

Cow's Milk Allergy in Infants with Atopic Eczema is Associated with Aberrant Production of Interleukin-4 During Oral Cow's Milk Challenge

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ABSTRACT

Objectives: A failure in the establishment and maintenance of oral tolerance in infancy may result in food allergy. To further assess the role of the intestinal immune system in cow's milk allergy (CMA), we investigated the systemic production of the pro-allergenic Th2 cytokine interleukin (IL)-4 and anti-allergenic cytokines IL-10, transforming growth factor (TGF)- β 1 and TGF- β 2 in infants suffering from atopic eczema with and without CMA during antigen elimination diet and oral antigen exposure.

Methods: 18 infants (mean age, 9.6 months; 95% confidence interval 8.1–11.1 months) with atopic eczema and CMA and 17 infants (mean age, 9.7 months; 95% confidence interval 8.6–10.9 months) with atopic eczema tolerant to milk as assessed by a double blind, placebo-controlled cow's milk challenge were investigated. Peripheral blood mononuclear cells were obtained during antigen elimination diet and during oral cow's milk challenge and stimulated with Concanavalin-A or cow's milk or

were left unstimulated. The cytokine concentrations were measured by enzyme-linked immunosorbent assay.

Results: During antigen elimination, the Concanavalin A-stimulated production of TGF- β 2 was significantly lower in infants with CMA as compared with infants without CMA: 129 pg/mL (interquartile ratio, 124–144 pg/mL) vs. 149 pg/mL (interquartile ratio, 133–169 pg/mL); $P = 0.016$. During oral antigen exposure, the immune responses in infants with CMA were characterized by significantly higher spontaneous production of IL-4 as compared with those without CMA: 12.0 pg/mL (interquartile ratio, 5.2–28.3 pg/mL) vs. 4.2 pg/mL (interquartile ratio, 1.5–7.6 pg/mL); $P = 0.018$.

Conclusions: Infants with atopic eczema and CMA exhibit markedly increased systemic pro-allergenic IL-4 responses on intestinal antigen contact, which may partially be explained by a defective ability to launch anti-allergenic TGF- β 2 responses. *JPGN* 39:529–535, 2004. **Key Words:** Cytokines—Th1—Th2—Th3—Tolerance. © 2004 Lippincott Williams & Wilkins

INTRODUCTION

Immune responses in the neonatal period are physiologically of the T helper (Th)2 type, a phenomenon explained as a consequence of the immunologic balance prevailing in utero (1). In healthy infants, an age-dependent decline in Th2 responses during the first months of life has been observed (2). The involvement of Th2 cytokines, such as interleukin (IL)-4, is well documented in the pathogenesis of fully established atopic disease (3–5) and, in accord with this, Th2 responses are augmented with age in infants who are to manifest atopy (2). The immunologic disturbances characteristic of atopy and atopic disease may be considered to occur at two distinct stages: the first phase, in which the initial T cell activation and failure to achieve tolerance

takes place, and the more recognizable, distinctly allergic second phase, in which an encounter with allergen triggers immunologic memory leading to symptoms and signs of allergy (6). The immunologic factors and defects in the first phase of atopic development that lead to the characteristic Th2-skewed imbalance in immune responsiveness in atopic disease remain elusive.

It has been hypothesized that atopic infants exhibit defective mechanisms of redirecting the originally Th2-skewed neonatal immunity (7). Accumulating evidence emphasizes the importance of gut-derived Th3-cells and Tr1-cells, secreting transforming growth factor (TGF)- β and IL-10, respectively, in suppressing potentially harmful pro-allergenic inflammatory responses and maintaining oral tolerance (8,9). In addition to its immunomodulatory properties, TGF- β promotes immunoglobulin (Ig)A production (10) and thereby enhances antigen exclusion in the gut, a phenomenon essential to the establishment and maintenance of oral tolerance. Extending work on experimental animal models (11), oral antigen administration has been shown to result in the development of circulating antigen-specific TGF- β 1-secreting cells in humans (12).

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Recent data suggest that the development of atopic disease may be causally related to lack of anti-allergenic immunoprotection provided by TGF- β at an early age, but the individual anti-allergenic properties of TGF- β 1 and TGF- β 2 have not been extensively investigated. Diminished spontaneous production of TGF- β 1 by peripheral blood mononuclear cells (PBMC) has been reported in subjects with atopic eczema (13). In addition, the strong genetic predisposition to atopic disease may in part be explained by a low-producer TGF- β 1 genotype associated with atopic eczema (14). According to recent observations (15), the disturbances in TGF- β 1 responsiveness are detectable already at birth and may thus at least in part be the result of immune skewing taking place in utero. After birth, TGF- β 2 in breast milk is associated with protection against atopic disease (16,17).

Dietary antigens, particularly those derived from cow's milk, often comprise the first triggers of allergic responsiveness in infancy. In addition to its role in atopic eczema (3,5), there are data indicating enhanced production of pro-allergenic IL-4 in promoting IgE-mediated food hypersensitivity in children (18). We hypothesized that early immune responses to dietary antigens might be involved in redirecting the infant's immunologic balance to a tolerogenic mode by promoting the production of anti-allergenic cytokines. To elucidate the question of whether distinct immune response patterns to dietary antigens can be observed in infants suffering from atopic eczema with and without cow's milk allergy, we investigated the PBMC cytokine production during a double blind placebo-controlled cow's milk challenge. We compared the immune response patterns during antigen elimination diet and oral antigen exposure. This enabled us to assess whether the observed differences could be explained by allergic status per se or by intestinal antigenic stimulation.

PATIENTS AND METHODS

In all, 35 infants having a mean age of 9.7 months (95% confidence interval, 8.8–10.6 months) participated in the study. The subjects were recruited from infants who were investigated because of a clinical suspicion of cow's milk allergy (CMA). To achieve some immunologic homogeneity in the study population, infants with only gastrointestinal symptoms were excluded from the study, and atopic eczema according to the criteria introduced by Hanifin was the second criterion of inclusion (19). Briefly, atopic eczema was confirmed if at least three of four major features were detected: pruritus, typical morphology and distribution, chronic or recurrent course and a family history of atopy. The infants were subjected to a double blind, placebo-controlled cow's milk challenge. After the challenge, 18 consecutive infants with a positive double blind, placebo-controlled cow's milk challenge were included in the study, and 17 consecutive infants with a negative challenge served as controls. The study protocol was approved by the local ethical board and informed consent was obtained from the parents.

Cow's milk elimination diet was commenced by removing all foods containing cow's milk protein from the diet and introducing a synthetic, amino acid-derived formula, Neocate (SHS Int., Liverpool, UK). In addition, egg, soy and cereal products were eliminated from the diet. Further alterations were made in the consumption of non-dairy solid foods if multiple food allergies were suspected. Clinical evaluation was performed using the SCORAD index (20) and after a clinical response to elimination diet was achieved, skin prick tests were performed as previously described by Isolauri and Turjanmaa (21). Briefly, the testing was performed on the volar side of the forearm with a 1-mm, one peak lancet (Allergologisk Laboratorium A/S, Horsholm, Denmark) with a shoulder to prevent deeper penetration. Histamine dihydrochloride 10 mg/mL (Allergologisk Laboratorium A/S, Horsholm, Denmark) served as the positive control; the negative control solution was provided by the same manufacturer. The tested antigens included cow's milk, wheat and egg, and the testing was performed with fresh extracts. Reactions were read at 10–15 minutes. Half of the histamine reaction size was considered positive on the condition that the mean diameter of the wheal was at least 3 mm and the negative control reaction was 0 mm. In addition, serum total concentration of IgE antibodies and the concentrations of antigen-specific IgE antibodies against cow's milk, egg and wheat antigens were measured using a UniCAP 100 automatic analyzer (Pharmacia & Upjohn, Uppsala, Sweden) according to manufacturer instructions.

After clinical resolution of symptoms during the elimination diet the patients were subjected to the double blind, placebo-controlled cow's milk challenge to confirm the diagnosis of CMA. Neocate served as the placebo formula and the test formula consisted of Neocate and 100 mg skimmed cow milk powder/L. On the first day of the challenge, increasing amounts (1, 5, 10, 50 and 100 mL) of the formula were given at 30-minute intervals until intake appropriate for age was reached. The challenge period was 1 week for both placebo and the test formula. The challenge was discontinued and the patient examined if a clinical reaction occurred. All patients were clinically assessed after each challenge corresponding to days 8 and 15 of the protocol. The assessment was performed double blind. On day 15 of the challenge, the clinical outcome was determined as positive or negative, and thereafter the code of the challenge formula was opened.

Five mL of blood was drawn on days 8 and 15 of the double blind, placebo-controlled cow's milk challenge and heparinized. Cell cultures were prepared from the samples. Briefly, peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation at 400g for 30 minutes at 20°C. A total of 6.25×10^5 isolated cells in 1 mL of RPMI 1640 containing antibiotics, glutamin and 10% human AB serum (culture medium) were cultured in a humidified 5% CO₂ atm at 37°C for 48 hours. The cultures contained either Concanavalin A (ConA, Pharmacia, Uppsala, Sweden) at a final concentration of 50 μ g/mL or a solution of dried cow's milk powder (Valio Finnish Co-operative Dairies' Association, Helsinki, Finland) at a final concentration of 1 mg/mL. Whole cow's milk was used because even though most patients with CMA react to several protein fractions in cow's milk, sufficient sensitivity is achieved with no single allergen (22). A control cell population was prepared containing the culture medium alone. The supernatants were collected and stored at –70°C for cytokine assays.

The concentrations of IL-4, IL-10, TGF- β 1 and TGF- β 2 were measured in duplicate from the culture supernatants using commercial sandwich enzyme-linked immunosorbent assays (ELISAs) specific for these molecules (R&D Systems Europe Ltd., Abingdon, UK). The detection limits of the IL-4 and TGF- β 2 assays were 0.13 pg/mL and 7.0 pg/mL, respectively. Activation of latent TGF- β and all determinations were made according to manufacturer instructions.

Data are expressed as medians with interquartile ranges (IQR) and comparisons between groups were made using nonparametric Mann-Whitney *U*-tests, as the distributions were skewed. χ^2 test was used in comparisons of proportions between groups.

RESULTS

The age of onset of symptoms and the initial SCORAD index were comparable between infants with and without CMA. By the time of the double blind, placebo-controlled cow's milk challenge, 1 of 18 (6%) of the infants with CMA and 5 of 17 (29%) of the infants tolerant to milk were partially breastfed ($P = 0.06$), but none of the infants were exclusively breastfed. Furthermore, 10 of 18 (56%) of the infants with CMA and 6 of 17 (35%) of the infants without CMA had received infant formula before the double blind, placebo-controlled cow's milk challenge ($P = 0.23$). Clinical characteristics of infants with and without CMA are presented in detail in Table 1.

The group of infants with CMA could be distinguished from those without CMA by detecting cow's milk specific IgE antibodies as assessed by a positive skin prick test or radioallergosorbent test (≥ 0.4 kU/L). However, infants with and without CMA were indistinguishable by the overall presence of specific IgE antibodies against food antigens, as presented in Table 2. In the double blind, placebo-controlled cow's milk challenge, 13 of 18 (72%) of the infants with CMA exhibited immediate hypersensitivity and 5 of 18 (28%) exhibited more delayed reactions. Clinical responses to the double blind, placebo-controlled cow's milk challenge in infants with CMA are presented in detail in Table 3.

The results concerning IL-4 and TGF- β 2 production in infants with and without CMA are presented in Tables 4 and 5, respectively. No differences between infants with and without CMA in production of TGF- β 1 or IL-10 were observed under any circumstances (data not shown).

The PBMC cytokine production patterns were investigated after the placebo challenge to reflect cytokine production during antigen elimination diet. This enabled us to assess the spontaneous production of cytokines without antigen exposure *in vivo* by measuring the concentrations of cytokines in the supernatants of unstimulated cell cultures. The spontaneous production of IL-4 appeared to be somewhat higher in infants with CMA as compared with infants without CMA, but the difference did not reach statistical significance (Table 4). Interestingly, the apparently high spontaneous production of IL-4 tended to be attributable to infants with immediate hypersensitivity, 14.2 pg/mL (IQR, 3.7–34.2 pg/mL), whereas in infants with more delayed hypersensitivity the production of IL-4 was 6.2 pg/mL (IQR, 5.8–12.4 pg/mL), which was comparable to infants without CMA, 6.0 pg/mL (IQR, 3.3–15.1 pg/mL) ($P = 0.38$ and $P = 0.68$ for infants with immediate and more delayed hypersensitivity, respectively). At this point the infants with and without CMA were indistinguishable with regard to production of TGF- β 2 (Table 5).

Cytokine production patterns were measured from cell cultures obtained during the placebo challenge and cow's milk challenge. The production of IL-4 in the cell cultures was markedly reduced on *in vitro* stimulation with cow's milk in infants with and without CMA alike. During the placebo challenge the concentration of IL-4 in infants with CMA was 0.5 pg/mL (IQR, 0.2–2.7 pg/mL) in milk-stimulated cultures and 10.2 pg/mL (IQR, 5.3–22.4 pg/mL) in unstimulated cultures ($P = 0.014$). In infants without CMA the respective concentrations were 1.8 pg/mL (IQR, 0.2–3.3 pg/mL) and 6.0 pg/mL (IQR, 3.3–15.1) ($P = 0.0087$). Similar results were obtained during cow's milk challenge in infants with CMA ($P = 0.0015$) and without CMA ($P = 0.063$) during cow's milk challenge (Table 4).

TABLE 1. Clinical history and characteristics of infants with and without cow's milk allergy

	Cow's milk allergy (<i>n</i> = 18)	Without cow's milk allergy (<i>n</i> = 17)	<i>P</i> value
Age (months)*	9.6 (8.1–11.1)	9.7 (8.6–10.9)	0.90
Gastrointestinal symptoms†	7/18 (39%)	5/17 (29%)	0.55
Age of onset of eczema* (months)	2.3 (1.6–3.1)	2.2 (1.4–2.9)	0.77
Initial SCORAD index‡	21 (11–36)	16 (6–24)	0.27
Eczema during exclusive breastfeeding†	13/18 (72%)	10/17 (59%)	0.40
Duration of exclusive breastfeeding* (months)	3.4 (2.7–4.0)	3.1 (2.2–4.0)	0.63
Overall duration of breastfeeding* (months)	5.8 (4.6–6.9)	4.6 (2.8–6.5)	0.24

* mean (95% confidence interval); Student's *t*-test; †Chi-square test; ‡median (interquartile range), Mann-Whitney *U*-test.

TABLE 2. Total and antigen-specific IgE antibodies as detected by skin prick tests or in serum (RAST) in infants with and without cow's milk allergy

	Cow's milk allergy (n = 18)	Without cow's milk allergy (n = 17)	P value
Serum total IgE (kU/L)*	38 (10–210)	10 (3–13)	0.0053
Skin prick positive to milk†	11/18 (61%)	1/16 (6%)	0.0008
Specific IgE antibodies to milk† (RAST ≥0.4 kU/L)	7/18 (39%)	4/17 (24%)	0.33
Positive RAST or skin prick to milk†	12/18 (67%)	4/16 (25%)	0.015
Skin prick positive to dietary antigens‡,§	12/18 (67%)	7/16 (44%)	0.18
IgE antibodies to dietary antigens‡,§ (RAST ≥0.14 kU/L)	10/18 (56%)	6/17 (35%)	0.23
Positive RAST or skin prick to dietary antigens‡,§	13/18 (72%)	10/17 (59%)	0.40
Suspected food allergy other than CMA†,§	4/18 (22%)	6/17 (35%)	0.39

* Median (interquartile range), Mann-Whitney U-test; †Chi-square test; ‡The tested dietary antigens included cow's milk, egg and wheat; §The suspected foods included egg, beef, wheat, oats, barley, rye, buckwheat, potato, carrot, blackberry, tomato, cauliflower and pineapple; appropriate elimination diets were commenced.
RAST = radioallergosorbent test.

Reciprocally, the production of TGF-β2 was enhanced on stimulation with cow's milk in vitro in both infants with and without CMA. In infants with CMA the concentration of TGF-β2 was 479 pg/mL (IQR, 418–571 pg/mL) in milk-stimulated cultures as compared with 141 pg/mL (IQR, 126–163 pg/mL) in unstimulated cultures obtained during the placebo challenge ($P < 0.0001$). The respective concentrations in infants tolerant to milk were 459 pg/mL (IQR, 387–551) and 139 pg/mL (IQR, 123–158) ($P < 0.0001$). During cow's milk challenge, similar results were obtained in both infants with CMA ($P < 0.0001$) and without CMA ($P < 0.0001$) (Table 5).

Stimulation with Concanavalin A in vitro appeared to have relatively little effect on the production of IL-4 (Table 4) and TGF-β2 (Table 5) with the exception of a significantly lower TGF-β2 response in infants with CMA as compared to those without CMA in samples obtained during the placebo challenge ($P = 0.016$).

Infants with and without CMA exhibited distinct cytokine response patterns during oral antigen challenge. The spontaneous production of IL-4 by PBMC, as assessed by the concentration of IL-4 in supernatants of unstimulated cell cultures obtained during the challenge, was significantly higher in infants with CMA as compared with those tolerant to milk ($P = 0.018$) (Table 4). This difference appeared to be the joint result of an

increase in IL-4 production in infants with CMA during the cow's milk challenge and a corresponding decrease in infants without CMA, although neither change was statistically significant ($P = 0.81$ and $P = 0.33$, respectively). Furthermore, a tendency for increased spontaneous production of IL-4 during the oral cow's milk challenge was observed in infants with both immediate and more delayed reactions. The concentrations of IL-4 were 19.4 pg/mL (IQR, 3.7–32.7 pg/mL) and 12.0 pg/mL (IQR, 7.3–17.9 pg/mL) for infants with immediate and more delayed reactions, respectively, as compared with 4.2 pg/mL (IQR, 1.5–7.6 pg/mL) for infants without CMA ($P = 0.051$ and $P = 0.063$, respectively). The spontaneous production of TGF-β2 during oral cow's milk challenge tended to be lower in infants with CMA ($P = 0.081$) (Table 5).

DISCUSSION

Our results may be interpreted in line with the traditional view that allergy and atopic disease are the consequence of contact with antigen resulting in pro-allergenic responsiveness, along with the central role attributed to IL-4 in atopic eczema and IgE-mediated food allergy. A report by Schade et al. (23) indicates that food allergy in infants with atopic eczema is associated with enhanced production of Th2 cytokines by circulating

TABLE 3. Symptoms during cow's milk challenge in infants with cow's milk allergy (n = 18)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total
Urticaria	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	13 (72%)
Aggravation of atopic eczema	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+	+	5 (28%)
Vomiting	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	2 (11%)
Diarrhea	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	1 (6%)
Wheezing	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	1 (6%)
Increased fussing	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	3 (17%)
Challenge discontinued (day)	1	1	1	1	1	1	1	1	1	1	1	1	4	7	7	7	7	7	–
Positive RAST or skin prick to milk	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	–	+	12 (67%)

TABLE 4. *The production of interleukin-4 (pg/mL) by unstimulated and stimulated (cow's milk or concanavalin A) peripheral blood mononuclear cells obtained during placebo and oral milk challenge in infants with and without cow's milk allergy*

	Placebo challenge			Oral milk challenge		
	CMA	P value	Without CMA	CMA	P value	Without CMA
Unstimulated	10.2 (5.3–22.4)	0.40	6.0 (3.3–15.1)	12.0 (5.2–28.3)	0.018	4.2 (1.5–7.6)
Cow's milk	0.5 (0.2–2.7)	0.59	1.8 (0.2–3.3)	1.1 (0.3–3.3)	0.76	2.0 (0.4–3.2)
Concanavalin A	9.4 (6.4–16.4)	0.98	9.2 (6.4–17.4)	11.2 (3.7–16.9)	0.97	9.2 (5.8–13.1)

CMA = Cow's milk allergy.
Data expressed as median (interquartile range).

T cells and tolerance on the other hand with low levels of Th2 cytokines after stimulating PBMC with cow's milk antigens in vitro as assessed using ELISA. Interestingly, however, a significant suppression of IL-4 production was observed when the cell cultures were stimulated with cow's milk in vitro in infants with and without CMA in this study. This appears to contradict with the traditional view on allergy.

Unstimulated cytokine production by PBMC is often regarded merely as background. Children with CMA, however, have been observed to exhibit increased spontaneous production of IL-4 (24) using single-spot ELISPOT assays. In addition, the existing data concerning stimulated IL-4 production by PBMC from food allergic individuals are somewhat ambiguous on closer examination. The studies demonstrating more pronounced IL-4 production in food allergic individuals are mainly conducted using ELISA assays after in vitro stimulation by nonspecific mitogens instead of the specific allergen (18,25). In contrast, milk antigen-specific T cell clones from adults and adolescents with atopic eczema and CMA have been observed to be activated in vitro by milk antigens but to produce little or no IL-4 on nonspecific stimulation as assessed by ELISA (26). A decreased number of IL-4 secreting cells as assessed by ELISPOT has been observed in cord blood mononuclear cells from infants of atopic mothers as compared to those with non-atopic mothers after stimulation with cow's milk antigens (15). Furthermore, in vitro stimulation of PBMC by food antigens has been observed to lead to significant responses in both allergic

and tolerant individuals (23,27), a finding consistent with our results. These observations may be explained by the fact that the PBMC cytokine response may be determined by the exact immunopathogenetic type of food allergy, as it has been suggested that immediate hypersensitivity reactions appear to be associated with an increase in in vitro IL-4 production as assessed using ELISA, whereas more delayed reactions often associated with atopic eczema are not (28). In this study, however, oral antigen exposure appeared to result in increased production of IL-4 in infants with immediate and more delayed hypersensitivity as compared with tolerant infants.

When antigenic stimulation took place via the physiologic route (i.e. through oral exposure) a more prominent difference in IL-4 production was observed between infants with and without CMA as compared to the elimination diet. Furthermore, infants with and without CMA tended to respond to oral antigen in an opposite fashion: infants with CMA exhibited an increase and those without CMA a decrease in IL-4 production, although neither change was statistically significant. The effect of oral antigen exposure on cytokine production in allergic patients has previously been studied by Andre et al. (29), who observed increased mitogen-stimulated IL-4 production after oral exposure as compared with elimination diet as assessed by ELISA. In this study, stimulation with cow's milk in vitro diminished IL-4 responses dramatically and in a practically identical manner in both infants with and without CMA, whereas oral cow's milk challenge

TABLE 5. *The production of transforming growth factor- β 2 (pg/mL) by unstimulated and stimulated (cow's milk or concanavalin A) peripheral blood mononuclear cells obtained during placebo and oral milk challenge in infants with and without cow's milk allergy*

	Placebo challenge			Oral milk challenge		
	CMA	P value	Without CMA	CMA	P value	Without CMA
Unstimulated	141 (126–163)	0.73	139 (123–158)	140 (119–155)	0.081	153 (140–166)
Cow's milk	479 (418–571)	0.41	459 (387–551)	451 (424–555)	0.34	494 (462–563)
Concanavalin A	129 (124–144)	0.016	149 (133–169)	141 (125–156)	0.14	146 (131–177)

CMA = Cow's milk allergy.
Data expressed as median (interquartile range).

appeared to increase IL-4 production in infants with CMA. Interestingly, there are data indicating a parallel increase in spontaneous lymphocyte proliferation on oral cow's milk challenge in children with CMA (30). It is well established that the intestinal immune system has complex immunomodulatory properties, some of which are presumably lacking in the PBMC pool. Increasing evidence suggests that the Th1/Th2/Th3 skewing of adaptive immune responses takes place in the intestine (9). Therefore, despite the difficulties involved in comparing processes occurring *in vivo* and *in vitro*, it is interesting to speculate that our results might be a reflection of defective intestinal immunomodulation presumably associated with CMA.

In contrast to the comparable spontaneous production of TGF- β 2 by PBMC during antigen elimination diet between infants with and without CMA, infants with CMA manifested with inadequate potential to launch anti-allergenic TGF- β 2 responses upon mitogenic stimulation as compared with infants without CMA. This suggests an underlying defect in the establishment and maintenance of oral tolerance by production of TGF- β 2 in infants with CMA. This conception was further supported by the fact that the spontaneous production of TGF- β 2 tended to be lower in infants with CMA than in infants without CMA during oral antigen exposure. TGF- β secreting cells are induced in the intestine and distributed systemically, where they presumably may be reactivated in various target organs. Our results may partially be explained by hypothesizing that the PBMC pool might be lacking cells or factors essential to this activation and the defects of the system may only be observed after artificial stimulation.

Consequently, a more plausible explanation for the observed patterns of cytokine production and previous findings on the development of Th2 responses during infancy presents itself. It may be argued that the enhanced production of pro-allergenic IL-4 in response to oral cow's milk challenge in infants with CMA is at least partially the result of insufficient anti-allergenic suppression, including the induction of TGF- β secreting cells, by the intestinal immune system. It is notable, though, that stimulation with cow's milk *in vitro* resulted in a threefold increase in the production of TGF- β 2 in infants with and without CMA, indicating that there is no constitutional defect in TGF- β 2 production capability *per se* in infants with CMA; perhaps the induction of TGF- β 2 producing cells is inadequate in the intestine. Indeed, it was recently reported that children with neither immediate nor delayed food allergy exhibit significant Th2 skewing amongst intestinal mucosal lymphocytes but the dominant intestinal abnormality in these patients is impaired generation of TGF- β 1 producing Th3 cells (31). Interestingly we found no effect on TGF- β 1 production by *in vitro* milk stimulation or oral milk challenge. Thus the individual anti-allergenic contributions of TGF- β 1 and TGF- β 2 remain elusive. The *in vitro*

studies indicating that TGF- β inhibits Th2 development were conducted using TGF- β 1 (32,33). Moreover, the Th3 cells induced in the murine model for oral tolerance have been observed to produce TGF- β 1 (11,12), but to our knowledge the production of TGF- β 2 has not been measured. On the other hand, TGF- β 2 has been implicated in experimental suppression of delayed type hypersensitivity reactions (34,35), of which food allergy manifesting as aggravation of atopic eczema may be considered a clinical example, and TGF- β 2 in breast milk appears to protect the infant from atopic eczema (16,17). It may thus be argued that perhaps TGF- β 1 has a more profound effect on naïve T cells and the development of the atopic responder phenotype, as demonstrated by the report by Hauer et al. (15) indicating diminished spontaneous and allergen-stimulated production of TGF- β 1 by cord blood mononuclear cells in infants with atopic mothers. In this study, however, the role of TGF- β was investigated in an active allergic condition, and there are data indicating that memory Th2 cells, already present in infants with CMA, are not subject to TGF- β 1 mediated suppression (36). Active suppression of IL-4 production associated with oral tolerance, however, appears to be associated with the production of TGF- β 2.

We thus interpret the discrepancy between responses to oral and *in vitro* antigenic stimulation to demonstrate the distinct features of encounter with antigens through the enteric route. These include intestinal antigen processing and the characteristic cell populations and cytokine milieu of the intestinal immune system (8). In a recent study, milk-specific mucosal human T-cell lines released Th2 cytokines but failed to release Th3 cytokines, including TGF- β , on stimulation with milk proteins (37). This observation is compatible with our current data, as it is well-established that after the priming of Th3 cells has taken place in the intestinal immune system, the gut-originating cells migrate to the periphery and are capable of suppressing inflammatory responses on reactivation (12,38). It may therefore be suggested that the primary defect in CMA might reside in the intestinal immune system and result in insufficient tolerogenic immunomodulation, including the induction of antigen-specific TGF- β secreting Th3 cells, which could establish and maintain tolerance locally and systemically, and that lack of such anti-allergenic protection may cause excessive Th2 responsiveness and allergic disease.

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