

Cytokine, chemokine and secretory IgA levels in human milk in relation to atopic disease and IgA production in infants

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The relationship between breast-feeding, IgA production and development of atopic disease in children is a matter of controversy. Some of this controversy might be due to individual differences in the composition of breast milk. The aim of this study was to relate the levels of cytokines, chemokines and secretory (S)-IgA antibodies in breast milk to the development of atopic manifestation and salivary IgA production in infants. Cytokine, chemokine and SIgA levels, as measured with enzyme-linked immunosorbent assay (ELISA), in colostrum and mature milk were analyzed in relation to the development of positive skin-prick tests (SPT), allergic symptoms and salivary IgA antibody production during the first 2 years of life in 53 infants. There was no association between levels of IL-4, -5, -6, -8, -10, -13, -16, IFN- γ , TGF- β 1, - β 2, RANTES, eotaxin or SIgA levels in the breast milk with either SPT-positivity, development of allergic symptoms or salivary IgA levels during the first 2 years of life in the infants. Thus, differences in the composition of cytokines, chemokines and SIgA in breast milk did not, to any major degree, affect the development of a positive SPT, atopic symptoms, nor salivary IgA antibody production during the first 2 years of life.

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The relationship between breast-feeding and development of allergy is complex and might depend on maternal allergic status (1). Breast-feeding is associated with decreased IgE levels in children of mothers with low IgE levels and with elevated IgE levels among children of mothers with high IgE levels (2). It is possible that the shared environment of mothers with high IgE levels and their infants might obscure any beneficial effect of breast-feeding seen in the low maternal IgE group. A more likely possibility, however, could be that the divergent effects on IgE production is due to qualitative differences in the composition of milk from allergic and non-allergic mothers. We have recently shown lower levels of IgA antibodies to ovalbumin (3), but higher levels of IL-4 (4), IL-8 and RANTES (5) in milk of allergic mothers than milk from non-

allergic mothers. Furthermore, low levels of total (6–8) and cow's milk specific IgA (6) antibodies in breast milk have been associated with an increased risk of developing a cow's milk allergy (CMA) in children. The concentration of TGF- β 1 is also lower in colostrum from mothers of infants with IgE-mediated CMA than from mothers of infants with non-IgE-mediated CMA (9). Finally, concentrations of both TGF- β 1 and TGF- β 2 are higher in colostrum from mothers of infants with postweaning, as compared with preweaning, onset of atopic disease (10).

Breast-feeding appears to modulate secretory (S)-IgA production in infants, even if the mode of this effect is controversial (11–13). We have earlier reported that the levels of IL-10 and TGF- β , that both regulate IgA production, correlate with the levels of SIgA in breast milk (4).

Furthermore, high levels of TGF- β in breast milk are associated with high levels of serum IgA antibodies to β -lactoglobulin (BLG) (9) and the presence of allergen-specific IgA secreting cells (10) in infants. However, the relationship between breast milk cytokines and mucosal IgA production in infants has not been studied previously. The primary functional role of IgA antibodies is to protect the epithelial surfaces and IgA is the principal isotype in mucosal tissues and secretions. Antigen-specific IgA prevents adherence and penetration of antigens, capable of provoking immune inflammatory responses, through the mucosal epithelium (14). Thus, determination of IgA in saliva is more relevant than analysis in blood.

The aim of this study was to evaluate if different levels of cytokines, chemokines and SIgA antibodies in breast milk affect the development of allergy and salivary IgA production in infants.

Materials and methods

Study population

Fifty-three mother/infant pairs participating in a prospective study regarding development of allergy were selected for this study based on availability of colostrum and mature milk samples. The parents received verbal and written information about the aim and design of the study. After having accepted participation, atopic history, i.e. allergic rhinoconjunctivitis, bronchial asthma or flexural, itchy dermatitis, was recorded by an experienced allergy research nurse for each mother during an initial interview. None of the mothers reported any autoimmune or other chronic disease. Twenty-four mothers had a history of atopic symptoms, while 29 had not. Colostrum ($n = 53$) was collected within 4 days postpartum, and mature milk ($n = 47$) at 1 month as described elsewhere (4). Briefly, the milk was collected in sterile plastic tubes, using a manual breast-pump, and immediately frozen at -20°C until analysis. After thawing, the breast milk fatty layer and cellular elements were removed by centrifugation at 680 g for 10 min at 4°C , followed by a second centrifugation of the supernatants at $10\,000\text{ g}$ for 30 min at 4°C . Some of the translucent whey was immediately used for measurement of IFN- γ , and the rest was stored in aliquots in plastic tubes at -20°C .

Saliva was collected from the buccal cavity of the infants, using a hand-pump connected to a thin plastic tube and immediately frozen at -20°C . Before analysis, the samples were heated

at 56°C for 30 min and then centrifuged at 5000 g for 15 min. The supernatants were stored in aliquots at -20°C . Long-term storage has earlier been reported not to have any effect on the antibody affinity (15), nor does long-term storage and repeated cycles of freezing and thawing alter the molecular weight of IgA (16). Saliva was collected at 3 ($n = 28$) or 6 ($n = 20$), and at 12 ($n = 47$) and 24 ($n = 38$) months of age. At the same time points, blood samples were drawn, skin-prick tests (SPT) were performed for common food and inhalant allergens, and questionnaires were answered regarding, e. g. atopic symptoms, breast-feeding, introduction of cow's milk and solid foods, presence of pets and smoking habits. In addition, a clinical examination regarding allergic symptoms was performed at 2 years.

Atopic dermatitis was defined as pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as three or more episodes of bronchial obstruction, at least once verified by a physician. Allergic rhinoconjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. SPTs were done in duplicate on the volar aspects of the forearms with thawed egg white, fresh skimmed cow's milk (lipid concentration 0.5%) (at 3 or 6 and 12 and 24 months), cat (at 12 and 24 months), birch and timothy (at 24 months) extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as a positive control, and albumin diluent was included as a negative control. The test was regarded as positive if the mean diameter was $\geq 3\text{ mm}$.

Detection of cytokines and chemokines in breast milk

The concentrations of IFN- γ , IL-4, IL-6, IL-10 and IL-13 were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits (CLB Pelikine Compact human IFN- γ , IL-4, IL-6, IL-10 and IL-13, Research Diagnostics Inc., Flanders, NJ, USA) according to the manufacturer's recommendation. The concentrations of TGF- β 1 and TGF- β 2 were analyzed with commercial ELISA kits from R & D (R & D System Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. TGF- β was only detectable in milk samples after acid treatment with $10\text{ }\mu\text{l}$ of 1.2 N HCl per $100\text{ }\mu\text{l}$ sample for 15 min followed by neutralization by adding $25\text{ }\mu\text{l}$ of 0.5 M HEPES/ 0.72 M NaOH per $100\text{ }\mu\text{l}$ of sample. This treatment activates latent TGF- β (17).

The concentrations of IL-5, eotaxin and IL-16 were analyzed using in-house ELISAs. Costar 3690 plates (Life Technologies, Täby, Sweden) were coated with 50 µl/well of 0.25 µg/ml monoclonal rat anti-human IL-5 (clone TRFK5, Pharmingen, Becton-Dickinson, Stockholm, Sweden), 4 µg/ml monoclonal mouse anti-human eotaxin (clone no. 43911.11, R & D Systems, Abingdon, UK), or with 0.6 µg/ml goat anti-human IL-16 (R & D Systems) diluted in carbonate buffer (pH = 9.2). The plates were incubated for 1 h at room temperature under continuous shaking and then at 4°C overnight for IL-5 and at room temperature overnight for eotaxin and IL-16. The subsequent incubations were performed in the dark at room temperature with shaking unless otherwise stated. After the incubations, the plates were washed four times with PBS-Tween 0.05%. Free plastic spaces were blocked with 100 µl/well of a blocking solution (CLB) or skimmed milk diluted 1/50 in PBS. Samples, standards and for controls, buffer solution (CLB) only, were added to the plates at 50 µl/well. Recombinant human IL-5 (Pharmingen), eotaxin or IL-16 (R & D Systems) diluted twofold (range 3.1–200 pg/ml for IL-5, 8–500 pg/ml for eotaxin and 31–2000 pg/ml for IL-16) in dilution buffer (CLB) were used as standards. For detection, 50 µl/well of a 1.0 µg/ml monoclonal biotinylated rat anti-human IL-5 antibody (clone JES1-5A10, Pharmingen), 180 ng/ml biotinylated goat anti-human eotaxin or 100 ng/ml biotinylated goat anti-human IL-16 (R & D Systems) were added, followed by 50 µl/well of streptavidin-conjugated poly horseradish peroxidase (HRP) (CLB) diluted 1/10 000 in dilution buffer (CLB). After incubation for 30 min and subsequent washing, 50 µl/well of the substrate TMB (Sigma-Aldrich) was added. The substrate reaction was stopped after 30 min by the addition of 50 µl/well of 1.8 M sulfuric acid. The optical density (OD) values were measured at 450 nm.

The concentrations of RANTES and IL-8 were analyzed on high-binding Immulon 4 plates (Dynatech Laboratories Inc., Chantilly, VA, USA). The plates were coated with 100 µl/well of 4 µg/ml monoclonal mouse anti-human RANTES or IL-8 (clone no. 21418.2 for RANTES and 6217.11 for IL-8, R & D Systems) diluted in PBS buffer and incubated at room temperature overnight. The subsequent incubations were performed at room temperature in the dark for 2 h, unless otherwise stated. The plates were then washed four times in PBS-Tween 0.05%. Free plastic spaces were blocked with 300 µl/well of PBS with 1% BSA and 5%

sucrose. After incubation and washing, 100 µl/well of samples, standards or for controls, buffer solution, PBS-Tween 0.05% with 0.1% BSA, only were added in duplicates to the plates. Recombinant human RANTES and IL-8 (R & D Systems) diluted twofold in dilution buffer (range 31–2000 pg/ml) were used as standards. For detection, 100 µl/well of 25 ng/ml biotinylated goat anti-human RANTES or goat anti-human IL-8 (R & D Systems) was added. After incubation and washing, 100 µl/well of streptavidin-conjugated HRP (Zymed Laboratories, San Francisco, CA, USA) diluted 1:5000 in buffer solution was added. After 20 min, 100 µl of the substrate o-phenylenediamine (Sigma Chemicals Co., St. Louis, MO) was added to each well. The substrate reaction was stopped after 20 min by the addition of 50 µl/well of 1.8 M sulfuric acid. The OD values were measured at 492 nm.

The concentrations of cytokines and chemokines were determined by correlating the OD of the samples to the OD of the standard curve. The OD values of the controls were subtracted from the ODs of the standards and samples. All samples and standards were analyzed in duplicate, and the mean values were used.

Quantitative determinations could be performed >67 pg/ml for IFN-γ, >5.6 pg/ml for IL-4 and IL-6, >19 pg/ml for IL-10, >3.2 pg/ml for IL-13, >125 and >250 pg/ml for TGF-β1 and -β2, >6.2 pg/ml for IL-5, >16 pg/ml for eotaxin, >125 pg/ml for IL-16 and >62 pg/ml for RANTES and IL-8.

The influence of breast milk components on the measured cytokine and chemokine concentrations was evaluated by adding known amounts of recombinant cytokines and chemokines to colostrum samples, which were then analyzed. The recovery was tested with a concentration expected to be in the middle of the linear part of the standard curve. The recovery was 87–100% for all cytokines and chemokines, except for eotaxin and IL-5, where the recovery was lower, i.e. 63–67% and 24–97%, respectively. The effects of freezing and centrifugation were also evaluated. Centrifugation before or after freezing or different centrifugation protocols did not affect the levels, nor did repeated freezing and thawing, except for IFN-γ, which became undetectable if frozen and thawed more than once. Therefore, IFN-γ in the milk samples was analyzed immediately after the centrifugations.

Detection of total IgA, SIgA, BLG and cat-specific IgA in saliva

For analysis of total IgA and total SIgA levels in saliva, high-binding plates were incubated at

37°C for 2 h and then for at least 3 h at 4°C with 200 µl/well of anti-human IgA diluted 1:1000 (Dakopatts AB, Täby, Sweden), or antisecretory IgA (Dakopatts AB) diluted 1:500 in PBS. The plates were blocked with 200 µl/well of PBS-Tween with 0.5% HSA (PHT) at 37°C for 2 h and then at 4°C overnight. Samples, 100 µl/well, were diluted 1:100 in PHT. For the standard curve, human IgA (Sigma Immunochemicals, Stockholm, Sweden) was diluted in seven steps. Antibody binding was detected by 100 µl/well of alkaline phosphatase-conjugated goat anti-human IgA antibodies (Sigma Immunochemicals), diluted 1:10 000 in PHT and 200 µl/well of FAST-pNPP substrate. The detection intervals were 40–2500 (µg/l) for total IgA and 16–1000 (µg/l) for SIgA.

For the detection of BLG and cat allergen (Fel d 1) specific IgA in saliva, high-binding ELISA plates (Costar 3590, Life Technologies) were coated with 200 µl/well of 20 µg/ml Fel d extract (ALK), or with 10 µg/ml BLG (Sigma Immunochemicals), diluted in PBS. Every second row was left uncoated as a control of unspecific binding. The plates were incubated overnight at room temperature and then blocked for 90 min at 37°C using 200 µl/well of 2% human serum albumin (HSA) (Behring Diagnostics, Marburg, Germany) diluted in PBS. Saliva samples were diluted 1:50 in PHT and 100 µl/well was added in quadruplicate to the plates (two coated and two uncoated wells). Saliva samples, containing high titres of specific IgA to cat and BLG, respectively, were diluted in eight steps in PHT and used as a standard curve. After incubation for 1 h in 37°C, 100 µl/well of a biotinylated mouse anti-human IgA (Sigma Immunochemicals), diluted 1:10 000 in PHT was added. After incubation for 1 h, 100 µl/well of streptavidin-poly HRP conjugate (CLB, Amsterdam, Netherlands), diluted 1:10 000 in 1% casein buffer (art. no. M2052, CLB, Amsterdam, Netherlands) was added, and the plates were incubated for 30 min. As substrate, 50 µl/well of TMB (Sigma Immunochemicals) was used and after 30 min 50 µl/well of 0.46 M H₂SO₄ was added to stop the reaction. The antibody levels in the samples were calculated from the OD of the reference curve and expressed as arbitrary units (AU). The OD of the blanks and the individual controls were subtracted from the OD values of the saliva samples.

The specificity of the methods were verified by blocking experiments, in which saliva samples were incubated overnight with BLG, Fel d 1 extract or ovalbumin to final concentrations of 0, 1, 10 and 100 µg/ml. The incubated samples were

centrifuged at 10 000 *g* for 15 min and the antibody levels in the supernatants were analyzed on plates coated with BLG or Fel d 1. Incubation with 100 µg/ml of the relevant antigen abolished 90% of the values, whereas no inhibition was seen when a non-relevant antigen (ovalbumin) was used.

Saliva samples from five patients with total IgA deficiency (kindly provided by Professor Lennart Hammarström, Huddinge University Hospital, Stockholm, Sweden) were used to verify the specificity of the methods. No IgA or SIgA could be detected in these samples, even at a 1:2 dilution.

Statistics

As the concentrations of cytokines, chemokines and antibodies were not normally distributed, groups were compared using the Mann–Whitney *U*-test rank test and correlations were analyzed with Spearman's rank order correlation coefficient test. The chi-square test was employed for categorical variables, and Fisher's exact test was used when the expected frequency for any cell was less than five. A probability level of <5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA).

Ethics

The Regional Ethics Committee for Human Research at the University Hospital of Linköping approved the study.

Results

The cumulative incidence of SPT-positivity at the 2-year follow up was 25% (*n* = 13/53). Eleven of the children developed a positive SPT to egg white and two of them were also SPT positive to cow's milk and two to birch. One child developed only a positive SPT to birch and one to cat. Ten infants developed atopic dermatitis during the first 2 years of life, one of them was also asthmatic and one child developed only asthmatic symptoms. Eight of the children with allergic symptoms had positive SPT. In seven of the infants, the allergic symptoms appeared while exclusively breast-fed while three developed symptoms during the weaning period and one after breast-feeding had ceased.

There was no association between the number of positive samples or the levels of the cytokines (Fig. 1), chemokines (Fig. 2), nor SIgA

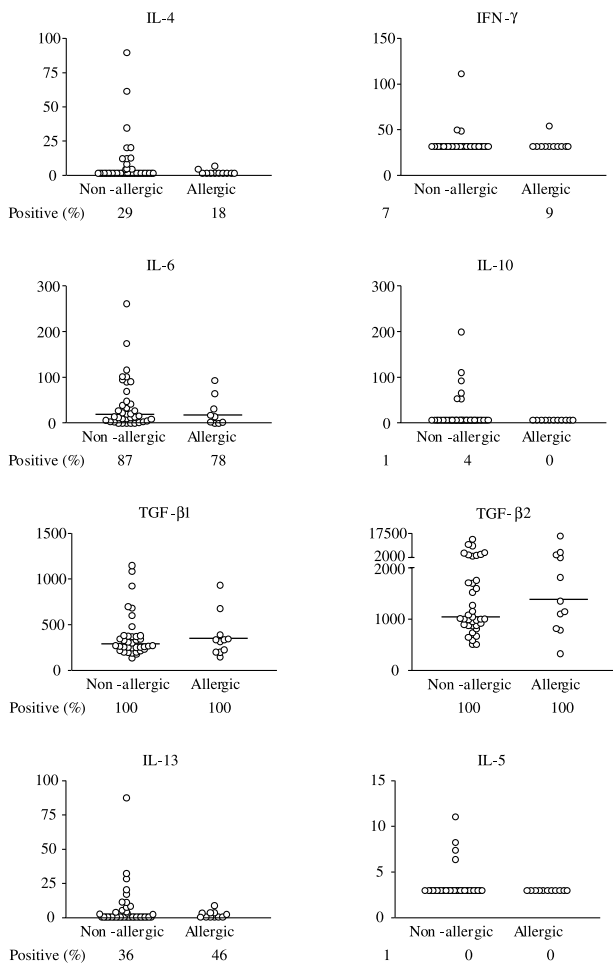


Fig. 1. The frequency (%) of positive samples (samples with levels above the limit of detection) and the levels of IL-4, IFN- γ , IL-6, IL-10, TGF- β 1, TGF- β 2, IL-13 and IL-5 (pg/ml) in colostrum of mothers whose infants became allergic or non-allergic up to 2 years of age. The medians are shown in those cases where 50% or more of the samples had detectable levels. No differences were observed between the groups.

antibodies (data not shown) in colostrum or mature milk and the development of either allergic symptoms (p-values ranged between 0.14 and 0.99) or positive SPT (data not shown) in the infants up to 2 years of age.

The different immune components in colostrum or mature milk and total IgA, SIgA, BLG or cat-specific IgA antibody levels in saliva of the infants did not correlate [p-values (0.11–0.96) ρ values (–0.24–0.15)]. The results for TGF- β 1 and SIgA are shown in Fig. 3.

Discussion

There was no association between the levels of any of the cytokines or chemokines, nor SIgA in breast milk and the development of either

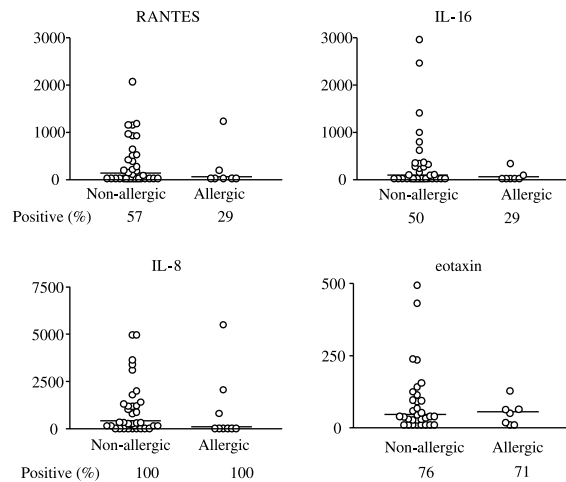


Fig. 2. The frequency (%) of positive samples (samples with levels above the limit of detection) and the levels of RANTES, IL-16, IL-8 and eotaxin (pg/ml) in colostrum of mothers whose infants became allergic or non-allergic up to 2 years of age. The medians are shown in those cases where 50% or more of the samples had detectable levels. No differences were observed between the groups.

positive SPT or allergic symptoms in the infants up to 2 years of age. We have earlier reported higher levels of the IgE-inducing cytokine IL-4 (4), as well as higher levels of RANTES and IL-8 (5), in breast milk from allergic mothers. Such differences do not, however, seem to influence the development of atopic diseases in the infants.

It has been suggested that the protective effect of breast-feeding is most pronounced in children of allergic mothers (1). On the other hand, breast-feeding is associated with decreased IgE levels in children of mothers with low IgE levels, but with elevated levels among children of mothers with high IgE levels (2). Furthermore, breast milk cell supernatants from allergic mothers have been reported to stimulate higher IgE production from cord blood mononuclear cells (18). These findings have not been confirmed, however, nor have we been able to observe any major differences between breast milk from allergic and non-allergic mothers on mitogen- or allergen-induced cytokine production by cord blood mononuclear cells (19).

TGF- β is the dominating cytokine in colostrum (4) and it has been suggested from animal studies to be an anti-inflammatory substance in milk (20). Furthermore, it has been reported to down-regulate IgE synthesis (21). High levels of TGF- β in breast milk did not protect against development of allergy in infants, confirming the results of previous studies (9, 10). However, Saarinen and colleagues reported higher levels of TGF- β 1 in colostrum of mothers whose children

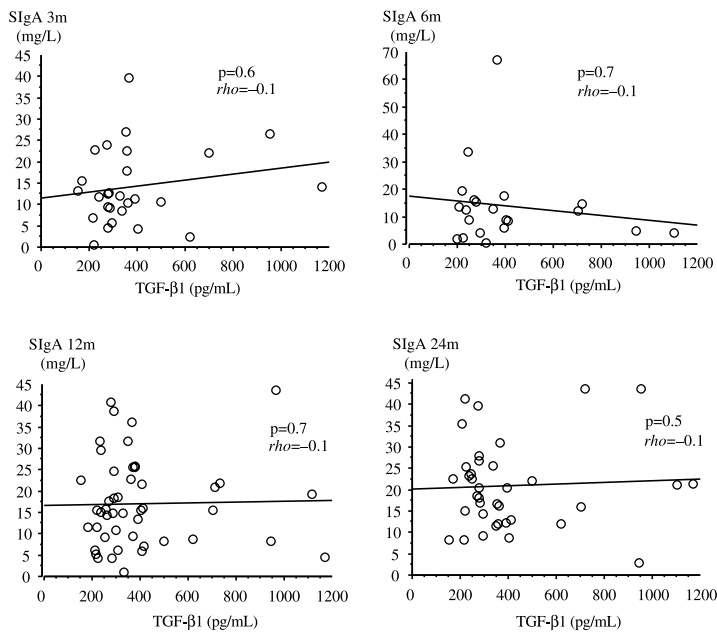


Fig. 3. The levels of SIgA (mg/l) in saliva at 3, 6, 12 and 24 months of age in relation to the levels of TGF-β1 (pg/ml) in colostrum of their mothers.

later developed non-IgE mediated CMA compared with those who developed IgE-mediated CMA (9). In the study by Kalliomäki and colleagues, high concentrations of colostral TGF-β were associated with postweaning, as compared with preweaning onset of atopic dermatitis (10). We were not able to perform such comparisons due to the low number of infants with allergic disease.

The lack of association between SIgA levels in breast milk and development of positive SPT or allergic symptoms confirms the results from a previous study (22). A protective effect of high IgA levels in breast milk against development of cow's milk allergy has been reported earlier (6–8). However, there were no cow's milk allergic infants in this study.

Although it seems unlikely that the levels of cytokines, chemokines or SIgA in breast milk affect the development of atopic disease to a major degree, other differences in composition of breast milk may be of importance. It has for instance been reported that the composition of polyunsaturated long-chain fatty acids in breast milk is associated with the development of allergic symptoms in infants (22).

High levels of allergen-specific IgA could, theoretically, prevent allergen absorption and thereby sensitization and subsequent development of an allergy. Low levels of salivary SIgA and transient IgA deficiency have been associated with an increased risk of an allergy (23, 24). The role of breast-feeding in the up-regulation of IgA production is controversial and it is possible that this is due to individual variations in the

composition of breast milk. The cytokine and chemokine levels in breast milk did not correlate with salivary IgA levels in infants up to 2 years of age, indicating that different levels of these factors are of minor importance for the production of IgA at mucosal sites. This is the first study on the relationship between breast milk cytokines and mucosal IgA production in infants. A previous study showed a positive correlation between the levels of colostral TGF-β1 and the serum levels of allergen-specific IgA and IgG antibody at 3–13 months (9). Furthermore, colostral TGF-β2 levels were higher in mothers whose infants had IgA secreting cells in peripheral blood at 3 months of age (10). It is possible that TGF-β in breast milk affects the systemic, but not the mucosal IgA antibody production, as the two are separately regulated (25). However, it is generally considered that most of the IgA producing cells in the circulation are transported to different mucosal sites. In the study by Saarinen and colleagues (9), the correlation between BLG-specific IgA antibody and TGF-β concentrations was based on approximately 210 pairs of colostrum and serum samples and the observed correlation was weak ($\rho = 0.2$). The smaller size of our study population would not have allowed the detection of such a small correlation.

In conclusion, the results from this study indicate that differences in the composition of cytokines, chemokines and SIgA in breast milk do not appreciably affect either the development of a positive SPT, atopic symptoms, or salivary IgA antibody levels during the first 2 years of life.

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