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# Breast-Feeding Is Associated With a Reduced Frequency of Acute Otitis Media and High Serum Antibody Levels Against NTHi and Outer Membrane Protein Vaccine Antigen Candidate P6

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**ABSTRACT:** Nontypeable *Haemophilus influenzae* (NTHi) causes acute otitis media (AOM) in infants. Breast-feeding protects against AOM and/or nasopharyngeal (NP) colonization; however, the mechanism of protection is incompletely understood. Children with AOM and healthy children were studied according to feeding status: breast-fed, breast/formula fed, or formula fed. Cumulative episodes of AOM, ELISA titers of serum IgG antibodies to whole-cell NTHi and vaccine candidate outer membrane protein P6, bactericidal titers of serum and NP colonization by NTHi were assessed. A lower incidence of AOM was found in breast- *versus* formula-fed children. Levels of specific serum IgG antibody to NTHi and P6 were highest in breast-fed, intermediate in breast/formula fed, and lowest in formula-fed infants. Serum IgG antibody to P6 correlated with bactericidal activity against NTHi. Among children with AOM, the prevalence of NTHi in the NP was lower in breast- *versus* nonbreast-fed infants. We conclude that breast-feeding shows an association with higher levels of antibodies to NTHi and P6, suggesting that breast-feeding modulates the serum immune response to NTHi and P6. Higher serum IgG might facilitate protection against AOM and NP colonization in breast-fed children. **(*Pediatr Res* 66: 565–570, 2009)**

Acute otitis media (AOM) is a common problem in infants and children. Nontypeable *Haemophilus influenzae* (NTHi) is one of the major causes of infections in the upper respiratory tract and middle ear (ME) (1). In most cases, this organism is carried in the nasopharynx (NP) without causing clinical symptoms. However, when the condition of the host is altered, NTHi may invade the ME, causing AOM (1). The protection from NTHi otitis media and NP carriage has been proposed to be associated with induction of protective immune responses to a number of antigenically conserved NTHi outer membrane proteins (OMPs), including P6 and to whole cell NTHi (2,3). Several studies reported that breast-feeding is associated with decreased frequency or duration of otitis media (4,5); however, the mechanism of protection is incompletely understood. It has been postulated that breast-feeding provides protection against AOM by interfering with the attachment of bacterial pathogens to NP epithelial cells (6,7). Various protective factors of breast milk, including secretory IgA antibodies, lactoferrin, oligosaccharides functioning as receptor analogues, etc., are thought to provide passive protection against NP colonization. However, clinical and epidemiologic studies have not confirmed the influence of breast-feeding on the prevalence of NP colonization with common bacterial pathogens, including NTHi (8,9). Moreover, this mechanism of passive protection does not explain the decreased risk of developing otitis media after the termination of breast-feeding (5).

Another possible mechanism might be the ability of breast-feeding to stimulate the immune response of infants (10–12). To our knowledge, no studies have thus far explored the potential role of breast-feeding in enhancing the infant's immune responses to NTHi. This study was designed to analyze serum antibodies to NTHi and OMP P6 and the frequency of AOM in breast- *versus* nonbreast-fed children. We hypothesized that breast-feeding may enhance the infant's humoral immune response to NTHi, and OMP P6, and this may correlate with a lower incidence of AOM and NP colonization by NTHi.

## METHODS

***General design.*** Two groups of children were studied. Information gathered included diet (breast-fed *versus* breast/formula fed *versus* formula fed) and the frequency of episodes of AOM. The children were assigned to breast-fed *versus* breast/formula fed *versus* formula-fed groups based on self-report of the mother at the time blood samples were taken, and no attempt was made to semiquantitate the proportion of breast *versus* bottle feeding in the mixed feeding group. Group 1 consisted of healthy and AOM children who were retrospectively identified from a 1990–1991 study done in a private pediatric practice in Rochester, NY, where serum samples had been collected at 2 and 6 mo of age. Group 2 was prospectively enrolled from the same private practice population in 2006–2007. In group 2, there were two subgroups: (a) children enrolled at 6 mo of age who were without previous episodes of AOM (group 2 healthy children) and (b) otitis prone children who underwent tympanocentesis (group 2 children with AOM). For all subjects, ears were examined by validated otoscopist pediatricians with pneumatic otoscopy. In group 1 healthy and AOM children, we determined the cumulative number of episodes of AOM from the birth until the time of a serum collection (for an antibody measurement). Children of age 2 mo were predefined as AOM children if they had more than or equal to one episodes of AOM. Children of age 6 mo were predefined as AOM children if they had

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**Abbreviations: AOM,** acute otitis media; **ME,** middle ear; **NP,** nasopharyngeal; **NTHi,** nontypeable *Haemophilus influenzae*; **OMP,** outer membrane protein


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more than or equal to two episodes of AOM. Group 2a healthy children had NP cultures and NP wash samples, and oropharyngeal swabs obtained every 3–6 mo between 6 and 24 mo of age. These subjects were not ill. Group 2b children with AOM were 6–24 mo old children who had AOM and were undergoing a tympanocentesis along with a sample of NP (swab and wash) and oropharynx (swab). The subjects were otitis prone, defined as three episodes in 6 mo or four episodes in 12 mo. The study was approved by the University of Rochester Research Subjects Review Board, and informed consent was obtained.

***Sample collection.*** Nasal washes and NP exudates were collected in group 2a healthy children and in group 2b children with AOM. ME fluids were collected in group 2b children with AOM. A nasal wash was obtained by irrigating the nasal cavity with 2 mL of sterile saline and collecting fluid from the nares. An exudate sample from the NP was obtained with a sterile cotton swab. The ME fluids were collected during tympanocentesis. Samples were diluted in 1 mL of sterile PBS.

***NP and ME cultures.*** The nasal wash, an exudate sample from the NP and ME fluid were cultured on blood agar with gentamicin and chocolate agar plates and in trypticase soy broth. NTHi was characterized by porphyrin test and X and V factor requirement. *H. influenzae* was distinguished from *H. haemolyticus* by the method of Murphy *et al.*, (13) including tests of hemolysis on blood agar and PCR of P6 amino acid variations.

***Detection of whole-cell NTHi IgG antibodies by ELISA.*** For the whole cell specific ELISA, we used a reference NTHi strain (86-028NP) obtained as a gift from Lauren O. Bakaletz, PhD (Ohio State University at Columbus, OH), which was recovered from the NP of a child with otitis media. Strain 86-028NP was grown on chocolate agar and then in brain heart infusion broth supplemented with hemin and NAD. The bacteria were harvested by centrifugation, washed with PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PCM). After washing, the pellet was suspended and then diluted with PCM to an optical density (OD) of 1 at 490 nm, and the NTHi preparation was used to coat 96-well plates. After blocking with 1% gelatin and washing, diluted serum was added to the wells, and the mixture was further incubated at room temperature for 1 h. Affinity purified goat anti-human IgG antibody conjugated to alkaline phosphatase was used as a secondary antibody. The reaction products were developed with PNP dissolved in diethanolamine buffer. The reaction was stopped by the addition of 2 M NaOH and was read by an automated ELISA reader using a 405-nm filter. In some experiments, the ODs of background and test wells were used to determine a sample's response index (response index = average sample OD/average background OD). In other experiments, titers for test samples were determined relative to a reference serum (pool of children's serum with high titers of specific NTHi and P6 antibody) run on the same plate, and values were expressed relative to the reference serum.

***Detection of P6-specific IgG antibodies by ELISA.*** P6-specific IgG antibody titers in the convalescent serum samples were determined by ELISA using purified recombinant P6. The P6 gene was cloned as previously described (14) resulting in a pure protein product. For group 1 samples, 96-well plates were coated with 0.25 $\mu$g/mL of P6 antigen in coating buffer (bicarbonate, [pH 9.4]) and the rest of the steps for the ELISA were performed as described earlier. For group 2 samples, 96-well plates were coated with 0.25 $\mu$g/mL of P6 antigen in coating buffer. After blocking with 3% skim milk and washing, diluted serum was added to the wells, and the mixture was further incubated at room temperature for 1 h. Affinity purified goat anti-human IgG antibody conjugated to horseradish-peroxidase was used as a secondary antibody. The reaction products were developed with TMB Microwell Peroxidase Substrate System, stopped by the addition of 1.0 M phosphoric acid, and read by an automated ELISA reader using a 450-nm filter. For all determinations, the level of the specific antibody present in the unknown fraction was determined by comparison to an internal reference serum (pool of human serum with high anti-P6 titers).

***Bactericidal assay.*** The serum samples collected from group 2b children with AOM in the acute period and 2–3 wk thereafter in the convalescent period, and serum depleted of antibodies against P6, were used in bactericidal reactions. The serum was heat inactivated at 56°C for 30 min to eliminate human complement. Each serum was assayed against the bacterial strain isolated from middle ear space of that child. Homologous NTHi strains were cultivated, harvested, and diluted to a concentration of $\sim 10^5$ CFU/mL. Twelve serial 2-fold dilutions of the serum to be tested (starting at 1:2) were mixed with precolostral calf serum complement and 20 $\mu$L of bacteria. After 60 min of incubation, the number of surviving bacteria was determined by plating 5 $\mu$L onto chocolate agar and counting the colonies. The bactericidal titer of the serum was defined as the inverse of the highest dilution that led to $\geq$50% bacterial killing and was compared with that of negative control serum. Appropriate controls were included in all experiments. To examine the contribution of P6 antibodies to serum bactericidal activity observed, we removed all P6 antibodies from available sera using aqueous phase precipitation. The efficacy of absorption was monitored by using increasing concentrations of P6 antigen in the sera until no antibody could be detected by ELISA.

***Statistics.*** In group 1, anti-whole-cell NTHi and anti-P6 IgG antibody levels are expressed as $\log_2$ of ELISA units/mL. In group 2, anti-P6 IgG antibody levels are expressed in ng/mL. Bactericidal activity is expressed as reciprocal bactericidal titers. For participant demographic data, frequency of AOM, frequency of carrier rate of NTHi, and serum antibody levels, $p$ values were calculated using Fisher exact test, Mann-Whitney test, or test of proportions, as appropriate. Kendall's rank correlation test was used for concordance between serum bactericidal activity and anti-P6 IgG titers in convalescent serum, and $p$ values of 0.05 were considered significant.

## RESULTS

***Study participation.*** Three hundred seventy-two children were studied from group 1 (healthy and AOM children); there were 180 children aged 2 mo and 192 children aged 6 mo. Among the 2 mo olds, there were 81 breast, 54 breast/formula, and 45 formula fed. Among 6 mo old, there were 45 breast, 47 breast/formula, and 100 formula fed. There were 128 children in group 2; 76 were in group 2a (healthy children) and 52 in group 2b children with AOM. In group 2a healthy children, 24 were breast, 25 breast/formula, and 27 formula fed; in group 2b children with AOM, the distribution was 9 breast, 24 breast/formula, and 19 formula fed.

***Incidence of AOM in relation to breast-feeding.*** In group 1, there was a significantly lower incidence of AOM in breast- *versus* formula-fed children at 2 mo of age, $p = 0.01$; (Table 1). At 6 mo of age, the incidence of AOM was again significantly lower in breast- *versus* formula-fed children ($p < 0.0001$), and in breast/formula *versus* formula-fed children ($p < 0.0001$) (Table 2).

***Serum IgG antibody levels to whole-cell NTHi in infants.*** Serum IgG antibody levels to whole-cell NTHi were measured in randomly selected samples from group 1 healthy children—2 mo olds ($n = 39$) and 6 mo olds ($n = 31$). Among 2 mo olds, the level of specific serum antibody to whole NTHi was significantly higher in the breast *versus* breast/formula ($p = 0.013$) and formula-fed groups ($p = 0.021$) (Table 3). Similarly, the levels of specific serum antibody levels among 6 mo olds in group 1 healthy children were significantly higher in the breast *versus* breast/formula ($p = 0.01$) and formula-fed groups ($p = 0.0008$) and in breast/formula *versus* formula-fed groups ($p = 0.0004$) (Table 3). In all three cohorts in group 1, the median levels of specific NTHi antibodies in 6 mo olds children were higher than those in 2 mo olds children ($p = 0.044$). In group 1 AOM children, there were fewer serum samples available than those in group 1 healthy children to measure the IgG antibody levels to whole-cell NTHi and P6, and these results were not sufficient for statistical analyses (data not shown).

**Table 1.** *Incidence of AOM in 2-mo-old infants in relation to breast-feeding (group 1)*

| AOM group | Breast-fed* | Breast/formula fed | Formula fed |
|---|---|---|---|
| Healthy ($n = 165$) | 79 (98%) | 48 (89%) | 38 (84%) |
| With AOM ($n = 15$) | 2 (2%) | 6 (11%) | 7 (16%) |

Values are the number of subjects (percentage).

* $p = 0.01$, the incidence of AOM in breast-fed *vs* formula-fed children.

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***Serum IgG antibody levels to NTHi OMP P6 in infants.*** In group 1 healthy and AOM children, in both 2 and 6 mo olds, there was a difference in specific serum IgG antibody levels to OMP P6 among the three feeding cohorts. In 2 mo olds, higher anti-P6 antibody levels to OMP P6 were measured in breast than in breast/formula ($p = 0.0008$) and formula fed ($p = 0.029$) infants (Table 4). Similarly, in 6 mo olds, significant differences in the specific anti-P6 antibody levels were measured between breast and breast/formula fed ($p = 0.022$) and between breast and formula fed ($p = 0.006$) infants.

**Table 2.** *Incidence of AOM in 6-mo-old infants in relation to breast-feeding (group 1)*

| AOM group | Breast-fed* | Breast/formula fed* | Formula fed |
|---|---|---|---|
| Healthy ($n = 116$) | 39 (87%) | 39 (83%) | 38 (38%) |
| With AOM ($n = 76$) | 6 (13%) | 8 (17%) | 62 (62%) |

Values are the number of subjects (percentage).

* $p < 0.0001$ comparing breast-fed *vs* formula-fed group and comparing breast/formula fed *vs* formula-fed group.

**Table 3.** *Serum antibody levels to whole NTHi in infants (group 1 healthy children)*

| | Breast-fed | Breast/formula fed | Formula fed |
|---|---|---|---|
| 2 mo olds ($n = 39$) | 2.00 ± 0.10* ($n = 15$) | 1.72 ± 0.07 ($n = 18$) | 1.52 ± 0.17 ($n = 6$) |
| 6 mo olds ($n = 31$) | 2.4 ± 0.37† ($n = 7$) | 1.85 ± 0.1‡ ($n = 14$) | 0.94 ± 0.12 ($n = 10$) |

Values are the mean ± SE of $\log_{10}$ of reference units.

* $p = 0.013$ comparing breast-fed *vs* breast/formula fed and $p = 0.021$ comparing breast-fed *vs* formula-fed groups in 2 mo olds.

† $p = 0.01$ comparing breast-fed *vs* breast/formula fed and $p = 0.0008$ comparing breast-fed *vs* formula-fed groups in 6 mo olds.

‡ $p = 0.0004$ comparing breast/formula fed *vs* formula-fed group in 6 mo olds.

**Table 4.** *Serum antibody levels to NTHi outer membrane protein P6 in infants (group 1 healthy children)*

| | Breast-fed | Breast/formula fed | Formula fed |
|---|---|---|---|
| 2 mo olds ($n = 40$) | 2.22 ± 0.09* ($n = 15$) | 1.8 ± 0.08 ($n = 19$) | 1.68 ± 0.18 ($n = 6$) |
| 6 mo olds ($n = 28$) | 2.48 ± 0.33† ($n = 6$) | 2.05 ± 0.1 ($n = 13$) | 1.86 ± 0.12 ($n = 9$) |

Values are the mean ± SE of $\log_{10}$ of reference units.

* $p = 0.0008$ comparing breast-fed *vs* breast/formula fed and $p = 0.029$ comparing breast-fed *vs* formula-fed groups in 2 mo olds.

† $p = 0.022$ comparing breast-fed *vs* breast/formula fed and $p = 0.006$ comparing breast-fed *vs* formula-fed groups in 6 mo olds.

***Serum IgG antibody levels to whole-cell NTHi in healthy children and with AOM.*** In group 1 healthy and AOM children, we selected (according to sera availability) a subset of 30 infants with no AOM and 30 children with AOM and measured antibody levels by whole cell specific ELISA using the reference NTHi strain 86-028. The response index was used as a measure of the strength of the immune response mounted by an infant. We observed a graded impact on convalescent serum antibody levels according to feeding status, with exclusively breast-fed infants having the highest convalescent antibody levels (*versus* formula fed only, $p = 0.03$), breast/formula fed being intermediate (*versus* formula fed, $p = 0.06$), and exclusively formula fed only being lowest (Fig. 1*A*). Compared with breast-fed infants, similar observations were made when the analysis was restricted to healthy children (breast *versus* formula fed, $p = 0.03$; breast/formula *versus* formula fed, $p = 0.05$; Fig. 1*B*) or with AOM (breast *versus* formula fed, $p = 0.05$; breast/formula *versus* formula fed, $p = 0.14$; Fig. 1*C*).

In children with AOM, we had sufficient paired acute and convalescent sera to compare antibody titers at the time of infection and 3 wk later. Sample size limited statistical analysis but a trend for increases in anti-P6 antibody were observed in all three feeding groups with the greatest increases in antibody among the breast *versus* the breast/formula *versus* the formula-fed infants (Table 5).

***Recovery of NTHi from NP cultures in healthy children and with AOM.*** In group 2a healthy children and group 2b children with AOM, the overall carrier rate of NTHi was 9% (7 of 76) and 44% (23 of 52), respectively, ($p < 0.0001$) (Table 6). In group 2a healthy children, the proportion of NTHi carriage was not different among breast (8%), breast/formula (12%), and formula-fed children (7%) ($p > 0.05$). In contrast, in group 2b children with AOM, the prevalence of NTHi was significantly lower in breast- *versus* formula-fed

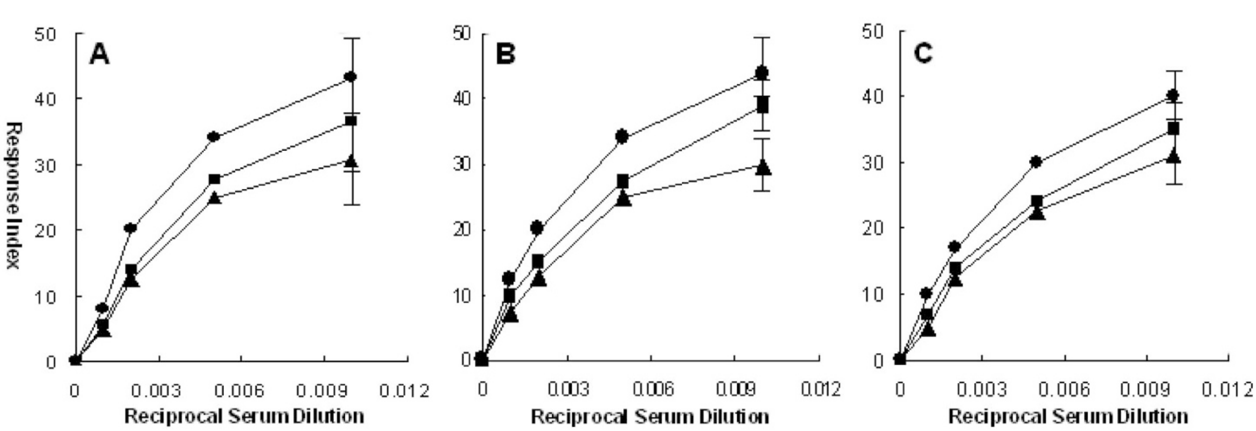


**Figure 1.** Serum IgG antibody levels to whole-cell NTHi in all children (*panel A*), healthy children (*panel B*), and children with AOM (*panel C*, group 1). (*A*) All AOM experience combined, (*B*) healthy children, and (*C*) children with AOM as a function of feeding status. ●, breast-fed; ■, breast/formula fed; ▲, formula fed.

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children ($p = 0.014$). In group 2b children with AOM, the proportion of breast-fed children carrying NTHi (11%) was similar to that in healthy children.

***Recovery of AOM pathogens isolated from ME fluid and inhibition of bactericidal antibody activity in children with AOM.*** Across the three feeding groups, the most common AOM pathogen was NTHi followed by *S. pneumoniae* and *M. catarrhalis*; none were *H. haemolyticus* (Table 7). The prevalence of NTHi in ME was lower in breast-fed children (22%), when compared with that in the middle ear from breast/formula (46%), and formula-fed children (58%).

Bactericidal titers, directed against the ME isolate of NTHi for each infected child, were determined in acute and convalescent serum of group 2b children with AOM. All acute sera had undetectable bactericidal activity, whereas convalescent sera had bactericidal antibody with the range from 1:2 to 1:16 (Table 8). The average bactericidal titers (mean ± SE) of the convalescent serum from breast-fed group (9 ± 2.2) tended to be higher than those from breast/formula (7.25 ± 2.1) and formula-fed groups (6.75 ± 2.13). Although significant differences among the groups were not achieved, the bactericidal titers correlated with the levels of P6-specific IgG antibodies in the convalescent serum ($p < 0.001$).

Twenty of 24 convalescent serum samples demonstrated 2- to 8-fold decreases in bactericidal activity after absorption of P6 antibody from the serum; four children demonstrated the same bactericidal activity as the original samples.

Among children with AOM caused by NTHi, the anti-P6 IgG antibody titers varied and were not related to the previous feeding status or to the number of previous episodes of AOM (Table 8). Some children (subjects 10, 14, 20, 21) with more than or equal to five episodes of AOM demonstrated high titers of anti-P6 IgG antibody (>3000 ng/mL) in convalescent serum. In contrast, two children (subjects 18 and 19) with three to five episodes of AOM demonstrated low titers of anti-P6 IgG antibody (<1000 ng/mL) in their convalescent serum.

**Table 5.** *The titers of P6-specific antibodies in acute* vs *convalescent serum in three feeding groups (mean ± SE)*

| | B (n−6) | BF (n−5) | F (n−4) |
|---|---|---|---|
| Acute sera | 1540 ± 266 | 1110 ± 276 | 964 ± 407 |
| Convalescent sera | 2574 ± 621 | 1899 ± 592 | 1690 ± 704 |
| *p* | 0.07 | 0.18 | 0.39 |

## DISCUSSION

In this study, we have shown that the incidence of AOM was sequentially lowest in breast, followed by breast/formula and the formula-fed infants. The results support other studies demonstrating that breast-feeding has a protective effect against acute and prolonged infections, including otitis media (15,16). The protective effect of breast-feeding against respiratory diseases, including otitis media, has been ascribed to passively transfer maternal antibodies, mainly secretory IgA, (1,7). A novel aspect of our work involved examination of another possible mechanism whereby breast-feeding might be protective against AOM—stimulation of higher serum antibody levels against a major otopathogen, NTHi. Indeed, we found that serum antibody responses to NTHi and an OMP vaccine candidate of NTHi, P6, were enhanced in breast compared with breast/formula and formula-fed infants. This observation supports the notion that breast-feeding facilitates immune responses in infants and suggests that feeding status may be an important covariate in studies that compare antibody levels among AOM groups.

Others have reported that children with AOM or NP colonization by NTHi develop an immune response to this pathogen (17). The NP is considered a reservoir for NTHi, and NP flora becomes established during the first year of life. In group 1 of our study, there were some children without previous AOM at 2 and 6 mo of age; however, they had an increase in serum antibody to whole NTHi and P6 suggesting that NP colonization by NTHi was an immunizing event.

We measured the serum antibody levels to a well-characterized NTHi strain. Several studies have reported that

**Table 7.** *Distribution of AOM pathogens isolated from middle ear fluid of children with AOM (group 2)*

| AOM pathogen isolated from middle ear | Breast-fed | Breast/formula fed | Formula fed |
|---|---|---|---|
| NTHi | 1 (11%) | 11 (46%) | 11 (58%) |
| *Streptococcus pneumoniae* | 5 (56%) | 8 (33%) | 5 (26%) |
| *Moraxella catarrhalis* | 1 (11%) | 0 (0%) | 2 (11%) |
| Others | 2 (22%) | 5 (21%) | 1 (5%) |
| Total | 9 | 24 | 19 |

Values are the number of subjects (percentage). Others: alpha *Haemoliticus streptococcus*, *Haemophilus parainfluenzae*.

**Table 6.** *Recovery of NTHi from NP cultures in healthy children and children with AOM (group 2)*

| | NP cultures* | | | |
|---|---|---|---|---|
| | Healthy | | With AOM | |
| Feeding status | NTHi (−) | NTHi (+) | NTHi (−) | NTHi (+) |
| Breast-fed ($n = 33$) | 22 (92%) | 2 (8%)† | 8 (89%) | 1 (11%)‡ |
| Breast/formula fed ($n = 49$) | 22 (88%) | 3 (12%)† | 13 (54%) | 11 (46%) |
| Formula fed ($n = 48$) | 25 (93%) | 2 (7%)† | 8 (42%) | 11 (58%) |

* NP cultures were obtained from nasal washes and/or nasal swabs. Values are the number of subjects (percentage). The percentages are for the two different populations: healthy children and children with AOM.

† $p > 0.05$, the difference in the proportion of NTHi (+) NP cultures across the three feeding groups.

‡ $p = 0.014$, the difference in proportion of NTHi (+) NP cultures in breast-fed *vs* formula-fed groups.

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**Table 8.** *Clinical data on children with acute nontypeable* Haemophilus Influenzae *otitis media and bactericidal activity in serum samples (group 2)*

| Group | Patient number | Age (mo) | Previous AOM | Serum bactericidal activity* | | Convalescent anti-P6 IgG titer (ng/ml)† |
|---|---|---|---|---|---|---|
| | | | | Convalescent | P6-absorbed convalescent | |
| Breast-fed | 1 | 11 | 1 | 2 | 2 | 788 |
| | 2 | 14 | 2 | 16 | 2 | 2800 |
| | 3 | 9 | 2 | 4 | 0 | 2147 |
| | 4 | 23 | 0 | 8 | 2 | 2803 |
| | 5 | 10 | 3 | 2 | 0 | 1275 |
| | 6 | 7 | 1 | 8 | 2 | 4942 |
| | 7 | 16 | 1 | 16 | 4 | 3490 |
| | 8 | 15 | 1 | 2 | 2 | 3508 |
| Breast/formula fed | 9 | 7 | 1 | 2 | 2 | 525 |
| | 10 | 11 | 8 | 16 | 2 | 7679 |
| | 11 | 12 | 2 | 4 | 0 | 1856 |
| | 12 | 8 | 2 | 8 | 2 | 3183 |
| | 13 | 11 | 2 | 2 | 0 | 2735 |
| | 14 | 9 | 5 | 8 | 2 | 3140 |
| | 15 | 13 | 3 | 16 | 4 | 3322 |
| | 16 | 14 | 1 | 2 | 2 | 651 |
| Formula fed | 17 | 14 | 3 | 8 | 2 | 2926 |
| | 18 | 11 | 3 | 4 | 0 | 779 |
| | 19 | 14 | 5 | 2 | 2 | 150 |
| | 20 | 17 | 5 | 16 | 2 | 3516 |
| | 21 | 16 | 5 | 16 | 4 | 3442 |
| | 22 | 7 | 3 | 2 | 0 | 1224 |
| | 23 | 12 | 0 | 4 | 4 | 1871 |
| | 24 | 21 | 2 | 2 | 0 | 1244 |

* All acute sera had no bactericidal activity. Data shown are reciprocal bactericidal titers. The bactericidal titer was defined as the serum dilution required for 50% killing of the inoculum of nontypeable *Haemophilus influenzae* after 60 min of incubation in the presence of complement. Each serum sample was assayed against the bacterial strain isolated from middle ear space of that same child.

† $p < 0.05$ comparing breast-fed *vs* breast/formula fed group and comparing breast *vs* formula-fed groups.

NP colonization causes children to develop antibody to homologous and heterologous NTHi strains (18). More recent studies demonstrated that all NTHi strains share antigenically conserved OMP P6 (3,19), and an antibody response to P6 may confer protection against NTHi, rendering infants less susceptible to AOM (2). The increase in levels of specific serum antibodies to whole-cell NTHi and to P6 in our study is consistent with these earlier results.

We demonstrated that children develop bactericidal antibodies to homologous NTHi strains isolated from ME fluid during AOM, similar to the findings by others (2,20). Two lines of evidence suggest that IgG antibody directed against highly conserved OMP P6 likely accounts for some bactericidal activity against NTHi. There was a reduction in bactericidal activity after removal of P6 antibodies, and P6-specific IgG antibodies and bactericidal titers correlated. Variable anti-P6 IgG responses among children with AOM caused by NTHi suggest that children also vary in their ability to mount protective responses to highly conserved OMP P6.

Specific serum antibodies are likely to protect against NP carriage and AOM provided that the antibody can reach the mucosal surface (21). If breast-feeding does enhance the serum antibody responses to NTHi, a protective effect would occur in support of this hypothesis. In group 2b, we observed decreased NP carriage in breast compared with nonbreast-fed infants with AOM. Thus, serum antibodies that are transudated to the NP during an upper respiratory infection could provide protection against NP carriage by NTHi, more so in breast-fed children. In the absence of inflammation, serum antibody transudation would be less and NP colonization would be impacted less. This proposal is supported by the observation that carriage of NTHi does not typically result in inflammation in the nasal passage (22).

Bernstein *et al.* (17) reported that passively acquired serum antibody in the newborn did not play a role in the prevention of NTHi NP colonization. However, during episodes of respiratory illness, there is inflammation in the upper respiratory tract mucosa (23) and an increase in NP flora (9). AOM is known to be accompanied by inflammation in the NP and ME, and this facilitates the transfer of circulating IgG antibodies into the NP and ME in the peak of the inflammatory response (21,24). Therefore, our observations are not inconsistent with earlier studies.

Increased naturally occurring NTHi-specific serum IgG responses may not completely prevent AOM in certain infants, as evidenced by episodes of AOM even in the breast-fed infants; however, a protective role of specific antibodies might be demonstrated in future studies and vaccine-induced antibody levels may be much higher than achievable by colonization and/or AOM. Importantly, among children with medium or high frequency of AOM, the levels of serum IgG antibody to whole-cell NTHi were significantly higher in the breast-fed group. Although NTHi-specific serum IgG responses did not prevent AOM in otitis-prone children, it is

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possible that these antibodies could facilitate faster resolution from AOM.

Others speculate the mechanism of protection against respiratory infections afforded by breast milk might be anti-idiotypic antibodies as well as T and B lymphocytes, which are transferred *via* milk (25). Breast milk also contains numerous anti-inflammatory factors, nucleotides, cytokines, growth factors, macrophages, and granulocytes in the milk that might stimulate the immune system of the infant (26). Increased responses of serum antibodies after vaccination with *Haemophilus influenzae* type b (Hib) conjugate vaccines, tetanus and diphtheria toxoids, BCG, live oral poliovirus, and pneumococcal vaccines have been shown in breast-fed infants (10–12). Nucleotides present in breast milk have been added to many infant formulas but were not present in the infant formulas used in 1990–1991 when our group 1 healthy and AOM children's sera were collected. Nucleotide supplementation has been shown to influence immune responses to vaccines (10,27).

It is likely that repeated contact with NTHi on the mucosal surface could stimulate the production of systemic IgG as well as local IgA responses to this pathogen. However, the presence of specific serum IgG antibodies in infants <6 mo of age could be due to maternal antibody passage. Specific IgG antibody usually decline by 6 mo of age, and in this study, the levels of these antibodies increased from 2 to 6 mo. These findings provide evidence that we were measuring an active infant's immune response in breast-fed children.

Our study has limitations. We did not confirm NP colonization by NTHi through NP cultures in group 1; therefore, we could not compare the specific antibody titers in children who were proven culture-positive *versus* culture-negative for NTHi. We made an assumption that enough infants were colonized with NTHi at least once during the sampling times based on NP carriage studies that have reported colonization rates ranging to >50% (9,28,29). To supplement this assumption, we did examine the overall rate of NTHi NP colonization in the group 2 population and found colonization rates among all enrolled infants to be 25.3% (33 of 130) as confirmed by culture. The rate of NTHi NP colonization most likely was higher, as evidenced by the presence of NTHi in 32% (47 of 147) of our culture-negative NP samples using a sensitive multiplex PCR technique (unpublished observation).

In conclusion, our findings suggest that breast-feeding plays a significant role in modulating serum antibody levels to NTHi and OMP P6 during the first 6 mo after birth. Transudation of serum antibody to NTHi and OMP P6 might protect against AOM during upper respiratory infections. This finding has implications for studies seeking a population-based serum correlate of protection against NTHi infection. The addition of nucleotides to a commercially available formula might produce the same effect as breast-feeding and deserves study.

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