

The Recognition Pattern of Sequential B Cell Epitopes of Beta-Lactoglobulin Does Not Vary with the Clinical Manifestations of Cow's Milk Allergy

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Key Words

β -Lactoglobulin · Epitope · Cow's milk allergy · Pin-ELISA

Abstract

Background: β -Lactoglobulin (BLG) represents one of the major allergens causing cow's milk allergy (CMA) – a disease with a wide spectrum of clinical symptoms. The aim of this study was to evaluate sequential B cell epitopes of BLG by the Pin-ELISA method. Furthermore, we wanted to investigate a possible association of the IgE recognition patterns in sera of patients with BLG sensitization and the type of clinical reactions following contact with cow's milk. **Methods:** Overlapping sequential decapeptides corresponding to the amino acid sequence of BLG were used in Pin-ELISAs specific for human IgE. Tested sera were from 14 individuals with CMA, 8 of them with a history of immediate systemic reactions and 6 with delayed skin reactions following contact with cow's milk. All of them showed specific IgE antibodies to BLG in the CAP-RAST. Control sera were from 5 healthy nonallergic individuals. **Results:** All sera from BLG-sensitized individuals showed IgE binding with one region of BLG corresponding to amino acids 95–113. Furthermore, individual sera showed reactions with two further regions, 12–27 and 124–135. Inhibition

of IgE binding to BLG with one soluble synthetic peptide confirmed the major epitope. No differences were found in the B cell epitope recognition pattern to BLG in the two groups of patients with CMA, characterized by acute systemic or delayed skin reactions. **Conclusions:** Using IgE Pin-ELISAs we were able to confirm previously described sequential B cell epitopes of BLG. However, the recognition pattern of one of the major cow's milk allergens is not predictive of the clinical type of reaction.

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Introduction

Cow's milk allergy (CMA) is one of the first clinical manifestations of an atopic disorder in childhood. Strictly speaking, CMA is an IgE-mediated type I immunological reaction, causing the systemic clinical symptoms of urticaria, bronchial constriction, angioedema and anaphylactic reactions following contact with cow's milk. However, further delayed or late immunological reactions mediated through cytokines and immunoregulatory cells are included in the clinical spectrum of CMA, e.g. atopic dermatitis [1–4]. Because of the wide range of clinical symptoms and differences in the definition of CMA, the published incidences vary from 0.1 to 7.5% [5, 6]. In northern Europe, the

incidence is reported to be 2.0–2.5% within the first 2 years of life [7–10].

The major allergens in cow's milk are β -lactoglobulin (BLG) and the caseins besides α -lactalbumin and bovine serum albumin.

BLG is the major whey protein and the most important antigen in cow's milk apart from the caseins [11]. It has no homologous counterpart in human milk [12]. For this reason, BLG is of special interest in the clinical context of CMA. BLG consists of 162 amino acids referring to a molecular weight of 18 kD. Among several known genetic variants, BLG A and B represent the main isoforms. The tertiary structure is characterized by the so-called ' β -barrel-structure' [13], which is stabilized by intrachain disulfide bridges [14, 15]. This structure represents a common feature in the group of lipocalin-proteins and shows a high allergenic potential. Many major allergens of animal origin belong to this group of proteins [16]. Cleavage of the intrachain disulfide bridges and thereby a loss of conformation of the molecule has little or no effect on its immunoreactivity. Therefore linear epitopes are mainly involved in its allergenicity [17, 18].

Several studies based on different techniques have been published dealing with the potential linear B cell epitopes of BLG [19–25]. Most of these studies showed B cell epitopes in the peptide region amino acids 19–30, 93–98 and 141–150 of bovine BLG.

The first aim of this study was to investigate sequential B cell epitopes of the BLG protein by the Pin-ELISA method and to compare the results with the published data base in order to evaluate the method. The second aim was to compare the IgE recognition patterns in sera of patients with BLG sensitization with the type of clinical reactions following their contact with cow's milk. This was of special interest regarding a possible predictive value of the IgE recognition pattern with respect to systemic, life-threatening, versus merely cutaneous reactions.

Materials and Methods

Purification of BLG

Commercially available BLG (L0130, Sigma) was further purified by Q-Sepharose anion exchange chromatography by means of the HighLoad System (Pharmacia). 130 mg BLG were dissolved in 4.0 ml 20 mM Tris-HCl pH 8.0 (buffer A) and applied onto a Q-Sepharose FastFlow column (1.0 $\times$ 10.5 cm, Pharmacia) equilibrated with the same buffer. BLG was eluted at a flow rate of 1.0 ml/min with a gradient from 0 to 350 mM NaCl (in buffer A) in 100 min, followed by a gradient from 350 to 500 mM NaCl in 30 min. Eluted proteins were detected by absorption at 280 nm (Uvicord SII, Pharmacia). Pooled fractions (mixture of isoforms A and B) were desalted (ultrafiltration

cell, YM-10 membrane, Amicon) and lyophilized. Purity and identity of BLG were checked by SDS-PAGE and by N-terminal amino acid sequencing using a gas-phase sequencer (Protein sequencer 477A/120A, Applied Biosystems).

IgE ELISA and Inhibition ELISA

IgE ELISA and inhibition ELISA were performed as described previously [26]. The synthetic peptide (SNPE, Germany) corresponds to the immunological reactive region of BLG (amino acids 95–113). Three different kinds of inhibition ELISAs were performed: (a) inhibition of patients' sera with native BLG (at a concentration of 1 mg/ml) prior to performing Pin-ELISAs; (b) inhibition with the synthetic peptide (at a concentration of 1 mg/ml) prior to performing Pin-ELISAs, and (c) inhibition with the synthetic peptide (at a concentration of 1 mg/ml) prior to performing IgE ELISAs.

Finally, inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(\text{control} - \text{test}) \times 100\%}{\text{control}}.$$

Epitope Scanning

A set of 156 decapeptides shifted by one amino acid was synthesized on polyethylene pins according to the known amino acid sequence of bovine BLG (Abimed, Germany). The first peptide started with the second amino acid of the BLG sequence and the last peptide ended with the penultimate amino acid. Specific binding of IgE to the immobilized peptides on the pins was measured by the ELISA method. All incubations of the pins with antibodies or reagents were performed in flat-bottom microtiter plates (Greiner, Germany). Pins were blocked with 250 μ l/well 10% fish gelatine in PBS-Tween for 6 h at room temperature, then washed 3 times with 200 ml PBS-Tween for 10 min each time. The blocked pins were incubated with human serum (diluted 1:200 in blocking buffer, 200 μ l/well, 4°C, over night). Bound IgE was detected by successive incubation with monoclonal mouse anti-human IgE (1:500, 200 μ l/well, 20°C, 2 h) and goat anti-mouse Ig conjugated with horseradish peroxidase (1:50, 200 μ l/well, 20°C, 1 h). Between all incubations pins were washed 3 times with 250 ml PBS-Tween. Finally, the pins were incubated with 200 μ l/well of 20 mg ABTS, 10 μ l 30% H₂O₂ in 40 ml 0.1 M citrate-phosphate buffer pH 5.0 for 60 min at room temperature. Absorption was measured at 405 nm with a microplate reader (Titertek Multiscan MCC/340, Flow). The pins were reused after stripping off the bound antibodies according to the manual of the Pin-ELISA kit.

Patients

Sera from 14 patients with CMA, 8 boys and 6 girls, aged between 6 months and 9 years were tested. The patients were divided in two groups: in group 1 all patients had a history of anaphylactic or immediate reactions following contact with cow's milk, like angioedema, bronchial constriction and/or urticaria. Group 2 consisted of patients with delayed cutaneous reactions following ingestion of milk. All patients had high levels of specific IgE to BLG (RAST class $\geq$ 2) determined by the CAP-RAST system (Pharmacia). Control sera were obtained from 2 healthy boys and 3 healthy girls aged 10 months to 3 years, who had no history of atopy or allergy to cow's milk and non-detectable levels of specific IgE to bovine BLG (RAST class 0). The groups of individuals were not age matched.

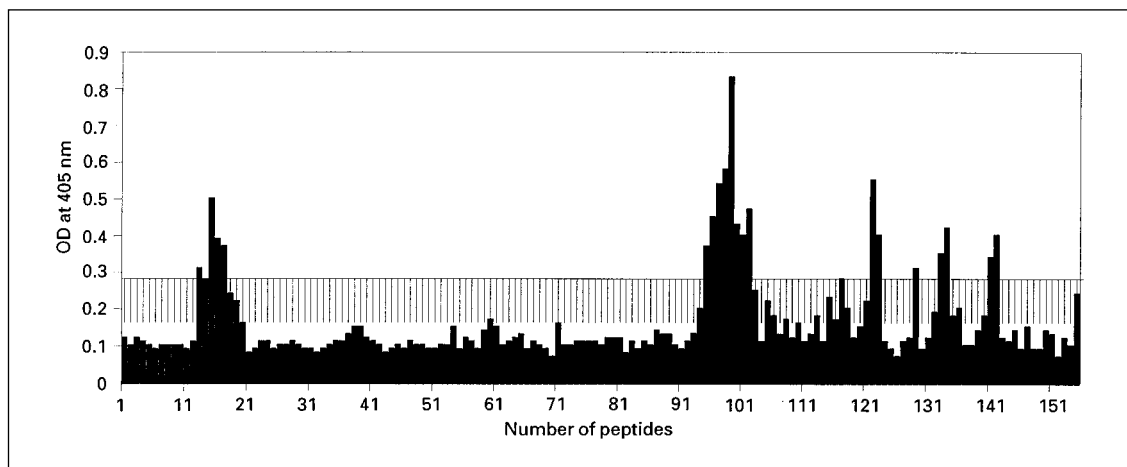


Fig. 1. Typical IgE Pin-ELISA scan of one highly allergic patient. The pins are labelled by the number of the first amino acid of the decapeptide according to the amino acid sequence. The hatched area indicates the range between the mean (lower border) and the first standard deviation (upper border).

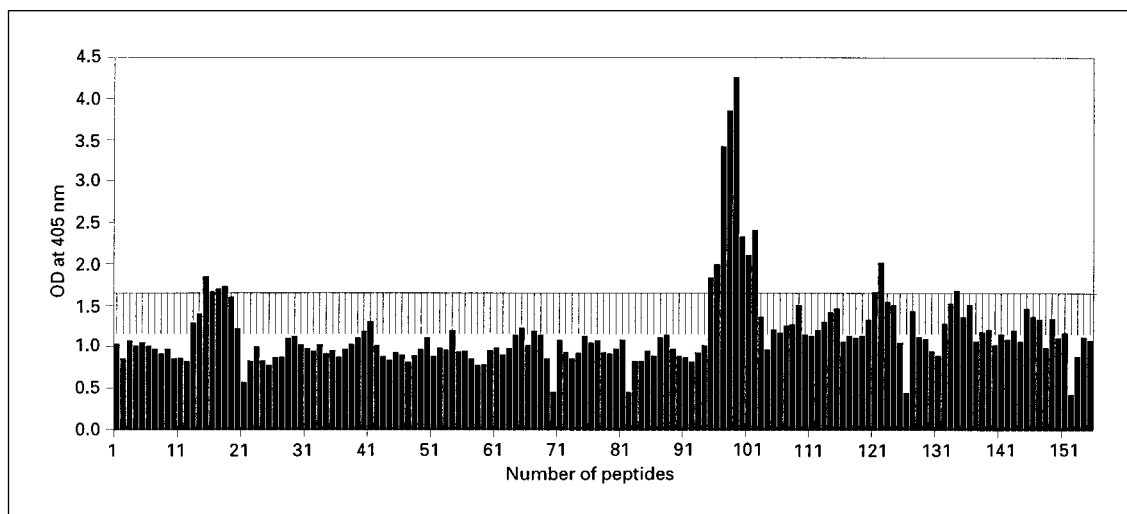


Fig. 2. Overlays of the IgE Pin-ELISA scans of all patients with CMA. For comparison, the measured absorption values of each scan were standardized by dividing by the mean absorption. The hatched area indicates the range between the mean (lower border) and the first standard deviation (upper border).

Results

Sera from 14 patients with sensitization to BLG and clinical symptoms of CMA and 5 healthy nonallergic controls were tested in Pin-ELISA assays for the presence of specific IgE to overlapping decapeptides of the BLG protein.

An absorption value higher than the mean absorption of the scan plus 1 SD was taken as a criterion for a positive rating. A typical scan of a highly sensitized patient is shown in figure 1.

Comparing the scans of all individuals we picked up a putative sequential B cell epitope, which was recognized by all individuals to an equal extent. This peptide comprises the protein region amino acids 94–113 and is referred to as the major sequential B cell epitope of BLG (fig. 2).

Two further putative epitopes, comprising the protein region amino acids 10–30 and 124–135, were only observed in a part of the sera studied. They are referred to as the minor sequential B cell epitopes of BLG (fig. 3).

1	11	21	31	
LIVTQTMKGL	DI <u>Q</u> KVAGTWY	SLAMAASDIS	LLDAQSAPLR	
41	51	61	71	
VYVEELKPTP	EGDLEILLQK	WENDECAQKK	IIAEKTKIPA	
81	91	101	111	
VFKIDALNEN	KVLVL <u>D</u> TDYK	KYLLFCMENS	AEPEQSLACQ	
121	131	141	151	161
CLVRT <u>P</u> EVDD	EAL <u>E</u> KFDKAL	KALPMHIRLS	FNPTQLEEQC	HI

Fig. 3. Amino acid sequence of bovine BLG. The major epitope is underlined twice and the two minor epitopes are underlined once.

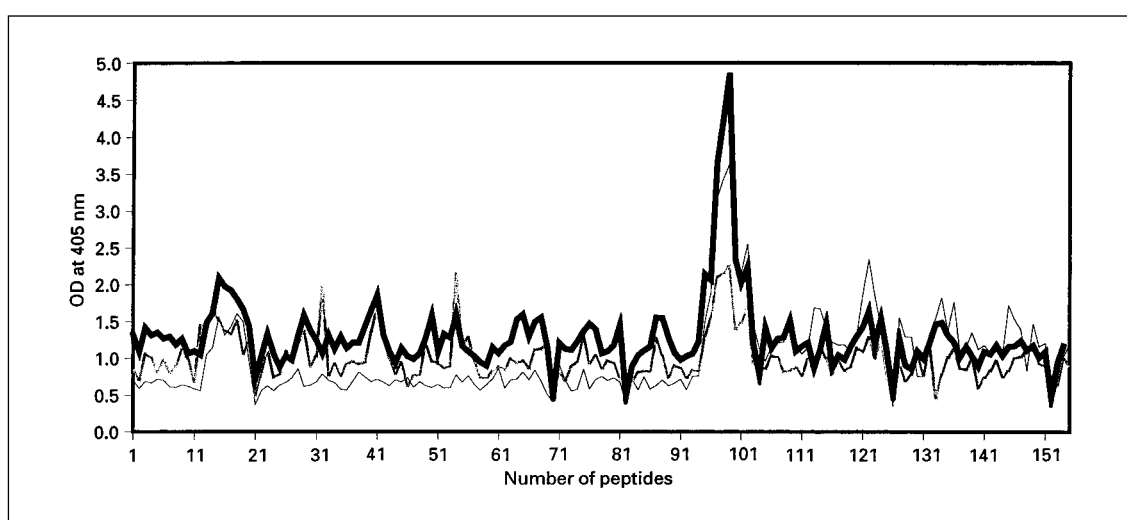


Fig. 4. Overlays of the IgE Pin-ELISA scans of patients with CMA and different clinical symptoms and controls. For comparison, the measured absorption values of each scan were standardized by dividing by the mean absorption. Black line: sera of group 1 (immediate systemic reaction). Gray line: sera of group 2 (delayed cutaneous reaction). Thin line: sera of healthy controls.

The B cell epitope recognition patterns in the groups of patients with acute systemic reactions following cow's milk contact (8 individuals) versus those with late or delayed cutaneous reactions (6 individuals) did not show any significant differences. This also applies for the epitope usage per se as for the individual signal intensity of single epitopes compared to the total signal (fig. 4).

Inhibition of IgE binding to BLG by one synthetic peptide (amino acids 95–113) was tested with all sera. The peptide inhibited IgE binding of all patients' sera (fig. 5).

In addition, Pin-ELISAs were performed with 2 sera after preincubation with the peptide, showing reduced inten-

sity of absorption in this region of BLG (fig. 6). To test for unspecific binding of patients' antibodies to the synthetic decapeptides, inhibition of 2 patients' sera by native BLG prior to Pin-ELISAs was performed. No absorption above the background level was seen (data not shown).

Discussion

CMA is one of the first and in some instances life-threatening clinical manifestations of an atopic disorder in childhood. Although the measurement of specific IgE to the most

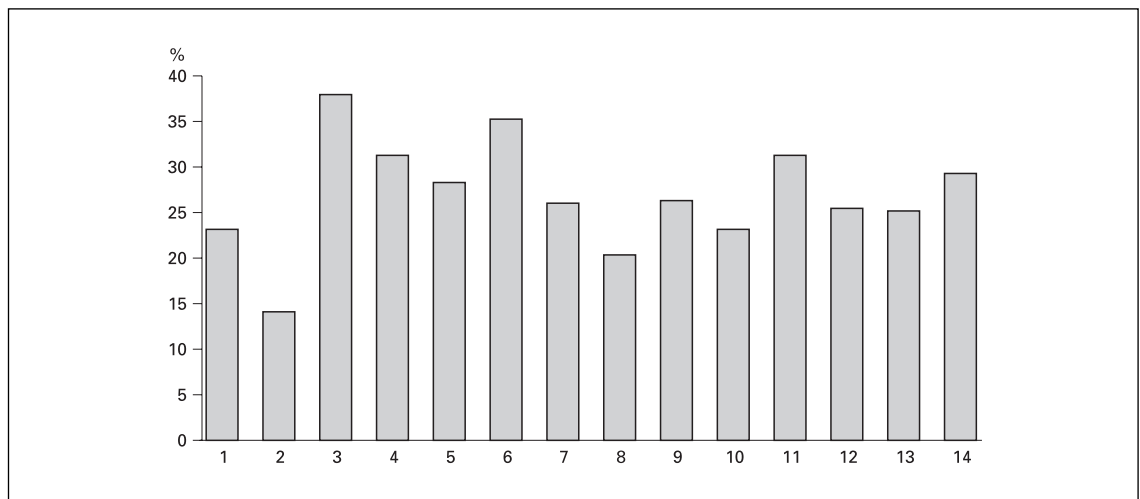


Fig. 5. Inhibition ELISAs. group 1: number 1–8; group 2: number 9–14. The synthetic peptide was used in a concentration of 1 mg/ml.

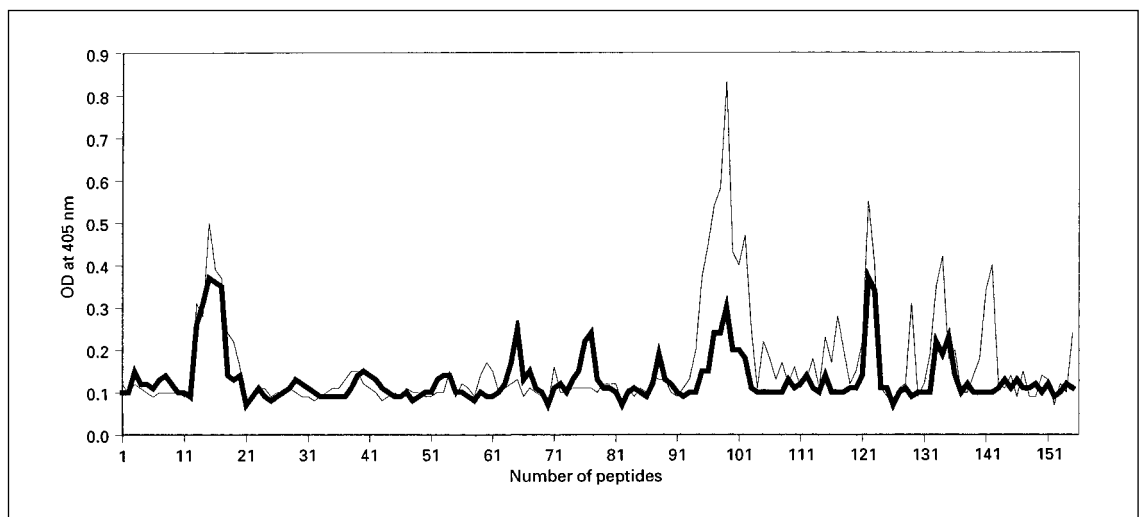


Fig. 6. IgE Pin-ELISA with serum of the same two patients. Thin line: native sera; thick line: after preincubation of the sera with the synthetic peptide in a concentration of 1 mg/ml.

important allergens in cow's milk (BLG and caseins) shows a high sensitivity in CMA diagnosis its specificity is rather low [27, 28]. This counts as well for the prediction of the kind of immune reaction after contact with cow's milk in sensitized individuals.

There is no clear predictive value of specific IgE levels for the risk of individuals to develop acute systemic reactions versus delayed or late-type skin reactions or no clinical reaction. Although children with atopic dermatitis often

show raised IgE levels to milk proteins, clinical symptoms are rarely proven by double-blind placebo-controlled food challenges [29, 30]. Therefore it seems likely that the skin reaction is caused by T-cell-mediated rather than IgE-mediated late-phase reactions, although the latter cannot be excluded.

Recently, it was shown that IgG antibodies to BLG might show specific profiles of epitopes with respect to the clinical reactivity of individuals [31]. Whereas an important

part of the IgG antibodies of allergic patients is directed towards epitopes of the native protein prone to destruction by peptic digestion, the natural antibodies of nonallergic subjects recognize epitopes unaffected by this denaturing process.

We were interested in B cell recognition patterns of sequential epitopes of BLG compared to the clinical reactivity of the sensitized individuals. BLG is of special interest as it represents one of the major allergens of cow's milk and is absent in human milk.

The previously described Pin-ELISA method had been improved for these studies by introducing a monoclonal antibody in order to reduce the background [26]. Using this Pin-ELISA method, we were able to pick up the protein region amino acids 95–113 which was recognized by all BLG sensitized individuals tested. This region corresponds to the epitope described by Ball et al. [23] who identified sequence 97–108 as a linear epitope using overlapping peptides. The major epitope is situated on the surface of the BLG molecule as deduced from its tertiary structure [32]. In a recent publication, Selo et al. [33] supposed that a major allergenic epitope of BLG could be located within or around the common sequence shared by peptides 97–108 and 102–124. The latter was shown to be stabilized by either hydrogen or disulfide bonds and to be present on the surface of the molecule, and hence accessible to antibodies. Our findings of a major epitope in this region with regard to the BLG allergenicity are in good agreement with these findings. Two further regions, amino acids 12–27 and 124–135, showed reactivity in a part of the sera tested. In fact, the allergenicity of sequence 124–134 – one of the sequences in common with α -lactalbumin – has already been suggested by the hydrophilicity profile of BLG and confirms the studies by Adams et al. [19], Tokita et al. [20] and Conti et al. [34]. In addition the same major epitope as well as the two minor epitopes were recognized by studying the IgE-binding capacity of four fragments of BLG obtained by CNBr cleavage in a study by Selo et al. [17]. In contrast, the epitopes found in this study overlap only partially with the epitopes described by Mizumachi et al. [21, 35]. However, the latter used various strains of mice and not human sera for their experiments.

With respect to these similarities, the character of the epitopes found as sequential epitopes and the value of the Pin-ELISA technique are confirmed.

Sera from individuals with no clinical history of food allergy and atopy, low levels of total IgE and no detectable specific IgE to cow's milk proteins (RAST class 0) showed reaction patterns in the IgE Pin-ELISA similar to those of the CMA patients. However, the signal intensities were low-

er. In contrast, their reactivity in the IgE ELISA was significantly lower compared with patient's sera, but a direct comparison of both ELISA methods is difficult since the IgE Pin-ELISA is a more qualitative method and a quantitative comparison of sera is difficult. In the Pin-ELISA, the competition between IgE and IgG is strongly reduced due to the high antigen loading of the pins (approximately 50 nmol/pin). On the other hand, in the IgE ELISA the antibodies compete for the limited amounts of antigen (approximately 4 pmol/well).

The striking feature of Pin-ELISA binding even in the absence of measurable specific IgE might reflect a level of sensitization in clinically nonaffected individuals that is too low for detection by standard IgE measurements. This would underline the sensitivity of the Pin-ELISA. To check for the possibility that antibody binding could be to some antigen other than BLG itself, we performed an additional inhibition ELISA using native BLG. No absorption to the Pin-peptides was seen.

The BLG B cell epitope recognition patterns in groups of healthy individuals, sensitized individuals with delayed or late cutaneous reactions and those with acute systemic reactions did not show any difference representing a predictive value of the method for the kind of clinical reaction. The same applies for the signal intensity to the whole protein as well as single epitopes. This is in contrast to the findings in IgG antibody testing [31].

We conclude from this that IgE antibodies to sequential B cell epitopes of BLG do not reflect the clinical reactivity of BLG-sensitized individuals. However, only linear epitopes were investigated in this study and different findings might result from the investigation of conformational epitopes. As well, further studies will have to clarify the meaning of specific T cell epitopes in the development of clinical reactivity to cow's milk.

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