

Human IgE-binding synthetic peptides of bovine β -lactoglobulin and α -lactalbumin. *In vitro* cross-reactivity of the allergens

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Summary The allergenicity of cow's milk whey proteins, purified by high performance liquid chromatography (HPLC), was examined by the radio-allergosorbent test (RAST) against the sera of children immediately hypersensitive to milk. β -lactoglobulin and α -lactalbumin bound specific IgE in the sera of 63% and 75% of these patients respectively. These allergens were tested for cross reactivity with each other by RAST inhibition. Both inhibited the binding of IgE, in the sera of allergic patients, to the other protein. Two possible determinant peptides, one from β -lactoglobulin and one from α -lactalbumin, were selected by computer prediction of antigenic sites and synthesized by the fluorenylmethoxycarbonyl (Fmoc)-polyamide method. The peptides were adsorbed to nitrocellulose discs and used in further RAST studies with sera from the allergic children. Both peptides bound specific IgE in the RAST assay.

INTRODUCTION

Cow's milk allergy affects about 2% of infants, although higher incidences have been reported (1). Clinical manifestations of cow's milk allergy include anaphylaxis, vomiting, diarrhoea, asthma, hives, angioedema, eczema, irritability and severe failure to thrive. It is assumed that immunological hypersensitivity (allergy) to milk protein antigens is the basis for these adverse reactions (2). Studies on the allergenicity of milk proteins by Crawford and Grogan showed that the most important allergenic fractions by serum-agar precipitation were α -casein, β -lactoglobulin and α -lactalbumin (3). Whilst the milk of all mammals contains α -lactalbumin, there is no counterpart in human milk for β -lactoglobulin and it is therefore not surprising that it is an allergen. The amino acid sequence of bovine α -lactalbumin has about 76% homology with human α -lactalbumin, which would suggest that it is unlikely to be an allergen (4). Baldo, however, reported that in subjects with true milk allergy,

the order of decreasing allergenic activity of the individual proteins was β -lactoglobulin, α -lactalbumin, caseins and bovine serum albumin (BSA) (5). The milk proteins used in that study were not isolated by a method capable of giving a high degree of purity, such as high performance liquid chromatography (HPLC). Several researchers have reported binding of specific IgE to peptides resulting from peptic-tryptic (6) and cyanogen bromide (CNBr) (7) digests of β -lactoglobulin. Huang *et al.* demonstrated with enzyme linked immunosorbent assay (ELISA) inhibition assays that certain tryptic and cyanogen bromide cleavage fragments were able to inhibit competitively the binding of rat β -lactoglobulin-specific IgE to bovine β -lactoglobulin (7). They also found that reductive cleavage of the intrachain disulfide bonds within β -lactoglobulin had little effect on its binding to rat IgE but eliminated the reactivity of both native β -lactoglobulin and its tryptic cleavage fragments with rabbit IgG antibody. It was demonstrated that it is possible to elicit reaginic binding of human

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Abbreviations used in this paper: BSA, bovine serum albumin; CNBr, cyanogen bromide; ELISA, enzyme linked immunosorbent assay; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline (pH 7.4); RAST, radio-allergosorbent test.

IgE to synthetic peptide fragments of codfish allergen M (8) and ragweed allergen Ra3 (9).

In this report we describe an investigation of the *in vitro* allergenicity and allergenic cross-reactivity of HPLC-purified β -lactoglobulin and α -lactalbumin. One putative allergenic peptide from each protein sequence, chosen by computer prediction of IgG-binding sites (10), was synthesized and tested for allergenicity by direct RAST assay.

MATERIALS AND METHODS

Sera

Serum samples were provided by the Allergy Clinic, Royal Children's Hospital, Melbourne and by Dr R. S. Baker, Sydney. Forty serum samples were screened for allergenic activity to defatted bovine milk using RAST. All 40 patients were children with a recorded history of an allergic reaction to ingestion of cow's milk. As most of the patients were young children, only small serum samples could be obtained. This meant that sera from different sets of patients had to be used for the various parts of this investigation (e.g. Fig. 1 and Tables 3,4,5). These individuals were found to be clinically milk sensitive by skin prick test and/or oral challenge. Table 1 gives details of clinical histories for six of the typical patients. Following preliminary RAST, a group of 12 sera with IgE apparently specific to α -lactalbumin and β -lactoglobulin were selected for further examination. Fetal cord serum and an allergic (but non-milk allergic) serum were used as negative controls.

Allergen purification

Crude β -lactoglobulin and α -lactalbumin (Sigma Chemical Company, St Louis, MO, USA) were purified by HPLC on an anion exchange column

(TSK-DEAE-5PW from LKB, Sweden). The proteins were chromatographed separately and both were eluted with a linear gradient of 0 (pH 9.0)–0.3 mol/L (pH 7.0) NaCl in 0.2 mol/L Tris-HCl. The purity of the eluted material was checked by reverse-phase HPLC on a Waters Associates microbondpak phenyl column. The proteins were eluted with a linear gradient of acetonitrile, 0–60% (v/v), in 0.05% v/v trifluoroacetic acid/water.

Polyacrylamide gel electrophoresis

Electrophoresis was performed using a discontinuous system of 12.5% sodium dodecyl sulfate-polyacrylamide gel in the presence of 2-mercaptoethanol as described by Laemmli (11). Proteins and molecular weight standards were stained with Coomassie Blue.

Prediction of antigenic determinants

Using a Hewlett Packard HP-85 computer, the amino acid sequences of α -lactalbumin and β -lactoglobulin were entered separately into the programs of Hopp and Woods (10) and Welling *et al.* (12). The prediction method of Hopp and Woods is based on calculated estimates of local hydrophilicity along the polypeptide chain and the assumption that hydrophilic regions are predominantly surface orientated and therefore potentially antigenic. More recently it has been shown that not all antigenic regions are hydrophilic and that not all hydrophilic regions are antigenic (12). Therefore, we also used the predictive method of Welling *et al.* (12) which is based upon empirical data derived from the frequency of occurrence of each amino acid in 20 known antigenic determinants.

Table 1. Clinical symptoms for six milk allergic children.

Patient code	Sex	Age (months)	Symptoms on ingestion of milk
Shi	M	24	vomiting, coughing, laryngeal oedema
Mac	M	14	circumoral urticaria, wheezing
Zek	M	13	flush, coughing, facial urticaria
Low	F	5	angioedema, circumoral eczema
All	F	14	urticaria, eczema
Pae	M	29	circumoral urticaria, pallor, abdominal pain, wheezing, rhinitis

Sera from these six patients were also used in obtaining some of the results in Figure 1.

Synthesis and purification of peptides

Peptides were synthesized by the Fmoc solid-phase polyamide method outlined by Sheppard (13). The resin used (Pepsyn KA 0.087 mequiv/g) and the Fmoc derivatives were supplied by Cambridge Research Biochemicals. Solid phase synthesis of the peptides was performed using the manual technique of Wade (14). An extra alanine residue was incorporated at the carboxy terminal of each peptide in order to increase the yield of coupling of the first amino acid of the required sequence. Alanine couples in high yield to the resin. We did not expect the extra alanine residue to affect the allergenicity of the peptide. Following cleavage from the resin, the peptides were desalted and purified by gel filtration on Biogel P4 (BioRad Laboratories, CA, USA). Peptide amino acid content was checked by amino acid analysis of their acid hydrolysates. Table 2 gives the amino acid sequence of each of the synthetic peptides as determined directly with a model 470 automatic gas-phase polypeptide sequencer (Applied Biosystems Inc., Foster City, CA, USA), essentially by the methods of Hewick *et al.* (15).

RAST

Paper discs The proteins were coupled to Whatman filter paper discs grade 44 (6 mm) using CNBr as described by Ceska *et al.* (16). Each disc was exposed to the equivalent of 100 μ g of protein. Standard RAST procedure followed the manufacturer's instructions (Kallestad Laboratories Inc., USA). Total counts of labelled anti-IgE were measured and were approximately 10 000 ct/min, equal to 100% of total radioactive uptake. All such percentages reported were the means of two replicates of duplicate determinations. Radioactive uptakes of 2% or less were considered to be negative.

Preparation of nitrocellulose discs Nitrocellulose discs (6 mm) were prepared from 0.1 μ m pore size nitrocellulose sheet (Schleicher and Shuell, Germany) with an office punch. The milk allergens and the synthetic peptides were dissolved at a concentration of 10 mg/mL in 1% w/v KOH in phosphate buffered saline (PBS) (50 mmol/L of sodium phosphate, pH 7.2 and 0.9% w/v NaCl) and applied to each of the discs. They were allowed to dry for at least 1 h and were blocked in 0.1% v/v Tween in PBS for a further hour. They were then washed three times (10 min each) in 2.5 mL of TAPS buffer (0.5% Tween 20, 0.05% BSA in PBS). The RAST procedure was carried out as described by Walsh *et al.* (17).

RAST inhibition Inhibition assays were performed as described by Walsh *et al.* (17).

RESULTS

HPLC of allergens

The major peak of each allergen eluted in the ion-exchange HPLC chromatogram was shown to be homogeneous in subsequent reverse-phase HPLC (results not shown). Only material corresponding to the major peak was used in subsequent assays. Polyacrylamide gel electrophoresis showed the HPLC-purified proteins as single bands at M_r 18 000 and 14 000 for β -lactoglobulin and α -lactalbumin respectively (18).

RAST and RAST inhibition

Figure 1 shows the results of RAST using sera from 12 patients carried out on HPLC-purified β -lactoglobulin and α -lactalbumin coupled to paper discs. The sera of two patients had little or no response to either allergen, four had reactivity against only one of the allergens whilst the

Table 2. Primary structures of the synthetic peptides of bovine β -lactoglobulin and α -lactalbumin.

Protein	Peptide*	Amino acid sequence**
β -lactoglobulin	41-52	H ₂ N-Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Ala-CO ₂ H
β -lactoglobulin	91-100	H ₂ N-Lys-Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys-Ala-CO ₂ H
β -lactoglobulin	124-134	H ₂ N-Arg-Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Ala-CO ₂ H
β -lactoglobulin	130-139	H ₂ N-Asp-Glu-Ala-Leu-Glu-Lys-Phe-Asp-Lys-Ala-CO ₂ H
α -lactalbumin	5-18	H ₂ N-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-Leu-Lys-Gly-Tyr-Ala-CO ₂ H

*Numbers refer to the positions of the residues in the protein sequences.

**The extra alanine residue at the carboxy-terminals was incorporated in order to increase the yield of coupling of the first amino acid of the required sequence.

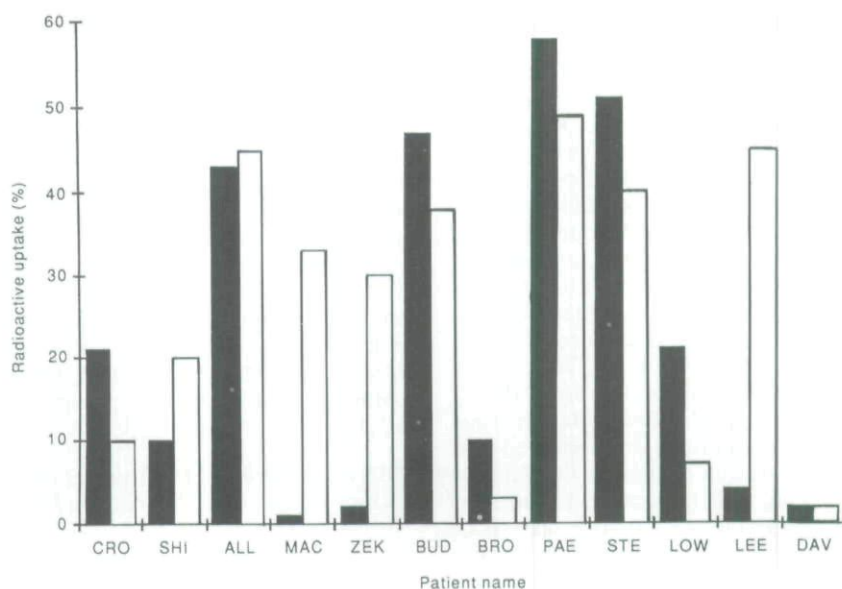


Fig. 1. Binding of bovine α -lactalbumin (□) and β -lactoglobulin (■) to specific IgE in the sera of 12 children hypersensitive to cow's milk.

sera of six patients had IgE against both β -lactoglobulin and α -lactalbumin.

In Table 3 results are given showing the ability of α -lactalbumin to inhibit the binding of IgE to β -lactoglobulin in six out of 10 sera. The results indicate that, at a level of 100 μ g, α -lactalbumin was able to inhibit the RAST reaction with β -lactoglobulin by as much as 83%. Similar results were found when IgE binding to α -lactalbumin was inhibited by β -lactoglobulin (Table 4). Control sera were negative in the RAST.

Allergenicity of peptides bound to nitrocellulose

The results in Table 5 show that the synthetic peptide 124-134 from β -lactoglobulin bound human specific IgE. The synthetic peptide 5-18 from α -lactalbumin also bound IgE from human sera. The three other peptides (Table 2) from β -lactoglobulin failed to bind IgE in any of the sera (data not shown).

Table 3. RAST inhibition of the binding of serum IgE to β -lactoglobulin by pre-incubation with 100 μ g of α -lactalbumin.

Serum	Radioactive uptake		RAST Inhibition (%)
	Uninhibited (%)	Inhibited (%)	
Web	13	6	54
Ken	32	31	0
Sle	25	23	0
Nic	22	21	0
Spe	12	2	83
Kit	27	7	74
Dal	13	7	46
Dic	17	17	0
Zen	24	12	50
Shi	9	6	33

Table 4. RAST inhibition of the binding of serum IgE to α -lactalbumin by pre-incubation with 100 μ g of β -lactoglobulin.

Serum	Radioactive uptake		RAST Inhibition (%)
	Uninhibited (%)	Inhibited (%)	
Web	11	7	36
Ken	21	6	71
Sle	11	2	82
Nic	23	12	48
Spe	9	3	27
Kit	13	14	0
Dal	17	17	0
Dic	4	2	0
Zen	33	15	55
Shi	14	4	71

Table 5. Radioactive uptakes of synthetic peptides and parent allergens by RAST assay.

Serum*	Uptake β -lactoglobulin (%)	Uptake Peptide 124-134 (%)	Ratio of uptakes of peptide to parent allergen (%)
	α -lactalbumin	peptide 5-18	
Wri	36	22	61
Nic	32	8	25
Tys	21	7	33
Hosk	27	10	37

100 μ g of parent allergen and synthetic peptides were exposed to the respective nitrocellulose discs.

*Sera were chosen which had high RAST uptakes for the parent allergens and of which there were sufficient quantities for the tests. Serum Nic also appears in Tables 3 and 4.

DISCUSSION

The results of this study show unambiguously that both β -lactoglobulin and α -lactalbumin, purified by HPLC, bind specific IgE present in the sera of individuals hypersensitive to cow's milk. The RAST results show that some of the sera had a high score ($> 20\%$) to α -lactalbumin but a low score to β -lactoglobulin. Where RAST results were low for both β -lactoglobulin and α -lactalbumin, it is possible that the patient was allergic to other milk proteins, for example, one of the caseins.

It is interesting that whilst cross-reactivity was found for β -lactoglobulin and α -lactalbumin over a range of sera for 10 patients, individual serum samples did not always show this in both directions. For example (Tables 3,4), serum Ken for which β -lactoglobulin inhibited the binding of IgE to α -lactalbumin but α -lactalbumin did

not inhibit the reaction of IgE with β -lactoglobulin. On the other hand for sera Kit and Dal α -lactalbumin inhibited the binding of IgE to β -lactoglobulin, but β -lactoglobulin did not do so in the reaction with α -lactalbumin. Similar observations were made by Walsh *et al.* in a study of cross-reactivity between different egg proteins (18). It is not known what is the cause(s) of this lack of reciprocity of inhibition of IgE binding by the two bovine allergens. It may be due to the involvement of conformational (discontinuous) epitopes in binding IgE, or the results may be due to the polyclonal population of IgE antibodies and to the unknown distribution of these in terms of their binding to each allergen. The high degree of cross-reactivity between two milk allergens is surprising in view of the fact that a computer search of sequence homologies between β -lactoglobulin and α -lactalbumin found only four regions where both proteins shared a common sequence of three amino acids.

Tokita showed that there appeared to be two major CNBr-peptides from β -lactoglobulin which exhibited antigenic activity (19). These were those of residues 41–61 and 125–145. Based on the findings of Elsayed and Apold (8) for codfish allergen M and Atassi and Atassi (9) for ragweed allergen Ra3, that IgE-binding determinants are also likely to bind IgG, we synthesized the peptides selected as being the most likely antigenic sites from the computer prediction programs. Mapping of hydrophilic regions in β -lactoglobulin showed that two out of three of the largest peaks were centred on residues 124 and 134. Mapping of the antigenic profile showed the major peak to be located at residues 124–134, consistent with the hydrophilic profile, and being included in a region which had been shown to exhibit antigenic activity by Tokita (19). As shown in Table 5, the serum sample Wri showed an antibody binding capacity to the peptide 124–134 which was 61% of the binding to whole β -lactoglobulin. The ability of IgE in human sera to bind specifically to synthetic peptides of allergens has been previously demonstrated. Using pooled sera, Elsayed and Apold were able to show that a synthetic peptide of codfish allergen M inhibited the binding of codfish-specific human IgE by as much as 67% (8). Atassi and Atassi successfully demonstrated, using RAST, that some of the peptides of ragweed allergen Ra3 exhibited considerable (e.g. 30% relative to Ra3) IgE-binding capacity (9).

Only the peptide regions of bovine α -lactalbumin which are not homologous with the human protein would be likely locations for allergenic determinants. Only relatively small fragments of the bovine protein are eligible for allergenicity as there is a high degree of homology between these proteins. Computer mapping using both antigenic and hydrophilic profiles of bovine α -lactalbumin showed that a major peak centred on residues 5–15, a region that also has the greatest sequence difference between the bovine and human proteins. Interestingly, for human α -lactalbumin, this region has a lower peak in both profiles than bovine α -lactalbumin. Hopp and Woods also identified residues 5–15 in bovine α -lactalbumin as being antigenic (20). Our study showed that peptide 5–18 from bovine α -lactalbumin indeed exhibited a high capacity to bind IgE in the sera of two allergic patients (Table 5).

The binding capacities of both the above peptides were considerable despite the view that peptides will have predominantly random conformations and thus possibly diminished affin-

ities for antibodies generated by the parent proteins (9). Furthermore, a portion of a peptide on the immunosorbent may be expected to have been rendered inactive due to attachment of the peptide via side chains that may also be essential for binding to the antibody. Such immunosorbent binding may also contribute to steric inaccessibility.

Although not exhaustive, this study revealed that for two major milk allergens the human IgE-binding epitopes appear to occupy continuous segments of the protein chain. In a study by Haddad *et al.* treatment of β -lactoglobulin with pepsin and pepsin-trypsin, under conditions simulating *in vivo* digestion, resulted in breakdown products which apparently bound specific IgE (6). Further synthetic studies of peptides overlapping the predicted regions will be required to define precise epitope boundaries for bovine α -lactalbumin and β -lactoglobulin. The approach reported here may be valid for a broader spectrum of allergens and, if so, will accelerate our understanding of the nature of allergenic epitopes and their interaction with the immune system.

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