The mucosal adhesion receptor α4β7 integrin is selectively increased in lymphocytes stimulated with β-lactoglobulin in children allergic to cow's milk

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Background: It has been shown in mice that the integrin α4β7 directs the migration of memory T cells into the gut-associated lymphoid tissue. However, little is known about T-cell homing mechanisms in children with food allergies.
Objective: We investigated the expression of this and other integrins in children with different manifestations of cow's milk allergy (urticaria, atopic dermatitis, and wheezing).
Methods: PBMCs were stimulated with β-lactoglobulin, 1 of the major allergenic proteins in cow's milk, and tetanus toxoid. Integrin expression was studied by flow cytometric analysis after 1 week of culture.
Results: We found significantly higher expression of the α4β7 integrin in cells from patients compared with control subjects with no allergies ($P = .005$) when β-lactoglobulin was used to stimulate the cells. α4β7 integrin was also expressed at significantly higher levels in β-lactoglobulin–stimulated cells than in tetanus toxoid-stimulated cells ($P = .005$). The αEβ7 and the α4β1 integrins were not upregulated by allergen stimulation. Most α4β7 integrin–expressing cells were identified as $CD4^+$ T cells.
Conclusion: These results show that α4β7 integrin expression after stimulation with β-lactoglobulin correlates with the presumptive site of cow's milk sensitization (ie, the gut-associated lymphoid tissue but not with the site of symptoms of cow's milk allergy). (J Allergy Clin Immunol 1999;103:931-6.)

Key words: *Food hypersensitivity, cow's milk, atopic dermatitis, lymphocyte homing, integrins, L-selectin, suppressor T lymphocytes, human*

Abbreviations used
AD: Atopic dermatitis
BLAC: β-Lactoglobulin
MFI: Mean fluorescence intensity
SI: Stimulation index
TT: Tetanus toxoid

The immune response to food antigens can lead to tolerance in most individuals or to allergic reactions in a minority of individuals. Although many factors controlling this outcome are unknown, T lymphocytes appear to control the response to food allergens. Blood lymphocytes have yielded some information about T cells in food allergy. PBMCs from patients with food allergy exhibit antigen-specific cell proliferation when stimulated in vitro with milk,[1,2] egg,[3,4] or peanut[5,6] antigens. PBMCs from many food-tolerant individuals, however, show the same feature. T_{H2}-like T cells are thought to be responsible for food-allergy reactions through stimulation of IgE synthesis and eosinophil recruitment, although evidence for this mechanism in food allergy is still under investigation.[7-9]

Integrins are heterodimeric-membrane proteins that mediate cell-cell or cell-matrix adhesion. T cells residing in the gut-associated lymphoid tissue are characterized by the expression of the integrin α4β7, which interacts with the mucosal endothelial ligand mucosal addressin cell adhesion molecule-1. This integrin directs naive T cells to Peyer's patches and memory T lymphocytes into intestinal but not pulmonary mucosal lymphoid tissue, including intestinal lamina propria.[10-12] The α4β7 integrin confers primary reversible adhesion and secondary stronger adhesive forces in the multistep process of adhesive interaction between lymphocytes and endothelium. Initial contact with endothelium in the gut, however, is established by L-selectin, which is also involved in migration to extraintestinal lymph nodes. The α4β7 integrin has been found upregulated on rotavirus-induced T-cell blasts, linking the immune response to rotavirus to the gut-associated immune system.[13] It has recently been reported that memory B cells responsible for the secretory IgA to this virus carry this integrin.[14] The integrin αEβ7 is specific for intraepithelial lymphocytes and binds to the epithelial adhesion molecule E-cadherin.[15] The α4β1-integrin (very late antigen-4) has been implicated in the recruitment of T cells to extraintestinal sites of inflammation, such as lungs and skin.[16,17] It interacts with the vascular cell adhesion molecule-1 and fibronectin.[18]

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TABLE I. Phenotype of PBMCs from patients with cow's milk allergy or control subjects 7 days after culture with BLAC or TT

	Patients with allergy		Control subjects	
	BLAC-stimulated	TT-stimulated	BLAC-stimulated	TT-stimulated
$CD3^+$	80.2 ± 8.1	75.6 ± 23.7	90.8 ± 1.7	81.6 ± 9.3
$CD3^+CD4^+$	61.7 ± 9.7	49.9 ± 23.6	56.9 ± 2.9	58.1 ± 7.9
$CD3^+CD8^+$	13.3 ± 2.6	24.3 ± 18.6	25.9 ± 2.9	19.2 ± 3.4

Values represent mean percentage ± SEM.

Here, we report the expression of the α4β7, αEβ7, and α4β1 integrins and L-selectin on β-lactoglobulin (BLAC)–stimulated T cells from patients with cow's milk allergy and healthy control subjects.

METHODS

Patients

Heparinized peripheral blood was obtained from 9 children with IgE-mediated cow's milk allergy. Patients were given a diagnosis as a result of either a positive milk challenge or a suggestive history of a recent anaphylactic reaction to cow's milk with elevated milk-specific serum IgE antibody levels and positive skin prick tests. The criteria for clinical reactivity to cow's milk according to the level of specific IgE were set as previously published.[19] Six age-matched children who were clinically tolerant to milk served as control subjects. This study was been approved by the Institutional Review Board of the Children's Hospital, and parents of the subjects gave informed consent.

Cell preparation and culture

PBMCs were isolated on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The cells were washed and resuspended at 4×10^6 cells/mL in RPMI 1640 medium with 5% autologous plasma, supplemented with 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Basel, Switzerland). PBMCs (10^5 cells per well) were cultured for 7 days in flat-bottom 96-well plates in the presence of purified BLAC (final concentration, 285 μg/mL) (Sigma Chemicals, Buchs, Switzerland) or tetanus toxoid (TT; Institut Pasteur, Lyon, France) 1:1000. The cells were then pulsed for 6 hours with 1 μCi ^{3}H-labeled thymidine (Amersham, Basel, Switzerland), harvested, and counted in a liquid scintillation counter (Beckman, Fullerton, Calif). Results of triplicate test conditions were expressed by average counts per minute and by stimulation index (SI = counts per minutes in the presence of antigen/counts per minute in media alone).

Alternatively, after 7 days of culture, BLAC (final concentration, 285 μg/mL) or TT (1:1000) stimulated cells were harvested for flow cytometric analysis.

Cell staining and flow cytometric analysis

Monoclonal antibodies recognizing a specific epitope of the dimer α4β7-integrin (Act-1) were provided by Leukosite (Cambridge, Mass). Phycoerythrin-conjugated mAb to CD3 (UCHT1), mAb to CD8 (DK25), and FITC-conjugated mAb to CD3 (UCHT-1) were purchased from Dako (Glostrup, Denmark). Monoclonal antibody 19H8, which recognizes a specific epitope of α4β1, was a gift from Dr B. McIntyre (MD Anderson Cancer Center, Houston, Texas). FITC-conjugated mAb to CD62L (FMC46) and to CD49d (44H6), the α4 integrin chain, were obtained from Serotec (Oxford, UK). Monoclonal antibody to αEβ7 (2G5) was purchased from Immunotech (Marseille, France). Isotype controls were IgG1 (1B7.11) and IgG2a (Immunocontact, Bioggio, Switzerland). Monoclonal antibody for second-step labeling to the unconjugated mAb Act-1 and 19H8 and were FITC-conjugated goat anti-mouse antibody (Tago, Burlingame, Calif) or FITC-conjugated goat anti-rat antibody (Caltag, Burlingame, Calif).

One hundred fifty thousand cells per condition were double-stained with mAb to CD4 and the corresponding receptor. For each step, cells were incubated on ice for 20 minutes with mAbs in 100 μL of PBA-buffer (1× PBS with 1% BSA [fraction V; Sigma Chemicals]) and then washed with 1 mL of PBA-buffer. For staining of unconjugated mAbs, additional steps were to the corresponding secondary antibody with the second color. Background was assessed by staining with an isotype control. Flow cytometric analysis with counts of 10^4 events were performed with an EPICSXL Coulter flow cytometer (Coulter, Hialeah, Fla). Mean fluorescence intensity (MFI) and the percentage of positive cells above the gate set with an isotype control were determined.

Statistical analysis

The MFI and cell percentages from various groups were analyzed by a nonparametric test for unpaired data (Wilcoxon rank sum test).

RESULTS

We investigated PBMCs from children with cow's milk allergy. Nine patients were studied (5 boys and 4 girls; median age, 28 months; range, 7 months to 9.3 years). All patients had positive cow's milk–specific IgE by CAP-RAST (median, 39.5 kU/mL; range, 3.9 to 52.7 kU/mL). Symptoms observed in these patients consisted of urticaria (8 patients), atopic dermatitis (AD; 4 patients), wheezing (3 patients), and gastrointestinal symptoms (1 patient). Patients with AD had either a suggestive maculopapular rash immediately after the challenge or worsening of AD after prolonged exposure. Six age-matched children who were milk tolerant were used as healthy control subjects.

PBMCs of patients and control subjects were stimulated with BLAC. The SI was determined after 7 days of culture. The mean SI in patients with allergy was 7.1 ± 6.1 and in control subjects was 2.7 ± 2.8 (P = not significant). The phenotype of the cell populations obtained after 7 days of culture with BLAC or the control antigen TT was determined in parallel by flow cytometry (Table I). With either antigen, most cells were $CD3^+$. An average of 80% of BLAC-stimulated $CD3^+$ cells from patients with allergy were $CD4^+$, and only a few were $CD8^+$. The average percentage of $CD3^+CD4^+$ cells in patients with allergy was comparable to control subjects (P = not significant), although the average percentage of $CD3^+CD8^+$ was significantly lower in patients with allergy than in control subjects (P = .02). The percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ in TT-stimulated cultures from patients with allergy and control subjects were similar and did not differ from BLAC-stimulated cells.

BLAC-stimulated cultures in patients with allergy express the α4β7 but not the α4β1-integrin.

In a preliminary experiment, sequential expression of

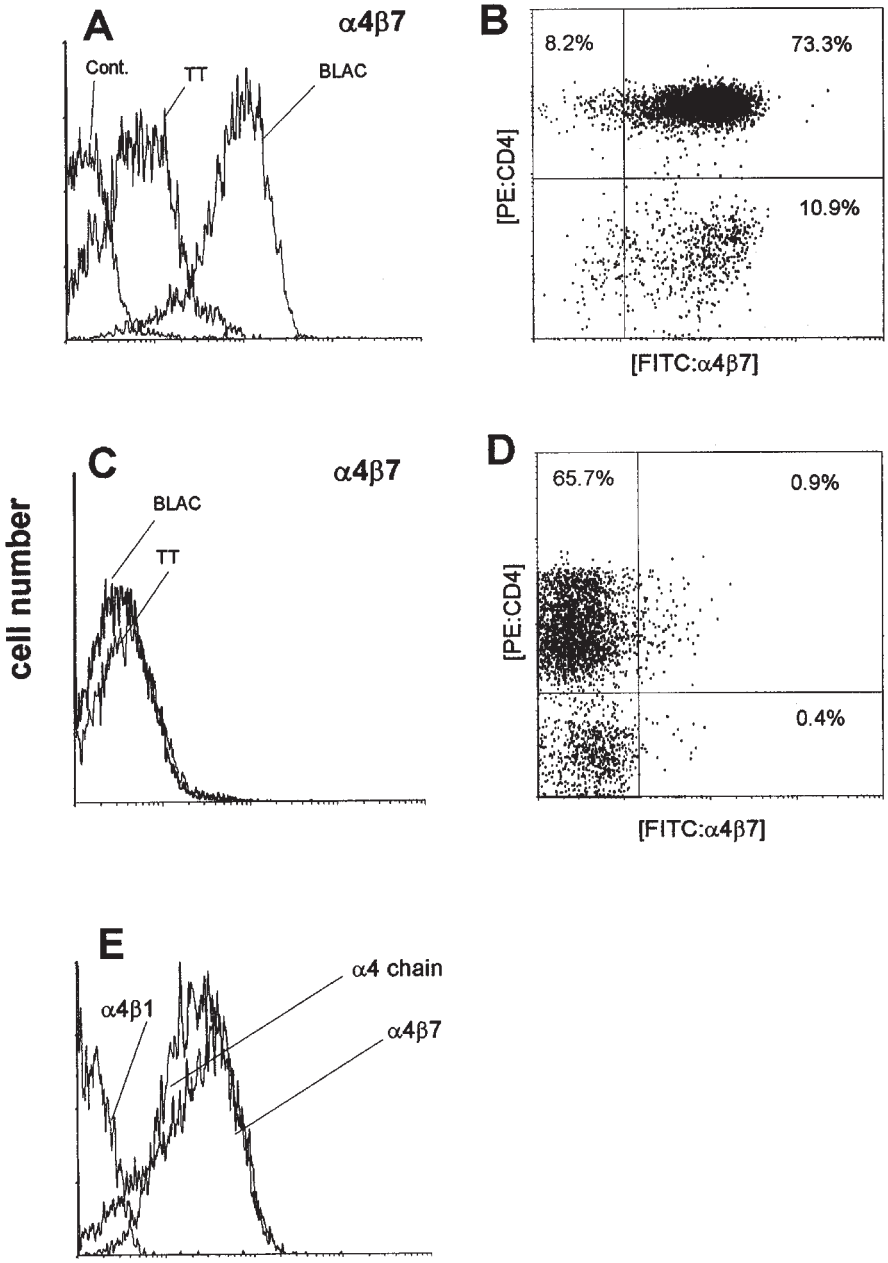

FIG 1. A, A representative experiment of α4β7 integrin expression in cells from a patient with cow's milk allergy cultured either in the presence of BLAC or TT. Gates were set with nonlabeled isotype control subjects followed by labeled second-step reagents. *Cont*, Isotype control. **B**, Dot plot from the same experiment with cells double-stained for CD4 and α4β7 integrin. **C** and **D**, Results from identical conditions with cells from a control subject. **E**, Overlay of α4β7, α4 chain, and α4β1 expression in a patient.

α4β7 and α4β1 was examined at 3, 8, and 15 days of culture (Table II). Results from 2 patients showed low fluorescence in cells cultured in the presence of either antigens (BLAC and TT) at 3 days, but high fluorescence at days 8 and 15 in BLAC-stimulated cells only. Experience from previous studies in which cells were not viable at 15 days of culture directed us to measure α4β7 expression at 8 days. Expression of α4β1 remained low at all times (data not shown).

TABLE II. Time course expression of α4β7-integrin expression in lymphocytes from 2 patients with cow's milk allergy cultured with BLAC or TT

	Day 3		Day 8		Day 15	
	BLAC	TT	BLAC	TT	BLAC	TT
Patient 1	14.9	13.7	30.4	18.7	33.8	25.3
Patient 2	15.9	17.9	35.0	21.1	51.2	32.2

All results are expressed in MFI.

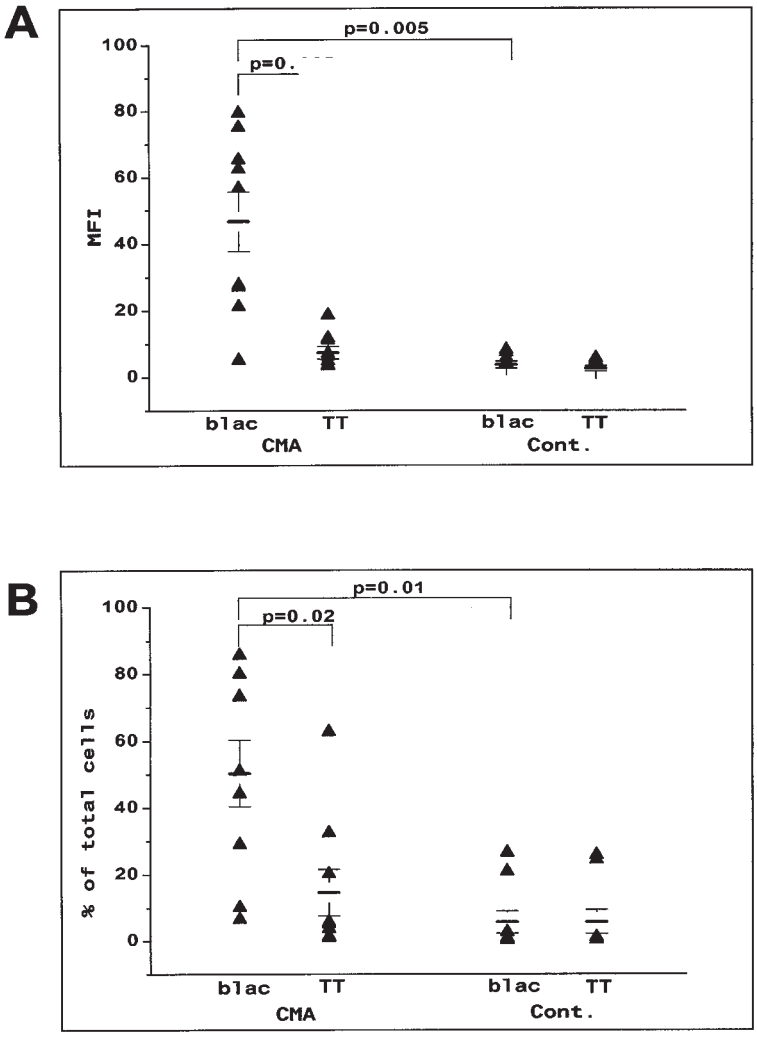

FIG 2. A, MFI of α4β7 integrin–positive cells from patients with cow's milk allergy (*CMA*) and milk-tolerant control subjects (*Cont*) after culture with BLAC or TT. **B**, Results from the same experiments expressed in percentages of total $CD4^+$ cells. Mean percentage values ± SEM.

Fig 1, *A* and *B*, show the results of a representative experiment with BLAC in a patient with cow's milk allergy. There was a shift of MFI in BLAC-stimulated cells, most of them being $\alpha4\beta7^{high}$, although TT-stimulated cells were mostly $\alpha4\beta7^{low}$ (Fig 1, *A*). Two-color staining with CD4 mAb and mAb to the α4β7-integrin (Fig 1, *B*) shows most $CD4^+\alpha4\beta7^+$ cells. Some $CD4^-$ cells, most likely $CD8^+$ cells, also expressed α4β7 integrin. Because most of the cells after culture with either BLAC or TT were $CD4^+$, expression of the α4β7 integrin was further measured on $CD4^+$ cells. In a control subject, α4β7 staining after stimulation with either antigen was negative (Fig 1, *C* and *D*). To verify specificity of the α4β7 dimer staining, cells cultured in presence of BLAC were stained with mAb to the α4 chain (CD49d) and with an antibody specific to the α4β1 dimer (clone 19H8). One representative experiment of 4 with cells from patients is depicted on Fig 1, *E*. A similar profile was observed with mAb to α4β7 and to the α4-chain (CD49d). However, expression of the α4β1 dimer was absent. These results confirm the specificity of our results for the α4β7 integrin staining.

The MFI for α4β7 integrin expression in $CD4^+$ T cells of patients with allergy and control subjects are shown in Fig 2, *A*. They were significantly higher in BLAC-stimulated cells from patients with cow's milk allergy than in control subjects (mean MFI, 46.7 ± 8.9 vs 3.7 ± 1.0; $P < .005$). In both groups, TT-stimulated $CD4^+$ T cells were $\alpha4\beta7^{low}$. In the group of patients with allergy, MFI for BLAC-stimulated cells were significantly higher than for TT-stimulated cells (mean MFI, 46.7 ± 8.9 vs 7.4 ± 1.9; $P < .005$). Similar results were obtained when the percentage of $\alpha4\beta7^+$ cells were determined (Fig 2, *B*).

No expression of αEβ7 integrin was observed in cultured cells from either patients with allergy or control subjects stimulated with either antigen (data not shown).

We finally explored the presence of L-selectin in cells obtained by either culture condition. The percentage of $CD4^+$ L-$selectin^+$ cells was consistently high (32.8% to 80.2%) and without statistically significant differences between the study conditions (data not shown).

DISCUSSION

The expression of α4β7 integrin in $CD4^+$ T cells was clearly increased in patients with allergy when compared with control subjects with no allergy 7 days after the stimulation of PBMCs with BLAC but not with TT. Thus α4β7 expression correlated with cow's milk allergy and stimulation with BLAC. The labeling for α4β7 appeared to be specific for α4β7 because staining for the α4 chain showed identical results, but largely negative results for α4β1. The increase in α4β7 staining was also selective in that neither α4β1 nor αEβ7 staining was increased in cells from patients with cow's milk allergy. The most likely explanation for this correlation is that only patients with cow's milk allergy have, in their circulating T-lymphocyte pool memory, T cells that recognize BLAC and that induce α4β7. It is intriguing that various anatomic sites and symptoms of cow's milk allergy expression (urticaria, AD, wheezing, gastrointestinal symptoms), all presumably at least in part IgE-dependent, were all associated with increased α4β7 levels. Only 1 patient had gastrointestinal symptoms. Thus α4β7 expression did not correlate with the site of allergy expression (skin, lung) or the symptom (urticaria, AD) but with the presumable site of allergen sensitization (ie, the gut-associated immune system). This is in contrast to the cutaneous lymphocyte-associated antigen, which correlates with the site of allergy expression (ie, skin).[17,20,21] Lymphocyte infiltrates have been objectivated in lesions of AD and might mediate a sequential response in the pathogenesis of the disease.[22,23] This response might be predominantly mediated by IgE in the early-phase and by lymphocytes in the late-phase. In patients with IgE-mediated food allergy, immediate symptoms are the predominant clinical features provoked by mast-cell mediator release.[24] One could argue that effector T cells in AD are required to migrate to the skin to induce late-phase symptoms of dermatitis, unlike regulatory T cells in milk allergy associated with α4β7. These T cells could induce allergic symptoms remote from the site of allergy expression by stimulating the synthesis of anaphylactogenic antibodies that are transferred to the site of allergy expression.

Most of the α4β7-reactive cells were identified to be $CD4^+$ T cells. A few α4β7-expressing cells appeared to be $CD8^+$. In cultures from some individuals, most BLAC-stimulated cells expressed α4β7. It is unlikely that all α4β7-reactive cells in these cultures were antigen specific or that all $\alpha4\beta7^+$ cells are gut-homing lymphocytes. Nevertheless, stimulation with BLAC represents a potent inducer of α4β7 in PBMCs from patients with food allergy. The α4β7 integrin was induced during culture because this integrin was expressed on 2% or fewer of freshly isolated T cells (data not shown), as reported.[25] The degree of T-cell proliferation does not directly correlate with integrin expression (Pearson correlation, 0.416). This is supported by the absence of a statistically significant difference in proliferation of cells from patients with allergy or control subjects, although there is a significant difference of integrin expression. This observation suggests that integrin expression in patients with allergy is upregulated by an antigen-specific trigger. There was no correlation between the CD8 numbers and integrin expression (correlation, 0.247). The mechanisms underlying the induction of α4β7 are largely unknown, but the present system offers an explorative assay system.

Lymphocyte proliferation induced by food allergen cannot distinguish between healthy subjects and patients with food allergy.[1] If α4β7 expression can be further validated and correlated with other forms of food-induced allergy, such as allergy to peanut, α4β7 expression in allergen-stimulated PBMC may be developed into a new diagnostic tool.

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