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Humoral immune response to *Bacteroides gingivalis* fimbrial antigen in mice

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SUMMARY

Bacteroides gingivalis fimbrial antigen incorporated into liposomes, but not in Tris-HCl buffer, significantly raised the levels of anti-fimbriae antibodies in serum, particularly of the IgG class, after oral primary and booster immunizations in BALB/c mice. An approximately linear relationship was observed between the dose of fimbrial antigen and the level of fimbriae-specific antibodies produced; antibody production reached its maximum at an immunization dosage of 500 μg of fimbriae per mouse. Fimbriae-specific antibody production was enhanced by use of a semi-synthetic adjuvant, a stearoyl derivative of sodium β-*N*-acetylglucosaminyl-(1→4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-stearoyl-(D)-*meso*-diamino-pimelic acid-(D)-amide-D-alanine (GM-53) in liposomes. High anti-fimbriae antibody levels in serum and saliva were maintained for several months in the mice that had received two orally administered boosters of fimbrial antigen with GM-53 in liposomes. Salivary anti-fimbriae antibody levels, particularly of the IgA class, were markedly raised.

INTRODUCTION

Attachment is an essential step in the initiation of most infectious diseases. Bacterial fimbriae are protein appendages which extend from the cell surface and serve to attach to body surfaces (Pearce & Buchanan, 1980). Attachment of *Escherichia coli* to porcine intestinal epithelium (Isaacson *et al.*, 1978) or human buccal epithelium cells (Abraham *et al.*, 1983; Guerina *et al.*, 1983; Weinstein & Silverblatt, 1983) was effectively blocked by antibody against type 1 fimbriae of *E. coli*. Furthermore, antiserum to type 1 fimbriae was shown to protect mice against pyelonephritis (Silverblatt & Cohen, 1979). Administration of purified type 1 fimbriae as a vaccine induced significant protection against colibacillosis in new-born pigs following challenge with *E. coli* that expressed type 1 fimbriae (Jayappa, Goodnow & Geary, 1985). A purified gonococcal P32 pilus vaccine was shown to raise not only pilus-specific antibody in serum but also specific secretory antibodies in seminal fluid and vaginal washes in immunized volunteers and to block the attachment of the bacteria from which the pilus was derived (McChesney *et al.*, 1982). These studies lead us to the concept that antibody-mediated blockage of attachment is correlated with protection from various infectious diseases on the mucosal surfaces.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween 20 and sodium azide; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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Some Gram-negative anaerobes, such as black-pigmented *Bacteroides* species and *Actinobacillus actinomycetemcomitans*, frequently found in the subgingival dental plaque, have been implicated in the pathogenesis of periodontal diseases. Evidence incriminates *Bacteroides gingivalis* as a major cause of adult periodontitis (Slots & Genco, 1984). Raised levels of serum IgG antibodies against *B. gingivalis* but not *A. actinomycetemcomitans* have been shown in patients with the severe form of adult periodontitis (Mouton *et al.*, 1981; Gmür *et al.*, 1986). The antibody response to *B. gingivalis* in the gingival crevicular fluid was also specifically elevated in patients with severe periodontitis, which was attributed to local antibody synthesis (Tew *et al.*, 1985). More recently, we have found that the number of plasma cells, which form antibodies specific for *B. gingivalis* fimbriae, increased markedly in the gingiva of patients with advanced adult periodontitis (Ogawa *et al.*, 1989). This observation serves as a good example to demonstrate the stimulation of the local immune system in localized chronic inflammatory tissue to a specific protein antigen of microbial origin. It may be of great benefit for good protection against pathogens associated with periodontal disease to elicit local immune responses to *B. gingivalis* fimbriae. In this study, we describe a humoral immune response to *B. gingivalis* fimbriae administered orally with a semi-synthetic adjuvant in liposomes in mice.

MATERIALS AND METHODS

Mice

Six-week-old male BALB/c mice were obtained from Charles River Japan (Atsugi City, Japan). They were maintained in a clean room of our animal facility.

Preparation of fimbriae from B. gingivalis *381*

B. gingivalis, strain 381, was grown in GAM broth (Nissui, Tokyo) supplemented with hemin (5 mg/litre; Wako Pure Chemical Industries, Osaka) and menadione (10 μg/litre; Wako) in an anaerobic chamber (Model 1024, Forma Scientific, Marietta, OH) containing 5% CO_2, 5% H_2 and 90% N_2, for 26 hr at 37°. Organisms were harvested by centrifugation at 10,000 *g* for 30 min at 25°.

Fimbriae were prepared on a large scale by modifications as described previously (Ogawa, Shimauchi & Hamada, 1989). Briefly, bacterial cells collected from a 16-litre culture (*c.* 120 g, wet weight) were suspended in 2 litres of 20 mM Tris-hydrochloride (Tris-HCl, pH 7·4), 0·15 M NaCl, and 10 mM $MgCl_2$, and aliquots (200 ml each) of the suspension were gently pipetted. The aliquots were combined, gently agitated further with a stirring bar for 15 min at 25°, and centrifuged at 10,000 *g* for 30 min at 25°. Solid ammonium sulphate was added to the supernatant to a 40% saturation. The precipitate was collected by centrifugation, and resuspended in 50 ml of 20 mM Tris-HCl buffer (pH 8·0). This material was dialysed against 25 litres of the same buffer. The dialysate was clarified by centrifugation at 10,000 *g* for 15 min, and the supernatant was applied to a column (5 × 15 cm) of DEAE–Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden), which had been equilibrated with the same buffer. The column was washed with 1·5 litres of 20 mM Tris-HCl buffer (pH 8·0) and eluted with a stepwise gradient of 0–0·15 M NaCl in 20 mM Tris-HCl buffer (pH 8·0). The fimbrial protein was eluted from the column at a concentration of 0·15 M NaCl of the elution buffer. The fractions containing the fimbrial protein were combined and concentrated by ammonium sulphate precipitation and dialysed against 10 litres of 20 mM Tris-HCl buffer (pH 8·0). The above procedures were repeated 10 times to obtain sufficient amounts of fimbriae; 1·3 g of the purified protein was obtained from a total of 160-litre culture (*c.* 1·2 kg, wet weight) of *B. gingivalis* 381. No other contaminating proteins were present as detected chromatographically, and by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 1·0-mm thick slab gel (12%), as described by Neville (1971). A single band of the 41 K protein was observed on SDS–PAGE gel.

Adjuvants

Sodium β-*N*-acetylglucosaminyl-(1→4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-stearoyl-(D)-*meso*-2,6-diaminopimelic acid-(D)-amide-D-alanine (GM-53) (Furuta *et al.*, 1986) a generous gift from Dr Shigeo Kawata, Research Laboratories, Dainippon Pharmaceutical Co., Osaka was used as an adjuvant.

Incorporation of fimbriae and adjuvant in liposomes

Lecithin (DL-α-phosphatidyl choline, dipalmitoyl, Grade I; Sigma Chemical Co., St Louis, MO) and cholesterol (Sigma), 30 μmoles each, were dissolved in 3 ml of chloroform in a 25-ml round-bottomed flask. Chloroform was then evaporated *in vacuo* at room temperature to leave a thin film consisting of lecithin and cholesterol on the inside wall of the flask. *B. gingivalis* fimbriae, with or without an adjuvant dissolved in 20 mM Tris-HCl buffer (pH 8·0), were added to the flask, and the mixture was shaken at 55°. The content of the flask was then ultrasonicated at 40° by use of an ultrasonic generator (Model UT-51; Sharp Electronic Inc., Osaka) to obtain a homogeneous suspension of small unilamellar liposome vesicles (Inoue, 1974). In some experiments, *B. gingivalis* fimbriae, dissolved in 20 mM Tris-HCl buffer (pH 8·0), were used as an immunogen, without being incorporated into liposomes.

Experimental design of oral immunization

Groups of 8–12 BALB/c mice were given the fimbriae orally with or without adjuvant, or liposomes alone, or Tris-HCl buffer (0·25 ml aliquot per mouse) with the aid of an intubation needle (Natsume Co., Ltd., Tokyo) on Days 0 and 1 as the primary immunization. The mice received the first and second booster administrations of the immunogen on Days 27 and 28, and on Days 123 and 124, respectively, in the same way as the primary immunization. The mice were bled from the inferior ophthalmic vein by use of a capillary glass tube on Day 33. Salivary samples were then collected from the mice treated with pilocarpine hydrochloride (Wako) under anaesthesia with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co.). These serum and salivary specimens were divided into small amounts and stored at −80° until use for examinations.

Enzyme-linked immunosorbent assay (ELISA)

The isotypes and the levels of anti-fimbriae antibodies in serum and saliva of mice were determined by ELISA according to the description by Voller, Bidwell & Bartlett (1980) with minor modifications. In brief, a solution of fimbriae (0·01 mg/ml) dissolved in 0·1 M carbonate buffer (pH 9·6) (100 μl per well) was added to the wells of 96-well flat-bottomed plates (M129; Dynatech Laboratories Ltd, Sussex). The plates were incubated overnight at 4°, and washed with phosphate-buffered saline (PBS) containing 0·05% Tween 20 and 0·2% sodium azide (PBST) to remove the unbound fimbriae from the wells. The plates were then incubated with 1% bovine serum albumin (BSA, Sigma) for 18 hr at 4°. PBST was used to dilute serum and saliva, and to wash plates. After washing, serum and saliva specimens (100 μl) were appropriately diluted and added to each well. After overnight incubation at 4°, the plates were washed three times, and alkaline phosphatase-labelled goat anti-mouse α-, γ- or μ-heavy chain-specific antibody (Zymed Laboratories Inc., San Francisco, CA) was added to the test wells. The plates were incubated at 37° for 2 hr, washed with PBST, and added with *p*-nitrophenylphosphate (phosphate substrate 104; Sigma) dissolved in diethanolamine solution (pH 9·8; 1 mg/ml) at a concentration of 100 μg per well. After incubation at 25° for 30 min, the enzyme reaction was stopped by adding 50 μl of 3 N sodium hydroxide, and the absorbance at 405 nm was read with a Titertek Multiskan MC photometer (Flow Laboratories Inc., McLean, VA). The values obtained from quadruplicate wells were averaged to obtain the mean values and their standard errors (SE).

For the determination of the antibody concentration in serum and saliva from the mice that had been orally immunized with *B. gingivalis* fimbriae, the 96-well flat-bottomed plates (Dynatech) were coated with 100 μl of goat anti-mouse α-, γ-, or μ-heavy chain-specific antibody (0·01 mg/ml) (Zymed) for 18 hr at 4°. After washing the plates with PBST, they were incubated in 100 μl of 1% BSA for 18 hr at 4° and washed again with PBST. Calibration curves were obtained using two-fold serial dilutions of purified mouse IgG (Zymed) for IgG standard, mouse IgA (mouse myeloma protein MOPC315; Cappel, Oraganon Tek-

nika Corporation, West Chester, PA) for IgA standard, and mouse IgM (TEPC185; Cappel) for IgM standard. Purified mouse IgG or monoclonal antibodies in hybridoma culture supernatants were incubated with plates for 18 hr at 4°. After washing again with PBST, alkaline phosphatase-labelled goat anti-mouse α-, γ-, or μ-heavy chain-specific antibody (Zymed) was added for 2 hr at 37°, followed by washing with PBST. *p*-nitrophenyl phosphate (Sigma) was then added to the plates. The degree of the reaction was determined by measuring the absorbance at 405 nm. The calibration curves and interpolation of unknown samples were obtained by the use of a personal computer (PC9801 vm; NEC corporation, Tokyo) using a program based on an equation of log-logit transformation (Rodbard, Bridson & Rayford, 1969) and fitted to a linear equation of the regression with correlation coefficients of higher than 96%. The titres of anti-fimbriae antibodies in serum or saliva were expressed as μg per ml or ng per ml, respectively.

Statistics

The significance of difference between means of groups was determined by the Student's *t*-test.

RESULTS

Humoral immune responses in mice following oral immunization with *B. gingivalis* fimbriae

Purified fimbriae of *B. gingivalis* 381 (500 μg per dose), incorporated into liposomes, were orally administered on Days 0 and 1, and 27 and 28. The immunized mice were found to have significantly raised levels of anti-fimbriae antibodies in serum (Fig. 1). However, fimbrial protein alone, without being incorporated into liposomes, did not induce detectable antibody production. Figure 2 depicts oral immunization with *B. gingivalis* fimbriae in liposomes resulting in a dose-dependent enhancement of the serum antibody responses in all three immunoglobulin isotypes, particularly in the IgG class, at a concentration range of 100–1000 μg of fimbriae per animal. The production of fimbriae-specific serum antibody reached its maximum at 500 μg of fimbriae per animal. In an attempt to obtain higher levels of antibody responses, we used a unique adjuvant material, GM-53, a stearoyl derivative of the disaccharide tetrapeptide isolated from *L. plantarum* cell wall. An adjuvant effect was only observed when given orally to BALB/c mice at the maximum dosage of 500 μg per capita with fimbriae in liposomes (Fig. 3). All oral immunization procedures hereafter were performed at a dosage of 500 μg of fimbriae, with or without the same dosage of GM-53.

Temporal changes in the levels of fimbriae-specific antibodies in BALB/c mice in response to oral immunization with *B. gingivalis* fimbriae

Figure 4 illustrates the temporal changes in the production of anti-fimbriae-specific antibodies following oral immunization of *B. gingivalis* fimbriae with or without GM-53 in liposomes. A difference in the level of fimbriae-specific IgM antibody was noted on Day 7 between groups given fimbriae with or without GM-53 (immunized group) and liposomes alone (control group), as shown by closed circles, open circles and open triangles, respectively. On Day 21, several days prior to the first

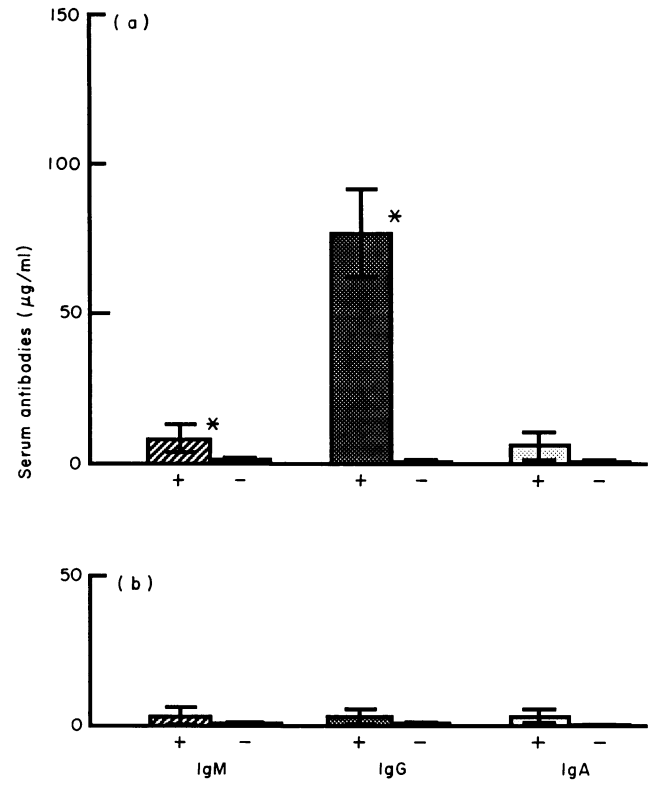

Figure 1. Production of anti-fimbriae antibody in BALB/c mice orally immunized with *Bacteroides gingivalis* fimbriae in either liposomes or Tris-HCl buffer. Two groups of 8–12 mice (male, 6-week-old) were orally administered on Days 0, 1, 27, and 28 with (+) or without (−) fimbriae (500 μg) in liposomes (a) or in the buffer (b). Serum anti-fimbriae antibody levels were determined by ELISA 5 days after the booster immunization. Values (mean ± SE) are expressed as micrograms antibody per millilitre of serum as described in detail in the Materials and Methods. * Statistical difference from the value for the control (liposomes or buffer alone) group, $P < 0.05$.

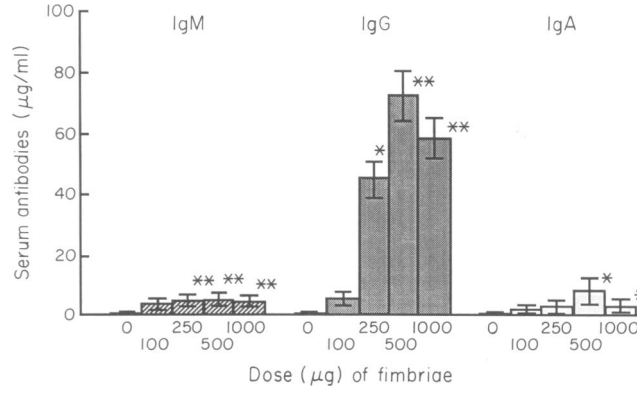

Figure 2. Dose–response relationship between *B. gingivalis* fimbriae and serum antibody titres in BALB/c mice. Mice were orally immunized with various doses of fimbriae following the protocol as described in the legend to Fig. 1. *, ** Statistical differences from the value for the liposomes alone (control) group, $P < 0.05$ and $P < 0.01$, respectively.

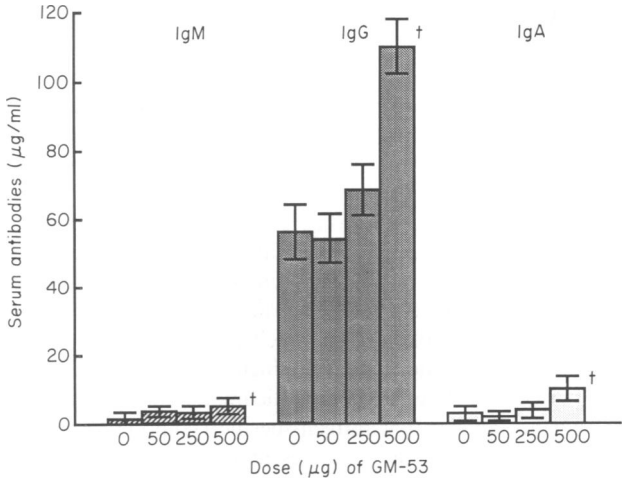

Figure 3. Effect of the dose of adjuvant, GM-53, on the induction of serum antibodies in BALB/c mice. *B. gingivalis* (500 μg) were orally administered with various doses of GM-53 in the mice following the protocol as described in the legend to Fig. 1. † Statistical difference from the value for the group given fimbriae alone, $P < 0.05$.

booster immunization, levels of anti-fimbriae IgG antibody in the mice orally immunized with fimbriae, with or without GM-53 in liposomes, was found to be raised. After the first booster immunization, significant differences between immunized and control groups continued up to almost 2 months. The higher levels of fimbriae-specific antibodies lasted much longer following the second booster immunization on Days 123 and 124. It

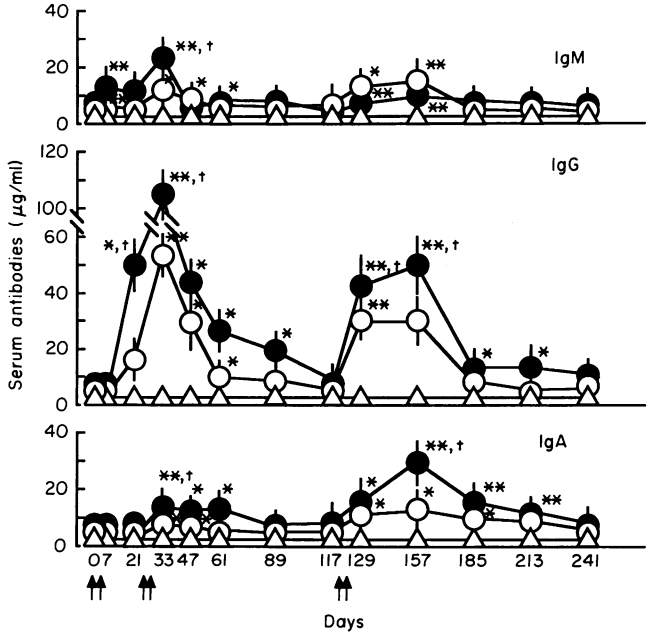

Figure 4. Temporal changes in the levels of serum anti-fimbriae antibodies following oral immunization with *B. gingivalis* fimbriae. Mice were immunized orally on Days 0, 1, 27, 28, and 123, 124 with 500 μg of fimbriae plus 500 μg of GM-53 (●), 500 μg of fimbriae alone (○), or liposomes alone (△). *, ** Statistical differences from the value for the control group, $P < 0.05$ and $P < 0.01$, respectively, † Statistical difference from the value of the group given fimbriae alone, $P < 0.05$.

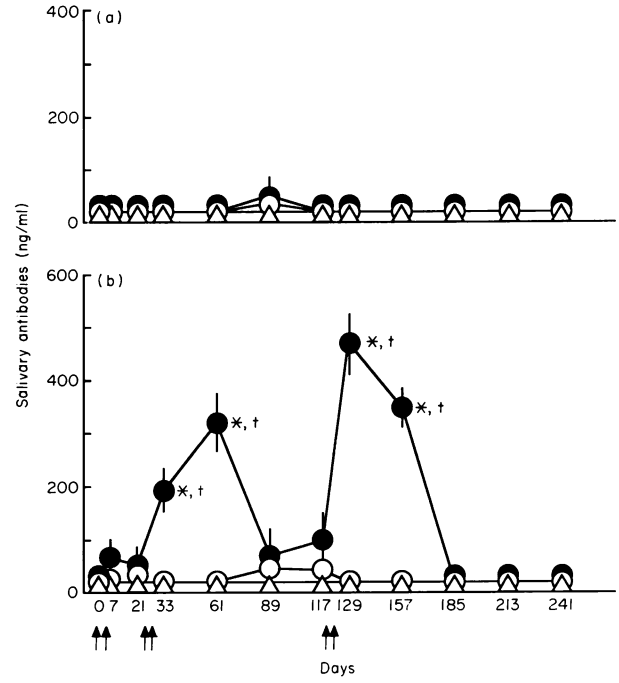

Figure 5. Temporal changes in the level of salivary anti-fimbriae antibodies following oral immunization with *B. gingivalis* fimbriae in BALB/c mice (a) IgG, (b) IgA. Mice were immunized orally on Days 0, 1, 27, 28, 123, and 124 with 500 μg of fimbriae plus 500 μg of GM-53 (●), 500 μg of fimbriae alone (○), or liposomes alone (control) (△). Values (mean ± SE) are expressed as nanograms antibody per millilitre of saliva, as described in detail in the Materials and Methods. * Statistical difference from the value for the control group, $P < 0.05$, † Statistical difference from the value of the group given fimbriae alone, $P < 0.05$.

was also found that the levels of fimbriae-specific IgA became much higher following the second booster immunization. Fimbriae-specific IgG was a major immunoglobulin isotype represented in the secondary and third immune responses. However, the levels of the IgG response following the second booster immunization with fimbrial antigen were lower than those following the first booster immunization. Oral administration of fimbriae with or without GM-53 in liposomes caused negligible production of fimbriae-specific IgG in saliva (Fig. 5). However, oral administration of fimbriae with GM-53 in liposomes induced fimbriae-specific IgA responses in saliva after the first booster immunization on Days 27 and 28, and after the second one on Days 123 and 124. The levels of fimbriae-specific IgA in saliva after the second booster immunization were much greater than those after the first one.

DISCUSSION

We have demonstrated here that oral administration of a fimbrial protein antigen, with or without a lipophilic derivative of a muramylpeptide, GM-53, in liposomes, markedly enhanced the production of serum and salivary anti-fimbriae antibodies in BALB/c mice. In the initial phase of our study, we found that oral administration of the purified 41-K fimbrial protein in Tris-HCl buffer did not give rise to detectable levels of antibody production. We, therefore, attempted to introduce liposomes as a vehicle for oral administration of the immunogen, because

earlier studies clearly indicated that the incorporation of antigen and adjuvant into liposomes did enhance the interactions of the antigen with immunologically competent cells (Van Rooijen & Van Nieuwmegen, 1979; Kramers *et al.*, 1980), and the fimbrial protein would be protected from degradation by proteolytic enzymes in gastric and intestinal juices. The incorporation of fimbriae into liposomes in our system results in a fimbriae-specific humoral immune response in BALB/c mice, and the magnitude of the response, as measured by ELISA, is dependent on the dose of fimbriae, an adjuvant, or both (Figs 1, 2 and 3). In this regard, Oka *et al.* (1981), and Chen & Keenan (1977) reported that the liposomes themselves exerted mitogenic effects on the lymphocytes of both BALB/c mice and humans. The adjuvant activity of the liposomes in the liposome–antigen complex was observed when diphtheria toxoid (Allison & Gregoriadis, 1974), serum albumin (Heath, Edwards & Ryman, 1976; Van Rooijen & Van Nieuwmegen, 1977, 1979), or γ-globulin (Van Rooijen & Van Nieuwmegen, 1978) was used as an immunogen. Suggestions are made for the use of liposomes as a practical immunoadjuvant with definite advantages in stimulating the humoral response when given orally with immunogen. Furthermore, it should be noted that GM-53 showed an adjuvant effect when given by oral administration in liposomes with fimbrial antigen (Fig. 3), while a weaker adjuvant effect was observed with the oral administration of another lipophilic derivative of muramylpeptides, namely, N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyl-L-lysine [MDP-Lys(L18)] (Kotani *et al.*, 1982) (data not shown). GM-53 and MDP-Lys(L18) were also reported to exhibit similar adjuvant activity when BSA was used as an antigen for immunization in liposomes under similar experimental conditions. However, plain MDP did not show significant adjuvant activity (Ogawa, Kotani & Shimauchi, 1986).

Mice were immunized with fimbriae in the presence or absence of GM-53 in liposomes by a series of three oral administrations, namely a primary and two booster regimes. These mice displayed weaker IgG and IgM responses in serum following the last series of immunization than those following the first one (Fig. 4). It was noted that mice exhibited the highest level of fimbriae-specific IgA response in saliva after the second booster immunization with fimbriae and GM-53 in liposomes, while negligible levels of salivary IgG specific for fimbriae were detected in mice that had fimbrial antigen orally administered repeatedly (Fig. 5). This finding clearly supports the concept of a common mucosal immune system (Mestecky & McGhee, 1987). At the same time, the result also indicates marked local IgA anamnestic response following oral immunizations, although several earlier studies had failed to show an IgA anamnestic response, as reviewed by Tomasi & Grey (1972). In this regard, Keren *et al.* (1982) demonstrated that rabbits immunized with live shigella exhibited a heightened IgA anamnestic response in intestinal secretions, which suggests the requirement of the local proliferation of the live bacteria in eliciting the maximum response. In our study, however, even purified fimbrial protein could stimulate immunocompetent cells of the murine intestine if the protein antigen was given with the entrapped form in liposomes with an appropriate adjuvant. It is also of interest to emphasize that anamnestic immune responses occurred very quickly after the two intermittent booster challenges, and that these responses continued for at least 5 months after the primary immunization of the fimbrial antigen (Figs 4 and 5). McChesney *et al.* (1982) reported that increased genital antibody levels, in particular the IgA class, were introduced by two intramuscular injections of a gonococcal pilus vaccine in human volunteers. The genital antibody response was found to follow the serum antibody response. Thus, they suggest that a parenteral gonococcal pilus vaccine may be effective in preventing mucosal infections with *Neisseria gonorrhoeae*.

It is of interest to note that parenteral administration of antigen results either in a preferential IgA response or, instead, induces tolerance to subsequent challenge with the antigen (Mestecky & McGhee, 1987). The variation in the type of host response can be explained by T-cell regulation; oral administration of antigen induces development of antigen-specific IgA T-helper cells in Peyer's patches (Richman *et al.*, 1981). Other experiments indicate that antigen-specific T-suppressor cells are formed in Peyer's patches and mesenteric lymph nodes following antigen feeding (MacDonald, 1982a, b), and these cells inhibit the development of IgG and IgM responses (Mattingly & Waksman, 1978). Taking these findings together, it appears that enhanced salivary IgA responses in this study were an outcome of stimulation of IgA helper T cells and IgG/IgM suppressor T cells in the mucosal tissues. However, it should be noted that serum IgG, as well as IgA antibodies specific for the fimbriae, were produced far more abundantly than as salivary IgA. This indicates again that different regulatory mechanisms function for the respective synthesis of serum and mucosal antibodies.

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