Immunoglobulin E epitope mapping by microarray immunoassay reveals differences in immune response to genetic variants of caseins from different ruminant species

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ABSTRACT

The allergenicity of the caseins (CN), one of the major allergens in cow milk, is well characterized and their immunoglobulin E (IgE)-binding epitopes have been identified. However, investigations about the allergenic potential of the genetic variants occurring in the caseins are lacking. Therefore, this study determined the influence of the genetic polymorphism on IgE binding to epitopes of bovine casein variants. Furthermore, differences in IgE binding between epitopes of goats and water buffaloes were analyzed. A set of 187 peptides, covering the previously identified sequential IgE-binding epitopes of α_{S1} -, α_{S2} -, β -, and κ -CN variants from cows and the corresponding homologous peptides of water buffaloes and goats, were synthesized and tested by means of peptide microarray for IgE binding, using sera from 16 cow milk-sensitized individuals. Seven of the 16 sera samples showed positive signals on microarrays and were included in this study. In 5 α_{S1} -CN variants (A, B, C, E, and I), the AA substitution or deletion affected the immunoreactivity of epitopes AA 4 to 23, AA 17 to 36, AA 83 to 102, AA 173 to 192, and AA 175 to 194, as well as of the variant-specific peptides AA 184 to 196, AA 187 to 199, AA 174 to 193, and AA 179 to 198, which were found to resist gastrointestinal digestion. Variation in IgE binding was further detected for peptides AA 103 to 123 and AA 108 to 129 of 3 β -CN variants (A¹, A², and B). The majority of sera showed IgE binding to α_{S1} -CN peptides of cows and the homologous counterpart of goats and water buffaloes. However, α_{S1} - and β -CN epitopes from goats and water buffaloes had lower immunoreactivity than those of cows, but, in some cases, higher or exclusive IgE binding was observed. The results of this study indicate that genetic variants of the caseins differ in their allergenicity. This might be useful in the search for a suitable protein source for cow milk-allergic patients. In addition, milk from water buffaloes and goats

harbor an allergenic potential due to cross-reactivity of IgE antibodies with cow milk caseins and are, therefore, not an acceptable alternative in the nutrition of cow milk-allergic patients.

Key words: casein variant, immunoglobulin E epitope, milk allergy, peptide microarray

INTRODUCTION

From a nutritional point of view, cow milk gains considerable attention, as it provides a wide range of essential nutrients, in particular proteins, fat, vitamins, and minerals. In contrast, cow milk belongs to the most common nutritive source of allergens during infancy and early childhood. Cow milk allergy (CMA) affects 2 to 3% of infants (Skripak et al., 2007) and approximately 0.1% of adults (Osterballe et al., 2005) who are typically sensitized to several cow milk proteins (CMP). The majority of children with CMA achieve tolerance toward cow milk, although the age of clinical recovery has differed among various studies (Savilahti and Savilahti, 2013). Reports suggest that in most children, CMA resolves by 3 to 5 yr of age, but outgrowing CMA can develop even later (Skripak et al., 2007; Savilahti and Savilahti, 2013). Cow milk allergy is mostly mediated by milk protein-specific IgE antibodies that react with the allergenic epitopes on milk proteins. The 4 caseins α_{S1} -, α_{S2} -, β -, and κ -CN were confirmed to be the major allergens in cow milk, with α_{S1} -CN found to be more allergenic than α_{S2} -, β -, and κ -CN (Shek et al., 2005; Gaudin et al., 2008). By using SPOT-membrane-based or microarray immunoassays and sera from CMA patients, a large number of IgE-binding epitopes 8 to 24 AA in length have been identified in the 4 caseins (Chatchatee et al., 2001a,b; Busse et al., 2002; Cerecedo et al., 2008; Lin et al., 2009). Recent studies demonstrated that peptides resulting from in vitro gastrointestinal digestion of milk proteins still contain some of these IgE-binding epitopes (Dupont et al., 2010a,b; Lisson et al., 2013, 2014). This might provide an explanation for the high sensitization rate to the caseins, even though they are very rapidly digestible proteins.

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Within the caseins, noticeable genetic variation has been identified, which is caused by substitutions or deletions of AA. So far, a total of 39 casein variants have been determined on the protein and DNA level, with important differences among species and breeds within the *Bos* genus (Caroli et al., 2009).

To date, the only treatment for CMA is strict avoidance of CMP. This is very difficult, as milk proteins are used as additives or processing aids and, therefore, a large number of food products contain remaining amounts of CMP. Furthermore, infant formulas based on CMP still may provoke allergic reactions in selected patients, as residual allergenic activity has been reported in partially and extensively hydrolyzed formulas (Ragno et al., 1993; de Boissieu et al., 1997; Hoffman and Sampson, 1997). Moreover, CMP hydrolysates are disadvantaged by their bitter taste and the requirement of additional emulsifiers. Thus, the identification of a suitable protein source as well as the search for options to eliminate the allergenicity in milk products or infant formulas is of great importance. Genetic variants of milk proteins are not considered when discussing the allergenic potential of bovine milk. However, detection of CMP variants with reduced allergenicity may contribute to find adequate alternatives for allergic patients. Genetic polymorphisms of milk proteins are of increasing interest in animal breeding because of their significant effect on milk production traits and on cheese-making properties (Martin et al., 2002; Heck et al., 2009). In addition, milk protein variants are also important in human nutrition, as they represent a reservoir of bioactive peptides (angiotensin I-converting enzyme inhibitory peptides, casomorphins, and caseinophosphopeptides), which affect the nutritional value of milk (Jinsmaa and Yoshikawa, 1999; Weimann et al., 2009; Tulipano et al., 2010).

On the basis of the allergenic epitopes previously identified, the present study investigated the influence of the genetic polymorphisms on the IgE-binding properties of epitopes from α_{S1} -, α_{S2} -, β -, and κ -CN variants. This study also aimed to determine if variant-specific casein peptides resisting *in vitro* gastrointestinal digestion still retain IgE-binding properties and differ in their allergenic potential. In addition, differences in IgE binding between epitopes of 2 mammalian species (goats and water buffaloes) were analyzed because there has been an increasing focus on the use of proteins from non-bovine milk as a possible alternative to cow milk.

MATERIALS AND METHODS

Sera from Patients with CMA

The screening was performed with sera from 16 patients (6 females, 8 males, and 2 unknown; 8 mo

to 52 yr old) who had a positive skin prick test reaction to cow milk or specific IgE antibodies to cow milk, or both. The sera were obtained from the University Hospitals Bonn and Marburg (Germany). The patients had milk-specific IgE antibodies ranging from 4.5 to >100 kU_A/L (where U_A = allergen-specific units), as measured with the ImmunoCAP System (Thermo Scientific, Freiburg, Germany). Only 7 (1 female, 5 males, and 1 unknown; 8 mo to 35 yr old; IgE >100 kU_A/L) of the 16 sera, which were characterized by an IgE titer ≥ 95.5 kU_A/L, showed positive signals on microarray and were included in the current study. This study was approved by the local ethical review committees of the Justus-Liebig-University Gießen and the University of Bonn, Germany.

Synthesis and Printing of Casein Peptides

To investigate the role of genetic polymorphism as well as interspecies differences on the epitope structure, a set of 187 peptides, 11 to 20 AA in length, covering the previously determined sequential IgE-binding epitopes of α_{S1} -, α_{S2} -, β -, and κ -CN variants from cows and the corresponding homologous peptides of water buffaloes and goats as well as casein peptide variants, which were shown to resist gastrointestinal digestion (Lisson et al. 2013, 2014), were synthesized as described below (Table 1). Moreover, 36 peptides were synthesized with a phosphorylated serine residue and 1 peptide with a phosphorylated threonine residue. A total of 9 of these 37 peptides were spotted on the microarray without the phosphorylation to study the influence of post-translational modifications on the immunoreactivity of the epitopes (Table 1). All peptides were synthesized in a stepwise manner on a cellulose membrane by using the SPOT synthesis technology (Frank and Overwin, 1996; Wenschuh et al., 2000). The peptide library and 3 control spots (human IgG, mouse IgE, and human IgE) were spotted in triplicate on each glass slide using a microarray printing system (JPT Peptide Technologies GmbH, Berlin, Germany). This gave a general microarray layout with 3 identical subarrays.

IgE-Binding Assays

The determination of peptide antibody binding was performed by RepliTope analysis (JPT Peptide Technologies GmbH). The array slides were first incubated with blocking buffer (SmartBlock; Candor Bioscience GmbH, Wangen, Germany) for 60 min. Then, they were placed into a microarray processing station (Tecan HS4800; Tecan Group Ltd., Männedorf, Switzerland) and incubated with 200 μ L of the patient's serum, diluted 1:100 in diluent buffer [SuperBlock Tris-buffered

Table 1. The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence ¹	Source of selected peptides ²
1	α_{S1} -CN B cow	1–15	RPKHPKHQGLPQEV	1
2	α_{S1} -CN goat	1–15	RPKHPINHRGLSPEV	1
3	α_{S1} -CN water buffalo	1–15	RPKQPIKHQGLPQGV	1
4	α_{S1} -CN B cow, water buffalo	17–36	<u>NENLLRFFVAPFPEVFGKEK</u> ³	1
5	α_{S1} -CN A cow	4–23	HPIKHQGLPQPFPEVFGKEK	2
6	α_{S1} -CN goat	17–36	NENLLRFVAPFPEVFRKEN	1
7	α_{S1} -CN B cow	34–48	KEKVNELSKDIGSES ³	1
8	α_{S1} -CN goat	34–48	KENINELSKDIGSES	1
9	α_{S1} -CN water buffalo	34–48	KEKVNELSTDIGSES	1
10	α_{S1} -CN B cow, water buffalo	49–63	<u>TEDQAMEDIKQMEAE</u> ⁴	1, 4
11	α_{S1} -CN D cow	49–63	TEDQ-pT-MEDIKQMEAE	2, 4
12	α_{S1} -CN E cow	49–63	TEDQAMEDIKKMEAE	2, 4
13	α_{S1} -CN H cow	45–59	GSESTEQMEAESISS	2
14	α_{S1} -CN goat	49–63	TEDQAMEDAKQMKAG	1, 4
15	α_{S1} -CN B cow	64–78	SISSSEEIVPNSVEQ ³	1
16	α_{S1} -CN F cow	64–78	SILSSEEIVPNSVEQ	2
17	α_{S1} -CN goat	64–78	SSSSSEEIVPNSAEQ	1
18	α_{S1} -CN water buffalo	64–78	SISSSEEIVPISVEQ	1
19	α_{S1} -CN B cow, water buffalo, goat	83–102	<u>KEDVPSERYLGYLEQLRLK</u> ³	1
20	α_{S1} -CN I cow	83–102	KDDVPSERYLGYLEQLRLK	2
21	α_{S1} -CN B cow	106–120	VPQLEIVPN-pS-AEERI ³	1, 4
22	α_{S1} -CN goat	106–120	VPQLEIVPK-pS-AEEQL	1, 4
23	α_{S1} -CN water buffalo	106–120	VPQLEIVPNLAEEQL	1, 4
24	α_{S1} -CN B cow, water buffalo	121–135	<u>HSMKEGIHAQQKEPM</u> ³	1
25	α_{S1} -CN goat	121–135	HSMKEGNPAHQKQPM	1
26	α_{S1} -CN B cow	139–154	<u>NQELAYFYPELFRQFY</u> ³	1
27	α_{S1} -CN water buffalo, goat	139–154	NQELAYFYPLFRQFY	1
28	α_{S1} -CN B cow	159–174	<u>YPSGAWYYYVPLGTQYT</u> ³	1
29	α_{S1} -CN goat	159–174	YPSGAWYYLPLGTQYT	1
30	α_{S1} -CN water buffalo	159–174	YPSGAWYYYVPLGTQYP	1
31	α_{S1} -CN B cow	173–192	YTDAPSFSDIPNPIGSENSE ³	1
32	α_{S1} -CN C, E cow, goat	173–192	YTDAPSFSDIPNPIGSENSG	1, 2
33	α_{S1} -CN water buffalo	173–192	YPDAPSFSDIPNPIGSENSG	1
34	α_{S1} -CN B cow	175–194	<u>DAPSFSDIPNPIGSENSEKT</u> ³	1
35	α_{S1} -CN C, E cow, goat	175–194	DAPSFSDIPNPIGSENSGKT	1, 2
36	α_{S1} -CN water buffalo	175–194	DAPSFSDIPNPIGSENSGKT	1
37	α_{S1} -CN B cow	184–196	NPIGSENSEKTTM ⁵	3
38	α_{S1} -CN B cow	187–199	GSENSEKTTMPLW ⁵	3
39	α_{S1} -CN C cow	187–199	GSENSGKTTMPLW ⁵	3
40	α_{S1} -CN B cow	180–193	SDIPNPIGSENSEK ⁵	3
41	α_{S1} -CN C cow	180–193	SDIPNPIGSENSGK ⁵	3
42	α_{S1} -CN B cow	174–193	TDAPSFSDIPNPIGSENSEK ⁵	3
43	α_{S1} -CN C cow	179–198	FSDIPNPIGSENSGKTTMPL ⁵	3
44	α_{S2} -CN A cow	1–20	<u>KNTMEHVSSEESIISQETY</u> ⁶	1
45	α_{S2} -CN B cow	1–20	KNTMEHVSSEESIISQETY	2
46	α_{S2} -CN goat	1–20	HKHMEHVSSEEPINIFQEI	1
47	α_{S2} -CN goat	2–21	HKMEHVSSEEPINIFQEIY	1
48	α_{S2} -CN water buffalo	1–20	KHTMEHVSSEESIISQETY	1
49	α_{S2} -CN A cow	13–32	<u>SIISQETYKQEKMAINPSK</u> ⁶	1
50	α_{S2} -CN A cow	14–33	<u>IISQETYKQEKMAINPSKE</u> ⁶	1
51	α_{S2} -CN C cow	13–32	SIISQETYKQEKMAINPSK	2
52	α_{S2} -CN C cow	14–33	IISQETYKQEKMAINPSKG	2
53	α_{S2} -CN A cow	31–45	-pS-KENLCSTFCKEVVR ⁷	1
54	α_{S2} -CN C cow	31–45	-pS-KGNLCSTFCKEVVR	2
55	α_{S2} -CN A cow	42–56	EVVRNANEEEYSIG-pS ⁷	1, 4
56	α_{S2} -CN C cow	42–56	EVVRNTNEEEYSIG-pS-	2, 4
57	α_{S2} -CN D cow	42–56	EVVRNANEEESAIVA	2, 4
58	α_{S2} -CN A cow	57–71	SSEESAEEVATEEVKI	1
59	α_{S2} -CN D cow	48–62	NEEESAEEVATEEVKI	2
60	α_{S2} -CN A cow	67–86	<u>EEVKITVDDKHYQKALNEIN</u> ^{4,6}	1
61	α_{S2} -CN A cow	83–100	<u>NEINQFYQKFPQYLQYLY</u> ⁷	1
62	α_{S2} -CN A cow	93–108	<u>PQYLYQYQGPVLPVNP</u> ⁷	1
63	α_{S2} -CN A cow	105–119	<u>VLNPWDQVKNRAVPI</u> ⁷	1
64	α_{S2} -CN A cow	117–131	<u>VPITPTLNREQLSTS</u> ⁷	1
65	α_{S2} -CN C cow	117–131	VPITPTLNREQLSIS	2
66	α_{S2} -CN A cow	130–144	<u>TSEENSKKTVDMEST</u> ⁶	1

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence ¹	Source of selected peptides ²
67	α_{S2} -CN C cow	130–144	ISEENSKKTVDMEST	2
68	α_{S2} -CN A cow	143–158	-pS-TEVFTKKTKLTEEEK ⁷	1
69	α_{S2} -CN A cow	157–172	EKNRLNFLKKISQRYQ ⁷	1
70	α_{S2} -CN A cow	165–184	KKISQRYQKFALPQYLKTVY ⁷	1
71	α_{S2} -CN A cow	169–188	QRYQKFALPQYLKTVYQHOK ⁷	1
72	α_{S2} -CN A cow	191–205	KPWIQPKTKVIPYVR ⁷	1
73	α_{S2} -CN A cow	7–26	VSSSEESIISQETYKQEKNM ⁵	3
74	α_{S2} -CN A cow	10–29	SEESIISQETYKQEKNM ⁵	3
75	α_{S2} -CN B cow	1–20	KNTMEHVFSSEESIISQETY ⁵	3
76	α_{S2} -CN B cow	3–22	TMEHVFSSEESIISQETYKQ ⁵	3
77	α_{S2} -CN B cow	9–21	SSEESIISQETYK ⁵	3
78	β -CN A ² cow, water buffalo	1–18	RELEELNVPGEIVESLSS ⁸	1
79	β -CN D cow	1–18	RELEELNVPGEIVESLSK	2
80	β -CN goat	1–18	REQEELNVVGETVESLSS	1
81	β -CN A ² cow	16–35	LSSEESITRINKKIEKFQ-pS ⁴	1, 4
82	β -CN A ² cow	18–37	SSEESITRINKKIEKFQ-pS-EE ⁴	1, 4
83	β -CN D cow	16–35	LSKSEESITRINKKIEKFQ-pS-	2, 4
84	β -CN D cow	18–37	KSEESITRINKKIEKFQ-pS-EE	2, 4
85	β -CN H ¹ cow	16–35	LSSEESITCINKKIEKFQ-pS-	2, 4
86	β -CN H ¹ cow	18–37	SSEESITCINKKIEKFQ-pS-EE	2, 4
87	β -CN C cow	16–35	LSSEESITRINKKIEKFQS	2, 4
88	β -CN C cow	18–37	SSEESITRINKKIEKFQSEK	2, 4
89	β -CN E cow	16–35	LSSEESITRINKKIEKFQ-pS-	2, 4
90	β -CN E cow	18–37	SSEESITRINKKIEKFQ-pS-KE	2, 4
91	β -CN goat, water buffalo	16–35	LSSEESITHINKKIEKFQ-pS-	1, 4
92	β -CN goat, water buffalo	18–37	SSEESITHINKKIEKFQ-pS-EE	1, 4
93	β -CN A ² cow, goat	34–50	Q-pS-EEQQQTEDELQDKIH ⁶	1, 4
94	β -CN C cow	34–50	QSEKQQQTEDELQDKIH	2, 4
95	β -CN E cow	34–50	Q-pS-KEQQQTEDELQDKIH	2, 4
96	β -CN water buffalo	34–50	Q-pS-EEQQQMEDELQDKIH	1, 4
97	β -CN A ² cow	42–56	EDELQDKIHPPFAQTQ ⁸	1
98	β -CN A ² cow	55–72	TQSLVYFPFGPIPN ⁸	1
99	β -CN A ¹ , B, C, F, G cow	55–72	TQSLVYFPFGPIHNSLPQ	2
100	β -CN H ² cow	55–72	TQSLVYFPFGPIPN ⁸	2
101	β -CN goat	55–72	AQSLVYFPFGPIPN ⁸	1
102	β -CN water buffalo	55–72	TQSLVYFPFGPIPN ⁸	1
103	β -CN A ² , water buffalo	70–84	LPQNIPLTQTPVVV	1
104	β -CN H ² cow	70–84	LPENIPPLTQTPVVV	2
105	β -CN goat	70–84	LPQNIPLTQTPVVV	1
106	β -CN A ² cow	83–97	VVPPFLQPEVMGVSK ⁸	1
107	β -CN H ¹ cow	83–97	VVPPFLQPEVMGVSK	2
108	β -CN H ² , I cow	83–97	VVPPFLQPEVLGVSK	2
109	β -CN goat	83–97	VVPPFLQPEIMGVPK	1
110	β -CN water buffalo	83–97	VVPPFLQPEIMGVSK	1
111	β -CN A ² cow, water buffalo	95–109	VSKVKEAMAPKHKEM	1
112	β -CN A ³ cow	95–109	VSKVKEAMAPKQKEM	2
113	β -CN goat	95–109	VPKVKETMVPKHKEM	1
114	β -CN A ² cow, goat, water buffalo	106–122	HKEMPFPKYPVEPF ⁸	1
115	β -CN A ³ cow	106–122	QKEMPFPKYPVEPF ⁸	2
116	β -CN B cow	106–122	HKEMPFPKYPVEPF ⁸	2
117	β -CN A ² cow, water buffalo	121–135	ESQSLTLTDVENLHL ⁶	1
118	β -CN B cow	121–135	ERQSLTLTDVENLHL	2
119	β -CN goat	121–135	ESQSLTLTDVEKLHL	1
120	β -CN A ² cow	135–149	LPLPLLQSWMHQPHQ ⁸	1
121	β -CN G cow	135–149	LPLLLLQSWMHQPHQ	2
122	β -CN water buffalo	135–149	LPLPLLQSWMHQPPQ	1
123	β -CN goat	135–149	LPLPLVQSWMHQPPQ	1
124	β -CN A ² cow, water buffalo	149–164	QPLPPTVMFPPQSVLS ⁸	1
125	β -CN B cow	149–164	QPLPPTVMFPPQSVLS	2
126	β -CN goat	149–164	QPLSPTVMFPPQSVLS	1
127	β -CN A ² cow, water buffalo	167–184	QSKVLPVPQKAVPYQRD ⁸	1
128	β -CN goat	167–182	QPKVLPVPQKAVPYQRD	1
129	β -CN A ² cow, goat, water buffalo	185–204	MPIQAFLLYQEPVLGPVRGP ⁸	1
130	β -CN A ² , A ¹ cow	114–124	YPVEPF ⁸	3
131	β -CN A ² cow	59–72	YVPFGPIPN ⁹	3
132	β -CN B, A ¹ cow	59–72	YVPFGPIHNSLPQ ⁹	3

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence ¹	Source of selected peptides ²
133	β-CN A ² cow	59–78	VYFPGPIPNLSLPQNIPPLT ⁹	3
134	β-CN A ² cow	61–80	PFPGPINSLPQNIPPLTQT ⁹	3
135	β-CN B, A ¹ cow	59–78	VYFPGPIHNSLPQNIPPLT ⁹	3
136	β-CN B, A ¹ cow	61–80	PFPGPINSLPQNIPPLTQT ⁹	3
137	β-CN A ² , A ¹ cow	110–125	PFPKYPVEPFOTESQSL ⁹	3
138	β-CN B cow	109–123	MPFPKYPVEPFOTESQ ⁹	3
139	β-CN A ² , A ¹ cow	108–127	EMFPKYPVEPFOTESQSLTL ⁹	3
140	β-CN A ² , A ¹ cow	110–129	PFPKYPVEPFOTESQSLTLTD ⁹	3
141	β-CN B cow	103–122	APKHKEMPFPKYPVEPFOTES ⁹	3
142	β-CN B cow	104–123	PKHKEMPFPKYPVEPFOTESQ ⁹	3
143	κ-CN A cow	1–15	QEQNQEPIRCEKDE	1
144	κ-CN F ² cow	1–15	QEQNQEPIHCEKDE	2
145	κ-CN A cow	9–26	IRCEKDERFFSDKIAKYI ⁸	1
146	κ-CN F ² cow	9–26	IHCEKDERFFSDKIAKYI	2
147	κ-CN A cow	24–43	KYIPIQYVLSRYPSYGLNYY ⁸	1
148	κ-CN A cow	25–44	YIPIQYVLSRYPSYGLNYYQ ⁸	1
149	κ-CN A cow	34–53	RYPYGLNYYQQKPVALINN ⁶	1
150	κ-CN A cow	49–68	ALINNQLPYPYAKPAAVR ⁸	1
151	κ-CN A cow	66–80	AVRSPAQLQWQVLS ⁸	1
152	κ-CN A cow	79–94	LSNTVPAKSCQAQPTT	1
153	κ-CN A cow	95–114	MARHPHPLSFMAIPPCKKNQ ⁸	1
154	κ-CN A cow	97–116	RHPHPLSFMAIPPCKKNQDK ⁸	1
155	κ-CN G ¹ cow	95–114	MACHPHPLSFMAIPPCKKNQ	2
156	κ-CN G ¹ cow	97–116	CHPHPLSFMAIPPCKKNQDK	2
157	κ-CN C, D cow	95–114	MAHHPHPLSFMAIPPCKKNQ	2
158	κ-CN C, D cow	97–116	HHPHPLSFMAIPPCKKNQDK	2
159	κ-CN I cow	95–114	MARHPHPLAFMAIPPCKKNQ	2
160	κ-CN I cow	97–116	RHPHPLAFMAIPPCKKNQDK	2
161	κ-CN A cow	111–126	KKNQDKTEIPTINTIA ⁸	1
162	κ-CN A cow	125–139	IA-pS-GEPTSTPTTEAV	1
163	κ-CN H cow	125–139	IA-pS-GEPTSTPTTEAV	2
164	κ-CN B, B ² , C, F ² , G ¹ , J cow	125–139	IA-pS-GEPTSTPTTEAV	2
165	κ-CN A cow	134–148	PTTEAVESTVATLED ⁸	1
166	κ-CN H cow	134–148	PITEAVESTVATLED	2
167	κ-CN B, B ² , C, F ² , G ¹ , J cow	134–148	PTIEAVESTVATLEA	2
168	κ-CN G ² cow	134–148	PTTEAVESTVATLEA	2
169	κ-CN F ¹ cow	134–148	PTTEAVESTVATLEV	2
170	κ-CN A cow	146–160	LED-pS-PEVIESPPEIN	1
171	κ-CN B, C, F ² , G ¹ , G ² , J cow	146–160	LEA-pS-PEVIESPPEIN	2
172	κ-CN F ¹ cow	146–160	LEV-pS-PEVIESPPEIN	2
173	κ-CN B ² cow	146–160	LEA-pS-PEVIESPPEIN	2
174	κ-CN E cow	146–160	LED-pS-PEVIEGPPEIN	2
175	κ-CN J cow	146–160	LED-pS-PEVIERPPEIN	2
176	κ-CN A cow	149–168	-pS-PEVIESPPEINTVQVTSTA ⁸	1
177	κ-CN B ² cow	149–168	-pS-PEVIESPPEINTVQVTSTA	2
178	κ-CN E cow	149–168	-pS-PEVIEGPPEINTVQVTSTA	2
179	κ-CN J cow	149–168	-pS-PEVIERPPEINTVQVTSTA	2
180	κ-CN A, E cow	125–137	IASGEPTSTPTTE ⁹	3
181	κ-CN B cow	125–137	IASGEPTSTPTTE ⁹	3
182	κ-CN A cow	146–157	LED-pS-PEVIESPP ⁹	3
183	κ-CN E cow	147–160	EDSPEVIEGPPEIN ⁹	3
184	κ-CN A cow	148–159	D-pS-PEVIESPPEI ⁹	3
185	κ-CN A, E cow	136–149	TEAVESTVATLED-pS ⁹	3, 4
186	κ-CN A, E cow	136–149	TEAVESTVATLED ⁹	3, 4
187	κ-CN B cow	134–150	PTIEAVESTVATLEASP ⁹	3

¹Peptides previously reported as IgE-binding epitopes are underlined. Phosphorylated peptides are indicated as -pS- or -pT-.

²1 = peptides described in cattle; 2 = bovine genetic variant; 3 = peptides resisting gastrointestinal digestion; 4 = peptides with different phosphorylation levels.

³Chatchatee et al. (2001a).

⁴Lin et al. (2009).

⁵Lisson et al. (2014).

⁶Cerecedo et al. (2008).

⁷Busse et al. (2002).

⁸Chatchatee et al. (2001b).

⁹Lisson et al. (2013).

Table 2. Sera of cow milk-allergic patients reacting with peptides of α_{S1} -, β -, and κ -CN variants and differences in IgE binding between variant-specific peptides and corresponding reference peptides¹

Patient serum ²	Age	Milk IgE [kU _A /L]	Peptide (AA no.)	Casein variant	IgE binding compared with reference peptides α_{S1} -CN B, β -CN A ² , and κ -CN A
2	35 yr	>100	4–23	α_{S1} -CN A	↓ ³
			17–36	α_{S1}-CN B	
			83–102	α_{S1}-CN B, I	↑ ⁴
			173–192	α_{S1}-CN B, C, E	No differences
			175–194	α_{S1} -CN C, E	No reaction to reference peptide
3	12 yr	>100	83–102	α_{S1}-CN B, I	↑
			173–192	α_{S1}-CN B	No reaction to peptide of α_{S1} -CN C, E
4	24 yr	>100	4–23	α_{S1} -CN A	↑
			17–36	α_{S1}-CN B	
			64–78	α_{S1}-CN B, F	No differences
			83–102	α_{S1}-CN B, I	↑
			173–192	α_{S1}-CN B, C, E	No differences
			175–194	α_{S1}-CN B, C, E	No differences
			180–193	α_{S1}-CN B, C, E	No differences
			184–196	α_{S1}-CN B	No corresponding peptide in α_{S1} -CN C, E
			187–199	α_{S1}-CN B	No reaction to peptide of α_{S1} -CN C, E
			179–198	α_{S1} -CN C, E	
			174–193	α_{S1}-CN B	Lower reaction compared with peptide 174–193 of α_{S1}-CN B
			55–72	β-CN A², A¹, B, C, F, G, H²	No differences
			106–122	β-CN A², A¹, B, C, F, G, H²	No differences
			110–125	β-CN A², A¹	No differences between 110–125 of β -CN A ² , A ¹ and 109–123 of β -CN B
			109–123	β -CN B	
			103–123	β -CN B	No reaction to corresponding peptide 108–129 of α_{S1}-CN A², A¹
5	8 mo	95.5	9–26	κ-CN A, F²	No differences
			17–36	α_{S1}-CN B	No reaction to peptide 4–23 of α_{S1} -CN A
6	10 yr	>100	4–23	α_{S1} -CN A	No reaction to reference peptide 17–36 of α_{S1} -CN B
			173–192	α_{S1} -CN C, E	No reaction to reference peptide 173–192 of α_{S1} -CN B
			175–194	α_{S1}-CN B, C, E	No differences
			174–193	α_{S1}-CN B	No reaction to corresponding peptide 179–198 of α_{S1} -CN C
7	16 yr	>100	173–192	α_{S1}-CN B, C, E	↑
			175–194	α_{S1}-CN B, C, E	↑
			179–198	α_{S1} -CN C, E	
			174–193	α_{S1}-CN B	Lower reaction compared with peptide 174–193 of α_{S1}-CN B

¹Reference proteins are shown in bold.²Serum 1 (age unknown; >100 kU_A/L, where U_A = allergen-specific units) showed no reaction with any of the casein variant peptides.³↓ = decreased binding.⁴↑ = increased binding.

saline (TBS); Pierce International, Rockford, IL], and with diluent buffer only as control for 2 h. Subsequently, the peptide microarray was washed 3 times with 50 mM TBS buffer, including 0.1% Tween 20 (pH 7.2). Specific human IgE binding was detected by incubation with SureLight allophycocyanin-labeled secondary anti-human IgE (Abcam, Cambridge, UK) at a final concentration of 1 µg/mL in diluent buffer. Control incubation with secondary antibody only was performed in parallel. Afterward, 3 washing steps with 50 mM TBS buffer, including 0.1% Tween 20 (pH 7.2) were carried out, which were followed by a washing step with 3 mM saline-sodium citrate buffer (pH 7.0). Finally, peptide microarrays were dried using a nitrogen stream.

Signal Detection and Data Analysis

The peptide microarrays were scanned with the GenePix Scanner 4300SL50 (Axon Instruments, Concord,

ON, Canada). Images were saved electronically in tagged image file format (*.tif). Image analysis was performed using the spot-recognition software GenePix Pro 6.1 (Axon Instruments), which showed the signal intensity (light units) as single measurements for each peptide. Each spot feature was analyzed for total intensity and background intensity. For data tables and diagrams, the mean value of all 3 instances on the microarray was calculated. In case the standard deviation divided by the mean value was larger than 0.5, the mean of the 2 closest values was used instead. Negative controls were used to define background noise. The fluorescent intensity of each peptide was determined by subtracting the mean intensity of the corresponding peptide spots from control incubation without serum. Immunoglobulin E binding was considered positive if signal intensities were above the lower limit of detection, meaning that ratios of signal-to-background noise intensities were greater than a minimum threshold of 1.5.

Figure 1. Immunoglobulin E binding pattern of cow milk-allergic patients to peptides of α_{s1} -CN (a), β -CN (b), and κ -CN (c) from cows, goats, and water buffaloes. The bars indicate the percentage of sera showing IgE binding to peptides (represented by AA residues, number, and species). c = cow; g = goat; wb = water buffalo.

Figure 2. Differences in IgE binding between epitopes (represented by AA residues, number, and casein variant) of α_{s1} -CN (a), β -CN (b), and κ -CN (c) variants from cows, using 7 sera with cow milk-specific IgE values >100 kU_A/L, where UA = allergen-specific units. Differences in AA sequence between the reference peptides and the mutated peptides are underlined. The mean fluorescence intensity (arbitrary units, AU) of the 3 subarrays on the microarray for each peptide is presented.

Figure 3. Differences in IgE binding between epitopes (represented by AA residues, number, and species) of α_{S1} -CN (a and b) and β -CN (c) from cows, goats, and water buffaloes, using 7 sera with cow milk-specific IgE values $>100 \text{ kU}_A/\text{L}$, where U_A = allergen-specific units. Modifications in AA sequence between peptides of the 3 species are underlined. The mean fluorescence intensity (arbitrary units, AU) of the 3 subarrays on the microarray for each peptide is presented. g = goat; wb = water buffalo.

RESULTS

Identification of IgE-Binding Epitopes in Patient Sera Samples

Among the sera samples tested in this study, IgE binding to at least 1 of the casein peptides occurred in all samples. A total of 46 casein peptides (27 α_{S1} -CN peptides, 13 β -CN peptides, and 6 κ -CN peptides) were recognized by IgE antibodies from the different sera samples (Figure 1a, b, and c). All sera showed IgE binding to α_{S1} -CN peptides. It was the most frequently detected milk allergen, whereas no significant IgE binding was found for α_{S2} -CN peptides. The α_{S1} -CN peptides AA 17 to 36, AA 139 to 154, AA 173 to 192, and AA 175 to 194 of cows, goats, and water buffaloes reacted with IgE antibodies from at least 50% of the patient sera samples and, thus, represented the major IgE-binding epitopes (Figure 1a). The α_{S1} -CN peptides AA 83 to 102, AA 159 to 174, AA 174 to 193, and AA 175 to 194 from cows were recognized by 3 of the 7 positive sera. The α_{S1} -CN peptides AA 4 to 23 (cow), AA 83 to 102 (goat and water buffalo), AA 179 to 198 (cow), and the β -CN peptide AA 16 to 35 (goat and water buffalo), as well as the κ -CN peptides AA 24 to 43 (cow) and AA 25 to 44 (cow) were identified in 2 sera samples (Figure 1a, b, and c). The other peptides of α_{S1} -, β -, and κ -CN, giving positive signals on the microarray, displayed only binding to individual sera, particularly to serum 4.

Only in 1 serum was an influence observed of the posttranslational modification on the immunoreactivity of the epitopes for the α_{S1} -CN peptide AA 106 to 120 of the water buffalo (Figure 3a). The IgE response to the peptide lacking the phosphorylated serine residue was 86% higher than to the phosphorylated peptides of cows and goats. The other sera showed no variation in the immunoreactivity between the peptides carrying phosphorylated serine residues or not.

Identification of Variant-Specific Differences on the Epitope Structure

The genetic variation affects the IgE-binding epitope structure of the caseins without inducing changes in IgE binding by the majority of sera (Table 2; Figure 2). Nevertheless, as indicated in Figure 2, some of the mutated epitopes led to abrogation or decreased or increased IgE binding in individual sera ($\geq 15\%$ of IgE binding remaining compared with the reference peptide).

In 4 α_{S1} -CN variants (A, C, E, and I), 1 deletion and 3 AA exchanges influenced the immunoreactivity of 5 epitopes (Figure 2a). Due to the deletion of AA 14 to 26 in α_{S1} -CN A, epitope AA 4 to 23 differs in 10 AA

from the reference epitope AA 17 to 36 in α_{S1} -CN B. Thus, for peptide AA 4 to 23 of α_{S1} -CN A, a loss of IgE binding could be observed in serum 5, a decrease in IgE binding in serum 2, and an increase in IgE binding in serum 4 compared with the reference peptide. Serum 6 reacted only with the peptide of α_{S1} -CN A, but showed no IgE binding to the reference peptide.

The AA substitutions characterizing epitopes AA 83 to 102 of α_{S1} -CN I as well as AA 173 to 192 and AA 175 to 194 of α_{S1} -CN C and E resulted in a loss of IgE binding in serum 3 and increased IgE binding in sera 2, 3, 4, and 7. In contrast to peptides AA 173 to 192 and AA 175 to 194 of α_{S1} -CN C and E, IgE binding to the corresponding peptides of α_{S1} -CN B was eliminated by the AA substitution occurring at position 192 (sera 2 and 6). In 3 sera, the AA exchanges had no significant effect on the immunoreactivity of epitopes AA 64 to 78 (serum 4), AA 173 to 192 (sera 2 and 4), and AA 175 to 194 (sera 4 and 6). Both the mutated peptide as well as the reference peptide displayed similar IgE-binding intensities on the peptide microarray (Figure 2a).

Five of the 7 peptides resulting from gastrointestinal digestion of α_{S1} -CN B and C showed positive IgE binding on the microarray in 3 sera (Figure 2a). The AA substitution did not cause a change in the immunoreactivity of peptides AA 180 to 193 of α_{S1} -CN B and C in serum 4. However, important differences in IgE binding were detected for peptides AA 187 to 199 and 179 to 198 of α_{S1} -CN C and the corresponding peptides of α_{S1} -CN B because sera 4, 6, and 7 revealed significantly higher or exclusive IgE binding to peptides of α_{S1} -CN B.

As indicated in Figure 2b, the highest signal intensity of the responses against the different β -CN peptides were almost 1,500% lower (700 versus 11,000 arbitrary units) compared with the highest signals obtained for peptides of α_{S1} -CN. Four AA exchanges occurring in epitopes AA 55 to 72 and AA 106 to 122 of 7 β -CN variants (A¹, A³, B, C, F, G, and H²) only induced positive IgE binding in serum 4 but without significant differences in the immunoreactivity between the mutated and the reference peptides.

The peptides AA 110 to 125 and AA 108 to 129 of β -CN A¹ and A², as well as AA 109 to 123 and AA 103 to 123 of β -CN B, which were found to resist gastrointestinal digestion of β -CN, reacted with IgE antibodies of serum 4 (Figure 2b). Significant variations in IgE binding could be demonstrated between peptides AA 103 to 122 and AA 104 to 123 of β -CN B, comprising the digested peptide AA 103 to 123, and peptides AA 108 to 127 and AA 110 to 129 of β -CN A¹ and A², representing the digested peptide AA 108 to 129, where peptides of variant A¹ and A² were not recognized by the IgE antibodies of serum 4.

Among the variant-specific κ -CN peptides, marked IgE binding was solely found for the κ -CN peptide AA 9 to 26 in serum 4. Nevertheless, the substitution of an arginine (R) by histidine (H) in position 10 of κ -CN F² resulted in no significant change in immunoreactivity, as the remaining IgE binding was less than 15% compared with the reference peptide of κ -CN A (Figure 2c).

Identification of Interspecies Differences on the Epitope Structure

Due to the high degree of similarity in AA sequence between the known IgE-binding epitopes of caseins from cows and caseins from goats and water buffaloes, differences in their IgE-binding potential were investigated. The majority of sera showed IgE binding to α_{S1} -CN peptides of cows and the homologous counterpart of goats as well as water buffaloes. Some sera bound exclusively to peptides of cows, water buffaloes or goats. As illustrated in Figure 3a, b, and c, only the immunoreactivities of α_{S1} - and β -CN peptides were affected by the variation in the epitope sequences occurring in the different species. In most sera, a significant modification in IgE binding between epitopes of cows and the other species occurred if the peptides differed from each other in at least 2 AA. Nevertheless, in some epitopes, 1 AA was sufficient to cause differences in the immunoreactivities between the epitopes of the species.

For several sera, marked diminished IgE binding ($\geq 15\%$ of IgE binding remaining compared with the reference peptides of cows) to α_{S1} -CN peptides AA 17 to 36 (sera 4 and 5), AA 64 to 78 (serum 4), AA 139 to 154 (sera 3 and 4) from goats as well as to α_{S1} -CN peptides AA 139 to 154 (sera 3 and 4) and AA 173 to 192 (serum 2) from water buffaloes was found (Figure 3a and b). In contrast, the α_{S1} -CN peptides AA 139 to 154 (sera 1 and 2), AA 173 to 192 (serum 7), and AA 175 to 194 (serum 7) of goats and water buffaloes were characterized by marked increased IgE binding compared with the peptides of cows.

Individual sera analyses recognized the cow α_{S1} -CN peptides AA 34 to 48 (serum 5), AA 121 to 135 (serum 5), AA 159 to 174 (sera 1, 3, and 4), and AA 173 to 192 (sera 2 and 3). However, they had no or less IgE binding to the homologous peptides of goats and water buffaloes. In contrast, 3 sera showed IgE binding solely to the α_{S1} -CN peptides AA 17 to 36 (serum 6), AA 173 to 192 (serum 6), AA 175 to 194 (serum 2), and AA 106 to 120 (serum 4) of goats or water buffaloes, or both, but not to the reference peptides of cows. In 3 sera, no significant differences in immunoreactivity were found between the α_{S1} -CN peptides AA 17 to 36 (serum 2), AA 173 to 192 (sera 2 and 4), and AA 175 to 194 (sera 4 and 6) of the 3 species.

For β -CN, significant IgE binding was seen for peptide AA 16 to 35 of goats and water buffaloes (sera 4 and 7), as indicated in Figure 3c. Additionally, serum 4 reacted with the β -CN peptide AA 55 to 72 of cows and water buffaloes. However, no reaction against the homologous peptide of goats could be observed. On the other hand, significant IgE binding was only detected for peptide AA 135 to 149 (serum 4) of goats.

DISCUSSION

Identification of IgE-Binding Epitopes in Patient Sera Samples

In this study, each individual serum had a heterogeneous IgE recognition pattern in terms of diversity and intensity of epitope binding. This agrees with previous observations and might be associated with clinical features of CMA, such as the course or the severity of allergic reactions (Savilahti et al., 2010; Wang et al., 2010). Most sera were monosensitized toward α_{S1} -CN, as IgE binding was mainly seen for α_{S1} -CN peptides, which is consistent with the fact that α_{S1} -CN has been described as the main allergen among the 4 caseins (Docena et al., 1996; Gaudin et al., 2008, Schulmeister et al., 2009).

The strongest fluorescence intensities and binding to most of casein peptides were obtained with serum 4. Nevertheless, for most samples, weak signal intensities on the peptide microarray were observed, but showed significant higher signals compared with noise. Conceivably, this is caused by IgG antibodies, which compete with the binding of serum IgE to the specific epitope peptides. It has been demonstrated that IgE and IgG antibodies recognize the same or similar epitopes on the various CMP (Chatchatee et al., 2001a,b; Savilahti et al., 2010). Due to this fact, it also might be that additionally to serum 4, further sera would react with other α_{S2} -, β -, or κ -CN peptides. Therefore, the possible competing interactions between IgE and IgG antibodies had to be considered in further analyses.

Identification of Variant-Specific Differences on the Epitope Structure

To date, the only treatment of CMA is strict avoidance of all CMP, including CMP-derived infant formulas and other dairy products. However, this carries the risk of inadequate nutrient intake, such as calcium or a negative calorie:protein ratio and AA composition (Fiocchi et al., 2010). Thus, the identification of a suitable protein source or safe cow milk substitutes is an important aim for pediatricians and nutritionists to ensure a balanced diet for CMA patients. Depending on the nature of the

allergy and as a consequence of the specific sensitization pattern of an individual, the best alternative diet has to be defined for each patient (Restani et al., 2009; du Toit et al., 2010). In the present study, it was demonstrated that the genetic polymorphism has influence on the immunoreactivity of some casein epitopes and peptides resisting gastrointestinal digestion, although with significant differences between individual sera (Table 2). A marked heterogeneity in IgE binding was seen for the variant-specific epitopes AA 4 to 23 of α_{S1} -CN A as well as AA 173 to 192 and AA 175 to 194 of α_{S1} -CN C and E because the deletions and substitutions resulted in abrogation or decreased or increased IgE binding in particular sera. This is of importance, as the corresponding epitopes AA 17 to 36 and AA 173 to 194 of the reference protein α_{S1} -CN B were identified as immunodominant IgE-binding regions, not only in this study but also by Chatchatee et al. (2001a), because they were recognized with the highest IgE-binding intensities in the majority of patients. Therefore, abrogated or increased IgE binding to both epitopes in α_{S1} -CN A, C, and E may change the allergenicity of the whole proteins in most patients. In contrast, epitope AA 83 to 102 of α_{S1} -CN I showed almost identical IgE binding between individual sera. The AA substitution led to a significant increase in IgE binding in 3 of the 7 sera. This is quite surprising, as the substituted AA, glutamic acid (E), and aspartic acid (D) are both polar, acidic, and have the same hydrophobicity and, consequently, no change in the immunoreactivity between epitope AA 83 to 102 of α_{S1} -CN I and B may be expected. However, in this epitope, the substitution is located at position 84 at the N terminus of the epitope, which confirms the observation of Cocco et al. (2003). They reported that substitutions of hydrophobic AA in the central portion of epitopes in α_{S1} -CN were most likely to induce a loss of antibody binding, whereas a small increase in IgE binding was observed for some substitutions at the periphery of the epitope.

For the majority of variant-specific peptides, no significant differences in IgE binding could be found compared with the corresponding reference peptides. This can be explained by the fact that the occurrence of only 1 AA substitution in these epitopes is not sufficient to alter their IgE-binding properties. In general, all sera showed variations in the IgE-binding intensities when peptides differed from each other by 2 AA. For example, with serum 4, more IgE binding was determined for peptides AA 173 to 192 > AA 175 to 194 > AA 174 to 193 of α_{S1} -CN B and for peptides AA 173 to 192 > AA 175 to 194 > AA 179 to 198 of α_{S1} -CN C. This indicates that for α_{S1} -CN, a minimum of 2 AA have to be changed to alter the immunoreactivity in the majority of epitopes, which is in agreement with the results of the mutational analysis of α_{S1} -CN by Cocco et

al. (2003). However, for the bulk of epitopes in β - and κ -CN, as well as in the peanut allergens Ara h1, Ara h2, and Ara h3, 1 AA substitution has been detected to be sufficient for inducing a significant modification in IgE binding (Burks et al., 1997; Stanley et al., 1997; Cocco et al., 2007; Han et al., 2008).

Some of the α_{S1} - and β -CN peptides, which were found to resist in vitro gastrointestinal digestion (Lisson et al., 2013, 2014), showed positive IgE binding on microarray immunoassay, suggesting that these digested low-molecular weight peptides retain an allergenic potential, as was also described for other food allergens (Bøgh et al., 2009, 2012; Martos et al., 2012). In the current study, peptides 13 AA in length, such as AA 184 to 196 of α_{S1} -CN B as well as AA 180 to 193 of α_{S1} -CN B and C, displayed significant IgE binding on the peptide microarray, even if immunoreactivity was lower compared with the longer peptides (e.g., AA 173 to 192 or AA 175 to 194). It is generally thought that peptides have to be approximately 3,500 Da (30 AA residues) in length and must contain at least 2 IgE-binding epitopes to induce cross-linking of the IgE receptors for stimulating an immune response (Huby et al., 2000). Nevertheless, it has been reported that immunization with free peptides as small as 6 to 14 AA long induced an acceptable antibody response (Atassi and Young, 1985; Muller, 1999). This was also demonstrated for the peanut allergen Ara h1, where peptides of less than 2,000 Da were able to sensitize and elicit allergic reactions (Bøgh et al., 2009, 2012). Results of the present study suggest that this could also apply to casein peptides. Thus, they probably do not need to survive the digestion process as large fragments to react with the immune system, which might contribute to the high sensitization rate to the caseins and explain why caseins are such potent allergens despite their excellent digestibility.

One limitation of the current study was the small number of tested sera, most of which reacted solely with α_{S1} -CN. Among the 16 sera used in this study, 7 displayed positive IgE binding on the microarray and only 2 sera exhibited positive IgE binding to β - and κ -CN peptides. Thus, it is entirely possible that some epitopes of β - and κ -CN variants differ in their allergenic properties when tested with other sera. All sera had milk-specific IgE ≥ 95.5 kU_A/L, meaning that low or lacking signals on the microarray were due to confounding factors in the methods rather than the quality of the sera. To evaluate differences in immunoreactivity between the variant-specific epitopes, further investigations with a larger number of patient sera known to have IgE against both proteins are needed.

In this study, decreased, abrogated, or even higher IgE binding to the immunodominant epitopes AA 17

to 36, AA 173 to 192, and AA 175 to 194 in α_{S1} -CN A, C, or E was identified (Table 2). For example, serum 2 showed significant lower IgE binding to peptide AA 4 to 23 of α_{S1} -CN A than to peptide AA 17 to 36 of α_{S1} -CN B, C, and E. Furthermore, peptide AA 175 to 194 of variant A, sharing the same sequence with peptide of α_{S1} -CN B, was no longer recognized from IgE antibodies of serum 2, whereas significant IgE binding was found to the corresponding peptide of α_{S1} -CN C and E. As a consequence, it is likely that the decreased and abrogated IgE binding to these 2 immunodominant epitopes in α_{S1} -CN A changed the allergenicity of the whole protein and, thus, milk from cows with genotypes α_{S1} -CN AA might be characterized by a lower or, as was detected for sera 4 and 6, higher allergenicity. This could be also relevant for cows carrying the genotype α_{S1} -CN CC and EE regarding the differences in IgE binding to epitopes AA 173 to 192 and AA 175 to 194. In the case of reduced allergenic potential, these protein variants might be suitable for the production of hypoallergenic milk or as a basis for infant formulas for a well-selected group of allergic patients monosensitized to α_{S1} -CN. This does not necessarily mean that whole milk has to be used, but rather individual milk protein fractions, which could be used for specific processed products. To achieve this aim, selection and breeding of particular cows with genotypes α_{S1} -CN AA, CC, or EE would be necessary. However, α_{S1} -CN A is rather common, but occurs with low frequencies in Holstein-Friesian and German Red cows (Erhardt, 1993). In contrast, α_{S1} -CN C is very common in all main breeds, with higher frequencies such as 0.45 in Jerseys or 0.2 in Pinzgauers and α_{S1} -CN E has been reported only in *Bos grunniens* (Caroli et al., 2009). There is already a kind of milk with a specific genotype on the market. Since 2003, A² milk, containing only the A² variant of β -CN, has been marketed in New Zealand and Australia because epidemiological evidence implies that A² milk is better for human health than A¹ milk (A2 Corporation Ltd., Auckland, New Zealand), although the European Food Safety Authority could not establish a relationship between A¹ milk and increased disease (EFSA, 2009). Because of the fact that IgE antibodies from individual patients also displayed increased binding to several mutated epitopes in the current study makes it difficult to consider specific genetic variants as a safe protein source for all patients with CMA.

Identification of Interspecies Differences on the Epitope Structure

In the quest to identify alternative protein sources for patients with CMA, there has been an increasing focus on the use of proteins from other species. Neverthe-

less, clinical cross-reactivity exists between milk from cows, sheep, goats, and water buffaloes (Järvinen and Chatchatee, 2009; Restani et al., 2009), which is due to the high degree of AA sequence homology between the proteins from cows and the other Bovidae (96.1% for buffaloes, 91.1% for sheep, and 87.6% for goats; Restani et al., 2009). Most patients who are sensitized to cow milk do not tolerate goat or sheep milk (Bellioni-Businco et al., 1999; Carroccio et al., 1999). In the present study, we found that several sera reacted with both cow peptides and the corresponding peptides of goats and water buffaloes, demonstrating cross-reactivity or co-sensitization. However, in the majority of sera, individual epitopes from goats and water buffaloes showed lower immunoreactivity compared with epitopes from cows, which suggests reduced allergenic activity of α_{S1} -CN from goat. These findings are in agreement with the results of other studies (Spuerger et al., 1997; Bevilacqua et al., 2001; Lara-Villoslada et al., 2004; Sanz Ceballos et al., 2009; Hodgkinson et al., 2012). The lower allergenic potential of goat milk could be attributable, at least in part, to the high genetic polymorphism of α_{S1} -CN that occurs also in goat milk proteins. In goat milk, genetic variants of α_{S1} -CN are characterized by high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and null quantities of this protein (Ballabio et al., 2011). Several studies demonstrated that goat milk with low or null α_{S1} -CN content has reduced allergenic potential. Ballabio et al. (2011) investigated the allergenic potency of goat milk with different α_{S1} -CN genotypes. They detected lower immunoreactions to α_{S1} -CN 0₁0₁ (null content of α_{S1} -CN) and 0₁F (reduced content of α_{S1} -CN) genotypes compared with goat milk samples containing α_{S1} -CN BB, BE, EE, or FF. By using guinea pig models (Bevilacqua et al., 2001; Sanz Ceballos et al., 2009) and murine models (Lara-Villoslada et al., 2004; Hodgkinson et al., 2012), it could be also shown that milk from goats producing low levels of α_{S1} -CN is potential less allergenic than milk from cows or goats containing higher levels of this protein. This may be one reason why some cow milk-allergic children are able to tolerate goat milk (Restani, 2004).

Moreover, in the present study, individual sera IgE only recognized α_{S1} -CN and β -CN peptides of goats or water buffaloes but not the corresponding cow milk peptides. This indicates higher allergenicity of both proteins from the nonbovine species and might explain the observation that some patients have a selective allergy to goat or sheep milk but not to cow milk, as has been reported by others (Alvarez and Lombardero, 2002; Bidat et al., 2003; Ah-Leung et al., 2006; Mori et al., 2013).

Interestingly, in serum 4, significant IgE binding was found to the α_{S1} -CN peptide AA 106 to 120 of water

buffaloes. In comparison with the corresponding peptides of cows and goats, showing no IgE binding in any serum, this peptide lacks a phosphorylated serine at position 115. Conflicting results exist about the role of phosphorylation in allergy. Cases et al. (2011) demonstrated that phosphorylation reduces the allergenicity of cow caseins in children with selective allergy to goat and sheep milk. In contrast, it has been assumed that the major phosphorylation sites act as important allergenic epitopes in the caseins, as dephosphorylation significantly diminish their allergenicity (Bernard et al., 2000), which is in concordance with the results of the present study. Twenty peptides were synthesized in phosphorylated and dephosphorylated form, but only 1 peptide (AA 106 to 120 of α_{S1} -CN) revealed differences in immunoreactivity. The other peptides displayed no significant IgE binding on the microarray immunoassay. A possible explanation is that 17 of the 20 peptides were from α_{S2} -, β -, and κ -CN and, therefore, not recognized by the IgE antibodies because most sera tested in this study were sensitized against α_{S1} -CN.

CONCLUSIONS

Genetic polymorphisms of caseins influence the allergenic potential of some immunodominant epitopes. Single AA substitutions or deletion resulted in a loss or decrease or increase in immunoreactivity in some α_{S1} - and β -CN epitopes with the tested sera. The genetic polymorphism of caseins should be taken into account in the search for a suitable protein source for CMA patients. However, a heterogeneous pattern of IgE binding to the variant-specific peptides was found between the individual sera. Thus, further clinical studies with more patient sera are needed to verify their suitability for CMA patients. The results of the present study confirm that the milk of goats and water buffaloes harbor an allergenic potential due to cross-reactivity between the proteins and are, consequently, not an appropriate replacement for patients with CMA. Nevertheless, these species are also characterized by genetic polymorphisms that affect the allergenic properties of their IgE-binding epitopes, as has been already shown for goat milk.

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