BIKEN JOURNAL Vol. 20 81-85, 1977

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

(Received December 23, 1976)

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 [^{3}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 μm 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 μg/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 CO_2-incubator for 48 hr at 37C. One μCi [^{3}H] thymidine (specific activity 2.0 Ci/mmole, Radiochemical Center, Amersham, Buckinghamshire, England) was added to each culture 24 hr before harvesting of the spleen cells. The incorporation of [^{3}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

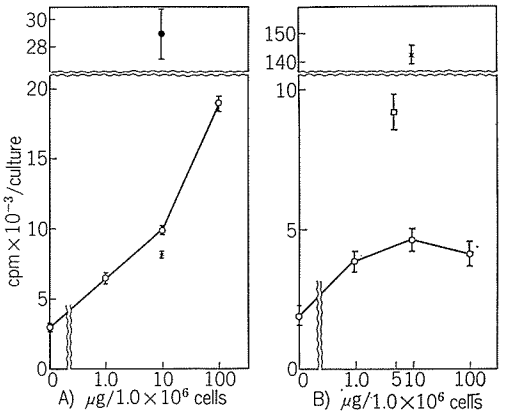

FIGURE 1. Mitogenic effect of the adjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutamine for spleen cells from guinea pigs (A) and ICR mice (B) (Dose-response curves). ○: MurNac-L-Ala-D-isoGln, ●: Phytohemagglutinin P (Bacto), □: Concanavalin A (Sigma), ×: Lipopolysaccharide B (Bacto). Expressed as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

closed colony mice and radom-bred albino guinea pigs, over a dose range from 1.0 μg to 100 μg/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 μg) 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 (μg)	Stimulation index±S. E.[i]	
		ICR mice	Guinea pigs
MurNAc-L-Ala-D-isoGln	0.1	1.08±0.23[a]	ND[k]
	1	2.02±0.20[a] (0.01)[j]	2.26±0.13[c] (0.001)
	10	2.42±0.22[a] (0.005)	3.41±0.04[c] (0.001)
			2.25±0.14[d] (0.001)
	100	2.17±0.22[a] (0.005)	6.58±0.18[c] (0.001)
		3.35±0.06[b] (0.001)	2.73±0.31[d] (0.001)
		2.35±0.12[g] (0.001)	3.55±0.27[e] (0.001)
		2.53±0.18[h] (0.001)	3.11±0.18[f] (0.001)
MurNAc-L-Ala-L-isoGln	0.1	1.18±0.04[a]	ND
	1	1.23±0.07[a]	ND
	10	1.33±0.03[a]	0.98±0.08[e]
	100	1.35±0.13[a]	1.31±0.03[e] (0.001)
		1.07±0.12[g]	
		1.10±0.08[h]	
MurNAc-L-Ala-D-Gln	10	1.16±0.07[b]	1.07±0.01[e]
	100	1.51±0.07[b] (0.001)	1.51±0.11[e] (0.01)
MurNAc-L-Ala-L-Gln	10	1.08±0.05[b]	1.11±0.02[f]
	100	1.20±0.02[b] (0.001)	1.24±0.03[f] (0.005)
MurNAc-L-Ala-D-Glu	10	1.72±0.16[a]	1.68±0.07[e] (0.001)
	100	2.02±0.14[a] (0.005)	1.97±0.07[e] (0.001)
MurNAc-L-Ala-L-Glu	10	1.01±0.07[b]	1.02±0.02[f]
	100	1.04±0.04[b]	1.08±0.04[f]
MurNAc-L-Ala-D-isoAsn	10	1.41±0.14[a]	0.89±0.08[e]
	100	1.32±0.07[b]	1.06±0.05[e]

[a,b] Spleen cells pooled from 4 to 5 ICR mice were used.

[c–h] Spleen cells obtained from individual guinea pigs or ICR mice were used to avoid possible allogenic stimulation.

[i] Ratio of [^{3}H] thymidine incorporation (cpm/10^6 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 [^{3}H] thymidine per 1.0×10^6 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.

[j] The difference between [^{3}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.

[k] 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-isoglutamine, 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.

ACKNOWLEDGMENTS

This research was supported in part by Grants in Aid for Scientific Research from the Ministry of Education, Science and Culture (Nos. 148149 and 112118).

REFERENCES

Adam, A., M. Devys, V. Souvannavong, P. Lefrancier, J. Choay, and E. Lederer. 1976. Correlation of structure and adjuvant activity of *N*-acetylmumramyl-L-alanyl-D-isoglutamine (MDP), its derivatives and analogues. Anti-adjuvant and competition properties of stereoisomers. Biochem. Biophys. Res. Commun. 72: 339–346.

Azuma, I., T. Taniyama, K. Sugimura, A. A.

Aladin, and Y. Yamamura. 1976a. Mitogenic activity of the cell walls of *Mycobacteria*, *Nocardia*, *Corynebacteria* and anaerobic coryneforms. Jpn. J. Microbiol. 20: 263–271.

Azuma, I., K. Sugimura, T. Taniyama, M. Yamawaki, Y. Yamamura, S. Kusumoto, S. Okada, and T. Shiba. 1976b. Adjuvant activity of mycobacterial fractions: adjuvant activity of synthetic *N*-acetylmuramyl-dipeptide and the related compounds. Infect. Immun. 14: 18–27.

Azuma, I., K. Sugimura, Y. Yamamura, S. Kusumoto, Y. Tarumi, and T. Shiba. 1976c. Adjuvant activity of synthetic cell-wall peptidoglycan subunits on monobenzenarsonate-*N*-acetyl-L-tyrosine and bacterial α-amylase in guinea pigs. Jpn. J. Microbiol. 20: 63–66.

Böyum. 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21 (Suppl. 97): 1–109.

Ciorbaru, R., J.-F. Petit, E. Lederer, E. Zissman, C. Bona, and L. Chedid. 1976. Presence and subcellular localization of two distinct mitogenic fractions in the cells of *Nocardia rubra* and *Nocardia opaca:* preparation of soluble mitogenic peptidoglycan fractions. Infect. Immun. 13: 1084–1090.

Cohen, J. J., G. E. Rodriguez, P. D. Kind, and P. A. Campbell. 1975. *Listeria* cell wall fraction: a B cell mitogen. J. Immunol. 114: 1132–1134.

Damais, C., C. Bona, L. Chedid, J. Fleck, C. Nauciel, and J. P. Martin. 1975. Mitogenic effect of bacterial peptidoglycans possessing adjuvant activity. J. Immunol. 115: 268–271.

Ellouz, F., A. Adam, R. Ciorbaru, and E. Lederer. 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Commun. 59: 1317–1325.

Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. 1975. Immunoadjuvant activities of synthetic *N*-acetylmuramyl-peptides or -amino acids. Biken J. 18: 105–111.

Kusumoto, S., Y. Tarumi, K. Ikenaka, and T. Shiba. 1976. Chemical synthesis of *N*-acetylmuramyl peptides with partial structures of bacterial cell wall and their analogues in relation to immunoadjuvant activities. Bull. Chem. Soc. Japan 49: 533–539.

Rook, G. A. W., and D. E. S. Stewart-Tull. 1976. The dissociation of adjuvant properties of mycobacterial components from mitogenicity, and from the ability to induce the release of mediators from macrophages. Immunology 31: 389–396.

Stewart-Tull, D. E. S., T. Shimono, S. Kotani, M. Kato, Y. Ogawa, Y. Yamamura, T. Koga, and C. M. Pearson. 1975. The adjuvant activity of a non-toxic, water-soluble glycopeptide present in large quantities in the culture filtrate of *Mycobacterium tuberculosis* strain DT. Immunology 29: 1–15.

Takada, H., S. Kotani, S. Kusumoto, and T. Shiba. 1977. Mitogenicity of cell walls and their components of various gram-positive bacteria. Jpn. J. Bacteriol. 32: 151. [In Japanese]

Tanaka, A., R. Saito, K. Sugiyama, I. Morisaki, S. Kotani, S. Kusumoto, and T. Shiba. 1977. Adjuvant activity of synthetic *N*-acetylmuramyl peptides in rats. Infect. Immun. 15: 332–334.

Yamamura, Y., I. Azuma, K. Sugimura, S. Kusumoto, and T. Shiba. 1976. Immunological activity of synthetic cell-wall peptidoglycan subunits. Proc. Japan Acad. 52: 58–61.