

Cow's milk protein-specific IgE concentrations in two age groups of milk-allergic children and in children achieving clinical tolerance

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Summary

Background IgE-mediated cow's milk protein allergy (CMPA) is usually outgrown in children by the age of 3 years. The immunological responses to cow's milk proteins in children who achieve tolerance, in comparison with those who remain allergic, however, are not well described.

Objective To compare the level of cow's milk protein-specific IgE among children with documented CMPA under the age of 3 years, another group over the age of 9 years (persistent allergy), and in another group of children in whom clinical tolerance developed.

Methods Stored sera from children with CMPA were analysed for IgE antibodies specific for whole cow's milk, casein, whey, α -lactalbumin (ALA), β -lactoglobulin (BLG), and bovine serum albumin (BSA) using the Pharmacia CAP System FEIA.

Results Within each group of CMPA children, the concentration of specific IgE antibody to casein proteins was not significantly different from that to whey proteins. However, children in the group with CMPA over 9 years of age had significantly greater concentrations of whole milk ($P=0.02$) and casein-specific ($P=0.04$) IgE antibodies compared with the group of children with CMPA under age 3 years. Children under the age of 3 years had a higher median concentration of casein-specific IgE (20.2 vs. 5.5, $P=0.04$) than another group of 11 children (mean age 3.5 years), who later lost their milk allergy. Out of 16 children who lost sensitivity to milk, 75% had milk-specific IgE levels below 14.3 kU_A/L (median, 1.3 kU_A/L).

Conclusions Although a dominant allergenic milk protein fraction was not identified within either of the two age groups, those with persistent CMPA over age 9 years had significantly elevated levels of milk and casein-specific IgE compared with younger children with CMPA.

Keywords: cow's milk, hypersensitivity reactions, IgE, double-blind placebo-controlled food challenges, casein, whey

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Introduction

Cow's milk protein allergy (CMPA) affects $\approx 2.5\%$ of children less than 3 years of age [1–4]. Symptoms of CMPA involve the skin, respiratory and gastrointestinal

tracts. About one-half of these reactions are mediated by specific IgE antibodies toward proteins in cow's milk (CM) [1]. The IgE antibodies mediating these reactions may recognize any of over 20 proteins identified in CM [5]. No particular protein in CM has been identified as a 'major allergen', neither the casein or whey fractions of CM has been clearly identified as being a major allergenic fraction [6]. Most children with CMPA have IgE antibodies to several of the proteins found in both of the fractions of CM [7].

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However, some studies have indicated that children with CMPA have higher concentrations of specific IgE antibody to proteins comprising the whey fractions of milk [8] while IgE directed to the proteins in the casein fraction appeared to be dominant in older children and adults with CMPA [9,10].

About 75% of children with CMPA 'outgrow' their sensitivity by their third birthday [1], but the immunological mechanisms determining the development of tolerance are unknown. Previous studies have shown that children who are destined to become tolerant have lower levels of CM-specific IgE antibody than those who were destined to remain clinically allergic [11,12]. Whether there is a relationship between differences in sensitivity to particular proteins in CM and the development of tolerance remains unknown.

The Pharmacia CAP System FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) is a method of measuring allergen-specific IgE and is reported to have improved sensitivity and a broader dynamic range compared with standard RAST; it has been shown to be useful in correlating symptomatic milk allergy with particular levels of CM-specific IgE [13]. Using the quantitative capability of the Pharmacia CAP System FEIA, we sought to measure the concentration of CM and milk-fraction (casein, whey, and the particular proteins in the whey fraction, namely α -lactalbumin[ALA], β -lactoglobulin[BLG] and bovine serum albumin[BSA]) specific-IgE among children with IgE-mediated CMPA. We selected stored sera from children with clearly defined IgE-mediated CMPA who were under age 3 years (in whom tolerance would be expected to occur in the majority), over age 9 years (a group in whom tolerance had not developed), and a group in whom tolerance to milk was known to eventually occur.

Materials and methods

Subjects

Subjects were selected from a referral population of over 360 children with atopic dermatitis who were evaluated for food allergy over a 16-year period. Subjects were eligible for inclusion in this study if they had IgE-mediated CMPA documented with a positive skin prick test to CM and by either: (1) a 'convincing history' of anaphylaxis (a severe sudden reaction involving 2/3 major organ systems of the skin, gastrointestinal tract and respiratory tree) following isolated exposure to CM protein; or (2) a positive response to double-blind, placebo-controlled food challenge (DBPCFC) with CM protein. Patients were selected (randomly for those under age 3 using a computer generated randomization program) among those who had stored serum samples available. Serum samples were obtained at the time of the oral food challenge or within 3 months of the episode

of anaphylaxis. Children also met at least one of the following additional criteria:

1. Group I: under age 3 at time of positive oral challenge or anaphylactic reaction (30 subjects).
2. Group II: over age 9 at the time of positive oral challenge or anaphylactic reaction (20 subjects).
3. Group III: lost reactivity to milk (11 patients with samples from before tolerance was achieved).
4. Group IV: 16 samples from patients after they lost sensitivity, with nine patients having paired samples.

Although some children had samples included in more than one group over their lifetime as noted above, this was not designed as a longitudinal study. The study was approved by the Joint Committee for Clinical Investigation of the Johns Hopkins Hospital and informed consent was obtained from all participants.

Evaluation of subjects

All subjects underwent a complete allergy evaluation at the time of entry including prick skin tests with glycerinated milk extracts (Greer Laboratories; Lenoir, NC, USA) and appropriate positive (histamine) and negative (saline) controls using a bifurcated needle as previously described [14]. A mean wheal diameter of greater than 3 mm was considered positive. DBPCFCs were performed as previously described [15,16] in the Paediatric Clinical Research Unit (RR30 or RR0052) under physician supervision. Briefly, up to 10 g of dehydrated milk or 100 ml of liquid milk were camouflaged in infant formula or a variety of foods and administered over 90 min. Two challenges were performed separated by 4 h; one challenge contained milk while the other was a placebo. Randomization and preparation of the challenges was performed by a dietitian. All reactions were scored as to type, severity, and duration and emergency medications were administered, as needed. To confirm non-reactivity, an open feeding of a larger, meal-sized portion of milk followed negative challenges.

Specific IgE

Sera were collected at the time of oral food challenge or within 3 months of CM protein-induced anaphylaxis and stored frozen at -20°C until analysed. Specific IgE antibody concentrations to milk, casein, whey, BLG, ALA and BSA were determined with the Pharmacia CAP System FEIA according to the manufacturer's instructions. The limit of the assay for undiluted sera is <0.35 to >100 kU/L.

SDS-PAGE

Samples of the same milk fractions used in manufacturing of the ImmunoCAPs were provided by Pharmacia & Upjohn

Diagnostic AB. Ten μg of protein of each fraction was loaded onto a precast 10–20% gradient gel (Catalogue No. OG-1020B, Owl separation systems, Woburn, MA, USA) and run at 40 mA for 1 h as described by the manufacturer and stained with Coomassie blue (0.25%).

Statistics

All statistical tests were two-sided, and a level of significance less than 5% was considered significant. Values below the lower limit of the assay were arbitrarily assigned a value of 0.34 $\text{kU}_\text{A}/\text{L}$ and those above the upper limit for undiluted samples as 101 $\text{kU}_\text{A}/\text{L}$. The Mann–Whitney *U*-test was used to compare median IgE concentrations at different ages, with loss of sensitivity and among the various groups. The Wilcoxon Rank Sign test was used to compare paired values among subjects who lost their milk sensitivity. Spearman's correlation coefficient was used to compare IgE levels with time that sera was in storage. Analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results

Subjects

The characteristics of the subjects are summarized in Table 1. A total of 64 patients were included in the study. All of the children were diagnosed with CMPA in the first 2 years of life and, after referral to the investigators, were offered yearly oral challenges to CM. Data is presented for the most recent challenges in patients in Group II. All but four patients in Group II were referred after age 3; these four children are included in both Group I and Group II. Nine patients were evaluated both before and after losing clinical

reactivity. All subjects had atopic dermatitis and a positive skin test to milk. Positive DBPCFCs resulted in symptoms affecting the skin (hives, angioedema, morbiliform eruptions), gastrointestinal tract (vomiting and diarrhea) and respiratory tract (dyspnea, cough, wheezing). The skin was the most common organ affected, and a majority of subjects had more than one organ system affected.

SDS-PAGE

The result of the SDS-PAGE separation of the milk fractions is shown in Fig. 1. The casein fraction had trace visible bands corresponding to the whey fractions (at 14 and 22 kD) and the whey fraction, although free from casein, had a small band corresponding to BSA (66 kD). The whey fractions, ALA and BLG, had no visible bands to indicate contamination with casein or BSA.

Specific IgE to CM and CM protein fractions

Sera were analysed after storage for 1–16 years (median, 5 years). Although IgE remains stable for decades when stored frozen [17], the theoretical risk of degradation of IgE was reduced by assuring that no aliquots were exposed to more than two cycles of thawing. In addition, there was no correlation between length of storage and CM-specific IgE (Spearman's correlation coefficient, 0.01; $P = 0.95$).

The median concentration of CM and CM protein-specific IgE in the groups are shown in Fig. 2 and in Table 2. In two children (one each from Groups I and II who had positive DBPCFCs to CM) CM-specific IgE was undetectable while there was a very low concentration of casein and whey-specific IgE (0.5–0.8 $\text{kU}_\text{A}/\text{L}$). All of the children with CMPA had detectable casein and whey-specific IgE; however, not all allergic children had detectable concentrations

Table 1. Characteristics of patients

Group	Subjects	<i>n</i>	Mean age (years)	Asthma (%)	Method of diagnosis		Symptoms during DBPCFC (%)		
					Challenge	History	Skin	Respiratory	GI
I	< 3 years	30	1.8	33	23	7	87	48	26
II	> 9 years	20	11.7	85	17	3	82	71	35
III	Destined to lose reactivity	11	3.5	55	8	3	100	63	25
IV	After losing reactivity*	16	6.8	38	13	3	92	23	8

*All were confirmed to have lost sensitivity by DBPCFC. The data for method of diagnosis and symptoms during DBPCFC refers to initial evaluations indicating milk allergy.

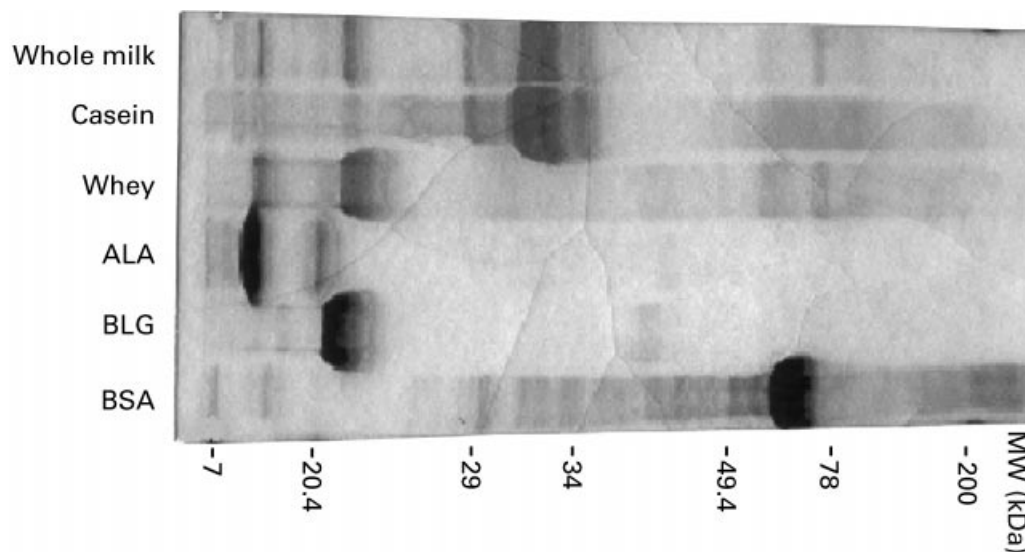


Fig. 1. SDS-PAGE using 10–20% gradient gel. See text for abbreviations.

of specific IgE to the whey components, BLG, ALA and BSA. BLG-specific IgE was undetectable in 7/30 (23%) in Group I, 7/20 (35%) in Group II and in 5/11 (45%) of the children in Group III. ALA-specific IgE was undetectable in 5/30 (17%) in Group I, 6/20 (30%) in Group II, and in 5/11 (45%) of Group III. BSA-specific IgE was undetectable in $\approx$ 50% of each group.

Comparison between groups

Children in Group I (a majority of whom would be expected to outgrow their allergy) had significantly lower median concentrations of CM and casein-specific IgE compared to those in Group II. Children in Group I had levels of whey-specific IgE which tended to be lower than those in Group II

($P=0.11$) but the levels of IgE to the specific whey fractions between these age groups were not different ($P=0.54$ – 0.77)(Fig. 2). In comparing the specific IgE concentrations in children in Group I to those who were to eventually become tolerant of CM (Group III), the median casein-specific IgE concentrations were the only ones significantly greater in Group I ($P=0.04$); however, the levels of IgE to all fractions except BSA ($P=0.78$) approached significance ($P=0.09$ to 0.26). The children in Group I had significantly higher median levels of specific IgE ($P=0.016$ – 0.0002) to all fractions except BSA ($P=0.17$) in comparison to those who had outgrown their sensitivity.

Group II had significantly higher median concentrations of specific IgE to whole CM, total casein and total whey proteins, but not to the individual whey proteins (ALA, BLG

Table 2. Comparison of median concentrations (kU/L) of milk and milk fraction specific IgE among the groups.

	Before losing sensitivity (Group III) $n=11$	After losing sensitivity (Group IV) $n=16$	
	Median (range)	Median (range)	<i>P</i> -value
Milk	4.0 (0.34–101)	1.3 (0.34–51.9)	0.22
Whey	4.5 (0.84–101)	0.8 (0.34–72.5)	0.06
Casein	5.5 (1.2–101)	1.9 (0.34–83.3)	0.19
ALA	1.5 (0.34–11.4)	0.34 (0.34–10.5)	0.17
BLG	1.0 (0.34–88.2)	0.34 (0.34–36.4)	0.39
BSA	1.4 (0.34–40.6)	0.34 (0.34–3.97)	0.15

and BSA) in comparison Group III ($P = 0.02-0.009$). Only the median concentration of BSA-specific IgE was not significantly greater ($P = 0.29$) in Group II compared to Group III (other fractions, $P = 0.02-0.0002$).

There was a trend for decreasing concentrations of milk and milk-fraction-specific IgE concentrations between Groups III and IV (Table 2). This was true also when paired samples from nine patients who lost their reactivity were compared (data not shown). Seventy-five per cent of the children in Group III had milk-specific levels of IgE below $14.3 \text{ kU}_A/\text{L}$ (median, $1.3 \text{ kU}_A/\text{L}$).

Comparisons within age groups

The median concentrations of specific IgE to each protein fraction within Groups I and II were compared. There was no significant difference in the median relative concentration of specific IgE for CM, casein or whey within each group ($P = 0.4-0.98$), thereby showing that there was not a dominant milk fraction to which IgE was binding. However, the median concentrations of milk, casein and whey-specific IgE were significantly greater than those to the whey fractions ALA, BLG and BSA.

Discussion

In general, we found that for any particular group of milk-allergic children, the relative concentration of specific IgE antibody levels measured with the Pharmacia CAP System FEIA were as follows: milk = casein = whey > ALA = BLG > BSA. Thus, measuring the specific IgE concentrations did not identify a single predominant milk allergenic fraction (casein or whey) within any age group, and a significant number of children with IgE-mediated milk

allergy would have been missed if only the ALA, BLG and/or BSA fractions were tested alone. To qualify as a 'major' allergen, over 50% of allergic subjects must have IgE antibody toward the protein in question [18]. Our findings are in general agreement with a number of previous reports which did not identify a single major milk allergen in children [7,19]. The older children, those with persistent milk allergy, had a higher level of IgE antibody to the casein fraction than the younger children (median 101 vs. $20.2 \text{ kU}/\text{L}$, $P = 0.04$). This finding adds support to the hypothesis that IgE binding to the casein proteins is more dominant in older patients who have not lost their reactivity to milk [9].

The majority of children with milk allergy, whether IgE-mediated or non-IgE-mediated, will outgrow their sensitivity [20]; but there has not been a definitive test, other than oral challenge with milk, to determine when this tolerance has developed. For infants and young children with IgE-mediated milk allergy, several workers have noted that children destined to lose reactivity had lower concentrations of serum milk-specific IgE than those who did not go on to become tolerant [11,12]. Hill *et al.* [12] noted a significantly lower concentration of serum IgE to CM, ALA, BLG and casein in children (median age 20 months) destined to become tolerant compared to those who did not achieve tolerance over a median follow-up period of 26 months. Those who achieved tolerance did not have a significant change in their milk or milk fraction-specific IgE levels over that time period. James and Sampson [11] measured casein and BLG-specific IgE levels in 29 children (median age at initial evaluation, 3 years) with immediate reactions to CM over a median follow-up period of 3.1 years. During that time, 11 children achieved clinical tolerance. They found that initial milk-specific IgE antibody concentrations were not significantly lower in patients ultimately becoming milk tolerant, but final casein and BLG-specific IgE concentrations were significantly lower ($P < 0.01$) in patients achieving clinical tolerance than in those remaining clinically reactive. Using the CAP System FEIA in the current study, patients with CMPA in the group over age 9 years (persistent allergy) had higher concentrations of milk, whey and casein but not ALA, BLG or BSA-specific IgE antibody compared to other children in the group who were destined to lose their reactivity. In addition, concentrations of specific IgE were higher to each component of CM tested, except for BSA, in comparing the group over age 9 to those who already became tolerant.

Since there was a broad range of overlap in CM-specific IgE concentration, it remains difficult to predict precisely which child will become tolerant. Sampson and Ho [13] determined that a milk-specific IgE level of less than $1 \text{ kU}/\text{L}$ had a 90% negative predictive value, and a level below $0.8 \text{ kU}/\text{L}$ had a 95% negative predictive value for clinical reactivity in children with eczema. In the present study,

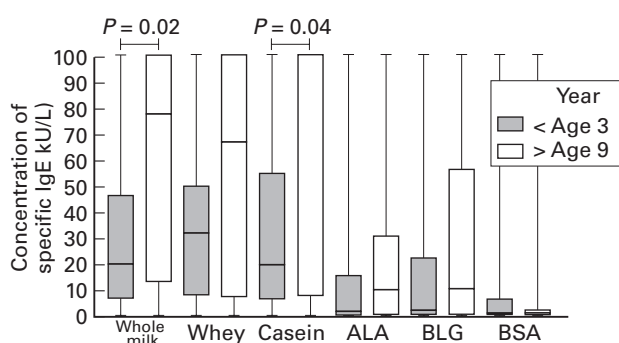


Fig. 2. Serum-specific IgE concentrations to whole milk and milk protein fractions among two age groups of children with CMPA (<age 3 years and >age 9 years). Comparisons of median levels for each tested substance were not significantly different except as indicated. The box extends from the 25th to the 75th percentile with a horizontal line at the median (50th percentile); whiskers indicate the range of values.

there was only a trend toward decreasing concentrations (Table 2) of specific-IgE antibody levels between the time before and after achieving clinical tolerance to CM ingestion (which may have been due to limited sample size). The group that became tolerant of CM (Group IV) had a median level of milk-specific IgE of 1.3 kU/L. This value is similar to that found in the previous study by Sampson indicating a low likelihood of a clinical reaction at a milk-specific CAP-System FEIA concentration under 1 kU/L.

In summary, the concentration of milk-specific IgE was lower in the group of children destined to outgrow milk allergy compared to those in another group with lasting allergy (over age 9 years). Utilizing the CAP System FEIA, no single dominant allergenic milk fraction was identified within any particular age group. However, the group of children with milk allergy over age 9 years had a significantly higher median concentration of CM and casein-specific IgE than in those under age 3 years. Children who outgrew their milk allergy (Group IV) had a lower level of milk-specific IgE than another group with persistent CMPA at age over 9 years (Group II) and testing for specific IgE antibodies to the milk fractions ALA, BLG and BSA did not add any further clinical information.

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