

Allergen-Specific IgE Antibodies against Antigenic Components in Cow Milk and Milk Substitutes

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Crossed radioimmuno-electrophoresis (CRIE) was used to study the presence of serum IgE against antigenic components of cow milk in 21 selected milk-allergic patients. The amount of each IgE specificity was estimated by a scoring system. The milk-allergic children had mainly IgE against α -lactalbumin, β -lactoglobulin, albumin and immunoglobulin, the four major proteins of bovine whey, as well as IgE against three casein components. A serum pool from 1000 normal adults had IgE against the same whey protein, but in smaller amounts, and no IgE against the casein components. Eight cow milk-based formulae, commonly used for infant feeding, and goat milk were studied by the same method. It was found that six of the milk substitutes did not differ significantly from cow milk in antibody binding, but the two hydrolysed casein products, Nutramigen® and Pregestimil®, consisted of such small molecules that the rabbit antisera could not precipitate the hydrolysed proteins in the gels on the CRIE plates. It was therefore not possible to study their IgE binding, if any, by this method.

Key words: allergens; cow milk; crossed radioimmuno-electrophoresis; IgE; infant milk formula; milk allergy.

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The simple theoretical solution to food allergy is avoidance of the offending foodstuff. This presupposes exact identification of the offender and knowledge of the composition of alternative feeds. For the infant reacting to cow milk there are problems in selecting appropriate feeds without allergenic components.

There is at present no laboratory method available that in a single test can differentiate between the cow milk-allergic patient and a normal individual. The allergic patient has increased levels of antibodies to cow milk, both IgG and IgE, but the same high levels are found in many normals (1, 3).

Milk is composed of a large number of antigenic components. Hansson & Månsson (4) found 16 antigens in cow milk and Löwenstein et al. (6) found 32 in bovine whey. Most of these are serum proteins and only a few are milk-specific. The major milk proteins: α -lactalbumin, β -lactoglobulin, and casein, have been purified and tested for

allergenicity in skin tests, passive cutaneous anaphylaxis, and other tests (2, 10). The tests could not, however, differentiate between a group of cow milk-allergic children and a group of children with non-milk allergies. Positive reactions in these tests did not show whether the reaction was due to the purified protein or to trace impurities.

The aim of this investigation was to study with the CRIE method the components of cow milk against which clinically selected cow milk-allergic infants and children have specific IgE antibodies. We have also studied the occurrence of these components in some common milk substitutes used in infant feeding and in goat milk.

MATERIAL AND METHODS

Patients

Based on clinical criteria, the patients were selected from different pediatric units in Den-

mark and West Germany, 12 children (7–80 months) from Denmark and eight children (1–60 months) and one adult from West Germany. The patients' milk allergy was confirmed by decrease or disappearance of symptoms on elimination of cow milk for 2–3 weeks, relapse during open challenge, and effect of repeated diet in accordance with recent review (11). In most cases one challenge with cow milk was sufficient. The patients were further characterised as specified in Table 1. All patients had high RAST class to cow milk, and most were genetically predisposed (questionnaire) having one or more close relative with clinically defined allergy.

Antigens

The antigens were preserved with 1.5 mM sodium azide and kept at -20°C . *Bovine whey*

(Bw) from one cow. Full cream mature milk was centrifuged for 1 h, 27,000 *g* at 5°C . The middle whey layer was siphoned off and centrifuged overnight under the same conditions. *Casein* (Bc) was prepared from 2 l commercial cow milk by centrifugation for 1 h, 27,000 *g* at 5°C . The whey plus casein was centrifuged overnight under the same conditions. The precipitate was washed 6 times with distilled water. Each time the precipitate was resuspended in 1 l distilled water and centrifuged for 1 h, 27,000 *g* at 5°C . The last two supernatants were immunochemically free from whey proteins (< 50 ng/ μl in rocket immunoelectrophoresis). Some of the casein was resuspended in 50 % glycerol and preserved. *Skimmed milk* was obtained commercially and preserved. *Boiled skimmed milk*: A sample of the skimmed milk was boiled at 100°C for 10 min. *Milk substitutes*: Commercial

Table 1
Patients allergic to cow milk¹

ID	Age (months)	Sex	Family ² disp.	Symptom ³	AI-RAST (class)	Total IgE ⁴ PRIST	Eos ⁵
2	17	M	M, G, C	A, AD	4	E	N
3	80	M	F/M	A, AD, R	4	E	E
4	7	M	M, B, S, G, C	G, U	4	E	E
5	51	M	M, B	AD	4	E	N
6	13	F	M	anaphylax. AD	4	N	E
7	59	M	B, C	AD, R	4	–	–
8	27	F	F, M	A, G, R	3	E	E
10	8	M	B, S	AD, G, R, U	4	E	–
11	56	M	M	G	4	E	E
12	10	M	B	AD, G, U	4	E	E
13	12	M	–	U	4	N	N
14	9	F	B	AD, G, U	4	E	N
	Age (years)				Paper RAST	kU/l	
15	5	M	+	A, AD	4	1,000	–
16	2	M	+	U	2	87	–
17	5	M	+	AD	3	1,000	–
18	1	M	+	AD	4	320	–
19	4	M	+	A, AD	4	287	–
20	28	F	–	U	2	10	–
21	4	M	+	AD	2	910	–
22	4	M	+	AD	4	2,160	–
23	5	F	–	A, AD	3	850	–

¹ Verified by milk diet/challenge.

² F/M: father/mother; B/S: brother/sister; C: cousins; G: grandparents; +: first degree allergic relatives.

³ A: asthma; AD: atopic dermatitis; G: gastrointestinal; R: rhinitis; U: urticaria.

⁴ E: elevated relative to normal range; N: non-elevated.

⁵ E: above $350 \times 10^6/\text{l}$; N: below.

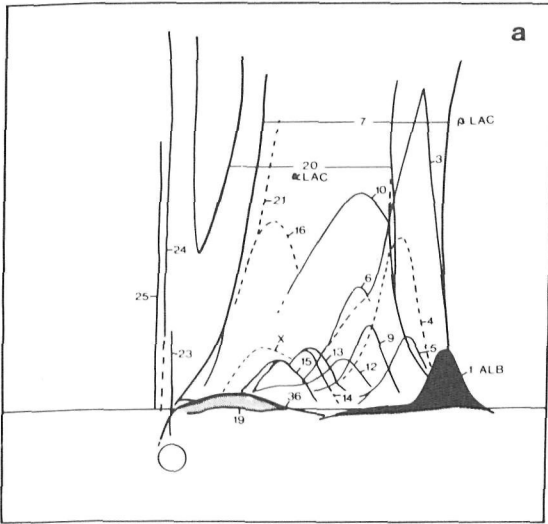


Fig. 1a. Reference precipitation pattern of 10 µl bovine whey into 180 µl anti-bovine whey. For conditions of electrophoresis, see Methods.

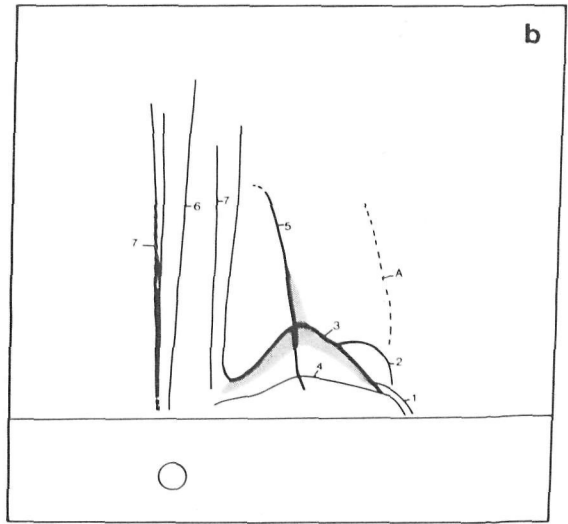


Fig. 1b. Reference precipitation pattern of 0.4 µl bovine casein into 75 µl anti-bovine casein. For conditions of electrophoresis, see Methods.

milk products (Acid Allomin®, Allomin®, Babymin®, Babymin B®, NAN®, Nidina®, Nutramigen® and Pregestemil®) were suspended in distilled water (0.1 g/ml) and centrifuged for 1 h, 30,000 *g* at 5°C, preserving the supernatants. Goat milk from one goat was treated as described for Bw.

Antibodies

Rabbit anti-bovine casein (anti Bc), Behring Werke AG, lot No. 117810 A. Rabbit anti-cow whey (anti Bw), DAKO, lot No. 018C, code Z 183. Radio-iodine labelled rabbit anti-human IgE (7).

Human sera

A pool of sera from 1000 healthy blood donors. Cord sera from 10 random newborns.

QIE methods (5)

CIE. 5 × 5 cm glass plates with 1% w/v agarose (HSA, Litex), 10 µl or 0.3 µl Bw in first dimension, 10 V/cm for 40 min. 0.4 µl Bc in first dimension 10 V/cm for 35 min. Second dimension 9 µl anti Bw/cm² or 3.75 µl anti-Bc/

cm², 2 V/cm overnight. **CLIE**, as CIE with 10–50 µl milk substitute in the intermediate gel. **CRIE**, as CIE, the cold-air dried gels were incubated with patient sera diluted 1:15 followed by radioiodine-labelled anti-IgE. Autoradiography for 10 days on Kodak XL 1 X-ray film at –20°C, followed by scoring of the amounts of radiostaining of the precipitates (14).

RAST

Al-RAST was performed according to (12, 13), paper-RAST by Phadebas® RAST and total IgE by Phadebas® IgE PRIST.

RESULTS

The reference precipitation pattern of the bovine whey/anti-whey system is shown in Fig. 1a. The precipitates are arbitrarily numbered 1 to 36. Albumin (Alb) No. 1, β-lactoglobulin (βLAC) No. 7, α-lactalbumin (αLAC) No. 20, and immunoglobulin (Ig), No. 36, were given the same numbers as in Löwenstein et al. (6). The other precipitates were not identified. Fig. 1b shows the reference precipitation pattern of the casein/anti-casein system. The casein pre-

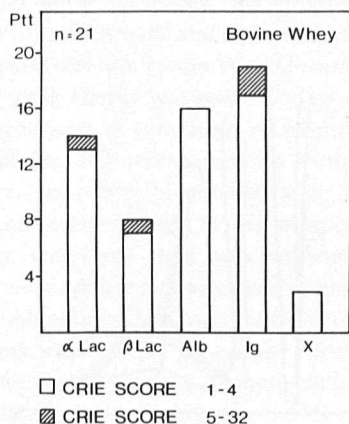


Fig. 2. Allergogram of bovine whey. CRIE scores of serum from 21 cow milk-allergic patients.

cipitates were arbitrarily numbered 1 to 7. The numbers were not related to the precipitates of Bw. Precipitate No. 3 in some cases split into two partially identical precipitates.

The sera of 21 defined milk-allergic patients were investigated individually by CRIE in the bovine whey system in two concentrations. All patients had IgE antibodies against albumin, α -lactalbumin, and β -lactoglobulin. The immunoglobulin precipitate also showed radiostaining. A serum pool from 1000 normal blood donors showed medium radiostaining with the same proteins in Bw as did sera from the allergic children. One unidentified protein X, not stained by Coomassie Blue and therefore not numbered in the reference precipitation pattern, showed weak radiostaining with three allergic sera. No other antigens bound IgE from these 21 milk-allergic patients. An allergogram (Fig. 2) illustrating the distribution of the IgE-binding towards the various antigens was constructed from the CRIE scores, subtracting the scores of the serum pool. The allergogram differentiates between medium and weak radiostaining according to the corrected scores.

The native precipitates of the casein system were incubated with 20 of the sera (excluding patient No. 13, insufficient serum). The 1000 donor serum pool did not react with any precipitate in the Bc system. The allergogram in

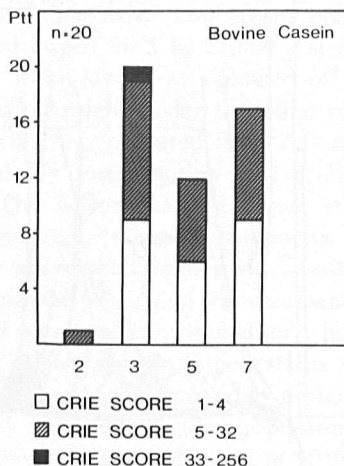


Fig. 3. Allergogram of bovine casein. CRIE scores of serum from 20 cow milk-allergic patients.

Fig. 3 shows the summation of the IgE binding to the casein antigens. All patients had IgE antibodies to precipitate No. 3, more than half had IgE antibodies against precipitates Nos. 5 and 7, and one patient had IgE antibodies against precipitate No. 2.

The 10 cord serum samples were tested in direct RAST and showed weak binding (RAST class 0-2) in the milk system. A pool made from equal amounts of the 10 cord sera showed medium radiostaining with the allergens in the bovine whey system, but not with those in the casein system (very weak binding to precipitate No. 5).

CLIE with the eight milk substitutes in the intermediate gel showed that six of the preparations contained the identified allergens in a form recognizable by the rabbit precipitating antibodies. Bovine immunoglobulin was identified in only two of the preparations. Pregestimil® and Nutramigen® did not contain any compound precipitated by the antibodies against Bw and Bc. CRIE was also performed on the milk substitutes using a serum pool from patients Nos. 5 and 12 and the radiostaining was scored. Fig. 4 shows that all the milk substitutes except Nutramigen® and Pregestimil® bound IgE to the whey components with about the same intensity. With respect to the casein components the IgE binding was more variable.

A similar study was made of skimmed milk

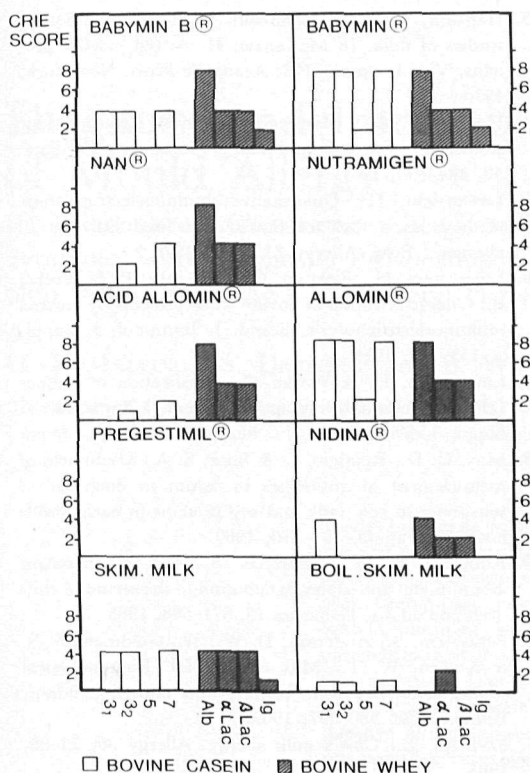


Fig. 4. The relative IgE-binding to antigenic components in various milk substitutes prepared from cow milk.

and boiled skimmed milk. Boiling changed the native antigenicity of the albumin and the immunoglobulins, and no precipitates were formed with the rabbit IgG; the antigenicities of α -lactalbumin and β -lactoglobulin were also changed. Boiling also greatly decreased the binding of IgE (Fig. 4).

CLIE with goat milk in the intermediate gel showed that goat milk proteins are sufficiently similar to cow milk proteins as to be recognized by rabbit precipitating antibodies in the Bw and the Bc systems. CRIE with goat milk as the antigen showed specific radiostaining of some precipitates in the two systems. This shows that the anti-milk IgE produced by the milk-allergic patients can also react with goat milk proteins even though the children have not had goat milk.

DISCUSSION

The bovine casein used in this study was immuno-

chemically free from whey proteins, but no attempts were made to ensure that all the casein was present. The bovine whey contained all the proteins not precipitable in a gravitational field of 27,000 g, and was thus not free from casein. This did not interfere with our study, as only the four whey proteins, albumin, α -lactalbumin, β -lactoglobulin and immunoglobulin were the important allergens. (The anti-bovine whey antibody could precipitate 32 antigens (6).) When proteins are transformed by proteolytic digestion and denaturing, they lose some of their antigenic determinants. Only proteins precipitated by the rabbit IgG on the CRIE plate were available for reaction with the patient's IgE. Transformed proteins might therefore not be available on the CRIE plates. Six of the milk substitutes were very much alike in allergenic content of whey proteins, and resembled fresh skimmed milk. Spray drying and other processing steps did not change the whey protein structures. The differences between the milk substitutes found in the reactions with anti-bovine casein were, at least partly, due to the way the milk substitute antigens were prepared, as the amount of casein in solution would depend on the micellar structure of each milk substitute. Boiling changed the antigenic structure of both whey and casein proteins. Thus the CRIE scores were also decreased. Two of the milk substitutes, Nutramigen® and Pregestimil®, based on hydrolysed casein, produced no precipitates with the two antibodies used in this study. The lack of CRIE scores does not necessarily reflect a complete lack of allergenicity, but the probability of having IgE-binding epitopes is reduced. Specific antibodies against each antigen (Nutramigen®, Pregestimil®, boiled skimmed milk, and goat milk) would be needed to perform CRIE studies of these in order to differentiate between a real lack and an apparent lack of allergens.

Earlier studies (1, 9) have shown that precipitating antibodies against milk components are more or less equally frequent in healthy and in milk-allergic children, their level decreases with age. We have shown the presence of CRIE radiostaining, due to IgE, in the serum pool collected from 1000 adults. There are indications that the amounts of antibody against milk proteins, rather than their presence or non-presence, might consti-

tute a difference between milk-allergics and non-allergics (8).

All tested milk substitutes except the two hydrolysed products reacted both with rabbit antibodies against cow milk and with human IgE antibodies. The same was true for goat milk. These findings emphasize the importance of not advocating the use of these products in milk-allergic children without testing the reactivity of the individual patient while under medical supervision.

CRIE scoring is a semi-quantitative way of describing the levels of specific IgE in serum. We have corrected the score for the binding produced by a 1000-donor pool. The nature of this binding needs further investigation, which is now in progress.

We also found CRIE radiostaining in the serum pool comprising 10 random cord blood samples. IgG can pass the placental barrier selectively while the newborn only has trace levels of IgE. The apparent content of milk-specific IgE in our cord sera was therefore surprising. We are in the process of studying this in greater detail.

Further study will be necessary to develop a good laboratory method for diagnosing cow milk allergy. In the meantime, this allergy must still be confirmed by withdrawal of and challenge with cow milk.

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