Nasopharyngeal Antibodies to Pneumococcal Pneumolysin in Children with Acute Otitis Media

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Pneumolysin, an intracellular protein toxin of all clinically relevant pneumococcal serotypes, is released in vivo during the autolysis of pneumococci and is believed to pave the way for intact pneumococci to invade and cause disease. Therefore, antibodies to pneumolysin should prevent its destructive function. We measured antibodies to pneumococcal pneumolysin in acute- and convalescent-phase nasopharyngeal aspirate samples of 120 children (median age, 2.5 years) with acute otitis media by enzyme immunoassay. Nasopharyngeal pneumolysin M (IgM) and IgG class antibodies to pneumolysin were rarely detectable, whereas IgA class antibody was detected often, occurred independently of serum IgA antibody in serum, and correlated with the presence of the secretory component in pneumococcal antibody, indicating local production of IgA antibodies. Nasopharyngeal IgA antibody to pneumolysin was detected in 93% of the children already in the acute phase of otitis. Twenty percent of the children developed at least a threefold rise in the pneumolysin-specific IgA antibody concentration by the convalescent phase of otitis, with the youngest at 6 months of age, regardless of the pneumococcal findings in the nasopharynx or middle ear fluid. We suggest that nasopharyngeal IgA antibody to pneumolysin can be produced early in life by pneumococcal colonization.

Streptococcus pneumoniae is the most prevalent bacterial pathogen in the middle ear fluid (MEF) of children with acute otitis media (AOM) (4, 11, 23) and is commonly found in nasopharyngeal mucosae (7). Pneumolysin, an intracellular protein toxin present in all clinically relevant pneumococcal strains (10), is released in vivo during the autolysis of pneumococcal cells (9). By causing extensive tissue damage (14), pneumolysin is believed to pave the way for intact pneumococci to invade and cause disease. Immunization with pneumolysin has been shown to result in the production of specific antibodies and the extended survival of mice challenged with S. pneumoniae (15), suggesting a protective role for these antibodies. Whether they act by neutralizing the toxin and thereby its inflammatory function is unknown. On the other hand, the detection of antibodies to pneumolysin in convalescing patients should represent a response to pneumococcal involvement in the disease, regardless of the capsular serotypes (10, 14).

We have recently studied the induction of nasopharyngeal antibodies to the capsular polysaccharides of S. pneumoniae in 120 children with AOM (22). S. pneumoniae-specific nasopharyngeal immunoglobulin M (IgM) and IgG class antibodies were rare, whereas IgA class antibodies to the capsular polysaccharides of S. pneumoniae were detected more often, occurred independently of IgA antibody in serum, and correlated with the presence of the secretory component (SC) in pneumococcal antibody, indicating local production of IgA antibodies. Children with pneumococci found in MEF samples developed nasopharyngeal IgA antibody responses to capsular polysaccharides more often than did children with pneumococci found only in the nasopharynx or not at all, indicating that the presence of S. pneumoniae in the middle ear was stimulative for nasopharyngeal antibody production. Responses were observed in children of all ages, with the youngest at 6 months of age. This supports the hypothesis that mucosal immunity to bacterial polysaccharides matures earlier than does systemic immunity (16).

In this study, our aim was to find out whether children with AOM have antibodies to pneumococcal pneumolysin in their nasopharyngeal aspirates (NPA). If so, we wanted to see which antibody classes appear, whether they are produced locally or are derived from serum, and how they correlate with age and findings of pneumococci in children with AOM.

MATERIALS AND METHODS

Patients. Altogether, 135 children with AOM, aged 3 months to 7 years 5 months (median, 2 years 6 months), were examined from 1990 to 1992 (21–23). Of the 135 children initially enrolled, 120 children with 120 episodes of AOM had both bacterial culture and pneumolysin PCR results and at least one acute- and/or convalescent-phase NPA antibody result available. Thus, they formed the patient group in this study. The age range of these 120 children was from 3 months to 7 years 5 months (median, 2 years 6 months); 22 were under 12 months, 29 were 12 to 23 months, and 69 were over 23 months of age.

Samples. MEF and NPA samples were obtained at the initial visit and (if an NPA sample was still obtainable) at the control visit 2 weeks later. Serum samples were taken at both visits. NPA samples were collected as described previously (22) and transported to the bacteriological laboratory, where the samples were cultured and the isolated bacteria were identified by standard methods (3) within 24 h. Pneumolysin DNA in MEF samples was detected by PCR (18, 23). After being stored at −70°C, NPA samples were centrifuged at 12,000 × g for 10 min at room temperature. The supernatant was used for measurements of pneumococcal antibodies and total IgA antibody.

Measurement of antibodies. The concentration of total IgA antibody was measured by the radial immunodiffusion technique of Mancini (LC-Partigen IgA, Behringwerke, Marburg, Germany). A quarter of the samples were analyzed for total IgA antibody both undiluted and after dilution (1:2) with 0.2 M dithiothreitol (Sigma, St. Louis, Mo.), which breaks the sulfide bonds between the two IgA monomers (1). The pneumolysin-specific antibodies in all 120 children were measured by enzyme immunoassay. Pneumolysin was produced in Bacillus subtilis by Pronab Chattopadhyay at the Laboratory of Molecular Bacteriology of our institute (20). The polystyrene microtiter plates sensitized for covalent binding of protein antigen (Covalink; Nunc, Roskilde, Denmark) were coated with 5 µg of pneumolysin in 1 ml of 0.2% glutaraldehyde–phosphate-buffered saline (PBS) and incubated at 22°C overnight. The plates were always

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used on the same day. Postcoating was done with 5% sheep serum in PBS. The enzyme immunoassay used to detect serum antibodies to pneumolysin (21) was modified for NPA samples. NPA samples were diluted 1:15 in PBS containing 10% fetal calf serum. To control nonspecific binding, NPA samples were laid out at 50 μl per well in duplicate both on antigen-specific plates and on plain postcoated (background) plates. For measuring the concentrations of IgA antibody and antibody containing the SC, monoclonal antisera to human IgA (Oxoid M26012; Unipath Ltd., Hampshire, England) and human SC (1-6635; Sigma) were used, with subsequent use of alkaline phosphatase-conjugated antiserum to mouse IgG (315-055-045; Jackson H & L, West Grove, Pa.).

The serum sample results were expressed as end point titers read at an optical density (OD) of 0.3, except for those of IgG to pneumolysin which were read at an OD of 0.2. The NPA sample results for pneumolysin-specific antibodies were calculated by subtracting the background OD from the specific OD and dividing by the concentration of total IgA antibody (in micrograms per ml; if below the detection limit of 4.5 μg/ml, a value of 2.25 μg/ml was used) in the same sample. If negative for both pneumolysin-specific IgA antibody and total IgA antibody, the sample was rejected.

Statistics. Kruskal-Wallis one-way analysis of variance was used to compare the geometric means of antibodies. The numbers of children in different subgroups were compared by using the chi-square test with the Yates correction or the two-tailed Fisher exact test when appropriate. The significance of correlation was determined with the t test.

RESULTS

Total IgA antibody. IgA antibody was present in the NPA samples of all age groups, but the concentrations varied greatly among individuals (data not shown). The mean concentration of total IgA antibody neither related to age nor differed between acute- and convalescent-phase samples. A quarter of the samples were analyzed for total IgA antibody both undiluted and after dilution (1:2) with 0.2 M dithiothreitol, and the concentrations obtained in both ways were identical (data not shown). This indicates that we measured the concentration of dimeric IgA antibody, likely of local origin.

Pneumolysin-specific IgA antibody. Pneumolysin-specific IgA antibody in NPA samples occurred independently of IgA antibody in serum (Fig. 1) (correlation coefficient, 0.06; \( P > 0.1 \), i.e., no significant correlation). In contrast, it was closely correlated with the presence of the SC in nasopharyngeal pneumolysin antibody in both the acute and convalescent phases; 95% of the samples positive for nasopharyngeal IgA antibody to pneumolysin were also positive for pneumolysin-specific antibody containing the SC. The correlation coefficient between IgA antibody and antibody containing the SC was 0.94.

In the acute phase, nasopharyngeal IgA antibody to pneumolysin was detectable in 93% of the children; in the convalescent phase, it was detectable in 90% of the children.

Pneumolysin-specific IgA antibody in relation to age. No significant age-related differences in the percentages of positive samples in either phase or between phases for any age group were observed (Fig. 2). However, geometric mean concentrations of pneumolysin-specific IgA antibody increased with age (Table 1).

Pneumolysin-specific IgA antibody in relation to \( S. \) pneumo- niae in MEF and NPA samples. To analyze the NPA antibody data in relation to pneumococcal culture and/or PCR findings of MEF and NPA samples, the children were divided...
into the following three etiological groups (Fig. 2): children with MEF samples positive for *S. pneumoniae* by culture and/or pneumolysin PCR (*n* = 41), children with NPA samples positive for *S. pneumoniae* only by culture (*n* = 27), and children with no pneumococci found in MEF or NPA samples (*n* = 52). The presence of pneumolysin-specific IgA antibody in the nasopharynx was not related to pneumococcal findings in the acute or convalescent phase (Fig. 2). Geometric mean concentration did not differ in relation to pneumococcal findings in either phase (data not shown).

When the acute- and convalescent-phase samples of each child were analyzed in pairs (Table 2) and a threefold or greater rise (based on the sensitivity of the assay) between acute- and convalescent-phase results was regarded as a response, 20% of the children developed a pneumolysin-specific IgA antibody response in the nasopharynx, regardless of the pneumococcal findings in MEF and/or NPA samples. The number of responders to pneumolysin did not differ among the three etiological groups (Table 2).

**Pneumococcal IgM and IgG class antibodies.** IgM and IgG class antibodies to pneumococcal pneumolysin were rarely detected in NPA samples. In the acute phase, IgM to pneumolysin was detected in one child and IgG to pneumolysin was detected in one child. In the convalescent phase, IgM to pneumolysin was detected in three children and IgG to pneumolysin was detected in one child.

**DISCUSSION**

In this study, antibodies to pneumococcal pneumolysin were detectable in the NPA samples of children with AOM. The main antibody class detected was IgA; it occurred independently of IgA antibody in serum and correlated with the specific SC, indicating local production of these antibodies. IgM and IgG class antibodies to pneumolysin were detected infrequently.

Protein concentrations in nasal secretions are known to vary both diurnally and from one individual to another, for instance, because of varying degrees of mucosal inflammation (19). However, the ratio of specific IgA antibody to total IgA antibody should remain relatively stable unless a specific antigen stimulus is involved. In our study, the variation between samples was increased by uncontrolled dilution with rinse-out PBS. Therefore, we chose to present our results as pneumolysin-specific IgA antibody in relation to total IgA antibody.

While nasopharyngeal IgA antibody to pneumococcal capsular polysaccharides was detected in one-third of the children (22), pneumolysin-specific IgA antibody was detected in nearly all of the children. This might have been due to a higher sensitivity of the assay for protein than for polysaccharide antibodies in vitro or to a real difference in the prevalence of such antibodies. In any case, the presence of pneumolysin-specific IgA antibody probably reflects the frequency of pneumococcal contact, beginning at an early age (2, 8). This is in concordance with the concept that children are able to produce antibodies to protein antigens earlier than they are to polysaccharides. In addition, mucosal immunity is thought to mature earlier than systemic immunity (16). The prevalence of nasopharyngeal IgA antibody to pneumolysin was high even among the youngest age group, although the mean concentrations of antibodies increased with age.

The acute-phase prevalence or amount of pneumolysin-specific IgA antibody was not related to the pneumococcal etiology of AOM in the children of our study. This is understandable if nasopharyngeal IgA antibody to pneumolysin is produced as soon as the child becomes colonized with *S. pneumoniae* for the first time, as we have suggested above. It is more difficult to understand why 20% of the children developed an IgA antibody response to pneumolysin regardless of the pneumococcal findings in NPA or MEF samples. This indicates that the middle ear is not important as an inductive site for pneumolysin-specific IgA antibody production in the nasopharynx, which contrasts with our previous findings for IgA antibody to pneumococcal capsular polysaccharides (22). It must be admitted that these MEF and NPA samples might have contained *S. pneumoniae*, although we were unable to detect any; for instance, only MEF samples were studied by PCR. However, we cannot exclude the possibility that the observed IgA antibody responses to pneumolysin were nonspecific or responses to nasopharyngeal inflammation associated with AOM. Thiol-activated bacterial toxins (pneumolysin, streptolysin O, lysteriolysin, perfringolysin, and alveolysin) have extensive primary amino acid sequence homology and show serological cross-reactivity (5). In our study, this kind of cross-reactivity is unlikely, since except for *S. pneumoniae* and group A streptococci, bacteria which produce these toxins are rarely if ever associated with otitis. From our data, group A streptococci were cultured in the MEF sample of one child, but she did not carry the same bacterium in her nasopharynx at the same time. Another reason for the nonspecificity observed might be the fact that pneumolysin is able to bind Fc fragments of antibody nonspecifically (13). Nevertheless, we did not see any nonspecific Fc binding when monoclonal antibody to Hib was tested by our pneumolysin enzyme immunoassay (unpublished observations).

Interestingly, human C-reactive protein (CRP) also has limited sequence homology to pneumolysin (5, 13). Although it is not known to what extent CRP exists at mucosal sites, it has been reported to be detected in histologic specimens from sites of experimentally induced inflammation (6). The hypothesis

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**TABLE 1.** Geometric mean concentrations of IgA antibody to pneumolysin in NPA samples positive for pneumolysin-specific IgA

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Acute phase</th>
<th>Convalescent phase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n = 108)</td>
<td>(n = 92)</td>
</tr>
<tr>
<td>&lt;12</td>
<td>0.071</td>
<td>0.115</td>
</tr>
<tr>
<td>12–23</td>
<td>0.133</td>
<td>0.120</td>
</tr>
<tr>
<td>≥24</td>
<td>0.168</td>
<td>0.221</td>
</tr>
<tr>
<td>Total</td>
<td>0.140</td>
<td>0.169</td>
</tr>
</tbody>
</table>

**TABLE 2.** Nasopharyngeal IgA antibody responses to pneumolysin in relation to pneumococcal etiology of otitis

<table>
<thead>
<tr>
<th>Etiological group</th>
<th>No. of responses/ no. of children</th>
<th>% of children with responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF+</td>
<td>7/35</td>
<td>20</td>
</tr>
<tr>
<td>NPA+</td>
<td>4/21</td>
<td>19</td>
</tr>
<tr>
<td>MEF – NPA−</td>
<td>9/42</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>20/98</td>
<td>20</td>
</tr>
</tbody>
</table>

*a* A threefold or greater rise in the specific antibody concentration between the acute- and convalescent-phase NPA samples was regarded as a response.
that anti-pneumolysin IgA antibody responses were partly induced by CRP is intriguing. However, it seems unlikely that we measured cross-reacting anti-CRP; the two short, noncontiguous amino acid sequences in the carboxyl-terminal end of pneumolysin with limited homology to CRP (12) correspond to peptides which have not been found to be reactive with the sera of patients with pneumococcal pneumonia (17). Therefore, one can only speculate that the 20% response rate to pneumolysin might be due to inflammation-associated, non-specific stimulation of pneumolysin-specific IgA-producing plasma cells present in the nasopharynges of a majority of the children already in the acute phase of otitis, possibly after lymphocyte migration through the adjacent lymphatic tissue and circulation (12). Although prospective studies are needed to understand the role of pneumolysin-specific IgA antibody at mucosal sites, we suggest that nasopharyngeal IgA antibody to pneumolysin can be produced in young children by pneumococcal colonization. Inflammation may further increase antibody production by non-specific activation of memory cells. Although the presence of nasopharyngeal IgA antibody to pneumolysin did not seem to protect the children in our study from pneumococcal otitis, pneumolysin-specific IgA antibody might have an important role as a neutralizing antibody in the prevention of extensive tissue damage by this destructive toxin of a common respiratory pathogen.

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REFERENCES