

Lymphocyte response to cow's milk proteins in patients with cow's milk allergy: relationship to antigen exposure

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The cellular immune response to cow's milk was measured in patients with challenge-proven cow's milk allergy (CMA), manifested with either gastrointestinal or skin symptoms. After 2–4 weeks on milk elimination, 44 children, mean (SD) age 15.7 (9.4) months, were challenged, and cow's milk-induced lymphocyte transformation was measured before the clinical challenge (Day 1) and / or one week later (Day 8). During the clinical challenge period, 17 (39%) patients showed gastrointestinal reactions, 9 (20%) had urticarial or eczematous skin eruptions, and 18 (41%) were negative to challenge. On Day 1, the mean [95% confidence interval] stimulation index for lymphocytes in patients manifesting CMA with gastrointestinal symptoms, 2.60 [1.60, 4.10], was significantly higher than that in patients with skin symptoms, 1.15 [0.60, 2.30], or patients with negative clinical challenge, 0.83 [0.64, 1.08], $F = 9.0$, $p = 0.001$. After the clinical challenge (Day 8), this cow's milk-induced lymphocyte proliferation response was abrogated. At the same time, CMA patients evidenced a significantly higher spontaneous lymphocyte proliferation response in RPMI medium-containing control cultures than those with negative clinical challenge. We conclude that in patients with CMA, the number of circulating cow's milk-sensitized lymphocytes is depleted or their function is impaired after clinical exposure to cow's milk antigens.

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Cow's milk allergy (CMA) is defined as an adverse reaction to cow's milk proteins that is mediated by immunologic mechanisms (1). It is diagnosed by clinical response to withdrawal and subsequent challenge with cow's milk (2). In addition to clinical criteria, objective measurements of the activation of immune mechanisms are mandatory.

The clinical reactions to milk provocation are not, however, uniform in patients with CMA. Patients have been divided into subgroups according to the organ system affected and also by onset of the reaction (3). To explain this heterogeneity it has been postulated that various immunopathogenic mechanisms are operative in CMA (4,5). CMA manifested with urticaria appearing immediately after ingestion of low doses of milk is associated with type I IgE-mediated hypersensitivity, for example positive skin prick tests or radioallergosorbent tests (RAST). Patients manifesting CMA

with gastrointestinal symptoms often show delayed hypersensitivity reactions (6). The precise pathogenic mechanisms leading to clinical symptoms without evidence of IgE-mediated reactions are as yet unknown. They have been the subject of several investigations, with conflicting results (7–9). Reasons for discrepancies might include differences in patient selection, challenge protocol, definitions of reaction time as well as the scheduling of immunologic measurements (4, 7, 10, 11).

The objective in the present investigation was to determine the cellular immune response to cow's milk proteins in CMA manifesting itself with gastrointestinal or skin symptoms, and to investigate whether the lymphocyte response is altered by an oral cow's milk challenge. The lymphocyte transformation test was used for detection of cell-mediated immune response but not as a diagnostic test.

Material and methods

Patients

The study population consisted of 44 children, aged from 2 to 44 months, with a clinical history suggestive of CMA. This included disappearance of symptoms following a diet devoid of cow's milk and their reappearance after reintroduction of cow's milk. Manifestations included diarrhea ($n=5$), loose stools ($n=13$), abdominal pain ($n=8$), urticaria ($n=8$), eczema ($n=10$), and respiratory symptoms ($n=3$). Four patients had experienced severe symptoms. Informed consent was obtained from the children's parents, and the study was approved by the Hospital's Committee on Ethical Practice.

Milk challenge protocol

The diagnosis of CMA is based on an adverse reaction to an oral cow's milk challenge, since skin testing or cow's milk-specific RAST are not specific for CMA (12). The diagnostic criteria applied here followed the current guidelines for the diagnosis of CMA (11, 13). The patients were placed on milk elimination diet for 2–4 weeks before oral milk challenge. During the elimination period patients were either on tolerated formulas (soy, protein hydrolysate) or were given calcium supplementation. They were admitted for a clinical cow's milk challenge to confirm the diagnosis. CMA is strongly suggested if the patient is free of symptoms of CMA while on the cow's milk elimination diet (11, 13). Only patients with highly suggestive symptoms were included to this study; those with symptoms persisting during milk elimination were excluded. After the disappearance of symptoms was confirmed by medical staff, the challenge was commenced in hospital. It was performed openly because placebo reactions have been shown to be rare in infants (10). The patients were followed up over the one-week period of the challenge and seen again one month after its commencement.

The children were given cow's milk in rising doses; on Day 1: 2, 5, 10, 20, 50 and 100 ml and on Day 2 the normal milk intake appropriate for age was commenced. The doses were given at two-hour intervals. The challenge was stopped and the patients were examined when any adverse reaction was noticed. The time of the reaction was defined as the time elapsing from the last given dose eliciting the specific symptoms.

Blood samples

For the determination of lymphocyte response, blood in heparin was drawn before commence-

ment of milk challenge (Day 1) and/or one week later (Day 8). On Day 1, samples were obtained from 30/44 patients and on Day 8 from 29/44 patients. In 15/44 cases samples were available on both occasions. The serum total IgE levels were measured and RAST performed to detect circulating cow's milk-specific IgE-antibodies before the challenge.

The lymphocyte transformation test

A modification of the whole blood micromethod for mitogen-induced lymphocyte transformation (13, 14) was employed. In brief, heparinized blood was diluted 1:7 with RPMI 1640 (Grand Island Biological Company, Grand Island, NY, USA) containing antibiotics. Blood / RPMI 1640 mixture (175 μ l) were placed in flat-bottomed wells of a microtitre plate. Thereafter, different dilutions of milk protein (dried milk powder, Valio Finnish Co-operative Dairies' Association, Helsinki, Finland) were added in 25 μ l of RPMI 1640. The control cultures received 25 μ l of plain medium. The final concentrations of milk protein ranged from 0.1 μ g/ml to 1000 μ g/ml in tenfold dilutions to find the optimal antigen concentration to detect cow's milk-sensitized patients. The total incubation time in humidified 5% CO₂ atmosphere at 37°C was 168 h. Sixteen to 18 hours before harvesting, 20 μ l of ¹²⁵I-labelled 5-iodo-2'-deoxyuridine (¹²⁵IUdR) (0.25 μ Ci, specific activity 2000 Ci/mmol, The Radiochemical Centre, Amersham, UK) was added to each well together with 5-fluoro-2'-deoxyuridine (final concentration 4×10^{-6} M) to enhance the uptake of ¹²⁵IUdR (16). All counts given were corrected according to the reference day of ¹²⁵IUdR.

The stimulation index was calculated according to the formula:

$$SI_{(C)} = \frac{[\times \text{cpm}_{(C)}] - D}{(E - D)}$$

$[\times \text{cpm}_{(C)}]$ = counts per min for cultures with antigen concentration C

C = antigen concentration from 0.1 μ g/ml to 1000 μ g/ml in tenfold dilutions

D = counts per min for background

E = counts per min for control cultures with RPMI 1640

The upper limit of negative SI was set at 1.85, corresponding to the (ln) mean + 2 SD of the SI for patients with negative challenge. Lymphocyte proliferation is also expressed as counts per min, background excluded.

Table 1. The age, cow's milk-specific RAST and serum total IgE of individual patients, and volume of cow's milk eliciting the specific symptoms and reaction time from the last given dose of patients positive to clinical cow's milk challenge.

Patient (initials)	Age ¹	Volume of milk eliciting symptoms	Reaction time	RAST	IgE ²
CMA (gi-symptoms)					
T.H.	2.9	10 ml	0.16 hours	0	7.6
J.H.	5.7	5 ml	0.16 hours	1.2	13.0
S.H.	12.5	200 ml	12.0 hours	0.4	161
T.S.	34.6	200 ml	12.0 hours	0	14.0
J.P.	7.7	200 ml	36.0 hours	0	–
S.L.	3.0	20 ml	4.0 hours	0	6.0
M.O.	31.3	100 ml	0.5 hours	0	9.1
S.K.	24.0	200 ml	4.0 hours	–	–
T.M.	15.0	200 ml	4.0 hours	0	53.0
M.K.	14.1	200 ml	12.0 hours	0	2.2
J.H.	8.8	100 ml	0.1 hours	–	12.0
H.S.	12.5	200 ml	4.0 hours	–	2.9
T.A.	13.1	200 ml	12.0 hours	0	7.0
V.S.	4.8	200 ml	12.0 hours	0	5.0
A.M.	15.4	200 ml	96.0 hours	–	0.5
A.J.	35.5	100 ml	4.0 hours	0	40.0
V.T.	15.1	100 ml	4.0 hours	0	3.0
CMA (skin-symptoms)					
J.L.	19.1	5 ml	0.1 hours	0	13.0
E.L.	6.5	20 ml	2.0 hours	0.4	347.0
O.P.	24.1	5 ml	0.5 hours	11.0	144.0
V.R.	14.1	5 ml	0.5 hours	–	4.0
J.L.	18.3	2 ml	0.1 hours	0	10.0
A.J.	10.0	200 ml	12.0 hours	0	40.0
O.P.	12.1	2 ml	0.5 hours	3.8	49.0
A.N.	6.7	200 ml	4.0 hours	0.9	–
L.J.	19.1	5 ml	0.1 hours	0	–
Negative challenge					
L.K.	10.0			0	–
P.R.	31.4			–	123.0
J.H.	13.9			0	187.0
P.R.	13.5			–	–
A.P.	6.0			0	0.8
M.V.	22.4			0	6.0
N.M.	17.7			0	55.0
V.P.	9.5			0	3.2
S.A.	20.9			0	1.1
M.S.	14.0			6.6	594.0
L.R.	17.8			0	–
T.H.	16.0			–	3.0
S.H.	10.5			0	6.0
A.S.	15.8			0	87.0
P.L.	18.7			–	2.3
O.K.	33.4			0.18	–
P.L.	43.9			0	0.6
K.N.	10.3			–	–

¹ months

² kU/L

Statistical analysis

Logarithmic transformations (In) were used because of skewed distributions of stimulation indices (SI). Geometric means are presented with 95% confidence intervals [95% CI]. Analysis of variance (ANOVA) by Tukey test and Student's two-tailed independent t-test were used to determine the statistical significance of differences in continuous variables. Repeated observations were studied using ANOVA for repeated measures and paired t-test. The Mann-Whitney U test was employed for comparison of medians and the Chi-square test to determine differences in proportions. The Spearman Rank correlation coefficient was employed to correlate the SIs with cow's milk-specific and serum total IgE-levels.

Results

Clinical characteristics of study patients

The oral milk challenge was positive in 26 (59%) and negative in 18 patients. Seventeen patients showed gastrointestinal symptoms (diarrhea, $n = 4$; loose stools, $n = 9$; vomiting, $n = 4$) and 9 manifested urticarial or eczematous skin eruptions. The patients with negative cow's milk challenge were treated as disease controls; 16/18 of them were found healthy after introduction of cow's milk to the diet and of the remaining two, one had recurrent respiratory infections and one congenital heart disease. Table 1 illustrates the age, cow's milk-specific RAST and serum total IgE of individual patients and the volume of milk eliciting the specific symptoms and the reaction time of patients positive to clinical cow's milk challenge. The patients with skin symptoms often reacted quickly, in 0.6 [0.16, 2.30] hours, and to lower volumes, median 5 (2, 200) ml, of cow's milk. Gastrointestinal manifestations often developed significantly later, in 3.7 [1.4, 10.0] hours, $p = 0.027$, after significantly larger amounts, median 200 (10, 200) ml of milk, $p = 0.0016$.

The mean [95% CI] age at the onset of symptoms suggestive of CMA was 3.6 [2.0, 5.0] and 5.0 [2.5, 7.5] months in patients with gastrointestinal symptoms and skin disorders, respectively, and 8.3 [4.4, 12.1] months in patients with negative challenges; $F = 3.4$, $p = 0.04$. However, at the time of the provocation the mean [95% CI] age of patients manifesting CMA with gastrointestinal, 10.1 [6.4, 15.8] months, or skin symptoms, 13.2 [9.2, 19.0] months, was comparable to that of patients with negative challenge, 16.1 [12.6, 20.6] months; $F = 2.185$, $p = 0.13$. Pairwise comparison by Tukey test showed that the differences in age were statistically not significant.

Table 2. The lymphocyte response before oral milk challenge (Day 1) in patients with cow's milk allergy, manifested with either gastrointestinal or skin symptoms, and in patients with negative clinical challenge. Cow's milk-induced (protein concentration 1000 µg/ml) response is expressed in stimulation index (SI) and in counts per minute (CPM); response in unstimulated cultures (RPMI) is expressed in counts per minute.

	Gastrointestinal symptoms (n = 14)	Skin symptoms (n = 6)	Negative challenge (n = 10)	Statistical value
SI	2.60 [1.60, 4.10]	1.15 [0.60, 2.30]	0.83 [0.64, 1.08]	F = 9.0 p = 0.001
CPM	230.0 [134.1, 396.0]	149.3 [67.2, 332.0]	105.6 [73.0, 152.8]	F = 2.90 p = 0.07
RPMI	49.4 [29.2, 83.0]	53.0 [31.1, 90.5]	47.2 [27.4, 84.2]	F = 0.03 p = 0.95

Cow's milk-specific RAST (>0.4 kU/L) was positive in only 15% of CMA patients with gastrointestinal symptoms and in those with negative clinical challenge, whereas 50% of those with cutaneous symptoms were shown to have cow's milk-specific IgE (Table 1). The mean [95% CI] total serum IgE values in patients manifesting CMA with gastrointestinal or skin symptoms and in those with negative challenge were 8.6 [4.0, 18.7], 30.0 [8.7,

104.0] and 10.8 [2.7, 43.3] kU/l, respectively; F = 1.30, p = 0.30.

Lymphocyte response before oral milk challenge (Day 1)

Cow's milk-induced response – Altogether 55% of patients with clinically positive milk challenge had a lymphocyte response within the sensitized range before the challenge. Table 2 shows that the lymphocyte SIs were significantly different in the three study groups. This distinction was caused by the high SI for CMA patients with gastrointestinal symptoms, whereas the difference between CMA patients who showed skin symptoms and those with negative challenge was statistically not significant (Tukey test).

The highest concentration of cow's milk proteins (1000 µg/ml) was optimal in proving the induced antigen-specific lymphocyte stimulation to detect the occurrence of sensitized lymphocytes (Fig. 1). There were only three patients whose lymphocytes also responded at low concentrations (0.1–10 µg/ml).

No significant correlation was found between the levels of the SIs and the cow's milk-specific RAST levels or the serum total IgE; rho = 0.014, p = 0.95 and rho = 0.11, p = 0.60, respectively.

Lymphocyte response in the absence of antigen

As depicted in Table 2, the lymphocyte response in RPMI-containing control cultures was comparable in the three study groups, indicating that no difference in spontaneous lymphocyte proliferation prevailed between the groups of patients and controls before the oral milk challenge. Hence the difference between SIs was caused directly by the cow's milk-specific lymphocyte response to cow's milk proteins.

Lymphocyte response after oral milk challenge (Day 8)

Cow's milk-induced response – On Day 8, the lymphocyte proliferation response was abrogated in all

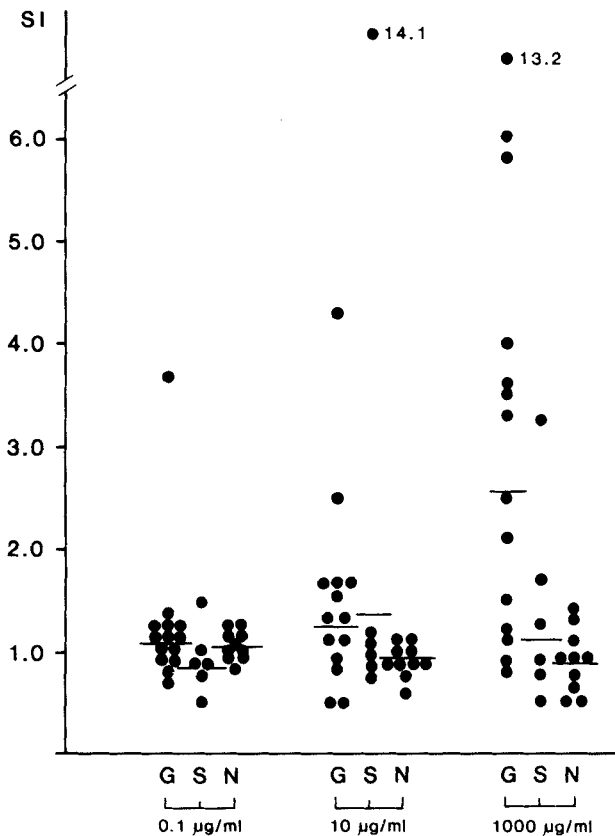


Table 3. ANOVA table for repeated measures: comparison of stimulation indices before and after oral challenge (repeated measure) in patients positive and negative to challenge (groups).

Source	df	Sum of squares	Mean square	F-test	p value
Group (A)	1	0.91	0.91	2.65	0.13
Subjects within groups	13	4.46	0.34		
Repeated measure (B)	1	2.82	2.82	25.07	0.0002
AB	1	0.97	0.97	8.57	0.01
B × subjects within groups	13	1.46	0.11		

but two patients with CMA. At this point, the mean SIs for patients positive and negative to challenge were 0.9 [0.7, 1.2] and 0.9 [0.8, 1.0], respectively, $t = 0.23$, $p = 0.82$.

The ANOVA table for the SIs is given in Table 3. At successive measurements the patients and controls, studied both before (Day 1) and after (Day 8) the clinical challenge, were shown to have different lymphocyte response: in patients positive to clinical challenge the mean SI decreased from

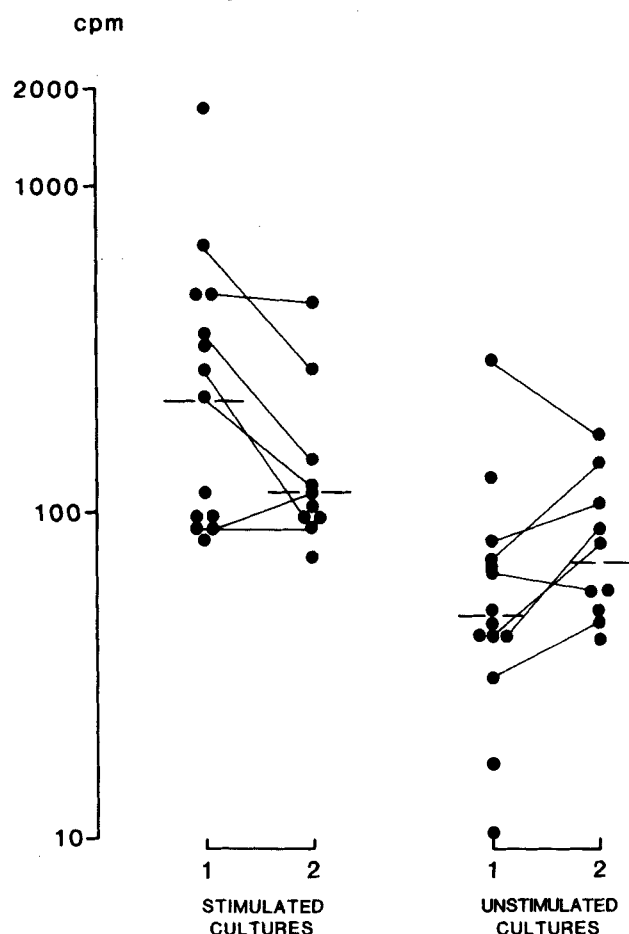


Fig. 2. The prechallenge (1) and the postchallenge (2) lymphocyte proliferative response (in counts per minute) in patients with cow's milk allergy manifested with gastrointestinal symptoms. Lymphocytes were induced by cow's milk (stimulated cultures) and cultured in the absence of antigen (unstimulated cultures). Points represent individual patients; those interconnected were evaluated on both occasions. Horizontal lines for geometric means.

Day 1 to Day 8, whereas in patients negative to challenge it remained unaltered.

Fig. 2 illustrates the change in cow's milk-induced lymphocyte proliferation (in cpm) from Day 1 to Day 8 in patients who manifested CMA with gastrointestinal symptoms; the mean [95% CI] lymphocyte proliferation decreased from 230.0 [134.1, 396.0] to 131.0 [88.3, 196.0]; $t = 2.4$, $p = 0.05$, reflecting depressed antigen-specific lymphocyte response.

Lymphocyte response in the absence of antigen – The patients who developed gastrointestinal symptoms showed raised spontaneous lymphocyte proliferation in RPMI-containing control cultures after the oral milk challenge (Fig. 2).

Discussion

CMA comprises a heterogeneous group of disorders, and there is evidence that distinct immunologic reactions to cow's milk proteins are involved (4, 17). CMA manifested with immediate skin eruptions has been connected with type I IgE-mediated hypersensitivity by elevated cow's milk-specific IgE or skin prick tests (17). The mechanisms underlying reactions where the clinical manifestations are localized in the gut seem not all to involve the type I hypersensitivity. The pattern of changes associated with tissue damage seen in cow's milk-sensitive enteropathy is suggestive of experimental graft-versus-host reaction with crypt hyperplasia, increased crypt cell production rate and increased intraepithelial lymphocyte count (18–20). Our results are in accord with these findings: the patients who manifested CMA clinically with gastrointestinal symptoms, frequently evidenced enhanced cow's milk-specific lymphocyte activation in the lymphocyte transformation test, which measures a cell-mediated hypersensitivity reaction (21).

Results of previous studies on lymphocyte response in CMA have varied considerably (7–9, 22). This might partly be due to different methodologies employed regarding milk challenge protocol as well as *in vitro* testing. Where isolated cells are stimulated, the proportions of cell types differ

from those in peripheral blood (23). To create conditions resembling those prevailing *in vivo*, we employed a method in which whole blood was used. In addition, we used whole cow's milk as source of antigens to reflect the antigenic experience of the patient. Furthermore, the results were analysed separately for CMA patients who showed gastrointestinal or skin symptoms.

The results of the present investigation indicate that contradictory findings in previous studies may also be attributable to clinical antigen exposure. It is noteworthy that in CMA patients with gastrointestinal symptoms the lymphocyte proliferative response was abrogated after the oral cow's milk challenge. This is in keeping with previous results on gluten sensitivity in patients with coeliac disease (24–26). Coeliac patients manifested cell-mediated immunity to gluten on gluten-free diet only (26). After the introduction of gluten to their diet the lymphocyte response was in the normal range. It was suggested that on normal diet sensitized lymphocytes may localize in the small bowel, being thus present in fewer numbers in the peripheral blood (26). A similar interpretation might apply to our results in CMA.

There may also be additional explanations for the phenomenon. The present study indicates that the function of cow's milk-specific lymphocytes could be impaired in CMA. Patients with CMA evidenced enhanced spontaneous lymphocyte-proliferative response *in vitro* after commencing the oral milk challenge. In addition, the cow's milk-induced lymphocyte transformation response was shown to be abrogated. This would indicate that a part of the cow's milk-sensitized lymphocyte population was preactivated *in vivo* during oral milk challenge. The number of resting lymphocytes would consequently be reduced in the peripheral blood in these patients after the introduction of cow's milk to the diet and the cells would not be able to respond to cow's milk *in vitro* one week later. It must be acknowledged that the amount of spontaneous proliferation was not high enough to explain alone the reduced T-cell function after clinical exposure to cow's milk antigens in patients with CMA. It must be assumed that the resting T-cells remaining in circulation were not able to respond *in vitro* to cow's milk after oral challenge as vigorously as before the challenge.

Taken together, our result reinforces the conception that in CMA manifested with gastrointestinal symptoms, the immune-system elimination of cow's milk antigens is defective. The inability to respond adequately to cow's milk antigens *in vitro* after oral exposure to cow's milk needs to be taken into consideration when immunologic evaluation of CMA patients is indicated.

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