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IMMUNOADJUVANT ACTIVITIES OF PEPTIDOGLYCAN SUBUNITS FROM THE CELL WALLS OF *STAPHYLOCCUS AUREUS* AND *LACTOBACILLUS PLANTARUM*[1]

SHOZO KOTANI, YOSHIRO WATANABE, TSUTOMU SHIMONO, FUMIO KINOSHITA, TOSHIHIKO NARITA, KEIJIRO KATO, DUNCAN E. S. STEWART-TULL[2] and ICHIJIRO MORISAKI

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

KANAE YOKOGAWA and SHIGEO KAWATA

Research and Development Division, Dainippon Pharmaceutical Co., Suita, Osaka

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SUMMARY Attempts were made to isolate and identify the unit chemical structure essential for manifestation of the immunoadjuvant activities characteristic of bacterial cell walls. The *N*-acetylmuramyl-peptide subunit monomers, N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(glycylglycyl)-L-lysyl-D-alanine from the cell walls of *Staphylococcus aureus* (FDA 209P) and *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelic acid and/or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine from those of *Lactobacillus plantarum* (ATCC 8014), were shown to be unit chemical entities with definite adjuvant activity both in stimulation of antibody production and in induction of delayed-type hypersensitivity to ovalbumin when administered to guinea-pigs as water-in-oil emulsions.

INTRODUCTION

As reported in the first paper of this series (Kotani et al., 1975), using cell wall lytic enzymes with different modes of action and substrate specificities, immunoadjuvant principles were solubilized with full retention of activities to stimulate both antibody-mediated and cell-mediated immune responses from *Staphylococcus aureus*, *Streptococcus pyogenes*, *Strepto-*

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2 Visiting professor on a Royal Society of London Travelling Fellowship. Present address: Microbiology Department, University of Glasgow, Glasgow, Great Britain.

coccus salivarius, *Streptococcus faecalis*, *Streptococcus mutans* and *Lactobacillus plantarum* as well as *Mycobacterium smegmatis*, *Corynebacterium diphtheriae* and *Actinomyces viscosus*.

In the present work the adjuvant-active, hydrosoluble higher molecular weight components (HMWC's) from the cell walls of *S. aureus* and *L. plantarum* were digested successively with peptidoglycan-degrading enzymes and possible changes in their adjuvant activities caused by these treatments were examined, to elucidate the unit chemical structure responsible for the immunoadjuvancy characteristic of bacterial cell walls. The enzymes used for this purpose were known to differ in their modes of action from the enzymes used to prepare the HMWC's.

MATERIALS AND METHODS

1. *Cell wall preparations used as starting materials*

Cells of *S. aureus* (FDA 209P) were grown with aeration in nutrient broth (Nissui Pharmaceutical Co., Tokyo), and harvested after cultivation for 20 hr at 37 C. Strain ATCC 8014 of *L. plantarum* was cultivated at 37 C in medium containing 2% glucose, 1% polypeptone, 1% meat extract, 0.5% NaCl, 0.2% yeast extract, 0.1 mM $MnSO_4$ and CH_3COONa in tap water, at pH 7.5 for 24 hr and then harvested.

Cells of *L. plantarum* (30 g wet weight) were washed thoroughly with physiological saline and suspended in 30 ml saline. After adding 30 g of glass beads of 0.17–0.18 mm diam. (Glasperlen, Kat. Nr. 2884, B. Braun Apparatebau, Melsungen, West-Germany) the cell suspension in 70 ml Braun flask was submitted to mechanical disruption (4,000 oscillations/sec) in a Braun Mechanical Cell Homogenizer. The disrupted cell suspension was separated from the glass beads, and the latter were thoroughly washed with physiological saline. The cell suspension and washings were combined and centrifuged at $700\times g$ for 10 min to remove unbroken cells and coarse debris. The sodium chloride was added to a final concentration of 1 M and the mixture was centrifuged at $9,000\times g$ for 30 min. The precipitated cell wall fraction was thoroughly washed with 1 M sodium chloride solution, suspended in 0.05 M phosphate-buffer, pH 7.0 and digested with trypsin (0.8 g/100 g wet weight of cell wall fraction) at 37 C for 4 hr. After removal of coarse debris by centrifugation at $800\times g$ for 15 min, the digestion mixture was centrifuged at $10,000\times g$ for 30 min. The sedimented cell walls were thoroughly washed with the same buffer and then with distilled deionized water, and lyophilized.

A purified specimen of the cell wall of *S. aureus* was prepared in essentially the same way.

2. *Peptidoglycan-degrading enzymes*

The source and mode of action of the Kyowa lytic #2 enzyme (Kyowa Fermentation Industry, Tokyo), are strikingly similar to those of the *Flavobacterium* L–11 enzyme (Kotani et al., 1975). This enzyme was purified by CM-cellulose column chromatography by the method described previously (Hirata, 1970; Hamada et al., 1971) for purification of the L–11 enzyme. A pure specimen of Mutanolysin, which appeared homogeneous on disc electrophoresis, was obtained as described elsewhere (Yokogawa et al., 1974). A specimen of the D-alanine-*meso*-diaminopimelic acid (Dpm) endopeptidase from the *Streptomyces* L–3 enzyme was obtained by DEAE-cellulose and CM-cellulose column chromatographies and isoelectric focusing with carrier ampholite (pH 7–10) by Dr. T. Katayama of this laboratory (Katayama, 1973). An exo-β-*N*-acetylglucosaminidase was prepared from pig epididymis by the method of Findlay, Levvy and Marsh (1958) and was a gift from Dr. H. Suginaka of this laboratory (Murayama, Kotani and Kato, 1968).

3. *Immunological and chemical methods*

The *N*-acetylhexosamine assay method of Reissig, Strominger and Leloir (1956) as modified by Ghuysen, Tipper and Strominger (1966) was used for detection of disaccharides of hexosamines by assay of *N*-acetylhexosamine. The other methods used here were essentially as described in the preceding paper (Kotani et al., 1975).

RESULTS

1. *Stepwise degradation of the cell walls of S. aureus and L. plantarum by successive treatments with different peptidoglycan-degrading enzymes*

Fig. 1 shows an outline of the procedures used for isolation of peptidoglycan subunits of various structural complexities from the cell

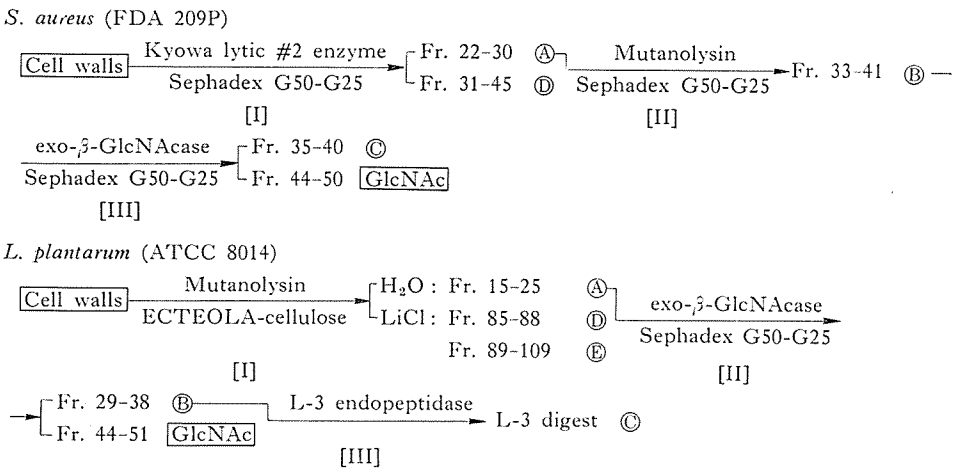

FIGURE 1. *Procedure for isolation of peptidoglycan subunits of different structural complexity from the cell walls of S. aureus (FDA 209P) and L. plantarum (ATCC 8014).*

walls of *S. aureus* and *L. plantarum.*

S. aureus (Fig. 2): Cell walls (999 mg) were incubated with the Kyowa lytic #2 enzyme (one cell wall lytic unit/mg of cell walls) in a total volume of 420 ml of 0.05 M phosphate buffer, pH 8.0, at 37 C. A small amount of chloroform was added as a preservative. After almost complete solubilization (95% reduction in optical density of the reaction mixture) on incubation for 24 hr, the solubilized material was concentrated under reduced pressure in a rotary evaporator and applied to a Sephadex G–50 column (1.9 $\phi \times$ 96 cm) connected in series with a Sephadex G–25 column (2.5 $\phi \times$ 96 cm) through a polyethylene capillary tube. These Sephadex gel columns were used in the same way for the other filtration experiments described below. The columns were developed with deionized water at a flow rate of about 50 ml/hr and fractions of 10 ml were collected. Aliquots of each fraction were analyzed for free amino-groups, total hexosamines and organic phosphorus. The first peak fraction (Nos. 22–30 in Fig. 2 [I], Fraction A) contained hexosamines and free amino-groups but very little organic phosphorus while the second peak fraction (Nos. 31–45, Fraction D) had a high phosphorus content. The yields of these two fractions were 251 mg and 355 mg, respectively. An aliquot (45 mg) of Fraction A was treated with 1.5 mg of purified Mutanolysin in 0.005 M sodium acetate buffer, pH 6.5 (45 ml) for 33 hr at 37 C. During this period 1.5 moles of reducing groups were liberated/mole of total glutamic acid residues of the substrate, suggesting that the glycan portion of Fraction A was completely cleaved to a disaccharide unit of the *N*-acetylglucosaminyl-β-(1 $\rightarrow$ 4)-*N*-acetylmuramic acid type (Ghuysen, Tipper and Strominger, 1966). Another aliquot of Fraction A was incubated in the same way without the enzyme. The test and control reaction mixtures were fractionated by gel filtration on Sephadex columns and their reducing sugar contents were determined. Fig. 2 shows that the molecular size of Fraction A decreased considerably during incubation with the endo-β-*N*-(, 6-*O*-di)-acetylmuramidase of Mutanolysin, indicating degradation into smaller fragments. Fractions, Nos. 33–41, were pooled and lyophilized (24.7 mg, Fraction B). An aliquot (7.5 mg) of Fraction B was then treated with excess exo-β-*N*-acetylglucosaminidase in 3.75 ml of 0.02 M citrate buffer, pH 4.4, at 37 C. After incubation for 29 hr, the reaction mixture was filtered through the connected Sephadex gel columns. Fractions, Nos. 35–40 and 44–50, were pooled

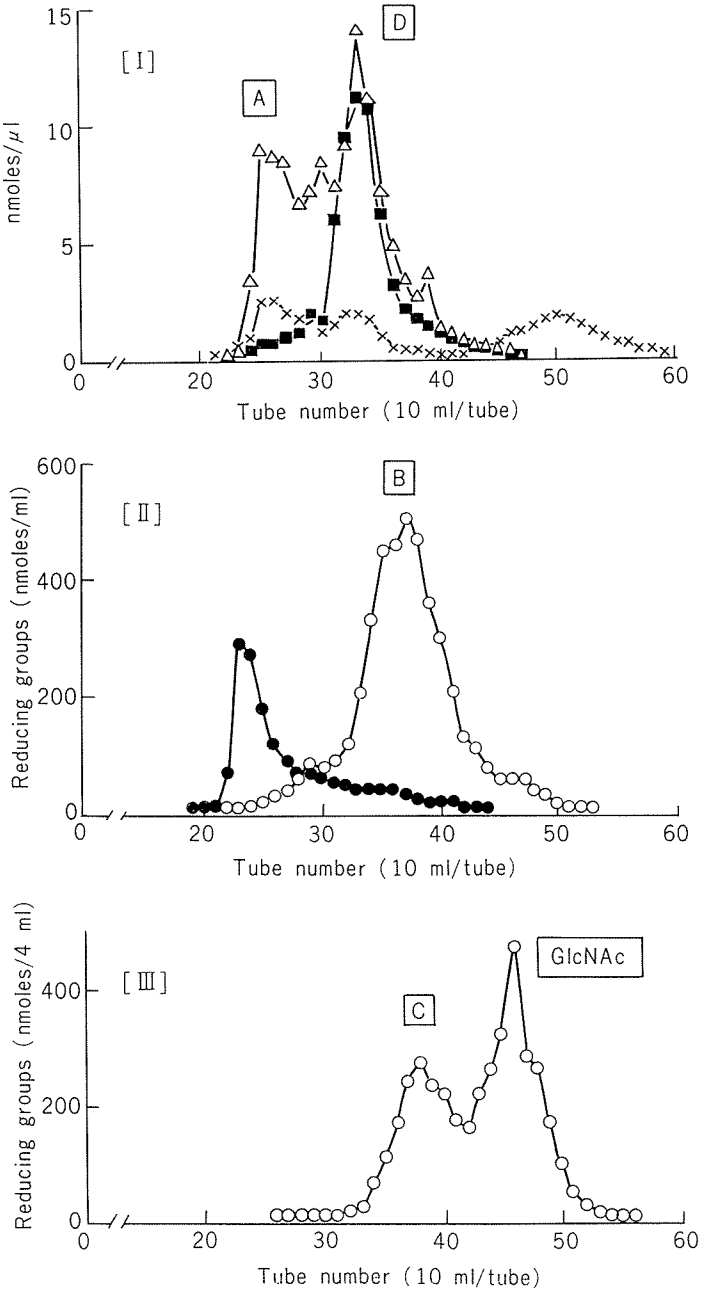

FIGURE 2. *Isolation of peptidoglycan subunits of varying structural complexity by fractionation of the digest of S. aureus (FDA 209 P) cell walls treated successively with Kyowa lytic #2 enzyme, Mutanolysin and exo-β-N-acetylglucosaminidase. Total hexosamines* (△——△), *free amino groups* (×——×), *organic phosphorus* (■——■), *with enzyme* (○——○) *and without enzyme* (●——●).

separately. The former (Fraction C) was lyophilized (6.75 mg), while the latter was subjected to paper chromatographic analysis and found to consist of a single *N*-acetylglucosamine, split off by the enzymic action. It may be added here that following the method of Reissig, Strominger and Leloir (1955) before treatment with exo-β-*N*-acetylglucosaminidase the preparation showed maximum chromogen formation after heating at 60 C for 30 min in borate buffer, while after the enzymic treament the maximum colour was obtained after heating for 7 min. This suggests that the glycosidic linkage of a disaccharide moiety of the peptidoglycan subunit in Fraction B was hydrolyzed.

L. plantarum (Fig. 3): A specimen of 3.0 g of cell walls after digestion with trypsin was solubilized with 170 μg of purified Mutanolysin in 200 ml of 0.005 M sodium acetate buffer, pH 6.5 at 37 C. The incubation mixture was overlaid with toluene as a preservative. After incubation for 24 hr the preparation was centrifuged to sediment insoluble materials (12.5 mg). The supernatant was concentrated to 10 ml under reduced pressure and lyophilized (3.0 g). The lyophilized material was dissolved in 30 ml of water, and desalted in 7.5 ml volumes by filtration through a Sephadex G-50 gel column (2.0 φ×75 cm) connected in series with a Sephadex G-25 gel column (2.5 φ×

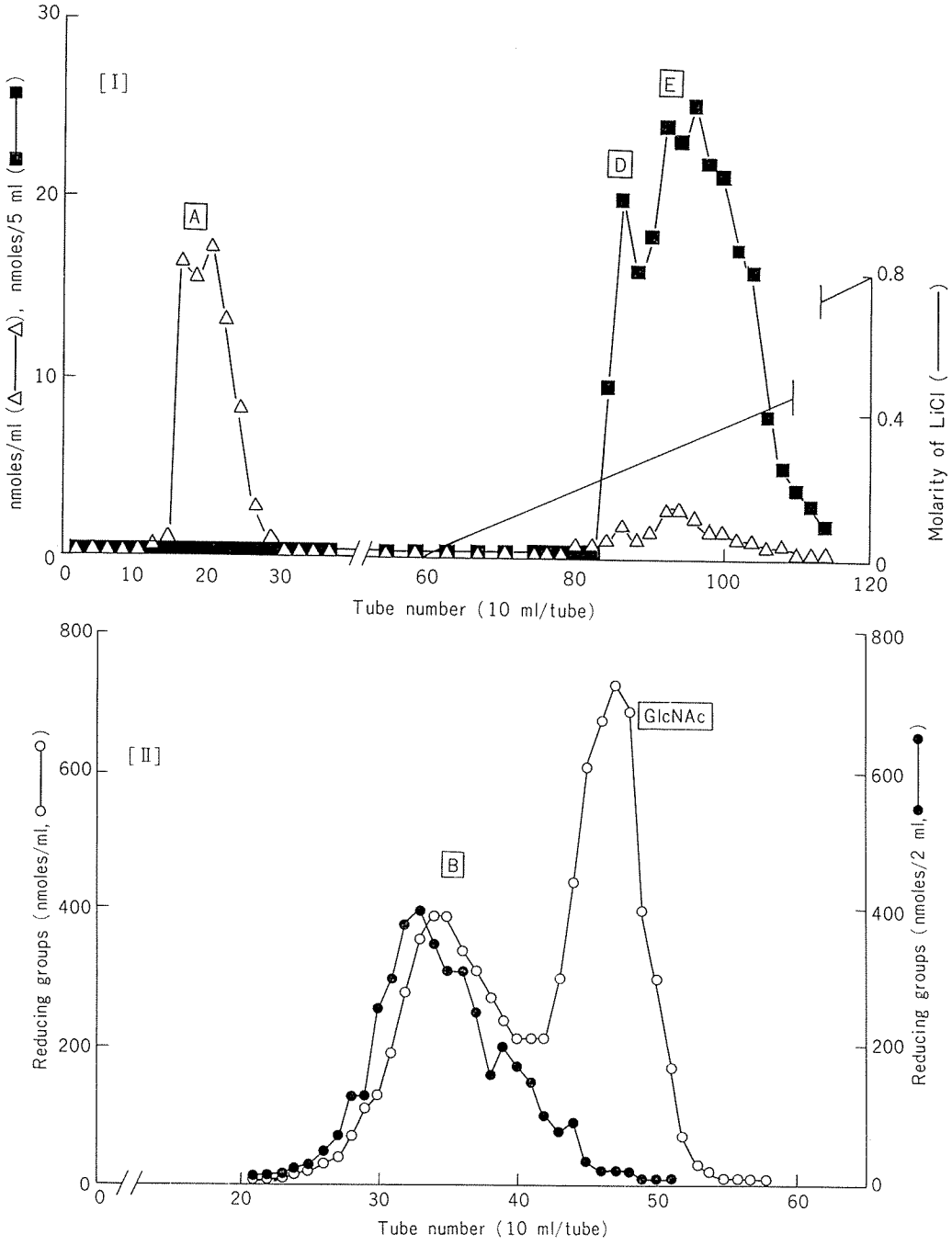

FIGURE 3. *Isolation of peptidoglycan subunits with or without associated nonpeptidoglycan moiety from Mutanolysin digest of L. plantarum (ATCC 8014) cell walls and treatment of the latter subunit with exo-β-N-acetylglucosaminidase. Reducing groups* (△——△), *methylpentoses* (■——■), *with enzyme* (○——○) *and without enzyme* (●——●).

75 cm). The desalted material was concentrated and lyophilized (2.88 g, 96% yield). An aliquot (1 g) of the preparation was dissolved in 3 ml of deionized water and applied to an ECTEOLA-cellulose column (3.6 $\phi \times 80$ cm). The column was developed first with deionized water and then with a linear gradient of 0 to 0.8 M LiCl solution, at pH 5.0. As shown in Fig. 3 [I], three portions differing in their contents of reducing groups and methylpentoses were obtained: Fraction A (Nos. 15–25) was eluted with water while Fraction D and Fraction E (Nos. 85–88 and 89–109, respectively) were eluted with LiC!. Fractions D and E were desalted by filtration through connected columns of Sephadex gels as described above. The yields of Fractions A, D, and E were 384 mg (38% yield), 76 mg (7.6%) and 311 (31.1%), respectively.

An aliquot (70.4 mg) of Fraction A was treated with excess exo-β-*N*-acetylglucosaminidase in 14 ml of 0.05 M citrate buffer, at pH 4.4. After incubation for 132 hr at 37 C, the digest showed maximum chromogen formation in the *N*-acetylhexosamine assay after heating for 7 min in borate buffer. It was then applied to connected columns of Sephadex G–50 and G–25 gel. The control mixture, incubated in the same way without enzyme, gave the maximum colour yield after heating for 30 min. Two peak fractions with reducing groups, Nos. 29–38 (Fraction B) and Nos. 44–51 (*N*-acetylglucosamine), were eluted successively with deionized water, while the control reaction mixture gave only one peak fraction, as shown in Fig. 3 [II]. Fraction B (5.4 mg) was incubated with sufficient purified L–3 endopeptidase to hydrolyze the D-alanine-*meso*-diaminopimelic acid linkages between neighbouring peptide subunits in 60 hr in 0.02 M phosphate buffer, pH 7.8, at 37 C. Free amino groups increased from 570 to 990 nmoles/mg of substrate during the incubation. Very little hydrolyzed material (Fraction C) was available so it was assayed for immunoadjuvant activity without further fractionation.

2. *Chemical structures of the peptidoglycan subunits isolated from the cell walls of S. aureus and L. plantarum by successive treatments with different peptidoglycan-degrading enzymes*

The analytical data on the amino acid and amino sugar compositions, amino-terminal and carboxyl-terminal amino acids (estimated by dinitrophenylation and hydrazinolysis, respectively) and chain lengths of the glycan portions (estimated by borohydride reduction) of the test peptidoglycan subunits with or without a non-peptidoglycan moiety ("special structure") are shown in Table 1. Other data on the non-peptidoglycan polymers are also included in this table. The chemical structures drawn in Figs. 4 and 5 are proposed for the peptidoglycan subunits with various structural complexities from the cell walls of *S. aureus* and *L. plantarum* respectively, on the basis of the analytical data presented above and information accumulated so far on the chemical characteristics of the cell walls of *S. aureus* (e.g. Kato and Strominger, 1968; Kato et al., 1968) and of *L. plantarum* (Matsuda, Kotani and Kato, 1968a; Matsuda, Kotani and Kato, 1968b). For instance, in the case of Fraction A from *L. plantarum* cell walls, about half the Dpm residues disappeared during dinitrophenylation of the fraction, indicating that one of the two moles of Dpm residues has at least one free amino-group. All the muramic acid residues were reduced to muramicitol by treatment with sodium borohydride, indicating that the glycan portion of this fraction consists of a disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid, with the latter at the reducing end. Fraction C from *S. aureus*, on the other hand, was shown to have one of the two moles of glycine residues as an amino-terminal amino acid, alanine as the carboxyl-terminal residue, and one mole of reducing muramic acid per mole of total glutamic acid residue as shown in Fig. 4.

TABLE 1. *Chemical structural analyses*[a] *of peptidoglycan subunits isolated from the cell walls of S. aureus (FDA 209P) and L. plantarum (ATCC 8014)*

Test fraction	Treatment[a]	GlcN[c]	Mur[c]	Ala	Glu	Lys	[Dpm]	Gly	M6P[b,c]	Murø[c]	Others[c]
S. aureus											
Ⓐ	[1]	0.99	0.98	2.53	1.00	1.06		1.99	—		P_0 (0.21)
	[2]	0.98	0.97	2.38	1.00	0.94		1.03			
	[3]	0.79	0.91	2.22	1.00	0.68		2.26			
Ⓑ	[1]	0.83	1.09	2.40	1.00	0.89		1.79	—		
	[2]	0.68	0.48	2.14	1.00	0.85		1.21			
	[3]	0.68	0	2.54	1.00	1.00		2.38		0.87	
	[4]	—	—	+	—	—					
Ⓒ	[1]	tr[e]	0.85	1.85	1.00	0.81		1.85	—		NH_3 (0.98)
	[2]	tr	0.68	2.44	1.00	0.92		0.90			
	[3]	tr	tr	2.55	1.00	1.01		1.82		1.11	
	[4]	—	—	+	—	—		—			
Ⓓ	[1]	1.94	0.97	2.72	1.00	0.76		1.95	—		P_0 (2.95)
	[2]	2.03	0.91	2.20	1.00	0.84		0.95			
	[3]	1.64	0.84	2.37	1.00	0.95		1.74			
L. plantarum											
Ⓐ	[1]	0.70	1.07	1.75	1.00		[1.17]		—		NH_3 (2.37), Rham (0.02), Glc (0.18), P_0 (0.24)
	[2]	0.75	0.54	1.43	1.00		[0.52]				
	[3]	0.66	tr	1.72	1.00		[1.14]			0.81	
Ⓑ	[1]	tr	1.04	1.48	1.00		[1.00]		—		
	[2]	tr	0.85	1.54	1.00		[0.58]				
	[3]	0	0	1.84	1.00		[1.23]			0.93	
	[4]	—	—	tr	—		—				
Ⓒ	[1]	tr	1.01	1.90	1.00		[1.34]		—		
	[2]	tr	0.47	1.55	1.00		[tr]				
	[3]	tr	0	1.85	1.00		[1.12]			+	
	[4]	—	—	+	—		—				
Ⓓ	[1]	3.85	0.91	1.57	1.00		[1.13]		+		Rham (7.68), Glc (12.8), P_0 (3.80)
	[2]	4.11	0.72	1.51	1.00		[0.58]				
	[3]	4.08	tr	1.59	1.00		[1.10]			0.96	
Ⓔ	[1]	1.08	0.91	1.75	1.00		[1.04]		+		NH_3 (2.36), Rham (3.12), Glc (15.2), P_0 (8.36)
	[2]	1.13	0.63	1.51	1.00		[0.55]				
	[3]	1.22	0	1.72	1.00		[1.12]			0.69	

[a] [1], None; [2], dinitrophenylation; [3], reduction with $NaBH_4$; [4], hydrazinolysis.

[b] Factors of 1.47 for muramic acid and of 3 for muramicitol were used to correct for destruction during acid hydrolysis.

[c] GlcN, glucosamine; Mur, muramic acid; M6P, muramic acid-6-phosphate; Glc, glucose; P_0, organic phosphorus; Murø, muramicitol; Rham, rhamnose.

[d] All data are expressed as moles/mole of glutamic acid residues.

[e] Trace.

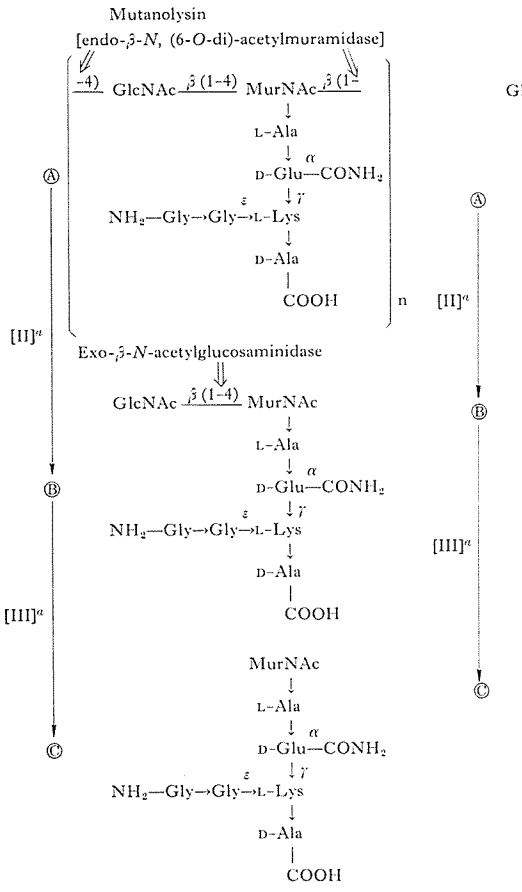

FIGURE 4. *Chemical structures of peptidoglycan subunits liberated from S. aureus (FDA 209P) cell walls by various peptidoglycan-degrading enzymes.* [a]*See Fig. 1. MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine.*

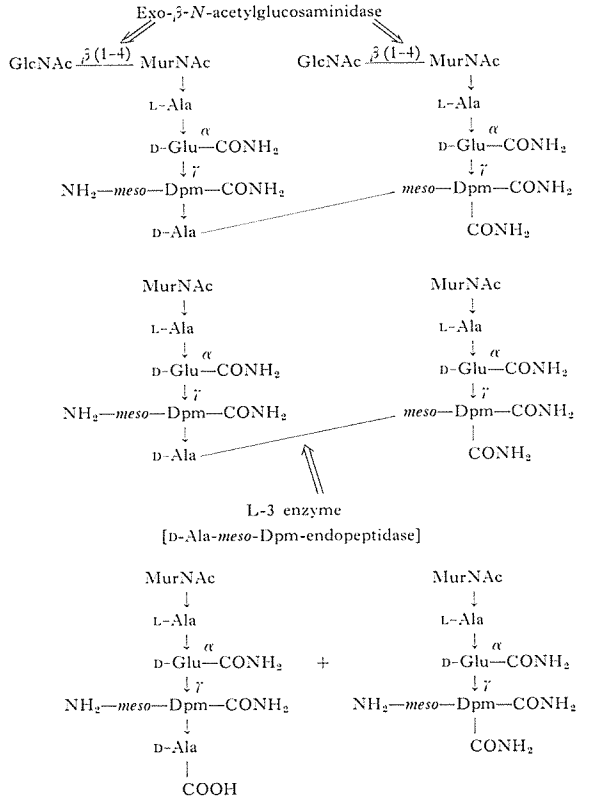

FIGURE 5. *Chemical structures of peptidoglycan subunits liberated from L. plantarum (ATCC 8014) cell walls by various peptidoglycan-degrading enzymes.* [a]*See Fig. 1. MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine.*

3. *Immunoadjuvant activities of peptidoglycan subunits isolated from the cell walls of S. aureus and L. plantarum*

It is apparent from Table 2 that N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-(glycylglycyl)-L-lysyl-D-alanine from *S. aureus* cell walls and *N*-acetylmuramyl-D-isoglutaminyl-*meso*-diaminopimelic acid and/or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine from *L. plantarum* cell walls, as well as the more complex peptidoglycan subunits, are definitely active in induction of delayed-type hypersensitivity and in stimulation of increased serum antibody levels to ovalbumin in guinea-pigs. In the case of the peptidoglycan subunits from *L. plantarum* cell walls, the minimum effective dose of Fraction C proved to be less than 25 μg/guinea-pig when administered as a water-in-oil emulsion.

DISCUSSION

The present study provided direct evidence

TABLE 2. *Induction of delayed-type hypersensitivity and stimulation of increasea antibody levels to ovalbumin in guinea-pigs by peptidoglycan subunits from S. aureus (FDA 209P) and L. plantarum (ATCC 8014) cell walls*

No. of experimental group	Test fraction	Dose (μg)	Corneal response (48 hr) Mean (Range)	Skin res ponse (48 hr) Mean±S.E.[a,g]	Antibody level (Ratio)[b] Mean±S.E.[g]	IgG_2 Mean (Range)
	S. aureus (FDA 209P)					
15	Ⓐ: [Disaccharide-hexapeptide[c]]$_n$	100	3.0	14.1±1.00**	6.7±1.22**	3.0
15	Ⓑ: Disaccharide-hexapeptide	100	2.6 (1.5-3.0)	11.5±0.76**	6.5±0.47**	2.9 (2.5-3.0)
15	Ⓒ: MurNAc-hexapetide	200	3.0	10.8±0.81*	3.4±0.71**	1.5 (1.0-2.5)
15	Ⓓ: Ⓐ-ribitol teichoic acid complex	100	3.0	10.0±0.91*	7.2±1.26**	3.0
	L. plantarum (ATCC 8014)					
15	Ⓐ: Disaccharide- tetrapaptide[d]-	100	3.0	10.4±0.13*	4.7±0.85**	2.1 (1.5-3.0)
16	tripeptide[e]-disaccharide	50	2.8 (2.0-3.0)	13.8±1.02**	2.3±0.08	2.1 (1.0-3.0)
15	Ⓑ: MurNAc-tetrapeptide-tripeptide-	200	3.0	11.5±0.76**	4.8±0.51**	2.4 (2.0-3.0)
15	MurNAc	100	2.9 (2.5-3.0)	14.8±1.02**	6.3±0.71**	2.5 (1.5-3.0)
15		50	2.5 (1.5-3.0)	10.6±0.24*	4.8±0.52**	1.9 (1.0-3.0)
15	Ⓒ: A mixture of MurNAc-tetrapeptide	200	3.0	9.8±0.98	2.9±0.50**	1.5 (0 -2.0)
17	and MurNAc-tripeptide	100	3.0	13.0±1.39**	4.0±0.96*	2.3 (1.5-3.0)
17		50	2.8 (2.5-3.0)	10.8±0.25	5.0±0.56	1.5 (1.0-2.0)
17		25	2.9 (2.5-3.0)	13.5±1.54**	5.1±0.91**	2.6 (1.0-3.0)
15	Ⓓ: Ⓐ-"Special structure 1"	100	2.7 (1.5-3.0)	14.4±1.47**	2.6±0.52**	2.9 (2.5-3.0)
16	Ⓔ: Ⓐ-"Special structure 2"	200	2.8 (2.0-3.0)	12.4±0.75**	2.7±0.19*	2.0 (2.0-2.0)
15		100	1.6 (1.0-2.0)	13.2±1.28**	3.1±0.27**	1.9 (1.5-2.0)
16		50	0.8 (0 -2.0)	10.0±1.26	1.4±0.37	1.2 (0 -3.0)
15	FICA-type control	—	0	6.9±0.78	[107±22][f]	0.3 (0 -2.0)
16	FICA-type control	—	0	8.0±0.42	[64±31]	0.3 (0 -1.0)
17	FICA-type control	—	0	6.5±1.09	[157±40]	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] N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-(glycylglycyl)-L-lysyl-D-alanine.
[d] L-alanyl-D-isoglutaminyl-*meso*-2, 6-diaminopimelyl-D-alanine.
[e] L-alanyl-D-isoglutaminyl-*meso*-2, 6-diaminopimelic acid.
[f] μg Antibody nitrogen/ml serum specimen.
[g] The difference between the test and respective control groups was significant at a level of 5% (*) or 1% (**), by the "Student"t-test.

that the unit chemical structure responsible for the adjuvant activities of bacterial cell walls in stimulation of both antibody-mediated and cell-mediated immune responses is an *N*-acetylmuramyl-peptide subunit monomer (the constituent diamino acid, L-lysine or *meso*-Dpm, is unimportant). After this study was finished, essentially similar findings were reported by Ellouz et al. (1974), independently from us, on the unit chemical structure essential for manifestation of immunoadjuvant activities. It is pertinent to add here that there seem to be some ambiguities and contradictions in their report with regard to the assessment of positive or negative adjuvant activity in the induction of delayed-type hypersensitivity.

It should also be pointed out that there are some seemingly conflicting results in our own studies; namely the cell walls of *Micrococcus lysodeikticus* and *Staphylococcus epidermidis* were found to be adjuvant-inactive in the study reported in the preceding paper (Kotani et al., 1975) although they contain a *N*-acetylmuramyl-peptide subunit, very similar in fundamental structure to that of *S. aureus* and *L. plantarum* cell walls, as a building block of peptidoglycans. Incidentally, Ellouz et al. (1974) reported that *N*-acetylmuramyl-tetrapeptide isolated from the cell walls of *S. epidermidis* (the strain name not mentioned in their report) had positive adjuvant activity. Further work is required on these discrepancies.

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