The Free Lipids of *Mycobacterium leprae* Harvested from Experimentally Infected Nine-banded Armadillos

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The free lipids of a sample of *Mycobacterium leprae* were extracted by a procedure designed to produce separate non-polar and polar fractions. The composition of these lipids was analysed semi-quantitatively by five special thin-layer chromatographic systems covering the total range of mycobacterial lipid polarities. In order of increasing polarity, the major lipids were dimycocerosates of phthiocerol A, phthiocerol B and phthiodione A, glycosyl phenolphthiocerol dimycocerosates and phospholipids, including monoacylphosphatidylinositol di- and pentamannosides. The diacylated forms of these latter lipids, found in most mycobacteria, were not present. The composition of the free lipids of the leprosy bacillus, surveyed over the total polarity range for the first time, showed that the patterns were particularly related to those of *Mycobacterium botis*, *Mycobacterium kansasii* and *Mycobacterium marinum*.

**INTRODUCTION**

Hansen's bacillus, the cause of human leprosy, has usually been given the name *Mycobacterium leprae*. Until recently, however, the scientific support for this name has been slender, since the organisms have never been grown in bacteriological media and human sources are limited. Production of biomass from experimental infection in the nine-banded armadillo has permitted a rapid recent increase in knowledge.

It is now fairly certain on immunological and chemical grounds that *M. leprae* is a member of the genus *Mycobacterium* but its relationship to other mycobacteria requires further investigation. Limited information from DNA hybridization suggests that it is not closely related to any of the known species (Imaeda et al., 1982; Athwal et al., 1984), though differences in genome size and gross composition of the DNA make this finding difficult to interpret.

A traditional approach to mycobacterial classification is the use of complex lipids as taxonomic markers (see, for example, Minnikin & Goodfellow, 1980; Minnikin, 1982; Dobson et al., 1985). A recently developed procedure (Dobson et al., 1985) makes it possible to separate, and roughly quantify, the complete polarity range of mycobacterial lipids from small samples. Its application to *M. leprae* is described here.

**METHODS**

A 20 mg freeze-dried sample (AB 14) of *M. leprae* was purified from an armadillo liver as described previously (World Health Organization, 1975). Free lipids were extracted by a two-stage procedure described previously (Minnikin et al., 1984; Dobson et al., 1985). Treatment with a biphasic mixture of petroleum ether and methanolic saline gave non-polar lipids in the upper petroleum ether layer. Further extraction of the lower layer and the residual cells with chloroform/methanol/water mixtures gave polar lipids. The non-polar lipid extract was taken to dryness, redissolved in chloroform (120 µl), and samples (20 µl) were applied to the corners of five 6.6 × 6.6 cm pieces of Merck 5554 silica gel TLC aluminium sheet. The polar extract was redissolved in 120 µl chloroform/methanol (2:1, v/v) and samples (20 µl) were applied to the corners of six similar TLC sheets. The spotted chromatograms were developed in two dimensions using the selected solvent systems (Dobson et al., 1985) given in Fig. 1, which also contains details of the spray reagents used for detection. As shown in Fig. 1, selected
standard lipids were chromatographed in one dimension on the sides of the two-dimensional TLC plates. For the solvent systems used for the analysis of the non-polar extract, portions of the chromatograms containing material migrating to the solvent front or remaining at the origin were cut out before spraying so that valuable lipid material could be saved for further study.

RESULTS AND DISCUSSION

A semi-quantitative profile of the free lipids of \textit{M. leprae} is shown in Fig. 1. The least polar solvent system (Fig. 1 \( a \)) demonstrates the presence of large proportions of waxes corresponding to dimycocerosates of phthiocerols \( A \) and \( B \) and phthiodiolone \( A \) (Draper et al., 1983; Minnikin et al., 1983, 1985b). Early studies demonstrated the possible presence of phthiocerol dimycocerosates in \textit{M. leprae} and lepromatous leprosy skin biopsies (Draper, 1980; Young, 1981, 1982) and a dimycocerosate of phthiocerol \( A \) was shown recently (Draper et al., 1983; Hunter \& Brennan, 1983) to have a structure different from that of the corresponding wax from \textit{M. tuberculosis}. Dimycocerosates of phthiocerol \( B \) and phthiodiolone \( A \) have not been reported previously in \textit{M. leprae} and their presence has been confirmed in a study of the mycolic acids of three preparations of \textit{M. leprae} from separate armadillos (Minnikin et al., 1985a). Analyses of the essential structures of diesters of the phthiocerol family from \textit{M. bovis}, \textit{M. kansasii}, \textit{M. marinum}, \textit{M. microti} and \textit{M. tuberculosis} (Minnikin et al., 1985b) and \textit{M. ulcerans} (Daffé et al., 1984) have confirmed that the wax from \textit{M. leprae} (Draper et al., 1983; Hunter \& Brennan, 1983) is structurally distinct.

No evidence was obtained for the presence of menaquiones in the non-polar extract of \textit{M. leprae}; the chromatographic mobility of menaquinone corresponds closely to that of vitamin \( K_4 \) (Fig. 1). Mycobacteria examined to date usually contain a dihydrogenated menaquinone with nine isoprene units (Minnikin, 1982). The minor component \( (X_1) \) in Fig. 1 \( a \) was not positively identified but it corresponds roughly in chromatographic mobility with fatty acid esters of cholesterol.

The dominant lipids in the next most polar TLC solvent system (Fig. 1 \( b \)), used to examine the non-polar lipid extract, were unesterified fatty acids. A major glycolipid, however, co-chromatographed with the standard glycosyl phenolphthiocerol dimycocerosate from other samples of \textit{M. leprae}. This lipid was accompanied by two minor glycolipids and traces of some unidentified components. The presence of a glycosyl phenolphthiocerol in \textit{M. leprae} was indicated by Draper (1980) and Young (1981, 1982) and the structure was determined by Hunter et al. (1982). The structure of the major glycosyl phenolphthiocerol from \textit{M. leprae} (Hunter et al., 1982) is distinct from those previously characterized from \textit{M. bovis}, \textit{M. kansasii} and \textit{M. marinum} (see Minnikin, 1982) and the present TLC system allows all of these lipids to be distinguished from each other (Dobson et al., 1985).

Free cholesterol migrates near the position of component \( X_2 \) in this chromatographic system (Fig. 1 \( b \)) but no positive identification was made. In a previous study (Asselineau et al., 1981) cholesterol comprised 30\% of the lipids remaining after saponification, the remainder being mycolic acids (3\%) and non-hydroxylated fatty acids (66-5\%). If components \( X_1 \) (Fig. 1 \( a \)) and \( X_2 \) (Fig. 1 \( b \)) corresponded to cholesterol esters and free cholesterol it is apparent that they are present in very low proportions. Cholesterol and its derivatives would be derived from host tissue and, while incorporation into bacterial cellular lipids is possible, the amounts present may vary depending on the procedure used for the isolation of \textit{M. leprae}. Cholesterol and its esters are never more than minor components of lipids from \textit{M. leprae} purified by the method used in this report (S. N. Payne, personal communication).

The third system (Fig. 1 \( c \)) used to examine the non-polar lipid extract is designed to detect the presence of acylated trehalase sulphates and trehalose dimycolates, the so-called 'cord-factors' (Minnikin, 1982; Dobson et al., 1985). The present \textit{M. leprae} sample (Fig. 1 \( c \)) did not contain components corresponding to either sulpholipids or cord-factors but three unidentified lipids \( (G_3, G_4, G_5) \) were observed. These latter lipids gave a brown colour with the \( \alpha \)-naphthol/sulphuric acid reagent for sugars; trehalase sulphates and cord-factors give a characteristic blue colour with this reagent. It has been claimed that sulpholipids are widespread in mycobacteria (Prabhudesai et al., 1981), including \textit{M. leprae} (Khuller et al., 1982), but
Fig. 1. TLC analysis of free lipids of *M. leprae*. (a–c) Non-polar extract and (d–f) polar extract. Abbreviations: A, B and C, dimycocerosates of phthiocerol A, phthiocerol B and phthiodiolone A, respectively; A', phthiocerol A dimycocerosates from *M. tuberculosis*; VK, vitamin K₁ (Sigma); X₁, X₂, unknowns; M, glycosyl phenolphthiocerol dimycocerosate from *M. leprae*; FA, fatty acids; G₁–G₃, unidentified glycolipids; S, acylated trehalose sulphate from *M. tuberculosis* H₃₇Rv; G', acylated trehalose from *M. tuberculosis* H₃₇Rv; DPG, diphasphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; MPIDM, mono-acylphosphatidylinositol dimannoside; MPIPM, mono-acylphosphatidylinositol pentamannoside; PIM, minor phosphatidylinositol mannannoside; P₁, P₂, unknown phospholipids; P₃, unknown phospholipid reacting also with periodate-Schiff reagent; ?, unknown lipids detected only with molybdophosphoric acid spray. Two-dimensional solvent systems (Dobson *et al.*, 1985): (a) petroleum ether (b.p. 60–80 °C)/ethyl acetate (49:1, v/v × 3), petroleum ether/acetone (49:1, v/v); (b) chloroform/methanol (24:1, v/v), toluene/acetone (4:1, v/v); (c, d) chloroform/methanol/water (100:14:0.8, by vol.), chloroform/acetone/methanol/water (50:60:2.5:3, by vol.); (e, f) chloroform/methanol/water (10:5:1, by vol.), chloroform/acetic acid/methanol/water (40:25:3:6, by vol.). Spray reagents (Dobson *et al.*, 1985): (a–e) 5% ethanolic molybdophosphoric acid followed by heating at 180 °C for 15 min; (f) α-naphthol/sulphuric acid for sugars.
Dhariwal et al. (1984) could not confirm these findings and unpublished studies in our respective laboratories have not detected sulpholipids in M. leprae. Cord-factors have usually been found in mycobacteria examined for their presence (Minnikin & Goodfellow, 1980) but since it has been argued that their role is metabolic rather than structural (Minnikin, 1982), it is not surprising that they were absent in the present M. leprae sample. Indeed if trehalose mycolates are key intermediates in mycolic acid biosynthesis and translocation (Minnikin, 1982), the very low metabolic activity of M. leprae might not require substantial proportions of such lipids. The unidentified lipids \(G_3-G_5\) (Fig. 1c) are similar in polarity to two more polar glycosyl phenolphthiocerol diesters characterized by Hunter & Brennan (1983).

The least polar TLC system developed for the analysis of the polar lipid extract (Fig. 1d) is designed to detect specific glycolipids such as characteristic glycopeptidolipids found in M. avium and related species and specific acylated trehaloses found in M. tuberculosis (Dobson et al., 1985; Minnikin et al., 1985c). These lipid classes were not detected in the present sample of M. leprae (Fig. 1d); the lipids detected reacted only with the universal molybdophosphoric acid reagent.

TLC of the most polar lipids from the M. leprae sample gave a pattern (Fig. 1e, f) which had some unusual features. The presence of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and mono-acylated forms of phosphatidylinositol di- and pentamannosides was typical of the majority of mycobacterial polar lipid patterns recorded so far (Minnikin & Goodfellow, 1980; Minnikin, 1982; Dobson et al., 1985). Mycobacteria, however, usually also produce diacylated forms of phosphatidylinositol di- and pentamannosides so it is probably significant that M. leprae appears to lack these lipids (Fig. 1e, f). Three unidentified phospholipids (\(P_1-P_3\)) were also observed in the M. leprae polar lipid pattern and one of these (\(P_3\)) also gave a blue colour with periodate-Schiff spray, indicating the presence of a \(\alpha\)-glycol grouping (Dobson et al., 1985). As shown in Fig. 1f, the polar lipids of M. leprae did not contain any phosphorus-free glycolipids.

The free lipid patterns from M. leprae show the presence of typical mycobacterial lipids, the most characteristic being dimycocerosates of the phthiocerol family (Fig. 1a), glycosyl phenolphthiocerol dimycocerosates (Fig. 1b) and phosphatidylinositol mannosides (Fig. 1e, f). The presence of these lipid classes had been indicated in previous studies (Draper, 1980; Young, 1981, 1982). The present finding of two, rather than the expected four, major phosphatidylinositol mannosides supports the observation of Young (1981) who found that four major sugar-positive spots were detected in M. tuberculosis, M. phlei and M. smegmatis but tissue-isolated M. leprae and M. lepraemurium gave only two of the four spots. It should be investigated whether this simplified pattern of phosphatidylinositol mannosides is related to growth in host organisms.

Taken in conjunction with the mycolic and fatty acid composition, which is typical of mycobacteria (Minnikin et al., 1985a; Draper et al., 1982), the free lipid patterns consolidate the position of the leprosy bacillus as a species of Mycobacterium. Close relationships with other mycobacterial species would be of practical interest in showing which cultivable species might be used as a model for M. leprae, or even as a possible vaccine. The free lipids in particular are similar to those of M. bovis, M. marinum and M. kansasii, all of which contain diesters of the phthiocerol family and glycosyl phenolphthiocerols (Minnikin et al., 1985b; Dobson et al., 1985; Hunter et al., 1982; Hunter & Brennan, 1983). The detailed structures of these characteristic lipids from leprosy bacillus and the three above species are quite distinct, however, indicating the integrity of M. leprae as a separate species.

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REFERENCES


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