

Cloning, Expression, and Mapping of Allergenic Determinants of α S1-Casein, a Major Cow's Milk Allergen¹

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Milk is one of the first components introduced into human diet. It also represents one of the first allergen sources, which induces IgE-mediated allergies in childhood ranging from gastrointestinal, skin, and respiratory manifestations to severe life-threatening manifestations, such as anaphylaxis. Here we isolated a cDNA coding for a major cow's milk allergen, α S1-casein, from a bovine mammary gland cDNA library with allergic patients' IgE Abs. Recombinant α S1-casein was expressed in *Escherichia coli*, purified, and characterized by circular dichroism as a folded protein. IgE epitopes of α S1-casein were determined with recombinant fragments and synthetic peptides spanning the α S1-casein sequence using microarrayed components and sera from 66 cow's milk-sensitized patients. The allergenic activity of α S1-casein and the α S1-casein-derived peptides was determined using rat basophil leukemia cells transfected with human Fc ϵ RI, which had been loaded with the patients' serum IgE. Our results demonstrate that α S1-casein as well as α S1-casein-derived peptides exhibit IgE reactivity, but mainly the intact α S1-casein induced strong basophil degranulation. These results suggest that primarily intact α S1-casein or larger IgE-reactive portions thereof are responsible for IgE-mediated symptoms of food allergy. Recombinant α S1-casein as well as α S1-casein-derived peptides may be used in clinical studies to further explore pathomechanisms of food allergy as well as for the development of new diagnostic and therapeutic strategies for milk allergy. *The Journal of Immunology*, 2009, 182: 7019–7029.

Immunoglobulin E-mediated allergies belong to the most common forms of immunologically mediated forms of hypersensitivity reactions to food (1). In sensitized individuals dietary intake of food can cause a variety of clinical manifestations reaching from oral allergy syndrome and gastrointestinal symptoms (e.g., vomiting, diarrhea) to skin, respiratory, and severe systemic manifestations such as anaphylactic shock (2–4). The development of food allergy shows a typical course (5). It starts early in childhood mainly against Ags encountered in the initial diet (e.g., cow's milk, eggs) and affects between 4% and 6% of children. Milk is one of the first food components introduced into the

diet and therefore represents one of the most important food allergen sources in terms of frequency and severity of allergic manifestations (6–9). The symptoms of cow's milk allergy are due to IgE-mediated activation of mast cells and basophils as well as to activation of allergen-specific T cells, and they comprise a plethora of gastrointestinal, skin, respiratory, and severe systemic manifestations such as death due to anaphylactic shock. Unlike in respiratory allergy, which proceeds untreated from mild (e.g., rhinoconjunctivitis) to severe manifestations (e.g., asthma), many milk-allergic children grow out their allergy, and the induction of tolerance against cow's milk allergens has already been described (10, 11). In this context it has been reported that allergen-specific CD4⁺CD25⁺ regulatory T cells may be involved in the induction of tolerance against cow's milk allergens in children who outgrew cow's milk allergy (12).

Furthermore, it has been suggested that the development of tolerance to cow's milk allergens is associated with a reduction of allergen-specific IgE levels and a reduction of IgE recognition of certain sequential epitopes (13, 14). Reduced sensitivities in the gut and the outcome of oral challenge tests are also reflected by reduced skin sensitivity to food allergens (15, 16).

Cow's milk contains >25 different proteins, but only the whey proteins α -lactalbumin, β -lactoglobulin, BSA, and lactoferrin, as well as the four caseins, have been identified as allergens (17). The casein fraction is composed of α S1-, α S2-, β -, and κ -casein, of which α S1-casein seems to be a major allergen according to IgE and T cell recognition data (18–21).

Here we constructed an expression cDNA library from bovine mammary glands and used IgE Abs from cow's milk-allergic patients to isolate a cDNA coding for α S1-casein and IgE-reactive α S1-casein fragments. The recombinant α S1-casein allergen was obtained by expression in *Escherichia coli*, purified, and characterized regarding its physicochemical and

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Table I. Characterization of milk-allergic patients^a

Patient	Age	Age Allergy Started	Sex	Country	Milk-Related Symptoms	SPT	CM	Challenge	CMA Outgrown	Other Allergies	Total IgE (kU/L)	CM sIgE (kU _A /L)	α S1-Casein (FI)
1*	61 yr	NK	F	A	NK	Pos	ND	ND	NK	Cat, WF, T, K, P	355	26	21.0
2*	64 yr	38 yr	F	F	U, Sys, GI	Pos	Pos	Pos	No	No	1,302	209.6	1,288.0
3*	42 yr	NK	F	A	OAS	Neg	ND	No	Mite, cat		148	15.3	17.5
4*	26 yr	NK	M	G	NK	ND	Pos	NK	NK		3,736	5.34	82.5
5	17 yr	NK	F	G	NR	ND	Neg ^d	NA	NK		926	4.12	29.0
6*	16 yr	NK	F	G	NR	ND	Neg ^d	NA	HE		433	12.4	4513.0
7*	14 yr	NK	F	G	NK	ND	NK	NK	NK		14,525	40.2	12,058.5
8*	13 yr	NK	M	G	RC, V	ND	Pos	NK	HE		866	7.8	210.0
9*	13 yr	11 mo	M	I	U, E, V, AS	Pos	NP ^c	No	PO, HE		909	147.4	12,444.5
10	11 yr	NK	M	G	NK	ND	NK	NK	NK		763	4.99	195.0
11*	11 yr	9 mo	F	I	U, AE, AS	Pos	NP ^c	No	Candida		ND	22.5	8,717.0
12*	10 yr	NK	F	G	Ap	Pos	Pos	NK	ND		1,432	44.8	3,785.5
13	10 yr	NK	F	G	U, V, D	Pos	Pos	NK	ND		653	10.4	893.0
14*	8 yr	NK	M	G	U, I	Pos	Pos ^d	NK	HE		455	3.77	973.0
15*	8 yr	NK	M	G	E, U, AS	Pos	Pos ^d	NK	HE		2,200	49.7	6,165.0
16	8 yr	NK	M	G	U, R, RC	Pos	Pos	NK	HE		1271	84.1	22,097.5
17*	7 yr	NK	M	G	U, R, AS	Pos	Pos ^d	NK	HE		399	17.8	836.5
18*	7 yr	NK	M	G	NK	ND	NK	NK	NK		2,275	20	4,931.5
19	7 yr	NK	M	G	Sys	Pos	Pos ^d	NK	NK		7,480	101	2,435.5
20*	6 yr	NK	NK	G	NK	ND	NK	NK	NK		246	6.09	356.0
21	6 yr	NK	M	G	NK	ND	NK	NK	NK		297	5.95	108.0
22*	5 yr	NK	M	G	NR	ND	Neg ^d	NA	HE		866	4.35	517.5
23	5 yr	NK	F	A	U, AS	Pos	ND	NK	GP, mite, cat, HE, PN		386	16.5	319.0
24	5 yr	NK	M	G	NK	ND	NK	NK	NK		252	27.9	1,595.0
25*	5 yr	NK	M	G	NK	ND	NK	NK	NK		2,760	6.44	1,000.5
26	5 yr	NK	M	G	NK	ND	NK	NK	NK		848	45.2	17.5
27*	4 yr	NK	M	G	U, I	ND	Pos ^d	NK	HE		56.6	6.19	3,658.5
28*	4 yr	NK	F	G	Sys	Pos	Pos ^d	NK	NK		974	98	19,178.0
29*	4 y	NK	M	G	NK	ND	NK	NK	NK		246	6.4	117.5
30*	4 y	NK	M	G	NK	ND	NK	NK	NK		1,894	3.81	87.5
31*	4 y	NK	F	G	Sys	Pos	Pos	NK	HE		489	62.5	8,847.5
32*	4 yr	NK	F	G	NK	ND	NK	NK	NK		116	6.94	943.0
33	3 yr	NK	M	G	U	Pos	Pos	NK	ND		137	24.6	3,986.5
34*	3 yr	NK	M	G	U, AE, R	ND	Pos ^d	NK	HE		59.1	4.13	27.5
35*	3 yr	NK	M	G	U, R, W	Pos	Pos ^d	NK	HE		59.1	4.13	123.0
36	3 yr	NK	M	G	NR	ND	Neg	NA	NK		10.9	5.81	1,025.5
37*	3 yr	NK	F	G	Sys	Pos	Pos ^d	NK	NK		125	11	1,474.0
38*	3 yr	NK	M	G	NK	ND	NK	NK	NK		26	5.19	61.5
39*	3 yr	NK	F	G	NK	ND	NK	NK	NK		1,718	4.15	2,480.0
40*	3 yr	NK	M	G	Sys	ND	ND	NK	NK		325	44.4	5,523.0
41*	3 yr	NK	F	G	NK	ND	NK	NK	NK		125	11	591.5
42*	3 yr	NK	F	G	NK	ND	NK	NK	NK		201	9.66	766.5
43*	3 yr	NK	M	G	NK	ND	NK	NK	NK		67.5	10.7	142.5
44*	2 yr	NK	M	G	RC, U, OAS	Pos	Pos ^d	NK	ND		1,466	82.7	36.5
45*	2 yr	9 mo	F	S	U (face)	Pos	Pos ^b	Yes	HE		ND	3.82	28.0
46*	2 yr	3 mo	F	S	U, AD, V	Pos	Pos ^b	No	HE, fish		ND	68.4	21,518.0
47*	2 yr	NK	M	A	AD, OB	Neg	ND	NK	Mite		134	9.3	620.0
48	2 yr	NK	F	G	NK	ND	NK	NK	NK		53.9	6.71	67.0
49	2 yr	NK	M	G	NK	ND	NK	NK	NK		>100	46.9	7,310.0
50*	2 yr	NK	F	G	NK	ND	NK	NK	NK		83.4	17.7	219.0
51*	2 yr	NK	M	G	NK	ND	NK	NK	NK		193	4.82	132.0
52*	2 yr	NK	M	G	NR	ND	Neg ^d	NA	NK		117	11.8	2,140.5
53	1 yr	NK	F	A	AD	NP ^c	ND	NK	NK		88.5	6.24	99.0
54*	1 yr	NK	F	A	AD, OB	Pos	ND	NK	EW, soja, nuts		217	16.5	118.0
55*	1 yr	5 mo	M	S	U, AE (face)	Pos	Pos ^b	No	HE, fish		13.5	8.05	16.5
56*	9 mo	9 mo	F	S	U, GI	Pos	Pos ^b	No	No		351	16.14	72.0
57	7 mo	7 mo	F	S	U, GI	Pos	Pos ^b	No	No		161	17.3	852.5
58	7 mo	7 mo	F	S	U, GI	Pos	Pos ^b	Yes	No		136	3.11	134.5
59*	4 mo	4 mo	M	S	U (face)	Pos	Pos ^b	No	No		2,000	13	52.5
60*	4 mo	4 mo	F	S	U	Pos	Pos ^b	Yes	No		60	9.97	27.0
61*	4 mo	3 mo	F	S	U (face)	Pos	Pos ^b	Yes	No		18.7	ND	30.5
62*	3 mo	3 mo	F	S	GI	Pos	Pos ^b	Yes	No		29	12.3	450.5
63*	3 mo	3 mo	M	S	U, AE	Pos	Pos ^b	Yes	No		51	16.6	882.5
64*	3 mo	3 mo	F	S	U	Pos	Pos ^b	Yes	NK		1,292	22.4	4,599.5
65	NK	NK	NK	A	NK	ND	NK	NK	NK		179	26.5	1,425.0
66*	NK	NK	NK	G	NK	ND	NK	NK	NK		1,129	66.4	13,629.5

^a Abbreviations: *, patients tested also for IgE reactivity to Cas1.2–Cas5.6; yr, years; mo, months; F, female; M, male; A, Austria; S, Spain; G, Germany; I, Italy; F, France; CM, cow's milk; SPT, skin-prick test; NA, not applicable; ND, not done; NK, not known; NP, not possible; NR, no reaction; Pos, positive; Neg, negative; FI, fluorescence intensity; kU_A/L, kilounits allergen-specific IgE per liter; kU/L, kilounits per liter; OAS, oral allergy syndrome; AD, atopic dermatitis; AE, angioedema; AS, asthma; Ap, abdominal pain; D, diarrhea; GI, gastrointestinal symptoms; I, itching; OB, obstructive bronchitis; R, redness; RC, rhinoconjunctivitis; Sys, systemic reaction; U, urticaria; V, vomiting; W, wheals; GP, grass pollen; PO, pollen; WF, wheat flour; T, tomato; K, kiwi; P, pork; HE, hen's egg; EW, egg white; PN, peanut.

^b Open challenge as described by Garcia-Ara et al. (22).

^c Challenge not possible as the reaction to cow's milk is too strong.

^d Double-blind placebo-controlled food challenge.

structural properties. IgE-reactive epitopes of α S1-casein were mapped using recombinant fragments and synthetic α S1-casein-derived peptides. The frequency of IgE recognition of α S1-casein and α S1-casein-derived synthetic peptides was determined by microarray technology using sera from cow's

milk-allergic patients. Furthermore, the allergenic activity of the intact allergen and α S1-casein-derived peptides was determined using rat basophil leukemia cells expressing the human Fc ϵ RI receptor, which had been loaded with allergic patients' sera. Our results indicate that α S1-casein contains several

Table II. Synthetic α S1-casein-derived peptides

Peptide	Sequence ^a	Length (aa)	pI	Molecular Mass (kDa)
Cas1 ^b	RPKHPIKHQGLPQEVLNENLLRFFVAPFPEV C	32	8.22	3.75
Cas1.2	VLNENLLRFFVAPFPEVFGKEKVNELSKDIGS	32	5.01	3.64
Cas2	FGKEKVNELSKDIGSESTEDQAMEDIKQMEAE S C	33	4.18	3.70
Cas2.3	EDQAMEDIKQMEAE S ISSEI V PNSVEQ K	30	3.89	3.38
Cas3	ISSSEI V PNSVEQKH I QKEDVP S ERYLG Y EQ L LR C	36	4.80	4.21
Cas3.4	VP S ERYLG Y EQ L LR L KK Y KVPQ L EIV P NS	30	9.31	3.57
Cas4	C LKK Y KVPQ L EIV P NSAEER L HS M KEGI H AQ Q KE	34	8.14	3.96
Cas4.5	RLHS M KEGI H AQ Q KE P MIGVNQELAYFY P E	30	6.07	3.55
Cas5	C PMIGVNQELAYFY P ELFRQFYQLDAYPSGAWYY V	35	4.14	4.24
Cas5.6	FYQLDAYPSGAWYYVPLGTQYTDAP S FS D I	30	3.42	3.44
Cas6	PLGTQYTDAP S FS D IPNP I GS E NS E KT T MPLW C	33	3.92	3.60

^a Cysteine residues added to facilitate coupling are indicated in boldface letters.
^b Cas indicates α S1-casein-derived peptide.

sequential IgE epitopes, but the isolated peptides were less potent than the complete allergen in triggering effector cell degranulation.

Materials and Methods

Patients and biological materials

Sera from children ($n = 62$; age 3 mo to 17 years; 27 females and 32 males, 2 unknown) and adults ($n = 4$; age 26–64 years; three women, one man) containing cow’s milk-specific IgE Abs (CAP- α FEIA; Phadia) were obtained from Austria ($n = 7$), Germany ($n = 44$), Italy ($n = 2$), Spain ($n = 12$), and France ($n = 1$). Table I summarizes the available

demographic, clinical, and serological characteristics of the patients. Information regarding symptoms after consumption of cow’s milk was available for 42 out of the 66 patients and for 16 patients it was known whether they had persistent cow’s milk allergy or if they had become tolerant (persistent, $n = 9$; tolerant, $n = 7$). Fourteen patients underwent double-blind challenge and 12 had received an open challenge as described by Garcia-Ara et al. (22). For 15 patients we knew the exact age when their cow’s milk allergy started. Only patient no. 2 became allergic when she was adult. The study was approved by the Ethics Committee of the Medical University of Vienna. Natural α -casein fractions containing a mixture of α S1- and α S2-casein were purchased from Sigma-Aldrich, and cow’s

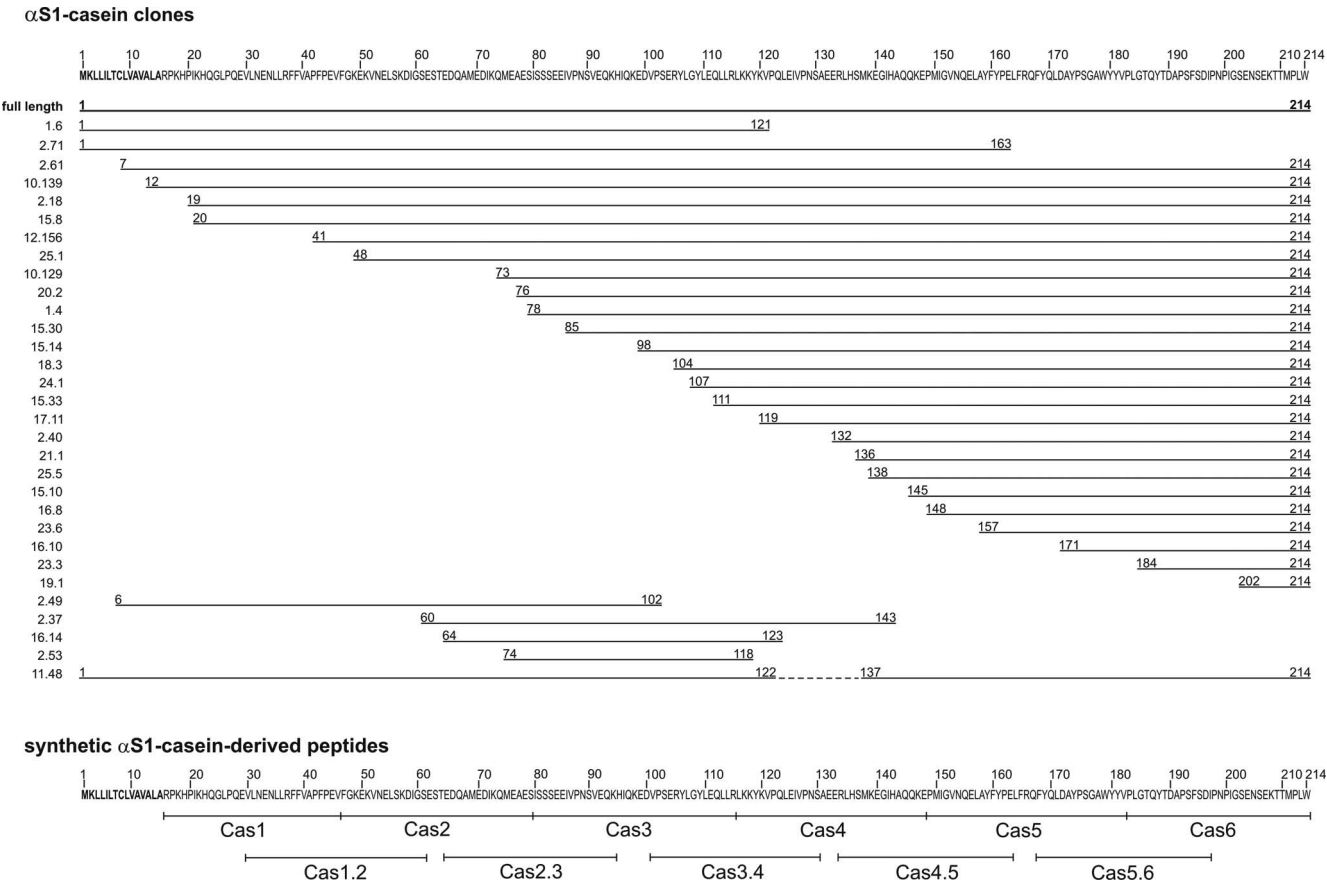


FIGURE 1. IgE-reactive recombinant α S1-casein fragments identified by screening of an expression cDNA library from bovine mammary glands. At the top the deduced amino acid sequence of α S1-casein (aa 1–214) is shown in the single letter code. Twenty-eight full-length cDNAs with identical sequence and 31 cDNAs coding for recombinant fragments (left margin, clone numbers) are shown. The cDNA of clone 11.48 codes for a protein variant with a deletion (broken line). At the bottom the location of synthetic α S1-casein peptides used for epitope mapping is shown. The DNA and deduced amino acid sequence of the α S1-casein clones were submitted to GenBank under the accession nos. EU221551–EU221581.

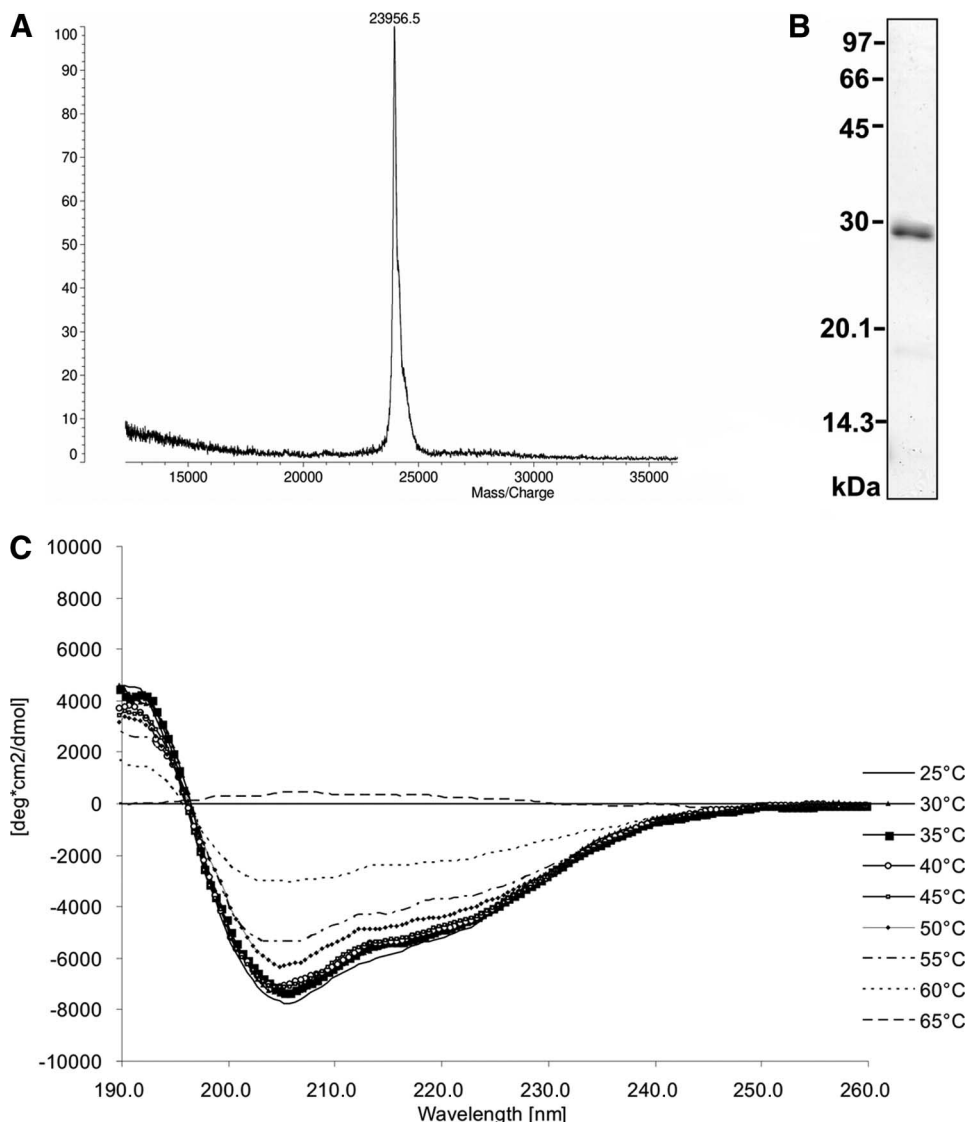


FIGURE 2. Biochemical and structural characterization of purified α S1-casein. **A**, Mass spectrometric analysis of purified α S1-casein. The mass/charge ratio is shown on the x-axis and the signal intensity is displayed on the y-axis as the percentage of the most intensive signal obtained in the investigated mass range. **B**, A Coomassie brilliant blue-stained SDS-PAGE containing purified α S1-casein is shown in **B**, where molecular masses are indicated in kDa in the left margin. Thermal unfolding of α S1-casein recorded by circular dichroism is displayed in **C**. The observed ellipticity (Θ , y-axis) was monitored from 190 to 260 nm at different temperatures (25–65°C).

milk (NÖM; 3.6% fat, pasteurized, batch 324103:43) was bought at the local market.

Construction and IgE screening of a bovine mammary gland cDNA library

Total RNA was isolated from bovine mammary glands obtained from a lactating cow using the NucleoSpin RNA L kit (Macherey-Nagel). Polyadenylated mRNA was separated using the NucleoTrap mRNA Mini kit (Macherey-Nagel). cDNA was obtained using oligo(dT)15 primers and the cDNA Synthesis System (Roche) following the manufacturer's instructions. The double-stranded cDNA was methylated, ligated to *Eco*RI linkers, digested with *Eco*RI, and inserted into dephosphorylated λ gt11 *Eco*RI-cut arms and packed into λ phages using the Lambda gt11/*Eco*RI/CIAP-Treated/Gigapack III cloning kit (Stratagene). The expression library (162,000 phages) was screened with a pool of sera from three cow's milk-allergic patients with a broad IgE reactivity profile to cow's milk allergens, and bound IgE Abs were detected with ¹²⁵I-labeled anti-IgE Abs (IBL International). Membranes were exposed to x-ray films (Kodak). Phage DNA of positive clones was amplified using λ gt11 forward primer and λ gt11 reverse primer (Stratagene), and the amplified PCR products were subjected to automated sequencing (VBC Genomics). The obtained nucleotide sequences were compared with the sequences deposited in the National Institutes of Health database (www.ncbi.nlm.nih.gov/BLAST/) and submitted to GenBank under the accession nos. EU221551–EU221581.

Expression and purification of full-length recombinant α S1-casein

The cDNA coding for the mature α S1-casein without N-terminal signal sequence and with C-terminal hexahistidine tag (α S1-casein) was obtained by PCR amplification using the PFU DNA polymerase system from Fermentas Life Sciences and the following oligonucleotides (MWG Biotech): α S1-casein 5', 5'-GCG GAT CCA CAT ATG AGG CCT AAA CAT CCT ATC AAG-3' (*Nde*I underlined); α S1-casein 3', 5'-CG GAA TTC CTG CAG AAC TCA GTG ATG ATG ATG ATG ATG CCA CAG TGG CAT AGT AGT CTT TTC-3' (*Eco*RI underlined; hexahistidine-encoding sequence is in italics); and the cDNA from the mammary gland cDNA library was used as a template. The PCR product was gel purified and cloned into the *Nde*I/*Eco*RI-digested expression vector pET17b (Novagen). The sequence was verified by automated sequencing (VBC Genomics).

E. coli strain BL21 (DE3) (Novagen) was transformed with the α S1-casein-pET17b construct. Protein expression was induced by the addition of isopropyl β -D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. Recombinant proteins accumulated in the inclusion body fractions. Bacterial cells were lysed, and proteins were solubilized in urea buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl (pH 8)) and purified by Ni²⁺-affinity chromatography (Qiagen). Purified α S1-casein was dialyzed against 10 mM sodium acetate (pH 5), and the protein concentration was determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories) using BSA as a standard. The purity was assessed by SDS-PAGE (23) and Coomassie staining (24).

Synthesis of α S1-casein-derived peptides

Peptides displayed in Table II were synthesized using Fmoc (9-fluorenylmethoxycarbonyl) strategy with HBTU ((2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) activation (0.1 mmol small-scale cycles) on an Applied Biosystems peptide synthesizer model 433A and purified as described (25).

The peptides Cas1, Cas2, Cas3, Cas4, Cas5, and Cas6 covered segments of α S1-casein, which according to surface prediction (26) contained sequences with high accessibility for Abs. In a second attempt for more detailed epitope mapping, peptides Cas1.2, 2.3, 3.4, 4.5, and 5.6 were made to overlap with the first six peptides (Table II).

MALDI-TOF mass spectrometry of α S1-casein

Laser desorption mass spectra were acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos and piCHEM). Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid) and α -cyano-4-hydroxycinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Circular dichroism (CD)³ analysis of purified α S1-casein

CD spectra were conducted on a Jasco J-810 spectropolarimeter fitted with a Jasco PTC-423S/L Peltier type temperature control system. Far-UV CD spectra were measured in a 2-mm pathlength quartz cuvette (Hellma) at a protein concentration of 0.1 mg/ml. Spectra were recorded from 190 to 260 nm with 1-nm resolution at a scan speed of 50 nm/min and resulted from averaging three scans. All measurements were performed in 1 mM sodium acetate (pH 5). The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength. For thermal denaturation, experiments of α S1-casein spectra were recorded at gradually increasing temperature (5°C steps) from 25°C to 95°C with a heat rate of 2°C/min.

IgE reactivity by immunoblot, dot blot, and microarray

For immunoblot analysis, 2 μ g/cm gel of α S1-casein was separated by SDS-PAGE and blotted onto nitrocellulose (27). For dot blot analysis 1 μ g of the protein was dotted onto nitrocellulose. Blots were incubated with sera from milk-allergic patients diluted 1/20 in PBST (PBS, 0.5% (v/v) Tween 20). Bound IgE Abs were detected with 1/15 diluted ¹²⁵I-labeled anti-human IgE Abs (IBL International).

IgE reactivity to microarrayed whole cow's milk extract, α S1-casein, a natural α -casein fraction, as well as to synthetic α -casein-derived peptides was determined as described in Nystrand (28). Whole milk extracts, recombinant and purified natural, as well as recombinant cow's milk proteins were spotted with a Nano-Plotter NP2 (Gesellschaft für Silizium-Mikrosysteme) onto capillary-flow membrane that was attached to ordinary microscope glass slides. The spotted microarrays were pre-wetted with 30 μ l of assay buffer, incubated with 25 μ l of undiluted sera of milk-allergic patients, washed with 30 μ l of assay buffer and assayed with 20 μ l of detecting Ab. Finally, the membrane was washed with 2 $\times$ 30 μ l of assay buffer. Bound IgE Abs were detected with a fluorescence-conjugated anti-IgE Ab and fluorescence intensities (FI) were measured at a wavelength of 670 nm. Cut-off level was set to a FI of 400 according to values gained with human serum albumin.

ELISA competition experiments

For the IgE competition experiments, α S1-casein in sodium carbonate buffer (pH 9.6) was coated onto ELISA plates (Nunc Maxisorb) at a concentration of 5 μ g/ml overnight at 4°C. Patients' sera diluted 1/5 in Tris-buffered saline containing 0.5% (v/v) Tween 20 (TBST) were preincubated overnight at 4°C either with 10 μ g/ml α S1-casein or with 10 μ g/ml each of the synthetic α S1-casein-derived peptides (Table II) (see Fig. 5A, Cas1–Cas6, and B, Cas1–Cas6 plus Cas1.2–5.6) (Mix) or, for control purposes, with TBST (not inhibited, NI). On the next day, the plates were washed five times with TBST and blocked with TBST at 37°C for 2.5 h. Preincubated patients' sera were exposed to ELISA plate-bound α S1-casein overnight. Bound IgE Abs were detected with a 1/1000 diluted monoclonal anti-human IgE Ab (BD Biosciences) and subsequently with a 1/1000 diluted HRP-labeled sheep anti-mouse IgG Ab (GE Healthcare). All determinations were conducted as duplicates.

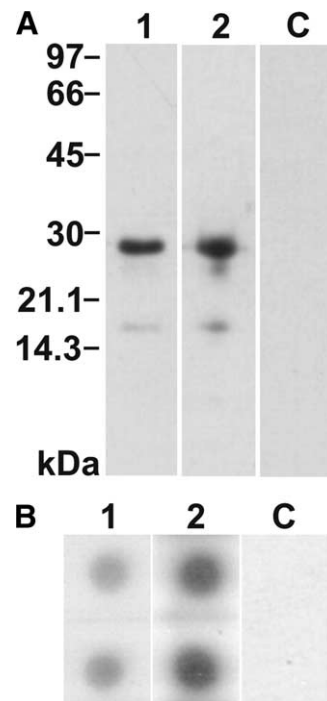


FIGURE 3. IgE binding of α S1-casein. IgE reactivity of nitrocellulose-blotted (denatured) α S1-casein by immunoblot (A) and dot blot analysis (B) under native conditions. Nitrocellulose-bound α S1-casein was incubated with the sera from two cow's milk-allergic patients (lanes 1 and 2) and a nonallergic individual (lane C), and bound IgE Abs were detected with ¹²⁵I-labeled anti-IgE Abs. Molecular masses are indicated in kDa.

Statistical analysis

Differences regarding IgE reactivity and induction of rat basophil leukemia (RBL) release among the patients with persistent vs transient cow's milk allergy were analyzed with the Mann-Whitney *U* test and were considered significant when *p* was <0.05 and have a trend toward significance when *p* < 0.1.

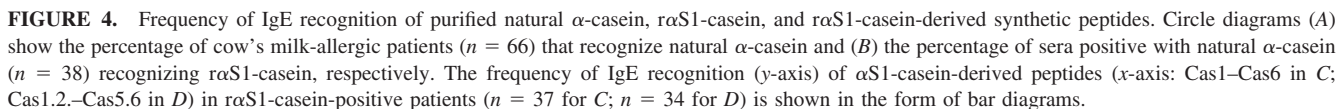
RBL assays

RBL cells (clone RBL-703/21) transfected with the human Fc ϵ RI receptor (29) were cultured in RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 100 μ M 2-ME, and 1% penicillin/streptomycin.

Cells were resuspended in culture medium (2 $\times$ 10⁶ cells/ml), and 50 μ l of this solution per well of a 96-well flat bottom microplate was pipetted. Sera from cow's milk-allergic patients were diluted 1/10 in culture medium and 50 μ l thereof was added to the cells incubated overnight at 37°C, 7% CO₂, 95% humidity.

Medium was removed and the plates were washed three times with 200 μ l/well Tyrode's buffer containing 0.1% (w/v) BSA or HSA, respectively. Then 100 μ l of the milk components (concentration, 0.3 μ g/ml) dissolved in Tyrode's buffer containing 50% D₂O and 0.1% (w/v) BSA/HSA was added to the cells and incubated for 1 h at 37°C, 7% CO₂, 95% humidity. Spontaneous release was determined by lysing cells in 1% Triton X-100 in Tyrode's buffer. Fifty-microliter aliquots of the supernatant were transferred to a fresh plate, mixed with 50 μ l of assay solution (0.1 M citric acid or sodium citrate (pH 4.5) plus 160 μ M 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide), and incubated at 37°C, 7% CO₂, 95% humidity for 1 h. The reaction was stopped by adding 100 μ l of glycine buffer (200 mM glycine, 200 mM NaCl (pH 10.7)) to each well. Fluorescence was measured at λ_{Ex} of 360 nm and λ_{Em} of 465 nm (where Ex is excitation and Em is emission) in a fluorescence microplate reader. Specific release was calculated using the formula: $[(F_{IS} - F_{ISp}) / (F_{TZ} - F_{ISp})] \times 100$, where F_{IS} is the fluorescence of the sample, F_{ISp} is the fluorescence of the spontaneous release, and F_{TZ} is the fluorescence of the total release. Values that exceeded 5% of the total release were considered as positive. Values obtained with buffer alone were subtracted.

³ Abbreviations used in this paper: CD, circular dichroism; kU/L, kilounits allergen-specific IgE per liter; RBL, rat basophil leukemia.



Isolation and characterization of cDNAs coding for IgE-reactive α S1-casein and α S1-casein fragments

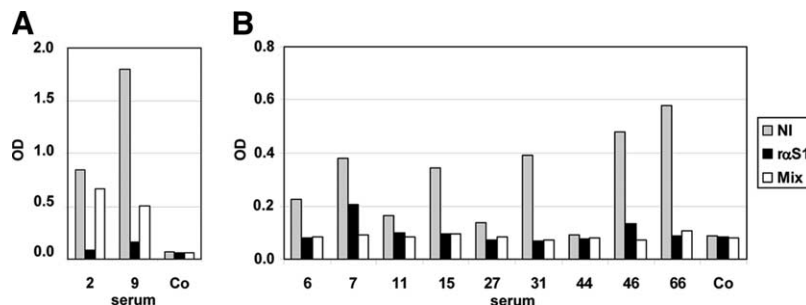
Expression, purification, and biochemical analysis of α S1-casein

The far-UV CD spectrum of purified recombinant α S1-casein recorded at 25°C (Fig. 2C, black line) with a minimum at 205 and 218 nm indicated a folded protein that contains a considerable amount of secondary structure (β -strands, β -turns, and random coiled structure). Thermal unfolding of α S1-casein was monitored at gradually increasing temperature (5°C steps) in a temperature range from 25°C to 95°C (Fig. 2C). During the heating process the allergen started to precipitate at ~60°C, but the remaining soluble fraction of α S1-casein still showed features of the folded protein. After cooling to 25°C it was possible to resuspend the protein, which showed again a considerable amount of secondary structure as observed for the untreated α S1-casein (data not shown).

raSI-casein is a major cow's milk allergen according to IgE reactivity

The IgE binding capacity of α S1-casein was evaluated first by immunoblot and dot blot analysis under denaturing and native conditions, respectively (Fig. 3). Sera from two cow's milk-allergic patients (Fig. 3, *lanes 1* and *2*) but not serum from the nonallergic individual (Fig. 3, *lane C*) showed comparable IgE reactivity with the denatured α S1-casein and with the native protein (Fig. 3*B*).

A detailed analysis of the IgE reactivity of α S1-casein was performed with sera from 66 cow's milk-allergic patients (Table I) using microarrayed components. IgE reactivity to cow's milk extract, a natural α -casein fraction, and α S1-casein was analyzed, and the percentages of reactive sera are displayed in Fig. 4, A and B. We found that 56.7% of the patients showed IgE reactivity to the natural α -casein fraction containing α S1- and α S2-casein (Fig. 4A). The vast majority (97.4%) of these patients showed IgE reactivity to α S1-casein (Fig. 4B).



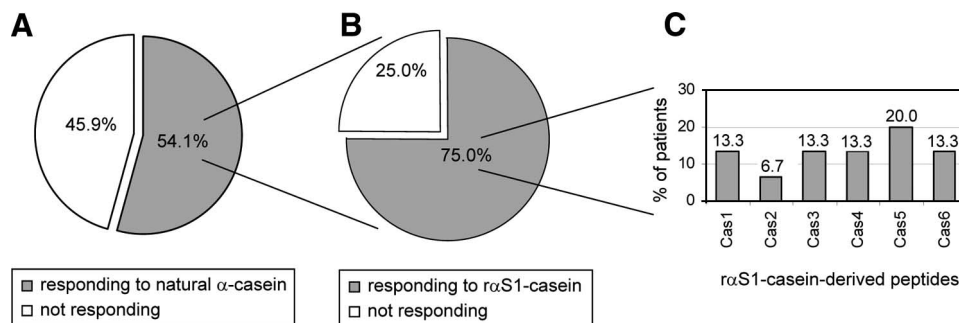


FIGURE 6. Frequency of degranulation induced in RBLs loaded with cow's milk-allergic patients' serum IgE with natural α -casein, r α S1-casein, and α S1-casein-derived synthetic peptides. Circle diagrams (A) show the percentage of sera from cow's milk-allergic patients ($n = 37$) that after loading to RBL cells induce degranulation in response to natural α -casein. B, Percentage of positive sera from A ($n = 20$), which induced degranulation in response to r α S1-casein. The frequency (y-axis) of degranulation in response to α S1-casein-derived peptides (x-axis, Cas1–Cas6) in positive patients from B ($n = 15$) is shown in C.

Most of our patients were children displaying high as well as low cow's milk allergen-specific IgE levels, but we found no evidence that this would affect IgE reactivity to α S1-casein. For example, patient no. 52 in Table I had 11.8 kilounits of allergen-specific IgE per liter (kU_A/L) cow's milk-specific IgE and 2140.5 FI specific for r α S1-casein, whereas patient no. 56 had 16.14 kU_A/L cow's milk-specific IgE and only 72 FI r α S1-casein-specific IgE.

Sequential IgE epitopes of r α S1-casein are distributed over the whole molecule

An in-depth analysis of IgE reactivity to microarrayed α S1-casein-derived peptides (Cas1–Cas6) showed that the IgE reactivity in the r α S1-casein-positive sera varied from 29.7% (Cas2) to 86.5% (Cas1) of the tested sera (Fig. 4C). Since each of the α S1-casein-derived peptides was recognized by IgE Abs, it appeared that IgE epitopes are distributed over the complete allergen. We therefore synthesized an additional five peptides (Cas1.2, Cas2.3, Cas3.4, Cas4.5, and Cas5.6), which overlap with the original six peptides and tested them for IgE reactivity with sera from 49 patients from whom serum samples were available (Table I, patient numbers with asterisks). The frequencies of IgE recognition of Cas1.2–Cas5.6 among the 34 patients positive with r α S1-casein ($n = 34$) are shown in Fig. 4D (Cas1.2, 70.6%; Cas2.3, 32.4%; Cas3.4, 44.1%; Cas4.5, 73.5%; Cas5.6, 55.9%), demonstrating that sequential IgE epitopes are indeed spread over the allergen. Interestingly, we found nine patients who showed IgE reactivity to α S1-casein-derived peptides but not to complete r α S1-casein. Seven of these patients reacted with the peptide Cas3, suggesting that this region may represent a cryptic epitope.

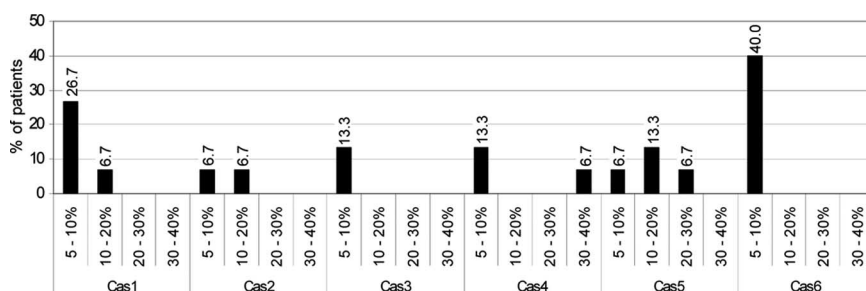
Using an ELISA competition experiment, we studied if r α S1-casein may also contain conformational IgE epitopes. For this purpose sera from two cow's milk-allergic patients with IgE

reactivity to the peptides were preadsorbed with the α S1-casein-derived peptides and the complete r α S1-casein allergen. IgE reactivity to r α S1-casein was strongly inhibited by the peptides in one patient but not in the other (Fig. 5A). Serum from a nonallergic person did not show IgE reactivity to r α S1-casein (Fig. 5A). We then preincubated sera from an additional nine patients with a mix containing each of the 11 casein-derived peptides (Table II), yielding a similar inhibition of IgE binding to r α S1-casein as compared with inhibition with the complete r α S1-casein (Fig. 5B).

Natural, recombinant α S1-casein and α S1-casein-derived synthetic peptides induce basophil degranulation only with sera from a subgroup of cow's milk-allergic patients

To assess the allergenic activity of the purified recombinant and synthetic milk components, RBL cells loaded with the same sera that had been tested for IgE reactivity were stimulated with the natural α -casein fraction, r α S1-casein, or the α S1-casein-derived synthetic peptides (Cas1–Cas6). We found that 54.1% of the sera that were positive in RBL assay with cow's milk ($n = 37$) also triggered RBL release upon exposure to the natural α -casein fraction (Fig. 6A). Most (i.e., 75%) of the latter sera also induced RBL release upon contact with r α S1-casein (Fig. 6B). Even the α S1-casein-derived synthetic peptides (Cas1–Cas6) induced basophil degranulation (Fig. 6C), but the extent of degranulation observed with the peptides was rather low (Fig. 7) and degranulation was observed only with 12 of the sera containing r α S1-casein-reactive IgE. Besides sera with IgE reactivity to r α S1-casein, Fig. 7 includes also sera that had reacted only with cryptic α S1-casein-derived peptides. It demonstrates that peptide-induced degranulation was mild because most sera induced <20% of mediator release. Only two sera gave a >20% release in conjunction with peptides (Cas4,

FIGURE 7. Percentages of α S1-casein-reactive sera ($n = 15$) giving a degranulation reaction with α S1-casein-derived peptides. The percentages of sera giving a degranulation reaction of defined magnitudes (x-axis, percentages of total release) with α S1-casein-derived peptides are displayed for those sera that had induced degranulation to r α S1-casein.



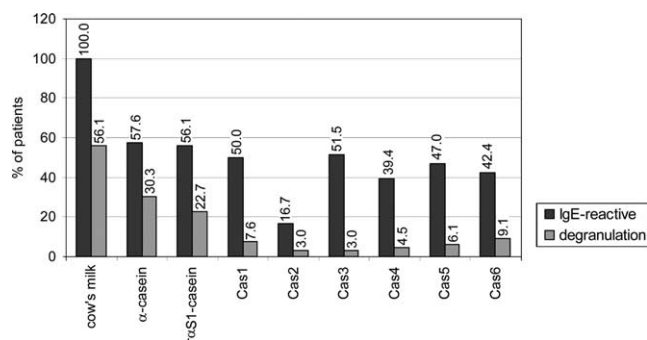


FIGURE 8. Comparison of the frequencies of IgE recognition and degranulation responses. The percentages of patients ($n = 66$) showing IgE reactivity to cow's milk, an α -casein fraction, r α S1-casein, as well as to synthetic α S1-casein-derived peptides (Cas1–Cas6) are represented by black bars and the percentages of sera giving a positive degranulation reaction in RBL assays are shown by gray bars.

Cas5). In fact, Fig. 8 shows that only a relatively low percentage of all tested sera (3.0–9.1%) caused degranulation in response to peptide exposure. The complete allergens were more potent in inducing RBL degranulation. We found that 30.3% of sera induced degranulation to the natural α -casein fraction and 22% to r α S1-casein (Fig. 8).

The additionally synthesized peptides Cas1.2–5.6 could not be tested in the RBL assays due to lack of sera.

Association of cow's milk-induced symptoms with IgE reactivity and basophil degranulation

In Table I demographic, clinical, and serological data of the 66 persons with cow's milk-specific IgE Abs are displayed. Fig. 9A shows the IgE reactivity and basophil degranulation due to cow's milk-, α -casein-, r α S1-casein-, and α S1-casein-derived peptides for 42 patients with known clinical symptoms. These patients were grouped according to their symptoms (group 1, no reaction; group 2, only gastrointestinal symptoms; group 3, gastrointestinal symptoms and other symptoms; group 4, only skin symptoms; group 5, skin and respiratory symptoms; group 6, severe, systemic reactions).

We found that individuals with cow's milk-specific IgE but no symptoms ($n = 5$) showed almost no mediator release upon stimulation with r α S1-casein, whereas almost all patients with severe systemic reactions ($n = 6$) showed mediator release after stimulation with r α S1-casein. In agreement with earlier studies we found that persons without reactions had much lower cow's milk-specific IgE levels (mean IgE level, 7.62 kUA/L) than did patients with systemic reactions (mean IgE level, 88.08 kUA/L) (Table I). The latter group also exhibited more frequent and stronger IgE reactivity to α S1-casein-derived peptides. α S1-casein was a major allergen for patients from most of the groups (i.e., groups 1–3, 5, and 6), but only 5 out of 13 patients with only skin symptoms showed IgE reactivity to this allergen.

Seven of our patients suffered from transient and nine from persistent cow's milk allergy (Fig. 9B). Peptide Cas2 was the only peptide that was recognized by serum IgE of patients in the persistent group but not from transient cow's milk-allergic patients (Fig. 9B). Interestingly, this peptide contains the sequence (aa 39–48) that has been identified earlier (14, 19). Patients who had not outgrown their cow's milk allergy showed more frequent and more intense IgE reactivity and mediator release to α S1-casein and α S1-casein-derived peptides than did patients who had outgrown their milk allergy. A statistically significant difference between these two groups was found for

cow's milk-, natural α -casein-, and r α S1-casein-induced mediator release ($p = 0.031$, 0.043 and 0.031, respectively), and for IgE reactivity we observed a trend toward significance ($p < 0.1$) in the cases of Cas2 and Cas6.

IgE reactivities to Cas1.2–Cas5.6 are also indicated for the different patients groups (Fig. 9) but could not be determined for each of the patients due to limited availability of sera.

Discussion

Many of the most common respiratory allergens are available as recombinant proteins for basic immunological studies, diagnostic purposes, and allergen-specific therapeutic approaches. In contrast, the molecular structures of a considerably lower number of food allergens have been revealed, and fewer recombinant food allergens resembling the structural and immunological properties of the natural counterparts are available. The recombinant α S1-casein reported in our study is the first recombinant cow's milk allergen that shows IgE reactivity and allergenic activity. The cDNA coding for α S1-casein has been isolated from a cDNA expression library constructed from cow's mammary glands with IgE Abs from cow's milk-allergic patients. The amino acid sequence deduced from the α S1-casein-encoding cDNA was identical with those that were earlier described in the context of dairy research. r α S1-casein was expressed in *E. coli* and could be purified as a folded protein that resembled a primarily β -fold structure as determined by CD spectroscopy. The r α S1-casein remained folded up to temperatures of 55°C and even after cooking could be resuspended as a folded protein. Accordingly, r α S1-casein was recognized by IgE Abs in its nondenatured form as well as after boiling and denaturing SDS-PAGE followed by immunoblotting. When we tested 66 cow's milk-sensitized patients (children, adults) from different European populations we found that 56.1% showed IgE reactivity to r α S1-casein, which therefore represents a major cow's milk allergen. None of the patients without IgE reactivity to natural α -casein showed IgE reactivity to r α S1-casein. Only one serum giving a positive reaction in the RBL assay with r α S1-casein failed to react with the natural casein preparation. It thus seems that r α S1-casein is highly specific for picking up α S1-casein-reactive patients. A potential advantage of r α S1-casein over natural α -casein preparations, which may also contain α S2-casein, could be that it allows to discriminate α S1-casein from α S2-casein-reactive patients.

α S1-casein is considered as a true food allergen, also classified as class I food allergen, which is comparable to the major fish allergen parvalbumin (31), the major peanut allergens (Ara h 1, Ara h 2, Ara h 3) (32), and the major shrimp allergen (33), and it can induce severe and life-threatening anaphylactic reactions. Interestingly, we found that >50% of patients with gastrointestinal symptoms, skin and respiratory symptoms, or severe systemic reactions, but fewer patients with only skin symptoms, showed IgE reactivity to r α S1-casein (Fig. 9A).

Most of the true food allergens have been reported to contain sequential IgE epitopes. In fact, using recombinant α S1-casein fragments as well as synthetic α S1-casein-derived peptides we were able to identify several continuous (i.e., sequential) IgE epitopes spanning almost the complete α S1-casein sequence. The most frequently recognized α S1-casein-derived synthetic peptides were Cas1 and Cas5, which were recognized by IgE Abs of 86.5% and 81.1% of the r α S1-casein-reactive patients. This finding is in agreement with earlier studies that identified regions covered by Cas1 and Cas5 as major B cell epitopes (18, 19, 34). Interestingly, Cas3 was recognized also by patients without IgE reactivity to complete r α S1-casein or the natural α -casein fraction, suggesting that this region contains a cryptic epitope that becomes accessible

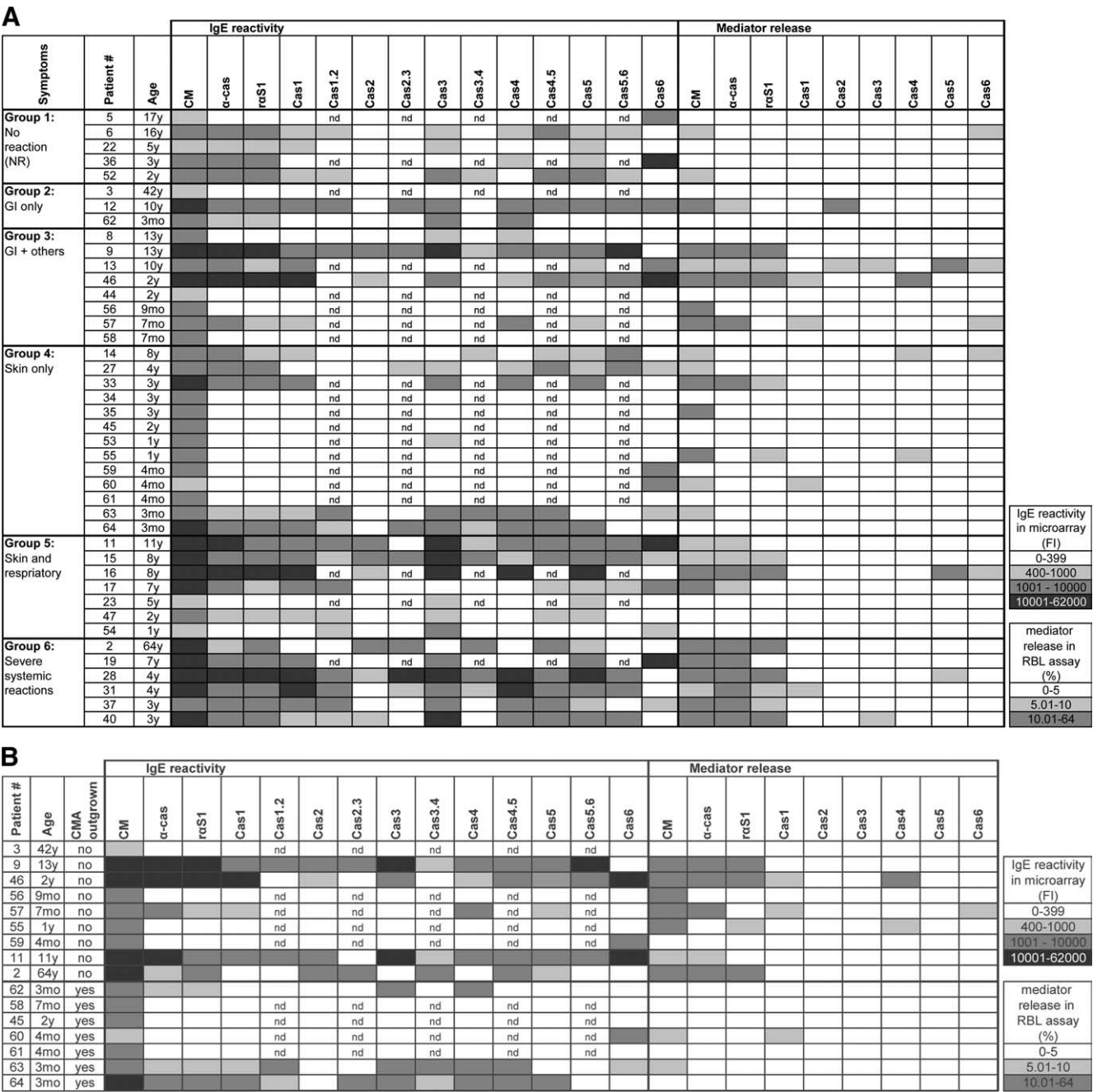


FIGURE 9. A, Association of milk-related symptoms with IgE reactivity and basophil degranulation. Patients were grouped according to their milk-related symptoms (group 1, no reaction; group 2, gastrointestinal symptoms only (GI only); group 3, gastrointestinal symptoms and symptoms in other organ systems (GI + others); group 4, skin symptoms only (skin only); group 5, skin and respiratory symptoms (skin + respiratory); group 6, severe systemic reactions. Intensities of IgE reactivities to microarrayed components (cow's milk, CM; natural α -casein, α -cas; recombinant α S1-casein, raS1-casein; α S1-casein-derived synthetic peptides, Cas1–Cas6; Cas1.2–Cas5.6) as well as the induction of degranulation by these components are indicated. B, IgE reactivity and basophil degranulation in persons with persistent and transient cow's milk allergy. The intensities of IgE reactivity and basophil degranulation in response to cow's milk (CM), natural α -casein (α -cas), recombinant α S1-casein (raS1-casein), and α S1-casein-derived synthetic peptides (Cas1–Cas6, Cas1.2–Cas5.6) are shown for each patient.

to IgE Abs after digestion of α S1-casein. In total, we found that 6 of the 66 sera contained IgE Abs to α S1-casein-derived peptides and induced RBL degranulation in response to peptides, although they did not react with natural α -casein, indicating the presence of cryptic epitopes that may be revealed after degradation of the allergen in the gastrointestinal tract.

Using IgE competition studies in which we preadsorbed sera from raS1-casein-reactive patients with the synthetic α S1-casein-derived peptides, we found that in 10 out of 11 patients the peptide

mix inhibited IgE reactivity to raS1-casein as well as the complete raS1-casein, indicating that most of the patients recognize sequential IgE epitopes.

Differences in recognition of specific epitopes have been described previously between patients with transient and persistent cow's milk allergy (14, 19, 35, 36). It appeared that patients with persistent cow's milk allergy showed more frequent and more intense IgE reactivity and mediator release to raS1-casein and α S1-casein-derived peptides than did patients who had outgrown milk

allergy. Peptide Cas2, which contains an earlier described sequence (14, 19, 34), was the only peptide that was recognized by serum IgE of patients in the persistent group but not from transient cow's milk-allergic patients.

Since the synthetic α S1-casein-derived peptides were rather small (<37 aa), we asked the question whether these IgE-reactive fragments contain a sufficient number of IgE epitopes to induce cross-linking of mast cell or basophil-bound IgE Abs. In fact, the number of IgE epitopes on an allergen is important for its allergenic activity (37) and relates directly to the capacity of a given structure to induce immediate inflammatory-type reactions in patients (38). To evaluate the allergenic activity of α S1-casein and the α S1-casein-derived synthetic peptides, we used basophil degranulation experiments. RBL cells that had been transfected with the human Fc ϵ RI were loaded with the very same sera that had been tested for IgE reactivity with the complete recombinant allergen and the synthetic α S1-casein-derived peptides. Approximately half of the sera containing IgE Abs to α S1-casein or to the natural α -casein fraction induced basophil degranulation upon exposure to the proteins, but only very few sera containing IgE Abs to the α S1-casein-derived peptides induced degranulation in response to the peptides. This result indicates that the α S1-casein-derived peptides induce only weak degranulation compared with the complete allergens, most likely due to the lack of a sufficient number of IgE epitopes. It may thus be assumed that primarily the intact α S1-casein or larger fragments thereof induce IgE-mediated symptoms of cow's milk allergy, whereas the peptides appeared less potent. Our results further suggest that not all cow's milk-allergic patients show vigorous basophil degranulation with the intact allergen, although they did exhibit IgE Ab reactivity. Similar observations have been made for respiratory allergens where discrepancies between the IgE reactivities of allergens and their ability to induce basophil degranulation and in vivo allergic inflammation have been found (39). These findings may explain why the occurrence of clinical manifestations of cow's milk allergy does not always correlate with the presence of IgE Abs specific for milk allergens (40). In fact, one of the major diagnostic problems regarding cow's milk allergy is that the presence of IgE does necessarily imply clinical manifestations, and expensive, labor-intensive, and potentially hazardous double-blind, placebo-controlled food challenges are required.

We found that five patients (nos. 5, 6, 22, 36, and 52) without clinical symptoms after cow's milk consumption showed lower IgE levels to cow's milk and failed to mount relevant degranulation when RBLs were loaded with their serum and exposed to α -casein, α S1-casein, and α S1-casein-derived peptides. In contrast, patients with severe systemic reactions showed strong degranulation. It is therefore possible that the RBL degranulation test may become useful as a diagnostic tool to discriminate patients without clinical symptoms. Unfortunately, all of the patients analyzed in our study exhibited IgE reactivity also to other cow's milk allergens besides α S1-casein. We therefore cannot answer the question whether patients lacking a substantial basophil degranulation in response to α S1-casein exposure will lack clinical symptoms of cow's milk allergy. Another important factor that needs consideration if one would like to use the RBL assays for the potential assessment of the clinical sensitivity of patients is that allergens are subjected to degradation in the gastrointestinal tract, which may lead to the destruction of IgE epitopes or even to the unmasking of cryptic epitopes. It may therefore be necessary to simulate this process before using the RBL assay for the assessment of allergenic activity. Finally, it must be considered that it is also difficult to decipher whether the clinical symptoms recorded for patients are exclusively IgE-mediated or T cell-related, as has

been reported for respiratory allergy to synthetic Fel d 1 peptides (41).

However, the availability of purified α S1-casein and α S1-casein-derived peptides should allow to further dissect the pathomechanisms of milk-induced food allergy in clinical studies. Furthermore, these molecules may allow the development of diagnostic tests and of novel therapeutic strategies for milk allergy.

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Disclosures

R.V. serves as a consultant for Phadia (Uppsala, Sweden) and Biomay (Vienna, Austria). The other authors have no financial conflicts of interest.

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