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Manabu Yanagita,*† Takachika Hiroi,* Noriko Kitagaki,* Shigeyuki Hamada,‡ Hiro-o Ito,§ Hidetoshi Shimauchi,† Shinya Murakami,† Hiroshi Okada,† and Hiroshi Kiyono2* 

To investigate the antibacterial activity of mucosal Th1 and Th2 immune responses induced nasally and orally, mice were immunized with mucosal vaccine containing fimbrial protein of Porphyromonas gingivalis, a causative agent for a destructive chronic inflammation in the periodontium, and cholera toxin (CT) as mucosal adjuvant. Nasal vaccine containing low doses of fimbriae (10 μg) and CT (1 μg) induced Ag-specific Th1/Th2-type response in CD4+ T cells in mucosal effector tissues, including nasal passage and submandibular glands, which accounted for the generation of Ag-specific IgA-producing cells. In contrast, oral immunization required higher amounts of fimbriae and CT for the induction of Ag-specific IgA responses. Fimbriae-specific IgA mAbs generated from submandibular glands of nasally immunized mice inhibited P. gingivalis attachment to and reduced subsequent inflammatory cytokine production from epithelial cells. These findings suggest that nasal vaccination is an effective immunization regimen for the induction of Ag-specific Th1 and Th2 cell-driven IgA immune responses that possess the ability to inhibit bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. The Journal of Immunology, 1999, 162: 3559–3565.

Induction of mucosal immune responses is achieved by the deposition of Ag via the mucosa (e.g., oral route) but not the systemic route (1). Further, mucosal immunization has been shown to induce Ag-specific immune responses in both mucosal and systemic compartments (1–3). Although systemic vaccination (e.g., i.m. injection) can induce effective immune responses in the systemic compartment, it does not result in the generation of Ag-specific mucosal immune responses. Considering bacterial infection, mucosal vaccination can offer two layers of immunity (e.g., mucosal and systemic immune responses) which can provide an effective barrier against invasion of pathogenic bacteria. It has been shown that delivery of soluble Ag alone is insufficient for the induction of maximum levels of Ag-specific immune response by mucosal vaccine. Thus, it is necessary to coadminister with mucosal adjuvant for the induction of mucosal immune responses including those of IgA isotype Ab (1–3).

Cholera toxin (CT), an exotoxin produced by Vibrio cholerae, is the most widely known immunogen and adjuvant for studying mucosal immunity (1–3). It is a strong mucosal adjuvant for enhancement of Ag-specific mucosal IgA and systemic IgG responses to mucosally coadministered protein Ag (4, 5). Previous studies have shown that CT elicits adjuvant responses by inducing Ag-specific Th2-type CD4+ T cells producing IL-4, IL-5, and IL-6, which are responsible for supporting Ag-specific IgA and IgG Ab production (4, 5). It has also been shown that CT can modulate a costimulatory molecule such as B-7 (6). For the development of effective mucosal vaccine, it is essential to consider the use of the adjuvant properties of CT.

Most mucosal diseases, especially chronic inflammation such as inflammatory bowel disease (7) and periodontal disease (8–11), share immunological similarity in terms of their pathological features. Intensive investigations have been carried out to understand the molecular and cellular mechanisms of disease development. Although destruction of immunological homeostasis between the mucosal immune system and oral-intestinal microorganism is involved in the development of these inflammatory diseases, we still do not know the exact molecular and cellular pathological process that initiates their mucosal chronic inflammation. However, Porphyromonas gingivalis, black Gram-negative anaerobe, has been at least identified as one of the causative microorganisms for the development of chronic inflammation in periodontium (8, 11). In this study, our investigation was aimed to characterize a potential

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3 Abbreviations used in this paper: CT, cholera toxin; AFC, Ab-forming cells; ELISPOT, enzyme-linked immunospot assay; LP, small intestinal lamina propria; NALT, nasopharyngeal-associated lymphoreticular tissue; NP, nasal passage; SP, spleen; PP, Peyer’s patch; DIG, digoxigenin; SMG, submandibular glands; S-IgA, secretory IgA; MCP-1, monocyte chemotactic protein-1.
of nasal immunization for the induction of *P. gingivalis*-specific Th1 and Th2 cells and of B cell responses in both mucosal and systemic compartments. Further, our study was expanded to examine whether nasal vaccine-induced Ag-specific mucosal response can provide a first barrier against bacterial attachment to mucosal epithelial cells and subsequent production of inflammatory cytokines.

**Materials and Methods**

**Mice**

BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan). Mice were maintained in barrier-protected animal facilities under pathogen-free conditions using ventilated microisolator cages in the experimental animal facility of the Research Institute for Microbial Diseases, Osaka University.

**Bacterial culture and Ag**

*P. gingivalis* 381 (laboratory strain) was grown in GAM broth (Nissui, Tokyo, Japan) supplemented with hemin (5 mg/L; Wako Pure Chemical Industries, Osaka, Japan) and menadione (10 mg/L; Wako) in anaerobic atmosphere containing 5% CO₂, 5% H₂, and 90% N₂ for 26 h at 37°C (12). Fimbriae of *P. gingivalis* 381 were prepared as described previously (12). In brief, cells were harvested by centrifugation at 8000 rpm for 15 min at 20°C. *P. gingivalis* were suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 10 mM MgCl₂, gently pipetted, and centrifuged at 8000 rpm for 15 min at 20°C. Solid ammonium sulfate was added to the supernatant to 40% saturation. The precipitate was collected and dialyzed, and then the supernatant was applied to a column (1.6 × 10⁴ ml) of DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). The column was eluted with a stepwise gradient of 0–0.15 M NaCl in 20 mM Tris-HCl buffer (pH 8.0), and solid ammonium sulfate was added to the fractions containing the fimbrial protein to 50% saturation. Precipitations were collected and dialyzed against 20 mM Tris-HCl buffer (pH 8.0). A single protein band of 41 kDa was observed following SDS-PAGE analysis as containing the fimbrial protein to 50% saturation. The purified fimbriae contained 381 were prepared as described previously (12).

**Immunization**

A group of mice were given nasal immunization with 10 μl of PBS containing *P. gingivalis* fimbriae (10 μg/mouse) and mucosal adjuvant CT (1 μg/mouse; Sigma Chemical, St. Louis, MO) (nasal vaccine). The other group of mice received oral vaccine containing a mixture of fimbriae (200 μg/mouse) and CT (20 μg/mouse) by an immunization protocol routinely used in our group (4). Each group of mice were nasally or orally immunized once per week for 3 consecutive weeks. Serum, saliva, nasal wash, and fecal samples were each collected at weekly intervals and monitored for IgM, IgG, and IgA anti-fimbriae Abs (13).

**Detection of Ag-specific Ab production by ELISA**

Ag-specific Ab titers in serum, saliva, and nasal wash were determined by ELISA using modified method as described previously (13–15). Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml fimbriae in PBS. Wells were blocked with Block Ace (SNOW BRAND, Sapporo, Japan). The plates were serially diluted in 10% Block Ace-PBS and transferred to individual well. Following 2 h of incubation, the plates were washed and reacted with detection Ab consisting of a horseradish peroxidase-labeled goat anti-mouse-IgG (1/2000 dilution of biotinylated goat anti-mouse-IgG) (Life Technologies, Gaithersburg, MD). Among different concentrations of fimbrial tested, a dose of 100 μg/ml gave the best Ag-specific T cell proliferation response. Culture supernatants of Ag-stimulated T cells were examined for the production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 by cytokine-specific ELISA. Ag-specific CD4⁺ T cell-derived cytokines were measured with murine cytokine ELISA kits (Amersham, Arlington Heights, IL).

**RT-PCR for cytokine-specific mRNA**

For detection of cytokine-specific mRNA (IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10) in Ag-specific CD4⁺ T cells, a standard RT-PCR amplification protocol was used (4, 19). CD4⁺ T cells were cultured at a density of 1 × 10⁶ cells/ml in the presence of 100 μg/ml fimbriae and T cell-depleted and irradiated (3000 rad) splenec feeder cells (1 × 10⁶ cells/ml) in flat-bottom 96-well microculture plates (Costar, Cambridge, MA). Among different concentrations of fimbrial tested, a dose of 100 μg/ml gave the best Ag-specific T cell proliferation response. Culture supernatants of Ag-stimulated T cells were examined for the production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 by cytokine-specific ELISA. Ag-specific CD4⁺ T cell-derived cytokines were measured with murine cytokine ELISA kits (Amersham, Arlington Heights, IL).

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For amplification of cDNA, a PCR program of 1 min at 95°C and 1 min at 60°C was used. The oligonucleotide primers specific for IL-5 (sense, 5′-GAA AGA GAC CTT GAC ACA GCT G-3′; antisense, 5′-GAA CTC TTG CAG GTA ATC CAG C-3′), IL-6 (sense, 5′-GAA CCA CAA CTG TAC AG-3′; antisense, 5′-AAA TCG GTG AGG AAG AAC TTG CAG GTA ATC CAG C-3′), IL-4, IL-2, IL-5, IL-6, and IL-10 by cytokine-specific ELISA. Ag-specific CD4⁺ T cell-derived cytokines were measured with murine cytokine ELISA kits (Amersham, Arlington Heights, IL).
TG-3’ and IL-10 (5’-ATG CAG GAC TTT GGT TGT TGG TGT TCA CAA A-3’) were prepared according to published results (20). The sequences of the other primers including β-actin, IFN-γ, IL-2, and IL-4 used for this study were already described in our previous paper (21). Following 35 cycles of amplification, the PCR products were then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining (0.2 μg/ml).

Generation of fimbriae-specific mouse IgA mAb

Female BALB/c mice were nasally immunized with fimbriae and CT as described above. From 3 to 4 days after final immunization, mononuclear cells isolated from SMG were fused with X63Ag8.653 myeloma cells by an established method. Hybridoma cells producing mouse anti-P. gingivalis fimbriae Abs were screened by using Ag-specific ELISA method as described above. Positively reacting hybridomas were selected and subsequently cloned three times by limiting dilution. The isolates of the selected mAbs were determined using a mouse mAb isotyping kit (Amersham). Hybridoma cells were cultured with 500 ml of serum-free medium (CELGROSSER-H, Sumitomo Pharm., Osaka, Japan). Culture supernatants were harvested and mixed with solid ammonium sulfate to 50% saturation. The precipitate was collected and dialyzed in PBS.

Cell culture

The KB cell line (derived from a human oral epidermoid carcinoma) obtained from the American Type Culture Collection (ATCC CCL17, Manassas, VA) was maintained in DMEM (Nikkken Seibutsu, Kyoto, Japan) supplemented with 10% FCS and 50 μg/ml gentamicin in 5% CO2 atmosphere at 37°C. KB cells (4.5 × 103 or 1.0 × 103) were seeded (200 μl or 1 ml) into tissue culture on 96-well or 48-well microtiter plates (IW AKI, Chiba, Japan) 24 h before the experiment.

[^3H]Thymidine-labeled bacterial adhesion assay

The assay of adhesion of P. gingivalis to KB cells was performed by the modified method of Mintz and Five-Taylor (22) as described previously. For radiolabeling of bacteria, the cells were cultured with 5 μCi of [3H]thymidine per ml (Amersham) for 24 h at 37°C in an anaerobic atmosphere containing 5% CO2, 5% H2, and 90% N2. Unincorporated [3H]thymidine was removed by centrifugation followed by the washings with PBS at 6000 rpm. The labeled 108 bacteria were suspended with 200 μl of DMEM containing fimbriae-specific IgA mAbs (50 μg/ml), purified mouse IgA (50 μg/ml, Pharmingen, San Diego, CA), or 10% saliva from mucosally immunized mice with fimbriae and CT and then added to the KB cell monolayer for 1.5 h of incubation at 37°C. The monolayers were rinsed four times with PBS and solubilized by incubation with 0.5 M NaOH-0.1% SDS overnight at 37°C. Solubilized cells were harvested, and the amount of radioactivity present was determined using the TopCount Microplate Scintillation Counter (Packard Instrument, Meriden, CT).

Measurement of cytokine produced by KB cells

For detection of inflammatory cytokines produced by P. gingivalis-infected KB cells, IL-6-, IL-8-, and MCP-1-specific ELISAs were used. P. gingivalis (5 × 103 bacteria) were incubated with 1 ml of DMEM in the presence or absence of fimbriae-specific IgA mAbs (50 μg/ml). P. gingivalis were added to the monolayer of KB cells and incubated with or without Ag-specific mAb at 37°C for 1.5 h. The monolayers were rinsed four times with PBS, replaced with fresh DMEM, and cultured for an additional 24 h. Culture supernatants were harvested and then subjected to analyses of IL-6, IL-8, and MCP-1 production by human cytokine ELISA kits (BioSource International, Camarillo, CA).

Results

Nasal immunization with fimbriae and CT induced higher levels of mucosal immune responses than did oral immunization

In our initial experiment, mice were nasally or orally immunized with fimbriae (10 or 20 μg) in the presence of mucosal adjuvant CT (1 or 20 μg) for the analysis of Ag-specific Abs in serum and secretions. In serum, a comparable level of Ag-specific Abs was induced in both groups. In the case of saliva and nasal wash, high levels of fimbriae-specific IgA responses were noted in mice received nasal vaccine. In contrast, lower levels of fimbriae-specific IgA responses were seen in mice immunized with oral vaccine. In fecal extracts, the levels of fimbriae-specific IgA in nasally immunized mice were much weaker than those detected in the oral vaccine group. These findings suggest that nasal vaccine containing 10 μg of fimbriae and 1 μg of CT is an effective immunization regimen for the induction of Ag-specific IgA Abs in saliva and nasal wash, but not intestinal secretion (Fig. 1).

Next we investigated whether Ag-specific Ig-producing cells were effectively induced in different mucosal and systemic compartments by nasal immunization. The numbers of Ag-specific IgA, IgG, and IgM Ab-forming cells (AFC) in the mononuclear cells isolated from SP, SMG, NP, NALT, PP, and intestinal LP of mice immunized nasally or orally with fimbriae and CT were enumerated by ELISPOT assay. Nasal immunization induced high numbers of Ag-specific IgA-producing cells in SMG (Fig. 2). Further, it was shown that mice receiving nasal vaccine had elevated numbers of fimbriae-specific IgA AFC in NP and NALT. However, nasal vaccination induced low numbers of Ag-specific IgA in intestinal LP. In comparison to nasal vaccine, oral vaccine effectively induced a high frequency of fimbriae-specific IgA AFC in intestinal LP. These findings demonstrate that nasal immunization with 10 μg of fimbriae and 1 μg of CT is effective for the induction of localized Ag-specific IgA responses in nasal and oral compartments, while oral immunization with 200 μg of fimbriae and 20 μg of CT results in compartmentalized Ag-specific IgA responses in intestine-associated tissues.

Nasal vaccine induced fimbriae-specific Th1 and Th2 type responses

Inasmuch as high levels of fimbriae-specific IgA responses were induced in mucosal immune compartments by nasal vaccination, it was important to examine the nature of fimbriae-specific T cell
responses (e.g., Th1 and Th2 type) induced in these nasally immunized mice. To characterize Ag-specific Th1 and Th2 responses, CD4+ T cells were isolated from NALT, NP, and SMG of mice given nasal vaccine and restimulated with fimbriae in vitro. Culture supernatants from fimbriae-stimulated CD4+ T cells were then examined for the presence of Th1 and Th2 cytokines by ELISA (Fig. 3). High levels of both Th1 (e.g., IFN-γ, IL-2) and Th2 (e.g., IL-5, IL-6, and IL-10) cytokines were detected in the culture supernatant harvest from in vitro fimbriae-stimulated CD4+ T cells isolated from mucosal effector tissues such as NP and SMG. In contrast, CD4+ T cells isolated from NALT, an IgA-inductive site for the upper respiratory tract, predominantly produced Th2 cytokines such as IL-4 but not other cytokines. To confirm these observations at molecular levels, Th1 and Th2 cytokine-specific RT-PCR was performed by using RNA samples extracted from the other aliquot of fimbriae-stimulated CD4+ T cells (Fig. 4). In NP and SMG, both Th1 and Th2 cytokine-specific mRNAs were expressed by in vitro restimulated Ag-specific CD4+ T cells. Among an array of Th1 and Th2 cytokines, PCR products representing IFN-γ, IL-6, and IL-10 resulted in high intensity bands. IL-4-specific mRNA was detected more prominently in NALT than in NP or SMG. These cytokine-specific mRNA results further confirmed the findings obtained by ELISA (Fig. 3). Taken together, these findings suggested that nasal immunization induces Ag-specific IFN-γ-producing Th1 type and IL-5-, IL-6-, and IL-10-secreting Th2 type CD4+ T cells in mucosal effector sites (e.g., NP and SMG) while IL-4-producing Th2 type cells are predominantly induced in IgA-inductive tissue (e.g., NALT).

Fimbriae-specific IgA Abs produced by SMG B cells inhibit bacterial adhesion to human epithelial cells

To investigate whether nasal vaccine-induced Ag-specific IgA Abs can inhibit bacterial attachment to oral epithelial cells, we developed a fimbriae-specific mAb-producing cell line from SMG isolated from mice nasally immunized with fimbriae and CT. In our initial experiment, it was shown that saliva from nasally immunized mice possessed some ability to reduce the attachment rate of P. gingivalis on epithelial cells (Fig. 5). To directly demonstrate

**FIGURE 2.** Enumeration of Ag-specific AFC in mucosal and systemic compartments of mice nasally (NI) or orally (OI) immunized with fimbriae and CT. Mononuclear cells were isolated from NALT, NP, SMG, PP, LP, and SP of mucosally immunized mice. IgM, IgG, and IgA isotypes of Ag-specific AFC were examined by ELISPOT. Results represent the values (mean ± SEM) for 10 mice in each experimental group.

**FIGURE 3.** Characterization of Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5, IL-6, and IL-10) cytokine synthesis by fimbriae-specific CD4+ T cells from NALT, NP, and SMG of mice immunized nasally with P. gingivalis fimbriae and CT. An identical experiment was repeated on three different occasions with similar results. Data represent means of triplicate wells. * and **, p < 0.05 and p < 0.01, respectively (compared with NALT).
the inhibitory function of fimbriae-specific IgA Abs, Ag-specific IgA mAbs were generated using B cells isolated from SMG of nasally vaccinated mice as a fusion partner source. Following extensive subcloning and screening from 87 clones, 4 hybridomas (5G4, 2D3, 2B2, and 5H3) with α heavy and κ light chains were selected and used for this experiment. When P. gingivalis organisms were treated with fimbriae-specific IgA mAbs, inhibition of attachment to KB cells was noted (Fig. 5). Although inhibition ability of bacteria to adhere to KB cells differed among these four fimbriae-specific IgA mAbs (30–70%), mAb 5G4 gave the highest inhibition titer at a concentration of 50 μg/ml IgA Abs. These results suggest that nasal immunization can induce immunologically active fimbriae-specific IgA Abs possessing ability to inhibit bacterial attachment to epithelial cells.

Nasally induced fimbriae-specific IgA inhibit inflammatory cytokine production

To further examine the immunological effect of nasal vaccine-induced fimbriae-specific IgA, we next investigated whether blockage of bacterial attachment to epithelial cells by fimbriae-specific IgA led to the reduction of inflammatory cytokine production. When levels of IL-6, IL-8, and MCP-1 production were examined as inflammatory cytokines, increased secretions of those cytokines were observed when KB cells were incubated with P. gingivalis. However, pretreatment of bacteria with fimbriae-specific IgA resulted in the partial blockage of inflammatory cytokine production by epithelial cells (Fig. 6).

Discussion

In the present study, nasal vaccine containing 10 μg of fimbriae and 1 μg of CT resulted in the high titers of Ag-specific IgA Abs in saliva and nasal wash (Figs. 1 and 2). In contrast, oral immunization with higher doses of fimbriae (200 μg) and CT (20 μg) resulted in the induction of Ag-specific IgA Abs predominantly in intestinal secretions. These findings suggest that a compartmentalized mucosal immune system is involved for such a distinctively separated dominant IgA response induced by nasal and oral immunization. Thus, nasal immunization is more effective for the induction of Ag-specific Ab response in the mucosal compartment of the upper part of body including nasal passage and oral cavity, while oral immunization is more effective in inducing intestinal immune responses. To this end, it was recently shown that nasal immunization is superior to oral administration for the induction of Ag-specific immune responses in the upper respiratory tract of human (23).

For the development of a mucosal vaccine, our present findings provide an important implication where one could select from two immunization regimens (e.g., oral or nasal) for the induction of the most optimal Ag-specific immune response in the intestinal tract or nasal/oral cavity, depending on the initial invasion site of different pathogenic microorganisms. Oral vaccine might be more suitable for various enteric infectious diseases caused by V. cholera, Salmonella, pathogenic Escherichia coli, Shigella, etc. In contrast, nasal vaccine would be beneficial for respiratory infectious diseases caused by influenza virus, adenovirus, respiratory syncytial virus, Streptococcus pneumoniae, etc. To this end, it has been shown that nasal immunization with influenza Ag and mucosal adjuvant CT (or heat-labile enterotoxin E. coli.) induced protective immunity (24). Most recently, nasal vaccine containing PsPA from S. pneumoniae and mucosal adjuvant mutant CT resulted in the generation of Ag-specific S-IgA and serum IgG responses that provide protection against the bacterial challenge (25). In addition to these respiratory infections, our present findings provide supportive evidence that nasal vaccination is also an effective immunization regimen for the induction of Ag-specific immune responses in the oral region. The oral cavity is immunologically considered to be a unique organ in which the mucosal and systemic origin of S-IgA and IgG Abs simultaneously provide two layers of protection via saliva and gingival crevicular fluids, respectively. Since

FIGURE 4. RT-PCR analysis of Th1 and Th2 cytokine-specific mRNA by fimbriae-stimulated CD4+ T cells from NALT, NP, and SMG of mice immunized nasally with fimbriae and CT. An identical experiment was repeated on three occasions with similar results. A representation of the results is described here.

FIGURE 5. Effects of nasally induced Ag-specific IgA Abs for the inhibition of P. gingivalis adhesion to epithelial KB cells. Prior to incubation with KB cells, P. gingivalis organisms were treated with an array of fimbriae-specific IgA mAb (5G4, 2D3, 2B2, and 5H3, 50 μg/ml) or saliva (1/10 diluted with culture medium) for 1.5 h at 37°C. These mAbs or saliva-treated P. gingivalis were subjected to in vitro adhesion assay using KB cells. The equation (cpm in adhesion of nontreatment P. gingivalis to KB cells – cpm in adhesion of mAb or saliva treatment P. gingivalis to KB cells)/(cpm in adhesion of nontreatment P. gingivalis to KB cells) × 100 was used to calculate the percent of reduction. Results represent the mean ± SEM of three separate experiments. *p < 0.05 (compared with a control of “Medium”).
high levels of fimbriae-specific S-IgA and systemic IgG are induced in the oral cavity by nasal immunization, a concept of nasal vaccine can be applied toward the development of a new immunoprophylaxis therapy in order to control infectious diseases in oral cavity.

CT has been shown to be an effective mucosal adjuvant for supporting induction of Ag-specific mucosal and systemic immune responses (1–3). Although the exact mode of the CT immunomodulator function is still not well understood, recent accumulated evidence suggests that mucosally coadministered CT is involved in the induction and regulation of Ag-specific Th1 and Th2 responses. It has been shown that CT inhibited IL-2 production and proliferation of Th1 cells but failed to down-regulate IL-4 production and associated Th2 cell proliferation (26). Further, orally administered CT has been shown to induce Ag-specific Th2 responses predominantly (4, 27). Our results also showed that nasal immunization with fimbrial protein and CT induces dominant Th2-type responses in NALT (Fig. 3). Among an array of Th2-type cytokines, a selective production of IL-4 was noted. This finding suggests that these IL-4-producing Th2-type CD4+ T cells may provide a molecular environment for preferential immunoglobulin class switching for IgA in NALT, an example of IgA-inductive tissue, since IL-4 has been shown to support TGF-β-induced IgA-specific class switching (28).

In NP and SMG, mucosal effector sites for the production of Ag-specific S-IgA Abs, Ag stimulation resulted in the induction of both Th1- and Th2-type cytokines. Interestingly, among different Th2 type cytokines, a group of IgA-enhancing cytokines that has been shown to induce differentiation of IgA-committed B cells to plasma cells such as IL-5, IL-6, and IL-10 (29–31) was preferentially produced by fimbriae-stimulated CD4+ T cells isolated from NP and SMG. In addition, the induction of Th1-type cytokines including IFN-γ and IL-2 by these Ag-specific CD4+ T cells may lead to creation of an optimal molecular environment for the maximum production of Ag-specific S-IgA Ab synthesis in NP and SMG. IFN-γ has been shown to enhance the induction of secretory component, an important poly-Ig receptor for the formation and transport of S-IgA by epithelial cells (32), while IL-2 can synergistically support IgA enhancing cytokine (e.g., IL-5) induced IgA+ B cell differentiation (33). Thus, it is possible that the coexistence of selected Th1 and Th2 cytokines produced by Ag-specific CD4+ T cells may provide a highly effective immunological environment for the production of Ag-specific S-IgA responses in mucosal effector tissues such as NP and SMG.

It was shown that *P. gingivalis* was capable of adhering to and invading epithelial cells (e.g., KB cells and human gingival epithelial cells) (34, 35). Further, fimbriae have been shown to play a central role for the initial step of adherence to and invasion of oral epithelial cells (36). If one can induce Ag-specific immune responses against *P. gingivalis* fimbriae, it might be possible to disturb cell to cell interaction between prokaryotic and eukaryotic cells leading to prevention of initial invasion by the pathological microorganism. A previous study has shown that mAb against fimbriae of *P. gingivalis* could almost block its adhesion to epithelial cells in vitro (37). Further, IgM isotype of mAb specific to hemagglutinating adhesion also inhibited cell to cell interaction between *P. gingivalis* and KB cells (38). These findings further supported a potential idea that induction of fimbriae-specific Ab leads to the inhibition of *P. gingivalis* attachment to mucosal epithelial cells. Our present study provides strong evidence that nasal vaccine containing fimbriae and mucosal adjuvant CT can induce high levels of Ag-specific S-IgA, in addition to serum-derived IgG Abs in external secretions (e.g., saliva), which can then interfere with bacterial attachment to epithelial cells (Fig. 5).

To determine the role of fimbriae-specific IgA Abs as an immunoprophylaxis molecule, a panel of Ag-specific mAbs was generated from SMG of mice nasally immunized with or without fimbriae and CT. It was shown that fimbriae-specific IgA mAbs were established from SMG of nasally immunized mice but not from the control group. These fimbriae-specific IgA mAbs possessed capability to inhibit binding of *P. gingivalis* to KB cells (Fig. 5). These findings further support the notion that immunological actions of salivary IgA are thought to inhibit adherence or growth of microorganisms on soft tissues (e.g., epithelial cells) and hard tissues (e.g., teeth). Although these Ag-specific IgA mAbs generated from mice receiving nasal vaccine have been shown to inhibit adhesion of *P. gingivalis* to KB cells, these effects did not result in complete blockage of bacterial adhesion (Fig. 5). These findings suggest that other bacterial surface molecules in addition to fimbriae might be involved in the bacterial adhesion. To this end, bacterial cell surface components such as hemagglutinin and capsule have been shown to be important molecules for adherence to epithelial cells (11). For the purpose of maximum inhibition of *P. gingivalis* attachment to epithelial cells, one might consider additional *P. gingivalis* cell adhesion molecules for the induction of Ag-specific S-IgA Ab responses by nasal immunization.

In this study, we also aimed to examine the possible influences of fimbriae-specific IgA Abs on the production of inflammatory
cytokines (e.g., IL-6, IL-8, and MCP-1) by *P. gingivalis*-stimulated KB cells. Since these cytokines are known as proinflammatory molecules, it was important to study the potential role of fimbrin-specific IgA antibody for the inhibition of these proinflammatory cytokine synthesis. To this end, IL-6 has been shown to be a multifunctional cytokine playing a central role in host defense mechanisms (39). IL-8 is known as the principal chemotactic factor for neutrophils, and MCP-1 has an essential role in host defense mechanisms (39). Our results also demonstrated that incubation of KB cells together with *P. gingivalis* results in the up-regulation of IL-6, IL-8, and MCP-1 synthesis (Fig. 6). As shown in the results demonstrated in Fig. 6, mAbs specific to fimbrins of *P. gingivalis* could inhibit the production of these inflammatory cytokines. These findings further emphasize the importance of the generation of Ag-specific IgA Ab, which possesses the ability to block the bacterial attachment to epithelial cells.

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**References**