Glycolipids of Recent Clinical Isolates of *Mycobacterium tuberculosis*: Chemical Characterization and Immunoreactivity

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Five distinct glycolipids were readily detected in isolates of *Mycobacterium tuberculosis*. Spectroscopic methods and chemical degradation techniques allowed the structural identification of four of these glycolipids. The specific phenolic glycolipid antigen previously characterized from the Canetti strain was found in all the strains examined, with identical structural features (triglycosyl phenol phthiocerol dimycocerosate). The other three glycolipids identified were acylated trehaloses: penta-acyl trehalose (containing phthienoyl substituents), tetra-acyl trehalose 2′-sulphate (with C_{40}–C_{50} hydroxyphthioceranoyl substituents) and diacyl trehalose 2′-sulphate (with C_{16} and C_{18} substituents). The two latter glycolipids as well as the phenolic glycolipid immunoreacted with whole-cell antiserum, indicating their surface location. The occurrence of these glycolipid antigens in recent clinical isolates suggests their possible utilization in the serodiagnosis of tuberculosis and the rapid identification of *M. tuberculosis* with specific antisera.

INTRODUCTION

Tuberculosis is still a serious public health problem today: as many as 8 million new cases occur in the world each year (Mason, 1986). As glycolipids from mycobacteria are antigenic, and often species-specific, a knowledge of the structure of *Mycobacterium tuberculosis* specific glycolipid antigens should be useful in developing serodiagnostic methods for tuberculosis (Papa *et al.*, 1988).

We have recently identified in *M. tuberculosis* (Canetti strain) a major phenolic glycolipid with a unique trisaccharide moiety (Daffé *et al.*, 1987) and related monoglycosyl derivatives (Daffé *et al.*, 1988*a*). We showed that antiserum prepared in rabbits against the major phenolic glycolipid bound its homologous antigen but not purified phenolic glycolipids from *M. bovis*, *M. kansasii*, *M. leprae* and *M. marinum* (Papa *et al.*, 1988). Furthermore, we have demonstrated the presence of other glycolipids in the Canetti strain and similar substances in wild strains of *M. tuberculosis* (Papa *et al.*, 1989). Accordingly, it seemed desirable to determine the structures of the compounds found in wild strains, to characterize the additional glycolipids of the Canetti strain and to evaluate their immunoreactivity.

METHODS

Strains and growth conditions. Mycobacterium tuberculosis CIPT 14-001-0059 (Pasteur Institute Collection) and ten recent clinical isolates of tubercle bacilli were selected for this study (Papa *et al.*, 1988). The clinical isolates were received at the Pasteur Institute National Reference Centre, and identified using conventional methods: they were typical strains of *M. tuberculosis* (Table 1). The best yield of glycolipid was obtained from mycobacteria harvested from the surface of the growth medium, a modified Middlebrook 7H9 broth (Papa *et al.*, 1988). All strains were grown in this medium for 4 weeks at 37 °C.

Table 1. *Mycobacterial strains*

Strain	Origin
CIPT 14-001-0059*	Canetti strain, pulmonary cavity, 1969, French patient
87-1146	Sputum, Brazilian patient
87-1236	Sputum, Portuguese patient
87-1346	Gastric lavage, French patient
87-1348	Gastric lavage, French patient
87-1350	Lymph node, North African patient
87-1352	Lymph node, French patient
87-1353	Sputum, Portuguese patient
87-1357	Gastric lavage, New Caledonian patient
87-1358	Gastric lavage, New Caledonian patient
87-1360	Lymph node, French patient

* CIPT, Collection de l'Institut Pasteur, Tuberculose. This is the original Canetti strain. It forms relatively smooth colonies, is niacin-negative, and causes progressive disease in guinea-pigs but not in rabbits.

Extraction and purification of glycolipids. Bacteria harvested from surface pellicles by centrifugation were suspended in chloroform/methanol (2:1, v/v) with continuous agitation for 2 d at room temperature; the residue was then re-extracted twice with the same solvent mixture. Pooled extracts were dried and then partitioned using diethyl ether/water (1:1, v/v); the ether phase was dried and the washed lipids were dissolved in boiling acetone (56 °C) and subsequently kept at 4 °C overnight. The dried acetone-soluble lipids were dissolved in chloroform and applied to a silicic acid column which was successively eluted with chloroform/methanol (98:2, 95:5, 93:7, 90:10, 80:20 and 70:30, v/v). The collected fractions were re-applied to a similar column, eluted likewise and analysed by thin-layer chromatography (see below). Further purification was performed by preparative thin-layer chromatography, the compounds being eluted from the gel by chloroform or chloroform/methanol (1:1, v/v). Final purification was achieved by reverse-phase chromatography using preparative C-18 material (Waters).

Analytical chromatographic techniques. Thin-layer chromatography of lipids eluted from column chromatography was performed using HP-K high-performance silica gel plates (Whatman). The non-polar lipids were analysed using both pure ethyl acetate and chloroform/methanol (98:2 or 96:4, v/v) as developing phases, whereas chloroform/methanol (80:20, v/v) or ethyl acetate/methanol (90:10, v/v) were used for analysing the more polar compounds. Glycolipids were detected by spraying with 0·2% anthrone in concentrated H_2SO_4 followed by heating at 110 °C. The trehalose-containing lipid spots slowly developed a characteristic blue-grey colour on heating the sprayed plates, whereas the phenolic glycolipid rapidly gave an intense blue-green colour. When heating was extended, the trehalose-lipid spots became reddish-purple, facilitating their detection.

Fatty methyl esters were analysed by thin-layer chromatography using dichloromethane as solvent (Daffé *et al.*, 1983).

Gas–liquid chromatography was performed using a Girdel chromatograph model G-30 equipped with a 25 m capillary column containing WCOT OV-1; compounds were separated with a temperature gradient of 180–280 °C (2 °C min^{-1}).

Miscellaneous analytical techniques. Alkaline hydrolysis of glycolipids was performed by heating a solution of the substance in methoxyethanol containing 5% (w/v) KOH and 12% (v/v) H_2O at 110 °C (Daffé *et al.*, 1983) overnight in order to obtain saponification of sterically hindered ester groups. The solution was acidified with 20% (v/v) sulphuric acid and fatty acids were extracted twice with diethyl ether. The ether solution was dried with sodium sulphate. Acids were esterified by diazomethane (Daffé *et al.*, 1983) and analysed by gas chromatography.

The water-soluble phase was desalted by passing through an ion-exchange column (MB3 Amberlite) and dried.

Acid hydrolysis of saccharide moieties was performed with 2 M-HCl at 110 °C for 2 h as previously described (Daffé *et al.*, 1987).

Trimethylsilyl derivatives of sugars were prepared according to Sweeley *et al.* (1963).

Desulphation was performed on glycolipids in diethylether/6 M-HCl (1:1, v/v) for 30 min at room temperature, the mixture being frequently homogenized by shaking. Petroleum ether (b.p. 50 °C) (1 vol.) and water (1 vol.) were added to the reaction mixture and the ether layer was dried and analysed by thin-layer chromatography. Dimycoloyl trehalose (cord-factor) was used as a negative control.

NMR spectra were obtained in $CDCl_3$ solutions with a Brucker AM 300 WB instrument at 23 °C. IR and UV spectra were recorded on Perkin-Elmer instruments, model 177 and Lambda 5 respectively.

Mass spectra were obtained by the electron-impact technique using a Varian MAT 311 A spectrometer as previously described (Daffé *et al.*, 1984).

Whole-cell immunization. Cells of *M. tuberculosis* (Canetti strain) were washed with sterile phosphate-buffered saline (pH 7·2), killed by ^{60}Co irradiation, suspended in the same buffer to an optical density of 0·3 (measured on a Coleman Junior spectrophotometer at 535 nm) and 0·5 ml of the suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Difco) for use as an immunogen. The water-in-oil emulsion (1 ml) was injected intradermally over a wide anatomical area into 4-month-old HY/CR rabbits (Charles River Laboratories, France). The animals were bled 4 weeks later in view of previous findings (Papa *et al.*, 1988).

Enzyme-linked immunosorbent assay (ELISA). The optimal coating doses of purified glycolipids were dissolved in 50 µl hexane, placed in the wells of polystyrene microtitre plates (CML Nemours, France) and dried overnight at 37 °C. The plates were blocked with 5% (w/v) bovine serum albumin in phosphate-buffered saline (100 µl per well), incubated at 37 °C for 2 h. Then 100 µl of whole-cell antiserum and 100 µl of the conjugate (anti-rabbit immunoglobulin G-β-galactosidase; Biosis, Compiègne, France) diluted in phosphated-buffered saline containing 0·5% bovine serum albumin was added to each well. The above steps were all followed by three washings with the buffer. The final reaction was obtained by the addition of 100 µl of the substrate reagent (*o*-nitrophenyl β-D-galactoside; Merck) for 1 h at 37 °C. Absorbance of product formed was measured at 420 nm with a Multiskan apparatus (Flow Laboratories).

RESULTS

In all crude lipid extracts of the ten recent clinical isolates tested, the major glycolipid detected by thin-layer chromatography had a mobility intermediate between those of dimycoloyl trehalose ('cord-factor') and monomycoloyl trehalose. Further, the glycolipid gave a colour typical of trehalose-containing lipids (see Methods), suggesting a sulphatide I structure (Dhariwal *et al.*, 1984). This major glycolipid and (after column chromatography) four other prominent glycolipids were detected (Table 2) in all the lipid extracts of every strain examined (Table 1). The relative mobilities and staining characteristics on thin-layer chromatography of the five glycolipids were not distinguishable between strains. One strain, 87-1350 (Papa *et al.*, 1988), was selected for chemical characterization of the glycolipids since its growth rate was slightly higher than that of the other clinical isolates examined. Its five glycolipids (Table 2) were compared with those previously studied in detail (Papa *et al.*, 1988) in the Canetti strain.

Structure of the least polar glycolipid

As previously noted (Daffé *et al.*, 1988*b*), some glycolipids escaped observation until the non-polar fraction was studied further. By using less polar eluents or after column chromatographic fractionation, glycolipid 1 from strain 1350 (Table 2) was easily detected and its grey-blue colour after spraying with anthrone and heating suggested a trehalose-containing lipid structure.

This glycolipid 1 was identified as a polyphthienoyl trehalose closely related to the compound we described in the Canetti strain of *M. tuberculosis* (Daffé *et al.*, 1988*b*), on the following bases. (1) It had the same relative mobility on thin-layer chromatography as the major polyphthienoyl trehalose of the Canetti strain. (2) It presented sharp bands in its IR spectrum at 750, 800 and 1640 cm^{-1}, and a strong absorption band at 219 nm in its UV spectrum, both data indicating the presence of an α,β unsaturated ester. (3) The ^{1}H-NMR spectrum of glycolipid 1 showed signals at 6·50 p.p.m. corresponding to protons of a double bond deshielded by conjugation with an ester group.

The water-soluble material resulting from alkaline hydrolysis of glycolipid 1 was analysed by gas–liquid chromatography after trimethylsilyl derivatization; the results indicated that trehalose was the only water-soluble component of this fraction. The coupling constant value $J_{1,2} = 4$ Hz indicated an α,α′ configuration for the trehalose.

Examination of the methyl esters (obtained after saponification followed by methylation) by thin-layer chromatography showed two spots, corresponding to straight-chain and polymethyl branched long-chain methyl esters. No hydroxylated analogues were found in the mixture. Gas–liquid chromatography showed that the straight-chain esters were hexadecanoic and octadecanoic methyl esters, whereas the branched-chain esters co-chromatographed with phthienoic esters (Fig. 1) having 27 and 30 carbon atoms. Again, these structures were like those observed in the Canetti strain (Daffé *et al.*, 1988*b*).

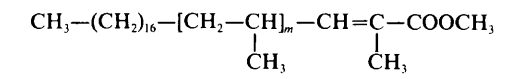

Fig. 1. Structures of the methyl phthienoates isolated from the apolar glycolipid 1 of *M. tuberculosis* strain 1350; $m = 2$ or 3.

Table 2. *Chromatographic characterization of glycolipids*

Developing phases used for thin-layer chromatography: chloroform/methanol (v/v) 99:1 (A), 95:5 (B), 90:10 (C) and 80:20 (D). The indicated solvents were selected according to the polarities of the various lipids, which co-chromatographed in the various solvents used.

				Occurrence in *M. tuberculosis*	
Glycolipid	R_F	Solvent	Identification	Canetti	Recent isolates*
1	0·50	A	Polyphthienoyl trehalose	+	+
2	0·54	B	Monoglycosyl phenol phthiocerol	+	ND
3	0·49	B	Triglycosyl phenol phthiocerol	+	+
4	0·35	C	Trehalose sulpholipid	+	+
5	0·44	D	Minor trehalose sulpholipid	+	+
6	0·14	D	Unidentified; most polar glycolipid	+	+

ND, Not detected.
* Strain 1350 and nine other clinical isolates (see Table 1).

The acylated positions in glycolipid 1 were identified by comparison of the ^{1}H-NMR spectra of the native and peracetylated glycolipid 1 to those of polyphthienoyl trehalose isolated from the Canetti strain (Daffé *et al.*, 1988*b*). Both spectra of native and modified glycolipid 1 were closely similar, if not identical, to the corresponding spectra of polyphthienoyl trehalose, suggesting a 2,2′,3′,4,6′-acyl trehalose structure for the native apolar glycolipid 1.

Structure of the phenolic glycolipid

Glycolipid 3 (Table 2) had the same chromatographic mobility as the triglycosyl phenol phthiocerol diester we have isolated from the Canetti strain (Daffé *et al.*, 1987). It also shared the same characteristic UV and IR absorption bands. UV absorption maxima were seen at 221, 273 and 280 nm and typical IR absorption bands were found at 1615, 1595, 1515 and 825 cm^{-1}, consistent with the phenolic structure (Daffé *et al.*, 1987, 1988*a*; Demarteau-Ginsburg & Lederer, 1963).

Comparison of the ^{1}H-NMR spectra of the intact and peracetylated glycolipid 3 with those of the triglycoside of the Canetti strain showed that they were the same compound. All the signal resonances were superimposable and identical values of coupling constants were found. It follows, then, that glycolipid 3 is a 2,3,4-tri-*O*-methylfucopyranosyl (α1→3)rhamnopyranosyl (α1→3)2-*O*-methylrhamnopyranosyl α1-linked to phenol phthiocerol dimycocerosate (Daffé *et al.*, 1987).

The minor related monoglycosyl phenol phthiocerol (mycoside B) previously characterized in the Canetti strain (Daffé *et al.*, 1988*a*) was not detectable in strain 1350, probably because of the small amount of the crude lipid extract used in this study since the latter strain produced tenfold less lipid material than the former.

Since identical data were obtained for glycolipids 4 and 5 whatever their origin (Canetti strain or 1350), the results to be presented refer to the glycolipids isolated from both strains.

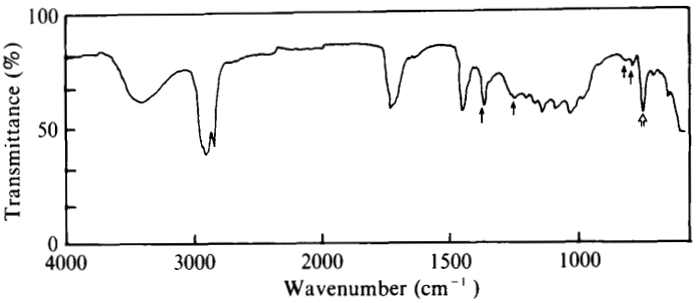

Fig. 2. IR spectrum of the major sulphatide (glycolipid 4) of *M. tuberculosis* strain 1350. Filled arrows point to the fine-structure characteristics of sulphatides with respect to the internal standard absorption band of $CHCl_3$ at 760 cm^{-1} (open arrow).

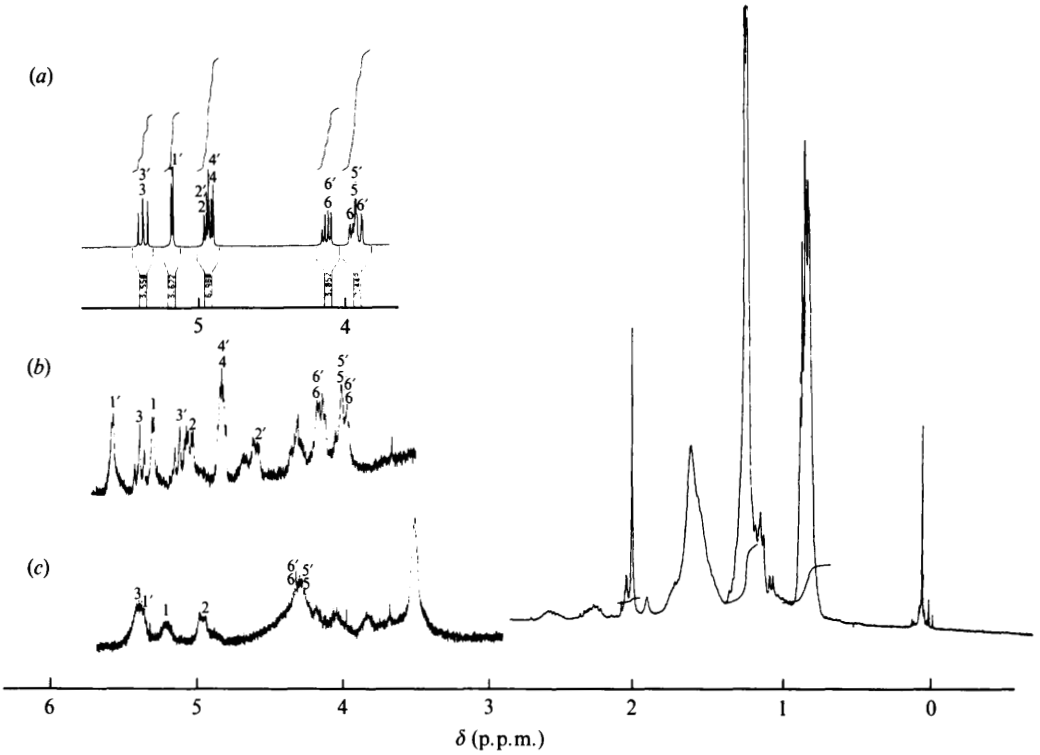

Fig. 3. ^{1}H-NMR spectra of the major sulphatide of *M. tuberculosis* strain 1350 (*a*), of its peracetylated derivative (*b*) and of peracetylated trehalose (*c*). 1,1′, 2,2′, etc., refer to protons located on carbons 1,1′, 2,2′, etc., of trehalose.

Structure of the major glycolipid

IR studies. The major glycolipid (compound 4, Table 2) exhibited a characteristic IR spectrum (Fig. 2): the presence of a deep absorption band at 1370 cm^{-1} due to a multiplicity of methyl branches, a weak band at about 808 cm^{-1} associated with absorption of α,α′-D-trehalose, and absorption bands centred at 1260 and 830 cm^{-1} typical of a sulphate group occupying a secondary equatorial position (Goren, 1984). Other absorption bands indicated the presence of hydroxyl group(s) (3500 cm^{-1}), ester function(s) (1740, 1170–1130 cm^{-1}) and polymethylenic chain(s) (725 cm^{-1}). Complete and rapid desulphation (by the method of Goren, 1984) induced the disappearance of the absorption band at 830 cm^{-1} but not of that at 808 cm^{-1}, and the significant change in the polarity of the glycolipid resulted in its migration with neutral lipids and polyacylated trehalose at the solvent front in thin-layer chromatography system C (Table 2).

NMR studies. ^{1}H-NMR spectroscopy (Fig. 3) was particularly informative. In the region of

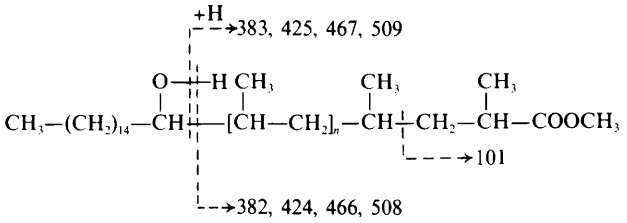

Fig. 4. The characteristic major fragments of methylhydroxyphthioceranates in mass spectrometry (electron impact ionization); $n = 6$ to 9.

0–1 p.p.m., the usual triplet assignable to the terminal methyl group protons centred at 0·87 p.p.m. was overlapped by a prominent doublet at 0·84 p.p.m. attributable to methyl branch protons. As compared to the broad singlet of the methylenic protons (1·25 p.p.m.), the number of methyl groups was estimated to be 0·4-fold that of methylene, suggesting the presence of highly branched fatty acyl substituents related to phthioceranyl and hydroxyphthioceranyl occurring in sulphatides (Goren *et al.*, 1971). In the region of anomeric and other deshielded protons (4·8–5·8 p.p.m., Fig. 3*a*) signals centred at 5·4, 5·2 and 4·9 p.p.m. were assignable to H-3, H-1 and H-2 protons of acylated trehalose, respectively, by comparison with the ^{1}H-NMR spectrum of polyacylated trehalose. The presence of a signal at 4·3 p.p.m. indicated that the 6,6′-positions of trehalose were acylated. It follows, then, that the probable location of fatty acyl substituents on trehalose are the 2,3,6 and 6′ positions, as previously proposed for the major sulphatide I of *M. tuberculosis* $H_{37}Rv$ on the basis of chemical degradations (Goren, 1984). The ^{1}H-NMR spectrum of the peracetylated glycolipid (Fig. 3*b*) as compared to that of peracetylated trehalose (Fig. 3*c*) or polyacylated trehalose (Daffé *et al.*, 1988*b*), confirmed the proposed structure. The occurrence of α,α′-trehalose was deduced from the coupling constant $J_{1,2} = 3{\cdot}8$ Hz and $J_{2,3} = J_{3,4} = 9{\cdot}6$ Hz. The presence of non-equivalent H-1 (5·3 and 5·6 p.p.m.) and H-3 (5·4 and 5·1 p.p.m.) suggested the location of the sulphate group in the 2′-position, in agreement with the chemical shift of H-2′ (4·6 p.p.m.).

Nature of the acyl substituents. Data from IR and NMR spectra of the major glycolipid suggested the occurrence of highly methyl-branched fatty acyl substituents. Examination of methyl esters (obtained after saponification followed by methylation) by thin-layer chromatography showed only one spot corresponding to hydroxylated long-chain methyl ester as judged by its migration, similar to that of *threo*-diunsaturated mycobacterial mycolates (2-branched, 3-hydroxy long-chain ester; see Daffé *et al.*, 1983). Comparison of the ^{1}H-NMR spectra (Fig. 3) to those of 6,6′-dimycoloyl trehalose (not shown) allowed us to conclude that no mycoloyl substituent was present in the glycolipid under study. The mass spectrum of the fatty methyl esters was very similar to that of hydroxyphthioceranates (Goren *et al.*, 1971). The cleavage pattern was readily interpretable as being due to a series of methyl esters having a common, essentially constant, C_{16} portion bearing the hydroxyl group and a variable multi-branched fragment having a homology pattern progressively increasing by 42 mass units (three carbon units) (Fig. 4). Very prominent peaks at m/z 88 and 101 indicated an α-branched structure. The other significant peaks were mainly seen at m/z 382 (the major component), 424, 466 and 508, corresponding to the principal charged fragments M-240 (minus palmitaldehyde, Fig. 4) and companion peaks of almost equal intensity at M − 239. Consequently, the major constituent of the fatty acyl substituents is a 17-hydroxy-2,4,6,8,10,12,14,16-octamethyldotriacontanoyl (C_{40}). The other components contain a total of 9, 10 and 11 methyl branches. These were the only types of fatty acids in the major sulphatide of strain 1350. In prevous studies, on strain $H_{37}Rv$ (Goren *et al.*, 1971), they constituted only 50% of the fatty acyl substituents of its major sulphatide I, the other fatty acyl moieties being methyl-branched and straight-chain compounds.

It is assumed that all the methyl-branched chiral centres of dextrorotatory phthioceranates and hydroxyphthioceranates are related to the L series (Goren *et al.*, 1971; Asselineau & Asselineau, 1978; Asselineau, 1982) and that the D configuration is the most probable for the hydroxyl-bearing asymmetric centre (Asselineau & Asselineau, 1978; Goren, 1984).

Table 3. *Reaction of purified glycolipid from M. tuberculosis (Canetti strain and wild strains) with Canetti whole-cell antiserum*

Glycolipid tested*	Antiserum dilution†
1	NR
2	1/250
3	1/2500
4	1/500
5	1/100

NR, Negative reaction.

* 1 to 5 refer to the glycolipids quoted in Table 2.

† Serum dilution giving an ELISA A_{420} value of 1·00. The purified antigens of all the strains tested were applied at exactly the same amount in the wells (100 ng per well); the titres were exactly the same for all, as indicated.

Structure of a minor sulphatide

Glycolipid 5 (Table 2) presented an IR spectrum very similar to that of the major sulphatide, glycolipid 4 (Fig. 2), indicating a sulphatide structure. Its ^{1}H-NMR spectrum showed that the triplet assignable to the terminal methyl groups occupied a common position at 0·87 p.p.m.; the doublet attributable to other methyl branches centred at 0·84 p.p.m. was absent from the spectrum, suggesting the occurrence of straight-chain fatty acyl substituents. In the region of anomeric and other deshielded protons (4·8–5·8 p.p.m.), signals were observed at 5·4, 5·3 and 4·8 p.p.m., indicating a 2,3-substituted trehalose structure, since the doublet at 5·3 p.p.m. (H-1) had a $J_{1,2}$ constant value similar to that of trehalose ($J_{1,2} = 4$ Hz). No significant signal was detected at 4·2 p.p.m.; this indicated that 6 and 6′ hydroxyls were not esterified.

Desulphation induced only partial removal of the sulphate group, whereas complete desulphation occurred for the major sulphatide in the same conditions. Such relative resistance to desulphation typified a minor polar sulphatide III as observed previously in strain H_{37}Rv (Goren, 1984).

The analysis of fatty acid methyl esters by gas–liquid chromatography showed that they consisted mainly of palmitate (C_{16}) and stearate (C_{18}); small amounts of higher homologues (C_{20}, C_{22}) were also present. The probable structure of glycolipid 5 is then a 2,3-diacyltrehalose 2′-sulphate. It would correspond to the more polar uncharacterized sulphatides observed in strain H_{37}Rv (Goren *et al.*, 1978).

The structure of the most polar glycolipid (compound 6, Table 2) is presently under study.

Immunoreactivity of the glycolipids

Inoculation of whole cells (Canetti strain) into rabbits produced IgG class antibodies (Papa *et al.*, 1988). The production of antibodies raised against each one of the glycolipids (Table 2) was monitored by an ELISA system (Table 3). Glycolipid 1 (polyphthienoyl trehalose) did not immunoreact with the antiserum. In previous work, glycolipids 2 and 3 (Table 2) – the monoglycosyl (Daffé *et al.*, 1988*a*) and the triglycosyl phenol phthiocerol diester (Daffé *et al.*, 1987) – were shown to be antigenic. In this work, the immunoreactivity of glycolipids 2 and 3 was confirmed (Table 3) and the sulphatides (glycolipids 4 and 5, Table 2) also proved to be antigenic (Table 3). The antisera obtained against the Canetti strain reacted similarly with the antigens isolated from the Canetti strain and with those extracted from the ten wild strains tested.

DISCUSSION

When a triglycosyl phenolic glycolipid was characterized in the Canetti and similar strains of *M. tuberculosis* (Daffé *et al.*, 1987) it was anticipated and confirmed that it was a useful antigen both for the identification of clinical isolates (Papa *et al.*, 1988, 1989) and the immunodiagnosis of tuberculosis (Torgal-Garcia *et al.*, 1988). The occurrence of the phenolic glycolipid antigen in wild strains of tubercle bacilli was detected using serological methods and its chemical structure was partially established (Papa *et al.*, 1989).

It has been already noted (Daffé & Lanéelle, 1988) that diacyl phenol phthiocerol was present in *M. tuberculosis* Type strain $H_{37}Rv$, but no glycosidic derivative of this lipid was detected. The same observation is true for type strains of *M. bovis* and of *M. marinum*. It was thus suggested that a wide survey of *M. tuberculosis* strains and clinical isolates could lead to the definition of neotypes.

The present study showed that the phenolic glycolipid partially characterized by thin-layer chromatography from wild strains of tubercle bacilli (Papa *et al.*, 1989) had exactly the same chemical structure as the triglycosyl phenolic glycolipid described in the Canetti strains.

During our previous studies other glycolipids were found in lipid extracts of tubercle bacilli (Papa *et al.*, 1988, 1989). The structures of three of them are now established, and they are found to be acylated trehaloses: a polyphthienoyl trehalose and two families of sulphatides (acylated trehalose 2′-sulphate). An antiserum prepared against whole cells of *M. tuberculosis*, Canetti strain, contained antibodies reacting with the sulphatides, but not with the non-sulphated acyl trehalose, from ten wild strains.

Thus the data both from thin-layer chromatography and from immunogenicity studies in the present work indicated without ambiguity that the glycolipid fractions found in Canetti strain (Daffé *et al.*, 1987, 1988*b*) were also found in wild strains of tubercle bacilli. Furthermore, the data confirmed the structures of trehalose containing glycolipids which were reported to be characteristic of virulent tubercle bacilli (Dhariwal *et al.*, 1984; Goren, 1984).

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