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SHORT COMMUNICATION

IMMUNOADJUVANT ACTIVITIES OF THE ENZYMATIC DIGESTS OF BACTERIAL CELL WALLS LACKING IMMUNOADJUVANCY BY THEMSELVES

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Previous works in this series (Kotani et al., 1975, 1977; Kotani, 1976) have shown that the cell walls from *Staphylococcus epidermidis* (ATCC 155), *Micrococcus lysodeikticus* (NCTC 2665) and *Arthrobacter* sp. (NCIB 9423) were exceptionally adjuvant-inactive, among the test walls from all 25 species (27 strains) of gram-positive bacteria whose peptidoglycans were the group A types (Schleifer and Kandler, 1972), in both induction of delayed-type hypersensitivity and stimulation of antibody production when injected with ovalbumin into the foot pads of guinea pigs as a water-in-oil emulsion. It has also been demonstrated (Kotani et al., 1977) that the cell walls of the group B peptidoglycan types obtained from five bacterial species such as *Corynebacterium poinsettiae* (NCPP 177) and other plant-pathogenic corynebacteria were devoid of the immunoadjuvancy.

To elucidate the reasons of inabilities as an immunoadjuvant of the above cell walls, the walls of *S. epidermidis* (ATCC 155), *M. lysodeikticus* (NCTC 2665) and *C. poinsettiae* (NCPP 177) were degraded into soluble " fragments " by treatment with appropriate cell wall lytic enzymes of different attack points on peptidoglycans, and the immunoadjuvant activities of the enzymatic digests were assayed. Two kinds of the peptidoglycan-degrading enzymes were used for this purpose; one of them was an endo-*N*-acetylmuramidase which hydrolyzed the β-1,4 glycosidic linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues in a glycan chain and the other was endopeptidases which cleaved the cross-links between the neighbouring stem peptide subunits in the peptide portion (Kato, 1975).

The cell wall specimens prepared as described in previous papers (Kotani et al., 1975; Perkins, 1971) were submitted to digestion with both Mutanolysin (Yokogawa et al., 1974; 1975) and the L-11 enzyme (Hamada et al., 1971) or L-3 enzyme (Katayama et al., 1976) under the conditions as shown in Table 1. A solubilized material separated by centrifugation at $10,000 \times g$ for 30 min was, without fractionation, examined for the immunoadjuvant activities as previously described (Kotani

TABLE 1. *Digestion of cell walls with peptidoglycan-degrading enzymes*

Constitutent	*S. epidermidis*		*M. lysodeikticus*		*C. poinsettiae*	
Enzyme (mg or U)	Mutanolysin 1.5 mg	L-11 55 U[a]	Mutanolysin 0.5 mg	L-11 18 U[b]	Mutanolysin 0.15 mg	L-3 0.25 mg
Cell walls (mg)	52.0 mg	32.2 mg	51.0 mg	41.2 mg	1.5 mg	1.5 mg
Buffer (pH, molar concentration)	Na-acetate 6.5, 0.005	Na-phosphate 8.0, 0.01	Na-acetate 6.5, 0.005	Na-phosphate 8.0, 0.01	Na-phosphate 6.5, 0.01	Na-phosphate 7.8, 0.01
Total volume (ml)	3.0	2.6	3.0	2.6	2.4	2.4
Reaction time (hr)	48	48	24	48	90	90
Insoluble residue (mg)	6.98	6.80	0.50	0.65	0.60	0.40

[a] Lytic units against *S. epidermidis* cell walls.
[b] Lytic units against *M. lysodeikticus* cell walls.

et al., 1975). The amount of solubilized cell walls was calculated by substracting the amount of insoluble residue from that of a cell wall specimen used for digestion.

Table 2 indicates that the enzymatic digest, either by the muramidase or the endopeptidase, of *S. epidermidis* cell walls showed the strong adjuvant activities in terms of positive corneal and delayed-type skin responses and elevation of precipitating antibody level to a test protein antigen (ovalbumin) when administered to guinea pigs as a water-in-oil emulsion. It has also become apparent, unexpectedly, that the solubilized product by the L-11 endopeptidase, but not the Mutanolysin digest, of *M. lysodeikticus* walls exhibited the weak but definite immunopotentiating effects which had never been recognized with the cell walls themselves isolated from this organism (We found that the walls from two strains other than NCTC 2665 were also devoid of the adjuvant activities). The cell walls of *C. poinsettiae*, on the other hand, did not show the immunoadjuvant activities even after solubilization with either the muramidase or the L-3 endopeptidase.

The results reported in this paper are of interest in view of the following facts. All available data on the chemical structures of bacterial cell wall peptidoglycans indicate that disaccharide tri- or tetra-peptide subunits are highly polymerized by extended glycan chain (e.g. the average chain length of a glycan part of *S. epidermidis* cell walls was reported to be around 13 hexosamine residues, Tipper, 1969), but they are not assembled in more than dimeric units (at most trimer) by cross-linking between the peptide subunits (Kato, 1975). Consequently, the endopeptidase digest of bacterial cell walls has been found to have on the whole a much bigger molecular size or a much more repetitive structure than the endo-*N*-acetylmuramidase digest. In this connection, we have found that the water-soluble adjuvant-active component(s) obtained from the endopeptidase digest of the cell walls from various gram-positive bacteria exhibited the immunoadjuvant activities to induce delayed-type hypersensitivity to ovalbumin and had definite arthritogenic activity in Lewis inbred rats, but the component(s) obtained by digestion with glycan-splitting enzymes of the same cell wall specimens failed to induce delayed-type anti-ovalbumin hypersensitivity and to produce arthritis in Lewis rats (Koga et al., 1976; Kohashi et al., 1976; Kotani et al., unpublished observation). The findings by Ciorbaru et al. (1976) would also be significant by indi-

TABLE 2. *Immunoadjuvant activities of the products solubilized by the action of endo-N-acetylmuramidase or endopeptidase on the adjuvant-inactive cell walls from S. epidermidis, M. lysodeikticus and C. poinsettiae*

Experimental group	Cell walls from	Enzymatic treatment with	Dose (μg)	Corneal response (48 hr)[a] Mean (Range)	Skin response (48 hr)[a] Erythema (mm)[f], Mean±S.E.	Induration[b] Mean±S.E.	Antibody level (ratio)[c] Mean S.E.
6	*S. epidermidis* (ATCC 155)	None	200	0	7±0.49	ND[g]	1.1 ±0.17
45		Mutanolysin	200	2.5(1.0–3.0)	18±3.2*[d]	2.4±0.34	4.4 ±1.1*
47		L-11	200	3.0	18±1.7*	2.9±0.14**	4.4 ±0.90**
3	*M. lysodeikticus* (NCTC 2665)	None	100	0	ND	ND	1.2 ±0.43
46		Mutanolysin	100	0	9±0.55	1.9±0.36	0.55±0.08
60		L-11	200	2.3(0.5–3.0)	15±1.2**	2.2±0.15**	3.6 ±0.85*
43	*C. poinsettiae* (NCPP 177)	None	100	0.3(0 –1.0)	6±1.7	1.5±0.17	0.65±0.13
60		Mutanolysin	100	0.5(0 –1.5)	11±0.64	1.8±0.03	1.6 ±0.24
62		L-3	100	0	9±2.0	1.4±0.13	0.08±0.015**
3 ~ 62	None (Freund incomplete type)	None	None	0 ~ 0.6(0 –2.0)	9±0.93 ~ 11±1.2	1.4±0.10 ~ 1.8±0.13	[62±20.3][e] ~ [167±17.8]

[a] Corneal test and skin test were performed 3 and 4 weeks after the sensitization, respectively.
[b] Ratio of double thickness of the skin injected with 100 μg ovalbumin/0.1 ml saline to that of the skin of the opposite side.
[c] Ratio of antibody nitrogen (μgN/ml serum) in the test group to that in the respective FIA group.
[d] The difference between the test and respective control group was significant at a level of 5% (*) or 1% (**) by the "Student" t-test.
[e] μg Antibody nitrogen/ml serum specimen.
[f] Average diameter of redness (mm).
[g] Not determined.

cating that the products solubilized by digestion with *Streptomyces albus* G (endo)peptidases of *Nocardia rubra* cell wall peptidoglycans were mitogenic, but the lysozyme digests were not. Further work is required to obtain the logical interpretation of the observations reported here.

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