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Muramyl Dipeptide-Induced Adjuvant Arthritis

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Muramyl dipeptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, induced adjuvant arthritis in WKA rats when injected in a water-in-oil emulsion prepared with Freund incomplete adjuvant (Difco), but not when emulsified with Drackeol and Arlacel A.

In 1956, a single intracutaneous injection of heat-killed tubercle bacilli incorporated in a water-in-oil emulsion was reported to induce an arthritis in rats which is now called adjuvant arthritis (15). Since then, mycobacterial wax D, bacterial cell walls of different species, their peptidoglycans, and fragments of the peptidoglycans were all found to be arthritogenic (2, 6, 8, 9, 14). Recently, among the peptidoglycan fragments tested, disaccharide-heptapeptide-disaccharide (*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanyl-*meso*-diaminopimelyl-D-isoglutaminyl-L-alanyl-*N*-acetylmuramyl-*N*-acetylglucosamine) was found to be the smallest arthritogenic structure (7, 12). Structures smaller than this, for instance, synthetic muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine), and products of enzymatic cleavage of glycan chains (-GlcNAc-MurNAc-) of peptidoglycans were not arthritogenic, though both MDP and the enzymic cleavage products were still active as adjuvants (6, 7, 11). For these reasons, it was thought that two or more disaccharide units were needed for the induction of adjuvant arthritis and the minimal structure necessary for adjuvant activity was not sufficient.

The present study shows that synthetic MDP can induce severe arthritis in rats. Female WKA rats, 10 weeks of age, from the Institute for Experimental Animals, Kyushu University, Fukuoka, Japan, were injected intracutaneously into left hind footpads with 0.1 ml of a water-in-oil emulsion containing 0.1 mg of MDP. To prepare the emulsion, we emulsified a phosphate-buffered saline (PBS), pH 7.2, containing MDP at a concentration of 2 mg/ml with an equal volume of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich., lot no. 636671 and 640223) using a sonicator (Sonifier, model W-200P, Branson Instruments Co., Stamford, Conn.). Animals were observed daily for 4 weeks after injection, and the degree of developed lesions and the days of onset were evaluated as described previously (26).

Arthritis occurred in about 50% of the rats injected with MDP (Table 1). Some of them showed particularly severe arthritis (Fig. 1). The ankle, tarsal, metatarsal, and phalangeal joints of all the legs were involved, and the tails were beaded. The day of onset was between 16 and 21 days. Rats injected with the emulsions containing no MDP or MDP with ovalbumin (100 μg) failed to develop arthritis. The site of MDP injection of rats that developed arthritis showed a marked swelling that became manifest about 1 week after injection. A correlation between the swelling and the development of polyarthritis was statistically significant (correlation coefficient, 0.9; $P < 0.005$). However, no correlation appeared to exist between the development of adjuvant arthritis and the weight of draining lymph nodes, which was shown to reflect the extent of granuloma formation by MDP (5). Rats developing adjuvant arthritis showed negative skin reactions when skin was tested with MDP (100 μg) 3 weeks after the injection.

We reported previously that MDP was active as an adjuvant in WKA rats (21). Though it has been suggested that the structure of MDP may not be sufficient for the induction of adjuvant arthritis (7, 12), the present study clearly shows that MDP did induce adjuvant arthritis. This finding may be of importance because of the following two points. First, it suggests that any attempts to use MDP with humans should be done cautiously. It was reported that 6% of the patients who had received *Mycobacterium bovis* BCG immunotherapy developed an arthritis which was thought to be adjuvant arthritis (22).

Second, it may contribute to our understanding of the pathogenesis of adjuvant arthritis. It appears certain that adjuvant arthritis develops as a result of some immunological response, probably of the delayed type (16, 25). However, responsible antigens are still obscure (3, 17, 19, 23, 25). No definite immunogenicity has been so far detected in MDP (1, 5). This was confirmed in the present study; MDP did not sensitize rats against MDP (Table 1). Therefore, although the question of whether MDP in some way can

TABLE 1. *Induction of adjuvant arthritis by MDP*[a]

Expt no.	Inoculum	Incidence	Skin reaction at 24 h[b]	Hind footpad thickness (mm; mean ± SD)		Wt of lymph node (mg; mean ± SD)	Arthritis score	Onset (days)
				Left	Right			
1	None	0/5	ND	4.9 ± 0.4	4.3 ± 0.4	ND	0	
	MDP	3/7		5.0	4.5		0	18–21
				5.0	4.5		0	
				5.5	5.0		0	
				8.0	4.5		0	
				8.5	4.0		6	
				9.5	6.5		10	
				10.0	7.0		10	
				7.4 ± 2.0	5.1 ± 1.1			
	MDP + ovalbumin	0/4		6.5 ± 0.9	4.9 ± 0.3		0	
2	None	0/5	2.0 ± 0.1	4.7 ± 0.4	4.3 ± 0.2	77 ± 13	0	
	MDP	3/5	2.1 ± 0.1	5.5	4.0	93	0	16–18
				5.5	4.5	114	0	
				6.5	4.8	87	8	
				11.0	9.0	149	14	
				11.0	7.0	130	13	
				7.9 ± 2.9	5.9 ± 2.1	115 ± 26		
	MDP + ovalbumin	0/4	4.1 ± 0.6	7.3 ± 1.2	4.4 ± 0.3	129 ± 20		

[a] Rats were injected with each inoculum into the left hind footpads. Freund incomplete adjuvant (Difco lot no. 636671) was used to prepare the inocula. The amount of MDP or ovalbumin was 100 μg. Three weeks later, the rats were skin tested either with 100 μg of MDP (control and MDP groups) or with 100 μg of ovalbumin (MDP + ovalbumin group), the thickness of the hind footpads was measured, the degree of polyarthritis was scored, and the weight of draining lymph nodes was weighed after sacrifice. For the MDP groups, the values of measurements of each rat were also shown. ND, Not determined; SD, standard deviation.

[b] Double skin thickness was measured. Numbers indicate mean thickness ± standard deviation in millimeters.

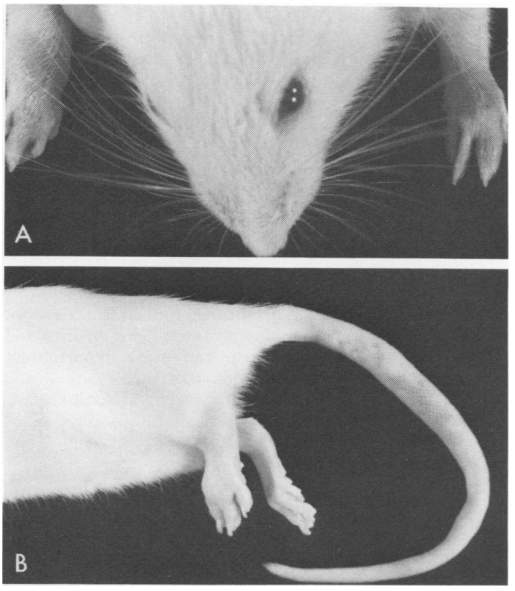

FIG. 1. *(A) Diffuse swelling of both digits and carpal regions. (B) Swelling and nodulation of the tail.*

become antigenic or even immunogenic in vivo still remains to be further studied, we are at the moment inclined to ascribe the mechanism of adjuvant arthritis induction not to the immunogenicity of MDP itself but to some self-antigen(s) generated by an action of MDP or to activities of MDP other than antigenicity (5, 13, 18, 20). Also, the possibility that MDP may induce virus infection cannot be excluded (4).

It is well known that the development of adjuvant arthritis is dependent on oil vehicles in which arthritogenic substances are injected (10, 24). This was confirmed in the present study (Table 2). Adjuvant arthritis was induced only when MDP-containing PBS was emulsified with an equal volume of Freund incomplete adjuvant (Difco) (Arlacel A-Bayol F, 8.5:1.5). A similar Freund-type emulsion prepared by mixing MDP-containing PBS with an equal volume of the mixture of Arlacel A (Atlas Powder Co., Wilmington, Del.) and Drackeol 6-VR (Penreco, Los Angeles, Calif.) (8.5:1.5) did not cause adjuvant arthritis (Table 2). This result contrasts with granuloma formation by MDP; both of the

TABLE 2. *Effect of oil vehicle on the induction of adjuvant arthritis by MDP*[a]

Oil vehicle	Inoculum	Incidence	Mean score	Onset (days)
Arlacel A-Drackeol (8.5:1.5)	MDP (100 μg)	0/26	0	
Incomplete Freund adjuvant (Difco)	None	0/4	0	
	MDP (100 μg)	5/6	10	18–25

[a] Rats were injected with inocula into left hind footpads. Freund incomplete adjuvant (Difco lot no. 640223 and 640223) was used to prepare the inocula.

above oil vehicles were effective to help MDP in granuloma formation (5). We cannot explain the reason why the use of the latter oil emulsion did not induce arthritis.

LITERATURE CITED

1. **Audibert, F., B. Heymer, C. Gros, K. H. Schleifer, R. H. Seidl, and L. Chedid.** 1978. Absence of binding of MDP, a synthetic immunoadjuvant, to anti-peptidoglycan antibodies. J. Immunol. **121**:1219–1222.
2. **Azuma, I., F. Kanetzuna, Y. Kada, T. Takashima, and Y. Yamamura.** 1972. Adjuvant-polyarthritogenicity of cell walls of Mycobacteria, Nocardia and Corynebacteria. Jpn. J. Microbiol. **16**:333–336.
3. **Berry, H., R. Willoughby, and J. P. Giroud.** 1973. Evidence for an endogenous antigen in the adjuvant arthritic rat. J. Pathol. **111**:229–238.
4. **Chang, Y., and C. M. Pearson.** 1978. Pathogenesis of adjuvant arthritis in rats. Arthritis Rheum. **21**:169–170.
5. **Emori, K., and A. Tanaka.** 1978. Granuloma formation by synthetic bacterial cell wall fragment: muramyl dipeptide. Infect. Immun. **19**:613–620.
6. **Koga, T., S. Kotani, T. Narita, and C. M. Pearson.** 1976. Induction of adjuvant arthritis in the rat by various bacterial cell walls and their water-soluble components. Int. Arch. Allergy Appl. Immunol. **51**:206–213.
7. **Koga, T., K. Maeda, K. Onoue, K. Kato, and S. Kotani.** 1979. Chemical structure required for immunoadjuvant and arthritogenic activities of cell wall peptidoglycans. Mol. Immunol. **16**:153–162.
8. **Koga, T., and C. M. Pearson.** 1973. Immunogenicity and arthritogenicity in the rat of an antigen from *Mycobacterium tuberculosis* wax D. J. Immunol. **111**:599–608.
9. **Koga, T., C. M. Pearson, T. Narita, and S. Kotani.** 1973. Polyarthritis induced in the rat with cell walls from several bacteria and two streptomyces species. Proc. Soc. Exp. Biol. Med. **143**:824–827.
10. **Kohashi, O., C. M. Pearson, F. J. W. Beck, and M. Alexander.** 1977. Effect of oil composition on both adjuvant-induced arthritis and delayed hypersensitivity to purified protein derivative and peptidoglycans in various rat strains. Infect. Immun. **17**:244–249.
11. **Kohashi, O., C. M. Pearson, Y. Watanabe, and S. Kotani.** 1977. Preparation of arthritogenic hydrosoluble peptidoglycans from both arthritogenic and nonarthritogenic bacterial cell walls. Infect. Immun. **16**: 861–866.
12. **Kohashi, O., C. M. Pearson, Y. Watanabe, S. Kotani, and T. Koga.** 1976. Structural requirements for arthritogenicity of peptidoglycans from *Staphylococcus aureus* and *Lactobacillus plantarum* and analogous synthetic compounds. J. Immunol. **116**:1635–1639.
13. **Nagao, S., A. Tanaka, Y. Yamamoto, T. Koga, K. Onoue, T. Shiba, S. Kusumoto, and S. Kotani.** 1979. Inhibition of macrophage migration by muramyl peptides. Infect. Immun. **24**:308–312.
14. **Paronetto, F.** 1970. Adjuvant arthritis by Corynebacterium rubrum. Proc. Soc. Exp. Biol. Med. **133**:296–298.
15. **Pearson, C. M.** 1956. Development of arthritis, periorthritis and periostitis in rats given adjuvant. Proc. Soc. Exp. Biol. Med. **91**:95–101.
16. **Pearson, C. M., and F. D. Wood.** 1964. Passive transfer of adjuvant arthritis by lymph node or spleen cells. J. Exp. Med. **120**:547–560.
17. **Quagliata, F., and J. M. Phillips-Quagliata.** 1972. Competence of thoracic duct cells in the transfer of adjuvant disease and delayed hypersensitivity. Evidence that mycobacterial components are required for the successful transfer of the disease. Cell. Immunol. **3**: 78–87.
18. **Staber, F. G., R. H. Gisler, G. Schumann, L. Tarcsay, E. Schläfli, and P. Dukor.** 1978. Modulation of myelopoiesis by different bacterial cell wall components: induction of colony-stimulating activity (by pure preparations, low molecular-weight degradation products, and a synthetic low-molecular analog of bacterial cell-wall components) in vitro. Cell. Immunol. **27**:174–187.
19. **Steffen, C., and G. Wick.** 1971. Delayed hypersensitivity reaction to collagen in rats with adjuvant-induced arthritis. Z. Immunitaetsforsch. Exp. Ther. **141**:169–180.
20. **Tanaka, A., S. Nagao, R. Nagao, S. Kotani, T. Shiba, and S. Kusumoto.** 1979. Stimulation of the reticuloendothelial system of mice by muramyl dipeptide. Infect. Immun. **24**:302–307.
21. **Tanaka, A., R. Saito, K. Sugiyama, I. Morisaki, S. Katani, S. Kusumoto, and T. Shiba.** 1977. Adjuvant activity of synthetic *N*-acetylmuramyl peptides in rats. Infect. Immun. **15**:332–334.
22. **Torisu, M., T. Miyahara, N. Shinohara, K. Ohsato, and H. Sonozaki.** 1978. A new side effect of BCG immunotherapy-BCG-induced arthritis in man. Cancer Immunol. Immunother. **5**:77–83.
23. **Waksman, B. H., C. M. Pearson, and J. T. Sharp.** 1960. Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. J. Immunol. **85**:403–417.
24. **Whitehouse, M. W., K. J. Orr, F. J. W. Beck, and C. M. Pearson.** 1974. Freund's adjuvant: relationship of arthritogenicity and adjuvanticity in rats to vehicle composition. Immunology **27**:311–330.
25. **Whitehouse, D., M. Whitehouse, and C. M. Pearson.** 1969. Passive transfer of adjuvant-induced arthritis and allergic encephalomyelitis in rats using thoracic duct lymphocytes. Nature (London) **224**:1322.
26. **Wood, F. D., C. M. Pearson, and A. Tanaka.** 1967. Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats. Int. Arch. Appl. Immunol. Allergy **35**:456–467.