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SHORT COMMUNICATION

ADJUVANT ACTIVITIES IN PRODUCTION OF REAGINIC ANTIBODY IN MICE OF BACTERIAL CELL WALL PEPTIDOGLYCANS OR PEPTIDOGLYCAN SUBUNITS AND OF SYNTHETIC *N*-ACETYLMURAMYL DIPEPTIDES[1]

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Bacterial cell walls or their peptidoglycans (PG) isolated from a variety of bacterial species have been shown to have strong adjuvant effects in production of humoral antibody and in induction of delayed-type hypersensitivity against protein antigen (Kotani, 1976). Recent studies have revealed that the minimum effective structure responsible for these immunoadjuvant activities of PG is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln) (Ellouz et al., 1974; Kotani et al., 1975a, 1975b; Audibert et al., 1976; Azuma et al., 1976a, 1976b; Yamamura et al., 1976; Adam et al., 1976; Tanaka et al., 1977). It was also found that although *N*-acetylmuramyl-L-alanyl-D-glutamic acid was only weakly active in guinea pigs (Kotani et al., 1975b; Adam et al., 1976), it was very active in mice in stimulating antibody production (Audibert et al., 1976) and in rats in inducing delayed-type hypersensitivity (Tanaka et al., 1977).

No information is available on whether reaginic (IgE) antibody production is stimulated by cell wall PG and PG subunits. Recently we found that production of IgE antibodies in mice against an acid extract from group A *Streptococcus pyogenes* cell walls or ovalbumin (OA) was remarkably enhanced by immunizing the mice with the antigen and streptococcal cell wall PG (Ohkuni et al., 1977). This paper describes studies on the possible adjuvant activities in stimulating IgE production to OA in mice of PG subunits isolated from *Staphylococcus epidermidis* cell walls with the PG-degrading enzymes and of synthetic

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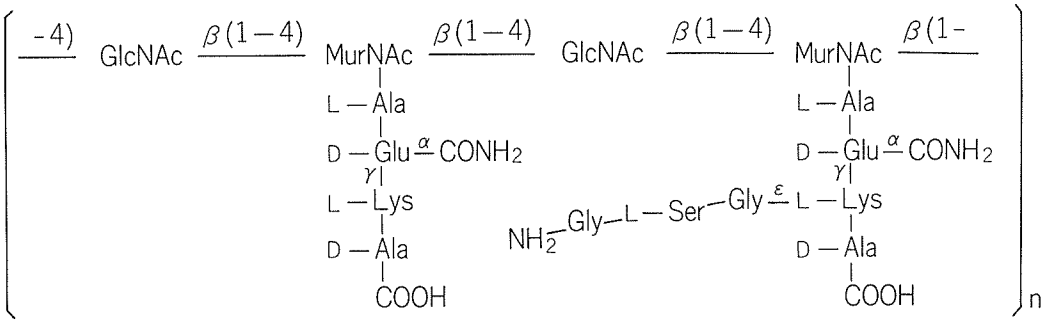
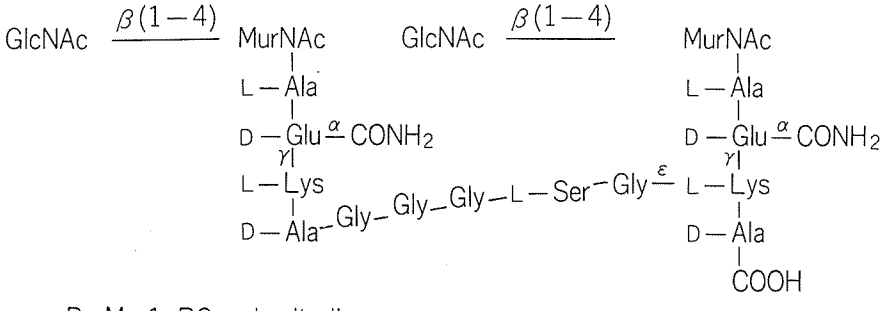
N-acetylmuramyl dipeptides.

Streptococcal cell wall PG was prepared as described previously (Ohkuni and Kimura, 1976) and used as a fine suspension made by sonication at 20 Kc for 7 min. The methods used for preparing PG subunits from *S. epidermidis* (ATCC 155) cell walls with enzymes degrading PG will be described in detail elsewhere, with analytical data on the isolated specimens. In brief, the cell wall preparation was extracted with 10% trichloroacetic acid at 4 C for 48 hr to remove a non-peptidoglycan moiety (glucosyl glycerol teichoic acid), and the resulting PG was digested with either SALE (*Staphylococcus aureus* lytic enzyme produced by a *Cytophaga* sp. B-30) endopeptidase (Yokogawa and Kawata, unpublished) or M-1 endo-*N*-acetylmuramidase (Yokogawa et al., 1975). The enzymatic digests were submitted to gel filtration. The SALE PG digest and the M-1 PG digest thus obtained, which differed in molecular structure as illustrated schematically in Fig. 1, were shown in a separate study to have full adjuvant activities in both induction of delayed-type hypersensitivity and stimulation of the circulating antibody level against OA, when administered as a water-in-mineral oil emulsion to guinea pigs (Kotani, 1976). In ICR mice and Lewis rats, however, although the SALE digest had definite adjuvant activity in both stimulation of humoral and



A. SALE PG subunit polymer



B. M—1 PG subunit dimer

FIGURE 1 A possible structure of the PG subunit polymer and dimer isolated from *S. epidermidis* cell walls with SALE endopeptidase and M-1 endo-*N*-acetylmuramidase, respectively. These structures are proposed on the basis of the results of amino acid and amino sugar analyses, and the hydrolytic actions of the enzymes.

cellular immune responses, the M-1 digest had scarcely any activity under similar assay conditions. Synthetic *N*-acetylmuramyl dipeptides were obtained as described in a previous paper (Kusumoto et al., 1976).

A/J mice of both sexes of 8 to 10 weeks old were immunized by an intraperitoneal injection of 1.0 mg OA (crystalline, grade V, Sigma) with 2.0 mg of each of the above test adjuvants dissolved in 1.0 ml of phosphate buffered saline (pH 7.2), and serum specimens were taken subsequently. A booster injection of 0.5 mg of OA alone was given 3 weeks later. Anti-OA IgE antibodies were determined in female Wister rats, weighing 150–200 g, by passive cutaneous anaphylaxis (PCA) (Ovary et al., 1975). The rats were sensitized by intradermal injection of 0.05 ml aliquots of 2-fold dilutions of the test antiserum specimen in saline and 48 hr later the rats were challenged intravenously with 0.5 ml of 2% Evans Blue (Wako Pure Chemical Industries, Osaka) containing 1.0 mg of OA. After 30 min, the diameters of the blue spot that appeared at the site of intradermal injection of test antiserum specimens were measured; a spot with a diameter of 5 mm or more was regarded as indicating a positive response. The PCA titers of serum specimens were expressed as the reciprocals of the highest serum dilutions giving a positive response.

As shown in Fig. 2, staphylococcal cell wall PG subunits prepared with SALE endopeptidase or M-1 endo-*N*-acetylmuramidase, synthetic MurNAc-L-Ala-D-isoGln and streptococcal cell wall PG all enhanced the primary response of anti-OA IgE antibody production. After the secondary injection of OA, the adjuvant effects of PG subunits, obtained either enzymatically or synthetically, were greater and the high PCA titers were maintained for at least 8 weeks from the first sensitization. There was no significant difference in the potencies of the three test PG subunits in stimulating IgE production, although they differed in structural complexity (see Fig. 1). Streptococcal cell wall PG seemed to be somewhat less active than PG subunits. *N*-Acetylmuramyl-L-alanyl-L-isoglutamine, on the other hand, did not show adjuvancy in IgE production, suggesting the importance of the D-configuration of the isoglutamine residue.

It is well known that the adjuvant activity of cell wall PG and their subunits, including mycobacterial wax D and synthetic MurNAc-L-Ala-D-isoGln, is exhibited most effectively when these materials are administered to guinea pigs in a Freund-type emulsion (i.e., a water-in-mineral oil emulsion). However, in mice MurNAc-L-Ala-D-isoGln was found to stimulate precipitating antibody production to bovine serum albumin when given subcutaneously as a solution in saline (Audibert et al., 1976; Chedid et al., 1976) or even when given orally with subcutaneously injected bovine serum albumin (Chedid et al., 1976) and OA (Kotani et al., 1976). The results reported here indicate that MurNAc-L-Ala-D-isoGln, like water-soluble PG subunits obtained enzymatically, is active in stimulating reaginic antibody production, using saline as a vehicle of administration.

Aluminum hydroxide gel, *Bordetella pertussis* vaccine and gram-negative bacterial lipopolysaccharide, have been found to be effective adjuvant-active substances for production of IgE antibody in experimental animals (Mota, 1964; Prouvost-Dann et al., 1972; Perini and Mota, 1973; Newburger et al., 1974; Danneman and Michael, 1976). Concanavalin A has also been shown to have an adjuvant effect on IgE production in mice (Gollapudi and Kind, 1975). In addition, helminth parasites have been reported to be effective potentiators of reaginic antibody formation in rats and mice (Orr and Blair, 1969; Petillo and Smith, 1973; Bradbury et al., 1974; Kojima and Ovary, 1975). Freund's complete adjuvant, however, does not have an adjuvant effect on IgE formation in rats (Mota, 1964). Further investigations are needed on whether the adjuvant effects of the above-mentioned substances and bacterial cell wall PG subunits or synthetic compounds have

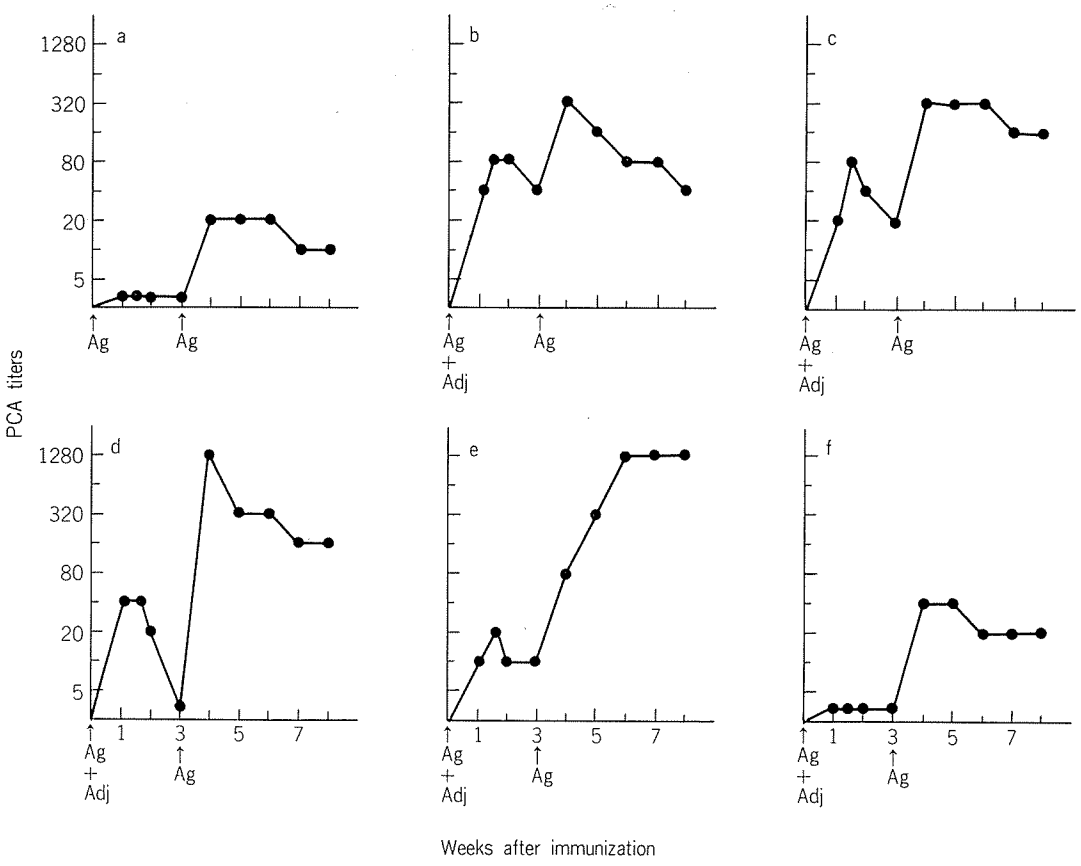


FIGURE 2 Production of reaginic antibody in A/J mice immunized by intraperitoneal injection of ovalbumin (1.0 mg) with *Str. pyogenes* cell wall PG, PG subunits isolated from *S. epidermidis* cell walls using PG degrading enzymes, or synthetic *N*-acetylmuramyl dipeptides as adjuvant (2.0 mg each). The dose of antigen in the secondary injection was 0.5 mg. The adjuvants added were a : none, b : streptococcal cell wall PG, c : SALE digest of *S. epidermidis* cell wall PG, d : M-1 digest of *S. epidermidis* cell wall PG, e : synthetic MurNAc-L-Ala-D-isoGln, and f : synthetic MurMAc-L-Ala-L-isoGln. Points are means of PCA titers of 5 serum specimens obtained from each experimental group at the indicated time after immunization.

similar mechanisms. Studies are in progress on the immunoadjuvancies of various analogues of MurNAc-L-Ala-D-isoGln other than MurNAc-L-Ala-L-isoGln in stimulating reaginic antibody production.

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