Current and Future Prospects for a Vaccine for Nontypeable *Haemophilus influenzae*

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Nontypeable *Haemophilus influenzae* is an important human respiratory tract pathogen that causes about 30% of otitis media in infants and children. This proportion is increasing as a result of pneumococcal conjugate vaccines. Because of the morbidity associated with otitis media, a strong rationale exists to develop strategies to prevent these infections. A challenge to developing a vaccine for nontypeable *H. influenzae* is the antigenic heterogeneity of several major surface antigens and the genetic heterogeneity among strains. Several research groups have identified conserved surface proteins and tested them as putative vaccines. A recent clinical trial with protein D, a conserved surface antigen, demonstrated partial efficacy in preventing *H. influenzae* otitis media. This important result provides a proof of principle for developing a vaccine to prevent otitis media caused by nontypeable *H. influenzae*. Several vaccine antigens for nontypeable *H. influenzae* are in development.

Introduction

Haemophilus influenzae includes encapsulated and nonencapsulated strains. The *H. influenzae* type b conjugate vaccines that consist of type b capsular polysaccharide conjugated to protein carriers successfully prevent invasive infections caused by encapsulated *H. influenzae* type b strains. However, these vaccines have no effect on infections caused by nontypeable *H. influenzae* because the latter strains lack a polysaccharide capsule. Nontypeable *H. influenzae* is an important and common human respiratory tract pathogen that causes otitis media in infants and children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease. Recent progress in vaccine development for nontypeable strains of *H. influenzae* has reinvigorated the field. This review provides an update on the status of vaccine development for nontypeable *H. influenzae*.

Spectrum of Disease Caused by Nontypeable *H. influenzae*

Nontypeable *H. influenzae* is a common and important respiratory tract pathogen in children and adults. Because the bacterium is present in the upper respiratory tract flora, estimating the true burden of disease caused by nontypeable *H. influenzae* requires rigorous methods. Simply isolating the organism in cultures of the respiratory tract does not establish causality.

Based on carefully performed studies, the primary clinical manifestations of infection caused by nontypeable *H. influenzae* are otitis media and sinusitis in infants and children [1••]. Indeed, nontypeable *H. influenzae* is an important cause of sinusitis in children and adults [2•,3]. In addition to upper respiratory tract infections, nontypeable *H. influenzae* also causes lower respiratory tract infections. The bacterium is the most common cause of exacerbations in adults with chronic obstructive pulmonary disease and also likely contributes to the pathogenesis of the disease by chronically colonizing the lower airways [4]. Furthermore, nontypeable *H. influenzae* causes community-acquired pneumonia in adults and may cause pneumonia in children in developing countries, although precise estimates are difficult [5].

In view of recent progress in vaccine development for nontypeable *H. influenzae* in the setting of otitis media and given the strong rationale for pursuing vaccines to prevent otitis media, this review focuses primarily on vaccines for otitis media caused by nontypeable *H. influenzae*.

Epidemiology and Disease Burden of Otitis Media

Otitis media is the most common bacterial infection in childhood, accounting for at least 16 million physician visits and more than 13 million antibiotic prescriptions annually in the

United States in 2000 [6]. Otitis media is a costly disease and is the most common reason why children receive antibiotics [7–12]. A subset of children experience recurrent and chronic otitis media [13]. These otitis-prone children have protracted middle-ear effusions that are associated with hearing loss and delays in speech and language development [6]. Insertion of tympanostomy tubes to correct chronic and recurrent otitis media is the most common indication for children to undergo general anesthesia. Preventing otitis media would have significant human and economic benefit, particularly for otitis-prone children.

Bacteriology of Otitis Media

Culture of middle-ear fluid obtained by tympanocentesis is the "gold standard" for identifying the etiology of acute otitis media. Based on cultures of middle-ear fluid, nontypeable *H. influenzae* is the second most common bacterial cause of otitis media after *Streptococcus pneumoniae*, causing 25% to 35% of episodes of acute otitis media [1••,14–17]. However, the bacteriology of otitis media is changing.

Since 2000, a majority of infants in many countries have received the 7-valent pneumococcal conjugate vaccine. Two studies have evaluated the effect of the widespread use of this vaccine on the distribution of pathogens causing otitis media. Casey and Pichichero [18] and Block et al. [19] compared the bacteriology of middle-ear fluids obtained by tympanocentesis from years prior to licensing of the pneumococcal conjugate vaccine to years during which most children received the vaccine. Both studies showed a significant increase in the proportion of acute otitis media caused by nontypeable *H. influenzae* in children failing initial antimicrobial therapy or with recurrent episodes coincident with widespread use of the vaccine. These findings were consistent with those of the Finnish Otitis Media Study Group, which reported an increase in otitis media due to nontypeable *H. influenzae* in vaccine recipients [20]. Another study by Revai et al. [21•] demonstrated that nasopharyngeal colonization by nontypeable *H. influenzae* and *Moraxella catarrhalis* are increasing with the widespread administration of pneumococcal conjugate vaccines. These observations highlight the increasing importance of nontypeable *H. influenzae* in otitis media and emphasize the need for continued surveillance of the bacteriology of otitis media [22,23]. Similar changes in nasopharyngeal colonization patterns are also observed in the setting of sinusitis in children and adults [2•,3].

Feasibility of Preventing Bacterial Otitis Media With Vaccines

Otitis media is a multifactorial disease. Predisposing factors include eustachian tube dysfunction, early colonization by nontypeable *H. influenzae* [24], immune dysfunction [25], and interactions between respiratory bacteria and viruses [26]. In view of these several determinants, the feasibility of preventing otitis media using a vaccination strategy is questionable.

Several lines of evidence indicate that a protective immune response can be generated to middle-ear bacterial pathogens. First, although the early experience with pneumococcal vaccines to prevent otitis media was disappointing because of lack of immunogenicity, it was clear that a good serum antibody response was followed by type-specific protection from middle-ear infection [27,28]. Therefore, if it is possible to overcome the poor immunogenicity of complex polysaccharide antigens in infants, prevention of otitis media due to *S. pneumoniae* by vaccination is a realistic goal. Second, the prophylactic administration to otitis-prone children of hyperimmune human immune globulin containing antibodies to pneumococcal polysaccharides significantly decreased the incidence of pneumococcal otitis media [29]. Third, bactericidal antibody to nontypeable *H. influenzae* was associated with protection from infection, and children developed a strain-specific protective immune response following otitis media [30]. However, the results of a recently published, randomized, prospective clinical trial provide the most compelling evidence for the feasibility of preventing otitis media caused by nontypeable *H. influenzae*, and are described in the following section [31••].

Protein D Pneumococcal Conjugate Vaccine

As reported by Prymula et al. [31••], the randomized, prospective clinical trial assessed a vaccine that contained polysaccharides from 11 different pneumococcal serotypes conjugated to protein D, a conserved surface protein of *H. influenzae*. A total of 4968 infants were randomized to receive the protein D conjugate vaccine or hepatitis A vaccine as a control. The vaccines were administered at ages 3, 4, 5, and 12 to 15 months. Children were followed for episodes of otitis media and underwent tympanocentesis and culture of middle-ear fluid for clinically documented otitis media.

The primary end point of the study was protective efficacy against the first episode of acute otitis media caused by pneumococcal vaccine serotypes. Of importance for the present discussion, the main secondary end point was protective efficacy against a first episode of otitis media caused by nontypeable *H. influenzae*. During the follow-up period, there were 44 episodes of *H. influenzae* otitis media in the protein D conjugate vaccine group compared with 68 such episodes in the hepatitis A vaccine control group, yielding an efficacy of 35.6%. This is the first clinical trial of a vaccine antigen that shows protective efficacy against *H. influenzae* otitis media and provides a proof of principle for a surface antigen inducing a protective immune response.

The immunogenicity of the protein D component of the vaccine was assessed in a subset of children in the study (149 children in the vaccine group and 148 in the control group). All children in the vaccine group subset

except one had measurable levels of antibody to protein by enzyme-linked immunosorbent assay compared with 23% of infants in the control group.

One mechanism by which the vaccine may have its protective effect is by reducing nasopharyngeal colonization. To assess the vaccine's effect on colonization by *H. influenzae*, nasopharyngeal cultures were performed on a subset of infants at age 15 to 18 months (177 in the vaccine group and 175 in the control group). There were 18 positive cultures in the vaccine group compared with 31 positives in the control group. Although these numbers reached statistical significance, a reliable conclusion regarding the vaccine's effect on colonization is not yet possible because of the small number of positive cultures and the fact that a single nasopharyngeal swab is not a reliable indicator of colonization. Additional studies are needed to determine the effect of the vaccine on nasopharyngeal colonization.

More work is needed to determine the mechanism of protection against *H. influenzae* induced by the protein D pneumococcal conjugate vaccine. A series of experiments with the chinchilla model of otitis media provided evidence suggesting that protection is antibody-mediated. Passive immunization of animals with serum from children in the trial induced protection in the model that paralleled the level of protection observed in immunized children [32•].

A more recent study further evaluated a potential protective mechanism of antibody responses to protein D. Protein D belongs to the glycerophosphodiester phosphodiesterase protein family [33•]. Toropainen et al. [34•] hypothesized that the protection induced by antibodies to protein D could be due to the inhibition of its enzyme activity. To test this hypothesis, pre- and postvaccination serum samples from infants who received the vaccine were studied in an enzyme-inhibition assay. Approximately 29% of the children who received the protein D conjugate vaccine showed a positive result after the enzyme-inhibition assay compared with no children in the control group. These results suggest that protective efficacy may be mediated by antibody inhibition of the enzyme activity of protein D. This enzyme is critical in the acquisition and incorporation of phosphorylcholine into the lipooligosaccharide of *H. influenzae* [35]. Phosphoryl choline is important for adherence and persistence of *H. influenzae* on the mucosal surface. Thus, it is intriguing to speculate that antibodies to protein D interfere with adherence by blocking the protein's enzymatic activity. It will be important to elucidate the mechanism of protection as the vaccine antigen is assessed in vaccine trials.

Additional clinical trials with protein D pneumococcal conjugate vaccines are being conducted. These results will be available in the next few years.

Approach to Vaccine Development

The observation that a surface protein (protein D) of *H. influenzae* can induce protective responses in humans has invigorated the field of vaccine development for nontypeable *H. influenzae*. A hallmark of nontypeable *H. influenzae* is the enormous genetic heterogeneity among strains. One result of this genetic heterogeneity is sequence heterogeneity of many of the major surface antigens. For example, the P2 porin protein is the most abundant protein on the bacterial surface, suggesting it may be an appealing vaccine antigen. However, the protein contains several surface loops that show sequence differences among strains, raising the possibility that P2 may not induce broadly reactive immune responses. Recent work suggests that the conserved regions of the protein may represent an effective approach [36,37]. Similarly, the lipooligosaccharide molecule is a prominent surface antigen that displays antigenic and phase variability among strains, creating obstacles to using this molecule as a vaccine antigen. However, a detoxified form of lipooligosaccharide using relatively conserved regions of the molecule has shown promise in animal models [38,39]. Therefore, one approach to developing vaccine antigens is to use antigenically conserved regions of abundant surface molecules to induce protective immune responses.

Another approach being taken by several research groups is to identify surface proteins that demonstrate sequence conservation among strains. In evaluating surface antigens as potential vaccine candidates, several characteristics should be considered. First, the antigen should express epitopes that are available for binding on the surface of the bacterium. Antibodies likely induce their protective responses by blocking adherence, directing complement-mediated killing, or opsonizing for killing; thus the antigen to which the antibodies are directed should be expressed on the bacterial surface during at least part of the bacterial growth cycle. A second characteristic of vaccine antigens is sequence conservation among strains. A successful vaccine antigen will induce a response that protects against all or most strains of the species. Ideally, the antigen is identical among strains. However, alternative approaches might involve using conserved regions of selected molecules or using a moderately conserved antigen from several selected strains in a vaccine preparation.

A third consideration for a vaccine antigen is phase variation and expression of the vaccine antigen in vivo. A putative vaccine antigen must be expressed by the bacterium during infection or colonization in the human host. Bacteria have a remarkable capability for turning off and on the expression of surface molecules under different growth conditions. A surface molecule that is expressed prominently when grown in vitro but whose expression is shut off under in vivo conditions would not be a very useful vaccine antigen.

A fourth consideration for a vaccine antigen involves the immunogenicity of the antigen. For an antigen to be effective, it must be capable of inducing an immune response in the target population. A vaccine for otitis media would need to be immunogenic in infants in the first several months of life because an episode of otitis

media during the first year of life is a risk factor for recurrent otitis media. It is not yet possible to predict which children will be otitis prone, but they are the children who will benefit most from a vaccine to prevent otitis media. Therefore, many authorities recommend universal vaccination for otitis media beginning in the first 2 months of life once effective vaccines are available [40,41].

Finally, a candidate antigen must induce a protective immune response. Identifying a correlate of protection to predict the likelihood of a putative vaccine antigen to induce a protective immune response is an important element in vaccine development. Several animal models have contributed important information in evaluating and prioritizing putative vaccine antigens for nontypeable *H. influenzae*, including a chinchilla model of otitis media, pulmonary clearance models in rats and mice, and nasopharyngeal colonization models in chinchillas and mice. In addition to animal models, serum bactericidal antibody to nontypeable *H. influenzae* was studied as a correlate of protection. Prospective studies of children have demonstrated that the presence of serum bactericidal antibody to nontypeable *H. influenzae* is strongly associated with protection against otitis media from that strain [30]. These methods are useful in characterizing vaccine antigens, but the true test of a vaccine antigen is how it performs in a well-designed clinical trial.

Candidate Vaccine Antigens

Several potential vaccine antigens in various stages of development are discussed briefly here. This is not an all-inclusive list, as other excellent vaccine antigens may also be viable candidates. In the following sections, the term "strains" refers to strains of nontypeable *H. influenzae*. Until an antigen demonstrates efficacy and safety in a clinical trial, one cannot predict with certainty whether the candidate antigen will be effective.

Haemophilus adhesin protein family (~ 155 kDa)

Haemophilus adhesin protein (Hap) is a surface adhesin that has homology with, but is separate and distinct from, IgA protease. Hap is an adhesin that is present in most or all strains, and has demonstrated protection in animal models [42].

HMW1 and HMW2 (~ 120–125 kDa)

High molecular-weight (HMW) proteins 1 and 2 are closely related adhesin proteins that are present in about 70% of strains. The proteins are homologous to the filamentous hemagglutinin, a vaccine antigen of *Bordetella pertussis*. HMWs contain conserved and heterogeneous regions [43].

H. influenzae adhesin (~ 115 kDa)

H. influenzae adhesin (Hia) autotransporter is an adhesin that promotes adherence to respiratory epithelial cells. Hia is expressed by nearly all strains that lack the HMW proteins.

D15 protein (~ 80 kDa)

The D15 protein is a target of human antibody and has been demonstrated to be protective in animal models.

HtrA (~ 46 kDa)

The HtrA heat shock protein has demonstrated protective efficacy in animal models.

P2 porin (~ 36–42 kDa)

P2 is a major surface protein comprising approximately half the protein content of the outer membrane. Although the protein contains heterogeneous surface loops, antibodies to conserved regions of the protein are bactericidal for multiple strains. In addition, mucosal immunization of mice induces antibodies that bind to surface epitopes of many strains, suggesting that conserved regions of P2 may be effective vaccine antigens [36,37].

Lipoprotein D (~ 42 kDa)

Lipoprotein D is a highly conserved lipoprotein that binds IgD and is present on the bacterial surface. Protein D, a form of the protein without the amino terminal lipid, is the carrier protein in the protein D pneumococcal conjugate vaccine discussed earlier [33•].

P5 fimbrin (~ 27–35 kDa)

P5 is an outer membrane protein A–like protein and an adhesin on the bacterial surface. A peptide corresponding to a surface-exposed loop induces protective responses in animal models [44].

Outer membrane protein P4 (~ 30 kDa)

P4 is a phosphomonoesterase that is conserved among strains. Antibodies to P4 are bactericidal for many strains and the protein induces protective responses in animal models.

Outer membrane protein 26 (~ 26 kDa)

Outer membrane protein 26 is a member of the Skp family of proteins, whose putative function is translocation of outer membrane proteins and lipooligosaccharide. This conserved protein induces protective responses in animal models [44,45].

P6 protein (~ 16 kDa)

P6 is a peptidoglycan-associated lipoprotein that is highly conserved among strains. Results from multiple animal models and indirect evidence from several clinical studies in adults and children indicate that P6 induces protective immune responses [46,47].

Protein E (~ 16 kDa)

This newly discovered surface protein binds IgD and acts as an adhesin to type 2 alveolar cells [48].

Type IV pilus (~ 14 kDa)

Type IV pilus mediates twitching motility and is involved in adherence and biofilm formation [49,50].

Lipooligosaccharide (~ 2.5–3.3 kDa)
A detoxified lipooligosaccharide vaccine induces protection in animal models. Such a vaccine will need to overcome the antigenic heterogeneity of lipooligosaccharide among strains.

Phosphoryl choline (184 Da)
Phosphoryl choline is an antigenic epitope on lipooligosaccharide of *H. influenzae* and on lipoteichoic acid of *S. pneumoniae*. The molecule is a virulence factor and immune responses to phosphoryl choline may prevent upper airway infections by both bacteria.

Conclusions
A vaccine to prevent otitis media caused by nontypeable *H. influenzae* would have significant human and economic benefit. The recent observation that a vaccine containing a conserved surface protein of *H. influenzae* yielded partial protection is a most important advance because the study provides a proof of principle of the feasibility of preventing *H. influenzae* otitis media by vaccination. Several additional vaccine antigens are in development. The successful vaccine for nontypeable *H. influenzae* infections will likely contain multiple antigens.

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