

A major continuous allergenic epitope of bovine β -lactoglobulin recognized by human IgE binding

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Summary

Hexapeptides of sequential overlapping sequences of β -lactoglobulin (BLG) were used to probe serum from children with immediate-type cow milk allergy for IgE binding to continuous epitopes of BLG in an enhanced enzyme-linked immunosorbent assay (ELISA). Six regions of IgE binding were identified on the BLG molecule and these were synthesized as dodecapeptides. Inhibition of IgE binding to whole BLG was used to confirm the BLG-specific binding of IgE to each of the synthesized peptides. One of the peptides, peptide 4, showed inhibition in an IgE anti-BLG radioimmunoassay to all 16 sera tested. The patterns of inhibition with the native BLG molecule and peptide 4 were significantly correlated ($P = 0.005$), suggesting that this peptide contains a major continuous IgE binding epitope of BLG.

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Introduction

Cow milk allergy (CMA) is a common problem in paediatrics affecting between 2 and 7.5% of infants [1–3]. Previous studies from this laboratory identified three groups of patients with CMA and described their immunological features [4]. One of these groups, infants with immediate cow milk allergy (group 1), have elevated circulating IgE antibodies to cow milk [5], and a high positive skin-prick test reaction to whole cow milk extract [6]. Adverse clinical reactions occur within 1 h and often within a few minutes of ingestion of relatively small volumes of cow milk in sensitized individuals.

Cow milk contains more than 25 proteins, some of which are known to be allergenic [7]. While casein is the most abundant protein in cow milk, β -lactoglobulin (BLG) is regarded as the dominant allergen [8,9]. BLG is a polypeptide which usually exists as a dimer (relative molecular mass (M_r) = 36 000) though at low pH it tends to form monomers of M_r = 18 000 (162 amino acids).

BLG is present in the milk of a range of species within which there are genetic variants (e.g. A, B and C), [10,11]. It is the only major cow milk protein type not produced by humans [9]. However, human milk can contain small amounts of bovine BLG from dietary intake [12,13].

The allergenicity of BLG may lie in its relative ability to resist acid digestion enabling some of the protein to remain intact after passing through the stomach [9]. This would increase the probability of intact BLG as well as digested fragments being absorbed as antigens, which in turn could lead to the generation of IgE antibodies to the intact BLG molecule and the digests. The amino acid sequence against which antibodies are generated is called the antigenic determinant or epitope. Epitopes can be of two types—'continuous' where the amino acids are sequential in the protein chain or 'conformational' where several amino acids are in close proximity due to the three dimensional structure of the protein.

A number of techniques have been employed to identify epitopes. Early investigations of continuous epitopes utilizing protein disruption methods, demonstrated that antibody binding to the fragments of BLG [14,15] and silk [16] still occurred. More recently, this has been supplemented by isolation and amino acid

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sequencing of the fragments to which the antibody bound. An early study of human IgE binding epitopes using these techniques was of cod fish protein [17]. These investigations led to the identification of an IgE binding peptide, allergen M, [18] which has been used for skin-prick testing in the investigation of cod fish allergy.

Early studies of the allergenic epitopes of BLG used similar methods of protein disruption [14,15] but without amino acid sequencing of the epitope containing-fragment. These studies suggested that some of the IgE binding to the BLG molecule may be to continuous rather than conformational epitopes. A computer algorithm has also been used to predict epitopes of BLG [19]. This algorithm is based firstly on the assumption that hydrophilic amino acids will tend to be located on the surface of the protein and therefore potentially antigenic, and secondly on the frequency with which certain amino acids occur in comparison with other known epitopes. From these studies potential epitopes were identified.

In this study a variation on a recently described technique [20,21] has been utilized whereby overlapping sequential hexapeptides of an antigen are synthesized on a solid support, and antibody binding to the peptides scanned using an ELISA system. From the scans specific IgE binding to the peptides of BLG have been identified. Dodecapeptides representing the areas of binding were synthesized and epitopes verified by their ability to prevent binding of IgE in the sera of cow milk allergic patients to native BLG [22].

Materials and methods

Radioimmunoassay of IgE binding

Allergic specific IgE antibodies to BLG were measured using a radioimmunoassay (RIA). [^{125} I] anti-IgE and washing solution was obtained from Pharmacia, Uppsala, Sweden (Phadebas RAST isotope and washing reagents). Standards were obtained from Kallestad Diagnostics (Chaska, MN 55318 USA). The RIA scoring scale used ranged from 0.18 to 17.5 Allercoat East Units (AEU)/ml. The BLG allergen discs were prepared by the method of Ceska *et al.* 1972 [23], using purified BLG (Sigma, Chemical Co., St Louis, USA).

Briefly, 50 μ l of the relevant serum was added to the allergen or reference discs in duplicate, and incubated at room temperature for 3 h with agitation. The tubes were then washed three times for 10 min with 2.5 ml of washing solution. Fifty microlitres of [^{125} I] anti-IgE solution was added to each tube and incubated overnight. The samples were washed three times as before, then counted on a gamma counter. The standard curve

was plotted and the results for the test samples read off the curve in AEU/ml.

Epitope scanning

Preparation of pins. The epitope scanning kits were obtained from Chiron Minotopes (Clayton, Victoria, Australia) and were used according to the manufacturer's instructions (Epitope Scanning Kit-Chiron Minotopes). Briefly, in this method the amino acids used have their α -amino group protected with the 9-fluorenylmethyloxycarbonyl (Fmoc) group. The surface of polyethylene pins are chemically modified to support a 'linker assembly' so that amino acids can be synthesized on the surface of the pin using the Fmoc chemistry. Repetitive cycling of the Fmoc-deprotection method enables the sequential addition of one amino acid at a time, with the terminal amino group being capped by acetylation [24]. As a control, additional hexapeptides were synthesized on additional pins, stripped off after completion, then analysed to confirm amino acid composition.

The A genetic variant of BLG was represented on these polypropylene pins as hexapeptides of overlapping sequences. Thus, starting from the N-terminal end, the first six amino-acids were sequentially coupled to the first pin. Attached to the second pin were the last four amino-acids of the previous pin followed by the next two amino-acids in the sequence, and so on. The set of peptides on the pins was then used to probe sera which contained IgE antibodies to BLG and control sera in an enhanced ELISA system.

Enhanced IgE ELISA. Binding of IgE to the hexapeptides on the pins was measured using a modification of the method of Walsh and Howden [20]. Briefly, incubation of the reagents with the pins was performed in flat-bottomed microtitre plates (Immunoplate, Maxisorp, Nunc, Roskilde, Denmark). Before use, the plates and pins were blocked with 4% horse serum (300 μ l/well) (CSL, Melbourne, Australia) at 37°C for 1 h, then washed three times with phosphate buffered saline at pH 7.1 containing 0.05% Tween 20 (PBS/Tween) for 5 min each time, with agitation at room temperature. The pins were then incubated with patients' sera (diluted 1:10 with PBS, 200 μ l/well), for 2 h at 37°C. Incubation with goat anti-human IgE (200 μ l/well, 1:2000, Bio-Rad Laboratories, Richmond, California, USA) was performed overnight at room temperature. The pins were washed with PBS/Tween and incubated with biotinylated donkey anti-sheep/goat Immunoglobulin (200 μ l/well, 1:4000) (affinity purified, Amersham International, Amersham, UK) for 1 h at 37°C. The pins were then

washed and incubated for a further hour at 37°C with avidin peroxidase (200 µl/well, 1:400) (Type VI, Sigma). Each wash, between incubations involved submerging the pins in 250 ml of PBS/Tween solution for 10 min then repeating the cycle three further times with fresh PBS/Tween.

The enzyme substrate combination used was *o*-phenylenediamine (OPD, 0.05% w/v), and hydrogen peroxide (0.01% in 0.1 M sodium citrate buffer, pH 5.0) (150 µl/well). Reaction continued for 10 min in the dark and was stopped with 50 µl/well of 4 M sulphuric acid. The absorbance was then read at 492 nm. The results of epitope scans were produced as bar graphs of absorbance against pin number. The pins were re-used following stripping of antibody [21].

Patients

Sera used in this study were from 14 patients, eight boys and six girls aged between 4 and 115 months (mean 75 months), referred to the Department of Allergy, Royal Children's Hospital, Melbourne for investigation of CMA. All patients aged 5 years or older (nine patients) had CMA confirmed by challenge in the Department. They developed urticaria (nine patients), some with rhinorrhoea (one patient) and abdominal pain (two patients), within 5–45 min of ingestion of 0.5–30 ml of cow milk. Five had the diagnosis confirmed on the basis of a history of an immediate reaction and a skin-test reaction of $> +4$ [6]. All 14 had a history of severe milk reactions (nine had eczema and asthma, two had eczema without asthma, three had asthma without eczema. Four of the children had had anaphylactic episodes with cow milk). The serum IgE levels to BLG were assessed using a solid phase radioimmunoassay (RIA) [23].

Additional serum samples were available from two patients (sera nos; 3, 4 & 6, 7), one 6 months, the other 15 months after the original test. Thus, a total of 16 individual sera were tested. Fifteen serum samples from CMA positive patients (> 3.5 AEU/ml to cow milk) were pooled. This positive pool (13.3 AEU/ml to BLG) was used to optimize the epitope scanning technique. The negative controls consisted of umbilical cord serum and serum samples shown to have very low levels of IgE antibodies to BLG (< 0.18 AEU/ml).

Affinity chromatography

β -lactoglobulin (A&B Sigma) was bound to cyanogen bromide-activated Sepharose 4B (Pharmacia Uppsala, Sweden) according to the manufacturer's instructions. The beads were poured into a column (1 cm $\times$ 20 cm) washed with PBS and were used to remove BLG specific

antibodies from the positive pooled sera as follows: 1 ml of pooled sera was applied to the top of the column, washed through with 9 ml of PBS and collected for use in the epitope scan.

Identification of IgE binding sequences

Using the ELISA absorbance scans, IgE binding hexapeptide sequences were selected by three methods:

- 1 The manufacturers of the epitope scanning kit suggest estimating the mean and standard deviation for those values below the median absorbance of antibody binding to pins. Thus pins which bound more than the mean plus three standard deviations of this group, have been reported to be indicative of significant IgE binding [21].
- 2 A serum with no detectable IgE antibodies was scanned and the absorbances were subtracted from the scans obtained with sera containing high levels of IgE to BLG.
- 3 Epitope scans were performed before and after affinity absorption of BLG specific antibodies from the positive pooled sera. A profile was produced by subtracting the absorption of the post-column serum from the native serum.

Synthesis of possible epitopes

Dodecapeptides representing the putative epitopes as identified above were synthesized on polyamide resin by the automated Fmoc method, then purified by reverse phase high performance liquid chromatography. An extra alanine residue was incorporated at the carboxyl terminal of each of peptides 3, 4, 5 and 6 in order to increase the yield of coupling of the first amino acid of the required sequence to the resin. The sequence of the product was then confirmed on a gas-phase sequencer (Applied Biosystem, Burwood, Melbourne, Australia).

Inhibition of IgE binding

The inhibitory capacities of BLG and of the six polypeptides representing putative epitopes was assessed using the method of Gleich *et al.* [22]. Briefly, 50 µl aliquots of serum were incubated in duplicate with 50 µl of each of the six peptides and BLG, all at 1 mg/ml (test) and PBS (control), for 3 h at room temperature with agitation. The BLG coated discs were added, and incubated for 3 h at room temperature with agitation. The discs were washed three times with 2.5 ml of PBS/Tween for 10 min each wash. Fifty microlitres of [^{125} I]

anti-IgE solution were added to each tube and incubated overnight. The discs were washed as before, and the radioactivity of each disc counted. The standards prepared at the same time as the above were plotted and the results read off the standard curve as AEU/ml. The inhibition by BLG or peptide was calculated in AEU/ml as a percentage of the PBS result as follows:

$$\text{Per cent inhibition} = \frac{(\text{Control} - \text{test}) \times 100}{\text{Control}}$$

Results

Sixteen sera from 14 patients with levels of IgE antibodies to BLG of 9 AEU/ml or greater were used. Epitope scans were obtained from four of the sera and from the pooled serum. Each gave a unique profile, but there were common peaks of reactivity, not necessarily present in every scan. In total six peptides were considered for further testing (Fig. 1). Specific reactivity to BLG epitopes was difficult to identify in individual scans alone (method 1 above), because of a multiplicity of peaks. Fig. 2a shows a typical scan from a BLG positive subject. In an attempt to minimize what was assumed to be background 'noise' a negative serum scan was subtracted from each of the scans of four positive sera (method 2, Fig. 2b). Although the resultant profiles appeared to have a number of defined peaks, the residual background still made the detection of significant peaks difficult. Method 3 which compared native serum and serum from which antibodies to BLG had been removed by absorption, minimized background and produced six defined peaks of reactivity (Fig. 2c). This allowed selection of six peptides that were further tested by inhibition of IgE binding to native BLG.

Inhibition of IgE binding

Inhibition studies were performed using the synthesized peptides. Figure 3 shows the inhibition by each of the synthesized peptides and BLG. Peptide 4 inhibited IgE binding of all the sera to BLG discs (at levels above 15% for 14 of the 16 individual sera and the pooled serum). There was a positive correlation in the degree of inhibition of the individual sera between that produced by whole BLG and peptide 4 (Pearson correlation coefficient $R = 0.652$, $P = 0.005$). The slope of the line was nearly unitary (1.0841). IgE binding to BLG was above the highest reference standard in two of the sera. Since there was insufficient serum to repeat the analysis, the level of inhibition could not be evaluated in AEU/ml, and was thus calculated in counts per

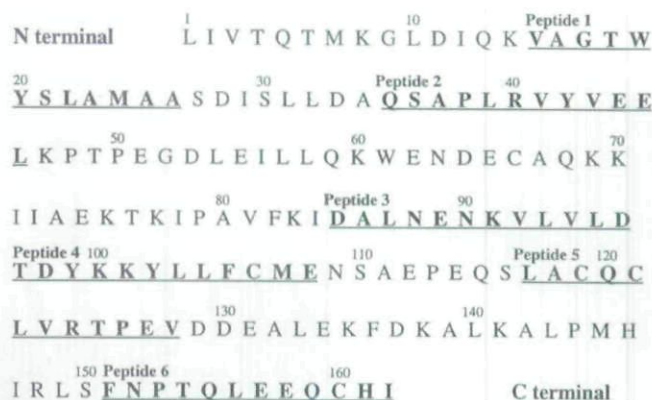


Fig. 1. The complete amino acid sequence of the monomer of bovine β -lactoglobulin (A variant) indicating, by underlining and bold lettering, the position of the six peptides identified for further testing.

minute (cpm), and reported as per cent inhibition of total cpm bound (%T).

Discussion

Inhibition (>15%) of IgE binding to BLG discs was demonstrated with peptide 4 in 14 of the 16 sera. The figure of 15% was chosen as a cut-off for appreciable inhibition rather than a higher figure as it is evident from the results that there are a number of epitopes on the BLG molecule. Therefore, it is possible that an individual will recognize more than one epitope. Thus binding to other epitopes must lower the per cent of inhibition which could be obtained with any one epitope. As well, the epitope appearing in the whole molecule may behave more efficiently (higher avidity) than the free epitope inhibition, driving the reaction towards the fixed epitope and reducing the potential for inhibition.

The other peptides inhibited sporadically although they all demonstrated inhibition with at least one serum sample (Fig. 3). This leads us to conclude that peptide 4 encompasses the major epitope of native BLG for IgE in humans. The other peptides tested can at best be regarded as containing minor epitopes.

Considerable differences were observed in the degree of inhibition by BLG on IgE binding to BLG bound to the paper discs (Fig. 3g). This observation is difficult to explain. A striking parallel pattern of inhibition was observed with peptide 4. Inhibition with individual sera demonstrated a significant correlation ($P = 0.005$) between the amount of inhibition that the native protein and peptide 4 were able to produce in solution. Variation in the degree of inhibition may be related to the affinity of the IgE antibody in the individual sera. If this is so, then the correlation suggests that the binding efficiency

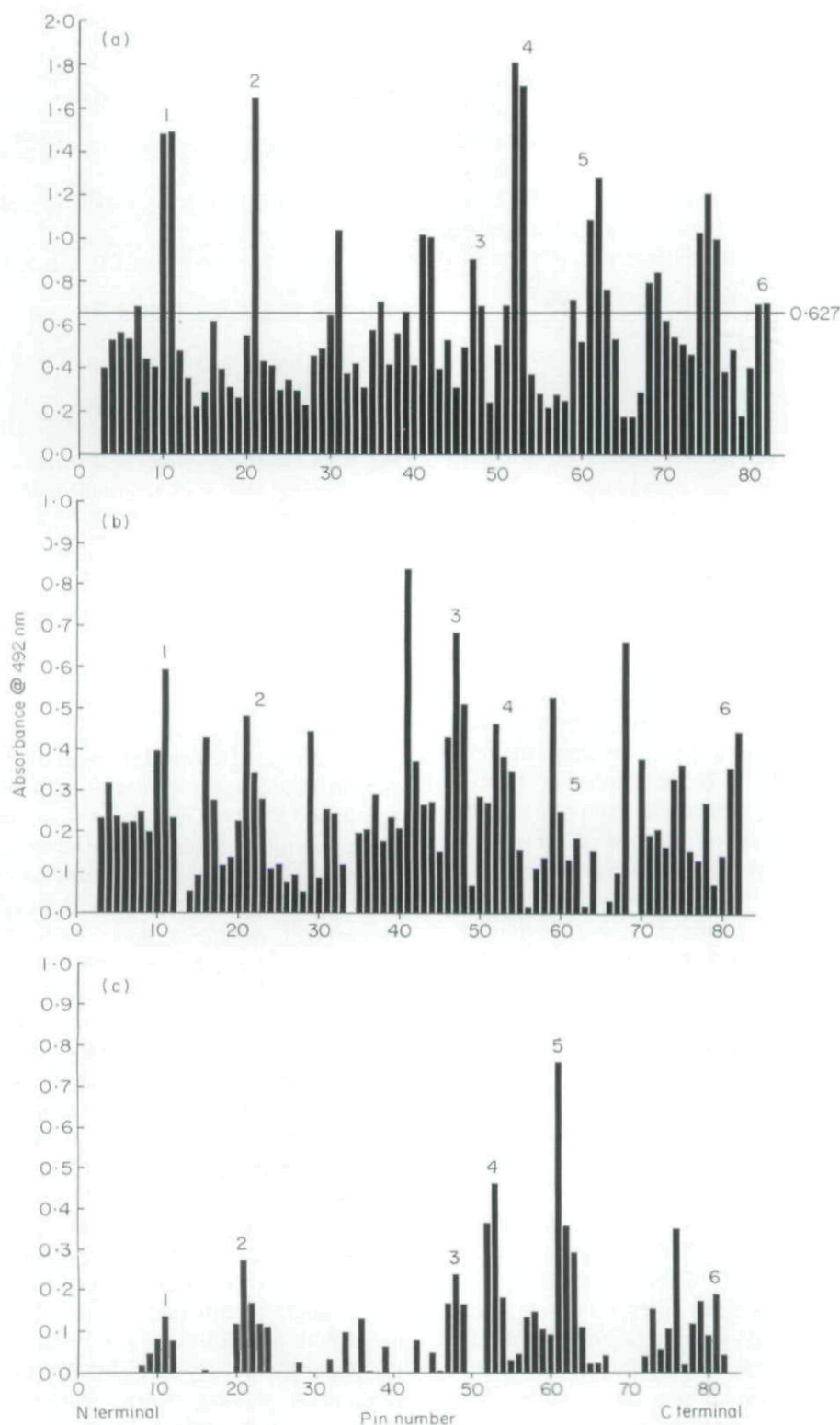


Fig. 2. Epitope scan (a) and profiles (b and c) obtained using the hexapeptides bound to pins in the IgE enhanced ELISA technique to detect anti-BLG peptides in human sera. (a) A scan of the serum of a patient with high levels of IgE to BLG. The horizontal line is drawn at the level calculated by method 1. (b) An epitope profile where the scan from a serum containing no IgE antibodies to BLG was subtracted from a scan of a serum containing high levels of IgE antibodies to BLG. (c) An epitope profile where the scan from the pooled serum containing IgE antibodies to BLG was subtracted from the scan of the same pooled serum after absorption of IgE antibodies to BLG.

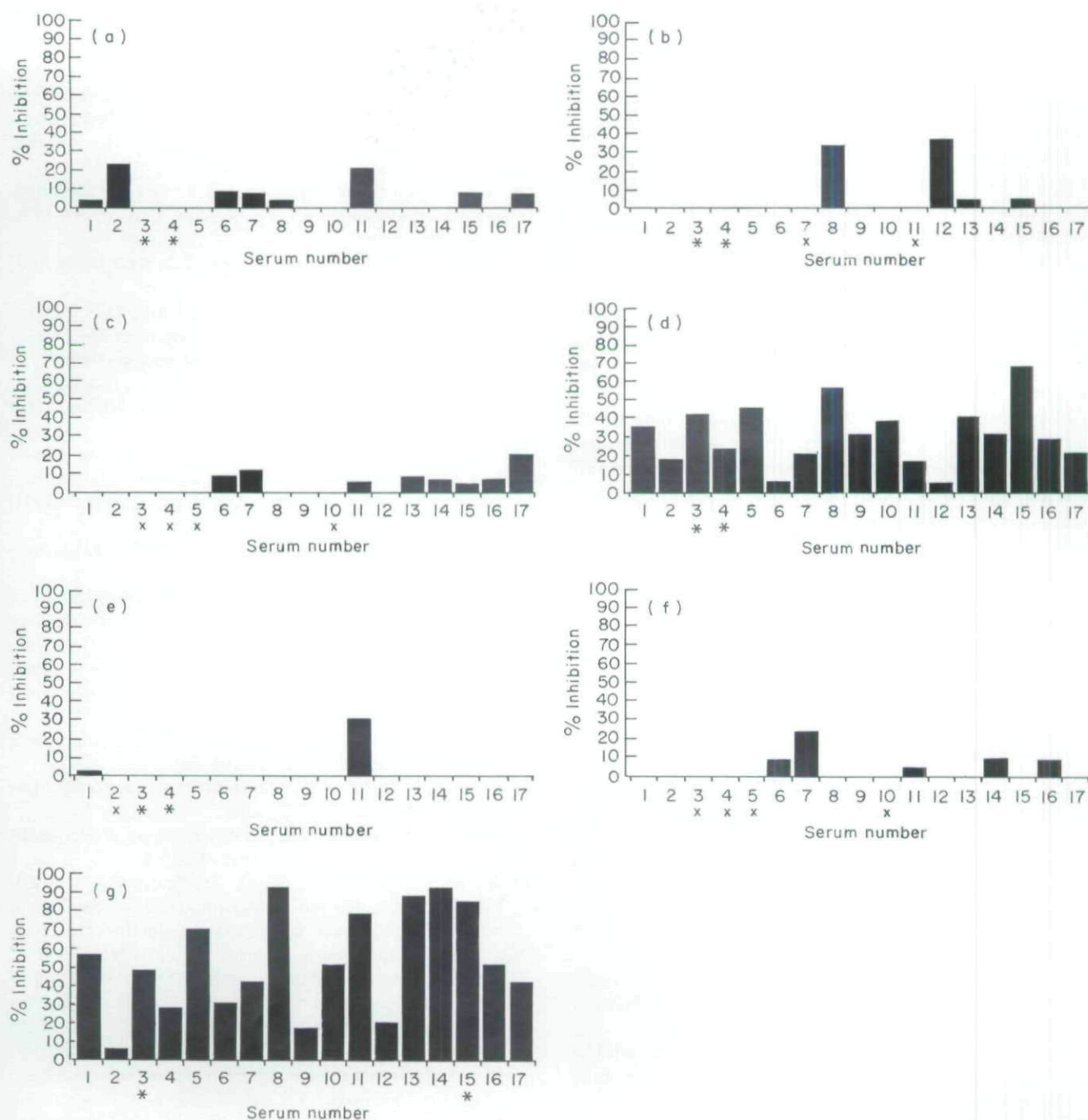


Fig. 3. RAST inhibition of the IgE binding of BLG by synthesized peptides; (a) Peptide 1. (b) Peptide 2. (c) Peptide 3. (d) Peptide 4. (e) Peptide 5. (f) Peptide 6. (g) β -lactoglobulin. x indicates insufficient serum for test. * indicates that the per cent inhibition is calculated as per cent total counts per minute (%T). Sera 3 and 4 are from the same patient at different ages. Sera 6 and 7 are in the same category. Serum 17 is the pooled serum, with high levels of IgE to β -lactoglobulin.

of peptide 4 and the native BLG molecule is similar (slope = 1.084).

Differences in the level of inhibition with peptide 4 could reflect variations in individual responses to minor epitopes on BLG or crossreactivity to epitopes on other

allergens. Peptides other than peptide 4, identified with the absorption technique demonstrated inhibition of IgE binding with only a few sera. Suggesting that these peptides are not recognized as allergic epitopes by all individuals. A possible limitation of this methodology

for exploring the epitopes of ingested allergens is the production of IgE antibody to internal epitopes. These buried epitopes may only be revealed to the immune system following gut digestion of the protein and absorption of peptides containing the epitopes. IgE binding to the native molecule would not occur therefore inhibition by the peptide would not be evident. These epitopes would thus not be revealed by the usual *in vitro* IgE binding assay or skin-test techniques which use the native BLG molecule. It may be that further *in vitro* and *in vivo* techniques utilizing the dodecapeptides will need to be developed before the clinical significance of these peptides can be assessed. We are also in the process of investigating the activity of peptide 4 conjugates in skin-prick tests.

If peptide 4 is confirmed as bearing a major IgE binding epitope of BLG, contributing to milk intolerance *in vitro* when it may be possible to disrupt the IgE binding epitope without denaturation of the protein structure, thus producing a hypoallergenic cow milk product without the need to digest the milk proteins extensively. This would have advantages in terms of taste and osmolality of the milk product compared with currently available hydrolysates.

In conclusion, we have defined a sequential peptide of 12 amino acids which appears to be a major IgE binding continuous epitope of native BLG.

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