

Monoclonal and polyclonal antibodies against casein components of cow milk for evaluation of residual antigenic activity in 'hypoallergenic' infant formulas

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Summary

Background Hydrolysed casein and whey protein formulas have been developed with the aim of preventing sensitization in infants at high risk of cow milk allergy. Subsequently these products have also been used for treatment of children with cow milk allergy. However, severe reactions have occurred in some allergic infants fed with these formulas raising doubts about their absolute safety and suggest the need for developing *in vitro* techniques for detection of eventual residual allergenic activity in such preparations.

Objectives Our purpose was to evaluate the usefulness of monoclonal and polyclonal antibodies against casein components (α , β and κ casein) as reagents for the detection of the residual antigenic activity of casein components in several hydrolysed formulas.

Methods The monoclonal and polyclonal antibodies were produced according to standard procedures by immunizing female Balb/c mice with casein fraction (a mixture of α , β and κ casein). ELISA assays were developed to test the specificity of the antibodies and to detect and evaluate the amount of residual antigenic activity of the casein components in hydrolysed formulas.

Results Use of polyclonal antiserum specific for casein allowed detection of residual antigenic activity of casein components in all partial hydrolysates and in the two extensive whey protein hydrolysates in the amounts ranging from 0.05 to 0.67% of total protein. No such activity was detectable in either the two extensive casein hydrolysates tested or the aminoacid based formula. The polyclonal antiserum proved to be more suitable than monoclonals for detecting residual antigenic activity in the hydrolysates. The monoclonal antibodies were directed against epitopes expressed on different casein components.

Conclusions In this study the ELISA inhibition assay with polyclonal antibodies specific for casein components of cow milk proved to be a sensitive method for estimating residual antigenicity in the hydrolysed formulas commercially available for infants with cow milk allergy suggesting their potential application for the quality control of hypoallergenic infant formulas.

Keywords: cow milk allergy, monoclonal and polyclonal antibodies, hypoallergenic formulas

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Introduction

Cow milk is probably the most common food allergen during infancy and early childhood and, in cow milk allergic

infants, circulating IgE specific for casein and whey proteins are responsible for many adverse reactions to milk [1]. The treatment of choice for cow milk allergy is avoidance of cow milk; substitutes such as soy formulas, hydrolysed cow milk casein and whey formulas, and elemental formulas are usually recommended. Casein and whey protein

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hydrolysates have been developed to decrease or eliminate epitopes which may induce an IgE response or react with specific IgE present in infants and children with IgE-mediated cow milk allergy.

Circulating IgE antibodies against protein fragments present in hydrolysed formulas have been found in allergic children [2]. This may explain the occasional occurrence of cases of severe reactions to hydrolysed formulas and raises doubts about their absolute safety [3–10]. These reactions may result from the presence in hydrolysates of small amounts either of residual intact proteins or of peptides retaining antigenic determinants.

Various *in vitro* methods have been employed to assess residual antigenic activity in cow milk protein hydrolysates: chromatographic analysis, radioallergosorbent test (RAST), RAST-inhibition, enzyme-linked immunosorbent assays (ELISA), sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and immunoblotting [11–14]. In the present study using antibodies to casein an ELISA inhibition assay proved to be very sensitive test for detecting and estimating the amount of residual antigenic activity in hydrolysed formulas.

Materials and methods

Reagents

Highly purified cow milk proteins (Sigma Chemical Co., St. Louis, MO, USA) all in the form of lyophilized powders with the following chemical characteristics were employed: casein fraction containing $\approx 90\%$ protein (α , β and κ casein); α -casein (purified chromatographically; $\approx 85\%$ by PAGE); β -casein (about 90% by electrophoresis); κ -casein ($\approx 80\%$ by PAGE); α -lactalbumin ($\approx 85\%$ by electrophoresis); β -lactoglobulin (crystallized three times).

Milk products

Milk products included Humana 1 (Humana, a cow milk formula; lot n. 03729 S27), Nidina HA (Nestlé, a partially hydrolysed whey formula; lot n. L-RLRAA), Vivena HA (Dieterba, a partially hydrolysed whey formula; lot n. 1683 6B), Aptamil HA (Milupa, a partially hydrolysed whey and casein formula), Nutrilon Pepti (Nutricia, an extensively hydrolysed whey formula; lot n. 124602881), Pepti Junior (Nutricia, an extensively hydrolysed whey formula with short-chain peptides; lot n. 836407086), Nutri Junior (Nutricia, aminoacid based formula; lot n. 2008 0092 41), Nutramigen (Mead Johnson, an extensively hydrolysed casein formula; lot n. S32642), Pregestimil (Mead Johnson, an extensively hydrolysed casein formula; lot n. S40983), Isomil (Abbott, a soy formula; lot n. 83467NR).

Preparation of anti casein monoclonal and polyclonal antibodies Casein specific monoclonal antibodies were prepared by injecting 4- to 6-week-old female Balb/c mice (Charles River Laboratories, Wilmington, MA, USA) intraperitoneally with 50 μ g of solubilized casein fraction emulsified 1:1 in Freund's complete adjuvant followed by two boosters with the same dose of immunogen at 14 day intervals. Three days before removal of the spleen the mice were inoculated with the same amount of immunogen in a phosphate-buffered saline solution (PBS) and the spleen cells were then hybridized with Ag8.X63 myeloma cells using 1500 MW polyethylene glycol. The fusion mixture was then diluted to 100 mL with HAT (Hypoxanthine, Aminopterin, Thymidine) medium and distributed on feeder layers of normal Balb/c mouse peritoneal exudate cells in 24-well Nunc plates (Gibco, Basel, Switzerland) [15]. Wells showing anti casein activity in their supernatants were cloned by limiting dilution. The cloned cells ($5\text{--}9 \times 10^6$ cells) were then injected in Balb/c mice 1 week after pre-treatment with 0.5 mL of pristane and the peritoneal liquid was harvested. The specificity of the monoclonals obtained was then tested and three, Mo50, Mo71, Mo169 were selected for study.

Casein specific polyclonal antiserum was prepared by injecting 4- to 6-week-old female Balb/c mice (Charles River Laboratories, Wilmington, MA) intraperitoneally with 100 μ g of solubilized casein fraction emulsified 1:1 in Freund's complete adjuvant followed by two boosters with the same dose of immunogen at 14 day intervals. Seven days after the second booster blood was drawn and the serum tested for casein-specific antibodies.

Specificity of polyclonal and monoclonal antibodies Specificity of the polyclonal and monoclonal antibodies against casein was evaluated by an ELISA inhibition assay as follows: round-bottom polystyrene microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with casein fraction by incubating each well overnight with 200 μ L of a solution containing 5 μ g/mL of casein fraction in 0.05 M carbonate-bicarbonate buffer pH 9.6. After three washings with 200 μ L of PBS containing 0.05% Tween 20, 100 μ L of an appropriate dilution of polyclonal or monoclonal antibodies in PBS containing 5% horse serum and 100 μ L of the casein fraction, α -lactalbumin or β -lactoglobulin in concentrations increasing by twofold from 78 to 5000 ng/mL (corresponding to an absolute protein concentration/well of 7.8–500 ng), were added simultaneously to the wells. After a 2 h incubation at room temperature followed by three washings, 200 μ L of an appropriate dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) in PBS containing 5% horse serum were added to the wells. After another 2 h incubation at room temperature followed by three washings, 200 μ L of

enzyme substrate solution (2 mg/mL o-phenyldiamine and 0.015% H₂O₂ in 0.1 M phosphate buffer pH 6.0) were added, absorbance at 492 nm recorded and the results expressed as optical density values.

To define the specificity of the polyclonal and monoclonal antibodies for the α , β and κ components of the casein fraction, the following assay was performed: 100 μ L of an appropriate dilution of antibodies in PBS containing 5% horse serum and 100 μ L of increasing concentrations of α or β or κ casein (0, 0.125, 0.250, 0.50, 1.0, 5.0, 10.0, 20.0 μ g/mL in PBS containing 5% horse serum) were added to the wells of a microtitre plate sensitized with the casein fraction (200 μ L of a solution containing 5 μ g/mL). The antigen-antibody reaction was detected by adding an appropriate dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG in PBS containing 5% horse serum, followed by addition of the enzyme substrate as described; absorbance at 492 nm was recorded. The percentage of antibody binding was calculated by assigning a value of 100% to the optical density observed when only antibodies were added to the wells.

Residual antigenic activity in the hydrolysed formulas The presence of proteins/peptides with potential antigenic activity in the partial and extensive hydrolysates was assessed as follows: 100 μ L of the appropriate dilution of polyclonal or monoclonal antibodies in PBS containing 5% horse serum and 100 μ L of total protein in concentrations increasing by twofold from 19.5–2500 μ g/mL in PBS containing 5% horse serum of each of the 10 formulas, as well as of α -lactalbumin and β -lactoglobulin as negative controls, were added to the wells of a microtitre plate, sensitized with 200 μ L of a solution containing 5 μ g/mL of casein fraction. The antigen-antibody reaction was revealed by adding an appropriate dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG in PBS containing 5% horse serum, followed by addition of the enzyme substrate as described; the absorbance at 492 nm was recorded. The results are expressed as optical density values.

Immunologically identifiable casein-related material (ICRM) in the hydrolysed formulas A standard dose-response inhibition curve was derived as follows: 100 μ L of the appropriate dilution of polyclonal antiserum and 100 μ L of casein fraction in concentrations increasing by twofold from 78 to 5000 ng/mL were added simultaneously to the wells of a microtitre plate coated with casein fraction. The percentage antibody binding (estimated by assigning a value of 100% to the optical density observed when only antibodies were added to the wells) was then plotted against each concentration of the casein fraction added.

The amount of immunologically identifiable casein-related material (ICRM) in the hydrolysates was estimated

as follows: 100 μ L of an appropriate dilution of the polyclonal antiserum were incubated with 100 μ L of hydrolysed formula in concentrations increasing by twofold from 19.5–2500 μ g/mL of total protein calculated considering the protein content in percentage of each formula. The percentage polyclonal antibody binding was calculated for those concentrations of the hydrolysates with optical density values within the range of the standard curve. The amount of ICRM was calculated for each formula by plotting the percentage binding of each selected total protein concentration on the standard curve. For each formula a regression curve was then calculated by plotting the total protein concentrations (x) against the corresponding amount of ICRM (y). The angular coefficient of the equation describing the regression for each formula was taken as the amount of ICRM contained in each formula and expressed as percentage of total protein.

Results

Specificity of antibodies for the casein fractions

The specificity of the monoclonal and polyclonal antibodies for casein was demonstrated by adding an appropriate dilution of antibodies and increasing concentrations of the casein fraction, α -lactalbumin or β -lactoglobulin simultaneously to the wells of a microtitre plate coated with casein fraction. As shown in Fig. 1, the inhibition of monoclonal and polyclonal binding was observed following incubation of antibodies with only the casein fraction. All three monoclonals showed the same inhibition pattern, so Fig. 1 shows only a representative experiment with Mo71.

In addition, Mo50 binding to the casein-coated microtitre plates was strongly inhibited when Mo50 was incubated with increasing concentrations of κ but not of α - or β -casein, except for a modest degree of inhibition with the highest concentrations of β -casein (Table 1). Similarly, Mo71 binding was significantly inhibited by the addition of β -casein as was that of Mo169 by the addition of α and κ casein (Table 1); some inhibition was observed at the highest concentrations of α or κ casein for Mo71 and of β -casein for Mo169. These findings suggest that Mo50, Mo71 and Mo169 recognize epitopes present, respectively, on κ , β , and on both α and κ -casein and also that the commercially available α , β and κ caseins are mutually contaminated, as previously shown by an immunoblotting technique [16].

Binding of the polyclonal antisera was strongly inhibited even at the lowest concentrations of α , β or κ casein. The greatest inhibition was observed with κ casein and, since the degree of purity of the single casein components was similar, this suggests that most antibodies were directed against κ casein.

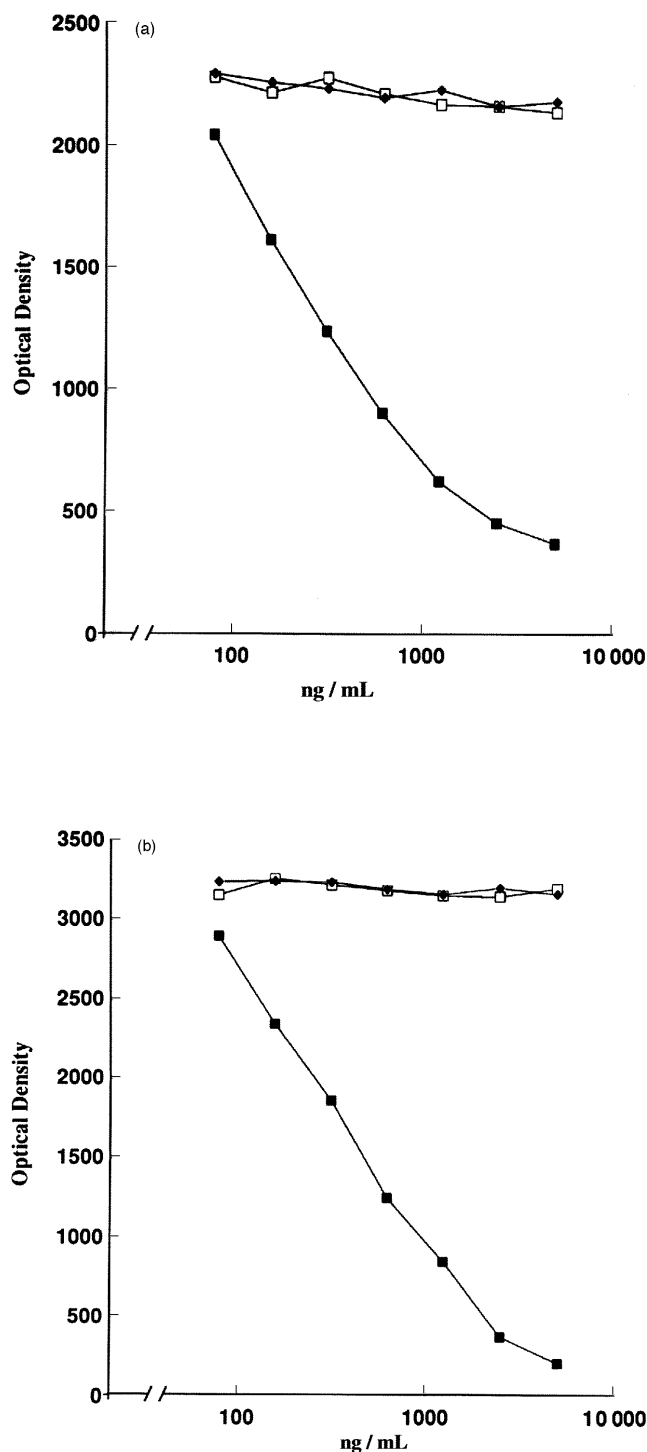


Fig. 1. Specificity of the monoclonal (a) and polyclonal (b) antibodies for the casein fraction. Concerning the former, a representative experiment with Mo71 is shown. Specificity was demonstrated by evaluating the inhibition of binding of Mo71 or of the polyclonal antibodies to the casein fraction coated-microtitre plates by increasing concentrations of casein fraction (■), or α -lactalbumin (□) or β -lactoglobulin (◆).

Residual antigenic activity in partial and extensive hydrolysed formulas

As expected, monoclonal and polyclonal antibody binding to the casein-coated microtitre plates was not inhibited when they were incubated with α -lactalbumin, β -lactoglobulin or a soy formula (Isomil). Strong inhibition of both monoclonal and polyclonal antibody binding was observed with all concentrations (from 19.5 to 2500 μ g/mL, corresponding to an absolute protein concentration/well of 1.95–250 μ g) of the non-hydrolysed cow milk formula (Humana 1) (Fig. 2a,b,c,d).

As for the polyclonal antiserum, marked inhibition was documented with all the partial and both extensive whey hydrolysates (Nutrilon Pepti, Pepti Junior) but none with either the two extensive casein hydrolysates (Nutramigen, Pregestimil) or the aminoacid based formula (Nutri Junior) (2d).

As for the monoclonal antibodies, binding inhibition of Mo71 was observed only with two (Nidina HA, Aptamil HA) of the three partial whey hydrolysates (2b), of Mo50 with all the partial whey hydrolysates (Vivena HA, Aptamil HA, Nidina HA) and one of the two extensive whey hydrolysates (Nutrilon Pepti) (2a); Mo169 binding was not inhibited by any hydrolysate (2c).

Amount of immunologically identifiable casein-related material (ICRM) in the hydrolysed formulas tested

A rough estimate of the amount of ICRM contained in the hydrolysed formulas is shown in Table 2. The partial whey hydrolysates (Vivena HA and Nidina HA) had the highest (respectively 0.67 and 0.57% total protein) content; similar amounts (respectively 0.08 and 0.07%) were found in the extensive whey (Pepti Junior) and partial whey/casein hydrolysate (Aptamil HA); the extensive whey hydrolysate (Nutrilon Pepti) contained the lowest amount (0.05%).

No ICRM was detectable in the extensive casein hydrolysates (Nutramigen, Pregestimil), the aminoacid based (Nutri Junior) or the soy Isomil formulas. ICRM in the non hydrolysed cow milk formula (Humana 1) accounted for 44.6% of total protein, an amount equivalent to that declared by manufacturers.

Discussion

In the present study we describe the production of monoclonal and polyclonal antibodies specific for casein as demonstrated by their marked reactivity against the casein fraction and lack of reactivity against α -lactalbumin and β -lactoglobulin. In addition, we show that the monoclonals recognize epitopes expressed on different casein components. In particular, the Mo50 antibody recognizes an

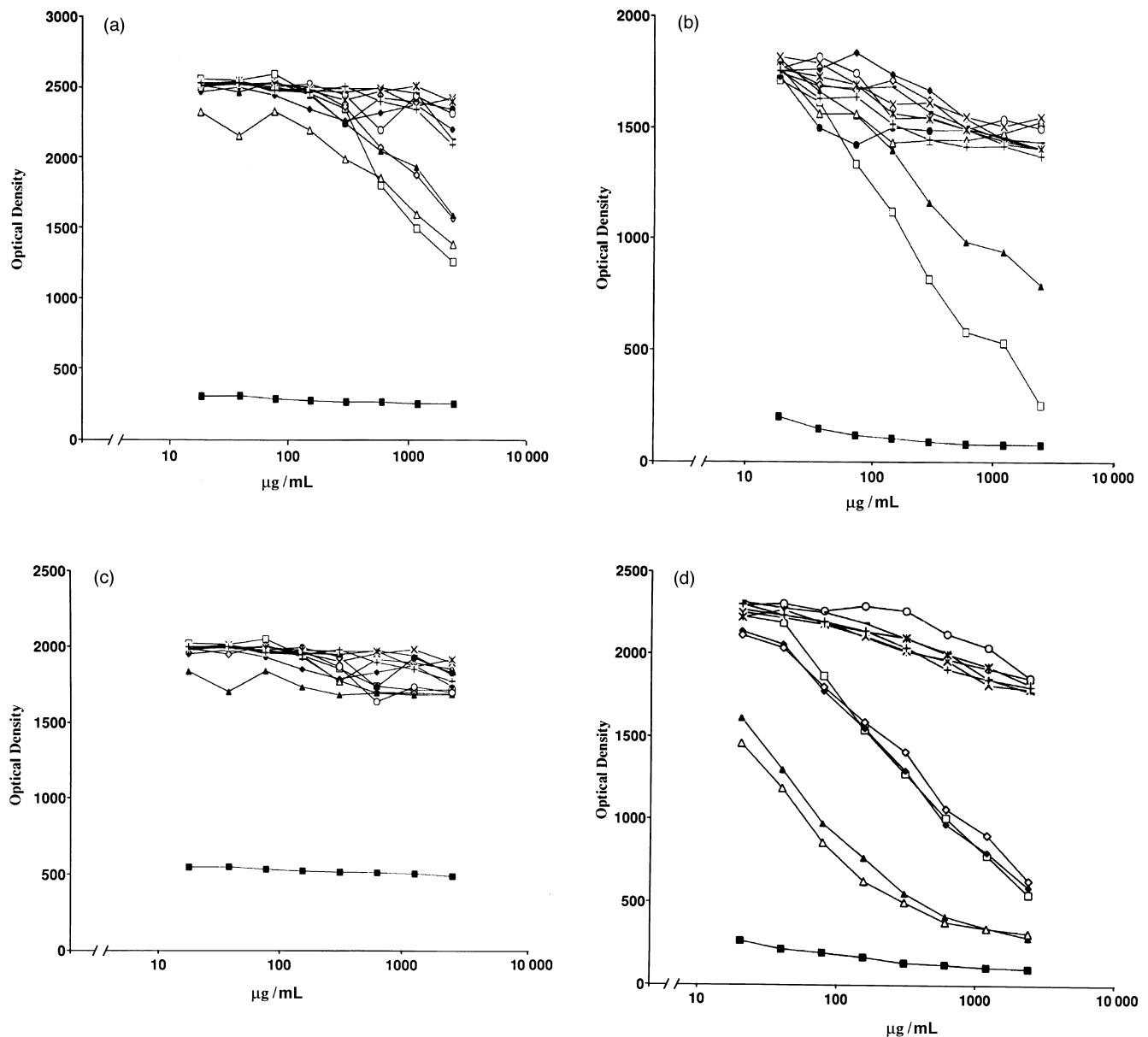


Fig. 2. Inhibition of binding of the monoclonals Mo50 (a), Mo71 (b), Mo169 (c) and polyclonal antibodies (d) to the casein fraction-coated microtitre plates by hydrolysed formulas. Monoclonal and polyclonal antibodies were incubated with increasing concentrations of the following: cow milk formula (Humana 1, ■), partial whey hydrolysates (Nidina HA, ▲; Vivena HA, △), a partial whey and casein hydrolysate (Aptamil HA, □), extensive casein hydrolysates (Nutramigen, ○; Pregestimil, ×), extensive whey hydrolysates (Pepti Junior, ◆; Nutrilon Pepti, ◇), and an elemental diet made of synthetic aminoacids (Nutri Junior, *). Isomil (a soy based formula, ●), α -lactalbumin (+) and β -lactoglobulin (–) were used as negative controls.

epitope present on κ casein, the Mo71 an epitope on β casein and the Mo169 an epitope common to both α and κ casein. The slight inhibition observed after incubation of Mo50 and Mo169 with the highest concentrations of β casein, and of Mo71 with the highest concentrations of α and κ casein probably results from mutual contamination of casein components as shown previously [16].

Binding of the polyclonal antiserum was strongly inhibited by addition of α , β or κ casein; the greatest inhibition was observed with κ casein and suggests that most of the polyclonal antibodies are directed against κ casein.

Variable amounts of immunologically identifiable casein-related material (ICRM), estimated by the percentage binding inhibition of polyclonal antiserum, were detected in five

Table 1. Assessment of the specificity of the monoclonal (Mo50, Mo71, and Mo169) and polyclonal antibodies for the casein components. The monoclonal or polyclonal antibodies were added either alone or with increasing concentrations of α , or β or κ casein to the wells of a microtitre plate sensitized with casein. The antigen-antibody reaction was revealed as described in the text. The results are expressed as percentages of monoclonal/polyclonal binding calculated by assigning a value of 100% to the optical density observed when only antibodies were added to the plate

Amount of α -, or β -, or κ -casein added with the monoclonal or polyclonal antibodies $\mu\text{g/mL}$	Mo50 Casein			Mo71 Casein			Mo169 Casein			Polyclonal Casein		
	α	β	κ	α	β	κ	α	β	κ	α	β	κ
0	100	100	100	100	100	100	100	100	100	100	100	100
0.125	108	103	104	95	100	97	90	97	104	61	54	31
0.25	111	100	100	95	26	97	78	95	96	45	40	20
0.5	102	96	88	88	20	97	63	94	82	32	26	12
1	106	90	76	81	16	90	62	88	70	24	19	5
5	102	82	40	64	13	55	19	89	35	15	10	2
10	102	72	24	40	13	38	16	80	21	7	5	1
20	98	60	12	34	13	35	17	76	11	—	—	—

of the eight hydrolysates tested, all three partial hydrolysates (Vivena HA, Nidina HA, Aptamil HA) and the two extensive whey hydrolysates (Pepti Junior, Nutrilon Pepti). No ICRM was found in the other three, two extensive casein hydrolysates (Nutramigen, Pregestimil) and one composed of free aminoacids (Nutri Junior). Two (Nidina HA and Aptamil HA) of the three partial hydrolysates inhibited Mo71 binding; all partial hydrolysates and one of the two

Table 2. Amount of immunologically identifiable casein related-material expressed as percentages of total protein in a cow milk non-hydrolysed formula (Humana 1) and in hydrolysed formulas, estimated by the degree of binding inhibition of polyclonal antiserum by each of the products listed below

Product	% of total protein
Humana 1	44.6
Aptamil HA	0.07
Nidina HA	0.57
Vivena HA	0.67
Pepti Junior	0.08
Nutrilon Pepti	0.05
Nutri Junior	ND
Nutramigen	ND
Pregestimil	ND
Isomil	ND
α -Lactoglobulin	ND
β -Lactalbumin	ND

ND, Not detectable.

extensive whey hydrolysates (Nutrilon Pepti) inhibited Mo50 binding; Mo169 binding was not inhibited by any hydrolysate. These findings suggest that polyclonals are more suitable than monoclonal antibodies for the detection of ICRM in hydrolysed formulas. Reactivity of Mo50 with Aptamil HA and Nidina HA and of Mo71 with Aptamil HA, was observed in a previous study using an immunoblotting technique on TCA-precipitated hydrolysed formulas [16]. However, with that technique, Mo50 also reacted with Pregestimil and Pepti Junior but not with Nutrilon Pepti; Mo71 reacted with Pepti Junior and Nutrilon Pepti. The high concentrations of the TCA-precipitated hydrolysed formulas loaded on gel and the possible effect of TCA-precipitation on the antigenic conformation of the protein may account for the difference in reactivity of the monoclonals observed in the two studies.

The results of the present study based on binding inhibition of polyclonal antiserum, suggest that the extensive casein hydrolysates tested are indeed depleted of most antigenic determinants of casein. Surprisingly, the partial and extensive whey hydrolysates were shown to contain variable amounts of intact casein epitopes suggesting that the procedures employed to separate casein from whey proteins are not satisfactory, or that the hydrolysis may uncover hidden casein-related antigenic determinants on whey proteins. The former hypothesis however, is more likely, as several reports have documented contamination of whey proteins with casein [13,14,17]. Although only small amounts of ICRM (from 0.05 to 0.67%) was found in hydrolysed formulas, the consequences of their daily ingestion could be clinically relevant in some allergic children.

According to our data, 80 mg of ICRM are present per 100 g of proteins, the equivalent of 700 g of Pepti Junior powder (protein content of 14%). This represents the formula intake of a 2-month-old infant in about 10 days and could explain the occurrence of anaphylactic reactions in some very sensitive allergic infants fed with extensively hydrolysed whey formulas. Indeed, anaphylactic reactions have also been reported in babies fed with the extensive casein hydrolysates tested (Nutramigen, Pregestimil) [3,5,6] which, according to our data, appear to contain no ICRM. Such reactions could be due to the presence of antigenic determinants of other allergenic proteins such as α -lactalbumin or β -lactoglobulin, or to qualitative or quantitative batch to batch variability of the casein content in the hydrolysates.

The failure of some hydrolysates to inhibit binding of the monoclonal but not of the polyclonal antibodies probably reflects modification of the antigenic determinants which occurs during hydrolysis, as well as the different specificities of the monoclonal and polyclonal antibodies. The former are antibodies with a single specificity, while the latter are a mixture of antibodies directed against multiple antigenic determinants on a molecule. Since the different processes used to prepare hydrolysed formulas are unlikely to affect all antigenic determinants of a protein in the same way and as the hydrolysates are likely to contain casein components in different proportions, the different degree of binding inhibition of the monoclonals observed with the hydrolysates tested and the higher proportion of hydrolysates inhibiting binding of the polyclonal than of the monoclonal antibodies are not surprising. Collectively these data suggest the potential usefulness of polyclonal antisera for the quality control of residual antigenic activity in hydrolysed formulas. However, due to their specificity, the monoclonals are more suitable for identifying specific antigenic determinants relevant to allergenicity of cow milk proteins.

So-called 'hypoallergenic formulas' are prepared on the assumption that hydrolysis results in low molecular weight peptides, and thus reduces or eliminates the antigenic/allergenic determinants of the protein. Indeed, evaluation of the molecular weight profile of the peptides in hydrolysed formulas combined with other immunochemical methods is currently used for the *in vitro* assessment of residual antigenic/allergenic activity: the more peptides of molecular weight above 1200, the less 'hypoallergenic' the preparation is considered. This cut-off is somewhat arbitrary and based on the assumption that peptides of lower molecular weight are not antigenic [18]. Such an assumption, however, is questionable as it is well known that peptides composed of as few as 5–10 aminoacids can react with specific antibodies [19]. Furthermore, many hydrolysates contain peptides with a molecular weight of more than 1200 [14,20]. Thus, the availability of a polyclonal antiserum directed

against cow milk proteins, which allows identification of epitopes with potential antigenic/allergenic activity, may be more suitable for the quality control of hypoallergenic formulas, than evaluation of their molecular weight profile.

A potential advantage of monoclonal over polyclonal antibodies resides in their selective specificity for a given epitope. Monoclonal antibodies directed against the antigenic determinants also recognized by serum IgE antibodies present in children with cow milk allergy could allow a new approach to the preparation of 'hypoallergenic' formulas. It is well known that some antigenic determinants within a protein are immunodominant compared with others and in predisposed subjects these could also act as the most allergenic. The availability of reagents like monoclonal antibodies specific for such determinants could permit their selective removal or modification and thus the preparation of safer and probably also more palatable hypoallergenic formulas.

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