Preliminary Serologic Evidence for a Pathogenic Role of Branhamella catarrhalis

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Branhamella catarrhalis has been suspected, on the basis of bacteriologic culture results, to have a pathogenic role in 60%-90% of cases of acute otitis media as well as in other upper respiratory tract infections. Serologic evidence of this role was obtained with use of an enzyme immunoassay to detect antibodies to B. catarrhalis. The presence of both IgG and IgA antibodies to Branhamella in the serum and/or middle ear fluid (MEF) of children with acute otitis media correlated with the isolation of B. catarrhalis from cultures of their MEF. An increase in titer of antibodies to Branhamella between acute-phase and convalescent-phase serum samples was found in 10 of the 19 children with otitis media from whom B. catarrhalis but no other pathogen was isolated from the MEF. Such an increase was found in none of the 14 children with otitis media caused by other organisms.

We reasoned that a true pathogen was likely to provoke an immune response, the demonstration of which would give further support to its pathogenic role. For this purpose, we developed an assay to detect antibodies to B. catarrhalis. The present study reports evidence of an antibody response in those patients with acute otitis media whose MEF culture results had implicated B. catarrhalis as the etiologic agent.

Materials and Methods

Patients. The subjects of the present study were selected from a group of 519 children with acute otitis media who visited the Departments of Otolaryngology and Pediatrics at the University Central Hospital of Oulu, Oulu, Finland, between October 1977 and December 1978 [8]. A child was considered to have acute otitis media if acute ear and/or upper respiratory tract symptoms were present and fluid could be aspirated by tympanocentesis from behind an inflamed eardrum. Patients who received antibiotics at the time of the visit or within the previous week or who had had an episode of otitis during the previous month were excluded from the study. One-fourth of the children had never had acute otitis media before the index episode, and well over one-half had experienced three or more episodes. Bacteriologic culture of the MEF yielded S. pneumoniae from 39% of the patients, Haemophilus influenzae from 12%, B. catarrhalis from 6%, Streptococcus pyogenes from 2%, Staphylococcus aureus from 5%, and Staphylococcus epidermidis from 14%
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(mainly those younger than two years of age), whereas 33% had negative cultures. We did not look for virus, *Chlamydia*, and *Mycoplasma*.

Three groups of children were chosen for the present study. The main study group consisted of all 19 patients from whose MEF *B. catarrhalis* had been cultured either alone or, in two cases, with *S. epidermidis* and from whom a blood sample had been drawn both in the acute stage of the disease and two to three weeks later. The age distribution (figure 1) of these 19 children was similar to that of all 519 children. In four children, all five to 12 months old, this episode was the first episode of otitis media, whereas nine had had three or more previous episodes. Of the 11 children considered to have bilateral otitis media, culture of MEF from the other ear was negative for seven children, whereas it yielded *B. catarrhalis* from three and *S. epidermidis* from one. All three children with *S. epidermidis* were younger than 12 months of age.

The second study group consisted of 11 children otherwise similar to the first group from whom only the MEF and acute-phase sera were available (table 1). The third (control) group consisted of 14 randomly chosen patients whose MEF samples grew *S. pneumoniae* (five patients), *H. influenzae* (five patients), *S. aureus* (two patients), group B streptococci (one patient), or *Candida albicans* (one patient) and from whom we had both acute-phase and convalescent-phase sera. Their age distribution is shown in figure 1.

*Branhamella* antigen and enzyme immunoassay. Ten strains of *B. catarrhalis* isolated from MEF samples from children enrolled in this study were used to prepare the *Branhamella* antigen for the enzyme immunoassay to detect antibodies to *B. catarrhalis*. The bacteria were grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates at 37 C in an atmosphere of 50%–70% CO₂ plus air. The overnight growth from an equal number of plates of each strain was harvested in 0.9% NaCl, mixed, and washed twice. The product was used as the *Branhamella* antigen.

For immunization of rabbits the antigen was further treated with 0.7 M formalin for 3 hr, washed twice, and resuspended in 0.9% NaCl to a concentration of 10⁶ cells/ml. Rabbits were immunized intradermally with the *Branhamella* antigen once a week for five weeks and were bled before and one week after the immunization. Control rabbits

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**Table 1.** Antibodies to *Branhamella* in middle ear fluid (MEF) of children with acute otitis media caused by *Branhamella catarrhalis* (as judged by MEF culture results).

<table>
<thead>
<tr>
<th>Age of patient (months)</th>
<th>Antibodies to <em>Branhamella</em>†</th>
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† Presence (+) or absence (−) of antibody activity in the enzyme immunoassay with *Branhamella* antigen.
were similarly immunized with a similarly prepared antigen consisting of a mixture of 10 strains of nontypable *H. influenzae* isolated from MEF or nasopharyngeal samples of children in this study and grown on chocolate agar plates.

The enzyme immunoassay was performed in microtiter plates (Dynatech Laboratories, Alexandria, Va.). The optimal dilution of the *Branhamella* antigen was determined by testing different dilutions of the cell suspension (1:100-1:5,000 in phosphate-buffered saline [PBS], pH 7.2) against serial dilutions of sera from rabbits immunized with this antigen. A dilution of 1:250 gave the highest IgG antibody titer (1:5,260) in the hyperimmune sera but a low OD (about 0.2) in premunination sera and in sera from rabbits immunized with *H. influenzae*. Antigen (150 μl) at this dilution was placed in each well, and the plates were incubated at 37 C for 5 hr before being washed three times with PBS containing 0.05% Tween 20 (PBS-Tween), pH 7.2. All serum samples were diluted in 4% PBS-Tween, starting with a dilution of 1:50 (if this dilution gave a negative result, no further dilutions were tested). The plates were incubated with 150 μl in each well of the serum dilutions for 1 hr at 37 C and washed with 0.05% PBS-Tween three times. Alkaline phosphatase-conjugated swine antibodies to rabbit IgG or antibodies to human IgG, IgA, and IgM (Orion Diagnostica, Helsinki, Finland) were diluted to 1:300, 1:400, 1:300, and 1:250, respectively, in 4% PBS-Tween. The conjugate dilutions (150 μl) were added to the wells and incubated at 37 C for 2 hr, after which the plates were washed with 0.05% PBS-Tween three times. Substrate solution (150 μl) (1 mg of p-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, Mo.] in 1 ml diethanolamine buffer containing 2 mmol of MgCl₂, pH 9.8), was added and incubated at 37 C for 30 min. The reaction was stopped by adding 50 μl of a solution of 2 N NaOH. ODs were measured at 405 nm with a multichannel photometer (Titertek Multiskan®; Eflab, Helsinki, Finland). Each assay was performed in triplicate; the results are given as reciprocal serum dilutions giving an OD of 0.5 in the enzyme immunoassay [9].

**Results**

*Antibodies to Branhamella in patient sera.* Figure 1, top, presents the titers of antibodies to *Branhamella* in the acute- and convalescent-phase sera of children with acute otitis media ascribed to *B. catarrhalis* on the basis of MEF culture results. Relatively high reciprocal titers (up to 1,000) were seen in these sera, and both the presence and the level of antibodies to *Branhamella* depended on the age of the child. No such antibodies were seen in children younger than 10 months of age. In the three children 10-12 months old, IgG antibodies to *Branhamella* were undetectable in the acute-phase sera but reached reciprocal titers of 100-210 in the convalescent-phase sera. In children older than one year, IgG antibodies to *Branhamella* were detected in acute-phase sera. In all of the children with IgG antibodies to *Branhamella* there was a change in the titer between the two serum samples; in nine children there was an increase, in five a decrease, in the titer.

IgA antibodies to *Branhamella* appeared at a somewhat later age. They were not found in the sera of any of the 10 children younger than two years of age but were present in eight of the nine children two to six years old. The reciprocal IgA antibody titers were lower (all <300) than the corresponding IgG antibody titers, and, like the IgG antibody titers, either increased or decreased in the acute-phase serum sample relative to the convalescent-phase serum sample. No IgM antibodies to *Branhamella* were detected in the sera studied.

In paired sera from children who had otitis media caused by bacteria other than *Branhamella* (figure 1, bottom), IgM and IgA antibodies to *Branhamella* were not found. IgG antibodies to *Branhamella* were present in seven of these 14 children and more often in older children. There were, however, no changes in the IgG antibody titer between the acute- and convalescent-phase sera, and the overall levels were lower (all reciprocals <400) than in the children who had otitis media from whose MEF *B. catarrhalis* was isolated.

We also looked for antibodies to *Branhamella* in MEF samples from which *B. catarrhalis* had been isolated (table 1). Antibody levels in these MEF samples could not be quantitated because the dilutions of the samples varied; for collection, the amount of fluid obtained from one ear was mixed with 1 ml of saline. The data in table 1 do show that both IgG and IgA antibodies were present in most of the MEF samples (eight of 11), but again IgM antibodies were not detected. IgG antibodies were regularly present in the MEF (as well as in the serum) of children older than one year of age and were occasionally found in the MEF of children as young as five months of age (at which
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Age serum antibody levels were very low. IgA antibodies were detected in the MEF of all six children older than 10 months of age and in the MEF of one eight-month-old child. The corresponding serum sample was negative for IgA antibodies in three of these infants (aged eight, 11, and 13 months). Thus, both classes of antibodies to *Branhamella* were detected in the MEF of one eight-month-old child. The corresponding serum sample was negative for IgA antibodies to *Branhamella*, but not IgM antibodies, to *Branhamella* in sera from patients with maxillary sinusitis for whom cultures had revealed no other pathogen. They also found such antibodies in the sera of healthy blood donors. However, they detected CF antibodies and recorded a change in the titers of the patients but not in the titers of the healthy blood donors. Lewis et al. [10] have demonstrated that IgG antibodies to *Neisseria* (possibly *B. catarrhalis*) were present during serous otitis media, both in the serum and in the ear effusion from which the same bacteria were also isolated.

In the present study we found IgG antibodies, but not IgA or IgM antibodies, to *Branhamella* in our control sera, obtained from children who had otitis media caused by pathogens other than *B. catarrhalis* (mainly pneumococci and *H. influenzae*). Both the frequency and the titers of these antibodies increased with the age of the child. No differences in IgG antibody titers were, however, seen between acute- and convalescent-phase sera. These findings are very similar to those of Brorson et al. [2]. It is probable that the presence of these antibodies indicates continuous immunogenic stimulation by *B. catarrhalis* organisms which are regularly present in the normal microflora of the upper respiratory tract. However, some of the children may have previously had *B. catarrhalis* infections—primarily otitis media—because most of the patients in this study had had several episodes of otitis media. Nevertheless, the constancy of the antibody titers and the absence of IgA antibodies to *Branhamella* mean that an acute infection with an immune response to *B. catarrhalis* is unlikely.

The titers of IgG antibodies to *Branhamella* were, as a rule, higher in the children whose MEF cultures grew *B. catarrhalis* than in the control children of the same age (figure 1). Either an increase or decrease in these titers was regularly recorded between the acute- and convalescent-phase sera, a difference which suggests an ongoing immune response to *B. catarrhalis* in the ear. Furthermore, the presence of IgA antibodies to *Branhamella* indicates a recent stimulus because IgA has a short $t\frac{1}{2}$ and was absent in the control children.

The decreasing antibody titers in some of the children could not be related to any observed parameter, such as age or the number of previous episodes of otitis media. However, it is reasonable to suppose that *B. catarrhalis* had been present in these children and had caused milder—unnoticed or unrecorded—upper respiratory tract infections before the eruption of otitis media. To determine whether this proposal is the correct explanation requires closer serologic follow-up of children with mild upper respiratory tract infections.

We do not know why IgM antibodies to *Branhamella* were not detected in any of the sera or MEF samples studied. This absence could indicate that the enzyme immunoassay is unable to detect these antibodies. However, we have easily detected IgM antibodies to several other antigens by similar enzyme immunoassays using the same conjugate of alkaline phosphatase and antibody to IgM [9]. We therefore consider it more likely that this antibody class is not part of the immune response to *B. catarrhalis* in the upper respiratory tract. To understand the reason for this lack of activity, it would be necessary to know to which antigens (lipopolysaccharide or protein) the measured antibodies are directed. A comparable enzyme immunoassay for antibodies to *Yersinia enterocolitica* primarily measured antibodies to the lipopolysaccharide [11].

The evidence we have for a pathogenic role of *B. catarrhalis* in acute otitis media is threefold. (1) The organism is often isolated from MEF in pure culture. In addition, it is often isolated with other bacteria such as pneumococci, *H. influenzae*, or *S. epidermidis* at the same low frequency as mixed infections or contaminants are found [8]. (2) MEF samples from which *B. catarrhalis* is cultured are
purulent to the same extent as are samples from pneumococcal otitis media, a similarity which suggests a bacterial infection. Because the gram-negative diplococci in these samples are seen in close association with or inside the polymorphonuclear leukocytes, contamination from the ear canal is unlikely [8]. (3) High levels of antibodies to Branhamella, increasing antibody titers, and the presence of IgA antibodies are all associated with the culture-positive episodes and are absent in otitis media caused by other pathogens. The evidence is comparable to the evidence we have for a pathogenic role of pneumococci in otitis media in children. In neither case do the data indicate whether the bacteria in question were the primary pathogens, and in fact viral infections often seem to have a role in predisposing the child to purulent otitis media of bacterial etiology. The mechanisms by which this predisposition might happen are numerous, such as local congestion of the mucosa obstructing the eustachian tube or suppression of the host's antibacterial defenses. Whether B. catarrhalis is more dependent on such predisposing factors than pneumococci cannot be determined with the present data. A careful analysis of clinical observations and comparative analyses of the occurrence of viral infection during and before otitis media due to these two pathogens might give some answers.

The recognition of B. catarrhalis as a pathogen in otitis media is important because β-lactamase is often found in clinical isolates [12-14]. β-Lactamase inactivates penicillin and ampicillin-amoxicillin, making them ineffective treatment regimens. In Finland 20% of B. catarrhalis strains isolated from the MEF of patients with otitis media presently produce β-lactamase, whereas all strains are sensitive to the common alternative regimens, erythromycin or sulfonamide-trimethoprim (authors’ unpublished observation). In Finland B. catarrhalis is the third most commonly encountered pathogen in the MEF, after only pneumococci and H. influenzae, which is also a potential β-lactamase producer.

References