RESEARCH ARTICLE

Bactericidal antibody response against P6, protein D, and OMP26 of nontypeable Haemophilus influenzae after acute otitis media in otitis-prone children

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Abstract

The bactericidal antibody response to three nontypeable Haemophilus influenzae (NTHi) outer membrane proteins (D, P6, and OMP26) was studied in 24 otitis-prone children (aged 7–28 months) after an acute otitis media (AOM) caused by NTHi. The study was carried out to understand the contribution of antigen-specific bactericidal antibody responses in the class of children who are most vulnerable to recurrent otitis media infections. Levels of protein D (P = 0.005) and P6 (P = 0.026) but not OMP26 antibodies were higher in bactericidal sera compared with nonbactericidal sera. For five (24%) and 16 (76%) of 21 bactericidal sera tested, removal of anti-protein D and P6 antibody, respectively, resulted in a two- to fourfold drop in bactericidal antibody. Antibodies to OMP26 did not make any contribution to the overall bactericidal activity in any serum samples. Eleven of 21 sera (52%) had bactericidal activity against a heterologous NTHi (86-028 NP) strain but the titers were significantly lower (P < 0.05) as compared to the homologous strains. Future studies of protein D, P6, OMP26, and other potential NTHi vaccine antigens should include studies of bactericidal antibody in children who are otitis prone as a possible correlate of protection.

Introduction

Acute otitis media (AOM) is a frequent disease of childhood, affecting millions worldwide each year (Quach et al., 2004). It is a leading cause of physician visits and a major contributor to healthcare costs and antibiotic use (Grevers, 2010). Nontypeable Haemophilus influenzae (NTHi) is one of the main causes of middle ear infections and especially in recurrent infections (Harabuchi et al., 1994). The carriage of NTHi is mostly asymptomatic; only when the condition of the host is altered, NTHi may invade the middle ear, causing AOM. Prevention of NTHi infections is important because otitis media (OM) is a major cause of conductive hearing impairment in children who are at the critical age for speech and language development (Giebink, 1989). Repeated or inappropriate antibiotic treatment for OM contributes to the emergence of antibiotic-resistant strains (Hou & Gu, 2003). Therefore, there is an urgent need to develop prophylactic vaccines to prevent these NTHi infections. A challenge to developing a vaccine for NTHi is the antigenic heterogeneity of several major surface antigens and genetic heterogeneity among strains. Several research groups have identified conserved surface proteins and tested them as putative vaccines (Akkoyunlu et al., 1991; Prymula et al., 2006; De Maria et al., 1996; Kyd et al., 2003).

There is evidence that bactericidal antibody provides protection from AOM caused by NTHi (De Maria et al., 1996; Yamanaka & Faden, 1992). Understanding the contributions of bactericidal antibody specific to potential vaccine antigens will help in the design of a novel vaccine, which could protect against NTHi infections. Protein D, P6, and OMP26 are three conserved outer membrane proteins of NTHi currently being considered as vaccine candidates against infections caused by NTHi. Protein D has demonstrated partial efficacy in preventing H. influenzae otitis media (Forsgreen et al., 2008) and this important result has provided proof of principle for developing a vaccine to prevent AOM caused by NTHi. P6 is antigenically conserved among both nontypeable
and type b *H. influenzae* strains and has been shown to elicit bactericidal antibodies (De Maria *et al.*, 1996; Murphy *et al.*, 1986) and is considered a vaccine candidate against NTHi infections. OMP26 has been evaluated for its potential as a vaccine candidate as it has been shown to enhance pulmonary clearance of NTHi in a rat model (Adhami *et al.*, 1999; Kyd & Cripps, 1998).

The aim of this study was to investigate the antigen-specific bactericidal antibody response, to protein D, P6, and OMP26 in otitis-prone children, defined as children who have experienced three episodes of AOM within 6 months or four episodes in 12 months. These are the most vulnerable children to recurrent AOM and most likely to benefit from vaccination. To accomplish the aim, specific antibodies were depleted from bactericidal sera and the contribution of antibody to each vaccine candidate protein in a particular serum sample was established.

**Materials and methods**

**Study design**

The patients constituted a consecutively studied series of 24 children; aged 7 months to 28 months (mean 16 ± 6 months) who were otitis prone. Otitis-prone children were defined as a subpopulation of children previously described to represent about 30% of all children who experienced AOM (Poehling *et al.*, 2007) and who have three or more episodes of AOM within 6 months or four infections within a year (Subcommittee on Management of Acute Otitis Media, 2004). From these 24 children, we had 17 acute serum samples collected at the time of diagnosis of AOM caused by NTHi and 20 serum samples from their convalescent stage. In total, 37 serum samples from 24 children used in the study, we had 10 paired (acute and convalescent) sera from children and the rest were either acute or convalescent. The window of collection of convalescent sera was 3–4 weeks post AOM. This time span was considered sufficient for the child to mount an antibody response. Informed consent was obtained for all study procedures as approved by the University of Rochester and Rochester General Hospital investigational review boards.

**Tympanocentesis**

All diagnoses of AOM for the defining event that caused the child to meet the definition of otitis prone was confirmed by tympanocentesis. Middle ear fluid (MEF) for culture was obtained by puncture of the inferior portion of an intact tympanic membrane (TM) with a 20-gauge spinal needle attached to a 3-mL syringe using a hand-held operating otoscope. If a small sample of MEF was obtained on aspiration, 0.5 mL of trypticase soy broth was aspirated through the spinal needle and then aliquoted and inoculated onto agar plates and into broth, as described in the following sections. To accomplish the aspiration, the sterile needle used in the procedure was punctured into a sterile blood culture bottle containing trypticase soy broth and 0.5 mL of broth drawn up into the needle and syringe in order to obtain the full MEF sample.

**Bacteriology**

MEF was cultured on chocolate agar plates and inoculated in brain heart infusion (BHI) with 15% glycerol and preserved at −80 °C. The NTHi strains were identified by standard laboratory procedures. An isolate was identified as NTHi based on colony morphology, porphyrin reactivity, and growth requirement for hemin and nicotinamide adenine dinucleotide using Haemophilus ID Quad plates (McCrea *et al.*, 2008). We have performed multilocus sequence typing to characterize the NTHi strains used for the study.

**Purification of P6, protein D and OMP26**

Recombinant protein D was obtained from GlaxoSmithKline (GSK, Rixensart, Belgium). The P6 plasmid was obtained from Tim Murphy, University of Buffalo and the OMP26 plasmid was obtained from Jennelle Kyd, University of Canberra, Australia. P6 and OMP26 were expressed in *E. coli* BL21 (DE3). P6 was expressed predominantly as inclusion bodies and purified under denaturing conditions. OMP26 was expressed in soluble fraction and purified under native conditions. P6 was expressed and purified using a modified published procedure (Bhushan *et al.*, 1997; Hotomi *et al.*, 2002). The purity of purified recombinant protein was assessed by SDS-PAGE.

**Bactericidal assay**

Thirty-seven samples were screened for bactericidal activity against the homologous NTHi strain isolated from MEF of that child. Twenty-one of the 30 serum samples were tested for bactericidal activity against a heterologous strain (86–028 NP provided as a gift from Lauren Baken, Ohio State University, Columbus, OH). The sera were heat inactivated at 56 °C for 30 min to inactivate human complement. The bactericidal assay was performed as published literature with some modification (Sabirov *et al.*, 2009; Neary & Murphy, 2006). Colonies of NTHi were inoculated in BHI broth supplemented with NAD and Hemin and grown to an optical density (OD) of 0.8 at 490 nm (log phase). The colony-forming unit (CFU) count was established at that particular OD. Homologous and heterologous NTHi strains were
cultivated, harvested, and diluted to $10^7$ CFU mL$^{-1}$ in PBS containing potassium, magnesium and calcium salts (PCMA). In 96-well plates, 50 µL of a serum sample was added, then serially diluted twofold 11 times leaving the last well without serum, which served as a control. Ten microliters of NTHi (10^5 CFU) and 500 µL of precoelostal calf serum were mixed as a source of complement followed by PCMA buffer to create a total volume of 2.5 mL. Fifty microliters of the complement and bacteria mixture was added to 50 µL of serum making the total reaction volume to 100 µL. After 60 min of incubation, the number of surviving bacteria was determined by plating 10 µL onto chocolate agar and counting the colonies. One adult and one pediatric serum pool with known bactericidal titers against NTHi strain (86-028 NP) were run during each experiment as in-house controls. Every test sample serum was run in duplicate and all sera were tested on two separate days to ascertain and confirm reproducibility of the data. Intra- and inter-assay variation was infrequent but when it occurred then the serum sample was retested on additional days, if necessary, to obtain a result that was completely reproducible in duplicate on two separate occasions.

The bactericidal titer of the serum was defined as the inverse of the highest dilution that led to $\geq 50\%$ bacterial killing and was compared with that of negative control (complement plus bacterium).

**Adsorption of anti P6, Protein D, and OMP26 antibodies from sera**

The bactericidal sera were later depleted for anti-P6, PD and OMP26-specific antibodies and used for bactericidal assay. For the absorption procedure, polystyrene beads were washed extensively with borate buffer (pH 8.0) and resuspended in 1 mL of Borate buffer. Recombinant protein D, P6, and OMP26 antigens were incubated with these beads overnight at room temperature. The beads were washed extensively, incubated in BSA/Borate buffer for 30 min at room temperature, then pelleted and incubated with 200 µL of patient sera for two hours at room temperature. The beads were centrifuged (200 g) and the supernatant was collected. The anti-protein D-, P6-, and OMP26-absorbed sera levels were measured by ELISA (mentioned in the following sections) and used for bactericidal assays. The reciprocal bactericidal titers were compared with unadsorbed sera to determine the bactericidal activity meditated by each of the specific antibodies. Adsorption experiments were run in duplicate and repeated on two different days. ELISA titers after absorption were found to be highly reproducible. Pre- and post-adsorbed sera were run together in same plate.

**Detection of P6-, Protein D-, and OMP26-specific IgG by ELISA**

Protein D-, P6-, and OMP26-specific IgG antibody titers in the acute and convalescent serum samples were determined by ELISA. Protein D, P6, and OMP26 recombinant proteins were coated on 96-well plate with the concentration of 0.25 µg mL$^{-1}$ each in coating buffer. After blocking with 3% skim milk, diluted serum samples were added to the wells, and the mixture was incubated at room temperature for 1 h. Affinity purified goat anti-human IgG antibody conjugated to horseradish–peroxidase was used as a secondary antibody. The reaction products were developed using TMB Microwell peroxidase substrate system, stopped by the addition of 1.0 M phosphoric acid, and read by ELISA reader at 450 nm. Protein D-, P6-, and OMP26-specific IgG antibody titers in the acute and convalescent serum samples were determined by ELISA as described in detailed elsewhere. (29). To provide quantitative results on antibody concentrations, the level of the specific antibody present in the unknown sample was determined by comparing it with an internal reference serum (pool of human serum with high anti-P6, Protein D, and OMP26 titers). The levels of IgG in the internal reference serum pool were quantitatively measured by using a human IgG ELISA quantitation kit with a known amount of antibodies concentration in the human reference serum (Bethyl laboratories). The IgG titers are expressed as ng mL$^{-1}$. Assay was validated for determining the amount of antibodies in the internal reference serum.

**Detection of whole-cell NTHi IgG antibodies by ELISA**

For the whole-cell-specific ELISA, for each child, we used their homologous NTHi strain isolated from MEF and a heterologous strain (86-028 NP). Homologous and heterologous strains were grown on chocolate agar and further inoculated into brain heart infusion broth supplemented with NAD and Hemin. The bacteria were grown to mid-log phase, harvested, and washed with PBS containing 0.15 mM CaCl$_2$, and 0.5 mM MgCl$_2$. The pellet was finally suspended and diluted to an OD of 1 at 490 nm. The NTHi preparation was used to coat 96-well plates. After blocking with 1% gelatin and washing, diluted serum was added to the wells, and the mixture was further incubated at room temperature for 1 h. Affinity purified goat anti-human IgG antibody conjugated to alkaline phosphatase was used as a secondary antibody. The reaction products were developed with PNP dissolved in diethanolamine buffer. The reaction was stopped by the addition of 2 M NaOH and was read by ELISA reader.
Titers against the homologous strain (Fig. 1).

Statistics

Paired T-test was used to compare the IgG titers between acute and convalescent serum samples. All other data were analyzed using Mann–Whitney (nonparametric) U-test. P value < 0.05 was considered as significant. Statistical analysis of correlation coefficients by linear regression ($r^2$) between ELISA titers and bactericidal titers was determined to measure the level of correlation between the assays.

Results

Bactericidal antibody

Bactericidal titers, directed against the homologous NTHi strains isolated from each infected child were measured. Among 17 acute serum samples from otitis-prone children, five sera (29%) were found to have any detectable bactericidal activity. In contrast, 16 among 20 convalescent serum samples (80%) had bactericidal activity. The mean bactericidal titers were 18.4 and 70.5 for acute and convalescent serum samples. The 21 bactericidal sera against homologous NTHi strains were also assayed against a heterologous strain (86-028 NP). Eleven sera (52%) were found to show bactericidal activity against the heterologous strain. However, the bactericidal titers [geometric means (GM)] were significantly lower ($P = 0.002$) with heterologous strain as compared to the titers against the homologous strain (Fig. 1).

Anti P6, protein D, and OMP26 serum IgG titers in acute vs. convalescent and bactericidal vs. nonbactericidal sera

We compared the serum IgG levels of protein D, P6, and OMP26 in paired acute and convalescent sera. The IgG titers (ng mL$^{-1}$) to protein D (Acute: GM 1058, CI = 294–3805, convalescent: GM 4010, CI = 2444–6580) and P6 (Acute: GM 1114, CI = 467–2656, convalescent: GM 2577, CI = 1436–4624) were significantly higher in convalescent compared with acute sera ($P = 0.029$, 0.048). The OMP26 IgG titers remained unchanged from acute to convalescent (acute: GM 723, CI = 235–223, convalescent: GM 785, CI = 460–1340) (Fig 2a). Usually, the IgG titers are increased in convalescent sera and the mounted immune response provides protection against the homologous strain. Figure 2b shows that the levels of protein D and P6 antibodies were significantly higher in bactericidal sera (Protein D GM = 3628; 95% CI; 2675–4921, P6 GM = 3140; 95% CI; 2233–4415) than nonbactericidal sera (Protein D GM = 1220; 95% CI; 464–2464, P6 GM = 1701; 95% CI; 1062–3725) [$P = 0.005$ (PD), 0.026 (P6)]. In contrast, the OMP26 antibody titers of bactericidal sera (OMP26 GM = 961.2, 95% CI; 463.2–1995) were not significantly different from nonbactericidal sera (OMP26 GM = 844.8 95% CI; 412.6–1730 ($P > 0.05$) (Fig 2b). We also analyzed the data to determine whether there was a correlation between individual serum IgG titers (protein D, P6, and OMP26) and the respective bactericidal titers. Despite higher antibody titers in bactericidal sera, a correlation between individual serum IgG titers and the respective bactericidal sera (Protein D GM = 3140; 95% CI; 2233–4415) as compared to nonbactericidal sera (GM = 673, 95% CI; 408.5–1071) against their homologous NTHi strain ($P = 0.012$). The IgG titers were also significantly higher for homologous strain (GM = 1531, 95% CI; 1089–1628) as compared to heterologous strain (GM = 635, 95% CI; 187–792).

Whole-cell (NTHi) serum IgG titers in bactericidal and nonbactericidal sera

Serum IgG antibody levels to whole-cell homologous and heterologous NTHi strains were measured and compared in bactericidal and nonbactericidal serum samples. Whole-cell ELISA represents the IgG titers (ng mL$^{-1}$) against all the surface proteins of NTHi. Figure 3a illustrates that the whole-cell IgG titers were significantly higher in bactericidal sera (GM = 1601, 95% CI; 1157–2214) as compared to nonbactericidal sera (GM = 673, 95% CI; 408.5–1071) against their homologous NTHi strain ($P = 0.012$). The IgG titers were also significantly higher for homologous strain (GM = 1531, 95% CI; 1089–1628) as compared to heterologous strain (GM = 635, 95% CI; 187–792).
Lower IgG titers against the heterologous NTHi strain reflects the antigenic heterogeneity on the bacterial surface and that the bulk of the immune response is directed against nonconserved antigens.

**Adsorption of Protein D-, P6-, and OMP26-specific antibodies from bactericidal sera**

Anti-protein D-, P6-, and OMP26-specific antibodies were selectively depleted from 21 bactericidal sera to assess their relative contribution in total bactericidal antibody titers. After adsorption, all sera were tested for residual antibody to protein D, P6, and OMP26 by ELISA to demonstrate the adsorption was complete (Fig 4). We also carried out cross-adsorption experiments to demonstrate that the adsorption was quite specific. For instance, Protein D and OMP26 ELISA titers were quantified in sera that were adsorbed for Protein P6 antibodies and results showed that there was no cross-adsorption (Supporting information, Fig. S1).

**Contribution of P6, Protein D and OMP 26 antibodies in total bactericidal activity**

To evaluate the functionality of the antibodies detected by ELISA, bactericidal activity of the antibodies was assessed, specific to each protein. The reduction in titer of one tube dilution in triplicates was represented as the reduction by twofold. For three (14%) of 21 bactericidal sera tested, adsorption of anti-protein D antibody resulted in a twofold reduction in bactericidal activity (Table 1). For 16 (76%) of the 21 bactericidal sera tested,
adsorption of anti-P6 antibody resulted in a two- to fourfold reduction in bactericidal activity (Table 1). We did not observe any contribution of OMP26 in bactericidal activity as the removal of OMP26 antibody did not change the bactericidal titers.

**Discussion**

This is the first study to assess the proportional contribution of serum bactericidal activity against three conserved outer membrane proteins of NTHi; protein D, P6, and OMP26 in otitis-prone children. We have previously reported bactericidal antibody responses against Protein D, P6, and OMP26 following AOM and NP colonization in nonotitis-prone children involved in our ongoing prospective studies (Pichichero et al., 2010). In non-otitis-prone children, bactericidal activity was observed against P6 and protein D but not OMP26 in most of the samples studied.

We have previously compared serum IgG, IgA, and IgM antibody responses measured by ELISA in otitis-prone children vs. nonotitis-prone children and found that otitis-prone children had lower serum antibody titers than nonotitis-prone children (Kaur et al., 2011). As otitis-prone children are the most vulnerable to the complications of AOM and would benefit most from preventative vaccination, in this study, we have evaluated the functionality of antibody induced in otitis-prone children by measuring bactericidal response against the same NTHi antigens Protein D, P6, and OMP26. Different from the total antibody results, we found that protein D less frequently induced bactericidal antibody compared with vaccine candidate P6 in otitis-prone children and OMP26 again failed to generate any bactericidal effect. Our finding with protein D is consistent with a prior report by Akkoyunlu et al. (1996). Those results along with other ongoing research in our laboratory is a step forward to understand the immunological differences in otitis-prone and nonotitis-prone children.

Bactericidal antibodies enhance killing by enhancing phagocytosis or by complement-mediated killing. Therefore, the term bactericidal antibodies are those functional antibodies in the sera that are associated with enhanced clearance of bacteria either by facilitation of opsonophagocytosis or by complement-mediated killing. Protein D, P6, and OMP26 are three unique protein antigens of NTHi that have the potential to elicit immune responses in children. Protein D is a carrier protein in the vaccine Synflorix and it thought to have contributed to the induction of protection against AOM caused by NTHi as suggested by Prymula et al. (2006). However, a correlate of protection did not emerge from that study and total serum antibody was not a correlate. The potential of bactericidal antibody to protein D to be a correlate of protection from AOM deserves future study. In contrast to protein D that showed bactericidal activity in 14% of children studied, the contribution of anti-P6 antibody to bactericidal activity in over 75% of otitis-prone children is noteworthy. P6 has not been administered to children as a vaccine but our results suggest that it has promise, especially in otitis-prone children because this protein stimulates bactericidal antibody response.

As a first step in this research, we measured bactericidal antibodies to homologous NTHi strains causing AOM. Our results were consistent with prior work showing that acute sera have infrequent bactericidal antibodies

| Table 1. Relative contribution of each antibody type in total bactericidal activity in all serum samples. For 3 (14.2%) of 21 bactericidal sera tested, adsorption of anti-protein D antibody resulted in a reduction in bactericidal activity. For 16 (76.19%) of the 21 bactericidal sera tested, adsorption of anti-P6 antibody resulted in a reduction of two- to fourfold in bactericidal activity. The antibodies to OMP26 were not found to be bactericidal in any of bactericidal serum sample tested in the study.

<table>
<thead>
<tr>
<th>Bacterial titers range</th>
<th>% of children showing twofold decline</th>
<th>% of children showing fourfold decline</th>
<th>% of children with no change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein D</td>
<td>8–256</td>
<td>14.2</td>
<td>85.8</td>
</tr>
<tr>
<td>P6</td>
<td>8–256</td>
<td>57.1</td>
<td>23.8</td>
</tr>
<tr>
<td>OMP26</td>
<td>8–256</td>
<td>0</td>
<td>100</td>
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Fig 4. Adsorption of P6-, Protein D-, and OMP26-specific antibodies from bactericidal sera. Twenty-one bactericidal serum samples were adsorbed for P6-, Protein D-, and OMP26-specific antibodies. Adsorbed serum samples were analyzed for above three antibodies. Figure 4 depicts the successful adsorption of protein D-, P6-, and OMP26-specific antibodies (P < 0.001). The protein D, P6, and OMP26 depleted serum samples were taken forward for the bactericidal assay.
but they develop in the convalescent state (Shurin et al., 1980; Yamanaka & Faden, 1992). Faden et al. (1989) and Bernstein et al. (1991) have reported in the past that the bactericidal antibody response is NTHi strain specific and provided little or no cross-protection in otitis-prone children with otitis media. We, therefore, also assessed bactericidal antibody directed to homologous and heterologous strains prior to and after an NTHi AOM. In contrast to prior results, we did find bactericidal activity against heterologous NTHi in otitis-prone children, albeit at lower titers as compared to the homologous strain. The differences between bactericidal titers to homologous and heterologous strains suggest a probable reason of failure to protect against new NTHi infections caused by heterologous strains.

The focus of our analysis of bactericidal antibody was on IgG. We have previously reported that IgG titers to Protein D and P6 were almost similar in acute and convalescent sera (Kaur et al., 2011), but in this study we found that IgG titers to protein D and P6 were significantly higher in convalescent sera. We found higher IgG anti-protein D and anti-P6 levels in bactericidal sera of otitis-prone children. IgG antibodies to P6 have been found to be bactericidal in an experimental animal system (De Maria et al., 1996) and to be associated, in part, with the bactericidal activity in children (Sabirov et al., 2009). We failed to observe any correlation between individual IgG titers and their respective bactericidal titers. Antigen-specific high-affinity antibodies are usually associated with bactericidal activity in nature. We speculate that a stronger correlation may have resulted if a high proportion of the IgG antibody generated as a result of natural infections in the otitis-prone children was of higher avidity. Vaccination might trigger the generation of high-avidity antibodies that can be associated with bactericidal activity and therefore may provide protection against NTHi-mediated AOM. We did not find any correlation between OMP26 IgG levels and bactericidal titers (data not shown), consistent with one previous report (Cripps & Otczyk, 2006). We found significantly higher whole-cell IgG titers in bactericidal sera as compared to nonbactericidal sera. The IgG titers were significantly higher for the homologous NTHi strain as compared with the heterologous strain. The results were quite comparable with the serum IgG titers for protein D and P6 in sera that were associated with bactericidal than nonbactericidal sera. This is an important aspect of studying the cross-protection against NTHi strains based on bactericidal serum antibodies. The lower bactericidal titers against a heterologous strain also reveal the contribution of nonconserved surface antigens in the bactericidal antibody response. In the past, serum IgG levels have been reported to be higher in bactericidal sera against *Moraxella catarrhalis* in healthy adults and children (Chen et al., 1999). However, it remains to be understood as to how much antigen-specific IgG is required to provide comprehensive protection against the spectrum of NTHi strains.

Among the 21 bactericidal sera, the antibodies to three proteins lead to a decline of at least two- to fourfold of bactericidal activity and remaining associated bactericidal activity must have been directed to other surface antigens of NTHi including high-molecular weight proteins (Barenkamp & Bodor, 1990), nonconserved porin proteins on the NTHi surface etc. (Neary et al., 2001; Sikkema & Murphy, 1992). Nevertheless, there might be other yet unidentified, conserved targets for bactericidal antibodies to NTHi. We are working further to map epitopes in conserved antigens of NTHi that are targets for antibodies associated with bactericidal activity in the pursuit of generating a multiepitope vaccine. This combination vaccine might establish a better correlate of protection and provide broad protection against a spectrum of NTHi strains. The children above 6 months were deliberately taken for the study to have a minimal effect mediated by maternal antibodies in the serum (Zinkernagel, 2001). Therefore, we assume that the bactericidal activity we detected in the otitis-prone children is conferred by their own immune system and not by passive transfer from mother.

In summary, the findings establish that some portion of bactericidal activity is directed to OMP P6 and to a lesser degree to Protein D in otitis-prone children following a naturally acquired AOM infection by NTHi. The contribution of other NTHi conserved antigens displayed on the NTHi surface is encouraged by our results. The lack of a correlation between bactericidal antibody titers and IgG titers suggests the need to establish a biologically relevant serologic surrogate to define a particular bactericidal titer, a threshold for defining a protective titer, which is critical for vaccine development.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** IgG titers of protein D in Unadsorbed and P6 adsorbed sera.

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