

Arthritogenic Activity of a Synthetic Immunoadjuvant, Muramyl Dipeptide

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A synthetic muramyl dipeptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, dissolved in saline only and applied subcutaneously to rats of the Lewis inbred strain, produced arthritis, clinically manifest by hind feet paresis but without apparent paw swelling in most cases. Histologically, the disease was characterized by edema and hyperemia of connective tissues, joint synovias, and tendon sheaths, with massive accumulation of inflammatory cell infiltrates composed mainly of lymphoplasmocytes and partly of neutrophil leukocytes. Fibrin exudation and fibrinoid necrosis in connective tissues were observed in the most severe cases. Synovial layers of the talocrural joint, especially on their villi, exhibited marked swelling or cell desquamation of the inner zone. Clinical symptoms of the disease disappeared spontaneously within 5 days after cessation of the treatment; also, histological examinations showed that the effects were well reversible. Our results prove that (i) muramyl dipeptide is the principal substance involved in the production of arthritis, (ii) there is no necessity for the presence of additional mycobacterial cell wall components, and (iii) the involvement of the oil moiety is not requisite for the production of arthritis.

An intensive search for the immunoadjuvant principal of mycobacteria in Freund complete adjuvant (FCA) led to the discovery of a minimal active structure, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) (2, 8). This low-molecular-weight compound was shown to produce experimental allergic encephalomyelitis (4), and recently Kohashi et al. (7) reported that MDP was able, under special conditions, to produce adjuvant polyarthritis in high-responder Lewis rats. However, the effect was strongly dependent on the kind and proportion of oil and emulsifier components of the incomplete adjuvant mixture. In fact, other authors (1, 4) failed to produce adjuvant polyarthritis under different experimental conditions. The role of the oil vehicle remains unclear, but it has been suggested (7) that formation of longer chains of MDP units (dimers or oligomers) could be stimulated in certain water-in-oil emulsions and that these would exert enhanced arthritogenic activity. It can also be supposed, however, that some of these components may act as a depot factor, causing prolongation of the contact of organism with MDP or changes in its pharmacokinetic patterns of elimination. It is known that MDP, injected intravenously or subcutaneously, is very rapidly eliminated in urine in unchanged form (10), but the elimination can be markedly prolonged by the incorporation of MDP into liposomes (I. Havlík, K. Mašek, I. Janků, M. Parant, and L. Chedid, *Int. J. Immunopharma-*

col., in press) or by its water-in-oil administration (10).

In this paper we have attempted, therefore, to find out whether or not MDP alone, dissolved in saline only, exerts its arthritogenic activity when rats are treated repeatedly. This approach was substantiated by the fact that MDP itself, without the oil moiety, proved to possess many biological activities (3, 14, 15).

MATERIALS AND METHODS

Animals. Conventional female Lewis rats (inbred strain LEW/CUB) and females of the AVN inbred strain (obtained from the Institute of Physiology, Czechoslovak Academy of Sciences, Prague), weighing 150 to 200 g at the beginning of the experiment, were used. The number of animals used is given in Table 1. The Lewis inbred strain was previously found to be extremely sensitive to the production of arthritis after intradermal injection of FCA, whereas the AVN inbred strain was highly resistant (16).

Administration of MDP. A synthetic compound, kindly supplied by M. Flegl (SPOFA, Prague), was dissolved in saline and daily applied subcutaneously into the neck area for intervals lasting from 9 to 32 days at doses of 0.1 to 2.0 mg per rat (see Table 1), always in volume of 0.5 ml.

Observation of animals and histological examinations. Animals were examined twice a day, at the time of administration and 6 h later, for signs of arthritis or any other effects.

Histological examinations were performed on randomly chosen animals at different stages of MDP administration (Table 1). Paws, talocrural joint, brain,

TABLE 1. Scheme of MDP administration, histology performed, and experimental findings of arthritis occurrence

Daily dosage regimen	Inbred strain (n)	No. of responders/ total	Minimal effective no. of dosages	Histology scheme
Controls (physiological saline soln; 0.5 ml, 31 days)	LEW (5)	0/5		1 rat after 31 doses
2 mg, 31 days	LEW (8)	8/8	7-8	2 rats after 22 doses 1 rat after 31 doses
1 mg, 31 days	LEW (7)	7/7	8-12	1 rat after 9 doses 1 rat after 31 doses 1 rat 28 days after termination of treatment
0.5 mg, 31 days	LEW (4)	4/4	20-21	
0.1 mg, 32 days	LEW (6)	0/6		1 rat after 32 doses
1 mg, 32 days	AVN (5)	0/5		1 rat after 32 doses

spinal cord, axillar and abdominal lymph nodes, spleen, thymus, bone marrow, liver, lungs, heart, jejunum, colon, ovaria, and eye (after 1 mg of MDP for 31 days) were examined. Tissues were fixed in AAF solution (Lillie) except central nervous system tissue, which was fixed in 5% buffered formaldehyde, and embedded in paraffin wax. Cut sections (5 to 8 μ m thick) were stained with hematoxylin and eosin, Trichrom (Masson), or periodic acid-Schiff or by the myelin-staining method (Lillie).

RESULTS

Description of clinical findings. Striking changes in the walking of animals, which could be simply described as pareses of both hind feet, were observed after repeated subcutaneous administration of MDP in saline. The occurrence of this effect was clearly dose dependent (Table 1): the shortest period necessary to produce the effect was 7 to 8 days with a dose of 2 mg of MDP; it was nearly the same for a dose of 1 mg, but it took about 20 to 21 days before any signs of impaired walking were noted with the 0.5-mg dose. The 0.1-mg dose of MDP remained ineffective even after 32 daily doses were applied. The severity of clinical symptoms tended to increase with increasing number of doses, especially with the highest dose of MDP used. Towards the end of the administration period, the animals were lying feebly on their backs or sides, apparently with no tendency to move freely about the cage. No obvious changes in the size of paws were seen, however, except in severe cases after the 2-mg application when mild edema of both hind paws and forepaws was produced. Slight body weight reduction was observed only in this group, the weight dropping from the preapplication value of 154 ± 6.8 g to 142.0 ± 3.4 g ($n = 5$) on day 18 of application (average $\pm 5\%$ limits of confidence). In the group given 1.0 mg of MDP ($n = 4$), the preapplication weight was 155.0 ± 13.0 g; on day 18 it

was 173.8 ± 27.1 g and was increasing steadily up to the end of application (31 days), at which time it was 181.3 ± 24.6 g. The most prominent sign of the disease (halting) disappeared spontaneously within 4 to 5 days after termination of the treatment, even after high doses of MDP.

The effect was found in the Lewis inbred strain only; the AVN inbred strain rats were not afflicted, even though animals were treated daily for as long as 32 days with a 1.0-mg dose.

Histological studies of feet (paw and talocrural joint). Connective tissues of the paw, synovia of intermetatarsal joints, parts of joint capsules, and tendon sheaths showed hyperemia, edema, and the presence of massive inflammatory infiltrates in which a preponderance of lymphoplasmocytes was typical. In some parts of infiltrated sites, a substantially increased amount of neutrophils was present. Fibrin exudation and fibrinoid necrosis were occasionally found within connective tissues. The talocrural joint showed irregular hyperemia, edema, and more or less dense foci of round cellular inflammatory infiltrates, with a preponderance of lymphocytes. The synovial layer, especially on its villi, exhibited swelling and even desquamation of cells of the inner zone (Fig. 1, 2, and 3). A similar picture was traced as early as day 9 of daily repeated application of the 1.0-mg dose of MDP, i.e., at the time of the earliest emergence of clinical signs of the disease. These alterations were transient, however, as shown by histological analysis performed on day 28 subsequent to the termination of MDP treatment lasting for 31 days at a dose of 1.0 mg daily; with the exception of a mild abundance of connective tissues of the joint capsule, no difference was detected compared with controls (Fig. 4).

In accordance with clinical observations, MDP proved to have no effect histologically at the lowest dose used (0.1 mg daily for 32 days)

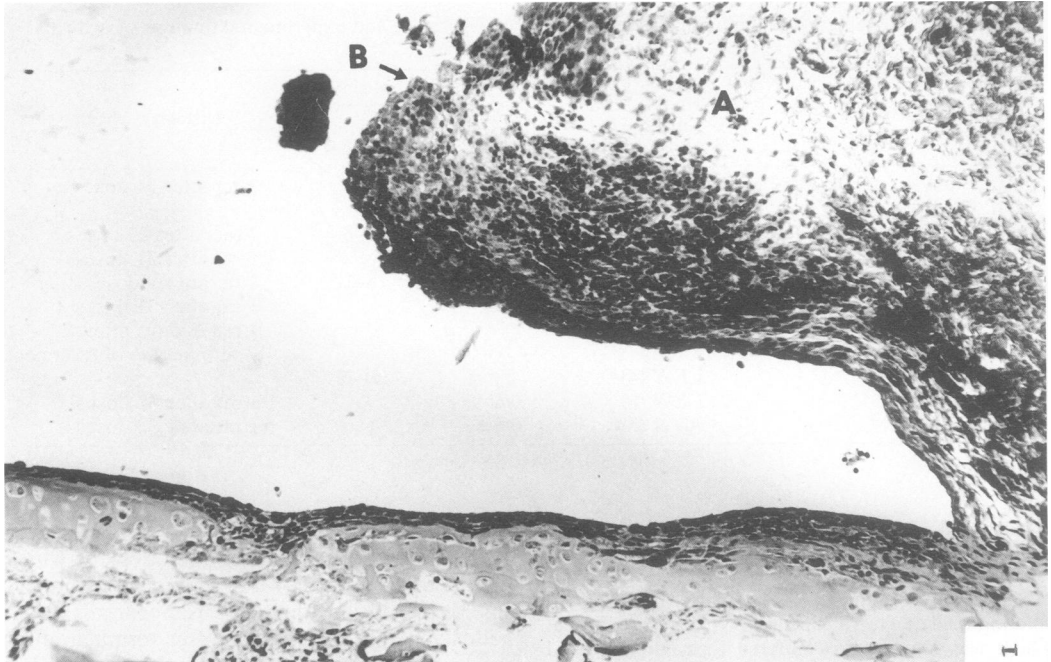


FIG. 1. Talocrural joint from a rat on day 22 of subcutaneous (neck area) daily treatment with a saline solution of MDP (2.0 mg/rat per day). Stained with hematoxylin and eosin; bar, 10 μ m. Synovial villus with marked edema and mononuclear inflammatory infiltration (A) is shown. Note the swelling and desquamation of synovial cells of the inner zone (B).

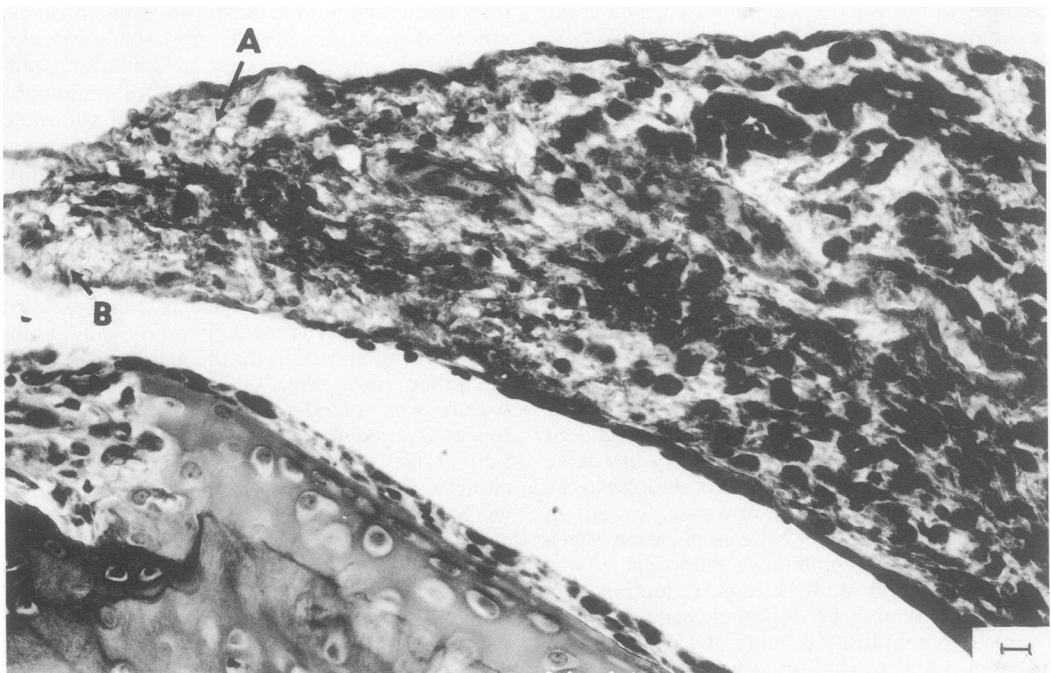


FIG. 2. Intermetatarsal joint from a rat on day 22 of subcutaneous (neck area) daily treatment with a saline solution of MDP (2.0 mg/rat per day). Stained with hematoxylin and eosin; bar, 10 μ m. Synovial villus with marked hyperemia and edema is shown: fibrin exudation (A) and fibrinoid necrosis (B).

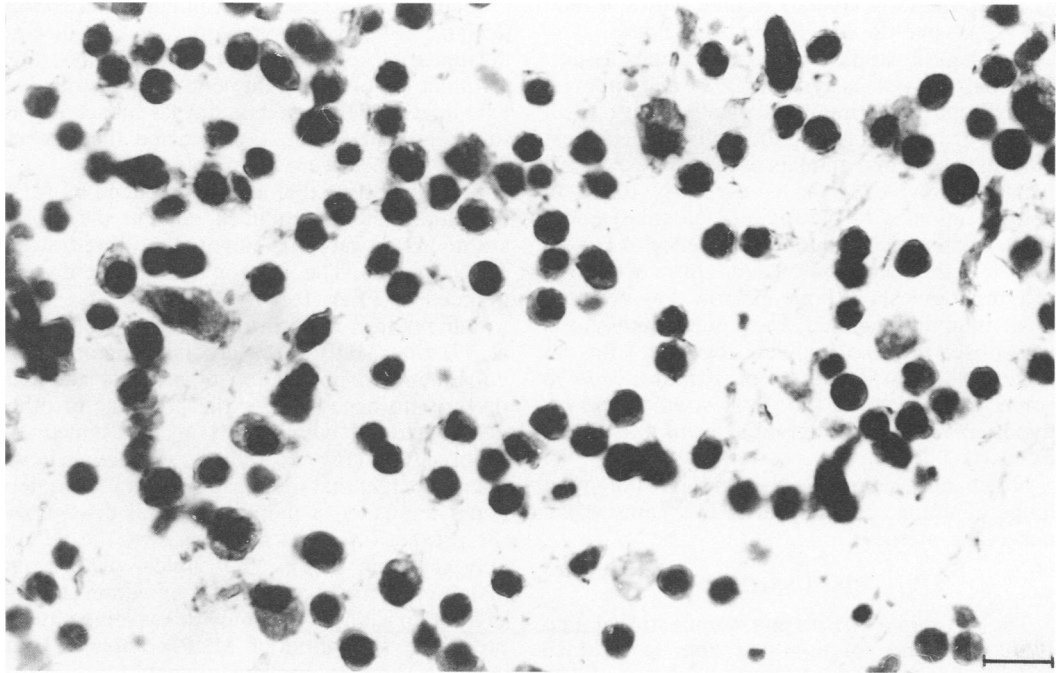


FIG. 3. Intermuscular connective tissue of the sole of the hind paw from a rat on day 22 of subcutaneous (neck area) daily treatment with a saline solution of MDP (2.0 mg/rat per day). Stained with hematoxylin and eosin; bar, 10 μ m. Inflammatory infiltrate consisting largely of lymphocytes and plasmocytes with a small amount of neutrophils is shown.



FIG. 4. Talocrural joint from a control rat. Stained with hematoxylin and eosin; bar, 10 μ m.

or in strain AVN animals treated with the relatively massive dose of 1.0 mg for 32 days.

Histological studies of extraarticular tissues. Spleens, as well as lymph nodes and thymus, were found to be immunologically active in all treated groups, including those given the lowest dose of 0.1 mg and animals of the nonresponsive inbred strain AVN, as is currently found in control animals. In the thymus, an enlargement of the cortex and frequent occurrence of Hassal bodies in the medula were seen. Mild activation of lymphopoiesis in bone marrow was detected in all animals examined, and a mild leukocytosis was observed also in liver, together with enlargement and activation of Kupffer cells in some rats, including the AVN strain. Irregular hyperemia of small interstitial veins was found in hearts.

No changes were observed in the histology of lungs, jejunum, colon, ovaria, eye, and central nervous system.

DISCUSSION

The present experiments demonstrated that MDP in saline solution was able to produce arthritis in rats of the Lewis inbred strain. The most prominent clinical observation was hind feet paresis, which was not accompanied by paw swelling in most cases. The histological findings could be summarized as mild edema and hyperemia of connective tissues, joint synovias, and tendon sheaths, with massive accumulation of inflammatory cell infiltrates composed mainly of lymphoplasmocytes and partly of neutrophil leukocytes. Connective tissues showed fibrin exudation and fibrinoid necrosis in most severe cases. Synovial layers of the talocrural joints, especially on the villi, exhibited swelling and even cell desquamation of the inner zone. On the basis of these findings, the changes can be classified as arthritis.

To a certain extent our results are in agreement with the findings reported by Kohashi et al. (7), who, however, administered MDP in a water-in-oil emulsion and found arthritis comparable to that produced by FCA (11, 12). However, in our experimental design, using MDP in saline only, we did not find all of the alterations typical for polyarthritis produced by FCA, namely, involvement of skeletal muscle, destruction of subchondral bone, osteoblastic activity of periosteum, or presence of mastocytes. Also, the clinical manifestation differed from that of FCA-induced arthritis, which characteristically is accompanied by extensive swelling of feet. In our studies the swelling was minimal and easily overlooked. Nor did we observe the marked reduction of total body weight or urethritis, balanitis, and diarrhea which are a prominent features of the FCA-induced disease.

It should be stressed that in our experimental design the clinical symptoms of the disease disappeared spontaneously within 5 days after termination of the treatment, and histological examination performed on day 28 subsequent to the treatment termination showed that the effects described were reversible.

It is interesting that arthritis induced by MDP in saline can be produced only in the inbred strain Lewis rats and not in the inbred strain AVN animals. The same is true for the disease induced by FCA (16).

Our findings confirm the view of Kohashi et al. (7) that MDP is the crucial component involved in the production of arthritis and that there is no necessity for the presence of other mycobacterial components as presumed by Wahl et al. (15). Moreover, we have shown unequivocally that the presence of the oil moiety is not requisite for induction of this disease. At the present time it is not clear why the oil and detergent can modify the clinical picture and histological findings found after administration of MDP in saline. This could be explained by the presumed formation of MDP complexes (micelle) in Difco incomplete adjuvant (4, 6, 7, 13), which may possess more pronounced arthritogenicity, at least as far as the severity of the disease is concerned. It cannot be excluded, however, that certain water-in-oil emulsions of MDP have some bearing on the pharmacokinetic behavior of MDP or on the formation of its tissue deposits. The critical factor for production of arthritis by MDP in saline is not only the overall dose injected, but also the time interval. It is known that most of the injected compound is very rapidly eliminated from the body; nevertheless, a small fraction is suspected to be present for a longer period of time (10; Havlík et al. in press). Experiments with an immunofluorescent compound which could clear up this question are in progress.

The exact mechanism of MDP-induced arthritis is not clear so far (9) and will require more detailed studies. The possible important role of the thymus in promoting the development of MDP-induced arthritis has been suggested recently by Kohashi et al. (5). It is noteworthy that in our present experiment, as well as in a number of previous ones in which MDP was given repeatedly in saline, constant enlargement of the thymus cortex was observed (K. Mašek, lecture, Int. Symp. Potent. Immune Respir. Vaccines, National Institutes of Health, Bethesda, Md., 1979).

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