Otitis Media in Children: Local Immune Response to Nontypeable Haemophilus influenzae

HOWARD FADEN, * LINDA BRODSKY, JOEL BERNSTEIN, JOHN STANIEVICH, DEBORAH KRYSOFIK, CYNTHIA SHUFF, JONG J. HONG, AND PEARAY L. OGRA

Department of Pediatrics and Otolaryngology, School of Medicine and Children's Hospital, State University of New York at Buffalo, Buffalo, New York 14222

Received 22 May 1989/Accepted 7 August 1989

Twenty children experienced 30 episodes of otitis media with effusion due to nontypeable (NT) Haemophilus influenzae in the first 2 years of life. The local and systemic immune responses to homologous strains of NT H. influenzae were determined by an immunodot assay. Strain-specific immunoglobulin G (IgG) antibody predominated in the middle ear fluid (MEF). It was detected in 91% of the children, compared with IgM in 48% (P < 0.005), IgA in 52% (P < 0.005), and secretory IgA in 18% (P < 0.005). The titer (log2) of NT H. influenzae-specific IgG antibody (mean ± standard error, 8.2 ± 0.1) exceeded the titers of IgM (3.4 ± 0.1), IgA (3.7 ± 0.1), and secretory IgA (1.2 ± 0.3). NT H. influenzae-specific antibody was detected exclusively in MEFs of individuals who possessed homologous serum antibody. Although antibody titers in MEF declined over time, serum antibody titers remained stable. These data suggest that immunity to NT H. influenzae in the middle ear, in part, reflects systemic immunity. Whereas local antibody disappears after resolution of the infection, systemic antibody persists.

Otitis media with effusion (OME) is among the most common illnesses that affect young children. Approximately 60 to 70% of children experience OME during the first 3 years of life, and at least 40% of them experience more than one episode (26). The incidence of OME is highest in the first 2 years of life and then declines (10). Presumably, immunity plays a role in the reduction of the frequency of OME.

Three bacterial species—Streptococcus pneumoniae, nontypeable (NT) Haemophilus influenzae, and Branhamella catarrhalis—are considered as the major share of recurrent episodes of OME (9, 11). Recent studies have suggested that NT H. influenzae consists of many different strains that may be characterized by their outer membrane proteins (13-16). Immunity to NT H. influenzae appears to be strain specific and directed to the outer membrane proteins (1, 5, 6, 8, 15, 17). Susceptibility and resistance to the development of NT H. influenzae OME have been associated with the presence and absence of serum bactericidal antibody (1, 8, 21). Less is known about the role of local immunity in NT H. influenzae OME.

The present study was designed to examine the local development of immunoglobulin G (IgG), IgM, IgA, and secretory IgA (sIgA) antibodies to homologous strains of NT H. influenzae in the middle ear during active infection. The study also analyzed humoral response to NT H. influenzae over a prolonged period of convalescence.

**MATERIALS AND METHODS**

**General design.** A total of 220 children were enrolled in a prospective study of otitis media. At enrollment, 39% were ≤6 months old, 32% were 7 to 12 months old, and 29% were >12 months old. At the time of entry into the study, 32% had experienced no OME, 51% had had one to five episodes of OME, and 17% had had more than five episodes of OME. During 3 years of monitoring the children, 320 tympanocenteses were performed for OME. Aerobic cultures of middle ear fluid (MEF) yielded NT H. influenzae in 23% of the cultures, S. pneumoniae in 14%, B. catarrhalis in 11%, and other organisms, such as coagulase-negative staphylococci and diphtheroids, in 30%. Twenty-seven percent of the effusions were sterile. Children less than 2 years old who experienced otitis media due to NT H. influenzae were evaluated for their immune responses to the homologous strain of NT H. influenzae. Blood was collected at the time of tympanocentesis and 1 month later for antibody determination.

At the time of tympanocentesis, the MEF was aspirated into an Alden-Senturia trap. The fluid was diluted in 2 ml of phosphate-buffered saline. A sample of the fluid was sent for culture. The remaining fluid was centrifuged at 1,000 × g, and the supernatant fluid was separated and frozen at −70°C until it was assayed for antibody by an immunodot assay. Serum collected at the same time was frozen at −70°C for antibody determination. Twenty children less than 2 years old who experienced 30 episodes of OME due to NT H. influenzae served as the basis for the present report.

**Bacteriology.** MEF was cultured on plates of phenylethyl alcohol, chocolate, MacConkey, and tryptic soy agar with 5% sheep blood and placed in brain heart infusion broth. Bacterial species were identified by standard laboratory procedures. H. influenzae was characterized by X and V factors and with serotyping. The organisms were frozen in 15% glycerol in nutrient broth at −70°C for subsequent studies.

**Isotypic antibody determinations.** The immunodot assay was used to determine levels of IgG, IgM, IgA, and sIgA antibodies to NT H. influenzae in serum and MEF. In order to perform the test, specific strains of NT H. influenzae were grown overnight in brain heart infusion broth supplemented with hemin (10 µg/ml) and adenosine dinucleotide (10 µg/ml). The bacteria were washed in phosphate-buffered saline, suspended in 1.0 ml of Hanks balanced salt solution with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and phenylmethylsulfonyl fluoride, and sonicated four times.
times for 15 s. The same antigen suspension was used throughout the study of the specimens from an individual patient. A 5-μl portion of the antigen suspension was placed on 0.45-μm-pore-size nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) and allowed to air dry in a 24-well plastic plate (Costar, Cambridge, Mass.). The paper was incubated in a blocking buffer (0.01 M Tris, 0.15 M NaCl, and 3% gel) for 1 h at room temperature on a rotator. The paper was rinsed with buffer. Appropriate twofold dilutions of the serum or MEF in buffer were added in 0.5-ml volumes and incubated for 1 h at room temperature on a rotating mixer. The lowest dilution of serum and MEF tested was 1:25. The processed paper was rinsed three times in buffer. Rabbit anti-human IgG (γ chain), IgM (μ chain), IgA (α chain), or secretory component conjugated with peroxidase (Accurate Chemical & Scientific Corp., Westbury, N.Y.) were diluted 1:500 in buffer, added in 0.5-ml volumes, and incubated for 1 h at room temperature on a rotator. The preparation was again rinsed in buffer three times. The nitrocellulose paper was next immersed in horseradish peroxidase color development solution (Bio-Rad) for 45 min in the dark. The reaction was stopped by the addition of and subsequent rinsing with water. The titer of antibody was expressed as the reciprocal of the highest dilution that produced detectable color. Each specimen was tested on three separate occasions. Paired samples were always tested at the same time. A negative and positive control were included in each assay. Negative controls included no antigen, no MEF, and hypogammaglobulinemic serum. A positive control consisted of antibody-positive serum.

Preliminary studies were conducted to determine the sensitivities and specificities of the reactions. The immunodot assay was able to detect 1 to 10 ng of immunoglobulin; IgG and IgA assays were the most sensitive, and IgM and slgA assays were the least sensitive. The binding of the conjugated antibody could be blocked with unconjugated antibody to the homologous immunoglobulin class but not with antibody to a heterologous immunoglobulin class. Because sonicates of whole bacteria were used as the antigen and because of the possibility that cross-reacting antibody might be present in the specimens, absorption experiments with homologous and heterologous strains of NT H. influenzae as well as strains of Staphylococcus aureus and Escherichia coli were conducted (Table 1). Antibody was absorbed by incubating 1 ml of serum with an equal volume of sonicated bacterial antigen at 4°C for 24 h with continuous mixing. The bacterial antigen was prepared from an 18-h growth of bacteria in 50 ml of brain heart infusion broth. The bacteria were concentrated to 1 ml and sonicated as described above. The entire absorption procedure was repeated a second time. The serum-bacterium mixture was centrifuged at 10,000 × g, and the supernatant was used as the absorbed serum. Unabsorbed serum was processed in a similar fashion except that bacteria were not used. Absorption with homologous strains of sonicated NT H. influenzae reduced the antibody titer from 8- to 128-fold, while heterologous strains of NT H. influenzae reduced the titer 2-fold or less (Table 1). Different bacterial species failed to absorb any NT H. influenzae antibody.

Table 1. Specificity of immunodot antibody determined by absorption with various bacterial species

<table>
<thead>
<tr>
<th>Subject</th>
<th>No absorption</th>
<th>Absorption with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous NT</td>
<td>Heterologous NT</td>
</tr>
<tr>
<td></td>
<td>H. influenzae</td>
<td>H. influenzae</td>
</tr>
<tr>
<td>1</td>
<td>25,600</td>
<td>2,200</td>
</tr>
<tr>
<td>2</td>
<td>51,200</td>
<td>1,600</td>
</tr>
<tr>
<td>3</td>
<td>12,800</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>12,800</td>
<td>1,600</td>
</tr>
</tbody>
</table>

*ND: Not done.

RESULTS

Population. Twenty children developed 30 episodes of inflammatory disease in the middle ear with an effusion infected with NT H. influenzae in the first 2 years of life. The children ranged in age from 3 to 23 months with a mean ± standard deviation of 14.6 ± 6.6 months. There were 11 males and 9 females. At the time of OME with NT H. influenzae, 9 of the children had previously experienced fewer than 5 episodes of OME (2 had only 1 episode), 13 had experienced 6 to 10 episodes, and 8 had experienced more than 10 episodes. Fifteen of the episodes were acute, i.e., present for 3 or fewer weeks, and 15 were nonacute, i.e., present for more than 3 weeks. The physical characteristics of the MEF were purulent in 22 cases, mucoid in 5 cases, and serous in 3 cases.

Serum IgG, IgM, and IgA antibody responses to homologous strains of NT H. influenzae. Antibody against infecting strains of NT H. influenzae in serum was studied first. IgG NT H. influenzae-specific antibody was present in acute and convalescent (30 days after the tympanocentesis) sera of all subjects. Sixty percent of the children manifested a twofold or greater rise in IgG antibody during convalescence. The acute and convalescent antibody titers (log_{2}) were 13.0 ± 0.1 and 13.8 ± 0.1, respectively (mean ± standard error [SE]; P = 0.05).

IgM NT H. influenzae-specific antibody was present in 18 of 19 acute serum samples and all convalescent serum samples. Only 25% of the subjects manifested a twofold or greater rise in IgM antibody titers during convalescence; 43% of the individuals had a drop in IgM titer during convalescence. The mean antibody titers in acute (8.0 ± 0.1) and convalescent (8.0 ± 0.1) periods were not significantly different.

Of 19 children, 16 had IgA specific antibody in the acute serum and 17 had IgA specific antibody in the convalescent serum. Thirty-six percent of the children exhibited a twofold or greater rise in IgA specific antibody during convalescence, although the antibody titer (log_{2}) during convalescence, 7.9 ± 0.1 (mean ± SE), was higher than that during the acute phase, 7.2 ± 0.2, the difference was not statistically significant.

Middle ear IgG, IgM, and IgA antibody responses to homologous strains of NT H. influenzae. Antibody against infecting strains of NT H. influenzae in MEF samples was studied next. IgG antibody to NT H. influenzae predominated in the middle ear. Significantly more children (91%) possessed IgG specific antibody than either IgM (48%, P < 0.005) or IgA (52%, P < 0.005) specific antibody (Table 2). Antibody titers (log_{2}) were 8.2 ± 0.1, 3.4 ± 0.1, and 3.7 ± 0.1 (mean ± SE), respectively. IgG, IgM, and IgA to NT H. influenzae were present in MEF only when concurrent serum antibody existed. However, not every child with serum antibody had specific antibody in the middle ear.

In order to assess, in a preliminary manner, the relative importance of the local production of NT H. influenzae-
specific IgA, the ratios of IgG NT H. influenzae antibody to IgG NT H. influenzae antibody in serum and in MEF were compared. The ratio of IgG antibody to IgG antibody titer in MEF was equal to or greater than the ratio in serum in 15 of 19 pairs. Thus, there was little evidence for the local production of IgA antibody directed against NT H. influenzae. Specific sIgA to NT H. influenzae was next evaluated in 17 MEF to further evaluate local immunity. sIgA specific for homologous strains of NT H. influenzae was detected in only 3 of 17 (18%) specimens. The sIgA antibody titer (log₂) was 1.2 ± 0.3 (mean ± SE).

The immune responses to homologous strains of NT H. influenzae in the right and left ears of 20 children who had bilateral OME were compared (Table 3). NT H. influenzae was recovered from both ears in 9 children and from a single ear in 10 children. The NT H. influenzae in the right and left ears of children with bilateral infections were the same, as determined by outer membrane protein characterization. The IgG specific anti-NTHI antibody titers in children with bilaterally infected ears were 7.6 ± 1.5 and 7.6 ± 1.6 (mean ± SE, P > 0.50). In contrast, the IgG specific anti-NTHI antibody titers in children with one infected and one sterile ear were 10.1 ± 1.3 and 7.8 ± 1.5, respectively (mean ± SE, P < 0.005).

The persistence of homologous IgG anti-NT H. influenzae antibody was next evaluated in six individuals who underwent second and third tympanocenteses within 1 to 4 months after the initial tympanocentesis that yielded NT H. influenzae. The serum antibody titer remained stable in the first two subjects over 3 to 4 months, while MEF antibody titers fell eightfold (Table 4). In subjects 3 and 4, the serum antibody titer rose over 2 months while the MEF antibody titer remained stable or disappeared. Subject 5 demonstrated a rise and subsequent fall in serum antibody titer, while the MEF antibody titer declined progressively over 2 months and ultimately disappeared. The last subject was most interesting because the same strain of NT H. influenzae, as identified by outer membrane proteins, persisted in the middle ear for 1 month. During that period, the antibody titer in the serum increased 8-fold and the antibody titer in the MEF increased 32-fold. Three months after the organism disappeared from the middle ear, the serum antibody titer remained stable but the titer in the MEF fell fourfold. These data suggested that specific antibody in the middle ear rose in the presence of active infection and disappeared when the infection resolved. In contrast, serum antibody titers remained relatively stable after the initial antibody response.

**TABLE 2. IgG, IgM, IgA, and sIgA antibody responses to homologous strains of NT H. influenzae in MEF**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. positive/no. tested (%)</th>
<th>Antibody titer (log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>21/23 (91)</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>IgM</td>
<td>11/23 (48)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IgA</td>
<td>12/13 (52)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>sIgA</td>
<td>3/17 (18)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Compared with value for IgG by chi-square analysis.  
* Mean ± SE.

**DISCUSSION**

NT H. influenzae is the most frequent cause of recurrent OME (9, 11). Protection against infection with NT H. influenza, as determined by a bactericidal antibody assay, appears to be strain specific, which was the primary reason for using homologous strains of NT H. influenzae to measure antibody response in the present study (1, 8). Much less is known about local protection against NT H. influenzae in the middle ear. The meager amount of MEF available for immunologic evaluation precluded the use of a relatively insensitive technique such as the bactericidal antibody assay; instead, an immunodot assay that was able to detect as little as 1 to 10 ng of immunoglobulin was utilized to measure NT H. influenzae-specific IgG, IgM, IgA, and sIgA antibodies to sonicates of whole organism. The use of a whole bacterial cell preparation may not be ideal. It has the advantage of being convenient but has the disadvantage of containing a variety of antigens, some of which may be cross-reacting antigens (28). However, preliminary absorp-
tion studies performed in this project were reassuring and demonstrated that homologous strains of NT H. influenzae removed 2- to 68-fold more antibody than did heterologous strains of NT H. influenzae, while different bacterial genera did not absorb any anti-NT H. influenzae antibody. Thus, the major portion of antibody detected by the immunodot assay represented homologous antibody.

The results from the present study demonstrated high titers of NT H. influenzae-specific antibody in the MEF of 91% of the children. The IgG class of NT H. influenzae-specific antibody predominated. IgM, IgA, and sIgA NT H. influenzae-specific antibodies were detected in 50% or less of the fluids. The titers of IgG antibody in MEF in the present study exceeded titers of immunoglobulins of other classes by 40- to 200-fold. Although the sensitivity of the antibody assay varied among the different immunoglobulin classes, the differences were insufficient to account for the degree of dominance of the IgG type of antibody. Specific immunoglobulins of the G, M, and A classes have been identified previously in MEF of children with pneumococcal and H. influenzae OME; organism-specific IgG antibody was similarly the more common immunoglobulin class detected in both types of infection (22, 23).

The origin of the homologous antibody in the middle ear was not elucidated entirely in the present study. In children with unilaterally infected bilateral MEFs, higher titers of NT H. influenzae-specific antibody were detected in the MEF with the organism while the titers were similar in the bilaterally infected ears. This suggested that the antibody was being produced locally in response to active infection. It is possible, however, that antibody diffused from the serum into the middle ear space more efficiently when the inflammatory process was associated with live bacteria. The presence of homologous antibody in the noninfected ear further suggested that this may have been derived from the serum antibody. In fact, antibody in MEF was present only in subjects who concurrently possessed serum antibody. Similarly, chinchillas rechallenged with NT H. influenzae in a previously noninfected ear following resolution of an infection with NT H. influenzae in the contralateral ear were protected against reinfection, presumably by serum antibody that diffused into the challenged ear (8). Passively administered serum anti-NT H. influenzae antibody also protected susceptible chinchillas against first-time challenge with NT H. influenzae in the middle ear space (1).

The functional capacity of the IgG specific antibody detected in MEF of children in this report is unknown. High levels of antipneumococcal antibody in MEF have been shown previously to be protective in experimental OME (4). In addition, the presence of organism-specific antibody in MEF early in the course of OME correlated to the rapid clearing of MEF in children with either pneumococcal or H. influenzae OME (24).

Although the middle ear is part of the respiratory tract, it is somewhat unique. The epithelial lining of the middle ear has less ciliated epithelium and fewer goblet cells than mucosal surfaces in other regions of the respiratory tract (12, 25, 27). Organized lymphoid follicles are absent. Absence of a normal bacterial flora may deprive the middle ear cavity of the kind of antigenic stimulation needed to change the epithelium into a more typical mucosal surface. Thus, the immune responsiveness of the middle ear cavity may not be characteristic of the local immunity present in other regions of the respiratory tract, and only after the development of OME may the mucosa undergo a transformation that causes it to resemble other regions in the respiratory tract. Further studies are necessary to carefully delineate the origin and function of antibody found in the middle ear space in humans with OME.

ACKNOWLEDGMENT

This work was supported in part by Public Health Service grant HD 19679 from the National Institute of Child Health and Human Development.

LITERATURE CITED