INFECTION AND IMMUNITY, Dec. 1986, p. 869–875
0019-9567/86/120869-07$02.00/0

Vol. 54, No. 3

Granuloma Formation and Hemopoiesis Induced by C_{36-48}-Mycolic Acid-Containing Glycolipids from *Nocardia rubra*

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Received 27 May 1986/Accepted 2 September 1986

As previously reported (I. Yano, I. Tomiyasu, S. Kitabatake, and K. Kaneda, Acta Leprologica 2:341–349, 1984), *Nocardia rubra*, one of the nonpathogenic actinomycetes, possesses three classes of mycolic acid-containing glycolipid, i.e., glucose mycolate, trehalose dimycolate, and trehalose monomycolate. The carbon chain length of their mycolic acids is shorter (C_{36-48}) than that in mycobacteria (longer than C_{70}), and the glycolipid consists of only α-mycolic acid. One intravenous administration of 500 μg of each purified glycolipid to ICR mice in the form of water-in-oil-in-water emulsion without any protein antigens caused prominent granuloma formation in the lungs, spleen, and liver. The lung index in the treated mice was about 3.5 times larger than that in the control mice (given water-in-oil-in-water emulsion only) at 1 week after the injection and then rapidly declined, while spleen and liver indices peaked at 2 weeks after the injection and persisted longer. The granuloma consisted of macrophages, some of which phagocytized glycolipid micelles, lymphocytes, monocytes, and neutrophils. In addition, many small hemopoietic islands were observed in the liver sinusoids, where various immature blood cells were trapped by the prominent cytoplasmic projections of Kupffer cells. The granuloma formation and hemopoiesis observed here are considered to be the most characteristic morphological expression of macrophage activation in these organs. This is the first report to show that such histological changes can be induced by chemically defined and homogeneous mycolic acid-containing glycolipids other than those of mycobacteria.

Granuloma formation is one of the most important pathological changes (2, 20) in chronic infectious diseases such as tuberculosis and leprosy (2, 16, 20). Many studies on experimental granuloma formation have been done with intact tubercle bacilli (1, 15), killed bacilli, or their cellular components (14). The granulomagenic activity of these bacilli is considered to be related profoundly to the immunoadjuvant activity of their highly characteristic cell wall components. Although muramyl dipeptide, which is the most-well-defined adjuvant component in bacterial cell walls (9, 19), is also thought to play an important role in tuberculosis, it is present in insufficient quantities to be regarded as the only adjuvant component in the development of tuberculosis immunity, because cavity formation, which is the most characteristic pathological change involved in tuberculosis, cannot be observed in general infections with other bacteria possessing the same peptidoglycan unit. On the other hand, mycolic acid-containing cell wall components such as cell wall skeleton or cord factor (trehalose dimycolate [TDM]), which exist characteristically in acid-fast bacteria, have been prepared from mycobacteria and extensively examined. Mycobacterial TDM P_3 was shown to possess significant granuloma-forming activity in the form of oil-in-water (o/w)-emulsified micelles combined with the cell wall skeleton (4, 5, 11), and recently, synthetic mycoloyl muramyl dipeptide was also reported to produce granulomas by itself in the form of water-in-oil-in-water (w/o/w) emulsion, the form prepared by further emulsification of water-in-oil (w/o) micelles with saline (22). To clarify the relationships between biological activities such as granuloma formation and the chemical structure of mycolic acid-containing cell wall components from natural sources, we administered mycolic acid-containing glycolipids, isolated from *Nocardia rubra*, one of the nonpathogenic actinomycetes, in the form of w/o/w emulsion without protein antigens to ICR mice. The glycolipids of *N. rubra* are suitable for this experiment because they have been chemically defined (23) and proved to be easier to use than those of mycobacteria for the following reasons. (i) While the latter includes several subclasses (α, β, γ, etc.) of mycolic acids (18), the former consists of only α-mycolic acid, so we can obtain chemically homogeneous glycolipids. (ii) The carbon chain length of the former mycolic acid is C_{36-48}, much shorter than that of the latter mycolic acid (longer than C_{70}).

In this study, we examined histologically the in vivo biological effects induced by such characteristic glycolipids of *N. rubra* on the lungs, spleens, and livers of mice.

MATERIALS AND METHODS

Preparation of glycolipids. The procedures for the preparation of purified glycolipids are described in detail in our previous paper (23), so we repeat them here only briefly. *N. rubra* M-1 was grown with shaking in medium containing 1% glucose, 0.2% yeast extract, and 0.5% peptone for 5 days at 30°C. The lipids were extracted from the harvested cells with chloroform-methanol (2:1, vol/vol) and developed on a thin-layer plate of silica gel (Analtech, Inc., Newark, Del.) with the solvent chloroform-methanol-acetone-acetic acid (90:10:6:1, vol/vol). Each glycolipid was recovered with chloroform-methanol (2:1, vol/vol), and the purification was repeated until a single spot was ultimately obtained. The glycolipids thus obtained were confirmed not to contain other cellular components, such as peptidoglycolipid (11), as contaminants by proving the hydrolyzed products to be only mycolic acids and trehalose (or glucose) and not ninhydrin-

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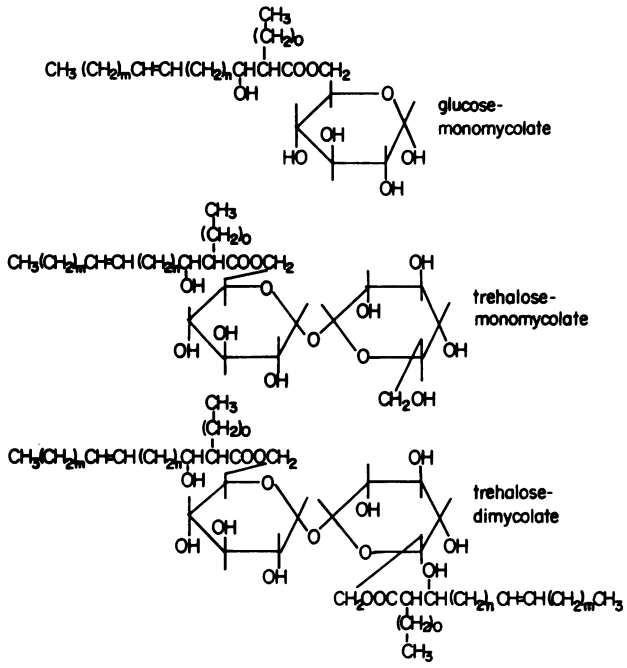

FIG. 1. Chemical structures of three major mycolic acid-containing glycolipids of *N. rubra*. m and n = 19 to 27; o = 9, 11, or 13; the total carbon number ranges from 36 to 48 (average, 44).

positive substances. Each purified glycolipid was chemically identified by several analytical procedures (23) as glucose mycolate, TDM, or trehalose monomycolate containing C_{36-48} α-mycolic acid (Fig. 1) and emulsified with 0.2% Tween 80–3% Freund incomplete adjuvant in phosphate-buffered saline to form w/o/w emulsion (22).

Granuloma formation. A 500-μg quantity of each glycolipid in the form of w/o/w emulsion was injected into the tail veins of ICR mice (female, 4 to 5 weeks old) (23). At 1, 3, 7, 14, and 28 days after the injection, the lungs, spleens, and livers were taken out and weighed. For the control, w/o/w emulsion without glycolipid was injected. In each group, eight mice were used. Their organ indices were calculated as follows to show the degree of granuloma formation (22): organ index = (organ weight/body weight) × 100.

Histological examination. For light microscopy, the organs were fixed in 10% Formalin, and the paraffin sections were stained with hematoxylin-eosin (H-E).

For electron microscopy, the lungs, spleen, and liver were perfusion fixed via the right ventricle, the vessels at the hilus, and a portal vein, respectively, with medium containing 1.5% glutaraldehyde in cacodylate buffer (pH 7.3), and postfixed in 1% OsO_4 in phosphate buffer. Epon thin sections were stained with saturated uranium acetate and then with saturated lead citrate and observed under a JEOL 100CX electron microscope.

RESULTS

Time course for lung, spleen, and liver indices (Fig. 2). The lung index showed a significant increase on day 3 after the injection of TDM, while that of the control showed no particular changes. It reached the maximum on day 7 and thereafter declined rapidly. On the other hand, the spleen index began to increase on day 3, reached a peak on day 14, and then decreased gradually. Both lung and spleen indices in TDM-treated mice were about 3.5-fold higher than those in control mice. The liver index in treated mice increased significantly on day 14; like the spleen index, it declined gradually. It was 1.3-fold higher than the liver index in control mice at its maximum point. The other two glycolipids (glucose mycolate and trehalose monomycolate) showed tendencies similar to those of TDM. The dose-dependent effect was observed up to 500 μg. At this dose (used here), toxicity in mice was low; glycolipid-treated mice showed a growth curve comparable to that of control mice.

Histological examination of granuloma formation. (i) Lungs. In H-E-stained specimens of the lungs of glycolipid-treated mice, a prominent cellular infiltration and disseminated granulomas were observed (Fig. 3a), while in control specimens, almost no changes were recognized. No fundamental differences were observed histologically among the three glycolipids. The granulomatous change in the lungs was maximum at days 3 to 7. The granulomas consisted mainly of macrophages, some of which resembled epithelioid cells in their appearance, monocytes, lymphocytes, and neutrophils (Fig. 3b). These cells were aggregated to form a nodular appearance or expanded diffusely along the alveolar walls. Oil droplets could be seen in the granulomas but not at a high frequency.

Electron microscopically, the granulomas consisted of macrophages containing numerous lysosomes and phagolysosomes, monocytes, and lymphocytes (Fig. 4a). They were packed together, and the collagen fibers often existed in the intercellular spaces of these cells (Fig. 4b). Some macrophages contained various numbers of glycolipid micelles in their phagolysosomes (Fig. 4b). Macrophages phagocytizing no glycolipid micelles were, however, also numerous. No degenerative or necrotic change was found inside the granulomas.

(ii) Spleen. In the spleens of glycolipid-treated mice, the granulomas, which were composed of many macrophages with chromatin-sparse nuclei and a large volume of cytoplasm, were observed 2 to 4 weeks after the injection. The boundary between the granulomatous portion and the surrounding reticular or lymphoid tissues was not very clear.

(iii) Liver. In the livers of glycolipid-treated mice, we recognized few granulomas showing a clear boundary on day 3, 2 to 4 granulomas on day 7, and 10 to 20 granulomas on day 14 per liver lobule section (Fig. 5a). On day 7, neutrophils and lymphocytes were also present to some extent; in the later stages, macrophages and monocytes were predominant (Fig. 5b and c). The granulomas were evenly distributed in a liver lobule section and were also formed in the Glisson sheath. At 4 weeks after the injection, the granulomas tended to fuse to each other.

Electron microscopically, various mononuclear cells and neutrophils were intermingled with one another to form granulomas, and some macrophages or Kupffer cells contained micelles. Besides the large granulomas, small cellular aggregates consisting of mononuclear cells were often observed in the sinusoids. No cytological damage was recognized in hepatocytes around the granulomas.

Histological examination of hemopoiesis. (i) Spleen. In the spleens of glycolipid-treated mice, a high frequency of megakaryocytes, a prominent lobulation of the nucleus, and a large volume of cytoplasm were observed, as compared with those in the spleens of control mice.

(ii) Liver. In the livers of glycolipid-treated mice, besides the granulomas, numerous small hemopoietic islands were observed in the parenchyma (Fig. 5a). In H-E-stained spec-

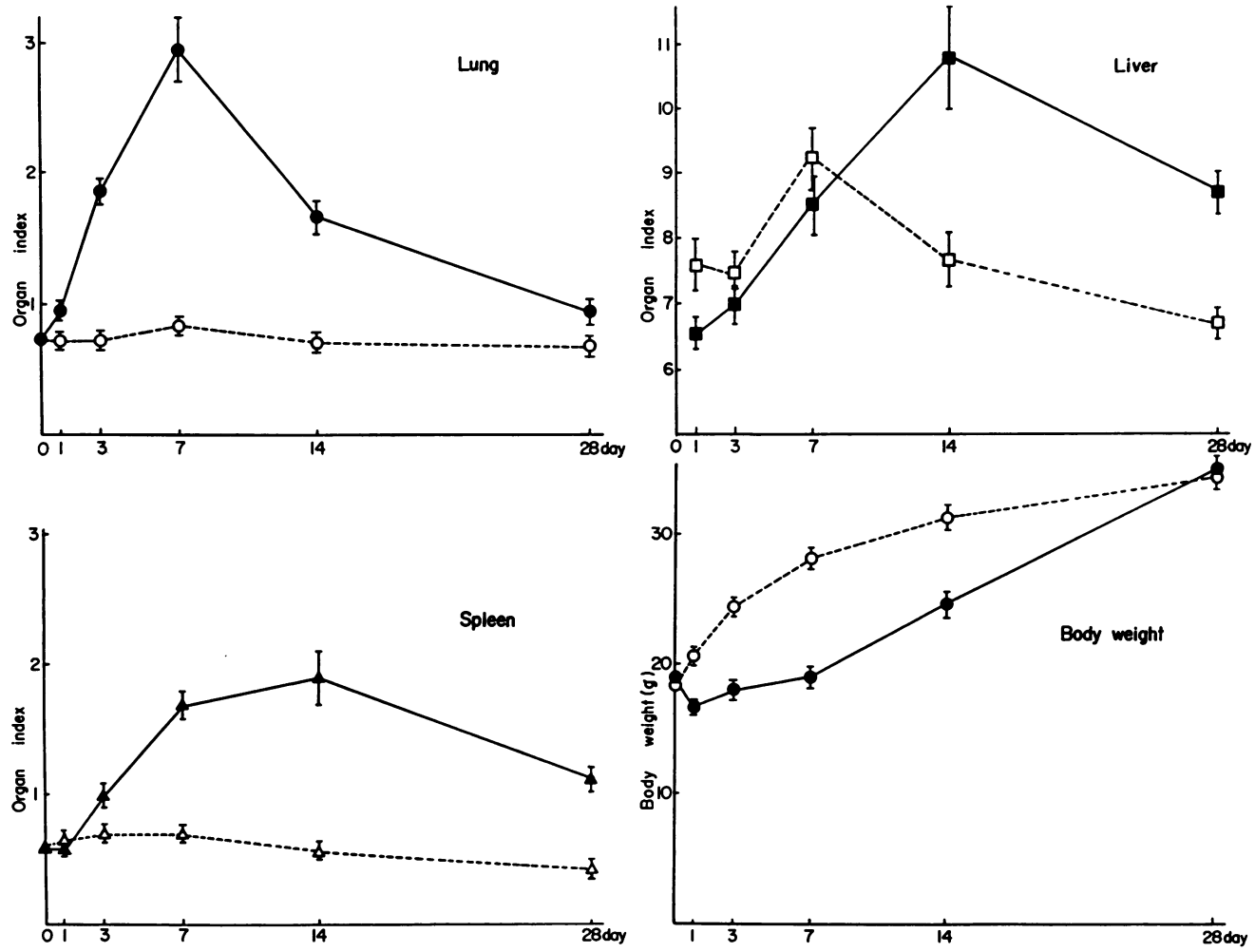

FIG. 2. Time course for lung, spleen, and liver indices and body weights of TDM (500 μg)-treated mice (solid lines) and control mice (dotted lines). Values are the means for eight mice.

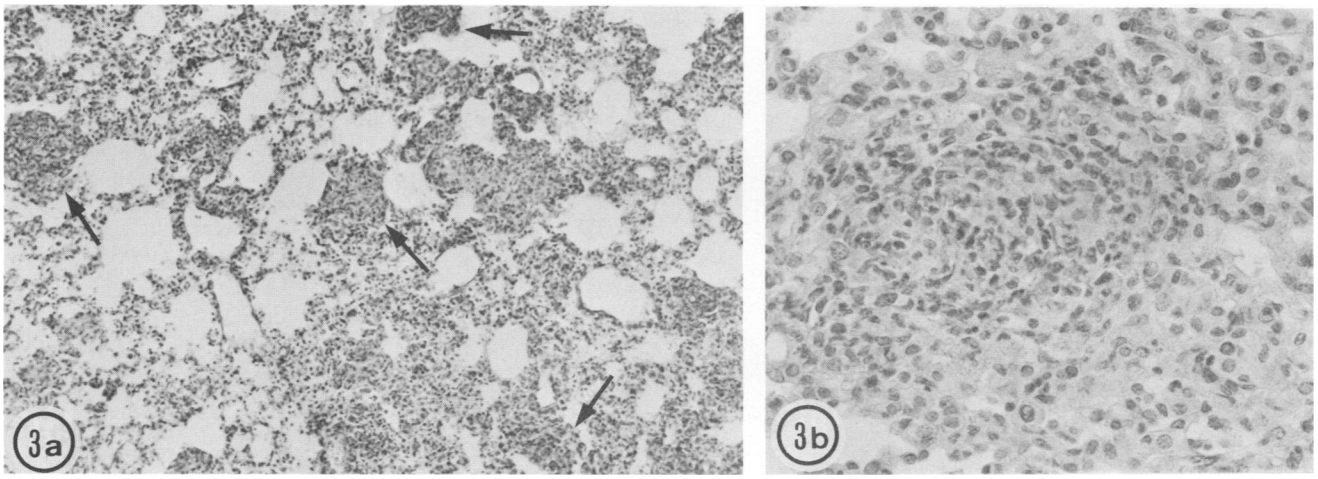

FIG. 3. (a) Lung granuloma formation (arrows) in an ICR mouse 1 week after the injection of 500 μg of TDM (×180). (b) Granuloma consisting of numerous macrophages, lymphocytes, and several neutrophils, tightly packed (×590). H-E staining.

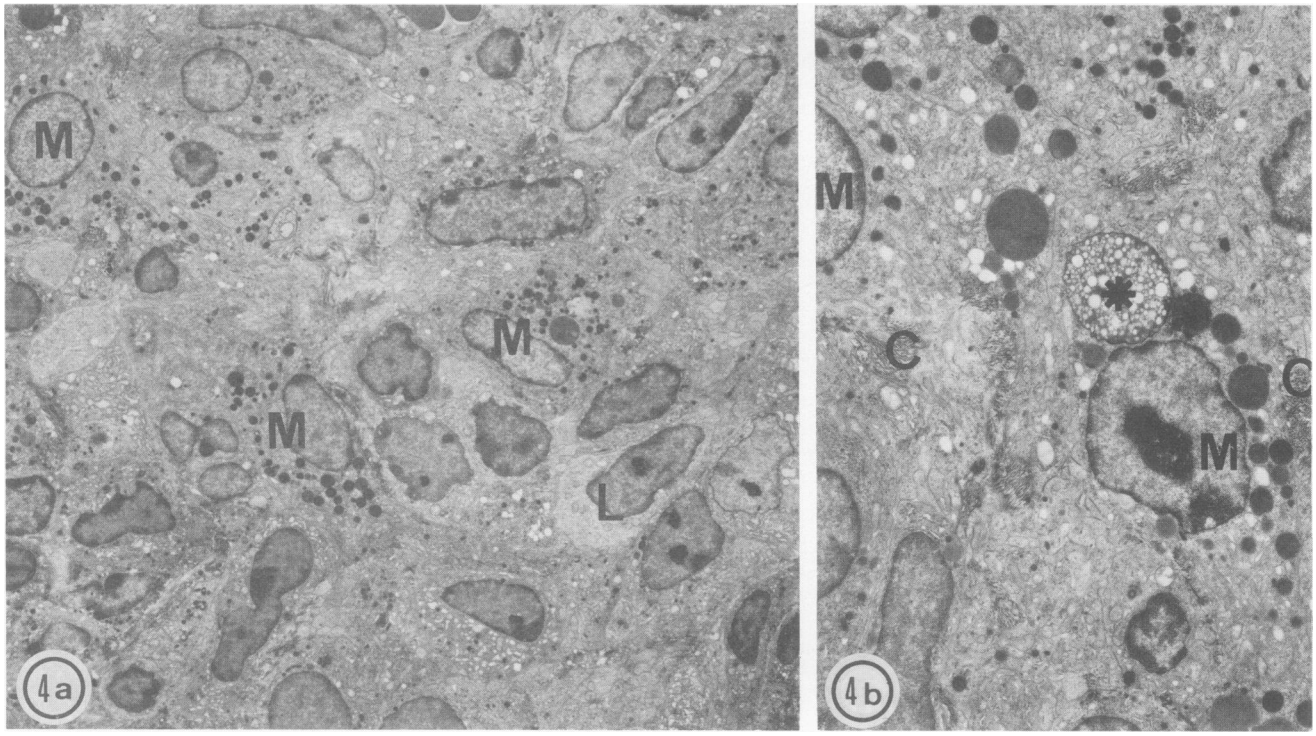

FIG. 4. (a) Lung granuloma (day 7) consisting of macrophages (M), monocytes, and lymphocytes (L) intermingling with each other and tightly packed (×2,000). (b) Macrophages possessing numerous lysosomes and sometimes phagocytizing various numbers of micelles (asterisk). Collagen fibers (C) are often intercalated between these cells (×3,600). ICR mice were treated with 500 μg of TDM.

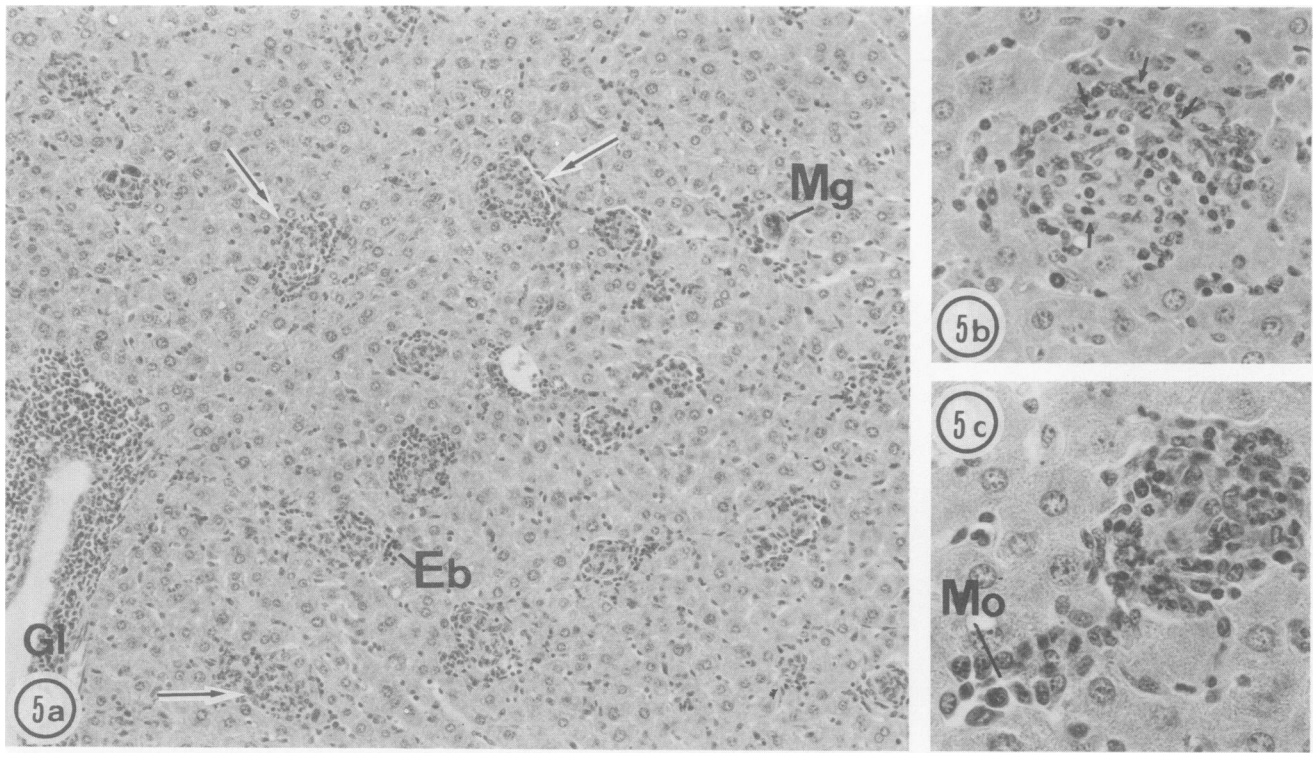

FIG. 5. (a) Formation of many granulomas (arrows) in the hepatic parenchyma and the Glisson sheath (Gl) (day 14). A megakaryocyte (Mg) and a cluster of erythroblasts (Eb) can also be seen in the parenchyma (×210). (b) Neutrophils (arrows) present on day 7 (×450). (c) Prominent macrophages or monocytes present on day 14. A cluster of monocytes (Mo) can also be seen in the sinusoid (×600). H-E staining. ICR mice were treated with 500 μg of TDM.

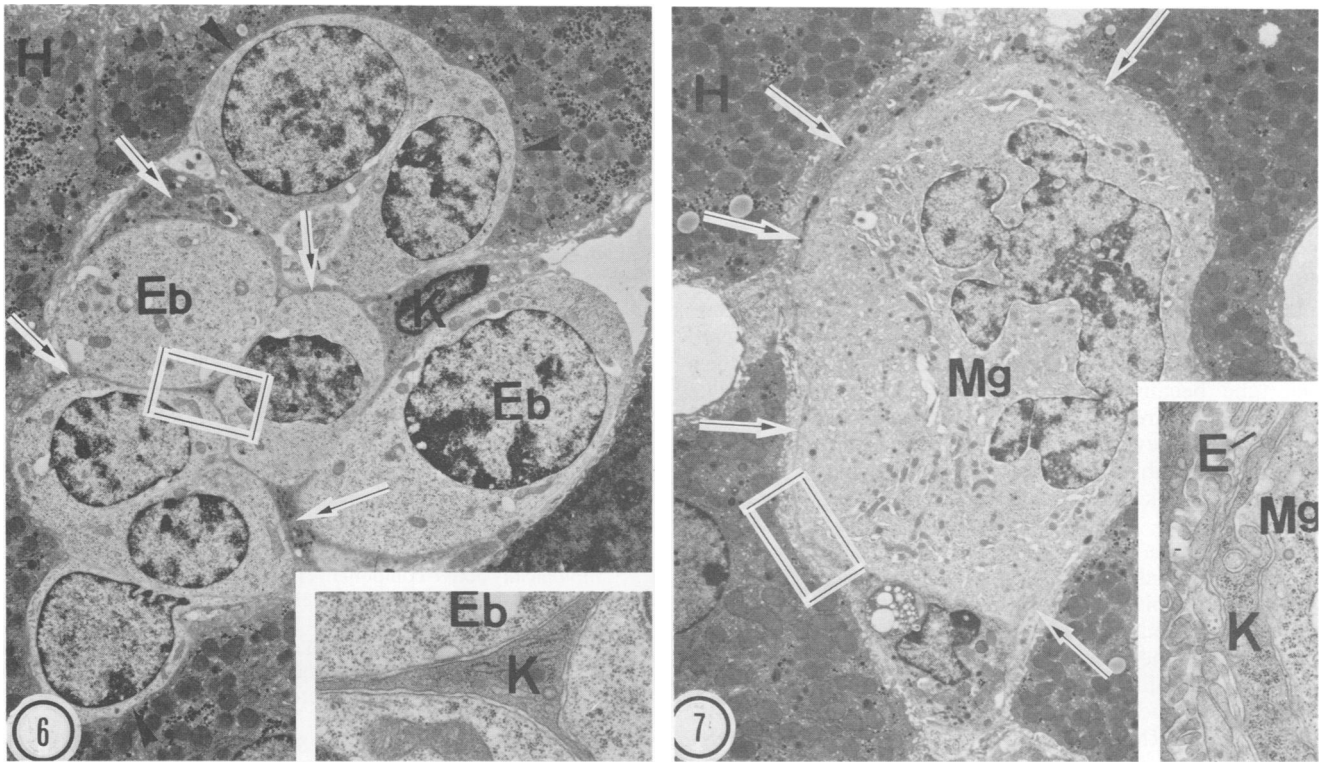

FIG. 6. and 7. 6. Cluster of erythroblasts (Eb) in a hepatic sinusoid (day 7) that are enclosed by prominent cytoplasmic processes (arrows) of a centrally located Kupffer cell (K). Some erythroblasts migrated into the space of Disse and made contact with hepatocytes (H) (arrowheads). ×2,600. Inset, ×17,000. 7. Megakaryocyte (Mg) in a sinusoid that is surrounded by long cytoplasmic projections (arrows) of a Kupffer cell (K) which contains micelles in the phagolysosome (day 7). H, Hepatocytes; E, endothelial cells. ×2,500. Inset, ×11,000. ICR mice were treated with 500 μg of TDM.

imens, they were seen as small cellular aggregates in the sinusoids. Electron microscopically, a group of erythroblasts, which were characterized by the nucleus with clumped heterochromatin and polyribosome-rich cytoplasm, were surrounded by the cytoplasm or cytoplasmic projections of centrally located Kupffer cells, which sometimes contained micelles (Fig. 6). Some of them migrated into the space of Disse and made direct contact with hepatocytes. They sometimes underwent mitosis. Megakaryocytes were also observed being enclosed by the long processes of Kupffer cells (Fig. 7) or making wide contact with Kupffer cells by cellular interdigitations. They occupied the sinusoidal lumen entirely. Monocytes (Fig. 5c) and neutrophils with their immature forms gathered in the sinusoids, adhering to the endothelium or to each other. They often made contact with Kupffer cells and sometimes underwent mitosis.

DISCUSSION

It is well known that many high- and low-molecular-weight components with various biological activities are associated with the cell wall of acid-fast bacteria, and many studies have been done about such components, particularly in mycobacteria. *N. rubra* is the one species of taxa related to mycobacteria that possesses weak acid fastness and no pathogenicity. We examined here the biological activities of the purified glycolipids of *N. rubra* as a simple model of natural mycolic acid-containing glycolipids because of their shorter and homogeneous mycolic acid moiety.

In this study, such glycolipids, composed of short α-mycolic acids, were shown to have a capability by themselves to induce obvious biological effects, as shown histologically, i.e., granuloma formation in the lungs, spleen, and liver and hemopoiesis in the spleen and liver, when injected intravenously in the form of w/o/w emulsion. Histologically, the granulomas were composed of macrophages, some of which resembled epithelioid cells, monocytes, lymphocytes and neutrophils intermingling randomly. The histological features and the short time course of the lung granulomas observed in this study resembled those induced nonspecifically by synthetic mycoloyl muramyl dipeptide in w/o/w emulsion (22), although immunological responses, such as delayed hypersensitivity to autologous proteins in mouse tissues, may have participated to some extent in the granuloma formation seen here.

This is the first report of granuloma formation in the liver caused by the administration of cellular components of acid-fast bacteria. The time course for granuloma development in the spleen and liver was different from that in the lungs; the response of the former came later and persisted longer. This difference may be a result of the different anatomical route used by the substances to reach the organs or, more possibly, of the different cytological nature of the macrophages located in each organ (3).

Interestingly, we also observed numerous small hemopoietic islands in the livers of glycolipid-treated mice. Various types of immature blood cells formed the homogeneous cellular accumulation in the sinusoids, being trapped by the cytoplasmic projections of Kupffer cells or adhering to endothelial cells; some of them migrated into the space of Disse, and some underwent mitosis. Thus, immature blood cells, coming into the liver possibly from the bone marrow or

the spleen, would be trapped by Kupffer cells or endothelial cells, undergo proliferation and differentiation there, and then gradually migrate out of the sinusoids. The observation of extrasinusoidal erythropoiesis reported in glucan-treated adult rats by Deimann and Fahimi (8) would represent the later stage of erythropoiesis. Furthermore, we observed a high population of megakaryocytes in the spleens of treated mice; this result suggests that enhanced blood cell production also occurred in the spleen.

The mechanism by which the mycolic acid-containing glycolipids induce such histological changes remains unknown. However, on the basis of histological observations, the primary site of action of these glycolipids is considered to be macrophages. The glycolipids persist in the cells for a long time because unusually long-chain fatty acids like mycolic acids are resistant to digestion by lysosomal enzymes. We believe that these glycolipids or their partially metabolized substances act on macrophages via their lysosomal or plasma membranes to release chemotactic factors (12, 24), colony stimulating factors (6, 17), etc., which participate in granuloma formation and hemopoiesis. It has been considered that granuloma formation (22, 24) and hepatic hemopoiesis (8, 10) represent the morphological expression of macrophage activation. Recently, the substances which increase the biological responses of the host, particularly the function of macrophages and lymphocytes, have been termed biological response modifiers (BRMs) (13). Several BRMs, such as glucan (7) and *Propionibacterium acnes* (21), induce hepatic granulomas, and glucan (8) and estriol (10) induce hepatic hemopoiesis. On the basis of histological similarities, we are convinced that the glycolipids of *N. rubra* also act as BRMs. The granulomatous changes induced by *N. rubra* glycolipids in this study are considered to be not pathological responses but the results of augmented physiological responses of mononuclear cells in the organ, because we observed no degenerative changes in the granulomas or in the parenchymas of the organs, and the changes were transient. While the mycolic acid-containing glycolipids of *N. rubra* possessed granuloma-forming activity similar to that of mycobacteria, they showed much less toxicity to the subjected animals, possibly because they are less hydrophobic than mycobacterial glycolipids, owing to their shorter mycolic acid moiety; this means that *N. rubra* glycolipids would be more suitable for practical use as BRMs.

In addition to the several characteristics of *N. rubra* glycolipids described above, there is another advantage; numerous *Nocardia* and *Rhodococcus* species other than *N. rubra* produce various glycolipids containing different mycolic acid structures ranging widely from C_{30} to C_{60}, leading to the possibility of investigating the relationship between the chemical structures of glycolipids and their biological activities. We have already observed that glycolipids with much shorter-chain (around C_{30}) mycolic acids show no granulomagenic activity (I. Yano, I. Tomiyasu, Y. Sumi, F. Kurano, K. Kato, and K. Kaneda, unpublished data).

We demonstrated in this histological study that purified mycolic acid-containing glycolipids of *N. rubra* cause a potent augmentation of macrophage responses in organs, and we expect that these substances will provide a simple and useful model for studying the biological activities of natural mycolic acid-containing glycolipids.

ACKNOWLEDGMENTS

We sincerely thank Kenjiro Wake, Toyko Medical and Dental University, for his kind permission to use the experiment in his laboratory. We also thank Sakae Gondaira and Sadao Imaizumi in our laboratory for technical help in the preparation of glycolipids and Kiyoyuki Motomatsu, Toyko Medical and Dental University, for technical help in the preparation of H-E stained specimens. Thanks are also due to Kenjiro Wake and Chieko Dan, Tokyo Medical and Dental University, and H. Dariush Fahimi, Heidelberg University, for their valuable comments on this work. We also acknowledge Yasuko Machiya for her assistance in the preparation of the manuscript.

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