

T-cell signal transduction in children with cow's milk allergy – increased MAP kinase activation in patients with acute symptoms of cow's milk allergy

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The precise immune mechanisms behind cow's milk allergy (CMA) are still unknown. Previously, the production of the cytokines TNF- α and IFN- γ in T cells from children with CMA has been shown to be decreased, and the production of IL-4 has been shown to be increased when compared to healthy children. As these aberrations in cytokine production may be associated with disturbances in cellular function, we investigated whether T-cell signal transduction is abnormal in children with CMA. For this purpose we evaluated the activation of the MAP kinase Erk2. Thirty-nine infants were included in the study. Of those with CMA, 13 had acute symptoms and 9 were free of symptoms due to a successful elimination diet at the time of the study. To activate T cells and to stimulate MAP kinase phosphorylation, peripheral blood mononuclear cells (PBMC) were incubated with Concanavalin A (ConA). The change in MAP kinase phosphorylation was measured by Western blotting. The increase in MAP kinase phosphorylation after stimulation with ConA for 5 min was significantly higher in cells from patients with acute symptoms of CMA than in cells from CMA patients free of symptoms or cells from healthy children. A time-course experiment showed that the change in MAP kinase phosphorylation was still increasing after 10 min incubation in cells from patients with acute symptoms of CMA. The increased MAP kinase activation was found to correlate positively with non-IgE mediated CMA in patients with acute symptoms of CMA.

Pamela Österlund¹, Maria von Willebrand^{1,2}, Leif C. Andersson² and Hanna Suomalainen¹

¹Helsinki University Central Hospital, Department of Dermatology, Helsinki, Finland, ²University of Helsinki, Haartman Institute, Department of Pathology, Helsinki, Finland

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Hanna Suomalainen, Docent, Helsinki University Central Hospital, Skin and Allergy Hospital, Department of Dermatology, PO Box 160, FIN-00029 HUCH, Helsinki, Finland
Tel.: (358) 9 4718 6404
Fax: (358) 9 4718 6560
E-mail: pamela.osterlund@helsinki.fi

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Cow's milk allergy (CMA) is defined as an immunologically mediated adverse reaction against cow's milk antigens (1). The symptoms of CMA commonly appear during the first months of life, within days or weeks after commencing feeding with a cow's milk-based formula, or even during exclusive breastfeeding (1–3). The prognosis of CMA, however, has been suggested to be favourable; more than 70% of affected infants can tolerate cow's milk without symptoms at the age of 3 years (4).

The production of several leukocyte-derived mediators has previously been shown to be

elevated or defective in infants with CMA (5–8). In our previous studies, production of IFN- γ and TNF- α was found to be deficient in peripheral blood mononuclear cells (PBMC) of infants with CMA (5, 8). These defects may influence the activation of lymphocytes, which may contribute to the breakdown in oral tolerance to cow's milk in these infants.

Stimulation of the antigen receptor of T lymphocytes initiates a complex intracellular signal transduction cascade that eventually reaches the nucleus resulting in gene transcription, expression of cytokines and cytokine receptors,

and finally cell division (9). The T-cell receptor itself does not possess tyrosine kinase activity. Instead, following stimulation of the receptor intracellular Src-family tyrosine kinases are activated. These tyrosine kinases phosphorylate special immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic domain of the T cell receptor. The phosphorylated ITAMs are then able to bind a number of cellular Src-homology 2 domain containing proteins, thus recruiting a variety of signaling molecules to the vicinity of the receptor and each other. These molecules include the adaptor protein Grb2, in complex with the guanine nucleotide exchange factor Sos, enabling Sos to activate Ras. Ras in turn can activate Raf, causing activation of the MAP kinase cascade (9). The MAP kinase cascade consists of 3 sequentially activated protein kinases (9). Catalytic activation of MAP kinase requires phosphorylation on tyrosine and threonine residues by a dual-specificity MAP kinase kinase, MAP2K (also known as Mek). MAP2K is itself activated by phosphorylation on serine/threonine residues by a MAP2K kinase (Raf or Mek) (10). Activation and increased phosphorylation of the MAP kinases occur within a few minutes after stimulation of the antigen receptor of the lymphocyte. Stimulation of the antigen receptor in T cells results in phosphorylation and activation of the MAP kinases Erk1 and Erk2 (9, 10). The activated MAP kinases then translocate to the nucleus where they phosphorylate transcription factors (11). This leads to expression of the IL-2 receptor and to IL-2 secretion, causing further activation of the T cells (11).

As production of both IFN- γ and TNF- α is deficient in PBMC from infants with CMA, we wanted to investigate whether there are aberrations in the initial signal transduction from the T-cell receptor to the nucleus in infants with CMA.

Patients and methods

Patients

Thirty-nine infants aged from 0.4 to 8.8 months were included in the study. The infants were followed-up prospectively from birth: one group because of their high risk for allergy (atopic or food-allergic sibling[s] and/or parent[s]) and the other group because of their low risk for allergy (no signs of atopic heredity in the family). They were followed-up for at least one year to detect any symptoms of atopic dermatitis or food allergy. CMA was diagnosed in 22 patients at the mean age of 4.4 months. The diagnosis of cow's milk

allergy was based on a positive response to oral cow's milk challenge. These infants were further divided into acute-phase CMA ($n = 13$) and CMA controlled with successful elimination diet ($n = 9$). 17 healthy age-matched children were studied as a control group; they had no symptoms suggestive to CMA or atopic eczema. The control group had no dietary restrictions at the time of testing. All the infants were born at term and had no other chronic diseases or infections at the time of the study. The parents gave written informed consent to the study, which was approved by the ethics committees of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki.

Cow's milk challenge protocol

The diagnosis of CMA was based on an adverse response to cow's milk challenge. After 2–4 weeks on a milk elimination diet the patients were subjected to a diagnostic and open cow's milk challenge if they were free of symptoms. The challenge was performed openly as placebo reactions have been shown to be rare in infants (12). During the elimination period cow's milk had been strictly eliminated from the infant's diet and also from the diet of the lactating mother. If the infants needed extra formula in addition to breastfeeding a tolerated formula (protein hydrolysate or amino-acid based formula) was given. The challenge was started with a drop of cow's milk on the skin or lips. Thereafter, cow's milk was given in increasing doses at one-hour intervals: on Day 1: 2, 10, 50 and 100 ml and on Day 2: the normal milk intake appropriate to the infant's age was commenced. The challenge was stopped immediately when any adverse reaction was noticed. The time of the reaction was defined as the time elapsed from the last given dose eliciting the specific symptoms. The patients were followed-up over the one-week period of challenge. All the elimination diets as well as cow's milk challenges were carried out based on clinical indications, not for research purposes.

Skin prick tests and RAST

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 diameter of the reaction was taken as an adequate histamine reaction. Reactions were read at 15 min and at least a half (3 mm) of the size of histamine was taken as a positive reaction. Commercial

RAST-method was used to measure cow's milk-specific IgE antibodies in patients' sera.

Preparation of peripheral blood lymphocytes

For lymphocyte induction, blood was drawn in heparin always at the same time each 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 (Sigma, Steinheim, Germany), antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml), and 2 mM L-glutamine (Sigma, Irvine, UK). After washing 3 times the cells were counted in a Bürker chamber. To induce T cells and increase MAP kinase phosphorylation, 1×10^6 lymphocytes were incubated in a humidified 5% CO₂ atmosphere at 37°C for 5 and 10 min together with 25 µg Con A at a final volume of 1.2 ml. A control cell population was cultured with RPMI-1640 only. The cells were stored frozen at -70°C.

Preparation of cell lysate

Cells (1×10^6 /sample) were lysed in a buffer containing 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10 µg/ml aprotinin and leupeptin and 1 mM Na₃VO₄ and clarified by centrifugation at 13 000 g for 15 min. After the centrifugation the supernatants were suspended in SDS sample buffer.

Reagents

Antibodies against phospho-p44/42 MAP kinase and p44/42 MAP kinase were from New England Biolabs, MA. Concanavalin A (Con A) -mitogen was from Pharmacia, Uppsala, Sweden.

Western blotting and quantification

Proteins were resolved by SDS/PAGE on 10% gels and transferred onto nitrocellulose filters. The antisera were used at 1 : 1000 dilution and the blots developed by the enhanced chemiluminescence technique (ECL lot, Amersham) according to manufacturer's instructions. The same nitrocellulose filters were first probed with antiphospho-MAP kinase antibodies, and then stripped and reprobed anti-MAP kinase antibodies. The bands were scanned and the density of the bands was quantified with the MacBas software. The amount of phospho-MAP kinase in each sample was divided with the total amount of MAP kinase protein in the same sample. The

results, i.e. the change in the adjusted amount of phospho-MAP kinase, is expressed as percentage, where the level without mitogen induction was labelled 100%.

Statistical analysis

The Kruskal-Wallis test and Mann-Whitney *U*-test were employed to determine the statistical significance of the differences between continuous variables. The Chi-square test was applied to determine differences in proportions. The analyses were carried out with NCSS2000 software.

Results

Clinical characteristics of CMA infants and controls

The study population ($n = 39$) consisted of 22 infants with challenge-proven CMA manifested either with urticarial or eczematous skin eruptions ($n = 7$), or gastrointestinal symptoms ($n = 1$) or both skin and gastrointestinal symptoms ($n = 14$). Of these CMA infants only 36% (8/22) had positive Skin Prick test or RAST to cow's milk. All those with positive Skin Prick test or RAST had urticarial or eczematous skin eruptions but only 43% of CMA patients with only skin symptoms reacted positively in Skin Prick test or had positive RAST to cow's milk. Of these 22 infants with CMA, 13 had acute symptoms and 9 were free of symptoms due to a successful elimination diet at the time of the study. 20 of these infants had generated symptoms already during exclusive breastfeeding, 11 infants before one month of age and 9 infants before 3 months of age. The geometric mean ages of the infants with acute symptoms of CMA were 3.5 [95% CI 2.9–4.7] months and that of the CMA infants free of symptoms 3.8 [2.9–7.3] months and that of the healthy infants 4.0 [3.4–5.3] months. The ages were comparable between these groups ($p = 0.42$, Kruskal-Wallis). A positive family history of atopy/allergy was detected in 95% (21/22) of the infants who had CMA and in 24% (4/17) of controls, $\chi^2 = 21.6$, $p = 0.000003$.

In clinical cow's milk challenge four patients manifested urticaria immediately after introduction of a drop of cow's milk on their skin and lips. The patients challenged perorally manifested as either urticarial or eczematous skin eruptions ($n = 9$), and reacted mainly in an early phase of the challenge, median 2.0 (range, 0.3–4) hours after beginning of the challenge in response to low volumes of milk, median total dose of 36 (range, 1–162) ml. Those with gastrointestinal

symptoms or slowly developing eczema ($n = 9$) more often showed delayed reactions after a median of 48 (range, 3–120) hours, in response to larger amounts of milk, median 211 (range, 37–2000) ml.

MAP kinase activation

Activation of the MAP kinase Erk2 (p42) was investigated by stimulating PBMC from infants with CMA and from healthy controls with ConA for 5 and 10 min, after which the cells were lysed and suspended in SDS-sample buffer. The samples were separated on 10% SDS/PAGE and analyzed by Western blotting with antiphospho-MAP kinase antibodies, which recognize only the phosphorylated, activated form of MAP kinase (Fig. 1b). The amount of MAP kinase protein in the lanes was analyzed by blotting the same filters with MAP kinase antibodies (Fig. 1a). The bands were scanned and quantified for statistical analysis of the results, and the amount of phospho-MAP kinase was adjusted for the amount of MAP kinase protein in the sample.

The increase in MAP kinase phosphorylation (percentage) after 5 minute incubation with ConA when compared with baseline phosphorylation (level without mitogen induction, 100%) (Fig. 1b) was significantly higher in cells from patients with acute symptoms of CMA when compared to cells from patients free of symptoms and to cells from healthy infants ($p = 0.02$, Kruskal–Wallis) (Fig. 2). A time-course experiment showed that the change in MAP kinase activation in cells from infants with acute-phase CMA was still

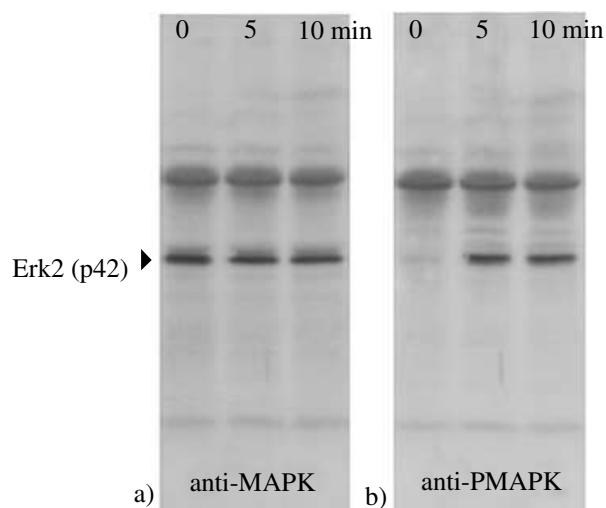


Fig. 1. The Western blot of total cell lysate from PBMC stimulated with or without mitogen ConcanavalinA for 5 or 10 min. The filter was blotted with anti MAP kinase (a) and anti phospho-MAP kinase (b) antibodies.

increasing after 10 min of mitogen induction. Cells from healthy and symptom-free infants reached their highest MAP kinase phosphorylation level after 5 min induction (Fig. 3). Statistically the differences in the medians after 10 min induction were still significant between these three study groups ($p = 0.03$, Kruskal–Wallis).

Correlation of increased MAP kinase activation with IgE-mediated responses to cow's milk

We then analyzed whether having IgE-mediated CMA, i.e. having positive Prick or RAST test to cow's milk proteins, had a relation to the increased MAP kinase activation. The increased MAP kinase activation was found to associate positively with the non-IgE mediated CMA in patients with acute symptoms of CMA (Table 1). Cells from infants having non-IgE mediated CMA showed an almost 2-fold increase in MAPK phosphorylation, while cells from infants with IgE mediated CMA showed a 1.3 fold increase, which is in the same range as the

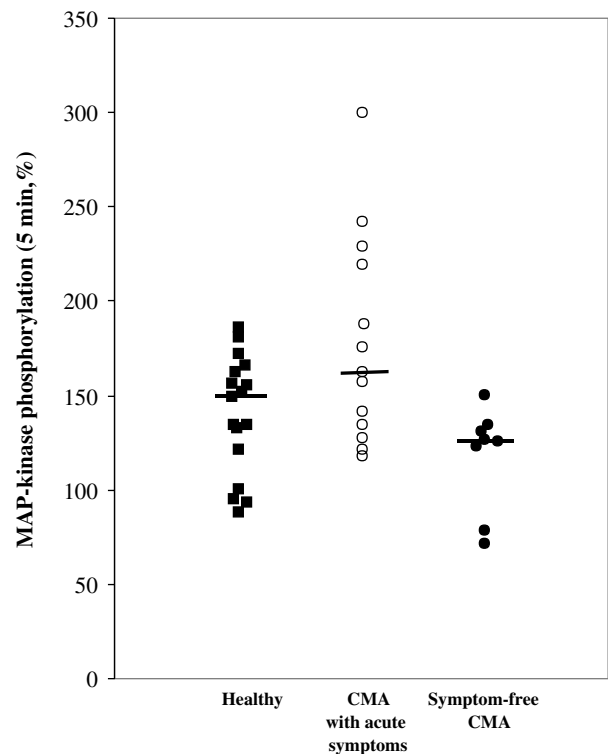


Fig. 2. The change in MAP kinase phosphorylation (%) after 5 min ConcanavalinA incubation (baseline without mitogen induction as 100%). The phosphorylation level in cells from patients with acute symptoms of cow's milk allergy (CMA) was significantly higher when compared to cells from patients without symptoms and to cells from healthy infants ($p = 0.02$, Kruskal–Wallis). The horizontal lines denote the median values: 150%, 163% and 126%, respectively.

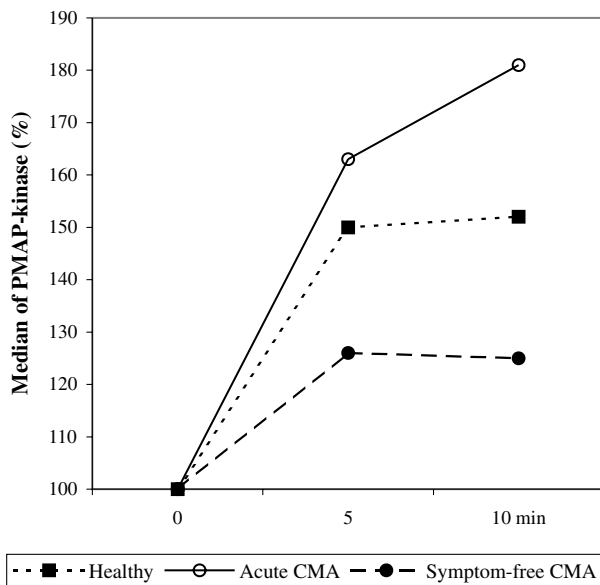


Fig. 3. The change in the MAP kinase phosphorylation after 5 and 10 min mitogen induction in cells from cow's milk allergic (CMA) infants with acute symptoms (full line), symptom-free CMA infants (broken line) and healthy infants (dotted line). The median and ranges: healthy group at 5 min 150[88–186]% and at 10 min 152[102–204]%, Acute CMA at 5 min 163[118–300]% and at 10 min 181[110–231]% and symptom-free CMA at 5 min 126[72–151]% and at 10 min 125[83–171]%.

increase in cells from healthy infants and infants with CMA but free of symptoms.

Discussion

In the present study, activation of the MAP kinase Erk2 was investigated by stimulating PBMC from CMA infants and healthy controls with ConA for 5 and 10 min. Our results show that the induction of MAP kinase phosphorylation after 5 min incubation with ConA was significantly stronger in cells from patients with acute symptoms of CMA, while cells from healthy and symptom-free infants showed a smaller response.

The mechanisms leading to development of food allergy are still poorly understood. IgE-mediated food allergy is better characterized but the mechanisms leading to a delayed-type food allergy still remain unresolved. According to the

results of our present study, in the acute phase of CMA, without a successful elimination diet, T cells of infants with CMA become more vigorously activated after mitogenic induction than T cells of healthy infants or those free of symptoms of CMA. These results suggest an overactivation of the immune system in infants with acute symptoms of CMA, when compared with healthy, age-matched children. The equal response in CMA infants free of symptoms and in healthy controls reflects that elimination of cow's milk proteins of the infant's diet has a normalizing effect on the function of the immune system, at least in responding to mitogenic stimulation. In a previous study by us it was shown that during cow's milk challenge, the infants allergic to cow's milk also developed a response to so called unrelated antigens like gliadin, although these patients were not allergic to wheat (13). The results of this previous study and our present one are well in agreement with each other. Together they suggest that overactivation of the immune system, or at least overactivation of T lymphocytes, may increase the risk of the allergic infant becoming sensitized to other foods. The immunological mechanisms behind these prolonged and severe symptoms of CMA in these infants might have been partly clarified in this study. The increased MAP kinase activation was found to correlate positively with non-IgE mediated CMA in patients with acute symptoms of CMA, although these subpopulations were quite small. The strong association of the non-IgE mediated disease with high activation of MAP kinase suggests that increased responsiveness of T cells to stimulation may mediate the prolonged immunoinflammatory disease.

Previously, extracellular signal-regulated kinases have been investigated in hyperproliferative cultured fibroblasts and peripheral blood lymphocytes from patients with psoriasis (14,15). These studies have demonstrated abnormalities in the MAP kinase pathway in hyperproliferative fibroblasts. These fibroblasts from psoriatic lesions were shown to express high MAP kinase activity (14). Also normal and nonlesional psoriatic skin

Table 1. Association of negative Skin prick-test or RAST to cow's milk and increased MAP kinase phosphorylation (median of PMAP kinase, %, baseline without mitogen induction as 100%) in acute-phase and symptom-free CMA infants

Total n = 22		IgE mediated n = 8(36%)	Non-IgE mediated n = 14(64%)	Stat. (Mann-Whitney)
Acute-phase n = 13 (6/7)	PMAP kinase 5 min	131	219	p = 0.007
	PMAP kinase 10 min	131	194	p = 0.005
Symptom-free n = 9 (2/7)	PMAP kinase 5 min	102	129	p = 0.5
	PMAP kinase 10 min	117	125	p = 0.8

was studied with MAP kinase antibodies by immunohistochemistry but no differences were found (14). Here we show that MAP kinase activity was comparable in samples taken from healthy infants and those with CMA but free of symptoms at the time of the study. The findings about the psoriatic lesions are well in agreement with our present ones showing that increased MAP kinase activity correlates with clinical disease activity.

Our clinical experience with the infants with acute symptoms of CMA showing increased MAP kinase activation is, that it may be impossible to get the clinically most severely reacting infants free of symptoms of CMA even by solely feeding them with amino-acid-based formula. These infants continuously show severe eczema or loose stools despite the elimination diet (unpublished data, HS). It is possible that in these infants there is an overactivation of the immune system behind this kind of high clinical reactivity. In these infants, it has also been difficult to find suitable solid foods for them later. The most severe cases are infants having only one to three solid foods in addition to the formula in their diet at the age of 1 year (unpublished data, HS).

Taken together, in the acute phase of cow's milk allergy without an elimination diet T cells of CMA infants become more activated after mitogen induction than T cells of healthy infants or those free of symptoms of CMA. We therefore hypothesize that if the symptoms of CMA are prolonged due to an inadequate elimination diet, it may lead to overactivation of T cells. This might lead to a prolonged immunoinflammatory process in the gut mucosa that can further widen the clinical allergic symptomatology.

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