Epithelioid Granuloma Formation by a Synthetic Bacterial Cell Wall Component, Muramyl Dipeptide (MDP)

Atsushi Tanaka, MD, and Kohzoh Emori, MD

A synthetic muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine) is a minimal essential structure that is contained generally in bacterial cell walls and is responsible for their many biologic activities such as adjuvant activity, pyrogenicity, and a capacity to confer resistance against bacterial and viral infections. We found that this MDP evoked dose-dependently massive organized epithelioid granulomas in guinea pigs, when injected in the form of Freund-type water-in-oil emulsion. Granuloma formation reached a peak at 3 weeks. A minimal effective dose of MDP was 0.1 μg. Essentially, no difference was observed qualitatively among granulomas evoked by MDP, MDP plus antigen, and killed tubercle bacilli incorporated in the emulsion. Quantitatively, however, MDP was stronger in its granulomagenic capacity than tubercle bacilli. Antigenicity of MDP was not detectable. These findings support our proposal that MDP may be a chemical structure in tubercle bacilli essential for epithelioid granuloma formation and that the MDP-induced epithelioid granuloma may be of a nonallergic nature. (Am J Pathol 1980, 98:733-748)

BACTERIAL CELL WALLS are generally composed of two structures, one specific for a given species and the other common to all the species. We found recently that a muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine), which is a part of the common structure of bacterial cell walls, a peptidoglycan portion, produced in guinea pigs and rats massive epithelioid granulomas indistinguishable from that produced by tubercle bacilli.[1] This MDP had been originally found as a minimal essential structure for adjuvant activity of bacterial cell walls in the studies using different fractions obtained from enzymic digests of cell walls of several bacteria.[2,3] It replaces tubercle bacilli in Freund complete adjuvant, and its adjuvant activity is much stronger than that of tubercle bacilli.[3] It was also found to cause other biologic phenomena such as pyrogenicity,[4] macrophage activation,[5-8,20,21,25,26] and resistance against bacterial and viral infections.[9,10]

In the present study the previous finding[1] of granuloma formation by MDP was confirmed and further extended; the time course of granuloma formation and the dose-response relationship were investigated. The tissue reactions to MDP were compared with those to tubercle bacilli.

From the Department of Biochemistry, Shimane Medical University, Izumo, and the Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

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Address reprint requests to Atsushi Tanaka, MD, Department of Biochemistry, Shimane Medical University, Izumo 693, Japan.

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Materials and Methods

Female Hartley guinea pigs weighing 500–600 g were obtained from a local breeder. Muramyl dipeptide (MDP) was supplied by Dr. Atsuro Inoue, of the Daiichi Pharmaceutical Company, Tokyo, Japan, and had been synthesized as described previously.[11]

MDP with or without a protein antigen (ovalbumin) was dissolved in a phosphate-buffered saline (PBS) and emulsified with either Drakeol 6VR containing Arlacel A (water: Drakeol: Arlacel A = 5:4:1) or with an equal volume of Freund incomplete adjuvant (Difco). When heat-killed tubercle bacilli (H37Rv) were used, they were suspended in PBS and then emulsified with the mixture of Drakeol and Arlacel A or the Freund incomplete adjuvant. A 0.2-ml portion of the resulting emulsion was injected intradermally into the left hind footpads.

Examinations of Lymph Nodes

Test materials incorporated in the emulsion were injected into the footpads, and excised draining lymph nodes (popliteal, inguinal, and flank) were weighed and subjected to histologic examination at various times.

For the examination by light microscopy, the draining lymph nodes were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin and eosin (H & E), with periodic acid–Schiff (PAS) or silver.

For the examination by electron microscopy, selected areas of the granulomas were cut into 1-mm blocks, fixed for 4 hours in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), washed in the cacodylate buffer, postfixed for 1 hour in phosphate-buffered 1% osmium textroxide, dehydrated in a graded series of ethanol, and embedded in Epon.

The blocks were thin-sectioned with an ultramicrotome. The sections were stained with uranyl acetate and lead citrate and were examined with a JEOL 100 C electron microscope.

Skin or Corneal Reaction

For the skin text 100 μg MDP dissolved in 0.1 ml PBS was injected intradermally. For the corneal test a PBS solution of MDP (10 mg/ml) was injected into the cornea. The guinea pigs were examined at 6, 24, and 48 hours.

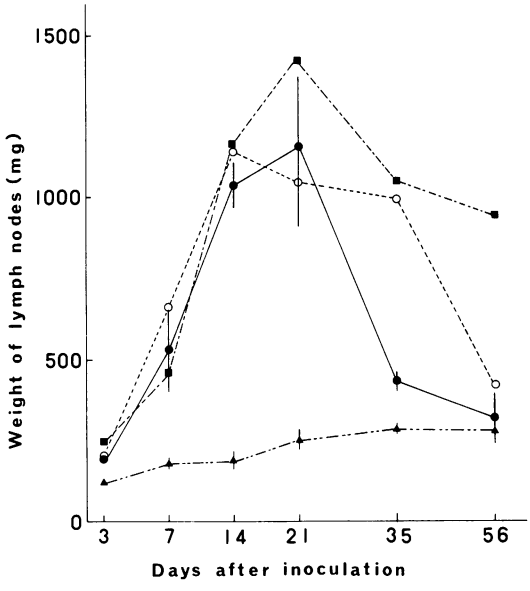
Results

Kinetics of Tissue Reactions Caused by MDP

Ninety-six guinea pigs were divided into 4 groups and given injections of either the water-in-oil (Drakeol, Arlacel) emulsion without test materials (control) or the emulsion containing MDP alone, MDP plus ovalbumin or heat-killed tubercle bacilli, respectively. A 100-μg amount of each material (MDP, ovalbumin, or tubercle bacilli) was incorporated in the emulsion. At different times after inoculation, 4 guinea pigs of each group were killed, and excised draining lymph nodes were weighed and examined microscopically.

Weight of Lymph Nodes

Draining lymph nodes of the 3 groups receiving the test materials enlarged markedly (Text-figure 1), with a peak at 3 weeks. Then they re-



TEXT-FIGURE 1—Weight of the draining lymph nodes measured in the course of time. Guinea pigs were injected with water-in-oil emulsion containing 100 μg MDP (●—●), 100 μg killed tubercle bacilli (■---■), or 100 μg MDP plus 100 μg ovalbumin (○---○). Control animals (▲---▲) were injected with water-in-oil emulsion alone.

gressed rapidly in the MDP group and gradually in the tubercle bacilli group. The maturity of epithelioid granuloma also reached its peak at 3 weeks in all 3 groups. The weight of regional lymph nodes paralleled granuloma formation, making it a good parameter with which to estimate the extent of granuloma formation. Lymph nodes of the opposite side did not show any significant increase in weight in all the 4 groups.

Histologic Findings

Epithelioid granulomas were evoked by MDP in the sites of injection, regional lymph nodes, and lungs. Other organs were not examined. We examined mainly the draining lymph nodes rather than the injection sites, because the nature and extent of granulomatous responses to MDP were more clear-cut in the draining lymph nodes than in the injection sites, since the tissue reaction to the control oil emulsion, which itself evokes macrophage infiltration to some extent, was minimal in the draining lymph nodes.[1]

Extensive granulomatous inflammations occurred in the draining lymph nodes of the test group receiving MDP in the oil emulsion (Figure 1A–D). At an early stage (on Day 3) a large number of polymorphonuclear leukocytes were seen, often with massive necroses around the oil droplets, particularly in the popliteal lymph nodes (Figure 1A). As time proceeded (on Day 7) the infiltration of macrophages became prominent (Figure 1B). Many of them phagocytized tiny oil droplets. They accumulated com-

pactly around the oil droplets and formed granulomas. Plasma cells were also seen. On day 14, solid, large and compact granulomas were formed, often throughout the entire lymph nodes, with small islands of lymphoid tissue remaining (Figure 2B). They became mature at 3 weeks. Epithelioid cells became organized into tubercles (Figure 1C). They had large, pale, oval nuclei with prominent nucleoli and abundant eosinophilic cytoplasms, the borders of which were not distinct (Figures 1C, 2A and C). In the granulomas infiltration of neutrophils was scarcely noticed. Occasionally, multinucleated giant cells were seen in the granulomas (Figure 2C). As the granulomas became more compact and extensive, the oil droplets became smaller in number and size, almost disappearing at 3 weeks. Five weeks after inoculation the granulomas showed a tendency to resolve, with the appearance of fibroblasts, fibrosis, and hyaline deposition (Figure 1D). No essential differences in the tempo of differentiation of macrophages into epithelioid cells, in the degree of maturity of appearing epithelioid cells, and in the nature and extent of formed granulomas were seen among granulomas induced by MDP (Figures 1A–D), tubercle bacilli (Figures 3A–D) and MDP plus ovalbumin (Figures 4A–D).

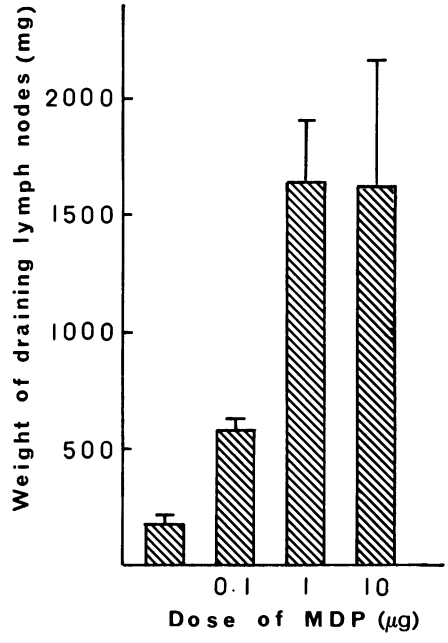
In contrast, no epithelioid granuloma was formed in the lymph nodes of the control group (Figure 5). Throughout the lymph nodes were scattered oil droplets, which were seen throughout the whole experimental period. Around the droplets were seen sometimes a few macrophages limited within sinuses. The nodal architecture was well preserved, with no infiltration of polymorphonuclear leukocytes.

Ultrastructural Findings

The cells composing MDP-induced granulomas had large, oval, euchromatic nuclei with margination of scanty heterochromatin and often large and prominent nucleoli (Figure 6). Their cytoplasm was abundant and often full of organelles, including lysosomes, mitochondria, rough endoplasmic reticulum, free ribosomes, and Golgi profiles. They were closely packed together and were sometimes interwoven with one another by interdigitation of cytoplasmic pseudopods. Phagosomes were not noticed in the cells.

Dose-Response Relationship

To investigate the relationship between the dose of MDP and the inflammatory reactions, we injected 0.1, 1, and 10 μg of MDP incorporated in the water-in-oil (Drakeol, Arlacel) emulsion into the footpads of guinea pigs (4 or 5 animals per group) and examined the draining lymph nodes 2 weeks later in terms of histologic characteristics and weight. MDP in

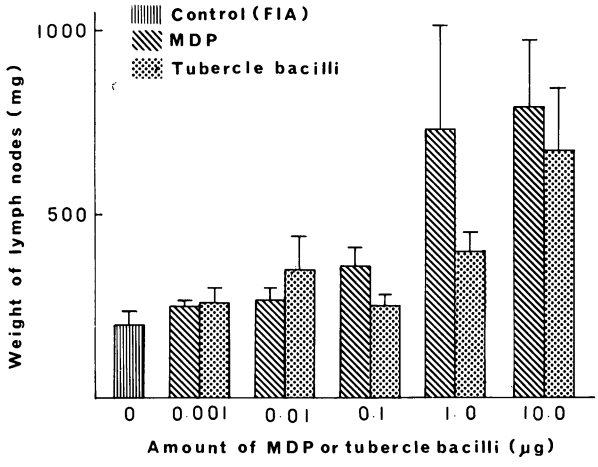


TEXT-FIGURE 2—Weight of the draining lymph nodes of guinea pigs given injections of water-in-oil emulsion containing different amounts of MDP 2 weeks previously. Drakeol and Arlacel A were used in the preparation of the emulsion.

doses of 0.1 μg caused a significant increase ($P < 0.01$) in the weight of the lymph nodes (Text-figure 2). Histologic examination showed massive epithelioid granulomas without necrosis in the draining lymph nodes of all the guinea pigs that had been given injections of 0.1 μg MDP (Figure 2B).The 1-μg dose of MDP seemed enough to cause maximal reactions, as assessed by the two parameters.

To compare its granulomagenic capacity with that of tubercle bacilli, 0.001, 0.01, 0.1, 1, and 10 μg of MDP or tubercle bacilli incorporated in the water-in-oil emulsion were injected into the footpads of 44 guinea pigs (4 animals per group). Freund incomplete adjuvant (Difco) was used this time. Inflammatory responses were slightly weaker than those caused by MDP incorporated in Drakeol and Arlacel A. The amount of 0.1 μg of MDP was the lowest effective dose (Text-figure 3). In 2 of 4 animals given injections of 0.1 μg MDP epithelioid granulomas were produced. One microgram of MDP evoked massive epithelioid granulomas in all the animals, and, again, this amount (1 μg) of MDP caused a nearly maximal inflammatory response.

In contrast, 1 μg of tubercle bacilli (dry weight) hardly evoked epithelioid granulomas on histologic examination and caused only a slight increase in the weight of the lymph nodes. For a definite epithelioid granuloma formation and a maximal lymph node swelling 10 μg of tubercle



TEXT-FIGURE 3—Weight of the draining lymph nodes of guinea pigs injected with water-in-oil emulsion containing different amounts of MDP or tubercle bacilli 2 weeks previously. Freund incomplete adjuvant (Difco) was used to prepare the emulsion.

bacilli was needed. The results indicate that MDP may be at least approximately 10 times stronger than tubercle bacilli to induce epithelioid granulomas. A parallelism was again seen between the weight of draining lymph nodes and granuloma formation.

Reaction to MDP Dissolved in Water

In contrast to MDP incorporated in the water-in-oil emulsion, MDP dissolved in PBS caused no granuloma formation in the draining lymph nodes or in the injection site.

Antigenicity of MDP

In order to test whether MDP possessed antigenicity, we divided 24 guinea pigs into 3 groups. The first group was given injections of 0.2 ml of a Freund-type emulsion containing 100 μg heat-killed tubercle bacilli in footpads; the second group with the same volume of the emulsion containing 100 μg of MDP, and the third group with the emulsion containing none. Two weeks later skin and corneal tests were performed with MDP as antigen. Both tests gave completely negative reactions in all 3 groups.

Discussion

For many years factors that induce epithelioid granulomas have been searched for but not yet well characterized. It is not well known yet how tubercle bacilli evoke epithelioid granulomas and which chemical structure(s) in tubercle bacilli is responsible for the epithelioid granuloma formation.[1,12,13,22]

Workers such as Anderson, Lederer, and Asselineau extracted lipids

from tubercle bacilli and fractionated them into many fractions.[14] Wax D (lipopolysaccharide) fraction among them is the one containing MDP.[15]

Delaunay, Asselineau, and Lederer reported that chronic granulomas containing epithelioid cells were formed by wax D in guinea pigs in 1951,[16] which was confirmed later by White et al[17] and by us (unpublished observation). Recently we found that MDP in the oil emulsion evoked massive organized epithelioid granulomas in guinea pigs.[1] The present study confirmed this; essentially no difference was observed qualitatively between granulomas evoked by MDP and those evoked by tubercle bacilli. Also, the rates of differentiation of macrophages into epithelioid cells were similar; both MDP and tubercle bacilli required 3 weeks for full differentiation. This seems important, since the rate of differentiation was found quite variable, depending upon the stimulating agent,[18,19] in other studies. MDP formed massive epithelioid granulomas also in rabbits (unpublished observation). The development of an epithelioid granuloma is shown to be a good model of the activation and differentiation of macrophages *in vivo*.[18] The present findings are, therefore, in accord with now accumulating data that MDP activates macrophages *in vitro* as well as *in vivo* [5–8,20,21,25,26] (unpublished data).

Quantitatively, MDP was stronger in granulomagenic activity than killed tubercle bacilli in guinea pigs under the present experimental conditions. The weight of the lymph nodes was found in the present study to be a good index of the extent of granuloma formation. Using this and histologic indexes, the minimal effective dose of MDP in evoking epithelioid granuloma was found to be 0.1 μg. To our knowledge, there has been reported no bacterial constituent, natural or synthetic, that could evoke such a massive organized epithelioid granuloma in so small an amount.

The facts that such a small amount was effective, that it was a synthetic substance, and that a 100-μg amount of a diastereoisomer of MDP, as well as other adjuvant-inactive MDP analogs, did not evoke epithelioid granuloma (unpublished observation) exclude the possibility of a contamination of bacteria or bacterial constituents other than MDP, a problem that appeared sometimes to be misleading in the past.[1,12,13,22] These results and the fact that MDP was stronger in the granulomagenic capacity than tubercle bacilli support our previously proposed view that MDP may be the structure in tubercle bacilli essential for epithelioid granuloma formation.[1]

A question may be raised: Why are tubercle bacilli particularly granulomagenic among many other bacteria that also contain MDP? Data are accumulating that show that MDP requires some lipidic factors to exert its granulomagenic capacity. MDP should be incorporated into the water-

in-oil emulsion to form the granuloma; MDP dissolved in PBS evoked no granuloma. Recently, we observed that a conjugate of MDP with a synthetic, branched-chain fatty acid of 30 carbon atoms produced granulomas in the draining lymph nodes of guinea pigs even when injected as a suspension in PBS (unpublished observation). In tubercle bacilli there are large amounts of lipids and branched-chain fatty acids, such as acids of the mycolic type or phthioic type.[23] Conceivably, in tubercle bacilli some such lipidic factors may act in some way to help MDP, or MDP-containing cell walls, or their fragments to be effective granulomagenic stimulants. Thus, it seems that MDP is essential for epithelioid granuloma formation but needs some lipidic factor for the expression of its granulomagenic capacity. This may explain why tubercle bacilli are particularly granulomagenic.

Another important point that has emerged in this study is that an immunologic reaction may not be involved in the epithelioid cell granuloma formation by MDP, though it was stressed that the formation of organized epithelioid cell granulomas was immunologic in nature.[13,24] A definite antigenicity of MDP had not been detected in guinea pig,[1] which was confirmed in the present study. Whereas 0.1 μg MDP in the water-in-oil emulsion did evoke the massive, organized epithelioid granuloma, a much larger amount (100 μg) of MDP in the emulsion could not render guinea pigs sensitized to MDP. Further, MDP possessing little or no antigenicity was much more granulomagenic than tubercle bacilli possessing strong antigenicity. Thus, the MDP-induced epithelioid granuloma formation cannot be explained on the basis of the antigenicity of MDP. A capacity other than antigenicity seems to be responsible for its granuloma formation. Such a capacity may be a macrophage-activating one. MDP was found to activate macrophages *in vivo* as well as *in vitro*, as measured by various parameters such as phagocytosis,[5,7] migration inhibition of macrophages,[6,8] a synthesis of collagenase, prostaglandin E, cyclic AMP,[21] a release of a factor enhancing antibody production,[20] production of an endogenous pyrogen,[25] production of lymphocyte-activating factor,[26] glucose oxidation, glucosamine incorporation, and spreading and attachment substrate (unpublished data). Intermediary contaminating lymphocytes that may release a macrophage-activating factor were found most probably not to be involved in this stimulation.[5-8] Finally, little difference was noticed in the extent and nature of the epithelioid granulomas evoked by MDP alone and those evoked by MDP plus antigen (ovalbumin), confirming previous data on rats.[1] Therefore, we infer that MDP-induced epithelioid granuloma formation may be nonallergic in nature.

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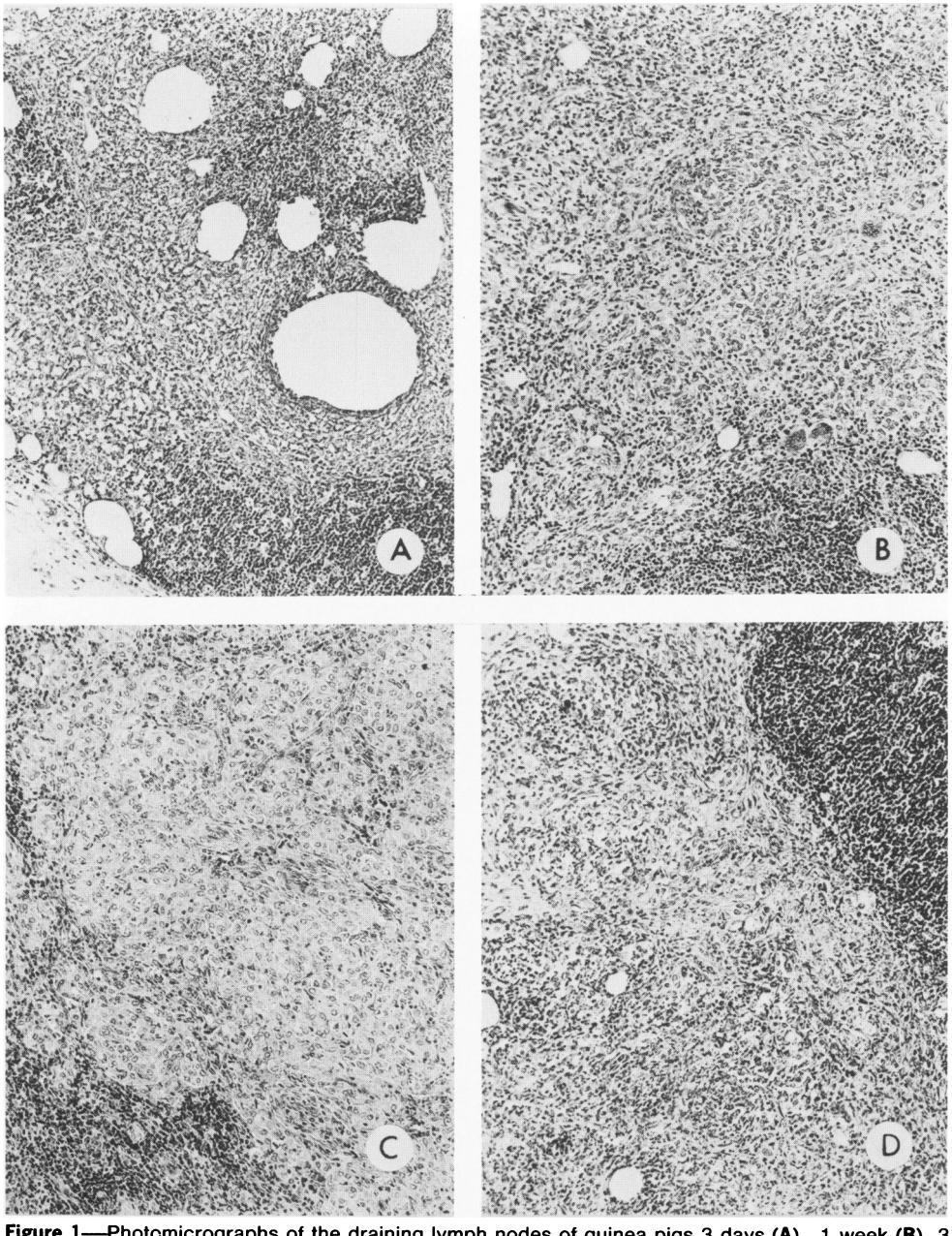


Figure 1—Photomicrographs of the draining lymph nodes of guinea pigs 3 days (**A**) , 1 week (**B**), 3 weeks (**C**), and 5 weeks (**D**) after the injection of the water-in-oil emulsion containing 100 μg MDP. Note necroses (**A**), multi-nucleated giant cells (**B**), and massive granulomas (**B** and **C**). (H&E, ×100)

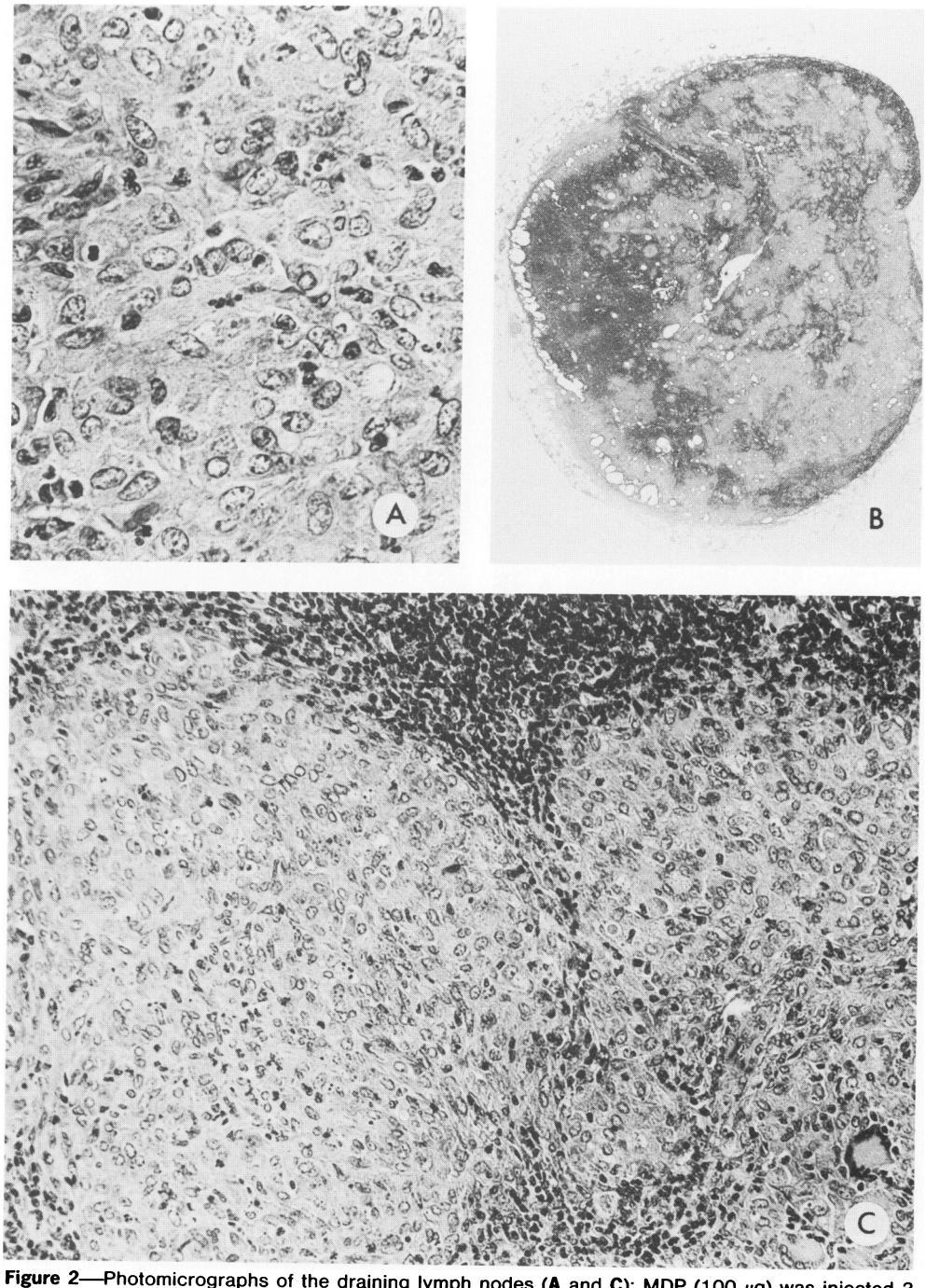


Figure 2—Photomicrographs of the draining lymph nodes (**A** and **C**); MDP (100 μg) was injected 3 weeks previously. **B**—MDP (0.1 μg) was injected 2 weeks previously. Note that the constituent cells of the granulomas are organized epithelioid cells (**A, C**) and that a very small amount (0.1 μg) of MDP can cause massive granulomas (**B**). (H&E, **A**, ×400; **B**, ×11; **C**, ×200)

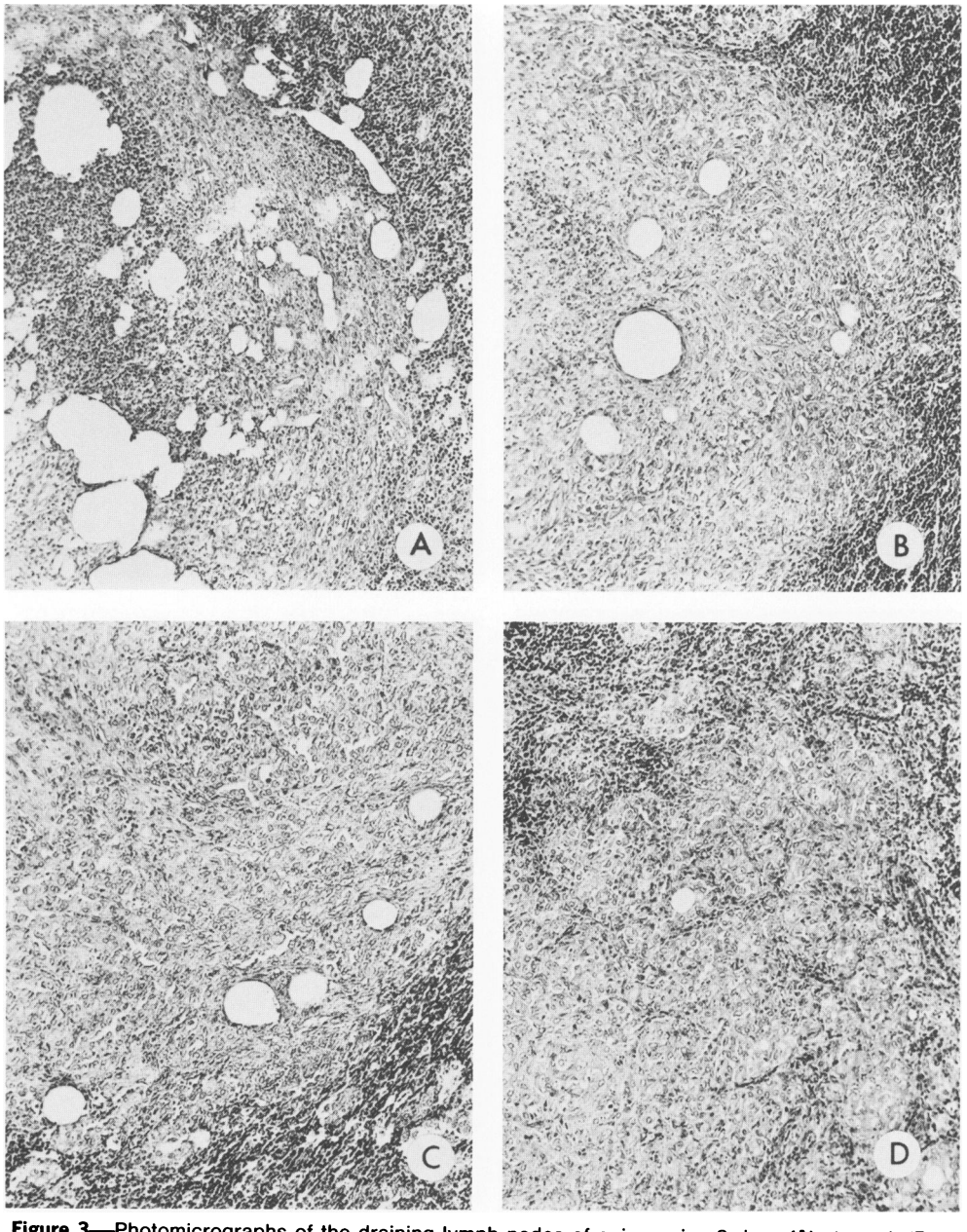


Figure 3—Photomicrographs of the draining lymph nodes of guinea pigs 3 days **(A)**, 1 week **(B)**, 3 weeks **(C)**, and 5 weeks **(D)** after the injection of the water-in-oil emulsion containing 100 μg tubercle bacilli. Note necroses **(A)** and massive granulomas **(B, C, D)**. Little differences are observable between the lesions shown here and those caused by MDP shown in Figure 1. (H&E, ×100)

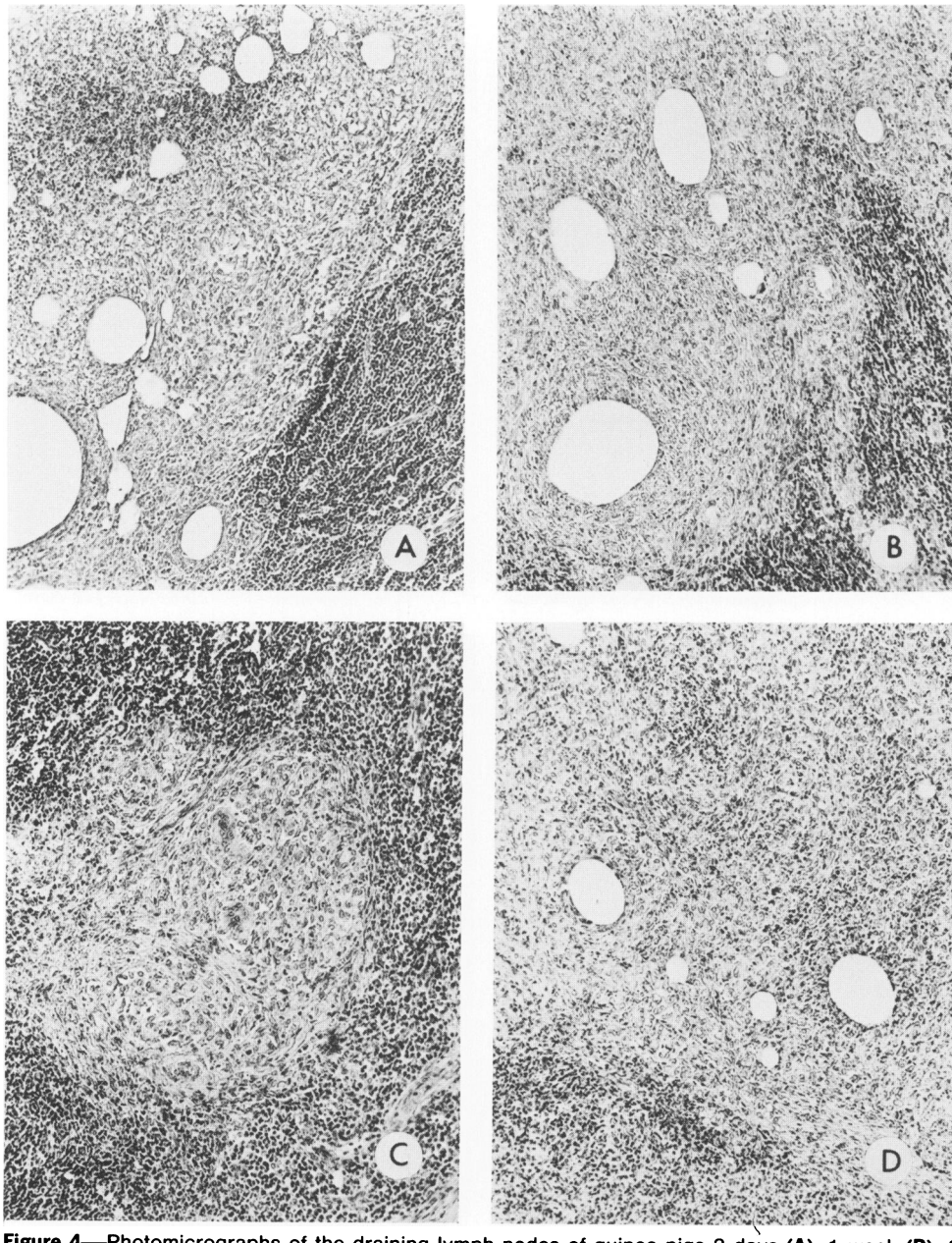
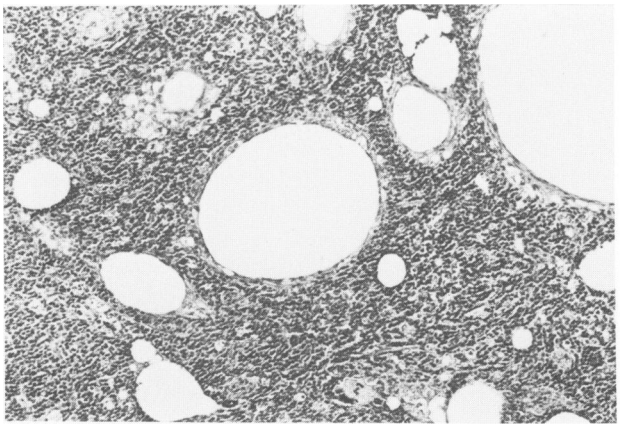


Figure 4—Photomicrographs of the draining lymph nodes of guinea pigs 3 days (**A**), 1 week (**B**), 3 weeks (**C**), and 5 weeks (**D**) after the injection of the water-in-oil emulsion containing 100 μg MDP and 100 μg ovalbumin. Note necroses (**A**), multinucleated giant cells (**C**), and massive granulomas (**B, C, D**). (H&E, ×100)



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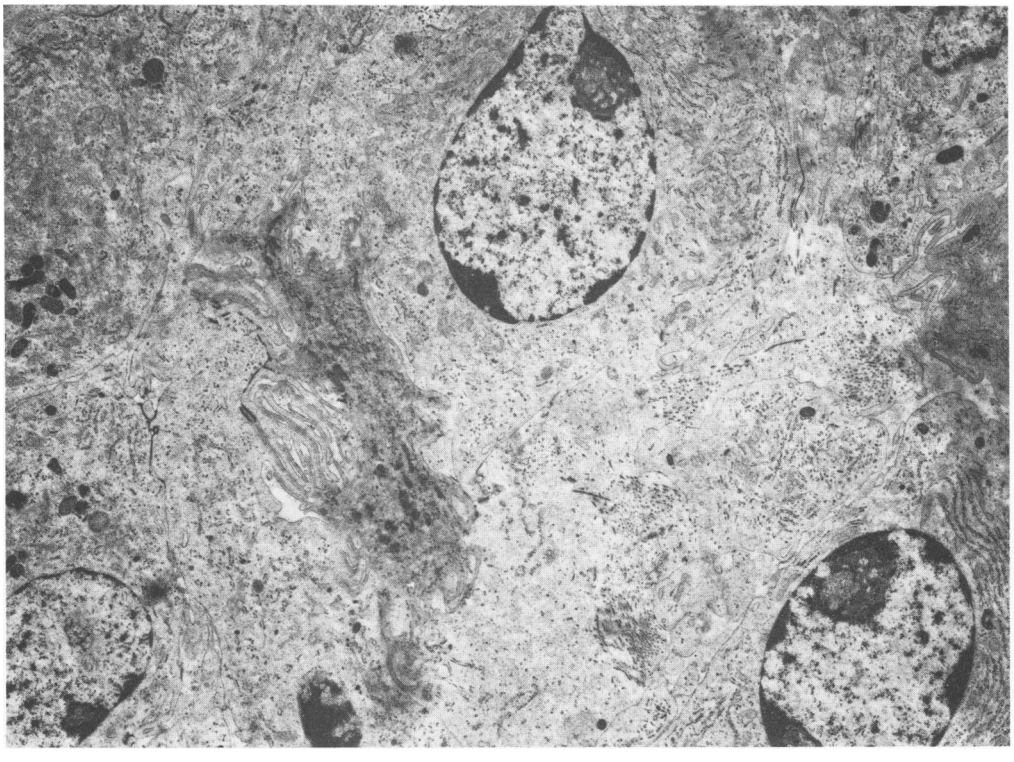

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Figure 5—Photomicrograph of the draining lymph nodes of control guinea pigs given injections of water-in-oil emulsion 3 weeks previously. Oil droplets of varying sized are encircled by a few macrophages (H&E, ×100) **Figure 6**—Electron micrographs showing epithelioid cells in the granuloma induced 3 weeks after the injection of MDP (100 μg). These cells have large euchromatic nuclei with prominent nucleoli. Cyctoplasms are extensive and filled with abundant rough endoplasmic reticulum (*upper right*) or with numerous lysosomes (*left*). The cells closely approximate one another. (×4400)

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