INFECTION AND IMMUNITY, Jan. 1977, p. 332-334

Vol. 15, No. 1
Printed in U.S.A.

Adjuvant Activity of Synthetic *N*-Acetylmuramyl Peptides in Rats

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Received for publication 14 July 1976

The minimal adjuvant-active structure in bacterial cell wall peptidoglycan for the induction of delayed hypersensitivity in rats (WKA) was the *N*-acetylmuramyl dipeptides (*N*-acetylmuramyl L-alanyl-D-isoglutamine and *N*-acetylmuramyl L-alanyl-D-glutamic acid).

Marked progress was recently made in the knowledge of chemical structures required for the immunological adjuvant effect of bacterial cell walls. A minimal effective structure for the adjuvant effect of inducing delayed hypersensitivity and enhancing antibody production was shown to be *N*-acetylmuramyl L-alanyl-D-isoglutamine (MurNAc-LAla-DIsoGln), when tested in a Freund-type emulsion in guinea pigs (2, 4, 7). In mice, MurNAc-LAla-DIsoGln was reported to enhance antibody production when given as a solution in saline (1) or as the Freund-type emulsion (7).

We have recently observed that the effectiveness of a mycobacterial trehalose lipid adjuvant, cord factor, was dependent upon the animal species used; cord factor was active as an adjuvant in mice (CFl and C57BL) and rats (SD) but inactive in guinea pigs (Hartley) when applied as the Freund-type emulsion (6). This observation prompted us to test the various synthetic cell wall muramyl peptides for their adjuvant activity in rats.

N-acetylmuramyl peptides were synthesized as described previously (5). To prepare the Freund-type emulsion, a mixture of Drakeol 6-VR and Arlacel A(4:1) was added to an equal volume of saline containing egg albumin and the muramyl peptides. When tubercle bacilli were used, they were suspended in Drakeol 6-VR before emulsification. Three-month-old WKA female rats (Institute for Experimental Animals, Kyushu University) were injected intradermally in the right hind footpad with 0.1 ml of the emulsion containing 0.1 mg of egg albumin and 0.1 mg of test materials.

Rats were tested by skin reaction 14 days after immunization. The skin test was performed on the flank by intradermal injection of 50 μg of antigen dissolved in 0.1 ml of saline. Double skin thickness of the test sites was measured at 4, 24, and 48 h.

Results are shown in Tables 1 to 4. MurNAc-LAla-DIsoGln caused a significantly greater induration of skin test sites than a control group at 24 and 48 h (Tables 1 and 2). In the other three experiments performed under the same conditions, this muramyl dipeptide caused similar delayed skin reactions (data not shown).

Kotani et al. found that *N*-acetylmuramyl-L-alanyl-D-glutamic acid (MurNAc-LAla-DGlu) was only weakly active in guinea pigs (4), whereas Audibert et al. found it to be very active in mice (1), although the latter investigators only measured antibody production. In the present study, it was found to be active in rats to about the same degree as MurNAc-LAla-D-IsoGln (Table 3). The increase in double skin thickness caused by MurNAc-LAla-DIsoGln and MurNAc-LAla-DGlu, as well as by tubercle bacilli, was usually not statistically significant at 4 h but was significant at 24 and 48 h (Tables 1 to 4). Corneal tests performed at the same time gave results essentially parallel to those of skin tests. The techniques and results of the corneal test will be published elsewhere. These results indicate that the muramyl dipeptides (MurNAc-LAla-DIsoGln and MurNAc-LAla-DGlu) caused the induction of delayed hypersensitivity in rats (WKA).

Concerning the adjuvant activity of the tetrapeptide LAla-DIsoGln-LLys-DAla to induce a delayed hypersensitivity, there is a discrepancy among investigators using guinea pigs; Lederer et al., Kotani et al., and Yamamura et al. found the tetrapeptide to be inactive (2, 4, 7), whereas Fleck et al. found it to be active (3). The present study showed that the tetrapeptide was totally inactive in rats (Table 4). MurNAc-LAla and MurNAc-LAla-L-IsoGln were both in-

TABLE 1. *Adjuvant activity of cord factor, tubercle bacilli, and N-acetylmuramyl-L-alanyl-D-isoglutamine*

Test substance (100 μg)	No. of animals	Skin reaction (mm, mean ± SD)[a]			
		Double skin thickness[b]			Erythema (24 h)
		4 h	24 h	48 h	
None	7	3.1 ± 0.2	3.0 ± 0.1	3.0 ± 0.1	0
Cord factor	6	3.6 ± 0.6	5.8 ± 1.6 ($P < 0.005$)	5.1 ± 1.6 ($P < 0.01$)	12.9 ± 1.7
Tubercle bacilli	8	4.3 ± 0.7 ($P < 0.005$)	6.9 ± 0.8 ($P < 0.001$)	5.8 ± 1.2 ($P < 0.001$)	13.3 ± 1.2
MurNAc-LAla-DIsoGln	7	4.0 ± 0.5 ($P < 0.005$)	5.1 ± 1.0 ($P < 0.001$)	4.3 ± 1.1 ($P < 0.025$)	9.0 ± 2.2

[a] SD, Standard deviation.
[b] Statistical evaluation was performed using Student's *t* test.

TABLE 2. *Adjuvant activity of various synthetic N-acetylmuramyl peptides*

Test substance (100 μg)	No. of animals	Double skin thickness (mm, mean ± SD)[a]		
		4 h	24 h	48 h
None	5	6.0 ± 0.9	5.0 ± 0.4	3.9 ± 0.4
Tubercle bacilli	5	6.2 ± 0.7	6.3 ± 0.7 ($P < 0.02$)	4.5 ± 0.4
MurNAc-LAla-DIsoGln	5	6.9 ± 0.5	7.5 ± 0.4 ($P < 0.001$)	7.3 ± 1.3 ($P < 0.001$)
LAla-DIsoGln	5	6.5 ± 0.5	4.4 ± 0.4	3.6 ± 0.5

[a] Statistical evaluation was performed using Student's *t* test. SD, Standard deviation.

TABLE 3. *Adjuvant activity of various synthetic N-acetylmuramyl peptides*

Test substance (100 μg)	No. of animals	Double skin thickness (mm, mean ± SD)[a]		
		4 h	24 h	48 h
None	4	4.4 ± 0.5	4.1 ± 0.8	3.1 ± 0.2
Tubercle bacilli	4	4.8 ± 0.8	6.4 ± 0.8 ($P < 0.02$)	5.0 ± 0.7 ($P < 0.01$)
MurNac-LAla-DGlu	5	4.9 ± 0.1	6.9 ± 1.2 ($P < 0.01$)	5.6 ± 1.5 ($P < 0.05$)
MurNAc-LAla-DGln	5	4.8 ± 0.7	5.2 ± 1.1	4.1 ± 1.0
MurNAc-LAla-LGln	5	4.6 ± 0.4	4.1 ± 0.7	3.4 ± 0.4
MurNAc-LAla-DIsoAsn	5	5.1 ± 0.2 ($P < 0.05$)	5.1 ± 0.5	3.9 ± 0.2

[a] Statistical evaluation was performed using Student's *t* test. SD, Standard deviation

TABLE 4. *Adjuvant activity of various synthetic N-acetylmuramyl peptides*

Test substance (100 μg)	No. of animals	Double skin thickness (mm, mean ± SD)[a]		
		4 h	24 h	48 h
None	4	4.7 ± 0.5	3.9 ± 0.2	3.8 ± 0.4
Tubercle bacilli	4	4.3 ± 0.2	5.9 ± 0.6 ($P < 0.001$)	5.5 ± 1.1 ($P < 0.05$)
LAla-DIsoGln-LLys-DAla	6	4.6 ± 0.5	4.5 ± 0.6	3.6 ± 0.5
MurNAc-LAla	6	3.9 ± 0.3	3.7 ± 0.4	3.3 ± 0.3
MurNAc-LAla-LIsoGln	6	4.5 ± 0.5	4.2 ± 0.3	3.6 ± 0.4

[a] Statistical evaluation was performed using Student's *t* test. SD, Standard deviation.

active, suggesting the importance of the presence and D-configuration of isoglutamine or glutamic acid adjacent to L-alanine for the activity (Table 4). Also, when D-isoglutamine in the active muramyl dipeptide MurNAc-LAla-DIsoGln was replaced by D-glutamine or D-isoasparagine, the resulting muramyl dipeptides caused no delayed skin reaction (Table 3).

From the data presented, it could be concluded that the minimal adjuvant-active structure in cell wall peptidoglycan for the induction of delayed hypersensitivity in WKA rats was the muramyl dipeptides (MurNAc-L-Ala-IsoGln and MurNAc-L-ALA-D-Glu).

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