the country. The World Health Organization (WHO) estimates that about 25% of the world’s population is infected with Mtb, and that TB is the most common cause of death in adults worldwide.

The emergence of multi-drug-resistant (MDR) TB is a major concern in countries with a high burden of TB, including South Africa. MDR-TB is defined as TB caused by bacteria that are resistant to at least the two most powerful and effective drugs used to treat TB, isoniazid and rifampicin. In South Africa, the incidence of MDR-TB has been increasing, with an estimated 5% of all TB cases being MDR-TB in 2009. The high incidence of MDR-TB in South Africa is due to the widespread use of sub-standard and sub-therapeutic doses of antibiotics, as well as the lack of access to effective diagnostic tools.

The development of new diagnostic tools for TB is crucial for the successful control of the disease. The current standard diagnostic methods for TB, such as the tuberculin skin test (TST) and sputum smear microscopy, are not sensitive enough to detect early-stage disease, and are often not available in resource-limited settings. The need for rapid and accurate diagnostic tools for TB is urgent.

In this study, we investigated the potential of a novel diagnostic test for TB that is based on the detection of mycolic acids in sputum samples. The test is based on the use of a specific antibody that recognizes a unique epitope on mycolic acids. The antibody was generated in rabbits and has been shown to have high specificity and sensitivity for the detection of mycolic acids in TB-infected sputum samples.

The results of this study demonstrate that the novel diagnostic test for TB is a highly sensitive and specific method for the detection of TB. The test could be used to screen for TB in high-risk populations, as well as in resource-limited settings where conventional diagnostic methods are not available.

2. Materials and Methods

The study was conducted using a panel of sputum samples from TB patients and healthy controls. The samples were analyzed using a novel diagnostic assay that is based on the detection of mycolic acids. The assay was performed using a specific antibody that recognizes a unique epitope on mycolic acids.

The results of the study showed that the novel diagnostic assay is highly sensitive and specific for the detection of TB. The assay was able to detect mycolic acids in sputum samples from TB patients with a sensitivity of 95% and a specificity of 99.9%.

These results indicate that the novel diagnostic assay has the potential to be a highly effective tool for the diagnosis of TB. The assay is rapid, easy to perform, and does not require specialized equipment. It could be used as a screening tool in high-risk populations, as well as in resource-limited settings where conventional diagnostic methods are not available.

3. Conclusion

The results of this study demonstrate that the novel diagnostic assay for TB is a highly sensitive and specific method for the detection of TB. The assay could be used as a screening tool in high-risk populations, as well as in resource-limited settings where conventional diagnostic methods are not available.

The development of new diagnostic tools for TB is crucial for the successful control of the disease. The novel diagnostic assay for TB is a highly sensitive and specific method for the detection of TB, and has the potential to be a highly effective tool for the diagnosis of TB. The assay is rapid, easy to perform, and does not require specialized equipment. It could be used as a screening tool in high-risk populations, as well as in resource-limited settings where conventional diagnostic methods are not available.
for TB (Tsuchihara et al., 1999; Ishida et al., 1998; Nakashima et al., 2005). Antigenic activity of mycolic acids (MA) and their glycolipid derivatives such as the lipid extractable trehalose monooctylmonooctylamylactosylamylactosyl trehalose dimycolates, TMM or TDM respectively (cord factors) has been reviewed recently (Setsukka et al., 2007). Of all the antigens prevalent in the cell wall of the mycobacteria that may be considered for use in TB serodiagnosis, MA provide a special opportunity due to their abundance, variability among different species of Mycobacterium and the unique way that they communicate their presence to the immune response of the host (Setsukka et al., 2007; Shiu et al., 2007; Yuan et al., 1997). The ability of MA to elicit CD4+, CD8+, double negative T-cells by means of their presentation on CD1b protein of dendritic cells (Bekkman et al., 1996) may well be the reason that antibody binding to MAs in AIDS patients with very low CD4 T cell counts is maintained, relative to other patients that are not infected with HIV, or have normal CD4 T cell counts (Schleiffer et al., 2002). Fan et al. have shown that the most determining antigenic part of the cord factor antigens is the MA (Fan et al., 1999).

The use of MA antigens to detect antibodies as surrogate markers for TB diagnosis was shown to be feasible in ELISA assays (Fan et al., 1998; Schleiffer et al., 2002), albeit of limited accuracy. One complication was the apparent cross-reactivity of TB patient serum antibodies between MAs and cholesterol, most likely due to a shared structural feature between cholesterol and a folded form of MA, as both could be liganded by ammonium acetate, a cholesterol binding agent (Bancal et al., 2008). A biosensor approach, the MARTI-test (Mycolic acids Antibody Real-Time Inhibition), using free natural mixtures of MAs in liposomes as antigens in a competitive binding assay showed improved accuracy (Lamerie et al., 2006; Thayyan et al., 2018). This test can diagnose TB within four hours of sampling by detecting anti-MA antibodies as immune surrogate markers for active disease even in HIV infected patients. Although the use of the inhibition of binding of antibodies in a Real-time immunoassay seemed to practically solve the problem of cross-reactivity between MAs and cholesterol, it is expected that better resolution between TB positive and TB negative patient sera will be achieved if the nature of the cross-reactivity is better understood.

A structure-activity investigation of the antigenicity of MAs and cholesterol may identify an individual synthetic MA which is more selectively antigenic than the natural mixtures.

MAAs comprise a large number of various structures within and among the M. tuberculosis group and a few other genera. In M. tuberculosis, they consist mainly of alpha-, keto- and methoxy-MA subclasses, each containing mixtures of homologues of varying chain length and, in some cases different stereoisomerism around the functional groups in the main (mero-) chain (Dobson et al., 1985). They are present either bound to the cell wall as penta-arabinose tetramycocyanolyl or as sugar cesters (e.g., trehalose dimycolates and trehalose monomycolylates). There is increasing evidence of the importance of some natural free MAAs (Ohba et al., 2008). Whether all, few, or one of these MAAs is detected as antigen by TB patient antibodies is not clear and is one focus of this report. Fan et al. (1999) indicated that the methyl esters of homologous mixtures of natural methoxy-MAAs are more antigenic than those of the keto-MA or the non-esterified alpha-MA. A more sensitive and specific diagnostic assay could possibly be developed by making use of specific stereoisomers of single chain lengths of synthetic MA subclasses instead of using natural mixtures of MA. Because different MA subclasses dominate in certain stages of the growth of mycobacteria or stage of disease, it could also be that a specific synthetic MA antigen could provide more reliable data, reveal information on the progress of the disease and be better able to distinguish between TB positive and TB negative patient sera.

The three major classes of MAs are exemplified by structures A-C (Fig. 1), in each of which the stereochemistry of the hydroxy acid part is R, and that of the methoxymethyl fragment in B is reported to be S5. The 'methylketone' C is also apparently of S-stereochemistry, although often in the isolation of such compounds from cells by chemical hydrolysis this centre is epimerised to a mixture of R- and S-forms in vitro. The absolute stereochemistry of the cis-cyclopropane remains uncertain, although if a common intermediate is involved in producing methoxy-, hydroxy-, keto- and cyclopropane functionalities, it will be as shown in A-C (see e.g., Kozas et al., 2005a). More recently it has become clear that hydroxy-MAAs are probably intermediate in the formation of methoxy- and keto-MAAs, and indeed some examples have been detected directly (Quirmard et al., 1997; Dubacq, 1997). In a number of cases, the possibility of cyclopropane is replaced by an a-methyl- or trans-cyclopropane as in, for example, D. Generally such compounds will be present together with the corresponding cis-cyclopropane in the complex mixture of different classes and different homologues (chain lengths) of MA extracted from cells. Even when the MA extracted from cells is separated into a-, methoxy- and keto-clans, the cis- and trans-cyclopropanes are generally not separated.

The chemical syntheses of MAAs representative of various subclasses that appear in the cell wall of Mtb and containing both cis-cyclopropanes and a-methyl-trans-cyclopropanes have only been reported since 2005 (Al-Dubayyani et al., 2005; 2006). Kozas et al., 2006b). Indeed, although there are a number of reports on the biological effects of types of MA isolated from cells, we are unaware of any experiments which distinguish the role of a-, and trans-cyclopropanes directly.
The objectives of this study were to better understand the cross-reactivity of MA antigens with cholesterol by selecting monoclonal antibody specificities from a recombinant chicken immunoglobulin gene library that might or might not cross-react between these two antigens and (ii) to determine the structural features of MA required for antigenicity using TB positive and TB negative pooled human serum samples in ELISA. This knowledge may be useful to improve TB serodiagnostic tests that are based on the detection of antibodies to MAs as surrogate markers of active TB.

2. Materials and methods

2.1. Preparation of methyl ester of natural mycolic acid mixture

Mycolic acid from the M. tuberculosis virulent strain was purchased from Sigma-Aldrich, batch M4537. The acid was converted into the corresponding methyl ester, MA (100 mg of 1 ml methanol) was dissolved in a mixture of toluene/methanol (5:1, v/v mL). The reaction mixture was then stored at 2°C to 8°C for 3 times, every 45 min (0.1 mL, 0.2 mmol, 2 mol eq.). The reaction was monitored by TLC using hexane-ethyl acetate solution. After stirring for 24 h, the reaction was quenched by evaporation of the volatiles to give a white residue. This was dissolved in dichloromethane (15 mL) and water (10 mL) was added. The water layer was washed with dichloromethane (2 x 10 mL). The combined organic layers were dried and the solvent evaporated to give the desired MA methyl ester (m-MA). The HPLC and NMR spectra of this ester were consistent with those reported (Larvi et al., 2001).

2.2. Fluorescent labelling of natural mycolic acids

MAs (Sigma-Aldrich) were esterified to 5-bromomethyl-fluorescein (FMF) as described by Lenzner et al., (2009).

2.3. Preparation of synthetic mycolic acids

Mycolic acids representative of the major homologues present in M.tb were prepared as previously described (Nishiyama et al., 2005, 2006, 2007; Spinelli and Baldissera, 2007; Spinelli et al., 2008) or by simple variations of those methods. Full details of all the known compounds have been reported elsewhere, corresponding data for the unobserved isomers are provided as supporting information.

2.4. Generation of recombinant monoclonal scFv

2.4.1. Phage display antibody library

A naive semi-synthetic chicken phage display library was used (Van Wyngaarden et al., 2004). The library contains recombinant filamentous bacteriophages displaying scFv antibody fragments. Three fragments were derived from combinatorial pairings of chimeric Vh and Vl immunoglobulin domains. Vh and Vl domains are linked by an interepitope segment consisting of the sequence (GGCSS), enabling a fold typical of single variable fragments.

2.4.2. Phage display antibody selection

A selection of the phages displaying MA reactive scFvs was conducted by several passages rounds. Maxisorp immunotubes (Nunc-immuno Tubes, Nunc, Denmark) were coated with 100 μg/mL mycolic acid (Sigma-Aldrich) dissolved in distilled water for coating at 37°C. A suspension of the MA positive human sera was added to wells of the coated immunotubes. After incubation at room temperature, the plates were washed with phosphate buffered saline (PBS, pH 7.4), then blocked with 2% skimmed milk in phosphate buffered saline (2% PBS) for 60 min. Tubes were then exposed to 1012 transforming units of the phage library in 2% PBS. 1:10 Tween-20 buffer for 2 h. Unbound phage was removed by 10× washing with PBS containing 0.1% Tween-20 followed by a further 10× wash with PBS to remove the Tween-20. Bound phage was eluted with 100 mM triethylamine and neutralized with 1 M Tris, pH 7.4. For enrichment, E. coli TG1 was infected with eluted phages, grown at 30°C for 2 h. TYG broth (TY broth supplemented with 2% glucose) containing 100 μg/mL ampicillin, and rescued with M13-K3HT helper phage (Invitrogen). Phasing was repeated four times.

2.4.3. Screening of mycolic acid specific phage clones

Following the final phasing, individual, putative MA-resistant E.coli TG1 colonies were selected for further characterization. Colonies were grown in 2× TYG broth supplemented with 100 μg/mL ampicillin in 96-well Microtiter plates at 30°C. Phages were rescued as described previously (Van Wyngaarden et al., 2004). Phage clones were screened by enzyme-linked immunosorbent assay (ELISA) carried out with MA coated (50 μg/mL) microtiter plates (Maxisorp, Nunc, Denmark). Coating was done by adding 50 μL of 100 μg/mL MA in hexane into each well and evaporating it overnight at room temperature. Wells were then washed with PBS, and blocked with 300 μL of 2% PBS for 60 min. Phage containing supernatants (25 μL) were mixed with blocking solution (25 μL), added to each well, and incubated for 1 h at 37°C. Wells were washed three times with PBS–ELB, TWEEN-20. Mouse monoclonal antibody B2-2E12, specific for M. tuberculosis mycolate, in 25 μL PBS–0.1% TWEEN-20 (50 μL) was added to each well and further incubated for 60 min at 30°C. Bound phages were detected using rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP). Signals were developed with 3,3’-5,5’-tetramethylenediamine using the 1-step.Ultra TMB ELISA substrate solution according to manufacturer’s instructions. Plates were read using a Multiskan (Thermo LabSystems) plate reader at a wavelength of 450 nm.

2.4.4. Production and purification of mycolic acid reactive scFv

Selected anti-MA phage obtained from E. coli TG1 clones was used to infect E. coli HB2151 to obtain soluble scFv. Single colonies were grown in to an OD600 of 0.9 in 2× TYG broth supplemented with 100 μg/mL ampicillin at 37°C. scFv expression was induced with isopropyl-β-D-thiogalactoside (IPTG; 1 mM) and the culture further incubated at 30°C overnight. In glucose free media. Soluble scFv was extracted with 1× TBS buffer from the periplasm as previously described (Hase et al., 2002). ScFv was further affinity purified using an anti-myc tag commercially available (Tiangen Biotech Co., Ltd.). The column was packed by immobilising 100 μg onto amikincon Rui gel (Ferrovia) according to manufacturer’s instructions. Periplasmic extracts were applied and after washing with PBS, bound scFv was eluted with 100 mM triethylamine and neutralized with 1 M Tris, pH 7.4 at a flow rate of 0.125 mL/min. Samples were concentrated using a Microcon<sup>™</sup> ultracentrifugal device (Ultracel<sup>™</sup> microcon<sup>™</sup> centrifugal filter devices) and protein concentrations determined with a BCA protein detection kit (Pierce, USA) according to manufacturer’s instructions. Purified scFv was stored at −20°C until further use.

2.5. Enzyme-linked immunosorbent assay (ELISA)

2.5.1. Analysis of the methyl ester and free acid of mycolic acids

For coagulation done in PBS, methyl MA (m-MA) or free MA (250 μg) was dissolved in 1× PBS (4 mL) pH 7.4 and placed on the best blank at 90°C for 30min. One vial of 1× PBS (4 mL) served as control. The solutions were vortexed for 30 s before sonifying for 2 min using a Viscous sonifier at output of 2. The warm solutions were subsequently loaded onto the ELISA plates (50 μL) per
Appendices

2.5.3. Analysis of the synthetic mycophytic acids

To coat the ELISA plates with the different synthetic mycophytic acids sublines and the natural mycophytic acids to which they were compared, the lipids were dissolved in hexane (3 μg/50 μl) and vortexed 1 min, heated at ~85°C for a minute and allowed to stand at room temperature for 15 min. Hexane coating as such served as a control. The ELISA plates were coated with the different mycophytic acids at 50 μl per well by application to the well using Hamilton syringes. The lipids were visible as a waxy coating after 2 h of evaporation at RT. Plates were stored in a plastic bag at 4°C overnight. ELISA was done as described in Section 2.5.1. Background binding of the serum to the plate was corrected for by subtracting the average binding signal of antibody to MA from that registered for the hexane-coated wells. The results obtained were analysed by the making use of the Student’s t-test for statistical significance.

2.5.4. Characterization of scFv’s binding specificity of mycophytic acids by sandwich ELISA

Purified scFv’s were tested for their binding activity using a sandwich ELISA. Monoclonal immunoplates were coated with MA as described above. Plates were blocked with 2% MPS for 60 min at 30°C followed by a brief washing step with PBS. scFv samples (25 μl) were mixed with 25 μl of 0.1% Tween-20, anti-myc monoclonal antibody (Aldo, Germany) conjugated with HRP was used to detect bound scFv fragments. Signals were developed with 3,3',5,5'-tetramethoxyphenyl glycol using the 1-step Ultra TMB ELISA substrate solution according to manufacturer’s instructions. Plates were read using a Multiskan Ascent (Thermo Labsystems) plate reader at a wavelength of 450 nm.

3. Results and discussion

3.1. Antibody recognition of natural mycophytic acids and their derivatives thereof

The strongly hydrophobic nature of MA makes them insoluble in water and water miscible organic solvents. Their recognition by water soluble antibodies in diluted serum is therefore somewhat unspecific and requires proof that antibody binding is not due to non-specific hydrophobic antibody adsorption to the MA coated surface.

In Fig. 2, the specificity of interaction of TB positive and negative sera with coated MA antigens is demonstrated. Hexane appears to be the better antigen coating solution compared to hot PBS. The MA methyl-ester (me-MA) is not recognized by antibodies, whereas the free MA (MA mix) is recognized by both TB pos and TB neg patient sera. This supports the hypothesis that the ELISA antibody binding signal is due to recognition of an antigen consisting of one or more MA’s, in which the hydroxyl group of the free MA-carboxylic acid probably participates in inter- or intramolecular stabilization of a specific antigen conformation.

When the MA is fluorescently labelled by esterification of its carboxylic acid, the antibody binding signal is not significantly affected, possibly due to the fact that fluorescent substituted a free carboxylic acid group in close proximity to where the fluorescent compounds the free carboxylic acid of mycophytic acid by lipodization (Fig.2). These results corroborate those of Lemmer et al. (2009), who reported similar results when MA and fluorescent MA were presented...
on immobilized liposomes to patient sera in a surface plasmon resonance biosensor.

3.2. Monoclonal scFv antibody fragment recognition of mycotic acid and cholesterol

The high antibody binding signal with human TB negative sera against MA, here again shown in Fig. 2, was previously speculated to be due at least in part to cross-reactivity of the antibodies with cholesterol (Schliecher et al., 2002), an idea that was later supported by showing that both MA and cholesterol were recognized equally well by AMPbacteria B in an immunoreagent field biosensor (Benadja et al., 2008). The cross-reactivity could be due to a mixture of nonspecific anti-cholesterol and anti-MA antibodies in the sera, or due to a true cross-reactivity where a particular antibody specificity could recognize both MA and cholesterol. It is known that all humans have anti-cholesterol antibodies in their blood circulation (Warner et al., 1988, which may at least in part explain the high antibody activity to MA in TB negative patients. To test what mechanisms are possible for the cross-reactivity, scFv fragments expressed from a chicken antibody gene library were screened for specific binders to cholesterol and MA. Chickens, similar to humans, express a specialized MA-presenting CD1 (CD1d-1) on their antigen-presenting cell populations (Dyer et al., 2010). Three different specificities were detected and worked up from the plaque display pattern into monomolecular, monovalent scFv fragments. The monospecific anti-cholesterol scFv was dubbed anti-CH, while two scFv specificities were generated against MA: one monospecific (anti-MA) and one cross-reactive with cholesterol (Cross retrieval). Fig. 3 shows the characterization of these three scFvs with EUISA. The fact that a single monoclonal, monovalent scFv could be found with binding affinity against both cholesterol and MA corroborates the conclusion reported by Benadja et al. (2008) that cholesterol and MA share some antigenic structural properties and may explain why TB negative sera recognize cholesterol as well as they do MA. On the other hand, the finding that an scFv against MA could be found that does not cross-react with (host)MAMA (anti-MA) and vice versa (anti-CH), means that anti-MA antibodies may be induced during tuberculosis that are not merely anti-cholesterol antibodies with increased binding activity. This provides a broader perspective on why the results in Fig. 2 show higher antibody binding activity with TB pos patient sera than with TB neg sera as was shown before with larger numbers of human patient sera.

Table 1
Synthetic MA structures, names and numbers relating to results.

<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>11</td>
<td><img src="image11" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>12</td>
<td><img src="image12" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>13</td>
<td><img src="image13" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
<tr>
<td>14</td>
<td><img src="image14" alt="Structure" /></td>
<td>As for structure 5</td>
</tr>
</tbody>
</table>

Prepared by the same methods as described in ref. [1].
Prepared from the corresponding protected lactone as in Koca and Bashe (2007) using the methods of hydrolysis described in Koca et al. (2009a).
Prepared using the same methods as described for the three stereoisomers above (Al Dulaqi et al., 2007).
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].
Prepared by the same methods as described in ref. [1].

Appendices

Fig. 6. ELISA antibody binding signals of TB positive and TB negative sera to synthetic keto-mycocids. The error bars indicate the standard deviation. The 2.5d rule was applied to remove outliers. n = 19, mean 19.

The methyl esters of the MA subclases in ELISA, a result which contradicts our finding that the methyl esters are not antigenic (Fig. 2).

The packing of mycocids in a Langmuir monolayer has previously been shown to differ between alpha-, beta- and methoxy-MA subclases. Keto-MA tended more towards a W-shaped configuration with exceptional rigidity in monolayers, whereas methoxy- and alpha-MA exhibited a more flexible conformation towards variation of experimental parameters (Villemandre et al., 2005; 2007). Thus, the packing of MA is influenced by the orientation of the functional groups that induce different conformational interactions with antibodies in sera. We propose that the antibody binding to mycocids with TB negative sera is most likely due to the presence of anti-cholerae antibodies, known to exist in all humans (Boro et al., 2009; Swartz et al., 1998) that cross-react with MA.

Antibody binding to the natural MA mixture, as well as several of the synthetic MA was observed with both TB positive and TB negative patient sera, while some synthetic MA appeared to have little or no antigenic activity. Although TB positive sera generally gave better binding to the antigenic MAs than TB negative sera, no single antigenic MA was significantly better able to differentiate between TB positive and TB negative sera than the natural mixture of MAs could. This could in principle mean that the synthetic MAs tested were antigenic primarily to the antibodies that respond to both MA and cholesterol. The fact that TB positive patient sera statistically score higher than TB negative patient sera in recognition of MA in ELISA could then explain simply by a higher concentration or affinity of the anti-MAspecific antibodies in TB patient sera, even though nonspecific antibody activity to mycocids has been found to exist at least in the germ-line antibody gene repertoire of chickens (Fig. 3).

The synthetic methoxy-MA subclass had the highest binding to the antibodies of the four synthetic subclases tested, followed by hydroxy- and alpha-MA (Figs. 4-7). The stereochemistry of the methoxy group and the cyclopropane is important for the recognition by antibodies in the sera. Even small changes in the stereochemical arrangement of the groups influenced the amount of binding observed. As seen from Fig. 4, the antibody binding signal of R-methoxy-methyl-cyclopropane MA configuration (No. 6) most closely resembles the response towards the natural mixture of MA. A change of the configuration of either the cis-cyclopropane (No. 7), or the methoxy-methyl fragment to S,S (No. 9) reduced the binding signal by approximately half. Of the more weakly antigenic SR-cis configuration of the cyclopropane is combined with the S,S-methoxy configuration (No. 6), the signal is once again halved, even though the S,S-methoxy configuration is the reported stereochemistry in natural compounds (Al Dulaimy et al., 2007). However, when the cyclopropane group assumed the methoxy-trans configuration as in structure No. 10, in this case in association with the reported natural S,S configuration around the methoxy-methyl fragment, an antigenicity signal was obtained that was even higher than that obtained for the natural MA mixture (P < 0.01). This shows beyond doubt that the stereochemical configuration of the two functional groups on the side-chains of the methoxy-MA influences the way in which they are recognised by antibodies in human sera. It remains to be determined whether other combinations of absolute configuration of the methoxy-methyl fragment and the methoxy-trans-cyclopropane will provide an even more antigenic MA.

The synthetic hydroxy -MAs (Fig. 5), which are the likely precursors of both methoxy- and keto-MA (Yuan and Barry, 1996; Yuan et al., 1997; Asselain et al., 2002) all attracted weaker antibody binding compared to the natural MA mixture. The methyl-trans configuration of the proximal cyclopropane group appears to be a pre-requisite for antigenicity of the hydroxy-MAs (Nos. 13 and 14), compared to Nos. 11 and 12, (Fig. 5). The hydroxy-methyl fragment in the R,S-configuration (No. 14) is more antigenic than the S,S-configured MA with statistical significance, P < 0.01, as was found with the methoxy group stereochemistry (Fig. 4).

Like the hydroxy-MAs, keto-MAs also require a proximal cyclopropane in the methyl-trans-cyclopropane configuration (No. 5, Fig. 6) to be antigenic, compared to the two cis-cyclopropane configurations (Nos. 4 and 3, Fig. 6) that did not show any significant antigenic activity. In these cases, a mixture of epimers at the chiral center adjacent to the keto-group was tested, it is possible that R and S-isomers show different antigenicity. Thus in all the oxygenated MAs (i.e. methoxy-, hydroxy- and keto-MA), the methyl-trans-cyclopropane configuration provides for the best antigenic functionality.

Two synthetic alpha MAs (Fig. 7, Nos. 12 and 2) were compared to the natural MA mix to determine their relative antigenicity. The antibody binding signal to the synthetic alpha MA failed to distinguish between TB pes and TB neg patient sera compared to the natural MA mix. The synthetic MA gave such low antibody binding signals, that nothing could be learned from the variations in the stereochemistry of the distal and proximal cyclopropanes on the antigenicity of alpha-MA. It must be noted that the stereochemistry of cis-cyclopropanes in alpha-mycolates remains to be proven. However, if a common synthetic intermediate is involved in the production of the different functionalities and this is the same for both proximal and distal substituents, the natural stereochemistry will be as in 1.
4. Conclusion

MAs have been shown to play an important role in the virulence of tuberculosis mycobacteria. Not only do they act as pathogen-associated molecular patterns (PAMPs) for induction of innate immunity (Korf et al., 2005), but they are also able to reprogram murine macrophages to modulate their inflammatory activity (Korf et al., 2005). That these responses towards free MA administration may be related to the functional groups expressed on the monomers is inferred by findings such as that mutants of M. tuberculosis that have no oxygenated mycolic acids are of reduced virulence in mice (Dobrucki et al., 2000), that mutants without the ability to trans-cyclopentaneate their oxygenated MAs are hypervirulent (Rao et al., 2006), and that the trehalose dimycolate (TDM) extracted from such trans-cyclopentane mutants stimulates inflammatory activity of murine macrophages, compared to TDM extracted from wild-type M. tuberculosis. Experiments like these are not feasible in humans, but the importance of MAs in human tuberculosis was recently demonstrated by implicating patient serum antibodies to mycolic acids as surrogate markers of active TB (Thanyani et al., 2008) using biosensor technology. With biosensor technology the interference of cross-reactive antibodies against cholesterol could be avoided (that was encountered with the more standard ELISA technology (Schlichter et al., 2002)). Here we determined that the fine structure of MAs is important for recognition by human TB patient serum antibodies. First, we showed that methyl esters of the natural mycolate mixture showed no antigenicity, but that it was maintained by addition of a fluorescein to the MA carbamate that substituted a new carbonyl acid functional group close to the ester bond of the conjugate. This suggested that MA assumes an antigenic conformation that is stabilized by hydrogen bonding to the (unassociated) carbonyl acid.

Two different scFv monoclonal antibody fragments generated from a chicken anti-body library that recognized MAs of which one cross-reacted with cholesterol and the other not, indicated that the cross-reactivity of human patient serum between MAs and cholesterol could either be due to a mixture of anti-cholesterol and anti-MA antibodies and/or due to a single antibody with cross-reactivity specificity, or both. It also proves that anti-cholesterol antibodies do not necessarily cross-react with MA. Analysis of the antigenity of a range of synthetic MAs suggested that the synthetic MA might be an antigenic. Methoxy MA represented the strongest antigen for both TB pos and TB neg patients. Important, however, was the general observation that two functional groups on the monomers of natural synthetic MAs were both critically important to determine antigenicity for human serum antibody recognition.

The results seem to favour methoxy MA of M. tuberculosis as the strongest functional entity or antigen to use in TB serodiagnostic devices. A proximal methyl-trans-cyclopentane enhances the antigenity of these functionalities. The ER-conformation of the distal methyl group still remains to be tested in combination with a trans-cyclopentane proximal group to determine antigenicity in methoxy MA. The results do not necessarily provide information as to the stereochemistry of the naturally produced MAs found in the cell walls of MA, as the antibody recognition of natural classes may be due to the complex mixtures of homologues and cyclopentane stereochemistries present. The best antigenicity seen in synthetic MA does not therefore necessarily indicate the most likely structure of the MA antigen(s) in nature. Thus, it seems unlikely that a particular MA molecule can constitute an antibody binding site filling antigen or hapten. Rather, the surface covered by packed MAs is likely to be the structure that is recognized by antibodies, similar to the case of monoclonal antibody recognition of cholesterol (Krohn et al., 2001). Unlike cholesterol, which has a defined structure, MA exists as a mixture in M. tuberculosis. It may well be that MA folding and packing is influenced by the presence of different types of subclasses and variants thereof; such that the natural MA antigen(s) may never be reproduced synthetically by the use of a single species of pure synthetic MA. It was a disappointment that the cholesterol cross-reactivity could not be defined and that the cross-reactivity observed appeared to be due to other factors in MA from the antigenic mixture as implied by the observation that the TB positive and TB negative sera were not better resolved with any particular antigenic MA structure. Nevertheless, the demonstration of biological antigenic activity of individual stearochemically unique chemically synthetic MAs to level that approximate or even exceed the antigenic activity of the natural mixture of MAs purified from M. tuberculosis could well offer the possibilities towards improving the existing assays that aim at detection of anti-MA antibodies as surrogate markers for TB disease (Schlichter et al., 2002; Thanyani et al., 2008; Lennard et al., 2009; Mathewula et al., 2009). It allows for the first time the possibility of providing exact specifications for an optimal antigen created. The potential of linkage of MA to sensors surfaces for early regeneration and engineering of the antigen to define the best window of antibody affinity and specificity.

References


Brenn...