Nasal Immunization Induces *Haemophilus influenzae*–specific Th1 and Th2 Responses with Mucosal IgA and Systemic IgG Antibodies for Protective Immunity

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To determine the efficacy of a mucosal vaccine against nontypeable *Haemophilus influenzae* (NTHi), mice were immunized nasally, orally, intratracheally, or intraperitoneally with NTHi antigen together with cholera toxin. Antigen-specific IgA antibody titers in nasal washes and the numbers of antigen-specific IgA-producing cells in nasal passages showed the greatest increases in mice immunized nasally. Cytokine analysis showed that interferon-γ, interleukin (IL)-2, IL-5, IL-6, and IL-10 were induced by nasal immunization, suggesting that Th2- and Th1-type cells were generated. Furthermore, bacterial clearance of a homologous strain of NTHi from the nasal tract was significantly enhanced in the nasal immunization group. These findings suggest that nasal immunization is an effective vaccination regimen for the induction of antigen-specific mucosal immune responses, which reduce the colonization of NTHi in the nasal tract.

Nontypeable *Haemophilus influenzae* (NTHi) is one of the major pathogens of otitis media with effusion (OME), sinusitis, and other respiratory tract diseases [1]. Because of the increased number of antibiotic-resistant strains of NTHi in recent years [2], the development of a vaccine against these bacteria is considered an important goal for public health. In patients with OME, NTHi is frequently isolated from the nasopharynx and the middle ear effusion. The incidence of colonization of the nasopharynx by NTHi is significantly greater in children with OME than in healthy children [3]. These findings suggest that nasopharyngeal colonization with NTHi is a prerequisite for the development of OME and that inhibition of nasopharyngeal colonization would be effective in preventing OME.

Our previous study showed that oral immunization of mice with formalin-killed NTHi increased salivary IgA antibody responses to this pathogen and enhanced nasopharyngeal clearance of live NTHi inoculated through the nose [4]. On the other hand, systemic immunization was not effective in reducing colonization of the nasopharyngeal and lower respiratory tract regions. Intra-Peyer's patch immunization with the major outer membrane proteins of NTHi, P1 and P6, significantly increased pulmonary clearance, compared with that in nonimmunized groups [5]. These results suggest that the enhancement of IgA antibody responses by mucosal vaccination could be an effective way to prevent NTHi infection, through reduction of colonization.

Several recent studies have shown that nasal immunization is an effective regimen to induce mucosal immune responses in the upper respiratory tract, including the nose and oral cavity (e.g., salivary gland) [6–10]. In the present study, we investigated Th1 and Th2 cytokine production and IgA immune responses induced by nasal immunization with cell membrane preparation from NTHi for the generation of protective immunity against NTHi.

Materials and Methods

Mice. Male BALB/c mice that were antibody negative for a panel of mouse virus and bacterial pathogens were obtained from Charles River Laboratories (Wilmington, DE) and were barrier maintained. The mice were kept in horizontal laminar flow cabinets and provided with sterile food and water. All mice used in this study were 8–12 weeks of age.

Antigen preparation from NTHi. NTHi (strain 76), which was

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All experiments described herein were approved by the competent local authorities. All procedures were in agreement with NIH guidelines for the handling of laboratory animals.

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isolated from the nasopharynx of a patient with OME at Oita Medical University, was stored at −80°C and used for the preparation of antigen and nasal inoculation [4]. The antigen was prepared by a method described previously [11]. In brief, NTHi was cultured overnight on chocolate agar at 37°C in a 5% CO_2 incubator. Bacteria were harvested by being scraped from the plate, suspended in EDTA buffer (pH 7.4), and incubated at 56°C for 30 min. The bacterial cells were then disrupted by sonication on ice, and the unbroken cells and debris were removed by centrifugation at 10,000 *g* for 20 min. The supernatants were pooled and centrifuged at 80,000 *g* for 2 h at 4°C. The clear, gel-like pellet was suspended in distilled water and lyophilized. The resulting powder, referred to as cell membrane preparation from NTHi (CM-Hi), was stored until used in the experiments [12].

Immunization and sample collection. Mice were immunized nasally, intratracheally, or intraperitoneally (ip) with 10 μg of CM-Hi together with 1 μg of cholera toxin (CT; List Biological Laboratories, Campbell, CA) as a mucosal adjuvant diluted in sterile PBS. The antigens, diluted in 10 μL of PBS, were inoculated into the nostrils (5 μL/nostril) by use of a pipette or into the trachea by intratracheal intubation connected to a microinjector under visualization with the aid of an electric otoscope. The procedures were performed under anesthesia with ip injection of 0.1 mL of a mixture containing 2 mg of ketamine and 0.2 mg of xylazine. For ip immunization, we diluted the antigens, including 10 μg of CM-Hi and 1 μg of CT, in 50 μL of PBS. Our previous studies demonstrated that oral immunization requires a higher dose of antigen and CT [13–15]; therefore, we orally immunized the other mouse group with 250 μg of CM-Hi and 10 μg of CT by gastric intubation without anesthesia. Prior to oral immunization, the mice were deprived of food for 2 h, and 30 min before immunization the mice were gavaged with 0.5 mL of a solution consisting of 8 parts Hanks' balanced salt solution and 2 parts 7.5% sodium bicarbonate by gastric intubation, to neutralize stomach acidity [13–15]. The vaccine was administered 3 times, on days 0, 7, and 14.

Saliva, fecal extract, and serum were collected at weekly intervals and monitored for *H. influenzae*–specific antibodies. Saliva samples were obtained following ip injection with 100 μL of 1 mg/mL pilocarpine (Sigma, St. Louis) diluted in sterile PBS to induce salivary secretion. Fecal extract samples were obtained by adding weighed pellets to PBS containing 0.01% sodium azide (1 mL/100 mg fecal sample) according to the method of deVos and Dick [16]. The pellet was vortexed and centrifuged, and the supernatants were collected for assay. On day 21, mice were sacrificed to obtain nasal wash, bronchoalveolar lavage fluid (BALF), and mucosal and systemic tissues. After removing the mandible, we gently flushed the nasal cavity from the posterior opening of the nose with 200 μL of PBS. Nasal washes flushing out from the anterior openings of the nose were collected. BALF was obtained by irrigation with 200 μL of PBS, by use of a blunted needle inserted into the trachea after tracheotomy. The recovered washes were centrifuged to remove cellular debris, and the supernatants were collected for ELISA (see below).

Preparation of single-cell suspensions. Single spleen-cell suspensions were obtained as described previously [17]. The nasal passages and lungs were carefully excised, teased apart, and dissociated with 0.5 mg/mL collagenase type IV (Sigma) to obtain single-cell suspensions. After we removed Peyer's patches, we stirred the small intestine in PBS containing 1 m*M* EDTA at 37°C for 30 min. The lamina propria lymphocytes were subsequently isolated by use of collagenase type IV. The mononuclear cells were then centrifuged over a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden), and cells at the interface of the 40% and 75% layers were collected [18].

Detection of H. influenzae*–specific antibodies by ELISA. H. influenzae*–specific antibody titers in nasal wash, saliva, BALF, fecal extract, and serum were determined by ELISA according to a previously reported method [14, 15]. An antigen preparation of CM-Hi was used as coating antigen (5 μg/mL). The color reaction was developed for 15 min at room temperature with 100 μL of 1.1 m*M* 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid in 0.1 *M* citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2 (Moss, Pasadena, MD). End-point titers were expressed as the reciprocal $\log_2$ of the last dilution giving an optical density (OD) at 414 nm of ⩾0.1 OD unit above the OD at 414 nm of negative control samples obtained from nonimmunized mice.

Detection of H. influenzae*–specific antibody-forming cells (AFCs) by ELISPOT assay.* Mononuclear cells obtained from mucosal and systemic tissues were suspended in complete medium (RPMI 1640 supplemented with 10 mL/L nonessential–amino acids solution, 1 m*M* sodium pyruvate, 10 m*M* HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 40 μg/mL gentamicin, and 10% fetal calf serum), and the numbers of *H. influenzae*–specific AFCs in various tissues were determined by use of ELISPOT assay with CM-Hi as a coating antigen [13]. The color reaction was developed at room temperature for 30 min with 1.6 m*M* 3-amino-9-ethylcarbazole in 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.05% H_2O_2 (Moss). The plates were washed with water and dried, and the numbers of *H. influenzae*–specific AFCs were quantified under a stereomicroscope.

H. influenzae*–specific CD4⁺ T cell responses.* The $CD4^+$ T cell subset was obtained from single spleen-cell suspensions by 2 cycles of positive-sorting with a magnetic bead separation system consisting of biotinylated anti-CD4 monoclonal antibody (MAb, clone GK1.5) and streptavidin microbeads (MACS; Miltenyi Biotec, Sunnyvale, CA) as described previously [19]. Purified splenic $CD4^+$ T cells (>95% purity) were cultured in complete medium, at a density of 2×10^6 cells/mL, with several dosages of CM-Hi (500, 200, 100, 10, 1 μg/mL) in the presence of recombinant interleukin (IL)-2 (10 U/mL; PharMingen, San Diego) and feeder cells (2.5×10^6 cells/mL). In preparation of feeder cells, splenic cells isolated from nonimmunized mice were treated with anti-mouse Thy 1.2 antibody and baby rabbit complement to deplete T cells and then were irradiated (3000 rads [30 Gy]) according to the standard method routinely used in our laboratory [13].

An antigen dose of 200 μg/mL gave optimal *H. influenzae*–specific $CD4^+$ T cell proliferative responses. To determine cytokine production by *H. influenzae*–specific $CD4^+$ T cells, we incubated 6-well cell culture plates (Corning Glass Works, Corning, NY) containing $CD4^+$ T cells (2×10^6 cells/mL), feeder cells (2.5×10^6 cells/mL), and CM-Hi (200 μg/mL) at 37°C in 5% CO_2. After being cultivated for 96 h, the cells were harvested for quantification of cytokine-producing cells by cytokine-specific ELISPOT assay, and the supernatants were collected for evaluation of secreted cytokine concentrations by ELISA.

Detection of cytokines by ELISA. Th1 and Th2 cytokines in

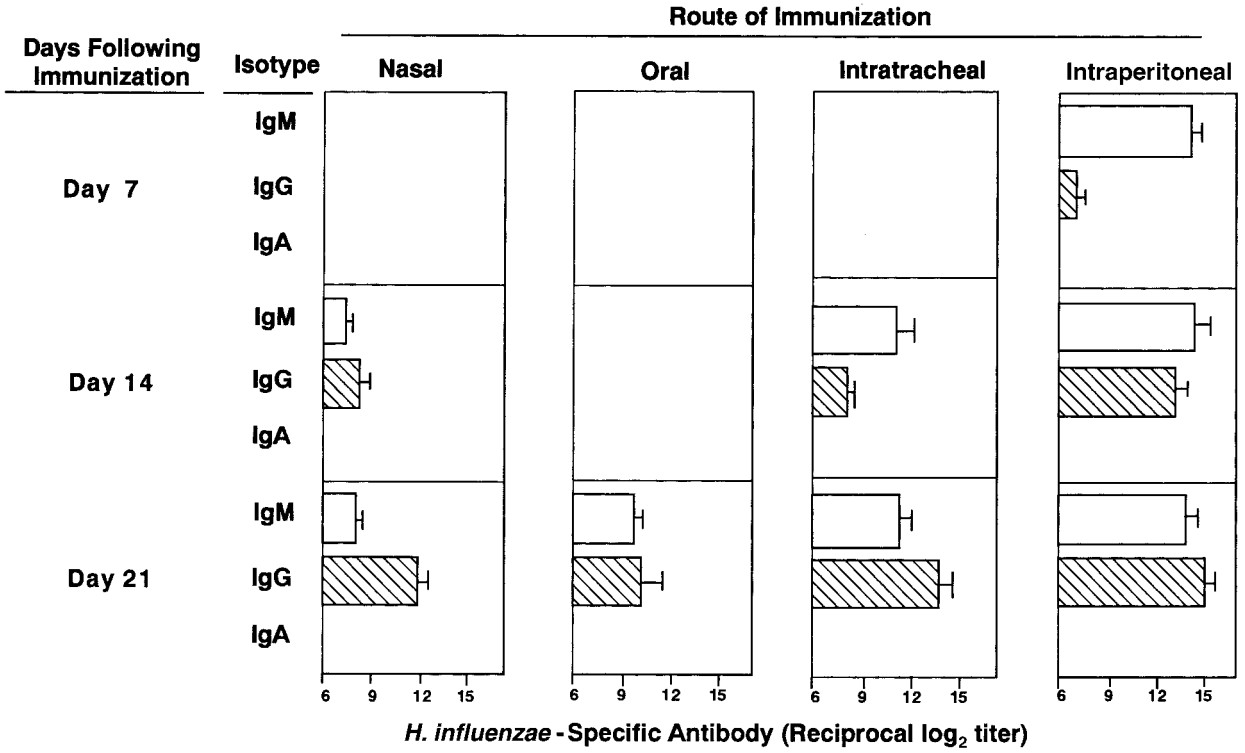

Figure 1. Nasal immunization induces *Haemophilus influenzae*–specific serum IgM and IgG but not IgA antibody responses. Mice were immunized mucosally or systemically with cell membrane preparation from nontypeable *H. influenzae* and cholera toxin. Isotype- and antigen-specific antibody titers were determined by end-point ELISA. Values represent mean antibody titer ± SE.

culture supernatants were determined by use of ELISA as described previously [19]. In brief, plates (ImmunoMaxiSorp; Nunc, Naperville, IL) were coated with 2.5 μg/mL of rat anti-mouse interferon (IFN)-γ (clone R4-6A2), IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A5) MAbs (PharMingen). Serial dilutions of culture supernatants or standard recombinant cytokines (PharMingen) were added in duplicate. For detection and quantification of secreted Th1 and Th2 cytokines, 0.2 μg/mL of biotinylated rat anti-mouse cytokine MAbs (clones XMG1.2, JES6-5H4, BVD6-24G2, TRFK4, MP5-32C11, SXC-1; PharMingen) for IFN-γ, IL-2, IL-4, IL-5, IL-6, or IL-10 and 1:4000 diluted horseradish peroxidase–labeled anti-biotin antibody (Vector Laboratories, Burlingame, CA) were used as the secondary antibody and detection enzyme, respectively. The color reaction was developed at room temperature for 15 min with 1.25 m*M* 3,3′,5,5′-tetramethylbenzidine containing 0.015% H_2O_2 (TMBE-1000; Moss) and was terminated with 0.5 *N* HCl. The ELISA could detect 20 pg/mL IFN-γ, 0.1 U/mL IL-2, 10 pg/mL IL-4, 2 U/mL IL-5, 1 ng/mL IL-6, and 0.5 ng/mL IL-10.

Cytokine-specific ELISPOT assay. To examine the numbers of IFN-γ–, IL-2–, IL-4–, IL-5–, and IL-6–producing cells, we performed a cytokine-specific ELISPOT assay according to a previously described method [18, 19]. In brief, 96-well nitrocellulose-based microtiter plates (Millititer HA; Millipore, Bedford, MA) were coated with appropriate anti-cytokine MAbs (see Detection of cytokines by ELISA, above) diluted in PBS. After being blocked with complete medium, the cells were added to individual wells and incubated for 20 h at 37°C in 5% CO_2. The plates then were incubated with the appropriate biotinylated anti-cytokine MAbs followed by horseradish peroxidase–conjugated goat anti-biotin MAbs (Vector Laboratories). The spots were visualized with 3-amino-9-ethylcarbazole substrate (Moss) and counted under a stereomicroscope.

Nasal challenge with live NTHi. The same strain of NTHi used for the preparation of CM-Hi was cultured on chocolate agar plates overnight at 37°C in 5% CO_2, removed by scraping, and resuspended in PBS (10^9 cfu/mL) for nasal challenge, as reported previously [4]. Mice were immunized with CM-Hi and CT as described above, and 10-μL aliquots of the live NTHi suspension were administered into the nose 1 week after the 3d immunization.

The same dose of live NTHi was also inoculated into nonimmunized mice; 12 h later, mice were sacrificed, and nasal washes were obtained by flushing the nasal cavity with 200 μL of PBS. Nasal washes were also obtained from nonimmunized mice that were not inoculated with NTHi, and the numbers of NTHi were counted, to determine whether contamination by *H. influenzae* other than the inoculated bacteria had occurred.

Since our previous study showed that intranasally inoculated NTHi were cleared from the nasal cavity after a few days and that the effect of mucosal immunization on the clearance of NTHi was most significant between 12 and 24 h after the inoculation with NTHi [4], the numbers of NTHi in nasal washes were examined 12 h after the inoculation with NTHi in the present study. Aliquots

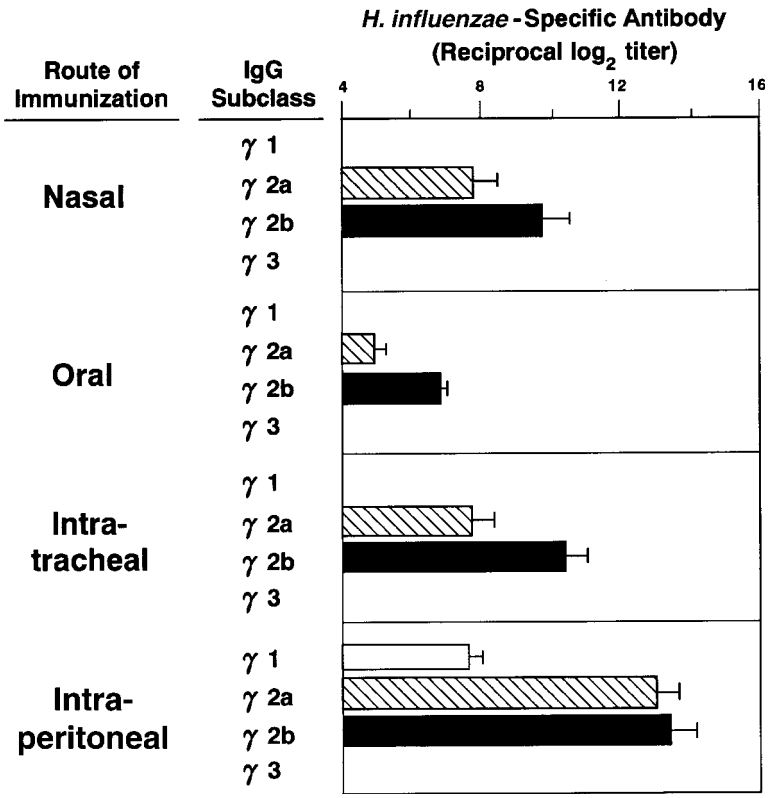

Figure 2. Characterization of *Haemophilus influenzae*–specific serum IgG subclass responses in mice nasally immunized with cell membrane preparation from nontypeable *H. influenzae* and cholera toxin. Antigen-specific antibody titers were determined by end-point ELISA. Values represent mean antibody titer ± SE.

of the recovered washes were diluted with PBS in 10-fold steps, and 10-μL aliquots of the diluted samples were spread on chocolate agar plates. The remaining recovered washes were centrifuged, and the supernatants were collected to determine antibody titers. After overnight incubation at 37°C in 5% CO_2, NTHi was identified by standard bacteriologic techniques, including Gram's staining and determination of their V and X growth factor requirements, and the numbers of colonies were counted [4]. The concentration of NTHi was expressed as colony-forming units per milliliter nasal wash.

Statistics. Comparisons between appropriate groups were performed by use of Student's unpaired *t* test and by analysis of variance followed by Fisher's least-significant–difference test. Differences with $P < .05$ were considered significant.

Results

Nasal immunization induces H. influenzae*–specific antibody responses in both serum and external secretions.* *H. influenzae*–specific serum IgM and IgG antibody titers were increased following nasal, oral, and intratracheal immunization, although the titers were lower than those induced by ip immunization (figure 1). IgG subclasses of *H. influenzae*–specific antibodies were assessed, and the dominant responses were seen for IgG2a and IgG2b subclasses in nasally, orally, intratracheally, and ip immunized mice. In general, the level of *H. influenzae*–specific IgG2b was greater than that of IgG2a (figure 2). Antigen-specific IgG1 subclass responses were not found in the mucosally immunized groups. In contrast, in addition to IgG2a and IgG2b subclasses, *H. influenzae*–specific IgG1 subclass responses were induced by ip immunization. *H. influenzae*–specific IgG3 antibodies were not detected in any of the groups.

H. influenzae–specific IgA antibody titers in external secretions were increased by mucosal immunization through nasal, oral, or intratracheal routes but not by ip immunization (figure 3). The IgA antibody titers in nasal washes and saliva were highest in mice nasally immunized with CM-Hi (10 μg) and CT (1 μg). On the other hand, *H. influenzae*–specific IgA antibody titers in BALF were highest in the intratracheal immunization group given CM-Hi (10 μg) and CT (1 μg). An increase in antigen-specific IgA antibody titer in fecal extracts was found only in mice orally immunized with 250 μg of CM-Hi in the presence of CT (10 μg). In our separate study, oral immunization with 50 or 100 μg of CM-Hi and 10 μg of CT did not induce *H. influenzae*–specific IgA responses in any samples of external secretions (data not shown). These findings further confirmed our previous finding that high doses of antigen and

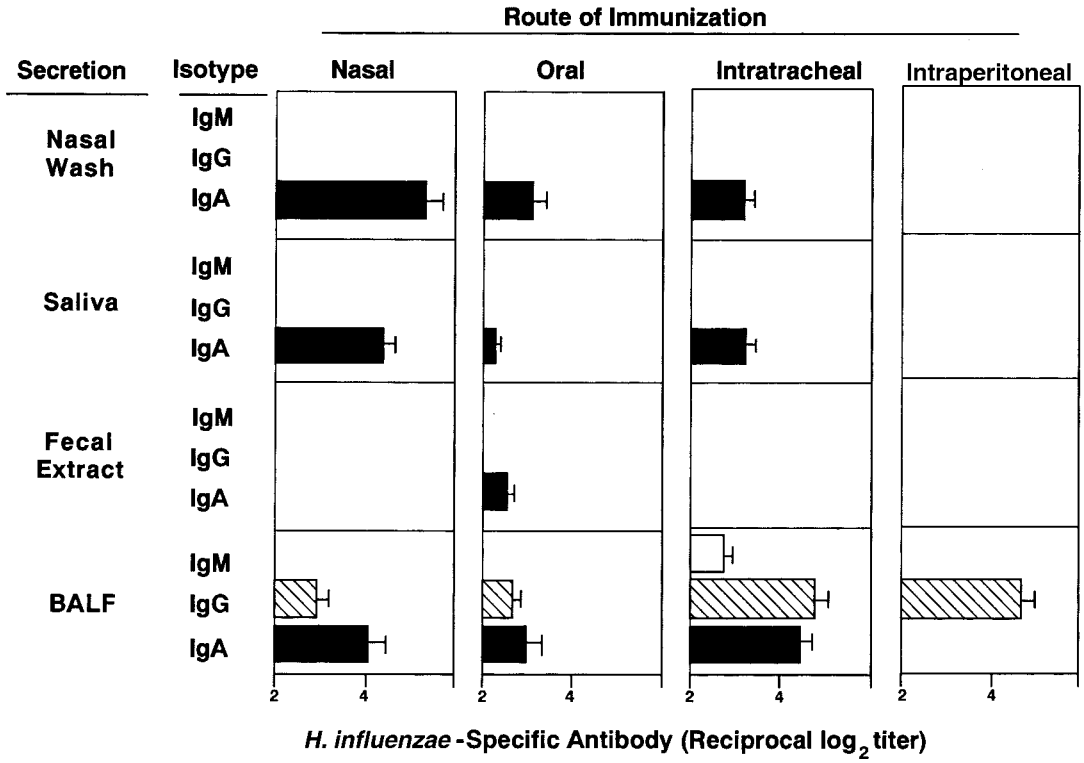

Figure 3. Induction of *Haemophilus influenzae*–specific IgA antibodies in nasal wash, saliva, and bronchoalveolar fluid (BALF) by nasal immunization with cell membrane preparation from nontypeable *H. influenzae* and cholera toxin. Isotype- and antigen-specific antibody titers were determined by end-point ELISA. Values represent mean antibody titer ± SE.

CT were required for the induction of antigen-specific IgA responses by oral immunization [13–15]. Systemic immunization (i.e., ip) with CM-Hi (10 μg) and CT (1 μg) resulted in the induction of *H. influenzae*–specific IgG antibodies in BALF. These findings demonstrated that nasal immunization with CM-Hi and CT is an effective regimen for induction of antigen-specific IgG antibody in serum and IgA antibody in mucosal secretions (including nasal wash, saliva, and BALF), respectively.

Nasal immunization–induced H. influenzae*–specific IgA antibody–producing cells in mucosal effector tissues.* Nasal immunization resulted in high numbers of *H. influenzae*–specific IgA AFCs in nasal passages (figure 4). Although oral and intratracheal immunization induced *H. influenzae*–specific IgA AFCs in the nasal passage, the numbers were smaller than those in the nasal immunization group. The numbers of antigen-specific IgA AFCs in the intestinal lamina propria were highest in the oral immunization group, whereas intratracheal immunization resulted in the highest numbers of *H. influenzae*–specific IgA AFCs in the lungs. In contrast, ip immunization did not induce antigen-specific IgA AFCs in any mucosal or systemic immune compartment. These results indicated that nasal immunization is the most effective way to induce antigen-specific IgA B cell responses in the nasal passages.

Nasal immunization induces H. influenzae*–specific Th1- and Th2-type CD4⁺ T cells.* Since nasal immunization with CM-Hi and CT has been shown to induce *H. influenzae*–specific mucosal IgA and systemic IgG antibody responses, the aim of our studies was to elucidate the nature of antigen-specific $CD4^+$ T cell responses (e.g., Th1 and Th2 cytokine production). Thus, $CD4^+$ T cells isolated from nasally immunized mice were restimulated with CM-Hi (200 μg/mL) in vitro, and culture supernatants were then harvested and analyzed for secreted cytokines by use of Th1 and Th2 cytokine–specific ELISA. IFN-γ and IL-2 production, as well as IL-5, IL-6, and IL-10 production, were noted as representative of Th1 and Th2 cytokines, respectively, in the culture supernatants harvested from wells containing $CD4^+$ T cells from nasal and intratracheal immunization groups (figure 5). In the oral immunization group, the levels of IFN-γ, IL-6, and IL-10 were lower than those in the nasal and intratracheal immunization groups. ip immunization induced the production of Th1 and Th2 cytokines. Further, the production of IL-4 was higher in the ip immunization group than in the nasal, oral, or intratracheal immunization groups.

To further confirm the results we obtained by use of cytokine-specific ELISA, we also used the Th1 and Th2 cytokine–specific ELISPOT assay to elucidate the frequency of cytokine-

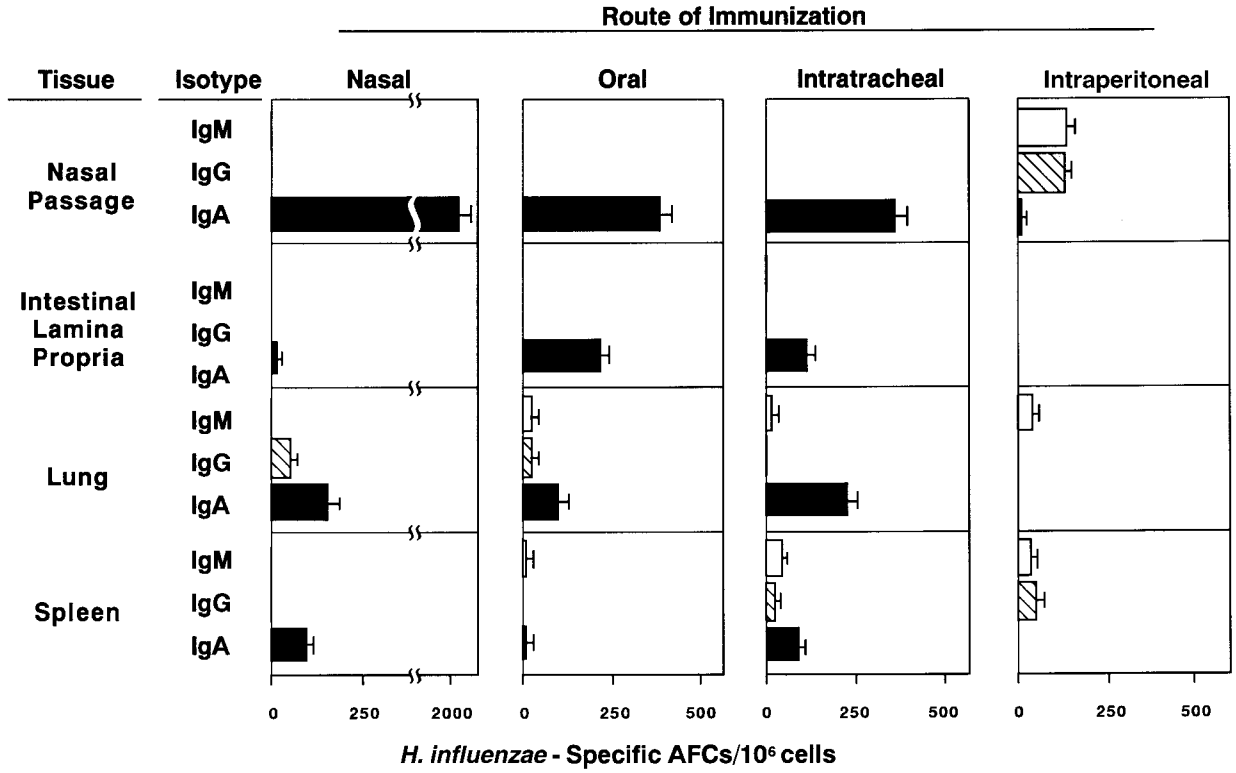

Figure 4. Nasal immunization induces *Haemophilus influenzae*–specific IgA antibody-forming cells (AFCs) in nasal passage. Nos. of *H. influenzae*–specific IgM, IgG, and IgA AFCs were determined by ELISPOT assay. Values represent mean no. of *H. influenzae*–specific AFCs ± SE. Each group consisted of 10 mice, and data are representative of 2 separate experiments.

producing *H. influenzae*–specific $CD4^+$ T cells. The cytokine ELISPOT assay revealed increased numbers of Th1 (e.g., IFN-γ, IL-2) and Th2 (e.g., IL-6) cytokine-producing $CD4^+$ T cells in mice immunized nasally (figure 6). Of interest, the numbers of these cytokine-producing $CD4^+$ T cells were higher in the nasal immunization group than in the other mucosally immunized groups. Systemic immunization also resulted in the induction of both Th1 and Th2 cytokine–producing $CD4^+$ T cells.

Mucosally induced H. influenzae*–specific immune responses provided better bacterial clearance activity than systemic immunization.* Since nasal immunization induced high levels of antigen-specific IgA antibodies in nasal wash and IgG antibodies in serum, it was important to examine whether these *H. influenzae*–specific immune responses contributed to the clearance of NTHi colonization in the nasal tract. As expected on the basis of the results described above (figures 1, 3, 4), nasal immunization resulted in high levels of *H. influenzae*–specific mucosal IgA and serum IgG antibody prior to challenge with NTHi (table 1). The levels of clearance of NTHi from nasal passages were highest in the nasally immunized mice, followed by the intratracheal and oral immunization groups. Thus, the numbers of NTHi cultured from nasal washes were lowest in the nasal immunization group (table 1).

In contrast to results from the mucosally immunized group, ip immunization resulted in increased *H. influenzae*–specific IgG antibody titers in serum but not in increased mucosal IgA (table 1), which confirmed our earlier results (figures 1, 3, 4). When the mice were challenged with NTHi, no bacterial clearance was observed, since the numbers of NTHi cultured from nasal washes were comparable to those of nonimmunized mice. *H. influenzae* was not present in nasal washes obtained from control mice that did not receive nasal inoculation with NTHi. These findings suggested that mucosal immunization, especially by the nasal route, is an effective vaccination regimen to provide protective immunity against NTHi infection.

Discussion

An important aspect of the present study, which distinguishes it from previous studies [6–10], is a direct demonstration of the induction of *H. influenzae*–specific IgA-producing cells in the nasal passages of nasally immunized mice. These findings indicated that, as with Peyer's patches in the intestinal tract, the upper respiratory tract contains an inductive site for the priming of immunocompetent cells to induce antigen-specific mucosal immune responses, and, as such, it has been termed a nasopharyngeal-associated lymphoreticular tissue (NALT) [20,

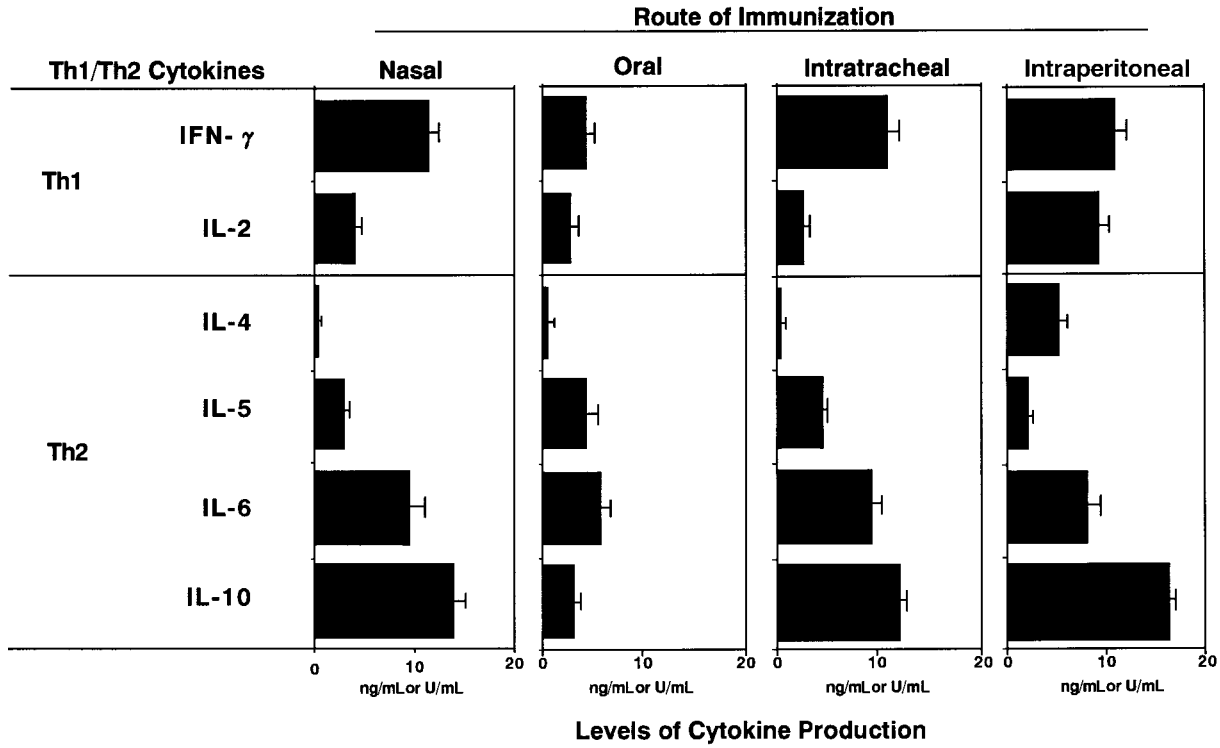

Figure 5. Th1 and Th2 cytokine production by *Haemophilus influenzae*–specific $CD4^+$ T cells isolated from spleen of nasally immunized mice. Cytokine levels in culture supernatants were determined by ELISA. Values represent mean cytokine concentration ± SE. Each group consisted of 10 mice, and data are representative of 2 separate experiments. IFN = interferon, IL = interleukin.

21]. Thus, IgA precursor B cells in NALT are primed and activated by nasal immunization and then disseminate to mucosal effector tissues, including nasal passage tissue, presumably via a common mucosal immune system [22]. Antigen-specific AFCs, predominantly of the IgA isotype, were detected in peripheral blood of rhesus monkeys 1 week after nasal immunization with *Streptococcus mutans* in the presence of CT B subunit [7].

Dense foci of lymphocytes were observed in the NALT of nasally immunized mice, and these cells correlated with the appearance of mucosal immune responses [9]. However, the precise inductive site(s) responsible for initiation of an antigen-specific IgA immune response after nasal immunization of mice is still uncertain. Dye inoculated into the nares of mice stained both the lung cavity and the small intestine, suggesting that antigens were both inhaled and swallowed [10]. Therefore, it was suggested that nasally administered antigens were taken up not only by NALT but also by bronchus- and gut-associated lymphoreticular tissues (BALT and GALT, respectively). On the other hand, a recent study showed that antibody responses in serum and saliva samples of mice immunized orally with bacterial antigens were much lower than those obtained from nasally immunized mice, suggesting that antibody responses induced by nasal immunization might not be due to antigen stimulation of lymphocytes in GALT [6]. The present study also showed that levels of mucosal IgA reactive to CM-Hi induced by oral immunization were much lower than those induced by nasal immunization, despite the use of 25- and 10-fold higher doses of antigen and CT, respectively (figures 3, 4).

In contrast to nasal immunization, intratracheal immunization induced higher systemic and lower mucosal antibody responses (figures 1–4). Those findings suggest that the nasal mucosa (e.g., NALT) is the major inductive site for nasal immunization, although some antigen-specific immune responses induced by nasal immunization may be associated with GALT or BALT lymphocytes stimulated by swallowed or inhaled antigen. With respect to the latter two sites, GALT lymphocytes were not shown to be a likely source of antigen-primed cells for the induction of *H. influenzae*–specific IgA responses because higher doses of CM-Hi and CT were required for oral immunization than for nasal immunization.

It has become clear recently that, although the common mucosal immune system is an important first barrier against infectious agents, the mucosal immune system may not be as common to all effector sites as initially thought. Instead, the common mucosal immune system appears to be compartmentalized, so that application of immunogens to one inductive site preferentially elicits antigen-specific IgA antibodies in adjacent

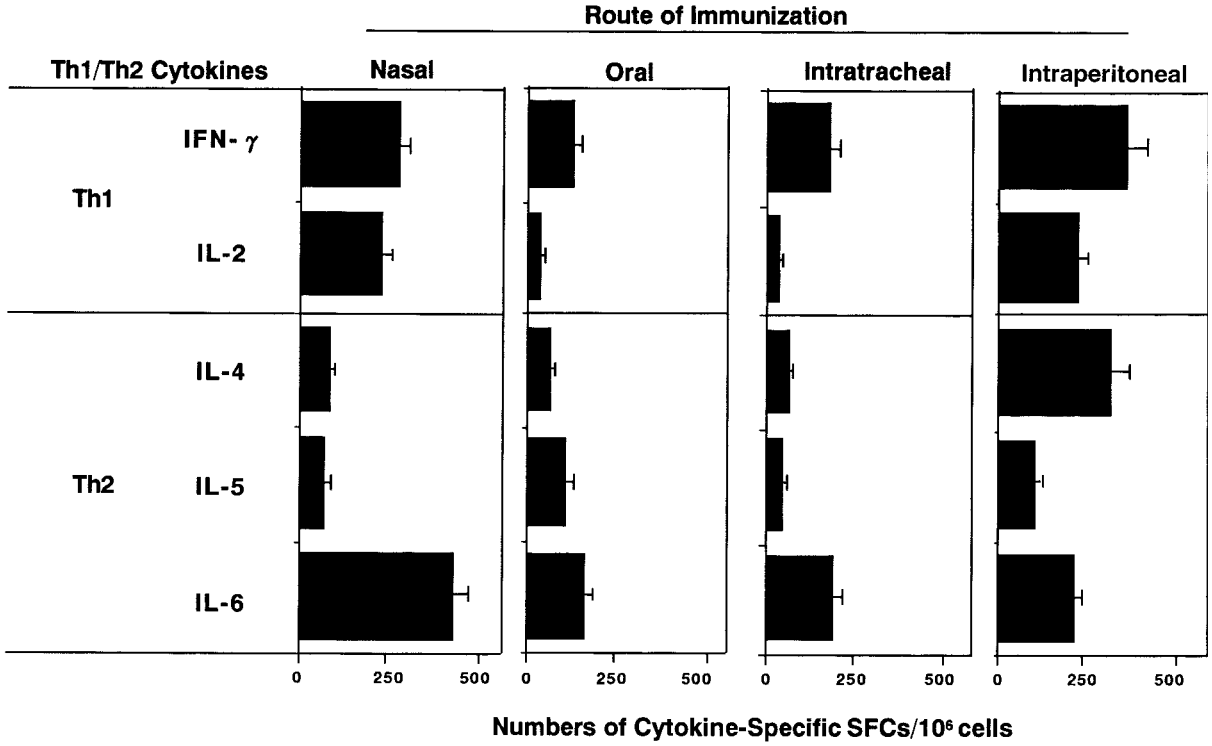

Figure 6. Induction of *Haemophilus influenzae*–specific Th1 and Th2 type $CD4^+$ T cells by nasal immunization. $CD4^+$ T cells purified from splenic mononuclear cells were cultured with cell membrane preparation from nontypeable *H. influenzae* for 4 days and then subjected to cytokine-specific ELISPOT assay. Values represent mean no. of spot-forming cells (SFCs) ± SE. Each group consisted of 10 mice, and data are representative of 2 separate experiments. IFN = interferon, IL = interleukin.

or immunologically and physiologically related effector sites [23, 24]. It was reported that the antigen-specific immune responses in saliva were significantly higher after nasal immunization than after oral immunization [6]. In the present study, we found that *H. influenzae*–specific IgA antibody titers in saliva and nasal washes were higher after nasal immunization with CM-Hi and CT than after either oral or intratracheal immunization. On the other hand, antigen-specific IgA antibody in fecal extract was detected only in the oral immunization group. The IgA antibody titer in BALF was highest in intratracheally immunized mice. Although these findings further demonstrated the occurrence of a compartmentalized common mucosal immune system (e.g., nasal → nasal passage, oral → intestine, and intratracheal → lung), it was interesting to note that relatively high numbers of *H. influenzae*–specific IgA AFC were always induced in the nasal tract by all three separate mucosal immunization routes. This suggests that antigen-specific IgA responses in the nasal mucosa may be the most affected by mucosal immunization, regardless of the route used.

Table 1. The effects of nasally induced *Haemophilus influenzae*–specific IgA and IgG antibodies on the clearance of nontypeable *H. influenzae* (NTHi) from the nasal passages.

Route of immunization	Mean no. of NTHi[a] (log_{10} cfu/mL)	*H. influenzae*–specific antibody titers[b]	
		Nasal wash IgA	Serum IgG
Nasal	1.7[c]	5.4	12.2
Oral	2.3[c]	2.8	10.8
Intratracheal	2.2[c]	3.2	13.5
Intraperitoneal	3.2	<2	15.2
Nonimmunized	3.8	<2	<7
Control	ND	<2	<7

NOTE. ND, not detected.

[a] Mice were mucosally or systemically immunized with cell membrane preparation from NTHi (CM-Hi) cholera toxin. Immunized and nonimmunized mice (5/group) were inoculated with live NTHi. Nasal washes were obtained 12 h after inoculation, and NTHi were counted. Nasal washes were also obtained from control mice, which were not challenged with NTHi.

[b] *H. influenzae*–specific antibody titers were assessed by ELISA with CM-Hi as coating antigen. Values are reciprocal log_2 antibody titers of end-point dilution.

[c] $P < .01$, compared with controls.

It has been shown that orally administered CT has immunomodulatory activity, by the induction of antigen-specific $CD4^+$ T cells secreting IL-4, IL-5, IL-6, and IL-10 (i.e., Th2-type cells, which provide help for serum IgG1, IgE, and mucosal IgA antibody responses) [15–19]. High levels of IL-4 production by antigen-specific $CD4^+$ T cells were observed in mice orally immunized with tetanus toxoid and CT [13]. In the present study, increased production of Th1 cytokines, such as IFN-γ and IL-2, were observed in addition to Th2 cytokines in splenic

$CD4^+$ T cells isolated from mice immunized mucosally with CM-Hi plus CT (figure 5). Mononuclear cells obtained from patients with arthritis or chlamydial infection also produce both Th1- and Th2-type cytokines in response to bacterial antigens [25, 26]. At present, we do not have any specific explanation for the discrepancy between the results of the present and previous studies. Although IL-4 induces differentiation of uncommitted T cells toward a Th2 phenotype [27], IL-4 knockout mice have normal mucosal IgA antibody levels [28].

Furthermore, orally administered recombinant *Salmonella* species elicited dominant antigen-specific Th1-type responses together with Th2-type cells producing IL-10 but not IL-4 or IL-5 [19]. Select cytokines (i.e., IL-6 and IL-10) from Th2-type cells are thought to play an important compensatory role in induction and regulation of mucosal IgA responses [28]. Since IFN-γ and IL-4 act as antagonists to each other [29], the production of high levels of IFN-γ might have led to reduced IL-4 production by splenic $CD4^+$ T cells in mucosally immunized mice in the present study. On the other hand, ip immunization resulted in the production of IL-4 in the presence of high levels of IFN-γ. Although we cannot readily explain this result, it has been reported that IL-4 production by splenic $CD4^+$ T cells was greater after ip immunization than after oral immunization [13]. In contrast, the production of IL-4 by splenic $CD4^+$ T cells was lower than that by $CD4^+$ T cells isolated from Peyer's patches, after oral immunization [13]. Thus, the route of immunization may affect the activation of Th2-type $CD4^+$ T cells, which then influence the differences in IL-4 production seen between mucosal and ip immunization.

Another possible explanation could be the nature of antigen used in these different studies (e.g., tetanus toxoid vs. CM-Hi). Although CM-Hi preparations used in the present study were enriched for proteins, these preparations contained some residual endotoxin (~0.2%). The increase of antigen-specific IgG2a and IgG2b subclass antibody responses and the lack of antigen-specific IgG1 subclass antibody response reflect this possibility, because carbohydrate-specific responses are generally associated with these IgG2 subclasses [30]. IgG2a and IgG2b MAbs are known to bind specifically to saccharide residues of *H. influenzae* lipopolysaccharide (LPS) [31]. The IgG antibody induced by LPS from *Salmonella minnesota* was strictly restricted to IgG2b [30]. Meningococcal LPS-derived oligosaccharides coupled to tetanus toxoid shift the IgG subclass distribution from mainly IgG1 toward the complement-activating subclasses IgG2a and IgG2b [32]. Furthermore, IFN-γ enhances the secretion of IgG2a by murine splenic B cells stimulated with bacterial LPS and concomitantly suppresses the production of IgG1 and IgG3 [33]. When mice were given IFN-γ at the time of immunization with *Pseudomonas aeruginosa* LPS, antigen-specific IgG2a antibody was increased, with a concomitant decrease in IgG1 antibody [34]. Thus, the polysaccharide moiety of CM-Hi may influence the outcome of antigen-specific IgG subclass responses.

In addition to the above two possibilities, the other explanation might be the nature of antigen-specific Th2 cells induced by mucosal immunization with CM-Hi and CT. The mucosal adjuvant CT induces IL-4 production by $CD4^+$ T cells [13], which leads to the promotion of IgG1 subclass switching and subsequent production of this subclass of specific antibody [35]. However, in the present study, the production of IL-4 was reduced despite the use of CT, and the IgG1 subclass response was not observed in mucosally immunized mice. An interesting possibility would be that mucosal immunization with CM-Hi and CT may induce level two–type Th2 cells producing selectively IgA-enhancing cytokines (e.g., IL-5, IL-6, and IL-10) without IL-4. These level two–type Th2 cells are often induced by mucosal immunization with intracellular bacteria (e.g., *Salmonella* species) that possess an ability to preferentially induce Th1-type responses. Oral immunization with recombinant *Salmonella* species resulted in the induction of concomitant antigen-specific mucosal IgA and systemic IgG2a antibody responses [19]. Thus, multiple stimulation signals provided by protein and polysaccharide moieties of the antigen and mucosal adjuvant CT may lead to activation of different cytokine cascades, which results in the coexistence of a unique subset of Th2 cells in the presence of IFN-γ–secreting Th1-type cells for the induction of mucosal IgA and different IgG subclass responses.

Secretory IgA (S-IgA) has an inhibitory effect on bacterial adherence [36, 37]. Salivary S-IgA has been shown to possess inhibitory activity toward various species of *Streptococcus,* and the inhibitory activity of S-IgA was correlated with the agglutinating activity [36]. The adherence of NTHi to human nasopharyngeal epithelial cells was markedly reduced by nasopharyngeal secretions containing NTHi-specific S-IgA antibodies [37]. In murine studies, salivary anti-adhesive activity and agglutination titers to NTHi were significantly greater in samples with antigen-specific IgA antibody induced by oral immunization with NTHi than in control samples [4]. Clinical investigations have also shown that the decreased nasopharyngeal colonization of NTHi was related to the occurrence of P6 (an outer membrane protein of NTHi)-specific S-IgA antibody in nasopharyngeal secretions [38].

Furthermore, it was recently shown that nasal immunization with P6 and CT resulted in the induction of *H. influenzae*–specific protective immunity [39]. In the present study, the clearance of NTHi from the nasal passage was enhanced by nasal, oral, or intratracheal immunization with CM-Hi, and the highest protective effect was observed when mice were immunized nasally. This bacterial clearance was associated with mucosal IgA responses in the nasal passages, since nasal immunization, compared with that in other groups, resulted in the highest *H. influenzae*–specific IgA antibody response. Although nasal immunization also resulted in the induction of antigen-specific IgG antibody responses, this antibody isotype may not be protective. Mice systemically immunized with CM-Hi and CT har-

bored high levels of antigen-specific serum IgG responses without mucosal IgA, which did not lead to the clearance of NTHi from the nasal passage.

In summary, the present study showed that nasal immunization was a more effective vaccination regimen than the other mucosal immunization methods examined for the induction of *H. influenzae*–specific mucosal immune response, which can reduce the colonization of NTHi in the nasal passages. The mucosal *H. influenzae*–specific IgA as well as systemic IgG2a and IgG2b responses induced by nasal immunization were mediated by antigen-specific $CD4^+$ Th2- and Th1-type cells, respectively. The present study suggested that nasal vaccination may be a useful strategy for prevention of OME and other upper respiratory tract diseases caused by NTHi.

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