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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

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SUMMARY Substitution of the L-alanine residue by L-serine and glycine in an *N*-acetylmuramyl-L-alanyl-D-isoglutamine, the minimum structural unit essential to the immunoadjuvancies characteristic of bacterial cell wall peptidoglycans, was shown to bring about significant increase and some decrease, respectively, in the adjuvant abilities of molecules to induce delayed-type hypersensitivity and to stimulate serum antibody levels to ovalbumin when administered to guinea pigs as a water-in-mineral oil emulsion. *N*-Acetylmuramyl-D-alanyl-D-isoglutamine, on the other hand, was found to be adjuvant-inactive.

INTRODUCTION

Ellouz et al. (1974) and Kotani et al. (1975b) have independently demonstrated by use of synthetic compounds and enzymatically obtained peptidoglycan subunits that the minimum structural entity responsible for the immunoadjuvancies, one of the most remarkable biological activities of bacterial cell walls, is *N*-acetylmuramyl-L-alanyl-D-isoglutamine. We further extended this discovery, studying the abilities of various synthetic analogues or diastereomers of the *N*-acetylmuramyl-L-alanyl-D-isoglutamine to induce delayed-type hypersensitivity and raise circulating antibody levels to ovalbumin when administered to guinea pigs as a water-in-oil emulsion. It was shown that the presence of D-isoglutamine residue linked to the *N*-acetylmuramyl-L-alanine was essential for the immunoadjuvancies of the molecule to appear (Kotani et al., 1975b). Confirmative studies on the above findings were reported by Azuma et al. (1976a) and Yamamura et al. (1976), using azobenzenarsonate-*N*-acetyl-L-tyrosin or a bacterial α-amylase as a test antigen, instead of ovalbumin. The immunoadjuvancy of synthetic muramyl peptides was further studied by Azuma et al.

(1976b) and Chedid et al. (1976) in mice, and by Tanaka et al. (1977) in rat.

In a separate paper we have reported that cell wall preparations isolated from several bacterial species whose peptidoglycans are of group B types (Schleifer and Kandler, 1972) lacked an immunoadjuvancy in the assay system where the cell walls of the group A peptidoglycan types, with rare exceptions, were found to be fully adjuvant-active (Kotani et al., 1977).

The cell walls of group B peptidoglycan types are known to be characterized 1) by replacement of the L-alanine residue adjacent to muramic acid with glycine or L-serine, and 2) by cross-linking between the α-carboxyl group of D-glutamic acid or *threo*-3-hydroxyglutamic acid residue and the carboxyl group of C-terminal D-alanine in the neighbouring peptide subunits through diamino acid or peptide containing diamino acid (Schleifer and Kandler, 1972). This work was thus undertaken to elucidate whether the replacement of the L-alanine residue with glycine or L-serine in adjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutamine could be responsible for inability of the cell walls of group B peptidoglycan types to stimulate both antibody-mediated and cell-mediated immune responses. A part of this work was reported in a preliminary form (Kotani et al., 1975c, 1975d).

MATERIALS AND METHODS

1. *Synthesis of N-acetylmuramyl dipeptides*

Three *N*-acetylmuramyl dipeptides (IIIa–c) listed in Table 1, i.e., *N*-acetylmuramyl-glycyl-, *N*-acetylmuramyl-L-seryl- and *N*-acetylmuramyl-D-alanyl-D-isoglutamine, were prepared in a manner similar to that described previously for *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Kusumoto et al., 1976). Thus, the *t*-butoxycarbonylamino acids were coupled with D-isoglutamine benzyl ester by dicyclohexylcarbodiimide—*N*-hydroxysuccinimide method to afford the protected dipeptides (Ia–c). These compounds were treated with trifluoroacetic acid to remove the *t*-butoxycarbonyl group, and then condensed with benzyl 4,6-*O*-benzylidene-*N*-acetyl-α-muramide by means of ethyl chloroformate to yield the corresponding protected *N*-acetylmuramyl dipeptides (IIa–c). The all protective groups were removed simultaneously by catalytic hydrogenolysis with palladium black in acetic acid to afford the free

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

	Synthesized compound	I R^1=Boc[d] R^2=Bzl[d]		II R^1=protected MurNAc[d] R^2=Bzl[d]		III R^1=MurNAc[d] R^2=H		
		mp (C)	$[\alpha]_D^{29}$[a]	Yield (%)	mp (C) (dec)	$[\alpha]_D^{28}$[b]	Yield (%)	$[\alpha]_D^{29}$[c]
a	R^1-Gly-D-Glu(OR^2)-NH_2[e]	112 -113	+ 5.7°	76	234	+81.5°	94	+33.3°
b	R^1-L-Ser-D-Glu(OR^2)-NH_2	73 - 75	+ 0.70°	84	197	+81.6°	66	+35.3°
c	R^1-D-Ala-D-Glu(OR^2)-NH_2	130.5-131.5	+19.2°	74	223	+83.5	quant.	+64.4°

[a] In ethyl acetate. [b] In dimethylformamide. [c] After 24 hr in H_2O

[d] Boc=*t*-butoxycarbonyl; Bzl=benzyl; MurNAc=*N*-acetylmuramyl; protected MurNAc=1-α-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetylmuramyl.

[e] Lot No. 33.

N-acetylmuramyl dipeptides (IIIa–c) in pure state.

2. *Assay of the immunoadjuvant activities*

The methods used were essentially as described in the first paper of this series (Kotani et al., 1975a), except that 1/75 M phosphate-buffered saline (pH 7.0) was used to prepare a water-in-oil emulsion, in place of plain physiological saline.

RESULTS

Groups of 5 female randomly-bred albino guinea pigs were injected in the left hind foot pad with 0.2 ml of a water-in-oil emulsion containing one mg of crystalline ovalbumin as an antigen and varying doses of test muramyl dipeptides. Two and three weeks after the sensitization, the induction of delayed-type hypersensitivity to ovalbumin was examined by corneal test. A few days after the second corneal test, skin reactions to the intracutaneous injection of 100 μg/0.1 ml ovalbumin solution were determined. Several days later, blood was drawn for estimation of circulating anti-ovalbumin precipitating antibody and examination of IgG_2-type immunoglobulin specific to ovalbumin.

The assay results on the immunoadjuvancies of test synthetic muramyl dipeptides are summarized in Table 2. The replacement of the L-alanine residue linked to muramic acid in the adjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutamine by L-serine resulted in significant increase in the immunoadjuvant activities of test muramyl dipeptides, in terms of all parameters examined: corneal and skin (erythema and induration) responses, circulating antibody levels and appearance of IgG_2-type immunoglobulin. Substitution of the L-alanine residue by glycin, on the other hand, brought about some reduction of the immunoadjuvancies of molecule, although fluctuations in the adjuvant potency were observed from experiment to experiment by unknown reason. It was also proved that the D-alanine which had never been found as the amino acid adjacent to muramic acid in bacterial cell wall peptidoglycans could not replace the L-alanine residue without causing disappearance of the adjuvant activities.

DISCUSSION

The results reported here indicate that the inabilities as an immunoadjuvant of the cell walls of group B peptidoglycan types, such as those of plant-pathogenic corynebacteria (*Corynebacterium poinsettiae*, *Corynebacterium betae* and *Corynebacterium insidiosum*), *Microbacterium lacticum* and *Eubacterium limosum* (Kotani et al., 1977), cannot satisfactorily be explained by the effect of replacement of the L-alanine residue linked to muramic acid in the peptidoglycan structure by glycine or L-serine. Exclusion of this possibility leads to an assumption that the involvement of the α-carboxyl group of the D-glutamic acid or *threo*-3-hydroxyglutamic acid in cross-linkings between the neighbouring peptide subunits might be responsible for the lack of adjuvancy in the walls of group B peptidoglycan types. A study to verify the latter possibility by use of synthetic muramyl peptides is in progress.

Recently Chedid et al. (1976) studied the adjuvant activities of different analogues and derivatives of *N*-acetylmuramyl-L-alanyl-D-isoglutamine on the humoral antibody response of Swiss mice to bovine serum albumin injected in saline, and found that the above immunoadjuvant activities of *N*-acetylmuramyl-L-alanyl-D-isoglutamine were not affected by replacement of the L-alanine residue by L-serine, but significantly decreased by substitution by glycine. Adam et al. (1976) further reported that *N*-acetylmuramyl-L-seryl-D-isoglutamine was fully active, but the glycine analogue was far less active than *N*-acetylmuramyl-L-alanyl-D-isoglutamine in both stimulation of antibody production and induction of delayed-type hypersensitivity, in the assay system with guinea pigs and ovalbumin as in our study. Thus there seem to be some, but not essential, discrepancies between the results of French groups and those of ours on the quantitative aspect of the effect of replace-

TABLE 2. *Comparison of the immunoadjuvant activities of N-acetylmuramyl-L-seryl-D-isoglutathose of N-acetylmuramyl-L-alanyl-D-isoglutamine in the induction of delayed-type hypersensitivity*

Experimental group	Test muramyl dipeptide	Dose (μg)	Corneal response (48 hr) Mean (Range)[h]
44	MurNAc-L-Ser-D-isoGln	100	3.0
48		50	3.0
48		25	3.0
49		12.5	3.0
49		6.25	3.0
49		3.13	2.7 (2.5-3.0)
61		1.56	2.6 (2.0-3.0)
61		0.78	2.4 (2.0-2.5)
61		0.39	2.3 (2.0-3.0)
44	MurNAc-Gly-D-isoGln	100[f]	3.0
65		100[f]	2.9 (2.5-3.0)
55		25[f]	3.0
63		25[f]	3.0
65		25[f]	2.9 (2.5-3.0)
68		25[g]	1.5 (0 -2.5)
68		25[g]	1.1 (0 -2.0)
55		6.25[f]	2.7 (2.0-3.0)
62		6.25[f]	0.6 (0 -3.0)
65		6.25[f]	2.6 (1.5-3.0)
55		1.56[f]	2.5 (0.5-3.0)
62		1.56[f]	0.9 (0 -2.0)
67		1.56[f]	0
61		0.39[f]	1.2 (0 -2.0)
67		0.39[f]	0.2 (0 -1.0)
44	MurNAc-D-Ala-D-isoGln	100	0.6 (0 -3.0)
46	MurNAc-L-Ala-D-isoGln	100	3.0
45		50	3.0
61		25	2.4 (2.0-3.0)
49		12.5	2.6 (1.0-3.0)
49		6.25	2.4 (0.5-3.0)
49		3.13	1.1 (0.5-2.0)
61		3.13	1.8 (1.0-3.0)
61		1.56	1.3 (1.0-2.0)
44	None (FIA control)	0	0.3 (0 -1.0)
45		0	0.3 (0 -1.0)
46		0	0.6 (0 -2.0)
48		0	0
49		0	0.4 (0 -1.0)
55		0	0.7 (0.5-1.0)
61		0	0.6 (0 -1.5)
62		0	0.5 (0 -1.0)
63		0	0.5 (0 -1.0)
65		0	0.3 (0 -1.0)
67		0	0
68		0	0.1 (0 -0.5)

[a] Average diameter of redness (mm).
[b] Ratio of double thickness of the skin injected with 100 μg ovalbumin/0.1 ml saline to that of the skin of
[c] Ratio of antibody nitrogen (μgN/ml serum) in the test group to that in the respective FIA control group.
[d] The difference between the test and respective control group was significant at a level of 5% (*) or 1%
[e] μg Antibody nitrogen/ml serum specimen.
[f] Lot. No. 33. [g] Lot. No. 160.
[h] 3.0-2.0 Strong, 2.0>-1.0 medium, and <1.0 negative or doubtful reactions, respectively.

mine, N-acetylmuramyl-glycyl-D-isoglutamine and N-acetylmuramyl-D-alanyl-D-isoglutamine with and stimulation of increased antibody level to ovalbumin in guinea pigs

Skin response (48 hr)		Antibody level (ratio)	IgG_2
Erythema (mm) Mean±S. E.[a]	Induration Mean±S.E.[b]	Mean±S.E.[c]	Mean (Range)[h]
18±1.0**[d]	2.9±0.14**	7.3 ± 0.76**	2.4 (2.0-3.0)
18±1.1**	3.8±0.19**	5.1 ± 0.38**	3.0
16±0.78**	3.5±0.21**	4.5 ± 0.50**	3.0
16±1.0**	2.4±0.19	3.0 ± 0.62*	1.3 (0 -3.0)
15±0.66**	3.2±0.11**	6.0 ± 0.29**	2.5 (2.0-3.0)
16±0.97**	2.9±0.19**	5.1 ± 0.37**	1.0 (0.5-3.0)
17±0.64**	2.1±0.10**	3.4 ± 0.26**	0
14±0.60**	1.9±0.08**	2.4 ± 0.85	0.6 (0 -2.0)
14±1.02	2.0±0.09**	2.4 ± 0.52*	0.4 (0 -1.0)
14±2.2	3.1±0.51	5.8 ± 2.2	1.0 (0 -2.0)
15±0.29**	2.5±0.16**	7.1 ± 1.3**	2.4 (2.0-3.0)
15±1.1**	2.1±0.04**	4.6 ± 0.66**	1.4 (1.0-2.0)
18±1.5**	2.2±0.04**	7.6 ± 0.52**	0.3 (0 -1.0)
12±1.6	1.9±0.12**	3.9 ± 0.47*	1.7 (0.5-3.0)
8±1.0	1.5±0.12	2.7 ± 1.2	0
10±0.74	1.8±0.16	3.5 ± 1.1	0.3 (0 -1.0)
12±1.1*	1.6±0.08*	3.4 ± 0.86*	0
13±0.77	1.9±0.21	0.90± 0.06	0.2 (0 -1.0)
14±0.91*	1.8±0.12*	3.1 ± 0.35*	0.4 (0 -1.0)
14±1.4*	1.8±0.10**	4.0 ± 1.4	0.2 (0 -1.0)
13±0.66	1.8±0.17	0.68± 0.24	0.4 (0 -2.0)
12±0.12	1.7±0.12	2.1 ± 0.47	0
11±1.1	1.7±0.15	0.77± 0.19	0
13±0.77	1.9±0.19*	1.3 ± 0.30	0
9±0.97	1.7±0.24	1.6 ± 1.3	0.6 (0 -3.0)
14±0.87	2.7±0.17	5.7 ± 0.44**	2.2 (1.5-3.0)
12±0.33**	2.4±0.38	4.9 ± 0.97*	2.0 (1.0-3.0)
13±0.98*	1.8±0.07**	3.7 ± 0.87*	0.8 (0 -1.0)
14±0.68*	2.6±0.22*	2.8 ± 0.73	0.6 (0 -2.0)
14±0.74*	2.4±0.30	1.9 ± 0.40	0.3 (0 -1.0)
10±0.20	1.9±0.32	1.6 ± 0.29	0.3 (0 -1.0)
16±2.6	2.1±0.31	3.1 ± 1.1	0.3 (0 -1.0)
13±0.92**	1.7±0.08*	2.2 ± 0.50	1.0 (0 -2.0)
9±0.13	1.7±0.26	[101 ±65.1][e]	0
9±0.52	1.6±0.10	[85 ±38.8]	0
9±0.53	1.8±0.13	[97 ±18.6]	0
11±0.63	2.1±0.24	[162 ±37.9]	0
10±1.0	1.8±0.19	[149 ±16.9]	0
9±0.78	1.3±0.06	[130 ±37.6]	0
9±0.57	1.4±0.07	[110 ±19.1]	0
11±1.2	1.6±0.05	[154 ±34.7]	0
10±0.69	1.6±0.12	[128 ±17.4]	0
12±0.45	1.3±0.11	[149 ±51.5]	0
11±0.41	1.4±0.09	[114 ±35.1]	0.1 (0 -0.5)
8±0.61	1.4±0.02	[59 ±26.2]	0

the opposite side.

(**) by the "Student" t-test.

ment of the L-alanine residue by glycine or L-serine on the immunoadjuvancies of molecules. It would be worth notice in this connection that a collaborative study with Dr. Y. Nagai and his colleagues (Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, Tokyo) has revealed that *N*-acetylmuramyl-L-seryl-D-isoglutamine and *N*-acetylmuramyl-glycyl-D-isoglutamine as well as *N*-acetylmuramyl-L-alanyl-D-isoglutamine worked as an effective adjuvant to a bovine myelin protein in the induction of experimental allergic encephalomyelitis in guinea pigs (unpublished observation).

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