

Residual antigenicity of hypoallergenic infant formulas and the occurrence of milk-specific IgE antibodies in patients with clinical allergy

Emerentia C. H. van Beresteijn, PhD, Ron J. G. M. Meijer,
and Daniël G. Schmidt, PhD *Ede, The Netherlands*

Background: Milk protein hydrolysates are frequently used in milk substitutes for children with cow's milk allergy. However, cases of hypersensitivity to commercially available hypoallergenic infant formulas based on milk protein hydrolysates have been reported.

Objective: Our purpose was to determine the immunologic response of milk protein-specific IgE and IgG in the serum of patients allergic to cow's milk against four commercially available hypoallergenic milk protein hydrolysates and eight infant formulas.

Methods: Antibody levels in patients' serum and milk protein-specific residual antigenicity of the hypoallergenic products were determined by indirect and competitive ELISA.

Results: Patients allergic to cow's milk had IgE and IgG antibodies to several protein fractions of cow's milk; intraindividual and interindividual variation in the concentrations of these antibodies was considerable. In general, IgE and IgG residual antigenicity of individual milk proteins in the hypoallergenic products was lower compared with that of the intact milk protein, but immunoreactive epitopes could still be detected in all products. Their number varied considerably among the individual milk proteins and also differed among products.

Conclusions: The individual sensitization pattern of the patient allergic to cow's milk and the milk protein-specific residual antigenicity might be considered as possible laboratory predictors of adverse reactions to hypoallergenic products. Their determination could be a useful preclinical screening test for pediatricians to select a formula adapted to the individual patient. (*J ALLERGY CLIN IMMUNOL* 1995;96:365-74.)

Key words: Cow's milk allergy, antigenicity, ELISA, cow milk hydrolysate formula, α -lactalbumin, β -lactoglobulin, bovine serum albumin, bovine IgG, casein, hypoallergenic

Cow's milk protein is probably the most common allergen during infancy and early childhood; the estimated prevalence of cow's milk allergy ranges from 0.5% to 7.5% in infants who are fed formulas based on cow's milk proteins.^{1,2} To benefit these children, milk formulas with reduced milk protein-associated allergenicity are used for therapeutic and recently also for preventive treatments. However, several cases of hypersensitivity to hypoallergenic infant formulas containing hydrolyzed casein and/or whey protein have been

Abbreviations used

AS:	Patients' serum
B-IgG:	Bovine immunoglobulin G
BSA:	Bovine serum albumin
DH:	Degree of hydrolysis
FAST:	Fluorescence allergosorbent test
α La:	α -Lactalbumin
β Lg:	β -Lactoglobulin
M _r :	Molecular mass
NCS:	Normal control serum
PBS:	Phosphate-buffered saline solution
S _r :	Repeatability standard deviation

From the Department of Nutrition and Department of Biophysical Chemistry, Netherlands Institute for Dairy Research.

Received for publication Sept. 14, 1994; revised Jan. 10, 1995; accepted for publication Jan. 12, 1995.

Reprint requests: E. C. H. van Beresteijn, PhD, Department of Nutrition, Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands.

Copyright © 1995 by Mosby-Year Book, Inc.
0091-6749/95 \$5.00 + 0 1/1/63274

reported³⁻¹⁰ and IgE-binding to components of these products has been shown in sera from children allergic to cow's milk.¹¹⁻¹⁵

Cow's milk allergenicity was initially attributed to β -lactoglobulin (β Lg) because it is present in cow's milk but not in human milk.^{16,17} However,

low concentrations of not only β Lg but also other bovine milk proteins such as casein and IgG (B-IgG) have been demonstrated in human breast milk or serum. This shows that antigenically active molecules derived from cow's milk are able to penetrate the intestinal epithelial surface.¹⁸⁻²³ Sensitivity to isolated milk proteins has repeatedly been reported,²⁴⁻²⁶ and specific IgE antibodies against all major milk proteins have been found in sera of subjects with this allergy.²⁷⁻²⁹ The quantitative IgE antibody responses to individual milk proteins, however, were shown to vary considerably among individual patients.²⁹

Currently, limited or extensive *in vitro* enzymatic hydrolysis of cow's milk proteins, followed by heat treatment and ultrafiltration through filters with a molecular mass (M_r) cutoff value between 5000 and 10,000 d, is used to reduce cow's milk antigenicity. Several food-grade proteinases are available for industrial use. The reduction in antigenicity strongly depends on the specificity of the enzyme and the molecular structure of the antigenic epitopes. As a consequence, residual antigenicity of the principal individual cow's milk proteins, namely casein, α -lactalbumin (α La), β Lg, bovine serum albumin (BSA), and B-IgG may be different in commercially available hypoallergenic products.

In this study the immunologic response of milk protein-specific IgE and IgG in the serum of patients allergic to cow's milk against hypoallergenic infant formulas was analyzed. We determined the relative distribution of IgE and IgG antibodies to the individual milk proteins in serum of patients with clinical symptoms of allergy to cow's milk by indirect ELISA. In various commercially available hypoallergenic products based on hydrolyzed whey protein or casein, the residual antigenicity of the individual cow's milk proteins was determined with a sensitive competitive ELISA. The molecular characteristics of the hydrolysates and of the protein part of the infant formulas were determined by physicochemical methods.

METHODS

Patient serum samples

We obtained sera from 215 children (age, 2 weeks to 3½ years) and five adults (age, 20 to 35 years) of either sex, suspected of having cow's milk allergy, by donations from the departments of pediatrics of the Academic Hospital of the Free University, Amsterdam, and of the Wilhelmina Children's Hospital, Utrecht, The Netherlands. For this study we selected sera of patients with a history of immediate reactions to cow's milk (skin and/or

gastrointestinal symptoms, urticaria) and elevated milk-specific IgE antibody concentrations (IgE fluorescence allergosorbent test [FAST] class ≥ 3 ; cow's milk, IgE Fast-Plus test; 3M Diagnostic Systems Inc., Santa Clara, Calif.). In addition, a positive elimination-provocation test result was a prerequisite for the children's sera, and a positive skin prick test response (wheal, ≥ 3 mm) or an incidental positive provocation for the adults. Sera collected from eight persons not allergic to milk proteins and with negative milk-specific IgE-FAST results were pooled and served as a normal control serum (NCS). The serum samples were stored at -20°C until further analysis.

Material

The proteins α La and β Lg (genetic variants A and B) were prepared from cheese whey by chromatography as described earlier.³⁰ BSA (A 4378) and B-IgG (I 5506) were purchased from Sigma Chemical Co. (St. Louis, Mo). Whole casein was prepared by isoelectric precipitation from skim milk with HCl at pH 4.6. The precipitated casein was thoroughly washed with distilled water, redissolved by the addition of NaOH to pH 7.0, and then lyophilized. A whey protein hydrolysate manufactured at our institute by enzymatic hydrolysis of spray-dried whey protein concentrate was filtered through a filter with a M_r mass cutoff value of 3000 d (3000 d hydrolysate) as described earlier.³¹

Four commercially available hydrolysates and eight infant formulas were investigated. For reasons of confidentiality the products were coded. Hydrolysates 1 to 4 and infant formulas 5 to 11 were based on hydrolyzed whey protein; formula 12 was based on hydrolyzed casein. Hydrolysate 1 was used for the production of both formulas 7 and 10. All products were designated as hypoallergenic by the manufacturer. For comparison the 3000 d hydrolysate was used; it did not elicit allergic reactions (e.g., anaphylactic shock or positive passive cutaneous anaphylaxis) in experimental animals.³¹

Immunologic characterization of patients' sera and hypoallergenic products

The relative distribution of IgE and IgG antibodies to the individual milk proteins in the serum samples was determined by indirect ELISA; the relative antigenicity of the various hydrolysates and infant formulas was determined by competitive ELISA. We used the ELISA method as described by Fritsché and Bonzon³² with slight modifications. The hypoallergenic products were dissolved in phosphate-buffered saline solution (PBS) (0.15 mol/L NaCl with 0.01 mol/L phosphate buffer, pH 7.4) containing 0.05% Tween-20 (Sigma) to a protein concentration of 10 gm/L (stock solution).

Indirect ELISA. Polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were used as solid support. Single wells were coated with 135 μL /well of antigen (individual milk proteins, 37 $\mu\text{g}/\text{mL}$ in 0.05 mol/L sodium bicarbonate buffer, pH 9.6) and incubated

overnight at 4°C. Residual free binding sites were blocked with 200 µl/well of 0.4% fish gelatin (Sigma) in PBS-Tween-20 for 1 hour at room temperature. Plates were incubated with 100 µl/well of patients' serum (AS) diluted in PBS-Tween-20 (1:25 for IgE binding and 1:1000 for IgG binding) for 2 hours at room temperature. The same dilutions of NCS were used as negative controls. The IgE and IgG antibodies that reacted with the plate-bound antigens were determined by use of either peroxidase-conjugated goat anti-human IgE (ε chain) or peroxidase-conjugated goat anti-human IgG (H and L chains) (Nordic Immunological Laboratories, Tilburg, The Netherlands). The anti-human IgE and anti-human-IgG antibodies were diluted 5000-fold in PBS-Tween-20 containing 0.4% fish gelatin and were incubated for 1 hour at room temperature. As enzyme substrate a freshly prepared solution or 1 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) in 0.05 mol/L citrate and 0.1 mol/L potassium phosphate buffer, pH 5.0, containing 0.012% hydrogen peroxide, was used. After the plates were incubated for 30 minutes at room temperature, 100 µl of 2N sulfuric acid was added. Optical densities were read at 490 nm on an automated ELISA plate reader (Dynatech MR 7000; Trade Tek, Rotterdam, The Netherlands). ELISA determinations were carried out in duplicate, and measurements were averaged.

Inhibition ELISA. Single wells of ELISA plates were coated with 135 µl of αLa, βLg, BSA, B-IgG, or casein (15 µg/ml in 0.05 mol/L sodium bicarbonate buffer, pH 9.6). In 1.5 ml glass vials serial ninefold dilutions of the stock solution of the hypoallergenic products were incubated overnight at 4°C in PBS-Tween-20 with a constant amount of AS (1.5% for IgE binding or 0.05% for IgG binding). Residual unbound IgE and IgG antibodies were determined by the ELISA method described by application of 100 µl of preincubated AS to each well of the coated ELISA plates. The percentage of inhibition was calculated in relation to the maximum inhibition (vials containing NCS instead of AS) and the minimum inhibition (vials without hypoallergenic product). For this purpose an inhibitor concentration of 1 gm/L was chosen because that was the highest concentration at which differences in inhibition of the various hypoallergenic products could still be detected and also the lowest concentration at which an almost 100% inhibition for all individual milk proteins could be attained.

Physicochemical characterization of hypoallergenic products

The average degree of hydrolysis (DH) of the protein component in each hypoallergenic product was calculated from the increase of the number of primary amino groups compared with that of unhydrolyzed whey protein or casein. We determined this number by the *o*-phthaldialdehyde method,³³ with *N,N*-dimethyl-2-mercaptoethyl-ammonium chloride instead of 2-mercaptoethanol.³⁴ For sample preparation the hydrolysates and infant formulas were dissolved in distilled water to a

protein concentration of about 5 gm/L. After 2 hours at room temperature the solutions were centrifuged for 7 minutes at 10,000 *g* to remove fat and other undissolved material. The intermediate layer was used to determine the DH of the samples. Despite the centrifugation step, most of the infant formulas still showed some turbidity, which would interfere with the determination of the DH. Therefore we measured the absorption at 340 nm not against water but against a blank containing the sample in the assay buffer without the *o*-phthaldialdehyde and *N,N*-dimethyl-2-mercaptoethyl-ammonium chloride. Because the number of primary amino groups of the protein preparations used for the manufacture of the hydrolysates was unknown, we determined the average number of primary amino groups per mole of protein in a large variety of whey protein concentrates and in casein. We found 17.6 (repeatability standard deviation [*S_r*] = 1.4 and *df* = 14) and 16.3 primary amino groups per mole of protein, respectively.

The hydrolysates and infant formulas were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis with the Phast system and high-density gels (Pharmacia, Uppsala, Sweden). The total protein concentration in the samples applied to the gels was approximately 2 gm/L. Two standard protein mixtures, Bio-Rad LMW standard (Bio-Rad, Richmond, Calif.) and BDH 44247 (BDH Laboratory Supplies, Poole, Dorset, U.K.), were used as *M_r* markers. After electrophoresis the proteins and peptides were stained with silver as described in the *Phast Gel Silver Kit Instruction Manual* (Pharmacia). This method is 100 times more sensitive than Coomassie blue staining.

M_r distributions of the peptides in the hydrolysates and infant formulas were determined by gel permeation chromatography on a Superdex-75 HR 10/30 column (Pharmacia/LKB, Uppsala, Sweden). Elution was done at room temperature with a 0.125 mol/L potassium phosphate-0.125 mol/L sodium sulfate buffer of pH 6.65. Peptide concentrations in the eluate were determined from the absorption at 220 nm. Calculations were made on the basis of an assumption of an exponential relationship between *M_r* and elution volume, and took into account apparent *M_r* regions based on the calibration with a series of reference compounds.³⁵ For sample preparation the products were dissolved in a 0.125 mol/L potassium phosphate-0.125 mol/L sodium sulfate buffer (pH 6.65) to a protein concentration of about 2 gm/L. Before application to the column the samples were centrifuged for 7 minutes at 10,000 *g* and then filtered through a Millipore filter cartridge (Millipore Corp., Bedford, Mass.) with pore size of 0.22 µm.

Reliability of results

All analytic determinations were done in duplicate, and results were averaged. The precision of the analytic methods, expressed as the *S_r*, was determined from duplicate measurements.³⁶ For the ELISA including between-well and between-plate variation, *S_r* was 1.77%

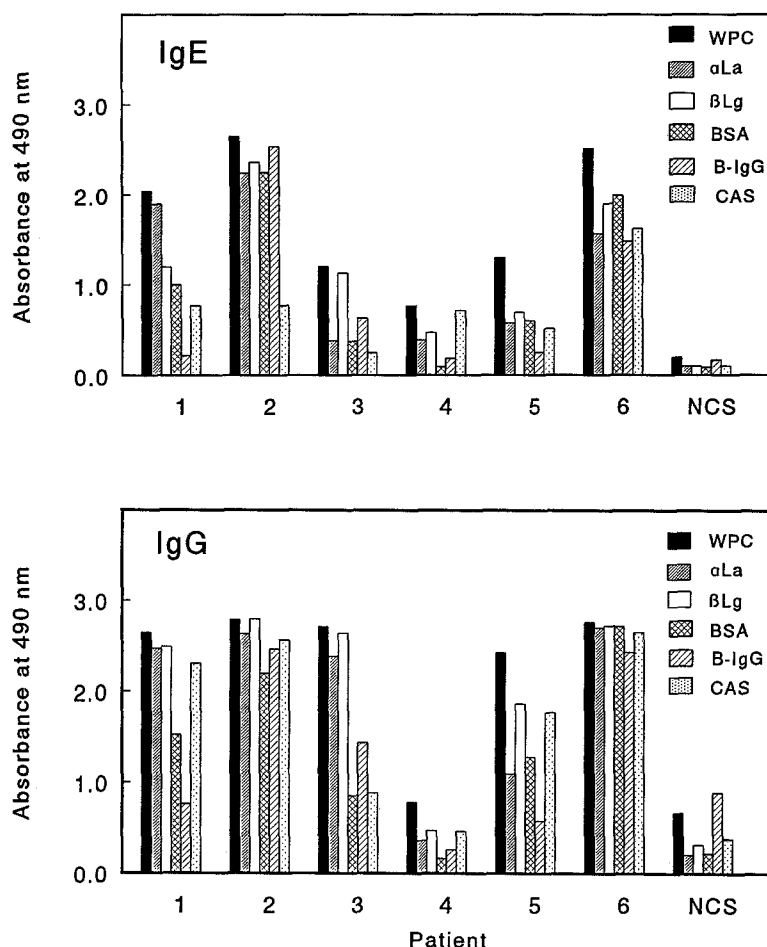


FIG. 1. Serum levels of specific IgE and IgG antibodies against casein (CAS), α La, β Lg, BSA, and B-IgG in six patients allergic to cow's milk and in NCS, expressed as A_{490} . The protein concentration of the coating antigen was 37 μ g/ml; sera were diluted 25-fold for IgE and 1000-fold for IgG.

($df = 19$); for the DH determination, S_r was 0.99% ($df = 48$); and for the M_r distribution, S_r was 0.29% ($df = 40$).

RESULTS

Patients' sera

IgE and IgG antibodies were found against α La, β Lg variant A, BSA, B-IgG, and casein in sera of patients allergic to cow's milk. As demonstrated by six typical examples in Fig. 1, the serum concentrations of these antibodies varied considerably within and among subjects. To facilitate a comparison, we used only one serum dilution (25-fold for IgE, 1000-fold for IgG). For a number of antibodies this resulted in ELISA values greater than 2, which indicates that quantitative differences were in fact even greater than shown in Fig. 1 (see also Fig. 2). No differences in antibody titers between the two genetic variants A and B of β Lg were found (data not shown). Control tests with NCS

showed low IgE binding to all milk proteins tested. Control tests on IgG binding were comparable except for a slightly higher binding to B-IgG, and as a consequence, to WPC. Positive values as found in the patient serum samples could not be attributed to nonspecific binding.

Hypoallergenic products

Residual antigenicity. Three serum samples (AS I, AS II, and AS III) with high titers of specific IgE and IgG antibodies against the individual milk proteins were selected to detect IgE- and IgG-binding proteins or peptides in the hypoallergenic products. One adult serum sample (AS I), which had high specific IgE binding to α La, β Lg, and casein (Fig. 2), was used to determine the residual IgE antigenicity of these proteins or their peptides in the hypoallergenic products. For analogous reasons AS II, another adult serum sample was used

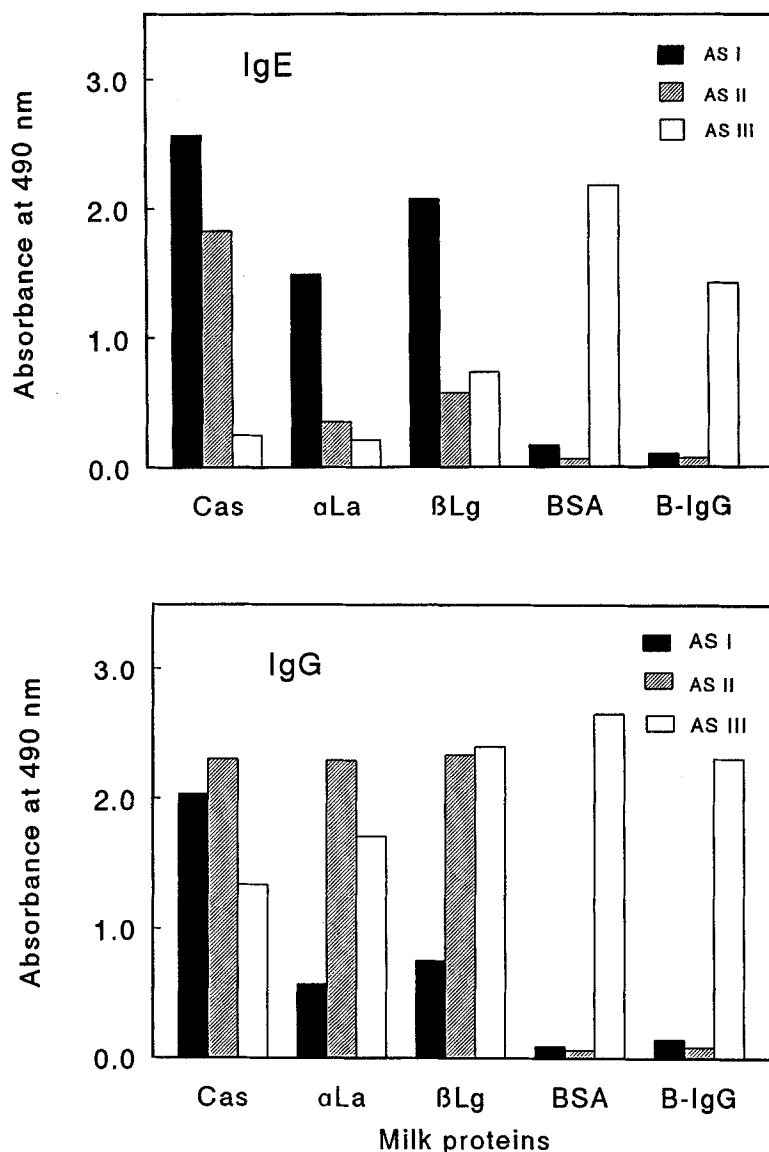


FIG. 2. Specific IgE and IgG binding to casein (*Cas*), α La, β Lg, BSA, and B-IgG of sera AS I, AS II, and AS III. The protein concentration of the coating antigen was 37 μ g/ml; sera were diluted 25-fold for IgE and 1000-fold for IgG.

to determine their residual IgG antigenicity. Residual IgE and IgG antigenicity of BSA and B-IgG were determined by use of AS III, consisting of the pooled sera of five children (Fig. 2).

Fig. 3 shows typical examples of the inhibition ELISA for BSA and β Lg for a number of the hypoallergenic products. The binding inhibition of IgE and IgG antibodies to the major milk proteins or their peptides is presented in Tables I and II. All tested products showed lower IgE-binding activity (Table I) than the intact individual milk proteins, but the reduction of antigenicity was strongly dependent on the type of protein. Most products retained a relatively high residual IgE antigenicity

of α La and β Lg, whereas that of BSA and B-IgG was low. Trace amounts of casein are always present in commercial whey preparations, and consequently casein IgE epitopes could be demonstrated in a number of products based on whey protein hydrolysates. For analogous reasons α La and β Lg epitopes could be demonstrated in the product based on casein hydrolysate (formula 12). It is also clear that IgE epitopes of α La, β Lg, and casein were present in the 3000 d hydrolysate, although this hydrolysate did not elicit allergic reactions (neither anaphylactic shock nor positive passive cutaneous anaphylaxis) in experimental animals.³¹

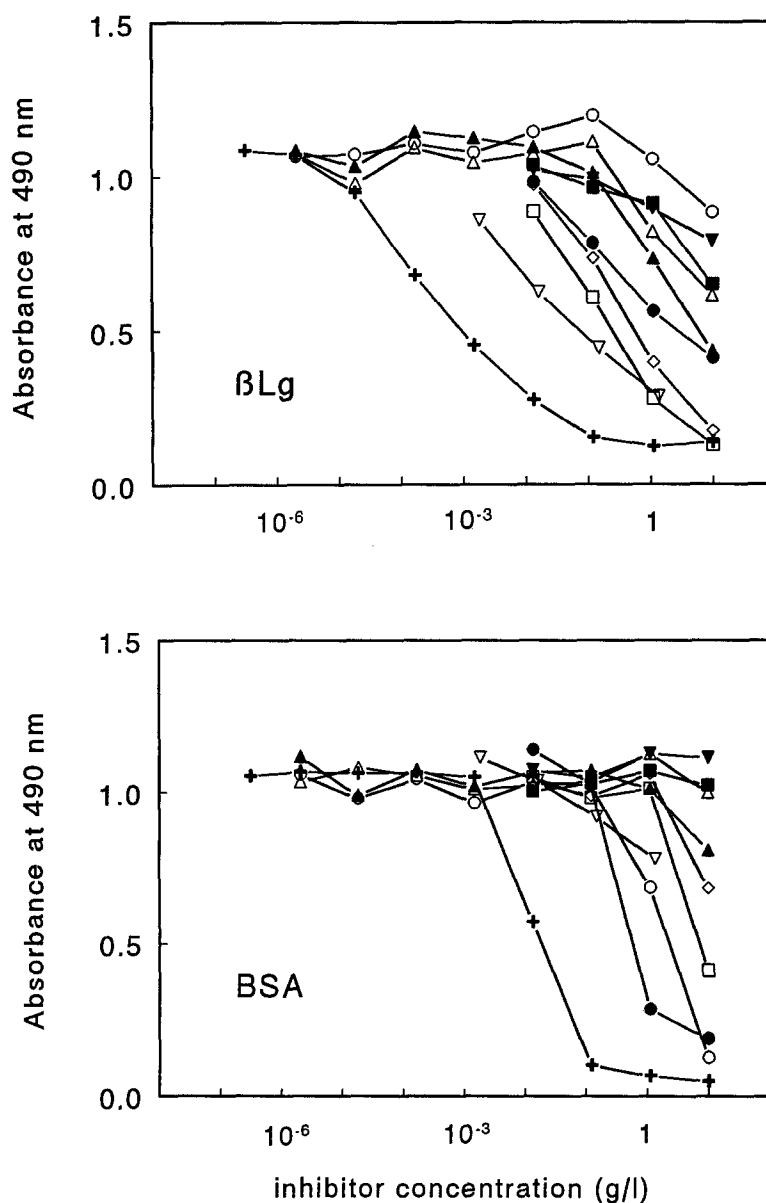


FIG. 3. Inhibition of human IgE binding to β Lg and BSA by WPC (+), hydrolysate 1 (▲), hydrolysate 2 (△), hydrolysate 3 (●), hydrolysate 4 (○), formula 7 (□), formula 10 (◇), formula 11 (▽), formula 12 (▼), and 3000 d hydrolysate (■). Protein concentration of the coating antigen was 15 μ g/ml. AS I was used to determine residual antigenicity against β Lg and AS III against BSA. Absorption values are from duplicate measurements.

The results of the experiments on the binding inhibition of IgG antibodies were comparable to those obtained on IgE antibodies except for quantitative differences. Residual IgG antigenicity of BSA and B-IgG appeared to be slightly higher in most of the hypoallergenic products.

Chemical characterization. The DH of the protein fraction varied from 1.3% to 52.0% in the various hypoallergenic products (Table III). In concordance, the electrophoretic patterns of the products did not show visible peptide bands except

those of formulas 8 and 11. In these two formulas the protein fraction had a DH of 6.3% and 1.3%, respectively, and contained some intact β Lg and peptides with M_r between 6000 and 8000 d; their concentrations were lower in formula 8. These observations parallel the results of the gel permeation chromatography analysis (Table III), which show the presence of 26.3% and 40.6% of peptides with M_r larger than 10 kd in the protein fraction of formulas 8 and 11, respectively. In all other samples this peptide fraction was less than 5%. The

TABLE I. Residual IgE antigenicity of (percent inhibition of IgE binding to) individual milk proteins of commercially available hypoallergenic whey protein hydrolysates and infant formulas based on hydrolyzed milk protein

Sample*	Inhibition of IgE binding (%)†				
	α La	β Lg	BSA	B-IgG	Casein
Whey protein concentrate	99	100	98	100	88
Hydrolysate 1	62	36	0	3	23
Hydrolysate 2	51	27	0	12	5
Hydrolysate 3	65	54	75	57	3
Hydrolysate 4	18	2	33	72	0
Formula 5	72	52	2	0	34
Formula 6	68	33	2	12	24
Formula 7	93	84	0	0	53
Formula 8	69	60	9	5	76
Formula 9	49	25	0	0	3
Formula 10	83	72	0	0	37
Formula 11	85	83	23	12	89
Formula 12	43	19	0	4	3
3000 d hydrolysate	37	17	0	1	29

*Hydrolysates 1 to 4 are hypoallergenic whey protein hydrolysates; formulas 5 to 11 are hypoallergenic infant formulas based on hydrolyzed whey protein; formula 12 is based on hydrolyzed casein. The 3000 d hydrolysate is whey protein hydrolysate after filtration with a M_r cutoff value of 3000 d.³¹

†AS I was used for determination of residual IgE antigenicity of α La, β Lg, and casein, and AS III for that of BSA and B-IgG. Concentration of the protein inhibitors was 1 gm/L.

TABLE II. Residual IgG antigenicity of (percent inhibition of IgG binding to) individual milk proteins of commercially available hypoallergenic whey protein hydrolysates and infant formulas based on hydrolyzed milk protein

Sample*	Inhibition of IgG binding (%)†				
	α La	β Lg	BSA	B-IgG	Casein
Whey protein concentrate	100	100	95	93	97
Hydrolysate 1	66	50	10	10	31
Hydrolysate 2	5	4	3	4	0
Hydrolysate 3	30	16	79	44	2
Hydrolysate 4	0	0	76	73	0
Formula 5	60	46	28	18	33
Formula 6	65	50	2	0	35
Formula 7	95	88	30	43	73
Formula 8	98	95	4	3	87
Formula 9	71	50	4	3	19
Formula 10	83	66	14	25	46
Formula 11	99	96	16	14	91
Formula 12	16	6	4	5	14
3000 d hydrolysate	15	6	0	3	0

*Samples are same as those described in Table I.

†AS II was used for determination of residual IgG antigenicity of α La, β Lg, and casein, and AS III for that of BSA and B-IgG. Concentration of the protein inhibitors was 1 gm/L.

DH values and the M_r distributions of the protein fraction in the samples of formulas 7 and 10 agreed with those of hydrolysate 1, from which these formulas were manufactured, demonstrating the reliability of their determination.

DISCUSSION

IgE-mediated allergic reactions caused by the consumption of infant formulas based on cow's milk proteins cannot be attributed to one specific protein. In agreement with other investiga-

TABLE III. DH and M_r distribution of four commercially available hypoallergenic whey protein hydrolysates and of milk protein hydrolysate present in eight hypoallergenic infant formulas

Sample*	DH (%)	M_r distribution (%)			
		> 10 kd	5-10 kd	3-5 kd	<3 kd
Hydrolysate 1	12.6	3.2	21.2	27.4	48.3
Hydrolysate 2	30.1	0.7	5.5	28.9	64.8
Hydrolysate 3	30.1	1.6	5.4	30.2	62.7
Hydrolysate 4	30.2	0.5	6.9	32.2	60.4
Formula 5	32.5	2.3	12.0	26.4	59.3
Formula 6	16.1	4.0	22.6	27.3	46.1
Formula 7	11.8	4.5	21.7	30.2	43.6
Formula 8	6.3	26.3	17.9	22.4	33.4
Formula 9	23.9	1.4	13.4	32.6	52.6
Formula 10	14.4	3.3	19.6	30.1	47.0
Formula 11	1.3	40.6	16.1	17.7	25.6
Formula 12	52.0	0.5	4.3	18.4	76.8
3000 d hydrolysate	—	0.5	9.4	27.5	62.6

*Hydrolysates 1 to 4 are hypoallergenic whey protein hydrolysates; formulas 5 to 11 are hypoallergenic infant formulas based on hydrolyzed whey protein; formula 12 is based on hydrolyzed casein. The 3000 d hydrolysate is whey protein hydrolysate after filtration with a M_r cutoff value of 3000 d.³¹

tions,^{27, 29} our ELISA studies, in which isolated proteins from cow's milk were used, clearly show considerable variations in the concentrations of IgE antibodies to the individual milk proteins within and among patients allergic to cow's milk. These results indicate differences in the individual patterns of sensitization to the various cow milk proteins.

Modern commercially available milk protein hydrolysates that are used to produce hypoallergenic infant formulas are the result of heat treatment, enzymatic cleavage, and subsequent ultrafiltration. During heat treatment the milk proteins are denatured and most conformational epitopes are eliminated. Enzymatic cleavage mostly eliminates sequential epitopes but may also eliminate conformational epitopes if conformation is not maintained after cleavage. Using different enzyme systems, we previously found that the reduction in antigenicity of the individual milk proteins is mainly determined by the molecular structure of the antigenic epitopes and enzyme specificity rather than by DH or M_r distribution.³⁷

Although the processing techniques of the various hydrolysates analyzed in this study are unknown, they differ in starting material, heat treatment, and choice of proteolytic enzymes. Heat stability and amino acid sequence of the antigenic epitopes differ among the individual milk proteins. This resulted, as our data indicate, in considerable variation in residual IgE antigenicity of the indi-

vidual milk proteins in the tested milk protein hydrolysates and infant formulas. In addition, residual IgE antigenicity of the individual milk proteins also differed among the hypoallergenic products. Apparently, immunoreactive epitopes of BSA and B-IgG are more easily eliminated by the current processing techniques than those of α La, β Lg, and casein; residual antigenicity of these latter proteins was considerably higher in most of the hypoallergenic products.

Cases of hypersensitivity to infant formulas based on hydrolyzed whey protein or casein have been reported.³⁻¹⁰ Our results suggest that the occurrence of milk protein-specific antibodies in serum and the protein-specific residual antigenicity of a hypoallergenic product might be valuable laboratory predictors for cow's milk allergy. Children highly sensitized to one or more of the milk proteins might have anaphylaxis after consumption of a hypoallergenic product in which a reasonable number of antigenic epitopes of those particular milk proteins are still present. Anaphylaxis is not apt to occur when these epitopes are absent. We found that the extensively hydrolyzed casein formula (formula 12) still has immunoreactive epitopes against α La. Therefore this product could cause anaphylaxis in children with high concentrations of IgE antibodies against this protein. The heterogeneous pattern of sensitization to the different milk proteins and their variable residual antigenicities in hypoallergenic products might ex-

plain why hypoallergenic products are well tolerated in some but not in all children allergic to cow's milk.³⁸

Residual antigenicity of α La, β Lg, and casein was higher in formulas 7 and 10 compared with hydrolysate 1, from which these formulas were manufactured. This might possibly be related to Maillard reactions in the products, as suggested by their yellow-brown hue.^{39, 40} Analyses of residual antigenicity should therefore involve the final product and not only the protein constituent of the product.

The results on IgE binding to various commercially available hypoallergenic products are in agreement with those reported by others.¹¹⁻¹⁵ However, in most studies RASTs and RAST inhibition with cow's milk allergen disks or skin prick tests were used, which made differentiation among the various milk proteins impossible. Differences in residual antigenicity of the individual milk proteins in whey protein hydrolysates produced with various enzyme systems have also been reported by others.^{37, 41}

The *in vitro* analysis of the interaction of hypoallergenic hydrolysate with preformed antibody is a sensitive method for estimating residual antigenicity. However, the results are affected by the proportion of residual epitopes recognized by the antiserum and by the avidity of the specific antibodies for these regions. As a consequence it should be realized that the results do not necessarily express the differences in residual antigenicity on a strictly quantitative basis. Estimation of the residual antigenicity of a hypoallergenic product should preferentially be carried out with the individual patient serum.

The ELISA method used in this study needs one epitope per protein molecule or per peptide fragment for binding to produce a positive test result. However, peptide molecules with only one epitope do not induce allergic reactions because two epitopes on the same protein fragment must bind to IgE on the surface of mast cells to promote mediator release. We previously found that the 3000 d hydrolysate, with some residual immunoreactive epitopes of α La, β Lg, and casein, was unable to induce IgE-mediated allergic reactions in sensitized experimental animals after intravenous administration of the hydrolysate.³¹ After oral administration of protein, hydrolysates undergo physiologic digestion. This may lead to a further reduction or even complete elimination of residual epitopes.³⁷ Therefore the clinical significance of the residual antigenicity in the tested hypoaller-

genic products certainly needs further investigation.

Animal studies are used as part of the preclinical screening of hydrolyzed formula, in which residual IgG antigenicity is often determined as a measure of residual IgE antigenicity.^{42, 43} We found clear differences between residual IgE and IgG antigenicity of the individual milk proteins in the hypoallergenic products. Because immediate sensitivity is based on interaction between IgE antibodies and mast cells, the relevance of residual IgG antigenicity of milk proteins for their allergenicity can be questioned.

From the results of our study we conclude that no single major allergen can be held responsible for cow's milk allergy and that all tested hypoallergenic products retain some residual antigenicity of one or more of the individual milk proteins. The individual sensitization pattern of the patient allergic to cow's milk and the milk protein-specific residual antigenicity might be considered as possible predictors of adverse reactions to hypoallergenic products. Their determination could be a useful screening tool for pediatricians to select a formula adapted to the patient.

We thank C. J. Slangen of our institute for performing gel permeation chromatography analyses and for statistical advice. We also thank A. C. Douwes, PhD, and G. J. van Kamp, PhD, from the Department of Pediatrics, Academic Hospital of the Free University, Amsterdam, and M. M. Panis, MD, of the Department of Pediatrics, Wilhelmina Children's Hospital, Utrecht, for donation of the patients' serum.

REFERENCES

1. Bahna SL. Control of milk allergy: a challenge for physicians, mothers and industry. *Ann Allergy* 1978;41:1-12.
2. Høst A, Husby S, Østerballe O. A prospective study of cow's milk allergy in exclusively breast-fed infants: incidence, pathogenic role of early inadvertent exposure to cow's milk formula, and characterization of bovine milk protein in human milk. *Acta Paediatr Scand* 1988;77:663-70.
3. Lifschitz CH, Hawkins HK, Guerra C, Byrd N. Anaphylactic shock due to cow's milk protein hypersensitivity in a breast-fed infant. *J Pediatr Gastroenterol Nutr* 1988;77:141-4.
4. Businco L, Cantani A, Longhi MA, Giampietro PG. Anaphylactic reactions to a cow's milk whey protein hydrolysate (Alfa-Ré, Nestlé) in infants with cow's milk allergy. *Ann Allergy* 1989;62:333-5.
5. Bock SL. Probable allergic reaction to casein hydrolysate formula. *J ALLERGY CLIN IMMUNOL* 1990;84:272.
6. Heyman MB, Stoker TW, Rudolph CD, Frick OL. Hypersensitivity reaction in an infant fed hydrolysed lactalbumin contained in a semielemental formula. *J Pediatr Gastroenterol Nutr* 1990;10:253-6.
7. Ellis MH, Short JA, Heiner DC. Anaphylaxis after inges-

- tion of a recently introduced hydrolyzed whey protein formula. *J Pediatr* 1991;118:74-7.
8. Sampson HA, Bernhisel-Braadbent J, Yang E. Safety of casein hydrolysate formula in children with cow milk allergy. *J Pediatr* 1991;118:520-5.
 9. Saylor JD, Bahna SL. Anaphylaxis to casein hydrolysate formula. *J Pediatr* 1991;118:71-4.
 10. Businco L, Lucenti P, Arcese G, Ziruolo G, Cantani A. Immunogenicity of a so-called hypoallergenic formula in at-risk babies: two case reports. *Clin Exp Allergy* 1994;24:42-5.
 11. Lau S, Wahn U. How allergenic are "hypoallergenic" formulas? In: Harms HK, Wahn U, eds. *Food allergy in infancy and childhood*. Berlin: Springer Verlag, 1989:173-6.
 12. Plebani A, Albertini A, Scotta S, Ugazio AG. IgE antibodies to hydrolysates of cow milk proteins in children with cow milk allergy. *Ann Allergy* 1990;64:279-80.
 13. Oldaeus G, Björkstén B, Einarsson R, Kjellman NIM. Antigenicity and allergenicity of cow milk hydrolysates intended for infant feeding. *Pediatr Allergy Immunol* 1991;4:156-64.
 14. Rugo E, Wahl R, Wahn U. How allergenic are hypoallergenic infant formulae? *Clin Exp Allergy* 1992;22:635-9.
 15. Dean TP, Adler BR, Ruge F, Warner JO. *In vitro* allergenicity of cow's milk substitute. *Clin Exp Allergy* 1992;23:205-10.
 16. Bell K, McKenzie HA. β -Lactoglobulins. *Nature* 1964;204:1275-9.
 17. Taylor SL. Immunologic and allergic properties of cow's milk proteins in humans. *J Food Protect* 1986;49:239-50.
 18. Kilshaw PJ, Cant AJ. The passage of maternal dietary proteins into human breast milk. *Int Arch Allergy Appl Immunol* 1984;75:8-15.
 19. Clyne PS, Kulczycki A. Human breast milk contains bovine IgG: relationship to infants colic? *Pediatrics* 1991;87:439-44.
 20. Mäkinen-Kiljunen S, Palosuo T. A sensitive enzyme-linked immunosorbent assay for determination of bovine β -lactoglobulin in infant feeding formulas and in human milk. *Allergy* 1992;47:347-52.
 21. Lovegrove JA, Osman DL, Morgan JB, Hampton SM. Transfer of cow's milk β -lactoglobulin to human serum after a milk load: a pilot study. *Gut* 1993;34:203-7.
 22. Maeda S, Morikawa A, Tokuyama K, Kuroume T. The concentration of bovine IgG in human breast milk measured using different methods. *Acta Paediatr* 1993;82:1012-6.
 23. Kuitunen M, Savilahti E, Sarnesto A. Human α -lactalbumin and bovine β -lactoglobulin absorption in infants. *Allergy* 1994;49:354-60.
 24. Goldman AS, Anderson DW, Sellers WA, Saperstein S, Kniker WT, Halpern ST. Milk allergy. I. Oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics* 1963;32:425-43.
 25. Kuitunen P, Visakorpi JK, Savilahti E, Pelkonen P. Malabsorption syndrome with cow's milk intolerance: clinical findings and course in 54 cases. *Arch Dis Child* 1975;50:351-6.
 26. Lebenthal E. Cow's milk protein allergy. *Pediatr Clin North Am* 1975;22:827-33.
 27. Gjesing B, Østerballe O, Schwartz B, Wahn U, Løwenstein H. Allergen specific IgE antibodies against antigenic components in cow milk substitutes. *Allergy* 1986;41:51-6.
 28. Otani H, Dong XY, Hara T, Kobayashi M, Kayahara H, Hosono A. Human serum antibodies to cow milk proteins in infants and children having clinically allergic symptoms. *Milchwissenschaft* 1989;44:131-4.
 29. Otani H, Dong XY, Hara T, Kobayashi M, Kayahara H, Hosono A. Specificities to cow milk proteins of human serum antibodies from clinically allergic patients. *Milchwissenschaft* 1989;44:267-70.
 30. Schmidt DG, van Markwijk BW. Enzymatic hydrolysis of whey proteins: influence of heat treatment of α -lactalbumin and β -lactoglobulin on their proteolysis by pepsin and papain. *Neth Milk Dairy J* 1993;47:15-22.
 31. van Beresteijn ECH, Peeters RA, Kaper J, Meijer RJGM, Robben AJPM, Schmidt DG. Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. *J Food Protect* 1994;57:619-25.
 32. Fritsché R, Bonzon M. Determination of cow milk formula allergenicity in the rat model by *in vitro* mast cell triggering and *in vivo* IgE induction. *Int Arch Allergy Appl Immunol* 1990;93:289-93.
 33. Church FC, Porter DH, Catignani GL, Swaisgood HE. An *o*-phthalaldehyde spectrophotometric assay for proteinases. *Anal Biochem* 1985;146:343-8.
 34. Frister H, Meisel H, Schlimme E. OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component. *Fresenius Z Anal Chem* 1988;30:631-3.
 35. Visser S, Slangen CJ, Robben AJPM. Determination of molecular mass distributions of whey protein hydrolysates by high-performance size-exclusion chromatography. *J Chromatogr* 1992;599:205-9.
 36. International Organisation for Standardization. Precision of test methods: determination of repeatability and reproducibility for a standard test method by interlaboratory tests. In: *ISO standards handbook 3: statistical methods*. 3rd ed. Geneva, Switzerland: International Organisation for Standardization, 1989:413-61.
 37. Ena JM, Beresteijn ECH van, Robben AJPM, Schmidt DG. Reduction of whey protein antigenicity by fungal proteinases and a pepsin/pancreatin combination. *J Food Sci* 1995;60:104-10.
 38. Ragno V, Giampietro PG, Bruno G, Businco L. Allergenicity of milk protein hydrolysate formulae in children with cow's milk allergy. *Eur J Pediatr* 1993;152:760-2.
 39. Otani H, Morita S, Tokita F. Studies on the antigenicity of the browning product between β -lactoglobulin and lactose. *Jpn J Zotech Sci* 1985;56:67-74.
 40. Otani H, Hosono A. Location of antigenic sites in a browning product between β -lactoglobulin and lactose. *Jpn J Zotech Sci* 1987;58:474-82.
 41. Asselin J, Hébert J, Amiot J. Effects of *in vitro* proteolysis on the allergenicity of major whey proteins. *J Food Sci* 1989;54:1037-9.
 42. Cordle CT, Mahmoud MI, Moore V. Immunogenicity of protein hydrolysates for hypoallergenic infant formulae. *J Pediatr Gastroenterol Nutr* 1991;13:270-6.
 43. Piacentini GL, Bertolini A, Spezia E, Piscione T, Boner AL. Ability of a new infant formula prepared from partially hydrolyzed bovine whey to induce anaphylactic sensitization: evaluation in a guinea pig model. *Allergy* 1994;49:361-4.