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Harabuchi, Y. ; Faden, H. ; Yamanaka, N. ; Duffy, L. ; Wolf, J. ; Krystofik, D. ; Williamsville, T.

Nasopharyngeal Colonization with Nontypeable *Haemophilus influenzae* and Recurrent Otitis Media

Yasuaki Harabuchi, Howard Faden, Noboru Yamanaka, Linda Duffy, Judy Wolf, Deborah Krystofik, and Tonawanda/Williamsville Pediatrics

Departments of Otolaryngology, Sapporo Medical College, Sapporo, and Wakayama Medical College, Wakayama, Japan; Department of Pediatrics, State University of New York at Buffalo School of Medicine, and Division of Infectious Diseases, Children's Hospital of Buffalo, Buffalo, New York

The relationship between nasopharyngeal colonization with nontypeable *H. influenzae* and recurrent otitis media was assessed in 157 children followed prospectively from birth through 12 months of age. Forty-nine (31%) became colonized. Nasopharyngeal secretory IgA (sIgA) reactive with the P6 outer membrane protein was detected in all colonized children. Reduction or elimination of the organism was associated with a better mucosal immune response (560 ± 864 units/ng/mL of sIgA) than was persistence in the nasopharynx (121 ± 81; $P = .04$). Forty colonized children (82%) and 61 noncolonized children (56%) developed otitis media ($P = .004$); colonized children were four times more likely to be classified as otitis prone ($P = .003$). The frequency of otitis media episodes was directly related to the frequency of colonization ($r = .42$, $P < .01$). These results demonstrate a strong relationship between nasopharyngeal colonization patterns and otitis media. The mucosal immune response may be important in elimination of potential pathogens from the respiratory tract.

Acute otitis media and otitis media with effusion are among the most common childhood illnesses. Middle ear disease accounts for 3.5% of all physician office visits, ~30% of pediatric office visits, and 5%–15% of well child examinations [1–4]. The incidence of middle ear disease is greatest in the first 2 years of life. By 1 year of age, 62% of children have had at least one episode and 17% have had three or more episodes [5].

Three bacteria are responsible for the vast majority of ear infections: *Streptococcus pneumoniae* causes between 20% and 50%, nontypeable *Haemophilus influenzae* between 27% and 37%, and *Moraxella catarrhalis* between 11% and 23% [6–9]. The relative distribution of these agents has undergone change over time. For example, nontypeable *H. influenzae* has become a more common cause of otitis media [7, 9], while the incidence of *M. catarrhalis* has increased >20-fold [8, 10]. The bacteria causing recurrent episodes of otitis media are the same that cause acute otitis media, but their relative frequency is different; for reasons not fully understood, nontypeable *H. influenzae* assumes a more prominent role in recurrent disease [11–15].

Children who experience recurrent episodes of otitis media have been classified as otitis-prone. Several factors that identify a child at risk for recurrent disease include having the first episode of otitis media early in life, having other family members with recurrent disease, enrollment in day care, and bottle feeding [4, 5, 16–18].

It is important to realize that nasopharyngeal colonization with a potential middle ear pathogen is the first step in the development of otitis media. The nasopharynx is colonized normally with numerous avirulent bacteria such as α-streptococci, nonhemolytic streptococci, *Neiserria* species, and diphtheroids [19]. Nontypeable *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* are also considered normal flora of the respiratory tract; however, unlike the previously mentioned organisms, these bacteria have the propensity for causing otitis media. Nontypeable *H. influenzae* has been recovered from the airways of 20%–80% of normal children [20–24]. Colonization rates increase from 20% in children <1 year old to >50% in children >5 [20]. Children characteristically carry a strain of nontypeable *H. influenzae* for several months, lose it, and acquire a new strain [25]. Children who are otitis prone are colonized with nontypeable *H. influenzae* more often than normal children [24, 26].

The present study was designed to investigate the relationship between nasopharyngeal colonization with nontypeable *H. influenzae* and recurrent episodes of otitis media in a population of children followed prospectively from birth through 1 year of age. The role of mucosal immunity on colonization was assessed by measuring the secretory IgA (sIgA) response to nontypeable *H. influenzae*.

Materials and Methods

General design. Children were enrolled within the first month of life without regard to sex, race, or social status. Chil-

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Informed consent was obtained from the parents at enrollment. The study had received prior approval from the institutional review board.

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Reprints or correspondence: Dr. Howard Faden, Division of Infectious Diseases, Children's Hospital of Buffalo, 219 Bryant St., Buffalo, NY 14222.

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dren with craniofacial abnormalities, genetic disorders, and immune deficiencies were excluded from the study. Information gathered at entry included family history for recurrent otitis media, allergy, and smoking. In addition, the number, age, and educational level of siblings were recorded.

Children were examined at regularly scheduled visits monthly through the first 6 months and at 8, 10, and 12 months of age in the offices of one group of pediatricians. Questions concerning diet (bottle vs. breast-feeding), recent and current illnesses, antibiotic exposure, and day care were recorded at each visit. Ears were examined with pneumatic otoscopy and tympanometry by the same group of practitioners for the duration of the study.

The diagnosis of acute otitis media was established by the presence of symptoms including fever, irritability, pulling at the ears, and tympanic membrane changes of increased thickness, bulging, loss of landmarks, decreased mobility, and a flat tympanogram, type B curve. Choice of antibiotic treatment was left to the discretion of the physician. Children were examined monthly after the diagnosis of otitis media until complete resolution of the process was observed. A new episode was diagnosed only when a previous one had completely resolved. Otitis media with effusion was diagnosed when fluid was detected in the middle ear in the absence of symptoms and unrelated to any prior episode of acute otitis media. The tympanic membrane was typically thin, exhibited decreased mobility, and produced a flat tympanogram. Treatment with antibiotics was not generally instituted for otitis media with effusion.

The cumulative number of episodes of otitis media was calculated at each visit. Children were considered otitis-prone if they experienced four or more episodes of otitis media or required placement of tympanostomy tubes for persistent middle ear effusions for ≥4 months by the age of 1 year.

Nasopharyngeal cultures. Nasopharyngeal cultures were obtained with a small rayon swab. Swabs were transported to the laboratory in transport medium, and specimens were cultured on trypticase soy agar with 5% sheep blood, chocolate agar, and MacConkey agar within 8 h of collection. Bacterial species were identified by standard laboratory procedures. Nontypeable *H. influenzae* was characterized by X and V factors and by the absence of agglutination with typing sera. Recovery rates of nontypeable *H. influenzae* on chocolate agar were shown to be equivalent to growth on selective media in a preliminary study of 408 nasopharyngeal cultures.

Nasopharyngeal secretions. Nasopharyngeal secretions were collected 1 month after documentation of the initial colonization with nontypeable *H. influenzae* by aspirating secretions with a small rubber catheter and rinsing the catheter with 1 mL of PBS. The sample fluids were transferred to the laboratory on ice and centrifuged at 2000 *g*; supernatants were filtered through a 0.45-μm filter and stored at −70°C until tested.

Determination of anti-P6 sIgA. A modification of the method of Munson and Granoff [27] was used to isolate P6 [28]. Coomassie blue–stained SDS-PAGE of the purified protein demonstrated a single band with a molecular mass of 16,000 Da.

An ELISA using avidin-biotin interaction was used to detect anti-P6 sIgA in nasopharyngeal secretions as described [28]. To standardize test values for potential dilutional differences between nasopharyngeal secretion samples, the level of anti-P6 antibody was adjusted to uniform sIgA levels by dividing them by the total sIgA concentration and expressed as units/ng/mL of total sIgA.

Table 1. Clinical characteristics of children colonized and not colonized by nontypeable *H. influenzae*.

Characteristic	Colonized (n = 49)	Not colonized (n = 108)	P
Boys	27 (55)	58 (54)	NS
Breast-fed	25 (51)	67 (62)	NS
Duration of breast-feeding, months	7.0 ± 3.7	6.9 ± 3.8	NS
Day-care enrollees	8 (16)	12 (11)	NS
Children with otitis media	40 (82)	61 (56)	.004
Age of first episode of otitis media, months	5.0 ± 2.8	6.4 ± 3.0	.017
Otitis-prone children	10 (20)	5 (5)	.003

NOTE. Data are no. (%) or mean ± SD as appropriate. NS, not significant.

Statistical methods. Data concerning antibody concentrations were log-transformed before statistical analysis. Overall, comparisons between appropriate groups were tested by Student's unpaired t test or the χ^2 test. Additional subanalyses were completed using appropriate nonparametric test procedures including the Mann-Whitney U test, Fisher's exact test, and Spearman's regression coefficient. $P < .05$ was considered significant.

Results

Population. One hundred fifty-seven white infants were enrolled and completed the 1-year evaluation. Eighty-five (54%) were boys and 72 (46%) girls. Ninety-two children (59%) were fed partially or fully with human milk for 7.0 ± 3.7 (mean ± SD) months. Of the study population, 13% were enrolled in day care. At 1 year of age, 101 children (64%) had a history of otitis media; 15 (9.6%) were classified as otitis-prone. The mean number of episodes of otitis media was 1.2 ± 1.2. The mean age of the first episode was 5.8 ± 3.0 months.

Nasopharyngeal colonization with nontypeable H. influenzae. Nontypeable *H. influenzae* was recovered from 49 children (31%). Initial colonization occurred between 2 and 12 months of age (mean, 6.0 ± 3.0). Colonized children carried the organism one to five times (median, two). Nontypeable *H. influenzae* disappeared within 1 month in 51% of the colonized children. Twenty-four (49%) of the colonized children carried the organism at more than one visit. The remaining 108 (69%) never became colonized.

Relationship between nasopharyngeal colonization with nontypeable H. influenzae and development of otitis media. Table 1 compares the clinical characteristics of children colonized and not colonized with nontypeable *H. influenzae*. There were no differences in sex, frequency or duration of

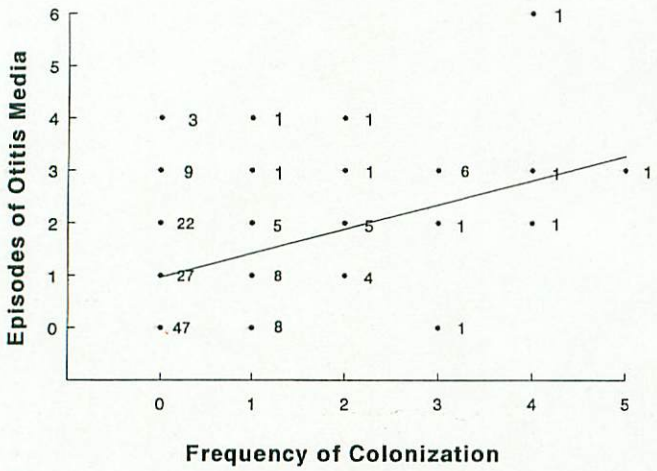

Figure 1. Relationship between frequency of colonization and episodes of otitis media. Spearman's regression coefficient, $R = .42$, $P < .01$.

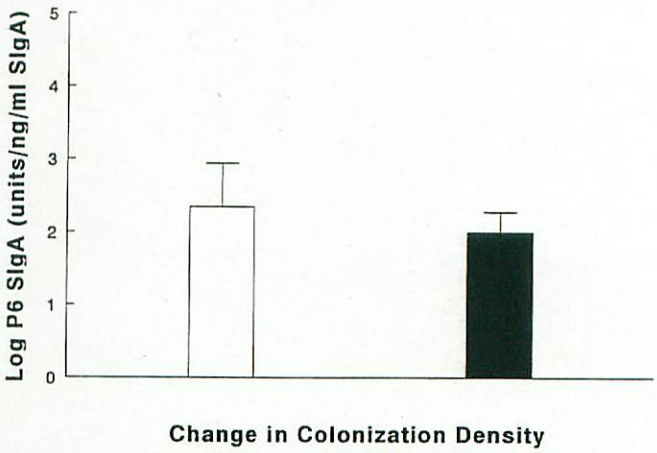

Figure 2. Secretory IgA (SIgA) P6 antibody response (log) and change in initial nasopharyngeal colonization. Open bar, decrease in colonization; solid bar, no change or increase in colonization. $P = .02$.

breast-feeding, or day care enrollment rate between groups. Similarly, the 2 groups did not differ with respect to family history of otitis media, allergy, or smoking. Forty (82%) of 49 children who became colonized developed otitis media compared with 61 (56%) of 108 children who had never been colonized ($P = .004$). Otitis media occurred significantly earlier in colonized children, 5.0 ± 2.8 months, than in noncolonized children, 6.4 ± 3.0 months ($P = .02$) and was related to the age of the initial colonization ($r = .43$, $P < .02$). More important, 10 (20%) of 49 colonized children but only 5 (5%) of 108 noncolonized children were classified as otitis-prone ($P = .003$). As seen in figure 1, the frequency of otitis media was directly related to the frequency of colonization with nontypeable *H. influenzae* ($r = .42$, $P < .01$). These data suggest that children who become colonized are at increased risk of developing recurrent otitis media.

Nasopharyngeal sIgA response to the P6 outer membrane protein of nontypeable H. influenzae. Nasopharyngeal secretions were obtained from 46 colonized children 1 month after the initial colonization with nontypeable *H. influenzae;* sIgA was detected in 42 samples. sIgA reactive with the P6 outer membrane protein of nontypeable *H. influenzae* was detectable in all of 42 nasopharyngeal secretions in which sIgA was detected. Nasopharyngeal secretions were also collected from 12 children who had never been colonized with nontypeable *H. influenzae;* samples were collected at the ages of 6 and 12 months. Specific sIgA antibody was undetectable in 11 (92%) of these 12 controls. These results suggest that the presence of nontypeable *H. influenzae*–specific sIgA indicates prior colonization with nontypeable *H. influenzae.*

Among colonized children, the level of P6 sIgA ranged between 15 and 3200 units/ng/mL of total sIgA (mean, 434 ± 755). Colonization decreased or disappeared within 1 month of the initial colonization in 30 children (73%) and was not changed or increased in 12 (27%). The mean antibody level in children in whom colonization decreased, 560 ± 864, was significantly higher than in children in whom colonization was not changed or increased, 121 ± 81 ($P = .04$; figure 2). Moreover, the antibody response was inversely related to the frequency of subsequent isolation of nontypeable *H. influenzae* ($r = -.40$, $P < .01$) and the number of episodes of otitis media ($r = -.38$, $P < .02$).

Because immunoglobulin levels increase with age, we next assessed the relationship between P6 sIgA level and age. As seen in figure 3, specific antibody levels were positively related to age ($r = .45$, $P < .01$). After controlling for age, the frequency of colonization was marginally related to P6 sIgA

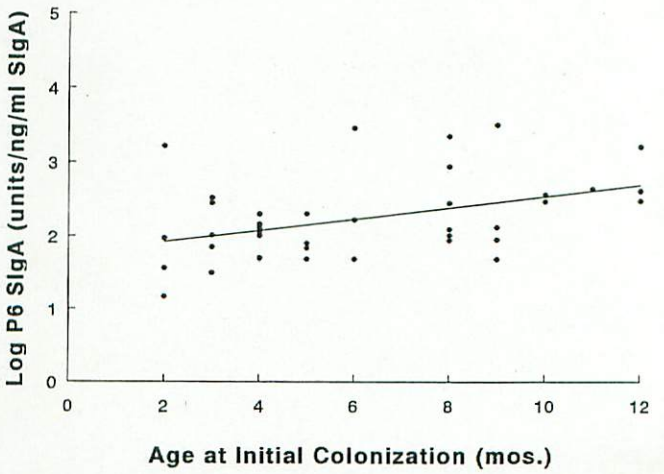

Figure 3. Relationship between age at initial colonization and secretory IgA (SIgA) response (log) to P6. Spearman's regression coefficient, $R = .45$, $P < .01$.

levels ($r = -.28$, $P < .06$). Similarly, controlling for age reduced the relationship between P6 sIgA levels and the frequency of otitis media ($r = -.34$); however, the relationship remained statistically significant ($P < .05$).

Discussion

Otitis media continues to be a major childhood disease. In the present study of 157 children carefully monitored through the first year of life, 64% of the subjects experienced one or more episodes of otitis media. In total, 15 children (9.6%) were classified as otitis prone. Because we hypothesized that nasopharyngeal colonization patterns with nontypeable *H. influenzae* would be related to the frequency of recurrent otitis media, nasopharyngeal cultures were obtained at nine scheduled monthly visits in the first year of life. Forty-nine children (31.2%) were colonized on one or more occasions. The incidence of colonization may have been higher if we had obtained cultures for each of the 12 months; however, the incidence observed in the present study is in agreement with earlier reports [20, 24]. Children colonized with nontypeable *H. influenzae* in the first year of life were at increased risk of developing otitis media compared with children who remained free of nontypeable *H. influenzae.* Otitis media occurred earlier in colonized children and was directly related to the age at the time of initial colonization. Perhaps the more convincing observation in this report was the fourfold higher otitis-proneness in colonized than in noncolonized children. We noted a direct relationship between the number of times a child was colonized and the number of episodes of otitis media.

The present study did not attempt to identify the etiologic agent of the otitis media through tympanocentesis. However, several earlier studies demonstrated relatedness of pathogens recovered simultaneously from the nasopharynx and middle ear [29–33]. It is highly unlikely that all episodes of otitis media were due to nontypeable *H. influenzae.* Much more likely is the possibility that increased colonization with potential pathogens identified a population of children who were at risk of developing recurrent otitis media [24, 26].

Children who are otitis-prone are born into families with an increased incidence of recurrent otitis media, suggesting a genetic predisposition [34, 35]. Recent studies suggest that children with recurrent otitis media may exhibit antigen-specific immunologic abnormalities. These children do not develop a normal antibody response to specific pneumococcal types [36–38]. Prellner et al. [34] demonstrated low serum levels of pneumococcal antibodies at birth in children destined to be otitis-prone, suggesting a maternal defect as well. In a longitudinal study of children from birth through 4 years, otitis-prone children also did not develop a normal age-related rise in serum antibody to the P6 protein of nontypeable *H. influenzae* despite repeated contact with the organism [28].

The present study examined the mucosal immune response to nontypeable *H. influenzae* because previous studies had suggested that serum antibody was not an important factor in nasopharyngeal colonization [25, 39]. An earlier study from our laboratory demonstrated that IgA and sIgA were the major immunoglobulin classes developed against nontypeable *H. influenzae* in the nasopharynx; in addition, the antibody appeared to be protective against colonization [40]. All children who had been colonized in the present study produced organism-specific local antibody. The study further demonstrated that a good local immune response was associated with elimination of the initial pathogen. Children who exhibited a poor mucosal immune response to *H. influenzae* may represent the same population of children identified in earlier studies who do not develop a normal serum antibody response to pneumococci or to *H. influenzae* [36–38].

P6 has been proposed as a possible antigen for vaccination against otitis media because it is antigenically conserved and functions as a target for bactericidal antibody [41]. Seroepidemiologic studies have demonstrated widespread seropositivity to P6 in the general population [28]. Otitis-prone children do not recognize P6 in a normal manner and do not develop an age-related rise in antibody concentration [28]. Thus, normal children will likely respond to a P6 vaccine favorably while otitis-prone children will respond less well. Because of the immunologic abnormality in otitis-prone children, an innovative approach to immunization would be needed to augment their immune response. However, a better understanding of the immunologic defect is needed before rational therapy can be instituted.

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