Otitis-prone Children Have Immunologic Deficiencies in Naturally Acquired Nasopharyngeal Mucosal Antibody Response after *Streptococcus pneumoniae* Colonization

Qingfu Xu, PhD, * Janet R. Casey, MD, † Emily Newman, BS, ‡, § and Michael E. Pichichero, MD*

**Objectives:** Acute otitis media (AOM) is the most common pediatric bacterial infection, and stringently defined otitis-prone (sOP) children have immunologic deficiencies. We recently found that nasopharyngeal (NP) colonization by *Streptococcus pneumoniae* (Spn) elicits a NP mucosal antibody response to vaccine candidate pneumococcal proteins that correlate with protection from AOM in non-sOP (NOP) children. Here, we sought to determine if sOP children experience significantly higher colonization rates with Spn than NOP children, develop lower naturally acquired NP mucosal antibody responses to those same pneumococcal proteins after colonization by Spn, and suffer greater frequency of AOM as a consequence.

**Methods:** NP samples were collected from 130 NOP and 45 sOP children during 270 healthy visits and 201 AOM visits between 6 and 24 months of age. Spn were identified by standard culture. NP mucosal IgG and IgA levels to vaccine candidate proteins pneumococcal histidine triad protein D, pneumococcal choline binding protein A (PcpA) and pneumolysin D1 were measured by quantitative enzyme-linked immunosorbent assay.

**Results:** sOP children had significantly higher colonization frequency by Spn (P < 0.0001) and significantly lower IgG and IgA levels to all 3 vaccine candidate proteins studied compared with NOP children (all P values <0.05) except IgG to Pld D1 (P = 0.31). Spn colonization in NOP children led to 2-fold to 5-fold increase in mucosal IgG and IgA levels to all 3 proteins (all P values <0.01), whereas Spn colonization in sOP children generally failed to elicit antibody responses (all P values >0.05). PcpA was unique in inducing significant increases in mucosal IgA (P = 0.02). When high mucosal IgG levels to all 3 proteins and IgA to PcpA were measured, they correlated with reduced AOM in sOP children.

**Conclusion:** sOP children experience significantly higher colonization rates with Spn, develop lower naturally acquired NP mucosal antibody responses to pneumococcal vaccine candidate proteins pneumococcal histidine triad protein D, PcpA and pneumolysin D1 after colonization by Spn, and suffer greater frequency of AOM if they do not generate high mucosal antibody to the studied proteins.

**Key Words:** *Streptococcus pneumoniae*, mucosal antibody, pneumococcal protein vaccine, acute otitis media, Otitis prone, pneumococcal histidine triad protein D, pneumococcal choline binding protein A, pneumolysin D1

*(Pediatr Infect Dis J 2016;35:54–60)*
have lower serum and bactericidal antibody titers against pneumococcal protein vaccine candidates PhtD, PcpA and PlyD1,27 as well as against nontypeable Haemophilus influenza (NTHI) outer membrane vaccine proteins28,29 compared with non-OP (NOP) children after nasopharyngeal (NP) colonization and AOM. We have shown that the reduced serum antibody response to pneumococcal proteins may be because of poor memory B-cell and T-helper cell generation.30,31 We have also shown that sOP children have lower epithelial cell repair responses during viral infection in the NP, and lower innate and adaptive inflammatory responses to respiratory viruses, which may contribute to increased frequency of AOM.12,13 Moreover, sOP children who have the above-described immune deficiencies may also respond poorly to routine pediatric vaccinations as proven by our demonstration that they fail to achieve protective antibody titers against diphtheria toxoid, tetanus toxoid, pertussis toxoid, filamentous hemagglutinin, pertactin and hepatitis B vaccines.34

The respiratory tract mucosal immune system provides the first line of defense against respiratory pathogens that invade at the NP epithelial surfaces. NP colonization is a necessary prerequisite for infections to develop from Spn colonization. Most recently, we found that higher mucosal antibody levels to 3 pneumococcal vaccine candidate proteins (PhtD, PcpA and Ply) correlated with reduced AOM caused by Spn in NOP children.17 Based on our prior work, we sought to determine if sOP children experience significantly higher colonization rates with Spn than NOP children, develop lower naturally acquired NP mucosal antibody responses to pneumococcal vaccine candidate proteins after colonization by Spn and suffer greater frequency of AOM as a consequence.

### METHODS

#### Subjects and Sample Collection

This study derives from a cohort of children prospectively enrolled during a 5-year time span (2006–2011) to evaluate immunity to Spn and NTHI NP colonization and AOM in young children. Prior studies from this cohort or subsets of this cohort have been previously published.12,27,30,31,32 Subsequent publications from our group have included this cohort with additional subjects as accrual of children into the study has occurred.15–17,31,32,34,40–46 Healthy infants without previous episodes of AOM were enrolled in a prospective study at 6 months of age from a middle class, suburban sociodemographic pediatric practice in Rochester, NY (Legacy Pediatrics). NP swabs and nasal wash (NW) samples were collected at routine well visits of children at 6, 9, 12, 15, 18 and 24 months of age. Besides the regular prospective healthy visits, parents were instructed to look for symptoms of AOM and to bring the child for evaluation whenever the children had suspicious symptoms as previously described.47 When symptoms of AOM occurred, the children were examined, and whenever they were diagnosed with AOM, tympanocentesis was performed as previously

### TABLE 1. Characteristics of Study Cohorts

<table>
<thead>
<tr>
<th></th>
<th>NOP (%)</th>
<th>OP (%)</th>
<th>P Values (OP vs. NOP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>130</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>60 (45)</td>
<td>15 (33)</td>
<td>0.16</td>
</tr>
<tr>
<td>Male</td>
<td>70 (55)</td>
<td>30 (67)</td>
<td></td>
</tr>
<tr>
<td>Breast feeding</td>
<td>40 (31)</td>
<td>8 (18)</td>
<td>0.20</td>
</tr>
<tr>
<td>Formula</td>
<td>50 (38)</td>
<td>27 (60)</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>40 (31)</td>
<td>30 (67)</td>
<td></td>
</tr>
<tr>
<td>Day care</td>
<td>40 (31)</td>
<td>15 (33)</td>
<td>0.6</td>
</tr>
<tr>
<td>Non–day care</td>
<td>80 (72)</td>
<td>30 (67)</td>
<td></td>
</tr>
</tbody>
</table>

### FIGURE 1.

Comparison of AOM episodes and NP colonization between NOP and sOP children. A, average episodes of AOM per child; B, AOM rates; C, Spn colonization; D, H. influenzae colonization; E, Mcat colonization; \(*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001\).
described\textsuperscript{35,48} to confirm the diagnosis with microbiologic culture for otopathogens. NP swab samples were concurrently obtained by inserting a cotton-tipped wire swab deeply into both nares. Identification of the major otopathogens [\textit{Spm}, \textit{NTHI} and \textit{Moraxella catarrhalis (Mcat)}] was done by standard culture methodology.\textsuperscript{49} NW samples were obtained by instilling 1 mL of sterile phosphate buffered saline and then aspirating from each nare yielding approximately 2 mL of material that was subsequently centrifuged at 3000 rpm (1100g) at 4°C for 10 minutes and the supernatants stored at −80°C until used for quantification of mucosal antibody by enzyme-linked immunosorbent assay (ELISA). All of the children received standard vaccinations including the PCV-7 or PCV-13 (Prevnar, Wyeth Pharmaceuticals, Collegeville, PA) at the appropriate age. The study was approved by the Institutional Review Board of Rochester General Hospital, and written informed consent was obtained from parents or guardians of all child subjects.

**Enzyme-linked Immunosorbent Assay**

PhtD, PcpA and PlyD1-specific antibody IgG and IgA concentrations and total IgG and IgA were determined in the NW by quantitative ELISA as previously described.\textsuperscript{15,17} To correct for differential dilution effects that occurred during NW sample collection, results were expressed as a ratio of specific IgG to total IgG or specific IgA to total IgA in the same sample (ng/μg) as described previously.\textsuperscript{15,17,50,51} For the purpose of statistical analysis, samples with undetectable specific antibody were arbitrarily assigned a value equivalent to half the lower limit of detection of the corresponding specific antibodies. Samples with a total IgG or IgA <0.05 μg/mL were excluded because in preliminary studies we determined that such samples were from children with a difficult or failed sampling process and had undetectable antigen-specific antibodies.

**Statistics**

Statistical analysis was performed with R Project version 2.13.2 (R Foundation for Statistical Computing, Vienna, Austria, www.r-project.org) using generalized estimating equations to fit a repeated measures logistic regression model, and graphs were made with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Antibody levels were expressed as geometric means (GM) with 95% confidence intervals of ratios of specific to total IgG or IgA. Because antibodies were found to correlate significantly with age, antibody levels were adjusted for age using a generalized estimating equation model, assuming a within-subject autoregressive correlation. Age-adjusted antibody levels between groups (colonization vs. noncolonization visits, OP vs. NOP children, AOM vs. health visits) were compared using nonparametric 2-tailed Mann–Whitney test. \( P < 0.05\) was considered to indicate statistical significance.

**RESULTS**

**Study Cohort**

This analysis involved a total of 471 NW samples collected during 270 healthy visits and 201 AOM visits from 175 children between age 6 and 24 months including 45 sOP and 130 NOP children. The characteristics of the children are shown in Table 1. By 2 years of age, all sOP children experienced AOM with an average of 4.6 episodes per child, whereas only 31% of NOP children

---

**FIGURE 2.** Comparison of mucosal antibody levels between NOP and sOP children. NP wash samples were collected from healthy NOP and sOP children colonized with \textit{Spm}. The ratios of pneumococcal specific to total IgG and total IgA were determined by ELISA and compared using Mann–Whitney test between sOP and NOP children. Lines represent GM with 95% confidence intervals. A, anti-PhtD, anti-PcpA and anti-PlyD1 IgG and B, anti-PhtD, anti-PcpA and anti-PlyD1 IgA. CN indicates culture negative; tIgG and tIgA, total amount of IgG and IgA.
experienced any AOM for an average of 0.4 episodes per child (Fig. 1A and B; \( P < 0.0001 \)).

**sOP Children Have Higher NP Colonization Rates With Spn**

NP colonization is a necessary first step in the pathogenesis of AOM. We first compared NP colonization frequency by Spn, along with the other dominant otopathogens *Haemophilus influenzae* and *Mcat* between sOP and NOP children during routine well child visits. We found from 9 months throughout 24 months of age, the sOP children had higher colonization rates than NOP children (Fig. 1C; overall \( P < 0.0001 \)). The sOP children also had higher colonization with *H. influenzae* from 9 to 24 months of age (Fig. 1D, and from 6 to 12 months of age (Fig. 1E). Overall, sOP children had significantly more frequent NP colonization with all 3 otopathogens.

**sOP Children Have Lower Levels of Naturally Acquired Mucosal Antibody in the NP**

We have previously found that children with AOM have significantly lower levels of mucosal antibody to PhdT, PcpA and PlyD1.\(^{17}\) We, therefore, anticipated that sOP children would have lower levels of natural-acquired mucosal antibodies to these proteins. To test this hypothesis, we compared NP mucosal antibody titers between sOP children and NOP children who were colonized by Spn in the NP. The results are summarized in Figure 2. Compared with NOP children, sOP children had a 1.5-fold lower GM of anti-PhdT IgG (3.31 vs. 4.94 ng/\( \mu \)g; \( P = 0.02 \)), 4.3-fold lower GM of anti-PcpA IgG (1.00 vs. 4.35 ng/\( \mu \)g, \( P < 0.0001 \)) and a 2.0-fold higher anti-PlyD1 IgG (1.15 vs. 0.57 ng/\( \mu \)g; \( P = 0.002 \) in the NOP children when Spn were colonized. There was a 4.8-fold higher anti-PhdT IgG (4.40 vs. 0.91 ng/\( \mu \)g; \( P < 0.0001 \)), a 4.6-fold anti-PcpA IgG (4.35 vs. 0.95 ng/\( \mu \)g; \( P < 0.0001 \)) and a 2.0-fold higher anti-PlyD1 IgG (1.15 vs. 0.57 ng/\( \mu \)g; \( P = 0.002 \)) in the NOP children when Spn were colonized.

**Nasopharyngeal Colonization by Spn Stimulates No or Much Less Mucosal Antibody Responses to All 3 Proteins in sOP Children**

According to our previous work,\(^{15-17,27,28,52}\) NP colonization is an immunizing event, eliciting serum and mucosal antibody responses to the 3 pneumococcal proteins we studied here. In this study, because we found that sOP children had significantly higher colonization with Spn, we sought to determine if higher mucosal antibody levels were elicited. We compared IgG and IgA levels to PhdT, PcpA and PlyD1 in the NP from sOP children who had Spn detected in their NP secretions at the time of sampling with those who were Spn culture-negative. In the NOP group (control), children with Spn colonization had significantly higher GM of specific IgG and IgA antibody titers to all 3 proteins compared with Spn culture-negative children (all \( P \) values <0.01; Fig. 2A and B). The GM of IgA in the NP of sOP children was 8.1-fold lower in anti-PhdT IgG (0.90 vs. 1.15 ng/\( \mu \)g; \( P = 0.31 \)) and 4.7-fold lower in anti-PcpA IgG (0.95 vs. 0.91 ng/\( \mu \)g; \( P < 0.0001 \)) and 2.6-fold lower in anti-PlyD1 (0.66 vs. 0.91 ng/\( \mu \)g; \( P < 0.0001 \)) and 4.7-fold lower in anti-PlyD1 (0.14 vs. 0.66 ng/\( \mu \)g; \( P < 0.0001 \); Fig. 2B).

**FIGURE 3.** Comparison of mucosal antibody levels between NP colonization and noncolonization by Spn in NOP (left) and sOP (right) children. NP wash samples were collected from age-matched healthy NOP and sOP children. The ratios of pneumococcal specific to total IgG and total IgA were determined by ELISA and compared using Mann–Whitney test between colonized and noncolonized NOP and sOP children. Lines represent GM with 95% confidence intervals. A, NOP, IgG; B, NOP, IgA; C, sOP, IgG; D, sOP, IgA. CN indicates culture negative; tIgG and tIgA, total amount of IgG and IgA.
Higher Mucosal Antibody Levels to PhtD, PcpA and Ply Correlate With Reduced AOM in sOP Children

We have previously found that higher mucosal antibody levels are associated with reduced AOM in NOP children. To determine if the mucosal antibody that was produced in sOP children correlated with protection from AOM, we compared NP mucosal antibody levels to PhtD, PcpA and PlyD1 between sOP children with Spn AOM and healthy sOP children asymptomatic colonized with Spn. We found that sOP children who developed Spn AOM had significantly lower mucosal IgG to all 3 studied Spn proteins compared with Spn colonized children who did not progress to AOM (Fig. 4A). The GM of antibody levels of sOP children with Spn AOM were 4-fold lower in anti-PhtD IgG (0.76 vs. 3.31 ng/µg; P < 0.0001), 3-fold lower in anti-PcpA IgG (0.33 vs. 1.00 ng/µg; P = 0.02), 4-fold lower in anti-PlyD1 IgG (0.23 vs. 0.90 ng/µg; P < 0.0001). sOP children with Spn AOM also had lower anti-PcpA IgA (0.71 vs. 2.66 ng/µg; P = 0.002) but not in anti-PhtD IgA (1.00 vs. 1.31 ng/µg; P = 0.49) nor anti-PlyD1 IgA (0.16 vs. 0.14 ng/µg, P = 0.72) compared with those of healthy children. The results indicate that sOP children who do develop higher IgG and/or IgA to the 3 proteins and IgA to PcpA have a reduced AOM frequency.

DISCUSSION

sOP children would highly benefit from pneumococcal vaccination because they are prone to middle ear infections and other pneumococcal infections such as pneumonia, have greater morbidity and consume a disproportionately high amount of health care expenditures. Therefore, it is gratifying to learn from the experiments reported here that sOP children who do respond after Spn NP colonization can produce levels of antibody to PhtD, PcpA and PlyD1 in the NP that correlate with protection from AOM. Nevertheless, consistent with our earlier work involving immune deficiencies of sOP children, we found that sOP children had significantly lower mucosal antibody levels despite more frequent NP Spn colonization because of the fact that NP colonization by Spn often failed in stimulating NP mucosal antibody responses to the pneumococcal proteins. Because of immunologic deficiencies in systemic and mucosal antibody responses after colonization with Spn and NTHI and routine pediatric vaccinations, sOP children likely have a substantially increased risk for invasive infections such as bacteremic pneumonia, bacteremia and meningitis. We have shown sOP children have more frequent nonbacteremic lobar pneumonia of presumed pneumococcal origin. However, we have not been able to identify more frequent Invasive Pneumococcal Disease because of the sample size of the sOP population. In addition, in a previous study, we have shown that passive human antibodies to PhtD, PcpA and Ply can reduce adherence of Spn to human lung epithelial cells and reduce NP colonization by Spn using a mouse model. Therefore, passive antibody prophylaxis might have a role as a therapeutic option for high-risk sOP children.

FIGURE 4. Comparison of mucosal antibody levels between sOP children with Spn AOM and asymptomatic Spn colonization. NP wash samples were collected from OP children with AOM caused by Spn and healthy sOP children colonized with Spn but without AOM. The ratios of pneumococcal specific to total IgG and total IgA were determined by ELISA and compared using Mann–Whitney test. Lines represent GM with 95% confidence intervals. A, anti-PhtD, anti-PcpA and anti-PlyD1 IgG; B, anti-PhtD, anti-PcpA and anti-PlyD1 IgA. tIgG and tIgA indicates total amount of IgG and IgA.
PhD, PcpA and PlyD1 as pneumococcal protein vaccine candidates have shown high immunogenicity in a mouse model and young children. We previously found that human antibodies to these 3 proteins reduce adherence to human lung epithelial cells and murine NP Spn colonization. Vaccination with PhD, PcpA and PlyD1 elicits protection against pneumonia in an infant murine model. Higher NP mucosal antibody titers correlated with protection of AOM in young NOP children.

Many prior studies have established that NP colonization with bacterial pathogens elicits serum and mucosal antibody responses. Holmlund et al reported that the increase in serum anti-antibody concentration in infants was associated with pneumococcal carriage but followed different kinetics depending on the antigen. Simell et al showed that children culture positive for Spn had significantly higher serum anti-CbpA and anti-Phd IgG. Prevaes et al reported that colonization with Spn and Staphylococcus aureus induced serum IgG against 14 pneumococcal proteins and 10 S. aureus proteins. Verkaik et al found that children at 6–24 months of age colonized by S. aureus had higher IgG and IgA levels to a number of staphylococcal proteins compared with noncolonized children. Zhang et al found that children 2–12 years of age who were culture-positive for Spn in their NP had higher IgG but not IgA in serum and saliva to CbpA and Ply but not pneumococcal surface adhesin A or pneumococcal surface protein A. Our group also previously reported that Spn NP colonization was an immunizing event for both systemic and mucosal-acquired immune responses.

However, in this study, we add new findings regarding sOP versus NOP children by showing significantly higher colonization rates with Spn in sOP children and significantly lower mucosal antibody levels that may explain why sOP children have higher Spn NP colonization events and thereby a higher number of Spn AOM events.

Our study has limitations. NP colonization was defined based on bacterial cultures at specific sampling times predetermined by our prospective study design at 3 month intervals between 6 and 24 months of age. Almost certainly there were subjects who had Spn NP colonization events before we commenced samplings at age 6 months and who had NP colonization events between the timing of scheduled samplings. We did not determine NP bacterial or viral loads, which have been proven to be critical variants influencing host immune responses. The antibody levels in this study were free antibody, which might be influenced by bacterial load, bacterial agglutination with antibody and soluble antigens (eg, Ply) released from Spn.

ACKNOWLEDGMENTS

This study was supported by the NIH NIDCD R01 08671 and an investigator-initiated grant via an agreement with Sanoh Pasteur. We thank the nurses and staff of Legacy Pediatrics; the collaborating pediatricians from Sunrise Pediatrics, Westfall Pediatrics, Lewis Pediatrics and Long Pond Pediatrics and the parents who consented and the children who participated in this long and challenging study. We also thank Dr. Anthony Almudevar (Department of Biostatistics and Computational Biology, University of Rochester Medical Center) for data statistical analysis, Jill Mangiafesto, Katerina Czup and Virginia Judson for assistance with ELISAs.

REFERENCES

17. Xu Q, Casey JR, Pichichero ME. Higher levels of mucosal antibody to pneumococcal vaccine candidate proteins are associated with reduced acute otitis media caused by Streptococcus pneumoniae in young children. Mucosal Immunol. 2015;8:1110–1117.