Histological Study of Local Skin Injected with Adjuvant-active and -inactive Muramyl Dipeptide (MDP)

(Histological Change in MDP Injected Skin)*

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SUMMARY In order to examine the relationship between local cellular responses at injected sites and adjuvant activities, adjuvant-active N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP-LD) or adjuvant-inactive N-acetylmuramyl-L-alanyl-L-isoglutamine (MDLP-LL) was injected intracutaneously into guinea pigs in the form of a water-in-oil-in-water emulsion (w/o/w). Countings of differential cell and acid phosphatase-positive cell were made on methacrylate-embedded thin sections of skin from the injected sites. Adjuvant-active MDP-LD in w/o/w induced the infiltration of polymorphonuclear leukocytes in the early stage, as well as local accumulation of activated macrophages characterized by strong positive acid phosphatase activity and morphological changes in epithelioid cells during the late stage. Adjuvant-inactive MDP-LL-w/o/w and w/o/w without MDP as a control also induced the aforementioned histological changes but to a lesser degree. Adjuvant activity of MDP was thus expressed as long lasting strong activation of macrophages and epithelioid cell granuloma formation at the injected sites.

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N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is a minimal structural unit which is essential for the induction of immune-adjuvant activities of bacterial cell walls.[1] When injected into guinea pigs or mice using mineral oil in the form of a water-in-oil emulsion, MDP enhanced both humoral and cellular immune responses[2-4] and nonspecific resistance to tumours.[5,6] Even if given subcutaneously or via the oral route as an aqueous solution containing antigens, MDP enhanced the humoral immune responses in mice.[7] Many studies on the mechanisms of action of MDP have shown its direct effects on T- and B-cells,[8] and its ability to induce generalized clonal expansion of lymphocytes as a "polyclonal activator".[9] Furthermore, many studies on macrophage activation *in vivo* and *in vitro*[10-12] have demonstrated that MDP released a variety of metabolic substances and induced microbicidal activities.[13] Tanaka and Emori[14] and Emori and Tanaka[15] found that MDP produced massive epithelioid cell granulomas in the regional lymph nodes of guinea pigs when injected in the form of a water-in-oil emulsion (w/o) without antigen, and stated that granuloma formation with MDP was caused by a nonallergic mechanism. Epstein,[16] however, emphasized the participation of an allergic mechanism in the epithelioid cell granuloma formation regardless of differences in the kind of inducing agents employed.

In the present study, we examined the local cellular reactions produced by intracutaneous injection of adjuvant-active N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP-LD) or adjuvant-inactive N-acetylmuramyl-L-alanyl-L-isoglutamine (MDP-LL). The aim of the study was to ascertain how the aforementioned activities of MDP are expressed with regard to histological features *in vivo*. In order to delay the removal of MDP from the injected sites, MDP was emulsified with small amounts of oil to make

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a water-in-oil-in-water emulsion (w/o/w).

MATERIALS AND METHODS

Female Hartley guinea pigs were used; they were purchased from Shizuoka Jikken Dobutsu Nokyo Shizuoka. The adjuvant-active substance, MDP-LD, and adjuvant-inactive MDP-LL were synthesized in our laboratory. The activities of these compounds were reported in a previous paper.[3] The MDP praparation was made in the form of w/o/w as follows: a given amount of MDP was dissolved in 0.03 ml of a phosphate buffered saline solution, 0.03 ml of Drackeol was added, and the mixture was homogenized in a Teflon homogenizer. Then 0.2 ml of 0.2% Tween 80-saline and 0.8 ml of the same solution were added and homogenized to make the w/o/w emulsion. The final oil volume was 3 per cent of the total volume.

Experimental schedule:

Exp. 1. In order to determine the most appropriate concentration of MDP for histological examination, 0.1 ml each of 100, 30, 10 and 1 μg of MDP-LD w/o/w, or w/o/w without MDP as a control, were injected intracutaneously into the backs of each of the animals.

Exp. 2. The MDP-LD group of animals was injected with 30 μg of MDP-LD-w/o/w (MDP-LD); the second group, with the same amount of MDP-LL-w/o/w (MDP-LL); and the last group, with w/o/w alone. Two 0.1 ml amounts of the samples were injected intracutaneously into two sites on the shaved back of each animal. At 24 and 72 hours and at seven and 14 days after the injections, the animals were sacrificed under ether anaesthesia, and the injected local skin was removed. Six skin specimens from each group were examined at each scheduled point in time.

Histological examination: Skin specimens 3 mm thick were fixed overnight at 4°C with periodate-paraformaldehyde fixative[17] and then rinsed in the buffer solution twice. After dehydration in 70, 90, 95 and 100% ethanol solutions each for 15 minutes with stirring, the specimens were embedded into a JB-4 methacrylate solution and cut into 2 μm-thick sections with a Sorvall JB-4 microtome. From each block, three serial sections were made. The first section was stained with Giemsa solution (1:50 dilution) for 60 minutes at 37°C; the second, with haematoxylin-eosin solution; and the last section, with acid phosphatase using the method of Burstone.[18] Using the Giemsa stained sections, the mean number of polymorphonuclear leukocytes (PMN), mononuclear cells (MN) including macrophages and lymphocytes, and the epithelioid cells infiltrating the dermis was counted as follows: the dermis, the centre of which contained the most intensive reaction sites, was divided into three layers of subepidermal, middle and deep layer, and the number of infiltrating cells in each layer was counted separately for each kind of cell mentioned above in 10 fields of 0.028 mm^2 The mean number for one field was calculated, and this value in the area of 0.028 mm^2 was converted into 0.1 mm^2. In figures, the number was expressed as the value in 0.1 mm^2 area. The intensity of acid phosphatase staining was divided into three grades, viz., strong positive (++), weak positive (+) and negative (−). The strong positive cells showed a deep red colour throughout the entire cytoplasm.

Statistical evaluation: The mean number of infiltrating cells in the MDP-LD or MDP-LL group was compared with that of the w/o/w control group and the P value was calculated.

RESULTS

Experiment 1: Cellular infiltration induced by various amounts of MDP-LD.

All of the 100, 30, 10 and 1 μg of MDP w/o/w emulsion and w/o/w alone induced local cellular infiltration in the dermis and in the hypodermis of the injected sites. The PMN in the dermis showed peak infiltration at 24 hours and then decreased in number, especially in the below 10 μg and w/o/w control group (Fig. 1). Many degenerative PMN were seen at 72 hours, and they had almost disappeared by the seventh day. The infiltrating cells consisted mainly of neutrophils,

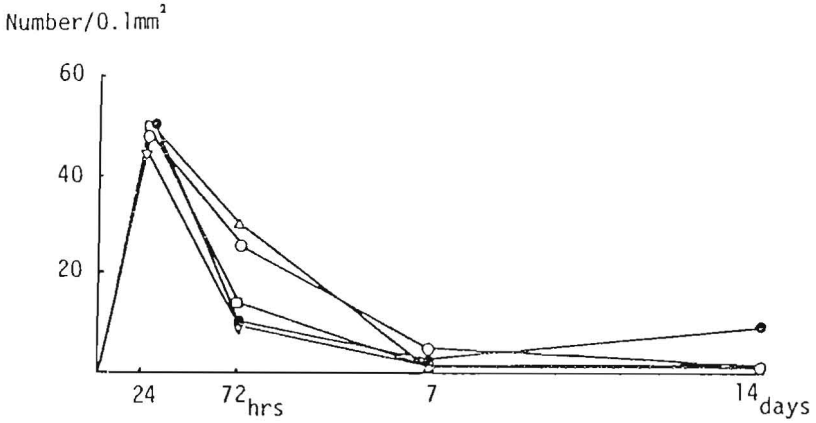

Fig. 1 The number of PMN infiltrated in the dermis injected with various doses of MDP-LD. Guinea pigs were injected intracutaneously in the back with 0.1 ml of w/o/w containing 100 μg (○——○), 30 μg(△——△), 10 μg (□——□), 1 μg (▽——▽) or w/o/w without MDP (●——●). Excisions of local skin were performed at 1, 3, 7 and 14 days after injection. Cell counting was made microscopically on JB-4 methacrylate embedded 2-μm-thick sections. The cell number is expressed as the mean value in 0.1 mm^2 area.

along with a small number of eosinophils and mast cells.

Inversely, infiltration of MN was delayed but reached the maximum number at 72 hours, maintaining the number until 14 days (Fig. 2). No difference in MN infiltration was clear among the three groups except in the group that receiving 100 μg of MDP, which showed a slight increase in the number of infiltrated cells. The enlarged MN, including epithelioid cells, appeared after seven days; their number was parallel with the number of doses of MDP Thus, the group receiving the 1 μg injection of MDP showed the lowest number, which was same as that for the w/o/w control. Acid phosphatase staining produced a few faintly stained cells at 24 hours, after which the number and the intensity of stainability increased until the end of the experimental period at 14 days (Fig. 3). The positive cells of those injected with over 10 μg in the MDP groups reached a number about twice as high as those in the groups injected with 1 μg of MDP or the w/o/w alone; the cell number corresponded to the grade of epithelioid-cell granuloma formation.

The hypodermis of the fat and vascular tissues was invaded by intensive cellular infiltrations, the number of which was too numerous to be counted. But the kinetics of infiltration of various cells was parallel with that of the dermis.

Based on the results of Exp. 1, it was decided to use 30 μg of MDP in Exp. 2.

Experiment 2: Local skin reaction produced with 30 μg of MDP-LD, MDP-LL and w/o/w.

1. Erythema and induration of injected skin sites. At 24 hours after injection, the local skin of all groups showed a weak but clearly visible redness which was most intense in the MDP-LD group (Fig. 4A). Thereafter, the erythema decreased, although it was still faintly visible until seven days after injection. Induration, which was measured by a double thickness of the folded skin, indicated two peaks at 24 hours and seven days, and this value was maintained until 14 days in the MDP-LD group (Fig 4B). All of the groups showed a not so intense but still clearly measurable increase in skin thickness, especially the MDP-LD group.

2. Histological features of the injected sites. From 72 hours to seven days later, the epidermis became hyperplastic, forming many layers of prickled cells; however, it also showed a slightly oedematous change by which the space between the prickled cells was somewhat dilated, and in some specimens only a few MN were infiltrated. All three groups showed these changes, which disappeared at 14 days. The blood vessels and lymphatic vessels in the dermis were dilated slightly and showed no manifestations of

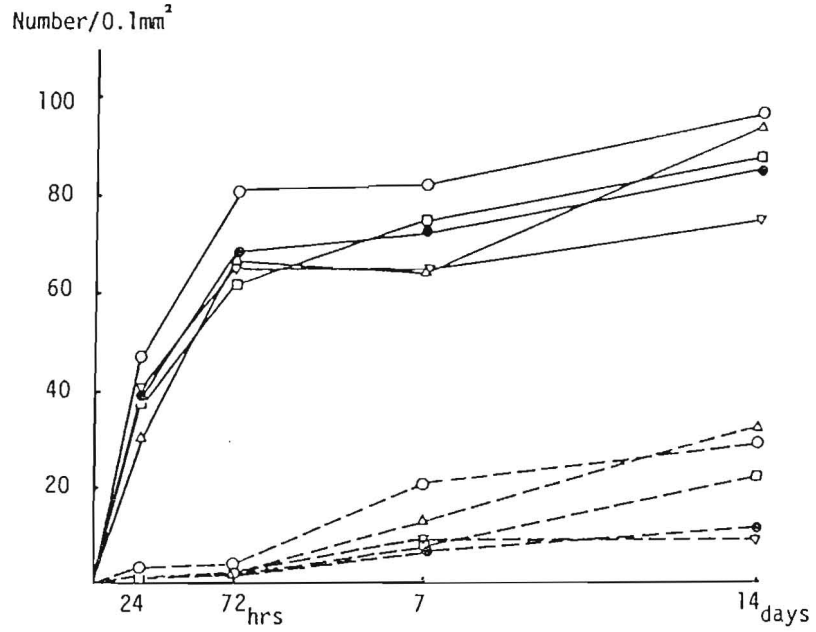

Fig. 2 The number of MN infiltrated in the dermis injected with various doses of MDP-LD. Total number of MN (————) and enlarged MN or epithelioid cells (– – –) were counted. (See Fig. 1 for legend).

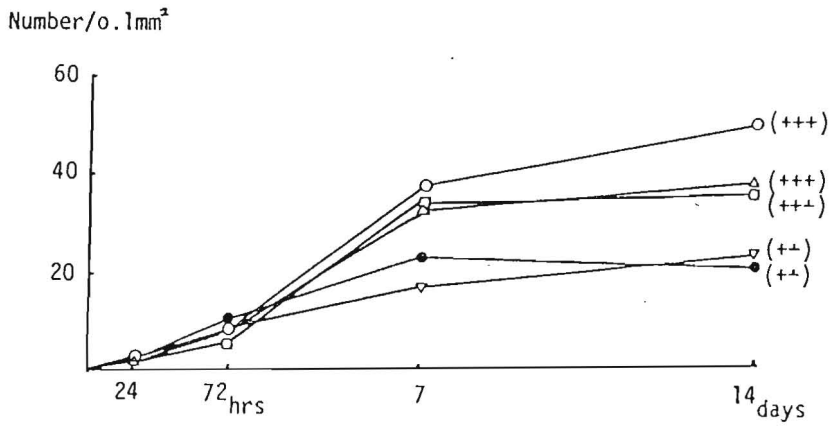

Fig. 3 The number of acid phosphatase positive cells infiltrated in the dermis injected with various doses of MDP-LD. The grade in the parentheses shows the intensity of granuloma formation in the dermis. (See Fig. 1 for legend).

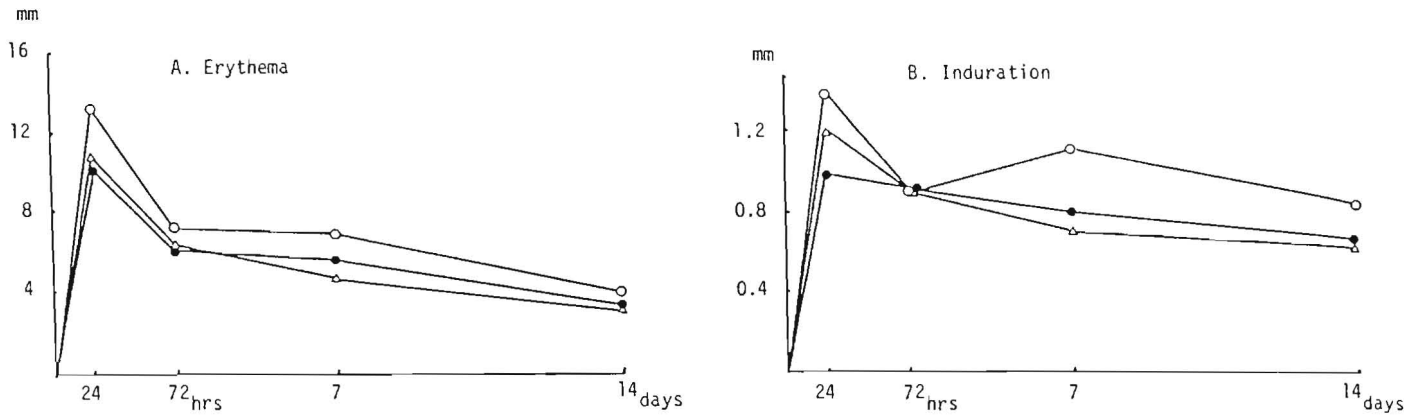

Fig. 4 Local skin reaction after intracutaneous injection with 30 μg of w/o/w containing MDP-LD (○——○), MDP-LL (Δ——Δ), or w/o/w (●——●). Erythematous reaction (A) was expressed as the diameter of the area showing redness. Induration (B) was expressed as the value of double thickness of the folded skin of injected sites minus that of uninjected sites. Each is the mean value of six injected sites.

degeneration of the vessel walls or haemorrhage, and no special cellular infiltration around the vessels. In the early stage, many PMN were found around the injected oil droplets. Eosinophils and mast cells were not distinct in these areas. Most of the PMN fell into degeneration at 72 hours, remaining only as broken nuclei. On the contrary, diffuse infiltration of MN, including macrophages and lymphocytes, was noted. Around the oil droplets, the infiltrating PMN were replaced in time by MN, which changed into large epithelioid-like or epithelioid cells and often phagocytized the injected oil droplets. Massive proliferation of the epithelioid cells was prominent at 14 days in the MDP-LD group. Although it was difficult to discriminate the macrophages and lymphocytes from the infiltrating MN cells, it appeared that about half the number of cells seen at seven days in all the groups and almost all of the cells observed at 14 days in the MDP-LD group belonged to the macrophage and epithelioid cells in comparison with the number of acid phosphatase-positive cells (Figs. 5-7).

The hypodermis, including the fat tissues and vessels, also showed massive cellular infiltration. The infiltrating cellular changes in the area ran parallel with those in the dermis but were more intensive and they phagocytized more oil droplets. In addition, many eosinophil leukocytes appeared to have been infiltrated at the later stage. These changes were also seen in the w/o/w control group, and there were no differences in cell species among the three groups; however, intensity was higher in the MDP-LD group than in the other groups.

After one week, many of the macrophages increased in size, and pale oval nuclei with prominent nucleoli and abundant eosinophilic cytoplasm were seen. Most of the cells were found distributed separately, except for some cells which were in contact with each other and had obscured cell borders. All of these cells were deeply coloured by acid phosphatase staining. The characteristic features of these cells

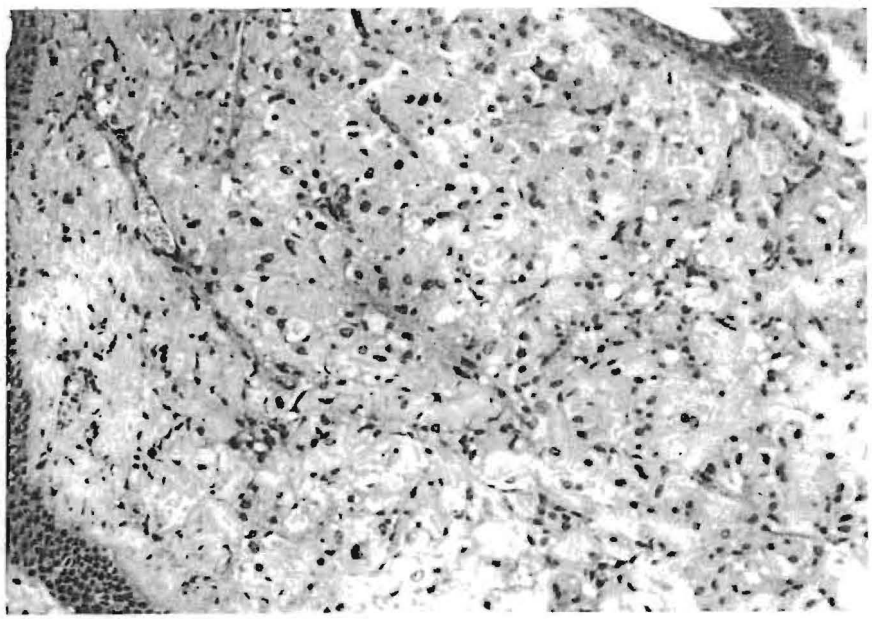

Fig. 5 Histological change in the dermis. MDP-LD (30 μg) in w/o/w was injected intracutaneously 21 days previously. The dermis in the centre of injected sites showed massive infiltration of enlarged macrophages and epithelioid cells. (Giemsa staining, x 100).

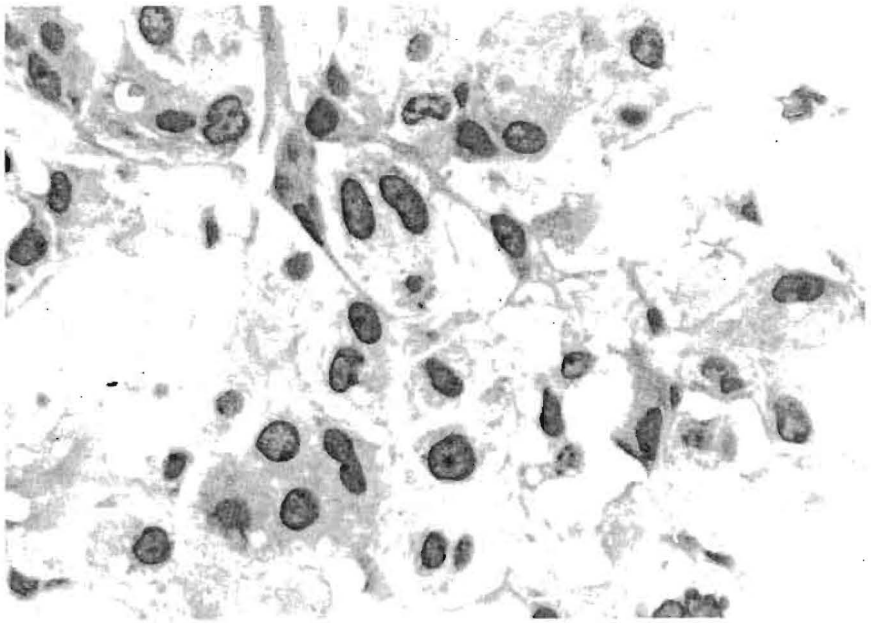

Fig. 6 Magnification of Fig. 5 Most of the cells had round or oval nuclei and abundant cytoplasm. Some cells contained oil vacuoles in their cytoplasm. (Giemsa staining, x 400).

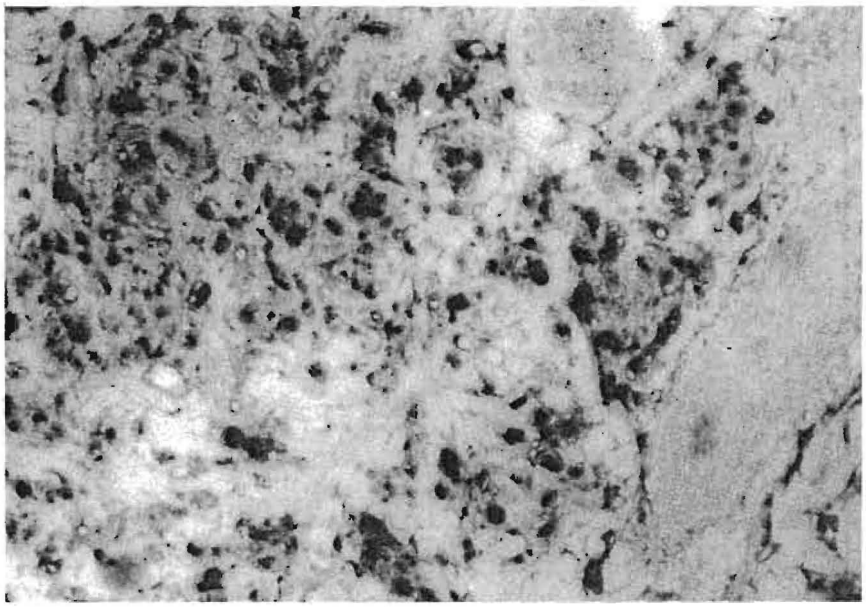

Fig. 7 Acid phosphatase-positive cells in the dermis at 21 days after injection with 30 μg of MDP-LD-w/o/w. Granuloma forming macrophages and/or epithelioid cells showed positive staining in the cytoplasm. Non-stained round or oval area in the stained cells are nuclei which were stained weakly with haematoxylin. (Acid phosphatase staining, x 100).

resembled those of the epithelioid cells; therefore, they were referred to as epithelioid cells.

The fluctuation in the number of infiltrating cells over the experimental time period is indicated in Figures 8 and 9. PMN infiltration in the early stage was highest in the MDP-LD group and lowered in the w/o/w control group and the MDP-LL group in respective order (Fig. 8). MN were found from the early stage and reached a maximum level at seven days (Fig. 9). The number of MN was highest in the MDP-LD group and lowest in the MDP-LL group, similar to that shown for PMN infiltration. Infiltration of epithelioid cells was also most intense in the MDP-LD group.

The acid phosphatase-positive cells, which consisted of activated macrophages and epithelioid cells, increased from 72 hours and reached a maximum at seven to 14 days in the MDP-LL and w/o/w group, but in the MDP-LD group the number continued to increase until 14 days (Fig. 10). The MDP-LD group showed not only the highest number of total acid phosphatase-positive cells but also a marked increase of strong positive stained cells, most of which were epithelioid cells.

DISCUSSION

It has been shown that MDP-LD is a minimal unit of adjuvant active components of bacterial cell walls.[1] Many reports have described the various activities of this compound.[2-4,8,9,13-15,19,20] Tanaka and Emori[14] and Emori and Tanaka[15] found that epithelioid-cell granuloma was produced in the regional lymph nodes when the footpads of guinea pigs were injected with MDP-LD as a w/o emulsion, and they emphasized that the production of epithelioid-cell granuloma was conducted by a non-allergic mechanism,[21] because MDP showed no antigenic activity.[15] They also found that the granuloma-forming activity was related to adjuvant activities.

The present study was attempted in order to clarify what kind of and how many cells would accumulate in the MDP-injected sites and what changes would occur in these infiltrating cells. In order to establish the relationship between the histological changes and adjuvant activities, MDP-LD, an adjuvant-

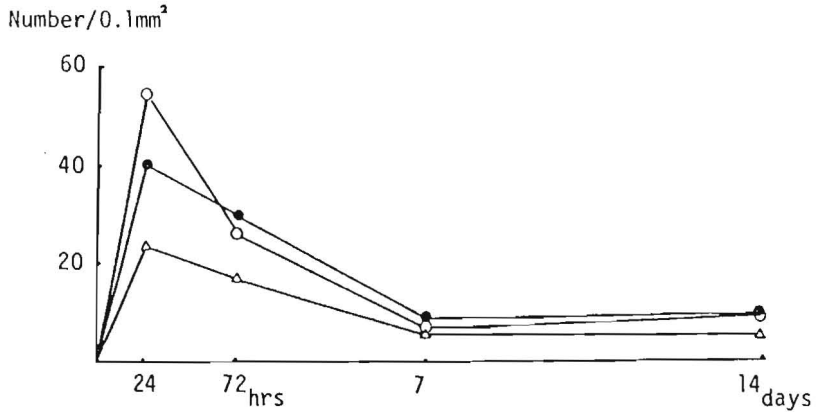

Fig. 8 The number of PMN infiltrated in the dermis. Guinea pigs were injected intracutaneously in the back with 0.1 ml of w/o/w containing 30 μg of MDP-LD (○——○), MDP-LL (Δ——Δ) or w/o/w alone (●——●). Local skin was excised at 24 and 72 hours, and 7 and 14 days after injection. Cell counting was made microscopically on JB-4 methacrylate-embedded 2-μm-thick sections.

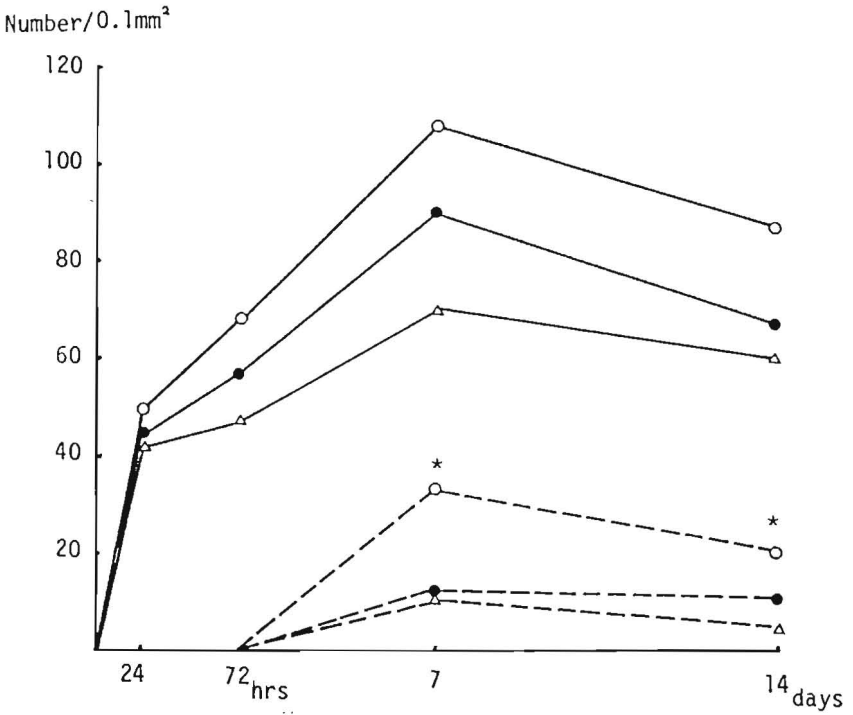

Fig. 9 The number of MN infiltrated in the dermis. Total number of MN (———) and enlarged MN or epithelioid cells (– – –) were counted. *$p < 0.05$ when compared to w/o/w group at same time. (See Fig. 8 for legend).

active substance, and MDP-LL, an inactive one, were used. It was reported that an aqueous solution of MDP was removed rapidly from injected sites and within a short time excreted largely into the urine.[22] When given as a w/o emulsion, MDP has been said to remain for a long time in the injected sites. In the present study, MDP was incorporated into w/o/w emulsion containing a minute volume of mineral oil rather than a large one such as that used in Freund's type w/o. A w/o/w emulsion rather than a w/o emulsion was used because the latter induces an intensive, nonspecific cellular reaction as a result of its high volume of oil; also the w/o/w emulsion was expected to minimize nonspecific cellular infiltrations without losing the depot effect of oil. The w/o/w emulsion indeed induced weak erythematous reactions in the local skin 24 hours after injection. The addition of MDP produced no effect on the erythematous reaction in intensity or duration, which corresponded to a zero or weak capacity of MDP to induce dilation of capillaries in the dermis. On the other hand, skin induration in the injected sites continued in all the groups, showing persistence of cellular infiltration in the dermis and hypodermis.

Upon histological examination, there were no indications of toxic effects of MDP such as degeneration of blood vessel walls, haemorrhage or perivascular cell infiltration resulting from the amounts used. Wachsmuth and Dukor[23] reported that prolonged administration of 100 to 1,000 mg/kg of nor-MDP into BALB/c mice showed no notable toxic or pathological findings. In the present experiments, using only 30 μg of MDP, local toxic effect appeared negligible.

Gisler *et al.*[24] found in their histological study of local cellular reaction that intracutaneous injection of guinea pigs with MDP in the form of saline solution caused a temporary accumulation of PMN and MN and their rapid disappearance, whereas MDP in the Freund's type w/o emulsion elicited an augmented and prolonged PMN infiltration. From these results, they considered that long lasting infiltration of granulocytes is a prerequisite to the development of cell-mediated immunity. Furthermore, Ohkawara *et al.*[25] and Goto *et al.*[26] reported that factors which were extracted from infiltrating PMN in the early stage accelerated lymphocyte proliferation. The present study showed that the infiltrated PMN began to degenerate at 72 hours and then decreased in number. In the present study, the PMN infiltration of the adjuvant-inactive

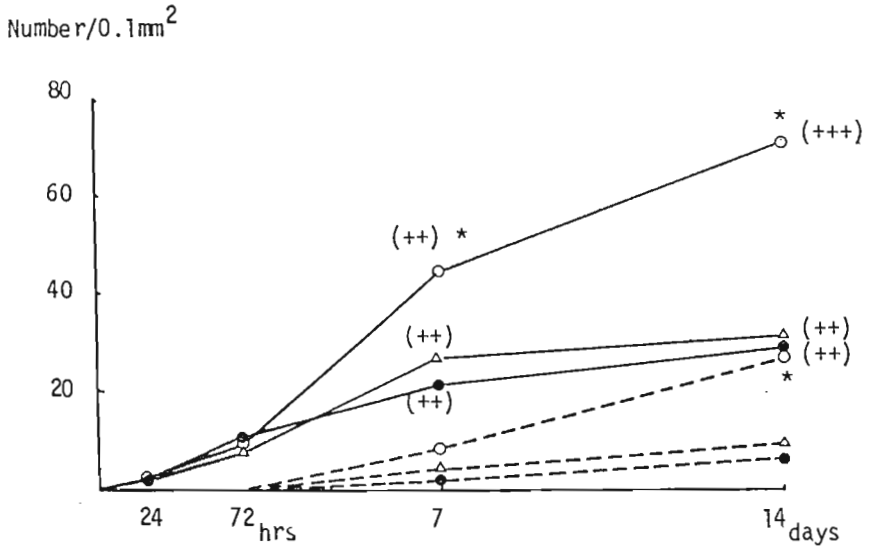

Fig. 10 The number of acid phosphatase-positive cells infiltrated in the dermis. Total number (———) and strong positive cell number (– – –) are shown. The grade in the parentheses shows the intensity of granuloma formation in the dermis. *$p < 0.05$ when compared with the w/o/w group at the same time. (See Fig. 8 for legend).

MDP-LL group showed the lowest number in the three groups at 24 and 72 hours, but the difference in the number compared with that of the w/o/w group was not statistically signifcant. It might be conceivable that PMN infiltration is a typical early reaction of nonspecific defense mechanisms against foreign bodies.

The appearance of large cells showing the features of epithelioid cells was the most conspicuous change in the local skin. These cells appeared from seven days after inoculation, and thereafter continued to accumulate and then form granuloma in both the dermis and hypodermis. Similarly, this change was found in all three groups, although the MDP-LD group showed more intensive infiltration of the cells. These large macrophages and/or epithelioid cells were coloured deeply by acid phosphatase staining and appeared to be in an activated state. Tanaka and Emory[14] found granuloma formation in the regional lymph nodes as a result of injections with adjuvant active-MDP without antigen in the form of w/o; however, this was not found when adjuvant-inactive MDP was used. In the present experiment, we demonstrated granuloma formation in the skin of injected sites with adjuvant-inactive MDP as well as active MDP, and even with w/o/w alone. The reason for the difference in granuloma formation in the regional lymph nodes in Tanaka's experiment and in the skin in our experiment is not clear. But one possibility is that local tissue is effective in producing immunological states, as the subcutaneous route is considered better than the intravenous route for the induction of delayed hypersensitivity. At any rate, adjuvant active MDP-LD in w/o/w produced the most intensive cellular infiltration, macrophage activation and epithelioid cell granuloma formation in the injected sites, thus suggesting that the adjuvant activity of MDP-LD resides in the activation of macrophages within the tissues.

It is well known that the w/o form of adjuvant substances is the most effective form for induction of humoral and cellular immunity. Many oil droplets were seen in the infiltrating macrophages in the collagenous tissue, especially in the hypodermis, throughout the entire period of this experiment. This finding suggests retention of MDP with mineral oil in local tissue. The w/o/w control group showed intensive PMN and MN infiltration, but to a lesser degree than that of the MDP-LD group. It is considered that mineral oil alone, even a small amount such as in w/o/w, might assist the local tissue in making provision for immunological reactions. The long lasting deposits of oil droplets themselves accentuate the yield of immunocompetent cells in the tissues and provide an effective place for the function of adjuvant activity of MDP-LD, the final result of which is production of more extensive macrophage activation and granuloma formation.

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