Studies on the Mycolic Acids from the Walls of *Mycobacterium microti*

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Mycobacterium microti walls contained three types of mycolic acids, very similar to those found in *Mycobacterium tuberculosis*. An α -mycolate with two cyclopropane rings, a methoxymycolate with one cyclopropane ring and a methoxyl group, and a ketomycolate with one cyclopropane ring and a keto group were partially characterized. The mycolates made up 34% (by weight) of the peptidoglycan–arabinogalactan–mycolate wall skeleton. Young exponential phase cultures and organisms harvested from mouse lungs contained high proportions of ketomycolates; older cultures had roughly equal proportions of keto- and methoxymycolates. The proportion of α -mycolates increased slightly with age of culture, but was always less than one-third of the total.

INTRODUCTION

Mycobacterium microti closely resembles *Mycobacterium tuberculosis* (Stanford & Grange, 1974). Since *M. microti* is pathogenic to rodents, but not man, it is valuable as a laboratory model in place of *M. tuberculosis*. Mycolic acids are 2-branched, 3-hydroxy fatty acids produced by mycobacteria and related organisms (Minnikin & Goodfellow, 1980). They mostly occur esterified to arabinose residues in the peptidoglycan–arabinogalactan–mycolate wall skeleton but are also found in some solvent-extractable lipids such as trehalose 6,6'-dimycolate (Lederer *et al.*, 1975; Asselineau & Asselineau, 1978). Although the mycolic acids of *M. tuberculosis* have been studied extensively (Etémadi, 1967; Minnikin & Polgar, 1966, 1967*a, b*; Asselineau *et al.*, 1969; Gensler & Marshall, 1977; Qureshi *et al.*, 1978), nothing is known about *M. microti* mycolic acids. We have therefore investigated the composition of the mycolic acids from walls of *M. microti* and have also studied variations in the proportions of individual mycolate species during growth.

METHODS

The media in which *M. microti* OV248 was grown were based on those of Ungar *et al.* (1962). In the glycerol-free medium, glutamine, L-asparagine and Triton WR1339 were omitted, but monosodium glutamate (8.0 g l⁻¹) and Tween 80 (0.5 ml l⁻¹) were added. In the modified Sauton medium, L-asparagine, citric acid, and Triton WR1339 were omitted, but monosodium glutamate (4.0 g l⁻¹) and Tween 80 (0.5 ml l⁻¹) were added. The pH of both media was adjusted with NaOH or HCl and the media were then autoclaved at 121 °C for 20 min. Bovine serum albumin (plasma fraction V; Armour & Co., Eastbourne, U.K.) solution, sterilized by filtration, was added to the sterilized media to give a concentration of 5.0 g l⁻¹ in each medium.

Mycobacterium microti was grown in 5-litre flasks on glycerol-free medium, pH 7.0. These cultures were grown for 15–18 d to an A_{450} of 0.7–0.9. Variations in the relative proportions of mycolates during growth were studied using several series of 200 ml cultures in 1-litre flasks. Bacilli were collected from cultures by centrifugation at 10 000 g for 10 min, washed twice with 0.05% (w/v) Tween 80, and freeze-dried.

Mycobacterium microti was also grown in mice (CBA strain). The mice were injected intravenously with 0.4 mg (approximate wet wt) of *M. microti*. After 8–10 weeks, the mice were killed and the bacilli were isolated from their lungs (Draper, 1971).

Preparation of methyl mycolates. Walls were prepared from freeze-dried bacilli by the method of Draper (1971); however, they were broken in suspension (about 5 mg ml⁻¹) in 0.1 M-Tris/HCl, pH 7.2, containing 0.05% (w/v) Tween 80 with an MSE 150 W, 20 KHz ultrasonic generator using a probe with a 2.8 cm² cross-section and a probe displacement of 8 µm for 20 min. Freeze-dried bacilli or walls were extracted for 24 h with chloroform/methanol (1:1, v/v) and methyl mycolates were produced by treating the whole defatted bacterial residue with methanol/toluene/concentrated H₂SO₄ (10:10:0.4, by vol.) at 50 °C (Minnikin *et al.*, 1975). Methyl mycolates were prepared from chloroform/methanol-extracted walls (wall skeletons) of *M. microti* by treatment with 0.5% (w/v) KOH in methanol at 37 °C for 4 d. The methyl mycolates were extracted into chloroform/methanol (2:1, v/v).

Thin-layer chromatography (t.l.c.). Methyl mycolates were fractionated on silica gel 60 plates (Merck) by developing six times with petroleum spirit (b.p. 40–60 °C)/diethyl ether (95:5, v/v). Methyl mycolates were detected on preparative thin-layer chromatograms by exposure to iodine vapour or on quantitative chromatograms by spraying with 0.5% (w/v) K₂Cr₂O₇ in 10% (v/v) H₂SO₄ and charring at 150 °C (Minnikin *et al.*, 1975). The intensity of charring was measured with a double-beam recording microdensitometer (model EL2, Mk IIIB; Joyce-Loebl & Co., Gateshead, U.K.).

Infrared spectroscopy. Infrared (i.r.) spectra of samples as films on rock salt discs were recorded using a Perkin-Elmer 457 spectrometer.

Nuclear magnetic resonance spectroscopy. Nuclear magnetic resonance (n.m.r.) spectra of samples dissolved in C²HCl₃ were recorded using a Varian EM-360 spectrometer with C¹HCl₃ as the internal standard.

Mass spectroscopy. Mass spectra were recorded using an AEI MS9 instrument with a direct insertion probe, an ionizing voltage of 70 eV, and a temperature range of 230–250 °C.

RESULTS AND DISCUSSION

Mycolates made up about 34%, by weight, of the wall skeleton of *M. microti* from glycerol-free medium, pH 7.0. The mycolic acid methyl esters from both isolated walls and defatted cells of *M. microti* were separated by t.l.c. into three components having the same mobilities as those of a sample of mycolic esters from *M. tuberculosis* (Minnikin & Polgar, 1967*a, b*). The least polar mycolates from *M. tuberculosis* are considered to have the general structure (I) (Minnikin & Polgar, 1967*a*). Having no oxygen functions other than the 3-hydroxy ester unit, they fall into the class of the so-called α-mycolates (Etémadi, 1967). The mycolates from *M. tuberculosis* having intermediate and greatest polarities have been found to be methoxy- and ketomycolates with the general structures (II) and (III), respectively (Minnikin & Polgar, 1967*b*). It is probable, therefore, that the mycolates from *M. microti* have related structures, and for the purposes of discussion they will now be described as α-, methoxy- and ketomycolates. Samples (40–60 mg) of each mycolic ester from *M. microti* were obtained by preparative t.l.c.

Each *M. microti* mycolate had i.r. absorbances at 1025 cm⁻¹ and 3060 cm⁻¹, attributable to cyclopropane rings (Cole, 1954). The n.m.r. spectra of the methoxymycolates had resonances at δ 3.0 (1H, broad) due to CHOCH₃ and δ 3.34 (3H, singlet) due to CHOCH₃, and the ketomycolate had a resonance at δ 1.07 (3H, doublet) due to CH₃CHCO. All three mycolates had broad resonances at δ -0.3 and δ +0.6 (2H and 6H for α, 0.7H and 2.1H for methoxy, 0.8H and 2.4H for keto), attributable to 1,3-disubstituted *cis*-cyclopropane rings; the methoxy- and ketomycolate n.m.r. spectra also showed broad resonances at δ 0.2 (1.2H for methoxy, 0.8H for keto) due to 1,2-disubstituted *trans*-cyclopropane rings (Minnikin, 1966; Longone & Miller, 1967). For the *M. microti* methoxy- and ketomycolate cyclopropane rings, the *cis* to *trans* ratios were estimated to be 7:3 and 4:1, respectively, compared with 5:1 and 1:2 in the corresponding mycolates in *M. tuberculosis* (Minnikin & Polgar, 1967*a, b*). In *M. tuberculosis* α-mycolate, as in *M. microti* α-mycolate, only *cis*-cyclopropane rings were present. The ratio differences in corresponding mycolates from *M. microti* and *M. tuberculosis* may reflect a species difference or be due to differences in culture conditions.

Mycobacterial methyl mycolates pyrolyse during mass spectrometry (Etémadi, 1967):

Peaks from the pyrolysis products predominated in the mass spectra. Mass spectra of *M. microti* methyl mycolates were quite similar to those of the corresponding compounds from *M. tuberculosis* (Minnikin & Polgar, 1966, 1967*a, b*). In both cases, the major straight-chain ester produced was methyl hexacosanoate (*m/e* 410). The spectrum of *M. microti* methyl α -mycolate had a single series of meroaldehyde peaks, *m/e* 712, 740, 768, 796, 824, 852 (the major component is italicized), and a series *m/e* 459, 487, 515, 543, due to fragmentation of the meroaldehydes. Similar series of peaks were present in the mass spectra of *M. tuberculosis* methyl α -mycolates (Etémadi & Lederer, 1965; Minnikin & Polgar, 1966, 1967*a*; Asselineau *et al.*, 1969; Qureshi *et al.*, 1978). We suggest that *M. microti* methyl α -mycolates are a single homologous series (structure I) with $x + y + z = 42, 44, 46, 48, 50, 52$. The *M. microti* methyl methoxymycolate spectrum had a major series of peaks, *m/e* 740, 768, 796, 824, 852, 880, due to meroaldehyde - CH₃OH. Two series (*m/e* 519, 547, 575, 603, 631, and 297, 325) were due to meroaldehyde cleavage adjacent to the methoxyl group. Loss of CH₃OH from the first of these two series gave another major series. These results, in conjunction with those reported for *M. tuberculosis* (Minnikin & Polgar, 1967*b*), indicate that the major series of *M. microti* methyl methoxymycolates has structure II a with $y + z = 27, 29, 31, 33, 35, 37$.

Fig. 1. (a) Growth of *M. microti* OV248 in glycerol-free medium, pH 7.0. (b) Variations during growth in the relative amounts of bound mycolates: α -mycolate (○), methoxymycolate (●) and ketomycolate (□).

A minor series of peaks (m/e 782, 810, 838, 866) was probably due to meroaldehyde — CH_3OH ions from a minor homologous series (structure IIb) with $y + z = 29, 31, 33, 35$, as in *M. tuberculosis* (Minnikin & Polgar, 1967b). The *M. microti* methyl ketomycolate mass spectrum had two series of meroaldehyde peaks similar to those found in the spectrum of the ketomycolate from *M. tuberculosis* (Minnikin & Polgar, 1967b): the major one (m/e 812, 840, 868, 896) and the minor one (m/e 826, 854, 882, 910). The overall structures of *M. microti* methyl ketomycolates are possibly similar to those of *M. tuberculosis* (III a and III b) with $x + y + z = 48, 50, 52, 54$. The major series (III b) in *M. tuberculosis* is the minor series in *M. microti*; this correlated with the n.m.r. results, which showed that *cis*-cyclopropane groups are more abundant in *M. microti* ketomycolates, but *trans* are more abundant in *M. tuberculosis* ketomycolates.

During the period of approximately exponential growth (5–15 d) in glycerol-free medium at pH 7.0 (Fig. 1a) the initially high proportion of ketomycolate decreased rapidly, with a somewhat smaller increase in methoxymycolate (Fig. 1b). The proportion of α -mycolate also showed an increase during the same period but as the rate of growth slowed down (15–30 d) the proportion of this component became fairly constant (Fig. 1). Similar changes in the relative proportions of mycolates occurred in *M. microti* grown in modified Sauton medium, pH 7.0. Since the pH of cultures increased by over one unit during growth, *M. microti* was grown in glycerol-free media with initial pH values of 6.0 and 8.0; these cultures grew more slowly than those initially at pH 7.0. However, at both pH 6.0 and 8.0, the changes occurring in the relative proportions of the three types of mycolates were similar to those which occurred in cultures at pH 7.0. At pH 6.0 and 8.0, respectively, the proportion of α -mycolate rose from 20% to 30% and from 14% to 27%. That of methoxymycolate rose from 14% to 25% and from 28% to 43%. In ketomycolate it fell from 66% to 45% and from 58% to 30%. Wall skeletons of *M. microti* from mouse lungs (two batches) had α :methoxy:ketomycolate ratios of 33:22:45 and 34:25:41.

The most striking change in the mycolic acid composition during growth is the apparent replacement of high proportions of ketomycolates in exponential phase cultures by methoxymycolates as stationary phase is approached (Fig. 1). Methoxymycolates are

considered to be synthesized from ketomycolates or their precursors, the methyl group being provided by methionine (Gastambide-Odier *et al.*, 1964). Alkylations involving a single carbon unit from methionine also are involved in the conversion of long-chain *cis*-octadecenoic acids either to methyl-branched acids such as tuberculostearic (10-methyloctadecanoic) acid or cyclopropane acids such as lactobacillic (11,12-methyleneoctadecanoic) acid (Lederer, 1969). It is very interesting to note that both tuberculostearic and lactobacillic acids tended to accumulate with age in the organisms producing them (for a review see Lechevalier, 1977). An explanation of this phenomenon may be that in resting cells relatively reactive *cis* double bonds are replaced by chemically more inert methyl branches and cyclopropane rings. It is possible that the conversion of the keto function in the mycolates of *M. microti* to a less reactive methoxy group is an analogous mechanism for the maintenance of envelope integrity in resting cells. *M. microti* obtained from mouse lungs, in containing considerably more ketomycolate than methoxymycolate, corresponds more closely in mycolic acid composition to actively growing exponential phase than to stationary phase cultures grown *in vitro* (Fig. 1).

Quantitative studies of variation in mycolic acid composition with growth conditions have not been widely reported. Michalec & Mára (1975) recorded variations in the proportions of two main unidentified components in *Mycobacterium bovis* BCG depending on origin and repeated lyophilization. An increase in growth temperature was shown by Toriyama *et al.* (1978, 1980) to lead to an increase in average chain length for the mycolates of *Mycobacterium phlei*.

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