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Enzyme Immunoassay of Specific Human IgE to Purified Cows' Milk Allergens

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An immunometric enzyme immunoassay for specific immunoglobulin E (IgE) against five purified cows' milk allergens, β -lactoglobulin, α -lactalbumin, bovine serum albumin, lactoferrin and whole casein fraction, has been developed. Allergens were immobilized on microtitration plates. After incubations with sera from allergic patients, specific IgE bound to the plastic were detected using a monoclonal anti-human IgE antibody labelled with acetylcholinesterase. Quantitative determinations were made by comparison with a dose-response curve obtained under the same conditions with standard total IgE. A quantification limit of 0.08 IU ml⁻¹ can thus be obtained with a coefficient of variation of lower than 5%. This allows specific IgE determinations of clinical significance in sera from allergic patients at a dilution of at least 1/10 (i.e. in a few μ l of serum) with good precision and reproducibility. The determinations of specific IgE in sera from 11 patients allergic to cows' milk showed an excellent correlation of this assay with clinical data.

Keywords: IgE, cows' milk, casein, β -lactoglobulin, α -lactalbumin, lactoferrin, bovine serum albumin

INTRODUCTION

Cows' milk is one of the most common food allergens. In newborn or young children, incidence rates for milk allergy (i.e. immediate-type reaction symptoms) as high as 0.5-7.5% have been described by Stintzing and Zetterstrom (1979) and Gerrard *et al.* (1973) respectively. Most other studies (Jacobsson & Lindberg, 1979; Host & Halken, 1990) have described an average incidence of 2-3% in the whole population of babies aged less than 1 year. β -Lactoglobulin (β -Lg), the main protein of whey, has been considered the major allergen for many years (Bleumink, 1968; Koritz *et al.*, 1987) since it is absent from human milk and is quite resistant to attack by digestive enzymes (Miranda & Pelissier,

1983; Kella & Kinsella, 1988; Reddy *et al.*, 1988). Moreover, β -Lg has been shown to have the highest ability among milk proteins to cross the intestinal mucosa (Marcon-Genty *et al.*, 1989). It is now established that other milk proteins can also sensitize babies with a predisposed genetic background, and they may then elicit an IgE-mediated response (Freier *et al.*, 1970; Baldo, 1984; Otani, 1989; Kaiser, 1990). However, no complete overview of the IgE response to the main purified cows' milk proteins has yet been described in terms of either specificity or quantitative determination in a large population of allergic patients.

The aim of this study was to develop a specific, very sensitive enzyme immunoassay for IgE to four whey proteins, β -Lg, α -lactalbumin (α -Lac), bovine serum albumin (BSA) and lactoferrin (Lf), and to the whole casein fraction (Cas). Since only very small volumes of sera from allergic patients are generally available, the tests were to be sensitive enough to require only a few μ l of serum in order to study the diversity of human IgE responses to milk proteins.

MATERIALS AND METHODS

Solid-phase enzyme immunoassay (EIA) was performed using Titertek microtitration equipment which included an automatic plate washer (Microplate Washer 120), an automatic dispenser (Autodrop) and a spectrophotometer (Multiskan MCC) set at 414 nm, all from Titertek Flow (Helsinki, Finland). Ninety-six well microtitre plates were purchased from Nunc (Immunonunc I 96F; Roskilde, Denmark).

High-pressure liquid chromatography (HPLC) was performed using a Waters Associates (Milford, MA, USA) chromatograph, equipped with two M 510 pumps, a WISP 712 automatic injector and a M841 double-beam UV detector set at 220 nm. Fractions were collected with a model 202 fraction collector (Gilson, Middleton, WI, USA).

Reagents

Unless otherwise stated, all chemicals were of analytical grade and were obtained from Sigma (St Louis, MO, USA).

Human serum albumin (HSA) was obtained from Centre National de Transfusion Sanguine (Les Ulis, France); free fatty acids were removed on active charcoal at pH 3 according to Chen (1967). After dialysis against water, purified albumin was freeze-dried.

Human sera were obtained from children presenting clinical symptoms of allergy and/or positive RAST tests to milk. Their mean age was 11 months.

Anti-rabbit IgG monoclonal antibodies (MAbs) (173, Metreau *et al.*, 1987) and anti-human IgE MAbs (BS17, Grassi *et al.*, 1986) were labelled with acetylcholinesterase (AChE). Purification of this enzyme from the electric organs of the electric eel, *Electrophorus electricus*, preparation of the tetrameric form and the labelling of antibodies in the Fab' form were performed as previously described by Grassi *et al.* (1989).

Buffers and reagents used for EIA were prepared as follows:

- (1) coating buffer: 0.025 M-ethylene diamine tetraacetic acid (EDTA), disodium salt, pH 9.3, as described by Campbell *et al.* (1987);
- (2) EIA buffer: 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.1% HSA, 0.4 M-NaCl, 0.001 M-EDTA and 0.001% sodium azide;
- (3) washing buffer: 0.01 M-potassium phosphate buffer, pH 7.4, containing 0.05% Tween 20;
- (4) Ellman medium: 7.5×10^{-4} M-acetylthiocholine and 5×10^{-4} M-dithio-bis-nitrobenzoate in 0.1 M-potassium phosphate buffer, pH 7, according to Ellman *et al.* (1961).

Preparation of Purified Milk Proteins

As shown in the Figure 1, whey and caseins were prepared by isoelectric precipitation at

pH 4.6 (Rowland, 1938) from the milk of one single cow, homozygous for the variant B of β -Lg.

Milk acidification was carried out by using 1 M-HCl; Cas was immediately separated by centrifugation, then dissolved in water by adjusting the pH to 7.5 with 1 M-NaOH, and re-precipitated. This operation was repeated three times in order to avoid contamination by whey proteins.

β -Lg, α -Lac and BSA were separated from the whey fraction by precipitation with 6 M-HCl at pH 2 in the presence of 7% NaCl, according to Maillart and Ribadeau-Dumas (1988). After centrifugation at 8000 rpm for 20 min, β -Lg was recovered in the supernatant, while the pellet contained concentrated fractions of α -Lac and BSA. Most of the β -Lg trapped in the precipitate was removed by washing the precipitate with 6% NaCl at pH 2. β -Lg in the supernatant was concentrated by salting it out with increasing concentrations of NaCl. At a concentration of ca. 30% NaCl at pH 2, β -Lg was precipitated. The two pellets (i.e. containing the β -Lg fraction and the other whey proteins) were then dissolved in the minimum amount of water, dialyzed against water and freeze-dried. Each fraction was dissolved in 20 mM-Tris-HCl buffer, pH 7 (10 mg ml⁻¹), then purified by fast protein liquid chromatography (FPLC) (Pharmacia system, Uppsala, Sweden) on an anion exchange column (Mono Q HR5/5). The elution was achieved with a 30-min linear gradient from 0 to 0.35 M-NaCl in 20 mM-Tris-HCl buffer, pH 7, at a flow rate of 1 ml min⁻¹, according to a method slightly modified from Andrews *et al.* (1985). Detection was performed by measuring absorbance at 280 nm. Each peak was collected, dialyzed against water and freeze-dried.

Lf was prepared on CM-Sephadex from concentrated whey. The desorption was performed with a linear gradient of 0.2–0.5 M-NaCl in a 50 mM-Tris-HCl buffer, pH 8. The fraction obtained was purified on a G 200 Sephadex column and was eluted with the same buffer containing 0.5 M-NaCl, according to Law and Reiter (1977). The final purified fraction was dialyzed against water.

The different whey proteins thus prepared were identified by amino acid composition analysis. For each protein, a final purification step was performed using reverse-phase HPLC (RP-HPLC) on a 300 A C-4 Vydac (250 × 4.6 mm internal diameter) column (SFCC/Shandon, Eragny, France). All sample preparations were passed through Millipore filters (Millex HV, 0.45 μ m). Elution was performed with a 30-min linear gradient of 25–40% buffer B (acetonitrile – 0.04% trifluoroacetic acid [TFA]) in buffer A (H₂O–0.1% TFA) at a 1 ml min⁻¹ flow rate, followed by a second 30-min linear gradient of 40–50% with the same buffers.

The purity of Cas was checked by RP-HPLC on the same column. Elution was then performed with a 40-min linear gradient of 25–45% buffer B in buffer A at a 1 ml min⁻¹ flow rate. The absorbance was measured at 220 nm.

Further immunochemical purity controls were carried out to check potential β -Lg contamination in the different purified protein fractions (see below).

Enzyme Immunoassays

Direct EIA were used for the determination of specific IgE. These tests were classical immunometric EIA, in which purified proteins were immobilized; specific IgE from allergic sera bound to these coated proteins were detected by anti human IgE MAb labelled with AChE. When sufficient volumes were available, sera showing positive responses (i.e. containing IgE specific to the whey proteins and/or to casein), were also analyzed by indirect EIA competitive inhibition tests to confirm direct determinations.

Wells of microplates were filled with 200 μ l of a 5 μ g ml⁻¹ solution of each of the purified allergens in coating buffer, left overnight at room temperature, then washed extensively with washing buffer. Plates were saturated by adding 300 μ l of EIA buffer to each well, sealed and stored at 4°C. They could be used 8 h later or kept for at least 2 months.

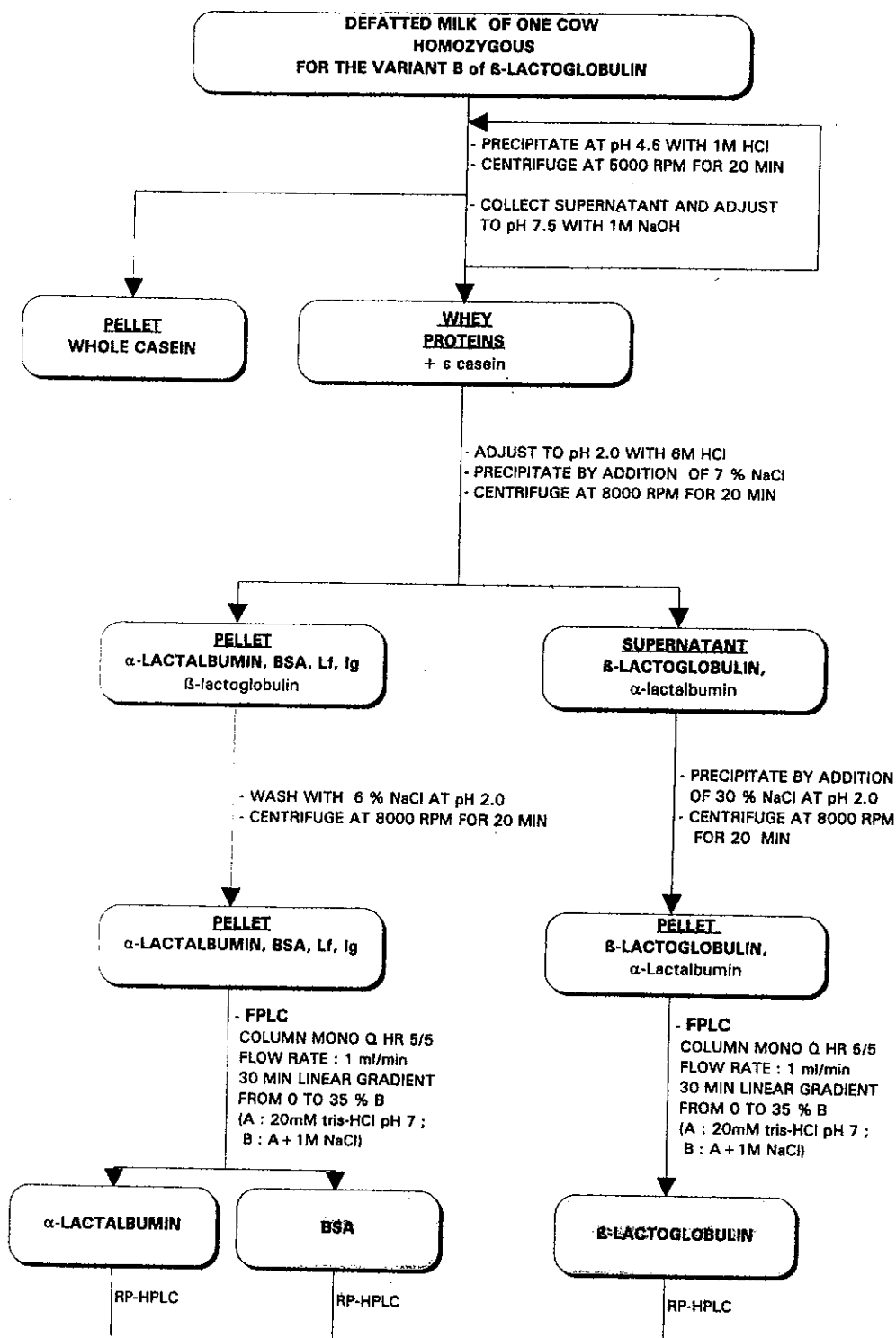


FIG. 1. Schematic procedure for the preparation and purification of allergens from milk from one cow homozygous for the variant B of β -Lg.

Just before use, plates were washed. For direct EIA of specific human IgE, 50 μ l of individual serum at convenient dilutions were distributed in each well, and left for 24 h at room temperature. Plates were then extensively washed, and 50 μ l of a solution of BS17-ACHe conjugate, at 3 Ellman units ml^{-1} , were added, and left incubating for 18 h at room temperature (1 Ellman unit is defined as the amount of enzyme inducing an increase of 1 absorbance unit during 1 min in 1 ml of Ellman's medium at 25°C, for a 1-cm path length. It corresponds to about 8 ng of enzyme). After washing, 200 μ l of Ellman's reagent were added and absorbance was measured at 414 nm after 1–3 h of enzymatic reaction. Negative controls were performed by using non-allergic sera on allergen-coated plates and some allergic sera on plates only saturated with HSA.

A similar protocol was used for the indirect competitive EIA, whether the immune serum was rabbit anti- β -Lg antiserum or a human allergic serum containing specific IgE. Immune sera (25 μ l at an appropriate dilution in EIA buffer) and inhibitors at increasing concentrations (25 μ l in EIA buffer) were first pre-incubated for 6 h at room temperature. The mixture was then delivered into antigen-coated wells. The further steps of the assay were then performed as previously described for direct EIA.

Most of the results were expressed in terms of absorbance units. However, for specific human IgE, quantification as IU (i.e. 2.4 ng) ml^{-1} was made by comparison with dose-response curves obtained with a total IgE assay performed with known standards of human IgE (Pharmacia Prist test kit), as described by Grassi *et al.* (1986). The corresponding immunometric assays of total and specific IgE were performed under identical conditions. An anti-human IgE MAb (clone LE27), complementary to the BS17-ACHe tracer, was used as capture antibody for the total IgE assay, instead of the immobilized, purified allergen preparation used for specific IgE determination.

RESULTS

Purified Allergens

Figure 2 shows the RP-HPLC chromatograms obtained from whey proteins and the casein fraction. β -Lg variant B, α -Lac, BSA and Lf each appeared as a unique peak. Cas was composed of four different molecules which were eluted in the order κ -, α S₂-, α S₁- and β -casein, as already described in similar chromatographic conditions by Visser (1991) and Strange (1991). The major caseins (i.e. α S₁-casein and β -casein) appeared as homogeneous (although not completely separated) peaks. Minor caseins α S₂-casein and, essentially, κ -casein, appeared to be more heterogeneous. The aim of this study was not to isolate each of the purified caseins, but to prepare 'pure' Cas, that is, a fraction free of whey proteins. Importantly, Figure 2(e) shows an absence of such contaminating peaks, particularly one corresponding to the major whey protein, β -Lg, which has a retention time of 36 min under these conditions. This chromatographic purity grade is in the range 97–98%, as spiking with 10% of another protein (e.g. β -Lg) gives a 4-cm high peak, while a 0.4-cm high peak can easily be detected.

Another way to check the absence of protein contamination by β -Lg was to use a specific competitive β -Lg immunoassay. Figure 3 shows the inhibition of the binding of specific rabbit anti β -Lg antibodies to immobilized β -Lg by increasing concentrations of the different purified milk protein fractions. The same 50% inhibition obtained with soluble β -Lg, was reached by adding 200 times more BSA and more than 500 times more α -Lac, Lf or Cas. Assuming that displacement was likely to be due to residual trace amounts of β -Lg in the protein preparations, these results demonstrated a level of contamination lower than 0.5 and 0.2% respectively.

Specific IgE Determinations in Allergic Patient Sera

Under the conditions of the assay, no false positives were observed with either control sera

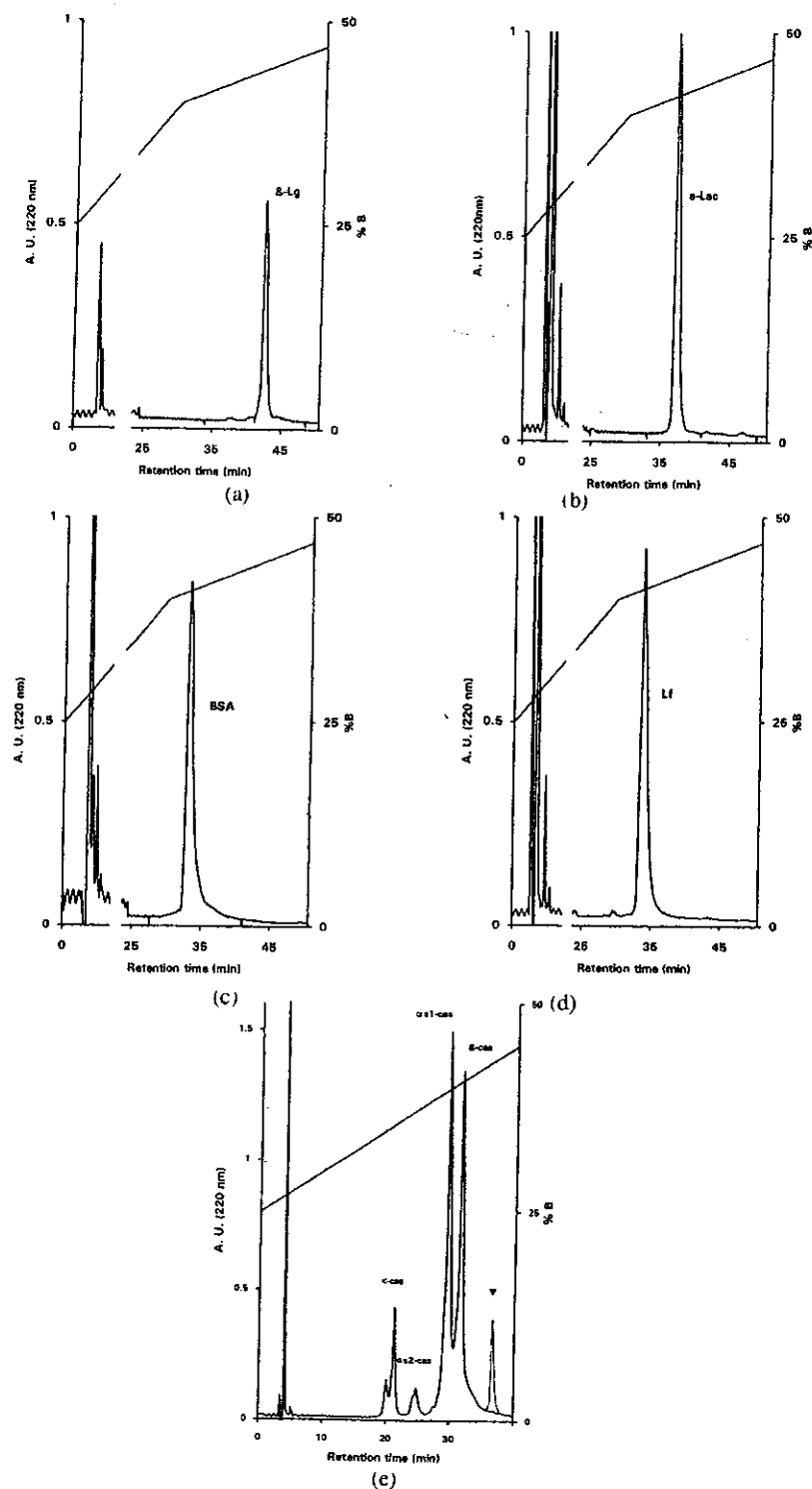


FIG. 2. RP-HPLC chromatograms of purified allergens: (a) β -Lg; (b) α -Lac; (c) BSA; (d) Lf; (e) Cas. The arrow in (e) indicates the elution of a spike of 10% β -Lg co-injected with the purified Cas. Without such spiking, no detectable peak can be observed at this retention time.

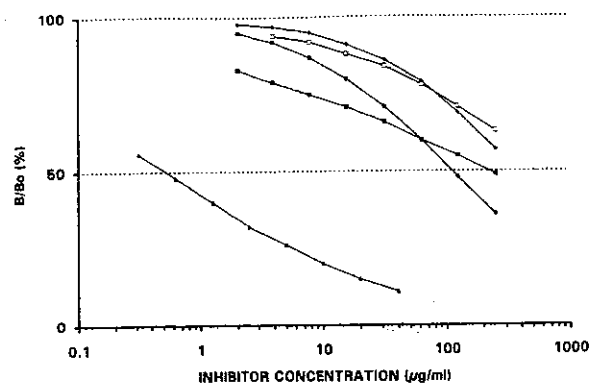


FIG. 3. Indirect EIA competitive inhibition tests. Increasing concentrations of purified proteins were pre-incubated with a specific rabbit anti- β -Lg antiserum in order to inhibit the binding of the anti- β -Lg antibodies to immobilized β -Lg. Fifty per cent inhibitory concentrations measure the immunological cross-reactivity and the contamination of purified fractions by residual β -Lg. ■, α -Lac; ●, BSA; ◆, Lf; ▲, β -Lg; □, Cas.

or allergic sera tested on plates coated with HSA. Specific IgE detection in human sera is possible at a concentration as low as 0.02 UI ml^{-1} . This detection limit is calculated as the dose corresponding to the average non-specific binding (absorbances measured with 12 control sera) plus $3 \times$ the standard deviation. The working range of the dose-response curve of total IgE extends from 0.02 to 10 IU of IgE, as shown in Figure 4. The precision profile, as determined on eight replicates/dose, is shown in the insert to Figure 4. A quantification limit of 0.08 IU ml^{-1} can thus be obtained with a coefficient of variation lower than 5%. This allows specific IgE determinations of clinical significance in allergic patient sera at a dilution of at least 1/10 (i.e. in a few μl of serum), with good precision and reproducibility.

The results of determinations of IgE specific to β -Lg, α -Lac, BSA, Lf and Cas in 11 different sera at various concentrations are reported on Table 1.

All the 11 sera tested were positive as judged by IgE response to at least one milk allergen. Three were monosensitive (Sera 1, 4 and 11), two were sensitive to two allergens (Sera 7 and 10), one was sensitive to three allergens (Serum 6), one to four allergens

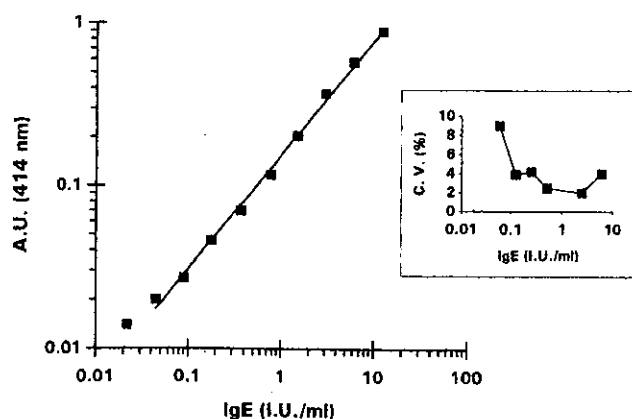


FIG. 4. Standard curve obtained with known concentrations of total standard IgE, and precision profile for different concentrations of specific anti- β -Lg IgE determined by comparison with the standard curve (eight replicates).

TABLE 1. Quantitative determinations of specific IgE to five purified cows' milk proteins in sera from 11 patients allergic to cows' milk

Serum no.	Specific IgE (IU ml ⁻¹)				
	Cas	β -Lg	α -Lac	BSA	Lf
1				88	
2	4.3	2	2.7	9	1.2
3	42	301	132	28	6.1
4					0.3
5	13	13	1.3	1.2	5.4
6	2.3	4.2			4.3
7		12		1	
8	29	40	14	15	2
9	71	21	9.4	26	
10				7.8	0.9
11		0.3			

(Serum 9) and four to all five purified allergens tested (Sera 2, 3, 5 and 8). All the purified proteins appeared to be potential allergens. Even a minor protein (in terms of concentration), such as Lf, present in milk in trace amounts (ca. 0.09 g l⁻¹), may sensitize atopic children. In serum 4 Lf was the only allergen recognized by IgE, despite its low specific titre. In addition, this test was the only one which correlated with clinical signs.

The results of indirect EIA-competitive inhibition tests on nine sera are presented in Figure 5. Increasing concentrations of the corresponding purified soluble allergens were pre-incubated with patient sera, and the remaining IgE binding capacity was tested with the same allergen preparation but coated on microtitration plates. Inhibition curves show the percentage of binding inhibition as a function of the soluble allergen added. In all cases, inhibition was observed, showing clear competition between soluble and immobilized allergen, indicating that the IgE antibodies were really specific to the purified immobilized proteins. The shapes of the curves obtained were clearly bimodal. A steep part was observed with low amounts of inhibitory soluble allergen, followed by a more or less flat plateau. In Figure 5(a), a concentration of 5 μ g ml⁻¹ of soluble β -Lg was able to inhibit 80% of IgE binding. At a concentration of 40 μ g ml⁻¹, a total inhibition was observed with all three sera tested, whatever their IgE titre was to β -Lg (301, 12 and 40 IU ml⁻¹ for sera 3, 7 and 8 respectively). Similar patterns were observed for Cas and Lf (Figure 5(b) and (e)), although inhibition appeared to be slightly lower. Total inhibition was possible for three sera, but only with higher concentrations of allergens. Inhibition patterns obtained for all six sera tested with BSA and α -Lac were clearly different. As shown in Figure 5(c) and (d), the first part of the curve, at low (less than 5 μ g ml⁻¹) allergen concentrations, reached only 50% of total inhibition. The second part of the curve was not a plateau, but straight lines with shallow slopes. At 40 μ g ml⁻¹ of soluble allergen, only 60–75% inhibition was observed.

DISCUSSION

Purified whey proteins, as analyzed by RP-HPLC, appeared as single, well-separated peaks. No contamination, by either other native proteins or degradation products, could be detected.

Cas was a mixture of single, pure isoforms of the different caseins (i.e. α S₁-, α S₂-, β - and κ -casein), with no contamination by whey proteins (particularly by β -Lg).

The chromatographic purity grade for these products was certainly better than 97%.

EIA inhibition curves (Figure 3) showed very low percentages of cross-reactivity,

displacement of the binding to immobilized β -Lg only being observed with high concentrations of inhibitors which might have no real significance. However, if these inhibitions were assumed to be specific then the amount of protein impurity such as β -Lg, the major milk allergen, was less than 0.2% and was most likely due to slight contaminations by residual traces of immunoreactive β -Lg in all purified allergens. Further observations on the specificity of the human IgE response (see Table 1) also confirmed this result: some sensitized patient sera appeared to be strictly monospecific, even to a minor protein. This indicated that antibodies did not recognize any contamination of the other allergens by this protein. As an example, serum 1 was strictly positive, with a very high IgE titre to BSA only. As there was no response with other allergens, it can be assumed that there was no consequent contamination of these proteins by BSA.

Specific IgE determination appeared to be sensitive and reproducible in a range of IgE concentrations of clinical significance, as shown in the curve and precision profile in Figure 4. No false positive results were observed with control sera from non-allergic humans. All 11 sera tested were from patients with clinical forms of allergy to cows' milk, and some of them had positive RAST tests to whole milk. All patients possessed IgE to at least one allergen, indicating that no false negative results occurred among these sera.

HSA was used as a saturating agent instead of the usual BSA as BSA was recognized as a milk allergen by eight out of the 11 patient sera. When sera positive to BSA were analyzed with HSA-coated plates, no significant binding occurred (i.e. there was no difference from the binding measured with sera negative to BSA, or with buffer instead of serum). As has already been reported for humans sensitized to BSA (Moneret-Vautrin *et al.*, 1991), the authors also concluded that with such sera, no significant immunological cross-reactivity was observed between HSA and BSA.

The aim of devising this EIA was to favour high sensitivity so that the lowest amounts of serum possible could be used. This is important as only small volumes of blood are available from young children. This immunometric EIA was devised with an excess of immobilized, purified allergen and an excess of AChE-labelled anti-human IgE MAb. Due to the high specific tracer activity, a saturating signal, in terms of absorbance, was obtained for relatively low IgE concentrations. As a consequence, the dynamic range of the assay was rather short compared with other classical, less sensitive assays. As a consequence, sample dilutions were required in order to quantify strongly positive sera.

A new capture assay for specific IgE has recently been proposed by Olivieri *et al.* (1993). It uses immobilized anti-human IgE MAb to capture serum IgE that are specifically detected by using biotinylated allergens, followed by streptavidin-peroxidase conjugate. In this case, too, the quantitative determination was made by comparison with a standard curve of total IgE incubated with a biotinylated anti-IgE MAb, complementary to the one employed as capture antibody. This assay has a wider dynamic range than the assay described in this study, with a linear response up to 50 IU IgE ml⁻¹. Its precision, expressed by the coefficients of variation, is similar to that reported here but the minimal detectable level, calculated in the same manner (mean background plus three standard deviations) is much higher: 0.15 IU IgE ml⁻¹ versus 0.02 IU. This increased sensitivity is very important for studying IgE specificity in food allergy, particularly in milk allergy in babies, since, as already discussed, it makes serum sample dilution possible and several assays can be performed on very small blood sample volumes.

The assay described in this study shows an excellent correlation with clinical data. Two sera (4 and 11), obtained from patients presenting allergic symptoms but having a negative RAST test result to whole milk, were found to be weakly positive (0.3 IU IgE ml⁻¹ to Lf and β -Lg) respectively. This demonstrated both the high sensitivity of the assay and the clinical relevance of the data thus obtained. Moreover, by using diluted patient sera, it might also be possible to avoid the interaction of potential blocking antibodies (i.e. non-IgE antibodies directed to the allergens). By using long reaction times (24 h for direct binding of patient serum to the immobilized allergen, 6 h for the pre-incubation of serum with

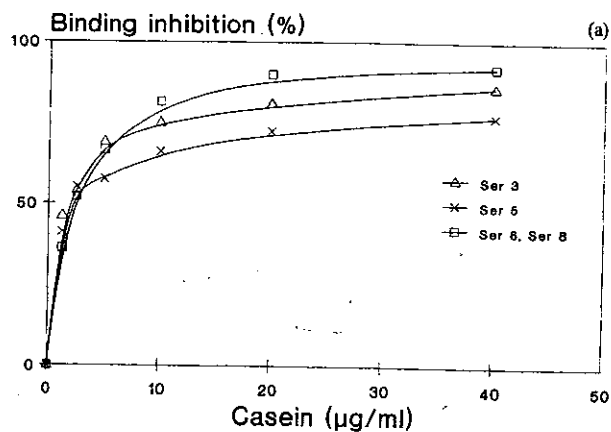
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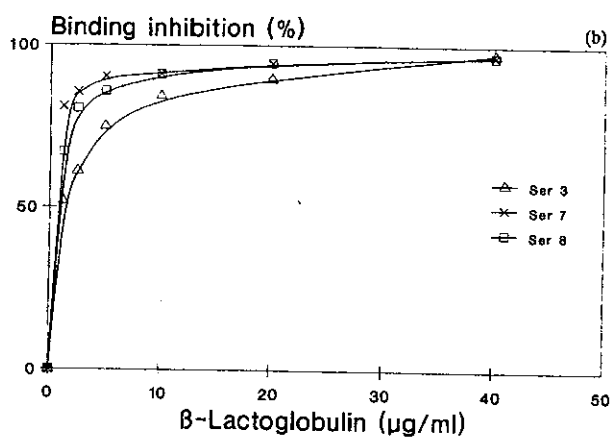
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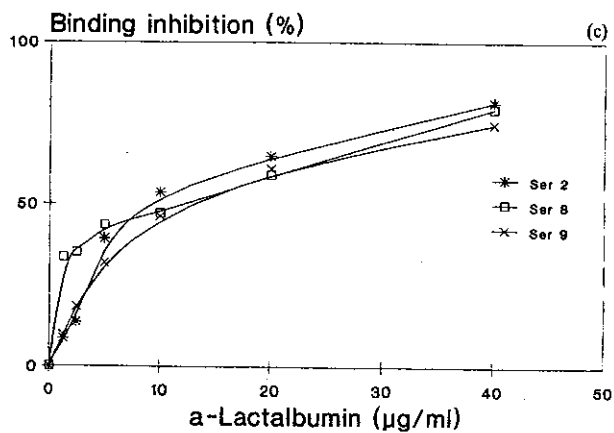
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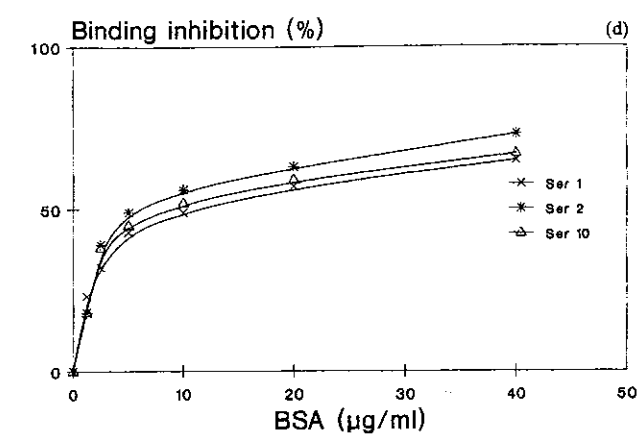
(a)



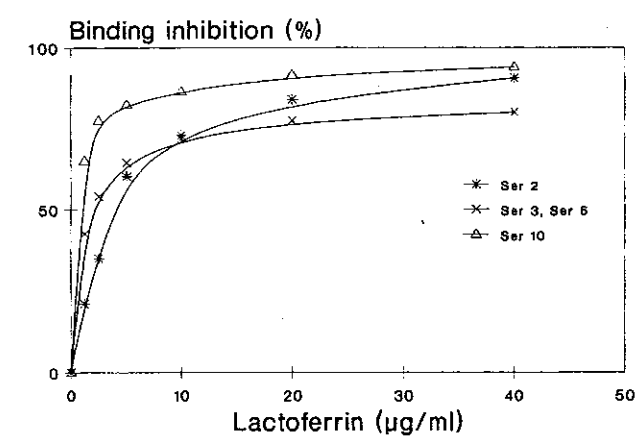
(b)



(c)



(d)



(e)

FIG. 5. Indirect EIA competitive inhibition tests. Human sera were pre-incubated with increasing concentrations of soluble, purified allergens in order to measure the inhibition of the specific IgE binding to the corresponding immobilized allergen. (a) Cas; (b) β -Lg; (c) α -Lac; (d) BSA; (e) Lf.

soluble allergens in the competitive inhibition test) and high amounts of immobilized purified allergens, a total fixation of specific IgE is expected. This is necessary to allow quantitative determination using total IgE standard as a reference (Grassi *et al.*, 1986). In indirect, competitive ELISA tests (Figure 5), at least 50% of the IgE binding to immobilized allergens is inhibited by addition of low concentrations of soluble proteins, thus demonstrating the homology between the epitopes recognized. In contrast, the remaining IgE binding to immobilized allergens needed high concentrations of soluble antigens to be displaced. This is particularly true with BSA and α -Lac, the less 'inhibitible' allergens, for which large excesses of soluble allergens are necessary to obtain only a partial complementary inhibition. This phenomenon suggests the appearance of modified epitopes during antigen interactions with the solid phase. Interestingly, the immobilized forms were strongly recognized by allergic human IgE antibodies, suggesting that subsets of IgE were essentially directed to epitopes specifically induced by the allergen binding to a plastic surface, and poorly represented in native proteins in solution. Immobilization on plastic appears to make epitopes accessible that are hindered in native soluble molecule. These epitopes could possibly be similar to those presented by partially degraded proteins with which the immune system of allergic patients was in contact. This result may also reflect a higher heterogeneity or changes in structure and conformation of BSA and α -Lac, depending on the environment (e.g. pH, presence of non-covalent ligands on albumin) and thus in their recognition by antibodies compared with β -Lg, Lf and even Cas. Obviously, these results also clearly showed that the specific IgE populations directed to the purified milk allergens were highly heterogeneous in fine specificity at the epitope level and in avidity, since some of the antibodies recognized the native form of the allergen while other populations were tightly bound to modified epitopes. In conclusion, the present immunometric EIA of IgE to purified milk proteins/allergens has a good precision and specificity. As for most of the available tests, the quantitative determination is made by comparison with a standard curve of total IgE captured by an anti-human IgE MAb complementary to the labelled one. The good reproducibility of this test permitted valuable comparisons in the IgE responses between sera and/or between allergens. This was most relevant for clinical significance. Finally, the high sensitivity of this test, requiring only very few μ l of diluted sera, has already permitted initial explorations of the fine specificity of individual human IgE responses.

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