Lack of correlation between colony morphology and lipooligosaccharide content in the *Mycobacterium tuberculosis* complex

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Rough and smooth colony variants of the *Mycobacterium tuberculosis* complex were compared with respect to their composition in trehalose-containing glycolipid antigens in view of the results of a recent investigation suggesting that the chemical basis of rough and smooth colony morphology in mycobacteria may reside in the occurrence of lipooligosaccharides. A careful chemical characterization of the individual glycolipids of the selected strains allowed the identification of the major glycolipids. The comparative study of the glycolipid content of the smooth Canetti strain, its spontaneous rough variant, and 16 additional strains of *M. tuberculosis*, *M. bovis* and *M. africanum* showed that the presence of lipooligosaccharides was not related to the morphology of the colonies.

Introduction

Mycobacterium tuberculosis is by far the most important of the mycobacterial pathogens and the one that has been responsible over the centuries for most of the mycobacteria-caused morbidity and mortality among human beings (Youmans, 1979). Consequently, notable efforts have been devoted to the tuberculosis elimination programme, which includes the early detection of the disease. In this respect, species or type-species-specific glycolipid antigens of *M. tuberculosis* have been chemically and serologically characterized (Cruaud *et al.*, 1989, 1990; Daffé *et al.*, 1987, 1989, 1991*a, b*; Lemassu *et al.*, 1991; Martin-Casabona *et al.*, 1989; Papa *et al.*, 1989*a, b*; Torgal Garcia *et al.*, 1988, 1989). The most antigenic of these (Daffé *et al.*, 1991*b*) belongs to the newest class of alkali-labile and phosphorus-free neutral glycolipids based on glycosidically linked acyl trehalose, the so-called lipooligosaccharides (LOS) (McNeil *et al.*, 1989). The highly antigenic LOS described in *M. tuberculosis* strain Canetti (Daffé *et al.*, 1991*b*) consisted of a lipononasaccharide with its own unique acyl amino epitope and a distinctive 6-*O*-methyl triacyl trehalose

unit. This antigen was found typifying the relatively smooth Canetti strain but was not detected in two typical rough strains of *M. tuberculosis*, namely H₃₇Ra and H₃₇Rv (Daffé *et al.*, 1991*b*). Interestingly, LOS have been characterized in three smooth variants of *M. kansasii* whereas the two examined rough strains of this species were devoid of the antigens (Belisle & Brennan, 1989). In view of these preliminary indications, the present study was undertaken in order to ascertain the possible correlation between the occurrence of LOS and the morphology of the colonies in the *M. tuberculosis* complex. Eighteen strains, including the smooth colony-forming Canetti strain and its spontaneous rough mutant were examined for their glycolipid content and the smoothness and roughness of their colonies. The purpose of using rough and smooth colony-forming strains derived from the Canetti strain was to have isogenic strains to test the relationship between colony morphology and glycolipid composition.

Methods

Strains and growth conditions. The strains of *M. tuberculosis*, *M. africanum* and *M. bovis* listed in Table 1 were grown on Sauton's medium (Sauton, 1912) as surface pellicles at 37 °C for 4 weeks. All the strains were examined for colony characteristics after 3–4 weeks of growth on Middlebrook 7H10 agar plates. A rough variety which

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Abbreviations: DAT, 2,3-diacyl trehalose; GPL, glycopeptidolipids; LOS, lipooligosaccharides; MMT, monomycocoyl trehalose; PGL, phenolic glycolipids.

appeared spontaneously from the smooth Canetti strain was picked directly from the plate and then subcultured on Middlebrook 7H10 agar plates to establish stability of its colony morphology.

Extraction and purification of glycolipids. Bacteria harvested from surface pellicles were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v) at room temperature for 1 week; the bacterial residues were then re-extracted twice for 2 d with the same solvent mixture.

Pooled extracts were dried and then partitioned using $\text{CHCl}_3/\text{H}_2\text{O}$ (1:1, v/v); the lower CHCl_3 phases were dried and the resulting washed lipids were dissolved in CHCl_3 (5 ml) and applied to either a silicic acid column (55 × 2.1 cm) or to a diethylaminoethyl (DEAE) cellulose column (58 × 2.5 cm). The columns were successively eluted with 100 ml CHCl_3 , $\text{CHCl}_3/\text{CH}_3\text{OH}$ (9:1, 8:2, 7:3, 1:1, v/v) and then with 100 ml $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65:25:4, by vol). For the DEAE column, further elutions were done with 100 ml $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4:1, v/v) containing NH_4OH (2%, w/v) and $\text{CH}_3\text{COONH}_4$ (0.01 M). The collected fractions (100 ml each) were dried and analysed by TLC.

Purified glycolipids were obtained by re-applying crude glycolipid fractions in CHCl_3 (2 ml) to a silicic acid column (50 × 1.2 cm) and eluting with the same solvent mixtures (10 ml each). Final purification was achieved by reverse phase chromatography using C-18 Sep-pak cartridges (Waters).

Analytical chromatographic techniques. TLC of chromatographic fractions was performed on precoated TLC plates of silicagel 60 on aluminium sheets (layer thickness 0.2 mm, 20 × 20 cm, Merck).

Native glycolipids were separated in polar solvent mixtures: $\text{CHCl}_3/\text{CH}_3\text{OH}$ (9:1, 7:3, v/v), $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (30:8:1, 30:12:1 and 65:25:4, by vol.). Glycolipids were detected by spraying with 0.2% anthrone in concentrated H_2SO_4 followed by heating at 110 °C. The Dittmer-Lester reagent (Dittmer & Lester, 1964) was used for revealing phosphorus-containing substances.

Fatty methyl esters were analysed using CH_2Cl_2 as previously described (Daffé *et al.*, 1983).

GLC was performed using a Girdel chromatograph (model G-30) equipped with a 25 m capillary column (0.22 mm i.d.) containing WCOT-OV1 (0.3 µm film thickness, Spiral, Dijon, France); fatty methyl esters were separated with a temperature gradient of 150–290 °C (2 °C min⁻¹) whereas acetate derivatives of methyl glycosides were analysed using a temperature gradient of 130–290 °C (2 °C min⁻¹).

Miscellaneous analytical techniques. Alkaline hydrolysis of glycolipids was performed as previously described (Daffé *et al.*, 1983). The water soluble material was desalted by mixing with Dowex 50WX4 resin (Fluka AG, Switzerland) (H^+), filtered and dried.

Acid hydrolysis was performed with 1 M-HCl in CH_3OH for 3 h at 80 °C. The solution was then evaporated under vacuum.

Sugars were analysed as their peracetyl derivatives obtained by an overnight reaction with acetic anhydride/pyridine (1:1, v/v) at room temperature and as their trimethylsilyl derivatives (Sweeley *et al.*, 1963).

NMR spectra were obtained in $\text{CDCl}_3/\text{CD}_3\text{OD}$ (2:1, v/v) with a Bruker 200 instrument at 23 °C. IR spectra were recorded on a Perkin-Elmer instrument model FTIR.

Mass spectra were obtained by the electron-impact technique using a Varian MAT 311A spectrometer; an ionizing voltage of 70 eV and a temperature range of 100–250 °C were used as previously described (Daffé *et al.*, 1984).

For radiolabelling experiments with ^{35}S , 1 mCi of $\text{Na}_2[^{35}\text{S}]\text{O}_4$ was added per litre of medium, the SO_4^{2-} content of the medium was lowered by replacing half of the MgSO_4 by MgCl_2 (Goren, 1970). Bacteria, appearing as veils of growth in the medium after 4 weeks of growth, were extracted and the lipid extract fractionated as described above. To locate the radioactivity on the TLC plates (Lemassu *et al.*, 1991), an automatic TLC linear analyser Berthold LB 2832 was used.

Desulphation was performed as previously described (Daffé *et al.*, 1989).

Results

Isolation of rough and smooth colonies from the Canetti strain of M. tuberculosis

From among the mass of smooth colonies on Middlebrook 7H10 agar plates that characterize the Canetti strain (Fig. 1a), a rough variety appeared spontaneously, a common occurrence reported in cultures of smooth colony-forming mycobacterial strains (Barrow & Brennan, 1982; Fregman *et al.*, 1962; Grange, 1973). Repeated subculturing of the rough variant resulted in a culture containing only rough colonies (Fig. 1b) whereas subcultures of smooth colonies failed to give smooth colonies solely. The mutation event which lead to the rough variety does not affect the biochemical characteristics of the strain (Table 1) suggesting that simple mutation events have occurred (Grange, 1973). Other strains of the tubercle bacillus were selected for the present study because of the morphology of their colonies and their biochemical features (Table 1).

Chemical characterization of LOS and other trehalose-containing glycolipids

Using the chemically defined lipoocta- (LOS-I), and lipononasaccharide (LOS-II) isolated from the Canetti strain (Daffé *et al.*, 1991b) as TLC standards, several glycolipids of similar polarity were detected in the crude lipid extracts of the examined strains (Fig. 2a). The TLC patterns of the various strains looked very similar and no obvious difference was noted between the lipid content of the various strains, regardless of the morphology of the colonies they formed (Fig. 2a). In order to analyse the distribution of the LOS in the selected strains, attempts were made to fractionate the crude extracts. Because of the similar polarity of the glycolipids under study, ion exchange chromatography was first applied, instead of adsorption chromatography. Using a DEAE column, it was possible to distinguish neutral from acidic glycolipids (Fig. 2b). By this procedure, it appeared that some glycolipids having chromatographic behaviour similar to that of LOS were in fact acidic compounds. These acidic glycolipids either contained phosphorus or could be labelled with ^{35}S and could also be desulphated suggesting that these acidic glycolipids were composed of phosphatidyl inositol mannosides and sulphatides.

To distinguish the multiglycosylated version of LOS from miscellaneous acyl trehaloses, the neutral glycolipids were further fractionated by chromatography on a

Fig. 1. Smooth colonies of *M. tuberculosis* strain Canetti (a) and rough colonies of its spontaneous mutant (b) on Middlebrook 7H10 agar plates. Bars, 1 mm.

silicic acid column and the purified glycolipids were analysed by ^1H -NMR and IR spectroscopy.

The least polar neutral glycolipid (Fig. 2b) was found to be identical to 6,6'-dimycoloyl trehalose ('cord factor') as it gave the same typical colour characteristic of this compound (Daffé *et al.*, 1989) and had the same chromatographic behaviour in all the solvent mixtures used.

A more polar neutral glycolipid (Fig. 2b) gave spectra similar to those obtained previously for 2,3-diacyl trehalose (DAT) (Lemassu *et al.*, 1991) and the fatty acyl substituents as well as the carbohydrate moiety were identified by GLC and were found to be identical to those of DAT. The compound with a slightly lower mobility than DAT (Fig. 2b, lanes 7 and 8) contained cyclopropane rings as deduced from its IR (absorption

bands at ν 3060 and 1020 cm^{-1}) and NMR (signals at δ 0.3, 0.1 and 0.6 p.p.m.) spectra. Analysis of its constituents gave trehalose and mycolic acids identified by TLC and GLC, and by TLC and mass spectrometry (Daffé *et al.*, 1983, Toubiana *et al.*, 1979), respectively. By comparison with an authentic standard 6-mycoloyl trehalose sample, it was deduced that the glycolipid under study was a monomycoloyl trehalose (MMT). Two other more polar glycolipids (Fig. 2b), detected in the Canetti strain and another strain of *M. tuberculosis* forming smooth colonies (Table 1), were identified by TLC and GLC of their constituents as LOS-I and LOS-II. The IR spectra of both purified LOS allowed the discrimination between the two glycolipids, i.e. LOS-II, but not LOS-I, exhibited the two characteristic absorption bands at 1690 and 1540 cm^{-1} consistent with the

Table 1. Biochemical characteristics and distribution of trehalose-containing glycolipids in smooth (S) and rough (R) variants of *M. tuberculosis*, *M. bovis* and *M. africanum*.

Strain*	Morphology	Biochemical tests†							Glycolipids‡		
		Niacin	TCH	PZA	Pyr	Lebek	Glcase	Nit	DAT	MMT	LOS
<i>M. tuberculosis</i>											
CIPT 140010001	R	+	+	—	—	—	+	+	+	+	—
CIPT 140010045	R	+	—	—	—	—	+	+	—	+	—
CIPT 140010068	R	+	—	—			—	+	+	+	—
CIPT 140010071	R	+	+	+					—	+	—
CIP 88628	R	+	—					+	+	—	+
CIPT 140010059	S	—	+	—	—	—	+	+	+	—	+
CIPT 140010059R	R	—	+	—	—	—	+	+	+	—	+
CIPT 140010060	S	—	+	—	—	—	+	+	+	—	+
CIPT 140010061	S	—	+	—	—	—	+	+	—	+	—
CIPT 140010062	S	—	+	—	—	—	+	+	—	+	—
<i>M. bovis</i>											
CIPT 140020001	S	—	—	+	+	+	—	—	+	+	—
CIPT 140020002	S	—	—	+	+			—	+	+	—
CIPT 140020005	R	—	+	+				+	—	+	—
CIPT 140020010	R	—	—	+				—	+	+	—
CIPT 140020011	R		—	+					—	+	—
CIPT 140040001	R	—	—	+	+	—	—	—	tr	+	—
<i>M. africanum</i>											
CIPT 140030001	S	—	—	—	+	+	—	—	—	+	—
CIPT 140030003	R	+	+	—			—	+	+	+	—

* Strains CIPT 140010001, -20001 and -30001 correspond to type strains of *M. tuberculosis* (ATCC 27294, H₃₇Rv), *M. bovis* (ATCC 19210) and *M. africanum* (ATCC 25420), respectively. The original Canetti strain is labelled CIPT 140010059 and Canetti-like strains are referred to as CIPT 140010060, -10061 and -10062. The spontaneous rough variant of the Canetti strain is labelled CIPT 140010059R. Strain CIPT 140010045 was received from Dr Szabo (Budapest, Hungary) and strain CIPT 140040001 corresponds to BCG Pasteur. ATCC, American Type Culture Collection, Rockville, MD, USA; CIPT, Collection Institut Pasteur Tuberculose, Paris, France; CIP, Collection Institut Pasteur, Paris, France.

† Biochemical tests: Niacin, detection of niacin; TCH, growth in the presence of thiophencarboxylic acid hydrazide (2 µg ml⁻¹); PZA, growth in the presence of pyrazinamid (100 µg ml⁻¹); Pyr, stimulation of growth by pyruvate; Lebek, microaerophily; Glcase, detection of β-glucosidase; Nit, detection of nitrate reductase.

‡ (+) indicates the detection of the glycolipids by TLC after fractionation of the crude lipid extracts by chromatography on a silicic acid column; tr, trace amounts.

presence of an aminosugar involved in the antigenicity of LOS-II (the characteristics of authentic LOS-I and -II had been established previously; Daffé *et al.*, 1991b). In a rough colony-forming *M. tuberculosis* strain (CIP 88628) a neutral glycolipid which had similar TLC and GLC properties as LOS-II was detected (data not shown). The purified glycolipid was shown to be a multiglycosylated lipooligosaccharide by NMR. Its glycosyl composition was found to consist of 2-O-methylfucose, 2-O-methyl rhamnose, rhamnose and glucose in a relation ratio 1:1:2:1. Hexa- and octadecanoic acids were the main fatty acids identified by GLC. It follows then that the glycolipid under study is a novel member of the LOS family.

Distribution of the LOS in the *M. tuberculosis* complex

LOS was detected in only a few strains of *M. tuberculosis* (Table 1). All the *M. bovis* and *M. africanum* strains were

devoid of LOS regardless of the morphology of the colonies, demonstrating that this class of glycolipid antigens is not the main factor implicated in the morphology of the colonies. This conclusion was confirmed by the comparative analysis of the glycolipids of the original (smooth colony-forming) Canetti strain and its rough colony-forming variant (Fig. 2b, lanes 1–6). The compounds co-migrating with LOS (Fig. 2, lane 5) were isolated from the mutant strain and analysed. Their IR as well as their constituents, i.e. the sugar and fatty acid derivatives, were found to be identical to those of LOS of the Canetti strain (established previously; Daffé *et al.*, 1991b). Incidentally, the phenolic glycolipids (PGL) previously characterized in the Canetti strain (Daffé, 1989; Daffé *et al.*, 1987, 1988, 1991a) were found in its spontaneous mutant (data not shown). In addition to the two isogenic strains, LOS was characterized in only one smooth and one rough colony-forming strain of *M. tuberculosis*. Again, it was observed that two strains of

Fig. 2. (a) TLC of the unfractionated lipids of selected strains of *M. tuberculosis*. Lanes: 1 and 3, smooth colony-forming strains; 2, 4 and 5, rough colony-forming strains; 6, mixture of authentic standard samples of LOS-I and II isolated from the Canetti strain. Developing solvent: $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (60:35:8, by vol.). (b) TLC of the crude lipid extracts of *M. tuberculosis* strain Canetti (lane 1), its spontaneous rough variant (lane 4) and H37Rv (lane 7). The corresponding neutral (lanes 2, 5 and 8) and acidic (lanes 3, 6 and 9) glycolipid-containing fractions eluted from DEAE columns with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (8:2, v/v) and with ammonium salt-containing solvents, respectively, are shown. Developing solvent: $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (30:12:1, by vol.). CF, 'Cord factor' (6,6'-dimycocoyl trehalose); SL, sulphatides; PIMs, phosphatidylinositol mannosides. The arrowheads indicate the solvent fronts. The plates were sprayed with anthrone followed by heating at 110°C for 5 min.

M. tuberculosis forming smooth colonies were devoid of LOS.

Distribution of miscellaneous acyl trehaloses

The acyl trehaloses (DAT or MMT) were more widely distributed in the tubercle bacilli than LOS (Table 1). DAT was detected and chemically characterized in some, but not all, strains examined herein (Table 1). MMT was detected in more strains of the *M. tuberculosis* complex than DAT (Table 1). However, the occurrence of both acyl trehaloses was not related to the morphology of the colonies. Similarly, 'cord factor' was detected in both smooth and rough colony-forming variants of the

Canetti strain (Fig. 2b), indicating that the presence of the ubiquitous glycolipid was not correlated with the smoothness and roughness of the tubercle bacillus colonies.

Discussion

The common objective of the earliest and the present-day investigations on mycobacterial lipids is the identification of specific components that might be implicated with the physiology of mycobacteria or biological events in the pathogenesis of diseases caused by these organisms. The dissociation of the tubercle bacilli into rough

and smooth colonial forms offered the opportunity to correlate the virulence of the strains, the morphology of the colonies and chemically defined compounds (Long, 1958). The most notable of the earlier work, conducted by Cooper (1930), suggested that an avirulent rough variant of the tubercle bacillus (BCG) produced more lipid than the smooth virulent type dissociated from the same culture. Further studies correlated the presence of a class of surface antigens, the so-called glycopeptidolipids (GPL) (Barrow & Brennan, 1982) or glycolipids (Fregman *et al.*, 1962) to the smooth morphology of *M. avium* and *M. intracellulare*, and *Mycobacterium* sp., respectively. With the finding of a spontaneous rough mutant that was identical to the parent smooth variant with respect to the GPL content (David *et al.*, 1987), the correlation between the morphology of the colonies of *M. avium* and the presence of GPL was questioned. In the case of *M. tuberculosis*, GPL cannot be detected; instead, PGL (Daffé, 1989; Daffé *et al.*, 1987, 1988, 1991a) and LOS (Daffé *et al.*, 1991b) were characterized in some of the strains. Although the specific PGL of *M. tuberculosis* were originally isolated from a smooth variant (Daffé *et al.*, 1987), their presence in other typical rough tubercle bacilli has since been demonstrated (Daffé *et al.*, 1989, 1991a; Papa *et al.*, 1989b). The spontaneous rough variant of the Canetti strain raised in this study also contains PGL, thus confirming that the occurrence of this class of glycolipid antigens is not related to the morphology of the tubercle bacilli. In contrast, LOS were found to occur on smooth variants of *M. kansasii*, but not in rough colony-forming strains (Belisle & Brennan, 1989), again suggesting a correlation between this type of mycobacterial glycolipids and the morphology of the colonies. However, LOS in *M. tuberculosis* has recently been well characterized (Daffé *et al.*, 1991b) and in this paper we show that both the rough and smooth isogenic variants of the Canetti strain contain this class of glycolipids. It remains possible that the quantity of LOS may affect colony morphology, but the absence of LOS in most strains of the *M. tuberculosis* complex examined, both rough and smooth colony-forming (see Table 1), clearly shows that this class of glycolipids is not the major factor affecting the morphology of these strains. In agreement with this finding is the observation that LOS are not major constituents of the smooth Canetti strain (2% of the crude lipid extract; Daffé *et al.*, 1991b).

The morphology of mycobacterial colonies has also been often correlated to the pathogenicity of the strains (Cooper, 1930; Collins & Cunningham, 1981; Schaefer *et al.*, 1970). In the case of the tubercle bacillus, the dissociation of the human tubercle bacillus H₃₇ into its extreme rough variants, virulent (Rv) and avirulent (Ra) (Dréa, 1953), shows that the earlier association between smooth and virulence on the one hand, and rough and

avirulence on the other hand (Cooper, 1930), is not always reliable. The similar behaviour of the Canetti strain and H₃₇Rv in experimental infections (Papa *et al.*, 1989b) confirms the concept that both rough and smooth variants may be virulent strains.

Beside these observations, another conclusion can be drawn from our data. Through the characterization of the different glycolipids, it appeared that the unfractionated lipid extracts of the tubercle bacilli contain complex mixtures of various classes of glycolipids. This may lead to misinterpretations of both serologic and chemical data if further fractionation and characterization are not done. TLC alone may not be enough as we found acidic and neutral glycolipids as well as trehalose derivatives and multiglycosylated compounds had co-eluted.

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