Detection of Trehalose Monomycolate in *Mycobacterium leprae* Grown in Armadillo Tissues

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(Received 20 May 1986; revised 19 August 1986)

Trehalose-6-monomycolate (TMM) was isolated from the lipids of armadillo-derived *Mycobacterium leprae*. Only meagre amounts of this glycolipid were recovered, but its structure was unequivocally established. Only α -mycolates were detected in the TMM by ²⁵²Cf plasma desorption mass spectrometry. Electron impact mass spectrometry showed the alpha branch to be principally C₂₀. Trehalose dimycolate (cord factor) was not detectable. Since we have also found TMM in *M. lepraemurium* and in every *Mycobacterium* species so far examined, we suggest that this glycolipid is truly ubiquitous amongst mycobacteria.

INTRODUCTION

Mycolate esters of trehalose should be ubiquitous amongst *Mycobacterium* species. The thesis has its origin in studies by Walker *et al.* (1973) on the biosynthesis of corynomycolic acid by *Corynebacterium diphtheriae*. This low-molecular-mass (32-carbon) alpha-branched β -hydroxy acid has been a useful model (Gastambide-Odier & Lederer, 1964) for studying late steps in the biosynthesis of the much more complex mycolic acids of mycobacteria (60 to 90 carbons), which are also alpha-branched, β -hydroxy acids (for reviews see Goren & Brennan, 1979; Minnikin, 1982; Takayama & Qureshi, 1984). According to Walker *et al.* (1973), a cell-free extract from *C. diphtheriae* condensed two molecules of [1-¹⁴C]palmitate via a Claisen-type condensation to produce 2-tetradecyl-3-keto-octadecanoic acid, which they reduced chemically to a mixture of erythro and threo corynomycolic acids:

On the other hand, when *C. diphtheriae* in culture condensed the labelled palmitate, the intermediate keto acid was immediately incorporated into a 6-monoester of trehalose, which was subsequently reduced biologically to trehalose-6-corynomycolate (Promé *et al.*, 1974; Ahibo-Coffy *et al.*, 1978). It seems likely that this sequence is also valid for mycobacteria.

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Abbreviations: MAT, trehalose-6-monoacetate-6'-monomycolate; TDM, trehalose-6,6'-dimycolate (cord factor); TMM, trehalose-6-monomycolate; TMS, *O*-trimethylsilyl.

Accordingly the completely assembled carbon skeleton of mycobacterial mycolic acid might also be expected to appear immediately as a trehalose derivative, potentially the precursor of trehalose-6-mycolate (TMM) (Kato & Maeda, 1974) and therefore of 'cord factor', trehalose-6,6'-dimycolate (TDM) (Kilburn *et al.*, 1982) (for reviews see Asselineau & Asselineau, 1978; Lederer, 1976, 1984).

If all of the mycolic acid synthesized by mycobacteria accumulated as trehalose derivatives, then the steady-state levels of these glycolipids and their contained mycolic acids would be enormous. In fact, they are meagre. Instead the bulk of the mycolic acids are found mainly esterified to the mycobacterial cell wall arabinogalactan (Azuma & Yamamura, 1963; Kanetsuna *et al.*, 1969; Bruneteau & Michel, 1968; Vilkas & Markovits, 1968). One may speculate from the studies just reviewed that the principal function of trehalose mycolates is to serve, either as such or as intermediates, to carry newly synthesized mycolic acid for transfer to growing cell wall. Indeed, important evidence in support of a trehalose mycolate 'carrier', potentially implicating trehalose-6-monoacetate-6'-monomycolate ('MAT') in this function was described by Takayama & Armstrong (1976). *Mycobacterium tuberculosis* H37Ra incorporated [^{14}C]acetate to produce MAT labelled in the mycolate moiety. After a chase with unlabelled acetate the radiolabel of this glycolipid declined with time and appeared instead in cell-wall-associated mycolate. It seems reasonable to anticipate, therefore, that all mycobacteria synthesize mycolic acid and incorporate it into cell wall via trehalose mycolate intermediates.

TMM was recently shown to be a direct precursor of TDM (Kilburn *et al.*, 1982), and TDM has already been found in *Mycobacterium lepraemurium* from bacteria grown *in vivo* (Goren *et al.*, 1979) as well as from those cultured in cell-free medium (Nakamura *et al.*, 1984). In anticipation that TMM might be the central substance leading from newly synthesized mycolic acid to cell wall and to TDM, our efforts targeted on finding and identifying these extractable glycolipids in *Mycobacterium leprae*. TMM had thus far evidently not been sought, whilst TDM had heretofore eluded detection in only this *Mycobacterium* species (Minnikin *et al.*, 1985). These glycolipids therefore represented a 'missing link', whose discovery could nevertheless be confidently expected based on the premises developed above.

METHODS

Lipids. A preparation designated 'crude *M. leprae* lipids' was kindly supplied to us by Dr P. J. Brennan. It was obtained from purified *M. leprae* recovered from irradiated infected armadillo livers (Draper, 1979; Hunter & Brennan, 1981). Lyophilized bacilli were extracted with 2:1 (v/v) $\text{CHCl}_3/\text{CH}_3\text{OH}$ at 50 °C for 15–18 h. Partitioning according to Folch *et al.* (1957) and recovery of the material from the CHCl_3 phase afforded the 'crude' product. Dr Philip Draper (National Institute for Medical Research, Mill Hill, London, UK) also kindly gave us 9.5 mg of 'wall lipids' isolated by 1:1 (v/v) $\text{CHCl}_3/\text{CH}_3\text{OH}$ extraction of purified cell walls from *M. leprae* (Draper, 1976).

Column chromatography and TLC. The 'crude *M. leprae* lipids' (see above) were subjected to column chromatography to seek enrichment of TMM or TDM; this was successful only with this sample. Short columns packed with Whatman DE23 DEAE-cellulose and CF1 long-fibre cellulose (1:2, w/w) were used in the neutralized acetate form (Goren, 1970a). As summarized in Fig. 1, 81 mg of the lipids were loaded in and washed with diethyl ether, and were eluted with diethyl ether/methanol (70:30, v/v). Fractions were monitored by TLC, and TMM-enriched fractions were combined for further enrichment. Sybron-Brinkmann Sil G-25 plates were used for analytical TLC. Plates were sprayed with 60% (w/w) H_2SO_4 containing 0.01% orcinol and heated at 130 °C for 4–5 min. TMM and TDM spots assume a characteristic clean blue-grey colour in this treatment (Dhariwal *et al.*, 1984). Samples of synthesized TMM (Liav & Goren, 1984) and TDM (Liav & Goren, 1980) were used as markers.

Preparative TLC of the considerably enriched material obtained from the DEAE-cellulose chromatographies was done on Sybron-Brinkmann 5 cm plates, developed successively in three solvent systems as described by P. Draper (personal communication): chloroform/methanol (90:10, v/v) 15 cm; chloroform/methanol/ H_2O (14:6:1, by vol.) 10 cm; and hexane/ether/acetic acid (80:18:2, by vol.) to the top of the plate (air dried after each development). The region containing TMM, visualized with iodine, was recovered and extracted with chloroform/methanol/ H_2O (80:20:0.5, by vol.) and the recovered lipids were re-extracted into wet diethyl ether. As judged from its IR spectrum (see below) the recovered TMM still contained some impurity, partially removed by extracting with hexane and then with CH_3OH . The methanol-insoluble lipids (about 400 μg) appeared to be almost pure TMM. For IR examination, neat samples of lipids were deposited on a NaCl microplate, and examined in a Beckman IR-9 spectrophotometer with beam condenser. The IR spectra of TMM and TDM are

Fig. 1. Isolation of TMM from Folch-washed 'crude *M. leprae* lipids'. About 1 g (dry wt) armadillo-derived *M. leprae* were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) at 50 °C. After washing with Folch upper phase the lipids in the CHCl_3 phase were chromatographed twice on cellulose/DEAE-cellulose (2:1, w/w). TMM from this enriched product was further separated by preparative TLC on silica gel plates by development with the three solvent systems shown and air drying between each development. About 0.6 mg TMM (still impure) was recovered from 81 mg 'crude *M. leprae* lipids'. It could be purified further by extractions with hexane followed by methanol and by HPLC.

very similar and possess unique features described below (Noll & Bloch, 1955; Noll *et al.*, 1956; Goren & Brokl, 1974).

HPLC. To remove stubbornly persistent contaminants from the isolated TMM, HPLC separation was kindly done for us by Dr K. Takayama and colleagues, who had earlier established the conditions for separating and isolating small amounts of TMM and TDM from cell-free reaction systems. The apparatus and conditions for further purifying about 500 µg of our nearly purified TMM were exactly as described before (Kilburn *et al.*, 1982); semi-synthetic TMM was used to calibrate the system.

Mass spectrometry. ^{252}Cf plasma desorption mass spectra were obtained on an instrument (see Macfarlane, 1983) which was built for the National Institutes of Health by Professor R. Macfarlane of Texas A&M, and subsequently modified by L. Pannell to change samples automatically. Samples dissolved in polar solvents were applied to the conductive surface of an aluminized Mylar film in a sample holder either by evaporation or electrospraying (see above reference). The sample holder was then placed (through a vacuum lock) in front of a ^{252}Cf source [about 10 µCi (370 kBq)]. The chamber is maintained at about 10^{-6} Torr (~ 1 mPa). Very high energy particles from the Cf source (100 MeV) impinge on the sample to generate a plasma containing desorbed molecules with attached sodium cations and these are accelerated down a 42 cm flight tube by a 10 kV grid. Flight times, which are proportional to the square root of the mass are stored in a computer and converted into masses. Each such ion is collected and added to others in the same channel to provide the mass spectrum. This mode of operation is useful principally to determine the molecular masses of the compounds under investigation.

In contrast, electron impact mass spectrometry with high temperature of the probe to volatilize the sample is useful for studying much less polar substances, i.e. lipid esters and permethylation products. It gives rise to fragments whose composition provides data about the intimate structure of the parent molecule (see, for example, Goren & Brennan, 1979).

Permethylation analysis. A portion of almost pure TMM from *M. leprae* was used for permethylation analysis to establish the position of the mycolate ester. In this technique, the free hydroxyl groups in the acylated trehalose are methylated, the product is hydrolysed with base to free the esterified hydroxyl(s) and the resulting methylated sugar is separated from the waxy mycolic acid and identified (see Noll *et al.*, 1956; Goren, 1970*b*). For the permethylation about 200 µg lipid was dissolved in 0.3 ml methylene chloride; 50 µl 2,6-di-*tert*-butyl pyridine and 50 µl methyl trifluoromethanesulphonate were added and the mixture was held overnight in a bath at 60 °C, under reflux. The mixture was evaporated under N_2 , dissolved in ethyl acetate, washed with dilute HCl, aqueous

Fig. 2. IR spectra of TMM samples: (a) semi-synthetic, (b) from *M. lepraemurium* and (c) from *M. leprae*. Neat samples were deposited on a NaCl microplate and spectra obtained in a Beckman IR-9 spectrophotometer with beam condenser. Arrows point to specific absorption bands associated with α -mycolates (3080 cm^{-1}), trehalose (808 cm^{-1}), and trehalose 6-esters ($1000\text{--}1200\text{ cm}^{-1}$). The IR spectra of three individual *M. leprae* TMM preparations were similar.

bicarbonate and H_2O , and the product was recovered. Our recent experiences with this elegant method described by Arnarp & Lönngren (1978) prove it to give high levels of methylation without inducing either hydrolysis or acyl migration (Liav & Goren, 1986).

Identification of the permethylated carbohydrate. (a) *Alkaline hydrolysis* of the permethylated TMM and separation of the methylated sugar from the K-mycolate were done as described by Noll *et al.* (1956). The methylated trehalose, dissolved in aqueous methanol, was then deionized with Bio-Rad mixed-bed resins and recovered.

(b) *Acid hydrolysis.* A portion of the (hepta-*O*-methyl) trehalose was dissolved in about 0.3 ml 2 M-HCl, sealed in a small tube and heated at 95°C for 18 h to hydrolyse the methylated sugar. The aqueous HCl was gently evaporated at about $40\text{--}50^\circ\text{C}$ with a stream of N_2 ; the residue was deionized after dissolving in water and taken to dryness again.

(a) *GLC* was used to identify both the methylated trehalose and the scission products obtained from it by acid hydrolysis. The methylated trehalose was examined without derivatization by GLC (2 mm $\times$ 183 cm column of SE30 at 200°C , N_2 carrier gas at 30 ml min^{-1}) and compared with an authentic sample of 2,3,4,6,2',3',4'-hepta-*O*-methyl trehalose (see Kato & Maeda, 1974; Goren *et al.*, 1976). After acid hydrolysis (as above) the products [as the *O*-trimethylsilyl (TMS) derivatives] were also examined by GLC (SE30, 125°C) using authentic TMS derivatives of 2,3,4-tri-*O*-methyl glucose and of 2,3,4,6-tetra-*O*-methyl glucose for comparison (Goren *et al.*, 1976; Liav & Goren, 1986).

RESULTS

Preliminary examination of P. Draper's 'wall lipids' (see Methods) by TLC gave no evidence that these contained either TDM or TMM. [We did not search for MAT, but having recently synthesized this rare diester (Liav & Goren, 1986) we plan in the future to search for evidence of MAT amongst various lipid extracts.] The more abundant 'crude *M. leprae* lipids' contained detectable amounts of what appeared to be TMM, but not of TDM.

Fig. 1 summarizes the procedures detailed in Methods for isolation of the 'enriched TMM'. Its IR spectrum is given in Fig. 2, in which the presumed TMM from *M. leprae* is compared with a like material obtained from crude lipids (kindly given to us by Dr P. Draper; see Draper & Rees, 1973, for their isolation) of *in vivo* grown (mice) *M. lepraemurium* (K. Dhariwal & M. Goren, unpublished results) and with semi-synthetic TMM. Clearly all of the absorption frequencies characteristic of trehalose monomycolate (arrowed) are found in the two products from the *in vivo*-derived bacteria: 3080 cm^{-1} [cyclopropane of the 'alpha-type' mycolic acids (Gastambide-Odier *et al.*, 1964)]; 808 cm^{-1} [a weak band specifically associated with trehalose (Goren,

(a) (b) (c) (d)

Fig. 3

Fig. 4

Fig. 3. TLC of TMM samples in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (75:25:4, by vol.): (a) semi-synthetic, (b) from *M. leprae*, (c) from *M. lepraemurium* and (d) from *M. microti*. The spots were visualized by spraying the plate with 60% (w/w) H_2SO_4 containing 0.01% orcinol and heating at 130 °C for 4–5 min. TMM assumes a characteristic blue-grey colour after this treatment.

Fig. 4. HPLC elution curves of TMM samples: (a) semi-synthetic and (b) from *M. leprae*. *M. leprae* TMM prepared as outlined in Fig. 1 was purified further on a Waters Associates Radial-pak C_{18} cartridge using a linear gradient of 0–60% hexane/2-propanol (2:1, v/v) in 2-propanol/water (9:1, v/v) at a flow rate of 2 ml min^{-1} . Fractions were collected at 1 min intervals after 6 min. The peaks at 25.2 and 26.3 min were the major peaks and nearly correspond to peaks obtained from the semi-synthetic TMM.

1970a)]; and a succession of about seven peaks between 1000 and 1200 cm^{-1} highly characteristic of trehalose 6-esters (Goren & Brokl, 1974). The spurious peaks in the region 1200–1400 cm^{-1} found in the spectrum of the product from *M. lepraemurium* indicate that it requires further purification. Fig. 3 shows a thin-layer chromatogram which compares the semi-synthetic TMM with the samples derived from *M. lepraemurium* and *M. leprae* and with a quite pure TMM that we isolated in abundance from *M. microti* (K. R. Dhariwal *et al.*, unpublished results).

To obtain a still purer sample, HPLC was carried out on about 400 μg of the *M. leprae* glycolipid (Fig. 4). The peaks at 25.2 and 26.3 min were very close to the principal peaks obtained for the semi-synthetic TMM sample. The effluents corresponding to the two peaks were combined and used for studying the composition of the mycolate components by electron impact mass spectrometry (see below).

²⁵²Cf plasma desorption mass spectrometry

Application of this technique to the semi-synthetic TMM (Fig. 5) allowed us to establish favourable conditions for obtaining informative spectra. Yet the quality of the spectra depends on variables that are often difficult to control or to recognize: in our interpretation the nature of

Fig. 5. ^{252}Cf plasma desorption mass spectra of trehalose monomycolates: (a) semi-synthetic and (b) from *M. leprae*. The semi-synthetic sample shows the presence of type I (dicyclopropane) and type IIa (methoxy) mycolates. Only type I are discernible in the spectrum of the *M. leprae* glycolipid.

the film sample produced by spraying was of critical importance, but was not consistently controllable. For example, successive samples formed from the semi-synthetic TMM in solution in acetone/ CH_3OH or in $\text{CHCl}_3/\text{CH}_3\text{OH}$ gave recognizable but nevertheless rather poor spectra during runs lasting 800 min each. In contrast, the spectrum in Fig. 5(a) was obtained during a 30 min scan from a (presumably identical) film also deposited from $\text{CHCl}_3/\text{CH}_3\text{OH}$ solution. With the much scarcer sample from *M. leprae* (also sprayed onto the Mylar from a $\text{CHCl}_3/\text{CH}_3\text{OH}$ solution) the spectrum in Fig. 5(b) was obtained during 5435 min. Nevertheless, it is an unequivocal spectrum. It was the only one successfully obtained with our limited sample.

The scan in Fig. 5(a) shows that the semi-synthetic TMM is characterized by two prominent series of mycolate substituents that are identified as types I and IIa according to the scheme of D. E. Minnikin (Davidson *et al.*, 1982). The series of type I are of the (*cis,cis*) dicyclopropane types, also referred to as 'alpha' mycolates (see below and reviews mentioned earlier). The IIa series (shaded peaks) contain a methoxyl group with an adjacent methyl branch as depicted by the structure

For the peaks at m/z 1601 and 1629, $x + y + z = 50, 52$.

The spectrum of the *M. leprae* lipid (Fig. 5b) shows essentially only one principal series of peaks (derived from the sodium-cationized molecules) at $M + 23 = 1457, 1485, 1513, 1541$ that correspond to trehalose esterified with a type I ('alpha') mycolate of the structure

in which $x + y + z = 46, 48, 50, 52$ (principal components 48, 50). The alpha branch is $C_{20}H_{41}$ as described below. These results are in accord with those obtained by Draper *et al.* (1982) for the type I mycolic acids recovered from defatted cells of (armadillo-grown) *M. leprae*, with principal $x + y + z = 48$. No other peaks definable with confidence can be recognized in our spectrum. *M. leprae* elaborates ketomycolates as well (Draper *et al.*, 1982) but we did not detect them in our TMM.

Characterization of the mycolate alpha branch: electron impact mass spectrometry

The *M. leprae* TMM purified by HPLC as described above was directly hydrolysed to provide the free mycolic acid. The methyl ester (obtained with CH_3N_2) was then examined by electron impact mass spectrometry. A principal spectrum was obtained at a temperature of about 260 °C in which a base (pyrolysis) peak was obtained at m/z 354 with a smaller peak at m/z 382; methylhexacosanoate (m/z 410) was imperceptible. Therefore, the mycolate alpha branch of *M. leprae* TMM is principally a C_{20} constituent, which yields a methyl docosanoate during the pyrolytic cleavage, with a smaller complement of tetracosanoate generated from a C_{22} branch. Etemadi (1967) established that under the pyrolytic conditions in the vicinity of the probe holding the sample, methyl mycolates undergo a reverse Claisen reaction in which the alpha branch, the alpha carbon and the carbomethoxy group are eliminated cleanly as a methyl ester whose size defines the alpha branch, viz.

Our results are largely in agreement with those reported by Kusaka *et al.* (1981) (who, however, did not find evidence for a C_{22} α -branch), by Asselineau *et al.* (1981), and by Draper *et al.* (1982), in whose studies both the C_{20} and C_{22} α -branches were described.

Characterization of the sugar-ester bond

As described in Methods, the TMM of *M. leprae* was permethylated, hydrolysed with alkali and the partially methylated trehalose recovered. By GLC it was found to be identical with authentic 2,3,4,6,2',3',4'-hepta-*O*-methyl trehalose prepared recently from semi-synthetic TMM (Liav & Goren, 1986). Both samples had identical retention times (10.8–10.9 min). Authentic 2,3,4,2',3',4'-hexa-*O*-methyl trehalose (Goren *et al.*, 1976) had a retention time of 15.8 min under the same conditions.

Acid hydrolysis of the hepta-*O*-methyl trehalose generated the two partially methylated glucose moieties. Their structures were determined by comparing them with authentic 2,3,4-tri- and 2,3,4,6-tetra-*O*-methyl glucose by GLC of the TMS derivatives. The following data were obtained:

Sample (TMS derivatives)	Retention time (min)			
2,3,4,6-Tetra- <i>O</i> -Me glucose	6.7,	7.1*		
2,3,4-Tri- <i>O</i> -Me glucose			8.2,	8.9*
Products from <i>M. leprae</i> TMM	6.7,	7.3*	8.2,	8.9*

* Anomers resolved.

Taken together with the results of mass spectrometry, these data prove that the glycolipid is a trehalose derivative acylated at a single 6-position with alpha (type I) mycolic acids bearing principally a C_{20} alpha branch, and therefore characteristic of *M. leprae*.

DISCUSSION

The sum of our evidence indicates that *M. leprae* synthesizes TMM *in vivo*. The most convincing evidence derives from the plasma desorption mass spectrum in which nearly exact

molecular masses of the glycolipid are defined, from IR spectrophotometry, from the pattern of the HPLC elution, and from the permethylation studies.

We judge that the maximum amount of TMM that we recovered is probably no more than 400 µg from 1 g (dry wt) of harvested *M. leprae*. In recent studies of *M. microti* cultured *in vitro* we recovered a minimum of 5 mg TMM per g (dry wt) of bacteria and an abundance of TDM as well (unpublished results). The *M. leprae* lipids, however, contained no trace of TDM that we could detect. It is of course possible that a careful separation of a considerably larger gross lipid sample may allow TDM to be detected and perhaps isolated. We were also unable to detect any sulphatides ('sulpholipids') behaving like those of *M. tuberculosis* (these are also trehalose esters). Minnikin *et al.* (1985) also reported that their *M. leprae* sample did not contain either sulpholipids or TDM.

The minuscule quantity of TMM that we were able to recover would suggest that fulfilling of its probable anabolic role in cell wall biosynthesis almost exhausts the amount of this glycolipid that is available to the cell at any given time and, therefore, it may be unavailable for TDM biosynthesis. It is in accord with current observations respecting the precursor/product relationship between TMM and TDM that the cell's economy hardly allows for any diversion of such an important substance (TMM) into what may be a biosynthetic 'dead end'. Finally, we suggest that the identification of TMM in *M. leprae* secures for this glycolipid the distinction of being truly ubiquitous among mycobacteria.

This investigation was supported by Grant AI-08401 from the United States-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases. We thank Drs P. J. Brennan and P. Draper for samples of *M. leprae* lipids and Dr Kuni Takayama for HPLC separation of the *M. leprae* TMM. We thank Nadia de Stackelburg for illustrations and Ms Shirley Downs for careful preparation of the manuscript. M. B. G. is the Margaret Regan Investigator in Chemical Pathology, National Jewish Center for Immunology and Respiratory Medicine.

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