

Value of immunologic tests in cow milk allergy

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The aim of this study has been to clarify the immunopathogenesis and diagnosis of cow milk allergy (CMA). Thirty-four children with symptoms suggestive of CMA had a challenge test with cow milk (CM) and an estimation of their immunological response. Nineteen of the 34 children reacted to CM challenge test. We measured serum levels of immunoglobulins G, A, M and E, complement fractions 3 and 4, class specific CM antibodies as well as the numbers of the different lymphocyte subsets and the responses of lymphocytes in whole blood to stimulation by phytohemagglutinin (PHA), concavalin A (ConA) and beta-lactoglobulin (BLG). Twelve (63%) of the children who reacted had high levels of IgE CM-specific antibodies; they had lower mean levels of serum IgA and lower lymphocyte stimulation indices with BLG and PHA than the rest of the children (37%) who reacted on CM, but were without IgE antibodies. Of these seven children, five gave elevated responses to stimulation with BLG, and in three of six the helper/suppressor lymphocyte ratio rose during the challenge test. Most (10) of 15 children who showed no reaction when challenged with CM at hospital, had earlier had cutaneous symptoms closely related to CM formula feeding. No single laboratory method was sufficient to discriminate between the children who reacted to CM and those who did not. The best combination of tests was measurements of CM-specific IgE and the index of lymphocyte stimulation with BLG. This combination had a sensitivity of 88% and specificity of 67% for predicting a clinical reaction.

Key words: cow milk allergy; cow milk antibodies; lymphocyte subsets; lymphocyte stimulation; beta-lactoglobulin; IgE.

Accepted for publication 4 November 1989

Cow milk allergy (CMA) is as great a challenge to practising physicians as to researchers. It is difficult to diagnose clinically, because of the great diversity and varying severity of the symptoms (18). Nor can abnormal immunologic reaction to CM be demonstrated in all cases (16). Immediate skin reactions to CM are often associated with IgE-specific antibodies (5, 11), but the relevance of tests showing activation of other types of immune mechanism is disputed. Observations suggesting involvement of these mechanisms include findings of increased levels of CM-specific antibodies of immunoglobulin classes G, A, and M (6, 7, 9), increased stimulation

of lymphocytes by beta-lactoglobulin (BLG) (17, 21), secretion of leukocyte migration inhibition factor by lymphocytes when in contact with BLG (1), serum complement reduction (14), depressed neutrophil chemotaxis (4), and reduced nonspecific suppressor cell activity (10, 21).

In the present study we performed several immunologic tests on a group of 34 children suspected of having CMA and compared the results of the tests with the outcome of a CM challenge test to demonstrate which laboratory tests are the most reliable for diagnosing of CMA and determining the types of immune reaction involved.

MATERIAL AND METHODS

Patients

Thirty-four consecutive patients with suspected CMA were studied at the Children's Hospital and the Hospital for Allergic Diseases of Helsinki University during 1 year. CMA was suspected because of cutaneous, gastrointestinal, and/or respiratory symptoms that had followed the introduction of CM into the diet and that disappeared when CM was eliminated. CM elimination had lasted at least 1 week before the challenge test. The median age of the children was 11 months (range 3–51 months) and the median duration of exclusive breast-feeding 3 months (range 0–12 months). The patients had been weaned to commercial adapted cow milk formulas. The families followed the feeding recommendations given by healthy baby clinics: fruit and vegetables were given from 3 months, cereals and meats from 5–6 months of age. The commercial formulas were used until the age of 9 months, and thereafter dairy milk was introduced. Challenge tests with CM were done in the hospital. They were started with a drop of adapted CM formula on the lips. If no reaction was seen within 1 h, 5 ml of formula was given orally. The amount was doubled every 4 h until the child showed symptoms or until the full feeding volume was reached in 72 h. The children were grouped according to age and time of appearance of symptoms (Table 1): Groups I and II, under and over 8 months of ages, respectively, had an immediate reaction appearing within 1 h of the challenge; Group III, all over 8 months, had a reaction more than 1 h after the start of the challenge; Groups IV and V were under and over 8 months, respectively, and had no clinical reactions. The limit of 8 months is arbitrary and was chosen on the basis of the age distribution of the study group. The number of samples studied with each individual test varied with the availability of facilities.

Methods

A venous blood sample was taken before the challenge test, and 8, 24 and 72 h after the start of the test. The samples were always taken at the same time of day (08.00 h and 14.00 h $\pm 1/2$ h). Total leukocyte count, differential count, levels of immunoglobulins G, A, M and E, CM antibodies, and complement fractions 3 and 4 were measured from each sample. Lymphocyte subsets were counted and whole blood stimulation tests were performed before challenge and 24 and 72 h after the start. We took the reference values for several immunologic tests from a group of 198 healthy children which we followed monthly during their first year and at the mean age of 28 months. Since the results of the tests may be greatly influenced by the age at first exposure to CM and the duration of CM feeding, the values of each child in the present study were compared with those of a healthy child with a similar history of CM feeding (19, 20).

Immunoglobulins were measured by single radial immunodiffusion and the values expressed as percentages of the mean for age.

Serum C3 and C4 levels were measured by single radial immunodiffusion and the values compared with those of 57 healthy children of ages similar to the study group.

Serum IgE was measured by the paper radioimmunosorbent test (PRIST) and CM-specific IgE antibodies by the radioimmunoabsorbent test (RAST) (Pharmacia, Uppsala, Sweden). Normal values for serum IgE were calculated from 434 samples taken from 198 healthy children. A RAST score ≥ 2 was regarded as supranormal.

Specific antibodies to CM in immunoglobulin classes G, A and M were measured by enzyme-linked immunosorbent assay (ELISA) (20). A serum standard with a high titer of CM antibodies was used as a reference and the antibody content of the infant sera is given as a percentage of the standard.

After separating lymphocytes by the Ficoll-Isopaque method the numbers of mature T lymphocytes, helper/inducer T cells, suppres-

sor/cytotoxic T cells, and B cells were measured by an indirect immunofluorescent method using monoclonal antisera OKT3, OKT4, OKT8, and OKIa1 (Ortho Diagnostic System, Rarita, NJ, USA) as a first layer, and then stained with FITC-conjugated goat anti-mouse IgG (Miles Lab, Elkhart, USA) (12, 19). The B cells were counted by direct immunofluorescence using polyvalent FITC-conjugated antisera to heavy chains (Hyland, Costa Mesa, USA and Behringwerke, Marburg, West Germany). Positively stained cells among 200 lymphocytes were counted in a phase-contrast microscope. As a control group we used 68 of the 198 children.

Whole blood was used in the lymphocyte stimulation tests with the mitogens phytohemagglutinin (PHA), concavalin A (ConA), and beta-lactoglobulin (BLG) by the method of Pauly (15). Unseparated blood was diluted 1:5 with RPMI 1640 (Gibco, NJ, USA) and fetal calf serum (Gibco, NJ, USA), glutamine (Orion Diagnostica, Espoo, Finland) and Hepes (Sigma, St Louis, USA) and then stimulated with PHA 33 µg/ml (Difco, Detroit, USA), ConA 50 µg/ml (Pharmacia, Piscataway, USA) and BLG 12.5 µg/ml (Sigma, St Louis, USA). The

samples were cultured in triplicate on microtiter plates (Falcon) for 72 h. 1 µCi ³H-thymidine (Amersham, Buckinghamshire, UK) in 10 µl was added to each well 7 h before the end of the incubation period. One sample, made with blood and RPMI only, served as a blank (unstimulated) test. We calculated the stimulation index by dividing the counts of the stimulated and unstimulated cultures. We used samples from healthy adult blood donors as controls to

ensure that the method functioned. We regarded PHA and ConA stimulation indices (mean -1SD) of less than 30 and 25 respectively, as pathological. A BLG stimulation index above 3 was regarded as positive. This value exceeded the mean ±2SD of adults.

Statistical methods

The mean values of samples taken 24 h after the start of the challenge test were compared between the different Groups (I-V) by the t test. Because of the skewness of the distributions, calculations of PHA, ConA indices, and IgE levels were made after logarithmic transformation. Fisher's exact test was used for comparing the distribution of abnormal values in Groups I to V and in children with and without IgE antibodies. The t test for dependent variables was used for repeat samples. The specificity and sensitivity of each variable and of combinations of the different variables for predicting positive clinical reactions in the challenge test were calculated by applying Bayes' theorem. Ages at first exposure to CM and duration of breast-feeding in the different groups were compared by the Wilcoxon rank sum test.

The study was approved by the ethics committee of the hospitals. The parents of the patients were informed about the design of the study and gave their consent.

RESULTS

The symptoms most frequently noted by the parents at home were erythematous (18) or

Table 1
Characteristics of the children in the different groups

Type of reaction to CM challenge:	Immediate		Delayed	None	
Age:	< 8 months	> 8 months	> 8 months	< 8 months	> 8 months
Group:	I	II	III	IV	V
Number of patients	6	8	5	4	11
Median duration of breast-feeding (months)	2.5	8.0	6.0	1.9	4.5
Median age at 1st exposure to CM (months)	2.0	6.5	3.5	1.5	4.5
Median age at CM challenge (months)	5.6	12.8	12.7	4.2	14.4
Positive heredity for atopy	3	5	4	2	7

Table 2

Results of laboratory examinations for the different groups 1 day after challenge. Means and $\pm$ SE are given or, if the geometric mean was used, 95% confidence limits

Type of reaction to CM challenge:	Immediate		Delayed	None	
Age:	< 8 months	> 8 months	> 8 months	< 8 months	> 8 months
Group:	I	II	III	IV	V
Number of patients	6	8	5	4	11
S-IgE U/ml	50(9-277)	30(6-161)	74(39-141)	21(4-106)	122(44-600)
S-IgG % (of mean for age)	58 $\pm$ 9	47 $\pm$ 4*	89 $\pm$ 14*	70 $\pm$ 25	91 $\pm$ 10*
S-IgA % (of mean for age)	140 $\pm$ 6	69 $\pm$ 25*	137 $\pm$ 32	86 $\pm$ 14	140 $\pm$ 22*

Groups compared (indicated by *) differed significantly ($P\leq0.05$).

urticarial (5) eruptions; seven had had respiratory symptoms (4 wheezing and/or 4 rhinitis) and seven intestinal symptoms (4 vomiting and/or 4 diarrhea) after taking CM formula. Other causes for submitting were growth failure (reduction of weight or relative height > 1SD) (13), frequent upper respiratory tract infections (7), or recurrent otitis media (8). The CM challenge performed in the hospital department evoked similar manifestations (erythema 13, urticaria 3, wheezing 4, rhinitis 2, vomiting 2, diarrhea 4) but symptoms were recognized by the medical staff in only 19/34 (56%). Fourteen (Groups I and II) showed symptoms within 1 h of the start of the challenge, four after skin contact with CM and 10 after drinking 5 ml of CM. Five showed clinical signs later (Group III) (Table 1). In 15 no symptoms were observed during the challenge test; 10 of them had

had cutaneous symptoms and five intestinal and two respiratory associated with CM formula feeding.

High values of total serum IgE were most frequent among older children who gave an immediate clinical reaction (Group II): 5/8 vs 2/6 of the younger children with an immediate reaction (Group I), 1/5 in those with a delayed reaction, and 4/15 in the non-reacting children. However, the logarithmic mean values of IgE were similar in all groups (Table 2).

Among the children who gave an immediate reaction, high levels of IgE antibodies to CM were seen most often in those over 8 months old (7/8). IgE antibodies to CM were more frequently high (RAST score ≥ 2) in patients with a positive challenge test (1 2/19, 63%) than in those with a negative test (3/15, 20%, $P<0.05$). The median score of the reacting

Table 3

Number of lymphocytes and their subsets one day after challenge. Means and SE are given

Type of reaction to CM challenge:	Immediate		Delayed	None	
Age:	< 8 months	> 8 months	> 8 months	< 8 months	> 8 months
Group:	I	II	III	IV	V
Number of patients	6	8	5	4	11
Lymphocytes $10^9/l$	7.4 $\pm$ 0.7	7.3 $\pm$ 0.6	7.2 $\pm$ 1.3	7.4 $\pm$ 1.2	5.8 $\pm$ 0.6
T cells $10^9/l$					
Total T	4.6 $\pm$ 0.5	4.6 $\pm$ 0.4	4.5 $\pm$ 1.0	4.6 $\pm$ 0.7	3.6 $\pm$ 0.8
Helper T	2.7 $\pm$ 0.2	2.9 $\pm$ 0.2	2.9 $\pm$ 0.4	2.9 $\pm$ 0.4	2.3 $\pm$ 0.5
Suppressor T	1.8 $\pm$ 0.3	1.5 $\pm$ 0.2	1.5 $\pm$ 0.3	1.5 $\pm$ 0.3	1.2 $\pm$ 0.3
Helper/Suppressor	1.6 $\pm$ 0.1*	1.9 $\pm$ 0.2*	2.0 $\pm$ 0.1*	2.0 $\pm$ 0.1*	1.9 $\pm$ 0.1
B cells $10^9/l$					
OKIa-cells	1.8 $\pm$ 0.3	1.6 $\pm$ 0.1	1.6 $\pm$ 0.3	1.6 $\pm$ 0.3	1.0 $\pm$ 0.2

Groups compared (indicated by *) differed significantly ($P<0.05$).

Table 4
Stimulation of lymphocytes in whole blood by mitogens 1 day after challenge

Type of reaction to CM challenge:	Immediate		Delayed	None	
Age:	< 8 months	> 8 months	> 8 months	< 8 months	> 8 months
Group:	I	II	III	IV	V
Number of patients	6	8	5	4	11
PHA stimulation index	169(69-418)*	164(50-536)*	33(20-82)*	63(17-225)	62(36-106)
ConA stimulation index	107(57-200)*	99(20-499)*	35(24-51)*	51 (9-288)	42(27- 73)
BLG stimulation index	4.5±1.7	3.1±2.2	1.6±0.5	1.1±0.1	1.6±0.4

Groups compared (indicated by *) differed significantly (*P* < 0.05).

patients was 2. The sensitivity of CM-specific IgE antibodies for predicting a clinical reaction was 63 % and the specificity 80 %.

In samples taken on admission the mean level of serum IgG was significantly lower in the children who responded positively to challenge than in those who gave no reaction ($72 \pm 7\%$ vs $99 \pm 10\%$, *P* < 0.05). After challenge, the older children who reacted immediately (Group II) had the lowest level of serum mean IgG and IgA (Table 2), but did not differ from the group as a whole in the mean level of IgM.

Mean levels of the complement fractions 3 and 4 were not lower in reacting groups, and

individuals with low levels were equally common among reacting and nonreacting children (5/19 and 1/19 vs 4/15 and 2/15 for C3 and C4 respectively).

No significant differences were found between the five study groups in the mean values of CM-specific antibodies of the IgG, IgM and IgA classes in samples taken either on admission or after challenge. The mean level of CM antibodies in the children of the present study group was similar to that of a matched group of healthy children in our prospective follow-up study (20).

The challenge caused a clear decrease (> 30 % of the prechallenge value) in IgM class antibodies in seven children with a reaction and in three with no reaction, and a clear increase (> 30 % of the prechallenge value) in the IgA class antibodies in five children with a reaction and in none with no reaction.

The mean numbers of lymphocytes did not differ between the groups (Table 3). After challenge, the number of lymphocytes decreased (> 30 %) in four children and increased in three. The decreases all occurred in children who reacted to the challenge. The mean numbers of the different lymphocyte subsets were similar in most groups both before and after challenge (Table 3). The helper/suppressor ratio was lowest in the children under 8 months old who had an immediate reaction (Table 3). A change of > 30 % in the absolute numbers of OKT3-, OKT4- or OKT8-positive cells was seen in samples from seven children during the challenge. A rise in helper cells was more frequent in the blood of children who had given a

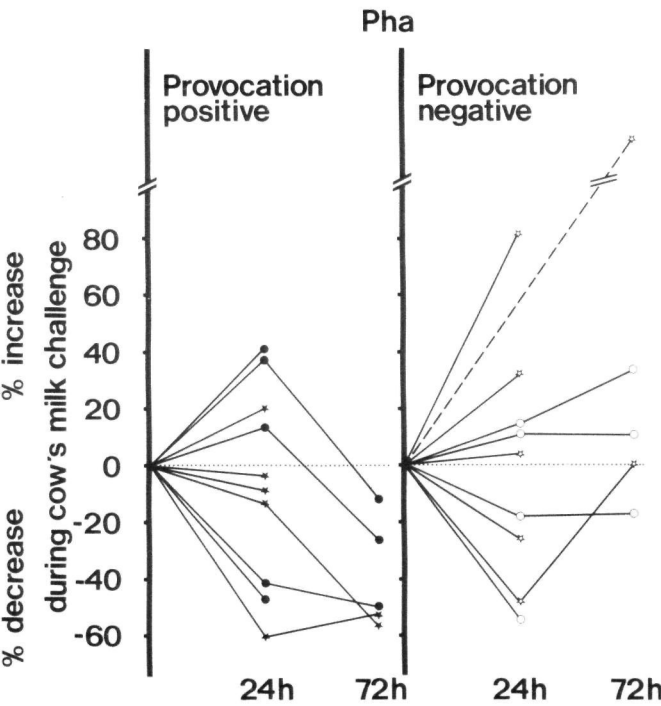


Fig. 1. Lymphocyte stimulation with PHA: the percentage change (from the prechallenge level) is shown 24 and 72 h after beginning the CM challenge.

Table 5

Results of laboratory examinations 1 day after challenge in children with a positive reaction with and without IgE antibodies to CM and in children with no reaction and without IgE antibodies to CM. Means and SE are given or if the geometric mean is used, mean and 95% confidence limit

Reaction	Positive	Positive	Negative
IgE antibodies to CM:	Positive	Negative	Negative
Number of patients:	12	7	12
Median duration of total breast-feeding (months)	9*	5*	3
Median age at 1st exposure to CM (months)	6	3	3
S-IgA %	85±17*	152±19*	133±19
PHA stimulation index	47(20-69)*	173(83-537)*	58(35-97)*
ConA stimulation index	42(20-89)†	105(60-182)†	42(26-84)†
BLG stimulation index	1.2±0.2*	4.7±1.3*†	1.6±0.3†

Groups compared (indicated by *) differed significantly ($P \leq 0.05$). The suggestive difference is indicated by symbol †.

positive reaction to the challenge test (3/12 vs 0/7; $P < 0.1$). The helper/suppressor ratio rose in samples from three children, who were all positive in the challenge test.

The whole blood lymphocyte stimulation test with BLG was positive in the samples from 6/15 children with a positive challenge test and from 2/12 of those who gave no reaction ($P < 0.1$). It was most often positive in children under 8 months who showed an immediate reaction (4/5 vs 1/5, 2/5 in other reacting children or 0/3, 2/9 in nonreacting children; $P < 0.1$). Some children in each group had low stimulation indices for PHA and ConA. After challenge the mean PHA and ConA stimulation indices were significantly higher for the children with an immediate reaction than for those with a delayed one (Table 4). The individual levels of lymphocyte stimulation with mitogen PHA (Fig. 1) and ConA varied similarly in children with and without a reaction to challenge.

Comparison between children who were positive and negative for IgE antibodies to CM

Of the 19 children who had a positive challenge test, those with IgE antibodies to CM had been weaned later than those without (Table 5). Of the 12 children with IgE antibodies to CM, seven had high level of serum IgE, and 4/8 had low stimulation indices with ConA. They had a lower mean level of serum IgA than the children without IgE antibodies (Table 5), but

similar levels of serum IgG and IgM. The mean levels of C3 and C4 did not differ. Many of the children (5/12) had increased levels of IgG antibodies to CM, whereas those reacting children who were without IgE antibodies had normal levels (increased in 0/7; $P < 0.1$). The lymphocytes of most reacting children (5/7) without IgE antibodies responded to stimulation with BLG, in contrast to samples from 1/8 with IgE antibodies reactivity ($P < 0.05$).

Combination of different laboratory methods for predicting CMA

The best combination of laboratory tests was measurements of IgE antibodies to CM and stimulation of lymphocytes with BLG. Either an increase in IgE antibodies and or an abnormal stimulation index was seen in 88% (14/16) of reacting children, and in 33% (4/12) with no reaction; both tests were negative in 12% (2/16) and in 67% (8/12), respectively. This gave a sensitivity of 88% and specificity of 67%.

DISCUSSION

As in earlier studies (2, 3), only about half (56%) of our patients suspected of having CMA, showed clinical manifestations during a CM challenge test. Among the reacting patients we tried to distinguish definite subgroups on the basis of age and mode of reaction to the test. Of the younger patients (< 8 months) who gave an

immediate reaction, the majority were without CM-specific IgE antibodies, but their lymphocytes were stimulated by BLG, whereas almost every child in the older group had IgE CM antibodies. The older children with a delayed reaction did not show any clear-cut immunologic mechanism. In the series as a whole, more than half had supranormal titers of IgE antibodies to CM. This finding confirms other reports of patients with CM allergy (5, 7, 11).

It has been claimed that in patients with IgE antibodies, IgG CM antibody titers are often low (8), but our results, like those of some other studies, show the opposite (6, 9). Among the children with a positive challenge test, high titers of IgG antibodies were more frequent in those with IgE antibodies than in those without. Among those with a negative challenge test, none without IgE antibodies had high IgG antibody titers. Thus, in patients with IgE antibodies, IgG antibodies may represent a secondary phenomenon caused by increased permeability of the intestine due to the IgE-mediated immune reaction in the gut.

Much has been argued about the possible importance in CMA of some immune mechanism other than that mediated by IgE (11, 18). In our series the children without IgE antibodies more often had a high stimulation index with BLG. On the whole, their cellular responses *in vitro* to stimulation were more vigorous. This type of reaction was more frequent in the younger children who had been weaned earlier to CM formula than in those weaned later, who had IgE antibodies. It has been speculated that early exposure to CM may lead to suppression of the IgE response (8) but at the same time provoke cellular responses which may later lead to symptoms.

An immune complex type of reaction with reduction of complement has been thought to be important in intestinal CMA (14). No such reaction was present as a clear entity among our patients; many had low levels of C3 and reduction of IgM antibodies during challenge, but these changes were also seen in children who did not react.

Because of the heterogeneity of the immunopathogenesis of CMA, no single laboratory

method is valid for diagnosing CMA. A combination of laboratory methods that measure different immune mechanisms may result in high sensitivity. Such a combination could be measurements of IgE CM antibodies and the lymphocyte stimulation index with BLG. In our study this combination had a sensitivity of 88% and a specificity of 67%.

ACKNOWLEDGMENTS

This research was supported by grants from the Foundation for Pediatric Research, the Foundation for Nutritional Research, and the Sigfrid Juselius Foundation.

The skillful technical assistance of Mrs Terttu Louhio is gratefully acknowledged.

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