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Adjuvant Activities in Production of Reaginic Antibody by Bacterial Cell Wall Peptidoglycan or Synthetic *N*-Acetylmuramyl Dipeptides in Mice

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This paper is concerned with the adjuvant activity in stimulatory immunoglobulin E production against ovalbumin (OA) by bacterial cell walls, cell wall peptidoglycan (PG), and their PG fragments and synthetic *N*-acetylmuramyl (MurNAc) dipeptides in A/J mice. A PG isolated from *Streptococcus pyogenes*, PG subunit polymer and dimer obtained from *Staphylococcus epidermidis*, and water-soluble fragments of cell walls or PG prepared from *Nocardia corynebacteriodes* and *Streptomyces gardneri* were found to enhance both the primary and secondary responses of anti-OA immunoglobulin E antibody production. It was suggested that the PG portion, either intact or highly degraded, was capable of enhancing the immunoglobulin E antibody production, and there was no need for the non-PG moiety or intactness of PG structure for the adjuvant activity. This finding was confirmed and extended by the use of synthetic MurNAc dipeptides. Among eight MurNAc dipeptides tested, MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-D-Gln, MurNAc-L-Ala-D-Glu, and MurNAc-L-Ser-D-isoGln were found active as an adjuvant in the stimulation of the primary and secondary reaginic anti-OA antibody production in a similar way to the cell wall PG and their fragments. None of the synthetic MurNAc-L-Ala-L-isoGln, MurNAc-L-Ala-L-Gln, MurNAc-L-Ala-L-Glu, and MurNAc-L-Ala-D-isoAsn, on the other hand, stimulated the anti-OA immunoglobulin E antibody production in either primary or secondary response, indicating the importance for the adjuvancy in immunoglobulin E production of the configuration of the glutamic acid residues adjacent to the L-Ala (or L-Ser) in muramyl dipeptides.

Bacterial cell walls or cell wall peptidoglycan (PG), when suspended with antigen in a mineral oil mixture, have been shown to have strong adjuvant effects in the production of humoral antibody, immunoglobulin M or G (IgM or IgG), and in the induction of delayed-type hypersensitivity (DTH) (1, 2, 10, 11, 14, 17, 22). Recent studies have revealed that the minimum effective structure responsible for the immunoadjuvant activities of bacterial cell wall PG is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln) (3-6, 11, 16, 28, 30). However, no information is available on whether reaginic (IgE) antibody production is stimulated by cell wall PG or PG subunits. We found that the production of IgE antibodies in mice against acid extracts from group A *Streptococcus pyogenes* cell walls or ovalbumin (OA) was remarkably enhanced by using streptococcal cell wall PG as adjuvant (H. Ohkuni et al., Abstr. 7th Int. Symp. Streptococci and Streptococcal Dis., Oxford, 1978, p. 35). We described further studies on the adjuvant activities in stimulatory IgE antibody production to OA in mice of PG subunits isolated from *Staphylococcus epidermidis* cell walls with the PG-degrading enzyme and of synthetic MurNAc-L-Ala-D-isoGln in a preliminary report (24).

This paper describes a further study of the minimum effective structure for the adjuvant activity of PG in stimulating IgE production to OA in mice.

MATERIALS AND METHODS

Animals. A/J mice, of both sexes, 8 to 10 weeks old, were used. The parent strains of A/J mice were

generously supplied by T. Tada, Chiba University, and bred in our laboratory. Female Wistar rats weighing 150 to 200 g were obtained from the Nippon Bio-Supply Center, Tokyo.

Test adjuvants. *S. pyogenes* cell wall PG was prepared as described previously (23, 24). *S. epidermidis* (ATCC 155) PG was extracted with 10% trichloroacetic acid at 4°C for 48 h to remove a non-PG moiety. The PG was digested with either M-1 endo-*N*-acetylmuramidase (31) or SALE endo-peptidase (S. Kawata and K. Yokogawa, unpublished data). The enzymatic digests were submitted to gel filtration. The M-1 PG digest and the SALE PG digest thus obtained were designated as SEPM and SEPS, respectively. SEPM and SEPS were assumed to be a dimer and polymer of disaccharide-stempeptide subunit with fragments of cross-linking pentapeptide consisting of glycine (4 mol) and serine (1 mol) as reported in the preceding preliminary report (24). The methods used for preparing these cell wall fragments will be described in detail, with analytical data on the isolated specimens. Other cell wall fragments, NCCM-I, NCCM-II and SGCM, were prepared by filtration through the columns of Sephadex G-50 and G-25 connected in a series of the M-1 enzyme digests of cell walls from *Nocardia corynebacteriodes* (ATCC 14898) and *Streptomyces gardneri* (ATCC 23911), respectively. The preparation methods and chemical analysis of these specimens are to be published in a separate paper, but the analytical data pertinent to the present study are presented in Table 1. On the basis of these data and the mode of lytic action of the M-1 enzyme, these cell wall fragments are assumed to be a complex of the PG subunits which resulted from hydrolysis of a glycan chain to an oligosaccharide unit, with a non-peptidoglycan portion whose chemical identity has not yet been elucidated.

TABLE 1. *Summary of chemical analyses of cell wall fragments isolated from N. corynebacteriodes and S. gardneri*[a]

Analysis[b]	NCCM-I	NCCM-II	SGCM
Peptidoglycan portion			
Mur	0.97	0.94	1.00
GlcN	0.99	0.84	1.46
Ala	1.76	1.73	1.57
Glu	1.00	1.00	1.00
A_2pm	1.05	0.93	1.28
Gly	—	—	1.04
Non-peptidoglycan portion			
GalN	—	—	0.68
Hexose (as Gal)	5.39	0.65	0.21
Pentose (as Ara)	3.53	0.12	0.13
Methyl pentose (as Rham)	1.72	0.19	0.13
Organic phosphorus (as KH_2PO_4)	—	—	1.25

[a] Data indicate molar ratios to glutamic acid residue. —, Trace or 0.

[b] GlcN, Glucosamine; GalN, galactosamine; A_2pm, 2,6-diaminopimelic acid; Gal, galactose; Ara, arabinose; Rham, rhamnose; Gly, glycine.

The following synthetic MurNAc dipeptides were obtained as described in a previous paper (18): MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-D-Gln, MurNAc-L-Ala-D-Glu, MurNAc-L-Ser-D-isoGln, MurNAc-L-Ala-L-isoGln, MurNAc-L-Ala-L-Glu, MurNAc-L-Ala-L-Gln, and MurNAc-L-Ala-D-isoAsn.

Antigen. As the antigen ovalbumin (OA) (crystalline, grade V; Sigma Chemical Co.) was used throughout the present study.

Immunization. A/J mice were immunized by an intraperitoneal injection of 1.0 mg of OA with 2.0 mg of each of the above test adjuvants dissolved in 1.0 ml of phosphate-buffered saline (pH 7.2), and the mice were bled from the orbital sinus. Serum specimens were taken subsequently. A booster injection of 0.5 mg of OA was given 3 weeks later.

Titration of anti-OA antibodies. Anti-OA IgE antibody titers in the sera of immunized mice were determined quantitatively by passive cutaneous anaphylaxis (PCA) (25) in Wistar rats. After depilation of the side abdominal skin, each 0.05-ml aliquot of antiserum of the twofold dilutions was injected intracutaneously; 48 h later, the animals were injected intravenously with 0.5 ml of 2% Evans Blue (Wako Pure Chemical Industries, Osaka, Japan) saline solution containing 1.0 ml of OA. The PCA was set up in duplicate. The mean diameters of the blue spots were measured. A blue spot with a diameter of 5 mm or more was regarded as indicating a positive response. The sera from the five animals in each group were mixed and used for PCA. The PCA titers of serum specimens were expressed as the reciprocal of the highest serum dilution giving a positive response. Hemagglutinating titers were measured by the passive hemagglutination technique (7).

RESULTS

Adjuvant activity of bacterial cell wall PG and cell wall or PG fragments. It was established that the PCA reaction in rats with mouse serum was due to the IgE antibody (25). The effects of heating and the fixing activity of anti-OA antibody for a 48-h PCA reaction in rat skin have also been studied in preliminary experiments. The skin-sensitizing activities of the antibodies found in the sera of mice decreased to 1/4 (1:80) by heating at 56°C for 1 h and to 1/8 (1:40) for 2 h. From the studies on the fixation of the antibodies to the skin of rats for over 20 days, it was suggested that the antibody in mouse serum detected in the 48-h PCA reaction might be IgE antibody. However, the determination of histamine release from rat peritoneal mast cells passively sensitized in vitro with the antibody of mouse serum was not performed.

The results of adjuvant activity of bacterial cell wall PG and cell wall or PG fragments are shown in Fig. 1, although the adjuvant activity of *S. pyogenes* PG and SEPS and SEPM from *S. epidermidis* PG have already been described in a previous report (24). *S. pyogenes* cell wall PG finely dispersed by sonication, and all of the

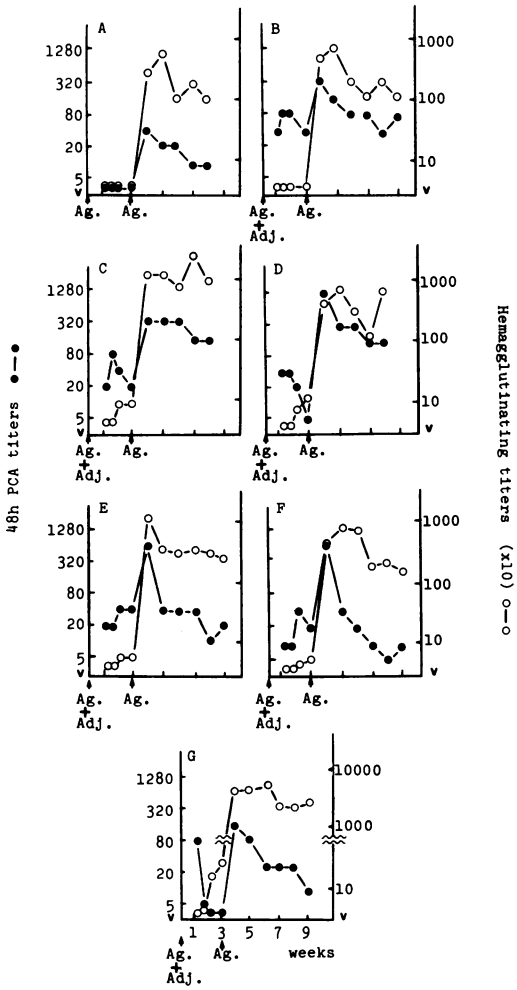

FIG. 1. *Production of reaginic antibody in A/J mice immunized by intraperitoneal injection of OA (1.0 mg) with S. pyogenes cell wall PG, PG subunits isolated from S. epidermidis, and water-soluble fragments of cell wall or PG prepared from N. corynebacteriodes and S. gardneri, using PG degrading enzyme as adjuvant (2.0 mg each). The dose of antigen in the secondary injection was 0.5 mg. Adjuvants added were (A) none; (B) S. pyogenes cell wall PG; (C) SEPS; (D) SEPM; (E) NCCM-I; (F) NCCM-II; and (G) SGCM. B, C, and D were described previously (24).*

water-soluble fragments of cell wall or PG, i.e., SEPS and SEPM from *S. epidermidis* PG, NCCM-I and NCCM-II from *N. corynebacteriodes* cell walls, and SGCM from *S. gardneri* cell walls, enhanced the primary response of anti-OA IgE antibody, although the activity of SGCM seemed to be less than that of the others. Anti-OA IgE antibody titers in the animals receiving the above adjuvants with OA reached 1:40 to 1:80 at 12 days and decreased at 2 to 3 weeks after the primary immunization, whereas antigen alone (a control group) was unable to produce the IgE antibody at all before the booster injection. After the secondary injection of OA, the adjuvant effects of the above preparations were maintained for at least 9 weeks from the first sensitization, although the potencies of NCCM-I, NCCM-II, and SGCM seemed to be less than those of other test specimens. The results suggest that the moiety is responsible for the adjuvant activity for IgE production, as already demonstrated in the stimulation of IgM and IgG antibody production and induction of cell-mediated immune responses (11, 14, 17), and that the non-peptidoglycan portion, variable from one species to another, and the polymerization of PG subunits through a long glycan chain are not necessary in the stimulation of IgE antibody.

Adjuvant activity of synthetic MurNAc dipeptides. Eight synthetic MurNAc dipeptide compounds were assayed for the adjuvant activity in the induction of IgE antibody, with OA as a test antigen. MurNAc-L-Ala-D-Gln, MurNAc-L-Ala-D-Glu, and MurNAc-L-Ser-D-isoGln as well as MurNAc-L-Ala-D-isoGln (24) stimulated the primary response of anti-OA IgE antibody in a similar manner to cell walls, cell wall fragments, or cell wall PG fragments (Fig. 2). In the secondary response, the adjuvant effects of the above muramyl dipeptides were more apparent, and the high PCA titers were maintained for the entire observation period, though the potency of MurNAc-L-Ala-D-Glu was less than that of the others. None of the synthetic MurNac-L-Ala-L-isoGln, MurNAc-L-Ala-L-Glu, MurNAc-L-Ala-L-Gln, or MurNAc-L-Ala-D-isoAsn, on the other hand, were found active as adjuvants in the stimulation of anti-OA IgE production in both the primary and secondary responses (Fig. 3). It is noteworthy that a lower production of anti-OA IgE antibody was observed in the animals receiving MurNAc-L-Ala-L-Gln and MurNAc-L-Ala-L-Glu with OA than in the control group injected with OA alone.

With the adjuvant activity in the production of anti-OA hemagglutinating antibody, less significant enhancing effects were noticed with all test adjuvants under the present experimental conditions, but in the secondary responses the production of hemagglutinating antibody seemed to be stimulated when SEPS, SGCM, MurNAc-L-Ala-D-isoGln, and MurNAc-L-Ser-D-isoGln were used as adjuvants.

DISCUSSION

A variety of water-soluble principles with adjuvant activity were isolated from cell walls or

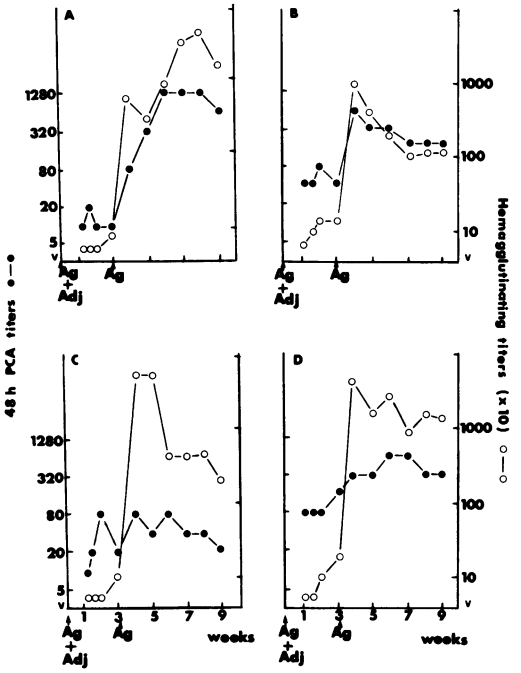

FIG. 2. *Production of reaginic antibody in A/J mice immunized by intraperitoneal injection of ovalbumin (1.0 mg) with synthetic MurNAc dipeptides as adjuvant (2.0 mg). The dose of antigen in the secondary injection was 0.5 mg. The adjuvants added were (A) MurNAc-L-Ala-D-isoGln; (B) MurNAc-L-Ala-D-Gln; (C) MurNAc-L-Ala-D-Glu; and (D) MurNAc-L-Ser-D-isoGln. A was described previously (24).*

whole cells of various gram-positive bacteria as well as mycobacteria and related organisms. They have been shown to exert strong stimulating effects in the production of humoral antibodies IgM and IgG and in the induction of DTH, when administered to test animals, especially guinea pigs, as a water-in-mineral oil emulsion with an antigen (1, 2, 10, 11, 14, 17, 22). These studies have been extended by identifying MurNAc-L-Ala-D-isoGln as the unit structural entity responsible for the immunoadjuvancies characteristic of bacterial cell walls or cell wall PG (3–6, 11, 16, 28, 30). No information, however, is available on whether reaginic antibody production is stimulated by cell walls or cell wall fragments.

We described a preliminary study on the adjuvant activities in the stimulatory production of IgE against OA in mice by PG fragments isolated from *S. epidermidis* cell walls and synthetic MurNAc-L-Ala-D-isoGln (24). This report is concerned with a further study, establishing that bacterial PG subunits or synthetic muramyl dipeptides of an appropriate structure and configuration have definite adjuvant effects in the formation of IgE antibody against OA in mice.

S. pyogenes cell wall PG finely dispersed by sonication and water-soluble cell wall or PG fragments isolated from *S. epidermidis, N. corynebacteriodes,* and *S. gardneri* by the use of PG-degrading enzyme enhanced the primary as well as the secondary responses of anti-OA IgE production, without the use of the oil mixture as a vehicle in administering it to mice. The data strongly suggest that the PG moiety, intact or degraded, is responsible for the adjuvant effects observed.

Studies were conducted to clarify the minimum effective structure and the structural specificity needed for the above immunoadjuvancies of PG, using a variety of synthetic muramyl dipeptides. MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-D-Gln, MurNAc-L-Ala-D-Glu and MurNAc-L-Ser-D-isoGln stimulated the responses in anti-OA IgE production equally well with cell walls, cell wall PG, or their fragments. In mice, MurNAc-L-Ala-D-isoGln was found to stimulate hemagglutinating or precipitating antibody production to bovine serum albumin when given subcutaneously as a solution in saline (4, 9) or

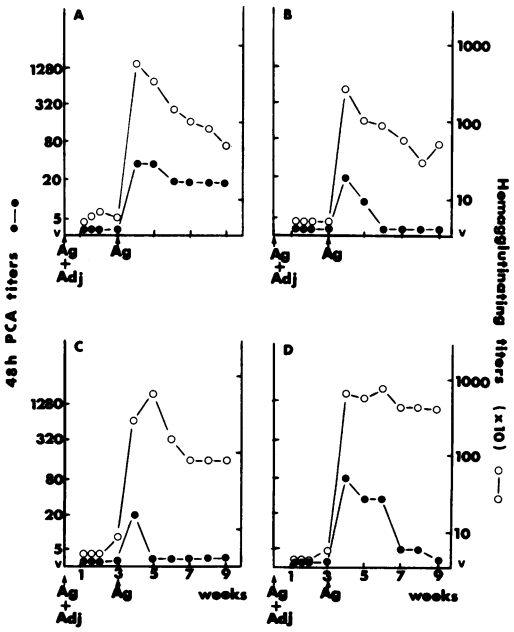

FIG. 3. *Production of reaginic antibody in A/J mice immunized by intraperitoneal injection of OA (1.0 mg) with synthetic MurNAc dipeptides as adjuvant (2.0 mg). The dose of antigen in the secondary injection was 0.5 mg. The adjuvants added were (A) MurNAc-L-Ala-L-isoGln; (B) MurNAc-L-Ala-L-Gln; (C) MurNAc-L-Ala-L-Glu; and (D) MurNAc-L-Ala-D-isoAsn. A was described previously (24).*

even when given orally with either bovine serum albumin (9) or OA (13) subcutaneously injected.

With regard to the adjuvant activities of analogs of MurNAc-L-Ala-D-isoGln, it was reported that MurNAc-L-Ala-D-Glu was only slightly active and that MurNAc-L-Ala-D-Gln was almost inactive as the adjuvant in stimulation of humoral immune responses and in the induction of DTH (3, 4, 11, 16) and experimental allergic encephalomyelitis (21) in guinea pigs when administered as a water-in-mineral oil emulsion. However, Tanaka et al. (28) found that MurNAc-L-Ala-D-Glu was active as the adjuvant in the induction of DTH in WSA rats under similar experimental conditions. Audibert et al. (4) and Chedid et al. (9) reported that MurNAc-L-Ala-D-Glu stimulated humoral immune response in Swiss mice or C3H mice when administered in saline with bovine serum albumin. Souvannavong et al. (26) also have reported that MurNAc-L-Ala-D-Glu, when injected in guinea pigs in Freund incomplete adjuvant with OA, can produce the humoral antibody and induce the DTH in the secondary response, but MurNAc-L-Ala-D-Glu was only slightly active as the adjuvant in the primary response. The adjuvant activity in the stimulation of anti-bovine serum albumin hemagglutinating antibody production, in female C3H mice, of MurNAc-L-Ala-D-Gln as well as MurNAc-L-Ala-D-Glu was recently reported by A. Tanaka (personal communication) and Chedid et al. (8). In this study, IgE antibody production was shown to be stimulated by the administration of MurNAc-L-Ala-D-Glu and MurNAc-L-Ala-D-Gln, as well as MurNAc-L-Ala-D-isoGln, in saline with OA. However, the adjuvant potency of MurNAc-L-Ala-D-Glu was less than that of MurNAc-L-Ala-D-Gln in the secondary response. The reasons are not clear now.

Furthermore, the L-alanine residue of MurNAc-L-Ala-D-isoGln could be replaced with some increase in the adjuvant potency by L-serine in anti-OA IgE formation, as in the induction of DTH and antibody production in guinea pigs to OA in a water-in-mineral oil emulsion (3, 15), in the stimulation of circulating antibody levels in Swiss mice to bovine serum albumin in saline (9), and in the induction of experimental allergic encephalomyelitis in guinea pigs (21).

None of the synthetic MurNAc-L-Ala-L-isoGln, MurNAc-L-Ala-L-Gln, MurNAc-L-Ala-L-Glu, or MurNAc-L-Ala-D-isoAsn showed adjuvant activity in anti-OA IgE antibody production in either the primary or the secondary response. The above finding indicates that the configuration of the glutamic acid residue adjacent to the L-alanine (or L-serine) is important for adjuvancy in IgE production as well as in stimulation of precipitating IgM or IgG class antibody formation and in induction of DTH.

It was described by Levine and Vaz (20) and Vaz et al. (29) that low doses of antigen were required for maximal reaginic antibody responsiveness. However, reaginic antibody formation was observed in the present study, in which a high dose (1 mg) of antigen was used with the test adjuvant. In this connection, Lehrer et al. (19) reported that the maximal reaginic response was obtained with high doses of antigen with a histamine-sensitizing factor extract from *Bordetella pertussis* as adjuvant. Furthermore, a high dosage of bovine serum albumin was also used in the production of hemagglutinating antibody when muramyl dipeptide was injected as adjuvant in an aqueous medium (4, 9). These discrepancies may reflect the differences in adjuvants used.

Adjuvant activity in the production of hemagglutinating antibody was not so marked with any test adjuvants. This fact could reflect the high dose of antigen used in the present experiment. It has been proposed by Tada and Okumura (27) that IgG production can turn off the synthesis of IgE in rats. Ishizaka and Okudaira, however, did not observe a suppression of IgE synthesis by IgG in mice (12). Our results also do not support the claim of Tada and Okumura, since the animals immunized with effective adjuvants plus antigen produced high titers of anti-OA hemagglutinating antibody, presumably of the IgG class, along with IgE antibody after the secondary immunization.

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