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Abrogation of nontypeable *Haemophilus influenzae* Protein D function reduces phosphorylcholine decoration, adherence to airway epithelial cells, and fitness in a chinchilla model of otitis media

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ABSTRACT

The pneumococcal polysaccharide conjugate vaccine which includes a nonacylated protein D carrier from *Haemophilus influenzae* has been recently licensed for use in many countries. While this vaccine is protective against nontypeable *Haemophilus influenzae* (NTHI)-induced acute otitis media (OM), the mechanism underlying this protective efficacy is not yet fully understood. Protein D/glycerophosphodiester phosphodiesterase (PD/GlpQ) is an outer membrane lipoprotein expressed by NTHI that has been ascribed several functions, including host cell adherence and phosphorylcholine (PCho) acquisition. We found that a *pd/glpQ* NTHI mutant exhibited reduced adherence to airway epithelial cells, diminished phosphorylcholine (PCho) decoration of biofilms, and compromised fitness during experimental acute OM compared to the parent strain. We also found that exposure of NTHI to antibodies directed against the vaccine formulation recapitulated the PCho decoration and NTHI adherence phenotypes exhibited by PD/GlpQ-deficient NTHI, providing at least two likely mechanisms by which the pneumococcal polysaccharide-PD/GlpQ conjugate vaccine induces protection from NTHI-induced OM.

1. Introduction

The bacterium nontypeable *Haemophilus influenzae* (NTHI) is a commensal of the human nasopharynx, as well as an opportunistic pathogen associated with infections of both the upper and lower airway. Along with *Streptococcus pneumoniae* and *Moraxella catarrhalis*, NTHI causes otitis media (OM) via ascension of the Eustachian tube and invasion of the normally sterile middle ear space [1]. NTHI is frequently associated with both acute and chronic OM, which can lead to hearing loss and consequent developmental delays [2]. Unlike bacterial species with well-characterized virulence factors, the pathogenicity of NTHI is mediated largely by its ability to colonize host epithelia and to evade the immune system. Specifically, dynamic expression of multiple adhesins, as well as sialylated and phosphorylcholine- (PCho-) decorated lipooligosaccharide (LOS) glycoforms in the NTHI outer membrane allow these bacteria to maintain long-term colonization of the nasopharynx and, during compromising conditions, infect the middle ear space [3–5].

In addition to NTHI, *Streptococcus pneumoniae* is a major causative agent of OM. Recent studies have reported middle ear fluid culture data that demonstrate 40–80% of bacterial isolates from OM patients are either *S. pneumoniae* or NTHI (for examples, see [6–9]). While vaccination with the 7-valent pneumococcal polysaccharide conjugate vaccine (Prevnar™/PCV7; Wyeth, New York, New York) has significantly reduced the occurrence of invasive pneumococcal disease, protection against OM has been modest in comparison (for example, see [10]). The pneumococcal conjugate vaccine recently licensed in Europe (Synflorix™/PHiD-CV; GlaxoSmithKline Biologicals, Rixensart, Belgium) is a 10-valent vaccine that employs a non-lipidated form of NTHI Protein D (PD) as the protein carrier component. In the pediatric pneumococcal otitis efficacy trial (POET) performed in the Czech Republic and Slovakia, PHiD-CV demonstrated 57% efficacy against pneumococcal OM caused by vaccine serotypes, and importantly, 35% efficacy against NTHI-induced OM [11]. These studies indicate that the ability of PHiD-CV to target two causative agents of OM holds the potential to provide more effective prevention of this ubiquitous pediatric disease.

Although the immunogenicity of NTHI PD as a polysaccharide carrier protein elicits PD-specific serum antibodies and affords pro-

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Table 1
Oligonucleotide primers used in this study.

Primer	Nucleotide sequence (5′→3′)	Amplicon
1	gaagcgcaggcgaaagatgt	*hpd/glpQ* genomic clone
2	gcctccttatttgaattctagg	*hpd/glpQ* genomic clone
3	ctaaaaactctataaaaatttaccgcactcttaaggagaaaatacttaatgattccggggatccgtcgacc	cassette with *hpd/glpQ*-homologous ends
4	ggtatgtttacccacggttgtgagggatattatttttattccttttaagaatgtaggctggagctgcttcg	cassette with *hpd/glpQ*-homologous ends
5	ggcgtcgaccccgattcccaaaatgtaag	*glpTQ* promoter
6	ggcggatccaatttttttctccaacttttta	*glpTQ* promoter
7	cacaaagcgaaattaggcgatact	*glpT* RT
8	aagtatttttctccttaagagtgcg	*glpT* RT
9	atggtaaagatattgctgctgaaa	*hpd/glpQ* RT
10	gggtctttttcttgtgtttctttc	*hpd/glpQ* RT

tection against NTHI-induced OM, the mechanisms underlying this protection are not well understood. PD exhibits sequence homology to the *E. coli* glycerophosphodiester phosphodiesterase GlpQ, and demonstrates similar enzymatic activity [12]. Recombinant PD/GlpQ can catalyze the cleavage of L-α-glycerophosphocholine (GPC) into choline and *sn*-glycerol-3-phosphate (G3P), an enzymatic function which is inhibited by incubation of PD/GlpQ with anti-PD/GlpQ antibodies [13]. However, the effects of antibody-mediated inhibition of PD/GlpQ activity on NTHI biology and pathogenesis, which ultimately leads to protection against NTHI-induced OM, have yet to be fully determined.

Various biological functions for NTHI PD/GlpQ have been observed, including promotion of NTHI adherence and internalization into host monocytes [14], acquisition of host-derived phosphorylcholine (PCho) for incorporation into NTHI LOS [15], and interference with host epithelial cell ciliary beating [16]. Furthermore, PD/GlpQ was found to be an important virulence determinant in a rat model of experimental NTHI-induced OM [17]. Here, we confirmed the conserved and stable expression of PD/GlpQ by NTHI. We have presented data demonstrating the role of PD/GlpQ in NTHI adherence and PCho decoration of LOS. Moreover, we showed that anti-PD/GlpQ antibodies attenuated these functions *in vitro*, and examined the impact of a PD/GlpQ deficiency on NTHI fitness in a chinchilla model of experimental OM. Together, our experiments have demonstrated mechanisms by which vaccine-directed antibodies likely protected from NTHI-induced OM, as has been observed in both experimental animal models and a pediatric clinical trial.

2. Experimental procedures

2.1. Construction and culture of bacterial strains

Unless otherwise indicated, NTHI was grown on chocolate agar or in brain heart infusion broth supplemented with NAD and hemin, each at a final concentration of 2 μg supplement/ml. Media were additionally supplemented with 20 μg kanamycin/ml, 200 μg spectinomycin/ml, 1 mg streptomycin/ml, and/or 15 μg ampicillin/ml when appropriate. The minimally passaged NTHI isolate strain 86-028NP, recovered from the nasopharynx of a child with chronic otitis media, was used for all functional analyses [18,19]. For luminescence experiments, we used the 86-028NP/pKMLN-03 (p*glpTQ*—lux) strain described herein in conjunction with strains 86-028NP/pKMLN-02 (p*pilA*—*lux*; [20], 86-028NP/pKMLN-1 (p*ompP2*—*lux*; [21], and 1885MEE/pKMLN-05 (p*ompP5*—*lux*; L.O. Bakaletz, unpublished).

To evaluate the role of PD/GlpQ in a well-characterized NTHI strain, a mutant with a nonpolar deletion of the *hpd/glpQ* gene was constructed in strain 86-028NP using a recombineering approach [22]. Briefly, the coding region of *hpd/glpQ* (plus an additional ∼870 kb upstream and ∼130 bp downstream) was amplified from 86-028NP genomic DNA by PCR using primers 1 and 2 (Table 1). This fragment was blunt-end cloned into pSMART LCKan (Lucigen, Middleton, WI) and transformed by electroporation into *E. coli* DH10B Electromax cells (Invitrogen, Carlsbad, CA). The resultant vector pRJ2 was then transformed into *E. coli* DY380. Primers 3 and 4, which contained 50 bp ends homologous to sequences that flank the *hpd/glpQ* coding region were used to amplify the region of pRSM2832 which encodes an *rpsL*; spectinomycin resistance (spec^R) cassette (Table 1). The PCR product was transformed into DY380/pRJ2, recombination was induced by temperature shift to 37 °C, and recombinants with cassette insertions were selected. A vector in which *hpd/glpQ* was interrupted by the *rpsL*; spec^R cassette, pRJ4, was isolated, verified by restriction digest, linearized using PvuI, and transformed by the MIV method [23] into an *rpsL* mutant of 86-028NP. Chromosomal recombinants were selected and transformed with the FLP recombinase-encoding vector pRSM2947. Removal of the *rpsL*; spec^R cassette from the endogenous *hpd/glpQ* locus was achieved by induction of recombinase expression with 200 ng tetracycline/ml for 2 h, followed by static incubation of cells overnight at 37 °C, 5% CO_2. The NTHI mutant that was isolated and utilized in subsequent studies contained a non-polar deletion of *hpd/glpQ* and a non-functional *rpsL* gene. The wild-type NTHI 86-028NP strain and the respective single *rpsL* mutant do not exhibit detectable phenotypic differences in *in vitro* growth or in relative ability to survive in the chinchilla middle ear (Mason et al., unpublished) and therefore the wild-type NTHI strain 86-028NP that carried a functional *rpsL* gene was utilized as the positive control in all experiments.

An 86-028NP-derived NTHI strain that expresses an *hpd/glpQ* promoter-driven luciferase reporter was generated from the previously reported *P. luminescens* luciferase-encoding vector pKMLN-01 [24]. After verification that *hpd/glpQ* was expressed in an operon with *glpT*, primers 5 and 6 were used to amplify the proximal ∼290 bp of sequence upstream of the *glpT* coding region (*i.e. glpTQ* operon promoter; Table 1), which contained all predicted regulatory sequences required for gene expression. The amplicon was cloned into pKMLN-01 as a BamHI to SalI fragment, replacing the *sap* promoter, then transformed into 86-028NP by standard electroporation methods [25,26]. The reporter plasmid pKMLN-03 was verified by restriction digest and observation of luminescent bacteria.

2.2. Growth assays

For standard growth curve analysis, 86-028NP parent and *hpd/glpQ* mutant strains were suspended in sBHI to an optical density (OD) at 490 nm of 0.65, diluted 1:6 into sBHI, and incubated statically at 37 °C, 5% CO_2. Growth was monitored every 30 min for changes in absorbance at an $OD_{600\,nm}$ over an 8 h period. For supplementation experiments, NTHI were suspended in 0.9% NaCl to the $OD_{490\,nm}$ as above, then diluted 1:20 into a chemically defined medium [27] with or without 10 mM L-α-glycerophosphocholine