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Mouse Models for the Study of Mucosal Vaccination Against Otitis Media

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Abstract

Otitis media (OM) is one of the most common infectious diseases in humans. The pathogenesis of OM involves nasopharyngeal colonization (NP) and retrograde ascension of the pathogen up the Eustachian tube into the middle ear (ME). Due to increasing rates of antibiotic resistance, there is an urgent need for vaccines to prevent infections caused by the most common causes of bacterial OM, including nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*. Current vaccine strategies aim to diminish bacterial NP carriage, thereby reducing the likelihood of developing acute OM. To be effective, vaccination should induce local mucosal mmunity both in the ME and in the NP. Studies in animal models have demonstrated that the intranasal route of vaccination is particularly effective at inducing immune responses in the nasal passage and ME for protection against OM. The mouse is increasingly used in these models, because of the availability of murine reagents and the existence of technology to manipulate murine models of disease immunologically and genetically. Previous studies confirmed the suitability of the mouse as a model for inflammatory processes in acute OM. Here, we discuss various murine models of OM and review the applicability of these models to assess the efficacy of mucosal vaccination and the mechanisms responsible for protection. In addition, we discuss various mucosal vaccine antigens, mucosal adjuvants and mucosal delivery systems.

1. Introduction

One of the most successful vaccines in human history is a mucosal vaccine, the oral poliovirus vaccine, and has been instrumental in eradicating this scourge from nearly the entire world. Another mucosal vaccine, FluMist, is similarly showing great promise against influenza. Thus, it is clear that improving the mucosal platform as a route for vaccination will be the next major advance in the field of vaccinology.

Otitis media (OM) is one of the most common infectious diseases in childhood and a frequent reason for prescribing antibacterials in infancy. Due to increasing rates of antibiotic resistance, there is an urgent need for vaccines to prevent bacterial OM infections. Mucosal administration of vaccines holds great promise as it allows induction of both mucosal and systemic immunity. However, for mucosal vaccination to be successful for OM, there are still major obstacles to be overcome. These include the diversity and substantial antigenic heterogeneity among the bacterial and viral pathogens that cause OM, and the low immunogenicity of mucosally

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This paper is dedicated to the memory of Professor Goro Mogi for his lasting contribution to the field of Mucosal Immunology.

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administered antigens [1]. Possible solutions to these problems might involve the use of highly conserved protective antigens for bacterial and viral pathogens, and the use of appropriate adjuvants and antigen delivery systems aimed at enhancing the immunogenicity of experimental mucosal vaccines. The mouse provides an optimal model to approach these issues, because of the availability of murine reagents and the existence of technology to manipulate murine models immunologically and genetically. Previous studies confirmed the suitability of the mouse as a model to study inflammatory processes in acute OM and for evaluating putative vaccine antigens directed against OM using novel mucosal adjuvants and mucosal delivery systems [2–6]. Here, we discuss various murine models of OM and review the applicability and limitations of these models to assess the efficacy of mucosal vaccination. In addition, we discuss the mechanisms responsible for protection following mucosal vaccination and various approaches to improve the immunogenicity of mucosal vaccine antigens.

2. Rational for mucosal vaccination against OM

OM often presents as a polymicrobial disease involving complex coinfection with viral and bacterial pathogens. Acute OM is usually caused by bacteria, most commonly by *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* (*S. pneumoniae*), and *Moraxella catarrhalis* (*M. catarrhalis*). Respiratory viruses, such as influenza, parainfluenza virus (PIV) and respiratory syncytial virus (RSV), are important co-pathogens and in most cases precede bacterial OM. The pathogenesis of OM involves nasopharyngeal (NP) bacterial colonization followed by retrograde ascension of the pathogen up the Eustachian tube into the middle ear (ME). Current vaccine strategies aim at diminishing bacterial NP carriage, thereby reducing the likelihood of developing acute OM. To be effective, vaccination should induce local mucosal immunity both in the NP and in the ME.

It is reasonable to believe that acute OM is a vaccine preventable disease. There is emerging evidence that immunological events are involved in the pathogenesis and resolution of acute OM. During OM, a large number of cells including lymphocytes and IgA-secreting cells are recruited to the middle ear [7,8]. In humans, immunization against influenza virus is associated with a reduction in viral disease as well as in acute OM [9,10]. Furthermore, the resolution of acute OM is correlated with the presence of specific ME mucosal and serum antibodies [11]. Taken together, these findings suggest that enhancement of specific immunity against respiratory pathogens can provide protection against OM.

Recent clinical studies have demonstrated that parenteral vaccine administration is effective at reducing OM caused by NTHi and *S. pneumoniae* [12–16]. The results from these studies suggest that serum antibody with bactericidal and opsonophagocytic activity can provide partial protection against OM. However, it is apparent that other immunization strategies need to be explored in order to enhance protection against OM. The human respiratory tract is an initial portal of entry for bacterial and viral pathogens that cause mucosal infections including OM, and, therefore, the mucosal immune response is likely to be important in protective immune responses against OM. At mucosal surfaces, secretory IgA (S-IgA) antibodies play a major role in protection. Local IgA responses in addition to serum antibody responses are induced through the use of mucosal vaccines whereas systemic immunization induces only limited or no S-IgA [17–19]. Other advantages offered by mucosal immunization compared to parenteral immunization include the ability to administer vaccines without a requirement for needles, thus improving patient compliance with vaccination schedules, and the capacity to induce immune responses capable of preventing infections at the site of exposure as well as at distant mucosal sites. Thus, optimal defense against major bacterial and viral pathogens of OM is likely to involve a mucosal route of vaccination.

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3. Overview of mucosal immunity

An overview of the mucosal immune system is depicted in Fig. 1. Mucosal immunity forms the first line of defense against most pathogens and consists of physicochemical barriers (mucous, epithelium), innate immune mechanisms, and adaptive host immunity, which at mucosal surfaces includes predominantly of S-IgA, $CD4^+$ T cells, and antigen-specific cytotoxic T-lymphocytes (CTLs) [20]. The mechanisms responsible for protection by mucosal S-IgA antibodies are distinct from those of serum antibodies [21], and include antiadhesive activity, agglutination, neutralization of biologically active antigens, enhancement of innate antibacterial activity, and inhibition of complement-dependent IgM- or IgG-mediated reactions. The mucosal immune system can be divided into two functionally distinct compartments: (1) inductive sites, where antigen is encountered and initial stimulation of naïve T and B lymphocytes occurs, and (2) effector sites, where B cells differentiate into IgA plasma cells and produce S-IgA antibody to protect local and distal mucosal sites. Both mucosal inductive and effector sites are integrated into a common mucosal immune system. Inductive sites for mucosal immunity consist of organized mucosal-associated lymphoid tissue (MALT) and local draining lymph nodes. MALT is subdivided according to anatomical location and includes nasal-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT), and gut-associated lymphoid tissue (GALT). NALT and Peyer's patches are though to be representative MALT in the respiratory and gastrointestinal tract, respectively. NALT is stimulated following intranasal (IN) administration of antigen, whereas Peyer's patches are stimulated following oral antigen administration. In humans, NALT consists of the Waldeyer's pharyngeal ring, which includes the adenoids and palatine tonsils. In addition, NALT-like structures consisting of lymphoid aggregates with follicle formation were identified in the human nasal mucosa of young children [22]. Rodents lack tonsils but have paired NALT on both sides of the nasopharyngeal (NP) duct, which are considered to be analogous to the Waldeyer's ring in humans [23].

Inductive sites contain all of the immunocompetent cells, including B cells, T cells, and antigen-presenting cells (macrophages, B cells and follicular dendritic cells) that are necessary for the development of effector and memory lymphocytes [19]. At mucosal surfaces, antigen is sampled by specialized microfold (M) cells in the lymphoid follicle-associated epithelium, which then delivers antigen to antigen-presenting cells. Antigen may also be sampled on mucosal surfaces by intra- and subepithelial dendritic cells, which migrate via draining lymph to local and regional lymph nodes where they present antigen to T cells. Naïve B and T lymphocytes enter MALT and lymph nodes via high endothelial venules (HEVs). After being primed to become memory B and T cells, they migrate from NALT and lymph nodes to the peripheral blood for subsequent extravasation into mucosal effector sites. The mechanism that guides lymphocytes back to the mucosa includes upregulation of expression of tissue-specific adhesion molecules and chemokine receptors on lymphocytes, which results in the attraction of IgA^+ B cells to various mucosal tissues. This mechanism explains why mucosal immunization at one site can result in the secretion of specific IgA antibodies at other mucosal (or glandular) tissues, a finding that led to the term, the "common mucosal immune system." [24].

The other important feature of the mucosal immune system is its "compartmentalization", such that there is a preferential distribution of responding cells to effector sites that are anatomically or physiologically related to the inductive sites that received the original antigenic stimulation [25]. The underlying mechanism for this is thought to involve selective expression of adhesions (in tissues) and chemokine receptors (on lymphocytes), which are directly involved in mucosal homing of effector B and T cells following stimulation of certain inductive sites. For example, following IN immunization, IgA^+ B cells and T cells that are generated in NALT enter the bloodstream, but they preferentially migrate back into the upper respiratory tract mucosa

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because they express L-selectin that interacts strongly with peripherial lymph node addressin, which is expressed by HEVs in the upper respiratory tract mucosa [26]. In contrast to NALT, α4β7 integrins and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) play predominant roles in the binding of primed lymphocytes to HEVs in the intestine following oral immunization [27]. This putative mechanism explains the induction of specific responses at mucosal sites where an antigen or pathogen was initially encountered, such as the upper respiratory tract following IN immunization [28,29] and the intestine following oral immunization [30]. Mucosal immunization also induces the production of serum IgA and IgG because mucosal dendritic cells can migrate and carry antigen to systemic inductive sites (*i.e.*, lymph nodes, spleen) [31], and because a fraction of B cells activated in the mucosa or in the mucosal draining lymph nodes express peripheral homing receptors [32].

Protective immunity to pathogens involves the activation of two classes of T cells: $CD4^+$ T helper (Th) cells and $CD8^+$ CTLs [33]. Activation of $CD4^+$ Th cells by foreign antigen leads to the secretion of appropriate cytokines for B-cell responses and immunoglobulin synthesis. Mature Th1 cells produce IL-2, IFN-γ, lymphotoxin (LT)-α, LT-β, and TNF- α, and mediate Th1 responses that are associated with IgG2a antibody responses in the mouse [34]. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and provide effective help for IgA, IgG1, IgG2b, and IgE responses. In addition to TGF-β, Th1-derived IL-2 and Th2-derived IL-5, IL-6, and IL-10 are important IgA-enhancing cytokines for the activation and clonal expansion of S-IgA^+ B cells and their terminal differentiation into IgA-producing plasma cells [34–36]. These plasma cells produce dimeric or polymeric forms of IgA which become S-IgA following covalent binding to secretory component that is synthesized by epithelial cells as part of the polymeric Ig receptor. The production of secretory component is up-regulated by both Th1 (IFN-γ) and Th2 (IL-4) cytokines [37–38]. Th1 type responses also up-regulate the function of CTLs that are believed to be major effectors for the elimination of infected cells [33]. Thus, induction of effective T cell immunity at mucosal sites involves both $CD4^+$ T cells that produce cytokines for activation of antibody production and macrophages, and CTLs for more effective pathogen killing at mucosal surfaces [39].

Mucosal inductive and effector sites are distinct in terms of their cytokine profiles. $CD4^+$ cells isolated from murine NALT demonstrate a Th0 cytokine profile, indicating that these T cells are capable of becoming Th1 or Th2 cells immediately after antigen exposure [40]. In contrast, the upper respiratory tract mucosa is considered to be a Th2 dominant site that supports the induction of IgA-producing cells. Of importance for vaccine development is the fact that various mucosal adjuvants influence the development of Th-1-type responses for protection against intracellular pathogens and Th-2-type responses required for protection against soluble antigens, allergens, and toxins [34].

3.1. Mucosal immune responses in the ME

Only a few immunocompetent cells are found in the normal ME mucosa, but acute or chronic inflammation results in significant recruitment of many immunocompetent cells including macrophages, T cells, B cells, dendritic cells and NK cells [8,41,42]. In an immune-mediated murine model of OM that was induced by challenge with keyhole limpet hemocyanin, many immunocytes were found to appear in the ME, including a dominant population of Mac-1^+ cells, as well as Th cells and IgG^+, IgA^+, and IgM^+ B cells. These findings suggest that immune responses can occur in the ME following appropriate antigenic or inflammatory stimulation. Kodama *et al*. [43] analyzed ME mucosa, NP mucosa and NALT from naïve mice for lymphocyte subset expression. In naïve mice, the composition of lymphocytes in ME mucosa was similar to that in NP mucosa, an example of an effector site, but was different from NALT, an inductive site. Both ME and NP mucosa contained large numbers of antibody-producing cells with a predominant IgA isotype. Another study by Kodama *et al*. [44] compared immunity

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in the ME, NP mucosa and NALT of mice after IN immunization with the P6 outer membrane protein of NTHi in the presence of cholera toxin (CT) as adjuvant. It was found that IN immunization induced P6-specific IgA antibody-producing cells in both the ME and NP. However, there were fewer numbers of specific antibody-producing cells in NALT. In addition, IN immunization induced in the ME mucosa CD4 T cells producing Th2 type cytokines (IL-5, IL-6, IL-10, TGF-β), which promote local IgA responses. We recently demonstrated that IN immunization with pneumococcal conjugate vaccine and IL-12 as a mucosal adjuvant induces a large number of IgA^+ cells in ME mucosa [45]. Two important conclusions relevant to immunization against OM emerge from the above studies. First, these findings suggest that ME mucosa has characteristics of a mucosal effector site similar to the nasal passage mucosa. Secondly, the evidence suggests that NALT-targeted immunization is an effective regimen for induction of protective IgA responses in the ME. IN administration has become a popular route of mucosal immunization in mice in recent years, and is particularly effective for the generation of antibody responses in the upper respiratory tract. A primary role of NALT in the generation of immune responses in the ME is suggested by the relative proximity of the two tissues, which is in line with a concept of "compartmentalization" of the mucosal immune system. In support of this, comparative studies of IN and intragastric routes of immunization in mice have shown that IN immunization is superior to intragastric immunization for the induction of antigen-specific IgA antibody responses in nasal passages [46–48]. This may also hold true for the induction of mucosal immunity in the ME, because the latter represents an anatomical extension of the NP. Taken together, these findings suggest the feasibility of inducing vaccine-specific mucosal IgA responses in the ME by IN immunization.

4. Animal models of OM

Animal models are considered to be an essential element in studying (1) the common pathogens of acute OM, (2) the role of pathogen-specific immune responses in resolution of acute OM, and (3) tests of strategies for vaccination against this disease in humans. An advantage of using animal models includes control over the animals and the microenvironment during disease progression, which enables repeated observations of variables, such as immunologic status, histological changes, and the function of the ME. Several animal models have been used to study OM. A summary of currently available animal models of OM secondary to bacterial infection is provided in Table 1. It should be noted that various rodent species demonstrate a similarity in response to challenge. Doyle [49] established the criteria for an organism to be considered pathogenic in a particular model: (1) the organism induces pathologies similar to those observed in patients with the disease; (2) the pathologies can be objectively documented by otomicroscopy, tympanometry and histopathology; and (3) the organism is shown to reproduce in the ME space. Acute OM models of both *S. pneumoniae* and NTHi have been developed in several species, but it has been difficult to develop an animal model for *M. catarrhalis*. The animals can develop acute OM, but the bacteria are rapidly cleared from the ME. Most human bacterial pathogens are not natural colonizers or pathogens in rodents, therefore the immune responses of animals following infection in ME or NP could be different from those in humans.

Various rodents, including mice, rats, gerbils, guinea pigs and chinchillas, have been used for induction of OM [63–65]. The structural features of the ME and Eustachian tubes in chinchillas and gerbils are similar, but both of these animals differ considerably from the rat [66]. Chinchillas and gerbils have large bulla, which is easily accessible for inoculation through the overlying skin and for serial sampling of ME fluid. The other advantages of chinchillas for an OM model include their general susceptibility to many human pathogens and the ability to induce OM after colonization of the NP [50]. However, in chinchillas, the tympanic membrane is not easily accessible for inspection with an ordinary microscope and for performing transtympanic challenge due to the elongated and S-shaped external auditory canal. In mice,

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rats, and guinea pigs, the structural features of the ME are similar but these animals have much smaller bulla compared to chinchillas and gerbils [63,66]. In these animals, pathogens can be inoculated through the tympanic membrane or through the bulla. The transbullar approach requires surgical exposure of the inferior bulla through an incision on the neck and blunt dissection of the soft tissue. One animal in particular that is now being increasingly studied in OM research is the mouse and this animal model is the focus of the present review. The mouse has several unique advantages over other species that are impossible to ignore. Experimental reagents for immunological studies are widely available, making it well suited for advanced research protocols. In addition, there is a broad range of transgenic and gene-deleted mouse strains to aid in delineation of the underlying genetic factors of host susceptibility to OM and specific pathophysiologic responses [67] (described in section 6). Furthermore, mice are inexpensive and easy to manipulate. Previous studies indicated the suitability of the mouse as a model for inflammatory processes in human acute OM, and thus, the results from the murine studies can be translated (with certain precautions) to humans [2–4;68].

4.1. Mouse models to assess protection against OM

Various models of ME infection and/or NP carriage, caused by common human pathogens, have been established in mice. These models have allowed an evaluation of candidate vaccine antigens and vaccine-specific immunity for prevention of bacterial and viral infections. In addition, individual mouse strains respond differently to infection, and this can result in unique patterns of susceptibility to infections in the NP and/or ME [69–70]. Melhus and Ryan [3] compared the propensity of BALB/c, Swiss-Webster and C57BL/6 strains of mice to develop acute OM and systemic disease after intrabullar inoculation of three OM pathogens. BALB/c mice were the most susceptible of the three strains, followed by C57BL/6 and Swiss-Webster mice. In addition, we recently evaluated development of OM-induced pneumococcal infection in BALB/c, BALB/c IFN-$\gamma^{-/-}$ and 129S1/SvImJ mice and found that 129S1/SvImJ mice were most susceptible to invasive disease following OM [45]. These findings indicate disparities in bacterial disease phenotype among various mouse strains. Such differences may help identify the gene(s) affecting host immune responses and host susceptibility to OM (described in Section 6). An approach to overcome strain variations in response to infection includes careful selection of specific mouse strains based on genetic background [67]. Furthermore, mice of the same genetic background may have unique susceptibilities to different bacterial pathogens. For example, Malley *et al.* [71] evaluated the susceptibility of C57BL/6 mice to development of bacterial carriage in the NP and the ME following IN inoculation of three capsular polysaccharide serotypes of pneumococci. In this study, the mice were more susceptible to pneumococcal serotypes 6B and 23F, and less susceptible to serotype 14. In various mouse models, the diagnosis of acute OM requires direct visualization of ME fluid behind the tympanic membrane and of tympanic membrane changes, such as dilated vessels, increased thickness, and reduced translucency by otomicroscopy. Both clinical symptoms (ME effusions, tympanic membrane appearance) and histopathology (Eustachian tube, ME) endpoints can be quantified. There are, however, certain limitations in using OM mouse models, described in Section 11.

4.1.1. IN inoculation model—In the IN inoculation model, initial bacterial colonization or viral replication occurs in the NP, which may be followed by invasion into the ME cavity. In this model, the portal of pathogen entry into the ME would thus resemble the disease process in humans. Anesthesia prior to IN inoculation and careful dosing of the inoculum (10 μl per adult mouse) allows minimal aspiration or swallowing of the inoculum, which in turn, makes the model highly reproducible. Whereas there are various established models of NP carriage that have been extensively used for the evaluation of the protective efficacy of IN vaccination, the actual spread of bacterial or viral infection into the Eustachian tube and ME has not been extensively studied. McCool and Weiser [72] evaluated the susceptibility of BALB/c, C57BL/

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6, and CBA/J mice to NP carriage after IN inoculation of *S. pneumoniae* strain P1121. Among these mice, BALB/c had the highest density and duration of colonization. Importantly, many aspects of this murine model resemble experimental P1121 pneumococcal carriage in humans, including the minimum colonizing dose (<10^4 CFU) and an average duration of colonization of several weeks [73]. Although development of OM is often sporadic following IN challenge with pathogen and infected ME show variability in bacterial numbers and inflammatory changes [74;our unpublished observations], Malley *et al*. [71] were able to induce pneumococcal infection in both NP and ME following IN inoculation of *S. pneumoniae,* as evidenced by positive NP and ME cultures. Similarly, after IN administration of a luciferase-expressing strain of *S. pneumoniae* that is known to efficiently colonize mucosal surfaces, McCullers *et al*. [75] confirmed the presence of pneumococci in NP and their subsequent expression in the ME by visualization of the bioluminescent bacteria. Imaging results showed that after pneumococcal IN inoculation, 100% of the mice developed NP colonization and 70% developed acute OM.

We have established a murine model of OM following IN infection of mice with type 14 *S. pneumoniae* for the evaluation of protective immune responses induced by mucosal vaccination [76]. We found progression of OM in normal mice following 5 daily IN inocula of pneumococci. Infection correlated with the presence of ME effusions and tympanic membrane changes. Time course studies showed that the optimal sampling time for measurement of bacterial carriage in NP and ME was day 8 after initial IN challenge. The possible mechanism responsible for ME infection could be induction of an inflammatory reaction at the Eustachian tube orifice in the NP following repeated exposure to *S. pneumoniae*.

Several studies confirmed the utility of this acute OM model for the evaluation of IN vaccine efficacy against NTHi [77] and pneumococcal disease [71,76]. IN vaccination prior to IN challenge was shown to confer protection against colonization in both NP and ME.

Several studies have utilized IN inoculation of viruses in order to induce OM. Meek *et al*. [78] developed a murine model of OM following IN inoculation of reovirus. The authors could detect reovirus antigen in the ME mucosa of infected animals, and histologic evidence of OM was found in half of all infected animals. Following infection, there were significant increases in B cell levels in the NP and Eustachian tubes. The number of infiltrating T cells, however, did not vary significantly from that in the uninfected control animals. Hirano *et al*. [79] demonstrated that IN inoculation of influenza virus induces inflammatory changes and increases in numbers of $CD4^+$ and $CD8^+$ T cells in the ME mucosa. McCullers *et al*. [75] infected pneumococcal-colonized mice with influenza virus and observed them for development of acute bacterial OM. These investigators found that 63% of virus-infected mice developed pneumococcal OM compared to 0% of mice mock-infected with PBS. Thus, virus infection allowed NP colonization to progress to OM. Hirano *et al*. [80] performed a study to further clarify the role of viral infection in development of OM. Mice were inoculated IN with influenza A virus and then examined for histological changes in the NP mucosa using a battery of lectins. Additionally, live NTHi or *S. pneumoniae* were inoculated into the NP after virus infection and the clearance of bacteria from the NP was examined. Influenza A virus infection changed the glycoconjugate composition of the NP mucosa. Moreover, similar to the findings of others [81,82], there was an increase in levels of NTHi and *S. pneumoniae* NP colonization in mice that had been infected with virus. Together, these findings could explain the enhanced inflammatory responses that were observed and the onset of bacterial OM following viral infection. Other mechanisms by which influenza virus could facilitate bacterial invasion include virus destruction of the respiratory epithelium, virus-induced immunosuppression, and up-regulated expression of molecules that bacteria utilize for epithelial attachment (*i.e.*, platelet-activating factor receptor) [83]. Gitiban *et al*. [84] developed a mouse model of RSV infection of the NP and Eustachian tubes following IN challenge. Sections from the NP and

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Eustachian tubes were examined for RSV antigen expression and for inflammation. It was found that the respiratory and olfactory mucosae of the nasal cavity and the ciliated epithelium of the Eustachian tubes were infected with RSV, and this correlated with inflammatory changes in the NP and Eustachian tube mucosa [85]. Appel *et al.* [86] demonstrated that in the murine upper respiratory tract, progression of infection with Sendai virus (SeV), the murine counterpart of human PIV, correlates with mucosal inflammatory changes. It should be noted that mice are a natural host for SeV, and this virus may cause severe morbidity in this species [87]. Klements *et al.* [88] demonstrated that following IN challenge with SeV, all mice developed inflammatory changes in the NP and sinuses, which were associated with positive viral cultures from nasal washes and increased levels of tissue macrophages, neutrophils, and $CD4^+$ and $CD8^+$ T cells compared to uninfected animals.

4.1.2. Direct inoculation of bacteria into the ME—Either transbullar or transtympanic inoculation of bacteria into the ME can cause OM. The advantage of these techniques is the ability to precisely and reproducibly inoculate an exact number of microorganisms and thereby guarantee induction of disease with a low amount of variation between individual animals. Direct inoculation of infectious organisms by either route also allows induction of unilateral OM. A disadvantage of these techniques is the fact that direct ME inoculation is an artificial route of infection as it bypasses NP colonization. Bacterial inoculation using the transbullar approach requires surgical exposure of the inferior bulla through an incision on the neck and blunt dissection of the soft tissue. The pressure within the tympanic cavity is equilibrated by making two microholes in the bulla. The injection volume into the bulla can be as much as 10 μl of fluid in adult mice. This technique requires surgical skill to avoid damage to the adjacent major blood vessels. Meltus and Ryan [3] investigated the course of ME infection in three strains of mice following intrabullar inoculation of *S. pneumoniae*, NTHi, or *M. catarrhalis*. Otomicroscopic changes were measured as well as numbers of bacteria in the ME and blood. BALB/c mice were the most susceptible to *S. pneumoniae* and NTHi infection. By day 3, 75% of mice had developed OM and 50% had a positive ME bacterial culture. There was no evidence of ME infection following inoculation with *M. catarrhalis.* The authors concluded that the course of acute OM depends upon the mouse strain, the bacterial strain, and the bacterial dose. We applied intrabullar inoculation of *H. influenzae* or *S. pneumoniae* to induce OM as confirmed by ME inflammation and the presence of culturable bacteria 3 and 7 days after inoculation [60,76]. Direct ME challenge with either pathogen induced a severe local inflammatory response by day 3, which was reduced by day 7. We also confirmed the utility of this acute OM model for the evaluation of IN vaccine efficacy against NTHi and *S. pneumoniae* disease (Table 1). Hirano *et al*. [89] utilized intrabullar inoculation of NTHi into the ME of wild-type and toll-like receptor 4 (TLR4)-deficient mice to investigate the mechanism of protective innate immunity via TLR in the ME. The severity of acute OM was assessed by the presence of ME effusions, the degree of tympanic membrane changes and inflammatory responses in the ME. TLR4-deficient mice demonstrated an increased severity of OM and delayed resolution compared to their immunocompetent counterparts. The authors concluded that innate immune responses induced via TLR4 play an important role in eradicating NTHi infection in the ME.

Transtympanic inoculation of bacteria is relatively easy to perform and includes microscopic insertion of a needle through the tympanic membrane. However, injected fluid often drains from the ME via the hole in the tympanic membrane, resulting in an inoculation volume that is less precise compared to intrabullar inoculation. In addition, the resultant hole in the tympanic membrane can be a conduit for contamination and enhanced drainage of ME effusions via the Eustachian tube due to pressure equilibration [74]. McCoy *et al*. [90] inoculated heat-inactivated *S. pneumoniae* transtympanically and assessed ME inflammation on day 3 by measuring the amount of fluid accumulation in the ME, number of cells in the ME fluid, and the thickness of the tympanic membrane. MacArthur *et al*. [91] similarly

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transtympanically inoculated heat-killed *S. pneumoniae* strain 6A and established the parameters of ME inflammation in a mouse model of OM. The most significant ME measures of inflammation were the amounts of ME effusions, tympanic membrane thickness changes, and numbers of inflammatory cells.

Thus, the mouse is a useful animal model for induction of ME infection. The method of choice for OM induction, *i.e*., IN or direct ME administration of pathogens, depends on the experimental needs of the investigator. Induction of OM using an IN challenge model is preferred for studies that involve evaluating protective immunity against NP colonization and subsequent infection in the ME. Direct ME challenge, on the other hand, might be preferred for evaluating the role of protective immunity against ME challenge or resolution of OM as well as to evaluate the extent of the inflammatory response within the ME cavity.

5. Inflammatory responses in the ME and nasal cavity

Various pathogens have been administered into the nasal passages or ME of mice to induce specific immunity (influenza virus) or for actual challenge after immunization (bacterial/viral pathogens). This can result in various degrees of inflammatory changes, from transient influx of neutrophils into the mucosal surface that results from inoculation of *S. pneumoniae* [92] to severe epithelial damage induced by influenza virus [93].

Important differences have been noted in the inflammatory responses induced in the ME or nasal mucosa following intrabullar or IN challenge with the same pathogen. Thus, IN inoculation of *S. pneumoniae* resulted in nasal carriage without local inflammation [94] or was accompanied by mild inflammation which resolved within 1 day [92,95]. A study by Sun *et al*. [96] provided histological evidence that IN pneumococcal challenge is not necessarily accompanied by inflammation in nasal passages and bacterial challenge induced an increase in specific antibodies in wild type, but not polymeric IgR deficient (pIgR$^{-/-}$) mice, *i.e*., there was no transudation of serum IgG into the respiratory tract. In contrast, direct ME inoculation of various strains of *S. pneumoniae* or NTHi results in extensive inflammation in the ME that includes the generation of effusion, infiltration of leukocytes into the mucosa and ME lumen, and mucosal hyperplasia [3,74]. The local ME inflammation lasts for 1–2 weeks [91]. The distinct inflammatory responses observed in these two anatomical sites could be explained, in part, by more effective clearance of bacterial pathogens from nasal passages than from the ME following challenge. The large surface area of nasal tissue and the wide opening of the upper airways into the NP likely facilitate bacterial clearance. In support of this, increasing the IN bacterial inoculation dose does not affect the probability of ascension of pathogen from the NP into the ME (our unpublished observations). In contrast, increasing of bacterial dose during direct ME challenge increases the frequency of OM and positive ME cultures [3].

Melhus and Ryan [3] examined the propensity of mice to develop bacteremia and succumb to systemic infection after intrabullar challenge with pathogen. Specifically, following intrabullar challenge with an invasive pneumococcal serotype 3 strain, all challenged animals succumbed within several days. Interestingly, in some mice, intrabullar challenge with strains of *S. pneumoniae* that are typically noninvasive as well as with encapsulated and even unencapsulated *H. influenzae* also resulted in systemic infection. In contrast, when 10-fold higher doses of *S. pneumoniae* type 3 were administered IN, all mice remained alive and exhibited nasal carriage without bacteremia or sepsis [94]. It should be noted that the ability of pneumococci to cause sepsis versus carriage after IN or ME challenge of mice is a function of both the mouse strain and the strain of pneumococci.

It should be noted that inclusion of mucosal adjuvants during IN vaccination can contribute to inflammatory responses in mucosal tissues. Previously, several studies showed the ability of CT and heat-labile enterotoxin (LT) [97,98] to induce inflammatory responses in nasal mucosa

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when administered IN. Van Ginkel *et al*. [98] demonstrated that CT induces a local inflammatory response in nasal passages, such as increased levels of IL-6 and IL-1β. This finding was supported by other observations that CT induces histological inflammation in nasal passages in a dose-dependent manner [97].

Thus, an inflammatory state can be induced in the ME and nasal passages either through live pathogen priming for immunization (*e.g.*, sublethal doses of influenza virus), actual pathogen challenge, or through use of mucosal adjuvants such as CT and LT. The resulting inflammation may breach the mucosal barrier and increase transudation of serum antibody across mucosal surfaces. These findings might be related to the apparent influence of inflammation on the efficacy of vaccination as well as protection upon challenge, a topic that will be discussed in detail below.

6. Genetically modified animals to study OM

Mouse models provide an advantage over other animal models in that there is an extensive genetic toolkit available for manipulating the mouse genome, which is well-characterized and demonstrates homology with the human genome [99]. Genetically altered mice have provided an opportunity to assess the role of various factors in the enhanced protection observed following mucosal administration of vaccine candidates, including the importance of innate immunity [89], mucosal and systemic antibodies [96,100,101], T-cell immunity [102–104], and cytokines [45](described in section 8).

An initial step in exploring the genes that predispose to OM may include screening of inbred strains of mice. Recently, Zheng *et al.* [99] used tympanometry in an attempt to identify mouse strains showing differing ME functions. Of the 61 genetically diverse inbred strains tested, 15 strains exhibited abnormal tympanograms and these differences were thought to be the result of genetic factors which predispose to OM. Specifically, the LP/J mouse strain was shown to have an ME condition that results in a predisposition towards increased susceptibility to OM. Further mapping of the differences among inbred strains of mice in their susceptibility to OM will ultimately allow cloning and characterization of OM susceptibility genes [68]. However, analysis of the inbred strains will be complicated by the apparent complex genetic basis, and the low penetrance, of the disease phenotype.

Existing technologies allow development of genetically engineered mice with targeted gene mutations which result in increased susceptibility to OM. These mutant mice provide a great opportunity for unraveling the factors that predispose to OM in genetically susceptible hosts. Progress in this area was recently demonstrated by development of mutant *Jeff* [105,106] and *Junbo* mice [107]. These animals exhibit spontaneous, chronic OM which is initiated by murine pathogens. In the future, these mice might also be useful as a model to study OM induced by common human pathogens.

7. Effects of mucosal vaccines in a murine model

Our intent is to review the recent work on mucosally administered vaccines to prevent bacterial or viral infections that lead to OM. A literature search of the MEDLINE database (July 1986–June 2007) was conducted using the terms mouse, OM, colonization, pneumococcus, NTHi, *M. catarrhalis*, respiratory syncytial virus, parainfluenza and influenza. The search was restricted to studies conducted in mice and published in English. Product literature and references to pertinent review articles were also evaluated. The present review lists previous studies in mice relevant to mucosal administration of vaccines aimed at preventing ME infection (Table 2) or NP colonization (Tables 3–5). Also included are our own data regarding the protective efficacy of IN immunization against murine OM induced by intrabullar challenge with NTHi or *S. pneumoniae* (Table 6). We demonstrated protection against experimental OM

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as evidenced by enhanced clearance of bacterial pathogens and reduced inflammatory responses in the ME, such as the presence of ME effusions and tympanic membrane changes. The reader will see that the models reported in these tables and discussed in the text have been restricted to IN administration of vaccine, because this route was mostly used in the assessment of efficacy of mucosal vaccination against NP carriage and experimental OM. Importantly, initial studies with various experimental IN vaccines conducted in the mouse were shown to closely predict success in man [140].

7.1. Candidate vaccine antigens

An effective vaccine formulation against OM must include bacterial antigens that are key targets for the immune system. Vaccine development is hampered by the extreme diversity of *S. pneumoniae* and NTHi capsular polysaccharides, and efforts are being made to identify protein surface antigens that are conserved among strains. Mucosally administered antiviral vaccines are also highly desirable since prevention of respiratory viral infection could be expected to substantially reduce the incidence of acute OM, which often develops as a complication of viral illness [141].

Several approaches have been used for induction of protective immunity in the ME and in nasal passages by mucosal vaccination. Studies in mice have demonstrated that IN vaccination is the most effective route for induction of protective immunity in the upper respiratory tract against OM. This approach is attractive because the nasal mucosa is the first portal of entry for most human pathogens and because the nasal mucosal environment does not induce degradation of antigens as opposed to the harsh environment in the gastrointestinal tract. One strategy employs whole killed or live attenuated pathogens as vaccines, in an attempt to induce broad protection. However, safety concerns with administration of whole microorganisms have prompted the use of capsular antigens and subunit outer membrane proteins (OMPs) with particular attention to virulence factors widely shared among strains.

In the mouse model, several targets have been identified as potential vaccine candidates against NTHi-mediated OM, including killed bacteria, OMPs, such as P4 and P6, core lipooligosaccharides (LOS), and the Hap adhesion protein [43,44,60,77,108–113,141–150]. Among these potential vaccine antigens, P6 is highly conserved among strains and is associated with crossreactive protection, whereas the other surface antigens demonstrate strain heterogeneity which limits their usefulness [151]. As a vaccine component, LOS is too toxic to be administered to humans, while detoxified LOS (dLOS or hapten) is not immunogenic. To overcome these problems, dLOS was covalently bound to antigenic proteins such as tetanus toxoid (TT) or a mutated form of diphtheria toxin. Such conjugate vaccines elicited long-lasting expression of antibodies with bactericidal activities against NTHi and *M. catarhalis*. Importantly, a dLOS-TT vaccine was the first investigational NTHi vaccine that has been tested and demonstrated to provide excellent immunogenicity in humans when administered systemically [12]. Moreover, IN immunization with the same conjugate significantly enhanced bacterial clearance in the mouse NP and thus holds promise for mucosal vaccination of humans [113]. The most effective vaccine might ultimately be one that includes several antigens of NTHi.

Capsular polysaccharide-based vaccines, including polysaccharide-protein conjugates have been shown to be effective in reducing *S. pneumoniae* infecton in humans and mouse models. However, the replacement by non-vaccine pneumocoocal serotypes causing OM after immunization has prompted the need for new pneumococcal vaccines. The possibility of using immunity to highly conserved and immunogenic pneumococcal surface proteins that may provide protection against all pneumococcal serotypes is currently being explored. Pneumococcal surface adhesion A (PsaA), pneumococcal histidine triad protein A (PhpA), pneumolysin, and pneumococcal protective protein A (PppA) are all conserved among *S.*

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pneumoniae strains [45,71,94,96,101–104,115,121,123,124,152,153]. In contrast, pneumococcal surface proteins A and C (PspA and PspC) demonstrate antigenic variability among different strains and immunization with these proteins elicits antibodies that cross-react with some but not all alleles. Alternatively, a combination of various proteins that have different protective functions may provide a broader protection. Finally, phosphorylcholine, a structural component of *S. pneumoniae* and NTHi, has also been evaluated for induction of cross-reactive protection against both pathogens[121].

Efforts to develop a *M. catarrhalis* vaccine have focused on killed bacteria [154], LOS [155], and OMPs, such as UspA2 [110]. A major obstacle in assessing potential vaccine antigens against *M. catarrhalis* includes the absence of a correlate of protection for this pathogen. A mouse model for the study of pulmonary clearance of *M. catarrhalis* is frequently used to study the effects of vaccination with *M. catarrhalis* antigens. In this model, mice are challenged by introducing bacteria into their lungs and the rate of clearance is followed as a measure of the immune response. IN administration of dLOS conjugated to mutated diphtheria toxin was found to induce mucosal and systemic antibodies and to enhance murine pulmonary clearance of *M. catarrhalis* [155].

Influenza vaccines tested in murine models include live topical vaccines, inactivated vaccines, and subunit vaccines consisting of antigens from pathogenic organisms, including the major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [47,156–164]. In addition, a number of influenza DNA vaccine candidates are in various stages of development, with the hope that this vaccine approach induces a broad spectrum of immunity against multiple strains of influenza virus.

RSV vaccines include live [135] or attenuated virus [165–168], and a number of subunit vaccines based on the fusion (F) and major surface glycoprotein (G) proteins [136,137,169–174]. G is an integral membrane protein responsible for cell attachment, and F is responsible for fusion of the viral envelope with the cell membrane [175].

PIV vaccines include live murine SeV which is the closest known homologue of human PIV-1 [176], and subunit vaccines consisting of the H and N envelope glycoproteins, as well as F protein [139]. Studies in mice have demonstrated that immune responses induced against SeV cross-react with human PIV-1 [176]. This suggests the use of SeV as a candidate for a human PIV-1 vaccine.

8. Protective mechanisms against ME and IN challenge

8. 1. Role of humoral vs. T-cell immunity

Murine model systems have been invaluable for discerning the mechanism of mucosal vaccine-induced protection against OM. Considerable efforts have been made to assess the role of T- and B-cell dependent mechanisms in protection against respiratory pathogens following IN exposure to either live [102–104] or killed unencapsulated *S. pneumoniae* [103] ("whole-cell vaccine"). Traditionally, humoral immunity has been thought to mediate primary protection against pneumococci. However, recent studies demonstrated that IN immunization with wild type whole-cell pneumococci [102,103] conferred protection against NP carriage in immunocompromised μMT mice, which do not produce mature B cells or serum antibody, but did not protect T-cell deficient nude mice. Similar protection was demonstrated after IN administration of pneumococcal polysaccharides [124]. The results of these studies suggest that protection against respiratory pathogens can be induced in the absence of antibody and requires the presence of $CD4^+$ T cells. Roche *et al.* [104] demonstrated that IN administration of a live nonencapsulated strain of *S. pneumoniae* to wild-type mice reduced NP colonization and conferred protection against systemic infection following IN challenge with a high dose

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of the parent strain. However, IN immunization failed to protect μMT mice or major histocompatibility complex class II-deficient mice which lack functional $CD4^+$ T cells. These findings indicate that protection is both antibody- and $CD4^+$ T cell-dependent. There are several potential explanations for these contrasting conclusions regarding the importance of antibody and T cells in protection of the respiratory tract from pneumococci. First, different experimental protocols were used - in some but not all cases, strong mucosal adjuvants such as CT or B subunit of CT (CTB) were used during vaccination [102,103,124]. In addition, it should be realized that μMT mice are known to produce mucosal (but not serum) IgA antibody [177], suggesting that use of μMT mice can not rule out a role for antibody-dependent mechanisms in vaccine protection. Thirdly, there are likely to be distinct protective immune responses to wild-type bacterial isolates [102,103] vs. attenuated mutant strains [104]. Encapsulation, for example, may obscure the immune response to underlying surface antigens. In support of this possibility, vaccination with attenuated mutant strains was shown to protect from subsequent IN challenge more efficiently than vaccination with wild-type isolates [104]. Similarly, differences may exist in the protective immune responses to killed [103] vs. live attenuated strains [104].

The precise mechanism by which $CD4^+$ T cells mediate protection against colonization remains to be determined and several possibilities have been raised [103]. One possibility is that the $CD4^+$ T cells responsible for protection are able to mediate optimal Th1 responses, similar to those shown to protect against intracellular bacteria. Another potential mechanism includes the presence in the whole-cell vaccines of TLR ligands, which can enhance Th1 responses. Finally, the potential contribution of the newly described Th17 T cell subset, which has been shown to mobilize neutrophils through granulopoesis and chemokine production [103], remains to be fully explored.

Thus, mature B cells are required for the protection against respiratory pathogens following IN immunization in at least some models. However, the findings also suggest an important role for T-dependent mechanisms even in the absence of B cells in protection against respiratory pathogens following IN immunization.

8.2. The role of mucosal and serum antibodies

One may speculate that the primary role of vaccines in limiting bacterial colonization at mucosal surfaces is to prevent initial infection. Secondarily, an increase in antibody concentrations caused by mucosal contact with pathogens in an immunized host could lead to a more rapid elimination of the pathogen from the mucosa. Mucosal immunization in various murine bacterial challenge models has been demonstrated to be effective at limiting acquisition of pathogens in the NP, ascension of pathogens from the NP to the ME, and pathogen replication in the ME. The analysis of protective immune responses in the ME fluids of infected mice is hampered by the difficulty in collecting ME fluids and their small volume. The concept of a compartmentalized mucosal immune system suggests that determination of specific antibodies in nasal fluids is the primary surrogate measure of immunity in the ME [25]. Likewise, immunization protocols that can induce protective immunity in the NP are expected to be effective in also activating protective immunity in the ME.

Studies using murine models have shown that IN and oral immunization is an effective regimen to induce expression of S-IgA and serum antibodies, as well as activation of mucosal Th cells, regulatory T cells and CTLs [43,178,179]. Co-administration of selected mucosal adjuvants favors the production of Th1- (IgG2a, IgG2b) and/or Th2-type (IgA, IgG1, IgE) antibodies, both in the mucosal and systemic compartments. Following IN immunization, the expression and function of antibodies in mediating bacterial clearance from mucosal surfaces as well as from the blood have been examined. In general, IN vaccination, which was effective in

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induction of nasal mucosal and serum antibodies, was also effective in the protection against carriage in ME or NP (Tables 2–6).

8.2.1. Protection against NTHi—Initial mucosal vaccination studies in mouse models were performed with whole killed NTHi or crude preparations of surface-exposed antigens (*i.e*., OMPs) as the immunogen, and the results from these studies established the parameters of effective immunity against NP infections as well as the potential of vaccination. For example, oral administration of formalin-killed NTHi was found to confer protection against nasal carriage by NTHi [142,143]. Similar protection was demonstrated after oral administration of OMP with GM-53, a chemically synthesized adjuvant [144]. Although this regimen was effective in inducing both mucosal and serum antibodies, the protective effect of oral vaccine was likely associated with mucosal IgA antibodies since oral immunization was more efficacious in a clearing nasal infection than subcutaneous immunization, which failed to induce mucosal antibodies [143]. Others have employed IN administration of OMP from NTHi [145,146], and two important conclusions resulted from those studies: (1) protection in the upper respiratory tract is correlated with the presence of specific IgA antibodies in nasal washes, and (2) nasal vaccine delivery was most efficacious in inducing mucosal IgA antibodies and in providing protection against nasal NTHi carriage compared to other routes of inoculation (oral, intra-tracheal, or systemic; [146]). IN administration of surface-expressed antigens from NTHi was used to elucidate the significance of immune responses to these antigens in clearing an infection. IN administration of the highly conserved NTHi P6 protein in the presence of adjuvant induced specific systemic as well as mucosal IgA responses in various effector sites, including nasal passages [44,148,150], Eustachian tubes [43] and the ME [44]. Hirano *et al.* [147] demonstrated that IN administration of detoxified NTHi lipooligosaccharide (dLOS)-tetanus toxoid (TT) conjugates induced specific mucosal IgA and serum IgG antibodies with a predominance of IgG1 antibodies (Th2-type). The murine model used in this study provided useful information regarding the kinetics of antibody expression and cellular responses following multiple administration of dLOS-TT. Thus, studies with IN immunized mice demonstrated enhanced bacterial clearance following IN or ME bacterial challenge [45,60,113]. Moreover, NTHi numbers in nasal washes [113,144] or ME washes [60] were associated with vaccine-specific mucosal IgA and serum IgG antibody titers.

8.2.2. Protection against S. pneumoniae—Wu *et al*. [94] demonstrated that IN immunization with heat killed pneumococci or pneumococcal lysates in the presence of CTB as an adjuvant elicited protection against carriage. Further studies using murine models showed that protective immunity against pneumococcal infection could be conferred by antibodies against conserved pneumococal surface proteins or by serotype-specific anti-capsular antibodies. Antibodies neutralize the activity of virulence factors and function as an opsonin to enhance bacterial clearance. IN immunization of mice with pneumococcal serotype 1 polysaccharide conjugated to TT or type 3 polysaccharide conjugated to diphtheria toxoid together with a nontoxic mutant of *Escherichia coli* or a glyceride-polysorbate based adjuvant induced mucosal and systemic responses, and serum IgG antibody levels were significantly higher than after systemic immunization [180]. IN immunization with pneumococcal serotype 9V polysaccharide conjugated to pneumococcal proteins (pneumolysin or autolysin), and CT as adjuvant, elicited high 9V IgG and IgA antibody responses in serum and tissue homogenates [181]. Recently, we demonstrated protection against OM in unimmunized mice if the pneumococci were first opsonized with antibody from ME fluid or serum obtained from immunized mice before intrabullar challenge [45]. Several studies using mouse models have demonstrated the importance of serum antibodies for prevention of invasive pneumococcal disease following NP colonization or ME infection [45,94,116]. In IN immunized mice, the levels of serum antibodies correlated with rapid pneumococcal clearance from the blood [181] and lungs [180] following intraperitonial or IN challenge, respectively. It is expected

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that serum antibodies which are protective in the systemic compartment could also be protective at mucosal surfaces. Indeed, systemic immunization with PsaA synthetic peptides induced peptide-specific serum antibodies and provided cross-protection against nasal challenge with pneumococci [182]. Thus, protection at mucosal surfaces following IN immunization may be mediated by both mucosal IgA and serum antibodies. Specific IgA antibodies play a key role in clearing bacterial pathogens from mucosal sites by inhibiting the uptake and colonization of microorganisms [183]. The protective function of serum antibodies, especially IgG2a and IgG3 isotypes, has been shown to be associated with increased complement-mediated bactericidal and opsonophagocytic activity [101], as well as with increased binding avidity compared to IgA antibodies [181].

8.2.3. Protection against M. catarrhalis—Jiao *et al.* [155] inoculated mice IN with a *M. catarrhalis* dLOS-protein conjugate and the results revealed a negative correlation between bacterial counts in the lung, and IgA and IgG levels in nasal washes, lung lavage fluids, and serum. These data indicate that local and/or systemic LOS-specific antibodies are associated with enhanced bacterial clearance from the mouse lung. Importantly, IN immunization resulted in a higher level of bacterial clearance compared to subcutaneous immunization, supporting an important role for mucosal antibodies in the observed protection. Mason *et al*. [110] demonstrated that IN administration of a mixture of rLP4/rLP6/UspA2 proteins in the presence of the RC259-AF adjuvant induced specific IgA responses in the respiratory tract as well as specific serum IgG and IgA responses. The serum IgG subclass distribution was predominantly IgG2a, representing a Th1-type response. Importantly, serum antibodies from immunized mice showed complement-dependent bactericidal activity toward several strains of *M. catarrhalis*. Hou *et al.* [154] induced specific nasal and serum antibody responses following injection of whole killed *M. catarrhalis* into NALT tissue. Intra-NALT immunization was shown to be more effective at inducing specific mucosal and serum responses compared with those induced using the IN route, and this is likely due to more efficient priming of immunocompetent cells in a mucosal inductive site.

8.2.4. Protection against viruses—With respect to prevention of virally-induced acute OM, mucosal vaccines have been shown to provide effective protection against influenza virus, RSV and PIV in the respiratory tract (Table 5). Vaccine-induced protection against viral infections is likely to be associated with both humoral (S-IgA, serum IgG antibodies) and cell-mediated (CTLs) immunity [157,135,184]. Renegar *et al*. [185] demonstrated that S-IgA prevents influenza virus-induced pathology in the upper respiratory tract but serum IgG antibody failed to prevent viral infection of the nose although it did neutralize newly replicated virus after infection had been initiated. Studies of heterosubtypic immunity, *i.e.*, crossreactive protection against different strains of influenza virus, also showed a correlation with S-IgA production (rather than cytotoxic T-cell reactivity) [186,187]. Others also demonstrated that passive transfer of IgA antibody, as well as IgG antibody, can confer protection against RSV infection in the upper respiratory tract [188]. The mechanism of IgA-mediated protection against viruses at mucosal surfaces may include both extracellular and intracellular neutralization [183,189]. IgG antibodies provide antiviral protection through direct neutralization as well as Fc receptor-mediated opsinophagocytosis [190]. IN immunization with recombinant SeV expressing the G glycoprotein of RSV induced SeV-specific and RSV-specific antibody-forming cells in lymph nodes, and a few antibody-forming cells in the bone marrow [191], and these increases were reflected by increased serum antibody levels [192]. Similarly, IN immunization of infant mice with human PIV-1 afforded protection against a subsequent challenge with SeV (mouse PIV-1) and this protection was associated with increased levels of specific antibodies in the serum [176]. Importantly, the response was completely cross-reactive between human PIV-1 and SeV. Several studies have compared the ability of mucosally and parenterally administered antiviral vaccines to induce mucosal *vs.*

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serum antibodies. Bastien *et al*. [170] demonstrated that both IN and intraperitoneal administration of an RSV G protein synthetic peptide mixed with CT induced specific IgG antibody in serum, whereas only IN administration induced specific IgA in nasal washes. Similarly, oral administration of an SeV-CT conjugate induced virus-specific IgA titers in nasal washes as well as specific antibodies in serum, and protected the upper respiratory tract against virus challenge with SeV [179]. In contrast, systemic immunization with the SeV-CT conjugate only induced virus-specific antibody responses in serum and failed to protect the upper respiratory tract against virus challenge. Thus, the enhanced protection against SeV conferred by the oral vaccine is likely to be due to specific S-IgA antibodies. Lovgren *et al*. [156] compared the induction of specific serum antibodies after IN, oral or subcutaneous administration of influenza glycoproteins in immunostimulating complexes (ISCOMs). Subcutaneous administration induced greater serum antibody titers compared to IN administration, whereas oral administration failed to induce serum antibody. The predominant IgG isotypes after IN or subcutaneous administration were IgG1 and IgG2a. Trudel *et al*. [166] demonstrated that IN administration of live RSV induced greater antibody titers in serum than those induced by intramuscular immunization, however, this difference could have been due to the fact that different adjuvants were used for IN and systemic vaccination. In addition to antibody-dependent protection, $CD8^+$ cytotoxic T cells and $CD4^+$ Th cells have been found to contribute to recovery from infection at mucosal surfaces by targeting internal viral proteins, but are not strong mediators of resistance to reinfection [166,193–196].

Considering the above, it is likely that mucosal specific IgA antibody protects against bacterial and viral pathogens at mucosal surfaces, whereas serum antibodies (as well as $CD8^+$ and $CD4^+$ T cells) mainly eradicate the pathogens after the infection has been initiated in the respiratory tract and/or ME [47,94,100,108,125].

8.3. Immunodeficiency models to understand the role of IgA in respiratory and ME immunity

The development of mice that are selectively deficient for expression of IgA ($IgA^{-/-}$ mice) or polymeric IgR (*i.e*., defective transport of IgA into the mucosal lumen) as a result of targeted genetic lesions has provided useful models to evaluate the role of IgA in upper respiratory tract and ME immunity [96,100,101,163,198].

Mbawuike *et al*. [163] demonstrated that in $IgA^{-/-}$ mice, IN immunization with a CTB-adjuvanted influenza vaccine provided protection against virus infection in the upper respiratory tract following IN challenge with a lethal dose of influenza virus. This finding challenged the notion that S-IgA is responsible for protection against virus infection in the respiratory tract. Similarly, IN immunization with live influenza virus conferred protection against heterosubtypic IN challenge in both $IgA^{-/-}$ and $IgA^{+/+}$ mice, as evidenced by reduced virus titers in nasal washes [164,198]. In contrast, IN immunization of $pIgR^{-/-}$ mice with inactivated influenza virus failed to confer protection against virus infection in the upper respiratory tract, in parallel with a decrease in nasal wash IgA titer [100]. Moreover, the importance of S-IgA in host defense was shown by the finding that IN vaccination with pneumococcal polysaccharide conjugated to diphtheria toxoid in the presence of IL-12 as an adjuvant protected $IgA^{+/+}$ but not $IgA^{-/-}$ mice against subsequent NP colonization with *S. pneumoniae* type 14 [96,101]. The same vaccination regimen similarly failed to protect $pIgR^{-/-}$ mice against nasal carriage, and this observation was associated with the absence of specific IgA antibody in nasal washes [96].

Recently, an effort has been made to present a unifying concept to explain apparently conflicting results obtained from various laboratories using IgA-deficient mice [199]. Differences in this regard may be related to the extent of inflammation induced during infection and the amount of transudated IgG antibody [199]. In general, it is the presence of inflammation that appears to determine whether IgA is necessary for any observed protection; that is, in the

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presence of inflammation, IgA-deficient mice are protected from infection, and in the absence of inflammation, IgA-deficient mice are not protected. This conclusion in supported by observations that S-IgA is not required for protection against IN challenge with *S. pneumoniae* type 3 or sublethal doses of influenza virus, or against intrabullar challenge with noninvasive pneumococci (unpublished observations), all of which result in significant inflammation and systemic infection. The latter effects facilitate passive transudation of serum IgG antibodies into mucosal secretions through damage to the epithelial layer and thus, compensate for the lack of IgA [60,185,199,200].

In essence, it is likely that IgA antibody provides an important first line of defense against infections of the respiratory tract. The presence of inflammation appears to determine whether IgA is necessary for any observed protection following IN or intrabullar challenge.

9. Mucosal adjuvants and delivery systems

Mucosal immunization suffers from two main problems: inefficient antigen retention and uptake at mucosal surfaces [201], and difficulties in eliciting immune responses to mucosally administered antigens [202]. With respect to oral immunization, the gastrointestinal tract prevents the absorption of many foreign molecules such as vaccine antigens [203]. Several strategies have been designed to improve the immunogenecity of vaccine antigens and to induce potent, long-lasting, and recallable mucosal immune responses manifested by expression of S-IgA antibodies in secretions, as well as specific antibody-secreting B cells and T effector cells in the relevant tissues [25]. These strategies include the use of purified synthetic adjuvants and delivery systems that can efficiently aid in uptake, processing, and presentation of vaccine antigens in mucosal tissues. Advantages include activation of only those elements of the local immune system that are required for protection, and limitation of the systemic distribution of the adjuvants. Novel adjuvants and delivery technologies are highly desirable for the successful development of mucosal vaccines against OM that have failed through the use of traditional approaches. Experiments in mice have revealed that balanced Th1- and Th2-type responses can be generated, offering the prospect of selectively inducing the most appropriate humoral or cell-mediated responses for protection against a particular infection.

The most widely experimentally used mucosal adjuvants in mice are CT and the closely related *E. coli* LT (Table 2), both of which can promote mucosal IgA responses, systemic IgG responses and CTL activation against co-administered antigens. Unfortunately, severe toxicity renders CT and LT unsuitable for clinical use in humans. To avoid toxicity, the isolated B subunits of CT [47,95,116,118,120,124] and LT [133] have been tested for their ability to augment immune responses against co-administered antigens. Both CTB and LTB are poor adjuvants when administered together with antigens by the oral route, although they display more significant activity when inoculated via the IN route [17]. In addition, genetically detoxified CT and LT mutants that have reduced toxicity, but retain adjuvanticity when given to animals by the IN route are now available [112,122,123,172]. It has been shown that CT primarily induces Th2 type responses characterized by $CD4^+$ T cells that produce IL-4, IL-5, IL-6 and IL-10 and by the production of IgA, IgG1 and IgE antibodies [1]. Use of LT has been reported to produce a less polarized response, and to activate both Th1 and Th2 cells [204].

Several investigators have tested CpG oligodeoxynucleotides (ODN) as mucosal adjuvants [109,148]. IN administration of CpG ODN together with the NTHi P6 antigen resulted in both Th1 responses (CTL and IFN-γ) and Th2 responses (IgA) at mucosal sites and Th1 responses systemically [109,148]. Following IN administration, CpG ODN also enhanced serum antibody responses to type 9V polysaccharide conjugated to inactivated pneumolysin, and these responses included mixed Th1-type and Th2 type antibodies [205]. Kodama *et al.* [150] recently coadministered P6 protein with Flt3 ligand, which mobilizes and stimulates

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dendritic cells and myeloid and lymphoid progenitor cells, and demonstrated efficient induction of specific mucosal and serum antibodies. Co-inoculation of pneumococcal polysaccharide conjugate vaccine with IN administered IL-12, generally thought to be a Th1-inducing cytokine, was found to induce high levels of both Th1- and Th2-associated antibodies locally and in the bloodstream [45,96,101].

A variety of mucosal antigen delivery systems have been developed to increase uptake of antigen at mucosal surfaces, including biodegradable nanoparticles [160] and biopolymers such as chitosan [134], liposomes [158,159,132], and ISCOMs [156,157,166,169]. Studies with recombinant bacterial [115,206] and viral [191] vector systems have demonstrated the feasibility of creating live delivery systems that express recombinant DNA encoding foreign antigens but without the ability to allow reversion to a virulent phenotype [115,206]. ISCOMs have shown the potential to induce a full range of humoral and CTL responses, both of which appear to be important for antiviral protection [156,157,166,169]. Other strategies include the incorporation of antigens into various microparticles [134,160] or membrane-bound vesicles [158,133,159], procedures that promote antigen uptake and provide protection against proteolytic digestion.

10. Mucosal immunization in early life

In humans, OM has its onset in the first year of life, and therefore, a vaccine to prevent OM must target young infants and newborns. However, mucosal vaccination in this target population may be problematic because of the relatively poor immunogenicity of many vaccine candidates during early life due to immature antigen presenting and T cell function. In previous murine OM models, the effect of vaccination was studied in adult animals, however, as pointed out by Gu *et al.* [12], the immunogenicity of vaccines in adults might not always predict their activity at a young age. Thus, recent efforts have been made to evaluate the immunogenicity of vaccines in mouse models of early life. For example, we demonstrated that neonatal IN vaccination with pneumococcal conjugate vaccine in the presence of IL-12 can induce protective immunity against *S. pneumoniae* [45,76]. This immunization regimen was shown to enhance the ability of infant mice to clear pneumococcal infection from both the ME and NP [45,76]. Inclusion of IL-12 during neonatal IN vaccination may partially overcome defective antigen presenting cell function and thereby enhance priming for antibody responses [36].

Another innovative approach has been explored to prevent NTHi-induced disease in young mice via maternal IN immunization [149]. Such a vaccination approach holds promise in stimulating antibody responses to the P6 protein and potentially preventing OM due to NTHi carriage in the mother, as well as OM in the infant through passive transfer of maternal antibodies across the placenta. Similarly, maternal IN immunization of mice with PspA and CTB was reported to significantly reduce nasal carriage of pneumococci in the offspring following IN challenge with pneumococci [152].

11. Limitation of the mouse model and mucosal vaccination strategy

How closely does the mouse immune response and the pathogenesis of ME disease resemble that in humans? Murine antibody responses and the stages of immune maturation are similar to those in humans [207]. Nevertheless, while it appears that induction of OM is reproducible in mice, the major bacterial (NTHi, *S. pneumoniae* and *M. catarrhalis*) and viral (influenza virus, PIV, RSV) pathogens for humans are not natural murine pathogens [64,85,208,209]. Since these microbes are exclusively human pathogens under natural conditions, the colonization patterns and the immune responses observed following infection of mouse ME and NP might be different from those of humans. This is a significant limitation to the usefulness of the rodent model. In order to overcome this limitation, multiple IN inoculations

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or direct inoculation into the ME space must be performed to induce disease (as described in section 4.1). Alternative approaches include using mutant bacterial strains that adhere to, and invade, murine mucosa more efficiently than wild type bacteria [210] and the use of genetically modified mice which demonstrate increased susceptibility to human pathogens. Even so, the observed inflammatory responses are often less prominent than those in humans. Therefore, transudation of serum antibody is also less efficient in the mouse compared to humans, where transudation of serum antibody appears to be very important. Nevertheless, both antibody and cellular systemic immune responses in the mouse mimic the human immune response sufficiently to be informative. Another limitation is that the mouse ME is not easily accessible for inoculation and for removal of fluid, and the small size precludes sampling of large quantities of ME effusion. An alternative is to use larger species such as chinchillas, guinea pigs, rats, or gerbils for induction of OM. However, despite the above disadvantages, the mouse model is still the most useful for testing hypotheses regarding immune responses and pathogenensis, although certain precautions should be taken in extrapolating results from murine studies to humans. Experience with oral vaccines has shown that vaccines which work perfectly in mice can fail to perform well in other animal species including humans [211]. It has to be noted that most of the vaccines in current use are parenteral vaccines and only very few mucosal vaccines have been approved for human use, primarily because the low immunogenicity of soluble antigens and the lack of safe and efficacious mucosal adjuvants [212]. In addition, it is uncertain whether a fully effective dose of vaccine can be administered mucosally. It has been difficult to gain approval for mucosal vaccines in humans with the notable recent exception of the IN FluMist influenza vaccine. However, progress in this field may allow increased development of needle-free vaccines against mucosally acquired pathogens, including those that lead to OM.

12. Conclusions and future directions

In our opinion, improving the mucosal platform as a route for vaccination will be the next major advance in the field of vaccinology. Studies in murine models have been instrumental in the development of many successful mucosal vaccines to prevent bacterial and viral infections that often lead to OM. Mucosal immunization studies, particularly those utilizing IN inoculation, have identified several vaccine candidates for key bacterial and viral targets against OM, and the types of immune responses associated with protection from infection in the ME. The progress in this field was demonstrated by the development of various nonreplicating subunit vaccines, DNA vaccines, recombinant replicating vaccines, and, microbial vector-based mucosal vaccines. Mouse models of NP colonization and ME infection have been used by several groups to show that IN vaccine administration with the use of appropriate adjuvants and antigen delivery systems can be effective for induction of protection against NP carriage and experimental OM. In addition, transgenic and knockout mice have been invaluable for discerning the mechanisms of mucosal vaccine-induced protection against OM and elucidating the roles of various components of a protective immune response. Over the next years, emphasis should be placed in several areas to advance the field of vaccine development:

1. Priorities for future mucosal vaccine development against OM should focus on multipathogen vaccines which include combinations of protective protein antigens from all three causative bacteria. The emphasis should be placed on development of strategies using antigens which are highly conserved among similar bacterial species as well as those shared among different bacterial species, such as phosphorylcholine. The induction of protective responses following mucosal administration of more than one antigen from similar pathogens should continue to be tested. The evaluation of combined bacterial-viral vaccine formulations should also continue to be an important area of investigation. Although IN vaccination appears to be an attractive route for

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prevention of OM, future studies should also focus on immunization by mixed, mucosal routes (nasal-oral) or a combination of mucosal and parenteral routes.

2. Novel mucosal adjuvants and mucosal delivery systems should continue to be developed as a means to enhance the efficacy of mucosal and systemic immune responses.

3. The immunogenicity of mucosal vaccines in infants needs to be further evaluated in the mouse models of early life and maternal immunization.

4. There is a need to develop a mouse model of OM caused by combinations of bacterial pathogens (NTHi and *S. pneumoniae*) and viruses. Such polymicrobial models will allow testing of multipathogen vaccines against OM. The efficacy of promising vaccine candidates against *M. catarrhalis* must also be assessed in a mouse pulmonary clearance model.

5. One of the priorities for research that can readily exploit mouse models includes further unraveling of the genetic factors that are involved in susceptibility to OM and that influence host immune responses to pathogens/mucosal vaccines. Current and future progress in developing mice with targeted gene mutations that result in increased susceptibility or spontaneous development of OM will provide significant new insights into the mechanisms responsible for disease progression and the requirements for protection.

If there is sufficient effort and resources devoted to resolving these issues, we predict that in the next decade or two, it will be possible to routinely utilize mucosal vaccines for prevention of OM so that this disease will ultimately represent a historical footnote and will no longer be a significant cause of human morbidity and mortality.

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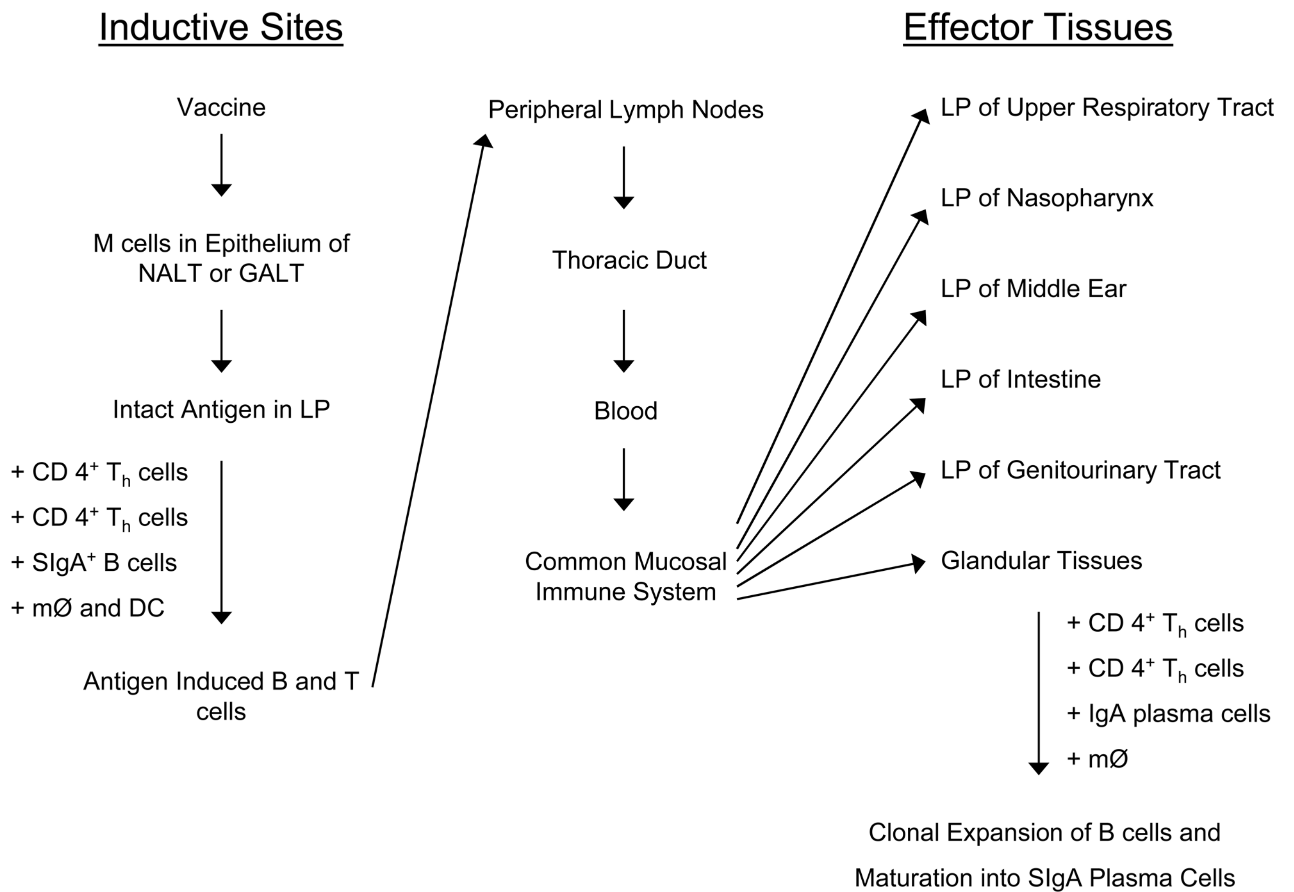

Fig. 1.
Induction of mucosal immunity following intranasal or oral administration of vaccine. The priming of antigen-specific, IgA-committed B cells occurs in the mucosal inductive sites and results in IgA antibody responses in effector tissues. NALT: nasal-associated lymphoid tissue; GALT: gut-associated lymphoid tissue; LP: lamina propria; SIgA: secretory IgA; MØ: macrophages; DC: dendritic cells.

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Table 1

Summary of animal models of OM[a]

Animal species	Chinchilla	Gerbil	Guinea pig	Rat	Mouse
Pathogen recovery frequency (%):	> 90	> 90	NA [53]	> 90	100 [60]
NTHi	> 90	> 09	100% [54]	> 90	100 [3]
SP	0	0	NA [55]	< 60	0 [3]
MC					
Pathology frequency (%):	> 90	> 90	100 [53]	> 90	100 [60]
NTHi	> 90	> 90	70 [56]	> 90	100 [3,61]
SP	< 60	< 60	>60 [55]	< 60	0 [3]
MC					
Inbred strains	−	+	+	++	+++
Genetic mutant models	−	−	−	+	+++
Experimental reagents	−	+	++	+++	+++
Naturally occurring OM	− [50]	+ [51,52]	+++ [57]	++ [58,59]	+ [62]
Cost	+++	+	++	+	+

[a]The data for chinchilla, gerbil and guinea pig models were adapted from Doyle, 1989 [49].

OM, otitis media; NTHi, nontypeable *H. influenza*; SP, *S. pneumoniae*; MC, *M. catarrhalis*; NA, not available.

−, absent; + to +++, minimal to highly significant.

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Table 2

Studies on intranasal vaccination to prevent ME carriage in mice

System	Comments	Reference
Nontypeable *H. influenzae*		
P6 (+ CT)	The protection was associated with enhanced ME mucosal and systemic responses as well as with reduced stimulation of TNF-α production in ME effusion.	[60]
P6 or rP6 (+ AdDP)	rP6 constitutes a promising vaccine candidate antigen. Vaccination with rP6 induced protection against OM and the mucosal response was stronger than to native P6.	[77]
S. pneumoniae		
KNP (+ CTB)	** Protection was demonstrated against both NP and ME colonization by three pneumococcal serotypes tested. The novel strategy of inexpensive vaccine for multiserotype protection.	[71]
PCV (+ IL-12)	*Neonatal immunization primed animals for enhanced ME mucosal and systemic antibody responses as infants, enhanced bacterial clearance from the ME, and increased survival against OM-induced invasive pneumococcal infection.	[45]
PCV (+ IL-12)	NALT-independent protection against NP carriage and ME invasion of challenged pneumococcal cells.	[76]

Papers of particular interest, published within 1986–2007 period of review, have been highlighted as:

* of special interest

** of outstanding interest

ME, middle ear; CT, cholera toxin; r, recombinant; AdDP, adamantylamide dipeptide; OM, otitis media; KNP, killed nonencapsulated pneumococci; CTB, B subunit of cholera toxin; NP, nasopharyngeal; PCV, protein conjugate vaccine; IL-12, interleukin-12; NALT, nasal-associated lymphoid tissue;

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Table 3

Studies on intranasal vaccination to prevent nasal carriage by NTHi in mice

System	Comments	Reference
Outer membrane protein-based vaccine		
rP6 (+ CT)	Study indicates the good immunogenicity of rP6 to induce specific immune responses and protection against NTHi-induced infection.	[108]
P6 (+ CpG ODN)	•• The first study to use non-toxic adjuvant CpG for inducing immunity against NTHi along with the P6.	[109]
rLP4/rLP6/UspA2 (+ RC529-AF)	• IN administration of a mixture of the NTHi (P6+P4) and *M. catarrhalis* (UspA2) proteins resulted in systemic and mucosal antibody responses to each protein and reduced NP colonization by NTHi.	[110]
rP4 or rP4/rP6 (+CT)	•• Used combination of two surface protein antigens of NTHi for induction of mucosal and serum antibody responses.	[111]
Hap (+ mutant CT)	The induced protection was associated with induced specific mucosal IgA and systemic antibodies.	[112]
Lipooligosaccharide-based vaccine		
dLOS-TT (+ CT)	The induced protection was associated with mucosal IgA and serum antibodies.	[113]

NTHi, Nontypeable *H. influenzae*; CpG ODN, synthetic oligodeoxynucleotide; LP4/LP6, lipidated P4/6; UspA, ubiquitous cell surface protein A; IN, intranasal; RC529-AF, chemically synthesized adjuvant in an aqueous formulation; CT, cholera toxin; Hap, *Haemophilus* adhesin protein; dLOS-TT, detoxified lipooligosaccharide conjugated to tetanus toxoid.

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Table 4

Studies on intranasal vaccination to prevent nasal carriage by *S. pneumoniae* in mice

System		Reference
Pathogenic and non-pathogenic bacteria		
KNP (+ CT or CTB)	•• The protection was demonstrated against both colonization in the NP and ME.	[71,114]
Live-attenuated bacteria	• Live *S. pneumoniae* attenuated through deletion of genes encoding virulence factors was effective in induction of cross-serotype protection. Protection did not depend on a response to the virulence factors such as C-Ps, pneumolysin and PspA.	[104]
rLactobacilli expressing PsaA	•• Recombinant lactobacilli expressing PsaA induced systemic and mucosal immune responses which where protective against pneumococcal nasal carriage.	[115]
Protein subunit-based vaccine		
PspA or 6B PS-TT (+ CTB)	IN but not systemic immunization is effective in inducing specific mucosal and serum IgA antibodies, and in providing the cross-protecton.	[116]
rPspA, rPsaA, rPneumolysin (+ CTB)	PsaA is better than PspA or pneumolysin in protection	[117]
PspA, PspC, PsaA, pneumolysin	•• Demonstrated that the best protein vaccines for prevention of infection may be those that include more than one protection-eliciting pneumococcal protein. Immunization with a mixture of PsaA and PspA elicited better protection against carriage by two pneumococcal strains than either protein alone.	[118]
PspA (+ IL-12)	IN administration of IL-12, known activator of Th1 and NK cells, augmented the production of both Th1- and Th2-associated antibody isotype both in mucosal and systemic compartments.	[119]
PspC (+ CTB)	Reduced the nasal carriage of a pneumolysin-minus mutant, but not wild-type strain.	[120]
Phosphorylcholine-KLH (+ CT)	•• Elicited cross-protection against nasal carriage by *S.pneumoniae* and NTHi. Provides a potential as a broad spectrum IN vaccine	[121]
PppA (+ MPL or CT mutant)	Elicited a high titer of serum antibody and reduced the recovery of challenged pneumococcal cells from nasal wash.	[122]
RPhpA (+ mutant CT)	Protection against NP colonization as well as against death and bacteremia following IN challenge with a heterologous pneumococcal strain	[123]
Polysaccharide -based vaccine		
C-Ps (+ CT or CTB)	• Cross-serotype protection against nasal carriage. Protection is independent of antibody but dependent on CD4+	[124]

KNP, killed nonencapsulated pneumococci; CTB, B subunit of cholera toxin; C-Ps, pneumococcal cell wall polysaccharide; PspA, pneumococcal surface protein A; PsaA, pneumococcal surface adhesion; PS-TT, polysaccharide, conjugated to tetanus toxoid; PspC, pneumococcal surface protein C; IL-12, interleukin 12; NK, natural killer cells; KLH, keyhole limpet hemocyanin; PppA, pneumococcal protective protein; MPL, monophosphoryl lipid A; rPhpA, pneumococcal histidine protein A

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Table 5

Studies on intranasal vaccination to prevent intranasal viral challenge in mice

System	Comments	Reference
Influenza		
Live and inactivated vaccines		
Live virus	IgA is primarily responsible for defense against virus.	[125]
Inactivated HA vaccine (+ CTB)	The degree of protecton against viral challenge correlated with levels of specific IgA and serum antibodies. Systemic immunization failed to induce nasal antiviral IgA antibodies.	[47]
Inactivated HA vaccine (+ CTB)	IN vaccination is more effective than parenteral immunization for providing cross-protection against nasal influenza infecton.	[126]
Inactivated trivalent vaccines (+ CTB)	Cross-protection against IN viral challenge associated with hemagglutinin-specific mucosal IgA	[127]
Inactivated vaccine (+ CTB)	• In the mice immunized primarily with later virus vaccine and boosted with another later virus vaccine, the cross-protection against challenge with the latest virus was provided more effectively than against challenge with the earliest virus. The strategy for inducing cross-protection against epidemic viral strain in humans.	[128]
Inactivated vaccine (+ CTB)	IN priming and IN boosting afforded the highest cross-protection, while combination of s.c. priming and IN or s.c. boosting afforded little cross-protection.	[129]
Inactivated virus (+ CTB)	• IN vaccination is superior to oral or systemic routes of vaccination in inducing specific antiviral IgA antibodies and protecting against IN viral challenge.	[130]
Inactivated vaccine (+ mutant CT)	Cross-protection against influenza correlated with nasal wash IgA.	[131]
Subunit and DNA vaccines		
Subunit antigen (in liposomes)	Incorporation of IN vaccine into liposomes enhanced mucosal IgA and serum IgG responses.	[132]
Subunit antigen (+ LTB)	•• Local protection against influenza virus may be mediated not only by IgA but also by mucosal IgG antibodies. Both IN and i.m. routes were effective in conferring the protection.	[133]
Chitosan-DNA, expressing influenza HA and NP	•• I.m. priming and IN or i.m. boosting conferred the equal protection against influenza virus challenge.	[134]
RSV		
Live and inactivated vaccines		
Live and inactivated virus	Primary immunization with live, but not inactivated, RSV enhanced the production of mucosal RSV-specific IgA upon challenge.	[135]
Inactivated virus	• Simultaneous oral and IN administration of inactivated RSV induced specific mucosal immunity, which protected the upper respiratory tract from viral replication.	[110]
Subunit vaccines		
F protein (+ CTB)	••Combined IN plus parenteral immunization provided the better protection from viral replication in the upper respiratory tract than IN or parenteral immunization alone. Association of the enhanced protection with the induction of mucosal IgA, serum IgG and neutralizing antibody.	[136]
Chimeric FG (+ CTB)	IN vaccination was superior to parenteral immunization in inducing the protection in the upper respiratory tract against RSV. The enhanced protection correlated with the presence of mucosal IgA antibody.	[137]
Parainfluenza		
Inactivated vaccine		
IN killed SeV + oral killed SeV (+CT)	Protection against carriage was associated with the presence of IgA antiviral antibodies in nasal wash. Neither IN or oral immunization alone conferred the protection or induced mucosal IgA.	[138]
Recombinant vaccine		
RVVs carrying either the HN or the F gene of Sendai virus	• IN but not i.p. immunization induced SeV specific IgA antibodies in nasal wash, and protected from viral replication in the nose. The resistance to SeV in the nose could be abrogated by the IN instillation of anti-mouse IgA but not of anti-IgG antiserum.	[139]

HA, hemagglutinin; s.c., subcutaneous; LTB, B subunit of heat-liable enterotoxin; i.m., intramuscular; NP, nucleoprotein; RSV, respiratory syncytial virus; F, fusion; protein; G, glycoprotein; SeV, Sendai virus; RVVs, recombinant vaccinia viruses; HN, hemagglutinin-neuraminidase.

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Table 6

Effect of intranasal immunization against otitis media (OM) induced by intrabullar challenge with nontypeable *H. influenza* (NTHi) or *S. pneumoniae*

	NTHi-induced OM[a]		***S. pneumoniae*-induced OM[b]**	
	Day 3	**Day 7**	**Day 3**	**Day 7**
	PBS/P6+CT	**PBS/P6+CT**	**PBS/PCV+IL-12**	**PBS/PCV+IL-12**
Mean concentration of bacteria in middle ear effusions (log_{10})	5.93/4.41[c]	4.64/3.56[d]	7.4/6.8[d]	4/0[d]
Middle ear bacterial carriage (%)	93/79	64/28	100/87	50/0[d]
Presence of middle ear effusions (%)	100/100	79/28[d]	100/100	50/37
Tympanic membrane changes (%)	93/87	21/0	87/75	25/0

Values obtained from the following publications: NTHi-induced OM (Sabirov *et al.*, [60]); *S. pneumonie*-induced OM (Sabirov and Metzger, [45]).

[a] Mice were inoculated IN with PBS or P6 and cholera toxin (P6+CT) prior to challenge with NTHi.

[b] Mice were inoculated IN with PBS or pneumococcal conjugate vaccine and IL-12 (PCV+IL-12) prior to challenge with *S. pneumoniae*. Mice were killed on days 3 and 7 after the intrabullar challenge.

[c] $P < 0.001$;

[d] $P < 0.05$ (analyzed by Mann-Whitney test or Fisher's exact test).