

Serum IgE Antibodies to Cow's Milk Proteins in Children With Differing Degrees of IgE-Mediated Cow's Milk Allergy: Analysis by Immunoblotting

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ABSTRACT

SDS-polyacrylamide minigel electrophoresis was performed on cow's milk (CM), four purified bovine proteins (α -casein, α -lactalbumin, β -lactoglobulin, and bovine serum albumin), four CM protein hydrolysate infant formulas (Good Start, Ultra Good Start, Alfaré, and Nutramigen), and a soy formula (Isomil). Separated proteins were then electrotransferred to nitrocellulose paper, and immunoblotting for IgE antibodies was performed using the serum from 21 children with IgE-mediated cow's milk hypersensitivity. These children had previously been divided into three groups based on patterns of skin prick test positivity to CM and CM protein hydrolysate formulas: group 1A—positive to CM only, group 1B—positive to CM and whey hydrolysate (Good Start), group 1C—positive to CM, whey hydrolysate (Good Start), and casein hydrolysate (Nutramigen). By history, open challenge, and double-blind, placebo-controlled food challenge (DBPCFC), the children either had topical (group 1A) or systemic (group 1B and 1C) reactions. Immunoblots of sera from these groups of children were compared in terms of the number of different proteins per patient to which they reacted and in terms of the intensity of reactions as determined by a grading scale. There were differences between each group for both parameters, although intensity of reactions to casein most clearly distinguished them from one another. These data further support the subclassification of children with IgE-mediated CM allergy into three skin test groups (1A, 1B, 1C) and two clinical groups (topical immediate reactors and systemic immediate reactors). The data also add further support to previous research by one of the authors (RHS) suggesting that allergists refine their approach to evaluating the CM-allergic child to include skin testing to CM protein hydrolysates as well as to whole CM.

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INTRODUCTION

ADVERSE REACTIONS to cow's milk (CM) are common in infancy and childhood, with a prevalence of 0.3%–7.5%.^(1–3)

In an effort to deal with this problem, a number of infant formulas have been produced through the hydrolysis of CM casein or whey proteins to produce low-molecular-weight peptides and amino acids that are less antigenic and, therefore, less allergenic. Some children with IgE-mediated CM allergy tolerate all of the hydrolysate formulas, and some highly sensitized children cannot tolerate any of the current commercially available hydrolysate formulas. Thus, it is evident that these formulas still carry a significant risk. Acute IgE-mediated reactions have been reported to Alfaré (whey hydrolysate, Nestlé),⁽⁴⁾ Good Start (whey hydrolysate, Carnation),⁽⁵⁾ Nutramigen (casein hydrolysate, Mead Johnson),^(6,7) Pregestimil (casein hydrolysate, Mead Johnson),⁽⁸⁾ and Alimentum (casein hydrolysate, Ross).⁽⁹⁾

Children with immediate IgE-mediated CM allergy can be subclassified into groups 1A, 1B, and 1C based on their skin prick test positivity profile to CM, whey, and casein hydrolysate formulas (Table 1). These groups also differ clinically by the types of reactions they have to CM as seen in their histories and with open CM challenges. Group 1A, topical reactors, develop mainly contact urticaria. Groups 1B and 1C, systemic reactors, develop generalized urticaria, rhinitis, stridor, wheezing, or shock. The groups also differ by their serum IgE levels to CM, casein, and β -lactoglobulin.^(10,11) In the present study, 21 children with these varying degrees of IgE-mediated cow's milk allergy had double-blind, placebo-controlled food challenges (DBPCFC) with CM and CM-hydrolysate formulas to verify their clinical reactivity. Using sodium dodecyl sulfate-polyacrylamide minigel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose and probing with sera from the 21 patients, we were able to demonstrate human IgE antibodies to individual CM proteins. The groups, as determined by skin testing, were compared to each other using the number of different proteins (i.e., α -casein, α -lactalbumin, β -lactoglobulin, and/or BSA) to which the patients' sera reacted on immunoblotting, and also using the intensity of those reactions. The results further support the division of patients into topical (group 1A) and systemic (groups 1B and 1C) reactors.

MATERIALS AND METHODS

Subjects

As part of a previous study by Schwartz et al., between January 1, 1989, and August 31, 1989, 26 consecutive patients, 8 new and 18 follow-ups, were evaluated for immediate IgE-mediated respiratory

TABLE 1. SUBCLASSIFICATION OF IMMEDIATE REACTORS TO COW'S MILK BASED ON PATTERNS OF SKIN PRICK TEST POSITIVITY TO COW'S MILK, A WHEY HYDROLYSATE, AND A CASEIN HYDROLYSATE INFANT FORMULA^{(10,16)*}

<i>Group</i>	<i>Prick skin test positivity^b</i>	<i>Clinical reactivity (by history)</i>	<i>Serum IgE to Cm (IU/mL $\pm$ SD)^c</i>
1A (<i>n</i> = 20)	CM only	Topical reactor, perioral contact hives	1.02 $\pm$ 0.87
1B (<i>n</i> = 17)	CM and whey hydrolysate	Systemic reactor, generalized hives, rhinitis, stridor, wheezing	10.6 $\pm$ 12.1
1C (<i>n</i> = 13)	CM, whey hydrolysate, and casein hydrolysate	Systemic reactor, more severe generalized hives, rhinitis, stridor, wheezing	29.3 $\pm$ 12.6

^aModified from ref. 9.

^bCM (Similac), whey hydrolysate (Good Start), and casein hydrolysate (Nutramigen) were prepared according to the manufacturer's instructions, frozen in aliquots for storage, and thawed for skin prick testing on the back.

^cANOVA shows significant differences between the three groups (*p* = 0.0004).

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(19/26, asthma) or cutaneous (25/26, contact and/or generalized urticaria) reactions to CM.⁽¹¹⁾ By history, all subjects had experienced at least three reactions involving acute wheezing or urticaria or both immediately after the ingestion of CM. Thus, they all had convincing histories for IgE-mediated CM allergy and as immediate reactors to CM corresponded to Hill's group 1 patients.⁽¹²⁾ These 26 patients were skin prick tested (SPT) to whole cow's milk 1:20 wt/vol (Hollister-Stier), a whey hydrolysate formula, Good Start H.A. (Carnation), and a casein hydrolysate formula, Nutramigen (Mead Johnson). Based on these results, Hill's group 1 immediate reactors were further subclassified into groups 1A, 1B, or 1C according to the pattern of reactivity on skin testing to the various formulas (Table 1).⁽¹²⁾ Previously, in a group of 50 children with IgE-mediated CM allergy, we found that the skin test reactivity also correlated with clinical reactivity, as indicated in Table 1, and significant differences in serum-specific IgE levels to CM were found between each group. Twenty-one of the 26 patients agreed to participate in the current study. Immunoblotting was carried out using serum from 9 patients (7 females, 2 males) classified as group 1A, 7 patients (7 males) classified as group 1B, and 5 patients (2 females, 3 males) classified as group 1C.

Controls

Serum from 7 subjects without clinical sensitivity to CM served as control sera to rule out nonspecific binding on immunoblot panels. Three subjects were nonatopic by history. Four subjects were known to be atopic (based on clinical history and positive skin tests). One of these was a patient with allergic bronchopulmonary aspergillosis (ABPA) with a total serum IgE level of 2000 IU/mL. An eighth control blot was developed using no serum at all but completing all other steps in the procedure.

A soy formula (Isomil-Ross) was used as a negative control both in the DBPCFC and on immunoblotting against which to compare the binding of IgE to CM and hydrolysate formulas. All but 1 patient (patient 7) were skin test negative to soy.

Double-blind, placebo-controlled food challenges

We performed DBPCFCs ($n = 126$) on 21 children (age 10 months–15 years) with varying degrees (group 1A = 9, group 1B = 7, group 1C = 5) of IgE-mediated cow's milk allergy. They were challenged in the morning, on separate days, with a randomized series of six formulas: CM (Similac), whey hydrolysates (Good Start, ultrafiltered Good Start, Alfaré—Nestlé product sold in Europe), casein hydrolysate (Nutramigen), and soy (Isomil) control. The children were not challenged with CM if they had a reaction to any of the CM protein hydrolysates. Orange-vanilla flavoring masked differences in taste. Starting with 1 mL, doubling amounts were fed every 10 min. Challenges were stopped when convincing objective signs appeared (contact or generalized urticaria, rhinorrhea and sneezing, vomiting, stridor, or wheezing) or after 318 mL of formula had been consumed. Reactions were treated with diphenhydramine or epinephrine or both. Challenges were conducted on the clinical research unit of Strong Memorial Hospital in the presence of a study nurse, one of the investigators (MSA or RHS), and a parent. Informed, approved consent was obtained in each case. The patient, parent, and medical personnel were blinded to the formula, which was selected randomly by a registered dietitian and prepared by dietary personnel.

Preparation of cow's milk and hydrolysate formulas

Cow's milk was prepared from nonfat dry milk (Carnation) according to the package instructions. CM protein hydrolysate formulas were prepared at double the recommended concentration as stated on the can because protein bands were not detectable at the standard concentration.

All formulas were defatted by centrifugation twice at 5000 rpm at 4°C for 15 min each time. Samples were then centrifuged in a microcentrifuge at room temperature at 15,000 rpm for 15 min, followed by aspiration of the aqueous layer and solid pellet.

Purified bovine proteins [α -casein, α -lactalbumin, β -lactoglobulin, and bovine serum albumin (BSA), Sigma Chemical Co., St. Louis, MO] were dissolved in distilled H₂O containing 0.02% sodium azide.

Serum IgE

Specific IgE (IU/mL) values to CM, casein, and β -lactoglobulin were determined by the 3M Fast-Plus method (3M Diagnostic Systems, Inc., Santa Clara, CA) on serum obtained before the first DBPCFC.

Gel electrophoresis

All samples were diluted with SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, containing 2% SDS, 6.25% β -mercaptoethanol, 10% glycerol, and 0.00125% bromophenol blue) and denatured at 100°C for 2 min. The optimal dilution for each sample was determined empirically so that a 5 μ L sample applied to each well would provide the optimal amount of protein. One microgram of each of the purified proteins (α -casein, α -lactalbumin, β -lactoglobulin, and BSA) was applied. Amounts applied to the other wells were 2.4 μ g for cow's milk, 40 μ g for Good Start and ultrafiltered Good Start, 47 μ g for Nutramigen, and 5.9 μ g for Isomil.

Electrophoresis was performed in a Mini Protean II Slab Cell (Bio Rad, Richmond, CA) using 300 V through the stacking gel and 200 V through the separating gel, employing a 4% acrylamide stacking gel and a 17% separating gel both at 37.5:1 acrylamide/bis-acrylamide ratio.

Separated proteins were transferred from the polyacrylamide gel onto nitrocellulose paper by electrotransfer overnight for 16–18 h at 60 V in a transfer buffer containing 25 mM Tris with 192 mM glycine and 14% methyl alcohol at 7°C. To confirm that transfers were adequate, several membranes were stained with amido black after transfer. Prior experimentation established that overnight transfer at low voltage was equivalent to shorter transfer periods at higher voltages.

After transfer, the nitrocellulose membranes were placed between 3 mm Whatman paper, wrapped in plastic wrap, and stored at 4°C. All membranes were used for immunoblotting with 2–4 weeks of storage.

Immunoblotting procedure

On removal from storage, the nitrocellulose membranes were prewet with phosphate-buffered saline (PBS) pH 7.4 and then blocked for 60–90 min in 0.5% porcine gelatin (Sigma Chemical Co., St. Louis, MO) in PBS, pH 7.4, with 0.05% Tween 20 and 0.001% merthiolate. They were then washed twice in PBS containing 0.05% Tween 20 for 5 min per wash and incubated for 2 h at room temperature in a 1:10 dilution of patient or control serum in PBS containing 0.5% porcine gelatin, 0.05% Tween 20, and 0.001% merthiolate. The membranes were washed three more times and incubated for 1 h at room temperature in a 1:2000 dilution of affinity-purified biotinylated goat antihuman IgE (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS containing 0.5% porcine gelatin, 0.05% Tween 20, and 0.001% merthiolate. Membranes were washed three more times in PBS without Tween 20 and incubated for 1 h in a 1:1000 dilution of horseradish peroxidase streptavidin D (ICN Biomedicals, Inc., Costa Mesa, CA) in PBS containing 0.5% porcine gelatin (without Tween 20 or merthiolate). The wash step was repeated with four exchanges of plain PBS, and the blot was then developed with amino ethyl carbazole by adding 25 μ L of 30% H₂O₂ to a 50 mL solution of peroxide buffer (6.8 g sodium acetate in 1L dH₂O, pH 5.5) with 2 mL of carbazole stock solution (500 mg carbazole in 50 mL acetone). The reaction was stopped by washing the developed blots in distilled water.

For 6 of the control patients (excluding the patient with ABPA), as well as 3 of the CM-allergic patients, the same procedure was performed using a 1:2000 dilution of biotin-labeled goat antihuman IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

Statistical analysis

Analysis of variance (ANOVA) with the Scheffe test was used to determine if there was a significant difference between each group for the average number of protein bands stained by sera and also for the average intensity score of each protein band stained by sera. A *p* value equal to or less than 0.05 was considered to be significant. Topical (group 1A) and systemic (group 1B plus 1C) reactors were compared as were groups 1A, 1B, and 1C.

RESULTS

Double-blind, placebo-controlled challenges

In group 1A ($n = 9$), 7 children had topical (acute perioral contact hives) reactions to CM. None reacted to any of the hydrolysates. Two no longer reacted to CM and had outgrown their CMA. One, known to be allergic to soy, had stridor and hives from Isomil.

In group 1B ($n = 7$), 7 children who were challenged with CM developed systemic acute reactions. Systemic reactions included rhinorrhea, cough, stridor, wheezing, or generalized urticaria. Three of 7 challenged with Good Start, 3 of 7 challenged with ultrafiltered Good Start, and 2 of 7 challenged with Nutramigen had reactions. A few hives and angioedema of the eyelid occurred with Nutramigen. None in group 1B reacted to Alfaré.

In group 1C ($n = 5$), 5 children had systemic reactions to Good Start, 5 reacted to ultrafiltered Good Start, 3 reacted to Nutramigen, and 1 reacted to Alfaré. Stridor, rhinitis, and wheezing occurred with Nutramigen (4–10 mL). The one reaction to Alfaré (158 mL) was mild—acute rhinorrhea and irritability. A 7-year-old girl who reacted to Alfaré also developed throat itching, rhinorrhea, wheezing, generalized hives, and repetitive vomiting when fed 4 mL of Alimentum (newest casein hydrolysate) randomized with two soy placebos in a revised approved protocol. For reasons of safety, the 5 children classified as 1C with SPT positivity to CM, Good Start, and Nutramigen were not challenged to CM. However, each child in group 1C reacted to one or more hydrolysate formulas. All reactions resolved spontaneously or were reversed with epinephrine or diphenhydramine or both except for 1 patient with a reaction to Alimentum (patient AR, group 1C), who required intravenous hydration because of repetitive vomiting.⁽⁹⁾

SDS-polyacrylamide gel electrophoresis

Coomassie brilliant blue staining demonstrated adequate separation of proteins in the CM and demonstrated adequate quantities of the purified bovine proteins and that diffusion across lanes did not occur (Fig. 1). No

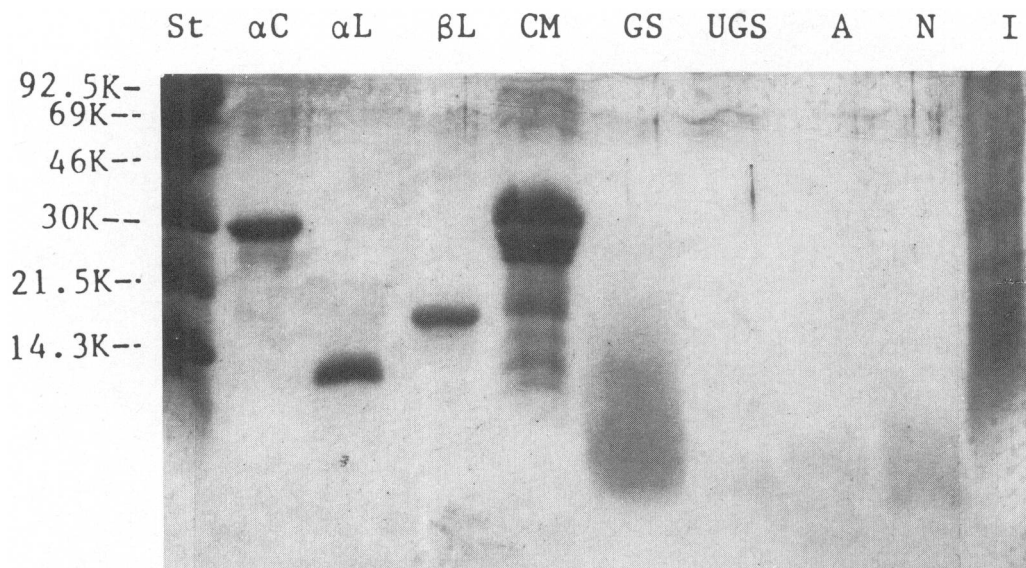


FIG. 1. SDS-PAGE gel stained with Coomassie brilliant blue showing adequate protein concentrations and adequate resolution without diffusion across lanes. St, standards: 92.5 K (phosphorylase B), 69 K (bovine serum albumin), 46 K (ovalbumin), 30 K (carbonic anhydrase), 21.5 K (trypsin inhibitor), 14.3 K (lysozyme). αC, alpha casein; αL, alpha lactalbumin; βL, beta lactoglobulin; CM, cow's milk; GS, Good Start; UGS, ultrafiltered Good Start; A, Alfaré; N, Nutramigen; I, Isomil.

distinct protein bands could be demonstrated by Coomassie blue staining for any of the hydrolysate formulas. Instead, there were large areas of protein deposition for each hydrolysate, with occasional areas of vague banding for Good Start. The molecular weight ranges over which protein deposition was observed were as follows: Good Start, 6000–22,500; ultrafiltered Good Start, 6000–14,500; Alfaré, 6000–11,000; Nutramigen, 6000–13,000. The purified bovine proteins resolved adequately at the expected molecular weight regions (α -casein, 31,000; α -lactalbumin, 14,200; β -lactoglobulin, 18,300; BSA, 67,000).

Electrotransfer

Transfer of the purified cow's milk proteins to the nitrocellulose paper was adequate as judged by amido black staining of the membranes, although there was slight inconsistency in the transfer of the α -lactalbumin and the β -lactoglobulin. There was consistent partial transfer of the broad protein band of Good Start, although there was no detectable protein on the nitrocellulose for the other three hydrolysate formulas by amido black staining.

Immunoblot analysis

A sample immunoblot from a patient with high serum IgE to all of the cow's milk proteins is shown in Figure 2. Of the 7 control sera, 6 showed no detectable binding of IgE to proteins on the nitrocellulose membrane, including the serum from a patient with ABPA containing 2000 IU/mL of IgE. One of the three atopic CM allergy-negative controls showed trace binding of IgE to casein. The immunoblot developed without serum showed no visible bands after development. Each of 4 normal control sera showed strong IgG bands to several of the CM proteins, as did 2 of the patient sera. Therefore, blots were not developed for IgG for the rest of the sera.

The scale used to grade the intensity of reactions is shown in Figure 3. When a faint reaction was visible but less intense than 1+, it was graded *trace* and, for the purposes of calculating average intensity scores, was assigned a value of 0.5.

Table 2 shows all 21 patients by group, their serum-specific IgE levels, and the results of IgE immunoblotting.

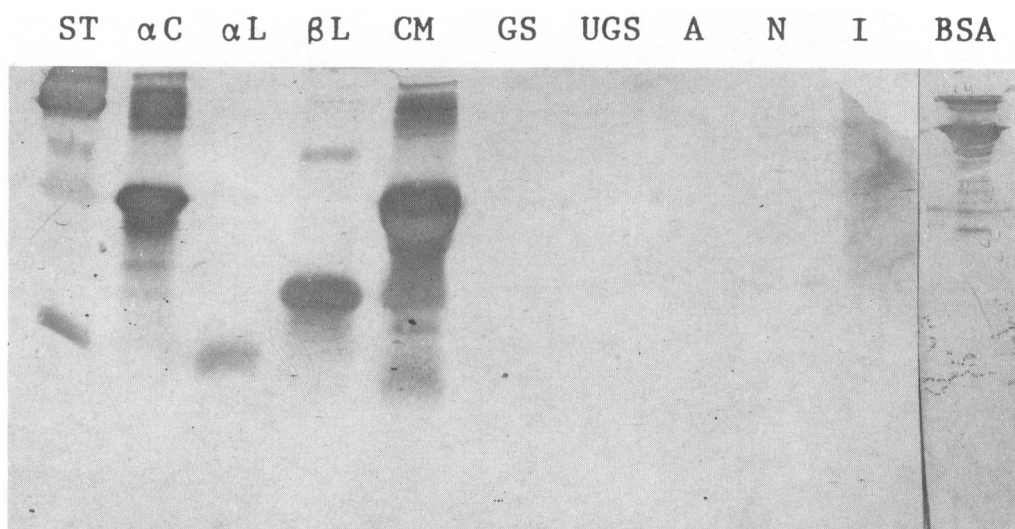


FIG. 2. Representative IgE immunoblot showing intense staining of α -casein, α -lactalbumin, β -lactoglobulin, and cow's milk proteins but no staining of Good Start, ultrafiltered Good Start, Alfaré, Nutramigen, Isomil. Bovine serum albumin (BSA) also stained with this patient.

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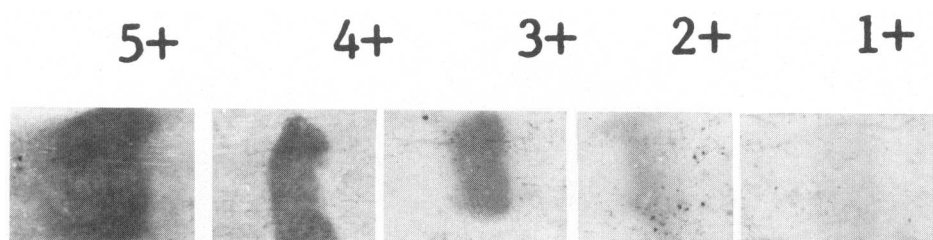


FIG. 3. Scale used to grade the intensity of reactions on immunoblotting.

Tables 3 and 4 show the average intensity score for all proteins combined for each patient group and the average number of proteins per patient giving some degree of positivity (regardless of intensity) for each patient group. Two patients in group 1A (patients 7 and 9) were excluded from the analysis because they were DBPCFC-negative to CM and all hydrolysates. Average intensity scores and number of proteins for group 1A

TABLE 2. STUDY PATIENTS BY GROUP WITH SERUM IGE LEVELS TO COW'S MILK, α -CASEIN, AND β -LACTOGLOBULIN AND RESULTS OF IMMUNOBLOTTING^a

Patient	Group	Serum-specific Ige levels (IU/ml)			Results of Immunoblotting (0-5+)			
		CM	α C	β L	α C	α L	β L	BSA
1. RP	1A	0.23	0.20	0.28	0	0	0	0
2. ER	1A	2.85	0.81	4.92	1+	0	Trace	Trace
3. EH	1A	0.32	0.20	0.27	0	0	0	0
4. JG	1A	1.59	0.46	1.13	0	0	0	Trace
5. BG	1A	19.28	7.57	11.94	3+	2+	3+	3+
6. LW	1A	3.13	2.52	1.46	Trace	0	0	Trace
7. JM ^b	1A	0.24	0.24	0.48	0	0	0	Trace
8. SK	1A	1.33	0.75	0.40	Trace	0	0	0
9. CM ^c	1A	0.76	0.74	0.41	Trace	0	0	Trace
10. MP	1B	14.49	10.45	2.16	3+	0	0	0
11. TM	1B	1.26	0.39	0.61	1+	0	0	0
12. MS	1B	5.80	5.89	0.59	2+	0	0	0
13. EL	1B	0.30	0.20	0.25	2+	Trace	Trace	0
14. BL	1B	10.86	2.68	2.41	2+	0	1+	1+
15. CC	1B	19.86	14.90	19.98	2+	0	2+	1+
16. GD	1B	17.23	4.06	6.05	2+	Trace	1+	3+
17. BL	1C	6.53	4.67	1.70	2+	Trace	Trace	Trace
18. AR	1C	44.0	44.0	44.0	3+	1+	2.5+	0
19. CS	1C	25.3	7.28	2.65	3+	Trace	1+	Trace
20. CF	1C	44.0	44.0	24.03	5+	1+	1+	2+
21. BD	1C	44.0	35.19	37.84	2+	Trace	Trace	Trace

^aCM, cow's milk; α C, α = casein; β L, β -lactoglobulin; α L, α = lactalbumin; BSA, bovine serum albumin.

^bNegative challenge to CM and hydrolysate formulas. Positive challenge to soybean formula. Known to be allergic to soybean.

^cNegative challenge to CM, hydrolysate formulas and to soybean formula control.

TABLE 3. TOPICAL (GROUP 1A) VS SYSTEMIC (GROUPS 1B AND 1C) REACTORS: NUMBER OF PROTEIN BANDS STAINED AND INTENSITY SCORES

	<i>Topical reactors group 1A (n = 6)</i>	<i>Systemic reactors group 1B + 1C (n = 12)</i>	<i>p value</i>
Average number of protein bands stained	1.17	2.92	0.011
Average casein intensity score	0.33	2.42	0.0002
Average α -lactalbumin intensity score	0.00	0.37	0.028
Average β -lactoglobulin intensity score	0.08	0.83	0.036
Average BSA intensity score	0.25	0.72	0.265

were influenced by 1 anomalous patient (patient 5, BG) who showed markedly more intense reactions to all proteins compared to other group 1A patients. Therefore, the results are expressed without this subject. Her clinical characteristics are described in the discussion.

Sera from systemic reactors (groups 1B and 1C) had IgE antibodies to more proteins ($p = 0.011$) and higher total intensity scores ($p = 0.001$) than topical reactors (group 1A). The intensity scores were significantly greater to casein ($p = 0.0002$), α -lactalbumin ($p = 0.028$), and β -lactoglobulin ($p = 0.036$) but not to BSA ($p = 0.265$) (Table 4).

ANOVA with the Scheffe test showed that group 1C had more lines than group 1A ($p < 0.004$). The intensity score for casein was significantly higher for group 1C vs 1A ($p < 0.0002$) and for group 1B vs 1A ($p < 0.005$). There were no differences for either parameter when comparing 1B vs 1C.

DISCUSSION

Cow's milk allergy is a significant problem with which parents, pediatricians, family practitioners, and allergists must deal. It is primarily a problem of infancy and childhood, and although it may persist into adulthood, the development of CM allergy in adulthood is rare.⁽¹³⁾ The previously cited prevalence figures for CM allergy (0.3%–7.5%)^(1–3) show considerable variation as they represent both non-IgE-mediated intolerance reactions and IgE-mediated allergic reactions. The prevalence of true IgE-mediated hypersensitivity reactions to CM is estimated at 1%–3%.⁽¹⁴⁾

DBPCFCs with CM and CM protein hydrolysates have supported the contention that children with IgE-mediated CM allergy, based on history and open challenges, could be divided into topical reactors (group 1A) manifesting contact urticaria and systemic reactors (group 1B and group 1C) manifesting rhinorrhea, stridor, wheezing, and urticaria.^(10,11) Group 1A patients reacted only to CM but not to any of the CM

TABLE 4. COMPARISON OF GROUPS 1A, 1B, AND 1C: NUMBER OF PROTEIN BANDS STAINED AND INTENSITY SCORES

	<i>Group 1A n = 6</i>	<i>Group 1B n = 7</i>	<i>Group 1C n = 5</i>	<i>A vs B p value</i>	<i>A vs C p value</i>	<i>B vs C p value</i>
Average number of protein bands stained	1.17	2.28	3.80	0.20	0.004	0.08
Average casein intensity score	0.33	2.00	3.00	0.005	0.0002	0.118
Average α -lactalbumin intensity score	0.00	0.14	0.70	0.48	0.0002	0.002
Average β -lactoglobulin intensity score	0.08	0.64	1.10	0.32	0.062	0.49
Average BSA intensity score	0.61	0.71	0.70	0.97	0.98	0.99

hydrolysate formulas. Group 1B and 1C children reacted to at least one hydrolysate formula, with children in group 1C reacting to more than one hydrolysate formula. Thus, children in groups 1B and 1C are more sensitive and more reactive.⁽¹⁵⁾

CM is composed of 80% casein and 20% whey.⁽¹⁶⁾ Although there are more than 25 different proteins in CM that may be allergenic, the 4 major allergenic proteins are β -lactoglobulin (whey protein), casein, α -lactalbumin (whey protein), and BSA. Human milk appears to be devoid of α_{s1} and α_{s2} -caseins and does not contain β -lactoglobulin.^(17,18) Perhaps these species differences contribute to the allergenicity of these proteins in humans.

The present study and those of others have shown that most children with IgE-mediated allergy are sensitized to more than one milk protein. The skin test and challenge data of Goldman et al.^(19,20) have been used to estimate the relative allergenicity of casein, α -lactalbumin, β -lactoglobulin, and BSA in children with CM allergy. Their group 1A patients ($n = 45$, no relationship to the current group 1A) with CMA (including IgE- and nonIgE-mediated adverse reactions) were skin tested and challenged orally with purified CM proteins (estimated 2%–3% or less contamination). Casein (27/45 = 60%) and β -lactoglobulin (23/37 = 62%) were responsible for more positive skin test reactions than α -lactalbumin (18/34 = 53%) and BSA (23/44 = 52%). Skin tests (mostly intradermal) were positive to one or more CM proteins in 60% (27/45) of children. Thus, 27 can be considered to potentially have had IgE-mediated CM allergy. In skin test-positive children, challenge tests were more frequently positive to casein (17/27 = 63%) than to β -lactoglobulin (12/23 = 52%), α -lactalbumin (10/20 = 50%), and BSA (11/26 = 42%). None of these are significantly different from the others when the data are analyzed by chi-square goodness of fit test. Although we have not skin tested our patients to the purified proteins nor have we challenged them with individual proteins, our data are most consistent with the notion that casein is the most antigenic and most allergenic.

Through previous work, one of the authors (RHS)^(10,11) has shown that clinical sensitivity to CM can be correlated with serum-specific IgE levels to CM and with patterns of prick skin test reactivity to CM and CM protein hydrolysate formulas (Table 1).⁽¹⁰⁾ It is reasonable to speculate that the degree of clinical sensitivity to CM correlates with the degree of reactivity to one particular protein in CM or to multiple proteins in CM. A markedly sensitive group 1C patient may have a high degree of reactivity to one protein in CM (high serum sIgE level) or have a lesser degree of reactivity to multiple CM proteins, leading in both cases to an equally high release of mediators with CM exposure. Thus, it is reasonable to look at these two parameters in comparing the three skin test groups of patients (1A, 1B, and 1C) and the two clinical groups (topical vs systemic reactors).

As one would expect and as seen in Table 3, systemic reactors had demonstrable IgE antibodies to more CM proteins than topical reactors, and the intensity of reactions to casein, α -lactalbumin, and β -lactoglobulin but not to BSA was greater for systemic reactors. As seen in Table 4, separation of the three skin test groups was less distinct, but definite differences were found. Group 1A patients could be best distinguished from group 1B ($p = 0.005$) and group 1C ($p = 0.0002$) by virtue of the lesser intensity of staining to casein. Groups 1A ($p = 0.004$) and 1B ($p = 0.08$) could be distinguished from group 1C by the lesser number of stained proteins. Group 1C could be distinguished from group 1B by more protein lines being stained ($p = 0.08$) and by more intense reactions to α -lactalbumin ($p = 0.0015$).

One group 1A patient's serum (patient 5, BG, Table 2) showed high degrees of binding to all proteins and had high serum-specific IgE antibody levels to CM, casein, and β -lactoglobulin. Thus, serologically, she looked like a group 1C patient. However, clinically, by definition she belonged to group 1A (positive SPT only to CM), and by DBPCFC she developed only contact urticaria (topical reactor) to CM and did not react to 10 ounces of each of the whey and casein hydrolysate formulas. This anomaly is difficult to explain. One possibility is that her gastrointestinal mucosa is impervious to food allergens. However, this does not explain her low level of skin reactivity. A high level of blocking antibody is another possible explanation. Indeed, in other studies, we have found her to have high serum IgG₄-specific antibodies to CM ($> 40 \mu\text{g/mL}$), α -lactalbumin ($> 40 \mu\text{g/mL}$), β -lactoglobulin ($> 40 \mu\text{g/mL}$), casein ($> 40 \mu\text{g/mL}$), egg ($> 40 \mu\text{g/mL}$), peanut ($> 40 \mu\text{g/mL}$), and wheat ($> 40 \mu\text{g/mL}$). The third possibility involves an aberration in her mast cells or mast cell receptors for IgE. She has a high serum total IgE (2992 IU/mL) and high specific IgE to other foods (peanut ≥ 44 IU/mL) and inhalants (ragweed ≥ 44 IU/mL). We speculate that her mast cell IgE receptors may be occupied by other food and inhalant antibodies, leaving room for only small amounts of

CM-specific IgE antibodies. Thus, she has a low level of sensitivity when skin tested to CM and when challenged with CM. Perhaps this is one mechanism of clinical tolerance.

One limitation to this study is the somewhat small sample size. There were only 9 patients in group 1A, 7 in 1B, and 5 patients in group 1C. Also, the potential for inconsistent transfer of proteins from the polyacrylamide gel to the nitrocellulose paper makes interpretation of intensity more difficult. However, although there may have been slight inconsistency in the transfer of α -lactalbumin and β -lactoglobulin, the transfer of proteins overall appeared to be very consistent. Another potential limitation is the possibility of nonspecific binding to protein bands, which was observed in one negative control serum. This was only a trace reaction, however, and all other controls as well as numerous CM-allergic patients in groups 1A and 1B showed no binding of IgE to the proteins. Therefore, this was not believed to be a factor.

Unfortunately, binding of IgE to proteins in the casein hydrolysate and whey hydrolysate formulas could not be detected. Many of these patients have clinical sensitivity to one or more of these formulas based on prick skin testing and DBPCFC and, therefore, have IgE antibodies to epitopes in these formulas. It is likely that the lack of sensitivity of this immunoblotting technique prevented the detection of the IgE antibodies. Therefore, our study does not answer the question of whether these antibodies are reacting with trace amounts of residual intact proteins or with antigenic epitopes remaining on small peptides present in the hydrolysates.

The data presented add further support to the division of CM-allergic patients into groups 1A, 1B, and 1C based on clinical sensitivity and skin test reactivity as determined by Schwartz et al.^(10,11) The clinical significance of this is that prick skin testing to CM, a whey hydrolysate, and a casein hydrolysate infant formula may be useful in determining the severity of the patient's CM allergy and in determining which formula(s) may be tolerated by the CM-allergic patient.

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