

Anti-betalactoglobulin IgG antibodies bind to a specific profile of epitopes when patients are allergic to cow's milk proteins

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Summary

Background We demonstrated recently that mite-allergic patients differed from healthy controls in the specificity of their IgG antibodies towards mite antigens.

Objective The present study investigates whether these discriminatory IgG responses could be associated with the expression and the evolution of clinical manifestations in allergy to cow's milk proteins.

Methods Antibody specificity was evaluated by comparing IgG-binding to native bovine beta-lactoglobulin (nBLG) and its products of pepsin hydrolysis (dBLG) using a solid-phase enzyme-linked immunosorbent assay (ELISA). Antibody specificity was further investigated in competitive ELISA using streptavidin-biotin technology with purified IgG fractions from selected subjects and specific mouse monoclonals raised against BLG.

Results IgG antibodies from CM-intolerant or allergic sera ($n = 222$) showed a higher degree of binding to nBLG than to dBLG, while control sera showed similar levels to both nBLG and dBLG ($n = 99$ children/65 adults). Sera from symptomatic patients, whether or not they contained IgE antibodies, demonstrated group-segregating capacities to compete with pooled purified IgG from each clinical class, and with selected murine anti-nBLG monoclonal antibodies for binding to n- and dBLG. Furthermore, this inhibitory capacity shifted dramatically in a small subset ($n = 14$) of children as they developed CM-tolerance.

Conclusion The IgG responses to BLG of CM-intolerant or allergic patients are very different from those of healthy controls, being characterized not only by increased titres but also similar patterns of modified specificity, including a marked preference for conformational epitopes.

Cross-competition experiments confirmed that the restricted specificity was clinically associated, appearing as an immunological signature, which allowed almost complete discrimination between patient groups. This phenomenon is a particularly promising diagnostic feature in this category of young patients where conventional tests usually only document the status of sensitization.

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Introduction

Cow's milk intolerance appears early in life, and now affects up to 3% of children in European countries,

owing to replacement of breast-feeding with bottle-feeding [1–3].

Clinical manifestations are well known [1,2] and are considered as atopic when specific IgE are demonstrated. Currently, diagnosis is confirmed by diet manipulation and occasionally by challenge tests. Detection of specific IgE antibodies against any of the milk allergens in children

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below the age of 2 years is often unsuccessful, due to the low sensitivity of current methodology.

Alternative markers of immunological hyperactivity to milk antigens such as IgG antibody levels have met with questionable success [4–12]. Some studies have related these anti-milk protein IgG simply to mucosal exposure by bottle-fed children [4–8], without any relation to symptoms, or differences between symptomatic and control groups. In contrast, other authors have demonstrated the predictive value of elevated anti-BLG IgG as indication of the successful outcome of an exclusion diet [9].

Yet others have reported higher anti-bovine BLG IgG levels in patients with active atopic dermatitis and consistently higher levels of IgG1 and IgG4 anti-BLG antibodies in infants with persistent cow milk allergy (CMA) [10,11]. Recently, the anti-BLG IgG was evaluated to be the most useful test for diagnosis of gastro-oesophageal reflux in CMA [12].

The reasons for these discrepancies have never been elucidated. Some studies have found significant associations between IgE and IgG antibodies: ranging from frequently associated detection [13], to significant correlation between isotype titres [14], and attribution of a predictive value to IgE/IgG ratios for monitoring or predicting the decline of clinical reactivity [15]. Other studies report no relationship at all between IgE and IgG antibody levels [16].

Finally, patients expressing high IgG responses to food allergens have been shown to be more likely to develop IgE antibodies to inhaled allergens later in life [17], indicating some kind of atopic predisposition even in the absence of specific IgE detection. We have recently demonstrated that IgG antibodies from adult patients, displaying symptoms of allergic rhinitis or asthma related to house dust mite allergy, expressed preferential recognition of some epitopes on the Der p 1 antigen [18]. This is compatible with the existence of a CD4⁺ T cell driving B cell production of IgG antibodies, in accordance with a Th₁ or Th₂ functional phenotype, the balance of which is disturbed for specific allergens in atopic subjects [19–21].

The present study investigates if and to what degree this discriminatory phenomenon can be observed, at the IgG antibody level, in CM-allergic children. Furthermore, the CM-allergy model offers the advantage of allowing a more objective approach to investigations of the clinical relevance of any new *in vitro* diagnostic test for allergy since the manifested symptoms of allergy disappear after diet manipulation.

Materials

Serum samples were obtained for routine purposes from inpatients at the Hôpital Universitaire des Enfants Reine

Fabiola, or attending the outpatient clinic of the hospital's chest department. Those from symptomatic patients with a *posteriori* identified cow's milk allergy were collected at the start of diagnostic procedure when both symptoms and milk exposition were present. Aliquots were frozen at –20°C for subsequent investigation. Allergic diagnosis was established on the basis of clinical history, the results of eviction diet, and a single-blind challenge test with cow's milk protein capsules.

A total of 222 patients suffering from persistent atopic manifestations, involving the skin, the gastrointestinal tract, or the upper airways was investigated. Among these patients, 14 children were investigated using a second serum sample collected at a distance varying from 2 to 12 months from the initial allergic episode, a period following the start of strict elimination diet. This diet was still ongoing at the time of second blood sampling for those described as intolerant ($n=8$), as deduced from recent unsuccessful assays of cow's milk reintroduction. They were nevertheless asymptomatic. The second subgroup ($n=6$) also comprised asymptomatic children who acquired full tolerance to milk ingestion, and were thus no longer undergoing the elimination diet at the time of the second blood sampling. Mean delays between both tests were similar in the two subgroups.

Control populations included:

- 50 patients with atopic manifestations unrelated to CM;
- 44 patients investigated for non-allergic reasons and devoid of any history of atopic manifestations;
- 65 healthy adult blood donors attending the Centre de Transfusion Albert Hustin (CHU St Pierre, Brussels).

Method

Specific IgE antibodies towards BLG

The presence of IgE antibodies against BLG was screened with a conventional radioallergosorbent test system (RAST) using ¹²⁵I-labelled antihuman IgE antibodies (Pharmacia-Cap System).

IgG binding to native and denatured BLG

Allergen preparations

Native protein purified lyophilized BLG antigen (Sigma®; NTL, Brussels, Belgium), comprising a mixture of variants A and B of bovine beta-lactoglobulin, crystallized four times, displayed two major bands (18 and 36 kDa on a sodium dodecyl-sulphate polyacrylamide gel. The solubilized antigen was filtered several times through a membrane (cut-off 10 kDa; Millipore®, Brussels, Belgium) to remove most contaminating traces of peptides possibly contained in the antigen extract (peptides can constitute up to 10% of the

total protein content, depending on the batch and storage conditions).

Digested protein

Five milligram of native protein (1 mg/mL in TRIS-HCl: 10 mM, pH 7.2) were added to insoluble pepsin bound to 300 mg of 4% cross-linked beaded agarose (activity: 170 U/mg dry agarose; Sigma, Dorset, UK) which had been washed twice with distilled water and then twice with the above described TRIS-HCl buffer. Digestion was carried out at 40°C for 3 h. The reaction was then stopped by centrifugation (5000 rpm for 10 min) which separated the solid-phase insoluble enzyme from the protein solution. The pH of the supernatant was raised to 7.0 by the addition of a neutralizing buffer (TRIS-HCl: 100 mM, pH 9.6).

Filtration (cut-off 10 kDa) was then performed several times to remove most remaining traces of the native protein. The concentration of the collected solution was evaluated by spectrophotometry and adjusted to the concentration of the native protein solution used in parallel for the coating procedure.

Specific IgG antibodies towards the native protein (nBLG) and its products of digestion (dBLG)

Specific IgG retention of nBLG and dBLG were evaluated in parallel by ELISA: 96-well polystyrene microplates (Nunc-Immunoplates; Gibco, Merelbeke, Belgium) were passively coated with nBLG and dBLG (10 µg/mL in bicarbonate buffer: 50 mM, pH 9.5) and additional non-specific binding was prevented by saturation with gelatin (Hemacel®, 3% v/v in normal saline; Hoechst, Belgium). After 30 min incubation with ethylene diamine tetraacetic acid (EDTA: 10 mM, pH 7.2), 100 µL of each serum, diluted 1:25 in phosphate buffered saline (PBS: 10 mM, pH 7.2) supplemented with 0.05% v/v Tween 20 (PBS-Tween buffer) were added to separate wells containing the different antigens and incubated overnight at room temperature.

After washing with the PBS-Tween buffer, 100 µL of a solution of peroxidase-conjugated protein A (Sigma; 0.1% v/v in PBS) were added and incubated for 2 h at 40°C; after a further washing the substrate solution (0.15% v/v H₂O₂, 0.3% w/v o-phenylenediamine in 0.2 M citric acid and 0.4 M sodium phosphate) was then added (100 µL/well); the enzyme reaction was stopped by the addition to each well of 100 µL of 2 M HCl. The binding activity of each serum's antibodies was determined by reading the absorbance at 490 nm on a microtitre-plate reader (Dynatech MR 5000; Dynatech Laboratories, Chantilly, VA, USA). Antibody titres were expressed in arbitrary units according to a calibration curve made with serial dilutions of hyperimmune pooled sera. The procedure was repeated twice for each serum. Non-specific binding was evaluated in parallel by

performing the same assay with microtitre-plates previously coated with gelatin (Hemacel, 3% v/v) only and no antigen. All assay conditions were identical allowing direct comparison of the results.

Competitive inhibition tests

IgG antibody purification and biotin labelling

Purification

IgG fractions were separated from either healthy control or CM-allergic pooled sera ($n = 12$ in each case) by affinity chromatography on recombinant protein G sepharose (Pharmacia Biotech; Roesdaele, Netherlands) [22], after depletion of IgG rheumatoid factor which could otherwise interfere with the net binding of labelled IgG. Depletion of IgG rheumatoid factor was achieved by preincubating the sera with packed sheep red cells sensitized with rabbit haemolysins. The purified IgG fraction was then dialysed against carbonate buffer (50 mM, pH 9.5) to eliminate the glycine buffer (10 mM, pH 2.0) used for elution during the purification process. The concentration of the IgG antibody solutions was determined by spectrophotometry, and RF depletion was confirmed by ELISA using human IgG as autoantigen.

Biotinylation of IgG fractions from selected pools of human sera and mouse monoclonal antibodies (MoAb) specific to nBLG

Twelve mouse monoclonal antibodies (MoAbs) specific to BLG were raised and developed from Balbc mice. They were highly reactive with nBLG and poorly if at all with dBLG in ELISA. Three were retained for appropriated selectivity (MC5, MC6, MC7). The pooled-IgG fractions and MoAb-ascite dilutions (adjusted to 100 µg/mL of IgG) were incubated for 4 h at room temperature with biotin (125 µg/mg of IgG antibody, in the described carbonate buffer). Excess biotin was then eliminated by dialysis against PBS (10 mM, pH 7.2). Stock solutions of labelled IgG were kept in PBS-gelatin buffer (1% v/v) containing 0.01% w/v sodium azide.

Competition between biotinylated pooled IgG fractions and individual sera for binding to solid phase bound nBLG and dBLG

Optimal conditions for competition had been pre-established to ensure convenient binding of labelled IgG, discriminant results between subject populations, and high reproducibility. Balance had to be found between the serum test dilution, the amount of antigen coating, and the amount of biotinylated IgG, which itself depended on the

labelling efficiency. The following protocol has been found to be reproducible.

96-well polystyrene microplates were passively coated with nBLG (0.3 µg/mL in bicarbonate buffer: 50 mM, pH 9.2) and additional non-specific binding was prevented by saturation with gelatin (Hemacel, 3% v/v); 100 µL of solutions containing individual sera and biotinylated pooled-IgG fraction diluted, respectively, 1:100 and 1:200 in PBS-Tween buffer containing EDTA (10 mM) and gelatin (Hemacel, 0.1% v/v), were transferred, after vigorous mixing, to coated plates and incubated overnight at room temperature; after the samples had been washed three times with PBS-Tween buffer, 100 µL of a solution of peroxidase-conjugated streptavidin (0.1% v/v in PBS-Tween buffer; Pierce: Polylab, Antwerpen, Belgium) was added and incubated for 3 h at 40°C. After an additional washing step, the binding activity was determined as described above. The binding activity or optical density (OD) displayed by the biotinylated pooled-IgG fraction alone, served as the reference value. The results are expressed as the percentage reduction of this OD value when individual sera are added to the biotinylated pooled-IgG fractions. Percentage binding inhibition was calculated as follows:

$$\% \text{ inhibition} = 100 \times \left(1 - \frac{\text{OD}_S - \text{OD}_{NS}}{\text{OD}_R - \text{OD}_{NS}} \right)$$

where OD_S, OD_R, and OD_{NS} represent, respectively, optical densities in the presence of serum, in the absence of serum (reference value), and corresponding to the non specific binding. Non-specific binding was evaluated in parallel by performing the same assay with microtitre-plates coated exclusively with gelatin (Hemacel 3% v/v). The blank was evaluated using coated plates and peroxidase-conjugated streptavidin.

As in some cases a protein matrix effect was visible, specially in the absence of competing serum, resulting in a reduction in the binding capacity of the biotinylated ligand, the values for maximal binding (OD_R) were always taken as those obtained with the least inhibitory sera.

Competition between biotinylated monoclonal antibodies and individual sera for binding to nBLG

Competition for binding to nBLG between biotinylated MoAb and individual sera was performed as described above under previously optimized conditions: such that the wells were passively coated with 5 µg/mL nBLG in bicarbonate buffer (50 mM, pH 9.5).

Biotinylated monoclonal antibodies and human sera were diluted 1:400 and 1:100, respectively, in the PBS-Tween buffer containing EDTA (10 mM) and gelatin (Hemacel, 0.1% v/v), supplemented with mouse pooled serum (1:100 final concentration). This was added to reduce interactions

between human natural antimouse antibodies and murine MoAb. The mouse sera had been preselected from hundreds of sera for their very low content of natural anti-BLG IgG from mice fed on a very low milk diet. The mouse sera were further adsorbed on nBLG sepharose to reduce potential interference with the binding activity displayed by the labelled MoAb. The remaining part of the procedure and the expression of the results are exactly the same as described above for purified IgG fractions.

Results

Anti-nBLG IgG-antibody titers in hospitalized CM-allergic children (Fig. 1)

Figure 1 shows two frequency histograms of anti-nBLG IgG-antibody titres, expressed in arbitrary units (AU), after natural logarithmic transformation. All subjects were children hospitalized for severe and recurrent atopic manifestations (respiratory: 57%; gastrointestinal: 55%; dermatological: 21%) suspected to be related to cow's milk.

Following a CM eviction diet, a dramatic improvement was immediately observed in 126 of the patients but in 50 patients there was no improvement at all. Linkage of the symptoms to milk ingestion was later confirmed by the reintroduction of milk, and by a single blind challenge test using milk protein capsules.

The two groups of patients expressed median titre values of 90 AU and 600 AU, respectively. A cut-off value of about 300 AU divided the two populations, illustrating the hyper-reactive character of this IgG response in acutely CM-allergic children. The two hospitalized populations (allergic and non-allergic) did not differ significantly in age (average ages being 5.6 months and 7.6 months, respectively; ranging from 1 to 48 months), sex ratio (35 m/50 vs 74 m/126), or target organ. Tests for specific IgE against cow's milk were positive in 1/50 and 16/126 patients, respectively. The presence or absence of specific IgE was not associated with any difference in anti-BLG antibody levels.

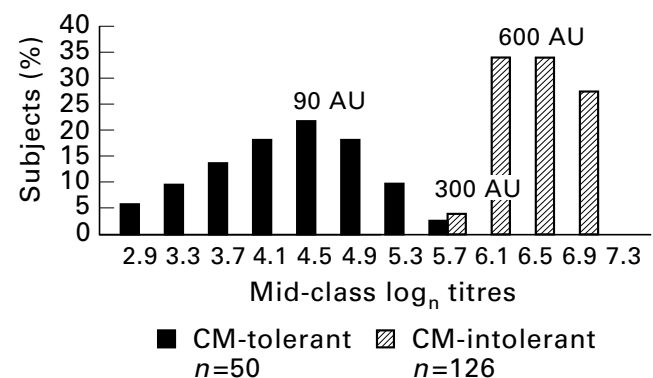


Fig. 1. Frequency histograms of anti-nBLG IgG antibody titres.

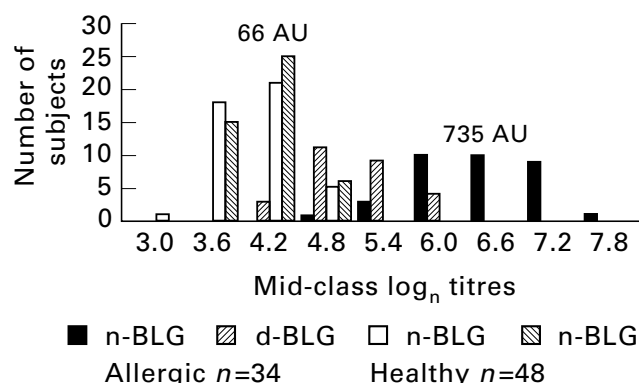


Fig. 2. Frequency histograms of anti-BLG Ig in allergic and healthy children.

Binding to nBLG and dBLG of IgG from allergic and healthy children (Fig. 2)

In this section we refer mostly to outpatients, who were usually less severely affected than the patients described above, and some of whom were already undergoing a CM eviction diet at the time of investigation. Figure 2 shows the frequency histograms for IgG-antibody levels, expressed in AU and after natural logarithmic transformation, directed against nBLG and dBLG, measured in parallel, in 34 CM-allergic children and 48 age-matched controls. The allergic group had mid-class values for anti-nBLG IgG antibodies ranging from 144 to 2180 AU with a median value of 742 AU. Antibody titres against the denatured protein, had mid-class values ranging from 85 to 498 AU with a median of 182 AU. In all 34 cases the values were lower for the denatured antigen, making the group response highly significant ($P < 0.0001$; paired *t*-test).

The control group of healthy children showed a mid-class value range of 28–215 AU with a median value of 77 AU for anti-nBLG IgG, significantly lower than those values for the allergic group ($P < 0.0001$; Student *t*-test).

The distribution range (33–274 AU) and median (69 AU) of IgG-antibody binding levels to the denatured protein are not distinct ($P > 0.9$; paired *t*-test) from those determined for the native protein.

Comparing the two populations, CM-allergic patients have significantly higher mean values of IgG antibodies directed both towards the native and denatured proteins, than their matched controls, although there is some overlapping of the two populations in the case of dBLG.

Specificity controls

As a control for the specificity of binding, representative sera were also tested for their IgG retention of native and

denatured forms of other proteins including: human albumin, tetanus toxoid, a pollen antigen extract (*Anthoxanthum Odoratum*) and Der p 1, the major house dust antigen [18]. No binding was observed with human albumin, and an independent level of binding with the other antigens.

The specific nature of this binding was confirmed by solid-phase adsorption experiments using column chromatography with the selected pooled sera, and relevant proteins, the subsequent recovery of the specific-antigen-binding-capacity of the eluted IgG fraction. Similar experiments showed that 1000 arbitrary units of pooled anti BLG IgG from allergic subjects corresponded to about 100 ng of purified IgG. In an ELISA adapted to study antigenic competition, it was demonstrated that the binding capacity of 1000 A. U of anti-BLG IgG from hyperimmunized rabbit could be reduced by 50% with 100 pg of native or denatured BLG (in solution). Using pooled sera from allergic subjects, an equivalent titer of 1000 AU needed about 300 pg/mL of nBLG and about 1200 pg dBLG to produce a similar reduction in binding capacity.

Competitive inhibition of nBLG and dBLG binding to biotinylated pooled IgG fractions (Fig. 3)

Figure 3(A–D) show the frequency histograms of percentage binding inhibitions obtained with individual sera from healthy and allergic children.

For this study, the allergic group comprised newly diagnosed cases of children with overt symptoms of CM-allergy.

Competition with pooled IgG from CM-allergic sera

Figure 3(A) shows the distribution of percentage binding inhibitions produced by individual sera from healthy and allergic children using the nBLG-binding capacity of biotinylated pooled IgG from allergic sera as reference. Two distinct populations/distributions were observed with only marginal overlapping between them.

CM-allergic sera ($n = 44$) showed mid-class percentage binding inhibitions ranging from 35 to 80% with a class median of 50%. Healthy control sera ($n = 44$) exhibited lower binding inhibitions ranging from 0 to 35% with a mid-class median of 25%.

The two populations were statistically very different ($P < 0.0001$; Student *t*-test).

Figure 3(B) shows data obtained in parallel experiments using the binding capacity for dBLG of the same allergic pooled IgG fraction as reference. Again two clearly separate distributions were observed for the two groups of sera. Percentage binding inhibitions exhibited by the allergic sera ranged from 45 to 85% with a mid-class median of 65% whilst those of the healthy control sera ranges from 0 to 55% with a median of 45%. The two (non-Gaussian)

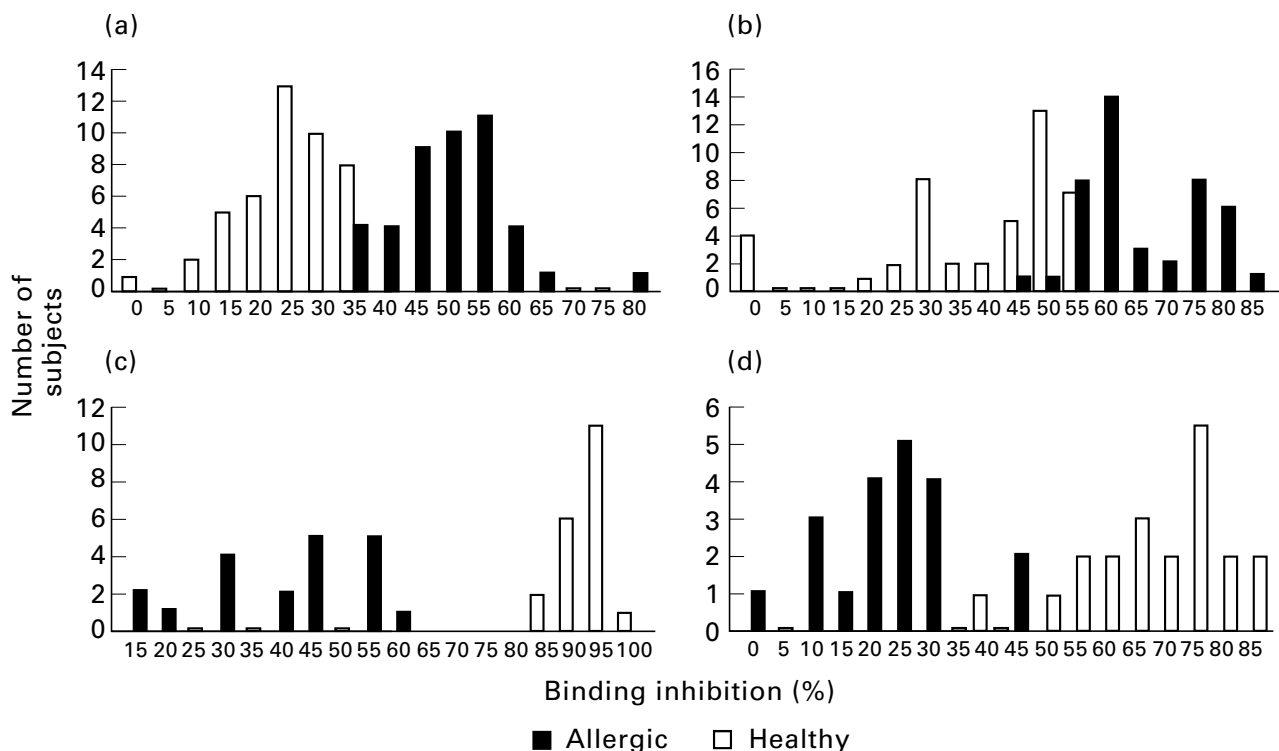


Fig. 3. Frequency histograms of percentage binding inhibitions.

distributions were again statistically very different ($P < 0.0001$; Mann–Whitney non-parametric test).

From allergic sera, the distribution was again clearly separated for the two groups.

Competition with pooled IgG from healthy sera

Figure 3(C and D) show the results of competitive binding experiments similar to those described above using, respectively, the nBLG- and dBLG-binding capacities of biotinylated pooled IgG from healthy sera as reference. Owing to a lack of availability of samples of IgG from healthy children, the pooled IgG used in these experiments were obtained from healthy adult blood donors. This substitution was made on the grounds that earlier studies had shown no qualitative differences between the anti-BLG antibodies of healthy children and those of healthy adults.

In Fig. 3(C) two distinct distributions are again observed corresponding to the individual sera from healthy and CM-allergic children. The relationship between the two nonoverlapping populations is, however, the reverse of that seen above. Allergic sera ($n = 20$) exhibited mid-class percentage binding inhibitions from 15 to 60% with a mid-class median of 40%. Sera from healthy children ($n = 20$) showed higher binding inhibitions ranging from 85 to 100% with a median class of 95%. The two populations were

confirmed to be statistically very different ($P < 0.0001$; Mann–Whitney non-parametric test).

Parallel experiments performed with individual sera from adult blood donors produced similar results (distribution, range, and median) to those obtained for the healthy children (data not shown), confirming that the behaviour of healthy sera was not age dependent.

A similar pattern of distributions is also seen in Fig. 3(D), referring to the capacity of sera from CM-allergic and healthy children to inhibit dBLG binding by healthy pooled IgG. Allergic sera again exhibited lower mid-class percentage inhibitions (ranging from 0 to 45%, with a class median of 70%). Once more, the inhibitory capacities of the two populations of sera were very significantly different ($P < 0.0001$) Mann–Whitney non-parametric test).

Evolution of binding inhibition and antibody level with acquired tolerance

A subgroup of 14 allergic children were tested before and some time after the initiation of what proved to be a successful CM-eviction diet. All had become completely asymptomatic at the time of the second test, but eight of them still presented relapses towards their allergic symptomatology when milk was reintroduced into their diet either accidentally or as a tolerance test. The other six patients

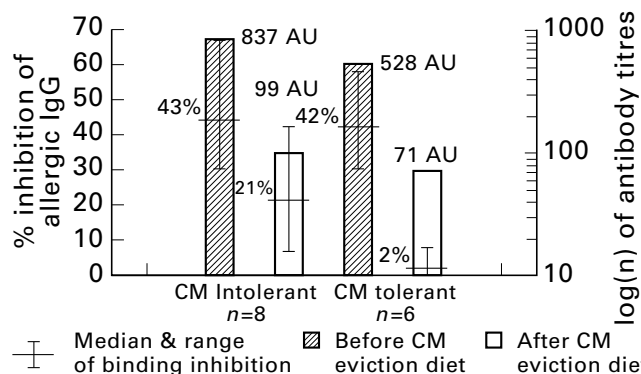


Fig. 4.

developed complete tolerance to CM, and could end the eviction diet.

Figure 4 summarizes the results for these two small series of patients. The first group of patients had started their diet with a median level of 837 AU of anti-nBLG IgG. This level declined to 99 AU at the time of the second testing. Their capacity of their sera to inhibit allergic IgG binding to nBLG ranged from 28%–binding inhibition to 67% (median 43%) at the start of the diet and reduced for all patients by the second test to 7%–43% (median 21%).

The second group of patients started their diet with an equivalent median level of anti nBLG IgG of 628 AU. This in turn was reduced to 71 AU at the time tolerance was acquired ($P < 0.05$ paired Wilcoxon non-parametric test), a level equivalent to that of the group of asymptomatic but still intolerant patients. However, what is important is the evolution of the capacity of their sera to inhibit nBLG binding by IgG from allergic children. Starting with percentage binding inhibition values equivalent to the first group of 32–60% (median 42%), these values fell to almost insignificant levels (0–8%; median 2%) by the time of the second test, making this group clearly very different from the asymptomatic but still intolerant group ($P < 0.005$; Mann–Whitney non-parametric test).

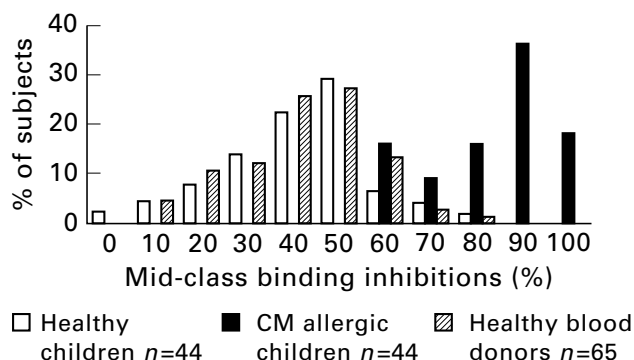


Fig. 5.

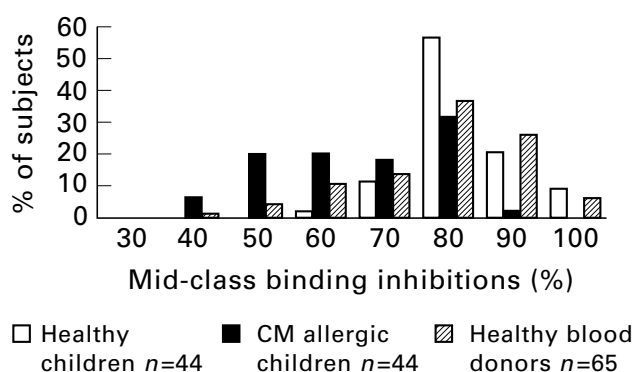


Fig. 6.

Inhibition of the binding capacity of biotinylated mouse monoclonal antibodies specific to nBLG

Of a total of 12 MoAb, three were selected for further study due to their potential for epitopic discrimination. Figures 5 and 6 illustrate the results obtained with two MoAb, designated M5 and M7.

Figure 5 shows the distribution of percentage binding inhibition produced by healthy and allergic sera with respect to the nBLG-binding capacity of biotinylated MoAb M5. Two distinct distributions are observed. CM-allergic sera ($n = 44$) showed mid-class percentage binding inhibition ranging from 50 to 100% with a median value of 85%.

Sera from healthy children ($n = 44$) exhibited mid-class values ranging from 0 to 80% with a median value of 50%. These two distributions were again significantly very different ($P < 0.0001$; Mann–Whitney non-parametric test) despite some overlapping which included about 25% of the allergic and 10% of the healthy child populations.

Sera from healthy blood donors ($n = 65$) showed a very similar distribution of percentage binding inhibitions to that of healthy children.

Similar experiments performed with MoAb M6 led to distribution pattern which were superimposable with those above (data not shown) indicating the non-exceptional character of the underlying specificity.

Interestingly, using M7 mouse monoclonal antibodies in the same experiments revealed once again separate distributions for the two groups of children (Fig. 6), but in the reverse sense: allergic sera had a lower inhibitory capacity than the control group, with mid-class binding inhibitions ranging from 40 to 90%, and with a median value of 65%, compared to 60–100%, with a median value of 80% for the control group. Here again healthy blood donors responded in the same way as the healthy children. The difference between the allergic and the control groups was significant ($P < 0.001$; Mann–Whitney non-parametric test). Since the relationship between these groups is opposite to that which would have been expected looking at antibody titres alone,

it emphasizes the importance of epitope specificity differences between allergic and control subjects.

Discussion

The present study focuses on the IgG-antibody response to BLG in a population of children clinically defined as CM allergic. Although not considered to be involved in the development of the observed symptomatology, IgG antibodies represent an accessible marker indicating the level of immune response against one of the major food antigens to which humans are exposed life long. At the time of maximum expression of allergic symptoms, leading to need for hospitalization, the level of these antibodies dramatically increased in almost all patients, independently of the levels of specific IgE. The direct relationship between levels of IgG antibodies to BLG and the success of eviction diet has led us to use them as an indicator for diet manipulations.

This is completely different from what could be observed in non-allergic subjects where the low level range of anti BLG IgG was clearly independant from milk consumption, as shown by the unchanged titres after a 2 week fasting cure in obese adults (personnal data).

Our results however, oppose those of several other studies [4–8]. It is possible that these discrepancies are the results of some methodological aspects of the *in vitro* testing that have been underestimated, such as the conservation of conformational epitopes in the bound antigen. The relative participation of conformational vs structural epitopes of the BLG protein in the IgG-antibody binding has been investigated here by using both relatively intact protein and the products of incomplete pepsin digestion as solid-phase adsorbed antigen. The results clearly document that in allergic patients an important part of their IgG antibodies is directed against epitopes of the native protein that are destroyed by peptic digestion. In contrast the natural antibodies of nonallergic subjects recognize epitopes unaffected by this denaturation process. Altered antigen structure can dramatically abrogate the clinical discriminant capacity of the test.

It should be emphasized that parallel determinations of antinative- and denaturated-BLG-IgG antibodies in healthy adults (18–65 years old) displayed exactly the same range and distributions of values here as those described for healthy children. This has also been extensively reported elsewhere [23].

Our results contradict those of Kohno *et al.* [24] who found preferential recognition of primary protein structures by IgG and IgE antibodies of patients with milk allergy, using alpha casein as the antigen reference. It should be pointed out, however, that alpha casein does not represent a single molecule [25] like BLG or alpha lactalbumin.

Heterogeneity of native molecules is likely to complicate specific discrimination by increasing the number of potential epitopes and thus the overlapping results. Our finding that there is a preference for recognition of native over digested allergen by IgG from allergic patients has now been extended from Der p1 and BLG to other allergens extracted from pollens and insect venom (Michils *et al.* submitted for publication). The clinical relevance of anti-BLG antibodies against denatured antigen is not documented here. Antigenic competition could be observed between milk hydrolysate solutions and denatured BLG for labelled IgG from CM allergies (data not shown).

Further precise specificity of the IgG antibodies involved was analysed through direct competition between IgG fractions that had been purified from a pool of subjects belonging to the same clinical category. The use of pooled sera was intended to provide a varied mixture of different individual specificities which could be used as a public general reference against individual patterns of immunodominance of individual sera. The homogeneity of the response within each clinical category indicates the restricted nature of a shared pattern of epitope recognitions. It was demonstrated here in a series of parallel experiments using labelled (pooled) IgG from each reference group against an extended series of individual sera obtained from allergic or non-allergic subjects. A stronger capacity to compete for binding to native or digested BLG by sera from allergic patients as compared with sera from healthy subjects could be predicted on the basis of differences in titres. This was indeed the case in competition experiments involving labelled (pooled) IgG from allergic subjects. In contrast, sera from non-allergic children are the more efficient competitors when challenged with labelled (pooled) IgG from healthy subjects, despite having near anti-BLG IgG levels about 8–10 times lower than those of allergic patients. The repeated correspondence, between results obtained in all cross-experiments, confirms the relationship between clinical status and the antigen binding specificity (cf *infra*). Moreover the same observations were made both for the native and digested antigen documenting further that even among conformational and structural epitopes types, belonging to a particular clinical category determines a specific set of immunodominant epitopes which are only marginally or not at all recognized by other clinical groups.

Further data to support the recognition of unique epitopes by the different clinical groups was generated by experiments using monoclonal antibodies as labelled references. Allergic and control sera could be distinguished on the basis of their particular specificities in the same way as labelled pooled IgG. All the monoclonal antibodies bound native BLG almost exclusively. This indicates that hyperimmunized mouse antibodies preferentially recognize native BLG epitopes that may represent part of a common set recognized

either by allergic or healthy humans, at least as part of their expressed specificities.

The observed evolution of antigen-binding specificities with the acquisition of relative or complete clinical tolerance documents the plasticity of the immunodominant epitopic profile. It appears to be independent of the antibody titer, which seems directly related to the level of antigen exposure, decreasing rapidly within a few weeks after initiation of an eviction diet. This is only visible for allergic subjects (cf *supra*). In contrast, a functional correlation exists between the clinical status and the immunodominant profile. Indeed, partially tolerant children still have similar anti-BLG IgG binding specificities to overtly allergic patients, but this is no longer the case when they become completely tolerant.

The specificity disclosed by the competitive binding test discriminates between these two groups of patients at a time where antibody titers are reduced to the level of those of asymptomatic subjects. The reasons behind these relationships are difficult to understand at the moment. We are presently unaware of any functional role for these IgG antibodies, although their specificity could be related to specificities expressed at other levels such as that of coexisting IgE antibodies. It has been documented in mice by Kolbe [26], that in cases where the antigen dose is low, the epitope specificities of IgG and IgE antibodies differed, preventing competitions therefore. With larger antigen doses administrations, and the subsequent decrease in IgE, class-restricted specificities were fused, allowing true competition in favour of IgG blocking activity.

It must be remembered that most of the allergic patients in this study had no detectable specific IgE, in spite of displaying recognizable atopic symptoms. Discussing the sensitivity of IgE tests in very young children is however, beyond the scope of the present study. What the present study shows is a common specificity pattern of anti BLG IgG in allergic patients as opposed to asymptomatic subjects.

It seems therefore that patients producing no or very low levels of specific IgE, may share some driving factor determining immunodominance at the level of their IgG antibodies. This study extends the scope of our previous observations obtained with house dust mite allergic patients in two ways: firstly, by confirming the association between an IgG immunodominant pattern and clinical status, and secondly by documenting this association in patients with no detectable levels of specific IgE against the involved antigen. If confirmed, these findings could help to re-evaluate the involvement of some allergens in some patients, where IgE tests are negative. Preliminary data obtained in cases of atopic eczema would appear to suggest this, since only a minority of the patients who showed IgG immunodominance against Der p 1 antigen had detectable

levels of anti Der p1 IgE (Laurence Baras- manuscript in preparation).

The influence of clinical status on the set of epitopes which are recognized as dominant somewhat challenges the current view that, at the B cell level, immunodominance is mostly dependent upon the molecular architecture of antigens [27–29]. It seems probable that many factors could alter the usual antigen presentation (e.g. infections, and available antibodies, in particular those transferred from the mother [30].

The mechanism which dictates the changing profile of epitopic dominance in cow's milk allergic patients remains unknown. Even if an eviction diet rapidly reduces antibody titre levels, the immunodominant profile remains longer (months thereafter at least). The production of antibodies against conformational epitopes would seem to be less easy to maintain over a period of time than antibodies against structural epitopes. This is deducible from the data from healthy controls who continue to absorb milk and thus remain chronically exposed to native antigens. It could question, for example, the structural integrity of the antigenic reserves used to maintain memory and allow sustained antibody renewal [31].

The relationship between epitopic structures belonging to a particular set of immunodominant epitopes is also unknown, but it would be usual for them to be involved in the allergen recognition process. It remains speculative to suggest that some combinations of antibodies could bind antigen more efficiently and allow or impede the bridging of adjacent Fc receptors, subsequently allowing or not stimulation of the relevant cell.

The present methodology linking the clinical ability to display allergic symptoms with an apparent epitopic specificity signature represents a completely new approach to the diagnosis of allergy. The extent of the specificity of the test requires further prospective investigation.

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