

Identification of casein as the major allergenic and antigenic protein of cow's milk

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The objective of this study was to analyze both the allergenicity and immunogenicity of cow's milk proteins. To this end, 80 milk-atopic patients were selected on the basis of the presence of cow's milk-specific IgE antibodies in serum and compatible clinical history. Fifteen patients allergic to other allergens and 10 nonatopic subjects were studied as controls. The specificity of serum IgG and IgE antibodies was determined by immunoblotting, employing both cow's milk and milk components, i.e., α - and β -casein, β -lactoglobulin, and α -lactalbumin separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The experiments showed that casein-specific IgE antibodies were present in all (80/80) sera examined; 10/80 showed reactivity to β -lactoglobulin, and 5/80 showed reactivity to α -lactalbumin. None of the 25 negative control sera analyzed showed the presence of specific IgE antibodies against milk proteins. These results were similar to those corresponding to the detection, by the radioallergosorbent test, of IgE antibodies against the milk components coupled to paper disks. All sera from milk-atopic patients also showed IgE reactivity against a high-molecular-mass fraction that hardly enters the gel. This fraction, after separation by gel filtration and treatment with β -mercaptoethanol and urea, was shown by SDS-PAGE analysis to be formed by casein monomers. All sera analyzed by immunoblotting reacted against the components corresponding to casein monomers. Inhibition of immunoblotting by adsorption with different milk components confirmed that those high-molecular-mass aggregates are formed by casein components. The results presented here strongly suggest that casein is the major allergenic component of cow's milk.

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Although not every disorder resulting from food intake has an immunologic origin, a large number of such diseases are associated with the immune system. Hypersensitivity reactions, mainly atopic allergy, are among them.

The mucosal immune system, especially that associated with the gastrointestinal tract, is covered by a specialized epithelium including both cells and lymphoid structures. It should be emphasized that most antigens enter the organism through feeding. The immune system of the intestinal mucosa can control the entrance of harmful agents; nevertheless, there are anomalies in susceptible individuals which may lead to disease (1), alimentary hypersensitivity being one such disease.

Allergy to cow's milk is within the wide range of

diseases caused by intolerance to foods. Atopic sensitization to this allergen is the most common food allergy. Its incidence varies, according to the different reports, between 1% and 7% during the first 2 years of life (2, 3). It is characterized by the presence of IgE specific for the allergen in the patient serum (4) and clinical symptoms (anaphylaxis, asthma, rhinitis, atopic dermatitis, urticaria, and gastrointestinal disorders).

Identification of antibodies of the IgE and IgG classes to cow's milk and some purified proteins had been attempted earlier by means of skin tests (2). Hoffman (5) used the radioallergosorbent test (RAST) for the detection of a specific IgE to whole milk and purified proteins, such as casein, β -lactoglobulin (β -Lg), and α -lactalbumin (α -La).

He concluded that β -Lg was the major allergen in cow's milk.

However, further studies on the allergenic behavior of hypoallergenic substitutes for cow's milk showed that casein was an important allergen (4, 6). This observation was supported by studies with RAST, skin test, histamine release, and blast transformation of lymphocytes, among other methods (4, 6–10).

The objective of the work presented here was the identification by immunoblotting of the major allergenic and immunogenic proteins of cow's milk in sera from atopic patients.

Material and methods

Sera

Sera of 80 patients atopic to cow's milk (age range: 3 months to 25 years, mean age: 6.19.) were divided as follows: 40/80 patients were between 3 months and 2 years old, 18/80 between 2 and 5 years old, 12/80 were between 5 and 12 years old, and 10/80 between 12 and 25 years old.

All patients had specific IgE seric antibodies (class 3 or higher as determined by RAST) against cow's milk proteins and its protein fractions, and had a clinical history and symptoms compatible with the disease such as atopic dermatitis and/or asthma and/or rhinitis.

Sera of 15 patients atopic to other allergens (*Dermatophagoides* or pollens) were studied; 10 sera of healthy subjects were used as negative controls. The pool of sera from patients atopic to cow's milk and an antiserum of mice hyperimmunized with cow's milk were used as positive controls. To this end, BALB/c mice were intraperitoneally inoculated with 20 μ g (0.3 ml) of proteins from skim milk in complete Freund's adjuvant. A series of four injections of 10 μ g of the same antigen in incomplete adjuvant was administered every 2 weeks. Antibody titers were determined by indirect enzyme-linked immunosorbent assay, with total cow's milk proteins. Sera were stored at -20°C until used.

Antigens

Commercial cow's skim milk, casein isolates, and serum proteins obtained from whole milk by isoelectric precipitation (11), and commercial fractions of total casein (Hammersten Quality, Research Organics, Inc.), β -Lg, α -La, β -casein, and α -casein (Sigma), were used.

Detection of specific IgE

Specific IgE in patient sera was determined with paper disks activated with CNBr conjugated with

whole milk or with different protein fractions, at 1 mg/ml concentration in 0.1 M carbonate/bicarbonate buffer, pH 8.5, according to the method of Ceska et al. (12). A volume of 50 μ l serum per disk was incubated for 12 h at room temperature. Specific IgE was detected with anti-IgE conjugated with alkaline phosphatase (Sigma), by incubating for 12 h at room temperature; *p*-nitrophenylphosphate (NPP, Sigma) was used as substrate.

Calibration was obtained with positive sera against *Dermatophagoides pteronyssinus* by Johansson's method (6) to quantify specific IgE in classes.

SDS-PAGE and immunoblotting

SDS-PAGE was performed with a Mini-Protean II (Bio-Rad) according to Laemmli (13), in 14% polyacrylamide gels. An amount of 35 μ g of protein were used per lane, and the run was carried out at 100 V for the stacking gel and at 150 V for 1 h for the running gel. Gels were fixed and stained with Coomassie brilliant blue G-250 or with silver stain.

The separated proteins were electrotransferred to 0.45 nm nitrocellulose membranes (SMI) for 1 h at 270 mA. The membranes were then blocked with PBS containing 5% horse serum and cut into 5-mm strips. Each strip was incubated with a patient serum or with a control serum diluted 1/5 in PBS with 5% horse serum, for 12 h at room temperature.

After washing, the strips were incubated for 12 h at room temperature with anti-IgE or anti-IgG antibodies conjugated with alkaline phosphatase 1/1000 (Sigma). Strips were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (5-BCIP/NBT, Sigma).

Immunoblotting inhibition was used to study the composition of high-molecular-mass components. To this end, the serum to be analyzed was adsorbed with different cow's milk proteins (1 mg/ml) for 3 h at room temperature. Commercially available casein, β -Lg, and α -La were used as inhibitors (Sigma).

Isolation of high-molecular-mass aggregates

Isolation was carried out by molecular exclusion chromatography, in a Pharmacia 26/10 column filled with Sephacryl S-300, loaded with 25 mg/ml of milk protein. Elution was performed with phosphate buffer, pH 8.4 (24 ml/h); fractions of 1.2 ml were collected. The run was studied by recording absorbance at 280 nm. Each peak was analyzed by SDS-PAGE.

This fraction was also disrupted by treatment for 2 h at 37°C with 5% 2-mercaptoethanol (ME) and

3 M urea and further analyzed by gel electrophoresis and immunoblotting.

Results

Fig. 1 shows the results of SDS-PAGE analysis of the proteins of skim milk and their components. Bands are observed with whole milk (lane 2) in the region between 14 kDa and above 150 kDa. These bands correspond to α -casein (32.4 kDa), β -casein (26.6 kDa), and κ -casein (19 kDa), and β -Lg (18 kDa) and α -La (14.2 kDa), which are the main protein fractions found within that range.

Bands corresponding to the range 60–70 kDa and those of molecular mass higher than 150 kDa appear only in the lanes corresponding to whole milk and casein (lanes 2 and 3, respectively). Moreover, the band corresponding to the highest

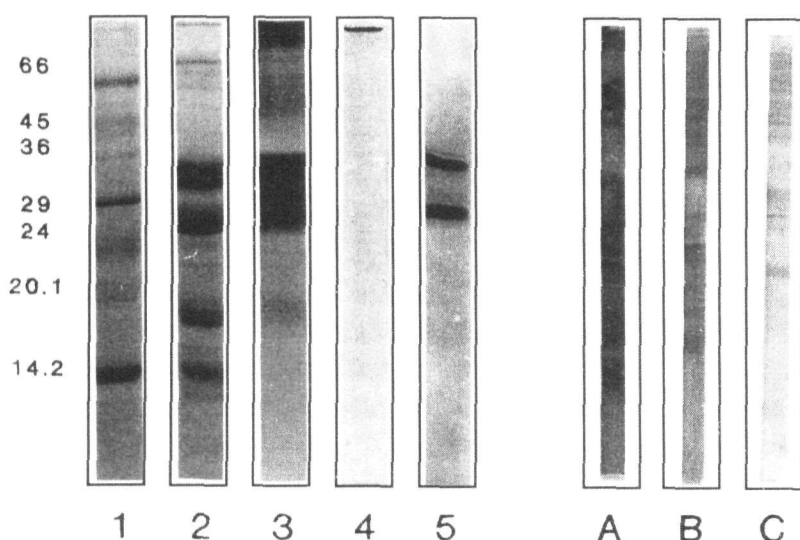


Fig. 1. Analysis by SDS-PAGE and immunoblotting: lane 1) molecular-mass markers; 2) skim milk; 3) casein; 4) high-molecular-mass aggregate isolated by exclusion chromatography; 5) high-molecular-mass fraction treated with 5% 2-ME and 3 M urea. Immunoblotting of milk sample: lane A) immunoblot developed with sera from hyperimmunized mice; B and C) IgG and IgE immunoblots developed with pool of sera from atopic patients, respectively.

molecular mass disappears in SDS-PAGE under reducing conditions with 2-ME (result not shown).

Study of the composition of high-molecular-mass components was performed by their isolation by molecular exclusion in Sephacryl S-300 (lane 4). This fraction, treated with 5% 2-ME and 3 M urea and analyzed by SDS-PAGE, shows the presence of bands in the regions corresponding to casein monomers (lane 5).

Immunoblotting analysis of skim milk is also shown in Fig. 1. Mouse antiserum revealed all protein fractions observed by SDS-PAGE (lane A), thus confirming that all milk proteins were transferred to the nucleopore membrane. The positive control (pool of sera from atopic patients) showed the presence of IgG antibodies to casein aggregates, casein fractions, and serum proteins (lane B). IgE reactivity is mainly directed against bands corresponding to high-molecular-mass components (lane C). On the other hand, no IgE reactivity against any component was detected with the negative control sera (not shown).

Fig. 2 shows the reactivity profiles for IgG and IgE, obtained with sera from 10 patients representative of the 80 atopic subjects included in this study. As can be seen, all of them exhibited IgG and IgE against the bands corresponding to masses higher than 150 kDa and 60–70 kDa, as well as against α - and β -casein fractions.

All sera (80/80) showed the presence of IgE antibodies against casein aggregates; 12.5% (10/80) also showed reactivity to β -Lg, and 6.2% (5/80) to α -La. Reactivity profiles for IgG were very different from those for IgE. Reactivity for IgG was found mainly in the regions of 200 kDa, 60–70 kDa, and casein fractions; some sera also exhibited reactivity against β -Lg or α -La. The reactivity profile for IgE differed greatly among the different sera. Nevertheless, all sera developed the band of the highest molecular mass and bands in the region of

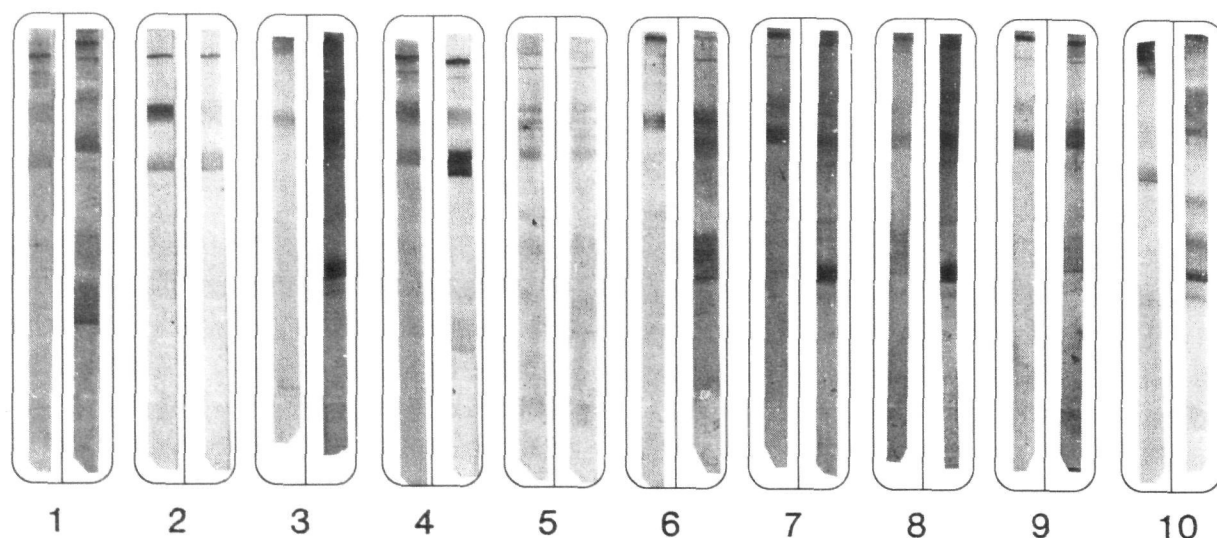


Fig. 2. Immunoblotting of patient sera. IgG (right) and IgE (left) immunoblots of unreduced skim milk developed with sera of 10 representative atopic patients.

60–70 kDa. Some sera exhibited reactivity to α - and β -casein (sera 4, 7, and 8), and a few against β -Lg and α -La (sera 3 and 5).

Table 1 shows the results of the specific IgE analysis corresponding to whole-milk β -Lg and α -La of the sera of the atopic patients studied. All of them (100%) were positive for milk and casein, and 17.5% for β -Lg and 5% for α -La.

Fig. 3 shows immunoblotting of the fractions corresponding to high-molecular-mass components, isolated by chromatography and treated with 2-ME and urea, and developed with sera from different atopic patients. Inhibition of the immunoblotting tests was performed to rule out the presence of other milk serum proteins that could be included in these aggregates. Lane A corresponds to the blotting of a RAST class 4 serum for milk proteins. Reactivity corresponding to high-molecular-mass aggregates, α - and β -caseins, α -La, and β -Lg is observed. Lane B shows the blotting of the same serum against proteins of high-molecular-mass aggregates (treated with 2-ME and urea); bands corresponding to aggregates which had not been completely disrupted, as well as α - and β -caseins, can be observed. Lanes C and D show the reactivity

profiles of the same serum inhibited with β -Lg or commercial casein, respectively. Lane C shows no variation in the reactivity profile; instead, when casein is the inhibitor (lane D), bands corresponding to monomers disappear and a certain reactivity remains in the high-molecular-mass region. Lanes 1–8 correspond to the blottings of eight sera from atopic patients against the fraction of high molecular mass, treated with 2-ME and urea. A remarkable individual variation in the reactivity profile may be seen, and bands in the α -casein, β -casein, and higher molecular mass aggregates regions are predominant.

Discussion

Cow's milk is the first element incorporated in the human diet, either replacing or supplementing breast-feeding.

Milk proteins may be divided into two groups: the casein fraction and the serum fraction. The casein fraction consists of a heterogeneous group of phosphorylated proteins, classified as α -, β -, and κ -casein, according to their electrophoretic mobility (14). They have in common the ability to form molecular aggregates, depending on pH and temperature; thus β -casein forms polymers containing up to six units, whereas α -casein can form polymers which contain up to 22 units (11). The serum fraction includes β -Lg, α -La, immunoglobulins, albumin, and enzymes in a lower proportion. The protein found in the highest amount is β -Lg; the albumin content seems to be identical to that found in serum (11).

In the study reported here, we have selected the immunoblotting technique, using 14% gels, since at that concentration it is possible to achieve an adequate separation of the different milk proteins, with no important overlapping or contamination with one another.

Previous studies with other tests concluded that β -Lg was the main allergen in cow's milk. Our RAST results suggest that casein is the major cow's milk allergen, since not all patients (with RAST of class 3 or 4 for milk) exhibited IgE specific for β -Lg.

The band in the region of higher molecular mass (more than 150 kDa), found to be positive for IgE in 100% of the sera of atopic patients, resulted from casein aggregation, as judged by its disappearance from both the gel and immunoblotting after reduction with 2-ME and urea. That band and those in the range 60–70 kDa were found to be the most immunogenic components, since they were positive for IgG in 100% of the patient sera.

To rule out the possibility that the reactivity detected in the region of 60–70 kDa resulted from bovine albumin, as had been reported by other authors (10), that protein was analyzed by

Table 1. Specific IgE analysis of patient sera

Specific IgE class	Patients %			
	Milk	Casein	β -Lg	α -La
4	28.70	15.00	0	0
3	71.25	66.25	6.75	1.25
2	0	18.75	9.25	2.50
1	0	0	1.50	1.25
0	0	0	0	0
Total	100	100	17.50	5

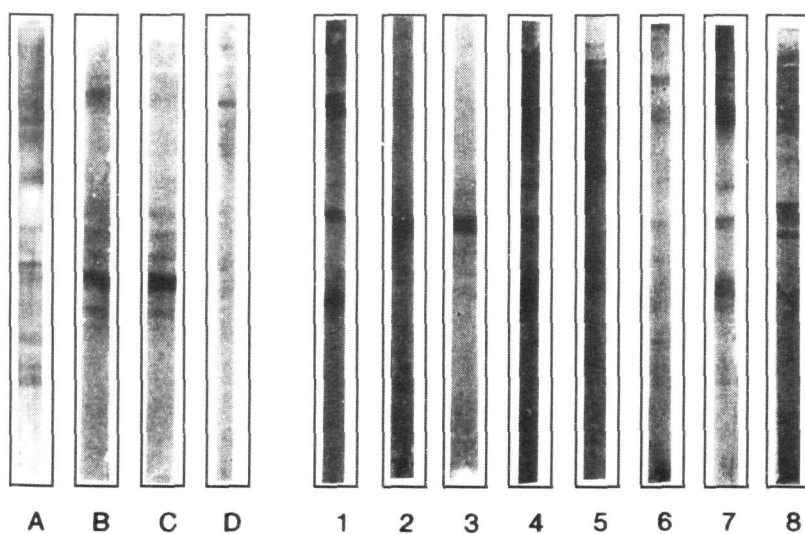


Fig. 3. Analysis by immunoblotting of IgE antibody reactivity of sera from atopic patients to milk and high-molecular-mass aggregates treated with urea and 2-ME. Serum of RAST-positive (class 4) patient atopic to cow's milk (A) and proteins from high-molecular-mass fraction (B) adsorbed with β -Lg (C) or casein (D). Lanes 1–8 correspond to sera from different patients studied with proteins of high-molecular-mass fraction.

SDS-PAGE and immunoblotting by using the pool of positive sera. The band corresponding to BSA could be easily seen in the gel stained with Coomassie brilliant blue, but no reactivity of either IgG or IgE was detected by immunoblotting (results not shown). From the results of immunoblotting and immunoblot inhibition, it can be concluded that high-molecular-mass aggregates consist mainly of casein monomers; moreover, β -Lg could not be detected within the aggregates. These results would suggest that the aggregation of casein monomers reveals new determinants that seem to be absent in the monomeric forms, since some sera show IgE reactivity only against aggregates, but not against their individual components (Fig. 2, samples 2, 3, 4, and 9).

Results of the determinations of specific IgE antibodies to whole milk and its fractions by RAST agree with those described for immunoblotting.

The immunoblotting technique used in this work is of major importance for studying and diagnosing atopy to cow's milk. Its use makes it possible to obtain an allergenic and antigenic profile for each serum; moreover, by studying a large number of sera, the relative importance of the different proteins included in the allergen can be assessed as well.

The results reported in this paper clearly indicate that in all sera analyzed, casein – as well as its high-molecular-mass aggregates – is the major allergen of cow's milk.

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