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Granuloma formation activity and mycolic acid composition of mycobacterial cord factor

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Abstract. We analyzed the mycolic acid composition of trehalose 6,6′-dimycolate (TDM) obtained from *Mycobacterium*, using thin layer chromatography, gas chromatography and gas chromatography-mass spectrometry. Utilizing TDM, whose structure was confirmed, granuloma formation in mice was investigated. All TDM used exhibited considerable granuloma formation activity in the lung and spleen. In particular, TDM from *M. bovis* showed the greatest activity and toxicity among mycobacterial TDM. We therefore discussed the relationship between the chemical structure and granuloma-forming activity of TDM, especially in relation to the structure of mycolic acid in TDM.
Key words. Trehalose 6,6′-dimycolate (TDM); cord factor; mycolic acid; granuloma formation; *Mycobacterium*; gas chromatography-mass spectrometry.

Cord factor was first recognized to be a toxic glycolipid produced by *M. tuberculosis* and to be related to the virulence of *Mycobacterium* [1]. Subsequently, it was demonstrated that cord factor was trehalose 6,6′-dimycolate (TDM) [2] and that avirulent *Mycobacterium* and Actinomycetes also had cord factor in their cell walls [3–5]. Trehalose 6,6′-dimycolate is now well-known as a potent immunomodulator possessing granuloma-forming activity [6] and anti-tumor activity [7, 8]. We also reported that cord factor isolated from *N. rubra* [9, 10] and *R. terrae* [11] had granuloma-forming activity. Furthermore, we demonstrated that there was marked variation in the granuloma-forming activity of mycoloyl glycolipids, depending on the carbohydrate moiety [12].

We also analyzed the mycolic acid composition of cord factor using thin layer chromatography (TLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) [13–16]. The results revealed that each species of *Mycobacterium* had a characteristic and complex composition of mycolic acids, but that *Nocardia* and *Rhodococcus* had only one subclass, α-mycolic acid. Moreover, cord factor from *Nocardia* and *Rhodococcus* possessed much shorter mycolic acid than that of mycobacterial cord factor.

Now, in this study, we have investigated the relationship between the chemical structure of mycolic acid in TDM and the granuloma-forming activity of TDM obtained from *Mycobacterium*.

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Materials and methods

We utilized the cells of *Mycobacterium bovis* BCG, *M. avium-intracellulare*, *M. kansasii* and *M. smegmatis* strains Jucho and Takeo for the experiment. Rapid growers were cultivated in a medium containing 1% glucose, 0.2% yeast extract and 0.5% peptone for 5 days at 30 °C. Slow growers were cultured at 35 °C for 3 to 5 weeks in Sauton medium or on slants containing Ogawa egg medium. The lipids were extracted from the harvested cells with chloroform-methanol (2:1 by volume) and separated by thin layer chromatography. The solvent system was chloroform, methanol, acetone and acetic acid (90:10:6:1 v/v). The purification of TDM was repeated until a single spot was obtained. Mycolic acid obtained by mild alkali hydrolysis was methylated and separated into subclasses by TLC (hexane and diethyl ether, 4:1 by volume). The molecular species of trimethylsilylated (TMS) methyl mycolate was determined by GC-MS system (Hitachi M-80B) with glass column (1% OV-101 on Gas chronQ, 0.3 m by 3 mm).

Purified trehalose dimycolate was emulsified with 0.2% Tween 80 and 3% Freund incomplete adjuvant in phosphate-buffered saline. Ten to 300 μg of TDM in the form of w/o/w emulsion was injected into the tail vein of ICR mice (male, 4 to 5 weeks old). For the control, w/o/w emulsion without TDM was injected. At 7 days after the injection, lungs, spleens and livers were taken out and weighed. The organ index was calculated to show the degree of granuloma formation as follows: organ index = (organ weight/body weight) × 100.

An F-test was used to determine significant differences between treated groups and w/o/w control groups.

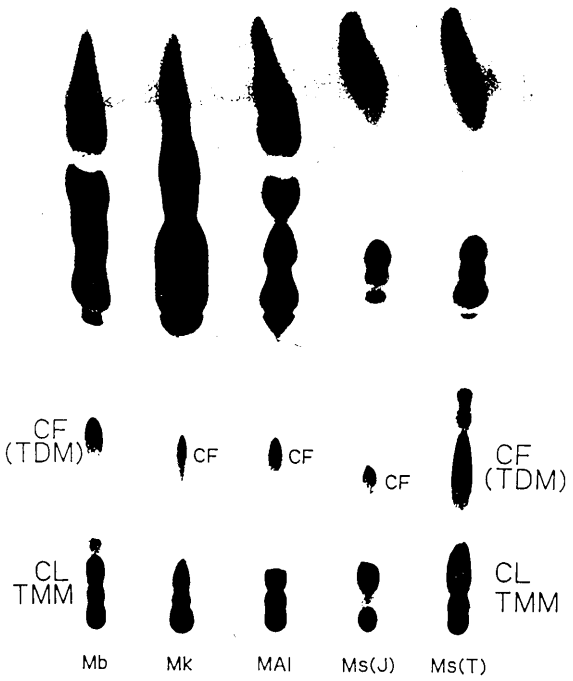

Figure 1. Thin layer chromatograms of chloroform-methanol soluble lipids from *Mycobacteriun bovis* BCG (Mb), *M. kansasii* (MK), *M. avium-intracellulare* (MAI), *M. smegmatis* Jucho (Ms(J)) and *M. smegmatis* Takeo (Ms(T)). CF(TDM): cord factor(trehalose dimycolate), CL: caldiolipin and TMM; trehalose monomycolate.

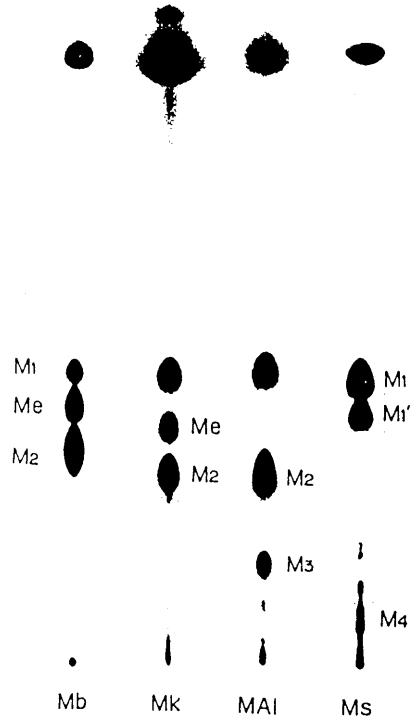

Figure 3. Thin layer chromatograms of the total fatty acid methyl esters from *M. bovis* BCG (Mb), *M. kansasii* (MK), *M. aviu-intracellulare* (MAI) and *M. smegmatis* (Ms). Mycolic acid subclass, M_1: α-, Me: methoxy-, M_2: keto-, M_3: dicarboxy-, M_1': α'-, M_4: epoxy-.

Results and discussion

Figure 1 shows the thin layer chromatogram of chloroform-methanol soluble lipid obtained from bacterial cells. Each species and strain had trehalose dimycolates as one spot on TLC. The general structure of TDM is shown in figure 2. While the carbohydrate moiety was trehalose in all TDM used in this experiment, the fatty acid moiety varied. Accordingly, methyl derivatives of alkali-hydrolyzed TDM were assayed by TLC and their TMS derivatives were analyzed by GC-MS. As shown in figure 3, *M. bovis* and *M. kansasii* had three subclasses: α-(M_1), methoxy-(Me), and keto-mycolic acids(M_2). *M. avium-intracellulare* had α-(M_1), keto-(M_2) and dicarboxy-mycolates(M_3) and *M. smegmatis* had α-(M_1), α'-(M_1') and epoxy-mycolates(M_4).

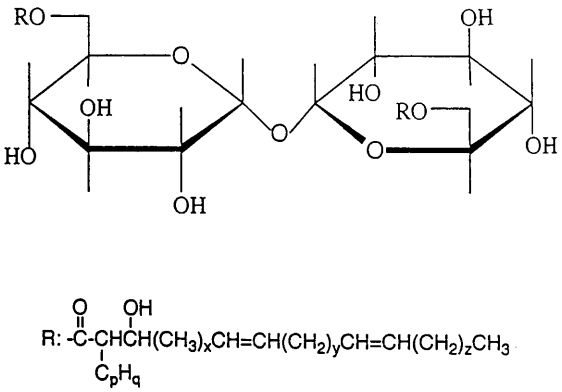

Figure 2. General structure of trehalose dimycolate R: mycolic acid residue.

The gas chromatographic analysis of TMS-methyl α-mycolates from four species of *Mycobacterium* gave characteristic patterns according to the total number of carbon atoms, as shown in figure 4. The total numbers of carbon atoms and double bonds were estimated by the mass spectra of the top of gas chromatographic peaks (fig. 5B, C). The M-15 ions produced by the loss of the methyl group from molecular ions of TMS methyl mycolate suggested the total carbon number of mycolic acid (fig. 5A). Fragment ion A resulting from C_{2-3} cleavage indicated the carbon and double bond numbers of the straight alkyl chain unit of TMS methyl mycolate. The main peaks of the gas chromatogram of mycolates from *M. smegmatis* had an odd number, which meant that their α-mycolates had one methyl branch on the straight alkyl chain unit. Fragment ion B produced by C_{3-4} cleavage indicated the carbon and double bond numbers of the α-branched chain unit. Fragment ion B and B-29 from *M. avium-intracellulare* were 483 and 454, respectively. They suggested that the branched chain at the 2-position was saturated C_{24}. The α-branched alkyl chain unit from *M. smegmatis* and *M. kansasii* was also saturated C_{24}. On the other hand, fragment ion B and B-29 from TMS methyl mycolate from *M. bovis* were 511 and 482, respectively. These

data suggested that the α-branched alkyl chain unit was $C_{26:0}$. Table 1 shows the summary of mycolic acid composition of four mycobacterial species obtained from GC-MS analysis. The total carbon numbers of α-mycolates of the experimental species were much greater than those from *Nocardia* and *Rhodococcus*, and all α-mycolates of the experimental group had two unsaturated bonds. Moreover, all mycobacterial species had a couple of subclasses of mycolic acid, while *Nocardia* and *Rhodococcus* had only one subclass, α-mycolic acid. One point of interest was whether the difference in fatty acid moiety of TDM had an effect on granuloma formation.

TDM purified from mycobacterial cells was prepared in w/o/w emulsion and injected into the tail vein of ICR mice. The previous paper [9] showed that the lungs of TDM-treated mice changed histologically between day 3 and 7, while the w/o/w control specimens did not. Cellular infiltration and disseminated granulomas were observed in the experimental tissues. These histological changes were paralleled by changes in lung weight. The maximal change was observed at seven days after injection. Therefore, organ weight was measured after seven days in order to estimate granuloma formation. The dose responses for granuloma formation by a single injection of TDM are shown in figure 6. A significant increase in lung and spleen weight was observed with the emulsion containing more than 30 μg TDM. Similar results for dose responses of TDM from other species were also obtained. Moreover, a marked increase in lung weight was observed following a single injection of 300 μg of TDM from all species examined (fig. 7). *M. bovis* TDM produced a significantly greater change in lung weight than that from other species. Similarly, all TDM induced an increase in spleen weight one week after injection. The TDM of *M. bovis* had less effect on spleen weight than on the lung. It is currently unclear why the degree of granuloma formation in the lungs and spleen showed a reverse correlation, since the previous data indicated an almost parallel effect on the lung and spleen. However, glucose monomycolate from *R. rhodococcus* 13161 had a strong effect on the lung but a weak one on the spleen [11]. Moreover, the time-dependent and dose-dependent patterns of organ index were a little different in the lung and the spleen. For example, the increase in spleen weight due to granuloma occurred later than that in the lung. Moreover, the affinity to organs was thought to be different depending on the nature of the TDM emulsion [17]. The speed and the quantity of TDM absorbed into tissues might be dependent on the organ and the chemical structure of TDM.

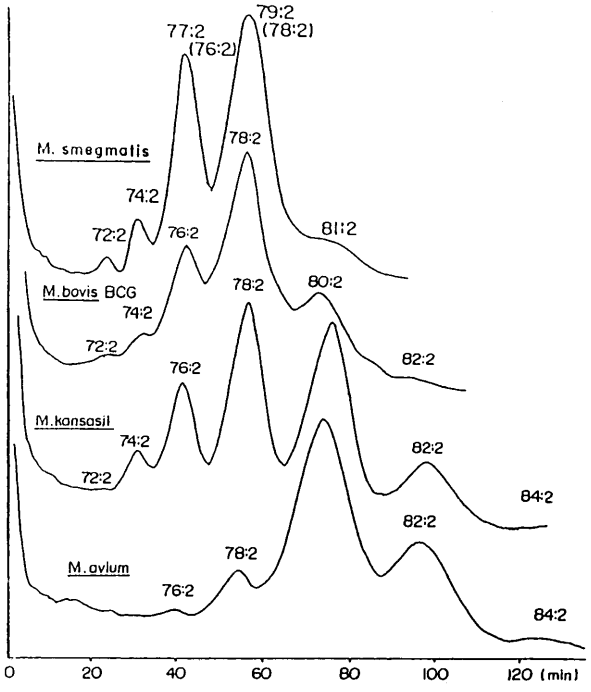

Figure 4. Gas chromatograms of TMS-methyl α-mycolate from *M. smegmatis*, *M. bovis* BCG, *M. kansasii*, *M. avium-intracellulare*. The carbon and double bond numbers of the ester at each peak were determined by GC-MS.

Figure 8 shows the weights of liver and body seven days after TDM injection. All values were significantly smaller than the controls. The TDM from *Nocardia* and *Rhodococcus* also had granuloma-forming activity on liver and induced an increase in liver weight. However, the peak of granuloma in livers was further delayed compared with those of lungs and spleens. It was generally recognized that mycobacterial TDM exerted a toxic effect on experimental animals, leading to death following severe weight loss [1, 18]. It was reported that the greater the toxicity of TDM produced, the greater was the decrease in body weight [11]. TDM-treated organ weight similarly remained the same or decreased for a few days, while the control organ weight increased day by day. *M. bovis* TDM, which had the strongest granuloma-forming activity in lungs, induced the largest decrease in body weight. It was thought that the toxicity of TDM dominated its granuloma-forming activity on the seventh day because it took much more than seven days to produce granuloma in livers.

In order to study the relationship between granuloma-forming activity and the total carbon number of the fatty acid moiety of TDM, the activity of *Mycobacterium's* TDM was compared with that from other species, such as *Nocardia* and *Rhodococcus*. Figure 9 shows lung indices obtained by TDM from various bacterial species which indicate the degree of granuloma formation. *Rhodococcus* and *Nocardia* had only one subclass of α-mycolic acids. TDM from R.13165 had no effect on the lung. α-Mycolic acids of R.13165 had carbon numbers lower than 40. The other TDM, possessing mycolic acids with carbon numbers greater than 40, had greater lung index values. All TDM used in this experiment had significant granuloma-forming activity and total carbon numbers of mycolic acids greater than 70. It was likely

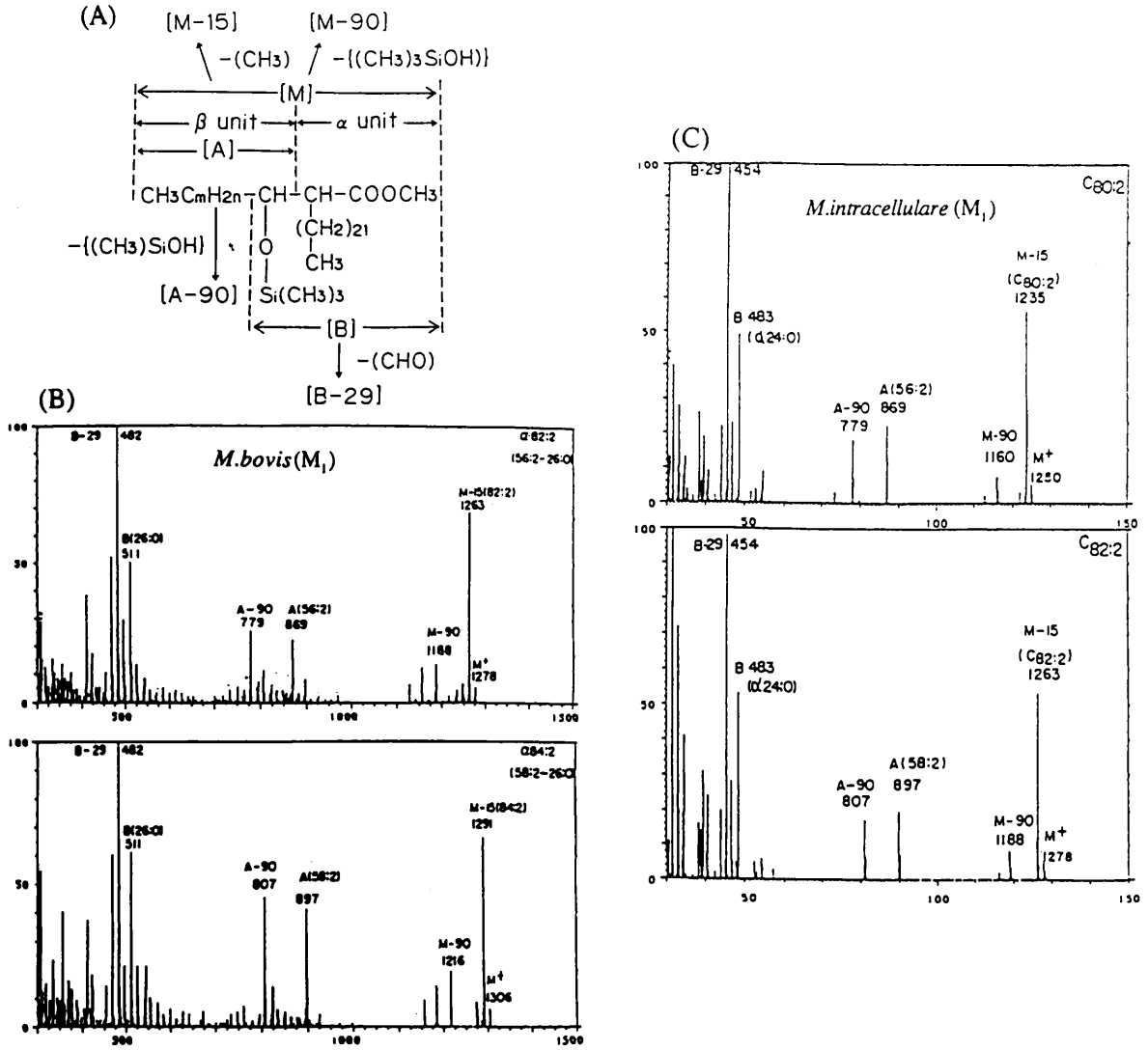

Figure 5. Mass fragmentation pattern (*A*) and mass spectra of TMS derivatives of methyl α-mycolate (M1) of *M. bovis* BCG (*B*) and *M. intracellulare* (*C*).

that the total carbon number of α-mycolic acid was influenced for the granuloma-forming activity of TDM among the genera investigated.

The index for *M. bovis* was much greater than the others and total carbon numbers of α-mycolic acid did not vary so much. However, the molecular species of mycolates constituting TDM was characteristic (fig. 3 and table 1). The ratios of mycolic acid subclasses were also diverse. α-Mycolic acid was minor in *M. bovis* but

Table 1. Summary of mycolic acid composition obtained from GC-MS analysis.

	Carbon number of α-mycolic acid		Mycolic acid
	total	α-branch	subclass*
M. bovis	78	26	M_1, Me, M_2
M. kansasii	78, 80	24	M_1, Me, M_2
M. avium-intracellulare	80	24	M_1, M_2, M_3
M. smegmatis	77, 79	24	M_1, (M_1'), M_4

* M_1: α-mycolic acid, M_1': α′-mycolic acid. Me: methoxy-mycolic acid; M_2: keto-mycolic acid; M_3: dicarboxy-mycolic acid; M_4: expoxy-mycolic acid.

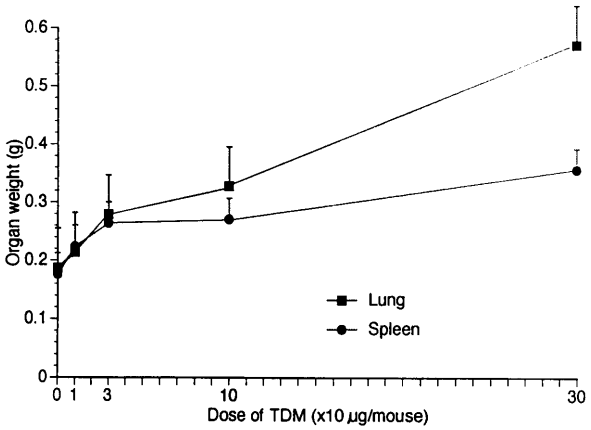

Figure 6. Dose response of TDM from *M. kansasii* for lung and spleen weight of mice.

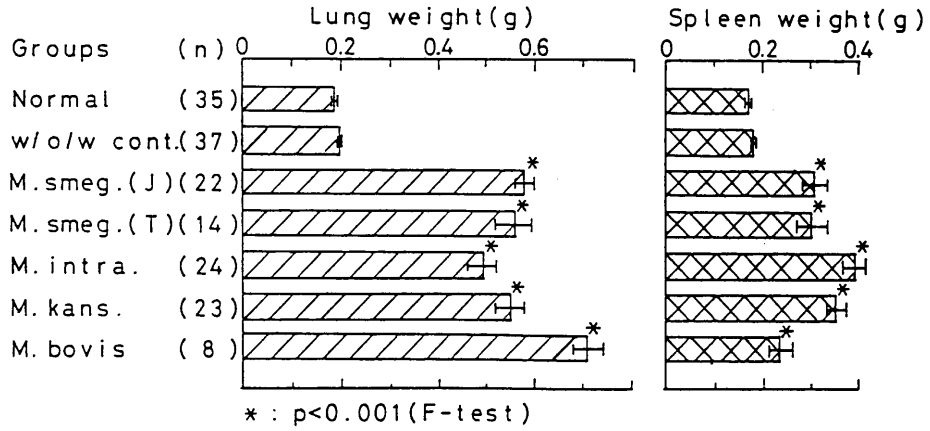

Figure 7. Effect of TDM (300 μg/mouse) from *Mycobacteria* on spleen and lung weight of mice 7 days after injection.

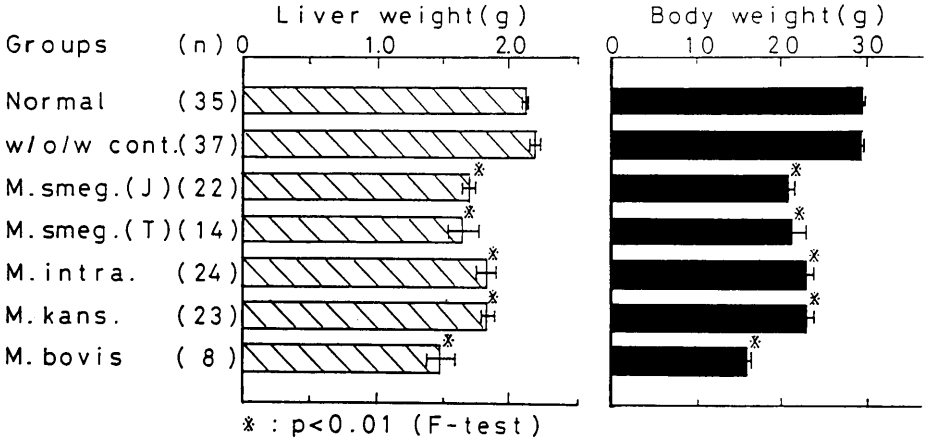

Figure 8. Effect of TDM (300 μg/mouse) from *Mycobateria* on liver and body weights of mice 7 days after injection.

major in *M. smegmatis*, *M. kansasii* and *M. avium-intracellulare*. Prome showed that TDM of *M. phlei* had three types in a combination of α-mycolic acid and oxygenated mycolic acid [19], which will be an important factor in the case of mycobacterial TDM. We observed that the total carbon numbers of methoxy- and keto-mycolic acids were larger by about 6 than that of α-mycolic acid in *M. bovis* (unpublished data). Toubiana also reported that oxygenated mycolic acids in *M. tuberculosis* were longer than α-mycolic acids [20]. It was noticed that the proportion of oxygenated mycolic acid (Me and M_2) was much larger than that of α-mycolic acid (M_1) in *M. bovis*, but that the proportion of those mycolates to the total mycolates was not so high in *M. kansasii*, in *M. avium-intracellulare* or in *M. smegmatis*. Accordingly, TDM of *M. bovis* might contain oxygenated mycolic acid with much longer total carbon number than α-mycolic acid.

Masihi [21, 22] demonstrated that the combination of monophosphoryl lipid A or muramyl dipeptide with TDM extracted from *M. bovis*, but not with that from *M. avium* or *M. phlei*, induced significant resistance to influenza virus. Bekierkunst [23] reported that the strongest response of tubercles in mouse lungs was induced by cord factor from *M. kansasii* and the weakest by cord factor from the BCG strain of *M. bovis*. Only TDM from *M. bovis* showed specific effects among mycobacterial TDM although it was not known to relate inversely. When we considered the difference between *M. bovis* TDM and other TDMs such as *M. kansasii*, *M. avium* or *M. phlei*, we noticed that only *M. bovis*'s α-mycolic acid had an α-branch with $C_{26:0}$ [24].

Our previous results showed that the specific carbohydrate moiety of mycoloyl glycolipid, such as trehalose and glucose, was essential for the granuloma-forming activity and also for the induction of a chemotactic factor from macrophages [25] and the induction of interleukin-1 and colony stimulating factor [26]. In this study we have demonstrated that TDM from *Mycobacterium* has potent granuloma-forming activity and that the total carbon number of mycolic acid in TDM was important for the activity. However, it is currently unknown why the intensity of the activity varied among mycobacterial TDM whose mycolic acids had a total carbon number of more than 70. Mycobacterial TDM can be distinguished as an antigen

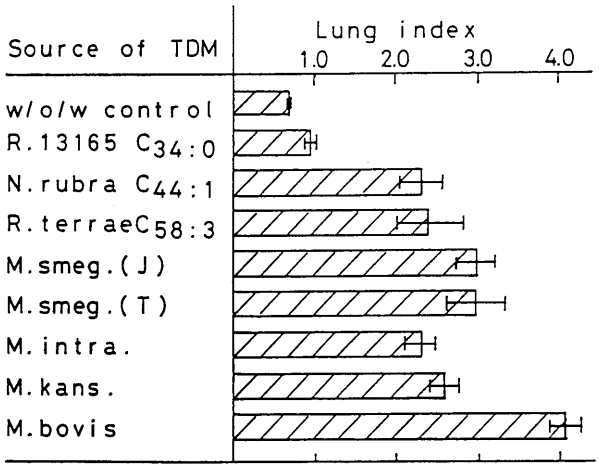

Figure 9. Comparison of lung indices induced by TDM (300 μg/mouse) from various bacterial species.

according to the mycolic acid subclass, that is according to the functional group of the straight alkyl chain of mycolic acid (unpublished data). It will be necessary, as far as the fatty acid moiety of TDM is concerned, to confirm the essential site for granuloma formation taking account not only of the total carbon number but also of the α-alkyl branch and the functional group of mycolic acids, and the ratio of mycolic acid subclasses.

1 Bloch H. (1950) Studies on the virulence of tubercle bacilli. Isolation and biological properties of a constituent of vilurent organisms. J. Exp. Med. **91:** 197–217

2 Noll H., Bloch H., Asselineu J. and Lederer E, (1956) The chemical structure of the cord factor of *Mycobacterium tuberculosis*. Biochim. Biophys. Acta **20:** 299–309

3 Yano I., Furukawa Y. and Kusunose M. (1971) Occurrence of acylated trehaloses in *Nocardia*. J. Gen. Appl. Microbiol. **17:** 329–334

4 Thomas D. W., Matida A. K., Silva C. L. and Ioneda T. (1979) Esters of trehalose from *Corynebacterium diphtheriae*: a modified purofocatin procedure and studies on the structure of their constituent hydroxylated fatty acids. Chem. Phys. Lipids **23:** 267–282

5 Silva C. L., Gesztesi J. L. and Ioneda T. (1979) Trehalose mycolates from *Nocardia asteroides*, *Nocardia farcinica*, *Gordona lentifragmenta* and *Gordona bronchialis*. Chem. Phys. Lipids **24:** 17–25

6 Bekierkunst A. (1968) Acute granulomatous response procedure in mice by trehalose-6,6′-dimycolate. J. Bacteriol. **96:** 958–961

7 Bekierkunst A., Wany L., Toubiana R. and Lederer E. (1974) Immunotherapy of cancer with nonliving BCG and fractions derived from *Mycobacteria*: role of cord factor (trehalose 6,6′-dimycolate) in tumor regression. Infect. Immun. **10:** 1044–1050

8 Lemaire G., Tenu J. P., Petit J. F. and Lederer E. (1986) Natural and synthetic trehalose diesters as immunomodulators. Med. Res. Rev. 6: 243–274

9 Kaneda K., Sumi Y., Kurano F., Kato Y. and Yano I. (1986) Granuloma formation and hemopoiesis induced by C_{36-48} mycolic acid containing glycolipids from *Nocardia rubra*. Infect. Immun. **54:** 869–875

10 Yano I., Tomiyasu I., Kaneda K., Kato Y., Sumi Y., Kurano S. et al. (1987) Isolation of mycolic acid-containing glucolipids in *Nocadia rubra* and their granuloma forming activity in mice. J. Pharmacobio.-Dyn. **10:** 113–123

11 Sawai H., Sumi Y., Kurano S., Gondaira S., Kato Y., Tomiyasu I. et al. (1987) Granuloma formation by the glycolipids containing mycolic acid in *Nocardia*, *Rhodococcus* and related *Actinomycetes* and their structure analysis. Yakugaku Zasshi **107:** 37–45

12 Natuhara Y., Oka S., Kato Y. and Yano I. (1990) Parallel antitumor, granuloma-forming and tumor-necrosis-factor-priming activities of mycolyl glycolipids from *Nocardia rubra* that differ in carbohydrate moiety: structure-activity relationships. Cancer Immunol. Immunother. **31:** 99–106

13 Yano I., Kageyama K., Ohno Y., Masui M., Kusunose M., Kusunose E. and Akimori N. (1978) Separation and analysis of molecular species of mycolic acids in *Nocardia* and related taxa by gas chromatography mass spectrometry. Biomed. Mass Spectrometry **5:** 14–24

14 Kaneda K., Naito S., Imaizumi S., Yano I., Mizuno S., Tomiyasu I. et al. (1986) Determination of molecular species composition of C_{80} or longer-chain-α-mycolic acids in *Mycobaterium* spp. by gas chromatography-mass spectrometry and mass chromatography. J. Clin. Microbiol. **24:** 1060–1070

15 Kaneda K., Imaizumi S., Mizuno S., Baba T., Tsukamura M. and Yano I. (1988) Structure and molecular species composition of three homologous series of α-mycolic acids from *Mycobacterium* spp. J. Gen. Microbiol. **134:** 2213–2229

16 Baba T., Kaneda K., Kusunose E., Kusunose M. and Yano I. (1988) Molecular species of mycolic acid subclasses in eight strains of *Mycobacterium smegmatis*. Lipids **23:** 1132–1138

17 Kurano S., Sugimoto N., Sumi Y., Sawai H., Kato Y., Kaneda K. and Yano I. (1987) Newly isolated glycolipids from *Rhodococuss terrae* cell wall and their granuloma forming activities. Yakugaku Zasshi **107:** 46–52

18 Silva C. L., Tincani I., Filho S. L. B. and Faccioli L. H. (1988) Mouse cachexia induced by trehalose dimycolate from *Nocardia asteroides*. J. Gen. Microbiol. **134:** 1629–1633

19 Prome J.-C., Lacave C., Ahibo-Coffy A. and Savagnac A. (1976) Separation and structural studies of the molecular species of monomycolates and dimycolates of α-D-trehalose present in *Mycobacterium phlei*. Eur. J. Biochem. **63:** 543–552

20 Toubiana R., Berlan J., Sato H. and Strain M. (1979) Three types of mycolic acid from *Mycobacterium tuberculosis Brevanne*: Implications for structure-fnction relationships in pathogenesis. J. Bacteriol. **139:** 205–211

21 Masihi K. N., Brehmer W., Lange W., Werner H. and Ribi E. (1985) Trehalose dimycolate from various mycobacterial species induces differing anti-infectious activities in combination with muramyl dipeptide. Infect.Immun. **50:** 938–940

22 Masihi K. N., Lange W., Brehmer W. and Ribi E. (1986) Immunobiological activities of nontoxic lipid A: enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. Int. J. Immunopharmac. **8:** 339–345

23 Bekierkunst A., Levij I. S., Yarkoni E., Vilkas E., Adam A. and Lederer E. (1969) Granuloma formation induced in mice by chemically defined mycobacterial fractions. J. Bacteriol. **100:** 95–102

24 Kaneda K., Imaizumi S. and Yano I. (1995) Distribution of C_{22}-, C_{24}- and C_{26}-α-unit-containing mycolic acid homologues in *Mycobacteria*. Microbiol. Immunol. **39:** 563–570

25 Matsunaga I., Oka S., Inoue T. and Yano I. (1990) Mycolyl glycolipids stimulate macrophages to release a chemotactic factor. FEMS Microbiol. Lett. **67:** 49–54

26 Oka S., Natsuhara Y., Kato Y., Kaneda K. and Yano I. (1989) Granuloma formation and cytokine-inducing activities of glycolipids containing mycolic acids from *Nocardia rubra*. Acta Leprologica **7**(Suppl. 1): 123–124