Local Immune Response Measured in Blood Lymphocytes Reflects the Clinical Reactivity of Children with Cow's Milk Allergy

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ABSTRACT. Study was made of immune responses in cow's milk allergy by a new immunoassay that measures total Ig-secreting cells and specific antibody-secreting cells during their maturation cycle in peripheral blood. These primed gut-associated lymphoid tissue-derived lymphocytes are assumed to reflect the intestinal immune responses. During diagnostic milk provocation, 15 patients had acute urticarial skin eruptions, eight patients had slow onset of eczema, and 15 showed symptoms from the gastrointestinal tract. A significant increase in IgM-secreting cells (means with 95% confidence intervals) from 382.2 (265, 552) to 621.4 (381, 1013)/ 10^6 cells, $p < 0.01$, but not IgA- and IgG-secreting cells was associated with acute urticaria. In patients with eczematous skin eruptions and gastrointestinal symptoms, the response involved all these Ig isotypes. The magnitude of the postchallenge Ig-secreting cell responses in patients with gastrointestinal symptoms in the IgM class [from 657.9 (428, 1012) to 3544.0 (1696, 7406)/ 10^6 cells, $p < 0.001$] and the IgA class [from 974.6 (590, 1610) to 2482.4 (1528, 4028)/ 10^6 cells, $p = 0.001$] significantly exceeded that of the patients with cutaneous symptoms. Notwithstanding the distinct increase in the total number of Ig-secreting cells, the specific antibody-secreting cell response specifically directed against β -lactoglobulin and α -casein was small and inconsistent. These findings indicate that immune exclusion of milk antigens is defective in cow's milk allergy. The quality and extent of the response varied in the three reaction types, suggesting that different immunopathogenic mechanisms are operative in cow's milk allergy. (*Pediatr Res* 28: 582-586, 1990)

Abbreviations

BLG, β -lactoglobulin
CA, α -casein
CMA, cow's milk allergy
ISC, Ig-secreting cells
sASC, specific antibody-secreting cells
ANOVA, analysis of variance
CI, confidence interval

CMA can manifest itself with gastrointestinal and extragastrointestinal symptoms. Patients have been assigned to clinical groups on the basis of the reaction onset time. Rapid reactions

with urticarial skin eruptions indicate the involvement of an immediate hypersensitivity mechanism. Slower-reacting patients often show symptoms from the gastrointestinal tract or have eczematous reactions (1). The pathogenetic mechanisms associated with these symptoms, however, remain uncertain.

Two essential prerequisites for clinical CMA have been proposed. First, the antigens must penetrate the intestinal mucosal barrier (2). Second, the absorbed antigens must evoke harmful immunologic responses. Intestinal permeability has been shown to be increased in CMA patients with gastrointestinal symptoms either at the acute stage of the disease or after a milk challenge, but not on an elimination diet (3, 4). These findings might reflect mucosal damage caused by local hypersensitivity mechanisms. In patients with cutaneous symptoms, however, modifications in the intestinal barrier remain elusive (4-7). Contradictory results may be attributable to different types of immunologic reactions operative in CMA, with varying components of local and systemic responses.

Exposure of gut-associated lymphoid tissue to antigens entering from the intestinal lumen stimulates intestinal lymphocytes, which migrate to local lymph nodes to mature (2). Primed lymphocytes are ultimately seeded to the gut through lymph and peripheral blood (8). These can be assessed as antibody-secreting cells in peripheral blood 1 wk after contact with the antigen (9, 10) to measure the immune response at the mucosal level. This approach was used here to evaluate the quality and extent of the immune response to cow's milk antigens during a diagnostic provocation test.

MATERIALS AND METHODS

Patients. The study comprised 38 children aged 3 wk to 34 mo consecutively admitted for a diagnostic milk provocation at the Department of Pediatrics, Tampere University Hospital.

The patients were examined on the basis of a previous clinical history suggestive of CMA. A precise relationship could be documented between ingestion of cow's milk and clinical symptoms, i.e. the symptoms had disappeared after elimination of cow's milk from the diet, and an unequivocal relapse had followed reintroduction of cow's milk to the diet. The symptoms were urticarial skin eruptions ($n = 13$); atopic eczema ($n = 15$); respiratory symptoms ($n = 2$); vomiting ($n = 6$); loose stools or diarrhea ($n = 12$); and poor weight gain ($n = 3$).

Total serum IgE and cow's milk-specific IgE antibodies were measured with the IgE RIA and Phadebas RAST kits, respectively (Pharmacia Diagnostics, Uppsala, Sweden). Additionally, the initial examination included determination of serum IgG, IgA, and IgM. Jejunal biopsies were taken in three cases; one 2-mo-old patient with severe diarrhea had partial villus atrophy.

Cow's milk was then eliminated from the diet for 4-6 wk, and a tolerated (soy or protein hydrolysate) formula was substituted in infants less than 1 y old. In older patients, calcium supple-

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mentation was given. The adequacy of the diet and compliance with treatment was followed and confirmed by a dietician. Patients with strong evidence of CMA substantiated by lack of symptoms and satisfactory weight gain on the elimination diet were enrolled in the study.

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 and blood samples. The provocation was started in hospital in patients free of any symptoms and signs of CMA or other illness. The following volumes of cow's milk were administered: d 1, 2, 5, and 10 mL; d 2, 20, 50, and 100 mL; d 3, normal *ad libitum* milk intake appropriate for age.

The patients were examined at the time of any adverse reaction to confirm the diagnosis and to determine the time of onset and the quality of reactions, as well as at 1 wk (d 8) and 1 mo. The patients were grouped according to their main complaints and symptoms at presentation and after the milk challenge. The patients were followed for at least 6 mo.

Blood in heparin was taken before starting the oral challenge (d 1) and 1 wk later (d 8). Day 8 was chosen to represent the maximal response after administration of the antigen on d 1 (9).

Control patients. Four children tolerated cow's milk; milk intake *ad libitum* could be introduced on d 3 with no reappearance of symptoms. Three additional infants were used as controls, and the blood sample was taken after successful introduction of cow's milk. The control patients were examined and followed in the same way as the study patients.

ELISPOT assay. ELISPOT, or ELISA plaque assay, is a solid-phase ELISA technique for the enumeration of antibody-secreting cells (11, 12). Lymphocytes are incubated in microtiter plate wells previously coated with antigen. Lymphocytes secreting specific antibodies against these are visualized as colored spots, using enzyme-conjugated antisera followed by an agarose overlay containing the substrate.

Reagents. The following antisera and enzyme-conjugated antisera were used: rabbit Ig to human IgA (α), IgE (ϵ -chains), and IgM (μ -chains) (Dakopatts a/s, Roskilde, Denmark); goat anti-human IgG (γ -chain specific) F(ab')₂ fragment of antibody, goat anti-human IgA (α -chain specific), IgG (γ -chain specific), and IgM (μ -chain specific) alkaline phosphatase conjugate F(ab')₂ fragment, goat anti-human IgE (ϵ -chain specific) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO).

5-Bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP, Sigma Chemical Co.) was used as substrate for alkaline phosphatase. The substrate solution was prepared at a final concentration of 2.9 mM BCIP in 1 M AMP (2-amino-2-methyl-1-propanol) buffer, pH 10.25 (12).

Lymphocyte isolation. Mononuclear cells containing mainly lymphocytes were obtained by Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) gradient centrifugation of heparinized venous blood. Isolated cells were washed three times with Hank's buffered salt solution (Flow Laboratories, Irvine, Scotland) and finally suspended ($1-2 \times 10^6$ cells/mL) in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) containing 10% FCS.

Antigen-specific immune response (sASC). To determine the number of milk antigen-sASC, BLG and CA were used as coating antigens. Trypsin and pepsin digests of BLG were included because intraluminal digestion may expose new immunogenic epitopes of the antigen (13, 14). Trypsin and pepsin served as controls.

Nunc-Immunoplate (MaxiSorp, Nunc, Roskilde, Denmark) wells were coated (2 h, 37°C) with the following concentrations of the antigens to enumerate sASC: BLG 20 μ g/mL (from bovine milk, 50 μ L per well at each stage); CA 20 μ g/mL (from bovine milk); trypsin-digested BLG (trypsin from bovine pancreas, type III) as a mixture of BLG 20 μ g/mL and trypsin 5 μ g/mL; pepsin-digested BLG (pepsin from porcine stomach mucosa) consisting

of BLG 20 μ g/mL and pepsin 2 μ g/mL; trypsin 10 μ g/mL; and pepsin 10 μ g/mL.

Uncoated binding sites were blocked with 1% BSA in PBS pH 7.4 (30 min, 37°C). Next, the lymphocyte suspension was applied and incubated for 2 h at 37°C. The antibodies secreted during this time were detected with alkaline phosphatase conjugated anti-human IgA, IgE, IgG, and IgM antisera diluted in 1% BSA-PBS pH 7.4 (overnight, 4°C). Finally, the BCIP substrate solution was applied in 0.6% agarose (50°C).

The plates were washed three times with PBS-0.05% Tween 20 between each incubation step; before applying the cells, they were washed with PBS.

Antigen nonspecific-immune response (ISC). The immune response was also assessed by the total number of ISC, which comprise all the lymphocytes secreting antibodies of a certain isotype, including those directed against milk antigens. The wells were coated (2 h, 37°C) with rabbit Ig to human IgA, IgE, and IgM, and goat anti-human IgG [F(ab')₂ fragment] diluted 1:100 in PBS pH 7.4. Subsequent steps were the same as in the determination of the sASC.

The sASC and ISC responses were determined in Ig classes IgA, IgE, IgG, and IgM, and were expressed per 10^6 cells plated.

Statistical analysis. Logarithmic transformations (ln) were applied because of skewed distributions in sASC and ISC counts. Therefore, geometric means are presented with 95% CI. ANOVA was used to determine the statistical significance of differences in continuous variables. A paired *t* test was applied in the comparison of repeated measurements. For comparison of several pairs of means, a series of 95% CI was constructed, using the Tukey test (15). The χ^2 test was applied to determine the differences in proportions.

RESULTS

Clinical characteristics of patients. The milk challenge test classified the study patients into three major groups. Fifteen patients had acute urticarial skin eruptions, eight patients had eczema, and 15 patients showed symptoms from the gastrointestinal tract. The mean (95% CI) reaction onset times were 1.5 (0.7, 3.3) h, 37.0 (13.5, 101.3) h, and 24.0 (12.3, 47.0) h, respectively, $p < 0.001$ (ANOVA). There was, however, some overlap between the groups. One patient with acute urticaria also had an episode of vomiting within 30 min of milk ingestion. In the eczema group, two patients had watery or loose stools but no vomiting after 10 and 72 h, respectively, from milk ingestion. Additionally, three patients with a predominance of gastrointestinal symptoms had slow onset of eczema. Respiratory symptoms were rare. Only one patient manifesting acute urticaria had wheezing within 30 min of consuming milk.

The mean (SD) age of the patients was 13.5 (7.5) mo. The study groups did not differ from each other or controls in age or clinical history before development of their symptoms. The mean age at onset of the symptoms ranged from 3.2 to 6.1 mo in study patients, and the mean duration of breast feeding between 5.0 and 5.5 mo in patients and controls. Elevated milk-specific IgE RAST values were significantly more frequent in children with cutaneous symptoms (in 54% of the patients with urticarial and 25% of the patients with eczematous skin eruptions) than in those with gastrointestinal symptoms (27%), and none of the control patients had elevated milk-specific IgE RAST values. The mean (95% CI) total serum IgE values were 54.9 (14, 207) kU/L in patients showing urticaria, 62.2 (10, 395) kU/L in patients with eczema, and 8.0 (3, 25) kU/L in patients showing gastrointestinal symptoms; $p = 0.04$ (ANOVA). All patients had normal total serum IgG, IgM, and IgA levels.

Total number of ISC. A distinct increase in the immune response was found to follow a clinically positive milk challenge as shown by a significant rise in the number of ISC (Fig. 1).

Six patients had severe symptoms: acute urticaria with edema, vesicles, and systemic symptoms ($n = 2$), or profuse diarrhea and

Fig. 1. The total number of ISC in the blood of patients with CMA manifested in urticarial or eczematous skin eruptions or gastrointestinal symptoms (log scale). Horizontal bars with vertical lines denote the geometric means with 95% CI. 1, prechallenge (d 1) values; 2, postchallenge (d 8) values. $\circ$ $p < 0.1$, $\star$ $p < 0.05$, $\star\star$ $p < 0.01$, $\star\star\star$ $p < 0.001$ compared with the prechallenge values (paired t test).

vomiting ($n = 4$). In these patients, the mean (95% CI) rise in the total number of ISC from 1960.6 (880, 4372) to 10 992.8 (3944, 30 668)/ 10^6 cells significantly exceeded that of other patients with a positive milk challenge [rise from 1502.7 (741, 3047) to 4068.4 (2805, 5896)/ 10^6 cells, $p = 0.03$]. The difference was due to a marked increase in the total number of IgM-secreting cells in patients with severe symptoms compared with those with milder symptoms ($p = 0.002$). Altogether, 93% of the patients with a positive milk challenge had a greater than 30% increase in IgM-secreting cells among peripheral blood lymphocytes, and this was found in 29% of the controls. The mean rise in the total number of IgM-secreting cells from d 1 to 8 in patients with CMA was 3062.7/ 10^6 cells with the 95% CI from 1252 to 4873/ 10^6 cells, whereas in controls the increase, 368/ 10^6 cells with the 95% CI ranging from -581 to 1316/ 10^6 cells, was not significant.

There were significant differences in the quality and extent of the immune response between the three reaction groups. In patients with acute urticaria, the predominant Ig class was IgM. The alterations in the number of IgA- and IgG-secreting cells were not statistically significant. In patients with eczematous skin eruptions and gastrointestinal symptoms, the response involved

all Ig classes (Fig. 1). The magnitude of the postchallenge ISC response also differed significantly between the study groups (IgM: $F = 7.67$, $p = 0.001$; IgA: $F = 4.89$, $p = 0.01$; IgG: $F = 8.53$, $p = 0.001$; ANOVA). This was due to the strong IgM and IgA component in the response in the patients with gastrointestinal symptoms (Tukey test). Additionally, the level of IgG-secreting cells in patients with eczema and gastrointestinal symptoms exceeded that of the patients with acute urticaria.

The total number of IgE-secreting cells did not increase significantly in any of the study groups. Positive ISC levels ($\geq 0.5/10^6$ cells) in the IgE class were found in two of 15 patients with urticaria, three of eight with eczema, and five of 15 with gastrointestinal symptoms.

In control patients, the total number of IgM-secreting cells [means (95% CI)] increased from 337 (75, 1485) to 388 (157, 960)/ 10^6 cells, IgA-secreting cells decreased from 686 (240, 1962) to 518 (358, 748)/ 10^6 cells, and IgG-secreting cells from 982 (273, 3533) to 410 (110, 1533)/ 10^6 cells.

sASC. In contrast to the distinct increase in the total ISC, the response specifically directed against BLG and CA was modest and covered only the IgM class (Table 1). In patients with acute urticaria, the rise in IgM-secreting cells against BLG and CA

Table 1. Levels of sASC against BLG and CA after cow's milk challenge in patients with CMA manifest in urticarial or eczematous skin eruptions or gastrointestinal symptoms, and in controls*

sASC/ 10^6 cells	Urticaria			Eczema			Gastrointestinal symptoms			Controls			χ^2 test		
	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG
BLG															
Negative†	33	93	80	25	88	75	7	74	73	71	72	72	$\chi^2 = 25.93$	$\chi^2 = 7.65$	$\chi^2 = 7.09$
+	27	0	20	63	12	12.5	7	13	7	29	14	14	$p = 0.002$	$p = 0.57$	$p = 0.63$
++	27	7	0	0	0	12.5	33	0	7	0	0	14			
+++	13	0	0	12	0	0	53	13	13	0	14	0			
CA															
Negative†	50	88	79	88	75	88	14	67	50	100	86	83	$\chi^2 = 20.91$	$\chi^2 = 5.18$	$\chi^2 = 7.28$
+	29	6	7	0	12.5	12	29	13	29	0	14	0	$p = 0.01$	$p = 0.82$	$p = 0.61$
++	14	6	7	0	0	0	14	13	14	0	0	17			
+++	7	0	7	12	12.5	0	43	7	7	0	0	0			

* Values are percent of patients in each reaction group. Urticaria, $n = 14$ –15; eczema, $n = 8$; gastrointestinal symptoms, $n = 14$ –15; controls, $n = 7$.

† Negative, <0.5 ; +, 0.5 – 2.49 ; ++, 2.5 – 4.99 ; +++, $\geq 5.0/10^6$ cells.

failed to reach statistical significance. In patients with eczema, the IgM-secreting cells to BLG but not to CA increased significantly [BLG: rise from 0.12 (0.03, 0.4) to 0.91 (0.2, 5.3)/10⁶ cells, $p = 0.01$]. There was a simultaneous increase in the number of IgM-secreting cells to trypsin- and pepsin-digested BLG [from 0.15 (0.04, 0.5), to 1.81 (0.2, 14.4)/10⁶ cells, $p = 0.03$ and from 0.08 (0.03, 0.2) to 1.03 (0.1, 7.87), $p = 0.004$, respectively].

The patients with gastrointestinal symptoms had somewhat higher sASC responses in the IgM class to BLG [0.48 (0.2, 1.2) to 6.50 (2.3, 18.6)/10⁶ cells, $p < 0.001$] and CA [0.13 (0.05, 0.3) to 2.32 (0.8, 6.9)/10⁶ cells, $p = 0.001$] than the other groups. This included responses to trypsin- and pepsin-digested BLG [0.22 (0.1, 0.6) to 5.80 (1.7, 20.0)/10⁶ cells, $p < 0.001$ and 0.27 (0.1, 0.8) to 6.83 (2.1, 22.0)/10⁶ cells, $p < 0.001$, respectively] and also to the enzymes used [trypsin: 0.13 (0.04, 0.4) to 4.07 (0.7, 23.1)/10⁶ cells, $p < 0.001$; pepsin: 0.07 (0.04, 0.1) to 2.06 (0.4, 9.6)/10⁶ cells, $p < 0.001$].

None of the study groups showed significant alterations in the number of IgA- or IgG-secreting cells against BLG or CA. In fact, in few patients did sASC in these Ig classes reach positive levels (Table 1). The sASC in the IgE class were negative in all patients.

DISCUSSION

Recent data support the notion that immune mechanisms are activated in CMA (1, 16). Our study extends these findings to indirect proof of local immune systems of the gut, the host's first line of defense against enteric antigens.

The activation of local immune responses can be directly determined from intestinal fluids. The application of such invasive methods to pediatric practice seems, however, undesirable. On the other hand, changes in the levels of antibodies in serum or secretions do not invariably follow a challenge, vaccination, or infection (8, 9, 17). In our study, we applied the ELISA plaque assay, which determines the amount of antibody-secreting cells among peripheral blood lymphocytes, in the diagnosis of CMA. The results confirm our previous findings (8, 18) that this is a useful means of evaluating the intestinal immune responses, and further extend this conclusion to hypersensitivity reactions. Although the method yielded promising results and the response thus measured correlated with disease activity, it seems too early to define the sensitivity and specificity of the test, inasmuch as a highly selected material was analyzed.

The postchallenge symptoms of our patients were consistent with previous observations (1, 19). The immediate urticarial reactions were associated with elevated serum IgE antibodies, whereas patients with gastrointestinal disease had intermediate or late reactions and lower IgE levels. The late-reacting patients developing mainly eczematous lesions showed high IgE levels. Although a distinct immune response at the mucosal level was measured in all reaction types, the quality and extent of the response varied, indicating that different immunopathogenic mechanisms may be operative in patients with CMA.

Under normal circumstances, oral immunization provokes a strong increase in IgA in the gut (20). The presence of specific IgA antibodies may block the uptake of antigen and in this way contribute to oral tolerance. We have previously demonstrated in adults a dramatic increase in IgA-secreting cells 1 wk after the onset of acute diarrhea (18) or after administration of oral, live *Salmonella typhi* Ty21a vaccine (9). IgM- and IgG-secreting cells were detected as well, but the responses were smaller than those for IgA. In children with CMA, the IgM component of the total ISC response exceeded that of the IgA and IgG classes. The kinetics of the intestinal antibody response could vary according to the type of the antigen. An alternative explanation might be the developmental deficiency of IgA in infants (21), when IgM could take over as the predominant Ig class in mucosal secretions. Perhaps also the route of antigen absorption, the amount of antigen absorbed, and the site of initial interaction between the

antigen and the mucosal immune system could influence the response.

Our study patients showed no increase in the total number of IgE-secreting cells. This suggests that the mucosal immune cell migration cycle does not necessarily apply to cells producing IgE-class antibodies in CMA, and that the local production of IgE is probably small. This is compatible with previous reports on inconsistent and low numbers of jejunal and rectal IgE immunocytes in children with food allergy and atopic eczema (22). Recent work demonstrates that the IgE-positive cells present in the intestinal mucosa are mast cells (23). Furthermore, there is substantial evidence that the disturbances in gut function during mucosal IgE-mediated reactions are dependent on mast cell degranulation (24).

Notwithstanding the distinct response in the ISC after milk challenge in patients with CMA, the response specifically directed against BLG and CA was small and inconsistent. Thus, only a fraction of the ISC were producing antibodies against BLG and CA. The targets of the rest of the ISC remain unknown but—although BLG and CA are clinically the most important antigens in cow's milk (25)—reactions to all of the milk proteins may occur. The response could also be directed against a number of bystander proteins gaining access to the body through damaged mucosa.

We found cells secreting IgM against trypsin- and pepsin-digested BLG, suggesting that some antigenic determinants may resist such treatment. On the other hand, new epitopes could be exposed by epithelial antigen processing, and it is not excluded that such fragments could be sensitizing. Surprisingly, we could also measure, in CMA manifested in gastrointestinal symptoms, a response against proteases. This may indicate that the response is polyclonal in the same way as in patients with acute diarrhea (18). We cannot, however, exclude the possibility that intact protein enzymes are transported from the intestinal lumen into the systemic circulation and cause further tissue injury. In newborn rabbits, enhanced uptake of exogenous trypsin has been shown to occur during the period of increased intestinal permeability (26).

The constant finding in CMA was that the sASC response was small, and that it became apparent in the IgM class only. Developmental IgA deficiency was probably not the cause of the lack of antigen-specific IgA responses, inasmuch as the same patients showed significant increases in the total number of IgA-secreting cells. Therefore, we suggest that this finding indicates an insufficient specific immune exclusion that may produce the symptoms in CMA.

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Announcement

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