

Clinical manifestations of cows' milk allergy in childhood. I. Associations with *in-vitro* cellular immune responses

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Summary

In this study 51 children who presented with symptoms of cows' milk allergy (CMA) were categorized clinically by their response to cows' milk challenge. Forty-two patients had unequivocal evidence of CMA and nine were milk tolerant. Of the patients with CMA two groups were identified. The first, made up of 32 patients, had immediate-type hypersensitivity reactions to milk associated with both positive skin-prick test (SPT) and RAST. The second group of 10 late reacting patients developed symptoms of CMA over several hours or days; significant increases in irritability, frequency of bowel actions, and rhinitis following milk ingestion were noted in this group. Leucocyte inhibition factor (LIF) produced in response to α -lactalbumin, β -lactoglobulin and α -casein was assessed in the immediate and late reacting CMA patients as well as in the milk-tolerant group. There was no difference in LIF production between the milk-tolerant group and those with immediate reactions. However, these two groups produced less LIF than the late reacting patients for α -lactalbumin ($P=0.02$), α -casein ($P=0.03$) and β -lactoglobulin ($P=0.05$). A clinical diary score card was found to be a useful instrument to assess the response of non-immediate reactions to milk ingestion.

Introduction

In a recently completed study of 100 infants and young children, less than 3 years of age, with unequivocal challenge evidence of cows' milk allergy (CMA) we identified three groups of children [1, 2]. The first, the immediate reactors, were IgE sensitized and demonstrated urticarial, angioedematous, and eczematous eruptions as well as mild symptoms of anaphylaxis and stridor within minutes of ingesting small volumes of milk. The second, a non-IgE sensitized group, developed vomiting and diarrhoea several hours after ingesting larger volumes of milk; they have features consistent with

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cows' milk protein intolerance (CMPI) or cow's milk enteropathy (CME). These were termed intermediate reactors. The third group of patients, late reactors, developed symptoms of diarrhoea, bronchitis and eczema more than 20 hr after commencing milk challenge with normal volumes of milk.

Subsequent investigations by ourselves and others have demonstrated the close association between immediate hypersensitivity reactions to cows' milk, and IgE type I hypersensitivity manifest by high positive skin-prick tests (SPT) and elevated IgE antibodies to cows' milk [1-5]. The diagnostic value of very high SPTs in identifying immediate reacting CMA infants has recently been described [6]. While it is relatively easy to define an immediate reaction to cows' milk clinically and immunologically, little work has been done on the later appearing manifestations of CMA.

Late reacting patients usually develop gastrointestinal, bronchitic and/or eczematous symptoms gradually several hours after ingesting normal volumes of milk; frequently these symptoms may not be of sufficient severity to be attributed to milk ingestion until some days have elapsed [1, 2, 7]. Minor *et al.* [8] and Ashkenazi *et al.* [9] suggested that lymphokine release, as a marker of T-lymphocyte hypersensitivity, may be related to some of the clinical features of CMA in these patients. Both these investigators reported a clear distinction between CMA positive and negative patients in the production of leucocyte migration inhibition factor (LIF). Khoshoo [10], in a study of infants with protracted diarrhoea, demonstrated that the leucocytes of patients with CMPI showed significant migration inhibition after *in-vitro* incubation with β -lactoglobulin. In these studies [8-10], detailed information regarding the volume of milk administered and the nature or time of onset of symptoms after milk challenge was not described. Furthermore, the possible influence of prolonged dietary milk exclusion on LIF production was not examined.

In the current study we hypothesized that immediate reactions to cows' milk were due to an IgE-mediated immune hypersensitivity mechanism, whereas non-immediate, i.e. intermediate or late reactions, were due to a T-cell-mediated response, which could be quantitated by measuring LIF production to milk challenge *in vitro*. In order to identify syndromes associated with these non-immediate reactions the clinical response to milk ingestion was assessed by quantifying symptom changes recorded on daily diary score cards.

Subjects and methods

Patients

One hundred and ten children had a history suggestive of CMA, which responded to elimination of cows' milk from their diet within 4 weeks. After at least 12 weeks on a milk-free diet, and 1 week before formal milk challenge, they attended the Royal Children's Hospital Allergy-Clinical Immunology Unit and had SPTs performed and blood taken for immunological studies. Patients less than 5 years of age with SPT ≥ 4 to milk protein were regarded as having immediate reacting CMA, in accordance with the results of our previous investigations [6].

The remaining patients were issued with daily diary score-cards that were completed for 7 days before and after formal milk challenge. In these patients milk intolerance caused by disaccharidase deficiency and other gastrointestinal tract diseases was excluded by duodenal biopsy, where relevant; milk aspiration, as a cause

of recurrent bronchitis, was excluded by demonstrating a reproducible relapse of symptoms following milk ingestion but not following the ingestion of non-cow milk infant formulas. Details of this protocol have been published elsewhere [1].

Milk challenge procedure

The following volumes of milk were administered:

day 1: 0.5 ml, 2.5 ml, and 5 ml at 10-min intervals, then 10, 20 and 30 ml at 30-min intervals;

day 2: 30, 60 and 120 ml at 30-min intervals;

day 3: normal volumes of milk, i.e. > 450 ml per day.

Patients attended a specialized unit on day 1 and day 2 of the challenge procedure, but subsequent volume increases were undertaken at home after discharge.

In the following discussion the volume of milk administered up to the time of a specific reaction has been documented. These milk challenge procedures were open. The time of onset of reactions was taken as the time from the beginning of the milk challenge procedure on day 1. Patients were reviewed at the time of any adverse reaction and at 1 week and 1 month after commencement of milk. Where reactions were doubtful, or developed more than 48 hr after milk challenge was commenced, the challenge was repeated on two more occasions before being regarded as positive; however, only the immunological and clinical data generated by the first positive challenge were included in analysing the results of this study. Patients who tolerated normal volumes of milk by day 7 and who maintained this intake until the 1-month follow-up were deemed milk tolerant. Patients who did not fulfil these strict criteria for CMA, or milk tolerance, were excluded from the study.

Skin tests

A single batch of whole cows' milk allergen extract 1/20 (w/v) was purchased from Dome-Hollister-Stier (Spokane, WA, U.S.A.; no. 3380) and was used throughout the study.

Skin-prick tests were performed by one specialist nurse as follows. One drop of the extract, control solution, and histamine were placed on the subject's back, and the point of a blood lancet was passed through the drop to indent the skin but not cause bleeding. The size of the skin weal elicited by the milk extract was compared with that of histamine acid phosphate, 1 mg/ml and the control solution. The area of weal and pseudopodia elicited by the histamine was designated as 3, and the reactions to milk graded by reference as follows [11]:

> 4 area of milk reaction = > twice the area of histamine standard;

4 area of milk reaction = twice the area of histamine standard;

3 area of milk reaction = area of the histamine standard;

2 area of milk reaction = half of the area of histamine standard;

1 area of reaction = one-quarter the area of histamine standard, or any reaction greater than control solution.

The weal of the control solution provided by Dome-Hollister-Stier on skin-prick test was deducted from that of the milk antigen before the test score was given [11].

RAST studies

Milk-specific IgE antibodies were measured with Phadebas RAST kits (Pharmacia, Uppsala, Sweden). The RAST scoring scale used was as advised by the manufacturers and graded from 0 to 4. These tests were done as part of the routine hospital immunology laboratory service and results were not available until after the milk challenge procedure was completed.

Lymphokine studies

The test used was a modification of the method of Clausen [12] and Minor *et al.* [8] mainly to permit optimal use of small volumes of blood. After initial centrifugation, plasma was removed and the pellet suspended to a final concentration of 0.6% dextran (T500)/saline to sediment the red cells. The red cell pellet to dextran-saline volume was 1:9. After 20 min the supernatant was removed, and the suspended cells washed three times in phosphate buffered saline (PBS), then once in PBS containing 10% horse serum. After final centrifugation, the cells were suspended at 1.54×10^8 cells per ml in Dulbecco's modification of Eagle's medium containing 10% horse serum.

The antigens used were β -lactoglobulin, α -casein and α -lactalbumin (Sigma, St Louis, MO, U.S.A.) at 10 mg/ml and human serum albumin (Commonwealth Serum Laboratories, Parkville, Australia) 24% solution. The negative control used PBS instead of antigen.

The agarose gel was 1% (Litex, Denmark) in Hanks tissue culture medium containing 10% horse serum. This solution (6 ml) at 47°C was pipetted into plastic petri-dishes (LUX no. 5220) and allowed to cool, following which 12 wells of 3-mm diameter were cut into the gel.

In order to perform the assay, 5 μ l of antigen were added to 50 μ l leucocyte suspension, mixed and incubated at 37°C for 30 min. After re-suspension, 10 μ l aliquots of the suspension were placed in each of these wells. The plates were incubated at 37°C overnight in a moist 5% CO₂ incubator.

The plates were fixed in 10% formalin, the agarose removed, and the cells adhering to the plastic stained with 0.5% toluidine blue for 30 sec. Two diameters, at right angles, of the migrated cells were measured and multiplied. In order to obtain the migration index the result from the wells with antigen were expressed as a percentage of the migration of cells from the same sample without antigen.

Assessment of response to milk challenge

The effect of formal milk challenge for the five patients older than 5 years of age with SPTs ≥ 4 was observed directly in the Allergy Unit.

For the other challenged patients the effect of milk ingestion was assessed clinically and from information recorded on diary score-cards. On these cards, parents were asked to record the volume of milk formula given, the number of bowel actions and vomit episodes each day, the presence of colic/irritable behaviour, wheezing/cough, rhinitis, skin rashes, and episodes of waking each night. Symptom frequency during the period on cows' milk was compared with a similar period without cows' milk in the pre-challenge week. The classification of patients into 'late reactors' or 'milk tolerant' was based on standard clinical history and the diary records.

As a second part of this investigation, following allocation of patients to the groups, diaries were scored according to the individual symptoms recorded and the number of bowel actions passed each day for the week before and the week after milk

challenge. A score of 1 was assigned to each of the above symptoms for each day on which it occurred. In addition, a score of 1 was assigned for each bowel action recorded per day. For each patient the sum of individual symptom scores for the week pre-challenge was subtracted from those recorded during the milk-challenge period, e.g. Table 2: patient 10 had four more days of rhinitis when ingesting cows' milk compared with the previous milk-free week. The change in the individual symptoms scores were compared in the patients previously grouped as tolerant or late reacting CMA on clinical assessment.

Statistics

Non-parametric methods, Mann-Whitney *U*-test, Chi-squared test, and Fisher's exact test, were used for comparing symptoms and immunological data between the different patient groups. The null hypothesis was rejected when P was < 0.05 . In evaluating the ability of the diary-derived symptom scores alone to discriminate reactors and non-reactors, a step-wise discriminant analysis programme minimizing Wilks Lambda (SPSS) was used.

Results

Patient groups

Group A—milk tolerant. Nine children tolerated normal volumes of cows' milk from day 1 to 7 according to the challenge protocol. A further 14 children developed symptoms after commencing cows' milk, which ceased after milk ingestion was discontinued. Because the symptoms may have been due to intercurrent viral illness or minor forms of CMA, these 14 children were excluded from the analysis as they did not fulfil the above criteria for milk tolerance on day 7. However, it was noted at the final 1-month assessment that these 14 children were ingesting normal volumes of milk.

Complete clinical and laboratory data were available on five girls and four boys whose ages ranged between 7 months and 109 months (median 18 months) Table 1.

Group B—immediate reactors. Two groups of patients were identified by using SPT or milk challenge. (i) Positive by SPT: of 63 patients with SPTs ≥ 4 , complete clinical and laboratory data were available on 27 patients < 5 years of age [6]; (ii) positive by challenge: there were five children older than 5 years of age who underwent formal cows' milk challenge. In four of these patients 0.5–5 ml of milk caused exacerbations of peri-oral urticaria and flares of eczema within 15 min of milk ingestion. Generalized urticaria developed in the remaining child 40 min after milk ingestion commenced (data not shown).

There were a total of 32 patients in this group of whom 15 were girls and 17 were boys—ages ranged from 8 to 68 months (median 26 months).

Group C—late reactors. There were 24 children who developed symptoms of CMA more than 24 hr after commencing challenge. Only eight boys and two girls, whose ages ranged between 8 months and 100 months (median 36 months), met the full criteria for late-onset CMA and had complete clinical and immunological data available. One child included in this group (patient 18) developed vomiting within 3 hr of ingestion of 60 ml cows' milk concealed in soy milk in a placebo-controlled study. Although her clinical features are more consistent with the intermediate group of patients identified in our earlier studies [1], for the purpose of this investigation she was regarded as suffering from non-immediate late-onset CMA.

Table 1. Clinical and immunological features in milk-tolerant patients

Patient no.	Age (months)	Milk ingested	Irritable days	bowel actions†	Rhinitis days*	α -casein‡	β -lactoglobulin‡	α -lactalbumin‡
1	52	1200 in 7 days	0	0	0	89.5	82.6	91.0
2	7	4000 ml in 7 days	-3	1	0	92.4	86.8	98.7
3	14	900 ml in 7 days	0	0	0	103.0	91.0	96.3
4	13	3000 ml in 5 days	3	4	-5	89.5	87.9	90.2
5	51	2400 ml in 7 days	-1	-7	0	120.4	104.7	98.7
6	18	3000 ml in 7 days	2	0	0	96.0	90.6	89.9
7	39	4200 ml in 7 days	0	0	0	93.5	87.9	97.7
8	10	2400 ml in 7 days	0	0	1	101.3	90.7	75.4
9	109	1500 ml in 7 days	0	0	1	108.7	98.6	84.7

* Days of symptoms post-challenge - Days of symptoms pre-challenge (see Subjects and methods section for explanation).

† Number of bowel actions post-challenge - Number of bowel actions pre-challenge.

‡ LIF score (% of control migration) to individual milk proteins.

Thus, of the 110 children initially tested for CMA, complete clinical and immunological data was available on 51 children. Their results form the basis of this study.

Analysis of symptom scores

In Tables 1 and 2 the effect of milk ingestion on the symptom scores in the milk-tolerant and late reacting CMA patients is shown.

The clinical scores before and after milk challenge were compared between the late reacting milk-allergic and non-allergic groups. No significant difference was demonstrated in the change in incidence of skin rash, vomiting, wakeful nights and cough, before and after the milk challenge between these two groups. However, differences were observed with irritability ($P=0.01$), the number of bowel actions ($P=0.02$), rhinitis ($P=0.01$), and the sum of the differences ($P=0.001$) between the groups, indicating a worsening of symptoms during milk ingestion for the CMA late reactors.

Discriminant analysis was employed to develop an algorithm that would separate the late reacting milk-allergic patients from those who were milk tolerant. One hundred per cent correct classification was achieved using all the individual parameters described above, except for the presence of skin rash, which did not add to the discriminating power of the algorithm.

Immunoglobulin data

All the patients with immediate reactions to cows' milk by skin test had SPTs > 4 (individual data not shown). For this group RAST score was 4 in 12 cases, 3 in 11 cases, 2 in 7 cases, and 0 in 2 cases. The median total IgE was 458 i.u./ml (range 12-9700).

Table 2. Clinical and immunological features in late reacting CMA patients

Patient no.	Age (months)	Milk ingested	Irritable days*	No. bowel actions†	Rhinitis days*	α -casein‡	β -lactoglobulin‡	α -lactalbumin‡
10	13	2300 ml in 5 days	3	5	4	84.2	95.0	104.4
11	9	1200 ml in 7 days	6	3	0	77.8	64.3	53.5
12	68	3100 ml in 6 days	6	0	6	57.8	62.8	71.3
13	66	1100 ml in 5 days	0	7	0	71.5	66.3	64.6
14	57	4000 ml in 7 days	6	2	3	92.9	65.0	68.0
15	36	550 ml in 7 days	2	6	4	108.7	107.3	94.4
16	92	(intermittent) 2000 ml in 6 days	4	5	3	—	86.8	74.6
17	17	1200 ml in 5 days	0	5	5	98.5	90.8	83.7
18	100	90 ml in 3 hr	1	1	0	86.8	64.4	59.5
19	8	500 ml	1	—2	3	82.8	77.5	75.8

* Days of symptoms post-challenge—Days of symptoms pre-challenge (See Subjects and methods section for explanation).

† Number of bowel actions post-challenge—Number of bowel actions pre-challenge.

‡ LIF score (% of control migration) to individual milk proteins.

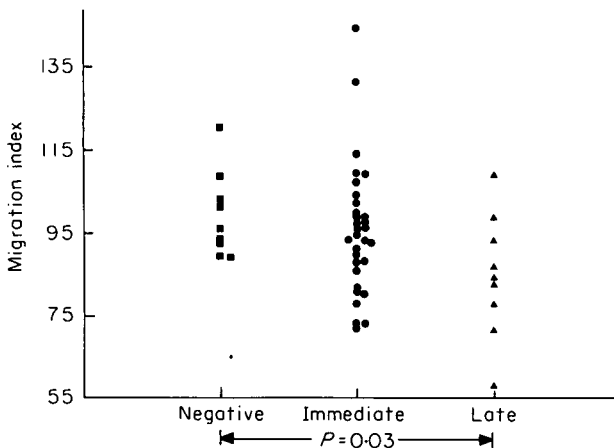


Fig. 1. Results of testing LIF production to α -casein in patients clinically tolerant to cows' milk (negative patients) and those with immediate or late reacting CMA.

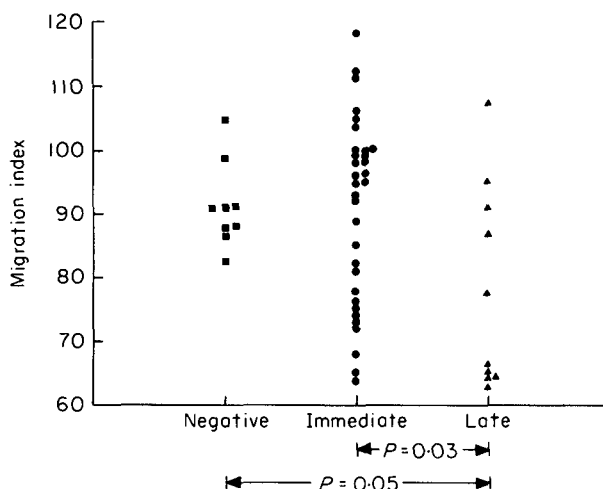


Fig. 2. Results of testing LIF production to β -lactoglobulin in patients clinically tolerant to cows' milk (negative patients) and those with immediate or late reacting CMA.

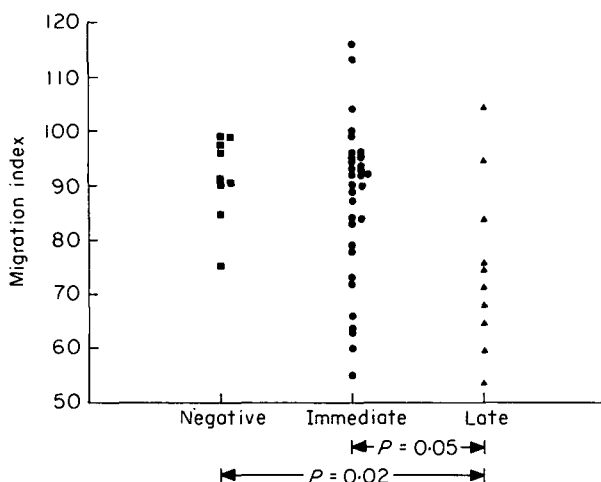


Fig. 3. Results of testing LIF production to α -lactalbumin in patients clinically tolerant to cows' milk (negative patients) and those with immediate or late reacting CMA.

For the milk-tolerant patients, RAST to milk was negative in all but one patient (patient 3), who was +3; this patient had SPT of 3 but the other patients were SPT negative. For the group, IgE ranges were <5–350 i.u./ml (median = 10 i.u./ml). Late reacting CMA patients were SPT negative, except for patient 13 who was SPT 1; two patients gave +1 RAST to milk but the remaining patients were negative. For the group, IgE range was <5–3440 i.u./ml (median 85 i.u./ml).

LIF results

In Figs 1, 2 and 3, LIF production for individual patients is shown for the three milk protein fractions used. While there is considerable overlap between all groups, there is

a statistically significant difference in LIF levels between cows' milk-tolerant and late-onset CMA for α -casein ($P=0.03$), β -lactoglobulin ($P=0.05$) and α -lactalbumin ($P=0.02$). A difference between immediate and late reacting CMA was observed for α -lactalbumin ($P=0.05$) and β -lactoglobulin ($P=0.03$). Minor *et al.* suggested LIF results $<80\%$ are abnormal [8]. Although a quarter of the immediate CMA patients, but none of the milk-tolerant ones, showed LIF levels $<80\%$ to α -lactalbumin and β -lactoglobulin, there was no statistically significant difference in the incidence of this abnormality between the immediate reacting CMA and milk-tolerant patients ($P=0.08$, Fisher's exact test).

Discussion

The results confirmed our original hypothesis in that patients with late reacting CMA show greater evidence of delayed type hypersensitivity (DTH), reflected by LIF production, compared with the other patient groups. Minor *et al.* [8], Khoshoo *et al.* [10] and Ashkenazi *et al.* [9] described similar *in-vitro* features of DTH to milk proteins in some children with CMA, although their findings are not strictly comparable with the results of this study. Minor *et al.*'s [8] patients were older and some had less well-defined symptoms attributable to CMA. Khoshoo *et al.*'s [10] population, while of similar age, presented with protracted diarrhoea; in addition their assay was not directly comparable with our assay. Ashkenazi *et al.* [9] described patients with similar clinical features to ours, but did not distinguish between patients with immediate- or late-onset reactions. Only Minor *et al.* studied LIF responses to the three proteins we investigated. He also found a higher incidence of abnormal responses to α -lactalbumin and β -lactoglobulin than to α -casein [7]. These findings suggest that these two proteins are more significant in the production of DTH than casein.

Our earlier studies highlighted the high incidence of diarrhoea and skin eruptions in patients with late-onset CMA [1]. In the current investigation additional features of CMA, including disturbed sleep, irritability and rhinitis, were significantly more frequent post-challenge than pre-challenge, emphasizing the multi-system effect of CMA. Initially we attributed these symptoms to the 'placebo effect' of asking parents to record data on diary cards [13]. However, the association of symptoms with the ingestion of cows' milk in CMA patients but not in those who were tolerant, as well as the demonstration of DTH abnormality in the group of patients who reacted, strengthens the significance of these clinical observations.

These preliminary findings must now be confirmed in a placebo-controlled investigation. Placebo-controlled studies have demonstrated eczema, urticaria, wheezing, vomiting, diarrhoea and anaphylaxis within minutes and up to 2 hr after ingestion of 8–10 g milk powder [14, 15]. This is equivalent to 60–70 ml cows' milk. In our experience, open challenge procedures in a controlled hospital setting more safely identify patients with this type of immediate reaction to these volumes of milk, and minimize the risk of anaphylaxis [1, 2]. However, some of our patients with late-onset CMA required up to 10 times this volume of milk daily for more than 48 hr before symptoms developed (Table 2). Milk powder equivalent to these volumes have been ingested by adults in capsules but this is not practical in infants and young children [16]. Despite these difficulties, placebo-controlled studies delivering appropriate volumes of milk protein now need to be undertaken to confirm our clinical and immunological findings.

Discriminant analysis of the diary score-card results allowed correct categorization of all patients. However, because the initial classification was by simple clinical evaluation, further studies will be required to confirm whether the algorithm derived from these results will correctly classify patients in the future. If the value of these score-cards is confirmed it will enable us to quantify symptoms attributable to late-onset CMA and subject them to appropriate statistical analysis. The validation of data recording methods of this type is necessary if the full spectrum of CMA is to be defined.

All the subjects in this study had been on a milk-free diet for at least 3 months before immunological investigations were undertaken, and none of the immediate reacting patients had previously ingested milk proteins in significant amounts. A third of the milk-tolerant patients had been on a milk-free diet for more than 3 years. These findings suggest that prolonged milk avoidance does not have a major influence on *in-vitro* detection of DTH in CMA patients. This is in contrast to the humoral immune response in patients with CMA, which may be heightened by milk ingestion and diminished by milk avoidance [3, 17]. These findings suggest that DTH to milk protein and late-onset CMA is associated with a long-lived antigen-sensitive lymphocyte population but the expression of DTH by these lymphocytes does not require ingestion of the antigen within the time-frame of this study.

At a hypothetical level we envisage that sensitized lymphocytes, from late-onset CMA patients exposed to milk antigen *in vivo*, generate a range of lymphokines leading to the clinical manifestations of CMA. In support of this, milk antigen has been identified in the systemic circulation following ingestion of cows' milk [18, 19]. This study has demonstrated circulating milk antigen-sensitized lymphocytes. Together these findings suggest that one of the factors leading to the wide range of symptoms described by our patients may be due to the local release of mediators from lymphocytes responding to circulating milk antigen.

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