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GELATION OF THE AMOEBOCYTE LYSATE OF *TACHYPLEUS TRIDENTATUS* BY CELL WALL DIGEST OF SEVERAL GRAM-POSITIVE BACTERIA AND SYNTHETIC PEPTIDOGLYCAN SUBUNITS OF NATURAL AND UNNATURAL CONFIGURATIONS

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SUMMARY Water-soluble adjuvant-active fractions prepared by digestion of the cell walls from several gram-positive bacteria with an endo-*N*-actetylmuramidase (Mutanolysin) were shown to gelate the amoebocyte lysate of *Tachypleus tridentatus*, the Japanese horseshoe crab. This finding was further extended by examination of the clotting activities on the amoebocyte lysate of a synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine and related compounds, either adjuvant-active or -inactive, demonstrating that there are in general close correlations between the abilities to initiate lysate gelation, immunoadjuvancies and pyrogenicities, although there are some exceptions.

INTRODUCTION

As one of the endotoxin-like properties of gram-positive bacterial cell wall peptidoglycans (Rotta, 1975; Heymer, 1975), peptidoglycan specimens prepared by extraction of some gram-positive bacterial cell walls with hot 10% trichloroacetic acid were shown to induce gelation of the amoebocyte lysate of *Limulus polyhemus*, the American horseshoe crab (the

Limulus assay), although the gelling activity was much weaker than that of *Escherichia coli* endotoxic lipopolysaccharide (Wildfeuer et al., 1975).

To elucidate the chemical structures responsible for gelation of the amoebocyte lysate by cell wall peptidoglycans, enzymatically solubilized cell wall materials from seven gram-positive bacterial species were submitted to the Limulus assay. A total of 21 different synthetic *N*-acetylmuramyl peptides and related compounds, with or without configurations inherent to natural peptidoglycans, were also assayed for initiation of the lysate gelation, with special reference to possible correlations with the pyrogenicities and immunoadjuvancies.

MATERIALS AND METHODS

1. *Preparation of the endo-N-acetylmuramidase digest of several gram-positive bacterial cell walls*

Streptococcus salivarius (IFO 3350), *Streptococcus mutans* (BHT) and *Lactobacillus plantarum* (ATCC 8014): A specimen of 100 mg of cell walls was incubated with 5.7 μg of purified Mutanolysin (M-1 enzyme, Yokogawa et al., 1974, 1975) in 4.5 ml of 0.005 M sodium acetate buffer, pH 6.5 at 37 C, with a few drops of toluene as a preservative. After a 24 hr incubation, an almost completely solubilized cell wall digest was centrifuged to sediment insoluble residue. The supernatant fluid was concentrated under reduced pressure, and submitted to gel filtration through a Sephadex G-50 column (1.5 $\times$70 cm) connected in series with a Sephadex G-25 column (1.5$\times$80 cm). The peak fractions eluated with water, in terms of reducing power and methylpentose content, were pooled and lyophilized.

Other cell wall digests (*Staphylococcus aureus*, FDA 209P, *Staphylococcus epidermidis* ATCC 155, *Micrococcus lysodeikticus* NCTC 2665 and *Actinomyces viscosus* ATCC 15987): The conditions adopted for preparation were essentially the same as those described in the preceding paragraph, except that a solubilized material obtained by removal of an insoluble residue was used as a test specimen without gel filtration. The amount of solubilized material was calculated by substracting the amount of insoluble residue from that of a cell wall specimen used for digestion.

2. *Synthetic N-acetylmuramy. peptides and related compounds*

The syntheses and properties of *N*-acetylmuramyl-peptides, -amino acids and peptides were reported previously (Kusumoto et al., 1976). Syntheses of 6-*O*-acetyl- and 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine and of *N*-acetylmuramyl-L-alanyl-D-glutamyl-glycine will be published elsewhere.

3. *Assay for gelation of the amoebocyte lysate of T. tridentatus*

Meticulous care was taken to prevent contamination of test specimens with extraneous endotoxic lipopolysaccharide: All glasswares were thoroughly cleaned and then heated in an oven at 250 C for 60 to 90 min. Test specimens were dissolved in pyrogen-free normal saline solution (an officinal), at a concentration of 5 mg/ml unless otherwise stated. If necessary, the solution was adjusted to pH 6.0 to 7.5 by addition of 0.1 N sodium hydroxide. The neutralized solution of the test specimen was then filtered through a membrane filter (MF-Millipore® VS with an average pore size 0.025 μm, Nihon Millipore Ltd., Tokyo) held on a 13 mm Syringe Filter Holder (Sartorius-Membranfilter GmBH, West Germany).

To 0.1 ml aliquots of Pre Gel (an amoebocyte lysate prepared from *T. tridentatus*, Teikoku Zoki Pharmaceutical Co., Tokyo) dissolved in ampoules with normal saline solution, 0.1 ml aliquots of serial dilutions of the filtered test specimen described above were added. Dilution and distribution of test specimens and dilutents were made by use of a pyrogen-free disposable plastic syringe with needle (TERUMO Co., Tokyo).

The ampoules were allowed to stand in an incubator at 37 C for one hr, and at room temperature at 5 min. For each set of test, the ampoules containing either 0.1 ml of the lysate and 0.1 ml normal saline solution or 0.1 ml of the lysate and 0.1 ml of a solution (1 ng/ml) of *E. coli* 0111-B4 endotoxin were set up as a minus- and plus-control, respectively. Gelling of the amoebocyte lysate was graded as follows: solid gelation (##), incomplete gelation (#), increased opacity and/or increased viscosity (+) and no reactions (−).

4. *Assay for the immunoadjuvancies and pyrogenicity test*

These were essentially as described in the preceding papers (Kotani et al., 1975a; 1976).

RESULTS

The assay for gelation of the amoebocyte lysate of *T. tridentatus* has been made on an endo-*N*-acetylmuramidase (Mutanolysin) digest of the walls from *S. aureus*, *S. epidermidis*, *Str. salivarius*, *Str. mutans*, *M. lysodeikticus*, *L. plantarum* and *A. viscosus*. The results are summarized in Table 1, with some of the results of the immunoadjuvancy assay. It is evident that all of the test cell wall digests did induce gelation of the amoebocyte lysate at the doses of 25 to 100 μg/0.2 ml assay mixture. The Mutanolysin specimen was found to be inactive at a dose of 250 μg/0.2 ml after filtration through a MF-Millipore® VS.

It is noteworthy that the Mutanolysin digest

TABLE 1. *Gelation of amoebocyte lysate of T. tridentatus by the adjuvant-active endo-N-acetylmuramidase (Mutanolysin) digest of several gram-positive bacterial cell walls*

Species (strain)	Gelation of the lysate (μg/0.2 ml assay mixture)						Immunoadjuvancy[a]				IgG_2
							Corneal response (48 hr)	Skin response (48 hr)		Antibody level (Ratio)	
	500	250	100	50	25	10	Mean (Range)	Erythema (mm) Mean± S.E.[b]	Induration Mean± S.E.[c]	Mean± S.E.[d]	Mean (Range)
Straphylococcus aureus (EDA 209P)	++	++	+	+	−			ND[f]			
Staphylococcus epidermidis (ATCC 155)	++	+	+	+	−		2.5 (1.0−3.0)	18±3.2	2.4±0.34	4.4 ±1.1**[e]	2.1 (1.0−3.0)
Streptococcus salivarius (IFO 3350)[g]	++	++	−				2.8 (2.5−3.0)	12±0.22	2.3±0.31	2.2 ±0.39	1.6 (0.5−3.0)
Streptococcus mutans (BHT)	++	++	+	−			2.8 (2.0−3.0)	ND	ND	2.9 ±0.65	2.3 (0 −3.0)
Micrococcus lysodeikticus (NCTC 2665)	+	+	+	+	−	−	0	9±0.55	1.9±0.36	0.55±0.08	0
Lactobacillus plantarum (ATCC 8014)	+	+	−				3.0	26±3.0*	2.9±0.15**	7.2 ±0.45**	3.0
Actinomyces viscosus (ATCC 15987)[g]		++	++	+	+	−	2.9 (2.5−3.0)	ND	ND	2.0 ±0.21*	2.4 (1.5−3.0)

[a] The amount of test specimens was 200 μg/guinea pigs with *S. epidermidis* cell wall digest. In other cases 100 μg were injected.

[b] Average diameter of redness (mm).

[c] Ratio of double thickness of the skin injected 100 μg test antigen (ovalbumin)/0.1 ml saline to that of the skin of the opposite control side.

[d] *Ratio of antibody* nitrogen in the test group to that in the respective Freund incomplete type control group.

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

[f] Not determined.

[g] Data on the adjuvancy were quoted from Kotani et al. (1975a), except those of the skin responses.

TABLE 2. *Gelation of amoebocyte lysate of T. tridentatus by N-acetylmuramyl peptides and related compounds, adjuvant-active or -inactive, and pyrogenic or not-pyrogenic*

Test material	Adjuvancy[e]	Pyrogenicity (μg)[f]	Test dose (μg/0.2 ml assay system)					
			500	250	100	50	25	10
MurNAc-L-Ala-D-Glu(L-Lys-D-Ala-OH)-NH_2	3+[a]	125[c]	++	++	−	−		
MurNAc-L-Ala-D-Glu(L-Lys-OH)-NH_2	3+[a]	16[c]	++	++	+	−	−	
MurNAc-L-Ala-D-Glu(OH)-NH_2	3+[a]	16[c]	++	++	++	−	−	
6-*O*-Acetyl-MurNAc-L-Ala-D-Glu(OH)-NH_2	3+[b]	63[b]	++	++	++	+	−	−
6-*O*-Stearoyl-MurNAc-L-Ala-D-Glu(OH)-NH_2	3+[b]	16[b]	++	+	+	−	−	
MurNAc-L-Ala-L-Glu(NH_2)-OH	0[a]	>250[c]	+	−	−	−		
MurNAc-L-Ala-D-Glu(NH_2)-OH	0[a]	>250[c]	+	−	−	−		
MurNAc-L-Ala-L-Glu(OH)-OH	0[a]	>250[c]	+	−	−			
MurNAc-L-Ala-D-Glu(OH)-OH	1+[a]	250[c]	+	+	−	−		
MurNAc-L-Ala-L-Glu(OH)-OH	0[a]	>250[c]	+	−	−			
MurNAc-L-Ala-D-Glu(OH)-Gly-OH	1+[b]	>250[b]	+	+	−	−		
MurNAc-L-Ala-D-Asp(OH)-NH_2	0[a]	>250[c]	−	−	−	−		
MurNAc-Gly-D-Glu(OH)-NH_2	3+[b]	500[b]	−	−	−			
MurNAc-L-Ser-D-Glu(OH)-NH_2	3+[b]	500[b]	−	−	−			
MurNAc-D-Ala-D-Glu(OH)-NH_2	0[b]	>1000[b]	−	−	−			
MurNAc-L-Ala-OH	0[a]	>250[c]	−	−				
MurNAc-D-Ala-OH	0[a]	>250[c]	−	−				
MurNAc-D-Glu(OH)-NH_2	0[a]	>250[c]	−	−				
L-Ala-D-Glu(L-Lys-D-Ala-OH)-NH_2	0[a]	>250[c]	+	−				
L-Ala-D-Glu(L-Lys-OH)-NH_2	ND[d]	ND	+	−				
L-Ala-D-Glu(OH)-NH_2	0[a]	ND	+	−				

[a] Data quoted from Kotani et al. (1975b).
[b] Unpublished observation.
[c] Data quoted from Kotani et al. (1976).
[d] Not determined.
[e] Adjuvant potency was arbitrarily graded from 0 (no adjuvant activity) to 3+ (strong adjuvancy) through 1+ or 2+ (weak, but definite activity).
[f] Minimum pyrogenic dose.

of *M. lysodeikticus* cell walls which was quite inactive in both induction of delayed-type hypersensitivity and simulation of antibody production, was found to gelate the amoebocyte lysate effectively, like other adjuvant-active cell wall digests.

Table 2 summarizes the results of assay for gelation of the amoebocyte lysate of *T. tridentatus* by synthetic *N*-acetylmuramyl peptides and related compounds, with the immunoadjuvancies and pyrogenicities, most of which had been examined in the previous studies (Kotani et al., 1975b; 1976) and some of which (the pyrogenicities) have been assayed in the present study. It can be seen from this table that there are close correlations between the activities to gelate the amoebocyte lysate, pyrogenicities and immunoadjuvancies of test synthetic compounds, with the following exceptions: *N*-acetylmuramyl-glycyl-D-isoglutamine and *N*-acetylmuramyl-L-seryl-D-isoglutamine were far less pyrogenic (the minimum pyrogenic dose is 500 μg for both) and inactive in gelling of the amoebocyte lysate even at the highest test concentration (500 μg/0.2 ml), in spite of the fact that the latter muramyl dipeptide was shown to be an immunoadjuvant more effective than *N*-acetylmuramyl-L-alanyl-D-isoglutamine, and the former muramyl dipeptide was found to be definitely adjuvant-active (Kotani et al., to be published). It may be worth mentioning that the adjuvant-inactive *N*-acetylmuramyl-D-alanyl-D-isoglutamine was not pyrogenic even at the highest test dose (one mg). As regards gelation of the amoebocyte lysate, all three muramyl dipeptides whose amino acid adjacent to the muramic acid residue is not L-alanine, but either glycine, L-serine or D-alanine, were proved to be inactive with the dose of 500 μg/0.2 ml assay mixture.

DISCUSSION

Wildfeuer et al. (1975) who reported the activy of the peptidoglycans of group A *Streptococcus pyogenes*, *S. aureus*, *S. epidermidis* and *M.lysodeikticus* cell walls claimed that the gelling activity of these peptidoglycans was completely destroyed by digestion with egg white lysozyme and consequently the possibility that the observed activity was due to contamination of test peptidoglycans with endotoxin could be ruled out. This seems to be in conflict with our findings reported here because, first, Mutanolysin which was used for preparation of water-soluble cell wall lysates shares with lysozyme an endo-*N*-acetylmuramidase activity although Mutanolysin has a wider cell wall lytic spectrum than egg white lysozyme, and secondly, synthetic *N*-acetylmuramyl di-, tri- and tetra-peptides of the configuration inherent to the peptidoglycans of the group A and L-Lysine type (Schleifer and Kandler, 1972) had a definite gelling activity. The reason for this discrepancy between Wildfeuer's study and ours is not known at present.

The possibility that the ability to gelate the amoebocyte lysate of test specimens observed in this study was due to contaminating exogeneous endotoxins seems to be excluded in view of the very close correlations between the gelling abilities, immunoadjuvancies and pyrogenicities on the one hand and the chemical structures on the other hand, as regarding synthetic compounds, although there are some exceptions as described above. In addition, use of the membrane filter of 0.025 μm average pore size, which was proved to be effective for an almost complete removal of endotoxic lipopolysaccharide, for preparation of test solutions, seems to exclude the possibility that extraneous endotoxins spoil the present experiment. It was also shown by a control experment that a dose (one μg/0.2 ml assay mixture) of polymyxin B, capable of neutralizing about ten times the minimum dose of Bacto-lipopolysaccharide B (Salmonella enteritidis) to induce lysate gelation, did not significantly affect the gelling activity of the minimum effective dose of *N*-acetylmuramyl-L-alanyl-D-isoglutamine or a Mutanolysin digest of *S. aureus* cell walls (cf. Niwa and Harada, 1976). This finding indicates that gelation of the amoebocyte lysate

reported in the present study is due to an inherent property of the bacterial cell wall peptidoglycan subunits.

It should be pointed out that the correlations between the ability to gelate the amoebocyte lysate, pyrogenicity and immunoadjuvancy turned out to be not perfect as reported on the correlation between immunoadjuvancy and pyrogenicity in a previous paper (Kotani et al., 1976): The Mutanolysin lysate of *M. lysodeikticus* cell walls were definitely positive in gelation of the amoebocyte lysate in spite of the fact that the same Mutanolysin digest was inactive in the immunoadjuvancy assay (a study in progress, however, revealed that the lysate of *M. lysodeikticus* cell walls by digestion with a partially purified Kyowa lytic #2 enzyme, endopeptidase, exhibited distinct immunoadjuvant activities in sharp contrast to the Mutanolysin endo-*N*-acetylmuramidase digest and the cell walls themselves; unpublished observation) and *N*-acetylmuramyl-L-seryl- and *N*-acetylmuramyl-glycyl-D-isoglutamine were found to be definitely active as an immunoadjuvant, but only weakly pyrogenic and inert in gelling of the amoebocyte lysate unlike *N*-acetylmuramyl-L-alanyl-D-isoglutamine. In the previous report, we speculated that there may be some connection between the mechanisms controlling the immunological responses of mammals to an antigenic stimulus and the mechanisms regulating the body temperature. In consideration of the present findings, this speculation might have to be somewhat revised.

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