Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes in β -lactoglobulin by alanine scanning analysis

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ABSTRACT

β -Lactoglobulin represents one of the major allergens causing cow milk allergy. Few studies have clearly evaluated immunological relationships between IgE- and IgG-binding epitopes of β -lactoglobulin. For characterization of immunological epitopes, peptides of 15 amino acids (AA) in length were synthesized. Immunoglobulin E- and IgG-binding epitopes were immunolabeled with individual sera from cow milk-allergic patients. Alanine scanning of immunodominant epitopes was used to identify the critical AA of IgE- and IgG-binding epitopes. The results showed that 4 IgE-binding epitopes were identified. Our initial data revealed IgE-binding epitopes at AA 17 to 31, AA 72 to 86, AA 92 to 106, and AA 152 to 166. Threonine 20, Met23, and Asp27 are the critical AA of IgE-binding epitopes. Two IgG epitopes were identified, which were located at AA 22 to 36 and AA 127 to 141. The critical AA of IgG-binding epitopes were Leu26 and Val31. Results obtained from this study will provide necessary information to alter the cDNA to encode a protein capable of activating milk-specific T cells, but with reduced IgE- or IgG-binding capacity.

Key words: β -lactoglobulin, cow milk allergy, epitope, critical amino acid

INTRODUCTION

Cow milk is one of the first foods introduced into an infant's diet, and cow milk allergy (CMA) is the most prevalent food allergy in the first year of life, affecting about 2.5% of infants (Bock, 1987; Saarinen et al., 1999). Cow milk proteins have been well characterized and divided into casein and whey fractions. Whey contains essentially β -LG and α -LA. β -Lactoglobulin is one of the first foreign antigens encountered by a

newborn child, as it does not exist in human milk. β -Lactoglobulin occurs naturally in the form of a 36-kDa dimer, with each subunit corresponding to a 162-residue polypeptide (Wal, 1998). It possesses 2 disulfide bridges and 1 free cysteine. It has no homologous counterpart in human milk. The relative resistance of β -LG to acid hydrolysis and to proteases allows some of the protein to remain intact after digestion, increasing the probability of absorption of both intact β -LG and digested peptides. β -Lactoglobulin, together with several allergens of animal origin, belongs to the lipocalin family, whose members share a similar tertiary (β -barrel) structure and high affinity for hydrophobic ligands, a characteristic that makes them potentially highly allergenic (Järvinen et al., 2001).

Cow milk allergy shows various clinical symptoms and involves different immunoglobulin antibodies. The immediate (type I) reaction based on IgE antibodies is considered to be the most common, at least in infants. The reaction may take place in the mucosa or in the circulation, and it is manifested soon after exposure. Typically, there is infiltration of eosinophilic leucocytes to the reaction site and into the peripheral blood. The detection of milk protein-specific IgG antibodies (Saalman et al., 1995) and duodenal lymphoid hyperplasia (Kokkonen et al., 1999), suggesting local production of IgG class antibodies, in children with CMA indicates that a type III reaction might also be involved. The clinical manifestations resulting from such reactions may be local intestinal or general (or both), with the latency (with respect to the ingestion of the relevant food allergen) lasting from hours to days, or even weeks (Hill and Hosking, 1995).

An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by antibodies, B cells, and T cells. Information regarding the immunodominant epitopes may be useful in the development of more specific diagnostic methods of CMA and eventually lead to safe, effective therapeutic approaches for patients with CMA. A region was defined as an epitope if it was statistically associated with reactive groups

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and recognized by at least 65% of reactive patients. In the present study, we aimed to map IgE and IgG epitopes of β -LG using sera from infant patients allergic to cow milk and found the critical AA for reducing the allergenicity of β -LG by alanine scanning analysis.

MATERIALS AND METHODS

Sera from Patients with CMA

Six CMA sera from 45 clinical examined infants were selected. Sera from 6 patients with documented cow milk hypersensitivity (median age at the time of collection of sera was 1 yr, range 6 mo to 3 yr) were used to identify IgE- and IgG-binding epitopes. Milk-specific IgE antibodies ranged from 25 to 100 kU/L as measured by the CAP System FEIA (Phadia Diagnostics, Uppsala, Sweden). Based on the clinical history of the infants, 6 had atopic dermatitis. Five non-milk-allergic children (aged 3 to 6 yr) with atopic dermatitis and allergy to other foods (i.e., tomato, shellfish, peanuts, and nuts) were used as control sera. Informed consent was obtained from the patients and their parents and the study was approved by Peking University Third Hospital (Beijing, China).

Synthesis of β -LG-Derived Peptides

For mapping and characterization of a dominant epitope and a critical AA, a library of peptides (15 AA in length) based on the sequence of β -LG were synthesized using the multiple method according to the Fmoc strategy (Cerecedo et al., 2008). The substitution of a single AA residue was done for a dominant epitope. All peptides were purified by C_{18} reverse-phase HPLC (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

IgE-Binding Assay

Immunoglobulin E ELISA was performed as described by Järvinen et al. (2001). Before immunological detection, the purified peptides were coupled with BSA. Then, the peptides that reacted with BSA were combined with 96-well plates. Peptides were incubated with individual serum samples for identifying IgE-binding epitopes or a serum pool for identifying IgE-binding AA diluted (1:10) in a solution containing Tris/NaCl (10 mM Tris/HCl and 500 mM NaCl, pH 7.5) and 1% BSA for 2 h at 37°C. The primary antibody was detected using monoclonal mouse anti-human-IgE (1:2,000; Pharmacia & Upjohn Diagnostics GmbH & Co. KG, Uppsala, Sweden) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining, then expressed as A450.

Between each step, plates were washed 5 times with Tris-buffered saline containing 0.1% Tween 20. Nonspecific binding was studied using serum from nonallergic individuals and allergic individuals (but not cow milk allergic). An epitope can be considered immunodominant if it is recognized by above 65% serum IgE from the majority of patients with cow milk hypersensitivity. Critical AA were defined as those that resulted in loss of IgE binding when substituted by an alanine residue.

IgG-Binding Assay

A modified IgG ELISA was performed as described earlier (Järvinen et al., 2001). Peptides were incubated with individual serum samples for identifying IgG-binding epitopes or the serum pool for identifying the critical AA from patients with cow milk hypersensitivity diluted (1:20) in a solution containing Tris/NaCl (10 mM Tris/HCl and 500 mM NaCl, pH 7.5) and 1% BSA for 2 h at 37°C. The primary antibody was detected using monoclonal goat anti-human IgG (1:3,000; Pharmacia & Upjohn Diagnostics GmbH & Co. KG) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining, and then expressed as A450. Between each step, plates were washed 5 times with Tris-buffered saline containing 0.1% Tween 20. Nonspecific binding was studied using serum from nonallergic individuals and allergic individuals (but not cow milk allergic). An epitope can be considered immunodominant if it is recognized by above 65% serum IgG from the majority of patients with cow milk hypersensitivity. Critical AA were defined as those that resulted in loss of IgG binding when substituted by an alanine residue.

RESULTS

Peptide Synthesis

To determine the AA sequence of IgE- and IgG-binding sites, 30 overlapping peptides spanning β -LG were synthesized, shown in Table 1. Each peptide was 15 AA long; the peptides were offset from each other by 5 AA. All peptides were purified by C_{18} reverse-phase HPLC.

Identification of IgE-Binding Epitopes

Each set of 30 peptides was probed individually with serum IgE from 6 different patients. The intensity of IgE binding to each peptide was determined as a function of that patient's total IgE binding to these 30 peptides, shown in Figure 1. Immunoglobulin E antibodies from 6 patients showed binding to at least 1 linear epitope. Thirty peptides were recognized by 6 sera. The peptide

Figure 1. The IgE-binding epitopes of β -LG. The intensity of IgE binding to each peptide was determined as a function of that patient's total IgE binding to these 30 peptides. Thirty peptides were recognized by 6 sera. The peptide coded as B01 at AA 17 to 31 was identified by IgE from 6 sera. The sequence at AA 72 to 86 was recognized by 5 of 6 sera. The sequences at AA 92 to 106 and AA 152 to 166 were identified by 4 of 6 sera. The peptides coded as B04, B07, B20, and B26 were recognized by 3 of 6 sera. Other peptides coded as B03, B05, B09, B10, B29, and B30 were also recognized by individual serum samples. OD = optical density. Error bars represent the SEM ($n = 4$).

coded as B01 at AA 17 to 31 was identified by IgE from 6 sera. Other peptides were tested in the same manner. The sequence at AA 72 to 86 was recognized by 5 of 6 sera. The sequences at AA 92 to 106 and AA 152 to 166 were identified by 4 of 6 sera. The peptides coded as B04, B07, B20, and B26 were recognized by 3 of 6 sera. Other peptides coded as B03, B05, B09, B10, B29, and B30 were also recognized by individual serum samples.

Table 1. The AA sequence of peptides synthesized

Code	Sequence	AA position in β -LG
B01	LIVTQTMKGLDIQKV	17–31
B02	TMKGLDIQKVAGTWY	22–36
B03	DIQKVAGTWYSLAMA	27–41
B04	AGTWYSLAMAASDIS	32–46
B05	SLAMAASDISLLDAQ	37–51
B06	ASDISLLDAQSAPLR	42–56
B07	LLDAQSAPLRVYVEE	47–61
B08	SAPLRVYVEELKPTP	52–66
B09	VYVEELKPTPEGDLE	57–71
B10	LKPTPEGDLEILLQK	62–76
B11	EGDLEILLQKWENGE	67–81
B12	ILLQKWENGECAQKK	72–86
B13	WENGECAQKKIIAEK	77–91
B14	CAQKKIIAEKTKIPA	82–96
B15	IIAEKTKIPAVFKID	87–101
B16	TKIPAVFKIDALNEN	92–106
B17	VFKIDALNENKVLVL	97–111
B18	ALNENKVLVLDTDYK	102–116
B19	KVLVLDTDYKKYLLF	107–121
B20	DTDYKKYLLFCMENS	112–126
B21	KYLLFCMENSAEPEQ	117–131
B22	CMENSAEPEQSLACQ	122–136
B23	AEPEQSLACQCLVRT	127–141
B24	SLACQCLVRTPEVDD	132–146
B25	CLVRTPEVDDEALEK	137–151
B26	PEVDDEALEKFDKAL	142–156
B27	EALEKFDKALKALPM	147–161
B28	FDKALKALPMHIRLS	152–166
B29	KALPMHIRLSFNPTQ	157–171
B30	HIRLSFNPTQLEEQC	162–176

Sera from non-milk-allergic children showed weak reaction with synthesized peptides (data not shown).

Identification of IgG-Binding Epitopes

The intensity of IgG binding to each peptide was determined as a function of that patient's total IgG binding to these 30 peptides, shown in Figure 2. Individual sera showed different IgG-binding ability to these 30 peptides. The sequence of AA 22 to 36 (B02), considered as an IgG-binding epitope, was recognized by the sera of 5 of 6 patients. The peptide at AA 127 to 141 (coded as B23), considered as an IgG-binding epitope, was identified by 4 of 6 sera. Also, the peptides at AA 42 to 56 and at AA 72 to 86 were recognized by 3 of 6 sera; B01, B08, B10, B15, B16, B18, B22, B26, B27, and B30 were all identified by 1 serum sample.

Characterization of the Critical AA of the IgE-Binding Epitope at AA 17 to 31

The AA essential to IgE binding in the epitope at AA 17 to 31 were determined by synthesizing duplicate peptides with single AA changes at each position. These peptides were then probed with pooled serum IgE from 6 patients with cow milk hypersensitivity to determine if the change affected cow milk-specific IgE binding (results are shown in Figure 3). The peptide at AA 17 to 31 with alanine substituted for threonine at position 20 was not recognized by pooled serum IgE. The peptide with alanine substituted for methionine at position 23 was not identified by pooled serum IgE. The peptide with alanine substituted for aspartic acid at position 27 was not recognized by pooled serum IgE. Compared with the control group (no AA substitutions), the peptide with alanine substituted for glycine at position 25 showed high intensity of IgE-binding abil-

Figure 2. The IgG-binding epitopes of β -LG. The intensity of IgG binding to each peptide was determined as a function of that patient's total IgG binding to these 30 peptides. Individual sera showed different IgG binding ability to these 30 peptides. The sequence of AA 22 to 36 (B02), considered as an IgG-binding epitope, was recognized by the sera of 5 of 6 patients. The peptide at AA 127 to 141 (coded as B23), considered as an IgG-binding epitope, was identified by 4 of 6 sera. Also, the peptides at AA 42 to 56 and at AA 72 to 86 were recognized by 3 of 6 sera. B01, B08, B10, B15, B16, B18, B22, B26, B27, and B30 were all identified by one serum sample. OD = optical density. Error bars represent the SEM ($n = 4$).

ity. The peptide with alanine substituted for leucine at position 26 showed low intensity of IgE-binding ability. The intensity of other peptides with alanine substituted for 1 of the AA residues showed no difference compared with the control group.

Characterization of the Critical AA of the IgG-Binding Epitope at AA 22 to 36

To identify the AA critical for IgG binding in the sequence of AA 22 to 36, a series of related peptides were synthesized with alanine substitutions at each

position of the 15-mer epitope and their IgG-binding abilities were evaluated (Figure 4). A significant loss of IgG binding was observed for 2 peptides, one with alanine substituted for leucine at position 26 and the other with alanine substituted for valine at position 31. Peptides at AA 22 to 36 with alanine substituted for isoleucine at position 28 resulted in low IgG-binding ability compared with the control group. The peptides with alanine substituted for glycine at position 33 also showed low IgG-binding ability. Substitution of other positions on this peptide did not cause a consistent decrease in IgG binding.

Figure 3. Single AA changes to peptide at AA 17 to 31 result in loss of IgE binding to this epitope. The peptide at AA 17 to 31 was synthesized with an alanine residue substituted for one of the AA in this peptide and probed with a pool of serum IgE from 6 patients with cow milk allergy. The letters across the bottom of each panel indicate the one-letter AA code for the residue normally at that position and the AA that was substituted for this residue. The numbers indicate the position of each residue in β -LG protein. The control group indicates the native epitope (no AA substitutions). OD = optical density. Color version available in the online PDF.

Immunological Relationship Between IgE- and IgG-Binding Epitopes

Ten AA overlapping regions (AA 22 to 31) were found to cross-react with IgE and IgG (shown in Table 2). The 10 AA were Thr, Met, Lys, Gly, Leu, Asp, Ile, Gln, Lys, and Val.

DISCUSSION

In the present study, we determined that multiple antigenic sites were predicted for the β -LG allergen. Thirty peptides synthesized are distributed throughout the protein. Recent evidence suggests a preferential variable heavy chain usage in IgE synthesis and direct switching from IgM production to IgE synthesis. This would suggest that epitopes responsible for antigen-specific IgE antibodies production may differ from those promoting antigen-specific IgG antibodies. Immunotherapeutic approaches using peptides representing IgG epitopes may be able to shift the balance of antigen-specific antibody production from IgE to IgG. We are currently identifying 10 AA overlapping regions at AA 22 to 31

Table 2. The AA sequence of β -LG¹

AA residue position and identity																			
10					20					30					40				
M	K	C	L	L	L	A	L	A	L	L	A	L	I	V	T	Q	D	L	K
A	A	S	D	I	S	L	L	D	A	Q	S	A	P	L	R	V	V	E	L
E	C	A	Q	K	I	I	A	E	K	T	K	I	P	A	V	F	K	I	D
F	C	M	E	N	S	A	E	P	E	Q	S	L	A	C	Q	C	L	V	E
M	H	I	R	L	S	F	N	P	T	Q	L	E	E	Q	C	H	I	R	E

¹The numbers indicate the position of each residue in β -LG protein. Bold sections indicate IgE-binding epitopes. Underlined sections indicate IgG-binding epitopes. Section with small capital letters indicates overlapping region between IgE- and IgG-binding epitopes.

Figure 4. Single AA changes to peptide at AA 22 to 36 result in loss of IgG binding to this epitope. The peptide at AA 22 to 36 was synthesized with an alanine residue substituted for one of the AA in this peptide and probed with a pool of serum IgE from 6 patients with cow milk allergy. The letters across the bottom of each panel indicate the one-letter AA code for the residue normally at that position and the AA that was substituted for this residue. The numbers indicate the position of each residue in β -LG protein. The control group indicates the native epitope (no AA substitutions). OD = optical density. Color version available in the online PDF.

and this region would be a feasible therapy target for patients with cow milk hypersensitivity.

Previous studies that used digested peptide fragments or synthetic peptides have reported different findings with respect to the major sequential IgE-binding regions on β -LG (Tokita, 1985; Adams et al., 1991; Ball et al., 1994; Sélo et al., 1998, 1999; Williams et al., 1998; Heinzmann et al., 1999). In this study, we identified IgE-binding epitopes of β -LG located at AA 17 to 31, AA 72 to 86, AA 92 to 106, and AA 152 to 166 by the serology method. The epitope at AA 17 to 31, located in the N-terminal region of β -LG, is significantly different from other families and generic proteins as the conserved sequence. Because this epitope is mainly composed of hydrophobic AA, it may locate in the inner β -LG spatial structure. The sequence at AA 152 to 166, located in the C-terminal of β -LG, is similar to other animal milk-derived proteins, and this phenomenon may explain the reason for cross-reaction among different animal milk proteins. The epitope at AA 92 to 106 may be consistent with the conclusions of Ball et al. (1994) and Heinzmann et al. (1999). Our initial data found IgE-binding epitopes located at AA 72 to 86 and AA 152 to 166; these have not been reported in other papers.

Järvinen et al. (2001) showed that the IgG-binding sequences generally colocalized with the IgE-binding epitopes, except for region AA 31 to 50 on β -LG. However, we identified the only overlapping region of IgE- and IgG-binding epitopes of β -LG located at AA 22 to 32. Two IgG-binding epitopes were recognized,

located at AA 22 to 36 and AA 127 to 141. The IgG-binding epitope at AA 22 to 36 is mainly composed of hydrophobic AA, so it may be located in the inner β -lactoglobulin spatial structure; moreover, this epitope has not been reported in previous studies.

CONCLUSIONS

Here, a series of AA was identified by alanine substitution scanning. Replacement of 1 basic AA for immunodominant epitopes by alanine resulted in loss of allergenicity and was considered as a critical AA for reducing the allergenicity of β -LG. The critical AA necessary for IgE and IgG binding in bovine β -LG were identified using pooled sera. Interestingly, a heterogeneous pattern of critical AA was found between IgE- and IgG-binding epitopes. Threonine at position 20, methionine at position 23, and aspartic acid at position 27 are the critical AA of IgE-binding epitopes in β -LG. Moreover, leucine at position 26 and valine at position 31 are the critical AA of IgG-binding epitopes in β -LG. This information is necessary to engineer recombinant proteins with decreased allergenicity for use in immunotherapeutic reagents for reversing CMA. Furthermore, knowledge of the AA positions of those that are not as critical for IgE binding may help in developing strategies to silence epitopes of other allergens. Because of the difficulties in time and funding, the number of tested patients' sera may be limited. In addition, the low incidence of cow milk allergy in China may increase the difficulty of collecting of cow milk-allergic patients' sera. However, this study is very important, as it provides basic data and theoretical methods for drawing general conclusions in the future. We will continue collect cow milk-allergic patients' sera to study the immunological mechanism of cow milk allergens in the next few years. Our data shows that it may be possible to mutate β -LG to no longer bind IgE or IgG. This raises the possibility that an altered β -LG gene could be used to replace its allergenic homolog in the β -LG genome.

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