Isolation of a Characteristic Phthiocerol Dimycocerosate from *Mycobacterium leprae*

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(Received 8 July 1982; revised 30 September 1982)

A characteristic mycobacterial wax, phthiocerol dimycocerosate, has been isolated from liver of armadillos experimentally infected with *Mycobacterium leprae*. The structure of this wax is generally similar to that produced by *Mycobacterium tuberculosis*, but the homologous phthiocerol and the mycocerosic acid components from *M. leprae* are significantly different from those of *M. tuberculosis*.

INTRODUCTION

Mycobacteria are capable of synthesizing a variety of complex lipids (Goren & Brennan, 1979) whose distribution is of interest in classification and identification (Minnikin & Goodfellow, 1980). A characteristic class of lipids consists of waxes composed of phthiocerol (I, $n = 20, 22$) esterified with multimethyl-branched long-chain acids (II, $x = 3; n = 14, 16, 19$ and $x = 4; n = 16, 18, 20$) for which the trivial names mycocerosic (Asselineau *et al.*, 1959) or mycoceranic (Polgar & Smith, 1963) acids are used. Phthiocerol dimycocerosate (PDIM) waxes were originally isolated from *Mycobacterium tuberculosis* (Noll, 1957) and are also possibly present in *Mycobacterium bovis* (Randall & Smith, 1964; Koul & Gastambide-Odier, 1977) and *Mycobacterium marinum* (Navalkar *et al.*, 1965). Chromatographic evidence for the presence of these lipids in spleen of armadillos infected with *Mycobacterium leprae* and in human lepromatous tissues has been obtained by Young (1981). This communication confirms the presence of PDIM in infected armadillo tissues and reports that chemical degradation of the *M. leprae*-derived lipid yields mixtures of components that are significantly different from those obtained from phthiocerol dimycocerosate isolated from *M. tuberculosis*.

Abbreviations: PDIM, Phthiocerol dimycocerosate; TBDMS, *t*-butyldimethylchlorosilane.

Fig. 1. TLC of constituents of PDIMs from *M. tuberculosis* and *M. leprae*. (a) Petroleum ether/acetone, 85:15 (v/v), three developments. (i) *M. leprae* phthiocerol; (ii) *M. tuberculosis* phthiocerol A, phthiocerol B, phthiodiolone A (in order of increasing mobility). (b) Petroleum ether/acetone, 98:2 (v/v), three developments. Ethylidene acetals of (i) *M. tuberculosis* phthiocerol A; (ii) *M. tuberculosis* phthiocerol B; (iii) *M. leprae* phthiocerol. (c) Petroleum ether/toluene, 98:2 (v/v). TBDMS ethers of mycocerosic alcohols from (i) *M. tuberculosis*; (ii) *M. leprae*.

METHODS

PDIM was isolated from bacteria-free supernatants from homogenates of *M. leprae*-infected armadillo liver (World Health Organization, 1980) prepared as follows: tissues were homogenized in 0.15 M-NaCl/2 M-Tris base (9:1, v/v) and centrifuged at 10000 *g* for 10 min. The alkaline supernatant fluid was neutralized with HCl and reduced in volume (40 °C *in vacuo*). Lipids were extracted by the procedure of Folch *et al.* (1957), using KCl in the aqueous phase to reduce accumulation of material at the interface [0.37% (w/v) KCl used to form 2 phases; the same total concentration in the washing solvent]. The washed lower phase was filtered and evaporated to a thick oil.

A column of silica gel (Hopkin & Williams MFC; 60–120 mesh) was prepared in light petroleum (b.p. 60–80 °C)/diethyl ether (1:1, v/v). The oil was mixed with a slurry of a small amount of silica gel in the same solvent mixture and applied to the column. The solvent mixture was also used to develop the column. Fractions eluted were purified further by preparative TLC using Merck 7731 silica gel G plates and light petroleum/diethyl ether/acetic acid (90:8:2, by vol.) as solvent to give a lipid (R_f 0.95) co-chromatographing with authentic PDIM from *M. tuberculosis* (kindly provided by Dr Mayer B. Goren, National Jewish Hospital, Denver, Co., U.S.A.) A further sample of PDIM was isolated from *M. tuberculosis* strain C (from the Central Veterinary Laboratory, Weybridge).

Efficient reductive deacylation was achieved by stirring PDIM samples with LiAlH_4 in dry diethyl ether for 2 h at room temperature. Separation of the ether layer, after sequential addition of ethyl acetate and water, gave phthiocerols and mycocerosic alcohols. Attempts to cleave PDIM under the following conditions gave minimal reaction (<10%): 10% (w/v) KOH in methanol/benzene (1:1, v/v) for 48 h at room temperature (Koul & Gastambide-Odier, 1977); methanol containing 5% (w/v) KOH, 2% (v/v) water and 20% (v/v) benzene under reflux for 3 h (Asselineau *et al.*, 1981); methanol/toluene/ H_2SO_4 (30:15:1, by vol.) at 75 °C for 16 h (Minnikin *et al.*, 1980); 0.25 M-sodium methoxide in CHCl_3 /methanol (1:1, v/v) at 20 °C for 1 h.

Phthiocerols (I) (<5 mg) were converted to ethylidene acetals (III) by reaction at room temperature overnight with acetaldehyde (0.5 ml) in toluene (1 ml) containing a crystal of toluene-4-sulphonic acid (Minnikin & Polgar, 1966). Mycocerosic alcohols (IV, R = H; <5 mg) were reacted at 75 °C overnight with TBDMS/imidazole/di-

Fig. 2. Gas chromatography of constituents of PDIMs from *M. tuberculosis* and *M. leprae* using a 1 m 2.5% OV-1 column isothermally at 280 °C. Values in brackets represent percentages of the total mixture and numbers of carbons (e.g. C₃₆) are those assigned to the underivatized parent molecules. (a, b) Ethylidene acetals of *M. tuberculosis* phthiocerol A and *M. leprae* phthiocerol. (c, d) TBDMS ethers of mycocerosic alcohols from *M. tuberculosis* and *M. leprae*.

methylformamide reagent (0.15 ml) as previously described (Minnikin & Patel, 1979). Analytical TLC of PDIM reduction products and their derivatives was performed using aluminium-backed silica gel sheet (Merck 5554); preparative TLC used 1 mm layers of Merck 7784 silica gel PF₂₅₄₊₃₆₆. Gas chromatography was performed with a Perkin-Elmer F11 flame-ionization instrument fitted with a stainless steel column (1 m × 2 mm i.d.) packed with 2.5% OV-1 methyl silicone on 80–100 mesh Chromosorb G AW-DMCS at 280 °C, with nitrogen as a carrier gas. Mass spectrometry was carried out using an A.E.I. MS9 instrument.

RESULTS AND DISCUSSION

The phthiocerol fraction from *M. leprae* was compared by TLC (Fig. 1a) with a standard sample from *M. tuberculosis* containing phthiocerol A, phthiocerol B and phthiodiolone A (Minnikin & Polgar, 1966). The similar mobility of the *M. leprae* phthiocerol to that of phthiocerol A suggested that both were methoxy diols having the general structure (I); phthiodiolone A (Minnikin & Polgar, 1967) has a keto group in place of the methoxy function and phthiocerol B has a terminal-CH(OCH₃).CH₃ unit (Minnikin & Polgar, 1966). This result was supported by conversion of the *M. leprae* phthiocerol to its ethylidene acetal (III) and comparison of this derivative (Fig. 1b) with similar derivatives from *M. tuberculosis* by TLC.

The only other long-chain components formed on reduction of *M. leprae* PDIM co-chromatographed (*R_F* 0.59) on TLC plates [solvent light petroleum/acetone, 95:5 (v/v), three developments] with mycocerosic alcohols (IV, R = H) prepared from PDIM isolated from *M. tuberculosis*. After conversion of these alcohols to TBDMS ethers [IV, R = Si(CH₃)₂.C(CH₃)₃] their chromatographic mobility (*R_F* 0.64) was again identical (Fig. 1c); derivatives of hexadecanol and elaidic alcohol had *R_F* values of 0.36 and 0.26, respectively. It is thus apparent that the fatty acid components of *M. leprae* PDIM are closely related to mycocerosic acids.

The results of gas chromatography of the ethylidene acetals of the phthiocerols and the

TBDMS ethers of the mycocerosic alcohols from both sources are shown in Fig. 2. It appears that the PDIM from *M. leprae* contains novel 30 and 32 carbon homologues of phthiocerol A. Mass spectrometry of the TBDMS ethers of the mycocerosic alcohol mixture from *M. tuberculosis* showed a peak at m/e 513 ($M-57$, Minnikin & Patel, 1979) corresponding to a main 32 carbon component (IV, $x = 4$, $n = 18$). The gas chromatographic data (Fig. 2) suggest that the mycocerosates of *M. leprae* PDIM are composed of a homologous 30, 32 and 34-carbon series (II, $x = 4$, $n = 16, 18, 20$), the latter being the main component.

The isolation from *M. leprae* of a characteristic PDIM wax with phthiocerol and mycocerosic acid components having a distinctive composition is of value in the classification of this pathogen. Taxonomic markers have been hard to find for *M. leprae*, mainly because it has not so far been grown on any bacteriological medium. *Mycobacterium leprae* also produces a phenolphthiocerol-based mycoside (Hunter & Brennan, 1981); this type of lipid has been found previously only in representative strains of *M. bovis*, *M. kansasii* and *M. marinum* (Goren & Brennan, 1979; Minnikin & Goodfellow, 1980). The presence of these two very characteristic mycobacterial lipids in *M. leprae* reinforces the idea that it is related to other mycobacteria, rather than more closely to corynebacteria as suggested by recent studies on nucleic acid composition (Imaeda *et al.*, 1982).

Thanks are due to the IMMLEP Steering Committee of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases for provision of infected armadillo tissue and a grant (T16/181/L4/29). G. Dobson is the recipient of a studentship from the British Leprosy Relief Association. Mass spectra were recorded by P. Kelly and S. H. Addison. Thanks are due to Dr R. H. Gigg for advice, and Dr M. B. Goren for advice and provision of a sample of PDIM.

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