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Antibody Response to *Haemophilus influenzae* Outer Membrane Protein D, P6, and OMP26 After Nasopharyngeal Colonization and Acute Otitis Media in Children

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Abstract

Development of natural antibodies to 3 nontypeable *Haemophilus influenzae* (NTHi) outer membrane proteins (D, P6 and OMP26) was prospectively studied in 168 children 6–30 months of age during NP colonization and AOM. IgG antibody to protein D, P6 and OMP26 increased with age ($p < 0.001$). Serum IgG responses to NP colonization were different for the 3 proteins: protein D responses occurred at a later age than P6, and OMP26 responses were minimal. For all 3 proteins serum antibody levels in the convalescent phase of AOM infection were not as high as after NP colonization. Antibodies to protein D and P6 but not OMP26 were bactericidal.

Keywords

Non-typeable *Haemophilus influenzae*; P6 protein; protein D; protein OMP26; Acute otitis media; carriage

INTRODUCTION

Nontypeable *Haemophilus influenzae* (NTHi) is currently the most frequent cause of episodic and recurrent acute otitis media (AOM) in children in the United States [1–3]. AOM and all respiratory bacterial infections begin pathogenesis with colonization of the nasopharynx (NP). However, carriage of NTHi is mostly asymptomatic; only when the condition of the host is altered, NTHi may invade the middle ear, causing AOM.

A vaccine against NTHi presents a different set of challenges compared with Hib vaccination because rather than a single dominant capsular antigen, NTHi strains express multiple outer membrane proteins (OMPs) [4–7]. Several of the OMPs of NTHi have been eliminated as vaccine candidates due to surface epitope heterogeneity, variable expression or other characteristics [7,8]. Desirable vaccine candidate antigens for NTHi should be

conserved among strains and immunogenic in children and adults. At this time one OMP of NTHi, protein D, has been incorporated into a commercialized vaccine product as a carrier of pneumococcal polysaccharide antigens. Administration of that conjugate vaccine resulted in a 35% reduction in AOM caused by NTHi [9]. Further proof of the efficacy of protein D as a vaccine ingredient for prevention of NTHi mucosal infections is needed and the study of other NTHi antigens is underway in many laboratories, anticipating the need for a multi-component vaccine to optimize protection at rates higher than protein D alone.

Two additional NTHi OMPs that are leading vaccine candidates are protein 6 (P6) and protein OMP26, since they possess the desirable features noted above [7,8]. For NTHi vaccine development it is important to know whether antibodies develop after natural NTHi exposure such as after asymptomatic NP colonization and after AOM. In the present study we hypothesized that NTHi NP colonization and AOM would represent immunizing events for potential OMP vaccine ingredients. To our knowledge, this is the first study to prospectively compare the development of natural antibodies to 3 NTHi outer membrane proteins D, P6 and OMP26 simultaneously in a cohort of children 6–30 months of age during NP colonization and AOM. The comparisons of interest we report here include: 1. Changes in the levels of protein D, P6 and OMP26-specific IgG antibodies in children as they increased from 6 to 30 months of age; 2. Changes in antibody levels following detected colonization of the NP with NTHi; 3. Differences in antibody levels in convalescence from NP colonization versus AOM; 4. Variations in individual antibody repertoire and responses in the study cohort following AOM; and 5. Differences in contribution of antibodies to protein D, P6 and OMP26 to bactericidal activity.

METHODS

General design

This report includes data for the 3.5 year time span June, 2006 to December, 2009 from children enrolled in a 5 year prospective study supported by the National Institutes of Deafness and Communication Disorders. Healthy children without previous episodes of AOM were enrolled from a middle class, suburban socio-demographic pediatric practice in Rochester, NY (Legacy Pediatrics). Healthy children had serum, NP and oropharyngeal (OP) cultures and NP wash samples obtained seven times, every 3–6 months, between 6 and 30 months of age (at age 6, 9, 12, 15, 18, 24, and 30 months). In addition, if a child developed symptoms compatible with AOM, they were examined by validated otoscopist pediatricians with pneumatic otoscopy and if middle ear infection was suspected a tympanocentesis was performed to confirm the diagnosis. At the time of the acute AOM diagnosis and three weeks later acute and convalescent serum, NP and OP cultures and NP wash samples were obtained. The study was approved by the University of Rochester and Rochester General Hospital Research Subjects Review Board and written informed consent was obtained for participation and all procedures.

We selected three NTHi OMPs to study. Protein D is a highly conserved antigen among NTHi strains [10]. It is a 43 kilodalton surface-exposed lipoprotein that has glycerophosphodiesterase. P6 has been described as a highly conserved OMP among NTHi strains. Immunization with P6 provides protection against AOM in the chinchilla model [11]. OMP26 is a highly conserved protein of NTHi that is associated with protection against NTHi infections after parenteral and mucosal immunization in the chinchilla and rat models that induced high levels of antibody [12,13].

Definition of AOM

AOM was diagnosed by pneumatic otoscopy by two of the authors (JC, MP), who are both validated otoscopists, when children with acute onset of otalgia had tympanic membranes (TMs) that were: (1) bulging or full; and (2) a cloudy or purulent effusion was observed, or the TM was completely opacified; and (3) TM mobility was reduced or absent.

Tympanocentesis

MEF for culture was obtained by puncture of the inferior portion of an intact 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 below.

Sample collection

At each sampling visit a cotton-tipped wire swab was inserted into both nares and a culture of the posterior nasopharynx was obtained; an OP culture was obtained by rubbing both tonsils and the posterior pharynx. Then 1 ml of sterile phosphate buffered saline was instilled and aspirated from both nares as a third sample for culture. Serum was obtained by venipuncture after application of ELMA cream for local numbing of the area.

Microbiology

MEF, NP, and OP samples were inoculated into trypticase soy broth, trypticase soy agar with 5% sheep blood plates, and chocolate agar plates. All samples were incubated at 37°C with 5% carbon dioxide. Bacteria were isolated according to the CLSI standard culture procedures. An isolate was further identified as *NTHi* on a similar basis as described by Murphy *et al.* [14] to include not only colony morphology, porphyrin reactivity, and growth requirement for hemin & nicotinamide adenine dinucleotide and Haemophilus ID Quad plates, but also by *ompP6* sequencing to distinguish *NTHi* from *H. haemolyticus*. [14]

Detection of OMP-specific antibodies by ELISA

Protein D, P6 and OMP26- specific antibody titers were determined by ELISA using purified recombinant protein D (provided as a gift from GlaxoSmithKline Biologicals, Rixensart Belgium), lipidated P6 (provided as a gift by Dr. Tim Murphy, University of Buffalo) and OMP26 (provided as a gift by Jennelle Kyd, University of Canberra, Australia). 96-well Nunc-Immulon 4 plates were coated with 0.25 µg–0.5 µg/ml of individual OMP antigens (100 µl/well) in coating buffer (bicarbonate, [pH 9.4]) and incubated overnight at 4°C. After washing the plates were blocked with 3% skim milk at 37°C for 1h (200 µl per well). After five washes, 100 µl of serum at a starting dilution of 1:100 (in PBS-3% skim milk) was added to the wells and diluted serially 2 fold. The mixture was incubated at room temperature for 1 hr. followed by the addition of affinity purified goat anti-human IgG, IgM or IgA antibody conjugated to horseradish-peroxidase (Bethyl Laboratories, Inc, Montgomery, TX) as a secondary antibody. The reaction products were developed with TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD), stopped by the addition of 1.0 molar phosphoric acid and read by an automated ELISA reader using a 450-nm filter.

To provide quantitative results on antibody concentrations, the level of the specific antibody present in the unknown sample was determined by comparison to an internal reference serum (pool of human serum with high anti-OMP titers). The levels of IgG, IgM and IgA in the reference serum were quantitatively measured by using a human IgG/IgA/IgM ELISA quantitation kit (Bethyl laboratories).

A Four-parameter logistic-log function was used to form the reference and sample curves. This ELISA was fully validated according to ICH Guidance. The assay lower limit of detection for protein D was 3.5 ng/ml for IgG, 4.5 ng/ml for IgM, and 8 ng/ml for IgA; for P6 it was at 1 ng/ml for IgG, 3 ng/ml for IgM, and 3 ng/ml for IgA; and for OMP26 it was at 4 ng/ml for IgG, 3 ng/ml for IgM, and 10.5 ng/ml for IgA. The inter-assay coefficient of variation was 20% for all antigens and secondary antibody combinations.

Bactericidal assay

Eleven sera were randomly selected from those with the greatest volumes to measure bactericidal activity pre and post absorption with protein D, P6 and OMP26. The sera were heat-inactivated at 56°C for 30 minutes to eliminate human complement. Each serum was assayed against the bacterial strain isolated from middle ear space of that child. Homologous *NTHi* strains were cultivated, harvested, and diluted to a concentration of $\sim 10^5$ CFU/ml. Twelve serial twofold dilutions of the serum to be tested (starting at 1:2) were mixed with precolostral calf serum complement and 20 μ l of bacteria. After 60 minutes of incubation, the number of surviving bacteria was determined by plating 5 μ l onto chocolate agar and counting the colonies. The bactericidal titer of the serum was defined as the inverse of the highest dilution that led to 50% bacterial killing and was compared to that of negative control serum. Appropriate controls were included in all experiments. To examine the contribution of protein D, P6 and OMP26 antibodies to serum bactericidal activity observed, we removed all protein D, P6 or OMP26 antibodies from available sera using polystyrene beads. For the absorption procedure, polystyrene beads were washed extensively with borate buffer (pH 8.5) and resuspended in 1ml of Borate buffer. Freshly prepared 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 minutes at room temperature, then pelleted and incubated with 200 μ l of patient sera for 2 hours at room temperature. The beads were centrifuged (200 $\times$ g) and the supernatant was collected.

The efficacy of absorption was monitored by using ELISA as described above. The reciprocal bactericidal titers were compared with unabsorbed sera to determine the bactericidal activity mediated by each of the specific antibodies. **We also carried out an experiment with cross adsorption of other antibodies and found that adsorption was quite specific.**

Multilocus sequence typing (MLST)

Bacterial genomic DNA was extracted from pure cultures of *NTHi* isolated from NP, OP or MEF samples (If children have AOM). The internal fragments of seven housekeeping genes of *NTHi* were amplified by PCR, using PCR Master Mix (Promega, 50 units/ml of Taq DNA Polymerase, 400 μ M dNTP, 3mM MgCl₂.) using primers described previously [15]. PCR conditions were as follows: initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 30 seconds, 50–55°C annealing for 30 seconds and 72°C extension for 30 seconds. Sizes of PCR products were checked by running 1.5% agarose gel electrophoresis stained with ethidium bromide. The size of PCR products was 50–100 bps larger than the fragments for typing. The PCR products were purified using the Exo-sap kit (USB Company) and identified by DNA sequencing. Sequencing analyses were performed on an ABI Prism 3730x/DNA analyzers with the same primers used for PCR product amplification.

Statistics

Two sample comparisons were performed using either paired t-test, two-sample t-test (using a log transformation where appropriate), the Mann-Whitney rank sum test or Fisher's exact test.

Testing for increasing antibody level with age (figure 2) was complicated by inconsistently represented time points, and by data dependence induced by repeated sampling by subject. We therefore used a modification of Kendall's tau statistic to measure within-subject concordance of level with age. Any pair of within-subject antibody level measurements which increases with age was concordant, and was discordant otherwise (neither applies in the case of ties). In the calculation N_c and N_d were the number of concordant and discordant within-subject pairs and $G = (N_c - N_d) / (N_c + N_d)$. The statistic G was calibrated as a correlation, with -1 and 1 representing perfect negative and positive associations, respectively, between age and antibody level measurement. The quantity $S = N_c / (N_c + N_d)$ represented the proportion of increasing pairs. If there was no concordance we expected $S = 1/2$, on average. To assess significance, we performed a bootstrap procedure by resampling subjects with replacement. This procedure permitted within-subject dependence. The bootstrap sample was then used to estimate 95% confidence intervals for S , and p -values against the null hypothesis $S = 1/2$.

For all testing, $p < 0.05$ was considered significant.

RESULTS

NP colonization and AOM events

During the 3.5 years of enrollment a total of 168 children were recruited into the study. There were 631 visits with 72 NTHi OP/NP colonization episodes documented in 47 (28%) children who were culture-positive for NTHi at one or more of the seven sampling visits (figure 1). Among the 47 NP colonized children, 31 (66%) of the children were NTHi culture-positive at one sampling visit, 10 (21%) at two visits, 4 (9%) at three visits and 2 (4%) at four or more visits. One hundred nineteen (72%) did not have NTHi detected by culture in the NP or OP at any of the 7 visits. Thirty-seven (79%) children experienced colonization that was detected by culture and cleared by the next sampling 3 to 6 months later. NP colonization was detected at the first sampling at 6 months of age in 10 (21%) children and at various times thereafter for the remainder of the subjects. Nine of the 47 colonized children (19%) experienced prolonged detected colonization of 6 months or longer with the same NTHi strain based on multi-locus sequence typing (subjects 1, 6, 7, 19, 20, 22, 37, 40, and 44; MLST data not shown). Because the study design called for NP/OP sampling at 7 specific times separated by 3 to 6 months, some NTHi colonization events were not detected by culture but most likely occurred as reflected in significant rises in specific antibody to one or more of the NTHi antigens studied. There were 28 NTHi AOM episodes in 18 children. Nine (50%) of the 18 AOM children experienced one AOM due to NTHi, 8 (44%) children experienced 2 AOM events due to NTHi and 1(6%) experienced 3 NTHi AOM events (Figure 1).

Natural acquisition of serum antibody to protein D, P6 and OMP 26 over time

Figure 2 shows a boxplot of the measured serum antibodies to protein D, P6 and OMP26 at 6, 9, 12, 15, 18, 24, and 30 months of age corresponding to visits 1–7 for the study cohort. For comparison the antibody levels of 26 adults (age 18 to 60 years) are also displayed. The antibody levels increase significantly over time to all 3 NTHi proteins ($p < 0.001$). Antibody levels among each of the 3 proteins at each of the 7 time points did not differ significantly. Compared to adults, antibody in the 24–30 month age groups combined was significantly lower than adults for protein D ($p < 0.001$).

Comparison of serum antibody levels to protein D, P6 and OMP26 in NP colonized versus non-colonized children at various ages

We compared the level of antibody to protein D, P6 and OMP26 in children who were NP culture-positive for NTHi at age 6, 9, 15, 18, and 24–30 months with children of the same age who were not NP culture-positive. The patterns were significantly different for the 3 proteins (Figure 3). For protein D no difference was identified between colonized and non-colonized children at age 6 or 9 months, but a significant difference was shown at age 15, 18, and 24–30 months. For P6, a significant difference was identified between colonized and non-colonized children at age 6, 9 and 15 months, but the difference was no longer significant at age 18, and 24–30 months, largely because of wide variation in antibody quantity among those children. For OMP26, no difference was seen between colonized and non-colonized children.

Comparison of convalescent serum IgG levels to NTHi outer membrane proteins D, P6 and OMP26 following an AOM or an NP colonization event

We hypothesized that an AOM event would stimulate a high quantity of antibody to the studied proteins in convalescence. It is known that NP colonization is a necessary first step in pathogenesis for AOM. Therefore we reasoned that NP colonization plus an acute infection in the form of AOM would result in higher antibody levels than NP colonization only. Unexpectedly we found that the levels of antibody to protein D, P6 and OMP26 following an AOM episode were generally low compared to those observed following NP colonization (Figure 4). The lower AOM convalescent antibody levels compared to colonization convalescent antibody levels was significant for P6 ($p < 0.001$) but not for protein D or OMP26. The analysis is complicated by the fact that the post AOM serum antibody levels were all obtained 3 weeks after an AOM event whereas the convalescent colonization antibody levels were obtained at the next scheduled visit for the child. Therefore a post colonization sample could have been obtained 3 to 6 months after the colonization event, at a time when some diminution of antibody likely occurred compared to the 3 weeks post AOM sampling. The differences in timing contributed to the variation in convalescent antibody levels after colonization.

Paired Acute and Convalescent Serum IgG, IgM and IgA antibody levels to NTHi outer membrane proteins D, P6 and OMP26 in children with AOM

The quantity of total immunoglobulin to protein D (IgG+IgM+IgA) in acute sera at the time a child presented for clinical care for AOM was 2082 ng/mL, for P6 it was 1422 ng/mL, and for OMP26 it was 1545 ng/mL, $p = 0.18$ for protein D vs. P6 (Figure 5). In the convalescent serum, the quantity of total immunoglobulin to protein D was 2872 ng/mL, for P6 it was 1108 ng/mL, and for OMP26 it was 2264 ng/mL, $p = 0.04$ for protein D vs. P6. The ratio of the three immunoglobulin classes (IgG to IgM to IgA) was not different for the 3 proteins. When assessed as a cohort the increases in IgG, IgM and IgA in acute vs. convalescent sera were not significant for any of the 3 proteins.

From the data displayed in Figure 4 for all three proteins it appears that in the convalescent phase the antibody levels following AOM infection are not as high as after NP colonization and from Figure 5 the acute to convalescent change in antibody concentration is not significant for the overall cohort. Therefore in Figure 5 we show the individual responses of children to all three proteins. When we examined the individual responses where paired sera were available we observed that about one-third of children showed a rising antibody response to one or more of the NTHi antigens, with IgM predominating in acute sera (suggesting a primary immune response), one-third had no change in antibody level between acute and convalescent sera, with IgG > IgM but relatively high IgM (suggesting that a primary response was occurring but the sampling missed the true onset of the immune

response) and one-third had a falling antibody level, with IgG >> IgM (suggesting that the samples were taken well past the onset of the primary immune response or that a secondary response had occurred). The absence of synchrony of the antibody responses to protein D, P6 and OMP26 is notable but at this time we have too few children to effectively analyze this effect.

Bactericidal activity of NTHi anti Protein D, P6 and OMP26 antibodies

To evaluate the functionality of the antibodies detected by ELISA, bactericidal activity of the antibodies was assessed, specific to each protein. In Table 1a the concentration of protein D specific antibody determined by the ELISA and the bactericidal activity of the total antibody to the homologous infecting AOM strain is shown and compared to the concentrations and bactericidal titers after absorption of all or nearly all of the protein D specific antibody. For 7 of the 11 sera tested absorption of anti-protein D antibody resulted in a significant drop in bactericidal antibody. Table 1b shows the results for P6 (9 of 11 sera showed a drop) and Table 1c shows the results for OMP26 (0 of 11 showed a drop). The number of sera with bactericidal antibody to protein D was significantly greater than OMP26 ($p=0.01$) and the bactericidal antibody to P6 was significantly greater than OMP26 ($p=0.001$). Antibody to protein D and P6 accounted for all of the detected bactericidal antibody in 5 (45%) of 11 studied children and at least 50% of the bactericidal antibody in the remaining 6 children. To study the specificity of the cross absorption, P6 and OMP26 ELISA titers were quantified in sera that were absorbed to remove Protein D antibodies; there was no cross absorption (data not shown). Similar experiments in duplicate were done with each sera on two different occasions to verify the specificity of absorption for the antibodies studied.

DISCUSSION

The systemic antibody response in children who experience NTHi colonization and AOM has not been well characterized. Progress in the development of an NTHi vaccine to prevent AOM is hampered by our gaps in knowledge of the immune response mounted by children who experience NTHi NP colonization and AOM. Studies in the past often did not have the advantage of current microbiology, molecular biology and immunology techniques, the antibody repertoire and functionality of antibody was not fully assessed, and/or the diagnostic accuracy of AOM and differentiation of AOM from OME—a distinctly different clinical condition, was often not made [16–32].

NP colonization by otopathogens among children has been studied in a study design similar to ours in the past. Faden et al [33] prospectively evaluated NP colonization by NTHi in a cohort of 200 children from birth to two years of age living in suburban Buffalo NY. NP colonization was detected in 44% of the children, with more frequent colonization detected in the first year of life compared to the second year of life. Frequent acquisition of NTHi strains with frequent clearing was observed in that study, similar to our findings. Faden et al included children from birth to 6 months of age when colonization occurred relatively frequently and his group sampled the NP more often than we did in the first year of life. This may account for our detection rate of 28% of evaluated children compared to their study. Indeed, we did observe significant increases in serum antibody to one or more of the NTHi antigens we studied occurring between study visits, suggesting that NP colonization events occurred without detection due to NP sampling frequency.

Importantly, the gradual acquisition of serum antibody in children over time to vaccine candidate antigens protein D, P6 and OMP26 demonstrates that the 3 proteins are immunogenic in infants 6 to 30 months of age. Such an observation is strongly supportive of the potential of these antigens to be useful in a vaccine against NTHi infection in children.

Previously, Akkoyun et al [34] evaluated naturally occurring protein D antibodies and found they were low in children below one year of age but rose between age 1 and 5 years. Yamanaka and Faden [35] prospectively studied the serum antibody levels to P6 in eight children at ages birth, 6 months, 1, 2, 4, 6, and 10 years old and eight adults. They found that levels increased over time and the difference became significant when 6 month olds were compared to four year olds.

Our group previously reported that NP colonization appeared to be an immunizing event in children relative to P6 protein [36]. The significant difference in antibody level among NP colonized compared to uncolonized children beginning at age 15 months for protein D and 6 months for P6 provides further evidence that NP colonization with NTHi is associated with stimulation of serum antibody to OMPs expressed by this bacteria. The absence of a significant increase in antibody to OMP26 in NP colonized compared to uncolonized children was unexpected since a gradual and significant rise in antibody to OMP26 was observed to occur as children increased in age from 6 to 30 months old. This observation will require further study. Our findings are in contrast to those by Spinola et al [21], who prospectively followed 3 children who attended a single day care center from infancy until early childhood obtaining NP cultures periodically. They noted that NP colonization in children with NTHi was a dynamic process with loss and acquisition of different strains occurring over time. Serum IgG directed to the OMPs of NTHi did not appear to change greatly over time, or to be correlated with NP colonization. In a study evaluating pneumococcal NP colonization and AOM, Virolainen et al [37] evaluated serum antibodies in children with AOM to pneumolysin, a pneumococcal protein that is a vaccine candidate. Eight of 10 children experienced a seroconversion in pneumolysin antibody levels following AOM due to pneumococci at a median age of 20 months old. Similarly, Rapola et al [38] studied the serum antibody response to pneumolysin and pneumococcal surface adhesion A (PsaA) in children with AOM age 2 months to 2 years. Antibody levels were compared among three groups: pneumococcal AOM, pneumococcal NP colonized and neither NP colonized nor AOM due to pneumococci. At the time of the sampling, children with NP colonization had the highest anti-PsaA antibody levels, children with a current AOM were next highest, children with no current but a past history of pneumococcal NP colonization or AOM were third highest, and lowest were those with no current or previous documented history of pneumococcal colonization or AOM. Wide variations in antibody levels were measured. The findings were similar with pneumolysin. Our study of NTHi antibody responses to protein D, P6 and OMP26 are in agreement with the studies of pneumococcal vaccine protein candidates. Age of the child and pre-existing antibody levels are important covariates in predicting an antibody response to NP colonization. This may prove true also for vaccination.

When we examined the isotypes of antibody in acute and convalescent sera surrounding an AOM for the subset of children where we had paired serum, we found IgG antibody predominated although IgM antibody levels were also elevated. This repertoire of antibody is most consistent with prior priming of the immune response before the AOM; otherwise we would have expected IgM to predominate in acute sera and a switch in Ig class to occur in convalescent sera to IgG predominant. Analysis of individual child data allowed us to observe that the cohort analysis masked differences in the antibody repertoire. The addition of more children to our study in the future may allow a clearer understanding of the proportion of children with various antibody response characteristics. In earlier work, Samukawa et al [39] studied the immune response to *S pneumoniae* surface protein A (PspA) and *M catarrhalis* OMP UspA in the sera of various age groups in the general population. In the first 2 years of life they found comparable amounts of IgG and IgM serum antibodies to both PspA and UspA whereas in adults IgG predominated. In contrast, when Virolainen et al [37] evaluated serum antibodies in children with AOM to pneumolysin they

found eight of 10 children experienced a seroconversion in pneumolysin antibody levels, all of the IgA class only.

Not all antibody elicited by natural exposure to NTHi may be functional; therefore the study of the contribution of antibody to protein D, P6 and OMP26 to bactericidal activity was of interest. Serum bactericidal antibody is associated with protection from AOM caused by NTHi [16, 40]. Previous work has shown that serum bactericidal antibody to the homologous strain persists after AOM and can protect against recurrent NTHi AOM infection by the homologous strain but cross-protection for other (heterologous) strains generally is not induced [16,40]. Forsgren has previously shown that antibody to protein D can be bactericidal [10]. Murphy et al [41] assessed the role of P6 as a target of bactericidal antibody and showed that in a pool of 6 adult sera depletion of P6 antibodies resulted in a reduction bactericidal activity. The absence of a bactericidal effect by OMP26 has been noted [42]. Therefore, our results are consistent with previous reports.

We did not find a significant correlation between bactericidal titers and ELISA antibody concentrations for P6, Protein D and OMP26 antibodies. Higher ELISA antibody titers did not consistently result in higher bactericidal titers. Particularly for OMP26 high ELISA antibody titers did not correlate with high bactericidal titers and the depletion of OMP26 specific antibodies did not change bactericidal titers. The disparity in antibody quantity measured by ELISA and bactericidal titers most likely is a reflection of the fact that ELISA measures antibody of low and high avidity whereas bactericidal titers largely reflect high avidity antibody [43, 44]. Also it may be that not all OMPs expressed by NTHi elicit bactericidal antibodies.

Our study has limitations. The difficulty of obtaining blood from young children repetitively between 6 and 30 months of age is considerable, particularly when coupled with additional blood sampling for acute and convalescent levels surrounding an AOM. Therefore, despite best efforts, we did not obtain blood samples from every child at every visit as designed. This created windows of missing data that we addressed as we could statistically, but the addition of more samples from more children is ongoing and may allow us to provide further light on some of the issues addressed in this report. We recognized the need to evaluate mucosal immune responses and cellular responses to NTHi NP colonization and AOM and to compare immune responses between children with absent or infrequent AOM with otitis prone children. Those studies are ongoing and will be reported subsequently. Our study cohort is drawn from a predominantly high socioeconomic population in a developed country and therefore our results may not be generalizable to children in developing countries with lower socioeconomic status where the NP colonization frequency and bacterial load may be higher.

In conclusion, this is the first study to compare antibody levels to three NTHi candidate vaccine OMPs in children following asymptomatic NP colonization and episodes of AOM. We found increasing levels of protein D, P6 and OMP26-specific IgG antibodies in children as they increased in age from 6 to 30 months of age. Increased antibody levels were specifically measured following detected NP colonization with NTHi for protein D and P6 but not OMP26. In convalescence from AOM children had lower overall IgG antibody levels than after asymptomatic NP colonization. Thus it appeared that AOM occurred in the context of a less robust immune response than following colonization. However the overall response did not reflect individual responses among the study cohort in that some children had a clear increase in antibody to protein D, P6 and/or OMP26 following AOM. Natural antibodies to protein D and P6 but not OMP26 elicited by NP colonization and AOM were bactericidal. Ongoing work in our laboratory includes the study of expression of these three

vaccine protein candidates on NTHi in each process of pathogenesis, i.e., carrier/colonization and causative pathogen of AOM.

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Figure 1.

Nasopharyngeal and/or oropharyngeal colonization episodes represented by a closed circle at visits 1–7 at 6, 9, 12, 15, 18, 24, and 30 months of age. AOM episodes are represented by a closed triangle.

Figure 2.

Serum IgG antibody levels to NTHi outer membrane proteins D, P6 and OMP26 in healthy children increases with age.

Boxplots of the geometric mean concentration (ng/ml) displayed as a bar, 25% ile and 75% ile of the data displayed as the lower and upper limit of the box and the 95% confidence interval displayed as a short horizontal dash of antibody in sera of children taken during 7 sampling visits at 6, 9, 12, 15, 18, 24 and 30 months of age. The number of sera included at each time point was 100, 88, 60, 59, 52, 43, and 8.

Figure 3.

Comparison of serum IgG antibody levels to NTHi outer membrane proteins Protein D, P6 and OMP 26 in NP colonized (□NTHi+) and non-colonized (●NTHi-) healthy children from 6 months to 24 months of age. * indicates that the differences in colonized vs non-colonized children were significant for Protein D at visit 15, 18 and 24–30 months with p value is equal to 0.04, 0.01 and 0.02 respectively and for P6 at 6, 9 and 15 months with p value 0.0003, 0.02 and 0.003 respectively.

Figure 4. Comparison of convalescent serum IgG levels to NTHi outer membrane proteins D, P6 and OMP26 following an AOM or an NP colonization event. X axis represents the number of cumulative data points. o = individual IgG level following an AOM event; x = individual IgG level following and NP colonization event

Figure 5. IgG, IgM and IgA antibody levels comparison to NTHi outer membrane proteins D, P6 and OMP26 in acute (top) and convalescent (bottom) sera of 9 children with NTHi AOM. Antibodies concentrations were summarized as geometric mean concentration with 95% confidence intervals.

Figure 6. Individual IgG, IgM and IgA antibody levels to NTHi outer membrane proteins D, P6 and OMP26 in acute and convalescent sera of 9 children with NTHi AOM.

Table 1

a. Bactericidal activity of NTHi anti Protein D antibodies				
Subjects	Anti PROTEIN D ELISA Ab titers (EU/ml)	Bactericidal titer* (Pre Adsorption)	Anti PROTEIN D ELISA Ab titers (post Protein D-Ab Adsorption) (EU/ml)	Bactericidal titer* (With Protein D Adsorbed Sera)
1	1100	8	200	4
2	76	8	40	4
3	1500	16	100	8
4	780	16	187	4
5	773	8	291	8
6	1130	32	<5	32
7	1260	4	<5	4
8	1275	64	150	32
9	5450	16	<5	8
10	8326	8	495	8
11	213	8	<5	4

b. Bactericidal activity of NTHi anti protein P6 antibodies				
Subjects	P6 ELISA Ab titers in sera (EU/ml)	Bactericidal titer* (Pre Adsorption)	Anti P6 ELISA Ab titers in sera (post P6-Ab adsorption) (EU/ml)	Bactericidal titer* (With P6 Adsorbed Sera)
1	4531	8	124	4
2	180	8	91	4
3	199	16	127	8
4	1265	16	226	16
5	1160	8	192	4
6	632	32	174	8
7	933	4	38	0
8	1348	64	128	16
9	>6000	16	40	16
10	1605	8	62	4
11	674	8	32	0

c. Bactericidal activity of NTHi anti OMP26 antibodies.				
Subjects	Anti OMP26 Ab titers (EU/ml)	Bactericidal titer* (Pre Adsorption)	Anti OMP26 Ab titers (Post OMP26-Ab Adsorption) (EU/ml)	Bactericidal titer (With OMP26 adsorbed sera)
1	260	8	<5	8
2	146	8	<5	8
3	281	16	<5	16
4	1110	16	265	16
5	207	8	<5	8
6	267	32	<5	32

c. Bactericidal activity of NTHi anti OMP26 antibodies.				
Subjects	Anti OMP26 Ab titers (EU/ml)	Bactericidal titer* (Pre Adsorption)	Anti OMP26 Ab titers (Post OMP26-Ab Adsorption) (EU/ml)	Bactericidal titer (With OMP26 adsorbed sera)
7	1600	4	866	4
8	840	64	<5	64
9	1800	16	172	16
10	1550	8	195	8
11	243	8	<5	8

* Bactericidal titer values are expressed as a reciprocal titer of the dilution where 50% bacterial killing was achieved.