

Evidence for eosinophil activation in cow's milk allergy

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To assist in identifying pathogenetic mechanisms in different clinical manifestations of cow's milk allergy (CMA), the involvement of eosinophil cells in immunoinflammatory reactions was evaluated. The study population comprised 28 patients, aged from 5.8 to 43.0 months, who had challenge-proven CMA, manifested either cutaneously ($n = 17$) or gastrointestinally ($n = 11$). A clinical cow's milk challenge was performed in hospital after a 4 week cow's milk elimination period. Eosinophil activation *in vivo* was studied by measuring the serum level of eosinophil cationic protein (ECP) before the oral cow's milk challenge, mean (SD) 27 (12) hours after commencing the challenge and one week later. These results were compared to those of 80 non-allergic age-matched controls. During the challenge, the level of ECP increased from 6.2 (4.5, 8.0) $\mu\text{g/L}$ to 20.0 (9.5, 30, 4) $\mu\text{g/L}$ in CMA patients with skin manifestations but not in those with gastrointestinal symptoms. The increase was shown to be transient. We conclude that eosinophil degranulation is an important immunologic mechanism leading to allergic inflammation in cutaneously manifested CMA.

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CMA is defined as an immunologically mediated adverse reaction against cow's milk antigens (1). The patients have been assigned to different subgroups on the basis of clinical manifestation or the onset of the reaction (2). There is evidence to suggest that different immunopathogenetic mechanisms may be operative in different clinical manifestations of CMA (1, 2). Characterization of such mechanisms may be helpful in the exploration of immunotherapy for the use in the treatment of food allergies.

Eosinophils are mainly tissue-dwelling cells; the blood-tissue ratio of eosinophils has been estimated to be 1:100 (3). They are presumably involved in normal immune defence as well as in specific morbid processes including parasitic infections and hypersensitivity reactions (3, 4). Eosinophil granules contain cationic proteins, which have been demonstrated *in vivo* and *in vitro* to be tissue-destructive (3). The lifespan and exact functions of eosinophils are not yet clear, and little is known of the precise mechanisms leading to activation and degranulation of these cells during hypersensitivity reaction. Detection of eosinophil-derived granular proteins reflects earlier activation and degranulation or cytolysis of eosinophils and

thereby provides evidence of involvement of these cells, even when intact cells are not detected (4).

Previous studies on eosinophil activation were concerned with adult HIV-infection and allergy; asthma, allergic rhinitis or suspected food hypersensitivity (5–7). Food allergies are a common manifestation of allergic symptomatology in infants, often first manifested as allergy to cow's milk (1, 8). In the present study we compared the eosinophil activation in patients with different clinical manifestations of CMA, by measuring the changes in serum concentrations of ECP during an oral cow's milk challenge.

Methods

Patients

The study comprised 28 children with a mean age of 13.7 [10.8, 17.48] months, who had challenge-proven cow's milk allergy, manifested cutaneously or gastrointestinally. These subgroups of CMA were studied in order to investigate whether eosinophil activation varies in patients who have different clinical manifestation of CMA. Patients with challenge-proven CMA were on cow's milk elimination diet, with additional dietary restric-

Table 1. The age, serum total IgE, cow's milk-specific RAST, volume of cow's milk eliciting the specific symptoms, reaction time from the last given dose, prechallenge and postchallenge ECP-level in patients with cutaneously and gastrointestinally manifested CMA

Patient	Age ¹	IgE ²	Cow's milk-specific RAST	Volume of milk eliciting symptoms (ml)	Reaction time	Prechallenge ³ ECP-level	Postchallenge ³ ECP-level
(initials)	(months)	(kU/L)			(hours)	(µg/L)	(µg/L)
CMA (skin-symptoms)							
A.A.	31.1	310.0	4.0	10.0	0.16	9.4	16.4
L.H.	6.2	—	1.0	5.0	0.16	6.7	11.9
V.H.	22.1	699.0	76.0	1.0	0.10	4.1	7.8
L.I.	7.6	2.3	0	200.0	72.0	8.0	14.0
T.J.	16.2	8.0	0	200.0	72.0	7.1	18.9
N.J.	6.0	255.0	3.4	50.0	0.16	3.5	12.1
J.K.	39.6	—	0	200.0	72.0	7.1	22.5
J.L.	10.3	3.2	1.2	10.0	2.0	8.8	15.5
A.M.	5.9	648.0	30.0	200.0	72.0	5.6	91.2
M.M.	13.5	29.0	0	5.0	0.10	3.8	8.7
M.N.	31.7	75.0	1.8	100.0	12.0	5.4	14.1
K.P.	13.6	18.0	0	5.0	0.16	2.8	6.3
M.S.	34.1	321.0	0	20.0	3.0	8.3	14.3
O.S.	11.1	2.9	0	200.0	4.0	3.3	7.9
J.S.	21.0	12.0	0	200.0	48.0	7.4	17.3
A.S.	12.3	—	1.0	200.0	72.0	11.5	25.2
V.S.	5.8	86.0	2.0	5.0	2.0	2.5	43.3
CMA (gi-symptoms)							
M.B.	7.2	8.9	0	100.0	3.0	6.3	4.2
H.H.	24.2	0.3	0	200.0	48.0	8.5	4.5
L.J.	10.8	51.0	1.0	100.0	4.0	9.4	9.4
A.K.	11.9	9.0	0	200.0	72.0	8.2	8.7
J.L.	8.1	373.0	0.8	100.0	2.0	4.1	4.5
K.L.	9.6	43.0	0	50.0	0.16	6.4	7.7
P.M.	7.6	—	0	200.0	72.0	6.4	3.4
A.M.	22.4	166.0	0	200.0	72.0	8.1	9.2
J.P.	16.2	2.4	0	50.0	8.0	7.8	2.7
S.S.	43.0	1.6	0	200.0	72.0	3.2	3.6
N.V.	9.6	27.0	1.2	200.0	48.0	3.0	3.1

tions for egg ($n = 12$), wheat ($n = 5$), and citrus fruits ($n = 11$) because of adverse reactions. In addition, 80 non-allergic children, aged from 6 to 36 months, were studied as controls. Informed consent was obtained from the children's parents and the study was approved by the Hospital's Committee on Ethical Practice.

Cow's milk challenge protocol

The diagnosis of CMA was based on a positive reaction to oral cow's milk challenge. In brief, after 4 weeks on milk elimination, patients were admitted for an oral cow's milk challenge. Cow's milk was given in rising doses at two-hour 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. If symptoms were suspected, the challenge was stopped and the patients were examined by the trained staff, and any adverse reaction was observed in hospital and always confirmed by

attending paediatrician. The time of the reaction was defined as the time elapsing from the last given dose eliciting the specific symptoms. The patients were followed over the one-week period of the challenge and were seen again one month later.

Blood samples – Blood was drawn before, and mean (SD) 27 (12) hours after commencement of the clinical challenge, and one week later from 17 CMA patients with cutaneous manifestations and 11 patients with gastrointestinal symptoms. During a follow-up period on cow's milk elimination diet, the blood was drawn from 15 patients with cutaneous and 11 patients with gastrointestinal CMA on two different occasions to detect the possible variation in serum ECP concentration during a cow's milk free diet. The levels of serum total IgE and cow's milk-specific radioallergosorbent test (RAST), were evaluated pre-challenge. Serum total IgE was available in 20 of 80 healthy controls.

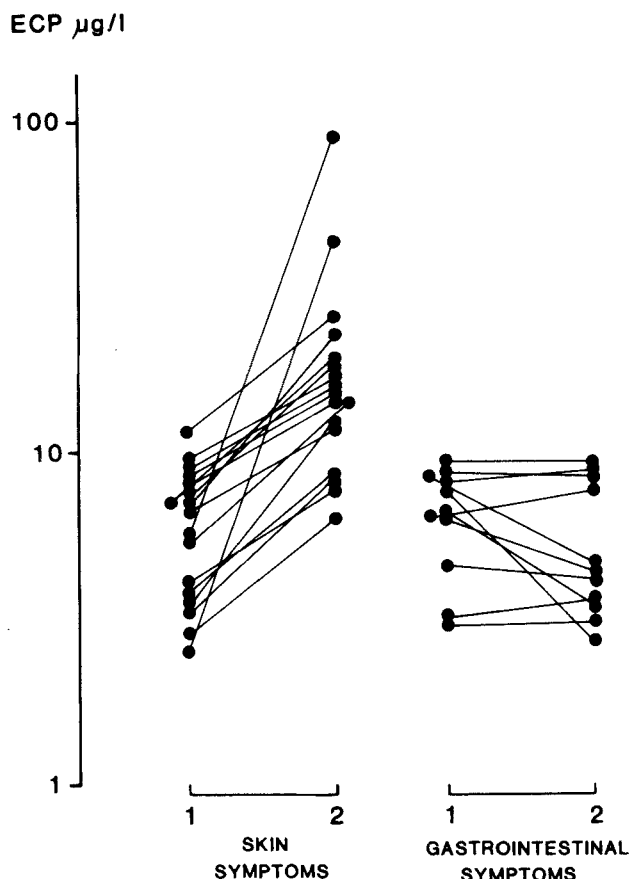


Fig. 1. The serum concentration of eosinophil cationic protein ($\mu\text{g/L}$) before the clinical cow's milk challenge (1) and mean (SD) 27 (6) hours later (2) in CMA patients with skin ($n = 17$) and gastrointestinal ($n = 11$) symptoms: ANOVA for repeated measures, $F = 7.1$, $p = 0.01$.

Serum concentration of ECP – The concentrations of serum ECP were measured with a commercial, human radioimmunoassay-Kit (Kabi-Pharmacia, Uppsala, Sweden) according to manufacturer's instructions. The results are expressed as $\mu\text{g/L}$.

Statistical analysis – ANOVA was employed to determine the statistical significance of differences in continuous variables. The repeated observations were studied using ANOVA for repeated measures. 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.

Results

Clinical and laboratory features – During the challenge, 17 patients showed cutaneous reactions (urticaria, $n = 6$; eczema, $n = 11$) and 11 gastrointestinal symptoms (loose stools, $n = 9$; diarrhea, $n = 2$). A positive family history of allergy was detected in 54% and 55% of these patient groups,

respectively. The mean serum IgE was 48 [11, 204] kU/L in the "skin" group, 14 [2, 110] kU/L in the "gastrointestinal" group, and 9 [4, 17] kU/L in healthy controls. Cow's milk-specific RAST was positive in 60% and 25% of the respective CMA groups. No child had peripheral blood eosinophilia.

The patients with cutaneously-manifested allergy reacted in mean 22.5 h with 95% confidence interval of [5.6, 39.5] hours, to small volumes of cow's milk, whereas gastrointestinal disturbance tended to appear in response to larger amounts of cow's milk (Table 1).

Comparison of serum ECP level between CMA patients with cutaneous or gastrointestinal manifestations and controls – The mean [95% CI] concentration of ECP in healthy controls was 10.9 [9.9, 11.9] $\mu\text{g/L}$. The two groups of patients were shown to have different responses during the clinical challenge (Fig. 1). The ANOVA for repeated measures confirms this; serial ECP measurements showed intergroup differences, with concentrations increasing in the patients with skin reactions, but stable or slightly falling in the group with gastrointestinal symptoms (Table 2). Repeat ECP test one week after the challenge showed the rise to have been transient; the ECP concentrations were 7.2 [5.6, 8.9] $\mu\text{g/L}$ and 6.4 [5.3, 7.5] $\mu\text{g/L}$ in patients with cutaneously and gastrointestinally manifested CMA, respectively. During the follow-up on milk elimination, ECP values in 26 measure-

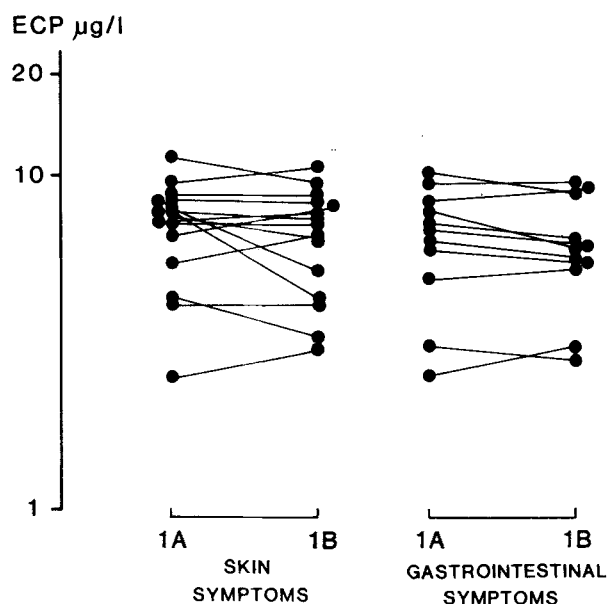


Fig. 2. The serum concentration of eosinophil cationic protein ($\mu\text{g/L}$) during the cow's milk elimination diet in CMA patients with skin ($n = 15$) and gastrointestinal ($n = 11$) symptoms. Measurements 1A and 1B are taken on two different occasions, the interval between them is 2–4 months.

ments remained unaltered, when measured on two different occasions (Fig. 2).

Discussion

In the present study, eosinophil activation *in vivo* was demonstrated during oral cow's milk challenge. Furthermore, the serum level of a eosinophil-derived soluble protein, ECP, was shown to discriminate CMA patients with skin and gastrointestinal manifestations; the sharp rise in the concentration of ECP was detected only in patients with cutaneous CMA. These results hence support previous suggestions that distinct immunopathogenetic mechanisms are involved in different subtypes of CMA, and reflect eosinophil degranulation as one cause of cutaneously manifest tissue damage due to cow's milk allergy.

The regulation of eosinophil growth and activation is mainly under the control of T-lymphocytes, in which at least the interleukin-3 and interleukin-5 play a central role (4). Eosinophils also express receptors for immunoglobulins A, G and E. It has been proposed that release of eosinophil components may be linked to earlier mast-cell activation (9). Although mast-cell-associated mediators are involved in eosinophil activation, they probably are less important in the case of young infants, because of immature and depressed mast-cell function (10). The existence of several regulatory pathways leading to eosinophil activation could explain the observed variation in the timing of the eosinophil response reported in different studies. Previously, the concentration of serum ECP has been shown to increase within hours after commencing the food challenge in adults with suggested food allergy (7). The rise in serum ECP in our patients was detected later, after a mean of 27 hours.

On the basis of *in vivo* and *in vitro* findings, eosinophil cells have been demonstrated to be toxic to other cells (11, 12). The involvement of the eosinophil cells in the immunoinflammatory reactions can be evidenced by measuring a number of proteins, which the cells have released from their granules after activation (4). ECP has been suggested to play a pathogenetic role in human allergic reactions, in HIV, asthma, inflammatory bowel disease, coeliac disease, IgE-mediated skin disorders and in several other diseases where the role of eosinophils is unresolved (5–7, 9, 13, 14). A correlation between serum ECP level and the number of eosinophils in peripheral blood has been observed previously in both healthy individuals and atopic patients, but not in patients with bronchial asthma (7, 15). Since the eosinophils are mainly located in tissues, it may be impossible in some occasions to measure their products in pe-

ripheral blood, which could be the most likely explanation for the controversial findings.

Our results do not exclude a localized, eosinophil-mediated inflammatory process in CMA with gastrointestinal expression. In a previous morphometric study the number of eosinophils in the lamina propria has been reported to be increased in patients with CMA (16). In accordance, in preweaned rat pups, exposure to cow's milk antigens resulted in enhanced endocytosis in association with elevated number of eosinophils in gut mucosa (17). The present study shows a slight decrease in the serum ECP concentration in CMA patients with gastrointestinal manifestations, which could account for increased local consumption of ECP in the gut. It nevertheless seems reasonable to suggest that the regulation of the gastrointestinal manifestations of food allergy is different and thereby other immunoglobulins than IgE, cytokines or chemoattractive factors may hence be involved, possibly in connection with a different timing of the reaction. The results of the present investigation reflect that the regulatory pathways leading to cutaneous or gastrointestinal expressions of cow's milk allergy probably are distinct. This is also in agreement with our previous finding that the quality and extent of humoral immune response varies in patients with different clinical manifestations of CMA (18).

The diagnosis of cow's milk allergy is based on clinical judgement, but objective evidence of the activation of immunological mechanisms is also essential. As skin prick tests and RAST are not specific indicators of clinical hypersensitivity (10), measurement of serum ECP during oral challenge may serve as a diagnostic tool for cow's milk allergy and probably also for other cutaneously manifested food allergies.

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