Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes on α_{s1} -casein by alanine scanning analysis

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ABSTRACT

α_{s1} -Casein represents one of the major allergens causing cow milk allergy. Few studies have clearly evaluated immunological relationships between IgE- and IgG-binding epitopes of α_{s1} -casein. This study aimed to map IgE- and IgG-binding epitopes of α_{s1} -casein by the serology method, and identify the critical amino acids of α_{s1} -casein by alanine scanning analysis. Our initial data revealed IgE-binding epitopes located in the sequences of AA 126 to 140, AA 6 to 20, AA 171 to 185, and AA 11 to 25. The sequences at AA 21 to 35, AA 56 to 70, and AA 161 to 175 were recognized by IgG antibodies. The alanine scanning analysis showed that IgE- and IgG-binding epitopes share the same critical AA: arginine at position 22 and phenylalanine at position 23. Results obtained from this study will provide necessary information to alter the cDNA to encode a protein with reduced IgE- or IgG-binding capacity.

Key words: α_{s1} -casein, cow milk allergy, epitope, critical amino acid

INTRODUCTION

Cow milk allergy (CMA) is the most common food allergy in young children, affecting approximately 2 to 7.5% of newborn infants. The majority of children outgrow their cow milk allergy by 3 to 4 yr of age; however, 15% of infants with IgE-mediated CMA retain their sensitivity into the second decade (Eggesbø et al., 2001). Symptoms of CMA can appear immediately or start several hours or even days after the intake of moderate to large amounts of cow milk or infant formula. A wide spectrum of clinical manifestations has been recorded with CMA, including gastrointestinal, respiratory, and cutaneous, as well as systemic anaphylactic symptoms. Clinical symptoms involve immediate or delayed reactions operating separately or together. Immediate reactions are mainly IgE dependent, lead-

ing to cutaneous, intestinal, or respiratory symptoms and, in some cases, to anaphylactic reaction (Sicherer, 2000). Delayed reactions happen after T-cell-dependent mechanisms and can be operative both at the skin and intestinal levels.

Casein has been well characterized as one of the major allergens in cow milk. Cow milk casein consists of 4 proteins: α_{s1} -CN, α_{s2} -CN, β -CN, and κ -CN, representing 32, 10, 28, and 10% of the total milk protein, respectively (Wal, 2004). α_{s1} -Casein, a single-chain linear phosphoprotein of 199 AA residues, is the most-abundant protein in cow milk and is thought to be the most potent among all casein proteins in inducing a specific IgE response (Bernard et al., 1998). This protein has only a small amount of secondary structure (α -helices or β -sheets) and lacks disulfide bonds, resulting in a reduction of tertiary interactions (Kumosinski et al., 1991).

An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by B cells (IgE and IgG antibodies), 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. In the current study, a region was defined as an epitope if it was statistically associated with reactive groups and recognized by at least 65% of reactive patients.

Some CMA immunodominant epitopes have been proposed as good markers of CMA (Järvinen et al., 2002). B-Cell epitopes were shown to be mostly of the sequential or continuous type, relatively short (12 to 16 AA), and located in hydrophobic parts of the molecule (Elsayed, 1993). The T-cell recognition patterns of α_{s1} -CN have been studied and 5 large peptides and 7 smaller peptides have been recognized by T cells generated from CMA patients (Elsayed et al., 2004a).

Because of the prevalence and severity of CMA in children, we aimed to reveal the immunological properties of sequential epitopes within α_{s1} -CN and identify the critical AA of the immunological binding region. The results will provide basic data for studying the

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structural properties of an allergen and more efficacious immunotherapeutic reagents in the future.

MATERIALS AND METHODS

Sera From CMA

Six CMA sera from 45 clinically examined children were selected. Sera from 6 patients with documented cow milk hypersensitivity (the 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 CAP System FEIA (fluorescent enzyme immunoassay; Phadia Diagnostics, Uppsala, Sweden). Based on the clinical history of the children, 6 had atopic dermatitis. Five non-milk-allergic children (aged 5–10 yr) with atopic dermatitis and allergy to other foods (i.e., tomato, shellfish, peanuts, and nuts) were used for 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 α_{s1} -CN-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 α_{s1} -CN, were synthesized using the multiple method according to the 9-fluorenylmethoxycarbonyl (Fmoc) strategy (Ruiter et al., 2006). The alanine substitution scanning of a single AA residue of a dominant epitope was used to identify the critical AA. All peptides were purified by C₁₈ reverse-phase HPLC (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

IgE-Binding Assays

An IgE ELISA was performed as described by Cocco et al. (2003). Before immunological detection, the purified peptides were coupled with BSA. Then, the peptides reacted with BSA were added to 96-well plates. Peptides were incubated with individual serum or the serum pool from patients with cow milk hypersensitivity 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, Uppsala, Sweden) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining, and then expressed as the optical density at a wavelength of 450 nm. 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 CMA). Critical AA were defined as those that resulted in loss of IgE binding when substituted by alanine residue.

IgG-Binding Assays

A modified IgG ELISA was performed as described previously (Okahata et al., 1990). Peptides were incubated with individual serum or the serum pool 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 room temperature. The primary antibody was detected using monoclonal goat-anti-human IgG (1:3,000; Pharmacia & Upjohn Diagnostics) conjugated to horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining, and then expressed as the optical density at a wavelength of 450 nm. 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 CMA). Critical AA were defined as those that resulted in loss of IgG binding when substituted by alanine residue.

RESULTS

Peptide Synthesis

To determine the AA sequence of IgE- and IgG-binding sites, 37 overlapping peptides spanning α_{s1} -CN were synthesized (shown in Table 1). By synthesizing peptides that were offset from each other by 5 AA, it was possible to identify individual IgE- and IgG-binding epitopes within the larger regions of the α_{s1} -CN molecule. Each peptide was 15 AA long. All peptides were purified by C₁₈ reverse-phase HPLC.

IgE-Binding Epitope

Each set of 37 peptides was probed individually with serum IgE from 6 different patients. An epitope can be considered immunodominant if it is recognized by above 65% serum IgE from the majority of patients with cow milk hypersensitivity. The intensity of IgE binding to each peptide was determined as a function of that patient's total IgE binding to these 37 peptides (shown in Figure 1). Immunoglobulin E antibodies from 6 patients showed binding to at least 1 linear peptide; 37 peptides were recognized by 6 sera. The serum IgE from patient no. 1 recognized peptides P01, P02,

Table 1. A library of peptides based on the sequence of α_{s1}-CN

Peptide number	Sequence synthesized	AA position in α _{s1} -CN
P01	RPKHPIKHQGLPQEV	1–15
P02	IKHQGLPQEVLNENL	6–20
P03	LPQEVLNENLLRFFV	11–25
P04	LNENLLRFFVAPFPE	16–30
P05	LRFFVAPFPEVFGKE	21–35
P06	APFPEVFGKEKVNEL	26–40
P07	VFGKEKVNELSKDIG	31–45
P08	KVNELSKDIGSESTE	36–50
P09	SKDIGSESTEDQAME	41–55
P10	SESTEDQAMEDIKQM	46–60
P11	DQAMEDIKQMEAESI	51–65
P12	DIKQMEAESISSEE	56–70
P13	EAESISSEEIVPNS	61–75
P14	SSSEEIVPNSVEQKH	66–80
P15	VVFNQEVQKHQKED	71–85
P16	VEQKHQKEDVPSEK	76–90
P17	IQKEDVPSEKYLGL	81–95
P18	VPSEKYLGLQQLLR	86–100
P19	YLGLQQLLRLLKKYK	91–105
P20	EQLLRLKKYKVPQLE	96–110
P21	LKKYKVPQLEIVPNS	101–115
P22	VPQLEIVPNSAEERL	106–120
P23	IVPNSAEERLHSMKE	111–125
P24	AEERLHSMKEGIHAQ	116–130
P25	HSMKEGIHAQQKEPM	121–135
P26	GIHAQQKEPMIGVNQ	126–140
P27	QKEPMIGVNQELAYF	131–145
P28	IGVNQELAYFYPELF	136–150
P29	ELAYFYPELFQRFYQ	141–155
P30	YPELFQRFYQLDAYP	146–160
P31	RQFYQLDAYPSGAWY	151–165
P32	LDAYPSGAWYYVPLG	156–170
P33	SGAWYYVPLGTQYTD	161–175
P34	YVPLGTQYTDAPSFS	166–180
P35	TQYTDAPSFSIPNP	171–185
P36	APSFSDIPNPISSEN	176–190
P37	DIPNPISSENSEKTT	181–195

P03, P16, P18, P26, and P35. The serum IgE from the remaining patients were tested in the same manner. The sequence at AA 126 to 140 was recognized by 6 of 6 sera from CMA patients. The sequence at AA 6 to 20, AA 76 to 90, AA 171 to 185 were recognized by 5 of 6 sera from cow milk patients. The sequence at AA 11 to 25 was recognized by 4 of 6 sera from CMA patients. Other peptides (P18, P07, P20, and P22) were recognized by few sera from CMA patients. Sera from non-milk-allergic children showed weak reaction with synthesized peptides, no data was showed in this paper.

IgG-Binding Epitope

The intensity of IgG binding to each peptide was determined as a function of that patient’s total IgG binding to these 37 peptides (shown in Figure 2); 37 peptides were recognized by 6 sera. The sequence of AA 21 to 35, AA 56 to 70, and AA 161 to 175 were recognized by 5 of 6 patients’ sera. In addition, the

peptides ⁸¹VPSEKYLGLQQLLR⁹⁵, ¹²⁶GIHAQQKEPMIGVNQ¹⁴⁰, ¹⁶⁶YVPLGTQYTDAPSFS¹⁸⁰, ¹³⁶IGVNQELAYFYPELF¹⁵⁰, and ⁴¹SKDIGSESTEDQAME⁵⁵ (where the beginning and ending superscripts represent the AA positions of the first and last AA, respectively) showed weak binding with IgG from CMA patients’ sera.

Characterization of the Critical AA of the IgE-Binding Epitope at AA 11 to 25

The AA essential to IgE binding in the epitope at AA 11 to 25 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 (the results are shown in Figure 3). Pooled serum IgE did not recognize this peptide when alanine was substituted for asparagine at position 19, arginine at position 22, and phenylalanine at position 23. In contrast, the substitution of an alanine for leucine residues at AA 20 and glutamine residues at AA 18 resulted in increased IgE binding. The intensity of IgE binding of other duplicate peptides showed no significant difference compared with native epitopes. Therefore, the AA residues of Asn19, Arg22, and Phe23 play important roles in the allergenicity of α_{s1}-CN.

Characterization of the Critical AA of the IgG-Binding Epitope at AA 21 to 35

To identify the AA critical for IgG binding in the sequence of AA 21 to 35, 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 after alanine was substituted for Arg22, Phe23, and Pro27. Substitution of leucine at position 21, phenylalanine at position 24, alanine at position 26, glutamic acid at position 30, glycine at position 33, and lysine at position 34 with alanine also resulted in lowered IgG binding. Substitution of other positions on this peptide did not cause a consistent decrease in IgG binding. Therefore, Arg22, Phe23, and Pro27 were considered as the critical AA of the IgG-binding epitope.

Immunological Relationship Between IgE- and IgG-Binding Epitopes

Five AA overlapping regions (AA 21–25, AA 171–175) were found to cross-react with IgE and IgG (shown in Table 2). In addition, a significant loss of IgE- and IgG-

Figure 1. Immunoglobulin E-binding epitopes of α_{s1} -CN. A total of 37 peptides were recognized by 6 sera. The x-axis represents the code of 37 peptides; the y-axis represents the optical density (OD). The sequence at AA 126 to 140, coded as P26, was recognized by 6 of 6 sera from cow-milk-allergic (CMA) patients. The sequences at AA 6 to 20, coded as P02; AA 76 to 90, coded as P16; and AA 171 to 185, coded as P35, were recognized by 5 of 6 sera from CMA patients. The sequence at AA 11 to 25, coded as P03, was recognized by 4 of 6 sera from CMA patients. Error bars represent the SEM ($n = 4$).

Figure 2. Immunoglobulin G-binding epitopes of α_{s1} -CN. The x-axis represents the code of 37 peptides; the y-axis represents the optical density (OD); 37 peptides were recognized by 6 sera. The sequences of AA21 to 35, coded as P05; AA56 to 70, coded as P12; and AA161 to 175, coded as P33, were recognized by 5 of 6 sera from cow-milk-allergic (CMA) patients. Error bars represent the SEM ($n = 4$).

Figure 3. Single AA changes to the peptide at AA 11 to 25, resulting in loss of IgE binding to this epitope. The peptide at AA 11 to 25 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 bar indicate the 1-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 the α_{s1} -CN protein. The control group indicates the native epitope (no AA substitutions). OD = optical density. Color version available in the online PDF.

Figure 4. Single AA changes to peptide at AA 21 to 35, resulting in loss of IgG binding to this epitope. The peptide at AA 21 to 35 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 bar indicate the 1-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 the α_{s1} -CN protein. The control group indicates the native epitope (no AA substitutions). OD = optical density. Color version available in the online PDF.

binding epitopes spanning α_{s1} -CN were both observed after alanine was substituted for arginine at position 22 and phenylalanine at position 23.

DISCUSSION

Several studies have indicated that IgE directed against casein fractions in cow milk appears to be dominant in older children and adults with CMA compared with younger children (Elsayed, 1993; Elsayed et al., 2004a). However, several other studies (Rowntree et al., 1985; Stöger and Wüthrich, 1993; Jenmalm and Björkstén, 1999; Sicherer and Sampson, 1999; Eysink et al., 2002) reported that atopic children have higher

levels of IgG subclass (particularly IgG4) antibodies to milk and egg than nonatopic children. Immunoglobulin G antibody responses to cow milk decreased with age from 1 yr onward. The question if IgG to foods might be useful as an early marker for the development of IgE-mediated allergy remains open (Homburger et al., 1986). In the current study, IgE- and IgG-binding epitopes of α_{s1} -CN were both identified. 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 which of the IgE-binding epitopes also bind IgG to find a feasible immunotherapeutic strategy for patients with cow milk hypersensitivity.

Table 2. The AA sequence of α_{s1} -CN¹

1 0																			
R	P	K	H	P	I	K	H	Q	G	L	P	Q	E	V	L	N	E	N	L
2 0																			
L	R	F	F	V	A	P	F	P	E	V	F	G	K	E	K	V	N	E	L
3 0																			
S	K	D	I	G	S	E	S	T	E	D	Q	A	M	E	D	I	K	Q	M
4 0																			
E	A	E	S	I	S	S	S	E	E	I	V	P	N	S	V	E	Q	K	H
5 0																			
I	Q	K	E	D	V	P	S	E	R	Y	L	G	Y	L	E	Q	L	L	R
6 0																			
L	K	K	Y	K	V	P	Q	L	E	I	V	P	N	S	A	E	E	R	L
7 0																			
H	S	M	K	E	G	I	H	A	Q	Q	K	E	P	M	I	G	V	N	Q
8 0																			
E	L	A	Y	F	Y	P	E	L	F	R	Q	F	Y	Q	L	D	A	Y	P
9 0																			
S	G	A	W	Y	Y	V	P	L	G	T	Q	Y	T	D	A	P	S	F	S
10 0																			
D	I	P	N	P	I	G	S	E	N	S	E	K	T	T	M	P	L	W	

¹The numbers indicate the position of each residue in the α_{s1} -CN protein. Amino acid sequences in bold indicate IgE-binding epitopes; underlined AA sequences indicate IgG-binding epitopes; italicized AA sequences indicate overlapping regions between IgE- and IgG-binding epitopes.

In previous papers, several studies had been carried out on IgE- and IgG-binding epitopes of α_{s1} -CN. Chatchatee et al. (2001) identified 6 major IgE-binding epitopes and 3 minor IgE-binding epitopes on α_{s1} -CN. Elsayed et al. (2004b) prepared 6 cyanogen bromide cleavage fragments and sought to locate the motifs critical for human-specific IgE and rabbit polyclonal IgG binding; the results showed that the peptides at AA 16 to 35 and AA 136 to 155 showed high IgE-binding affinity, and the peptides at AA 1 to 18 and AA 181 to 199 showed high IgG binding (Chatchatee et al., 2001). Ruiter et al. (2006) synthesized 32 overlapping peptides (18-mers) spanning the α_{s1} -CN molecule to identify T-cell epitopes; the results showed that 4 main regions (AA residues 43–66, 73–96, 91–114, and 127–180) in the α_{s1} -CN molecule were immunogenic to T cells, of which AA residues 133 to 156 spanned the immunodominant part. In the current study, 5 IgE-binding epitopes were recognized. Our initial data revealed IgE-binding epitopes located in AA 126 to 140, AA 6 to 20, AA 171 to 185, and AA 11 to 35; the epitope at AA 6 to 20 has not reported in other studies; this may due to different serology in different countries. However, the sequences at AA 76 to 90, AA 126 to 140, and AA 171 to 185 are consistent with the results found by Ruiter et al. (2006). Moreover, we found the IgG-binding epitope at AA 21 to 35 located in the N-terminal end of α_{s1} -CN, the epitope at AA 161 to 175 located in the C-terminal end of α_{s1} -CN, and in addition, the epitope at AA 56 to 70 located in the central region of the protein. This confirms the conclusion reached by Elsayed et al. (2004b) that IgG-binding epitope regions on α_{s1} -CN mainly reside at the N-terminal and C-terminal end of the protein; the linear epitopes were identified as the sequences at AA 1 to 18 and AA 181 to 199.

In the search for critical AA for IgE binding, alanine scanning analysis was used for AA substitution because alanine has relatively small neutral AA that do not significantly alter the charge or solubility of peptides. This approach has been used successfully for other allergens (Rabjohn et al., 1999; Robotham et al., 2002; Elsayed et al., 2004b). Cocco et al. (2003) showed that single or multiple AA substitutions within IgE-binding epitopes result in reduced binding of milk-specific IgE antibodies by patients' sera. The critical AA in α_{s1} -CN are most commonly hydrophobic. Similar hydrophobic residues appear to be critical for IgE binding in the peanut allergen Ara h1 (Shin et al., 1998), whereas on obvious type of AA was apparent in Ara h2 and Ara h3 (Stanley et al., 1997; Rabjohn et al., 1999). In addition, AA in the center of the epitope appear to be more important for IgE binding in α_{s1} -CN than those found at the periphery of the epitope. This was also observed

for the shrimp allergen Pen a 1 and the walnut allergen Jug r 1 (Lehrer et al., 2002; Robotham et al., 2002). On the basis of IgE- and IgG-binding epitopes recognized by our group, we randomly selected 1 overlapping region to identify the critical AA by alanine scanning analysis. Surprisingly, we found the same critical AA within IgE- and IgG-binding epitopes. A significant loss of IgE and IgG binding on α_{s1} -CN were both observed after arginine at position 22 and phenylalanine at position 23 were substituted by alanine. Determination of AA critical for IgE and IgG binding could provide crucial information for altering α_{s1} -CN cDNA to encode a protein with reduced IgE- and IgG-binding capacity. Such mutated proteins or peptides could be used in future immunotherapeutic approaches for this disease. Our study indicated that modification of arginine at position 22 and phenylalanine at position 23 could decrease the allergenicity and antigenicity of α_{s1} -CN. The results could be used in the future for developing safe immunotherapeutic agents for food allergies, or modified, recombinant proteins need to be engineered that will not bind serum and mast cell IgE; this possibility is actively being pursued in our laboratories.

Because of the difficulties in time and funding, the number of tested patients may be low. In addition, the low incidence of CMA in China may increase the difficulty in the collection of CMA patients' sera. However, this study is very important for providing initial results; it provides basic data and theory for drawing general conclusions in the future. We will continue collect CMA patients' sera to study the immunological mechanism of cow milk allergens in the next few years.

CONCLUSIONS

Our data show that it may be possible to mutate α_{s1} -CN to no longer bind IgE or IgG. This raises the possibility that an altered α_{s1} -CN gene could be used to replace its allergenic homolog in the α_{s1} -CN genome. The same AA residues between IgE- and IgG-binding epitopes may provide important information for elucidation of the association between IgG and IgE antibody generation.

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