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Middle Ear and Eustachian Tube

Chapter 88

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Despite the development of a variety of antibiotics and the improvement of sanitary conditions, otitis media (OM), such as acute otitis media (AOM) and otitis media with effusion (OME), is still one of the most prevalent infectious diseases in children and accounts for a significant medical cost. Further, the bacteria that commonly cause OM are increasingly showing antibiotic resistance. Therefore, the development of a vaccine against those bacteria is considered an important goal for public health.

Many studies have demonstrated that immunologic reactions are involved in the occurrence of OM. Elevated specific serum antibodies, mainly IgG, inactivate pathogenic bacteria and resolve the infection. Infants with recurrent episodes of OM have been shown to have significantly decreased IgG antibody levels against pneumococcal capsular polysaccharide (Prellner *et al.*, 1989). It has also been reported that children with recurrent OM have decreased serum IgG2 subclass levels (Freijd *et al.*, 1985). These findings indicate that systemic humoral immunity protects the middle ear from bacterial infection and that enhancement of the immunity might be effective to prevent OM. However, the protective efficacy of parenteral administration of pneumococcal polysaccharide vaccine was disappointing (Karma *et al.*, 1985). The poor effect of the vaccine suggests that reformulation of the immunizing preparation and use of different routes of immunization, such as oral and intranasal immunization, may enhance the protective efficacy against OM.

Mucosal immunization induces the production of secretory IgA (S-IgA) in external secretions via mucosal immune system. In earlier studies, Mogi *et al.* (1974) isolated S-IgA from pooled middle ear effusions (MEEs) and revealed that the antigenicity and subunit structure of S-IgA are identical to those of S-IgA obtained from other external secretions such as saliva, nasal secretion, colostrum, and bronchial fluid. Ogra *et al.* (1974) demonstrated that specific antibody activity in MEEs against mumps, measles, rubella, and poliovirus is limited to S-IgA. Further, intraduodenal or intratracheal immunization induced antigen-specific IgA-forming cells in the tympanic mucosa (Watanabe *et al.*, 1988). These findings suggest that the middle ear might be a potential organ to act as an effector site of the mucosal immune system.

The purpose of this chapter is to review recent available data regarding the mucosal immune system equipped in the middle ear and microbiologic as well as immunologic aspects of OM, and to discuss the efficacy of mucosal vaccines for OM.

MICROBIOLOGY OF OTITIS MEDIA

Streptococcus pneumoniae, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* are the most common causative bacteria for AOM as well as OME. Because those pathogens ascend into the middle ear from the nasopharynx through the eustachian tube, nasopharyngeal colonization with those bacteria is considered the prerequisite for OM. In fact, most of the pathogens cultured from MEEs are identical to those found in the nasopharynx (Kurono *et al.*, 1988). The carriage rate of NTHi in the nasopharynx is higher in patients with OME than in healthy children, and the intensity of the colonization is associated with the occurrence of this disease. Ueyama *et al.* (1995) investigated the presence P6 gene DNA of *H. influenzae* in nasopharyngeal secretions

by PCR and demonstrated that the incidence was significantly higher in patients with OME than that in controls. They also reported that P6 gene DNA was detected in all nasopharyngeal secretions of patients with OME who had P6 gene DNA in MEEs. Those findings suggest that microorganisms in the nasopharynx, as well as those in the middle ear, play an important role in the pathogenesis of OM. Further, Rayner *et al.* (1998) demonstrated the presence of bacterial mRNA in MEEs by an RT-PCR-based assay. Because bacterial mRNA has a half-life measured in seconds to minutes, detection of bacteria-specific mRNA would be evidence that metabolically active organisms are present. The results showed that all specimens having DNA of *H. influenzae* detected by PCR but negative by conventional culture method were positive by RT-PCR, indicating the presence of viable, metabolically active, intact bacteria in some culture-negative cases of OME.

Recent studies regarding microbiology of OM have focused on identification of bacterial adherence factors such as *H. influenzae* fimbriae (Hif), high-molecular-weight (HMW) adhesion proteins, and pneumococcal surface adhesion A (PsaA). Hif is classified into the major (HifA) and minor (HifD and HifE) subunits, and both the major and minor subunits were required for adherence of *H. influenzae* to oropharyngeal epithelial cells (van Ham *et al.*, 1995). However, immunologic and structural characteristics vary among NTHi (McCrea *et al.*, 1998). In contrast, HMW adhesion proteins, HMW1 and HMW2, are members of a family of highly immunogenic proteins and are common to 70% to 75% of NTHi strains (Barenkamp and St Geme, 1996), suggesting the possibility of developing vaccine against diseases caused by NTHi including AOM and OME.

Lipooligosaccharide (LOS) is known to be a virulence factor of NTHi. DeMaria *et al.* (1997) examined the relative virulence of a parent NTHi strain and two different LOS-deficient mutants by evaluating the ability of those strains to colonize the nasopharynx and to induce OM subsequent to transbullar inoculation in chinchilla model. They found that the abilities of mutants to induce OM and to persist in the middle ear were significantly decreased compared with the parent NTHi. Thus, the presence of intact LOS molecules appears to be critical to the virulence of NTHi in inducing OM. Moreover, Sun *et al.* (2000) reported that detoxified LOS-protein conjugates from NTHi elicited a significant rise of anti-LOS antibodies with bactericidal activity and inhibitory activity of the adherence of NTHi. Those data indicate the availability of detoxified LOS as a vaccine for OM.

Most cases of AOM are preceded by a viral upper respiratory infection. Okamoto *et al.* (1993) detected genomic sequences of RSV in the samples of MEE obtained from patients with OME by RT-PCR. In those patients from whose nasopharynx RSV was isolated, the viral sequences were highly detectable in MEEs. Recently, Pitkaranta *et al.* (1998) reported that viral RNA of human rhinoviruses, RSV, and coronaviruses was detected in 48% of MEEs, 62% of nasopharyngeal aspirate samples, and in 57% of bacteria-negative MEE samples collected from children with AOM at the time of diagnosis. Heikkinen *et al.* (1999) also examined the prevalence of various respiratory viruses in MEEs of 456 children with AOM. They detected RSV in 74% of samples, parainfluenza viruses in 52%, and influenza viruses in 42% of MEE, suggesting that RSV was the principal virus invading the middle ear during AOM. These findings highlight the importance of common respiratory viruses in predisposing young children to AOM and in causing this disease.

Wadowsky *et al.* (1995) examined the relationship between viral infection and isolation of bacterial pathogens from the middle ear. They intranasally inoculated influenza viruses into adult subjects and found that *S. pneumoniae* was isolated from 15% of subjects on day 6, whereas the bacteria was not isolated before the virus challenge. Belshe *et al.* (1998) reported that live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine reduced the incidence of AOM during the epidemic of influenza. In an experimental study using mice, influenza A virus infection changed the glycoconjugate sequence of the surface of nasopharyngeal mucosa, and increased the colonization of *H. influenzae* and *S. pneumoniae* inoculated after the virus infection (Hirano *et al.*, 1999). Suzuki and Bakaletz (1994) demonstrated the synergistic effects between adenovirus and NTHi in inducing experimental OM. The attachment of P5-fimbriated NTHi to respiratory epithelial cells was significantly enhanced by RSV infection (Jiang *et al.*, 1999). Those results indicate that viral infection might be the sole etiology of OM in some children and that preceding viral infection alters the defenses preventing bacterial colonization and invasion into the middle ear.

IMMUNE RESPONSES DURING OTITIS MEDIA

Serum antibodies, mainly IgG, induced during OM are pathogen specific. All patients with OME who lacked serum IgG activity against the organism causing the second episode possessed the antibody activity against the first strain at the time of the second episode (Faden *et al.*, 1989). On the other hand, Yamanaka and Faden (1993) reported that certain children, who are classified as otitis prone, do not respond well to P6, whereas non-otitis-prone individuals are more likely to develop a significant antibody response to P6. P6 is highly conserved among strains of NTHi and serves as a target for bactericidal antibody. DeMaria *et al.* (1996) demonstrated that subcutaneous immunization of chinchillas with P6 elevated serum bactericidal antibody activity against homologous as well as a heterologous NTHi isolate and reduced the incidence of NTHi culture-positive middle ear fluids after post-transbullar challenge with live NTHi. Further, Yamanaka and Faden (1993) reported that the concentration of P6-specific IgG in MEE was directly related to the concentration of anti-P6 antibody in serum and that the concentration of P6-specific IgG in MEE was inversely related to the number of bacteria in MEE. Those findings

suggest that systemic immune responses may account for the resolution of OM.

Although specific IgG antibodies are protective against the invasion of bacterial antigen into the middle ear, some evidence suggests that systemic immune system may be involved in inducing or sustaining MEEs. Ueyama *et al.* (1997) reported that when chinchillas were inoculated with live *S. pneumoniae* after systemic immunization with killed bacteria of the same strain, 7 out of 20 animals developed chronic OME. These findings suggest that the formation of immune complexes induces immune-mediated OM and is associated with the persistence of inflammatory reactions in the middle ear.

The presence of antigen-specific IgA antibodies in MEEs of patients with AOM as well as OME suggests that the mucosal immune system in the middle ear plays a role in the pathogenesis of those diseases. Liu *et al.* (1975) found that IgA levels in MEEs were significantly higher in culture-negative samples than in culture-positive samples, and that bacterial recovery rate was related inversely to the increase in the levels of IgA and IgG in MEEs. Sloyer *et al.* (1974) reported that specific IgG, IgM, and IgA antibodies against causative pneumococcal serotype were found in 27% of MEEs of patients with AOM, and that specific IgA antibodies were detected more often in MEEs than in serum. On the other hand, Yamanaka and Faden (1993) measured antibody activities in MEEs to P6 of NTHi and reported that IgG specific to P6 was detected more frequently than IgM, IgA, and S-IgA antibodies. These findings suggest that the local as well as systemic immunity is associated with the immune responses in the middle ear cavity and the pathogenesis of OM.

THE ROLE OF CYTOKINES IN OTITIS MEDIA

Inflammatory cytokines such as IL-1 and TNF-α were found in MEEs of children with OME, and the levels of those cytokines were higher in younger children than in older children (Yellon *et al.*, 1991). Schousboe *et al.* (2001) investigated the relationship among microorganisms, endotoxin, and inflammatory mediators in OME and found positive correlation between endotoxin and inflammatory cytokines such as TNF-α and IL-1β in culture-positive MEEs as well as in culture-negative ones, suggesting endotoxin-induced local production of TNF-α and IL-1β in the middle ear. Watanabe *et al.* (2001) further investigated the relationship between endotoxin and the production of IL-1β. They developed a murine model of OME by intratympanic injections with endotoxin derived from NTHi and with recombinant IL-1β. Anti-IL-1 receptor antibodies inhibited the pathologic changes induced by endotoxin and by recombinant IL-1β. These results suggest that IL-1β is associated with the middle ear inflammation induced by endotoxin. Further, IL-1β induces the production of IL-8, which is associated with the migration of neutrophils and the secretion of mucin from goblet cells (Smirnova *et al.*, 2002).

Inflammatory cytokines were detected in nasopharyngeal secretions. Healthy children, 1 to 3 years of age, were found to have significantly higher levels of IL-1β and TNF-α in nasopharyngeal secretions compared with healthy adults. In contrast, children with recurrent episodes of OM had significantly lower levels of IL-1β than healthy children (Lindberg *et al.*, 1994). The results indicate that nasopharyngeal cytokine activity is protective for middle ear infection and that the defect in cytokine production might be responsible for defective immune reactivity.

The increase of IgA antibodies in MEEs indicates the induction of mucosal immune response in the middle ear during OM. Mucosal immune response is regulated by immunoregulatory cytokines such as IL-2, IL-4, IL-5, and TGF-β. IL-2 and IL-4 function primarily as general immune activators in immunoglobulin production and stimulate B cells already committed to IgM, IgG, or IgA to differentiate into plasma cells. IL-5 preferentially stimulates the proliferation and production of IgA-B cells already committed to this isotype. TGF-β stimulates class-switching of IgM-B cells to an IgA phenotype. Bikhazi and Ryan (1995) investigated the expression of those immunoregulatory cytokines associated with the production of different antibody isotypes in experimental acute and chronic OME using *in situ* mRNA hybridization. They found that cells producing IL-2 and IL-4, but not IL-5, were present during acute OME. However, in chronic OME, IL-2- and IL-4-producing cells were less prevalent, but cells producing IL-5 were numerous. Cooter *et al.* (1998) demonstrated the presence of TGF-β in the middle ear, and showed that TGF-β1 and TGF-β2 levels in MEEs are elevated in association with a history of previous tympanostomy tube placements and chronic mucoid effusion. Those results are consistent with the enhancement of IgG production in acute OME and increased local production of IgA during chronic OME. Further, the findings indicate the induction and enhancement of mucosal immune responses during the inflammatory process in the middle ear.

INNNATE IMMUNE SYSTEM IN THE TUBOTYMPANUM

There are few immunocytes in the normal tubal and middle ear mucosa of humans and animals (Ichimiya *et al.*, 1990) and immune cell recruitment requires antigen stimulation (Matsune *et al.*, 1996). However, the middle ear cavity of normal human and laboratory animals is sterile, although numerous immunocompetent cells as well as a variety of pathogens are found in the middle ear during OM. These findings imply that there are highly effective antimicrobial defense systems, so called the innate immune system, protecting the tubotympanum other than the acquired immune system. The system consists of the mucociliary function of the tubotympanum and the molecules secreted by its epithelial cells, which create a highly effective barrier against invading pathogens and help to maintain the sterility of the middle ear cavity. Included in the molecules of the innate immune

system are lysozyme, lactoferrin, defensins, and members of the collectin family of the surfactant proteins such as SP-A and SP-D (Bevins, 1999). Increased production of those molecules in MEEs suggests the importance in the pathogenesis of OM.

Lysozyme has potent antibacterial properties because of its ability to disrupt bacterial cell walls, and it is an important participant in host defense at mucosal surfaces, pleural fluid, and in leukocytes (Leitch and Willcox, 1998). Although there are no data available as to the role of lysozyme in the protection of the tubotympanum, it is possible that tubotympanal lysozyme, together with other antibacterial factors, may protect these tissues. Lim *et al.* (2000) demonstrated that lysozyme itself did not kill NTHi, but preexposure of bacteria to lysozyme enhanced bactericidal activity of β-defensins.

Lactoferrin is an iron-binding glycoprotein found in the milk and exocrine secretions of mammals including those of eustachian tube and middle ear mucosa. Lactoferrin synergistically interacts with immunoglobulins, complement, and neutrophil cationic proteins against gram-negative bacteria by inflicting damage to the outer membrane (Ellison, 1994). Lactoferrin is also active against NTHi, and the proteolytic activity was first discovered when strains of NTHi were cultured in the presence of human milk whey (Qiu *et al.*, 1998). Recently, it has been demonstrated that the proteolytic activity of lactoferrin causes Hap adhesin of NTHi strains to lose their ability to adhere to epithelial cells and removes most of the IgA protease inhibitor from the outer membrane of those bacteria (Plaut *et al.*, 2000).

Defensins are endogenous antimicrobial peptides widely distributed among the plant and animal kingdoms. The peptides represent elements of the ancestral immune system predating lymphocytes and immunoglobulins (Lehrer and Ganz, 1999). The vertebrate defensin family can be divided into α- and β-defensins. β-defensins are primarily expressed by the epithelial cells of skin, kidneys, and tracheobronchial lining of nearly all vertebrates, where they can be released upon microbial invasion or upregulated by stimulation with lipopolysaccharide and TNF-α (Stolzenberg *et al.*, 1997). Boe *et al.* (1999) demonstrated the presence of human β-defensin-1 mRNA in the tympanic membrane and meatal skin by RT-PCR and *in situ* hybridization studies. Recently, Moon *et al.* (2002) showed the expression of β-defensin-2 in the human middle ear mucosa and human middle ear epithelial cell line. Further, they demonstrated that its expression is induced by proinflammatory stimuli such as IL-1α, TNF-α, and lipopolysaccharide. Although the involvement of these defensins in the defense of the eustachian tube and middle ear is yet to be proven, the potent microbicidal properties of defensins induced in the human middle ear epithelial cell line suggests that they may play a role in the pathogenesis of OM.

SP-A and SP-D can bind to bacteria, fungi, and viruses via their carbohydrate groups causing them to agglutinate. This promotes phagocytosis by alveolar macrophages (Nepomuceno *et al.*, 1997). Several investigators have demonstrated the expression of surfactant proteins SP-A and SP-D in the eustachian tube and middle ear epithelium (Dutton *et al.*, 1999; Paananen *et al.*, 1999). Although the role of SP-A and SP-D in the defense of the tubotympanum remains to be proven, it is likely that a deficiency in these molecules may contribute to the pathogenesis of OM.

Mucins are also associated with the innate immune system of tubotympanum. Mucins are high-molecular-weight glycoproteins that constitute the major component of mucus secretions in all mucosal surfaces including the eustachian tube and middle ear. Mucus secretions protect and lubricate the epithelial surface and trap bacteria and viruses for mucociliary clearance. The mucus blanket and the periciliary fluid seromucin are critical for the proper functioning of the mucociliary system. Moreover, mucins act as receptors for a variety of bacteria including *H. influenzae* and are associated with bacterial adherence to epithelial surface. Thus, mucins play an important role in the mechanical clearance of bacteria.

THE MUCOSAL IMMUNE SYSTEM IN THE TUBOTYMPANUM

Because the pathogens colonizing in the nasopharynx invade into the middle ear mucosa via the eustachian tube and cause OM, the nasopharynx is equipped with a specific immunologic defense system. Several studies have demonstrated that increased levels of S-IgA in nasopharyngeal secretions are associated with the decrease of the colonization of middle ear pathogens such as *S. pneumoniae* and *H. influenzae* in the nasopharynx (Kurono *et al.*, 1991; Harabuchi *et al.*, 1994). The inhibitory effect of IgA antibodies against nasopharyngeal colonization was confirmed by animal experiments (Suzuki *et al.*, 1998; Kurono *et al.*, 1999). Oral as well as intranasal immunization of mice with outer membrane proteins of NTHi significantly enhanced antigen-specific IgA antibody titers in nasal wash and the ability to clear the same strain of live NTHi inoculated into the nasopharynx. On the other hand, subcutaneous immunization affected neither IgA antibody titer in nasal wash nor nasopharyngeal colonization by NTHi. DeMaria *et al.* (1996) also reported that parenteral immunization with P6 did not alter the extent or duration of nasopharyngeal colonization by NTHi. These results indicate that IgA antibodies in nasopharyngeal secretions inhibit the adherence of middle ear pathogens to nasopharyngeal mucosa and facilitate the clearance of those bacteria from the nasopharynx.

It is well acknowledged that S-IgA antibody is induced by the mucosal immune system, which is anatomically and functionally divided into two compartments: inductive sites and effector sites. The inductive sites include the gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), and nasal-associated lymphoid tissue (NALT). IgA precursor B cells in the inductive sites are primed and activated by mucosal immunization and disseminate throughout the mucosal effector sites such as nasal mucosa, nasopharyngeal mucosa, and salivary glands via the

common mucosal immune system (McGhee and Kiyono, 1994). NALT has been identified as an inductive site of rodents, and the embryologic as well as immunologic characteristics recently have been investigated (Fukuyama *et al.*, 2002). In humans, the adenoids and palatine tonsils are considered to act as NALT. Quiding-Järbrink *et al.* (1995) showed that intratonsillar vaccination induced a substantial immune response such as IgG and IgA antibody-producing cells in tonsils. Further, primary immunization in one palatine tonsil followed by a second immunization of both palatine tonsils evoked a larger immune response in the primed tonsil. Sakamoto *et al.* (1998) demonstrated that the numbers of P6-specific IgA-producing cells in the adenoids were significantly correlated with IgA antibody titers in nasopharyngeal secretions. Those findings suggest that human palatine tonsils and adenoids serve as not only inductive sites but also effector sites of the mucosal immune system in the nasopharynx.

Recently, several studies demonstrated that middle ear mucosa has a function as an effector site of the mucosal immune system. Kodama *et al.* (2000) and Suenaga *et al.* (2001) examined the characteristics of the lymphocytes in the middle ear mucosa of mice at the single-cell level after intranasal immunization with P6 together with cholera toxin **(Fig. 88.1)**. They found the induction of P6-specific IgA-producing cells into the middle ear mucosa and a similar cell population of T/B cells, $CD4^+/CD8^+$ cells, and αβ T cells between the middle ear mucosa and nasal mucosa after intranasal immunization. Further, the presence of a certain amount of γδ T cells were observed in both middle ear and nasal mucosa, whereas no γδ T cells were found in the other lymphoid tissues such as NALT, cervical lymph nodes, spleen, or Peyer's patches. Those findings suggest that the middle ear mucosa has the same function to nasal mucosa as an effector site of the mucosal immune responses induced by intranasal immunization. Kodama *et al.* (2000) further investigated the T-cell responses in the middle ear mucosa of mice after intranasal immunization with P6. The data showed an increase of memory T cells as well as P6-specific IgA-B-cell in the middle ear mucosa after intranasal immunization. *In vitro* stimulation with P6 resulted in a proliferation of purified $CD4^+$ T cells obtained from the middle ear mucosa of immunized mice, and those T cells expressed Th2 cytokine mRNA. Moreover, $CD4^+$ T cells enabled IgA-B cells derived from NALT to differentiate into IgA plasma cells in the middle ear mucosa. Those findings suggest that antigen-specific IgA-B cells primed in NALT might home to the middle ear mucosa and differentiate into IgA-producing plasma cells with the help of Th2 cells recruited to the middle ear mucosa.

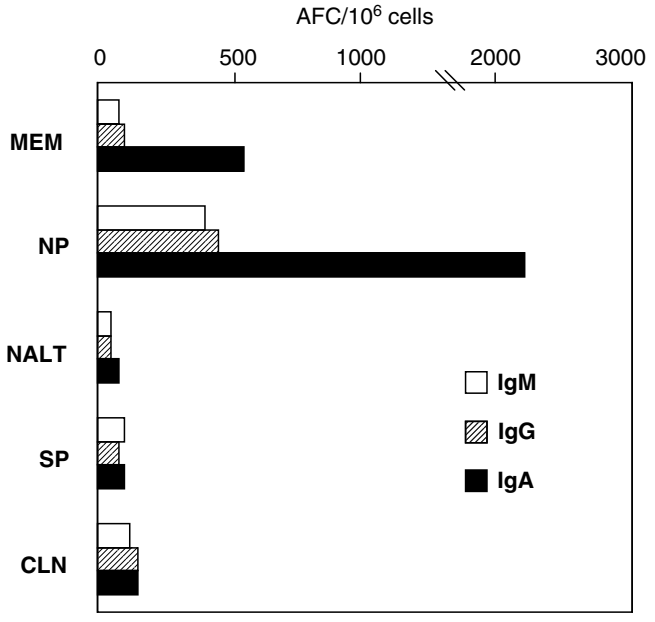

Fig. 88.1. Induction of P6-specific antibody-forming cells in the middle ear after intranasal immunization with P6 together with cholera toxin. Modified from Kodama *et al.* (2000). MEM, middle ear mucosa; NP, nasal passage; NALT, nasal-associated lymphoid tissue; SP, spleen; CLN, cervical lymph node.

APPLICATION OF MUCOSAL VACCINE FOR OTITIS MEDIA

Prevention of OM is complicated by the multifactorial nature of the disease. Predisposing factors include eustachian tube dysfunction, early nasopharyngeal colonization by NTHi, immune dysfunction, and others. Further, the bacterial strains causing OM, such as *S. pneumoniae* and NTHi, are extremely diverse. Therefore, effective vaccines for OM must possess surface epitopes of microbial antigens that are common among strains and able to elicit protective antibodies.

Because NTHi lack capsular polysaccharide, antigenic determinants of NTHi are outer membrane proteins. Among the outer membrane proteins, P6 is a highly conserved peptidoglycan-associated lipoprotein present in all strains of NTHi. Kyd *et al.* (1995) have demonstrated that intra-Peyer's patch immunization with P6 followed by an intratracheal boost induced P6-specific $CD4^+$ T-helper cell proliferation in lymphocytes isolated from the mesenteric lymph nodes and increased antibody response in both serum and bronchoalveolar lavage fluid. However, the absence of enhanced pulmonary clearance in some animals suggests that the route of immunization might have affected the induction of mucosal immune responses in respiratory tract responsible for the pulmonary clearance of NTHi. To develop a useful vaccine delivery system to prevent OM, Kurono *et al.* (1999) compared the mucosal immune responses in mice immunized with NTHi antigen intranasally, orally, and intraperitoneally. The results showed that antigen-specific IgA antibody titers in nasal washes and the numbers of antigen-specific IgA-producing cells in nasal passages were significantly increased in intranasally immunized mice when compared with the other groups **(Fig. 88.2)**. Cytokine assay showed that IFN-γ, IL-2, and IL-6 were predominantly produced in intranasal immunization,

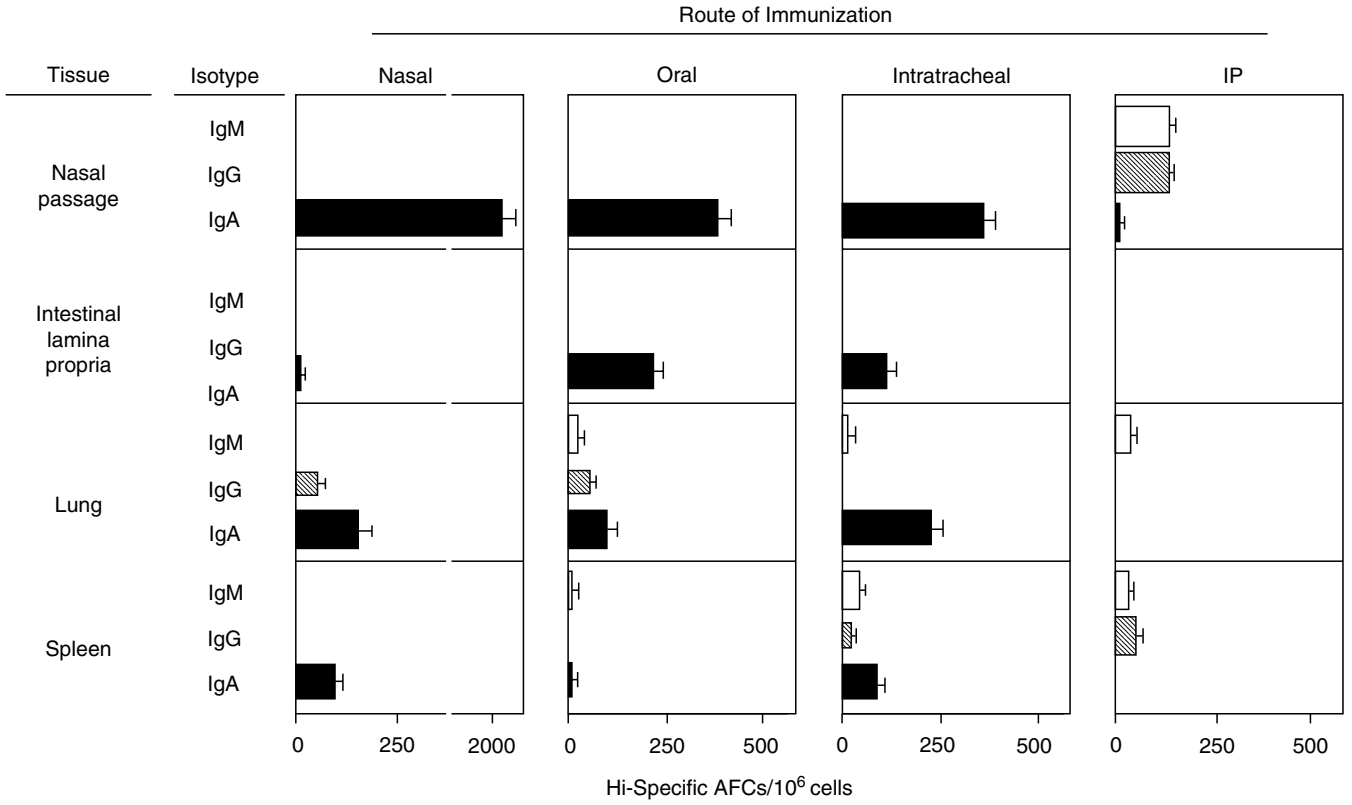

Fig. 88.2. Induction of *H. influenzae* (Hi)-specific antibody-forming cells in mucosal tissues after intranasal, oral, intratracheal, and intraperitoneal (IP) immunization with outer membrane complex of Hi. Antigen-specific IgA antibodies were predominantly induced in nasal passage by intranasal immunization. Modified from Kurono *et al*. (1999).

suggesting that Th2-as well as Th1-type cells were involved in the induction of antigen-specific immune response. 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 intranasal immunization is an effective vaccination regimen for the induction of antigen-specific mucosal immune responses in the upper respiratory tract.

Hotomi *et al.* (1998) investigated the immune responses induced by intranasal immunization with P6. They found that intranasal immunization of mice with P6 together with cholera toxin evoked a good mucosal IgA as well as a systemic IgG response against P6 and increased the number of anti-P6-specific antibody-producing cells in the nasal mucosa of immunized mice. The protective effects of intranasal immunization were confirmed *in vitro* by the enhancement of nasopharyngeal clearance of NTHi and the inhibition of adherence of NTHi to cultured human epithelial cells. In another experiment, they reported that intranasal immunization with recombinant P6 together with cholera toxin also

Table 88.1. Bacterial Clearance and Occurrence of OME

Vaccination and Day Postchallenge	No. of NTHi Cultured from MEEs (CFU/ml)	Presence of MEEs Cases/Total (%)	No. of Culture-Positive MEEs/Total Ears (%)
Day 3			
Control	8.6×105	14/14 (100%)	13/14 (92.8%)
P6+CT	2.6×104	14/14 (100%)	11/14 (78.5%)
Day 7			
Control	4.4×104	11/14 (78.5%)	9/14 (64.2%)
P6+CT	3.7×103	4/14 (28.5%)	4/14 (28.5%)

Modified from Sabirov *et al*. (2001).

induced NTHi-specific mucosal and systemic immune responses (Hotomi *et al.*, 2002). Sabirov *et al.* (2001) assessed the effect of intranasal immunization for the protection against NTHi-induced OM in mice. They found that the clearance of NTHi from the middle ear was remarkably enhanced and the incidence of NTHi-induced experimental OM was significantly reduced by intranasal immunization with P6 together with cholera toxin **(Table 88.1)**. Further, immunized mice showed lower concentrations of TNF-α in MEEs. These findings strongly suggest that P6 is a good potential vaccine candidate and intranasal vaccination with P6 affords protection against OM caused by NTHi.

Recently, the efficacy of a newly developed live attenuated intranasal influenza vaccine was investigated in 1602 children (Belshe *et al.*, 1998). The vaccine reduced not only culture-positive influenza virus but also the incidence of febrile AOM, indicating that prevention of viral infection is an effective way to prevent the development of AOM. Further, the results suggest that intranasal vaccine might be a useful vaccine delivery system to prevent OM.

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