Structures of the glycopeptidolipid antigens of *Mycobacterium abscessus* and *Mycobacterium chelonae* and possible chemical basis of the serological cross-reactions in the *Mycobacterium fortuitum* complex

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*Mycobacterium abscessus* and *Mycobacterium chelonae*, two members of the *Mycobacterium fortuitum* complex, contain five major glycolipids. A combination of NMR spectroscopy, fast atom bombardment mass spectrometry and chemical degradation was used to elucidate their structures. All the compounds belong to the family of glycopeptidolipids. A 6-deoxy-α-L-talosyl unit, which may bear one or two acetyl groups, invariably occupies the site of glycosylation on the threonine residue in the various compounds. A 3,4-di-O-methyl- or 2,3,4-tri-O-methyl-α-β-rhamnosyl unit modifies the alaninol end of the diglycosylated molecules. Both species also contain a multiglycosylated compound consisting of α-L-rhamnosyl-(1 → 2)-3,4-di-O-methyl-α-L-rhamnosyl linked to alaninol, which belongs to the class of new variants of glycopeptidolipids recently described. Using an ELISA, the latter glycolipid as well as the diglycosylated ones (not previously reported to be antigenic), were shown to react with the serum raised against the whole lipid antigens of *M. chelonae*. A comparative serologic study of the native and chemically modified glycopeptidolipid antigens allowed the identification of their epitope as the 3,4-di-O-methyl-α-L-rhamnosyl residue. Similar experiments conducted on the glycopeptidolipids isolated from the serologically cross-reacting species *M. peregrinum* led to the conclusion that the epitope identified in *M. chelonae* and *M. abscessus* was involved in the cross-reactions and demonstrated the existence of a second haptenic moiety in the glycolipids of *M. peregrinum*, the 3-O-methyl-α-L-rhamnosyl unit. In addition to this latter non-shared epitope, the recently described sulfated glycopeptidolipid antigen of *M. peregrinum* did not react with the *M. chelonae* serum, thus further explaining the difference in the seroreactivity within the complex.

**Keywords:** *Mycobacterium abscessus*, *Mycobacterium chelonae*, glycopeptidolipids, *Mycobacterium fortuitum* complex, antigen

**INTRODUCTION**

The most frequently isolated rapidly growing mycobacteria of clinical importance are members of the *Mycobacterium fortuitum* complex composed of non-chromogenic, closely related strains (Good, 1985; Woods & Washington, 1987). Clinical manifestations of infection with these organisms include disseminated disease, cutaneous lesions, post-surgical infections, pulmonary disease and a gamut of miscellaneous infections (Woods & Washington, 1987). Although the identification of the isolates at the species and biovariant level by conventional biochemical tests is not obvious, DNA-DNA hybridiza-

Abbreviations: 2D-COSY, two-dimensional chemical-shift correlated spectroscopy; FAB, fast atom bombardment; GPL, glycopeptidolipid; MIKE, mass-analysed ion kinetic energy; TMS, trimethylsilyl.

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tion studies demonstrated that the complex consists of four different genomic species: *M. fortuitum*, *M. peregrinum*, *M. abscessus* and *M. cheloneae* (Baess, 1982; Kusunoki & Ezaki, 1992; Lévy-Frebault et al., 1986). The recognition of different antimicrobial susceptibilities for the various species within the *M. fortuitum* complex (Swenson et al., 1985; Wolinsky, 1992) makes their specific and easy recognition important.

In the past few years, several families of specific mycobacterial glycolipid antigens have been identified and have proved to be useful in the identification of the mycobacterial pathogens (for a review see Brennan, 1988). In the members of the *M. fortuitum* complex, two types of glycolipids have been identified by Tsang et al. (1984): alkali-labile acyltrehalose-containing compounds in *M. fortuitum* and alkali-stable glycopeptidolipids (GPLs) in *M. peregrinum*, *M. cheloneae* and *M. abscessus*. Sero-agglutination and antibody adsorption studies led to the conclusion that the lipids of the two latter species are antigenically identical whereas *M. peregrinum* may share some antigenic motifs with them, but is not identical. In agreement with this observation, chemical studies of the species-specific glycolipids established that *M. peregrinum* and *M. cheloneae* synthesize GPLs (López Marín et al., 1991, 1992a, b; Tsang et al., 1984) whilst *M. fortuitum* elaborates acyltrehalose-containing glycolipids (Gautier et al., 1992; Besra et al., 1992; Sempere et al., 1993). However, the structure tentatively proposed for the most polar GPL of *M. cheloneae* (Tsang et al., 1984) has been recently questioned by mass spectrometry, which indicated a different structure for this molecule, including a different location of the oligosaccharide moiety (López Marín et al., 1992b). These results might suggest that different compounds were examined in the two independent studies. Thus, in the present study, conventional chemical analyses were combined with fast atom bombardment (FAB) mass spectrometry and NMR to arrive at the complete structures of all the GPLs of both *M. abscessus* and *M. cheloneae*. Furthermore, the question of the antigenicity of the different purified GPLs and the chemical basis of the serological cross-reactions in the *M. fortuitum* complex was also addressed.

**METHODS**

**Strains and growth conditions.** The type strains of *Mycobacterium cheloneae* NCTC 946, *M. peregrinum* ATCC 14467, *M. fortuitum* ATCC 6841 as well as the previously described strains of *M. abscessus* IP 81 0192 and IP 82 1894 and *Mycobacterium cheloneae* 'miscellaneous' IP 810 292 (Lévy-Frebault et al., 1983) were grown on Sauton's medium (Sauton, 1912) as surface pellicles in 250 ml glass flasks at 33 °C for 1 or 2 weeks. These pellicle growth conditions allowed an easy harvest of cells by pouring off the medium, when the pellicles remained attached to the flasks.

**Extraction and purification of the surface antigens.** Bacteria harvested from surface pellicles were extracted first with chloroform/methanol (1:2, v/v) at room temperature for 4 d; the bacterial residues were then re-extracted with chloroform/methanol (2:1, v/v) for 1 week. Pooled extracts were concentrated, partitioned using chloroform/water and the chloroform phases were dried. The resulting washed lipids were separated on a Florisil column (32 × 3 cm, 60–100 mesh) with increasing concentrations of methanol in chloroform (López Marín et al., 1991). Final purification of the GPLs was achieved by high performance liquid chromatography (HPLC) on a reverse phase column (Nucleosil C18, 300 × 7.5 mm, Biochem. Champniers, France) as previously described (López Marín et al., 1992b). Fractions were monitored by TLC on silica-gel-coated plates (G-60, 0.25 mm thickness, Merck) developed with chloroform/methanol (90:10, v/v) or with chloroform/methanol/water (65:25:4 or 60:12:1, by vol.). Sugar-containing compounds were visualized by spraying plates with 0.2% (w/v) anthrone in concentrated H2SO4, followed by heating.

**Immunological procedures.** Rabbit antisera were obtained by intradermal injection (in multiple sites) of 1 ml of a water-in-oil emulsion prepared by mixing 0.5 ml PBS (pH 7.2) containing 5 mg of the washed crude lipid extract (described above) of either *M. peregrinum* or *M. cheloneae* and 0.5 ml Freund's incomplete adjuvant. The emulsion was injected into 3-month-old white New Zealand rabbits. Three weeks later, the animals received a booster dose with 3 mg of crude lipid extract of the corresponding species in water-in-oil emulsion as described above and were bled at 2-week intervals.

For ELISA lipids were suspended by sonication at concentrations of 12, 25, 50 and 100 µg ml⁻¹ in cyclohexane. Samples (50 µl) of the suspension were applied to the wells of a polystyrene microtitre plate (Nunc-ImmuNo Plate I) and allowed to evaporate at 37 °C overnight. The wells were blocked with 200 µl of a 3% (w/v) BSA solution in PBS at 37 °C for 2 h. The rabbit sera were diluted (1:500, v/v) in PBS containing 0.3% BSA and added to the plates followed by a 2 h incubation at 37 °C. After five further washings, 100 µl alkaline phosphatase conjugated to whole anti-rabbit IgG (Sigma) diluted 1:1000 in 3% BSA in PBS was added to each well followed by a 2 h incubation at 37 °C. After washing the plates five times with PBS, 100 µl p-nitrophenyl-phosphate substrate (Sigma) was added, followed by a 30 min incubation at 37 °C. Absorbance was read at 405 nm with a Multiskan apparatus (Flow Laboratories).

**Miscellaneous analytical techniques**

Alkaline borohydride reductive cleavage. Alkaline borohydride reductive cleavage (β-elimination) of GPLs and alkaline deacylation of crude lipid extracts and GPLs were performed according to Brennan & Goren (1979).

Sugar characterization. Samples were routinely hydrolysed with 1 M trifluoroacetic acid solution at 110 °C for 1 h (no additional compound was obtained with a 2 M trifluoroacetic acid treatment for 4 h). The hydrolysates were then partitioned between chloroform and water. The aqueous phase was dried under N₂ and analysed both on TLC silicagel-coated plates (Merek) with chloroform/methanol (7:3, v/v) as solvent, and by gas chromatography (GC) of their trimethylsilyl (TMS) derivatizes (Sweeley et al., 1963). Authentic standards of 3-O-methylrhamnose and 3,4-di-O-methylrhamnose were obtained from the hydrolysis of the already described GPLs (López Marín et al., 1991).

The D or L configuration of the monosaccharides was determined after their demethylation by BCl₃ (Bonner et al., 1960). The retention times on GC analysis of the TMS derivatives of
their (−)-2-butyl glycosides were compared to those of (−)-2-butyl-1-rhamnoside and 6-deoxy-1-taloside, and (±)-2-butyl-1-rhamnosides and 6-deoxy-1-talosides (Gerwig et al., 1978).

GC was performed on a Girdel G-30 apparatus equipped with a fused silica capillary column (25 m length × 0.22 mm internal diameter) coated with OV-1 (0.3 mm film thickness). A temperature gradient of 100–280 °C (2 °C min⁻¹) was used for the separation of silylated monosaccharides, whereas isothermal conditions (150 °C) were chosen for butyrglycosides.

Identification of the amino compounds. Amino compounds were identified after treatment of GPLs with 6 M HCl at 110 °C for 16 h. The hydrolysates were partitioned between chloroform and water. The aqueous phases were concentrated and analysed by TLC on pre-coated cellulose plates (Merck) with 1-butanol/ethanol/water (4:1:1, by vol.) as solvent. Threonine was differentiated from allo-threonine using the upper phase from a mixture of 1-butanol/water/acetone/30% aqueous ammonia (8:6:1:1, by vol.) as the developing solvent (Shaw & Fox, 1953).

The amino-acid configuration was determined on chiral HPTLC plates (Merck 14285) developed with acetone/methanol/water (10:2:2, by vol.) according to Günther (1988). Spots were detected by spraying plates with 0.2% (w/v) ninhydrin in acetone followed by heating.

Spectroscopy and spectrometry. Infra-red spectra of film samples were recorded on a Perkin-Elmer FTIR 1600 spectrophotometer.

1H-NMR spectra were obtained in CDCl₃ or CDCl₃/CD₃OD (98:2, v/v) on a Bruker AM 300 WB instrument at 25 °C. 13C-NMR spectra were obtained in CDCl₃/CD₃OD (9:1, v/v) on a 62.9 MHz Bruker WM250 apparatus using a Bruker J-mod sequence. The chemical shift reference used was that of tetramethylsilane. The homonuclear two-dimensional chemical-shift correlated (2D-COSY) spectra were performed using the previously described pulse sequence and parameters (Daffé & Servin, 1989).

Mass spectrometry was performed on a ZAB-HS reverse geometry mass spectrometer (VG Analytical, Manchester). FAB spectra were generated by an 8 keV xenon atom beam. Samples were dissolved in chloroform/methanol (1:1, v/v). This solution (1 μl) was mixed on the probe tip with 1 μl of meta-nitrobenzyl alcohol and 1 μl of a 10% solution of NaI in H₂O. Ten scans of 10 s per decade were accumulated to obtain a spectrum. The resolution of the instrument was set to 1500. Mass-analysed ion kinetic energy (MIKE) spectra were measured by electrostatic voltage scanning (800 eV s⁻¹), while keeping the parent ion resolution to 1500. Between 20 and 40 scans were stored and accumulated for each experiment.

RESULTS

Comparative TLC analysis of the GPLs of M. abscessus and M. cheloneae

When the crude lipid extracts of M. abscessus and M. cheloneae were compared with those from M. peregrinum by TLC (Fig. 1a), several apolar and polar spots were detected which rapidly gave an intense blue-green colour with anthrone. As indicated elsewhere (Daffé et al., 1989), such a response is characteristic of glycolipids other than acyltrehaloses (whose spots slowly develop a characteristic blue-grey colour on heating the anthrone-sprayed plates). In addition, while acyltrehaloses are alkali-labile, examination of the TLC profiles after deacylation of the lipid extracts (not shown) indicates that most of the glycolipids under study are alkali-resistant. Similar results were obtained with M. abscessus and M. cheloneae in agreement with a previous finding (Tsang et al., 1984).

Purification of the major glycolipids and characterization of the amino and the sugar components

The native glycolipids were purified by adsorption chromatography, followed by HPLC on a reverse phase column and five major compounds were obtained (Fig. 1b).

Strong acid hydrolysis (6 M HCl) released, from all glycolipids, four amino compounds which were identified, on cellulose and chiral TLC plates, as D-phenylalanine, D-allo-threonine, D-alanine and alaninol (assumed to be L as in known C-mycosides; Voland et al., 1971) by comparison with authentic standards and well-characterized components obtained by hydrolysis of GPLs isolated from M. peregrinum (López Marín et al., 1991).

On milder acid hydrolysis (1 M trifluoroacetic acid), GPL I liberated 6-deoxytalo side and 2,3,4-tri-O-methylrhamnose. GPLs II, III and V released 6-deoxytalo side and 3,4-di-O-methylrhamnose. In addition to these two latter monosaccharides, rhamnose was detected in the hydrolysis products of GPL IV. The absolute configuration of the sugars was determined after de-O-methylation by BCl₃ (Bonnet et al., 1960) according to Gerwig et al. (1978). Rhamnose resulting from the de-O-methylation as well as 6-deoxytalo side were converted into their (−)-2-butyl glycosides and the TMS derivatives were analysed by GC on a capillary column. Two peaks corresponding to (−)-2-butyl-1-rhamnoside and (−)-2-butyl-6-deoxy-1-taloside were identified from each GPL, indicating that the different partially O-methylated rhamnosyl residues as well as the rhamnosyl and 6-deoxytalo side units belonged to the L-series.

Analysis of the sugar moieties

Analysis of the 1H- and 13C-NMR spectra of the purified GPLs (Fig. 2) indicated the number of sugar residues, amino compounds and methoxyl groups per molecule. Two anomic signals were observed in the spectra of GPLs I, II, III and V while three such signals were seen in the anomic region of GPL IV. Similarly, four signals assignable to NH-protons and to >CH-NH-carbon signals were observed in all the 1H- and 13C-NMR spectra (Fig. 2). The number of methoxyl protons and carbon signals were also consistent with the nature of the sugars liberated by mild acid hydrolysis and suggested the presence of an additional methoxyl group on the lipid chain (Daffé et al., 1983; López Marín et al., 1991). Interestingly, sharp singlets (3H, each) assignable to acetyl protons were observed in the 1H-NMR spectra of Glycolipids of M. abscessus and M. cheloneae

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GPLs I–IV. Two acetyl groups were present in GPLs I, II and IV, and one such group was observed in the spectrum of GPL III. Comparative TLC of the native and deacetylated GPLs (Fig. 1b, c) confirmed the above results: de-O-acetylated GPLs II and III gave the same Rf as that of GPL V, demonstrating that GPL II and III correspond to di- and mono-acetylated forms of GPL V, respectively.

The location of acetyl groups was established by 2D-COSY (Daffé & Servin, 1989). The 2D-COSY spectra of GPL IV showed a cross peak between the resonance of H-1 at 5.0 p.p.m. and that of H-2 at 4.1 p.p.m. From the H-2 resonance a connectivity to the H-3 resonance at 4.7 p.p.m. was observed and likewise, from H-3 to H-4 resonance at 4.9 p.p.m. The H-4 resonance showed a connectivity to the H-5 resonance at 3.6 p.p.m. which showed a cross peak allowing the resonance of H-6s to be located at 1.2 p.p.m. Similarly, the resonances of the other sugar protons were determined (Table 1). The chemical shifts of H-3 and H-4 resonances of sugar A clearly indicated that the two acetyl groups were located on C-3 and C-4 of this sugar unit, causing the deshielding of the corresponding proton resonances (Daffé & Servin, 1989).

Analysis of the 2D-COSY spectrum of GPL I established a similar location of the acetyl groups (data not shown). For the acetylated diglycosylated molecules (GPLs I–III) it was obvious that the acetyl groups were located on the 6-deoxytalosyl residue since the other sugar component is already substituted at both positions 3 and 4 by methoxyl groups, i.e. the 3,4-di-O-methylrhamnosyl residue. In contrast, for GPL IV, two possible locations of the acetyl groups exist: the 6-deoxytalosyl and the rhamnosyl residues. The precise location of the acetyl groups in this latter GPL was found by mass spectrometry (see below).

To locate the sugar units on the threonine or/and the alaninol residues, β-elimination was applied to the various GPLs and the resulting GPLs were purified, hydrolysed and their constituents identified. 6-Deoxytalose and allo-threonine were no longer detected in any of the GPLs after β-elimination. Alanine, phenylalanine, alaninol and an amino compound migrating as 2-aminobutyric acid were liberated by strong acid hydrolysis of the β-eliminated glycolipids. GPL I gave after β-elimination followed by mild acid hydrolysis 2,3,4-tri-O-methylrhamnose while GPLs II, III and V liberated 3,4-di-O-methylrhamnose. In addition to this latter sugar, rhamnose was also detected in the acid hydrolysis products of

![Fig. 1. TLC (solvent: chloroform/methanol, 9:1, v/v) of (a) the crude lipid extracts of M. peregrinum (lane 1), M. chelonae (lanes 2 and 3) and M. abscessus (lanes 4 and 5), (b) the purified GPLs I-V of M. abscessus (lanes 6–10, respectively) and (c) the deacylated GPLs I-V (lanes 11–15, respectively).](image-url)
**Fig. 2.** NMR spectra of GPLs from *M. abscessus*. (a) $^1$H-NMR spectrum of GPL I in CDCl$_3$; the assignment of the H-1 signal resonances was done by 2D-COSY. (b) Portion of the J-modulated $^{13}$C-NMR spectrum of GPL IV in CDCl$_3$/CD$_3$OD. $R = -\text{CH}_3$, $R' = \text{rhamnosyl residue}$. 

$\beta$-eliminated GPL IV. Thus, in all the GPLs, a 6-deoxytalosyl residue is linked to the threonine residue.

**Sequencing of the amino compounds and the disaccharide**

Although the nature of the amino compounds suggested the classical peptide sequence usually occurring in GPLs (Brennan, 1988), sequencing of the amino compounds was done by FAB–MIKE spectrometry (López Marín et al., 1991, 1992a, b). Table 2 shows the main data obtained from the analysis of the mass spectra. The mass unit differences between the $m/z$ values of peaks corresponding to cleavages between amino acid residues with charge retention on either the fatty acyl-containing moiety ($A_2$, $A_3$, b- and c-type fragmentations) or the glycopeptide moiety ($Y_2$- and $Y_3$-type fragmentations) established the predicted sequence (Table 2) and confirmed the chemical and NMR data (number and location of the sugar residues, acetyl and methoxyl groups). Furthermore, the $m/z$ values of peaks corresponding to b- and c-type fragmentations showed that the rhamnsoyl residue of GPL IV was not acetylated and was linked to the 3,4-di-O-methyl-rhamnosyl unit attached to the alaninol end. Thus, the $1 \rightarrow 2$ glycosidic linkage between the two rhamnosyl
Table 1. Chemical shifts of the ring sugar protons of the three sugar residues (labelled A, B and C) of GPL IV as determined by 2D-COSY

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<th>Sugar ring proton</th>
<th>Chemical shift, δ (p.p.m.)</th>
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<tr>
<td></td>
<td>A</td>
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<td>H-1</td>
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<td>H-2</td>
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residues, the only possibility, was deduced. The 13C-NMR spectrum (Fig. 2b) supported this conclusion.

Structure of the lipid moiety
Two main pseudomolecular ion [M + Na]+ peaks differing by 28 mass units were observed in the FAB mass spectrum of each GPL. Additional minor peaks, 2 mass units lower, indicated the occurrence of unsaturated homologues in the acyl chain, a result supported by NMR spectroscopy (see Fig. 2a). Analysis of the FAB–MIKE spectra of both major peaks demonstrated that the corresponding compounds differed in the chain length of the fatty acyl moiety, i.e. identical m/z values for peaks attributed to Y2- and Y3-type fragmentations (see Table 2) and 28 mass units difference for the remaining peaks. Thus, from the various m/z values, the molecular masses of the two major fatty acyl constituents of the GPLs were deduced (409 and 437). The presence in the NMR spectra of a methoxyl group on the fatty acyl chain (Fig. 2) allowed us to propose a methoxylated hexacosanoyl and octacosanoyl structure for these two main components.

Anomeric configuration of the sugars
The anomeric configuration of the 6-deoxytalosyl and rhamnosyl residues could not be established by either 1H- or proton-uncoupled 13C-NMR; the literature shows that both α- and β-rhamnosides have small 3J1,2 values and exhibit variable C-1 chemical shifts. In contrast, the 1JCH values were shown to be useful in differentiating between the two anomers (Kasai et al., 1979). By measuring this

Table 2. Assignments of the major peaks observed in the FAB–MIKE spectra of the different glycopeptidolipids isolated from M. chelonae and M. abscessus.

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<td>881</td>
<td>999</td>
<td>981</td>
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<td>343</td>
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<td>981</td>
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Glycolipids of *M. abscessus* and *M. chelonae*

Seroreactivity of the lipid extracts

The whole lipid extracts from *M. abscessus*, *M. chelonae* and *M. peregrinum* reacted readily in ELISA with the serum raised against the homologous lipid antigens (Fig. 3). Similar seroreactivities were observed for lipids from *M. abscessus* and *M. chelonae* against the anti-*M. chelonae* serum. In contrast, the reactivity of *M. peregrinum* lipids with anti-*M. chelonae* serum was about half that of the homologous pair, i.e. *M. chelonae* and *M. abscessus* (Fig. 3a). Likewise, the reactivity of *M. chelonae* and *M. abscessus* lipids with anti-*M. peregrinum* serum was only one-tenth that of the homologous lipid antigens (Fig. 3b). Deacylation of the lipid extracts did not diminish their seroreactivity (data not shown), thereby demonstrating that the alkali-labile compounds (acyltrehaloses, phospholipids) did not significantly contribute to the seroreactivity and suggesting that the alkali-stable molecules were the antigenic substances of the crude lipid extracts.

**Fig. 3.** Reactivity in ELISA of the total lipid antigens of *M. chelonae* (●), *M. abscessus* (▲) and *M. peregrinum* (□) against a rabbit anti-(whole lipid of *M. chelonae*) serum (a), and against a rabbit anti-(whole lipid of *M. peregrinum*) serum (b). The reactivity of the lipids against the rabbit pre-immune serum (PIS) is shown (-x-). The sera were diluted in PBS (1:500).

Among the alkali-stable lipids, multiglycosylated GPLs are known to be antigenic molecules (Brennan, 1988) and it was thus interesting to test the seroreactivity of the

**Fig. 4.** Reactivity in ELISA of the native and β-eliminated (*) GPLs of *M. abscessus* (a) and of *M. peregrinum* (b) against a rabbit anti-(whole lipid of *M. chelonae*) serum and of the native GPLs of *M. peregrinum* and their β-eliminated products (*) against a rabbit anti-(whole lipid of *M. peregrinum*) serum (c). The reactivity of the GPLs against the rabbit pre-immune serum is shown (PIS). Results represent the arithmetic mean for two determinations and for 4.0 nmol product per well. The sera were diluted in PBS (1:500).
purified GPLs of *M. chelonae* and *M. abscessus* to determine the antigenicity of the new variant (multiglycosylated) GPL (GPL IV) as compared to that of, virtually non-antigenic, diglycosylated GPLs (Brennan, 1988).

**Seroreactivity of the purified GPLs of *M. chelonae* and *M. abscessus* using an anti-*M. chelonae* lipid serum**

Comparison of the seroreactivity of the individual GPLs of *M. abscessus* by ELISA, using an anti-*M. chelonae* lipid serum (Fig. 4a) showed different seroreactivities for the various compounds, thereby demonstrating that antibodies are directed against carbohydrates rather than the lipopeptide moiety. GPLs II, III and V (which are diglycosylated compounds) were the most reactive compounds while GPL I exhibited the lowest seroreactivity. β-Elimination of the GPLs did not diminish their seroreactivity, suggesting that the haptenic moiety of GPLs resides in the sugar residue(s) linked to the alaninol end. These data demonstrate that the seroreactivity depends, not on the degree of glycosylation, but on the nature of the sugar residues and suggest that the haptenic moiety of the examined GPLs is the di-O-methylrhamnosyl unit. The low reactivity of GPLs I and IV, which contained modified version of this sugar residue, suggests that its free -OH on position 2 is probably implied in the seroreactivity.

As *M. peregrinum* synthesizes 3,4-di-O-methylrhamnosyl-containing GPLs (whose structures are shown in Fig. 5) and presents serological cross-reactions with sera raised against both *M. chelonae* and *M. abscessus* (Jenkins et al., 1971; Tsang et al., 1984), it was interesting to test the seroreactivity of the individual GPLs of *M. peregrinum* against both the homologous and the anti-*M. chelonae* sera to look for a specific marker(s).

**Seroreactivity of the purified GPLs of *M. peregrinum* using an homologous serum**

The seroreactivity of the various GPLs of *M. peregrinum* (P-I–IV) with the homologous serum is shown in Fig. 4(c). The GPL containing the 3,4-di-O-methylrhamnosyl unit in duplicate (GPL P-I, Fig. 5) was again the most seroreactive. In contrast to what was observed with the antigens of *M. chelonae* (Fig. 4a), β-elimination of the major GPL of *M. peregrinum* (P-I) strongly affected the reactivity of the resulting antigen, thereby suggesting that antibodies were also raised against the 3-O-methylrhamnosyl residue. The reactivity of the GPL containing the 3-O-methylrhamnosyl unit in duplicate (P-II, Fig. 5) supports this hypothesis. This observation may thus explain in part the very low reactivity of the GPLs from *M. chelonae* (devoid of this sugar residue) with the anti-*M. peregrinum* lipid serum (Fig. 3b).

**Seroreactivity of the purified GPLs of *M. peregrinum* using an anti-*M. chelonae* lipid serum**

Using an anti-*M. chelonae* lipid serum, GPL P-I from *M. peregrinum*, which consists of 3,4-di-O-methylrhamnosyl(1 → 2)-3,4-di-O-methylrhamnosyl (1-alaninol) was as reactive as the most reactive GPL of *M. chelonae* (GPL II) whilst the substitution of position 2 of the 3,4-di-O-methylrhamnosyl residue by a sugar residue (GPL P-II, P-III, Fig. 5) or a sulfate group (GPL P-IV, Fig. 5) either reduced or completely abolised the seroreactivity (Fig. 4b).

The occurrence in the three species *M. chelonae*, *M. abscessus* and *M. peregrinum* of a common recognized motif, i.e. the 3,4-di-O-methylrhamnosyl unit may be at the origin of the serological cross-reactions within the *M. fortuitum* complex. It has to be pointed out that the sulfated GPL (P-IV, Fig. 4) was found to be weakly but definitively seroreactive with the anti-*M. peregrinum* serum (Fig. 4c), but...
not with the anti-\textit{M. chelonae} serum (Fig. 4b), thus showing a clear specificity within the \textit{M. fortuitum} complex.

**DISCUSSION**

The recognition that environmental mycobacteria were opportunistic pathogens led to the development of methods allowing their easy identification for therapeutic purposes, as their response to various drugs is known to depend on the species and subspecies (Good, 1985; Wolinsky, 1992). Among these, members of the \textit{M. avium} complex are the most frequently isolated strains (Good, 1985; Woods & Washington, 1987) and have been most extensively examined. Serological and chemical studies of their surface-exposed lipid antigens (the typing antigens of Schaefer) has proved that they consisted of GPLs (Brennan, 1988). Further studies demonstrated that other opportunistic mycobacterial pathogens such as members of the \textit{M. fortuitum} complex are endowed with glycopeptidolipids allowing their identification (Tsang et al., 1984). The proposed basic structure of these GPLs (Lanéelle & Asselineau, 1968; Vilkas & Lederer, 1968) consists of a common lipopeptide core bearing a rhamnosyl unit at one end of the molecule and either mono- or haptenic oligosaccharides (composed of characteristic sugar combinations) linked to the threonine residue (Brennan, 1988). In 1984, Tsang et al. proposed a trisaccharide structure attached to threonine in the GPLs of \textit{M. chelonae} (Tsang et al., 1984). However, based on the recent demonstration of the existence of structural variants in GPLs of \textit{M. peregrinum} (López Marín et al., 1991, 1992a), mass spectrometry has been used to discriminate between these variants (López Marín et al., 1992b). The latter study arrived at a different structure for the most polar decylated GPL of \textit{M. chelonae}. In view of this discrepancy, the present study was undertaken. The application of conventional chemical degradation methods and modern analytical techniques to the purified multiglycosylated GPL of \textit{M. chelonae} and \textit{M. abscessus} clearly demonstrate that they belong to the family of the new variants of GPLs (López Marín et al., 1991), which are characterized by the presence of an oligosaccharide appendage on the alaninol rather than on the threonine residue. The location of acetyl groups on positions 3 and 4 of the 6-deoxyhexosyl unit suggests that the different location of the oligosaccharide moiety is not due to the acetylation of the usual glycosylation site of this sugar (i.e., position 2), but probably results from the difference of the enzymic system occurring in the two mycobacterial groups and related strains.

The occurrence of both apolar diglycosylated and new variant (multiglycosylated) GPLs posed the question of their immunogenicity, since the high antigenicity attached to conventional GPLs was assumed to be exclusively due to the threonine-linked oligosaccharide moiety (Brennan, 1988). The data from seroreactions analysed in the present work demonstrate that both diglycosylated and new variants GPLs are antigenic molecules. The chemical basis of their seroreactions was tentatively defined as being due to the sugar moiety linked to the alaninol end, while that attached to the threonine residue may contribute to the immunogenicity. The identical seroreactions observed with whole lipid antigens or purified GPLs of \textit{M. chelonae} and \textit{M. abscessus} confirm the structural data showing that the two species elaborate GPLs exhibiting identical structures. In contrast, lipid antigens of \textit{M. peregrinum} differ in their structure from those of the former species. The observed serological cross-reactions between the two groups of species of the \textit{M. fortuitum} complex (Jenkins et al., 1971; Tsang et al., 1984) had been tentatively attributed (López Marín et al., 1992a) to the occurrence in the GPL-containing species of the \textit{M. fortuitum} complex of a common haptenic moiety (GPL P-I and GPL IV). In addition to this fact, the data herein presented demonstrate that the serum raised against the lipids of \textit{M. chelonae} reacts with other purified GPLs of \textit{M. peregrinum} (P-I and P-II), thus enhancing the cross-reactivity.

**ACKNOWLEDGEMENTS**

The authors are grateful to Dr D. Promé for performing the FAB mass spectra.

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**Received 27 August 1993; revised 16 November 1993; accepted 1 December 1993.**