

# Protein D of *Haemophilus influenzae*: A Protective Nontypeable *H. influenzae* Antigen and a Carrier for Pneumococcal Conjugate Vaccines

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Protein D (PD) is a highly conserved 42 kDa surface lipoprotein found in all *Haemophilus influenzae*, including nontypeable (NT) *H. influenzae*. PD is involved in the pathogenesis of respiratory tract infections, in the context of which it has been shown to impair ciliary function in a human nasopharyngeal tissue culture model and to augment the capacity to cause otitis media in rats. A likely mechanism indicating that PD is a virulence factor is its glycerophosphodiesterase activity, which leads to the release of phosphorylcholine from host epithelial cells. PD has been demonstrated to be a promising vaccine candidate against experimental NT *H. influenzae* infection. Rats vaccinated with PD cleared NT *H. influenzae* better after middle ear and pulmonary bacterial challenge, and chinchillas vaccinated with PD showed significant protection against NT *H. influenzae*-dependent acute otitis media. In a clinical trial involving children, PD was used as an antigenically active carrier protein in an 11-valent pneumococcal conjugate investigational vaccine; significant protection was achieved against acute otitis media not only caused by pneumococci but also caused by NT *H. influenzae*. This may have great clinical implications, because PD is the first NT *H. influenzae* antigen that has induced protective responses in humans.

*Haemophilus influenzae* is an important human-specific pathogen colonizing the mucosa of the upper respiratory tract. *H. influenzae* isolates can be divided into 2 major groups: the encapsulated strains, many of which cause invasive diseases, and the nonencapsulated, nontypeable (NT) strains, which are responsible for the majority of mucosal *H. influenzae* infections. Encapsulated forms can penetrate the epithelium of the nasopharynx and invade blood capillaries, with their polysaccharide capsule allowing them to resist phagocytosis and complement-mediated lysis in nonimmune hosts. Six serotypes (a–f) have been identified; *H. influenzae* serotype b (Hib) is the leading cause of invasive infec-

tions in children. This was the case until the implementation of widespread vaccination programs, initially with capsular polysaccharide vaccines and, recently, with conjugate vaccines to overcome the limitation that polysaccharide vaccines are poorly immunogenic in children aged <2 years. In regions of the world where a conjugate vaccine is not introduced, sepsis, meningitis, and pneumonia caused by Hib are still a major burden of disease.

NT *H. influenzae* strains are rarely associated with invasive disease in healthy children and adults, but they are associated with respiratory tract infections in both populations. These strains are frequently (up to 40% of the time) the cause of acute otitis media (AOM) in children. In addition, NT *H. influenzae* strains are also commonly isolated in purulent secretions of patients with cystic fibrosis and chronic obstructive pulmonary disease. The burden of disease caused by NT *H. influenzae* justifies immune prophylaxis preferentially by active vaccination. A number of NT *H. influenzae* proteins have been evaluated in preclinical studies for their potential as vaccine antigen. Identifying ideal NT

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*H. influenzae* vaccine candidates has not been easy, because NT *H. influenzae* demonstrates extensive sequence and antigenic variation among gene products interacting with the immune system, such as outer membrane proteins, adhesins, lipopolysaccharides, and secreted virulence factors [1–4].

Protein D (PD), a surface lipoprotein highly conserved among capsulated and noncapsulated strains of *H. influenzae*, has shown protection against *H. influenzae* AOM in a chinchilla model [5]. PD also has the potential to protect children against *H. influenzae*, as was shown in a randomized, double-blind efficacy study in which PD was conjugated with pneumococcal capsular polysaccharides [6]. This study confirmed that using *H. influenzae*-derived PD as a carrier protein for pneumococcal polysaccharides allowed protection against not only pneumococcal otitis but also AOM due to NT *H. influenzae*. Our article reviews the properties of PD and its potential applications as an antigenically active carrier protein for conjugate vaccines.

### PD IS AN OUTER MEMBRANE PROTEIN THAT IS ANTIGENICALLY CONSERVED AND EXPRESSED BY ALL *H. INFLUENZAE* STRAINS

PD was originally defined as a surface-exposed outer membrane protein of *H. influenzae* and was detected by its ability to bind to human IgD myeloma protein 4490 [7]. It was later shown by Sasaki and Munson [8] that PD did not bind IgD but rather was a target of the unique IgD myeloma protein.

PD, with an apparent molecular weight of 42 kDa, was found in all 127 *H. influenzae* strains, including encapsulated serotypes a–f and NT *H. influenzae* strains, which were analyzed by Western blot using 3 different mouse anti-PD monoclonal antibodies and the human IgD myeloma 4490 [9]. The number of PD molecules was estimated to be 2800 molecules per bacterial cell. Thus, these studies suggested that PD is antigenically conserved, surface located, and present in most (if not all) *H. influenzae* strains, making it an attractive vaccine candidate.

### LIMITED ANTIGENIC DRIFT IN THE GENE ENCODING PD

DNA sequence analysis of the region encoding PD revealed an open reading frame of 1092 bp encoding a putative protein of 346 amino acids with a calculated molecular weight of 41,821 Dalton [10]. A very limited diversity of PD among 14 *H. influenzae* strains was found (table 1) [11–16]. The sequences were >97% identical on both the nucleotide and deduced amino acid levels, and the substitutions were relatively evenly distributed across the gene. Moreover, there seems to be limited drift in the PD gene, as shown by sequencing of the PD gene of *H. influenzae* isolated in the lung specimens from patients with persisting bronchitis [14].

**Table 1. Diversity of protein D in *Haemophilus influenzae* strains.**

Strain	Serotype	Accession number <sup>a</sup>	Identity with consensus sequence of protein D, %	
			DNA	Protein
772	Nontypeable	M37487	99.7	100
86-028NP	Nontypeable	CP000057	99.6	100
3639	Nontypeable	Z35656	99.2	99.7
3640	Nontypeable	Z35657	99.5	99.7
6-7626	Nontypeable	Z35658	98.1	98.1
A850079	Nontypeable	X90495	99.7	100
A850053	Nontypeable	X90493	98.9	99.1
A850047	Nontypeable	X90489	98.9	99.1
A850048	Nontypeable	X90491	97.2	98.2
MinnA	b	L15200	99.6	99.4
HK695	b	Z35660	99.6	99.4
Eagan	b	Z35659	99.6	99.4
NCTC8468	b	Z35661	97.5	98.1
Rd KW20	d	L42023	99.1	98.9

**NOTE.** From [11–16].

<sup>a</sup> Accession numbers are from GenBank.

### PD IS A VIRULENCE FACTOR INVOLVED IN THE PATHOGENESIS OF RESPIRATORY TRACT INFECTIONS

The surface localization and lack of antigenic drift made it highly interesting to investigate whether PD was involved in the virulence of *H. influenzae*. An isogenic mutant lacking the ability to express PD was constructed [17]. No phenotypic differences in lipopolysaccharide expression, protein profiles (apart from PD), or growth rates between the PD mutant and the NT *H. influenzae* wild-type strain were found. However, ~100 times more colony-forming units of the mutant were required to cause infection in the middle ears of rats in an otitis model. Viable NT *H. influenzae* were isolated from the middle ears of rats with or without diagnosed AOM 4 days after challenge at similar frequencies for both wild-type strain 772 and the PD-deficient mutant (A.F. and H.J., unpublished data). These results indicated that PD was not essential for survival of *H. influenzae* in the middle ear of rats, but it enhanced the severity of the disease.

The PD-deficient NT *H. influenzae* strain was also compared with its isogenic wild-type strain with regard to its ability to cause damage in a human nasopharyngeal tissue culture model. Culture specimens inoculated with *H. influenzae* showed a decrease in ciliary activity beginning after 12 h. The impairment of ciliary function caused by the PD-expressing strain was significantly greater than that caused by the PD-negative mutant ( $P < .01$ ) [18]. After 48 h of incubation, the PD-expressing strain caused a significant loss of cilia. No difference in survival in the culture medium or in the adhesion to adenoid tissue

was observed between PD-producing or nonproducing NT *H. influenzae*. These results suggested that PD is involved in the pathogenesis of upper respiratory tract infections due to NT *H. influenzae*, probably by enhancing functional and morphological damage to ciliated epithelial cells. It is becoming increasingly accepted that NT *H. influenzae*, which is usually regarded as a noninvasive pathogen, can enter nonciliated epithelial cells [19, 20]. NT *H. influenzae* has been shown to be diffusely present in the respiratory epithelium and subepithelial layers of the lungs of patients with chronic obstructive pulmonary disease or cystic fibrosis [21]. This was also true in the human nasopharyngeal tissue culture model, in which NT *H. influenzae* adhered near the junctions between nonciliated cells and, subsequently, localized intra- and intercellularly [18]. Viable intracellular NT *H. influenzae* has been recovered from epithelial cells and macrophages from the adenoids of children undergoing adenoidectomy because of nasal obstruction [22], and some strains can resist macrophage-mediated killing [23], indicating that NT *H. influenzae* probably avoids the immune system. Therefore, it was interesting to observe that PD expression was shown to promote the adherence and internalization of NT *H. influenzae* into human monocytic cells [24].

### **PD IS A LIPOPROTEIN AND A GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE (GLPQ)**

When sequencing the PD gene from the Hib strain MinnA [12], it was discovered that PD was 67% identical to the gene encoding GlpQ of *Escherichia coli* that catalyzes the hydrolysis of glycerophosphodiester [25, 26]. Indeed, PD had enzymatic activity similar to that of GlpQ of *E. coli* [11, 12]. In *E. coli* and most other bacterial species, the glycerophosphodiesterase encoded by *glpQ* is periplasmic [25, 26], whereas in *H. influenzae*, it is membrane bound. This membrane localization of PD could be explained by fatty acids linked to a cysteine residue in a consensus sequence for bacterial lipoproteins [27, 28]. A mutated form of PD that lacked the cysteine residue could not be acylated and became hydrophilic. The nonacylated form of PD was shown to be secreted into the periplasmic space of *E. coli*. These results strongly indicate that the acylation of PD determines its hydrophobicity and that PD is an outer membrane-anchored lipoprotein [27].

### **CHOLINE ACQUISITION CONTRIBUTES TO *H. INFLUENZAE* VIRULENCE**

Choline acquisition is important for *H. influenzae*, because choline can be incorporated into its lipooligosaccharides as phosphorylcholine. Glycerophosphorylcholine, which is an abundant degradation product of eukaryotic membrane-associated phospholipids, can be hydrolyzed into glycerol 3-phosphate and choline by PD because of its GlpQ activity. By this mechanism,

PD facilitates the acquisition of choline directly from host epithelial cells [29]. The phosphorylcholine-decorated lipooligosaccharide serves as a ligand for the platelet-activating factor receptor of bronchial epithelial cells. More-recent data demonstrate that the binding of the platelet-activating factor receptor by NT *H. influenzae* initiates receptor coupling to a pertussis toxin-sensitive heterotrimeric G protein complex, resulting in a multifactorial host cell signal cascade and bacterial invasion [30, 31]. Phosphorylcholine also promoted the establishment of stable biofilm communities of NT *H. influenzae* in a chinchilla model of AOM [32]. Moreover, phosphorylcholine is a well-known constituent of teichoic acid in *Streptococcus pneumoniae* and is a critical determinant of the inflammatory activity of this particular species [33].

### **PD IS PROTECTIVE IN ANIMAL MODELS**

Initial experiments with rats revealed that vaccination with PD induced high levels of serum IgG and IgA antibodies and significant bactericidal activity against homologous and heterologous strains [34]. A rat middle ear clearance model developed by Kyd et al. [35] was used to evaluate the potential of PD as a protective antigen against NT *H. influenzae*. Rats were vaccinated on day 0 via intestinal Peyer's patches, followed by an intratracheal boost on day 14. An intrabullar challenge with live NT *H. influenzae* was performed on day 21, and bacteria were counted after 4 h. Rats vaccinated with PD tended to clear NT *H. influenzae* more efficiently, compared with rats that received aluminum orthophosphate ( $P = .083$ ).

The protective effect of PD was also studied in a rat pulmonary clearance model. Rats were vaccinated in the middle ear clearance model, as described above. Pulmonary challenge was performed on day 21 after intestinal vaccination, and 4 h after challenge, bacteria were counted in bronchoalveolar lavage samples and lung homogenates. The clearance of NT *H. influenzae* observed in the group that was vaccinated with PD was highly significant, compared with that in the nonimmunized group ( $P < .001$ ). Differences of  $>1$  log NT *H. influenzae* colony forming units recovered 4 h after challenge were observed either in bronchoalveolar lavage samples or in lung homogenates [35].

A pronounced activity of PD antibodies was shown in the chinchilla AOM model [5, 36], a highly reproducible viral-bacterial superinfection animal model. A dual infection step was developed to mimic a human NT *H. influenzae* infection, in which *H. influenzae* is an opportunistic pathogen. Juvenile chinchillas were first challenged intranasally with adenovirus (serotype 1) and, subsequently, with NT *H. influenzae* to coincide with maximal viral damage of the eustachian tube mucosa. AOM began to develop 7 days after NT *H. influenzae* challenge. The AOM induced in chinchillas according to this

model shows many similarities with the polymicrobial nature and natural disease in children.

To evaluate the protective capacity of anti-PD antibodies, chinchillas were injected intracardially with chinchilla anti-PD serum 1 day before challenge with NT *H. influenzae*. Compared with sham vaccination (placebo) of animals, delivery of anti-PD serum by passive transfer reduced the incidence of middle ear effusion ( $P \leq .001$ ) [37]. In another viral-bacterial coinfection study, passive transfer of a pediatric human serum pool, generated against an 11-valent investigational vaccine composed of pneumococcal capsular polysaccharide conjugated to PD, conferred ~34% ( $P \leq .001$ ) protection against development of ascending NT *H. influenzae*-induced AOM [36].

## PD IS IMMUNOGENIC IN HUMANS

Human serum antibodies to nonlipidated PD were measured by ELISA. The mean IgG level was slightly above the detection limit in infants aged <6 months, was decreased to almost undetectable levels in infants aged 6 months to 1 year, and increased steadily with age in children up to 5 years of age. After a plateau in the 5–10-year age group, the IgG level increased steadily with age in persons up to 20 years of age and slowly decreased with age in the subsequent age groups. Children aged 6–12 months had a significantly higher mean anti-PD IgM antibody concentration than did infants aged <6 months. Slightly increased IgM antibody levels were found in the remaining age groups. The IgA antibody concentrations were at background levels in infants aged <1 year. The levels were slightly higher in the 1–10-year age group and increased with age throughout life [34].

## PD: AN ANTIGENIC CARRIER PROTEIN IN A CONJUGATED PNEUMOCOCCAL VACCINE

The 2 leading bacterial pathogens causing AOM are *S. pneumoniae* and NT *H. influenzae*. Both are also recognized as a major cause of lower respiratory tract infections. Vaccines containing plain capsular polysaccharides of *S. pneumoniae* have been used for decades, but they show poor immunogenicity in children. However, a vaccine (Prevnar; Wyeth) containing polysaccharides from 7 serotypes—each conjugated to CRM197, a nontoxic mutant of diphtheria toxin that is an immunogenically inactive carrier—has shown high efficacy in young children against invasive pneumococcal disease and intermediate efficacy against AOM caused by vaccine pneumococcal serotypes [38]. An intrinsic disadvantage of the CRM197-based vaccine is that the same carrier, namely CRM197, is used in Hib and meningococcal conjugate vaccines and, therefore, might have a negative impact on the immunogenicity of the other conjugate vaccines injected concomitantly and on the pneumococcal conjugate vaccine itself [39]. For those reasons and because of its properties, such as surface localization, high

degree of conservation, wide distribution, and pathogenicity, as well as the promising preclinical results with PD, it was decided to use PD in a nonacylated form as antigenically active carrier protein in a new 11-valent pneumococcal conjugate vaccine. Thus, PD is expected to function as a carrier protein with antigenic potential, resulting in dual protection against *S. pneumoniae* and NT *H. influenzae*.

The immunogenicity and safety of the new investigational 11-valent pneumococcal vaccine with PD as a carrier was tested in a randomized, single-blind, controlled phase II study involving 154 healthy Finnish infants [40]. Vaccine was given at 2, 4, 6, and 12–15 months of age. Pneumococcal antibody concentrations after receipt of the first 3 doses varied from 1.26 to 4.92  $\mu\text{g/mL}$ , depending on the serotype and study group. It was concluded that the PD conjugate pneumococcal vaccine was immunogenic and safe in infants.

In a randomized, double-blind efficacy study, Prymula et al. [6] assessed the efficacy of the investigational pneumococcal polysaccharide vaccine in preventing AOM caused by both *S. pneumoniae* and NT *H. influenzae*. The primary end point was protective efficacy against the first episode of AOM caused by vaccine serotypes. Analysis showed that the PD conjugate vaccine provided a significant reduction (33.6%) in the overall incidence of AOM. Vaccine efficacy against AOM caused by pneumococcal vaccine serotypes and NT *H. influenzae* was 52.6%–57.6% and 35.3%, respectively. The vaccine also reduced the frequency of infection due to all vaccine-related cross-reactive pneumococcal serotypes (including 6A and 19A) by 65.5%. No increase in the incidence of AOM caused by pneumococcal nonvaccine serotypes or other bacterial pathogens was recorded over the study period. The PD-based vaccine also appeared to reduce the rate of nasopharyngeal carriage of both pneumococci and NT *H. influenzae*, but the number of samples was too small to draw any definite conclusion regarding whether the vaccine had this effect on colonization. After receipt of the fourth dose of vaccine, vaccine-serotype pneumococci were isolated in specimens from 6% of the infants in the PD conjugate group and from 11% of control subjects, and *H. influenzae* was isolated in specimens from 10% of the infants in the PD conjugate group and from 18% of infants in the control group. This is an important finding, because the success of current conjugate vaccination programs against Hib, pneumococci, and meningococci is, in part, attributable to the lowered rate of transmission of the bacteria.

The overall efficacy of vaccination against AOM episodes caused by pneumococcal vaccine serotypes was remarkably similar for the 11-valent PD conjugate vaccine and the 7-valent conjugate vaccines in a Finnish trial, in which there was a 56%–57% decrease in the incidence of episodes linked to the vaccine strains [41, 42]. However, after vaccination with the 7-valent CRM197 conjugate vaccine, an increase in the incidence of

episodes of AOM due to nonvaccine pneumococcal strains or other pathogens was recorded [42, 43]. There was no evidence of such replacement phenomenon in the patient population included in the 11-valent pneumococcal PD conjugate study, but this may have been because of the relatively short observation period for this vaccine [6].

## DISCUSSION

Vaccines based on plain capsular polysaccharides of *S. pneumoniae* have been beneficial mostly in adults for decades. In 2000, a vaccine containing polysaccharides from 7 serotypes conjugated to a nontoxic mutant of diphtheria toxin was introduced and was shown to be immunogenic in children. This review reports encouraging data supporting the use of *H. influenzae*-derived PD as a carrier protein for pneumococcal conjugates to provide protection against pneumococcal- and NT *H. influenzae*-induced AOM.

PD was antigenically and genetically conserved despite its localization on the surface of all *H. influenzae* strains studied. It has been shown to be a crucial virulence factor in animal models [17, 18]. The limited antigenic drift of PD during persistent infection [14] may be linked to its important function in choline acquisition from epithelial cells through its glycerophosphodiesterase activity. Choline is subsequently incorporated into lipooligosaccharide as phosphorylcholine, allowing NT *H. influenzae* to become more adherent and invasive. These findings, together with the observation that the number of anti-PD antibodies increases from childhood through early adulthood, support the hypothesis that PD may be a good candidate to initiate an immune response in vaccine against all *H. influenzae* strains.

In the chinchilla otitis model that closely mimics human AOM infection, PD was found to induce significant protection both after active vaccination with PD and after passive transfer of a pediatric serum pool generated against an 11-valent vaccine composed of pneumococcal polysaccharides conjugated to PD [5, 36]. In a large pediatric trial, the same 11-valent pneumococcal polysaccharide vaccine conjugated to PD provided a 35.3% reduction in the prevalence of AOM caused by NT *H. influenzae* [6]. This finding is definitively clinically significant, because a Finnish trial registered an increase in the number of episodes of AOM caused by NT *H. influenzae* after vaccination with 2 different 7-valent pneumococcal conjugate vaccines not containing PD [42]. Considering the postlicensure experience with a pneumococcal conjugate vaccine using CRM197 as carrier protein in the United States, where NT *H. influenzae* has become the predominant cause of recurrent or refractory AOM due mostly to  $\beta$ -lactamase-producing strains [43], the additional protection provided by a PD conjugate vaccine candidate against NT *H. influenzae* will most likely have a major health impact in the near future.

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