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Breastfeeding 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 non-breast 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.

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 (OMP), 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).

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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 epidemiological 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 vs. non-breast 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 vs. breast/formula fed vs. formula fed) and the frequency of episodes of AOM. The children were assigned to breast fed vs. breast/formula fed vs. formula fed groups based on self report of the mother at the time blood samples were taken and no attempt was made to semi-quantitate the proportion of breast vs. 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 months of age. Group 2 was prospectively enrolled from the same private practice population in 2006-2007. In group 2, there were 2 subgroups: (a) children enrolled at 6 months 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 months were pre-defined as AOM children if they had ≥ 1 episodes of AOM. Children of age 6 months were pre-defined as AOM children if they had ≥ 2 episodes of AOM. Group 2a healthy children had NP cultures and NP wash samples, and oropharyngeal swabs obtained every 3-6 months between 6 to 24 months of age. These subjects were not ill. Group 2b children with AOM were 6-24 months 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 3 episodes in 6 months or 4 episodes in 12 months. 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 TF 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 enzyme-linked immunosorbent assays (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 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 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 hr. 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 2M NaOH and was read by an automated ELISA reader using a 405-nm filter. In some experiments, the optical densities (OD) 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 μg/ml of P6 antigen in coating buffer (bicarbonate, [pH 9.4]) and the rest of the steps for the ELISA were performed as described above. For group 2 samples, 96-well plates were coated with 0.25 μg/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 hr. 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 weeks thereafter in the convalescent period, and serum depleted of antibodies against P6, were employed 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 ~10^5 CFU/ml. Twelve serial twofold dilutions of the serum to be tested (starting at 1:2) were mixed with precolostral calf serum complement and 20 μl of bacteria. After 60 min of incubation, the number of surviving bacteria was determined by plating 5 μl 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 ≥ 50% bacterial killing and was compared to 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. *P* values of .05 were considered significant.

RESULTS

Study participation

372 children were studied from group 1 (healthy and AOM children); there were 180 2 month and 192 6 month olds. Among the 2 month olds, there were 81 breast, 54 breast/formula, and 45 formula fed. Among 6 months 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 vs. formula fed children at 2 months of age, $P = .01$; (Table 1). At 6 months of age, the incidence of AOM was again significantly lower in breast vs. formula fed children ($P < .0001$), and in breast/ formula vs. formula fed children ($P < .0001$) (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 month olds (n = 39) and 6 month olds (n = 31). Among 2 month olds, the level of specific serum antibody to whole NTHi was significantly higher in the breast vs. breast/formula ($P = .013$) and formula fed groups ($P = .021$) (Table 3). Similarly, the levels of specific serum antibody levels among 6 month olds in group 1 healthy children were significantly higher in the breast vs. breast/formula ($P = .01$) and formula fed groups ($P = .0008$), and in breast/formula vs. formula fed groups ($P = .0004$) (Table 3). In all three cohorts in group 1, the median levels of specific NTHi antibodies in 6 month olds children were higher than those in 2 month olds children ($P = .044$). 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).

Serum IgG antibody levels to NTHi outer membrane protein P6 in infants

In group 1 healthy and AOM children, in both 2 and 6 month olds, there was a difference in specific serum IgG antibody levels to OMP P6 among the three feeding cohorts. In 2 month olds, higher anti-P6 antibody levels to OMP P6 were measured in breast than in breast/formula ($P = .0008$) and formula fed ($P = .029$) infants (Table 4). Similarly, in 6 month olds significant

differences in the specific anti-P6 antibody levels were measured between breast and breast/formula fed ($P = .022$) and between breast and formula fed ($P = .006$) infants.

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 (vs. formula fed only, $P = .03$), breast/formula fed being intermediate (vs. formula fed, $P = .06$), and exclusively formula fed only being lowest (Fig. 1A). Compared to breast fed infants, similar observations were made when the analysis was restricted to healthy children (breast vs. formula fed, $P = .03$; breast/formula vs. formula fed, $P = .05$; Fig. 1B) or with AOM (breast vs. formula fed, $P = .05$; breast/formula vs. formula fed, $P = .14$; Fig. 1C).

In children with AOM, we had sufficient paired acute and convalescent sera to compare antibody titers at the time of infection and 3 weeks later. Sample size limited statistical analysis but a trend for increases in anti-P6 antibody were observed in all 3 feeding groups with the greatest increases in antibody among the breast vs the breast/formula vs 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 ($P < .0001$) (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%) ($P > .05$). In contrast, in group 2b children with AOM, the prevalence of NTHi was significantly lower in breast vs. formula fed children ($P = .014$). 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 3 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%), as 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 ($P < .001$).

20 of 24 convalescent serum samples demonstrated 2- to 8-fold decreases in bactericidal activity after absorption of P6 antibody from the serum; 4 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 ≥ 5 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 3-5 episodes of AOM demonstrated low titers of anti-P6 IgG antibody (< 1000 ng/ml) in their convalescent serum.

DISCUSSION

In the present 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 to 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 co-variate 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 were some children without prior AOM at 2 and 6 months of age, yet 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 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 prior 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 to non-breast 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 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 less than 6 months of age could be due to maternal antibody passage. Specific IgG antibody usually decline by 6 months of age, and in the present study the levels of these antibodies increased from 2 months to 6 months. 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 vs. 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 months 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 breastfeeding and deserves study.

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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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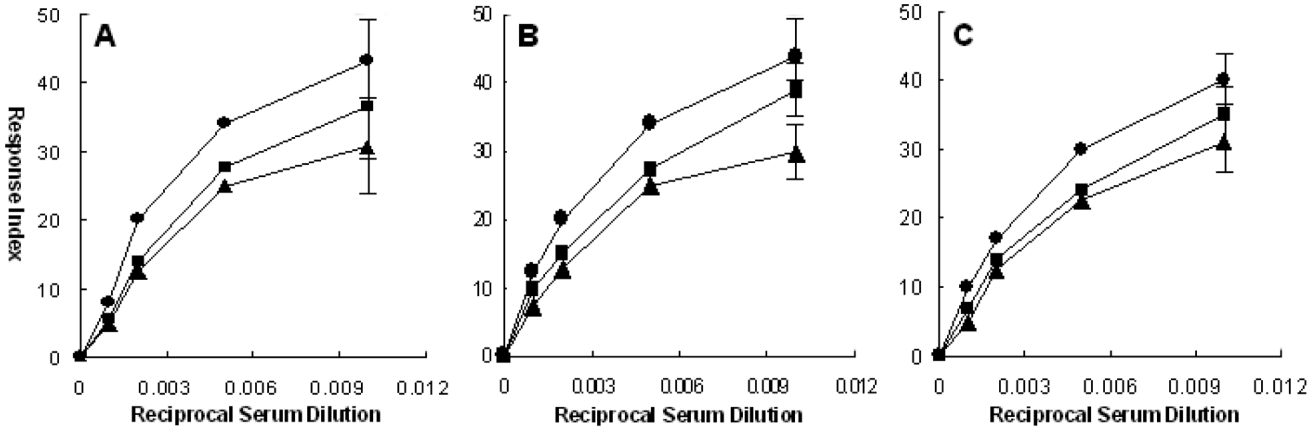

FIGURE 1.

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 and formula fed (■); Formula fed (▲).

TABLE 1

Incidence of AOM in 2 Month Old Infants in Relation to Breastfeeding (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%)

AOM: acute otitis media. Values are the number of subjects (percentage).

* $P = .01$, the incidence of AOM in breast fed vs. formula fed children.

TABLE 2

Incidence of AOM in 6 Month Old Infants in Relation to Breastfeeding (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%)

AOM: acute otitis media. Values are the number of subjects (percentage).

* $P < .0001$ comparing breast fed vs. formula fed group and comparing breast/formula fed vs. formula fed group.

** $P < .0001$ 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 month olds(n=39)	2.00 ± 0.10 (n=15) *	1.72 ± 0.07 (n=18)	1.52 ± 0.17 (n=6)
6 month olds (n=31)	2.4 ±0.37 (n=7) **	1.85 ±0.1 † (n=14)	0.94 ± 0.12 (n=10)

NTHi: nontypeable *H. influenza*. Values are the mean ± SE of log $_{10}$ of Reference Units.

* *P* = .013 comparing breast fed vs. breast/formula fed and *P* = .021 comparing breast fed vs. formula fed groups in 2 month olds.

** *P* = .01 comparing breast fed vs. breast/formula fed and *P* = .0008 comparing breast fed vs. formula fed groups in 6 month olds.

† *P* = .0004 comparing breast/formula fed vs. formula fed group in 6 month 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 month olds (n=40)	2.22 ±0.09* (n=15)	1.8 ± 0.08 (n=19)	1.68 ± 0.18 (n=6)
6 month olds (n=28)	2.48 ±0.33** (n=6)	2.05 ±0.1 (n=13)	1.86 ±0.12 (n=9)

NTHi: nontypeable *H. influenza*. Values are the mean ± SE of $\log_{10}$ of Reference Units.

* $P = .0008$ comparing breast fed vs. breast/formula fed and $P = .029$ comparing breast fed vs. formula fed groups in 2 month olds.

** $P = .022$ comparing breast fed vs. breast/formula fed and $P = .006$ comparing breast fed vs. formula fed groups in 6 month olds.

TABLE 5

The titers of P6-specific antibodies in acute vs. convalescent serum in 3 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 value	0.07	0.18	0.39

TABLE 6

Recovery of NTHi from NP Cultures in Healthy Children, and Children with AOM (group 2)

Feeding status	NP Cultures*			
	Healthy		With AOM	
	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%)

NTHi: nontypeable *H. influenzae*; NP: nasopharyngeal; AOM: acute otitis media.

* 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.

** $P > .05$, the difference in the proportion of NTHi (+) NP cultures across the 3 feeding groups.

† $P = .014$, the difference in proportion of NTHi (+) NP cultures in breast fed vs. formula fed groups.

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%)
S. pneumoniae	5 (56%)	8 (33%)	5 (26%)
M. catarrhalis	1 (11%)	0 (0%)	2 (11%)
Others	2 (22%)	5 (21%)	1 (5%)
Total	9	24	19

AOM: acute otitis media; NTHi: nontypeable *H. influenzae*; S. pneumoniae: *Streptococcus pneumoniae*; M. catarrhalis: *Moraxella catarrhalis*; Others: alpha *Haemoliticus streptococcus, Haemophilus parainfluenzae*. Values are the number of subjects (percentage).

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 months	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 minutes 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.

**$P < .05$ comparing breast fed vs. breast/formula fed group and comparing breast vs. formula fed groups.