

Immunologic disturbances in cow's milk allergy, 1: delayed maturation of suppressor activity

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To assist in identifying pathogenetic mechanisms in different subtypes of cow's milk allergy (CMA), the function of immunoregulatory T-lymphocytes was studied. The study population consisted of 23 patients, mean [95% confidence interval] age of 25.6 [19.5, 33.6] months, who had challenge-proven cow's milk allergy manifested with either skin ($n = 9$) or gastrointestinal ($n = 14$) symptoms; in addition, 13 age-matched disease controls were studied. Patients with challenge-proven CMA were rechallenged to establish whether they had acquired clinical tolerance to cow's milk. The suppressor activity of isolated lymphocytes was measured *in vitro* by a cell coculture at rechallenge and in 10/23 patients at diagnosis. At diagnosis, patients with CMA ($n = 10$) showed a decreased mean [95% CI] suppressor activity, induced by either Concanavalin A, 7 [–2, 15]%, or cow's milk, 3 [–8, 14]% as compared with disease controls ($n = 13$), 19 [15, 24]% and 24 [17, 31]%; $F = 7.1$, $p = 0.004$ and $F = 6.7$, $p = 0.005$, respectively. At rechallenge the suppressor activity, induced both by Concanavalin A and cow's milk, reached the level of disease controls only in patients who had acquired clinical tolerance to cow's milk ($n = 13/23$), but not in those retaining CMA ($n = 10/23$). Our results indicate that the maturation of suppressor function is delayed in CMA, which might be of primary importance in the etiopathogenesis of CMA.

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The mechanisms underlying the development of food allergies are poorly understood. Two important considerations may be operative: firstly, the antigen must penetrate the mucosal defence barrier, and secondly, it must evoke harmful immune response (1). In infants, antigenic macromolecules encountered by the enteric route are absorbed across the small-intestinal mucosa (2). At this point, many functions of the inexperienced immune system are immature, and this may impede efficient counteracting antigen-specific suppressive mechanisms (3–5). Specific T-cell-mediated suppression is thought to be mainly responsible for keeping the host in a non-allergic state (6, 7).

CMA frequently comprises the first manifestation of allergic pathology, since cow's milk is invariably the first foreign protein consumed in early infancy. CMA is usually considered a temporary, antigen-specific condition (8, 9). Recent data have

presented evidence that it may also be the first manifestation of allergic symptoms (10). In this study we tested the hypothesis that functional maturation of immunoregulatory lymphocytes could be delayed in patients who have CMA. For this purpose, we studied the activity of non-specific and cow's milk-induced suppression *in vitro* at the time of the diagnosis and/or at re-challenge.

Methods

Patients

The whole study population consisted of 36 children, mean [95% CI] age of 18.1 [13.9, 23.6] months. Twenty-three patients had challenge-proven CMA, manifested by either urticarial or eczematous skin eruptions ($n = 9$) or gastrointestinal symptoms (loose stools, $n = 9$; diarrhea, $n = 3$; vomiting, $n = 2$). These subgroups of CMA were

studied in order to investigate whether suppressor activity varies in patients who have different clinical manifestation of CMA. The diagnosis of CMA was based on a positive response to oral cow's milk challenge, since skin testing and the cow's milk-specific radioallergosorbent test are not specific tests for CMA (11). Patients with challenge-proven CMA were on cow's milk elimination diet, with additional dietary restrictions for egg ($n = 3$), wheat ($n = 4$), and citrus fruits ($n = 9$) because of adverse reactions. In addition, 13 age-matched children who were free of symptoms or signs of CMA or atopic eczema were studied as disease controls. They were electively admitted for examinations [urinary tract examinations ($n = 1$); oesophageal pH recording ($n = 2$); endocrinological ($n = 2$) or neurological ($n = 1$) examinations] or had had symptoms suggestive of CMA, (vomiting, $n = 2$; diarrhoea, $n = 2$; loose stools, $n = 3$; eczema, $n = 2$), but were clinically negative to the oral cow's milk challenge ($n = 7$). These patients had no dietary restrictions at testing. None of the patients was receiving systemic corticosteroid therapy.

Cow's milk challenge protocol

After 2–4 weeks on milk elimination, patients were admitted for the diagnostic cow's milk challenge. The challenge was performed openly since placebo reactions have been shown to be rare in infants (12). During the elimination period the patients had consumed a tolerated formula (soy or protein hydrolysate) or were given calcium supplementation. The challenge was started in hospital. Cow's milk was given in rising doses at 2-h intervals: on *Day 1*: 2, 5, 10, 20, 50 and 100 ml, and on *Day 2*: the normal milk intake appropriate for age was commenced. The challenge was stopped and the patients were examined by a paediatrician, and any adverse reaction was observed in hospital and always confirmed by the same person (EI). The time of the reaction was defined as the time elapsing from the last given dose eliciting the specific symptoms. The patients followed over the one-week period of challenge were seen again one month later. They were on milk elimination diet until the clinical rechallenge was performed, after a follow-up of 4–18 months.

Blood samples

Blood in heparin was drawn for the suppressor-cell assay immediately before the commencement of the oral milk challenge, always at the same time, at 8.00 a.m. At diagnosis, samples from 10 patients and 13 disease controls were available for this as-

say. At rechallenge, samples from 23 patients were examined. Also the serum total IgE levels and cow's milk-specific radioallergosorbent test were taken prechallenge.

Suppressor cell assay

Concanavalin A (Con A) activation of human lymphocytes provides a means of examining the capacity of suppressor function (13). After induction with Con A, T-cells are capable of inhibiting the T-cell proliferative response of an unrelated person as assessed by co-culture (13, 14).

The analyses were performed blind, always by the same person (HS). A modification (15) of the method described by Shou, Schwartz and Good was used (16). In brief, mononuclear cells containing mainly lymphocytes were isolated, immediately after the blood was drawn, by Ficoll-Hypaque gradient centrifugation at 400 g for 30 min at 20°C. To induce suppression, 6.25×10^5 isolated lymphocytes in one ml of RPMI 1640 containing antibiotics, glutamin and 10% human AB serum were cultured in a humidified, 5% CO₂ atmosphere at 37°C for 48 h together with Con A (Pharmacia, Uppsala, Sweden) at a final concentration of 25 µg/ml, and cow's milk protein solution (final protein concentration of 1 µg/ml dried milk powder (Valio Finnish Co-operative Dairies' Association, Helsinki, Finland), while a control cell population was generated only with RPMI 1640. For the last 30 min of the induction period 75 µg (50 µl) of Mitomycin C (Sigma Chemicals, St. Louis, Mo., USA) was added to each tube to terminate the DNA synthesis of the cells. Subsequently the cells were washed four times with RPMI 1640, and manually counted under a light microscope. The concentration of intact-looking cells was finally adjusted to 32000 cells/ml in RPMI 1640 with 10% human AB serum.

As responder cells, lymphocytes always from the same healthy male donor were used. Mononuclear cells were isolated and their concentration adjusted to 2.5×10^5 cells/ml in RPMI 1640 with 10% human AB serum. The responder cells were stimulated with phytohaemagglutinin, concentration 125 µg/ml (PHA, Difco Laboratories, Detroit, MI, USA) in the presence of either RPMI 1640-induced control cells, Con A-induced or cow's milk-induced cells. The quadruplicate cultures containing 14×10^3 cells, responder/induced cell ratio 8:1, in a total volume of 200 µl were incubated in a humidified 5% CO₂ atmosphere at 37°C for 72 hours. Eighteen hours before harvesting, 20 µl of ¹²⁵I-labelled 5-iodo-2'-deoxyuridine (¹²⁵IUdR) (25 µCi, specific activity 2000 Ci/mmol, The Radiochemical Centre, Amersham, UK) was added to

Table 1. Age, volume of milk eliciting symptoms, reaction time from last given dose, cow's milk-specific RAST and serum total IgE of individual patients at diagnostic challenge and at rechallenge.

Patient (initials)	Diagnostic challenge					Rechallenge		
	Age ¹⁾	Volume of milk eliciting symptoms	Reaction time	RAST	IgE ²⁾	Age	Volume of milk eliciting symptoms	Reaction time
CMA (gi-symptoms)								
J.L.	2.6	100 ml	12.0 hours	–	20.0	10.4	Recovered	
K.R.	9.7	10 ml	0.1 hours	0	3.1	17.1	Recovered	
V.K.	15.6	100 ml	24.0 hours	–	7.0	22.0	Recovered	
E.N.	10.0	100 ml	0.3 hours	–	88.0	15.8	Recovered	
M.R.	3.8	50 ml	0.16 hours	0	–	9.6	Recovered	
J.H.	3.0	50 ml	0.10 hours	–	4.8	24.3	Recovered	
O.K.	15.5	100 ml	4.0 hours	0	26.0	45.5	Recovered	
S.K.	6.0	2 ml	2.0 hours	0	2.7	43.6	Recovered	
A.M.	5.3	100 ml	4.0 hours	–	0.5	15.3	200 ml	48.0 hours
T.O.	3.0	200 ml	72.0 hours	–	0.7	44.3	200 ml	48.0 hours
S.S.	16.0	200 ml	12.0 hours	0	1.6	45.7	200 ml	72.0 hours
S.M.	14.3	200 ml	12.0 hours	0	4.3	36.0	200 ml	12.0 hours
S.K.	3.0	100 ml	4.0 hours	–	–	24.7	200 ml	24.0 hours
S.A.	11.0	100 ml	12.0 hours	0	1.6	48.0	200 ml	72.0 hours
CMA (skin-symptoms)								
L.H.	9.3	50 ml	2.0 hours	–	144.0	21.7	Recovered	
Ju.K.	9.0	200 ml	7.0 hours	0	–	56.9	Recovered	
Ja.K.	3.9	100 ml	4.0 hours	0.4	228.0	8.2	Recovered	
V.A.	13.8	200 ml	72.0 hours	1.9	74.0	48.0	Recovered	
M.T.	3.5	200 ml	12.0 hours	–	13.0	40.3	Recovered	
J.K.	5.2	2 ml	0.10 hours	2.0	120.0	14.3	5 ml	0.16 hours
S.M.	6.5	200 ml	72.0 hours	0	105.0	28.4	200 ml	72.0 hours
T.M.	6.8	100 ml	4.0 hours	0	53.0	37.4	200 ml	12.0 hours
K.L.	9.5	20 ml	0.5 hours	–	59.0	42.5	200 ml	72.0 hours

¹⁾ months²⁾ kU/L

each well together with 5-fluoro-2'-deoxyuridine (final concentration of 4×10^6 M) (17). The cultures were harvested with a multiple cell-culture harvester (1295-001, Pharmacia Wallac, Turku, Finland). The radioactivities (in counts per minute, cpm) were measured with an automatic gamma-counter (Wizard 1470, Pharmacia Wallac, Turku, Finland), and the percentage of suppression calculated according to the formula:

$$1 - \frac{\text{net cpm of mitogen-stimulated responder cells in the presence of con A- or cow's milk-induced cells}}{\text{net cpm of mitogen-stimulated responder cells in the presence of RPMI-induced cells}} \times 100$$

To reduce the effect of mixed lymphocyte culture (MLC) on the results, the total number of cells in the co-culture was low. The MLC was also taken into consideration when comparing the cpm of induced cells to the cpm of cells cultured in plain medium using the formula set on above.

Statistical analysis

ANOVA was employed to determine the statistical significance of differences in continuous variables. A two-tailed Student's t-test was applied to compare differences between groups. The Mann-Whitney U test was employed for comparison of medians. The Chi-square test was applied to determine differences in proportions. For comparing several pairs of means, a series of 95% confidence interval [95% CI] was constructed using the Tukey test.

Results

Initial challenge

Initial challenge clinical characteristics – The geometric mean [95% CI] ages of the patients, 9.8 [6.7, 14.5] months, and disease controls, 9.8 [6.6, 14.4] months, were comparable at the time of the study; $F = 0.087$, $p = 0.77$. A positive family history of allergy was detected in 75% of patients who had CMA compared to 36% of controls, $X^2 = 7.8$, $p = 0.02$. The mean [95% CI] serum total IgE levels in patients manifesting CMA with skin or

CON A-INDUCED SUPPRESSION (%)

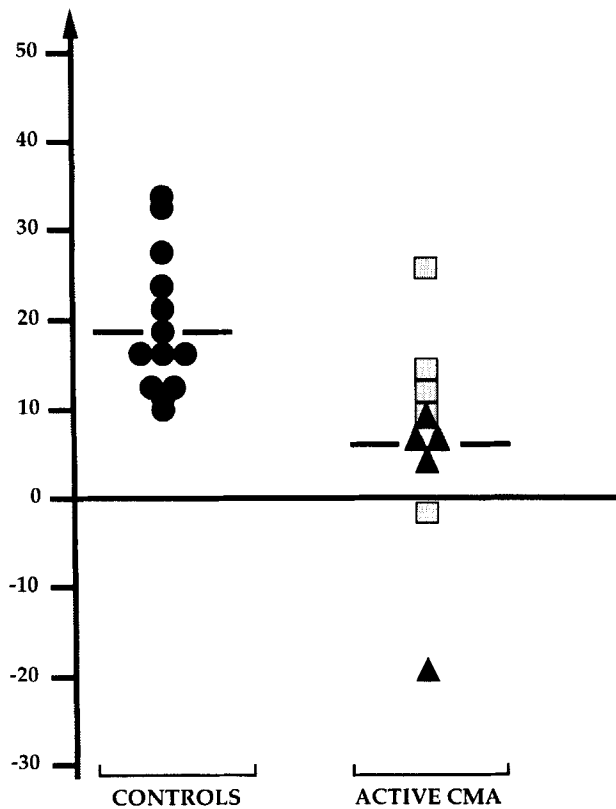


Fig. 1. Concanavalin A-induced suppression (per cent) in controls and in patients with active cow's milk allergy, manifested either with skin (□) or gastrointestinal (▲) symptoms at the initial challenge. Horizontal bars denote the geometric means. Comparison between three groups: $F = 7.08$, $p = 0.004$.

gastrointestinal symptoms, and in disease controls were 77.1 [37.4, 158.9] kU/l, 4.4 [1.7, 11.5] kU/l, and 10.8 [2.9, 40.1] kU/l, respectively; $F = 12.0$, $p = 0.0003$. Cow's milk-specific RAST was detected in 80% and 0% of CMA patients with skin and gastrointestinal symptoms, and in 20% of controls, respectively; $X^2 = 5.1$, $p = 0.08$. The serum total IgE and cow's milk-specific RAST from individual patients are described in Table 1.

Patients who manifested CMA cutaneously ($n = 9$) reacted to a median (range) 100 (2, 200) ml of cow's milk after a mean [95% CI] 5.8 [1.3, 26.3] h. Those with gastrointestinal symptoms ($n = 14$) showed adverse reactions to 100 (2, 200) ml of cow's milk after 2.8 [0.8, 9.8] h. Five patients reacted immediately after ingesting cow's milk, four with vomiting, and one with urticaria. The differences between patients with cutaneously and gastrointestinally manifested CMA, or RAST-positive and RAST-negative patients were statistically not significant. The reaction time from the last given dose and the volume eliciting symptoms from individual patients are described in Table 1.

Non-specific suppressor activity at initial challenge – As depicted in Fig. 1, the Con A-induced suppression was different in CMA patients with cutaneous or gastrointestinal manifestations and disease controls. The distinction between the study groups was attributable to the low suppressor activity in patients with CMA manifested cutaneously or gastrointestinally (Fig. 1). Lack of suppression was comparable in patients manifesting CMA with either skin or gastrointestinal symptoms. In addition, lack of suppression was indistinguishable in patients with skin symptoms, who were RAST-positive or RAST-negative to cow's milk.

Cow's milk-induced suppressor activity at initial challenge – Cow's milk-induced suppressor function was different in CMA patients with cutaneous or gastrointestinal manifestations and disease controls (Fig. 2). The distinction between the study groups was attributable to the low suppressor activity in the two subgroups of CMA. In addition, lack of suppression was indistinguishable in pa-

COW'S MILK-INDUCED SUPPRESSION (%)

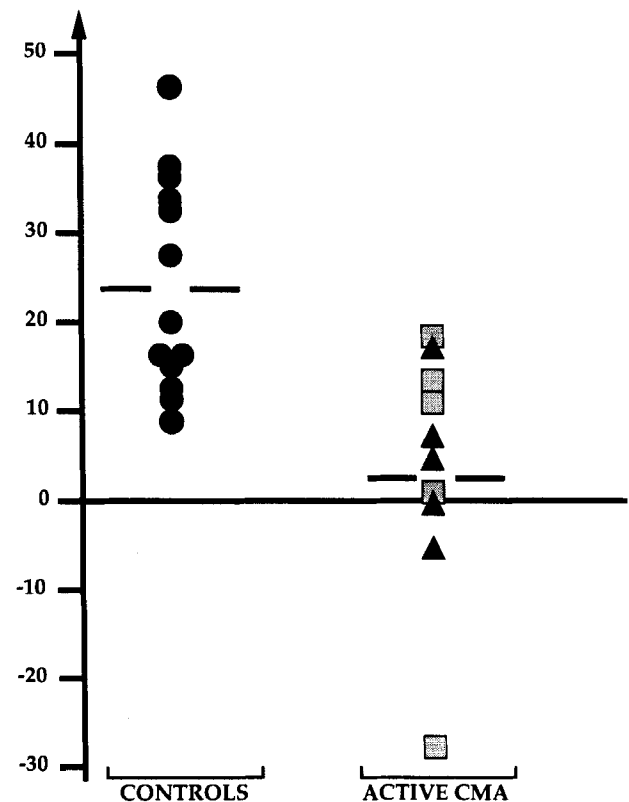


Fig. 2. Cow's milk-induced suppression (per cent) in controls and in patients with active cow's milk allergy, manifested either with skin (□) or gastrointestinal (▲) symptoms at the initial challenge. Horizontal bars denote the geometric means. Comparison between three groups: $F = 6.75$, $p = 0.005$.

CON A- INDUCED
SUPPRESSION (%)

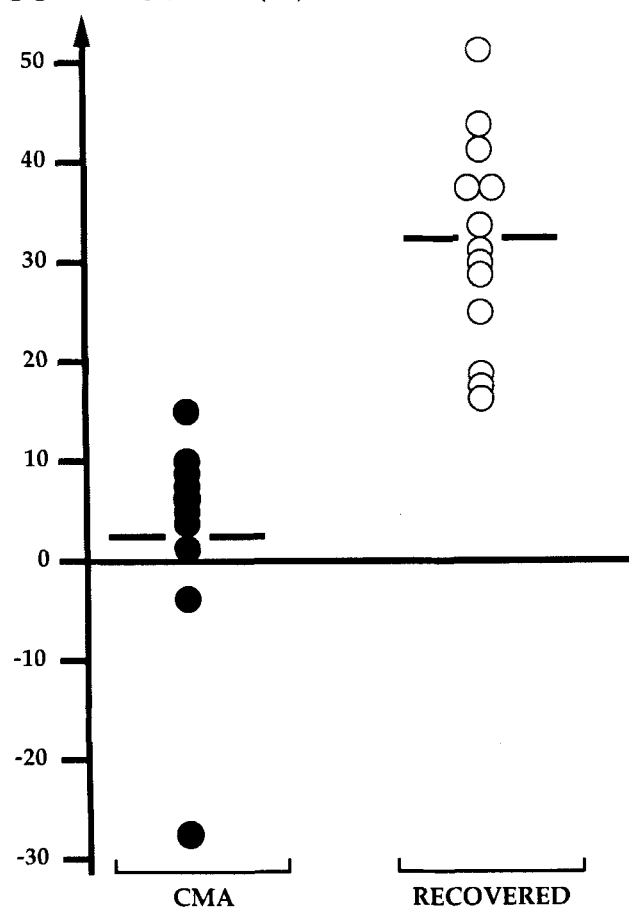


Fig. 3. The results of Concanavalin A-induced suppression (per cent) at rechallenge in children who had recovered from cow's milk allergy (recovered) and in patients retaining allergy (active CMA). Horizontal bars denote the geometric means. $F = 38.3$, $p = 0.0001$.

tients with skin symptoms, who were RAST-positive or RAST-negative to cow's milk.

Reassessment

Clinical characteristics – Of the 23 patients tested at the re-challenge, 13 had achieved clinical tolerance to cow's milk, whereas 10 children still reacted clinically. The ages of the children with a positive and negative re-challenge were 29.0 [19.0, 44.5] and 23.2 [15.6, 34.6] months, respectively; $F = 0.71$, $p = 0.41$. The clinical reactions to cow's milk were in general milder than at the initial challenge. In addition, the symptoms developed later, after larger amounts of milk, than at the diagnostic challenge.

Non-specific suppressor activity – At re-challenge, the percentage of Con A-induced suppression was different in patients who had recovered from CMA and in those retaining allergy (Fig. 3). When com-

paring these to the levels observed in controls (Fig. 4), the difference of 16% with 95% CI [2,30] % was statistically significant in patients retaining allergy. The 10 patients tested on both occasions, at the initial and the re-challenge, showed normalized Con A-induced suppressor activity when they had clinically recovered from CMA, but depressed suppressor function if retaining allergy.

Cow's milk-induced suppressor activity – Like the non-antigen-specific suppression, the percentage of cow's milk-induced suppression was different in patients who had recovered from CMA and in those retaining allergy (Fig. 3). When comparing these to the levels observed in controls (Fig. 1), the difference of 32% with 95% CI [20,44] % was statistically significant in patients retaining allergy, but comparable in children who had achieved clinical tolerance to cow's milk, 2 [–10, 14] %.

**COW'S
MILK- INDUCED
SUPPRESSION (%)**

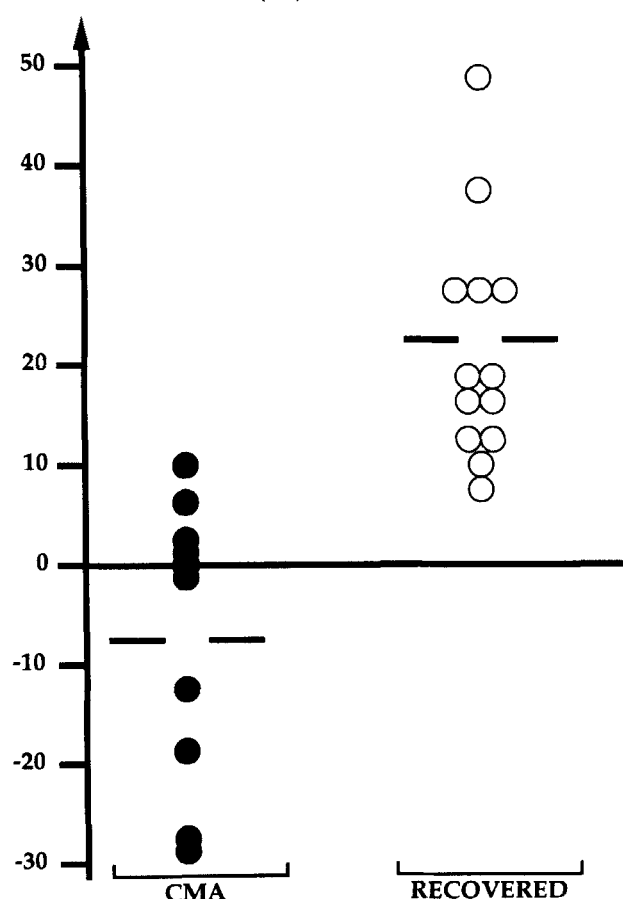


Fig. 4. The results of cow's milk-induced suppression (per cent) at rechallenge in children who had recovered from cow's milk allergy (recovered) and in patients retaining allergy (active CMA). Horizontal bars denote the geometric means. $F = 26.9$, $p = 0.0001$.

Discussion

The present investigation suggests a novel potential pathogenetic mechanism for the development of CMA. We have demonstrated that T-cell-mediated suppression was defective in patients with active CMA, as compared with age-matched controls. After a follow-up period on cow's milk elimination diet, the suppressor function reached the level of disease controls in patients who had acquired clinical tolerance to cow's milk, but not in those retaining allergy. These results suggest that suppressor activity has an important role in the development and maintenance of oral tolerance in humans.

Although small but immunologically significant amounts of macromolecules are absorbed in antigenic form across the mucosal barrier and gain access to the immune system of the body, allergic reactions to dietary constituents are uncommon (18, 19). In health, the usual consequence is antigen-specific hyporesponsiveness, oral tolerance (19, 20).

Oral tolerance has been extensively studied in experimental animals, but the immunologic events leading to the development of hyporesponsiveness in humans are unknown (21). Oral tolerance has been shown to be transferrable; serum from a tolerant mouse can induce tolerance in an antigenically inexperienced mouse when fed with the respective antigen (22). Oral tolerance can also be abrogated, when suppressor T-cells are depleted with cyclophosphamide or 2'-deoxyguanosine, or when reticuloendothelial system is activated during a graft-versus-host reaction (23-25). Thus evidence from animal experiments has given rise to the conception that antigen-specific suppressor T-cells or an appropriate balance of suppressor and helper T-cells are of primary importance for the induction of oral tolerance (19, 21). Although previous investigations have been performed mainly in rodents, there is no reason to assume that the phenomenon "oral tolerance" does not exist in man (26). This conception is strongly reinforced by the results of the present investigation. The level of active suppression is high in healthy infants, descending to the adult level during the second year of life (27, 28), which is considered a physiologic phenomenon. The inhibitory activity dampens the immune responses, and thereby protects the infant until other effective suppressive mechanisms are developed (27, 28).

CMA is defined as an immunologically mediated adverse reaction against cow's milk antigens (29). The patients have been assigned to different subgroups on the basis of clinical manifestation of CMA or the onset of the reaction (30). The factors

that determine the target organ in CMA to be skin, gastrointestinal tract or respiratory system, are currently not known. There is evidence to suggest that different immunopathogenetic mechanisms may be operative in these subgroups (30). In support of this notion we have previously shown that the quality and extent of gut humoral immune response varies in patients manifesting CMA with urticaria, eczema or gastrointestinal symptoms (31). Therefore, it was interesting to find out that lack of suppressor activity was comparable in patients with different clinical manifestation of CMA. Whether this defect is a specific phenomenon for CMA remains to be shown.

There are several explanations for depressed suppressor activity in patients with CMA. It is possible that the generation of suppressor T-cells is impaired in patients with active CMA; analogous results have been presented in patients with atopic eczema (32, 33). However, since CD4+ and CD8+ subsets as well as macrophages may be involved in the generation of suppression, the absolute numbers of these cell types were not compared in this study. It is also possible that an imbalance between CD4+ and CD8+ T-cells may lead to enhanced helper T-cell activity, which was detected as lack of suppression in patients with active CMA. Studies in rodents have also suggested that induction of contrasuppressor T-cells could explain the defective oral tolerance against specific antigens (19). However, our follow-up study strongly suggests that there is a delay in the maturation of suppressor activity in these patients.

We have previously shown by ELISPOT method that oral cow's milk challenge induces a high non-antigen-specific immune response in patients with CMA (31). At the same time, the specific immune response against cow's milk antigens is defective, especially in the IgA class, indicating a defective immune exclusion of cow's milk antigens. We have also demonstrated that a specific immune response to cow's milk antigens has been matured in patients, who have clinically recovered from CMA (34). The results of the present investigation may give an explanation for the findings detected by the ELISPOT. Since the humoral immune response is regulated by T-lymphocytes and T-cell-derived soluble factors, it is understandable that defects in T-cell function may disturb also the humoral arm of the immune response. Clearly, further investigations are needed to elucidate the exact mechanisms behind the impaired suppressor function in CMA.

Taken together, the results of the present study indicate that maturation of non-antigen-specific and antigen-specific suppression may be one determinant for the normal development of oral toler-

ance. We conclude that the delay in the maturation of suppressor activity results in a breakdown of oral tolerance and might be an important factor in the etiopathogenesis of CMA.

Acknowledgements

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