

TGF- β in human milk is associated with wheeze in infancy

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Background: Cytokines secreted in human milk might play important roles in newborn health and in the development of infant immune responses. We investigated the relationship of the concentration and dose of cytokines in human milk to infant wheeze at 1 year of age.

Objective: Our objective was to test whether the cytokines in milk could account for some of the apparent protective effect of breast-feeding against wheeze in the first year of life.

Methods: Data on breast-feeding and infant wheeze were collected prospectively from birth to 1 year from 243 mothers participating in the Infant Immune Study in Tucson, Arizona. Breast milk samples obtained at a mean age of 11 days postpartum were assayed by means of ELISA for concentrations of TGF- β 1, IL-10, TNF- α , and the soluble form of CD14. The dose of each cytokine was assessed for a relationship with wheeze in bivariate and logistic regression analyses.

Results: Increasing duration of breast-feeding was significantly associated with a decreased prevalence of wheeze ($P = .039$). There was wide variability in levels of each cytokine in milk, as well as variability between women in the amount of each cytokine produced. There was a significant inverse association between the dose of TGF- β 1 received through milk with the percentage of wheeze ($P = .017$), and the relationship was linear ($P = .006$). None of the other cytokines showed a linear relationship with wheeze. In multivariate analyses the risk of wheeze was significantly decreased (odds ratio, 0.22; 95% CI 0.05-0.89; $P = .034$) with increasing TGF- β 1 dose (long breast-feeding and medium-high TGF- β 1 level compared with short breast-feeding and low TGF- β 1 level).

Conclusion: This analysis shows that the dose of TGF- β 1 received from milk has a significant relationship with infant wheeze, which might account for at least some of the protective effect of breast-feeding against wheeze. (*J Allergy Clin Immunol* 2003;112:723-8.)

Key words: Breast-feeding, wheeze, cytokines, TGF- β , human milk, infancy

There is much evidence that breast-feeding protects against wheeze in infancy in both developing and industrialized countries.¹⁻⁸ Several components of human milk have been postulated as conferring this protective effect. Breast-feeding might provide an immediate line of defense against infectious agents, which compensates directly for the immaturity of the newborn immune system to resist infection.⁹ Protection might also be achieved through the myriad of factors in milk, including bioactive enzymes, hormones, growth factors, and immunologic agents, that augment and stimulate the development of immature host defense.¹⁰⁻¹³ Breast-fed infants might also be less exposed to infection or differ in other ways from formula-fed infants. Thus, it is not clear which of the many components of this complex biologic fluid account for the protective effect.

It has only recently been shown that human milk contains cytokines.¹⁴ Cytokines are small soluble glycoproteins that act in autocrine-paracrine fashions by binding to specific cellular receptors, operating in networks, and orchestrating immune system development and function.¹⁵ Because these cytokines are present at picogram quantities, they were initially viewed as insignificant byproducts of milk secretion. However, early milk has an abundance of cytokines at a time when neonatal organ systems are immature, suggesting that these bioactive components of milk might be important in neonatal development.

TGF- β 1 is one of the most abundant cytokines in human milk.¹⁶ It is thought that TGF- β might play a role in lung morphogenesis¹⁷ in early infancy and act on secretory IgA production¹⁸ in the intestinal mucosa of the infant to serve in defense against infection. There is considerable variability in the concentrations of TGF- β in human milk,¹⁹ and thus diminished secretion of this cytokine might contribute to the development of wheeze.^{20,21} To our knowledge, the relationship of TGF- β 1 in milk to the occurrence of wheeze has not been studied.

Our objective was to determine whether there is a relationship between TGF- β 1 levels in milk and wheeze in infancy and to determine whether the relationship could account for some of the apparent protective effect of breast-feeding against wheeze. Other factors in milk considered for association with wheeze were the anti-inflammatory cytokine IL-10, the pro-inflammatory cytokine TNF- α , and soluble CD14 (sCD14), the soluble form of

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The Infant Immune Study is funded by a grant from the National Institutes of Health (AI42268) and the Arizona Disease Control Research Commission. Dr Oddy is supported by a National Health and Medical Research Council Public Health Fellowship Award and received travel awards from the National Health and Medical Research Council of Australia, the Ian Potter Foundation, and the International Society for Research into Human Milk and Lactation to conduct this research and present the results.

Received for publication March 3, 2003; revised June 27, 2003; accepted for publication July 2, 2003.

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0091-6749/2003 \$30.00 + 0
doi:10.1067/mai.2003.1747

Abbreviation used
sCD14: Soluble CD14

the innate immune receptor for LPS, a component of bacterial cell walls. These components were assessed because they are produced by the macrophages and epithelial cells (ie, the primary cells in human milk),¹³ they exist at detectable levels in human milk, and they influence the behavior of other cells, tissues, or both. Thus, any effects of a given cytokine could be tested for specificity by comparison with the other immunologically active components.

METHODS

The Infant Immune Study is a prospective study of the development of immunologic markers of asthma risk in infancy and early childhood. It was designed to investigate potential immunologic mechanisms that might underlie the stronger relationship of maternal asthma to asthma in the child relative to paternal asthma. Healthy women between 32 and 35 weeks' gestation were invited to participate in the study. Only women who planned to obtain care for their newborns through collaborating pediatric practices were eligible. Before the infant's birth, information was obtained regarding maternal health during pregnancy, demographic characteristics, and the respiratory health of both parents.

When the child was 1 year of age (mean age, 1.05 ± 0.12 years), parents completed a questionnaire about their child's respiratory health. Data on wheeze were obtained by asking, "Has his/her chest ever sounded wheezy or whistling?" If the answer was yes, the parents were asked how often this occurred on a 5-point scale from "very rarely" (1) to "on most days" (5). Children given a score of 2 were considered occasional wheezers, whereas those with scores of 3 or more on this scale were defined as having frequent wheeze. Although data on wheeze were also obtained at the time of well-child visits, more than 50% of the children had missing information for 2 or more well-child visits, and the last of these questionnaires was administered at 9 months, thus leaving out the last quarter of the first year of life. Only children who were at least 1 year old with wheeze data from the 12-month questionnaire were included.

Data about infant feeding were obtained by means of parental questionnaire administered either during a well-child visit or by means of telephone at 2 weeks and 1, 2, 3, 4, 6 and 9 months. Breast-feeding duration was considered as a continuous variable (in weeks) and in categories (never breast-fed or breast-fed <1 week, ≥ 1 week- <3 months, 3- <6 months, ≥ 6 months with formula introduced, and ≥ 6 months with no formula introduced). Duration of any breast-feeding and the age at introduction of other milk-formula (signifying the end of exclusive breast-feeding) were highly correlated. This analysis uses duration of any breast-feeding because the primary focus was the concentration of cytokines in milk and the dose of cytokines to the recipient infant throughout the duration of breast-feeding.

Potential confounders considered in the analysis included sex, gestational age (<37 vs ≥ 37 weeks), maternal smoking at 1 year (any vs none), birth weight (≤ 6 lbs vs >6 lbs), exposure to other children (presence of any older siblings or attendance at daycare with other children before 3 months), maternal education (≤ 12 vs >12 years), and maternal history of physician-diagnosed asthma (yes vs no). Allergy in the mother was not included in multivariate models both because of colinearity with maternal asthma and the finding that maternal atopy shows no relationship with cytokine levels in milk.²²

Milk specimen collection and processing

A milk sample was collected from each breast-feeding mother at a home visit in the first 2 weeks after delivery (mean age, 11 days postpartum). Collection was standardized to reduce potential diurnal variability in cytokine measurements. Specimens were obtained within 2 hours of the first feeding in the morning, defined as 6 AM to 8 AM. Mothers were asked not to feed from the breast used for specimen collection for 2 hours before collection. The entire content of one breast was collected by using a LACT-E electric low-pressure breast pump (donated by Ameda-Egnell Corp) and transferred into a sterile 60-mL disposable Grad-U-Feed Bottle until the milk stopped flowing or the bottle was full. The sample was maintained at room temperature and processed within 4 hours of collection.

The milk volume and creamocrit level were determined, and then a whole milk aliquot was collected. The remaining sample was centrifuged at 1800 rpm for 12 minutes, after which the lipid layer and aqueous fractions were removed. An aliquot of the aqueous fraction was filtered (0.45- μ m Acrodisc, Gelman Sciences). All fractions were stored at -70°C until assayed for cytokines. The filtered aqueous fraction was used for cytokine determinations, with the lipid layer frozen for other studies.

Milk cytokine ELISA assays

Filtered aqueous milk fractions were assayed for TGF- $\beta 1$, TNF- α , IL-10, and sCD14 levels with quantitative colorimetric sandwich cytokine ELISA kits (R & D Systems). All fractions were assayed undiluted, except sCD14 and TGF- $\beta 1$, which were assayed at dilution factors of greater than 2000 and 1.4, respectively. The thresholds of detectability for the assays were as follows: TGF- $\beta 1$, 31.2 pg/mL; IL-10, 7.8 pg/mL; TNF- α (high-sensitivity assay), 15.6 pg/mL; and sCD14, 250 μ g/mL. Before assay for TGF- $\beta 1$, acid treatment (100 μ L of 1N HCl per 500 μ L of milk for 10 minutes at room temperature) and neutralization (100 μ L of 1.2 N NaOH per 0.5 mol/L Hepes per 500 μ L of milk) of an aliquot of each milk sample was performed to activate latent TGF- $\beta 1$ to the immunoreactive form. Early difficulties in processing TGF- $\beta 1$ limited the sample size for these analyses. The coefficients of variation for intra-assay and interassay, respectively, are the following: TGF- $\beta 1$, 7.5% and 12.7%; IL-10, 8.5% and 15.6%; sCD14, 6.4% and 7.4%; and TNF- α , 17.6% and 14.9%.

Cytokine values were log transformed to provide a normal distribution. In addition, because most of the milk cytokines were correlated with the time since delivery, all were age adjusted as follows. Log-transformed cytokine values were regressed with time since delivery, and the residuals were saved. Values were then standardized to the mean time since delivery (11 days) by using the coefficients from the regression and the residuals. The concentration of each cytokine was calculated in picograms per milliliter of milk, with the exception of sCD14, which was calculated in micrograms per milliliter. An estimated dose was calculated by multiplying the antilogged values of each age-adjusted cytokine concentration by the duration of breast-feeding in weeks to assess the total amount of each cytokine received throughout the course of breast-feeding. The estimated dose was classified into tertiles (high, medium, and low).

Statistical analysis

The relationship between breast-feeding and wheeze in the first year of life was assessed by using contingency tables and the χ^2 test. Spearman nonparametric tests were conducted to examine correlation coefficients between cytokines. The relationship of the concentration of each cytokine in milk to wheeze was assessed by dividing the concentrations into tertiles with trend χ^2 tests. Infants never breast-fed were excluded from these analyses (rather than including them in the lowest category for cytokine dose) to ensure the rela-

tionships observed were not an artifact of any breast-feeding. Finally, logistic regression analyses were conducted to determine the odds for any wheeze associated with cytokine dose in milk after adjustment for potential confounders. A final categorization of concentration and duration of breast-feeding was calculated to reflect short or long duration of feeding (divided at the median in weeks) and low versus medium-high cytokine concentration. The regression results are expressed as odds ratios with 95% CIs. In all analyses a *P* value of less than .05 was regarded as statistically significant. Approval by the Institutional Review Board of the University of Arizona in Tucson, Arizona, was sought and obtained.

RESULTS

The Infant Immune Study population had a high rate of breast-feeding, with 63% breast-fed for 3 months or longer and 48% breast-fed for 6 months or longer (Table I). The age at introduction of formula was significantly related to the duration of breast-feeding (*P* < .0005), with means being 0.3 weeks, 2 weeks, 4 weeks, and 11 weeks for those breast-fed less than 1 week, less than 3 months, 3 to 6 months, and 6 months or more, respectively.

Of the 243 children with complete data at 12 months, 40% (*n* = 97) had wheezed at least once. Most of these children wheezed rarely (*n* = 53), with 29 wheezing occasionally and 15 wheezing frequently.

The proportion of children experiencing wheeze decreased with increased breast-feeding, from 65% for infants never breast-fed to 44% for infants breast-fed less than 6 months to 34% for infants breast-fed for 6 months or longer (*P* = .039, Pearson χ^2 ; *P* = .014, trend χ^2). The mean duration of breast-feeding in the group never wheezing was 24 weeks versus 19 weeks in the wheezing group (*P* = .029, *t* test).

Table II presents the mean age-adjusted concentration (and 95% CIs) for TGF- β 1, IL-10, TNF- α , and sCD14 in the milk samples, and the estimated mean dose of each cytokine received from milk by the infant during the entire course of breast-feeding (Table II). There were significant positive correlations (Table III) between certain cytokine concentrations in the milk, such that mothers with high levels of some cytokines also had high levels of other cytokines. Specifically, there was a significant correlation between TGF- β 1 and both TNF- α and sCD14.

The relationship between wheeze and the concentration of each cytokine in the milk was assessed, with the group never breast-fed removed from analysis. None of these relationships achieved statistical significance (data not shown); the association closest to statistical significance was that with concentration of TGF- β 1 (*P* = .180). When considered as a dose (Fig 1), TGF- β 1 was the only cytokine significantly associated with wheeze (*P* = .017). Furthermore, TGF- β 1 was the only cytokine that showed a linear relationship with the percentage of children with wheeze (*P* = .006, trend χ^2).

Table IV gives the results of multivariate analysis with logistic regression. As shown in the model, the risk of wheeze decreased with both increasing duration of breast-feeding and with increasing TGF- β 1 concentration, with a dose response evident (*P* = 0.053, Pearson

TABLE I. Participant characteristics

Breast-feeding variable	
Breast-feeding duration, % (n)	
Never breast-fed	7.4 (17)
Breast-fed <1 wk	15.7 (36)
Breast-fed $\geq$ 1 wk-<3 mo	13.9 (32)
Breast-fed 3 mo-<6 mo	14.8 (34)
Breast-fed $\geq$ 6 mo	29.1 (67)
Breast-fed $\geq$ 6 mo-never formula	19.1 (44)
Outcome variable	
Wheeze ever, % (n)	
No	60.1 (146)
Yes	39.9 (97)
Potential confounding variables	
Exposure to other children, % (n)	62.1 (151/243)
Male sex, % (n)	52.7 (128/243)
Maternal asthma, % (n)	20.9 (49/235)
Maternal smoking at 1 y, % (n)	11.8 (27/228)
Maternal education, mean y $\pm$ SD	15.0 $\pm$ 2.5
Maternal age, mean y $\pm$ SD	28.6 $\pm$ 6.2
Mean gestational age, mean wk $\pm$ SD	39.5 $\pm$ 1.3
Birth weight of infant, mean lbs $\pm$ SD	7.7 $\pm$ 1.0

χ^2). Only infants with both a long duration of breast-feeding and a medium-high concentration of TGF- β 1 had significantly lower odds of wheeze in infancy (*P* = .034) relative to children with a short duration of breast-feeding and low TGF- β 1 concentration. The model was adjusted for sex, gestational age, maternal smoking, exposure to other children, maternal education, and maternal asthma. The only other factor that was a significant predictor of wheeze was exposure to other children (odds ratio, 3.66; 95% CI, 1.49-8.98; *P* = .005). In children with older siblings (*n* = 55), long breast-feeding with a medium-high concentration of TGF- β 1 was associated with significant protective effects against wheezing (*P* = .016) relative to children with siblings in the short breast-feeding and low TGF- β 1 group. In children with no older siblings (*n* = 61), the protective effect of long breast-feeding with a medium-high concentration of TGF- β 1 was not apparent (*P* = .180).

DISCUSSION

This analysis shows that the dose of TGF- β 1 received from milk is significantly associated with infant wheeze: the more TGF- β 1 an infant consumes, the less likely he or she is to wheeze in the first year of life. This effect persisted after consideration of breast-feeding duration, suggesting that at least some of the protective effect of breast-feeding against wheeze might be attributable to the presence of this cytokine in the milk.

Human milk has been reported to contain a large amount of TGF- β (1365.7 pg/mL in colostrum and 728.1 pg/mL in mature milk), a multifunctional polypeptide that acts on a host of different cell types.²³ In addition, mRNAs that code for TGF- β 1 and TGF- β 2 exist in human milk cells.¹⁶ Furthermore, it has been proposed that passage through the acidic pH of the infant stomach

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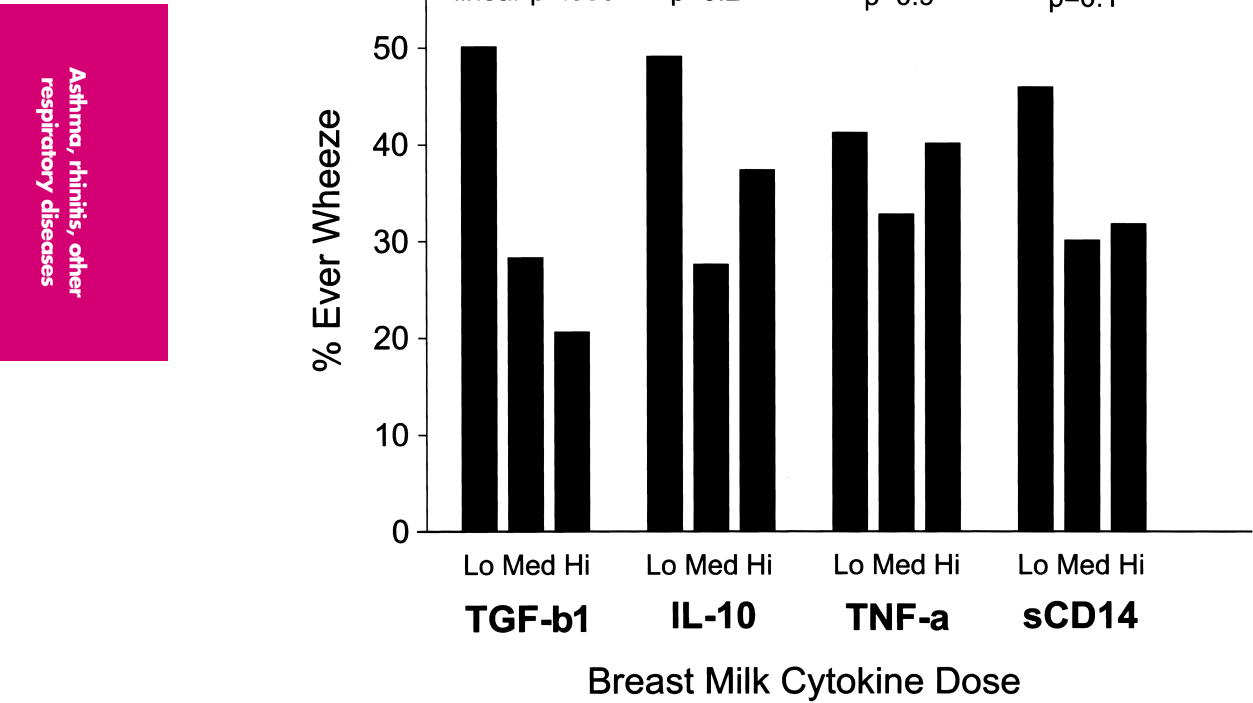


FIG 1. Percentage with wheeze in the first year of life by tertiles of cytokine dose (χ^2 analysis).

TABLE II. Mean concentration and dose of cytokines in human milk

Cytokine	Total n*	Geometric mean concentration (95% CI)	Mean dose received (95% CI)
TGF-β1 (pg/mL)	142	787 (731-844)	17,293 (14,951-19,636)
IL-10 (pg/mL)	190	10.16 (8.46-11.67)	205.51 (164.71-246.31)
TNF-α (pg/mL)	204	6.15 (5.34-6.96)	130.53 (105.31-155.76)
sCD14 (μg/mL)	243	17.29 (16.46-18.12)	388.23 (347.91-428.55)

*All milk cytokine concentrations were age adjusted. The number of subjects with data for each cytokine varies depending on the volume of milk and the number of cells in each specimen.

might free active moieties of TGF-β1,²³ thereby exposing the neonatal intestine to high concentrations of activated TGF-β1. In the gut, TGF-β1 has a potent immunosuppressive action that might prevent sensitization to food allergens and might inhibit T-cell activation.²⁴ In addition, TGF-β1 acts with IL-10 to increase IgA production by naive B cells¹⁴ and to promote specific IgA production,¹⁸ which might reduce susceptibility to both enteric and respiratory infections.

A second mechanism of TGF-β1 might involve effects on infant lung development. Models using in vivo treatment of fetal mouse lungs with epidermal growth factor and TGF-β1 have been shown to enhance biochemical and structural development, suggesting that TGF-β1 in conjunction with epidermal growth factor might regulate the signaling mechanisms of proliferation and differentiation of lung cells.²⁵ Additionally, these growth factors play a role in the regulation of surfactant proteins in the lung.²⁶ The biologic effects of TGF-β are mediated by type I and II receptors and demonstrate differential regu-

lation and localization of type I relative to type II. The isoforms exhibit distinct but overlapping patterns of expression during lung morphogenesis, implying several possible roles for TGF-β during lung development.²⁷

There is preliminary evidence in human studies that breast-feeding might foster lung development. In a different population our group assessed the relationship of feeding practices to lung function among 124 infants enrolled at birth with pulmonary function testing before age 6 months. Independent of known confounders, we found that infants not breast-fed at age 1 month had a 7-fold increase (95% CI, 2.3-21.9; $P = .0002$) in the risk of having low values of an indirect index of airway conductance (derived from the shape of the breathing curve). Furthermore, 27% (95% CI, 9.6-40.9) of all cases with low values for this index could be attributed to a lack of breast-feeding.²⁸ This finding has direct relevance to the study of wheeze in infancy because we have also shown that infants with reduced lung function early in life had an increased risk of lower respiratory tract illness before

TABLE III. Correlation coefficients (Spearman) between cytokine concentrations in milk

Measure	sCD14	TNF- α	IL-10
TNF- α	0.302* (n = 203)		
IL-10	-0.132 (n = 188)	0.080 (n = 178)	
TGF- β 1	0.383* (n = 141)	0.529* (n = 132)	0.053 (n = 121)

*Correlation is significant at less than .0005 (2-tailed).

their first birthday.²⁹ This suggests that initial airway caliber, length, or both (and perhaps the lung parenchyma structure) could predispose infants to wheezing in association with common viral respiratory infections.

It might be argued that cytokines in milk will be digested before they can exert any effects on the recipient infant. However, milk cytokines encounter a large mucosal surface in the infant's alimentary tract before reaching the stomach, where no proteolysis occurs. Pepsin production, the only gastric proteolytic enzyme, is delayed in the first week of life,³⁰ and a large number of antiproteases are found in milk. Furthermore, during the first week after delivery, the neonatal gut is "leaky," facilitating passage through the mucosa at the time cytokine levels are highest^{25,31} and subsequent movement to distal organs.³² There are animal³³ and human³⁴ studies showing cells of maternal origin in systemic circulation. In particular, Letterio et al^{35,36} have shown that when TGF- β was fed to TGF- β -null mice, the cytokine was found distributed intact to lung tissue. Thus, some of these agents likely survive passage through the gut.

There are certain limitations to our work. First, only one milk sample was collected. It is well established that milk composition differs with increasing parturition, throughout the day and at each feeding. Our analysis has assumed that although the levels of a particular cytokine will vary, a mother who produces large amounts at one time will likely produce large amounts throughout lactation, relative to other lactating mothers. There might, in fact, be wide variability for each mother in milk cytokine levels both over time, throughout the day, and within a feeding. Having multiple cytokine measures taken over time from mothers who vary in the exclusivity of breast-feeding would permit verification of the assumption underlying this work. In addition, we might lack sufficient power to detect effects of other cytokines, particularly IL-10, which is produced in lower amounts and yet showed a slight (though nonlinear) relationship with wheeze.

However, the study has several strengths. Prospective assessment clarifies the temporal relationships between exposure to the cytokine in milk and later wheeze. Our measure of wheeze has been used in previous studies and is valid.³⁷ Furthermore, the fact that 63% of the infants in the study were breast-fed provides a large enough group to be confident of results. Nevertheless, it is premature to know how the findings will pertain to later asthma because the majority of children who wheeze in infancy do not go on to have asthma.³⁸ However, we did observe an increased risk of current asthma at 6 years with one or more wheezing lower respiratory tract illnesses in the

TABLE IV. Multivariate logistic regression analyses of wheeze

TGF- β 1 dose group	Wheeze ever* OR (95% CI)	P value
Short breast-feeding duration/low concentration of TGF- β 1	1.00†	
Short breast-feeding duration/medium-high concentration of TGF- β 1	0.94 (0.27-3.26)	.922
Long breast-feeding duration/low concentration of TGF- β 1	0.44 (0.11-1.81)	.255
Long breast-feeding duration/medium-high concentration of TGF- β 1	0.22 (0.05-0.89)	.034‡

OR, Odds ratio.

*n = 116 in model.

†Overall Pearson χ^2 value of .053. Sex, gestational age, maternal asthma, maternal education, and maternal smoking were removed from the model because of nonsignificance. Exposure to other children remained in the model (odds ratio, 3.66; 95% CI, 1.49-8.98; P = .005).

‡Relative to short breast-feeding and low concentration of TGF- β 1.

first year of life.³⁹ Our finding that exposure of infants to other children was a much larger risk factor for report of wheeze at 1 year than dose of TGF- β 1 is not surprising given that the protective effects of breast-feeding are particularly evident in low-income populations. If anything, the effect we observed might be even greater among infants exposed to multiple risks for wheeze in infancy.

This study suggests that bioactive factors in milk, specifically TGF- β 1, provide protection against wheeze in infancy. With the exception of one study of cow's milk protein allergy,⁴⁰ this is the first study of the effects of a cytokine in human milk on the health of the infant. There remains much to be learned about the spectrum of these agents in milk and the long-term molecular-biologic consequences of their actions in the infant. The effects of milk cytokines on the maturation and functions of the epithelium, mucosal or submucosal leukocytes, and other specialized cells and structures in the alimentary and respiratory system should be particularly targeted for investigation.

We thank the study staff responsible for the collection of the data presented here. Sincere thanks are extended to all study families, without whose participation this research could not have been conducted.

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