Pediatr Allergy Immunol 1999: 10: 186–190
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PEDIATRIC ALLERGY AND IMMUNOLOGY
ISSN 0905-6157

Defective tumor necrosis factor-α production in infants with cow's milk allergy

Österlund P, Järvinen K-M, Laine S, Suomalainen H. Defective tumor necrosis factor-α production in infants with cow's milk allergy. Pediatr Allergy Immunol 1999: 10: 186–190. © Munksgaard, 1999

As an aid to clarifying the role of immune mechanisms in the development of cow's milk allergy (CMA) in suckling infants, we studied the capacity of peripheral blood mononuclear cells (PBMC) to produce tumor necrosis factor-α (TNF-α) *in vitro*. The study population consisted of 43 infants, aged 0.12–11.2 months; of these, 31 had challenge-proven cow's milk allergy manifested with either skin or gastrointestinal symptoms or both. In addition, 12 healthy infants were studied as controls. The spontaneous, unstimulated and mitogen-induced production of TNF-α and interferon-γ (IFN-γ) by isolated peripheral blood leukocytes was evaluated. TNF-α and IFN-γ production of PBMC was significantly lower in infants with cow's milk allergy than in healthy children. Our results indicate that, in infants with CMA, the function of TNF-α-producing cells is defective. This might disturb the development of oral tolerance and thereby lead to cow's milk allergy. These results may help to clarify the etiopathology of CMA.

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Key words: tumor necrosis factor α; oral tolerance; cow's milk; allergy

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Accepted 7 June 1999

Cow's milk allergy (CMA) is frequently the first manifestation of allergic pathology, because cow's milk is invariably the first foreign protein consumed in early infancy. CMA is usually considered a temporary, antigen-specific condition (1). Immediate (type I) hypersensitivity does not characterize all cases of CMA in infants. Many of the patients show symptoms of delayed T-cell-mediated hypersensitivity (type IV) (2). CMA is an example of a condition with breakdown in development of oral tolerance. The mechanisms underlying regulation of the development of oral tolerance, a state of immunologic hypo-responsiveness, are well studied in animals, but poorly understood in humans. At birth, the immune system is inexperienced and immature, as can be seen from several phenotypic markers of immaturity (3). In infancy, large amounts of antigenic macromolecules encountered by the enteric route are absorbed across the small intestinal mucosa (4). Despite this, hypersensitivity reactions against food antigens are not very common in infants (5).

Tumor necrosis factor-α (TNF-α), produced by activated macrophages and T lymphocytes, is one of the proximal mediators of the inflammatory response (6). Natural induction of TNF-α is protective, but overproduction may be detrimental (7). Synergistically with interferon-γ (IFN-γ), it has many pro-inflammatory activities, including upregulation of class II major histocompatibility complex (MHC) expression on epithelial and dendritic cells and macrophages, adhesion molecule expression, and T-cell proliferation (8,9). These effects are important steps in the propagation of a T-lymphocyte-driven immune response (10). We therefore studied whether maturation of TNF-α-producing leukocytes was disturbed in infants with challenge-proven CMA. For this purpose, the capacity of mitogen-induced peripheral blood mononuclear cells (PBMC) to produce TNF-α and IFN-γ was evaluated *in vitro*.

Patients and methods

Patients

The whole study population consisted of 43 infants, their geometric mean age being 3.2 months (95% CI 2.5–4.0). Of these infants, 31 had symptoms strongly suggestive of CMA; urticaria or eczematous skin eruptions (n = 17)

or gastrointestinal symptoms (n = 1), or both skin and gastrointestinal symptoms (n = 13). The control group comprised 12 age-matched healthy infants, who were without symptoms or signs of CMA or atopic eczema. These patients had no dietary restrictions at testing. All the infants were born at term and had no other chronic diseases or infections at the time of the study. The study protocol was approved by the Ethical Committee of the Skin and Allergy Hospital of the Helsinki University Central Hospital.

Cow's milk challenge protocol

The diagnosis of CMA was based on an adverse response to cow's milk challenge. The challenge was performed openly, since placebo reactions have been shown to be rare in infants (11). During the elimination period, cow's milk had been strictly eliminated from the diet, and the lactating mother was also on the avoidance diet. Eleven infants with CMA received breast milk and additionally tolerated formula, 19 received breast milk, tolerated formula and solid foods appropriate for their age, and one infant with CMA received tolerated formula and solid foods. The challenge was performed as described earlier (2). The time of the reaction was defined as the time elapsing from the last administered dose which elicited specific symptoms. The patients were followed up over the one-week period of challenge and all the infants in this study were followed up through the next year, to detect any allergies that might develop in the group of healthy infants during that time.

Blood samples

For lymphocyte induction, blood was drawn in heparin, always at the same time in the morning. Mononuclear cells consisting mainly of lymphocytes were isolated by Ficoll–Hypaque gradient centrifugation at 400 *g* for 30 min at 20°C. The cells were resuspended in RPMI-1640 containing 5% fetal bovine serum, antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml), and 2 mM L-glutamine. After washing three times, the cells were counted visually in Bürker chambers under a light microscope.

Skin-prick tests

Skin-prick tests were performed on the volar side of the forearm using a commercial cow's milk extract (ALK, Denmark). Histamine dihydrochloride 10 mg/ml (ALK, Denmark) was used as a positive control and at least a 5 mm reaction diameter was taken as an adequate histamine reaction. Reactions were read at 15 min, and at least a reaction half of the size of histamine reaction.

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

To induce lymphocytes and monocytes, 5.0×10^5 isolated cells in 1 ml of RPMI-1640 containing antibiotics and glutamine were cultured in a humidified 5% CO_2 atmosphere at 37°C for 48 h together with Concanavalin A (Con A, Pharmacia, Uppsala, Sweden) at a final concentration of 25 μg/ml. A control cell population was cultured with RPMI-1640 only. The supernatants were collected and stored frozen at –70°C for later determination of cytokines. Subsequently, the TNF-α, IL-4 and IFN-γ produced during lymphocyte induction were determined from the stored frozen and subsequently thawed supernatants using a commercial ELISA kit (CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions. The results of different runs were equalized, employing the comparison of standard curves, and are expressed as pg/ml.

Statistical analysis

ANOVA was employed to determine the statistical significance of differences between continuous variables.

Results

Clinical characteristics of CMA infants and controls

The mean ages of the infants with CMA and controls were comparable at the time of the study; $F = 0.06$, $p = 0.8$. Of the 31 infants with CMA, 10 manifested urticaria immediately after introduction of a drop of cow's milk on their skin or lips. Other patients with CMA developed either urticarial or eczematous skin eruptions (n = 17), or gastrointestinal symptoms (n = 2) or both cutaneous and gastrointestinal symptoms (n = 2) during the challenge (Table 1). The patients challenged perorally reacted to a median total dose of 162 ml (range 7–2000) of cow's milk at a mean of 44.3 h (range 0.5–168) after beginning the challenge. The median of the last dose eliciting symptoms was 100 ml (range 5–200) of cow's milk and the mean time from the last dose until the symptoms appeared was 3.1 h (range 0.5–21). Table 1 shows the individual symptoms, skin-prick test reactions and doses eliciting the symptoms of CMA during the challenge.

Spontaneous TNF-α, IL-4 and IFN-γ production in unstimulated cultures of peripheral blood mononuclear cells

Spontaneous TNF-α production of peripheral blood mononuclear cells was significantly lower in infants with CMA than in healthy infants ($F = 8.6$, $p = 0.005$; ANOVA) (Fig. 1). The production of IL-4 and IFN-γ did not differ in the two groups.

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

TNF-α production of peripheral blood mononuclear cells induced with Con A was significantly lower in infants with CMA than in healthy controls ($F = 6.2$, $p = 0.017$; ANOVA) (Fig. 1). The mean of stimulated production in infants with CMA did not even reach the level of spontaneous production in healthy infants: 40.9 pg/ml and 49.5 pg/ml, respectively (Fig. 1). In infants with CMA, the IFN-γ production of peripheral blood mononuclear cells induced with Con A was also strikingly lower than in healthy infants ($F = 19.3$, $p = 0.0001$; ANOVA) as also demonstrated previously (2). IFN-γ production was low in the entire group of CMA infants; no correlation was found between IFN-γ production and the time of the onset of reaction during the challenge ($F = 0.012$, $p = 0.914$). IL-4 production was almost undetectable in both groups.

Discussion

In the present investigation, we have assessed cytokine production in peripheral blood mononuclear cells in healthy infants and in those with cow's milk allergy. We have shown that TNF-α production of PBMC was significantly lower in infants with CMA than in healthy control infants. Our results suggest that TNF-α may have a role in the achievement of oral tolerance to dietary antigens in humans.

It has previously been demonstrated that the capacity to produce cytokines, especially TNF-α and IFN-γ, is physiologically low in newborn infants (12). This finding has been attributed to the virgin antigenic experience of the neonate (13). In a healthy infant, the production of IFN-γ has been shown to increase with age (2). This increases the class II MHC expression on the infant's antigen-presenting cells, which subsequently increases the activation of lymphocytes and enables them to produce cytokines more effectively. An increased production of IL-4 and decreased or normal production of IFN-γ in atopic diseases has been demonstrated in earlier studies (11,14–18). In suckling infants, depressed antigen presentation and T-cell activation, due to weak TNF-α and IFN-γ production, may weaken the immune response to dietary antigens, which may lead to development of food allergies until the infant develops mature suppressive mechanisms (2,3).

In early infancy, the gastrointestinal barrier is immature and the intestine absorbs more macromolecules than the mature intestine (4). Normally, this may lead to activation of T cells and macrophages, which start to produce cytokines such as IFN-γ and TNF-α (19). In the present study, even the spontaneous, unstimulated production of TNF-α was significantly lower in the infants with CMA, and may have led to failure of its protective effects. Upon stimulation, TNF-α production reached only about the level of its spontaneous production in healthy infants.

In recent papers, TNF-α has been shown to affect the immature dendritic cells by differentiating them to more mature ones with an enhanced antigen-presenting capacity by expression of T-cell co-stimulatory and adhesion molecules (20,21). Optimal TNF-α production seems to be important in the regulation of the infant's

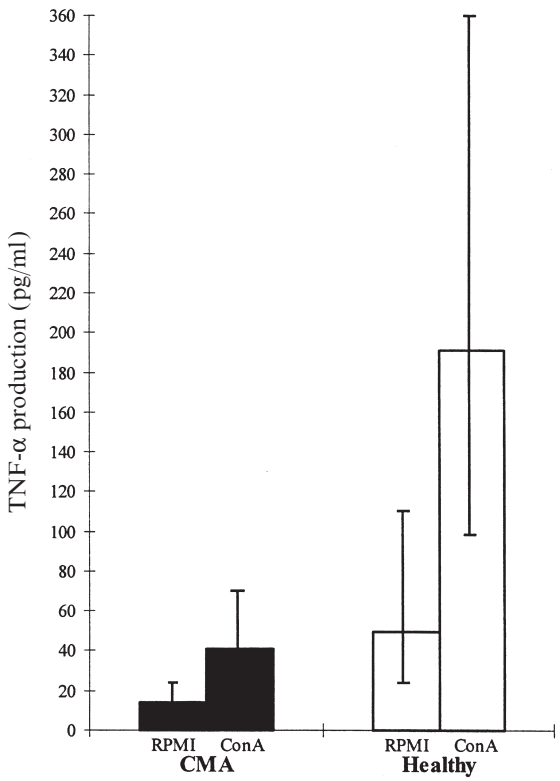

Fig. 1. The *in vitro* tumor necrosis factor-α (TNF-α) production (pg/ml) of peripheral blood mononuclear cells, in plain medium (RPMI) and induced by Concanavalin A (Con A), in infants with cow's milk allergy (CMA) and in healthy infants. Vertical bars denote confidence intervals (95% CI).

Table 1. The age, result of skin-prick test with cow's milk and cow's milk challenge reaction type, symptoms, total and last amounts of cow's milk eliciting symptoms, total reaction time and reaction time from the last dose of milk in patients with cutaneously, gastrointestinally and both cutaneously and gastrointestinally manifested cow's milk allergy (CMA)

			Cow's milk challenge					
Patient	Age (months)	Skin-prick test with cow's milk	Reaction type	Symptoms	Total amount of milk eliciting symptoms (ml)	Last amount of milk eliciting symptoms (ml)	Total reaction time (h)	Reaction time from the last dose (h)
Skin symptoms								
HR	8.5	+	Immediate	Urticaria	Drop	Drop	0.1	0.1
KS	4.3	−	Immediate	Eczema	112	50	2.3	0.5
KV	3.5	−	Immediate	Eczema	52	30	2.0	0.5
MA	5.8	+	Delayed	Eczema	162	100	24.0	21.0
ML	7.2	+	Immediate	Urticaria	Drop	Drop	0.1	0.1
MO	1.1	−	Delayed	Eczema	52	10	96.0	2.0
NM	7.4	+	Immediate	Urticaria	Drop	Drop	0.1	0.1
PA	3.4	−	Immediate	Eczema	10	10	0.5	0.5
PA	0.1	−	Immediate	Eczema	10	10	0.5	0.5
RF	2.3	−	Delayed	Eczema	162	100	4.7	1.7
RF	6.7	−	Delayed	Eczema	162	100	4.7	1.7
RF	4.3	−	Delayed	Eczema	162	100	4.7	1.7
RJ	1.9	−	Delayed	Eczema	62	50	3.0	1.0
RL	2.3	+	Immediate	Urticaria	Drop	Drop	0.2	0.2
RL	4.7	+	Immediate	Urticaria	Drop	Drop	0.2	0.2
RL	3.6	+	Immediate	Urticaria	Drop	Drop	0.2	0.2
VA-M	3.0	−	Immediate	Urticaria	22	10	2.7	0.1
Gastrointestinal symptoms								
TT	0.4	−	Delayed	Vomiting	360	200	2.0	0.5
Both skin and gastrointestinal symptoms								
AA	9.4	+	Delayed	Eczema	962	100	66.0	7.0
AA	2.2	+	Delayed	Eczema	962	100	66.0	7.0
AA	7.7	+	Delayed	Eczema	962	100	66.0	7.0
HL	8.5	+	Immediate	Urticaria	Drop	Drop	0.1	0.1
IR	2.4	−	Delayed	Eczema and diarrhea	7	5	3.8	1.3
IT	11.6	−	Delayed	Eczema	1162	100	72.0	3.0
JK	0.4	−	Delayed	Eczema and diarrhea	262	100	30.0	1.0
KA	7.3	+	Immediate	Urticaria	Drop	Drop	0.1	0.1
NR	4.3	−	Immediate	Urticaria	Drop	Drop	0.1	0.1
PS	4.6	−	Immediate	Urticaria	Drop	Drop	0.2	0.2
RN	0.7	−	Delayed	Diarrhea	2000	100	144.0	2.0
RR	0.6	−	Delayed	Eczema	1335	100	168.0	3.0
RR	1.7	−	Delayed	Eczema	1335	100	168.0	3.0

macrophage and T-cell function. Therefore, lack of it may dramatically disturb the development and maintenance of oral tolerance to dietary antigens by weakening the activation of antigen-presenting cells and decreasing the communication between activated dendritic cells and the target T cells (21). If the infant's T-cell function is depleted or only weakly induced because of low TNF-α production, exposure of a child to foreign proteins might cause a local or systemic immuno-inflammatory reaction (4). This is also in agreement with our recent finding that TNF-α production *in vitro* in human milk leukocytes is decreased in mothers with a CMA infant (22). In that study, we suggested that since the infant's own cytokine production is weak, breast milk-derived TNF-α has a role in helping the immature cells in the suckling infant's gut to develop adequate communication between antigen-presenting cells and target T cells until the infant's own TNF-α- and IFN-γ-producing cells are mature.

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 add important information towards clarifying the mechanisms of development of food allergy and atopic diseases in suckling infants.

Acknowledgments

This work was partially supported by the Yrjö Jahnsson Foundation (personal grant to K.-M.J.). We thank Ms Marjo Leponiemi for excellent technical assistance.

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