

ORIGINAL PAPER

Transforming growth factor- β_1 and interleukin-10 in breast milk and development of atopic diseases in infants

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Clinical and Experimental Allergy

Summary

Background Precise relationship between breastfeeding and infant allergy is poorly understood. **Objective** Aim was to quantify TGF- β_1 and IL-10 in colostrum and mature milk from allergic and non-allergic mothers and to verify relationship with allergic disease development.

Methods Mothers (13 allergics, nine controls) of 22 newborns participated to prospective study on development of children atopy. Colostrum and mature milk were assayed for TGF- β_1 and IL-10 by ELISA. Children underwent paediatrician evaluation at 6 months of life.

Results Data are presented as median values and range. A significant difference in concentration of TGF- β_1 between colostrum (330, range 0–3400 pg/mL) and mature milk (215, range 0–2400 pg/mL) was observed in samples from allergic mothers ($P = 0.015$). In mature milk TGF- β_1 was significantly lower in allergic (215, range 0–2400 pg/mL) than in non-allergic mothers (1059, range 0–6250 pg/mL) ($P = 0.015$). IL-10 was weakly expressed without significant differences between allergic (4.8, range 0–42 and 9.5, range 0–42 pg/mL in colostrum and in mature milk) and non-allergic mothers (0, range 0–42 pg/mL in colostrum and 0, range 0–42 pg/mL in mature milk). After 6 months 46% infants from allergic mothers, but none from controls, presented atopic dermatitis.

Conclusion TGF- β_1 was significantly less secreted in mature milk of allergic mothers, while no difference in IL-10 was found. Particular cytokine patterns in milk could influence development of atopic diseases. Further immunological studies in this field are necessary.

Keywords allergy, breast milk, children, IL-10, TGF- β_1

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Introduction

The immune system of newborn infant is not fully developed and during this period babies are more susceptible to bacterial and viral infection as well as to allergic sensitization to environmental allergens [1]. The breast milk provides the infant with nutrients for growth and development and immune protection to compensate for the immature defence mechanisms [2]. However, little information is available on the fact that allergic status of the mother *per se* could influence the composition of her breast milk. Allergic mothers are commonly advised to breastfeed their babies in order to reduce the infants' risk to develop atopic diseases. Literature reviews, however, show that the allergy preventing effect of breastfeeding has not been established beyond doubt [3, 4]. The immuno-protection conferred by human milk seems to vary from mother to mother [4]. For instance, it is possible that the allergic disease of the mother may influence the breast

milk composition, and such variation in composition may in turn affect the maturation of the defence mechanisms of gastrointestinal tract and thus the health status of the breastfed infant [4].

It is unknown which of the many different components of breast milk might be responsible for the hypothesized differential effects of milk of allergic and non-allergic mothers [5, 6]. Leucocytes, cytokines, immunoglobulins, and fatty acids have all been suggested as potential candidates [6]. Furthermore, human milk contains several components protecting the infant against infections, including antibodies and viable lymphocytes [2] and several cytokines [7]. Cytokines have the capacity to exert both pro-inflammatory and anti-inflammatory effects, but little is known as to their concentrations and associations in breast milk. Data from studies investigating the cytokine pattern, and particularly TGF- β_1 , in human milk reported not univocal results: some showing no difference in TGF- β_1 [8–10], other reporting differences in TGF- β_2 levels in

colostrum or in mature milk between allergic and non-allergic mothers [11].

The aim of the present prospective study was to perform an investigation to quantify TGF- β_1 and IL-10 in human milk, comparing their concentrations in colostrum and mature milk in allergic and in non-allergic mothers. Further aim was to assess in a short-term follow-up the relationship between atopic status of the mothers, presence of these cytokines in the milk, and following development of infant allergic diseases.

Material and methods

Patients

From February to March 2005 the mothers of 22 newborns were invited to participate in a prospective study on the development of immune responses of their children in relationship with the particular composition of mothers' milk. None of the mothers had any autoimmune or chronic disease, except eventual allergic diseases, i.e. asthma, rhinoconjunctivitis, and atopic dermatitis. Atopic status was verified in each mother by skin prick tests (SPTs) performed by standardized extracts for foods (egg, milk), pollens (Grass mix, *Parietaria judaica*), house dust mites (*Dermatophagoides pteronyssinus* and *farinae*), animal dander (cat, dog), alternaria, as well as a saline negative control and a histamine control (10 mg/mL) (Stallergenes, Milan, Italy). Mothers were divided in cases with allergic diseases ($n = 13$) and without allergic diseases ($n = 9$) according to personal history, clinical data, physical examination, and SPT results.

At the end of sixth month of life babies returned to hospital and they underwent to a paediatric examination in order to evaluate presence of signs and symptoms of atopic dermatitis or wheezing.

Collection and processing of breast milk

The milk samples were collected from each mother in the morning from 8:00 to 10:00 hours with a manual breast pump (ISIS Avent, Suffolk, UK); the specimens were preserved into sterile plastic tubes at room temperature and processed within 4 h after collection.

Colostrum was collected within 3 days postpartum and mature milk at 1 month of life. Whole milk aliquots were centrifuged at 500 *g* for 12 min, after which the lipid layer and aqueous fraction were removed. Aliquots of the aqueous fraction were filtered (0.45 μ m) and stored at -70°C until assayed for cytokines.

Milk cytokine ELISA assays

Breast milk was filtered and prepared for assay of TGF- β_1 and IL-10 as previously described [12]. Thresholds of

detectability for the assays were as follows: TGF- β_1 31.2 pg/mL and IL-10 7.8 pg/mL, with $< 5\%$ intra- and $< 7\%$ inter-assays CV. Before assay for TGF- β_1 the sample must be treated with 1 N HCl to adjust to pH 3 (20 μ L 1 N to 500 μ L sample); the acidified sample was incubated for 15 min at room temperature and neutralized with 1 N NaOH (15 μ L) and immediately tested. The treatment was performed to activate latent TGF- β_1 to the immunoreactive form.

Cytokine IL-10 and TGF- β_1 in milk samples were detected by ELISA according to the manufacturer's instructions, continuously shaking the plate during incubation. We started with titration tests to setup a protocol optimized for our application. Detection of IL-10 and TGF- β_1 was performed with a dual antibody sandwich ELISA evaluated after performing spiking experiments (Biosource, Camarillo, CA, USA). Tetramethylbenzidine and hydrogen peroxide were used as substrates in a peroxidase reaction. Plates were read at 450 nm. The concentration of each cytokine was calculated in picograms per millilitre of breast milk.

Statistical analysis

Data were analysed by parametric and non-parametric tests both confirming the same level of significance. Differences between two unpaired groups were evaluated by the independent samples *t*-test for normally distributed variables and by the Mann-Whitney *U*-test for variables not normally distributed. For all analyses *P*-values < 0.05 were regarded statistically significant.

Ethical approval

The study was approved by the local Hospital Ethical Committee and written informed consent was obtained by child's parents or guardians.

Results

Data are presented as median values and range. The two groups of mothers were comparable for aging, alimentary habits, and pregnancy course. All babies presented good health at the birth and correct growth at the sixth month of life. At 6-month control visit, babies were almost all exclusively breastfed. Only four (two in every group) received supplementation with formula. At 6-month control six infants presented atopic dermatitis. None of the children presented signs or symptoms of wheezing. The six babies (6/13%, 46%) who developed atopic dermatitis were all from allergic mothers. The cytokine TGF- β_1 was detected in the majority of breast milk samples of allergic and non-allergic mothers (Fig. 1). A significant difference in concentration of TGF- β_1 between colostrum (330, range 0–3400 pg/mL) and mature milk (215, range

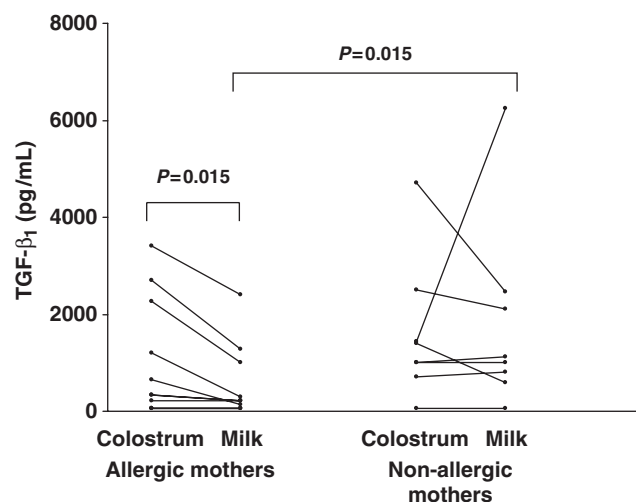


Fig. 1. Concentration of TGF- β_1 present in the milk of allergic and non-allergic mothers. Data are showed as single patient values in colostrums and in mature milk.

0–2400 pg/mL) was observed in samples from allergic mothers ($P=0.015$). A significant difference ($P=0.015$) was evident by comparison of the mature milk from allergic and non-allergic mothers, being TGF- β_1 significantly lower in the samples from allergic (215, range 0–2400 pg/mL) than in the samples from non-allergic mothers (1059, range 0–6250 pg/mL). No difference in TGF- β_1 present in colostrum was observed between allergic (330, range 0–3400 pg/mL) and non-allergic mothers (1200, range 0–4700 pg/mL). Interestingly, four out of six children who developed atopic dermatitis received breast milk with no TGF- β_1 level both in colostrum and in mature milk.

The cytokine IL-10 was detected in few of all breast milk samples from allergic and non-allergic mothers. There was no significant difference in IL-10 concentration between the two groups and no difference within the same group between colostrum and mature milk (Fig. 2). IL-10 was moderately but not significantly higher in the samples of allergic mothers (4.8, range 0–42 pg/mL and 9.5, range 0–42 pg/mL in colostrum and in mature milk, respectively) than in the samples of non-allergic mothers (0.0, range 0–42 pg/mL both in colostrum and in mature milk).

Discussion

The results of this preliminary study confirm the presence of secreted cytokines such as TGF- β_1 and IL-10 in colostrum and breast milk. Interestingly, in our study population TGF- β_1 seems to be less secreted in the milk of allergic mothers already in the first days of life. Moreover, only in the allergics there was a significant decrease of this cytokine from colostrum to mature milk. Consequently, at 1 month there was a significant difference in concentration of TGF- β_1 between allergic and non-allergic

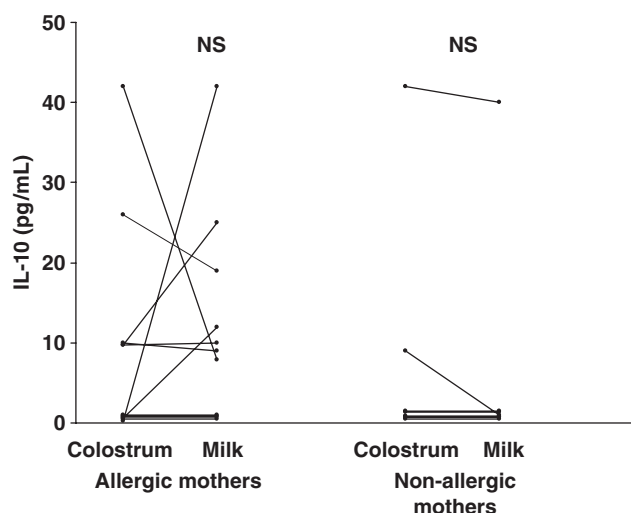


Fig. 2. Concentration of IL-10 present in the milk of allergic and non-allergic mothers. Data are showed as single patient values in colostrums and in mature milk.

mothers, being significantly reduced in the formers. Furthermore, at the 6-month follow-up 46% of children born from allergic mothers, but none from controls, presented symptoms compatible with atopic dermatitis.

Data from literature demonstrated that the concentration of TGF- β_1 was significantly higher in colostrum compared with mature milk [10, 13, 14], showing however a considerable variability between individual women [14]. Discrepancies between these evidences and data from our own study may be partly explained by the fact that in our study the mature milk was obtained when the infant was one instead that 3 months old [13]. In fact in an animal model it has been shown that TGF- β_1 and TGF- β_2 levels are higher in early milk and decrease during the weaning period [15]. Furthermore, previous studies reported no difference in the TGF- β_1 levels in colostrum or in the mature milk between mothers with or without allergic diseases [8–10]. However, in another paper milk TGF- β_2 levels were lower in allergic mothers than in non-allergic mothers, which is accordingly with our findings about TGF- β_1 [11]. All these evidences lead to consider that the individual variability and particularly the reduced TGF- β_2 or TGF- β_1 concentration in the breast milk of mothers with allergic diseases may interfere with the development of the mucosal immune system of the breastfed infant [11].

During the early infancy TGF- β_1 may prevent sensitization to food allergens [16], promote specific IgA production in human subjects [13] and might inhibit T cell activation acting synergistically with IL-10 to promote specific IgA production [17]. The influence of human milk TGF- β_1 and/or IL-10 to the class switch of immunoglobulins towards IgA class [17] has probably a crucial effects on development of the immature gastrointestinal tract by influencing IgA production and oral tolerance induction [13]. The authors found higher levels of TGF- β_1 and TGF-

β_2 in colostrum of mothers whose infants had specific IgA production [13]. In another study [9] the concentration of TGF- β_1 in colostrum samples from mothers of infants with IgE-mediated milk allergy was lower than from mothers of infants without IgE-mediated milk allergy and from mothers of control subjects. Authors suggested that in infants prone to have milk allergy, the TGF- β_1 content of mother's colostrum may promote IgG-IgA antibody production and inhibit IgE- and cell-mediated reactions to milk [9]. These findings are in line with our data, which show that children born from allergic mothers and who are more prone to develop atopic diseases (atopic dermatitis in the 46% at 6 months), are exposed to significantly reduced amounts of TGF- β_1 both in colostrum and in mature milk. We could speculate that this reduced exposure could affect the mucosal immune system and the following development of allergy. Of course also in our population it would be of extreme interest to verify as in the papers by Saarinen et al. [9] and Kalliomaki et al. [13] the effect of cytokine exposure on IgA levels both in milk and in infants. Unfortunately, in this pilot study drawings from infants were not considered in the study planning.

The role of TGF- β_1 on development of immune response is further sustained by animal models, which showed that TGF- β_1 knockout mice die of a widespread inflammatory disease after weaning [18], suggesting that this cytokine could be important as anti-inflammatory substance in breast milk. Furthermore, TGF- β_1 in conjunction with epidermal growth factor might regulate mechanisms of proliferation and differentiation of lung cells [19]. Therefore, it seems that the reduced production in milk might contribute to the development of wheezing: TGF- β_1 in milk was demonstrated to be associated with wheeze in infancy with a significant inverse association between the dose of TGF- β_1 received through milk and the percentage of children who wheezed [12].

All these evidences lead to consider the importance of milk cytokines on the maturation and function of epithelial, inflammatory, and structural components not only of the intestinal but also of the respiratory system [12, 20].

In our study we also evaluated IL-10, which according to the literature was detected both in colostrum and milk even if in very few samples and with a weak expression from both the groups [8, 20]. The same low levels were detected in previous study [14, 20], one of which showing that IL-10 was higher in the colostrum in the first day after the delivery, but significantly decreased after 3 days [20]. In our samples no difference was observed both in colostrum and in mature milk. IL-10 has been shown to present a very broad spectrum of anti-inflammatory and anti-allergic effects, having the capacity to inhibit the expression of many of the inflammatory genes that are abnormally expressed in allergic diseases [21]. In line with

this it has been shown that a defect in IL-10 synthesis may result in exaggerated and more prolonged inflammatory responses in asthmatic airways [21, 22], with an increase of eosinophilic inflammatory response to allergen in an animal model [23]. In our study we found low levels of IL-10 and no difference between groups. Therefore, it could be argued that IL-10 may have a secondary and limited role in healthy conditions but could become relevant after allergen stimulation as it has been shown in the animal model [23].

In conclusion, in our study TGF- β_1 was significantly less secreted especially in mature milk of allergic mothers. We would speculate that the children born from allergics are probably exposed to a particular cytokine pattern in milk which could contribute to predispose the children to the development of atopic diseases. Therefore, the different composition of the breast milk could be of relevance to partly explain the controversy regarding the protective role of breastfeeding against allergy. Obviously, studies involving a larger population and a longer period of observation are needed.

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