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Granuloma Formation by Muramyl Dipeptide Associated with Branched Fatty Acids, a Structure Probably Essential for Tubercle Formation by *Mycobacterium tuberculosis*

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Muramyl dipeptide, which does not induce epithelioid granuloma when injected alone dissolved in phosphate-buffered saline, could induce extensive granulomas in guinea pigs when chemically conjugated with branched, but not linear, fatty acids. Peptidoglycan fragments of *Staphylococcus epidermidis* could evoke epithelioid granulomas when incorporated in a water-in-oil emulsion. These findings suggest the importance of a lipid bound to muramyl dipeptide for granuloma formation. In view of the fact that mycobacteria uniquely contain large amounts of branched fatty acids, it was proposed that the complex of muramyl dipeptide and branched fatty acids, mostly mycolic acids, is a structure in tubercle bacilli responsible for tubercle formation.

Tuberculosis was once a significant medical and social problem worldwide and remains so in many developing countries. Tubercle (epithelioid granuloma) formation, a hallmark of tuberculosis, still remains enigmatic (1, 3, 5, 10, 32). In many granulomatous diseases such as sarcoidosis and Crohn's disease, even the etiologic agent(s) is totally unknown (1, 5, 10, 32). Although the etiologic agent for tuberculosis is known, a chemical structure responsible for granuloma formation is still unclear (1, 3, 5, 10, 32), and the establishment of this structure would greatly contribute to our understanding of granulomatous diseases in general.

We found that a muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine) present in a bacterial cell wall common structure, peptidoglycan (6, 8, 16), activated macrophages (31) and if injected in a water-in-oil emulsion evoked epithelioid granulomas (9, 29). Since granulomas evoked by MDP were indistinguishable from those evoked by tubercle bacilli and MDP was stronger than tubercle bacilli in the granulomagenic capacity, we presumed that MDP may be a structure essential for granuloma formation by tubercle bacilli (29). The obvious question is, then, why do many other bacteria containing MDP evoke no epithelioid granulomas?

In the present study we found that conjugates of MDP with α-branched, but not linear, fatty acids were remarkably granulomagenic, even if not incorporated in the water-in-oil emulsion. Also, soluble subunits of cell wall peptidoglycan derived from *Staphylococcus epidermidis* could evoke granulomas when incorporated in the water-in-oil emulsion. These findings indicate the importance of a close association of MDP with some lipids for epithelioid granuloma formation and suggest that the chemical structure in tubercle bacilli essential for epithelioid granuloma formation is the complex of MDP and mycolic acids.

MATERIALS AND METHODS

Animals. Female outbred Hartley guinea pigs weighing 400 to 600 g were obtained from a local breeder.

6-*O*-Acyl muramyl peptides. Chemical structures and abbreviations of 6-*O*-acyl derivatives of muramyl dipeptides (6-*O*-acyl MDPs) used in this study are shown in Table 1. The different acyl groups were conjugated with MDP at the 6 position of muramic acid. Many kinds of 6-*O*-acyl-MurNAc-L-Ala-D-isoGln (acyl MDP) with different acyl groups were obtained through the synthetic routes already reported (17).

Nocardomycolic acid (N-myc) was extracted from *Nocardia rubra*. This acid is an α-branched and β-hydroxylated fatty acid containing 51 carbon atoms and was conjugated with MDP at the 6 position of muramic acid (28).

Water-soluble peptidoglycan fragments. Cell wall peptidoglycans were obtained by the conventional methods, namely, by extraction of isolated cell walls with 10% trichloroacetic acid at 4°C for 18 h from *S. epidermidis* (ATCC 155) to remove nonpeptidoglycan moieties. This peptidoglycan fragment (molecular weight, 10,000 to 11,000) designated as SECS a-1 was obtained by gel filtration on columns of Sephadex G-50 and G-25 connected in series of *S. epidermidis* cell walls solubilized by the SALE endopeptidase and by chromatography with an ECTEOLA-cellulose column of the Sephadex fraction (23).

Injection of 6-*O*-acyl MDP and SECS a-1. 6-*O*-Acyl MDPs insoluble in water were finely suspended by sonication in Dulbecco phosphate-buffered saline (PBS; pH 7.2) containing 0.1% Tween 80. 6-*O*-Acyl MDPs suspended in 0.2 ml of PBS containing 0.1% Tween 80 were injected intracutaneously into the left hind footpads of guinea pigs. SECS a-1 was dissolved in PBS (1 mg/ml) and emulsified with an equal volume of incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The emulsion containing 100 μg of SECS a-1 in 0.2 ml of PBS was injected intracutaneously into the left hind footpads of guinea pigs.

Examination for granuloma formation. Tissue reactions to 6-*O*-acyl MDP injected into the left hind footpad of guinea pigs were estimated by using two parameters, i.e., the weight and the histology of the draining lymph nodes. Two weeks after injection, the draining lymph nodes were excised, weighed, and subjected to the histological examina-

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TABLE 1. Chemical structures and abbreviations of synthetic acyl MDPs

6-O-Acyl MDPs	Abbreviation
$CH_3(CH_2)_{28}$—CO-MurNAc-L-Ala-D-isoGln	L30-MDP
$CH_3(CH_2)_{13}$ / $CH_3(CH_2)_{13}$ > CH-CO-MurNAc-L-Ala-D-isoGln	B30-MDP
$CH_3(CH_2)_{13}$ / $CH_3(CH_2)_{13}$ > CH-CO-MurNAc-L-Ala-D-isoAsn	B30-MDP(D-isoAsn)
$CH_3(CH_2)_{21}$ / $CH_3(CH_2)_{21}$ > CH-CO-MurNAc-L-Ala-D-isoGln	B46-MDP
OH | $CH_3(CH_2)_{22}$-CH / $CH_3(CH_2)_{21}$ > CH-CO-MurNAc-L-Ala-D-isoGln	BH48-MDP
OH | $CH_3(CH_2)_{22}$-CH / $CH_3(CH_2)_{21}$ > CH-CO-MurNAc-L-Ala-D-isoAsn	BH48-MDP(D-isoAsn)
OH | $CH_3(CH_2)_{22}$-CH / $CH_3(CH_2)_{21}$ > CH-CO-MurNAc-L-Ala-L-isoGln	BH48-MDP(L-isoGln)
OH | $CH_3(CH_2)_{22}$-CH / $CH_3(CH_2)_{21}$ > CH-CO-MurNAc-L-Ala-L-isoGln-L-Lys-D-Ala	BH48-MDP-L-Lys-D-Ala

tions. The draining lymph nodes were used to estimate tissue reactions (weight and histology), because the tissue reactions in the draining lymph nodes were more clear-cut than those in the footpad and could be expressed quantitatively as described previously (9, 29).

RESULTS

Figure 1 shows the time course of the tissue reactions to the aqueous suspension of B30-MDP injected into the footpads. The draining lymph nodes, significantly enlarged already at day 3, reached their greatest weight at 2 to 3 weeks and returned to the level of day 3 at 5 weeks. Figure 2 shows a dose-response relationship. Amounts ranging from 0.01 to 0.1 μg of B30-MDP per injection evoked no detectable tissue reaction, whereas the injection of 1 to 10 μg of B30-MDP caused granulomas which were demonstrable by histological examinations (Fig. 2 and 3A and B). Roughly the same dose-response relationship was obtained previously with killed tubercle bacilli incorporated in the water-in-oil emul-

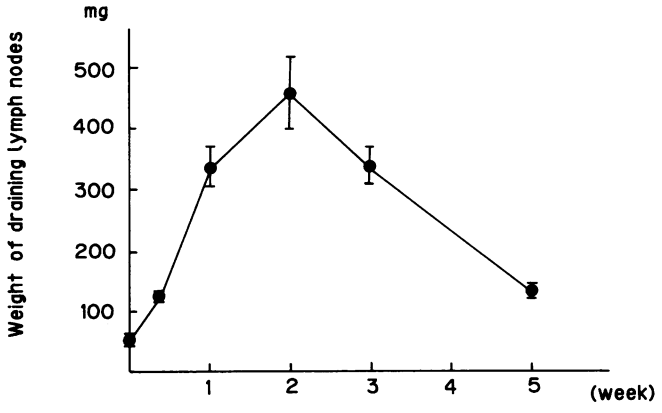

FIG. 1. Time course of tissue reactions of the draining lymph nodes to B30-MDP as measured by weighing the draining lymph nodes. A 100-μg amount of B30-MDP, suspended by sonication in 0.2 ml of PBS containing 0.1% Tween 80, was injected into the left hind footpads of female Hartley guinea pigs weighing 400 to 500 g. The draining lymph nodes (popliteal, inguinal, and flank) were excised on days 0, 3, 7, 14, 21, and 28 after the injection of 100 μg of B30-MDP. Cumulative data are shown from all experiments performed. For the time points, 4, 3, 4, 12, 10, and 3 guinea pigs were used, respectively. Circles and bars show mean ± standard error.

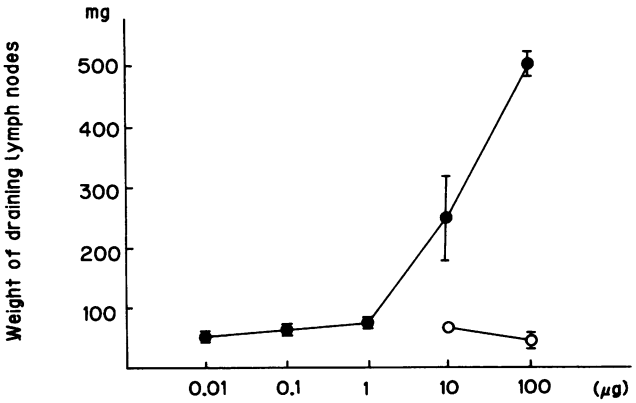

FIG. 2. Dose-response curves of tissue reactions of the lymph nodes draining the left hind footpad injected with B30-MDP. Different amounts of B30-MDP were injected, and the draining lymph nodes were excised and weighed on day 14 postinjection. Three guinea pigs were used for each point. Circles and bars show mean ± standard error. ●, B30-MDP; ○, B30-MDP(D-isoAsn), which is a conjugate of B30 and an adjuvant-inactive MDP analog containing D-isoAsn in the place of D-isoGln. Results obtained 7 days after injection also showed similar curves (data not shown).

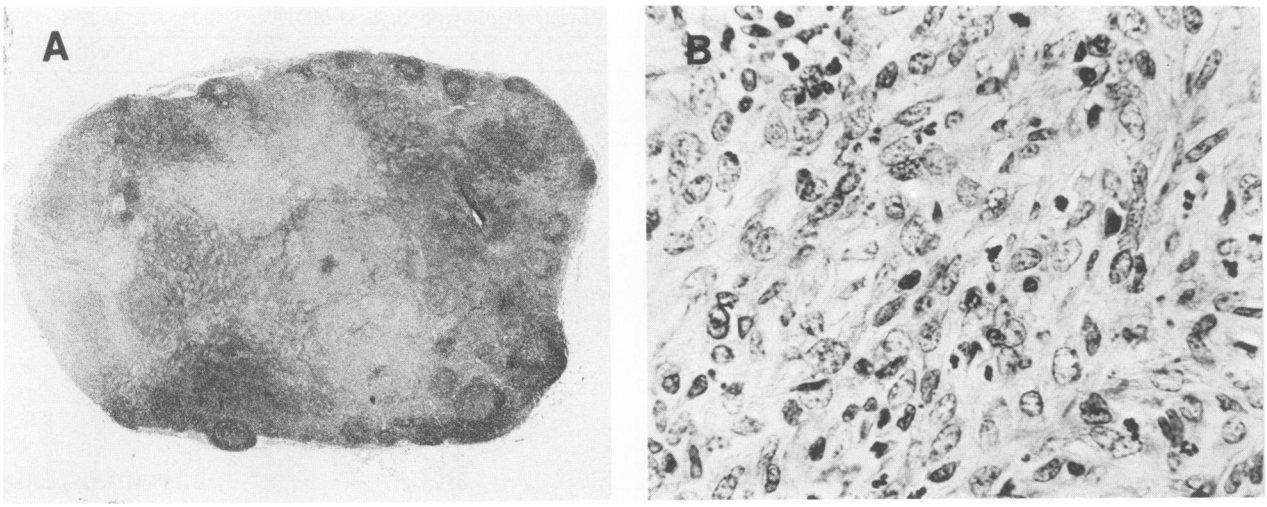

FIG. 3. Tissue reactions of the draining lymph nodes to B30-MDP. B30-MDP was similarly prepared and injected as described in the legend to Fig. 1. (A) Granuloma developed 1 week after the injection of 1 μg of B30-MDP (×15). (B) Granuloma developed 2 weeks after the injection of 10 μg of B30-MDP (×400). Note the epithelioid cells constituting the granuloma.

sion (29). The granulomas caused by B30-MDP were extensive, sometimes with intensive perilymphadenitis. Cells composing granulomas were mostly macrophages and immature epithelioid cells. A polymorphonuclear leukocyte infiltration was also seen. Neither MDP alone dissolved in PBS nor a mere mixture of B30 and MDP induced granulomas (Table 2). Thus, the effect of chemical conjugation of the α-branched fatty acid, B30, to MDP on granuloma formation in the draining lymph nodes was dramatic.

To confirm the effect of the conjugation of α-branched fatty acids on granuloma formation, MDPs similarly conjugated with other synthetic α-branched fatty acids or naturally occurring N-myc (17, 28) were examined for their granulomagenic capacity. The conjugated MDPs (B46-MDP, BH48-MDP, N-myc–MDP) and a similar conjugate, BH48-MDP-L-Lys-D-Ala, all evoked similar, extensive granulomas in the draining lymph nodes when injected in the form of fine suspensions in PBS (Table 2; Fig. 4A and B). Conjugates of branched fatty acids with adjuvant-inactive MDP analogs, such as BH48-MDP(D-isoAsn), BH48-MDP(L-isoGln), and B30-MDP(D-isoAsn) (Table 1) did not evoke granulomas (Table 2; Fig. 4C).

In sharp contrast to these MDP conjugates with α-branched fatty acids, a similar derivative of MDP conjugated with a linear fatty acid (L30-MDP) was devoid of granulomagenic capacity (Table 2; Fig. 4D). Also, a mixture of L30-MDP and B30-MDP(D-isoAsn) did not induce granulomas (Table 2).

The bacteria cell walls suspended in PBS did not induce granulomas (data not shown). However, SECS a-1 isolated from the cell walls was granulomatogenic when injected along with the mineral oil. SECS a-1 (100 μg) was incorporated in a water-in-oil emulsion and injected into footpads of guinea pigs. SECS a-1 thus injected induced massive epithelioid granulomas 2 weeks after the injection indistinguishable from those evoked by tubercle bacilli in a water-in-oil emulsion (Fig. 5A and B).

TABLE 2. Various activities of acyl MDPs

Test compound[a]	No. of animals	Mean ± SE of swelling of draining lymph node (mg)	Granuloma formation[b]	Adjuvant effect[c]
No treatment	5	60 ± 11	−	−
PBS	4	52 ± 6	−	−
MDP	5	68 ± 8	−	+
L30	4	59 ± 7	−	−
L30-MDP	4	76 ± 5	−	+
B30	5	63 ± 6	−	−
B30-MDP	22	412 ± 27	+	+
B30-MDP(D-isoAsn)	5	65 ± 5	−	−
B30 + MDP[d]	3	68 ± 4	−	
B30-MDP(D-isoAsn) + L30-MDP[d]	3	80 ± 6	−	
B46-MDP	3	351 ± 100	+	+
BH48-MDP	10	160 ± 24	+	+
BH48-MDP(L-isoGln)	6	89 ± 12	−	−
BH48-MDP(D-isoAsn)	10	77 ± 6	−	−
BH48-MDP-L-Lys-D-Ala	10	166 ± 17	+	+
N-Myc–MDP	10	127 ± 7	+	+

[a] Suspensions or solutions of these compounds (100 μg) were similarly prepared and injected as described in the legend to Fig. 1. Unconjugated MDP dissolved in PBS was injected as a control. Cumulative data from all the experiments in which the draining lymph nodes were excised 2 or 3 weeks after injection are shown.

[b] + or −, Presence or absence, respectively, of granulomas on microscopic examinations of the draining lymph nodes.

[c] + or −, Compound is active or inactive, respectively, as adjuvant, as revealed in several experiments made in our laboratory (15).

[d] B30 + MDP and B30-MDP(D-isoAsn) + L30-MDP indicate mixtures of 50-μg amounts of each compound.

DISCUSSION

In the present study we found that MDP conjugated with different kinds of α-branched long-chain fatty acids evoked extensive granulomas without being incorporated in a water-in-oil emulsion, though the MDP conjugate-induced granulomas contained smaller numbers of mature epithelioid cells than did the granulomas induced by tubercle bacilli or MDP incorporated in a water-in-oil emulsion.

In contrast to these MDP conjugates with α-branched fatty acids, an MDP derivative conjugated with a linear long-chain fatty acid (L30-MDP) did not induce granuloma (Fig. 4D; Table 2). An amount of L30-MDP as large as 100 μg caused no granuloma in the draining lymph nodes, whereas 1 μg of B30-MDP could evoke granuloma. This difference strongly

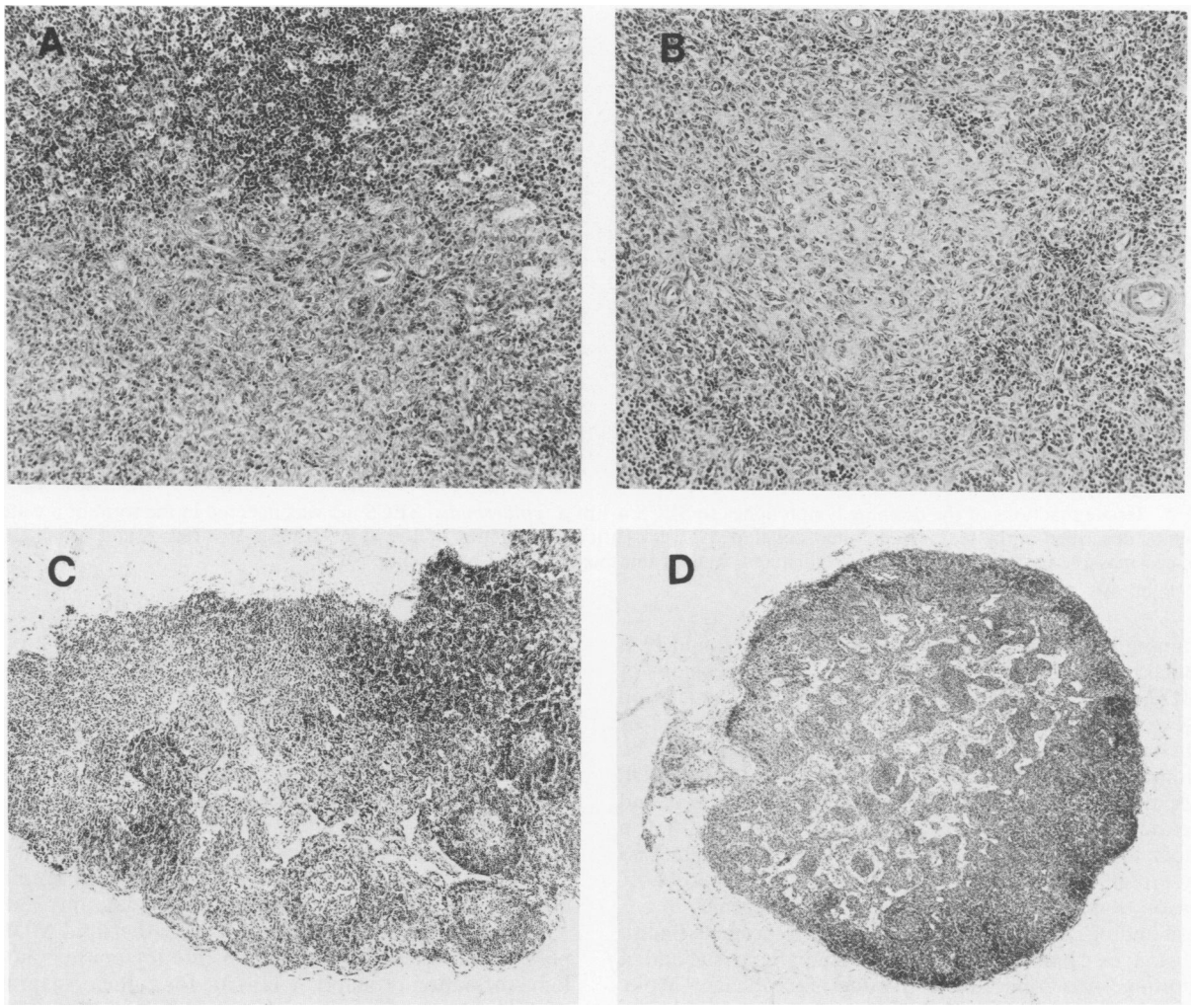

FIG. 4. Tissue reactions of the draining lymph node to acyl MDPs. Acyl MDPs were similarly prepared and injected as described in the legend to Fig. 1. Granuloma developed 2 weeks after the injection of 100 μg of BH48-MDP (A) and N-myc MDP (B) (×100). The granulomas contain many epithelioid cells. Histological changes seen 2 weeks after the injection of 100 μg of BH48-MDP(D-isoAsn) (×60; C) and 3 weeks after the injection of 100 μg of L30-MDP (×30; D). No inflammatory reactions were observable.

suggests the importance of branching of fatty acids conjugated with MDP for granuloma formation in the draining lymph nodes.

We previously found that MDP activated guinea pig macrophages (31). Furthermore, we have recently observed that MDP and its analogs capable of activating macrophages, but not those incapable of such activation, could evoke epithelioid granulomas when injected in the water-in-oil emulsion (unpublished data). This result appears to suggest that a granulomagenic substance must be able to activate macrophages. In accord with this observation, conjugates of branched fatty acids with adjuvant-inactive MDP analogs, such as BH48-MDP(D-isoAsn), BH48-MDP(L-isoGln), and B30-MDP(D-isoAsn), which did not activate macrophages, did not evoke granuloma (Table 2; Fig. 4C).

We recently found that DNA replication of macrophages was markedly inhibited when the macrophages were activated by MDP (22). This fact suggests that MDP stimulates macrophages to differentiate into epithelioid cells (this possibility will be discussed in more detail separately). It was also reported that MDP induced a dose-dependent increase in the number of bone marrow macrophage progenitor cells (33), caused monocytosis (13), and exerted a chemotactic activity for macrophages (24). All these immunopharmacological activities of the MDP molecule may contribute to its capacity to induce epithelioid granulomas. Likewise, lymphokines generating constantly in the tissue which are considered to play an important role in granuloma formation may act in a way similar to that of MDP to form granulomas.

Thus, MDP should be either incorporated in the water-in-oil emulsion or conjugated with branched fatty acids to be granulomagenic. MDP did not produce granulomas when injected alone in PBS, merely mixed with branched fatty acids, or conjugated with a linear fatty acid. Although L30-MDP evoked no granuloma in the lymph nodes, it activated macrophages to the same extent in vitro as did MDP or B30-MDP (data not shown). The reason for the inability of L30-MDP to evoke granuloma in the draining lymph nodes despite its capacity for activating macrophages is not clear. The ester bond between B30 and MDP, but not that between L30 and MDP, would be resistant to the esterases, because of the steric hindrance by the large aliphatic side chain at the α position. Another possibility is that B30-MDP might more easily reach and be deposited in

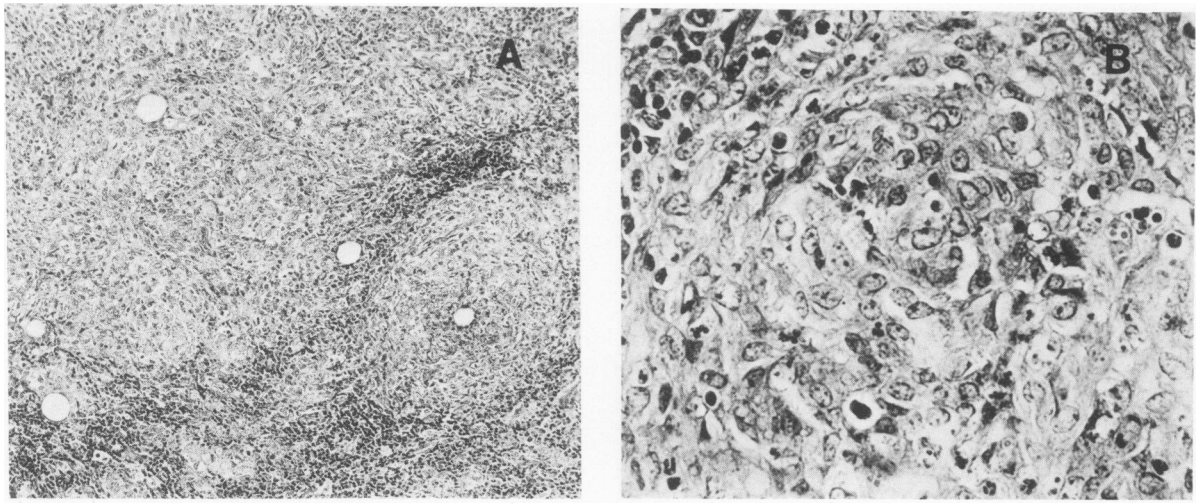

FIG. 5. Tissue reactions of the draining lymph nodes to SECS a-1 of *S. epidermidis*. SECS a-1 was injected in the form of a water-in-oil emulsion as described in the text. Granulomas developed 2 weeks after the injection of 100 μg of SECS a-1 (×100; A and ×400; B). SECS a-1 induced massive epithelioid granulomas indistinguishable from those induced by tubercle bacilli.

the draining lymph nodes than L30-MDP. There could be a difference in the physical states between the suspensions of B30-MDP and L30-MDP. In any case, a close association with scarcely metabolizable lipid, such as a mineral oil or branched fatty acids, appears essential for MDP to be granulomagenic. Such a close association should make MDP localize in the tissue. To be able to localize in the tissue and not be degraded rapidly was considered essential for a substance to be granulomagenic (10). MDP dissolved in PBS is very rapidly excreted in the urine (25), which is probably the reason that MDP as such is not granulomagenic.

These findings and considerations led us to propose that (i) the substances capable of being deposited in the tissue and of proliferating, attracting, and activating macrophages evoke epithelioid granulomas, and (ii) in tubercle bacilli, MDP, which is closely associated with α-branched fatty acids, is probably a structure essential for granuloma formation. The latter proposition is made because in tubercle bacilli, branched fatty acids such as mycolic acids are present in close association with an MDP moiety characteristically, abundantly, and uniquely in wax D and cell walls, granulomagenic fractions characterizing these bacteria (2, 7, 12). Admittedly, mycolic acids are not directly bound to the MDP moiety in tubercle bacilli (2, 12). However, mycolic acids are closely associated with the MDP moiety in tubercle bacilli, since they covalently bind to the MDP moiety through the bridge of the polysaccharide portion, forming a mycolic acid-polysaccharide-MDP complex (2, 12). The α-branched fatty acids used in the present study, though much smaller than mycolic acids, mimic mycolic acids in that they possess such a large aliphatic side chain at the α position and are much larger than the fatty acids so far detected in mammals. It may be interesting to remember that in the past, a branched fatty acid called phthioic acid once attracted much attention due to a claim that it was a granulomagenic factor in tubercle bacilli (10, 32).

MDP is commonly present in peptidoglycan of cell walls of bacteria which are parasitic on mammals and belong to the group A type of Schleifer and Kandler's classification (27). However, these bacteria do not always evoke granulomas. If MDP can become granulomagenic when closely associated with scarcely metabolizable lipids as mentioned above, then the cell walls or their peptidoglycan fragments of these bacteria incapable of producing granulomas should become granulomagenic when closely associated with such lipids, because peptidoglycans of such bacteria contain the MDP moiety. This possibility was indeed the case. When a peptidoglycan fragment, SECS a-1, obtained from *S. epidermidis* as an example for such bacteria, was injected in the water-in-oil emulsion, it did evoke epithelioid granulomas (Fig. 5A and B). Also, three other MDP-containing peptidoglycan fragments of different sizes were all granulomagenic if incorporated in the water-in-oil emulsion (data not shown).

The hypothesis presented here that the lipid-MDP complex in tubercle bacilli is responsible for epithelioid granuloma formation probably holds true for guinea pigs, rats, and rabbits, but not for mice, because MDP in the water-in-oil emulsion evoked extensive epithelioid granulomas in the first three animals (9, 29) but not in mice (30). For mice, the cord factor was reported to be granulomagenic (4), although it was not granulomagenic in rabbits (19), rats, and guinea pigs (unpublished data), and a purified cord factor was reported to be less granulomagenic than a crude one in mice (18). It is of interest, however, to note that cord factor is also a complex containing mycolic acids (mycolic acid trehalose ester), can activate macrophages of mice, and acts as an adjuvant in mice (26).

We found recently that granuloma formation by MDP required no T-cell participation (21, 30). Therefore, on the one hand, it is conceivable that the proposed granulomagenic chemical entity in tubercle bacilli (the lipid-MDP complex) can evoke granuloma without T-cell involvement. On the other hand, however, the granulomagenic chemical entity probably acts as a potent immunological adjuvant in tubercle bacilli, because an association with lipids such as branched fatty acids or liposomes often makes MDP very potent as adjuvant (11, 14, 15). Thus, although MDP can activate macrophages without lymphocytes in vitro (20), it should also be able to activate macrophages via T cells in vivo, if antigens coexist. Therefore, we consider that the proposed structure, the lipid-MDP complex, in tubercle bacilli probably exerts its granulomagenic capacity directly (non-immunologically) as well as indirectly (immunologically via lymphokine production), when it acts as a

granulomagenic factor in tubercle bacilli and evokes tuberculous granulomas, since tubercle bacilli contain large amounts of antigens.

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