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IMMUNOADJUVANT ACTIVITIES OF SYNTHETIC *N*-ACETYL-MURAMYL-PEPTIDES OR -AMINO ACIDS[1]

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SUMMARY A variety of *N*-acetylmuramyl-peptides (or -amino acids) were prepared by condensation of benzyl *N*-acetyl-4, 6-*O*-benzylidene-α-muramide with various peptide (or amino acid) benzyl esters by the dicyclohexylcarbodiimide—*N*-hydroxysuccinimide or ethylchlorocarbonate—*N*-methylmorpholine method and removal of the protecting groups by hydrogenolysis. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine was identified as the minimum structural entity essential for the immunoadjuvant activities characteristic of bacterial cell walls. Consequently *N*-acetylmuramyl-L-alanine was not adjuvant active. The tetrapeptide portion of adjuvant-active *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine proved to be inert, at least in induction of delayed-type hypersensitivity.

The possible adjuvant activities of various analogues or diastereomers of the above *N*-acetylmuramyl-dipeptide and related compounds were studied. *N*-Acetylmuramyl-L-alanyl-D-glutamic acid exhibited weak, but definite adjuvancy, but none of the others, including *N*-acetylmuramyl-L-alanyl-L-isoglutamine, *N*-acetylmuramyl-L-alanyl-D-glutamine and *N*-acetylmuramyl-L-alanyl-D-isoasparagine, had any adjuvant activity. This clearly indicated the importance of the configuration of the glutamic acid residue or its amides, i.e. the presence of the D-isoglutamine residue in the *N*-acetylmuramyl-dipeptide, for manifestation of adjuvant activities in stimulation of both antibody-mediated and cell-mediated immune responses. Neither *N*-acetylmuramyl-D-isoglutamine nor *N*-acetylmuramyl-D-alanine had any adjuvancy.

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INTRODUCTION

The preceding paper of this series (Kotani et al., 1975b) showed that *N*-acetylmuramyl-peptide subunit monomers isolated from the cell walls of *Staphylococcus aureus* and *Lactobacillus plantarum* by successive treatments with different peptidoglycan-degrading enzymes are the unit chemical structures responsible for manifestation of adjuvant activities to stimulate increased serum antibody levels and induce delayed-type hypersensitivity to ovalbumin when administered to guinea-pigs as water-in-oil emulsions.

This work was undertaken to confirm and extend the above findings and to elucidate the minimum or simplest chemical structure essential for the immunoadjuvant activities characteristic of bacterial cell walls. For this purpose the abilities of a variety of synthetic *N*-acetylmuramyl-peptides or -amino acids with natural or unnatural configurations, to stimulate or modify the humoral or cellular immune responses to ovalbumin in guinea-pigs were examined.

MATERIALS AND METHODS

1. *Synthesis of N-acetylmuramyl-peptides (or -amino acids)*

The fourteen *N*-acetylmuramyl-peptides (Va-n) listed in Table 1 were synthesized to test their possible immunoadjuvant activities. The syntheses of these compounds will be reported in detail elsewhere, but briefly the methods used were as follows.

The tetrapeptide moiety, i.e. N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-benzyloxycarbonyl-L-lysyl-D-alanine benzyl ester, was synthesized as follows. Coupling of *t*-butyloxycarbonyl-L-alanine with benzyl D-isoglutaminate, using dicyclohexylcarbodiimide—*N*-hydroxysuccinimide as a condensation reagent, afforded *t*-butyloxycarbonyl-L-alanyl-D-isoglutamine benzyl ester. This was then hydrogenolyzed to the free carboxyl peptide. This was condensed, applying the mixed anhydride method with ethyl chlorocarbonate, with N^{ε}-benzyloxycarbonyl-L-lysyl-D-alanine benzyl ester, which had been prepared from N^{α}-*t*-butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine and benzyl-D-alaninate by the dicyclohexylcarbodiimide method to give a protected tetrapeptide (IIa). Compound IIa was treated with trifluoroacetic acid to remove the *t*-butyloxycarbonyl group, and then condensed with the protected *N*-acetylmuramic acid (I), i.e., benzyl 2-acetamido-4, 6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (Flowers and Jeanloz, 1963), by the dicyclohexylcarbodiimide—*N*-hydroxysuccinimide method to afford the fully protected product (IVa). Removal of the protecting benzyl and benzylidene groups from IVa by hydrogenolysis in acetic acid with palladium black catalyst yielded *N*-acetylmuramyl-tetrapeptide (Va).

Other *N*-acetylmuramyl-peptides or -amino acids (Vb-n) were prepared by similar procedures, except that sometimes ethyl chlorocarbonate—*N*-methylmorpholine was used instead of dicyclohexylcarbodiimide—*N*-hydroxysuccinimide.

2. *Assay of the immunoadjuvant activities of synthetic N-acetylmuramyl-peptides or -amino acids*

The methods used were essentially as described in the first paper of this series (Kotani et al., 1975a).

RESULTS

1. *Immunoadjuvant activities of synthetic N-acetylmuramyl-peptides or -amino acids*

The adjuvant activities of synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysine, *N*-acetylmuramyl-L-alanyl-D-isoglutamine and *N*-acetylmuramyl-L-alanine were tested. Table 2 shows that *N*-acetylmuramyl-L-alanyl-D-isoglutamine was the minimum chemical entity for manifestation of a definite adjuvant effect to induce delayed-type hypersensitivity and to stimulate increased serum antibody levels. The minimum effective dose of *N*-acetylmuramyl-L-alanyl-D-isoglutamine was found to be around 12.5–25 μg/guinea-pig when administered as a water-in-oil emulsion with crystalline ovalbumin as a test antigen. *N*-Acetylmuramyl-L-alanine showed no adjuvancy.

The possible adjuvant activities of the tetrapeptide portion of the above adjuvant-active *N*-acetylmuramyl-tetrapetide was also

TABLE 1. *Yields and melting points of N-acetylmuramyl- and t-butyloxycarbonyl-peptide or -amino acid derivatives*

	Synthesized compound	II R=Boc, R^1=Z, R^2=Bzl	IV R=protected MurNAc, R^1=Z, R^2=Bzl			V R=MurNAc, R^1=H, R^2=H
		mp (C)	Method	Yield (%)	mp (C) (dec.)	Yield (%)
a	R-L-Ala-D-Glu(R^1)-L-Lys(R^1)-D-Ala-OR^2	191–194.5	A	76	221	85
b	R-L-Ala-D-Glu(R^1)-L-Lys-OR^2	133–137	A	87	221–224	79
c	R-L-Ala-D-Glu(OR^2)-NH_2	137.5–138	A	75	226.5–227.5	94
			B	79	224–225	
d	R-L-Ala-OR^2	—	A	77	215.5–217	67
e	R-D-Ala-OR^2	—	A	76	211–213	82
f	R-L-Ala-L-Glu(OR^2)-NH_2	133.5–134.5	A	83	243–245	92
g	R-L-Ala-D-Glu(OR^2)-OR^2	106.5–107	A	70	211	93
h	R-L-Ala-L-Glu(OR^2)-OR^2	52.5– 53.5	A	75	208	95
i	R-L-Ala-D-Glu(NH_2)-OR^2	143.5–144.5	A	87	246–248	85
j	R-L-Ala-L-Glu(NH_2)-OR^2	136–136.5	A	98	240	86
k	R-D-Glu(OR^2)-NH_2	—	A	80	243–245	92
l	R-L-Ala-D-Asp(OR^2)-NH_2	125.5–126.5	B	69	222–223	81
m	R-L-Ala-D-Ala-NH_2	128–128.5	B	83	284.5–285.5	90
n	R-L-Ala-D-Glu(OR^2)-NH_2[a]	137.5–138	B	77	248–249	73

[a] α-Methylglycoside.

Boc=*t*-butyloxycarbonyl, Z=benzyloxycarbonyl, Bzl=benzyl.

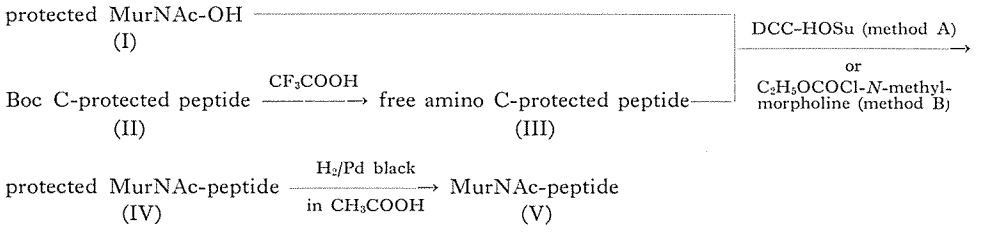

TABLE 2. *Induction of delayed-type hypersensitivity and stimulation of increased serum antibody levels to ovalbumin in guinea-pigs by synthetic N-acetylmuramyl-peptides*

No. of experimental group	Test material	Dose (μg)	Corneal response (48 hr) Mean (Range)	Skin response (48 hr)[a] Mean±S.E.[d]	Antibody level (Ratio)[b] Mean±S.E.[d]	IgG_2 Mean (Range)
	MurNAc-L-Ala-D-Glu(-L-Lys-D-Ala-OH)-NH_2					
13		200	2.8 (2.0-3.0)	11.7±0.46**	2.1±0.38*	2.0 (2.0-2.0)
13		100	2.6 (1.5-3.0)	11.8±0.97*	2.0±0.36*	2.1 (1.0-3.0)
13		50	2.7 (2.0-3.0)	12.1±0.56**	2.3±0.20**	2.3 (1.0-2.5)
18		25	2.8 (2.5-3.0)	12.4±0.97	1.5±0.14*	2.5 (1.0-3.0)
19		12.5	0.6 (0 -2.0)	4.4±0.40	0.9±0.26	0
	MurNAc-L-Ala-D-Glu(-L-Lys-OH)-NH_2					
13		100	2.6 (1.5-3.0)	11.7±1.21*	2.9±0.54**	1.9 (1.0-2.5)
		25	3.0	13.3±0.54**	3.7±0.34**	1.9 (1.0-3.0)
	MurNAc-L-Ala-D-Glu(-OH)-NH_2					
13		100	2.7 (1.5-3.0)	12.5±0.57*	2.6±0.16**	2.1 (1.0-3.0)
13		50	2.4 (1.0-3.0)	11.7±0.20**	2.5±0.19**	1.9 (1.0-2.5)
18		25	2.7 (1.0-3.0)	11.2±1.33	1.5±0.13*	2.4 (1.0-3.0)
18		12.5	2.0 (2.0-2.0)	10.8±0.25	1.3±0.25	1.8 (0.5-3.0)
13	MurNAc-L-Ala-OH	200	0	8.0±0.76	1.0±0.36	0.8 (0.5-1.5)
13		100	0	10.4±0.38	1.6±0.17	0.5 (0 -1.0)
	H-L-Ala-D-Glu(-L-Lys-D-Ala-OH)-NH_2					
13		200	0	5.4±2.34	1.5±0.23	0
19	MurNAc	200	0	10.7±0.26	1.0±0.08	0.9 (0 -1.5)
13	FICA-type control	—	0	7.1±0.91	[104±19][c]	0
18	FICA-type control	—	0.2 (0 -0.5)	10.7±0.75	[458±54]	1.2 (0.5-2.0)
19	FICA-type control	—	0	5.5±1.41	[168±15]	0

[a] Average diameter of redness (mm).

[b] Ratio of antibody nitrogen (μg/ml of serum specimen) in the test group to that in the respective control group.

[c] μg Antibody nitrogen/ml serum specimen.

[d] The difference between the test and respective control groups was significant at a level of 5% (*) or 1% (**), by the "Student" t-test.

TABLE 3. *Immunoadjuvant activities of various analogues of N-acetylmuramyl-L-alanyl-D-isoglutamine and related compounds*

No. of experimental group	Test material	Dose (μg)	Corneal response (48 hr) Mean (Range)	Skin response (48 hr)[a] Mean±S.E.	Antibody level (Ratio)[b] Mean±S.E.	IgG_2 Mean (Range)
20	MurNAc-L-Ala-L-Glu(OH)-NH_2	200	0.2 (0 -1.0)	ND[d]	1.0±0.21	0.2 (0 -1.0)
21		200	0	4.7±2.06	0.9±0.27	0.1 (0 -0.5)
21	MurNAc-L-Ala-D-Glu(NH_2)-OH	100	0.3 (0 -1.0)	8.7±0.66	1.4±0.16	0
21	MurNAc-L-Ala-L-Glu(NH_2)-OH	100	0.8 (0 -1.0)	8.7±1.22	1.5±0.44	0.3 (0 -1.0)
21	MurNAc-L-Ala-D-Glu(OH)-OH	100	1.8 (0.5-3.0)	9.3±1.54	2.8±0.88	0.9 (0 -2.5)
21	MurNAc-L-Ala-L-Glu(OH)-OH	100	0.3 (0 -1.0)	9.3±0.34	1.7±0.42	0.3 (0 -0.5)
22	MurNAc-L-Ala-D-Asp(OH)-NH_2	100	0.1 (0 -0.5)	10.3±1.80	0.8±0.15	0
22	MurNAc-L-Ala-D-Ala-NH_2	100	0.3 (0 -0.5)	9.3±0.18	1.3±0.09	0
22	MurNAc-D-Glu(OH)-NH_2	100	0.2 (0 -1.0)	10.4±0.61	1.7±0.44	0.1 (0 -0.5)
21	MurNAc-D-Ala-OH	200	0	9.3±0.58	1.6±0.61	0.8 (0 -2.5)
20	FICA-type control	—	0	6.0±3.0	[174±49][c]	0.1 (0 -0.5)
21	FICA-type control	—	0	5.5±1.41	[168±15]	0
22	FICA-type control	—	0.3 (0 -1.0)	9.5±0.47	[184±15]	0

[a] Average diameter of redness (mm).
[b] Ratio of antibody nitrogen (μg/ml serum specimen) in the test group to that in the respective control group.
[c] μg Antibody nitrogen/ml serum specimen.
[d] Not determined.

tested. This compound lacking the *N*-acetylmuramic acid residue, showed no activity, at least in induction of delayed-type hypersensitivity.

2. *Possible adjuvant activities of analogues of N-acetylmuramyl-L-alanyl-D-isoglutamine or N-acetylmuramyl-L-alanine*

As summarized in Table 3, none of the test analogues of the adjuvant-active natural *N*-acetylmuramyl-dipeptide, including *N*-acetylmuramyl-L-alanyl-L-isoglutamine, *N*-acetylmuramyl-L-alanyl-D-glutamine and *N*-acetylmuramyl-L-alanyl-L-glutamine showed no adjuvant activity, at least under the present experimental conditions. *N*-acetylmuramyl-L-alanyl-D-glutamic acid, however, was found to exhibit weak, but distinct activities to develop a positive corneal response and to stimulate increased serum antibody levels to ovalbumin in guinea-pigs. Attempts to obtain adjuvant-active *N*-acetylmuramyl-amino acid synthesized by replacement of the L-alanine residue of *N*-acetylmuramyl-L-alanine by D-alanine or D-isoglutamine were unsuccessful.

DISCUSSION

While this study was in progress Ellouz et al. (1974) reported independently, but in harmony with our results that *N*-acetylmuramyl-L-alanyl-D-isoglutamine synthesized by Merser and Sinaÿ (1974) was at least as adjuvant-active as the monomeric unit of the peptidoglycan, while synthetic *N*-acetylglucosaminyl-β-(1→4)-*N*-acetylmuramyl-L-alanine (Merser and Sinaÿ, 1973) showed no activity.

The findings reported here and by Ellouz et al. (1974) that L-alanyl-D-isoglutaminyl-L-lysyl D-alanine lacks the ability to induce delayed-type hypersensitivity seem to be inconsistent with the observation of Fleck et al. (1974) that this tetrapeptide was the smallest structure required for induction of delayed-hypersensitivity. The major difference between these studies was that in the former studies, ovalbumin was used as antigen, while azobenzenearsonate-*N*-acetyl-L-tyrosine was used in the latter. It should also be noted that the peptide monomer used in the study of Fleck et al. was not synthetic, but a natural product obtained, using *Bacillus licheniformis* amidase, from a disaccharide-peptide monomer of the *Escherichia coli* peptidoglycan. However, the tetrapeptide specimen used by Ellouz et al. was also a natural product obtained by treatment of a disaccharide-tetrapeptide from *E. coli* with *Myxobacter* amidase.

Assays of the immunoadjuvant activities of unnatural *N*-acetylmuramyl-dipeptides showed that the D-configuration of the glutamic acid residue and amidation of the α-carboxyl group of this amino dicarboxylic acid seem to be very critical for manifestation of the immunoadjuvancy of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, since the replacement of the D-isoglutamine residue by the L-isoglutamine or D-isoasparagine or amidation of the γ-carboxyl group instead of the α-carboxyl group of the D-glutamic acid residue was found to abolish the adjuvant activities of the *N*-acetylmuramyl-dipeptide completely. It may be added here that in a recent collaborative study with Drs. I. Azuma, I. Taniyama and Y. Yamamura, Department of Internal Medicine, Osaka University Medical School, it was shown by the method of Marbrook that the *N*-acetylmuramyl-di, -tri and -tetrapeptide described above, but not *N*-acetylmuramyl-L-alanine stimulated the *in vitro* production of antibody to sheep erythrocytes in mouse spleen cells.

Finally, with ragard to the synthesis of *N*-acetylmuramyl-peptides or -amino acids, it should be added that the free tetrapeptide corresponding to IIa (see Table 1) was prepared by Lefrancier and Bricas (1967) using Woodward's reagent K, and N^{α}-(*N*-acetylmuramyl-L-alanyl-D-α and γ-glutamyl)-L-lysyl-D-alanyl-D-alanine was also synthesized using the same Woodward's condensation reagent by Lanzilotti, Benz and Goldman (1964). However, the method described in the present report for preparation of *N*-acetylmuramyl-peptides or -amino acids has the advantages that it is simple

and inexpensive and can be used for preparation of *N*-acetylmuramyl-L-alanyl-D-isoglutamine in large quantity and for preparation of analogues of natural *N*-acetylmuramyl-peptides or -amino acids.

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REFERENCES

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.

Fleck, J., M. Mock, F. Tytgat, C. Nauciel, and R. Minck. 1974. Adjuvant activity in delayed hypersensitivity of the peptidic part of bacterial peptidoglycans. Nature 250: 517–518.

Flowers, H. M., and R. W. Jeanloz. 1963. The synthesis of 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (*N*-acetylmuramic acid) and of benzyl glycoside derivatives of 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (muramic acid). J. Org. Chem. 28: 2983–2986.

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

Kotani, S., Y. Watanabe, T. Shimono, F. Kinoshita, T. Narita, K. Kato, D. E. S. Stewart-Tull, K. Yokogawa, and S. Kawata. 1975b. Immunoadjuvant activities of peptidoglycan subunits from the cell walls of *Staphylococcus aureus* and *Lactobacillus plantarum*. Biken J. 18: 93–103.

Lanzilotti, A. E., E. Benz, and L. Goldman. 1964. Total synthesis of N^{α}-[1-(2-acetamido-3-*O*-D-glucosyl)-D-propionyl-L-alanyl-D-α- and γ-glutamyl]-L-lysyl-D-alanyl-D-alanine, and identity of the γ-glutamyl isomer with the glycopeptide of a bacterial cell wall precursor. J. Amer. Chem. Soc. 86: 1880–1881.

Lefrancier, P., and E. Bricas. 1967. Synthèse de la subunité peptidique de peptidoglycane de la parois de trois bacteries gram-positif et de peptides de structure analogue. Bull. Soc. Chim. Biol. (Paris). 49: 1257–1271.

Merser, C., and P. Sinaÿ. 1973. Synthesis of the repeating disaccharide unit of the glycan moiety of the bacterial cell wall peptidoglycan. Tetrahedron Lett. 1029–1032.

Merser, C., and P. Sinaÿ. 1974. Chemical synthesis of bacterial peptidoglycans. Abstr. Intern. Symp. on Bacterial Immunostimulants (Chemical structures, Mechanism of action, Applications) held at the Pasteur Institute, Paris, on Oct. 14 and 15. p. 9.