BIKEN JOURNAL Vol. 20 125-130, 1977

SHORT COMMUNICATION

EFFECTS OF CHEMICAL MODIFICATIONS OF THE GLUTAMIC ACID RESIDUE IN *N*-ACETYLMURAMYL PEPTIDES ON THE IMMUNOADJUVANCIES OF THE MOLECULES

SHOZO KOTANI, FUMIO KINOSHITA, YOSHIRO WATANABE, ICHIJIRO MORISAKI, TSUTOMU SHIMONO and KEIJIRO KATO

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

TETSUO SHIBA, SHOICHI KUSUMOTO, KAZUHIRO IKENAKA and YUZO TARUMI

Faculty of Science, Osaka University, Toyonaka, Osaka

(Received July 14, 1977)

In studies on the immunoadjuvant activities of the cell walls of over 35 species of gram-positive or acid-fast bacteria, we found that the walls of *Corynebacterium poinsettiae*, *Corynebacterium betae*, *Corynebacterium insidiosum*, *Microbacterium lacticum*, *Eubacterium limosum*, *Micrococcus lysodeikticus*, *Staphylococcus epidermidis*, *Arthrobacter atrocyaneus* and *Arthrobacter* sp. had little or no adjuvancies to stimulate humoral and cellular immune responses. In contrast other cell walls had definite adjuvant activities both in induction of delayed-type hypersensitivity and in stimulation of serum precipitin levels towards a test protein antigen when administered to guinea pigs as a water-in-mineral oil emulsion (Kotani et al. 1975a; 1977a).

A survey on the chemical structures of the cell walls without adjuvant activity listed above showed that their peptidoglycans had a common structural feature (Schleifer and Kandler, 1972); namely, the α-carboxyl groups of the D-glutamic acid residues in their stem peptide subunits were either combined with glycine (*M. lysodeikticus*), glycine amide (*A. atrocyaneus*) or D-alanine amide (*Arthobacter* sp.) or involved in cross linkings between two neighbouring stem peptide subunits (the walls from *C. insidiosum* and other bacteria whose peptidoglycans are group B type according to the classification of Schleifer and Kandler). An exception was *S. epidermidis* cell walls, which had no adjuvant activity by themselves, but which became highly active when solubilized with either endo-*N*-acetylmuramidase or endopeptidase (Kotani et al., 1977b). In contrast, in the cell walls with adjuvant activity and those of *S. epidermidis*, the α-carboxyl groups of D-glutamic acid residues in the peptidoglycans are not combined with amino acids, their amides or peptides (Ghuysen, 1968; Schleifer and Kandler, 1972).

On the basis of the fact that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (or -D-glutamic acid) is the minimum structure required for the immunoadjuvancies of bacterial cell walls (Ellouz et al., 1974; Kotani et al., 1975b; Audibert et al., 1976; Azuma et al., 1976b;

Yamamura et al., 1976; Adam et al., 1976; Tanaka et al., 1977), we studied the effects of chemical modifications of the D-glutamic acid residues in *N*-acetylmuramyl dipeptides and *N*-acetylmuramyl tetrapeptide on the immunoadjuvant activities of the resulting molecules.

For the syntheses of test *N*-acetylmuramyl peptide derivatives with modified D-glutamic acid residues (**IIIa-f,** see Table 1 for this and the following symbols), we used the same principles as in previous work on *N*-acetylmuramyl dipeptides (Kusumoto et al., 1976). The peptide portions were prepared by stepwise elongation from the C-terminal with dicyclohexylcarbodiimide or 1-succinimidyl ester, using *t*-butoxycarbonyl (Boc) and benzyl groups for protection of amino and carboxyl groups, respectively. However, some modifications were required for the syntheses of **Ib** and **Ie;** namely, since glycine amide and its peptides are so soluble in water that they are difficult to handle, we prepared a tripeptide containing glycine ethyl ester, i.e., ethyl Boc-L-alanyl-γ-benzyl-D-glutamylglycinate, and converted it to **Ib** by hydrogenolysis followed by ammonolysis. Another difficulty was encountered in the synthesis of **If** for

TABLE 1. *Yields and physical constants of t-butoxycarbonyl and N-acetylmuramyl peptide derivatives*

Synthetic compound	**I** R^1=Boc R^2=Bzl, R^3=Z		**II** R^1=protected MurNAc R^2=Bzl, R^3=Z			**III** R^1=MurNAc R^2=R^3=H	
	mp (C)	$[\alpha]_D$[a]	Yield (%)	mp (C) (dec)	$[\alpha]_D$[e]	Yield (%)	$[\alpha]_D$[j]
a) R^1-L-Ala-D-Glu(OR^2)-Gly-OR^2	107	+6.53°[b]	74	220	+67.6°[f]	60	+38.5°[k]
b) R^1-L-Ala-D-Glu(OH)-Gly-NH_2	183[m] (dec)	−10.5°[m]	77	243	+99.6°[g]	94	+38.1°
c) R^1-L-Ala-D-Glu(OR^2)-D-Ala-NH_2	182.5-183.5 (dec)	−4.98°	72	275	+83.0°[h]	98	+47.9°
d) R^1-Gly-D-Glu(OR^2)-Gly-OR^2	syrup[c]		79	203	+73.2°[g]	95	+41.9°
e) R^1-L-Ala-D-Glu(L-Lys(R^3)-D-Ala-OR^2)-Gly-OR^2	173-174[d]	+0.50°	75	220	+55.8°[i]	90	+18.6°
f) R^1-L-Ala-D-Glu(NHC_4H_9-*n*)-NH_2	194.5-195 (dec)	−8.49°	82	280-282	+88.2°	90[l]	+31.2°

[a] c 2 in *N*, *N*-dimethylformamide (DMF) at 26-28 C. [b] c 1 at 19 C. [c] H-Gly-D-Glu(OBzl)-Gly-OBzl HCl salt (mp 133-134 C) derived from **Id** gave satisfactory results on elemental analysis. [d] with softening at 167 C. [e] c 0.5 in DMF at 25-28 C [f] at 19 C. [g] c 1. [h] c 0.1. [i] in *N*, *N*-dimethylacetamide. [j] c 0.5 in H_2O at 26-28 C after 24 hr. [k] at 15 C. [l] precipitated from methanol-absolute ether; mp 183-185 C (dec). [m] dicyclohexylammonium salt. Boc=*t*-butoxycarbonyl; Bzl=benzyl; Z=benzyloxycarbonyl; protected MurNAc=1-α-*O*-benzyl-4, 6-*O*-benzylidene-*N*-acetylmuramyl; MurNAc=*N*-acetylmuramyl.

construction of a branched part; we used the following novel procedure to avoid α, γ-transpeptidation at the glutamic acid residue in preparation of the key intermediate benzyl Boc-D-glutamylglycinate (**IV**). When Boc-D-glutamic anhydride was treated with benzyl glycinate and then dicyclohexylamine was added, the desired α-peptide (**IV**) precipitated in fairly good yield (43%) as its amine salt, while the isomeric γ-peptide, benzyl Boc-γ-D-glutamylglycinate remained in solution. Condensation of the γ-carboxyl group of **IV** with benzyl N^{ε}-benzyloxycarbonyl-L-lysyl-D-alaninate afforded the branched tetrapeptide, which was treated with trifluoroacetic acid and then coupled with Boc-L-alanine to give **Ie.**

The Boc groups in the peptides (**Ia-f**) were removed with trifluoroacetic acid and the product was treated with 1-α-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetylmuramic acid 1-succinimidyl ester (**V**) to yield the protected *N*-acetylmuramyl peptides (**II**). The water soluble ambident tripeptide, L-alanyl-D-glutamylglycine amide, obtained from **Ib** gave better results when treated with 5-norbornene-2,3-dicarboxyimidyl ester of protected muramic acid in aqueous tetrahydrofuran, than the 1-succinimidyl ester (**V**).

The final hydrogenolytic deprotection of **IIa-f** proceeded satisfactorily, as described previously, and **IIIa-f** were isolated as hygroscopic colorless solids by lyophilization. Further details of the synthesis will be reported in a separate paper (Kusumoto, Ikenaka and Shiba, in preparation).

To assay the immunoadjuvant activities of test muramyl peptides, the methods previously described (Kotani et al. 1975a) were followed with some modifications (Kotani et al., 1977d). In brief, groups of 5 female albino guinea pigs were injected in the left hind footpad with 0.2 ml of water-in-mineral oil emulsion containing one mg of ovalbumin and test muramyl peptide specimens equivalent in moles to 50 and 100 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine per animal.

The results summarized in Fig. 1 show that combination of the α-carboxyl group of the D-glutamic acid residue in either *N*-acetylmuramyl-L-alanyl-D-isoglutamine (the α-amide of D-glutamic acid) or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine with glycine results in considerable decrease of the immunoadjuvancies of the respective molecules in both induction of delayed-type hypersensitivity towards ovalbumin and stimulation of serum anti-ovalbumin precipitating antibody levels. Combination of the α-carboxyl group of the D-glutamic acid residue with glycine amide or D-alanine amide, on the other hand, caused no or only slight reduction in the adjuvancies of the molecules. We found further that *N*-acetylmuramyl-glycyl-D-glutamyl-glycine was almost completely devoid of adjuvancies, indicating that the low adjuvancies of *N*-acetylmuramyl-glycyl-D-isoglutamine (Adam et al., 1976; Kotani et al., 1977c) were further reduced by blocking the α-carboxyl group of the D-glutamic acid residue. Incidentally we found that the γ-butylamide of *N*-acetylmuramyl-L-alanyl-D-isoglutamine retained only a part of the adjuvant activities of *N*-acetylmuramyl-L-alanyl-D-isoglutamine to stimulate both humoral and cellular immune responses, although the dose-response relationship was irregular.

The aim of this work was to find out whether the absence of immunoadjuvant activities in the cell walls of the bacterial species listed in the opening paragraph of this paper are due to combination of the α-carboxyl groups of the D-glutamic acid residues in their peptidoglycans with amino acids, or their amides or peptides. The present findings suggest that the inhibitory effects of binding a single amino acid or amide cannot completely explain the absence of immunoadjuvancies of the cell walls. Binding of the α-carboxyl groups of the D-glutamic acid residues in the peptidoglycans of group B types with amino acids or peptides involved in cross-linking probably results in stronger inhibitory effects on the adjuvancies of the cell walls than binding with a single amino acid or amide.

In a separate study we found that when *M.*

lysodeikticus cell walls, which have no adjuvant activity by themselves, were solubilized with L-11 endopeptidase, but not mutanolysin endo-*N*-acetylmuramidase, they developed weak but definite immunopotentiating activities (Kotani et al., 1977b). This difference is probably because the inhibitory effect of combination of the α-carboxyl groups of the D-

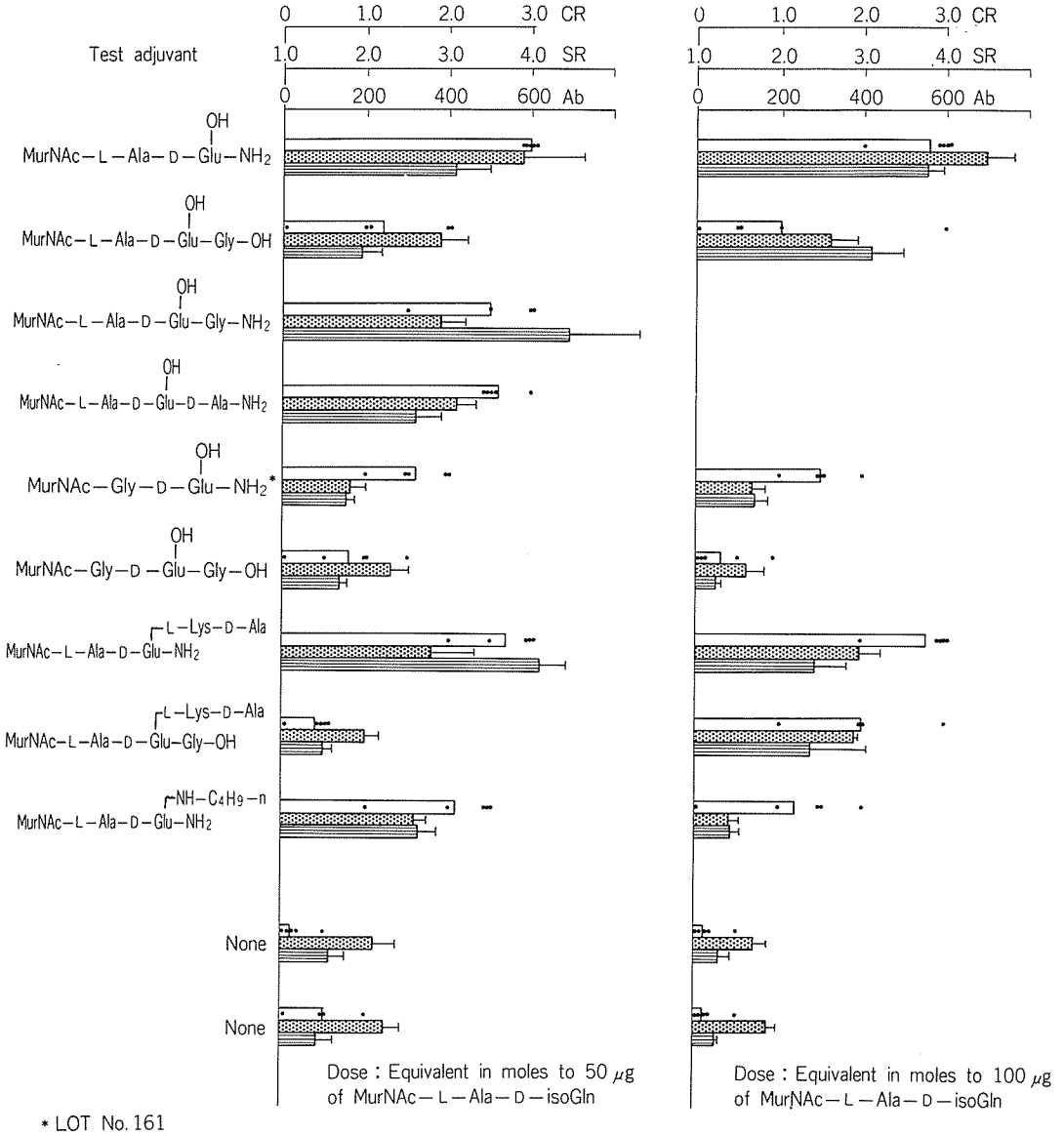

FIGURE 1 Changes in immunoadjuvancies by chemical modifications of the D-glutamic acid residues in the adjuvant-active *N*-acetylmuramyl peptides. CR, ☐ corneal response (48 hr reading), SR, ▨ skin response (induration, 48 hr reading), Ab, ▤ serum antibody level (μg antibody nitrogen/ml serum, determined by the quantitative precipitin reaction), ⊢Σ⊣ +1 standard error, and ● the corneal response of an individual animal.

glutamic acid residues in *M. lysodeikticus* walls with glycine is greater in the mutanolysin digest than in the endopeptidase digest, since the immunoadjuvancies of bacterial cell walls can sometimes be enhanced by solubilization with appropriate peptidoglycan-degrading enzymes and since the adjuvancies of constituent muramyl peptide subunits are more effectively exhibited in a form polymerized through a rather long glycan chain than in the form of monomers or dimers (Kotani, 1976).

The finding that the walls of *A. atrocyaneus*, in which the α-carboxyl groups of the D-glutamic acid are combined with glycine amide, exhibit detectable adjuvant activities (Kotani et al., 1977a) seems to be consistent with the results reported in this paper.

It was shown in previous studies (Adam et al., 1976; Kotani et al., 1977c) that replacement of the L-alanine residue by glycine in *N*-acetylmuramyl-L-alanyl-D-isoglutamine caused considerable decrease in adjuvancies of the molecule. This fact, together with the present finding that *N*-acetylmuramyl-glycyl-D-glutamyl-glycine has very weak adjuvant abilities, strongly suggests that the inabilities of cell walls with group B type peptidoglycans to act as immunoadjuvants may be explained by the combined inhibitory effects of replacement of the L-alanine residue adjacent to the muramic acid residue by glycine and combination of the α-carboxyl group of the D-glutamic acid residue with amino acids or peptides in cross-links with neighboring stem peptide subunits. It should be noted, however, that the cell walls of *E. limosum*, which had no adjuvant activities, have peptidoglycans in which the amino acid adjacent to the muramic acid redue is L-serine and that replacement of the L-alanine residue by L-serine caused a definite increase in the immunoadjuvancies of the molecule (Kotani et al., 1977c).

Audibert et al. (1977) reported that *N*-acetylmuramyl-L-alanyl-D-glutamyl-glycine and the γ-methylamide of *N*-acetylmuramyl-L-alanyl-D-isoglutamine did not show immunoadjuvancies in assay systems with either Hartley guinea pigs or Swiss common stock mice. There does not seem to be essential discrepancies between their results and ours, though we found that *N*-acetylmuramyl-L-alanyl-D-glutamyl-glycine was less active than an equimolar amount of *N*-acetylmuramyl-L-alanyl-D-isoglutamine but had definite adjuvant activity and that the γ-butylamide of *N*-acetylmuramyl-L-alanyl-D-isoglutamine exhibited low, but detectable, immunoadjuvancies.

ACKNOWLEDGMENTS

This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 148149, 112118 and 211318).

REFERENCES

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

Audibert, F., L. Chedid, P. Lefrancier, J. Choay, and E. Lederer. 1977. Relationship between chemical structure and adjuvant activity of some synthetic analogues of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP). Ann. Immunol. (Inst. Pasteur) 128C: 653–661.

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

Azuma, I., K. Sugimura, Y. Yamamura, S. Kusumoto, Y. Tarumi, and T. Shiba. 1976a. Adjuvant activity of synthetic cell-wall peptidoglycan subunits on monoazobenzenearsonate-*N*-acetyl-L-tyrosine and bacterial α-amylase in guinea pigs.

Jpn. J. Microbiol. 20: 63–66.

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.

Ghuysen, J.-M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. 32: 425–464.

Kotani, S. 1976. Biological activities of bacterial cell wall peptidoglycans and their subunits, with special reference to the immunoadjuvant actions (a review). J. Jpn. Biochem. Soc. 48: 1081–1107. [In Japanese]

Kotani, S., F. Kinoshita, I. Morisaki, T. Shimono, T. Okunaga, H. Takada, M. Tsujimoto, Y. Watanabe, K. Kato, T. Shiba, S. Kusumoto, and S. Okada. 1977d. Immunoadjuvant activities of synthetic 6-*O*-acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine with special reference to the effect of its administration with liposomes. Biken J. 20: 95–103.

Kotani, S., T. Narita, D.E.S. Stewart-Tull, Y. Watanabe, K. Kato, and S. Iwata. 1975a. Immunoadjuvant activites of cell walls and their water-soluble fractions prepared from various gram-positive bacteria. Biken J. 18: 77–92.

Kotani, S., Y. Watanabe, F. Kinoshita, K. Kato, H. R. Perkins. 1977b. Immunoadjuvant activities of the enzymatic digests of bacterial cell walls lacking immunoadjuvancy by themselves. Biken J. 20: 87–92.

Kotani, S., Y. Watanabe, F. Kinoshita, K. Kato, K. H. Schleifer, and H. R. Perkins. 1977a. Inabilities as an immunoadjuvant of cell walls of the group B peptidoglycan types and those of arthrobacters. Biken J. 20: 1–4.

Kotani, S., Y. Watanabe, F. Kinoshita, I. Morisaki, K. Kato, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. 1977c. The effect of replacement of L-alanine residue by glycine, L-serine or D-alanine in an *N*-acetylmuramyl-L-alanyl-D-isoglutamine on immunoadjuvancies of molecules. Biken J. 20: 39–45.

Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. 1975b. 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 syntheses of *N*-acetylmuramyl peptides with partial structures of bacterial cell wall and their analogs in relation to immunoadjuvant activities. Bull. Chem. Soc. Jpn. 49: 533–539.

Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36: 407–477.

Tanaka, A., R. Saito, K. Sugimura, 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. Sugiyama, S. Kusumoto, and T. Shiba. 1976. Immunological activity of synthetic cell wall peptidoglycan subunits. Proc. Japan Acad. 52: 58–61.