Intact molecular characterization of cord factor (trehalose 6,6'-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry

Yukiko Fujita,¹ Takashi Naka,¹ Michael R. McNeil² and Ikuya Yano¹

Correspondence

Yukiko Fujita
y-fujita@bcg.gr.jp

¹Japan BCG Central Laboratory, 3-1-5 Matsuyama, Kiyose-shi, Tokyo 204-0022, Japan

²Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA

Cord factor (trehalose 6,6'-dimycolate, TDM) is a unique glycolipid with a trehalose and two molecules of mycolic acids in the mycobacterial cell envelope. Since TDM consists of two molecules of very long branched-chain 3-hydroxy fatty acids, the molecular mass ranges widely and in a complex manner. To characterize the molecular structure of TDM precisely and simply, an attempt was made to determine the mycolic acid subclasses of TDM and the molecular species composition of intact TDM by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for the first time. The results showed that less than 1 µg mycolic acid methyl ester of TDM from nine representative species of mycobacteria and TDM from the same species was sufficient to obtain well-resolved mass spectra composed of pseudomolecular ions $[M + Na]^+$. Although the mass ion distribution was extremely diverse, the molecular species of each TDM was identified clearly by constructing a molecular ion matrix consisting of the combination of two molecules of mycolic acids. The results showed a marked difference in the molecular structure of TDM among mycobacterial species and subspecies. TDM from *Mycobacterium tuberculosis* (H₃₇Rv and Aoyama B) showed a distinctive mass pattern and consisted of over 60 molecular ions with α -, methoxy- and ketomycolate. TDM from *Mycobacterium bovis* BCG Tokyo 172 similarly showed over 35 molecular ions, but that from *M. bovis* BCG Connaught showed simpler molecular ion clusters consisting of less than 35 molecular species due to a complete lack of methoxymycolate. Mass ions due to TDM from *M. bovis* BCG Connaught and *Mycobacterium kansasii* showed a biphasic distribution, but the two major peaks of TDM from *M. kansasii* were shifted up two or three carbon units higher compared with *M. bovis* BCG Connaught. Within the rapid grower group, in TDM consisting of α -, keto- and wax ester mycolate from *Mycobacterium phlei* and *Mycobacterium flavescens*, the mass ion distribution due to polar mycolates was shifted lower than that from the *Mycobacterium avium*-*intracellulare* group. Since the physico-chemical properties and antigenic structure of mycolic acid of TDM affect the host immune responses profoundly, the molecular characterization of TDM by MALDI-TOF mass analysis may give very useful information on the relationship of glycolipid structure to its biological activity.

Received 27 April 2005

Revised 15 June 2005

Accepted 12 July 2005

INTRODUCTION

Cord factor (trehalose 6,6'-dimycolate, TDM) is a characteristic wax-like glycolipid of the genus *Mycobacterium* and related taxa such as *Corynebacterium*, *Rhodococcus* and *Nocardia* (Bloch, 1950; Noll *et al.*, 1956). The structure of TDM, initially found as a virulence factor of the highly virulent cord-forming *Mycobacterium tuberculosis*, has been

investigated vigorously, and the glycolipid has been shown to be distributed widely as a potent immunomodifier among non-virulent mycobacteria and related micro-organisms (Minnikin, 1982; Rastogi *et al.*, 2001; Ryll *et al.*, 2001a). The molecular characteristics of TDM are fundamentally related to the structure of the component mycolic acids, and in general, increasing the average carbon number of mycolic acids has been considered to increase both the hydrophobicity and the virulence (Dubnau *et al.*, 2000; Gotoh *et al.*, 1991; Liu *et al.*, 1996; Ueda *et al.*, 2001). In the mycobacterial cell envelope, together with arabinogalactan mycolate and trehalose monomycolate, TDM forms an integral part of the cell wall skeleton, resulting in highly

Abbreviations: 2,5-DHB, 2,5-dihydroxybenzoic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; TBAH, tetrabutylammonium hydroxide; TDM, trehalose 6,6'-dimycolate. Mycolic acid designations: (0), saturated; (1), monocyclopropanoic or monoenoic; k, keto; (k + H₂), reduced keto; m, methoxy; w, wax ester.

hydrophobic cell surface properties and acid-fastness (Barry *et al.*, 1998; Dubnau *et al.*, 2000; Liu *et al.*, 1996). For the cells of the host animal, both the carbon chain length and structure of mycolic acids of TDM have been found to be closely related to toxicity or intracellular survival, thereby constituting potential virulence mechanisms (Gotoh *et al.*, 1991; Liu *et al.*, 1996). Particular attention has been paid to the virulence mechanisms, including potent inhibition of phagosome-lysosome membrane fusion (Crowe *et al.*, 1994; Spargo *et al.*, 1991), which was shown in *in vitro* systems; Th-1 immunopotentiating properties, including IFN- γ production *in vivo*, have also been investigated (Oswald *et al.*, 1997; Ryll *et al.*, 2001b). Recently, mycoloyl glycolipids have been reported to be a unique lipid antigen restricted by the CD-1 antigen presentation molecule (Beckman *et al.*, 1994; Moody *et al.*, 1997, 1999; Niazi *et al.*, 2001). Furthermore, it has been found that human patient sera (IgG) against TDM from either *M. tuberculosis* or the *Mycobacterium avium-intracellulare* group recognized each respective TDM from the species used as the antigen source (Enomoto *et al.*, 1998; Pan *et al.*, 1999), suggesting that the underlying IgG antibodies were directed not against the carbohydrate backbone, but against the mycolic acid moiety. Therefore, determination of the precise molecular structure of TDM is essential, particularly focussing on the mycolic acid moiety. However, as described in the introduction of our recent paper on trehalose monomycolate (Fujita *et al.*, 2005), the complex structure of mycolic acids has hindered the study of structure-activity relationships, despite the use of various analytical techniques.

In our previous paper, we reported for the first time the direct MALDI-TOF MS analysis of trehalose monomycolate, a precursor of TDM biosynthesis, from 11 species of mycobacteria (Fujita *et al.*, 2005). By the introduction of MALDI-TOF mass analysis of the intact glycolipid, the molecular characterization of ester-bound mycoloyl glycolipid of the mycobacterial cell envelope became possible. Although MALDI-TOF MS is a powerful tool, the structure of the intact TDM, with two molecules of mycolic acids, is very complex and its analysis would be very laborious. Therefore, in the present study, we first isolated the mycolic acid subclasses of TDM from nine species of representative mycobacteria and identified them by MS, and then based on these results we directly analysed the intact TDM from the same species of mycobacteria by MALDI-TOF MS for the first time.

METHODS

Bacterial strains. Nine strains representative of human-virulent and non-virulent, or rapid- and slow-growing, mycobacteria were selected as follows, *Mycobacterium tuberculosis* H₃₇Rv (ATCC 27294), *Mycobacterium tuberculosis* Aoyama B (ATCC 31726), *Mycobacterium bovis* BCG Tokyo 172 (ATCC 35737), *M. bovis* BCG Connaught (ATCC 35745), *Mycobacterium intracellulare* serotype 4 (ATCC 35767), *Mycobacterium intracellulare* serotype 16 (ATCC 13950), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium phlei* (ATCC 11758) and *Mycobacterium flavescens* (ATCC 14474).

Growth conditions. *M. tuberculosis*, *M. bovis* (including BCG strains), *M. phlei* and *M. flavescens* were grown at 37 °C on Sauton medium as surface pellicles until early stationary phase. *M. avium-intracellulare* group and *M. kansasii* were grown on Middlebrook 7H9 medium with shaking at 37 °C until early stationary phase.

Extraction, isolation and purification of TDM. The procedures as described for trehalose monomycolate in our previous paper (Fujita *et al.*, 2005) were used.

Analysis of mycolic acid methyl esters from TDM.

Mycobacterial TDM without wax ester mycolic acid was hydrolysed with 1.25 M NaOH in 90 % methanol at 70 °C for 1 h, and the resultant mycolic acids were extracted with n-hexane after acidification with HCl, followed by methylation with benzene/methanol/H₂SO₄ (10:20:1, by vol.). For the separation of keto- and wax ester mycolate subclasses, TDM containing wax ester mycolic acid was added to 5 % tetrabutylammonium hydroxide (TBAH, 1 ml) and hydrolysed at 100 °C for over 8 h, then mixed with water/CH₂Cl₂/CH₃I (10:20:1, by vol.) (Minnikin, 1988). After centrifugation, the lower organic phase was kept and washed with 1 M HCl and water. Finally, the resultant mycolic acids were extracted with diethyl ether after acidification with glacial acetic acid, followed by reduction of ketomycolate to hydroxymycolate with tetrahydrofuran (2 ml) and NaBH₄ (10 mg) at 37 °C overnight. Mycolic acid methyl esters derived from each TDM were fully separated into subclasses by threefold TLC with benzene as solvent. For reasons of health and safety, experiments using benzene were done with care, in a high-power negative-pressure chamber.

Sample preparation for MS. For MALDI-TOF mass analysis, mycolic acid methyl esters, TDM and 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrix were dissolved in chloroform/methanol (2:1, v/v) at a concentration of 1 mg ml⁻¹ (mycolic acid methyl esters and TDM) or 10 mg ml⁻¹ (2,5-DHB). Samples and matrix (5 µl each) were mixed and applied onto the sample plate as 1.5 µl droplets. The samples were then allowed to crystallize at room temperature.

Mass spectrometric analysis. MALDI-TOF mass spectra (in the positive mode) were acquired as described by Fujita *et al.* (2005).

We determined the molecular mass of mycobacterial TDM based on the quasimolecular mass ions [M+Na]⁺ by the reflectron mode with higher resolution power. Since TDM is a high-molecular-mass (≥ 2500 Da) glycolipid, C₁₇₂H₃₃₀O₁₇ [in the case of diketo 80-(monocyclopropanoic or monoenoic)-mycoloyl TDM], there are marked differences between the nominal and accurate mass numbers in one molecule of TDM. If we calculate the mass of the above TDM, the nominal mass number becomes [(C × 172) + (H × 330) + (O × 172)] = 2666, while the accurate number is [(12.0107 × 172) + (1.00794 × 330) + (15.9994 × 172)] = 2670.4504, the difference being 4.4504. Therefore, we had to correct each molecular (nominal) mass number, and then determine the numbers of C, H and O experimentally.

Structural analysis of TDM. Based on the analytical results of mycolic acids obtained after alkali or very mild alkali hydrolysis of parent TDM, the possible and most abundant combination(s) of mycolic acids were deduced.

RESULTS AND DISCUSSION

TLC of purified TDM

TLC analysis of purified TDM from each mycobacterial strain showed slightly different *R_F* values with the chloroform/methanol solvent systems (Fig. 1). *R_F* values of TDM

Fig. 1. Thin-layer chromatogram of purified TDM. TDM (50 μ g) from: 1, *M. tuberculosis* H₃₇Rv; 2, *M. tuberculosis* Aoyama B; 3, *M. bovis* BCG Tokyo 172; 4, *M. bovis* BCG Connaught; 5, *M. kansasii*; 6, *M. avium-intracellulare* serotype 4; 7, *M. avium-intracellulare* serotype 16; 8, *M. phlei*; 9, *M. flavescens*. Developing solvent, chloroform/methanol/water (90:10:1, by vol.), one time; visualization, 9 M H₂SO₄ spray, followed by heating at 200 °C for 15 min. The arrow indicates the solvent front.

from the *M. tuberculosis* complex and *M. kansasii* were the same, while the R_F values of TDM from mycobacterial species containing wax ester mycolic acid, such as the *M. avium-intracellulare* group, *M. phlei* and *M. flavescens*, were slightly higher than those of TDM which does not contain wax ester mycolic acid.

TLC of mycolic acid methyl esters from TDM

TLC analysis of purified mycolic acid methyl esters of TDM from *M. tuberculosis* H₃₇Rv and *M. avium-intracellulare* serotype 4 showed distinctive R_F values by threefold development with benzene as the solvent (Fig. 2). Mycolic acid methyl esters of TDM from *M. tuberculosis* H₃₇Rv showed three spots corresponding to α -, methoxy- and ketomycolic acid methyl esters, respectively. *M. tuberculosis* Aoyama B, *M. bovis* BCG Tokyo 172 and *M. kansasii* showed a similar pattern, although the relative amount of each mycolic acid subclass differed among the species. In contrast, *M. bovis* Connaught lacked methoxymycolic acid methyl ester entirely. On the other hand, mycolic acid methyl esters of TDM from the *M. avium-intracellulare* group (serotype 4 and 16), *M. phlei* and *M. flavescens* obtained after conventional alkali hydrolysis showed four or more spots on TLC, corresponding to α -, keto- and dicarboxymycolates and secondary alcohol liberated from wax ester mycolate. Since by this procedure, we cannot separate the keto- and wax ester mycolates on TLC in their intact form, we introduced very mild alkali hydrolysis and NaBH₄ reduction techniques. As a result, the mycolic acid methyl esters of TDM from the above four species of mycobacteria were separated into three spots corresponding to α -, wax ester- and reduced keto (k + H₂)-mycolic acid methyl esters, respectively.

Fig. 2. Thin-layer chromatogram of mycolic acid methyl ester subclasses from TDM of mycobacteria. 1, Mycolic acid methyl ester mixture of TDM from *M. tuberculosis* H₃₇Rv; 2–4, α - (2), methoxy- (3) and keto- (4) mycolic acid methyl ester of TDM from *M. tuberculosis* H₃₇Rv; 5, mycolic acid methyl ester mixture of TDM from *M. avium-intracellulare* serotype 4; 6–8, α - (6), wax ester (7) and reduced keto (k + H₂)- (8) mycolic acid methyl ester of TDM from *M. avium-intracellulare* serotype 4. Developing solvent, benzene, three times. Other experimental conditions were as indicated in Fig. 1.

MALDI-TOF MS analysis of mycolic acid methyl esters of TDM from nine representative species of mycobacteria

The MALDI-TOF mass spectra of mycolic acid methyl esters from TDM showed major clusters of mass ions due to $[M+Na]^+$ (M is the molecular mass of mycolic acid methyl esters). When TDM from the *M. avium-intracellulare* group, *M. phlei* and *M. flavescens* were very mildly alkali hydrolysed, methylated and reduced with NaBH₄, the resultant reduced keto (k + H₂)-mycolate showed pseudomolecular ions higher by two mass numbers than the parent ketomycolate.

As shown in Fig. 3, positive MALDI-TOF mass spectra of α -mycolic acid methyl esters of TDM from *M. tuberculosis* H₃₇Rv showed the most abundant mass ion at m/z 1201 due to C₈₀ α -mycolate. Mass ions due to C₇₈, C₈₂, C₈₄ and C₈₆ α -mycolates were also detected clearly. On the other hand, the mass spectra of methoxy- and ketomycolates were shifted higher, by about 100 a.m.u., corresponding to an increase of 5–7 carbon atoms and introduction of one oxygen atom. Thus, the most abundant mass ions are at m/z 1289 and 1317 due to C₈₅ and C₈₇ methoxymycolic acid and at m/z 1315 due to C₈₇ ketomycolic acid. The carbon numbers of methoxy- and ketomycolate ranged between C₈₀ and C₉₂ in the former and between C₇₉ and C₉₁ in the latter in TDM from *M. tuberculosis* H₃₇Rv. Essentially the same composition of subclasses and molecular species of mycolic acids were demonstrated in the case of TDM from *M. tuberculosis* Aoyama B.

Fig. 3. MALDI-TOF mass spectra of mycolic acid methyl esters of TDM from *M. tuberculosis* H₃₇Rv: (a) α -, (b) methoxy-, (c) ketomycolic acid methyl ester. Samples were dissolved in chloroform/methanol (2:1, v/v) at a concentration of 1 mg ml⁻¹ applied on the sample plate as 0.75 μ l droplets. 2,5-DHB was used as matrix. The accelerating voltage was 20 kV (TOF ion trapping; positive reflectron mode).

Mycolic acid methyl esters of TDM from *M. bovis* BCG Tokyo 172 differed markedly from those of TDM of *M. tuberculosis*, although the subclass compositions were very similar, consisting of α -, methoxy- and ketomycolic acids. The MALDI-TOF mass spectra of α -mycolic acid methyl esters of TDM from *M. bovis* BCG Tokyo 172 showed that they consist of even carbon numbered series (C₇₆ to C₈₄) of α -mycolate centring at C₇₈ (m/z 1173). Mass ions due to odd carbon numbered α -mycolates cannot be detected practically. Methoxymycolic acid of TDM from *M. bovis* BCG Tokyo 172 consisted of odd (and even) carbon numbered (C₈₁ to C₉₀) acids centring at C₈₅ (m/z 1289), while ketomycolic acids consisted of even (C₈₀ to C₈₈) and odd (C₈₃ to C₈₉) carbon numbered series acids centring at C₈₆ (m/z 1301). On the other hand, *M. bovis* BCG Connaught strain lacked methoxymycolic acid entirely, as reported by

Minnikin *et al.* (1984), although pseudomolecular mass ions due to α -mycolic acid centring at C₇₈ (m/z 1173) and ions due to ketomycolic acid centring at C₈₄ (m/z 1273) were clearly demonstrated. Thus, the molecular species composition of both α - and ketomycolic acids of TDM from *M. bovis* BCG Tokyo 172 and Connaught were almost identical. The molecular species composition of α -, methoxy- and ketomycolates of TDM from *M. kansasii* resembled those of *M. tuberculosis*, but the molecular species composition of each subclass of TDM from *M. kansasii* was shifted lower by 2 carbon units.

Differing from *M. tuberculosis*, *M. bovis* and *M. kansasii*, the TDM of the *M. avium*-*intracellulare* group possessed characteristic α -, keto- and wax ester mycolic acid subclasses (Fig. 4). Since the R_F values of keto- and wax ester mycolic acid methyl esters were almost identical on TLC, and further, wax ester mycolates were broken down to dicarboxylic acid and secondary alcohol by the conventional

Fig. 4. MALDI-TOF mass spectra of mycolic acid methyl esters of TDM from *M. avium*-*intracellulare* serotype 4: (a) α -, (b) reduced keto ($k+H_2$)-, (c) wax ester mycolic acid methyl ester. Experimental conditions were as indicated in Fig. 3.

alkali hydrolytic procedure, we methylated the free mycolic acids obtained after very mild alkali hydrolysis with TBAH, followed by NaBH₄ reduction without cleavage of intramolecular ester bond of wax ester mycolate (Minnikin, 1988). The resultant (k + H₂)-mycolic acid methyl ester derived from ketomycolate was separated clearly from wax ester mycolic acid methyl ester on TLC. Major molecular species of α -mycolate of TDM from *M. avium-intracellulare* serotype 4 were identified as C₈₀ (*m/z* 1201), C₈₂ (*m/z* 1229) and C₈₃ (*m/z* 1243) α -mycolate, indicating an α -mycolate composition of TDM resembling those of other slow-growing mycobacterial species. The major molecular species of (k + H₂)-mycolate of TDM were C₈₅ (*m/z* 1289) and C₈₇ (*m/z* 1317), and those of wax ester mycolate of TDM were also C₈₅ (*m/z* 1303) and C₈₇ (*m/z* 1331), suggesting that these two subclasses are in precursor-product relationships biosynthetically. Mass spectra of mycolate of TDM from *M. avium-intracellulare* serotype 16 also showed essentially the same results.

On the other hand, *M. phlei* and *M. flavescens*, belonging to the rapidly growing mycobacteria and also to a group producing α -, keto- and wax ester mycolates, showed a distinctive pattern in their mass spectra. The molecular species composition of α -mycolic acid of TDM from both *M. phlei* and *M. flavescens* was similar and again possessed C₈₀ α -mycolate most abundantly, with a smaller amount of shorter and longer homologues. It was also noted that (k + H₂)-mycolate of TDM from *M. phlei* contained saturated even and odd carbon numbered molecular species

abundantly centring at C₈₂, besides ketomonocyclopropanoic (or monoenoic) mycolates. In the cases of *M. phlei* and *M. flavescens* the carbon chain length of reduced keto (k + H₂)- and wax ester mycolate was almost identical and much shorter than those of the *M. avium-intracellulare* group, centring at C₈₀ to C₈₃.

Table 1 and Table 2 summarize the results of comparative MALDI-TOF mass analysis of mycolic acid of TDM from the nine species of mycobacteria. Total carbon and cyclopropane ring (or double bond) numbers of mycolic acids were determined by the mass ions of α -, methoxy-, keto- and wax ester mycolic acid, respectively.

Exceptionally in TDM from *M. phlei*, very mild alkali hydrolysis and reduction products of ketomycolic acids yielded a large amount of even and odd carbon numbered hydroxymycolates with saturated carbon chains, although their detailed structure is not known (whether straight-chain or methyl-branched mero acids exist).

In this study, we first introduced very mild alkali hydrolysis with TBAH followed by NaBH₄ reduction to isolate wax ester mycolate in an intact form and to detect ketomycolate as a reduced form (k + H₂). Since the effect of such procedures on the intact molecules of mycolic acids has not been fully investigated, we compared the alkali hydrolysis products, especially ketomycolate obtained by classical and novel techniques. The results showed both ketomycolates to be essentially the same.

Table 1. MALDI-TOF MS data of the individual types of mycolic acid methyl ester from TDM of representative species of the *M. tuberculosis* complex

Values represent the pseudomolecular mass [M + Na]⁺ of mycolic acid methyl ester. The mass values of the major homologues are shown in bold.

Species	Mycolic acid type*	Total number of carbons in mycolic acid:																		
		74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
<i>M. tuberculosis</i> H ₃₇ Rv	I			1145	1159	1173	1187	1201	1215	1229	1243	1257	1271	1285						
	II							1219	1233	1247	1261	1275	1289	1303	1317	1331	1345	1359	1373	1387
	III						1203	1217	1231	1245	1259	1273	1287	1301	1315	1329	1343	1357	1371	
<i>M. tuberculosis</i> Aoyama B	I			1145	1159	1173	1187	1201	1215	1229	1243	1257	1271	1285						
	II							1233	1247	1261	1275	1289	1303	1317	1331	1345	1359	1373	1387	
	III						1203	1231	1245	1259	1273	1287	1301	1315	1329	1343	1357	1371		
<i>M. bovis</i> BCG Tokyo 172	I			1145		1173		1201		1229		1257								
	II							1233		1261	1275	1289	1303	1317	1331	1345	1359			
	III						1217		1245	1259	1273	1287	1301	1315	1329	1343				
<i>M. bovis</i> BCG Connaught	I	1117		1145	1159	1173	1187	1201	1215	1229		1257								
	III				1175		1203	1217	1231	1245	1259	1273	1287	1301	1315	1329	1343	1357	1371	
<i>M. kansasii</i>	I			1145		1173	1187	1201	1215	1229		1257								
	II							1219	1233	1247	1261	1275	1289	1303	1317	1331	1345	1359		
	III						1203		1231	1245	1259	1273	1287	1301	1315	1329	1343			

*I, α -Dicyclopropanoic or dienoic; II, methoxy; III, keto monocyclopropanoic or monoenoic.

Table 2. MALDI-TOF MS data of the individual types of mycolic acid methyl ester from TDM of the *M. avium*–*intracellulare* group and rapidly growing mycobacteria

Values represent the pseudomolecular mass $[M+Na]^+$ of mycolic acid methyl ester. The mass values of the major homologues are shown in bold.

Species	Mycolic acid type*	Total number of carbons in mycolic acid:															
		75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	
<i>M. avium</i> – <i>intracellulare</i> serotype 4	I				1173	1187	1201	1215	1229	1243	1257	1271			1299		
	III								1247	1261	1275	1289	1303	1317	1331	1345	
	V						1233	1247	1261	1275	1289	1303	1317	1331		1359	
<i>M. avium</i> – <i>intracellulare</i> serotype 16	I				1173	1187	1201	1215	1229	1243	1257	1271					
	III						1219	1233	1247	1261	1275	1289	1303	1317			
	V				1205	1219	1233	1247	1261	1275	1289	1303	1317	1331			
<i>M. phlei</i>	I				1173	1187	1201	1215	1229	1243							
	III				1191	1205	1219	1233	1247	1261							
	IV				1193	1207	1221	1235	1249	1263	1277	1291					
<i>M. flavescens</i>	V		1177	1191	1205	1219	1233	1247	1261	1275	1289	1303					
	I		1145		1173		1201	1215	1229		1257						
	III		1163	1177	1191	1205	1219	1233	1247	1261	1275	1289					
	V		1177	1191	1205	1219	1233	1247	1261	1275	1289	1303					

*I, α -Dicyclopropanoic or dienoic; III, keto monocyclopropanoic or monoenoic; IV, keto saturated (tentatively identified, based on mass spectrometric data, although the structure should be clarified); V, wax ester.

MALDI-TOF MS analysis of TDM

Generally, mycobacterial TDM consisted of a diverse mycolic acid molecular species with 60–90 carbon atoms; thus the mass spectra of TDM showed wide ranges of distribution of mass ions. Based on the precise information on the mycolic acid composition of each TDM from nine representative species of mycobacteria, we have deduced possible combinations of two molecules of mycolic acids and TDM molecular structure.

M. tuberculosis complex and *M. kansasii*, belonging to the slow-growing mycobacteria, produce α -, methoxy- and ketomycolic acid subclasses, among which there is a difference of five to eight carbons between α - and polar mycolates. Therefore, in one cluster of mass ions, ions due to di- α -mycolyl TDM were detected in the lowest mass range, ions due to α - and methoxy- or α - and ketomycolyl TDM in the middle, and ions due to dimethoxy-, methoxy- and keto- or diketomycolyl TDM in the highest mass range.

Fig. 5(a) shows positive MALDI-TOF mass spectra of TDM from *M. tuberculosis* H₃₇Rv. Diverse pseudomolecular ions (mass ion, $[M+Na]^+$) of TDM from both *M. tuberculosis* H₃₇Rv and Aoyama B strains were detected, ranging from m/z 2601 (due to di- α 78-mycolyl TDM) to m/z 3013 [due to methoxy (m)92 and keto (k)91(1)-mycolyl TDM] [(1) means monocyclopropanoic or monoenoic]. The characteristic feature is the normal and almost symmetric distribution of mass ions in these species centring at m/z 2773. Although this ion at m/z 2773 is the most abundant among the quasimolecular ions of TDM from *M. tuberculosis*, there

were possibly multiple combinations of two molecules of mycolates. Based on the analytical results of pre-separated mycolic acid, ions at m/z 2773 was probably due to α 78- and m89-, α 80- and m87- or α 82- and m85-mycolyl TDM, as shown in Table 3. In the lower mass ranges, ions due to di- α -mycolyl TDM predominate, while in the middle mass ranges, ions due to α - and methoxy- or α - and ketomycolyl TDM were abundant. On the other hand, in the higher mass ranges, ions at m/z 2887 seemed to be due to m85- and k89(1)-, m87- and k87(1)- or m88- and k86(1)-mycolyl TDM, although there were numerous minor components with combinations of molecular species other than the major ones. Thus, in the TDM from *M. tuberculosis*, three groups of mass ion distributions, due to di- α -, α -, and polar and dipolar mycoloyl TDM, were demonstrated.

TDM from *M. bovis* BCG Tokyo 172 and *M. bovis* BCG Connaught substrains differed from that of *M. tuberculosis*, although the subclass compositions of mycolic acids of *M. bovis* BCG Tokyo 172 and *M. tuberculosis* were similar, consisting of α -, methoxy- and ketomycolates, as shown in Fig. 6(a). However, TDM from *M. bovis* BCG Connaught lacked methoxymycolate completely, and therefore the distribution patterns of mass ions of TDM from *M. bovis* BCG Tokyo 172 (Fig. 6a) and BCG Connaught (Fig. 6b) differed distinctively. Mass patterns of TDM from *M. bovis* BCG Connaught showed a clear biphasic distribution of mass ions, consisting of a combination of α - and ketomycolate (or di- α -mycolate) at lower mass ranges (m/z 2573–2757) and of diketomycolate at higher ranges (m/z 2745–2955). The most abundant ion among the former

Fig. 5. MALDI-TOF mass spectra of TDM from *M. tuberculosis* H₃₇Rv (a) and *M. kansasii* (b). Spectra of TDM ranging from *m/z* 2600 to *m/z* 3000. Experimental conditions were as indicated in Fig. 3.

group was *m/z* 2701 due to α 76- and k86(1)-, α 78- and k84(1)- or α 80- and k82(1)-mycolyl TDM and that of the latter was *m/z* 2829 due to k84(1)- and k86(1)- or di-k85(1)-mycolyl TDM. On the other hand, TDM from *M. bovis* BCG Tokyo 172 showed a more complex mass pattern due to the additional occurrence of methoxymycolate. The most abundant mass ion shifted to higher mass ranges and was observed at *m/z* 2843, probably due to k84(1)- and k87(1)-mycolyl TDM.

TDM from *M. kansasii* also possessed α -, methoxy- and ketomycolate, and again mass ion distribution of TDM was biphasic, as shown in Fig. 5(b). At the lower mass ranges, ions due to di- α -, α - and methoxy- or α - and ketomycolyl TDM were clearly demonstrated, centring at *m/z* 2743 [due to α 78 and k87(1)- or α 80- and k85(1)-mycolyl TDM] or at *m/z* 2771 [due to α 78- and k89(1)-, α 80- and k87(1)- or α 82- and k85(1)-mycolyl TDM]. On the other hand, at the higher mass ranges, ions due to diketomycolyl TDM predominated at *m/z* 2829 [due to di-k85(1)-mycolyl TDM], *m/z* 2857 [due to k85(1) and k87(1)-mycolyl TDM] or *m/z* 2885 [due to k85(1) and k89(1)- or di-k87(1)-mycolyl TDM]. TDM with the highest molecular mass (*m/z* 2957) in *M. kansasii* was identified as m90- and k89(1)-mycolyl TDM.

Differing from the *M. tuberculosis* complex and *M. kansasii*, the *M. avium*-*intracellulare* group showed a unique mass ion distribution due to the occurrence of TDM containing wax ester mycolate besides α - and ketomycolates, as shown

in Fig. 7. The lowest mass range of TDM from *M. avium*-*intracellulare* serotype 4 (Fig. 7a) consisted mainly of small amounts of ions due to di- α -mycolyl TDM with C₈₀, C₈₂ and/or C₈₃ (*m/z* 2657 due to di- α 80-, *m/z* 2685 due to α 80- and α 82-, *m/z* 2713 due to di- α 82-, *m/z* 2727 due to α 82- and α 83-mycolyl TDM) (see Table 4). In the higher mass ranges, a large amount of ions due to TDM containing keto- and wax ester or di-wax ester mycolate were demonstrated clearly [*m/z* 2845 due to k85(1)- and wax ester(w)85-, *m/z* 2861 due to di-w85-, *m/z* 2873 due to k87(1)- and w85-, *m/z* 2889 due to w85- and w87-mycolyl TDM]. In the middle mass range, a large amount of ions due to TDM containing α - and keto- or α - and wax ester mycolate were detected distinctively [*m/z* 2759 due to α 80- and w85-, *m/z* 2785 due to α 83- and k85(1)-, *m/z* 2787 due to α 80- and w87- or α 82- and w85-mycolyl TDM]. Thus, in the case of TDM from *M. avium*-*intracellulare* serotype 4, the distribution of mass ions of TDM showed a triphasic pattern (*m/z* 2629–2727, 2731–2829 and 2845–2943). A similar pattern was also observed in the case of TDM from *M. avium*-*intracellulare* serotype 16 (Fig. 7b). The mycolic acid subclass compositions of the TDM from *M. avium*-*intracellulare* serotypes 4 and 16 were almost the same, but the mass ion distribution of these types of TDM differed substantially. The mass ion distribution of TDM from *M. avium*-*intracellulare* serotype 16 was shifted lower than that of serotype 4, and TDM with di- α -mycolate molecules (*m/z* 2657 due to di- α 80-mycolyl TDM) predominated.

Table 3. Most probable combination of mycolic acids constructing TDM from representative species of the *M. tuberculosis* complex

Molecular ions of TDM with intensities $\geq 30\%$ of the highest intensity observed are listed. α , α -Dicyclopropanoic or dienoic; m, methoxy-; k, keto monocyclopropanoic or monoenoic mycolic acid.

Mass of TDM*	<i>M. tuberculosis</i>						<i>M. bovis</i> BCG				<i>M. kansasii</i>		
	H ₃₇ Rv			Aoyama B			Tokyo 172		Connaught				
2657	$\alpha 78 : \alpha 82$	$\alpha 80 : \alpha 80$		$\alpha 78 : \alpha 82$	$\alpha 80 : \alpha 80$								
2673									$\alpha 76 : k 84$	$\alpha 78 : k 82$			
2685	$\alpha 78 : \alpha 84$	$\alpha 80 : \alpha 82$									$\alpha 80 : \alpha 82$		
2687	$\alpha 78 : k 83$	$\alpha 80 : k 81$	$\alpha 82 : k 79$								$\alpha 76 : k 85$	$\alpha 78 : k 83$	$\alpha 80 : k 81$
2699	$\alpha 78 : \alpha 85$	$\alpha 80 : \alpha 83$											
2701							$\alpha 78 : k 84$		$\alpha 76 : k 86$	$\alpha 78 : k 84$	$\alpha 80 : k 82$		
2707	m80 : m81												
2713	$\alpha 80 : \alpha 84$	$\alpha 82 : \alpha 82$											
2715	$\alpha 78 : k 85$	$\alpha 80 : k 83$	$\alpha 82 : k 81$	$\alpha 84 : k 79$					$\alpha 76 : k 87$	$\alpha 78 : k 85$	$\alpha 78 : k 85$		
2717					$\alpha 78 : m 85$		$\alpha 78 : m 85$	$\alpha 80 : m 83$					
2729	$\alpha 78 : k 86$								$\alpha 78 : k 86$	$\alpha 80 : k 84$			
2731					$\alpha 78 : m 86$								
2743					$\alpha 78 : k 87$				$\alpha 78 : k 87$	$\alpha 80 : k 85$	$\alpha 78 : k 87$	$\alpha 80 : k 85$	
2745	$\alpha 78 : m 87$	$\alpha 80 : m 85$					$\alpha 78 : m 87$	$\alpha 80 : m 85$	k82 : k82				
2757	$\alpha 78 : k 88$	$\alpha 80 : k 86$					$\alpha 80 : k 86$		$\alpha 80 : k 86$				
2759	$\alpha 78 : m 88$				$\alpha 78 : m 88$						$\alpha 78 : m 88$	$\alpha 80 : m 86$	
2771											$\alpha 78 : k 89$	$\alpha 80 : k 87$	$\alpha 82 : k 85$
2773	$\alpha 78 : m 89$	$\alpha 80 : m 87$	$\alpha 82 : m 85$		$\alpha 78 : m 89$	$\alpha 80 : m 87$	$\alpha 82 : m 85$	$\alpha 80 : m 87$	k82 : k84				
2785	$\alpha 80 : k 88$	$\alpha 82 : k 86$			$\alpha 80 : k 88$								
2787					$\alpha 80 : m 88$				k82 : k85				
2789								m83 : k84					
2799											$\alpha 80 : k 89$	$\alpha 82 : k 87$	
2801	$\alpha 80 : m 89$	$\alpha 82 : m 87$	$\alpha 84 : m 85$		$\alpha 80 : m 89$	$\alpha 82 : m 87$	$\alpha 84 : m 85$	k84 : k84	k82 : k86	k84 : k84			
2803											m83 : k85		
2813	$\alpha 82 : k 88$	$\alpha 84 : k 86$											
2815	$\alpha 82 : m 88$				$\alpha 82 : m 88$				k82 : k87	k84 : k85			
2817								m83 : k86	m85 : k84				
2827	$\alpha 82 : k 89$	$\alpha 84 : k 87$			$\alpha 82 : k 89$	$\alpha 84 : k 87$							
2829	$\alpha 82 : m 89$	$\alpha 84 : m 87$						k84 : k86	k84 : k86	k85 : k85	k85 : k85		
2831					m83 : k87	m85 : k85	m87 : k83	m83 : k87					
2843	$\alpha 84 : m 88$				$\alpha 84 : m 88$			k84 : k87	k84 : k87	k85 : k86			
2845					m85 : k86						m86 : k85		
2847					m83 : m88	m84 : m87	m85 : m86						
2855	$\alpha 84 : k 89$				$\alpha 84 : k 89$								

Table 3. cont.

Mass of TDM*	M. tuberculosis		M. bovis BCG		M. kansasii
	H ₃₇ Rv	Aoyama B	Tokyo 172	Connaught	
2857	α84 : m89	α84 : m89	k86 : k86	k85 : k87	k85 : k87
2859	m85 : k87	m85 : k87			
2871	k86 : k87		k86 : k87	k86 : k87	
2873	m85 : k88	m85 : k88	m87 : k86	m87 : k86	m88 : k85
2885				k87 : k87	k85 : k89
2887	m85 : k89	m85 : k89	m87 : k87		
2889	m85 : m89	m87 : m87			
2901	m87 : k88	m89 : k86	m88 : k87		
2903	m87 : m88	m87 : m88			
2915		m87 : k89	m89 : k87		m88 : k87
2917	m87 : m89	m87 : m89	m88 : m88		

TDM from *M. phlei* and *M. flavescens*, both members of the chromogenic rapid grower group of mycobacteria, also possessed α-, keto- and wax ester mycolic acids. However, differing from that of the slow grower group, the distribution ranges of mass ions of TDM from *M. phlei* and *M. flavescens* were narrower and shifted to lower values between m/z 2629 due to α78- and α80-mycolyl TDM and m/z 2847 due to w84- and w85-mycolyl TDM, respectively (Fig. 8). The mass ion distributions of these types of TDM were monophasic, since the carbon chain lengths of the keto- and wax ester mycolates were shorter and the mass numbers due to α- and polar mycolates were close each other. Therefore, more mass ions were detected in one cluster of pseudo-molecular ions. Since the TDM from *M. phlei* contains abundant unique keto saturated mycolates ranging from C₇₈ to C₈₅, the cluster ion composition of its TDM was more complicated than that of the other rapidly growing mycobacteria (Fig. 8a). Five duplicated mass ions are included in one cluster ion group, namely: (1) di-α-mycolyl TDM, (2) α- and keto- monocyclopropanoic (or monoenoic)-mycolyl TDM, (3) α- and keto- saturated mycolyl TDM [or α- and wax ester, diketo- monocyclopropanoic (or monoenoic)-mycolyl TDM], (4) keto- monocyclopropanoic (or monoenoic) and keto- saturated mycolyl TDM (or keto- monocyclopropanoic (or monoenoic) and wax ester mycolyl TDM) and (5) diketo- saturated mycolyl TDM (or keto- saturated and wax ester, di-wax ester mycolyl TDM). The most abundant mass ion of TDM from *M. phlei* was observed at m/z 2719, possibly due to k80(1) and k82(0)- [0 means saturated (tentatively identified, based on mass spectrometric data, although the structure should be clarified)], k81(1)- and k81(0)-, k82(1)- and k80(0)-, k80(1)- and w81-, k81(1)- and w80- or k83(1)- and w78-mycolyl TDM. Further, this cluster contained m/z 2713 due to di-α82-mycolyl TDM, m/z 2715 due to α80- and k83(1)- or α82- and k81(1)-mycolyl TDM, m/z 2717 due to α82- and k81(0)-, α80- and w82-, α82- and w80-, k80(1)- and k82(1)- or di-k81(1)-mycolyl TDM, and m/z 2721 due to k80(0)- and k82(0)-, di-k81(0)-, k80(0)- and w81-, k81(0)- and w80-, w78- and w82- or di-w80-mycolyl TDM, respectively. Similar multiple ion clusters were also observed in every ion cluster group commonly and characteristically, indicating that the molecular species composition of TDM from *M. phlei* was more diverse than that of the other mycobacterial species studied.

In contrast, since TDM from *M. flavescens* (Fig. 8b) did not possess significant amounts of keto saturated mycolate, the mass pattern was simpler than that of TDM from *M. phlei*. The abundant mass ion in TDM from *M. flavescens* was observed at m/z 2717, possibly due to k80(1)- and k82(1)- or di-k81(1)-mycolyl TDM. Further, this cluster contained m/z 2715 due to α80- and k83(1)- or α82- and k81(1)-mycolyl TDM, m/z 2719 due to k78(1)- and w83-, k80(1)- and w81-, k81(1)- and w80- or k82(1)- and w79-mycolyl TDM, respectively. Although similar multiple ion clusters were observed as in the case of TDM from *M. phlei*, the multiplicity of cluster ion composition was lower. Therefore, the

Fig. 6. MALDI-TOF mass spectra of TDM from *M. bovis* BCG Tokyo 172 (a) and *M. bovis* Connaught (b). Spectra of TDM ranging from m/z 2600 to m/z 3000. Experimental conditions were as indicated in Fig. 3.

Fig. 7. MALDI-TOF mass spectra of TDM from *M. avium-intracellulare* serotype 4 (a) and 16 (b). Spectra of TDM ranging from m/z 2600 to m/z 3000. Experimental conditions were as indicated in Fig. 3.

Table 4. Most probable combination of mycolic acids constructing TDM from representative species of the *M. avium–intracellulare* group and rapidly growing mycobacteria

k', keto saturated (tentatively identified, based on mass spectrometric data, although the structure should be clarified); w, wax ester mycolic acid. Other details are given in the legend to Table 3.

Mass of TDM	<i>M. avium–intracellulare</i>				<i>M. phlei</i>				<i>M. flavescens</i>			
	Serotype 4		Serotype 16									
2657			$\alpha 80 : \alpha 80$									
2659					$\alpha 78 : k81$							
2661					$\alpha 78 : k'81$	$\alpha 78 : w80$	$\alpha 80 : w78$		$k78 : k80$			
2675					$\alpha 78 : k'82$	$\alpha 80 : k'80$	$\alpha 78 : w81$		$k78 : k81$			
2677					$k80 : w78$				$k78 : w80$	$k80 : w78$		
2685			$\alpha 80 : \alpha 82$									
2689									$k78 : k82$	$k80 : k80$		
2691					$k80 : k'80$	$k81 : w78$						
2693					$k'80 : k'80$	$k'81 : w78$						
2703					$\alpha 80 : k'82$	$\alpha 82 : k'80$	$\alpha 78 : w83$	$\alpha 80 : w81$	$k80 : k81$	$k78 : k83$	$k80 : k81$	
2707					$k'80 : k'81$	$k'80 : w80$	$k'82 : w78$	$w78 : w81$				
2713			$\alpha 82 : \alpha 82$									
2715			$\alpha 80 : k83$		$\alpha 80 : k83$	$\alpha 82 : k81$				$\alpha 80 : k83$	$\alpha 82 : k81$	
2717					$\alpha 82 : k'81$	$\alpha 80 : w82$	$\alpha 82 : w80$	$k80 : k82$	$k81 : k81$	$k80 : k82$	$k81 : k81$	
2719					$k80 : k'82$	$k81 : k'81$	$k82 : k'80$	$k80 : w81$	$k81 : w80$	$k83 : w78$	$k78 : w83$	$k80 : w81$
2721					$k'80 : k'82$	$k'81 : k'81$	$k'80 : w81$	$k'81 : w80$	$w78 : w82$	$w80 : w80$		$k81 : w80$
2729	$\alpha 80 : k84$	$\alpha 82 : k82$										$k82 : w79$
2731			$\alpha 80 : w83$							$k80 : k83$	$k81 : k82$	
2733					$k81 : k'82$	$k82 : k'81$	$k83 : k'80$	$k80 : w82$	$k81 : w81$	$k82 : w80$	$k80 : w82$	$k81 : w81$
2743	$\alpha 80 : k85$		$\alpha 82 : k83$							$k82 : w80$	$k81 : w81$	$k82 : w80$
2745					$\alpha 82 : w82$	$k81 : k83$	$k82 : k82$					$k83 : w79$
2747					$k82 : k'82$	$k83 : k'81$	$k80 : w83$	$k81 : w82$	$k82 : w81$	$k83 : w80$	$k80 : w83$	$k81 : w82$
2749					$k'82 : k'82$	$k'80 : w83$	$k'81 : w82$	$k'82 : w81$	$w80 : w82$	$w81 : w81$		$k82 : w81$
2759	$\alpha 80 : w85$		$\alpha 80 : w85$	$\alpha 82 : w83$	$\alpha 82 : w83$	$k82 : k83$					$k82 : k83$	
2761											$k81 : w83$	$k82 : w82$
2763					$k'81 : w83$	$k'82 : w82$	$w80 : w83$	$w81 : w82$			$k83 : w81$	
2771			$\alpha 82 : k85$									
2773			$k83 : k83$									
2775					$k82 : w82$	$k83 : w82$				$k82 : w83$	$k83 : w82$	
2777										$w81 : w83$	$w82 : w82$	
2785	$\alpha 83 : k85$											
2787	$\alpha 80 : w87$	$\alpha 82 : w85$	$\alpha 80 : w87$	$\alpha 82 : w85$								
2789			$k83 : w83$		$k83 : w83$							
2791					$w82 : w83$					$w82 : w83$		

Table 4. cont.

Mass of TDM	<i>M. avium</i> – <i>intracellulare</i>		<i>M. phlei</i>	<i>M. flavescens</i>
	Serotype 4	Serotype 16		
2801	α83 : w85	k83 : k85		
2805		w83 : w83		
2813	α83 : k87			
2815	α82 : w87	α82 : w87		
2817		k83 : w85		
2819		w82 : 85		
2829	α83 : w87	k85 : k85		
2831	k85 : w84	k85 : w84		
2833		w83 : w85		
2845	k85 : w85	k85 : w85		
2859		k84 : w87		
2861	w85 : w85	k85 : w86		
2873	k87 : w85	k86 : w85		
2889	w85 : w87			
2901	k87 : w87	w85 : w87		
2917	w87 : w87			

molecular species composition of TDM from *M. flavescens* was simpler than that of TDM from *M. phlei*.

Conclusion

TDM is the most prominent and best-studied mycolic-acid-containing glycolipid of mycobacteria. The structure varies greatly among mycobacterial species, and the mycolyl moiety has been linked to toxicity, antigenicity or intracellular survival, thereby constituting potential virulence or immunostimulating mechanisms. In order to establish the structure–activity relationship, simple and precise analysis of the high-molecular-mass glycolipids is essential.

To establish the intact molecular structure analysis of TDM by MALDI-TOF MS, we first analysed mycolic acid subclasses after alkali hydrolysis of TDM. The results showed marked differences in subclasses and molecular species composition of mycolic acids according to the mycobacterial species. α-Mycolic acid was a ubiquitous component among the mycobacterial species, with carbon numbers ranging from C₇₄ to C₈₈ and with two cyclopropane rings or equivalent double bonds. Ketomycolates were also widely distributed, with carbon numbers ranging from C₇₆ to C₉₁, but the ranges of the carbon number of ketomycolates differed greatly among the species. Methoxymycolates were a more characteristic component in particular species of mycobacteria such as *M. tuberculosis*, *M. bovis* BCG Tokyo 172 and *M. kansasii*. Wax ester mycolates were also specific for the *M. avium*–*intracellulare* group and chromogenic rapid growers such as *M. phlei* and *M. flavescens*, differing in the ranges of distribution of carbon numbers.

In the case of slow growers such as *M. tuberculosis*, *M. bovis* BCG, *M. kansasii* and the *M. avium*–*intracellulare* group, chain length of keto-, methoxy- or wax ester mycolate was much longer than those of α-mycolate, while in the case of rapid growers such as *M. phlei* and *M. flavescens*, the chain lengths of both α- and polar mycolates were similar.

The carbon numbers of keto- and wax ester mycolic acid were almost identical, this reflecting the metabolic precursor–product relationship between the former and the latter, since the wax ester mycolates are synthesized directly from ketomycolates by a direct biological Baeyer–Villiger type oxidation system (Toriyama *et al.*, 1982).

Based on such precise information on mycolic acids and their possible combination of two molecules of mycolic acids, we deduced the molecular structure of each TDM from the direct MALDI-TOF mass spectra. MALDI-TOF mass analysis enabled us to reveal whole molecular structures of TDM without a degradative procedure, especially the carbon, cyclopropane ring or double bond numbers, and oxygenated functions such as carbonyl, methoxy or intramolecular ester group (although the positions or configurations of cyclopropane rings or double bonds should be determined further). The MALDI-TOF mass fingerprint of the whole TDM molecule from a given mycobacterial

Fig. 8. MALDI-TOF mass spectra of TDM from *M. phlei* (a) and *M. flavescens* (b). Spectra of TDM ranging from m/z 2600 to m/z 3000. Experimental conditions were as indicated in Fig. 3.

strain appears to be a very promising tool for rapid analysis, facilitating the identification of mycobacterial species and the elucidation of the relationship between structure and immunopotentiating activity.

REFERENCES

- Barry, C. E., 3rd, Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A. & Yuan, Y. (1998). Mycolic acids: structure, biosynthesis and physiological functions. *Prog Lipid Res* 37, 143–179.
- Beckman, E. M., Porcelli, S. A., Morita, C. T., Behar, S. M., Furlong, S. T. & Brenner, M. B. (1994). Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature* 372, 691–694.
- Bloch, H. (1950). Studies on the virulence of tubercle bacilli; the relationship of the physiological state of the organisms to their pathogenicity. *J Exp Med* 92, 507–526.
- Crowe, L. M., Spargo, B. J., Ionedo, T., Beaman, B. L. & Crowe, J. H. (1994). Interaction of cord factor (α,α' -trehalose-6,6'-dimycolate) with phospholipids. *Biochim Biophys Acta* 1194, 53–60.
- Dubnau, E., Chan, J., Raynaud, C., Mohan, V. P., Lanelle, M. A., Yu, K., Quemard, A., Smith, I. & Daff, M. (2000). Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Mol Microbiol* 36, 630–637.
- Enomoto, K., Oka, S., Fujiwara, N., Okamoto, T., Okuda, Y., Maekura, R., Kuroki, T. & Yano, I. (1998). Rapid serodiagnosis of *Mycobacterium avium*-intracellular complex infection by ELISA with cord factor (trehalose 6,6'-dimycolate), and serotyping using the glycopeptidolipid antigen. *Microbiol Immunol* 42, 689–696.
- Fujita, Y., Naka, T., Doi, T. & Yano, I. (2005). Direct molecular mass determination of trehalose monomycolate from 11 species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* 151, 1443–1452.
- Gotoh, K., Mitsuyama, M., Imaizumi, S., Kawamura, I. & Yano, I. (1991). Mycolic acid-containing glycolipid as a possible virulence factor of *Rhodococcus equi* for mice. *Microbiol Immunol* 35, 175–185.
- Liu, J., Barry, C. E., 3rd, Besra, G. S. & Nikaido, H. (1996). Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *J Biol Chem* 271, 29545–29551.
- Minnikin, D. E. (1982). Lipids: complex lipids, their chemistry, biosynthesis and roles. Part I: physiology of the mycobacteria. In *The Biology of the Mycobacteria*, pp. 95–184. Edited by C. Ratledge & J. Stanford. London: Academic Press.
- Minnikin, D. E. (1988). Isolation and purification of mycobacterial wall lipids. In *Bacterial Cell Surface Techniques*, pp. 125–135. Edited by I. C. Hancock & I. R. Poxton. Chichester: Wiley.
- Minnikin, D. E., Parlett, J. H., Magnusson, M., Ridell, M. & Lind, A. (1984). Mycolic acid patterns of representatives of *Mycobacterium bovis* BCG. *J Gen Microbiol* 130, 2733–2736.
- Moody, D. B., Reinhold, B. B., Guy, M. R. & 9 other authors (1997). Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* 278, 283–286.
- Moody, D. B., Reinhold, B. B., Reinhold, V. N., Besra, G. S. & Porcelli, S. A. (1999). Uptake and processing of glycosylated mycolates for presentation to CD1b-restricted T cells. *Immunol Lett* 65, 85–91.
- Niazi, K., Chiu, M., Mendoza, R. & 8 other authors (2001). The A' and F' pockets of human CD1b are both required for optimal presentation of lipid antigens to T cells. *J Immunol* 166, 2562–2570.

- Noll, H., Bloch, H., Asselineau, J. & Lederer, E. (1956). The chemical structure of the cord factor of *Mycobacterium tuberculosis*. *Biochim Biophys Acta* **20**, 299–309.
- Oswald, I. P., Dozois, C. M., Petit, J. F. & Lemaire, G. (1997). Interleukin-12 synthesis is a required step in trehalose dimycolate-induced activation of mouse peritoneal macrophages. *Infect Immun* **65**, 1364–1369.
- Pan, J., Fujiwara, N., Oka, S., Maekura, R., Ogura, T. & Yano, I. (1999). Anti-cord factor (trehalose 6,6'-dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses. *Microbiol Immunol* **43**, 863–869.
- Rastogi, N., Legrand, E. & Sola, C. (2001). The mycobacteria: an introduction to nomenclature and pathogenesis. *Rev Sci Tech* **20**, 21–54.
- Ryll, R., Kumazawa, Y. & Yano, I. (2001a). Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids – a review. *Microbiol Immunol* **45**, 801–811.
- Ryll, R., Watanabe, K., Fujiwara, N., Takimoto, H., Hasunuma, R., Kumazawa, Y., Okada, M. & Yano, I. (2001b). Mycobacterial cord factor, but not sulfolipid, causes depletion of NKT cells and upregulation of CD1d1 on murine macrophages. *Microbes Infect* **3**, 611–619.
- Spargo, B. J., Crowe, L. M., Ionedo, T., Beaman, B. L. & Crowe, J. H. (1991). Cord factor (α,α -trehalose 6,6'-dimycolate) inhibits fusion between phospholipid vesicles. *Proc Natl Acad Sci U S A* **88**, 737–740.
- Toriyama, S., Imaizumi, S., Tomiyasu, I., Masui, M. & Yano, I. (1982). Incorporation of ^{18}O into long-chain, secondary alcohols derived from ester mycolic acids in *Mycobacterium phlei*. *Biochim Biophys Acta* **712**, 427–429.
- Ueda, S., Fujiwara, N., Naka, T., Sakaguchi, I., Ozeki, Y., Yano, I., Kasama, T. & Kobayashi, K. (2001). Structure-activity relationship of mycoloyl glycolipids derived from *Rhodococcus* sp. 4306. *Microb Pathog* **30**, 91–99.