J. Biochem. 120, 663-670 (1996)

Relationship between Induction of Macrophage Chemotactic Factors and Formation of Granulomas Caused by Mycoloyl Glycolipids from *Rhodococcus ruber* (*Nocardia rubra*)

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Received for publication, May 23, 1996

Mycoloyl glycolipids cause granulomas in the lungs, liver, and spleen of mice, but the mechanism is not fully understood. To understand the role of macrophage chemotactic factors (MCFs) in granuloma formation, we prepared various mycoloyl glycolipids with different carbohydrate moieties: trehalose dimycolate (TDM), glucose mycolate (GM), mannose mycolate (MM), and fructose mycolate (FM) from *Rhodococcus ruber*, and examined the relationship between their MCF induction in peritoneal macrophages and the extent of granuloma formation. The molecular mass of each glycolipid was confirmed by fast-atom-bombardment mass-spectrometry. TDM or GM caused granulomas in the lungs, spleen, and liver of ICR mice, but MM and FM did not. The culture supernatant of peritoneal macrophages stimulated with TDM or GM increased macrophage migration, whereas MM and FM had no chemotactic activity. The activity of interleukin-1 (IL-1) in the supernatant was increased equally by each glycolipid and was therefore not related to chemotaxis. Tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were not detected in the four supernatants. The TDM-induced MCF was heat-stable, trypsin-labile, and undialyzable. Furthermore, we separated two MCF active fractions from the supernatant of TDM-stimulated macrophages by gel filtration. These factors acted on macrophages but not on neutrophils. Our results suggested that macrophages recognize the sugar moieties of mycoloyl glycolipids and may, in response, generate a MCF that may play an important role in the macrophage or monocyte recruitment which is essential prior to granuloma formation.

Key words: chemotactic factor, granuloma, macrophage, mycoloyl glycolipid.

Granulomatous inflammation is the most characteristic pathological change caused by an acid-fast bacterial infection, particularly by *Mycobacterium tuberculosis*. This type of change is induced usually 3 to 6 weeks after infection through complicated immunological processes, including cellular recruitment and proliferation at the site of infection. Mycoloyl glycolipids, such as cord factor (trehalose-6,6′-dimycolate; TDM), are bacterial components that can induce granulomas without protein antigens (*1*). Therefore, mycoloyl glycolipids are useful in studies on the mechanism of granuloma formation. TDM was first isolated as a major lipid component of *M. tuberculosis* (*2*), and it has many biological activities, including granuloma-forming activity (*1, 3, 4*). Mycoloyl glycolipids have been found widely as cell-wall components of acid-fast bacteria such as *Nocardia, Rhodococcus*, and *Gordona*, and their structures vary greatly. We analyzed the structures of mycoloyl glycolipids isolated from these and other mycobacteria-related species, and studied their granuloma-forming, cytokine-inducing, and antitumor activities (*5-11*).

In this study, we used mycoloyl glycolipids isolated from *Rhodococcus ruber* cultivated on various carbon sources. TDM and glucose-6-monomycolate (GM) can be obtained when *R. ruber* is cultivated with glucose as the carbohydrate source. However, when cultivated with mannose or fructose, mannose-6-monomycolate (MM) or fructose-6-monomycolate (FM), respectively, is produced instead of GM. For TDM, GM, MM, and FM, all with different carbohydrate moieties, we investigated their *in vivo* priming of tumor necrosis factor (TNF) and their antitumor activities (*8*), finding that the carbohydrate moiety affected their biological activities. It is not known which cells recognize the structure of mycoloyl glycolipids and what responses are provoked by these cells.

Macrophages are the main cellular component of granulomas. In the early phase of granuloma formation, macrophages accumulate to remove stimuli such as mycobacterial cells or their components and are activated to release several cytokines such as interleukin-1 (IL-1) and TNF-α, which are involved in granuloma formation (*12-14*). Thus, the accumulation of macrophages caused by chemotactic factors released from other macrophages, lymphocytes, and endothelial cells is the first and key event in granuloma formation. Some chemotactic factors for monocytes/mac-

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Abbreviations: FM, fructose-6-monomycolate; GM, glucose-6-monomycolate; MCF, macrophage chemotactic factor; MM, mannose-6-monomycolate; TDM, trehalose-6,6′-dimycolate.

rophages induced in experimental granulomas or human tuberculous granulomas have been identified (*15-17*). Details of the involvement of the carbohydrate moieties of mycoloyl glycolipids, which are potent granuloma-inducing agents, in the induction of macrophage chemotactic factors (MCFs) have not yet been reported yet, and it is not known what kind of MCFs are produced.

We postulated that macrophages recognize the carbohydrate moieties of mycoloyl glycolipids and respond by releasing MCFs during granuloma formation. Here, we studied granuloma formation caused by various glycolipids, and not only MCFs but also other cytokines from macrophages stimulated with these mycoloyl glycolipids to reveal the relationship between the structures of mycoloyl glycolipids and their *in vitro* cytokine-inducing activities. Furthermore, we characterized the MCFs.

MATERIALS AND METHODS

Preparation and Isolation of Mycoloyl Glycolipids from R. ruber—Mycoloyl glycolipids were prepared and purified essentially as described previously (*5, 18*). In brief, *R. ruber* M-1 strain was cultivated in a medium containing 1% D-glucose or, instead, D-mannose, or D-fructose as the carbohydrate source for 3 days at 30°C. After the cells had been harvested by centrifugation, the lipids were extracted by the method of Folch *et al.* (*19*), and then separated by thin-layer chromatography (TLC) on a silica gel with a mixture of chloroform/methanol/acetone/acetic acid (90 : 10 : 6 : 1, v/v/v/v) as the development solvent. The plates were sprayed with 50% H_2SO_4, and then heated at 180°C for 20 min, or dipped in iodine vapor to visualize the lipid bands. The glycolipids were repeatedly purified on preparative thin-layer plates until a single spot was obtained.

Analysis of the Isolated Glycolipids—The component sugars were identified by silica gel TLC with phenol/water/ethanol/acetic acid (50 : 22 : 3 : 3, v/v/v/v) as the solvent, after alkali hydrolysis with 0.5 N NaOH in methanol/water (1 : 1, v/v), and by gas-chromatography mass-spectrometry (GC/MS) of alditol acetates obtained on 2 M trifluoroacetic acid hydrolysis at 120°C for 2 h, $NaBH_4$ reduction, and acetylation with acetic-acid-anhydride/pyridine (1 : 1, v/v). The molecular species of mycolic acids were determined by GC/MS, using trimethylsilylether derivatives of methyl esters, as reported previously (*5*). The molecular weights of the intact mycoloyl glycolipids were determined by fast-atom-bombardment mass-spectrometry (FAB/MS) with a JEOL-SX 102 double focusing mass spectrometer (JEOL, Tokyo).

Granuloma Formation Caused by Mycoloyl Glycolipids—Male ICR mice of 4-5 weeks old were purchased from SLC (Shizuoka). Granulomas were caused by the injection of 300 μg of a glycolipid in the form of a water-in-oil-in-water (W/O/W) emulsion into the tail vein of mice as previously described (*6*). At 3, 7, 14, and 28 days after the injection, the lungs, spleens and livers were removed and weighed. As a control, a W/O/W emulsion without a glycolipid was injected. Organ indices were calculated as follows to express the degree of granuloma formation: organ index = (organ weight/body weight) $\times$ 100.

Preparation of Mouse Peritoneal Macrophages—Mouse peritoneal macrophages were prepared as described previously (*18*). Briefly, 2 ml of 10% proteose peptone was injected intraperitoneally into male ICR mice (6-8 weeks old). Four days later, peritoneal exudate cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) with 1 mU/ml heparin, and then washed twice by centrifugation with RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) containing 100 μg/ml streptomycin and 100 U/ml penicillin G. Then, peritoneal macrophages were prepared by the method of Kumagai *et al.* (*20*), and suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium with or without 10% heat-inactivated fetal calf serum (FCS., Gibco, Grand Island, NY, USA).

Preparation of a Culture Supernatant of Macrophages Stimulated with a Mycoloyl Glycolipid—In the standard procedure, macrophages (1×10^6 cells) were plated onto 24-well plates coated with 1 μg of a glycolipid or not, and incubated without FCS at 37°C for 24 h. Thereafter, the culture supernatants were collected, passed through a filter (pore size, 0.22 μm, Millipore, Milford, MA, USA), and used for the assaying of macrophage migration, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF). For the IL-1 assay, the samples were dialyzed against RPMI 1640 medium overnight. For the time-course study, the culture supernatants were collected after various incubation times.

Estimation of Chemotactic Activity—The macrophage migration rate was estimated using a Boyden's chamber (Neuro Probe, Pleasanton, CA, USA) equipped with a 5-μm-pore polycarbonate filter, 10 μm thick (Nucleopore, Pleasanton, CA, USA) as described previously (*18*). The number of macrophages in the upper chamber was 2×10^5. After incubation of the chamber for 90 min at 37°C in humidified air, the filter was removed, dried, and stained with Giemsa's stain. The chemotactic activity was expressed as the number of cells in five randomly selected microscopic fields at a magnification of $\times$400. A total of 20 fields per filter was counted. In the neutrophil migration assay, mouse neutrophils were prepared from peritoneal exudate cells induced by an injection of thioglycolate 12 h earlier, and chemotactic activity was evaluated by Boyden's method with a 3-μm-pore polycarbonate filter, 10 μm thick. A checkerboard assay was performed as described previously (*18*).

Estimation of IL-1, TNF-α, and GM-CSF Activity—IL-1 activity was evaluated with mitogen-stimulated C3H/HeJ mice (4 weeks old, SLC) in a thymocyte proliferation assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT., Wako Pure Chemical Industries, Osaka) (*21*). Briefly, 1×10^6 thymus cells were incubated with a dialyzed culture supernatant of macrophages and a suboptimal dose of phytohemagglutinin at 37°C for 48 h. After incubation of the cells, MTT was added to a final concentration of 1 mg/ml and the cells were incubated for 2 h. Then, the cells were lysed with sodium dodecyl sulfate and *N,N'*-dimethylformamide (Wako). After overnight incubation, the absorbance of the suspension at 550 nm was measured. TNF-α activity was assayed as the lysis of mouse fibroblast L-929 cells (*22*). GM-CSF activity was assayed by the method of colony formation in soft agar (*23*). Recombinant mouse IL-1α (Genzyme, Boston, MA, USA), recombinant mouse TNF-α (Genzyme), and recombinant mouse GM-CSF (Endogen, Cambridge, MA, USA) were used as positive controls.

Treatment of the Culture Supernatant of TDM-Stimu-

lated Macrophages with Heat, Trypsin, or Dialysis—Culture supernatants of macrophages stimulated with 1 μg/well TDM or without TDM were incubated with 2.5 μg/ml porcine pancreatic trypsin (Wako) at 37°C for 60 min, and then treated with 100 μg/ml soybean trypsin inhibitor (Wako). As a control, only trypsin inhibitor was added after incubation without trypsin. The heat stability of MCF activity was examined by incubation of the culture supernatants at 37, 56, or 100°C for various times. The effect of dialysis was determined by dialyzing the culture supernatants against RPMI 1640 medium overnight at 4°C.

Separation of MCF Activities on a Sephacryl S-200 Column—The culture supernatant of macrophages incubated without FCS and with 1 μg/well TDM was concentrated with an ultrafiltration membrane (YM 3; Amicon, Danvers, MA, USA), and then dialyzed against PBS (pH 7.4) at 4°C overnight. Thereafter, 0.5 ml of the dialyzed sample was placed on a 1.0×40-cm column of Sephacryl S-200 gel (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) equilibrated with PBS (pH 7.4). The column was eluted at the flow rate of 20 ml/h with PBS (pH 7.4) at 4°C. After the protein concentration of each 1-ml fraction had been determined by the method of Bradford, using bovine serum albumin as a standard (*24*), MCF and IL-1 activities were assayed. Molecular weights were estimated with bovine serum albumin (molecular weight, 67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease (13,700) as markers.

RESULTS

Isolation and Characterization of Mycoloyl Glycolipids from R. ruber—The extractable lipids obtained from *R. ruber* M-1 accounted for 3% of the packed cell weight. As shown in Fig. 1, TLC of the lipids from *R. ruber* M-1 showed that two classes of glycolipids (trehalose monomycolate and TDM) were found in all cells regardless of the carbohydrate source. Furthermore, GM, MM, or FM was isolated from *R. ruber* M-1 grown with (D)-glucose, (D)-mannose, or (D)-fructose, respectively. The structures of these new glycolipids were tentatively identified on GC/MS analysis of the carbohydrate moieties and mycoloyl residues after acid methanolysis or alkali hydrolysis. The molecular weights of the individual glycolipids determined by FAB/MS analysis showed that they were monomycoloyl glycolipids possessing mycolic acids ranging from C_{36} to C_{60} and centered at C_{44} or C_{46}, with one or no double bands (Fig. 2). It was noted that the mycoloyl glycolipids thus obtained differed only in the carbohydrate moiety and the most abundant molecular species of mycolic acid was $C_{46:1}$ among the mycoloyl glycolipids, commonly. The putative structures of these glycolipids are shown in Fig. 3.

Granuloma Formation and MCF Induction by Mycoloyl Glycolipids with Different Carbohydrate Moieties—In mice treated with TDM or GM, the lung and spleen indices increased until 7 days after the injection, when they were maximum (Fig. 4, A and B). Thereafter, the lung index decreased rapidly and the spleen index decreased gradually. The liver index in mice treated with TDM was higher than that in the controls on day 14, and then decreased gradually (Fig. 4C). In mice treated with GM, the liver index was the highest on day 28. The increase in body weight of mice treated with TDM or GM was less than that of the controls until day 28 (Fig. 4D). In mice treated with MM or FM, however, the organ indices and body weight were not significantly different from those in the controls at any time.

After the injection of TDM or GM as a W/O/W emulsion into mice, typical granulomas were found in sections of the lungs on microscopic observation, as reported previously (*8*). MM and FM did not induce granulomas, although the alveolar septa were thickened somewhat because of mild infiltration by inflammatory cells in mice injected with MM. Similar results were observed for the liver and spleen.

Macrophage migration was greatest in the culture supernatants incubated with 1 μg/well TDM for 12 or 24 h, and the migration was slightly decreased when the incubation was continued for 48 h (Fig. 5). At all times, activity was absent in the culture supernatants of unstimulated macrophages. We have deduced that a macrophage migration factor induced by GM is chemotactic for macrophages by means of a checkerboard assay (*18*). Here, we reexamined whether or not the macrophage migration toward the factor induced by TDM involved chemotaxis or chemokinesis (random migration). The macrophage migration activity depended mostly on the concentration gradient of the sample between the lower and upper chambers (Table I). Little activity was detected when the lower and upper chambers contained equal concentrations of the sample.

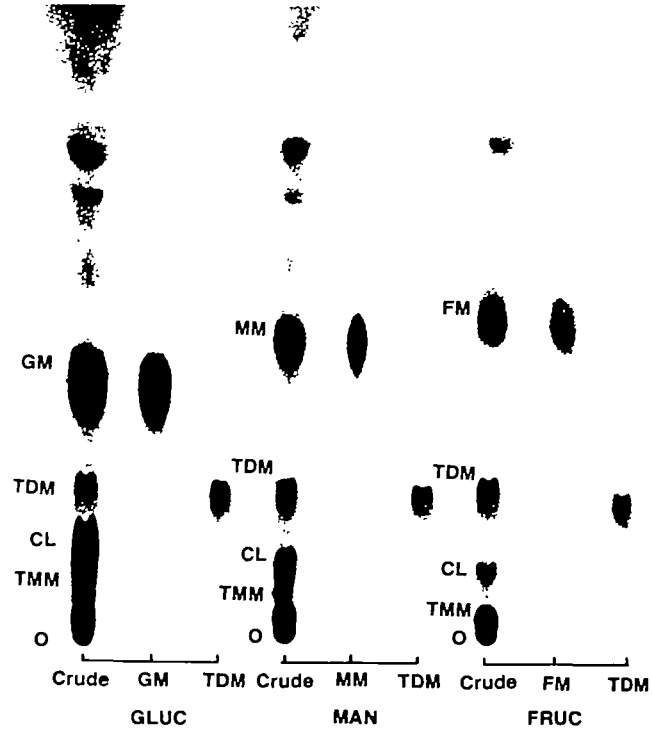

Fig. 1. **Thin-layer chromatography of mycoloyl glycolipids from *R. ruber* grown in a medium containing various carbohydrate sources.** The plates were developed with a solvent system of chloroform/methanol/acetone/acetic acid (90 : 10 : 6 : 1, v/v/v/v), and charred after 16-N H_2SO_4 spraying. GM, glucose monomycolate; MM, mannose monomycolate; FM, fructose monomycolate; CL, cardiolipin; TDM, trehalose dimycolate; TMM, trehalose monomycolate; and Crude, crude extract. O indicates the origin. GLUC, MAN, and FRUC indicate cells grown in a medium containing (D)-glucose, (D)-mannose, and (D)-fructose, respectively.

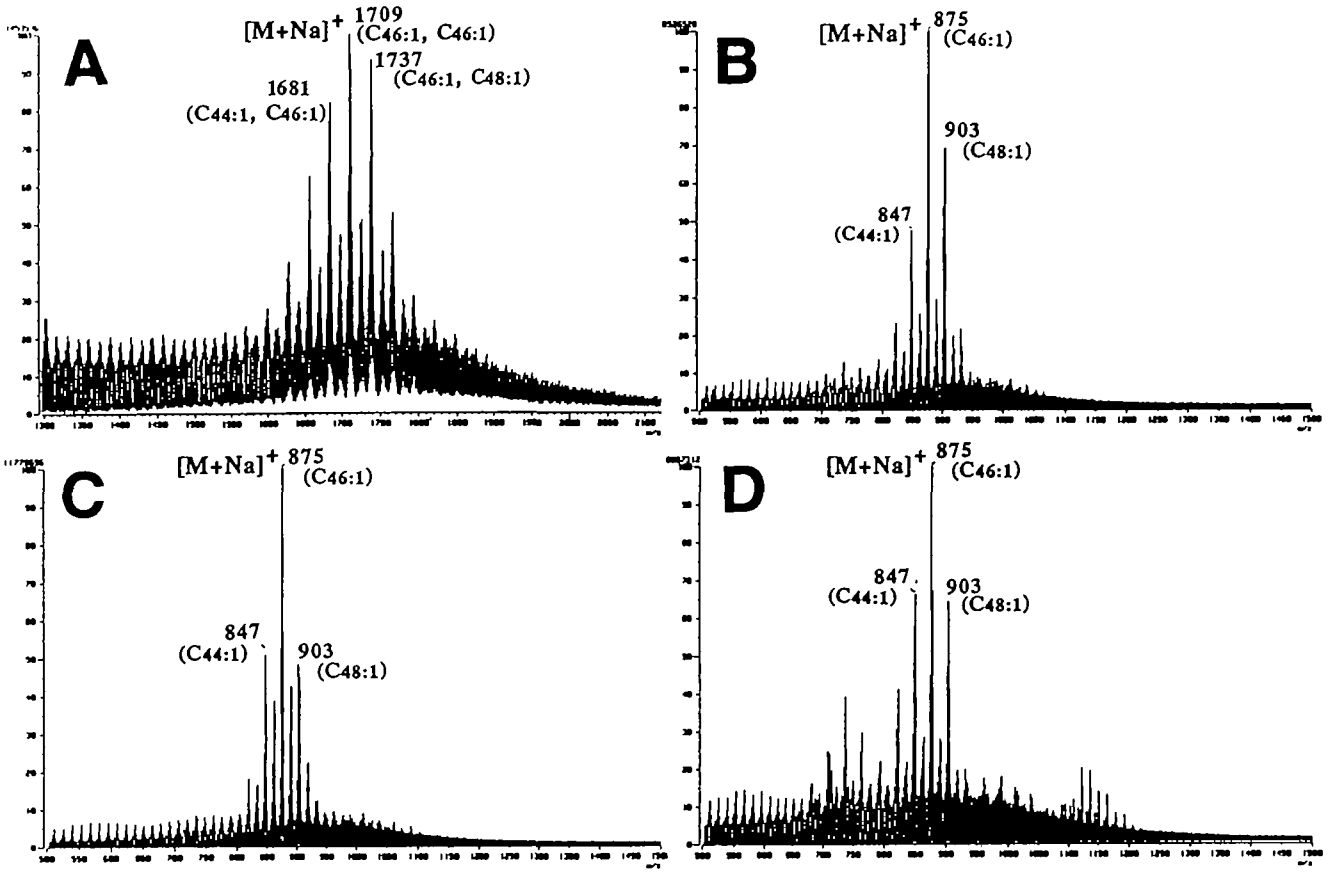

Fig. 2. **Positive FAB/MS spectra of mycoloyl glycolipids from *R. ruber*.** (A) TDM, (B) GM, (C) MM, and (D) TDM.

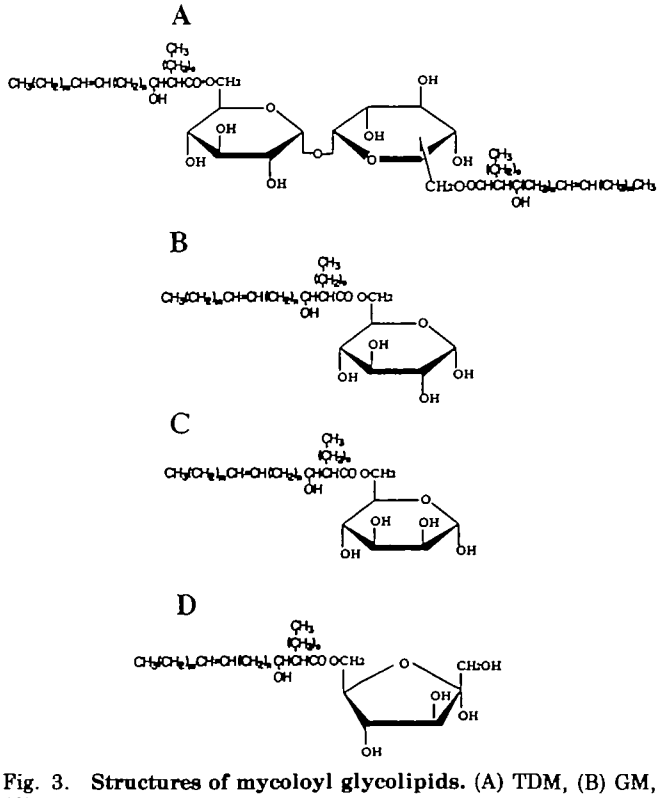

Fig. 3. **Structures of mycoloyl glycolipids.** (A) TDM, (B) GM, (C) MM, and (D) FM.

Macrophage migration activity was due mainly to chemotaxis of macrophages, not to random migration.

When we examined MCF induction by different mycoloyl glycolipids, we found that TDM and GM induced MCF activity, but that MM and FM did not (Table II). Our previous study showed that when the concentration of TDM or GM is 1 μg/well, MCF activity is greatest; 5 μg/well or more of TDM or GM inhibits MCF induction (*18*). This inhibition may be due to the cytotoxicity of mycoloyl glycolipids, because the viability of the macrophages examined with crystal violet was decreased (viability at 25 μg/well TDM, 80.9%; 125 μg/well, 60.9%). Similar changes in cell viability were observed when macrophages were incubated with MM or FM. Macrophages incubated with any glycolipid at 1 μg/well were fully viable. Therefore, cytotoxicity of the mycoloyl glycolipid was not the reason for the lack of MCF induction by MM or FM.

We assayed the activities of IL-1, TNF-α, and GM-CSF in the culture supernatants, but TNF-α and GM-CSF were not detected (Table II). Various levels of IL-1 activity were detected in the culture supernatants of macrophages treated with the mycoloyl glycolipids. However, the profile of IL-1 activity did not match that of MCFs in the supernatants. For example, IL-1 activity was found in culture supernatants of macrophages incubated with MM or FM, where there was no MCF activity. We examined whether or not mouse recombinant IL-1α, TNF-α, and GM-CSF are chemotactic for mouse peritoneal macrophages. IL-1α, TNF-α, and GM-CSF were not chemotactic for mouse peritoneal macrophages within the concentration ranges of

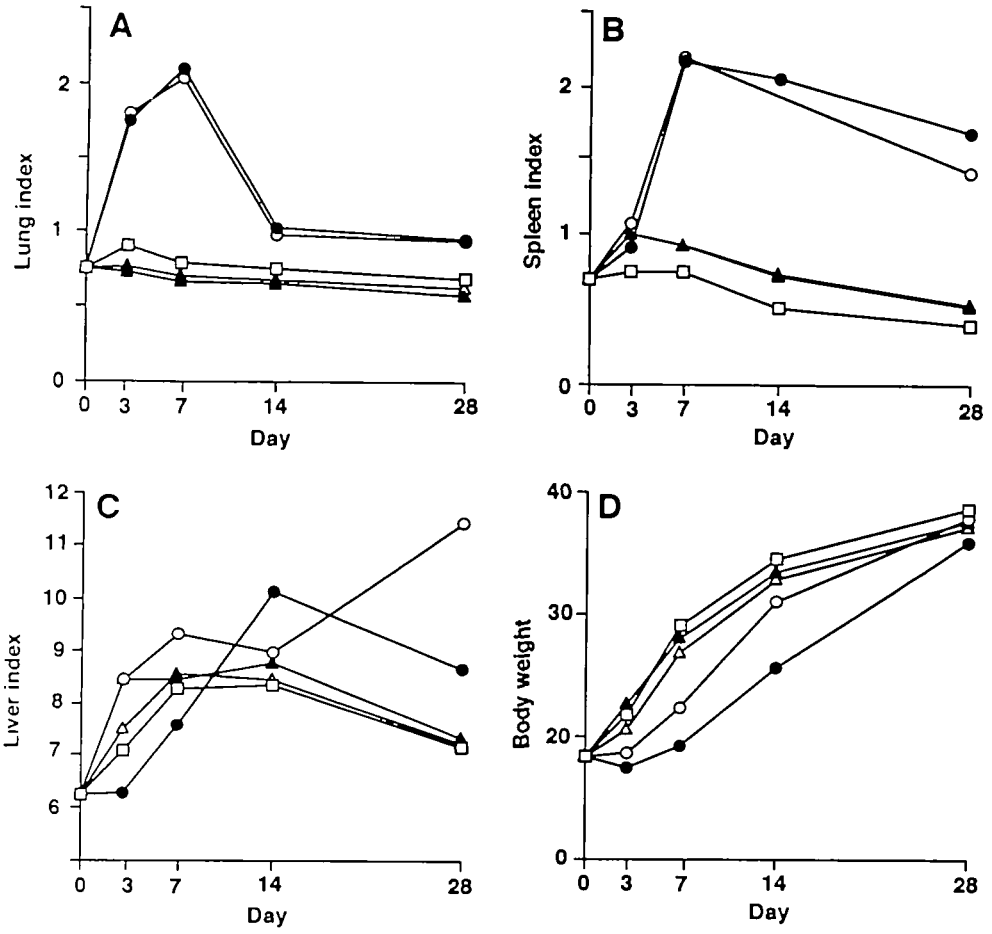

Fig. 4. **Time courses of (A) lung, (B) spleen, and (C) liver indices, and (D) body weight.** Closed circles, open circles, closed triangles, and open triangles indicate the organ indices and body weights of mice treated with TDM, GM, MM, and FM, respectively. Open squares show the organ indices and body weights of control mice.

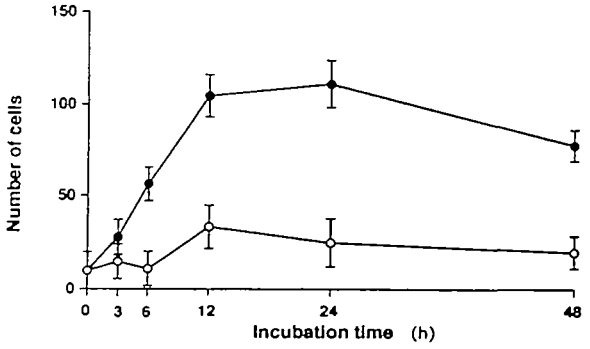

Fig. 5. **Time course of the induction of macrophage chemotactic activity.** Closed and open circles indicate macrophage migration activities in the culture supernatants of macrophages incubated or not incubated with 1 μg of TDM for 0-48 h, respectively. The activity is shown as the mean number of cells ± the standard deviation.

1.0×10^{-1} to 1.0×10^{4} U/ml, 1.0 to 1.0×10^{5} pg/ml, and 1.0×10^{-1} to 10 ng/ml, respectively.

Characterization of MCF Induced by TDM—MCF induced by TDM was stable when incubated at 37°C for 60 min or less, and there was only a slight decrease in MCF activity on incubation at 56°C for 30-60 min. Incubation at 100°C for 10 min abolished the MCF activity completely (Fig. 6A). MCF activity also disappeared on digestion with 2.5 μg/ml porcine pancreatic trypsin (Fig. 6B). Dialysis of

TABLE I. **Checkerboard assay of macrophage migration caused by supernatants treated with TDM.** Macrophage migration activity is expressed as the mean number of migrated cells ± the standard deviation.

Dilution of supernatant in lower chamber	Dilution of supernatant in upper chamber		
	1	1/2	1/4
1	21±13	107±24	199±40
1/2	4± 2	10± 2	66± 7
1/4	2± 1	3± 2	9± 2

TABLE II. **Macrophage migration, IL-1, TNF-α, and GM-CSF activities in supernatants incubated with a mycoloyl glycolipid:** [a]Mean number of cells ± standard deviation. The migration activity of the negative control was 55 ± 14 cells. [b]Mean absorption at 550 nm ± standard deviation. When RPMI medium was used as a negative control, the IL-1 activity was 0.16. [c]n.d., not detected (less than 30 pg/ml). [d]n.t., not tested. [e]—, colonies not detected (less than 5 ng/ml).

Mycoloyl glycolipid (μg/well)	Activity			
	Macrophage migration[a] (cells)	IL-1[b]	TNF-α	GM-CSF
No glycolipid	52±14	0.160±0.002	n.d.[c]	—[e]
TDM (1)	150±26	0.253±0.010	n.d.	—
GM (1)	132±10	0.253±0.008	n.t.[d]	—
MM (1)	57±12	0.236±0.004	n.t.	—
FM (1)	37±10	0.195±0.004	n.t.	—

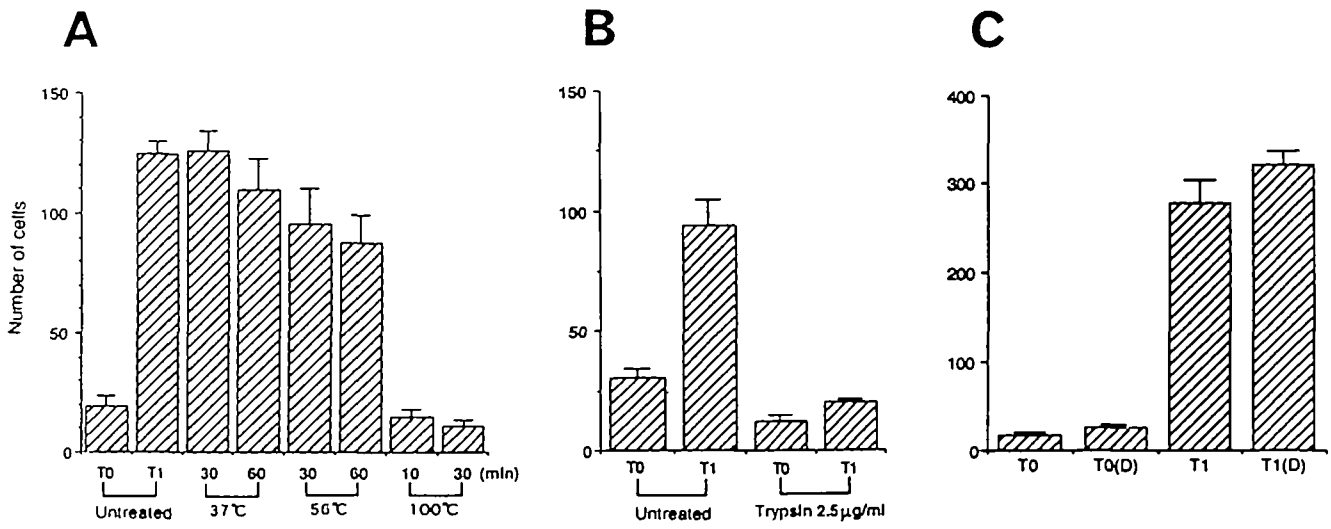

Fig. 6. **Effects of heat, digestion with trypsin, and dialysis upon the macrophage chemotactic factor induced by TDM.** Mouse peritoneal macrophages were incubated at 37°C for 24 h without TDM (T0) or with 1 μg of TDM (T1) in serum-free medium. A: Effect of heat treatment on MCF activity induced by 1 μg of TDM. B: Effect of treatment with trypsin. C: Effect of dialysis. Dialyzed samples are denoted by "D". Macrophage migration activity is shown as the mean number of cells ± the standard deviation.

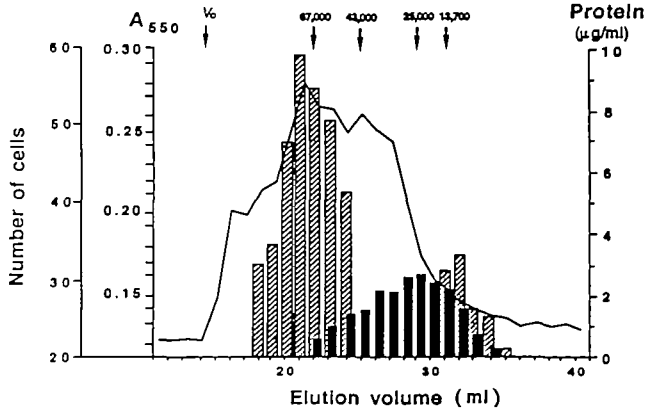

Fig. 7. **Elution profiles of TDM-induced macrophage chemotactic factors on a Sephacryl S-200 column.** Hatched and shaded bars show macrophage chemotactic and IL-1 activities, respectively. The continuous line shows the concentration of protein. Arrows indicate the void volume (Vo) and the molecular markers: bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsin A (25,000), and ribonuclease (13,700).

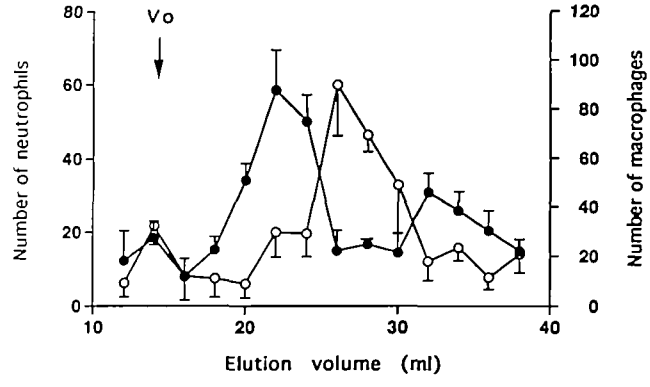

Fig. 8. **Migration activities of fractionated samples for macrophages and neutrophils.** The chemotactic activities for macrophages and neutrophils of samples fractionated on a Sephacryl S-200 column were assayed. Closed and open circles show the migration activities for macrophages and neutrophils, respectively. The arrow indicate the void volume. The activity is shown as the mean number of cells ± the standard deviation.

the culture supernatants incubated with or without TDM did not affect the MCF activity (Fig. 6C).

Gel filtration separated the MCF activity into two peaks (Fig. 7). The estimated molecular weights of the two peak materials were 80,000 and 12,000, and these factors are designated as 80K and 12K MCF, respectively. IL-1 activity was eluted as a broad peak that did not coincide with that of either of the MCF activities. To determine whether or not the 80K MCF was an aggregate of the 12K MCF, we collected the 80K MCF and repeated the gel filtration. Only the MCF with a molecular weight of 80,000 was detected. Therefore, the two factors seemed to be different.

Several MCFs reported earlier are chemoattractants for neutrophils (*25-27*). Neutrophil migration activity was eluted between 24 and 30 ml, but the peak of the activity did not coincide with any of the peaks of MCF activity (Fig. 8).

DISCUSSION

Mycoloyl glycolipids are ubiquitous cell-wall components of acid-fast bacteria such as *Mycobacterium, Nocardia, Rhodococcus, Gordona,* and *Corynebacterium.* The major mycoloyl glycolipids from *R. ruber* (formerly *Nocardia rubra*) grown in a medium containing (D)-glucose as a carbon source have been chemically defined. TDM and GM cause prominent granulomas in lungs, spleen, and liver of mice (*6, 8*). When *R. ruber* was grown in a medium containing (D)-mannose or (D)-fructose as a carbon source, MM or FM was synthesized besides trehalose monomycolate and TDM, respectively (Fig. 1). All these glycolipids

possess similar mycolic acid carbon-number ranges centered at C_{46}, as judged on FAB/MS analysis (Fig. 2).

Granuloma formation can be induced by various materials such as whole cells of tubercle bacilli (*12, 14*), eggs of *Schistosoma mansonii* (*16, 28*), mycoloyl glycolipids such as TDM (*1, 6, 8, 9*), and cytokines bound to beads (*13*). Mycoloyl glycolipids are the only factors that can induce granulomas without a protein antigen among the defined mycobacterial surface components. However, the essential structure for granuloma formation is not well understood. We have hypothesized that the different granuloma-forming activities of mycoloyl glycolipids with various carbohydrate moieties are due at least in part to the release of MCFs from macrophages stimulated with the glycolipids. Our results showed that mycoloyl glycolipids causing granuloma formation also stimulated macrophages to release MCFs. The results suggested that recognition of the carbohydrate moieties of mycoloyl glycolipids by macrophages is crucial for MCF induction and the granuloma formation that follows.

IL-1, TNF-α, and GM-CSF have been described as potent chemoattractants for monocytes/macrophages (*26, 27, 29*), but the assaying of these cytokine activities in the culture supernatants of macrophages stimulated with mycoloyl glycolipids revealed no TNF-α or GM-CSF activity. IL-1 activity was detected without any association with MCF activity. MCF activity was not necessarily found in the same pattern as IL-1 activity in the fractions separated by gel filtration on Sephacryl S-200. In addition, mouse peritoneal macrophages were not sensitive to mouse recombinant IL-1α, TNF-α, and GM-CSF. Such insensitivity has been reported for rat alveolar macrophages (*30*). These results indicated that the MCFs induced by mycoloyl glycolipids were distinct from IL-1, TNF-α, and GM-CSF.

Therefore, to characterize the TDM-induced MCF, we examined the effects of various procedures. The TDM-induced MCF was fairly heat-stable, trypsin-labile, and not dialyzable, so it is proteinous, and not a low-molecular-weight chemoattractant like leukotrienes (*31*) or *N*-formylmethionyl peptide (*32*). At least two MCFs induced by TDM were isolated and both were specific for macrophages. Neutrophil migration activity was found in the culture supernatant of TDM-stimulated macrophages. However, its peak and distribution did not coincide with those of MCF activity. MCFs specific for monocytes/macrophages are released from these cells. Among such MCFs, macrophage inflammatory protein 1 (MIP-1), monocyte chemoattractant protein 1 (MCP-1), and RANTES seem to play important roles in granuloma formation (*15–17*). These factors are gene products of the supergene family encoding chemokines and their molecular weights are 8,000–10,000 (*33*). Therefore, the 12K MCF observed in this study may be related to or possibly even identical with JE, a mouse homolog of MCP-1. MCF with a molecular weight of 650,000 has been partially purified from granulomas induced by *S. mansonii* eggs (*28*). An 80K MCF has not yet been reported, so it may be a newly identified MCF.

Here, we described mycoloyl glycolipid-induced MCFs released from macrophages. The structure–activity relationship for MCF induction suggested that MCF induction plays a key role in granuloma formation. Therefore, we assumed that when a host organism is infected by acid-fast bacteria, continuous stimulation by a mycoloyl glycolipid induces both the accumulation of macrophages in the early stage of inflammation and further stimulates the migration of macrophages to the inflammatory site through the release of more MCFs from stimulated macrophages. At least two MCFs were induced by TDM. These MCFs are not identical to IL-1, TNF-α, or GM-CSF, but the 12K MCF might be an MCF already identified. The purification, complete characterization, and clarification of the physiological roles of the MCFs induced by TDM are now in progress.

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