

Milk-induced urticaria is associated with the expansion of T cells expressing cutaneous lymphocyte antigen

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Background: Two forms of IgE-mediated skin reactions, atopic dermatitis (AD) and urticaria, have been associated with milk allergy. The reason for these distinct reactions is poorly understood. T cells expressing cutaneous lymphocyte antigen (CLA), a unique skin-homing receptor, are known to play an important role in AD. In contrast, the role of lymphocytes in patients with milk-induced urticaria is unclear.

Objective: The expression of the skin-specific homing receptor CLA after in vitro milk protein-specific stimulation was investigated to determine whether T-lymphocyte homing to the skin plays a role in food-induced urticaria.

Methods: Fourteen patients with milk-induced urticaria but no evidence of AD were included in the study and compared with 6 children with milk-induced AD, 6 children with milk-induced gastrointestinal diseases, and 6 nonatopic and 6 atopic individuals without milk allergy. PBMCs were cultured in the presence or absence of caseins or tetanus toxoid. T-cell proliferation was determined, and T-cell phenotyping was performed by means of flow cytometry with anti-CD4, anti-CD8, and anti-CLA mAbs.

Results: After in vitro stimulation with caseins, PBMCs from patients with milk-induced urticaria and AD had a significantly greater percentage of CD4⁺ T cells expressing CLA than patients with milk-induced gastrointestinal symptoms and atopic or nonatopic control subjects. After tetanus stimulation in vitro, no significant difference between the groups was observed. T cells from both patients with milk-induced urticaria and control subjects proliferated well in response to caseins and tetanus.

Conclusion: Lymphocytes expressing CLA are selectively activated in patients with milk-induced urticaria and may play an important role in the pathogenesis of this disease. Expression of CLA is not unique to milk-induced inflammation in the skin of patients with AD and milk allergy. (*J Allergy Clin Immunol* 2002;109:688-93.)

Key words: Acute urticaria, cutaneous lymphocyte antigen, lymphocyte, allergen, cow's milk, homing receptor, skin

Abbreviations used

AD:	Atopic dermatitis
APC:	Allophycocyanin
CLA:	Cutaneous lymphocyte antigen
DBPCFC:	Double-blind, placebo-controlled food challenge
RT:	Room temperature

Cow's milk is the most common cause of food allergy in the first year of life, with approximately 2.5% of newborns experiencing allergic reactions to it during this time.¹⁻⁴ Two forms of IgE-mediated skin reactions, atopic dermatitis (AD) and urticaria, have been associated with milk allergy. The reason for these 2 distinct reactions is poorly understood.

Urticaria is reported to be one of the most common symptoms of milk allergy in young children.⁵ Children with milk-induced urticaria typically experience symptoms anywhere between a few minutes to 2 hours after ingestion. Mast cells are generally viewed as key elicitors of acute urticaria. The reaction is due to allergen-induced cross-linkage of mast cell membrane-bound IgE. Vasoactive and chemotactic mediators are released, leading to localized edema and leucocyte infiltrations.^{6,7}

AD also has been associated with IgE-mediated mast cell activation, with release of preformed mediators and newly generated cytokines.^{8,9} In addition, it is well established that T cells play an important role in AD. Lymphocytes expressing T_H2 cytokines account for the vast majority of cells found in eczematous lesions.¹⁰ Memory T cells that infiltrate the skin express a unique skin-homing receptor designated cutaneous lymphocyte antigen (CLA).¹¹ After in vitro stimulation of PBMCs with casein, patients with AD and milk allergy had a significantly greater percentage of CLA⁺ T cells than control subjects with milk-induced gastrointestinal diseases or nonatopic, healthy control subjects.¹²

In contrast to AD, the role of lymphocytes in the pathogenesis of acute allergen-induced urticaria is less well defined. Urticaria is an acute reaction caused by activation of cutaneous mast cells. It was hypothesized that lymphocytes from patients with eczematous skin lesions would express CLA, whereas lymphocytes from patients with urticaria would not. The present study investigated whether T cells from the peripheral blood of patients with milk-induced urticaria express CLA after allergen stimulation.

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TABLE I. Characterization of the study groups

	No. of patients	Age (y), mean (range)	Cow's milk-specific IgE, mean (range)
Patients with urticaria	14	5 (2-11)	17.73 (<0.35->100)
Patients with AD	6	3 (1-7)	45.34 (1.39->100)
Patients with GI symptoms	6	8 (3-13)	0.88 (<0.35-62.40)
Atopic control subjects	6	4 (1-10)	1.67 (0.43-5.30)
Nonatopic control subjects	6	34 (25-55)	<0.35

GI, Gastrointestinal.

METHODS

Study population

Five different groups were included in the study. The first group was composed of 14 children with milk-induced urticaria and no history of AD. The second group included 6 children with milk-induced AD, and the third group included 6 children with milk-induced gastrointestinal symptoms. The fourth group, 6 children with sensitization to cow's milk but negative double-blind, placebo-controlled food challenge (DBPCFC) results, was used as an atopic control group. The fifth group included 6 nonatopic adults. In all patients and control subjects cow's milk-specific IgE levels were determined by using the Pharmacia CAP System FEIA (Pharmacia-Upjohn). These cow's milk-specific IgE levels, as well as the age range of the study groups, are shown in Table I. All patients included in the study were seen in our food allergy clinic and had a convincing clinical history. In all children with cow's milk-specific IgE levels of less than 15 kU_A/L, the diagnosis was confirmed by means of DBPCFC. Children with cow's milk-specific IgE levels of greater than 15 kU_A/L were excluded from oral food challenges because it was previously shown that these IgE levels elicit greater than 95% positive predictive values for positive clinical reaction.¹³ In detail, in 4 of 14 children in the urticaria group, in 1 of 6 children in the AD group, in 5 of 6 children in the gastrointestinal symptom group, and in all children in the atopic control group, DBPCFCs were performed. Informed consent was obtained, and the study was approved by the Institutional Review Board of Mount Sinai School of Medicine.

Isolation of PBMCs

PBMCs were purified from heparinized blood by means of density-gradient centrifugation on Ficoll-Paque Plus (Amersham Pharmacia) for 30 minutes at 400g at room temperature (RT). Cells from the interface were washed 3 times for 10 minutes at 450g at RT with RPMI medium (Mediatech) supplemented with L-glutamine, penicillin, and streptomycin (Gibco).

Lymphocyte proliferation

Isolated PBMCs (2×10^6 /mL) were cultured in triplicate in flat-bottom 96-well plates (200 μ L/well) in the presence or absence of antigen in AIM V (Gibco) supplemented with L-glutamine and amphotericin B (Gibco) to assess proliferation. The following antigens were used: α -casein (500 μ g/mL), β -casein (500 μ g/mL, Sigma), and tetanus toxoid (5 μ g/mL, List Biological Laboratories). After 7 days of culture (37°C, 5% CO₂ atmosphere), cells were pulsed with 1 μ Ci of tritiated thymidine (ICN Pharmaceuticals) per well, collected with a cell harvester 8 hours later (Tomtec), and counted in a scintillation counter (Wallac Oy). Stimulation indices were calculated as the quotient of stimulation in the presence and absence of antigen (in counts per million).

Cell culture

Isolated PBMCs (2×10^6 /mL) were cultured in 24-well plates with or without casein (500 μ g/mL α - and β -casein in equal

amounts, Sigma) or tetanus toxoid (5 μ g/mL, List Biological Laboratories) in AIM V medium (Gibco) supplemented as described above. Cells were harvested after 7 days of culture (at 37°C in a 5% CO₂ atmosphere) to perform T-cell phenotyping.

Flow cytometry

The phenotype of antigen-stimulated and nonstimulated cells was determined by means of flow cytometry with FITC-labeled anti-CLA, allophycocyanin (APC)-labeled anti-CD4, and APC-labeled anti-CD8 mAb (BD PharMingen). As a negative isotype control, FITC-labeled recombinant IgM and APC-labeled mIgG1 were used. Harvested lymphocytes were incubated with mAb for 30 minutes at 4°C in the dark. After washing 3 times with PBS (10 minutes, 250g, RT), cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometric Systems). Analysis was performed with a gate for lymphocytes.

Statistical analysis

Data were tested for normal distribution. When normally distributed, the Student *t* test was performed with Graph Pad Prism software. Differences associated with *P* values of less than .05 were considered significant.

RESULTS

The expression of the skin-specific homing receptor CLA on T cells was determined after in vitro milk protein stimulation to investigate the role of lymphocyte homing to the skin in milk-induced urticaria. We studied PBMCs from 5 different subject populations: (1) a group of children with milk-induced urticaria; (2) a group of children with milk-induced AD; (3) a group of children with milk-induced gastrointestinal symptoms; (4) a group of cow's milk-sensitized atopic children without milk allergy; and (5) a group of nonatopic, healthy adults. The children with milk-induced urticaria had no history of AD.

The expression of CLA was examined on CD4⁺ and CD8⁺ lymphocytes after 7 days of culture in medium alone or after stimulation with casein. In the medium culture there was no difference in the CLA expression among the pediatric study groups (Fig 1). However, the nonatopic control group, which consisted of adults, had a significantly higher expression of CLA in CD4⁺ cells than the pediatric study groups (*P* < .002 in comparison with children with urticaria and *P* < .05 in comparison with the 3 other groups; Fig 1, A). The adult control group also had a slightly higher expression of CLA on CD8⁺ cells, which was significant in comparison with that seen in children with AD and atopic control subjects (*P* < .05; Fig 1, B).

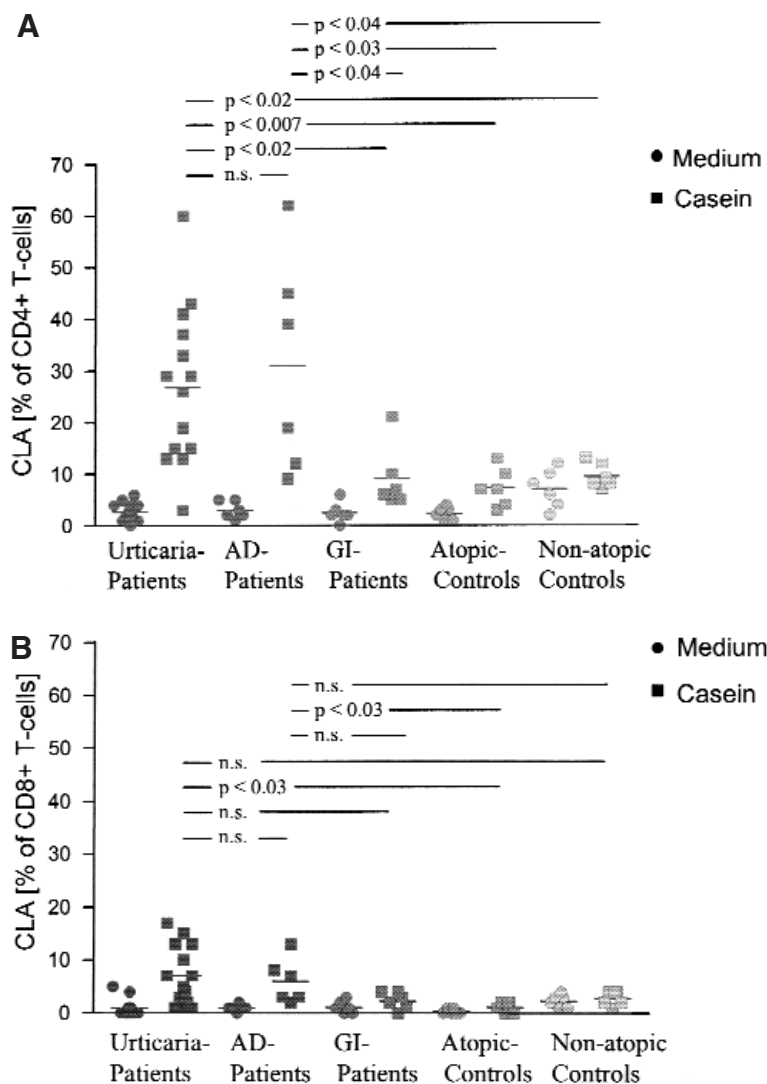


FIG 1. CLA expression on CD4⁺ (A) and CD8⁺ (B) T cells in children with milk-induced urticaria (n = 14), AD (n = 6), or gastrointestinal symptoms (n = 6), as well as in atopic control subjects without milk allergy (n = 6) and nonatopic adult control subjects (n = 6). Cells were analyzed after 7 days of culture in medium alone or after in vitro stimulation with casein. *GI*, Gastrointestinal; *n.s.*, not significant.

After casein stimulation, the percentage of CLA in CD4⁺ cells rose significantly in the children with urticaria ($P < .0001$; Fig 1, A, and 2). A similar increase was observed in children with milk-induced AD ($P < .03$; Fig 1, A). A small but significant increase was also observed in the children with milk-induced gastrointestinal symptoms ($P < .05$; Fig 1, A) and the atopic control group ($P < .01$; Fig 1, A). However, the level of CLA in CD4⁺ T cells after casein stimulation was markedly higher in patients with milk-induced skin symptoms, urticaria, or AD compared with that seen in either the children with milk-induced gastrointestinal symptoms ($P < .02$ and $P < .04$; Fig 1, A), the atopic group ($P < .007$ and $P < .03$; Fig 1, A), or the nonatopic control group ($P < .02$ and $P < .04$; Fig 1, A). The increase of CLA cannot be explained through differences in IgE antibody levels.

No correlation between IgE levels and CLA was observed in the patients with milk-induced skin symptoms (data not shown). The percentage of CLA in CD8⁺ cells also rose significantly in the children with urticaria ($P < .0008$; Fig 1, B) but was significantly higher only compared with that in the atopic control subjects ($P < .03$; Fig 1, B).

Lymphocytes were also stimulated with a nonspecific antigen to show the antigen specificity of the CLA increase after casein stimulation. After stimulation with tetanus toxoid, no differences in the expression of CLA on CD4⁺ or CD8⁺ cells was observed among the 5 study groups (Fig 3). In addition, the expression of CLA on CD4⁺ lymphocytes after casein stimulation in the patients with urticaria was significantly higher than the expression after tetanus stimulation in all 5 groups (Fig 3).

As shown in Table II, PBMCs from all 5 study groups proliferated well in response to casein and tetanus. Although the children with milk-induced urticaria and AD appear to have higher stimulation indices for α - and β -casein, the differences were not statistically significant (Table II).

DISCUSSION

In the present study we were able to show that PBMCs from children with milk-induced urticaria show an activation of CLA⁺ lymphocytes after in vitro milk protein stimulation similar to that seen in children with milk-induced AD. CLA is thought to be a skin-specific homing receptor. It has been reported that CLA is expressed on 85% of lymphocytes in inflammatory skin lesions but only in 5% of cells in extracutaneous sites.¹⁴ In the peripheral blood CLA is expressed on only 10% to 23% of CD4⁺ T cells and in 9% to 15% of CD8⁺ T cells.¹⁴ The expression of CLA on T cells increases with age, ranging from less than 1% in cord blood to over 10% in adults.¹⁵ This explains the higher levels of CLA expression under baseline conditions in our nonatopic adult control group.

T cells migrating into the skin are highly enriched for CLA⁺ memory-effector T cells.¹⁶ It was shown previously that after casein stimulation in vitro, children with AD and milk allergy had a significantly higher percentage of CLA⁺ T cells than control subjects with milk-induced gastrointestinal disease or nonatopic, healthy control subjects.¹² However, a relative increase was observed in all study groups, and children with AD had significantly higher baseline values than children with gastroenteropathy.¹² It could be speculated that the higher levels in infants with eczema might be due to these differences in baseline levels. In the present study a relative increase after in vitro casein stimulation also was observed in all study groups. However, there were no differences in the baseline levels among children with eczema, urticaria, or gastroenteropathy or atopic control subjects. This might be explained through differences in the study design. In the study by Abernathy-Carver et al¹² freshly isolated peripheral blood T cells were used for baseline values, whereas in the present study cultured lymphocytes without allergen stimulation were used. We can conclude from the present study that differences in baseline levels cannot explain the significantly higher levels of CLA⁺ T cells seen after allergen-specific stimulation in children with milk-induced urticaria or AD. Moreover, the only group with significantly higher baseline levels in the present study was the nonatopic adult group, and this group showed no increase after casein stimulation.

The results from the present study emphasize that lymphocyte infiltration into the skin not only plays an important role in milk-induced AD but also in milk-induced urticaria. E-selectin serves as the ligand for CLA.¹⁷ E-selectin is absent in the skin of nonatopic, healthy individuals.^{18,19} In patients with AD, however, E-Selectin was observed in nonlesional skin and is even more pronounced in lesional skin.¹⁸ E-selectin expres-

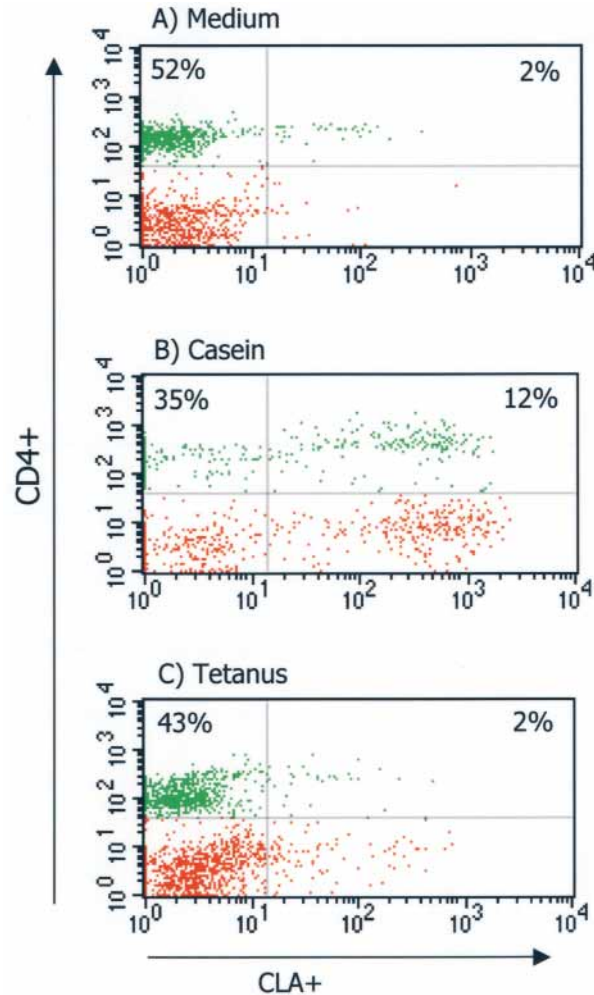


FIG 2. Flow cytometric analysis of CD4⁺ cells for CLA in PBMCs cultured in medium only (A), casein (B), or tetanus toxoid (C). Shown is one representative patient with milk-induced urticaria. Casein induces a higher percentage of CLA⁺ CD4⁺ cells.

sion can be upregulated by TNF- α . Concomitant with the increase of adhesion marker expression, an increase in the number of TNF- α -containing cells was observed in the skin of patients with AD.¹⁸ In addition, it was shown that TNF- α secretion from milk-stimulated PBMCs was significantly increased in children with milk-induced cutaneous symptoms.²⁰ Although histamine does not affect E-selectin expression,^{19,21} it enhances the TNF- α -induced expression of E-selectin in a concentration-dependent fashion.²¹ Taking these findings together, it can be speculated that allergen exposure in children with milk-induced AD is followed by an expression of CLA on lymphocytes paralleled by an upregulation of its ligand, E-selectin, through TNF- α and histamine. This then allows lymphocytes to migrate to the skin. Although TNF- α secretion from milk-stimulated PBMCs was also found in children with milk-induced gastrointestinal symptoms,²⁰ most of these PBMCs do not express CLA and therefore are not likely to migrate into the skin.

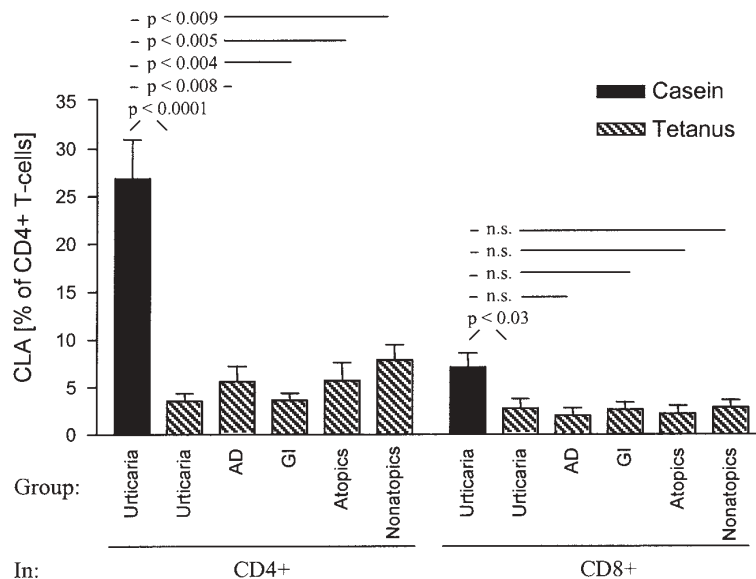


FIG 3. CLA expression on CD4⁺ and CD8⁺ T cells in patients with milk-induced urticaria (n = 14), AD (n = 6), or gastrointestinal symptoms (n = 6), as well as in atopic control subjects without milk allergy (n = 6) and nonatopic control subjects (n = 6). Cells were analyzed after 7 days of culture in casein or tetanus toxoid. GI, Gastrointestinal; n.s., not significant.

TABLE II. Lymphocyte proliferation in children with milk-induced urticaria, AD, and GI symptoms as well as in atopic and nonatopic control subjects

	α -Casein	β -Casein	Tetanus toxoid
Patients with urticaria (n = 14)	21.2 $\pm$ 5.9	23.5 $\pm$ 7.3	5.7 $\pm$ 1.7
Patients with AD (n = 6)	16.2 $\pm$ 6.5	16.6 $\pm$ 8.0	4.9 $\pm$ 2.4
Patients with GI symptoms (n = 6)	4.9 $\pm$ 2.0*	22.9 $\pm$ 16.7	8.7 $\pm$ 6.6
Atopic control subjects (n = 6)	3.0 $\pm$ 0.5†	13.3 $\pm$ 7.0	6.4 $\pm$ 2.6
Nonatopic control subjects (n = 6)	3.9 $\pm$ 1.3‡	2.7 $\pm$ 1.0§	6.1 $\pm$ 3.2

Shown is the stimulation index (mean $\pm$ SEM). No statistically significant difference was observed. However, in comparison with the urticaria group the following trends were found: * P = .1, † P = .06, ‡ P = .08, and § P = .08.

Looking at PBMCs in patients with milk-induced acute urticaria, the findings are very similar. E-selectin expression is markedly upregulated in lesional and less so in non-lesional skin of patients with acute urticaria.²² In parallel, an upregulation of TNF- α expression was found.²³ In addition, most of the children with milk-induced cutaneous symptoms who showed an increased TNF- α secretion from milk-stimulated PBMCs were children with urticaria.²⁰

Although our results demonstrate an increased percentage of peripheral blood T cells expressing CLA in infants with milk-induced urticaria, and it is known that E-selection is upregulated in lesional skin,²¹ the final proof of the pathogenic importance of T lymphocytes in milk-induced urticaria will require skin biopsy. To our knowledge, the microscopic morphology of skin biopsy specimens in children with milk-induced urticaria is not known. However, in an extensive study of 108 patients with diverse types of urticaria, including 20 patients with acute and 49 patients with chronic urticaria, Haas et al²⁴

showed that a mononuclear cell infiltrate was present in all types of urticaria, with a significant difference between involved and uninvolved skin. In patients with acute urticaria, mononuclear cells were most prominent, and in 40% of patients with chronic urticaria, the infiltrate consisted of mononuclear cells only. In addition, T lymphocytes were found in the cellular infiltrate of patients with chronic idiopathic urticaria.²⁵

Viewing the present findings together with recent data from the literature, the mechanisms distinguishing the 2 forms of IgE-mediated skin reactions associated with milk allergy, AD, and urticaria, are still not understood. Allergen-specific CLA expression on lymphocytes cannot explain the reason for these 2 distinct reactions. Lymphocytes are able to upregulate CLA after milk-specific stimulation in both patients with milk-induced AD¹² and patients with milk-induced urticaria. However, the role of lymphocytes in food allergen-induced urticaria will need to be further clarified.

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