

Large number of CD19⁺/CD23⁺ B cells and small number of CD8⁺ T cells as early markers for cow's milk allergy (CMA)

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Assessment of activation of immune mechanisms is valuable in the early diagnosis of cow's milk allergy (CMA). The purpose of this study was to evaluate peripheral blood lymphocyte subclasses in children suspected of having CMA and healthy infants in order to detect an early marker for food allergy. Altogether 47 breast-fed infants, aged from 0.4 to 10 months were followed-up prospectively from birth because of atopic heredity. Twenty-three of the infants were healthy and 24 infants had a strong suspicion of and later challenge-proven cow's milk allergy. Leucocyte subsets were determined from peripheral blood mononuclear cells by flow cytometry. In response to a clinical cow's milk challenge, seven infants developed urticaria, 11 infants had eczema, three patients had loose stools, diarrhoea or vomiting and three infants had eczema and diarrhoea, loose stools or vomiting. The total percentage of B cells and also the proportion of B cells bearing a low-affinity IgE receptor as a marker for activation were significantly higher, whereas the percentage of CD8⁺ T cells was significantly lower in infants with challenge-proven CMA than in healthy controls. These results imply that infants with active CMA have a defect in regulation of B-cell function. Further, they suggest that imbalance of the ratio of suppressor and helper T cells might be an important factor in the etiopathogenesis of CMA. Our results show that large numbers of activated CD19⁺ B cells and low numbers of CD8⁺ T cells could be considered as early markers for food allergy since they are already detectable in peripheral blood during the earliest symptoms of CMA.

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Cow's milk allergy (CMA) is an adverse reaction to cow's milk antigens that is mediated by immune mechanisms (1). Since there are no diagnostic *in vitro* tests, CMA is still diagnosed by the clinical response to withdrawal and subsequent oral challenge with cow's milk. The activation of immune mechanisms should be demonstrated in order to meet the definition.

Assessment of the activation of immune mechanisms is important in the early diagnosis of CMA. Little is known about the significance of activation markers in children with early symptoms of CMA. The diagnosis of food allergy is nowadays time consuming, because of the long elimination period and

the oral challenge procedure. Therefore, a marker reflecting CMA would be useful as a tool in accelerating the diagnosis process.

Subjects and methods

Subjects

The study population comprised 47 breast-fed infants with atopic heredity (atopic sibling and/or parent(s)). Twenty-three of the children were healthy and 24 had symptoms suggestive of CMA (10 cutaneous, three gastrointestinal and 11 both cutaneous and gastrointestinal symptoms). At the time of the

study, 14 of the 24 infants with suspicion of CMA showed symptoms of CMA and 10 had shown symptoms earlier but were currently symptom-free, because of an adequate elimination diet. Of the healthy infants, eight (35%) were exclusively breast-fed and 15 had also cow's milk-based formula and solid foods appropriate for age. Ten (42%) of the 24 infants with later challenge-proven cow's milk allergy were exclusively breast-fed and 14 (58%) also had cow's milk-based formula and solid foods.

Study protocol

This was a follow-up study. All the infants were selected because of atopic heredity. They were enrolled in the study at geometric mean (95% CI) 1.2 (1.5–2.7) months after birth. They were examined clinically and blood samples were taken at the beginning of the study and after one, three and six months of follow-up. Infants with infections were excluded. Current allergic symptoms were recorded. The infants with suspicion of cow's milk allergy and their breast-feeding mothers were put on a cow's milk elimination diet. In addition, the classical allergens (fish, egg, peanuts, citrus fruits and tomato) were eliminated from the mother's diet. The diagnosis of food allergy was later confirmed by an open cow's milk challenge.

The study protocol was approved by the Ethical Committee of the Skin and Allergy Hospital of Helsinki University Central Hospital.

Flow cytometry

Diluted, heparinized blood was layered over density-gradient medium (Ficoll-Hypaque, Pharmacia, Sweden) and centrifuged at 400 g at room temperature for 20–30 min. Mononuclear cells were collected and washed in RPMI 1640 medium (Sigma-Aldrich CO Ltd., UK) containing penicillin (100 IU/ml), streptomycin (100 µl/ml), glutamine (600 µl/ml), and 5% fetal calf serum.

Thereafter, 1×10^6 lymphocytes/ml were incubated with monoclonal antibodies against CD3, CD4, CD5, CD8, CD19, CD23 (low-affinity IgE-receptor), and CD45 (Beckton Dickinson Immunocytometry Systems, Mountain View, CA, USA). The numbers of cells positive for the surface markers used were expressed as percentages of the cells within the gate.

Light microscopy

Venous blood-EDTA samples were drawn onto slides and stained with May-Grünwald-Giemsa. The numbers of different leucocytes were counted under

the light microscope. The results were expressed as percentage of the cell type per 100 leucocytes.

Cow's milk challenge protocol

After elimination of cow's milk for two to four weeks, patients were admitted for oral cow's milk challenge. On day one, cow's milk was given in increasing doses (1, 10, 50 and 100 ml) at one hour intervals, and on day two, 200 ml and then the normal milk intake appropriate for age was commenced. If any adverse reaction appeared, the challenge was stopped and the patients were examined by a paediatrician or a dermatologist.

Statistical analysis

The Mann-Whitney U test was employed to determine the statistical significance of differences between continuous variables. Logarithmic transformations were used because of the skewed distributions.

Results

Clinical characteristics of study patients

The geometric mean (95% CI) age of the healthy infants was 4.0 (3.8–6.0) months and of the infants with CMA 3.3 (3.0–4.5) months. The difference between the groups was not statistically significant.

During the open cow's milk challenge, seven infants developed urticaria, eleven infants eczema, three patients diarrhoea, loose stools or vomiting and three infants eczema and diarrhoea, loose stools or vomiting. The patients reacted to a median total dose of 112 (range, 7 to 2000) ml of cow's milk at a mean of 37 (range, 0.5 to 168) hours after commencement of the challenge. The median dose eliciting symptoms was 75 (range, 5 to 200) ml of cow's milk and the mean time from the last dose until symptoms appeared was 2 (range, 0.5 to 7) hours.

Results of flow cytometric analysis

The percentages of lymphocytes expressing the surface markers CD19 (pan B), CD23 and both CD19 and CD23 (double-positive) were significantly higher in the infants with CMA than in healthy controls (Fig. 1). The percentage of CD19/CD23 double-positive B cells was higher in the children with cutaneous symptoms only (18.5, SD 6.0) than in those showing both cutaneous and gastrointestinal symptoms (12.9, SD 4.4), $P = 0.027$.

The mean percentage of CD19⁺ B cells co-expressing CD5 antigen was significantly higher in the infants currently showing-symptoms of CMA (11,

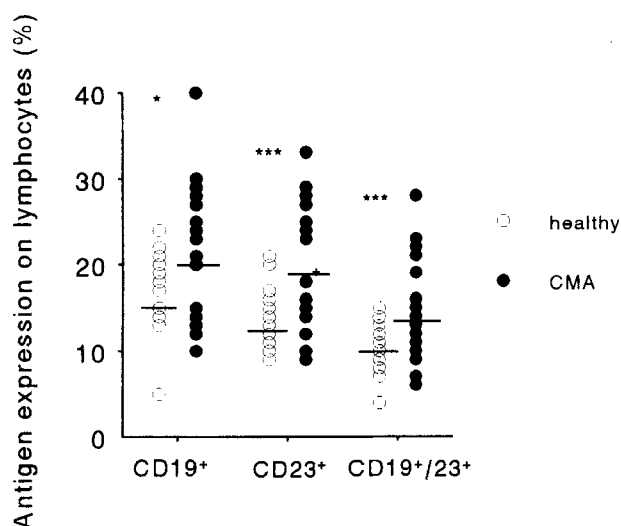


Fig. 1. The amounts of surface antigens CD19⁺, CD23⁺ and CD19⁺/23⁺ expressed as percentages of lymphocytes differed significantly between healthy infants and those allergic to cow's milk. *) $P < 0.01$, ***) $P < 0.005$, — mean.

SD 2.6) than in those with a suspicion of CMA but already symptom free at the time of the study, because of an adequate elimination diet (8, SD 1.9), $P = 0.03$. The proportion of CD5-positive B cells in these asymptomatic infants later confirmed to have CMA was comparable to that in healthy infants (data not shown).

The percentage of CD8⁺ cells was significantly lower in the infants with CMA than in the healthy infants (Table 1). The percentage of CD4 positive cells in the two study groups was comparable (Table 1).

Light microscopy

The geometric mean (95% CI) number of eosinophils was comparable in the healthy infants and in those with cow's milk allergy, 3.7 (3.1–6.3)% and 1.7 (2.7–6.3)%, respectively ($p = 0.85$).

Discussion

In the present study, we have shown that the percentage of B cells and their surface expression of low-affinity IgE receptors were higher in the infants with early symptoms CMA than in the healthy controls. In addition, the percentage of CD8⁺ cells was significantly lower in the infants with CMA than in the healthy controls.

Our results show that the number of B cells is increased in active CMA. Furthermore, they demonstrate these cells to be activated. These findings are

in agreement with previous studies indicating that the total number of antibody-secreting B cells, measured by the solid-phase enzyme-linked immunoassay (ELISPOT), has been shown to be increased during oral cow's milk challenge in children with CMA (2). At this point, the antigen-specific response was shown to be defective.

The leucocyte antigen CD23 (Fc ϵ RII), a low-affinity receptor for IgE, is a 45 kilodalton molecule expressed on B lymphocytes following activation by a number of stimuli (3). Increased levels of CD23 or soluble CD23 (sCD23) have been observed in various immune-mediated disorders, such as atopic dermatitis (4) and asthma (5). sCD23 is involved in the regulation of IgE synthesis (6) and CD23 may also be involved in presentation of antigens to T cells and in cell adhesion (3). sCD23 may be useful for the early diagnosis of atopic disorders (7). The incidence of atopic disorders has been found to increase together with the increase in cord blood sCD23, which suggests that the rise in sCD23 may precede the increase in IgE synthesis (7). The only agent capable of inducing the expression of CD23 is IL-4 (6). Hence, expression of CD23 may be inhibited by IFN- γ (6), the generation of which has been previously shown to be almost undetectable in infants with CMA (8). It therefore appears likely that over-expression of CD23 in CMA may also reflect decreased production of IFN- γ or an imbalance between the production of IL-4 and IFN- γ . In the present study we have shown evidence that lymphocytes are already activated and expression of CD23 increased during very early symptoms of CMA.

The CD5 leucocyte antigen is expressed on both T and B cells. In early life, B cells bearing the surface CD5 marker predominate, but in adults they represent only 27% of the peripheral blood B lymphocytes, reflecting the immaturity of the immune system in childhood (9). These cells can generate polyreactive, low-affinity antibodies, mainly of IgM class (10). In the present study, the significantly higher proportion of these cells in babies with

Table 1. Mean percentages (SD) of surface marker expression by flow cytometry on peripheral blood lymphocytes in healthy and cow's milk-allergic children.

Surface marker	Healthy child	Allergic child	Statistics
CD4 ⁺ (%)	42.6 (7.2)	44.7 (8.8)	$p=0.65$
CD8 ⁺ (%)	23.1 (4.5)	18.3 (5.0)	$p<0.001$
CD5 ⁺ (%)	79.0 (10.1)	77.6 (7.5)	$p=0.30$
CD5 ⁺ /19 ⁺ (%)	7.6 (3.0)	9.3 (2.8)	$p=0.15$
CD5 ⁺ 19 ⁺ /CD19 ⁺ (%)	57.7 (11.6)	58.3 (8.6)	$p=0.85$

symptoms of CMA than in healthy infants mirrors disease activity and continuous antigenic load, resulting in the non-antigen-specific response of an infant with an immature immune system (11).

Our present finding of lower numbers of CD8⁺ T cells in patients with CMA is analogous to the results presented for patients with atopic eczema (12). According to the study of Chandra and Baker, the numerical and functional deficiency of suppressor T cells precedes the development of atopic eczema (12). Suppressor cell activity has also been demonstrated to be lower in children with CMA (13). According to our present results, low suppressor activity could result partly from a low number of suppressor cells. Evidence from animal and human experiments has given rise to the notion that an appropriate balance between suppressor and helper T cells is important for the induction of oral tolerance (13, 14). Thus impaired generation in combination with low activity of suppressor cells may lead to a breakdown of oral tolerance. This may also be important in the etiopathogenesis of CMA.

In conclusion, the large number of activated CD19⁺ B cells, as well as small number of CD8⁺ T cells, are early, although non-specific, markers of food allergy. These characteristics are detected in peripheral blood even during the earliest symptoms indicative of cow's milk allergy. We suggest that the measurement of these markers may be useful in the differentiation of CMA from other non-immunological diseases, e.g. functional disorders of the gastrointestinal tract.

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