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Author(s)	Takada, Haruhiko; Kotani, Shozo; Kusumoto, Shoichi et al.
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## SHORT COMMUNICATION

MITOGENIC ACTIVITY OF ADJUVANT-ACTIVE *N*-ACETYLMURAMYL-L-ALANYL-D-ISOGLUTAMINE AND ITS ANALOGUES

HARUHIKO TAKADA and SHOZO KOTANI

Department of Microbiology, Osaka University Dental School, Joan-cho, Kita-ku, Osaka

SHOICHI KUSUMOTO, YUZO TARUMI, KAZUHIRO IKENAKA and TETSUO SHIBA

Faculty of Science, Osaka University, Machikaneyama-cho, Toyonaka, Osaka

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It has been reported that cell walls or their peptidoglycans isolated from *Bacillus megaterium* and *Escherichia coli* (Damais et al., 1975), *Listeria monocytogenes* (Cohen et al., 1975), *Nocardia rubra* (Ciorbaru et al., 1976), those from various bacterial species belonging to *Mycobacterium*, *Nocardia*, *Corynebacterium* and anaerobic coryneforms (Azuma et al., 1976a) and a peptidoglycolipid from mycobacterial cell walls (Rook and Stewart-Tull, 1976) significantly stimulated the DNA synthesis in mouse or rabbit lymphoid cells as a mitogen. More recently, experiments in our laboratory have also demonstrated that cell walls and/or peptidoglycans from *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus mutans*, *Lactobacillus plantarum* and *Actinomyces viscosus* definitely enhanced the incorporation of [<sup>3</sup>H] thymidine by spleen cells of ICR mice and guinea pigs (Takada et al., 1977).

In the study reported here, a synthetic cell wall peptidoglycan subunit, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, and its analogues were tested for their mitogenic effect on spleen lymphocytes from mice and guinea pigs, to

elucidate the minimum chemical structure essential to the mitogenic or blastogenic activity of bacterial cell wall peptidoglycan.

Syntheses of an immunoadjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its analogues were described in previous papers (Kusumoto et al., 1976; Kotani et al., 1975). These muramyl dipeptides were dissolved in a Medium RPMI 1640 "NISSUI" (Nissui Seiyaku Co., Tokyo) and filtered through a membrane filter of 0.22  $\mu$ m average pore size (Millipore, Cat. No. GSWP02500, Millipore Corp., Bedford, Mass., U.S.A.) to minimize possible contamination of test specimens with extraneous endotoxic lipopolysaccharides which are known to have powerful mitogenic effect. Spleen lymphocytes were separated by Conray 400 (Daiichi Pharmaceutical Co., Tokyo)—Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) method (Böyum, 1968) from minced spleen suspensions obtained from either male ICR mice (CLER Japan Inc., Tokyo), 6-12 weeks old, or randomly-bred female albino guinea pigs of 250 g-450 g. With guinea pigs, all experiments were carried out by use of spleen lymphocytes obtained

from individual animals in order to avoid possible allogenic stimulation. Most experiments with ICR mice, on the other hand, were made using splenocytes pooled from 4 to 5 animals, since the amount of splenocytes available from one mouse was very limited. However, to check the possibility that allogenic effect could intervene in the mitogenic effect of muramyl peptides on pooled splenocytes from a not-inbred ICR mice, some ICR mice experiments were done on spleen lymphocytes derived from individual animals.

The spleen cells were suspended in a Medium RPMI 1640 supplemented with 10% fetal bovine serum (Lot 40551056, Flow Laboratories, Rockville, Md., U.S.A.) and containing Penicillin G 100 U/ml and streptomycin 100  $\mu\text{g}/\text{ml}$  to give  $10^6$  cells/ml medium. One ml aliquots of the spleen cell suspension were incubated with 0.1 ml portions of test specimen solutions in a  $\text{CO}_2$ -incubator for 48 hr at 37°C. One  $\mu\text{Ci}$  [ $^3\text{H}$ ] thymidine (specific activity 2.0 Ci/mmmole, Radiochemical Center, Amersham, Buckinghamshire, England) was added to each culture 24 hr before harvesting of the spleen cells. The incorporation of [ $^3\text{H}$ ] thymidine in the spleen lymphocytes was counted with a Liquid Scintillation Spectrometer (ALOKA LSC-673, Aloca Co., Tokyo) in the usual way. Bacto-Lipopolysaccharide B (from *Salmonella enteritidis*, control 578031, Difco Laboratories, Detroit, Mich. U.S.A.), Bacto-Phytohemagglutinin P (control 607029) and Concanavalin A (Lot 115C-7060, Sigma Chemical Co., St. Louis, Mo., U.S.A.) were used as reference mitogens.

It can be seen from Fig. 1 that a synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine which had been shown to be adjuvant-active in both induction of a delayed hypersensitivity and stimulation of increased serum antibody level to ovalbumin when administered to guinea pigs in a form of water-in-oil (mineral oil) emulsion (Ellouz et al., 1974; Kotani et al., 1975; Azuma et al., 1976b, c; Yamamura et al., 1976) exhibited a definite mitogenic effect on spleen lymphocytes from both ICR

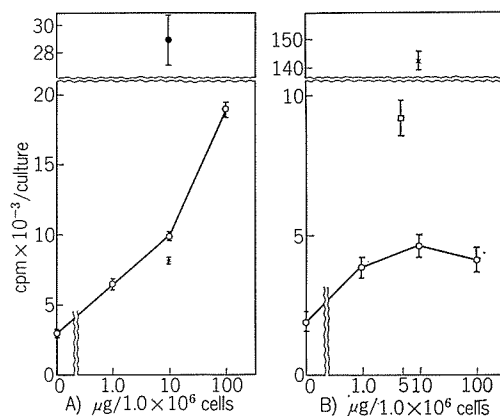


FIGURE 1. Mitogenic effect of the adjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutamine for spleen cells from guinea pigs (A) and ICR mice (B) (Dose-response curves). ○: MurNac-L-Ala-D-isoGln, ●: Phytohemagglutinin P (Bacto), □: Concanavalin A (Sigma), ×: Lipopolysaccharide B (Bacto). Expressed as the mean of thymidine incorporation and standard error (the vertical bar) as determined in five individual cultures.

closed colony mice and random-bred albino guinea pigs, over a dose range from 1.0  $\mu\text{g}$  to 100  $\mu\text{g}/10^6$  cells. Splenocytes from guinea pigs appeared to be more reactive to the mitogenic effect of the adjuvant-active muramyl dipeptide than those from ICR mice.

The assay was then made on the possible mitogenic activity of either adjuvant-inactive or weakly-active analogues of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Kotani et al., 1975; Adam et al., 1976). The results are summarized in Table 1. It has been revealed that adjuvant-inactive analogues, that is to say, *N*-acetylmuramyl-L-alanyl-L-isoglutamine, *N*-acetylmuramyl-L-alanyl-D-glutamine, *N*-acetylmuramyl-L-alanyl-L-glutamine and *N*-acetylmuramyl-L-alanyl-D-isoasparagine, were not mitogenic, although some increase in the incorporation of thymidine was noticed with the spleen cells incubated with the above muramyl dipeptides (100  $\mu\text{g}$ ) except -D-isoasparagine

TABLE 1. Effect of various synthetic muramyl dipeptides on the incorporation of thymidine into the spleen lymphocytes from ICR mice and random-bred albino guinea pigs

Preparation added	Dose ( $\mu$ g)	Stimulation index $\pm$ S. E. <sup>i</sup>	
		ICR mice	Guinea pigs
MurNAc-L-Ala-D-isoGln	0.1	1.08 $\pm$ 0.23 <sup>a</sup>	ND <sup>k</sup>
	1	2.02 $\pm$ 0.20 <sup>a</sup> (0.01) <sup>j</sup>	2.26 $\pm$ 0.13 <sup>c</sup> (0.001)
	10	2.42 $\pm$ 0.22 <sup>a</sup> (0.005)	3.41 $\pm$ 0.04 <sup>e</sup> (0.001)
			2.25 $\pm$ 0.14 <sup>d</sup> (0.001)
	100	2.17 $\pm$ 0.22 <sup>a</sup> (0.005)	6.58 $\pm$ 0.18 <sup>c</sup> (0.001)
		3.35 $\pm$ 0.06 <sup>b</sup> (0.001)	2.73 $\pm$ 0.31 <sup>d</sup> (0.001)
		2.35 $\pm$ 0.12 <sup>g</sup> (0.001)	3.55 $\pm$ 0.27 <sup>e</sup> (0.001)
MurNAc-L-Ala-L-isoGln	0.1	2.53 $\pm$ 0.18 <sup>h</sup> (0.001)	3.11 $\pm$ 0.18 <sup>f</sup> (0.001)
	1	1.18 $\pm$ 0.04 <sup>a</sup>	ND
	10	1.23 $\pm$ 0.07 <sup>a</sup>	ND
	100	1.33 $\pm$ 0.03 <sup>a</sup>	0.98 $\pm$ 0.08 <sup>e</sup>
		1.35 $\pm$ 0.13 <sup>a</sup>	1.31 $\pm$ 0.03 <sup>e</sup> (0.001)
		1.07 $\pm$ 0.12 <sup>g</sup>	
		1.10 $\pm$ 0.08 <sup>h</sup>	
MurNAc-L-Ala-D-Gln	10	1.16 $\pm$ 0.07 <sup>b</sup>	1.07 $\pm$ 0.01 <sup>c</sup>
	100	1.51 $\pm$ 0.07 <sup>b</sup> (0.001)	1.51 $\pm$ 0.11 <sup>c</sup> (0.01)
MurNAc-L-Ala-L-Gln	10	1.08 $\pm$ 0.05 <sup>b</sup>	1.11 $\pm$ 0.02 <sup>f</sup>
	100	1.20 $\pm$ 0.02 <sup>b</sup> (0.001)	1.24 $\pm$ 0.03 <sup>f</sup> (0.005)
MurNAc-L-Ala-D-Glu	10	1.72 $\pm$ 0.16 <sup>a</sup>	1.68 $\pm$ 0.07 <sup>e</sup> (0.001)
	100	2.02 $\pm$ 0.14 <sup>a</sup> (0.005)	1.97 $\pm$ 0.07 <sup>e</sup> (0.001)
MurNAc-L-Ala-L-Glu	10	1.01 $\pm$ 0.07 <sup>b</sup>	1.02 $\pm$ 0.02 <sup>f</sup>
	100	1.04 $\pm$ 0.04 <sup>b</sup>	1.08 $\pm$ 0.04 <sup>f</sup>
MurNAc-L-Ala-D-isoAsn	10	1.41 $\pm$ 0.14 <sup>a</sup>	0.89 $\pm$ 0.08 <sup>c</sup>
	100	1.32 $\pm$ 0.07 <sup>b</sup>	1.06 $\pm$ 0.05 <sup>c</sup>

<sup>a, b</sup> Spleen cells pooled from 4 to 5 ICR mice were used.

<sup>c-h</sup> Spleen cells obtained from individual guinea pigs or ICR mice were used to avoid possible allogenic stimulation.

<sup>i</sup> Ratio of [<sup>3</sup>H] thymidine incorporation (cpm/10<sup>6</sup> cells) in stimulated cultures added with test preparations to that in the respective control cultures without mitogen. All determinations were carried out in quintuple cultures. The incorporation of [<sup>3</sup>H] thymidine per 1.0  $\times$  10<sup>6</sup> cells in control cultures was within the range of 1,914 cpm to 26,863 cpm in ICR mice, and within the limits of 1,371 cpm to 3,313 cpm in guinea pigs, respectively.

<sup>j</sup> The difference between [<sup>3</sup>H] thymidine incorporation in stimulated cultures and that in the respective control cultures was significant at the level of P values indicated in parentheses, by the "Student" t-test.

<sup>k</sup> Not determined.

analogue. *N*-acetylmuramyl-L-alanyl-D-glutamic acid, weakly adjuvant-active in guinea pigs and definitely adjuvant-active in WKA female rats (Tanaka et al., 1977), on the other hand, showed a mitogenic effect, significant

but less than *N*-acetylmuramyl-L-alanyl-D-iso-glutamine, on ICR mice and guinea pig spleen lymphocytes. An adjuvant-inactive -L-glutamic acid analogue proved to lack the mitogenicity.

Azuma et al. (1976b) reported that *N*-acetylmuramyl-L-alanyl-D-isoglutamine, the same synthetic preparation that we used in the present study, was inactive as mitogen on spleen cells from C57BL/6J mice, although the data cited in their paper showed that the test muramyl dipeptide, but neither L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine nor 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, had some stimulatory effect on the incorporation of thymidine in the splenocytes. Damais and others (1975) found that the monomer of the *E. coli* peptidoglycan was devoid of mitogenicity on spleen cell suspensions of AKR mice. Ciorbaru et al. (1976) also reported that "polymers" of peptidoglycan subunits linked by extended glycan chain which were obtained from *Nocardia rubra* cell walls by use of *Streptomyces albus* G endopeptidase were mitogenic, but the products solubilized by lysozyme, containing monomeric or dimeric peptidoglycan subunits, were not. There was another paper (Rook and Stewart-Tull, 1976) reporting that adjuvant-active glycopeptides obtained from a culture supernatant fluid of *Mycobacterium tuberculosis* strain DT, which seemed to be either monomeric or dimeric peptidoglycan subunits (Stewart-Tull et al., 1975) had no effect on Balb/c inbred mice spleen cells, while peptidoglycolipids from the same or other strains of *M. tuberculosis*, were definite mitogens.

The observations cited in the preceding paragraph, indicating that a repetitive structure consisting of not a few peptidoglycan subunits may be a necessary requisite for stimulation of (B-derived) lymphocytes, do not seem to be in harmony with the results described here. The possibility that the observed stimulatory effect of the adjuvant-active muramyl dipeptide on ICR mice spleen cells is affected by the

allogenic effect, since ICR mice are not an inbred strain, could be excluded by the finding that MurNAc-L-Ala-D-isoGln, but not MurNAc-L-Ala-L-isoGln, was definitely mitogenic on splenocytes obtained from individual ICR mice. A possible explanation for the above discrepancies, therefore, would be that ICR mice used in this study were high responders to the mitogenic effect of monomeric peptidoglycan subunits. It should be pointed out that with our assay conditions, the mitogenic effect of adjuvant-active muramyl dipeptide monomer was found to be weaker than that of either cell walls or peptidoglycans. The close correlation between the mitogenicity and immunoadjuvancy of a variety of muramyl dipeptides, whose configurations are inherent or not inherent to bacterial cell wall peptidoglycans, indicates that the mitogenic effect of adjuvant-active synthetic compounds was a real one.

The findings reported here would imply that the minimum structure required for the mitogenic activity of bacterial cell wall peptidoglycans is a part of the peptidoglycan subunit monomer, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (or -D-glutamic acid), and that a repetitive structure of the monomers serve to enhance the mitogenicity of the monomer. Further experimentation, however, is necessary to explain fully the discrepancies described above.

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