

Leucocytes in human milk and lymphocyte subsets in cow's milk-allergic infants

Järvinen K-M, Suomalainen H. Leucocytes in human milk and lymphocyte subsets in cow's milk-allergic infants.
Pediatr Allergy Immunol 2002; 13: 243–254. © 2002 Blackwell Munksgaard

The breast-fed infant ingests an average of 10^8 leucocytes per day, with breast-feeding often continuing for several months. The precise role of human milk leucocytes is still unresolved. Breast-feeding has been recommended for infants at high risk of allergy to prevent or delay the development of food allergies and atopic eczema. However, studies dealing with distinct immunologic factors in the mother's milk, and their effect on health status or development of allergies in the infant, are scarce. We evaluated the relationship between the cellular composition of human milk and development of cow's milk allergy (CMA) in the breast-fed infant. Leucocyte subsets in the breast-fed infants were also measured. The study population comprised 61 breast-feeding mothers and their infants. Thirty-nine mothers each had a cow's milk-allergic infant, 10 had an infant with atopic dermatitis without CMA, and 12 mothers had a healthy infant. Leucocyte subsets in mothers' milk were counted using a light microscope and confirmed by flow cytometry. In infants, peripheral blood lymphocyte subsets were determined by flow cytometry and were correlated with the health status of the breast-fed infant and leucocyte composition of the mother's milk. Human milk was found to be a non-homogenous morphological entity. In the milk of mothers of infants with CMA, the proportion of macrophages was significantly smaller than in the mothers with infants without CMA ($p = 0.036$, t -test). Mothers with high proportions of neutrophils in their milk ($>20\%$) had significantly more often infants with CMA than did those with low proportions of neutrophils ($p = 0.02$; Fischer's exact test). Eosinophils comprising $>1\%$ of milk cells were only detected in the mothers who had infants with CMA. Furthermore, the proportions of $CD4^+$ T cells were positively correlated with the proportion of milk macrophages and negatively with the percentage of milk neutrophils and eosinophils. The proportions of total B cells and those expressing CD23, a low-affinity immunoglobulin E receptor, were positively correlated with the proportions of neutrophils and eosinophils in mother's milk and negatively with the percentage of milk macrophages. To conclude, the composition of breast milk in some mothers is abnormal and correlates with a diagnosis of CMA in a breast-fed infant. This may provide a new and interesting insight into the development of food allergies.

**Kirsi-Marjut Järvinen and
Hanna Suomalainen**

Helsinki University Central Hospital, Skin and Allergy Hospital, Department of Dermatology, Helsinki, Finland

Key words: human milk; leucocytes; oral tolerance; cow's milk allergy; light microscopy; flow cytometry; infants; breast-feeding

Kirsi-Marjut Järvinen, MD, PhD, Mount Sinai School of Medicine, Div. of Pediatric Allergy and Immunology, Box 1198, One Gustave L-Levy Place, NY10029, USA
Tel: +1-212-241-4908
Fax: +1-212-426-1902
E-mail: kirsi.jarvinen@mssm.edu

Accepted 18 October 2001

During breast-feeding, milk leucocytes and cytokines ingested have the potential to influence the immune system of the suckling infant. Human milk contains leucocytes that differ in morphology from their counterparts in the peripheral blood (1). The breast-fed infant ingests an average of 10^8 leucocytes per day with breast milk, with breast-feeding often continuing for

several months (2). The predominant cellular component of human milk leucocytes is the macrophage, comprising ≈ 60 – 90% of the total number of cells (1,2). According to the literature, neutrophils are rare in human milk (8– 28%) from breast-feeding mothers (3). However, some authors report a proportion of neutrophils as high as 40– 60% (1,3). Only 3– 9% of human milk

leucocytes are lymphocytes, the majority of which are T cells (3). In addition, occasional monocytes (1), $\approx 2\%$ eosinophils (4), and few basophils (4) are found. The precise role of human milk leucocytes is still unknown.

In humans, milk cell viability has been demonstrated for up to 4 h following neonatal ingestion, with viability being independent of osmolality and protein concentration of milk (5). This is a result of the relatively neutral pH of the stomach of a newborn and the buffering capacity of milk (5). Milk leucocytes are able to resist trypsinization (6), and milk macrophages tolerate large variations in environmental pH, temperature, and osmolality (3,5,7–9). In animal studies, milk leucocytes have been shown to enter the circulation, spleen, and lymphoid tissue of the newborn (7–9). Furthermore, human milk cells infused into implanted fetal small intestinal loops in mice were found to transverse the human small intestinal transplant intact and migrate into the tissues of the mice (10). With milk lymphocytes from dams immunized with tetanus toxoid deposited in the duodenum of their offspring, priming to vaccine was detected (8). These results can be most reasonably explained by the natural transmission of viable milk lymphocytes to the suckling neonate and their short-term utilization by the infant. In the gut of breast-fed infants, viable milk leucocytes may release mediators, possibly harming the mucosa in a manner similar to that seen in asthma (11).

In the literature, the effect of breast-feeding on reduction in incidence of atopic disease and cow's milk allergy (CMA) in the first year of life still remains unresolved (12–14). Our previous and other recent studies suggest that the cytokine and chemokine composition in human milk vary between mothers with infants with CMA and those with a healthy infant (15,16), and between non-atopic and atopic mothers (17,18). These findings support the concept that the effect of breast-feeding on the development of allergy may depend on the composition of mothers' milk, and variations in immunologically active constituents of human milk may partly explain the contradictory results found in investigations of the relationship of breast-feeding to allergic symptoms.

The cellular composition of mothers' milk has not been assessed in association with maternal atopy or outcome of the infant, except in one study, which reported a larger number of eosinophils in atopic mothers (4). The purpose of the present study was to assess the leucocyte composition of human milk in relation to the

health status or to development of food allergies in the breast-fed infant.

Subjects and methods

Subjects

The study population comprised 61 breast-feeding mothers and their 61 infants. They were recruited between August 1995 and August 1998 from the Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki (among those referred owing to suspicion of having CMA, or among infants who were prospectively followed-up from birth in a study cohort because of having an older sibling with food allergies), and from the Helsinki City Maternity Hospital, Helsinki (among volunteering non-atopic families). The clinical investigations and challenges were all performed by two investigators (K.-M. J. and H. S.). Thirty-nine mothers each had a cow's milk-allergic infant, 10 had an infant with atopic dermatitis without CMA, and 12 mothers had a healthy infant. The clinical characteristics of the infants and their mothers are represented in Table 1 and Table 2. Generally, the families recruited represent middle/higher social classes from an urban environment.

At the time of collection of the samples, the geometric mean age of the infants with CMA was 2.1 months [95% confidence interval (CI): 1.5–2.9], of those with atopic dermatitis was 2.4 months (95% CI: 1.3–4.3), and of healthy infants was 2.1 months (95% CI: 1.2–3.8), comparable in each group. The geometric mean age at onset of symptoms of CMA was 1.1 months (95% CI: 0.8–1.4). A diagnosis of CMA had not yet been made in all the patients at the time of sample collection, but it was confirmed later at a geometric mean age of 4.8 months (95% CI: 4.0–5.9). The duration of exclusive breast-feeding was comparable in infants with CMA, geometric mean age 3.2 months (95% CI: 2.7–3.9), in infants with atopic dermatitis, geometric mean age 3.0 months (95% CI: 1.6–5.4), and in the healthy infants, geometric mean age 1.9 months (95% CI: 0.8–4.9).

The mothers received no medication during the study, and those suffering from mastitis during the preceding 4 weeks were excluded. The infants were full-term and had no chronic diseases, except allergy, and no infection whilst visiting the clinic. They were followed-up until at least 1 year of age for detection of symptoms of atopic dermatitis or food allergy.

Table 1. Clinical characteristics of the healthy and atopic infants and of their mothers

Child no.	Maternal age (years)	Parity*	Maternal smoking	Maternal/paternal atopy	Age at start of complementary foods (months)	Exclusively breast-fed at onset of symptoms
Healthy infants						
1	37	I/1	No	-/-	5	NA
2	27	I/2	No	-/-	4	NA
3	27	II/2	No	+/-	6	NA
4	28	I/2	No	+/-	4	NA
5	34	I/1	No	-/-	4	NA
6	31	III/3	No	+/+	4	NA
7	30	II/2	No	-/-	4	NA
8	27	I/2	No	+/+	4.5	NA
9	38	I/1	No	-/-	4	NA
10	30	I/1	No	-/-	4	NA
11	38	II/2	No	-/+	5	NA
12	30	I/1	No	-/-	5	NA
Atopic infants						
1	31	I/2	No	+/+	5	No
2	24	I/2	No	+/+	4	Yes
3	35	I/1	No	-/-	4	No
4	32	II/2	No	+/+	4	No
5	33	III/3	No	+/-	4	Yes
6	28	II/2	No	-/-	5	Yes
7	24	II/2	No	-/+	5.5	No
8	20	I/2	No	+/+	5	No
9	36	I/1	No	+/+	2.5	Yes
10	27	I/1	No	+/-	5	No

*Expressed as order of birth/total number of children.

NA, non-applicable.

Because the infants displayed symptoms suggestive of CMA, or CMA had already been diagnosed, all the mothers of these infants were on an elimination diet: foods suspected to cause the symptoms in the infant – e.g. egg, fish, tomato, citrus fruits, strawberries, chocolate, peanuts and nuts, cow's milk, cereals, and (if necessary) several other foods – were eliminated from the mothers' and the infants' diet until the infant was either free of symptoms or until the mother and her infant already were on a 'few foods only' diet. Of the food-allergic infants, 21/39 showed no symptoms of food allergy during the elimination diet, and in 18/39 symptoms remarkably diminished but minor symptoms similar to food allergy were detected despite the fact that the mothers were on a very restricted diet. The healthy infants had a diet corresponding to their age.

The mothers gave written, informed consent for participation in the study, which was approved by the ethics committees of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki.

Skin prick tests (SPTs)

SPTs were performed using a commercial cow's milk extract (Soluprick; Allergologisk Laboratorium A/S, Horsholm, Denmark) on

the volar side of the forearm. Histamine dihydrochloride, 10 mg/ml (Soluprick), was used as a positive control. Reactions were read at 15 min and regarded as positive if a wheal appeared with a diameter at least half the size of that produced by histamine, which was at least 5 mm in diameter.

Cow's milk challenge protocol

Because open food challenges have been shown to be appropriate in infants (19), the diagnosis of CMA was confirmed by an open cow's milk challenge. After elimination of cow's milk for 2–4 weeks, patients were admitted for an oral cow's milk challenge. The challenge was started with a drop of adapted formula (Tutteli; Valio Ltd, Helsinki, Finland) on the skin and/or lips. On day 1, cow's milk was given in portions of increasing volume (1, 10, 50, and 100 ml) at 1-h intervals. On day 2, 200 ml of milk was given, followed by normal milk intake corresponding to the infant's age. The challenge was continued beyond 1 week to reveal any late-phase reactions. If any adverse reaction appeared, the challenge was stopped and the patients were examined by an experienced pediatrician or a dermatologist (K.-M. J. and H. S.). The diagnosis was based on typical symptoms, their amelioration on a cow's milk-elimination diet, and re-appearance on a cow's

milk-containing diet. Symptoms regarded as positive were anaphylaxis, urticaria, eczema, vomiting, and diarrhea. The reactions that occurred within 1 h of the last dose were defined as immediate and those that occurred thereafter were defined as delayed.

Light microscopy of human milk leucocytes

In 21 of the 61 mothers, more than one sample was analyzed, giving a total number of 94 milk specimens assessed, but only one sample (the earliest one) from each mother was included in the final analysis. The breasts were washed with warm water before collection of milk using a manual breast pump. The samples were always collected in the morning (the second feeding of the day) and before the baby was breast-fed.

Immediately after collection of the samples, 20–50 ml in volume, they were centrifuged at 400 g at room temperature for 15 min to separate the fat layer. Fat and supernatant were removed, and leucocytes were collected and washed for 15 min in RPMI-1640 containing 5% fetal bovine serum and antibiotics (penicillin and streptomycin). After being washed three to four times in RPMI-1640, the cells were re-suspended in RPMI-1640 containing 5% fetal calf serum and antibiotics, and were visually counted in a Burker chamber. Any samples with fewer than 1×10^5 cells/ml, unless exceptionally large in volume (>50 ml) were discarded. Subsequently, 1×10^5 leucocytes were attached to each slide by cytopspin centrifugation. The object glasses were precoated with Vectabond (Vector Laboratories Inc, Burlingame, CA, USA) to increase attachment

Table 2. Clinical characteristics of the cow's milk-allergic infants and their mothers

Child no.	Maternal age (years)	Parity*	Maternal smoking	Maternal/paternal atopy	Age at start of complementary foods (months)	Exclusively breast-fed at onset of symptoms
1	31	II/3	No	+/+	5	Yes
2	36	III/3	No	+/-	4	No
3	35	III/3	No	+/+	6	No
4	33	III/3	No	+/+	5	Yes
5	30	II/2	No	+/+	4.5	Yes
6	32	II/2	No	+/+	5	Yes
7	28	I/1	No	-/-	6	Yes
8	35	IV/4	No	+/-	5	Yes
9	31	II/2	No	+/+	5	Yes
10	28	II/2	No	+/-	3.5	No
11	32	III/3	No	-/+	5	Yes
12	28	II/2	No	+/+	4	Yes
13	32	II/2	No	+/?	5	Yes
14	29	II/2	No	+/+	4	No
15	38	I/1	No	-/+	5	No
16	29	II/2	No	-/-	5	Yes
17	31	II/2	No	-/-	6	Yes
18	32	III/3	No	-/+	4	Yes
19	33	III/3	No	+/-	6	Yes
20	33	III/3	No	+/+	5	No
21	30	I/1	No	-/-	4	No
22	30	I/1	No	-/-	5	Yes
23	28	I/1	No	+/-	5	Yes
24	33	II/2	No	+/-	5	Yes
25	29	II/2	No	+/+	4.5	Yes
26	29	I/1	No	+/-	5	Yes
27	29	II/2	No	-/+	4	Yes
28	34	II/2	No	-/+	5	Yes
29	22	I/1	No	-/?	5	No
30	30	II/2	No	+/-	5	No
31	34	I/1	No	+/-	6	Yes
32	27	I/1	No	+/-	4	Yes
33	39	II/2	No	+/-	3	No
34	31	III/3	No	+/+	5	Yes
35	30	II/2	No	+/-	5	Yes
36	36	III/3	No	+/+	5	No
37	30	II/2	No	+/-	6	Yes
38	28	VI/6	No	+/-	5	Yes
39	29	I/1	No	-/-	5	No

*Expressed as order of birth/total number of children.

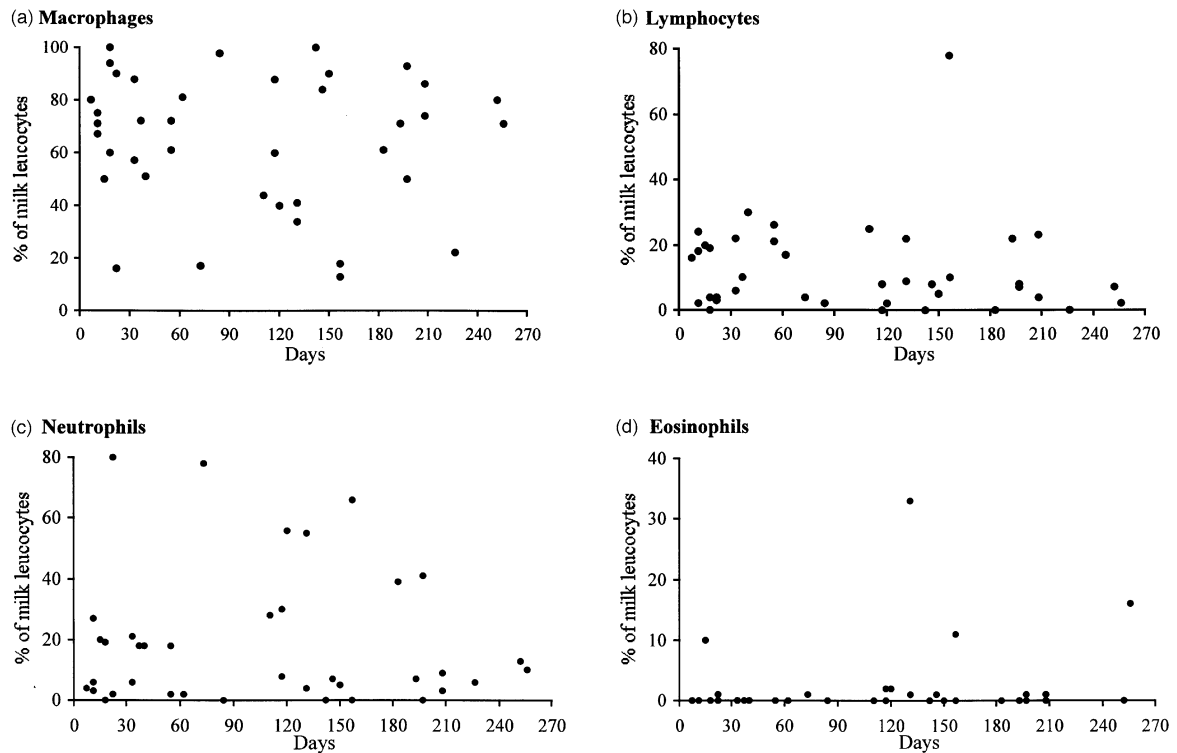


Fig. 1. The percentages of different leucocytes, as determined by using light microscopy, in mothers' milk as a function of lactational duration.

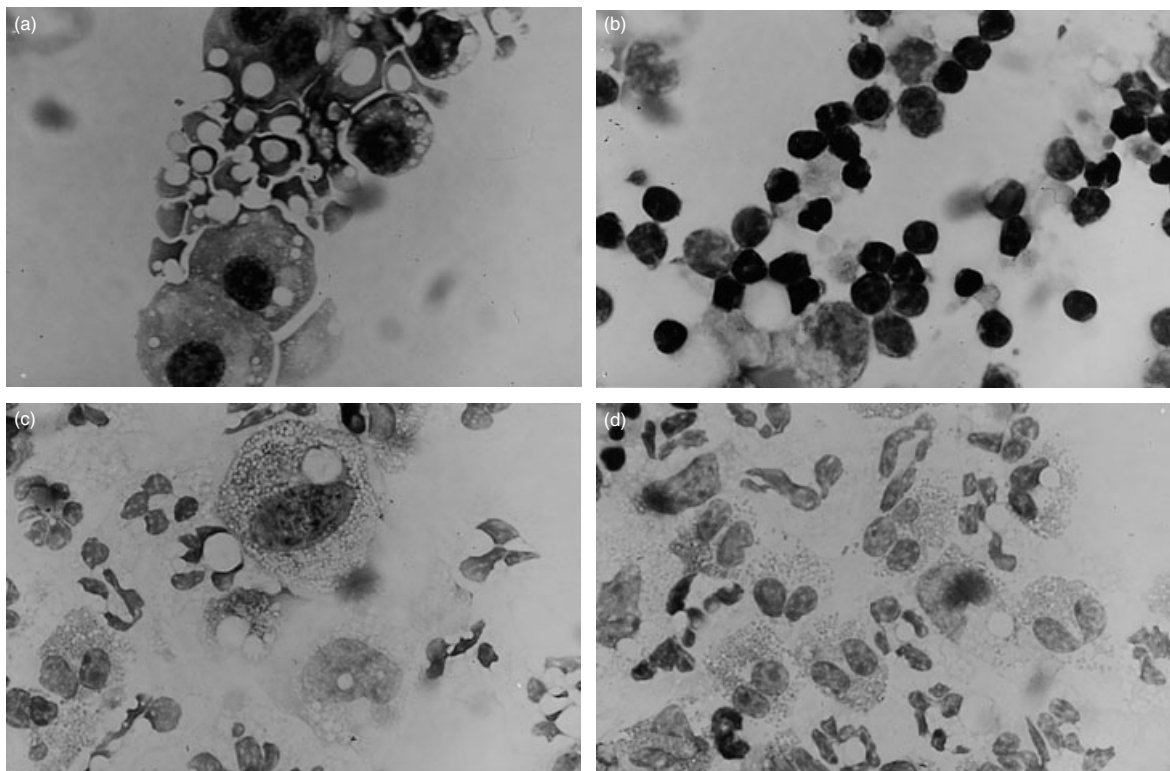


Fig. 2. Examples of different human milk composition in four mothers. A mother with a healthy infant and mainly macrophages (a) in her milk. Three mothers with cow's milk-allergic infants and predominantly lymphocytes (b), neutrophils (c), or eosinophils (d) in their milk.

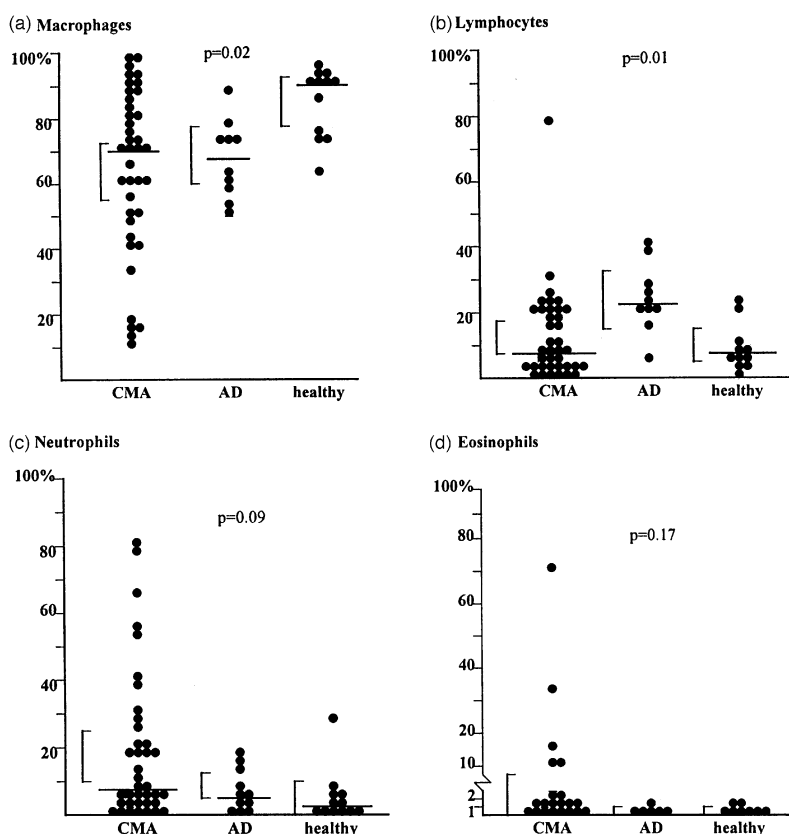


Fig. 3. The percentages of different leucocytes, as determined by using light microscopy, in the milk of mothers with infants showing cow's milk allergy (CMA) or atopic dermatitis (AD), or in those with healthy infants. Horizontal bars denote median and 95% confidence intervals (Kruskal–Wallis test).

of human milk leucocytes to the glass. The samples were finally stained with May–Grünwald–Giemsa, and the numbers of macrophages, monocytes, lymphocytes, neutrophils, and eosinophils were counted under a light microscope, by two investigators independently. The results are expressed as the percentage of that cell type per 100 leucocytes. Any sample containing erythrocytes and/or bacteria was discarded.

Light microscopy of the mothers' peripheral blood leucocytes

At the time of collection of milk samples, venous blood samples (anti-coagulated in EDTA) from mothers were drawn onto slides and stained with May–Grünwald–Giemsa. The numbers of different leucocytes were counted under light microscopy. Results are expressed as the percentage of each cell type per 100 leucocytes.

Flow cytometry of human milk leucocytes and infants' peripheral blood lymphocytes

After the isolation of milk leucocytes described above, flow cytometric analysis of milk leucocytes was performed to study their expression of different surface markers. During the same visit, venous blood from infants was drawn and

heparinized. Peripheral blood mononuclear cells were obtained by Ficoll–Hypaque (Pharmacia AB, Uppsala, Sweden) centrifugation of the blood diluted 1:4 in RPMI-1640. The cells were re-suspended in RPMI-1640 containing penicillin/streptomycin, glutamine, and 5% fetal calf serum, washed three times, and visually counted in Burker chamber. Milk and peripheral blood cell suspensions at a cell concentration of $1 \times 10^6/\text{ml}$ in RPMI-1640, which contained antibiotics, glutamine, and 5% fetal calf serum, were incubated at 4°C for 15 min with a saturating concentration of fluorescent monoclonal antibodies against CD45, CD14, CD3, CD4, CD8, CD19, CD23, and CD16 (Becton-Dickinson Immunocytometry Systems, Mountain View, CA, USA), according to the manufacturer's instructions (20). Flow cytometric analysis was then performed, with the same instrument settings used in every analysis.

Statistical analysis

Because of the skewed age-distribution, logarithmic transformations were used. The results are expressed as geometric means and CI. As the distributions of the leucocyte counts were skewed

and the variances could not therefore be stabilized by logarithmic transformations, the differences between the three groups of infants were studied by Kruskal–Wallis analysis. Appropriate cut-off points for the leucocyte counts were sought by attempts at different possibilities. Fischer's exact test was used to study the independence of two grouping variables. The Mann–Whitney *U*-test was used for comparisons of lymphocyte subsets in infants. Correlation between two variables was calculated by using Spearman's rank correlation coefficient. The analyses were carried out using S plus 4 software (MathSoft, Inc., Seattle, WN, USA) and Statview 4.0 (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Clinical characteristics of the study patients

The SPT was positive with cow's milk in 13/39 infants with CMA, but in none of the 10 infants with atopic dermatitis nor in the 12 healthy infants. In the clinical cow's milk challenge, 22 infants manifested CMA with an immediate reaction and 17 with a delayed reaction. Twelve of these children manifested local urticaria immediately after introduction of a drop of cow's milk on their skin or lips. Sixteen other infants developed eczema, four gastrointestinal symptoms, and seven reacted both cutaneously and gastrointestinally to per-oral administration of cow's milk.

Total cell counts

The milk samples contained a mean of 0.4×10^6 cells/ml, yielding a total number of 10×10^6 milk cells for further analyses as the total volume of each sample was at least 25 ml. The mean number of leucocytes in milk was comparable for the mothers of infants with CMA, atopic dermatitis, or for mothers of healthy infants ($p = 0.058$, Kruskal–Wallis). The mean number of milk leucocytes did not correlate with maternal atopy.

Differential cell count of milk leucocytes

After careful removal of any visible fat layer in the milk specimens and several washes, membrane/fat globules were rarely detectable. The results from differential cell counts were comparable between two independent investigators. The proportions of leucocyte subtypes did not change significantly during the breast-feeding period (Fig. 1). In human milk, the large, lipid-filled macrophage was the predominant cell (Fig. 2a). In the mothers with infants without CMA

(healthy infants or infants with atopic dermatitis only), the composition of milk was markedly more constant between the individuals than in those with cow's milk-allergic infants. Figure 3 shows the proportions of leucocyte subsets in milk from mothers of infants with CMA or atopic eczema, and in those with healthy infants. In the milk from mothers of healthy infants, the proportion of macrophages was significantly higher than in milk from those with infants with CMA, and the proportions of neutrophils and eosinophils were very low. In comparison, the milk from mothers of infants with CMA or atopic dermatitis without CMA contained proportionally fewer macrophages. Mothers with high proportions of neutrophils in their milk ($>20\%$) had significantly more often infants with CMA than did those with low proportions of neutrophils ($p = 0.02$, Fischer's exact test). A proportion of eosinophils representing $>1\%$ of milk cells was detected only in the mothers of infants with CMA. There was no correlation between the composition of breast milk and clinical symptoms or type of reaction (immediate vs. delayed) (data not shown). The milk from mothers of babies showing atopic eczema but no CMA contained proportionally fewer macrophages and a greater number of lymphocytes than that of mothers of healthy infants.

The cellular composition of milk was the most abnormal in mothers whose infants showed symptoms of food allergy despite their mothers' extensively restricted diet (eliminating cow's milk, cereal, egg, tomato, fish, nuts, and some vegetables). A significantly smaller proportion of macrophages (mean 55%, range 10–90) and a greater proportion of eosinophils (mean 6%, range 0–72) was seen than in mothers whose infants became free of symptoms during the elimination diet (macrophages: mean 72%, range 16–100, and eosinophils: mean 2%, range 0–33; $p = 0.03$ macrophages, and $p = 0.03$ eosinophils) (Mann–Whitney *U*-test). One mother had 78% lymphocytes, another 80% neutrophils, and a third had 72% eosinophils in milk (Fig. 2, b–d, respectively). Their infants developed severe cow's milk and cereal allergy during the first few months of life, despite being fed exclusively with breast milk.

Although only one sample from each mother was included in the analysis, the preliminary data on the comparison of consecutive milk samples from the same mother during breast-feeding showed that the cellular composition of breast milk is relatively constant (data not shown). The cellular composition of human milk was comparable between the atopic and non-atopic mothers.

The highest levels of eosinophils in milk were found in the spring in three atopic mothers allergic to birch pollen, although high levels were also found out of season in three mothers. The proportions of lymphocytes, neutrophils or eosinophils in human milk did not correlate with their proportions in peripheral blood. Interestingly, large proportions of eosinophils (8–18%) were found in the peripheral blood of four mothers who had very few eosinophils in their milk.

Cellular composition of human milk and risk of an infant of developing CMA

The risk of the breast-fed infant developing CMA was moderately high if the mother's milk fulfilled at least two of the following criteria: <91% macrophages, <52% macrophages, >5% neutrophils, and at least 1% eosinophils [sensitivity 0.72, specificity 0.64, odds ratio 4.5 (95% CI 1.5–13.5), $p = 0.01$].

Differential cell count of mothers' peripheral blood leucocytes

The mean proportions of blood monocytes, lymphocytes, neutrophils, eosinophils, and basophils were comparable and within the normal range in these three study groups of mothers.

Surface marker expression of milk cells

The expression of surface markers was examined among the milk leucocytes to determine the number of different cell types in 14 milk samples and to confirm the findings of light microscopy (Fig. 4). The proportion of cells expressing CD14 was consistent with the proportion of macrophages verified by differential cell counting ($p = 0.02$, $Rho = 0.63$, Spearman's rank correlation test). Similarly, the percentages of cells expressing CD3 or CD19, and those expressing CD16, correlated with the proportions of lymphocytes ($p = 0.04$, $Rho = 0.6$) and neutrophils ($p = 0.04$, $Rho = 0.59$), respectively, in light microscopy (Spearman's rank correlation test).

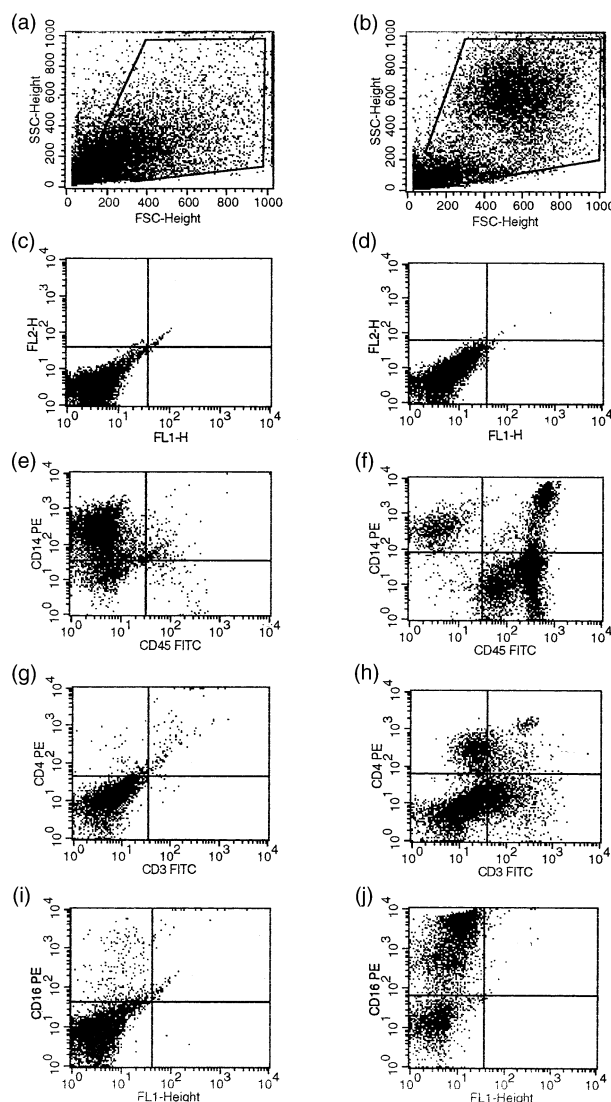


Fig. 4. Example of CD14, CD45, CD3, and CD16 expression of human milk leucocytes. (a) and (b) Forward- and side-scatter of human milk leucocytes. (c) and (d) Isotype controls. Mother (of a healthy infant) with predominantly macrophages in her milk (a), (c), (e), (g), (i); mother (of an infant with cow's milk allergy) with a significant proportion of lymphocytes and neutrophils in her milk (b), (d), (f), (h), (j).

Lymphocyte subsets in infants' peripheral blood

Venous blood samples from 22 infants with CMA and from seven infants without CMA were

Table 3. Proportions of different lymphocyte subclasses in the peripheral blood of infants with or without cow's milk allergy (CMA)

	Infants with CMA	Infants without CMA	p-value*
CD4 (%)†	39.0 (36–45)	43 (29–55)	0.683
CD8 (%)†	18.5 (17–21)	23 (19–29)	0.049
CD19 (%)†	21.0 (19–25)	16 (13–21)	0.036
CD23 (%)†	23.0 (19–25)	14 (11–17)	0.0099
CD19 ⁺ 23 (%)†	15.5 (12–25)	11 (–4 to 44)	0.119

*Mann–Whitney *U*-test.

†Values are expressed as median (95% confidence interval) in seven infants with CMA and 22 infants without CMA (12 healthy infants and 10 with atopic dermatitis exclusively).

Table 4. Correlation between different leucocytes in mothers' milk and T- and B-lymphocyte sub-classes in infants' peripheral blood

	Mothers' milk			
	Macrophages*	Lymphocytes*	Neutrophils*	Eosinophils*
	Infants' blood			
CD4	0.004 (0.55)	0.73 (-0.06)	0.003 (-0.55)	0.014 (-0.163)
CD8	0.290 (-0.2)	0.70 (0.08)	0.370 (0.18)	0.740 (0.25)
CD4/CD8 ratio	0.002 (0.58)	0.39 (-0.16)	0.003 (-0.56)	0.180 (0.001)
CD19	0.017 (-0.45)	0.49 (-0.13)	0.002 (0.58)	0.001 (0.58)
CD23	0.075 (-0.33)	0.67 (-0.08)	0.027 (0.42)	0.066 (0.47)
CD19 ⁺ 23	0.120 (-0.29)	0.68 (-0.07)	0.022 (0.44)	0.044 (0.50)

*Values represent p-values (Rho) when correlations were sought between different milk leucocytes and parameters in peripheral blood in seven infants with cow's milk allergy (CMA) and 22 infants without CMA (12 healthy infants and 10 infants with atopic dermatitis exclusively) (Spearman's rank correlation test).

obtained. The infants with CMA had a significantly smaller proportion of CD8⁺ T cells ($p=0.049$, Mann-Whitney U -test) and a higher proportion of cells bearing CD19 ($p=0.036$) and CD23, a low-affinity immunoglobulin E (IgE) receptor ($p=0.0099$), than the infants without CMA (Table 3). Furthermore, the proportions of CD4⁺ T cells and the CD4/CD8 ratio were positively correlated with the proportion of milk macrophages ($p=0.004$ and $p=0.002$, respectively) (Spearman's rank correlation test), and the proportions of CD4⁺ T cells were negatively correlated with the percentage of milk neutrophils and eosinophils ($p=0.003$ and $p=0.014$, respectively) (Table 4). In addition, the total proportions of B cells and those expressing CD23 positively correlated with the proportions of neutrophils ($p=0.002$ and $p=0.022$, respectively) and of eosinophils ($p=0.001$ and $p=0.044$, respectively) in mother's milk, whereas the proportion of CD19⁺ B cells was negatively correlated with the percentage of milk macrophages ($p=0.017$) (Table 4).

Discussion

As demonstrated in this study, the cellular composition of human milk is not homogenous. We have shown that a small number of macrophages and large numbers of neutrophils and/or eosinophils in the milk correlates with development of CMA, and the number of lymphocytes positively correlates with development of atopic dermatitis, in a breast-fed infant. Furthermore, these abnormalities in mothers' milk were associated with an imbalance in the CD4⁺/CD8⁺ T-cell ratio and disturbed B-cell function in the infants, which were previously found to be associated with the development of CMA (21).

In the present study, owing to careful removal of any visible fat layer of milk specimens and several washes, very few fat globules were detectable on slides, enabling an accurate dis-

tinction between different cell types. Keeney et al. confirmed, by flow cytometry, that neutrophils predominated in normal human milk collected on the second post-partum day (22). This finding was not confirmed by light microscopy or flow cytometry in the present study, or in one conducted previously by Wirt et al. (23). However, the estimation of macrophages in flow cytometry may be low because CD14 is partially shed from stimulated human monocytes (24), suggesting that flow cytometry may be inferior to light microscopy for assessing numbers of monocytes/macrophages in milk. In the present study, the macrophage was the predominant leucocyte present during the first 2–3 days post-partum in milk from mothers with a healthy infant, and proportions of other cell types such as lymphocytes, monocytes, and eosinophils were low, in agreement with previous findings (1,4). However, in mothers with cow's milk-allergic infants, the proportion of human milk macrophages was significantly smaller, and the proportions of lymphocytes, neutrophils, and eosinophils were larger than in mothers of healthy infants and larger than figures reported in the literature. Such cellular compositions were therefore regarded as abnormal. These light microscopy findings with conventional stains were confirmed by monocyte/macrophage, lymphocyte and neutrophil-specific markers in flow cytometry. Neutrophils were abundant in several human milk samples, together with erythrocytes and bacteria in the microscopic evaluation, although all mothers with clinical mastitis had been excluded. We therefore suggest that this sub-clinical mastitis may be common in breast-feeding mothers, and may attribute to the large proportion of neutrophils found in previous studies (1). In our study, samples containing erythrocytes and bacteria were excluded from the final analysis.

The present study suggests that dietary antigens in human milk may not be the only factors in

milk contributing to sensitization of the newborn to foods. Vassella and colleagues have shown that the milk of atopic mothers contains a relatively high number (4%) of eosinophils (4). In the present study also, some of the atopic mothers who had an infant with CMA had high proportions of eosinophils in their milk, which gives an overwhelming total number of $6-70 \times 10^6$ eosinophils ingested by such an infant each day. In the gut of the suckling infant, eosinophils will finally break by cytolysis (even without any activation process), leading to de-granulation, following which highly cytotoxic mediators, including eosinophilic cationic protein, will be released. In asthma, these eosinophil-derived proteins have been shown to cause damage to airway epithelium (11). The fate and actions of eosinophils and their mediators in the gut of the infant remain to be investigated.

The presence in colostrum of human milk polymorphonuclear cells is obscure. Infection in the mammary gland has been associated with an increase in their number. They are functionally exudate cells with locomotive, adherence, microbicidal, and stimulated respiratory burst capabilities lower than those of their counterparts in blood (25). They therefore do not seem to provide significant anti-infective protection to the breast-fed infant (25), despite the lactoferrin they release. These characteristics are typical for activated neutrophils, which in the alimentary tract of the infant might substitute for neutrophils that cannot be recruited from the intra-vascular compartment owing to decreased L-selectin and yet, because of prior activation, serve a non-inflammatory role. In general, the neutrophilic granulocyte is a cytotoxic and potentially tissue-injuring cell that participates in the destructive processes and symptoms seen in a variety of inflammatory diseases, including asthma.

It has been recently hypothesized that the first 2 years of life is an important period for immune deviation (26). We found lymphocytes in considerably high numbers, especially in the milk of mothers with an infant with CMA or atopic dermatitis. It is known from previous studies that the majority of lymphocytes in human milk comprise T cells (4), 50% of which are of the helper subtype (23), but their T helper (Th)1/Th2 ratio is unknown. Considering the important role of human milk-derived memory cells in aiding the inexperienced immune system of the infant, the possibility that the polarization of milk lymphocytes might affect the immune deviation of the infant is a fascinating, but to date unconsidered, issue.

The precise function of human milk macrophages is unknown. In the gut of a newborn, the total absence of secretory immunoglobulin A (sIgA) production severely impairs the antibody-mediated elimination of a potential allergen. It has been speculated that maternal sIgA, and maternal macrophages as a storage for IgA, partly compensate for this deficiency (27). Consistently, a smaller proportion of milk macrophages might indirectly lead to deficient antigen elimination. Previously, we suggested that the central role of human milk macrophages might be in the regulation of oral tolerance in the gut mucosa of the suckling infant. This was based on the finding that expression of human leucocyte antigen (HLA)-DR and production of tumor necrosis factor- α by human milk macrophages is normally high, but defective in those mothers with a cow's milk-allergic infant, suggesting deficient function of this predominant milk leucocyte (15,28). This might cause an imbalance in the antigen exclusion and local cytokine environment in the gastrointestinal mucosa of the infant, and thus may play a role in the delay of development of oral tolerance.

Although it is not known how leucocyte migration to the mammary gland is regulated, it has been shown that this migratory process is the result of a selective homing process, at least for lymphocytes (29). The presence of eosinophils in high numbers to some mothers' milk remains unexplained, as in others even large proportions of eosinophils were found in peripheral blood, with none detected in milk. It has recently been reported that the concentrations of interleukin-8 (IL-8) and RANTES were higher in breast milk from allergic compared to non-allergic mothers, which may be responsible for the traffic of eosinophils from the maternal circulation to the breast milk (18).

CMA is not attributable to a single cause. The development of allergies is known to be affected by numerous factors, including parental smoking, family size, pets, the house dust mite, and, according to more recent findings, the gut microflora (30,31). Recent studies have shown that the function of the epithelial barrier, infections, intestinal and peripheral cytokine environment, the age at introduction of cow's milk formulas, and cytokine and IgA composition of mother's milk may affect the development of CMA (13,15,32-34). However, other still-unknown factors (intrinsic as well as extrinsic) may have an important bearing on sensitization. In short, breast-feeding may normally provide the newborn with many advantages, but in some mothers the composition of their milk is abnor-

mal and is associated with a diagnosis of CMA in a breast-fed infant. This may provide a new and interesting insight into the development of food allergies, which remains to be confirmed by future studies.

Acknowledgments

We thank Dr Kaisu Juntunen-Backman for providing the facilities and Dr Anna-Liisa Järvenpää and Dr Arja Hakulinen for recruitment of low-risk families and collection of samples. We also thank Carol Norris for language revision. This work was partially supported (personal grant) by the Allergy Research Foundation in Finland (to K.-M.J. and H.S.), by the Orion Research Foundation, and by the Clinical Research Institute of Helsinki University Central Hospital (K.-M.J.).

References

1. HO FCS, WONG RLC, LAWTON JWM. Human colostrum and breast milk cells. A light and electron microscopic study. *Acta Paediatr Scand* 1979; 68: 389–96.
2. OGRA SS, OGRA PL. Immunologic aspects of human colostrum and milk. II. Characteristics of lymphocyte reactivity and distribution of E-rosette forming cells at different times after the onset of lactation. *J Pediatr* 1978; 92: 550–5.
3. CRAGO SS, PRINCE SJ, PRETLOW TG, MCGHEE JR, MESTECKY J. Human colostrum cells. I. Separation and characterization. *Clin Exp Immunol* 1979; 38: 585–97.
4. VASSELLA CC, HJÄLLE L, BJÖRKSTEN B. Basophils and eosinophils in human milk in relation to maternal allergy. *Pediatr Allergy Immunol* 1992; 3: 28–32.
5. PAXSON CL, CROSS CC. Survival of human leukocytes. *J Pediatr* 1979; 94: 61–4.
6. CERIANI RL, TAYLOR-PAPADIMITRIOU J, PETERSON JA, BROWN P. Characterization of cells cultured from early lactation milks. *In Vitro* 1979; 5: 356–62.
7. SEELIG LL, HEAD JR. Uptake of lymphocytes fed to suckling rats: An autographic study of the transit of labelled cells through the neonatal gastric mucosa. *J Reprod Immunol* 1987; 10: 285–97.
8. TUBOLY S, BERNATH S, GLAVITS R, KOVACS A, MEGYER Z. Intestinal absorption of colostrum lymphocytes in newborn lambs and their role in the development of immune status. *Acta Vet Hung* 1995; 43: 105–15.
9. JAIN L, VIDYASAGAR D, XANTHOU M, GHAI V, SHIMADA S, BLEND M. *In vivo* distribution of human milk leucocytes after ingestion by newborn baboons. *Arch Dis Child* 1989; 64: 930–3.
10. XANTHOU M. Human milk cells – Review. *Acta Paediatr* 1997; 86: 1288–90.
11. VENGE P, PETERSON CGB. Eosinophil biochemistry and killing mechanisms. In: MORLEY J, COLDITZ I, eds. *Eosinophils in Asthma*. New York, London: Academic Press, 1989. pp. 163–77.
12. WRIGHT AL, SHERRILL D, HOLBERG CJ, HALONEN M, MARTINEZ FD. Breast-feeding, maternal IgE, and total serum IgE in childhood. *J Allergy Clin Immunol* 1999; 104: 589–94.
13. SAARINEN KM, JUNTUNEN-BACKMAN K, JÄRVENPÄÄ AL, et al. Supplementary feeding in maternity hospitals and the risk of cow's milk allergy: A prospective study of 6209 infants. *J Allergy Clin Immunol* 1999; 104: 457–61.
14. SAARINEN UM, KAJOSAARI M. Breastfeeding as prophylaxis against atopic disease: Prospective follow-up study until 17 years old. *Lancet* 1995; 346: 1065–9.
15. JÄRVINEN K-M, LAINE S, SUOMALAINEN H. Defective tumour necrosis factor- α production in mother's milk is related to cow's milk allergy in suckling infants. *Clin Exp Allergy* 2000; 30: 637–43.
16. SAARINEN KM, VAARALA O, KLEMETTI P, SAVILAHTI E. Transforming growth factor- β 1 in mothers' colostrum and immune responses to cow's milk proteins in infants with cow's milk allergy. *J Allergy Clin Immunol* 1999; 104: 1093–8.
17. BÖTTCHER MF, JENMALM MC, GAROFALO RP, BJÖRKSTEN B. Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr Res* 2000; 47: 157–62.
18. BÖTTCHER MF, JENMALM MC, BJÖRKSTEN B, GAROFALO RP. Chemoattractant factors in breast milk from allergic and nonallergic mothers. *Pediatr Res* 2000; 47: 592–7.
19. ISOLAURI E, TURJANMAA K. Combined skin prick and patch testing enhances identification of food allergy in infants with atopic dermatitis. *J Allergy Clin Immunol* 1996; 97: 9–15.
20. MACEY MG. Immunophenotypic analysis of lymphocytes and leukaemias. In: MACEY MARION G, ed. *Flow Cytometry. Clinical Applications*. Oxford: Blackwell Scientific Publications, 1994. pp. 67–100.
21. JÄRVINEN K-M, ARO A, JUNTUNEN-BACKMAN K, SUOMALAINEN H. Large number of CD19⁺/CD23⁺ B cells and small number of CD8⁺ T cells as early markers for cow's milk allergy (CMA). *Pediatr Allergy Immunol* 1998; 9: 139–42.
22. KEENEY SE, SCHMALSTIEG FC, PALKOWETZ KH, RUDLOFF HE, LE B-M, GOLDMAN AS. Activated neutrophils and neutrophil activators in human milk: Increased expression of CD11b and decreased expression of L-selectin. *J Leukoc Biol* 1993; 54: 97–104.
23. WIRT DP, ADKINS LT, PALKOWETZ KH, SCHMALSTIEG FC, GOLDMAN AS. Activated and memory T lymphocytes in human milk. *Cytometry* 1992; 13: 282–90.
24. BAZIL V, STROMINGER JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J Immunol* 1991; 147: 1567–74.
25. BUESCHER ES, MCLLERAN SM. Polymorphonuclear leukocytes and human colostrum: Effects of *in vivo* and *in vitro* exposure. *J Pediatr* 1993; 17: 424–33.
26. YABUHARA A, MACAUBAS C, PRESCOTT SL, et al. TH2 polarized immunological memory to inhalant allergens in atopics is established during infancy and early childhood. *Clin Exp Allergy* 1997; 27: 1261–9.
27. BERNT KM, WALKER WA. Human milk as a carrier of biochemical messages. *Acta Paediatr Suppl* 1999; 430: 27–41.
28. JÄRVINEN K-M, JUNTUNEN-BACKMAN K, SUOMALAINEN H. Relation between weak HLA-DR expression on human breast milk macrophages and cow milk allergy (CMA) in suckling infants. *Pediatr Res* 1999; 45: 76–81.
29. SLADE HB, SCHWARTZ SA. Mucosal immunity: The immunology of breast milk. *J Allergy Clin Immunol* 1987; 80: 346–56.
30. STRACHAN DP. Family size, infection and atopy: The first decade of the 'hygiene hypothesis'. *Thorax* 2000; 55 (Suppl. 1): S2–10.
31. KALLIOMÄKI M, KIRJAVAINEN P, EEROLA E, KERO P, SALMINEN S, ISOLAURI E. Distinct patterns of neonatal gut

- microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001; 107: 129–34.
32. HEYMAN M, DESJEU JF. Cytokine-induced alteration of the epithelial barrier to food antigens in disease. *Rev Ann N Y Acad Sci* 2000; 915: 304–11.
33. SCHADE RP, VAN IEPEREN-VAN DIJK AG, VAN REIJSEN FC, et al. Differences in antigen-specific T-cell responses between infants with atopic dermatitis with and without cow's milk allergy: Relevance of TH2 cytokines. *J Allergy Clin Immunol* 2000; 106: 1155–62.
34. JÄRVINEN K-M, LAINE S, JÄRVENPÄÄ A-L, SUOMALAINEN H. Does low IgA in human milk predispose the infant to the development of cow's milk allergy? *Pediatr Res* 2000; 48: 457–62.