

Defective tumour necrosis factor- α production in mother's milk is related to cow's milk allergy in suckling infants

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Summary

Background The precise role of leucocytes in human milk is still unresolved.

Objective To assist in clarifying the immune mechanisms involved in the development of CMA in suckling infants, we studied the role of immunoregulatory leucocytes and their mediators in human breast milk.

Methods The study population consisted of 43 lactating mothers and their infants, aged 0.25–8.0 months, followed-up prospectively from birth. Of these mothers, 27 had an infant with challenge-proven cow's milk allergy manifested with either skin ($n=23$), gastrointestinal ($n=2$) or skin and gastrointestinal symptoms ($n=3$). Sixteen mothers with a healthy infant served as controls. We evaluated the spontaneous and mitogen-induced tumour necrosis factor- α (TNF α) and interferon- γ (IFN γ) production of human milk leucocytes and isolated peripheral blood lymphocytes *in vitro* with a commercial ELISA kit.

Results TNF α production of breast milk leucocytes was significantly lower in the mothers with a cow's milk-allergic infant, whereas IFN γ production of these cells was comparable in the two groups.

Conclusion Our results suggest that in the breast milk of mothers having an infant with cow's milk allergy, the number and function of TNF α -producing cells is defective. This might lead to a disturbance in the development of oral tolerance and thereby to the development of CMA in suckling infants. These novel results may help in clarifying the etiopathogenesis of CMA.

Keywords: cow's milk allergy, human milk, oral tolerance, tumour necrosis factor- α

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Introduction

Colostrum and milk from healthy mothers have been studied extensively. In human milk, the predominant cellular component is the breast milk macrophage, which from its morphology appears to be activated [1]. According to the literature, other milk cells include 40–60% of neutrophils and 5–27% of lymphocytes, the majority of which are T cells [1,2], occasional monocytes [1] and few eosinophils and basophils [3]. Human milk also contains cytokines such as interleukin (IL) -1 [4], IL-6 [5], IL-8 [6], IL-10 [7] and

tumour necrosis factor- α (TNF α) [8]. Human milk mononuclear cells have also been demonstrated to have a potential, upon stimulation, for production of other cytokines, such as IL-4 and interferon- γ (IFN γ) [9]. Although evidence is lacking about the direct influence of breast milk cytokines on the recipient, breast milk is believed to impart specific immune advantages to the neonate through enhancement or induction of the still developing neonatal immune system [10].

TNF α is a regulatory lymphokine produced by activated macrophages and T lymphocytes [11]. It is found in breast milk in sufficient quantities to potentially affect the recipient infant [8]. This factor has numerous effects on the function of the immune system. TNF α , among other factors,

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has been found to act synergistically with IFN γ to upregulate HLA class II expression [12]. By activating macrophages, it augments the number of receptors for TNF α on the cell surfaces, making the cells more responsive to TNF α and thus further increasing their activation and secretion of TNF α [13]. TNF α production also induces adhesion molecule expression [14], chemotaxis of monocytes [15] and intra-epithelial lymphocyte proliferation and migration [16]. We have previously demonstrated that in mothers whose infant has CMA, HLA-DR expression of breast milk macrophages is lower than in those with a healthy infant, reflecting decreased activity of these cells [17]. To obtain further information about the function of milk leucocytes and their possible role in the regulation of the development or breakdown of oral tolerance in suckling infants, we evaluated the capacity of mitogen-induced human breast milk leucocytes to produce TNF α and IFN γ *in vitro*.

Materials and methods

Patients

The study population comprised 43 breast-feeding mothers and their infants. All the infants were followed-up prospectively from birth: one group because of their high risk for allergy (atopic or food-allergic sibling(s) and/or parent[s]), and the other group because of their low risk for allergy (no signs of atopic heredity in the family). They were followed-up for at least 1 year to detect any symptoms of atopic dermatitis or food allergy. The diagnosis of cow's milk allergy (CMA) was based on a positive response to oral cow's milk challenge. The control patients had had no dietary restrictions at testing. All the infants were born at term and had no other chronic disease or infection at the time of study. The mothers had no medication at the time of the study and those with mastitis during the previous 4 weeks were excluded from the study. The mothers with atopic constitutions had no symptoms of hay fever, eczema, urticaria or asthma at the time of the study.

The mothers gave written informed consent to the study, which was approved by the Ethics Committee of the Skin and Allergy Hospital of the Helsinki University Central Hospital.

Cow's milk challenge protocol

After a 2–4-week cow's milk elimination diet, patients were admitted for diagnostic cow's milk challenge. This was performed openly, since placebo reactions have been shown to be rare in infants [18]. During the elimination period, cow's milk was strictly eliminated also from the mothers' diet.

The mothers were given calcium supplementation and if

the infants needed extra formula in addition to the breast milk, a tolerated formula (protein hydrolysate or amino-acid based formula) was given. During the challenge, cow's milk was given in increasing doses at 2-h intervals: on day 1: 2, 10, 50 and 100 mL and on day 2: the normal milk intake appropriate for age was commenced. When any adverse reaction was noticed, the challenge was stopped and the patients were examined by the same paediatrician or dermatologist. The time of the reaction was defined as the time since the last dose eliciting the specific symptoms. The patients were followed over the 1-week period of challenge and all the infants in this study were followed up for the next year, to detect possible development of any allergies during that time.

Collection of human milk and preparation of cell suspensions

The breast was washed with warm water and fresh human milk was collected by manual expression or with a breast pump, always at the same time between 08.00 and 11.00 h. The volume of each milk sample was measured, and it was immediately centrifuged (400 g at room temperature for 15 min) to remove the lipids and the whey. The leucocytes were then collected and resuspended in RPMI 1640 (Sigma-Aldrich Co Ltd, UK) containing 5% fetal calf serum (FCS) and penicillin (100 IU/mL), streptomycin (100 μ L/mL) and glutamine (600 μ L/mL). After four washes, the cells were visually counted in Bürker chambers under a light microscope.

Blood samples

Blood was drawn into heparin for lymphocyte induction and cytospin preparations, always at the same time as the breast milk samples were taken. Mononuclear cells, mainly lymphocytes, were isolated by Ficoll-Hypaque gradient centrifugation at 400 g for 30 min at 20 °C. The leucocytes were resuspended in RPMI 1640 containing 5% FCS, antibiotics (penicillin and streptomycin), and glutamine. After washing three times, the cells were visually counted by eye in Bürker chambers under a light microscope.

Cytospin preparations

Cytospin preparations of milk cells were obtained with a sample application of 1×10^5 cells (Cytospin3, Shandon, Life Sciences International Ltd, Runcorn, UK). The slides were treated with VectapondTM (Vector Laboratories, Inc, Burlingame, CA, USA) before sample application, to improve the fixation of breast milk cells. The numbers of macrophages, monocytes, lymphocytes, neutrophils and eosinophils were evaluated on slides stained with May–Grünwald–Giemsa.

Flow cytometry

Flow cytometric analysis enables enumeration of leucocyte subsets. For this purpose, leucocyte suspensions from human milk and peripheral blood at a cell concentration of $1 \times 10^6/\text{mL}$ in RPMI 1640, with antibiotics, glutamine and 5% FCS, were incubated at 4°C for 15 min with a saturating concentration of phycoerythrin-labelled monoclonal antibodies against CD14 and CD8, and fluorescein isothiocyanate-labelled antibodies to CD45, CD3, CD4 and CD19 (Beckton Dickinson Immunocytometry Systems, Mountain View, CA, USA) according to the manufacturer's instructions [19]. Flow cytometry was then performed, using the same instrument settings in every analysis. The leucocytes were gated to study the numbers of cell subsets within the gate. The numbers of cells positive for the surface markers are expressed as percents of the cells within the gate.

Determination of TNF α , IL-4 and IFN γ production during cell induction

To induce breast milk and peripheral blood lymphocytes and breast milk macrophages, 6.25×10^5 isolated cells in 1 mL of RPMI 1640 containing antibiotics, glutamine, and 10% fetal calf serum were cultured in a humidified, 5% CO₂ atmosphere at 37°C for 48 h with Concanavalin A (Con A, Pharmacia, Uppsala, Sweden) at a final concentration of 25 $\mu\text{g}/\text{mL}$; a control cell population was generated with RPMI 1640 only. The supernatants were collected and stored at -70°C for later determination of TNF α , IFN γ and IL-4. Subsequently, the TNF α , IL-4 and IFN γ produced during lymphocyte induction were determined from the thawed supernatants with commercial ELISA kits (CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions. The results of the different runs were equalized, employing the comparison of standard curves, and are expressed as pg/mL.

Statistical analysis

Because of skewed distributions, logarithmic transformations were used. ANOVA was employed to determine the statistical significance of differences in continuous variables. The Mann-Whitney *U*-test was employed for comparison of medians. The Chi-square test was applied to determine differences in proportions. The repeated observations were studied by using a paired *t*-test.

Results

Clinical characteristics of infants with CMA and controls

The study population consisted of 27 breast-feeding mothers who had an infant with challenge-proven CMA, manifested

as either urticarial or eczematous skin eruptions ($n = 22$), gastrointestinal symptoms ($n = 2$) or both skin and gastrointestinal symptoms ($n = 3$), and 16 lactating mothers with their age-matched healthy infants, who were free of symptoms or signs of CMA or atopic eczema. The geometric mean age of the infants with CMA was 1.9 [95% CI 1.3–3.8] months and of the healthy infants was 2.2 [1.2–3.1] months, $F = 0.06$, $P = 0.8$. The mean geometric age at the onset of the symptoms of CMA was 1.5 months [95% CI 1.1–2.9]. A positive family history of allergy was detected in 88.9% of the infants who had CMA and in 87.5% of controls, $\chi^2 = 2.0$, $P = 0.20$. Of the lactating mothers, 18/27 (67%) of those whose infant had CMA and 8/16 (50%) of those with a healthy infant had an atopic constitution, $\chi^2 = 1.0$, $P = 0.28$.

Patients who had CMA, manifested as either urticarial or eczematous skin eruptions ($n = 13$), mainly reacted in an early phase of the challenge, mean 1.3 h (range, 0.5–3 h) after the start of the challenge in response to low volumes of milk, median 10 mL (range, 10–22 mL). Those with gastrointestinal symptoms or slowly developing eczema ($n = 14$) more often evidenced delayed reactions after a mean of 70 h (range, 2.5–168 h), in response to larger amounts of milk, median 360 mL (range, 100–2300 mL).

Light microscopy of human milk leucocytes

The total number of human milk leucocytes per millilitre of milk was comparable in mothers whose infants were allergic to cow's milk and in those with a healthy infant. The proportion of macrophages was smaller in the breast milk of mothers having an infant with CMA, geometric mean 62% [95% CI, 50–75] than in those with a healthy infant, 81% [74–89], $P = 0.048$ (ANOVA). The mothers of allergic infants seemed to have larger proportion of monocytes (9 vs 5%) and neutrophils (9 vs 5%) in their breast milk. The numbers of lymphocytes (3 vs 2%), and eosinophils (2 vs 2%) were comparable in the mothers with an allergic infant and in those with a healthy infant, respectively.

Flow cytometry of human milk and peripheral blood leucocytes

Breast milk cells mainly consisted of CD14⁺ cells in mothers with a CMA infant and in those with a healthy infant, geometric mean 76% [95% CI, 69–84] and 68% [55–83] of cells, respectively ($P = 0.69$, Mann-Whitney *U*-test). CD4⁺ and CD8⁺ T cells as well as CD19⁺ B cells each comprised only 1–2% of breast milk cells and their proportions were comparable in these two groups. In the peripheral blood, the proportions of CD14⁺ cells as well as different lymphocyte subsets were comparable and within normal range in these two groups of mothers.

Spontaneous TNF α , IL-4 and IFN γ production of human milk leucocytes

Spontaneous TNF α production of human milk leucocytes, measured from unstimulated cultures, was significantly lower in the mothers whose infant was allergic to cow's milk than in those with a healthy infant, geometric mean 4.3 pg/mL [95% CI, 2.0–9.5] and 22.4 pg/mL [8.2–60.7], respectively ($P=0.02$, Mann–Whitney U -test) (Fig. 1). Unstimulated IL-4 production was undetectable in most samples in these two groups (data not shown). Spontaneous IFN γ production was very low in the breast milk of mothers with an allergic infant as well as in those with a healthy infant, geometric mean 1.1 pg/mL [95% CI, 0.2–6.3] and 0.6 pg/mL [0.002–1.7], respectively ($P=0.42$, Mann–Whitney U -test).

Con A-induced TNF α , IL-4 and IFN γ production of human milk leucocytes

Con A-induced TNF α production was significantly lower in the breast milk of mothers whose infant was allergic to cow's milk than in that of mothers with a healthy infant, geometric mean 7.9 pg/mL [95% CI 3.9–15.7], and 46.6 pg/mL [20.6–105], respectively ($P=0.004$, Mann–Whitney U -test; Fig. 1). IL-4 production was undetectable in most milk samples in these two groups (data not shown). The IFN γ production of Con A-stimulated human milk leucocytes was

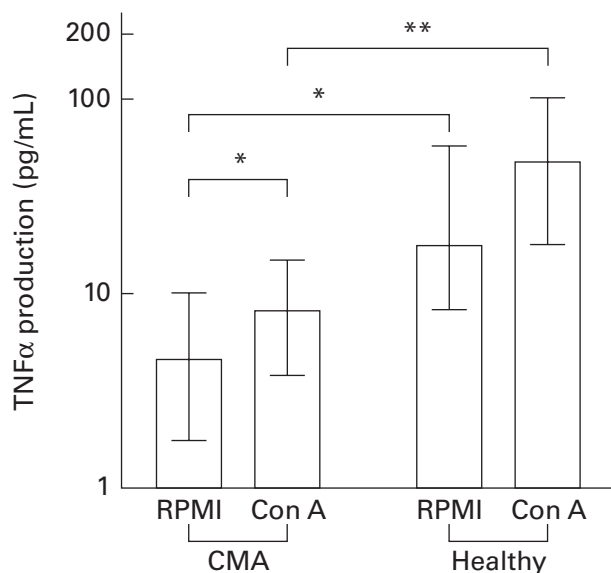


Fig. 1. The *in vitro* production (pg/mL) of TNF α of breast milk cells, from mothers whose infant has cow's milk allergy (CMA) or from mothers who have a healthy infant when incubated in plain medium (RPMI) or induced by Concanavalin A (Con A). Horizontal bars denote the 95% confidence intervals. * $P<0.05$, ** $P<0.005$

comparable in the breast milk of mothers with an allergic infant and in those with a healthy infant, geometric mean 4.6 pg/mL [95% CI 1.4–15.4] and 10.6 pg/mL [2.55–44.8], respectively ($P=0.14$, Mann–Whitney U -test).

Comparison of spontaneous and Con A-induced TNF α production of human milk leucocytes between groups

In mothers of a healthy infant, the increase in the TNF α production of breast milk cells after stimulation with Con A was considerable when compared with unstimulated production, although not statistically significant, $P=0.09$, $t=-1.98$. In mothers with an infant with CMA, there was a statistically significant rise in the TNF α production of breast milk cells after stimulation when compared with the unstimulated production, $P=0.019$, $t=-2.62$. Despite this increase, the amount of TNF α production did not reach even close to the level of unstimulated production of TNF α of breast milk cells from mothers with a healthy child.

Spontaneous TNF α , IL-4 and IFN γ production of peripheral blood leucocytes

Spontaneous TNF α and IFN γ production was comparable in mothers with an allergic infant and in those with a healthy infant (Table 1). Spontaneous IL-4 production was low in both groups (data not shown). Atopic mothers had significantly higher spontaneous TNF α production, geometric mean 28.8 pg/mL [95% CI, 12.3–68.0], than the nonatopic mothers, 21.8 pg/mL [14.0–33.8] ($P=0.029$, ANOVA).

Con A-induced TNF α , IL-4 and IFN γ production of peripheral blood leucocytes

Con A-induced production of TNF α and IFN γ were comparable in mothers whose infants had CMA and in those with a healthy infant (Table 1). IL-4 production was very low in both groups (data not shown). Con A-induced TNF α production of the atopic mothers tended to be higher and the IFN γ production lower than that of nonatopic mothers, but this difference was not statistically significant.

Discussion

In the present investigation we have correlated the cytokine production of the maternal milk leucocytes with the allergic status of the suckling infant. We have shown that, in the breast milk of mothers having an infant with CMA, TNF α production of leucocytes was significantly lower than in those with a healthy infant. Our results imply that lack of TNF α production of breast milk leucocytes might have a role in the development of CMA in the suckling infant. They further suggest that TNF α may play a central role in the

Table 1. Spontaneous and Con A-induced TNF α and IFN γ production of peripheral blood mononuclear cells of mothers with a healthy child and of those with a cow's milk-allergic child

Cytokine	Peripheral blood of mothers with:		P-value
	Healthy child	Cow's milk-allergic child	
TNF α RPMI	30.6 [16.0, 58.4]	23.6 [11.4, 48.9]	0.51
TNF α Con A	216 [119, 393]	347 [267, 450]	0.15
IFN γ RPMI	21.2 [9.2, 48.9]	7.0 [2.8, 17.9]	0.71
IFN γ Con A	1203 [421, 3435]	1762 [1086, 2861]	0.54

Values are geometric mean [95% CI] expressed as pg/mL. P-value was calculated using the Mann–Whitney U-test.

achievement of oral tolerance to dietary antigens in the human infants.

TNF α is a regulatory lymphokine produced by activated macrophages and T lymphocytes [11]. It is present in human milk in concentrations high enough to suggest that it could be physiologically active [8]. In early life, there is no evidence of intragastric protein digestion [20], and high concentrations of antiproteases, α_1 -antichymotrypsin, and α_1 -antitrypsin, which impede proteolysis, are present in human milk [21]. Previous human studies have shown that urinary excretion of lactoferrin, IgA, and secretory component, present in high amounts in human milk, is higher in infants fed breast milk than in those fed formula, suggesting that immune factors in breast milk may be absorbed in the gut of the suckling infant and secreted in the urine [22]. Therefore, there is also evidence that breast milk-derived TNF α can persist in the recipient long enough to be biologically active.

The precise role of antigen-presenting cells in the induction of oral tolerance is yet to be determined. It has been claimed to be likely that both mucosal dendritic cells and gastrointestinal epithelial cells play important roles in that process at least according to rodent models [23]. Dendritic cells have been shown to pick up and process antigen, but paradoxically as immature dendritic cells to be unable to effectively present the processed antigen to T cells [24]. There is some evidence that TNF α may have an important role in the maturation process [25,26]. Further, there is evidence that the antigen-presenting property of dendritic cells is downregulated and the T-cell stimulating function is upregulated by neighbouring macrophages in lamina propria and in lungs [27,28]. According to Holt *et al.* this effect was mediated by TNF α [28]. In the same study, the rats displayed greatly enhanced immune responses to all inhaled antigens if the macrophages were removed from the lung alveoli [28]. Enhanced antigen-presenting cell activity, which is characteristic for an immature dendritic cell, has previously been suggested to interfere with the induction of oral tolerance [29]. Based on these previous and our novel

findings, we would like to hypothesize that if there was lack of TNF α production by breast milk leucocytes, the important step of switching of immature dendritic cells to mature ones might be downregulated. It is therefore tempting to speculate that the lack of TNF α in breast milk could downregulate the differentiation of dendritic cells in the suckling infant, which might play a role in the development of CMA. TNF α has numerous effects on the network of cellular interactions and therefore other possibilities may also be considered for TNF α in the induction of immune responses.

The present study does not identify how the low production of TNF α interferes with the induction of oral tolerance. According to previous animal studies, there is evidence that clonal anergy of T cells occurs in oral tolerance. This is, however, preceded by a transient phase of T-cell priming, characterized by an activation of antigen-specific T cells [30]. Since tolerance induced by oral antigen is antigen-specific, it must follow that interaction of T cells with its specific antigen occurs during the induction phase of oral tolerance [30]. We would like to speculate that the first weeks of breast-feeding may be an important period for the priming of neonatal T cells, while foreign antigens are introduced in small amounts to the infant for the first time.

Whereas local production of TNF α may play a physiological role in regulating antigen presentation by dendritic cells, high systemic levels of TNF α , such as in chronic inflammatory diseases and autoimmune diseases [31,32], may be detrimental. Thus it seems obvious that moderate production of this cytokine is required to maintain the homeostasis. In the present study, the considerably high TNF α production of breast milk cells in mothers with a healthy infant suggests that the cells producing TNF α are more numerous and functionally more active in breast milk after *in vivo* activation in the mother. Furthermore, the cells are even capable of augmenting their cytokine production upon stimulation. Since in healthy mothers with a healthy infant, the TNF α production of breast milk cells is regulated to the adequate level by *in vivo* activation in the mother, it

does not increase significantly after *in vitro* stimulation. On the contrary, in mothers with an infant allergic to cow's milk, the unstimulated TNF α production was remarkably lower. This suggests that in mothers with a cow's milk-allergic infant, the number and the *in vivo* activation of the cells producing TNF α is defective. Further, these cells are also poorly activated after *in vitro* stimulation and their TNF α production does not reach even close to the level of unstimulated production of TNF α of breast milk cells from mothers with a healthy child. This may be due to their poor capacity to produce mRNA for TNF α inside the cell or to secrete the prepared cytokine out of the cell. Although Con A is not the best mitogen to induce macrophages [33], it was selected to induce TNF α production in a mixed cell population in human breast milk, usually comprising macrophages and lymphocytes. In the present study, we have measured the capacity of breast milk leucocytes to produce TNF α after incubation with or without mitogen. The levels of TNF α were not measured in the breast milk itself. Since epithelial cells are a source of TNF α , its concentration in breast milk itself may be somewhat different from what has been found in the present study.

Finally, we wish to emphasize that atopic diseases and food allergy are not due to a single defect, but are regulated by numerous factors. Notwithstanding, we think that our findings may add information important for clarifying the mechanisms involved in the development of food allergy and atopic diseases in suckling infants.

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