

ORIGINAL ARTICLE

Microarray and allergenic activity assessment of milk allergens

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Summary

Background Cow's milk is one of the most common causes of food allergy affecting approximately 2.5% of infants in the first years of their life. However, only limited information regarding the allergenic activity of individual cow's milk allergens is available.

Objective To analyse the frequency of IgE reactivity and to determine the allergenic activity of individual cow's milk allergens.

Methods A nitrocellulose-based microarray, based on purified natural and recombinant cow's milk allergens was used to determine IgE reactivity profiles using sera from 78 cow's milk-sensitized individuals of varying ages. The allergenic activity of the individual allergens was tested using patients' sera for loading rat basophil leukaemia cells (RBL) expressing the α -chain of the human receptor Fc ϵ RI.

Results Using the microarray and the RBL assay, cow's milk allergens were assessed for frequency of IgE recognition and allergenic activity. Moreover, the RBL assay allowed distinguishing individuals without or with mild clinical reactions from those with severe systemic or gastrointestinal symptoms as well as persons who grew out cow's milk allergy from those who did not.

Conclusions Component-resolved testing using milk allergen microarrays and RBL assays seems to provide useful additional diagnostic information and may represent a basis for future forms of prophylactic and therapeutic strategies for cow's milk allergy.

Keywords Basophil degranulation, cow's milk allergy, diagnosis, microarray, recombinant allergen

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Introduction

Cow's milk is one of the most common causes of food allergy in the first years of life with around 2.5% of infants showing adverse reactions to cow's milk [1–3]. Symptoms of cow's milk allergy (CMA) range from mild to severe reactions and involve the skin, respiratory tract, gastrointestinal tract and in the worst case appear as life-threatening systemic reactions. The diagnosis of cow's milk allergy as well as the assessment and prediction of the severity of symptoms is particularly difficult for

infants because only small amounts of serum can be obtained for serological diagnosis and it is often difficult to perform provocation tests [4, 5]. Owing to the difficulties of obtaining sufficient serum samples and the lack of highly pure allergens, data regarding the prevalence of IgE recognition of individual cow's milk allergens are incomplete and sometimes controversial [6]. Furthermore, there is little information regarding the allergenic activities of individual cow's milk allergens.

Cow's milk contains numerous proteins of which eight have been characterized as allergens. They include α -lactalbumin (Bos d 4), β -lactoglobulin (Bos d 5), bovine serum albumin (BSA) (Bos d 6), and lactoferrin from the

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whey fraction, α S1-casein (Bos d 8 α -S1), α S2-casein (Bos d 8 α -S2), β -casein (BC) (Bos d 8 β), and κ -casein (KC) (Bos d 8 κ) from the casein fraction (Bos d 8) [6].

Here we report the establishment of a miniaturized chip assay which contains highly pure recombinant cow's milk allergens and natural milk allergen preparations for the analysis of the IgE reactivity profiles in cow's milk-allergic patients. The milk allergen array allowed us to detect the allergen profiles and frequencies of IgE reactivities towards a panel of nine recombinant cow's milk allergens/allergen fragments with as little as 25 μ L of serum. In order to assess the allergenic activity of the individual allergen components, a basophil degranulation test was developed which was based on rat basophil leukaemia (RBL) cells expressing the human Fc ϵ RI. These cells could be loaded with small volumes of serum and allowed us to test the allergenic activity of the individual allergen components.

Materials and methods

Clinical description of patients

Sera from 78 cow's milk-allergic children and adults (Table 1) were obtained from several European countries. Patients were selected according to a positive case history, positive skin-prick reactions, and/or determination of specific IgE to cow's milk extract using the ImmunoCAP System (Phadia, Uppsala, Sweden). Among the patients, three age groups could be defined (3 months–5 years, $n = 53$; 6–14 years, $n = 16$; 16–70 years, $n = 9$) (Table 1). The analysis of the serum samples was performed after patients were made anonymous with approval of the Ethics Committee of the Medical University of Vienna.

Natural and recombinant allergens, antibodies

Purified natural milk proteins (AC, α -casein; BC, β -casein; KC, κ -casein; ALA, α -lactalbumin; hALA, human α -lactalbumin; BLGA, β -lactoglobulin variant A; BLGB, β -lactoglobulin variant B; BSA; SSA, sheep serum albumin; HSA, human serum albumin (HSA); Lf, lactoferrin) and casein fractions (GC, goat casein fraction; CC, cow casein fraction; SC, sheep casein fraction) were purchased from Sigma-Aldrich (Vienna, Austria). Milk samples from cow (CM), sheep (SM), goat (GM), and mare (MM) were bought at a local market and stored at -20°C until analysis. Human milk (HM) samples were obtained from a lactating woman [7].

Complementary DNAs (cDNAs) coding for mature α -lactalbumin (rALA), β -lactoglobulin (rBLG), α S1-(raS1C), α S2-(raS2C), β -(rBC), and κ -casein (rKC) and for three fragments spanning the whole sequence of BSA [rBSAF1: amino acid (aa) 1–199, rBSAF2: aa 200–389, rBSAF3: aa 390–590] without N-terminal signal sequences and with C-

terminal hexahistidine tags were obtained from a mammary gland cDNA library by IgE immunoscreening or PCR amplification [8]. Recombinant proteins were expressed in the *Escherichia coli* strain BL21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA, USA) and purified using Ni-NTA resin affinity columns (Qiagen, Hilden, Germany). Recombinant parvalbumin (parv) was produced as reported [9].

Rabbit anti-sera were raised against each of the purified recombinant milk allergens (Charles, River, Kissleg, Germany). The purity of natural cow's milk allergens was tested by ELISA using monospecific rabbit anti-sera raised against purified recombinant cow's milk allergens. Anti-sera were diluted 1 : 5000 in Tris-buffered saline. Bound IgG antibodies were detected with a 1 : 1000 diluted horseradish peroxidase-labelled anti-rabbit IgG antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK). All determinations were carried out in duplicates and are expressed as mean values.

Allergen microarray analysis

For measurement of IgE reactivity, 0.1–0.15 ng/spot of whole milk extracts, casein fractions, and purified as well as recombinant cow's milk proteins were spotted with a Nano-Plotter NP2 (Gesellschaft für Silizium-Mikrosysteme mbH, Großberkmannsdorf, Germany) onto a capillary flow membrane that was fixed to an ordinary microscope glass slide as described by Nystrand [10]. After spotting, the microarrays were pre-wetted with 30 μ L of assay buffer (phosphate buffer, pH 7.5), then incubated with 25 μ L of undiluted sera, washed with 30 μ L of assay buffer, and assayed with 20 μ L of detecting antibody. Finally, the microarrays were washed two times with 30 μ L of assay buffer and bound IgE antibodies were detected with a fluorophore-conjugated anti-IgE antibody as fluorescence intensities (FI), measured at a wavelength of 670 nm (GenePix 4000B Axon Instruments; Molecular Devices, Sunnyvale, CA, USA). The cut-off level was set to FI = 400 based on values gained with spotted HSA that was used as a negative control. Variation studies were performed to test the reproducibility of the microarray. The pooled total CV (coefficient of variation, 1 SD expressed as percent of the mean) for positive results (> 400) in the microarray was 18%, which includes chip manufacturing lot variation, run variation, and chip variation.

Measurement of cow's milk-specific immunoglobulin G by enzyme-linked immunosorbent assay

Cow's milk allergen-specific IgG antibodies were measured in sera from patients who grew out or did not grow out cow's milk allergy by ELISA as described previously [11]. Instead of purified allergens, cow's milk extract ($c = 100 \mu\text{g/mL}$) was coated to the plates and a 1 : 1000 diluted monoclonal anti-human IgG antibody

Table 1. Characterization of milk-sensitized patients

Patient	Sex M/F	Country	Milk-related symptoms	SPT CM	Challenge	CMA outgrown	Other allergies	Total IgE (kU/L)	Specific IgE to CM (kU A/L)
A: A1-A53 3 months-5 years (n = 53)	29/22	S (n = 15)	No reaction (n = 2)	Pos (n = 20)	Pos* (n = 17)	Yes (n = 9)	GP (n = 1)	8.36-3295	<0.35 to > 100
	NK (n = 2)	A (n = 8)	GI (n = 1)	Neg (n = 7)	Pos [†] (n = 6)	No (n = 6)	M, C (n = 1)	MV: 161	MV: 10.7
		G (n = 30)	GI + others (n = 5)	ND/NK (n = 26)	Neg* (n = 1)	NK (n = 38)	HE (n = 7)	(n = 49)	(n = 49)
			Skin (n = 15)		Neg [†] (n = 2)		HE + various others (n = 6)		
			Resp (n = 1)		ND/NK (n = 27)		No (n = 11)		
B: B54-B69 6-14 years (n = 16)			Skin + resp (n = 5)				NK (n = 27)		
			Sys (n = 4)						
			NK (n = 20)						
	10/5	G (n = 14)	No reaction (n = 1)	Pos (n = 9)	Pos (n = 4)	No (n = 2)	HE (n = 6)	14.1-14 525	3.77 to > 100
	NK (n = 1)	I (n = 2)	GI + others (n = 3)	ND (n = 7)	Pos [†] (n = 3)	NK (n = 14)	HE + various others (n = 1)	MV: 866	MV: 18.9
C: C70-C78 16-70 years (n = 9)			Skin (n = 1)		NP (n = 2)		Candida (n = 1)	(n = 15)	(n = 16)
			Skin + resp (n = 4)		ND/NK (n = 7)		NK (n = 8)		
			Sys (n = 2)						
			NK (n = 5)						
	3/6	G (n = 3)	No reaction (n = 2)	Pos (n = 4)	Pos (n = 2)	No (n = 3)	HE (n = 1)	148-3736	1.3 to > 100
D: D54-D69 16-70 years (n = 9)			OAS (n = 1)	Neg (n = 2)	Pos* (n = 1)	NK (n = 4)	M, C (n = 1)	MV: 433	MV: 5.34
		F (n = 1)	Resp (n = 1)	ND (n = 3)	Neg [†] (n = 2)	NA (n = 2)	PO, AH, M (n = 1)	(n = 7)	(n = 7)
		S (n = 1)	Skin (n = 2)		ND/NK (n = 4)		Meat, C (n = 1)		
			Sys (n = 1)				C, WF, T, K, P (n = 1)		
			NK (n = 2)				GP, dog (n = 1)		
NK (n = 3)									

*Open challenge as described by Garcia-Ara et al. [28].

†Double-blind placebo-controlled food challenge.

f, female; m, male; A, Austria; F, France; G, Germany; I, Italy; S, Spain; NK, not known; GI, gastrointestinal symptoms; OAS, oral allergy syndrome; Resp, respiratory symptoms; Skin, skin symptoms; Sys, systemic reactions; SPT, skin prick test; CM, cow's milk; CMA, cow's milk allergy; Pos, positive; Neg, negative; ND, not done; NA, not applicable because patients were asymptomatic; NP, not possible; AH, animal hair; C, cat; GP, grass pollen; HE, hen's egg; K, kiwi; M, mite; PO, pollen; P, pork; T, tomato; WF, wheat flour; MV, median value.

(Sanquin, Amsterdam, the Netherlands) was used for detection. Differences in cow's milk-specific IgG levels were analysed using the Wilcoxon–Mann–Whitney *U*-test and the Program SPSS (2008, version 16.0 SPSS Inc., Chicago, IL, USA).

Rat basophil leukaemia assays

The allergenic activity of the milk components was tested using RBL cells expressing the human receptor FcεRI. RBL cells were loaded with patients' sera and exposed to the allergen preparations [8, 12].

Results

Establishment and characterization of an allergen chip containing microarrayed milk components

In total, 27 milk samples (dots 1–9, 11, 12, 14–29) and for control purposes HSA (dots 13) and an unrelated allergen, recombinant carp parvalbumin (dots 10), were spotted in duplicates on a nitrocellulose membrane as shown in the application scheme in Fig. 1a. Purified IgE (dots 0) was spotted as a position marker. The correct immobilization of the allergens on the nitrocellulose chip was checked at a wavelength of 635 nm (Fig. 1b). The use of a fluorescence-labelled detection antibody allowed determining the FI of the anti-IgE labelling at a wavelength of 670 nm. According to the colour intensities of the fluorescence signals, the specific IgE concentrations of the samples could be ranked (i.e. white > red > orange > yellow > green > light blue > dark blue) and semi-quantitative results were obtained.

In pilot experiments, we compared IgE reactivity profiles by chip analysis and by ELISA for certain sera, of which larger volumes were available, and obtained comparable results with both tests (data not shown).

Figure 1c shows representative images of microarray results obtained with sera from three cow milk-allergic patients and with the serum from a non-allergic individual. Serum IgE from patient B68, a 13-year-old boy with persistent cow's milk allergy who suffered from urticaria, eczema, vomiting, and asthma after milk consumption, reacted with most of the allergens except BSA and lactoferrin. The serum did not react with SSA, parvalbumin, and HSA (Fig. 1c). Similar results were obtained with the serum from patient B63, a 9-year-old girl, who suffered mainly from gastrointestinal symptoms and with the serum from patient A18, a 22-month-old girl, who suffered from urticaria, atopic dermatitis, and vomiting (Fig. 1c). Both sera showed IgE reactivity to most of the spotted components. The IgE reactivity profiles depicted in Fig. 1c have been obtained with small serum volumes of approximately 25 µL. The specificity of the method was demonstrated by the lack of IgE reactivity to the spotted

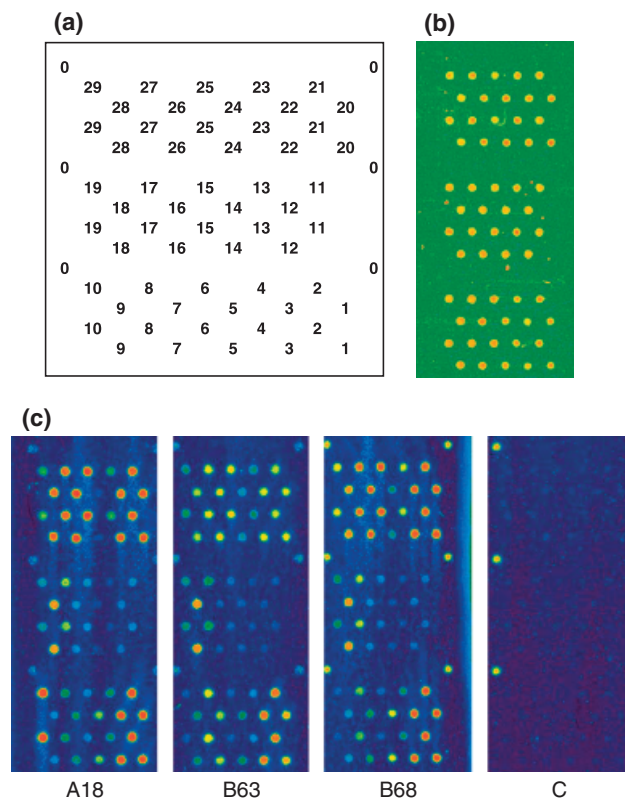


Fig. 1. Microarray. (a) Application scheme of microarrayed milk components: 0: IgE; 1: GM; 2: CM; 3: SM; 4: HM; 5: MM; 6: LF; 7: BLGA; 8: BLGB; 9: rBLG; 10: parv; 11: BSA; 12: SSA; 13: HSA; 14: rBSAF1; 15: rBSAF2; 16: rBSAF3; 17: hALA; 18: ALA; 19: rALA; 20: GC; 21: CC; 22: SC; 23: KC; 24: rKC; 25: BC; 26: rBC; 27: AC; 28: raS1C; 29: raS2C. (b) Visualization of protein dots on a microarray slide at a wavelength of 635 nm. (c) Detection of IgE-reactive protein spots at a wavelength of 670 nm after incubation with sera from milk allergic patients (A18, B63, B68) and a non-allergic individual (C) and visualization with a fluorescence-labelled anti-IgE antibody.

components when serum from a non-allergic person (C) was used (Fig. 1c).

Frequencies of immunoglobulin E reactivity to microarrayed milk allergens

A summary of the frequencies of IgE reactivities to microarrayed milk allergens is shown in Fig. 2. In total, 78 sera (Table 1) were tested for IgE reactivity to microarrayed components. Seventy-one of those sera were positive in the cow's milk ImmunoCAP, one serum was negative and six sera were not tested by ImmunoCAP measurements. The black bars in Fig. 2a, c, and e show the percentages of IgE reactivities for all tested patients. Results from each of the three age groups are also displayed.

Among the whole milk extracts those from cow ($n = 66$; 84.6%), sheep ($n = 63$; 80.8%), and goat ($n = 53$; 67.9%) were most frequently recognized. MM and HM were less

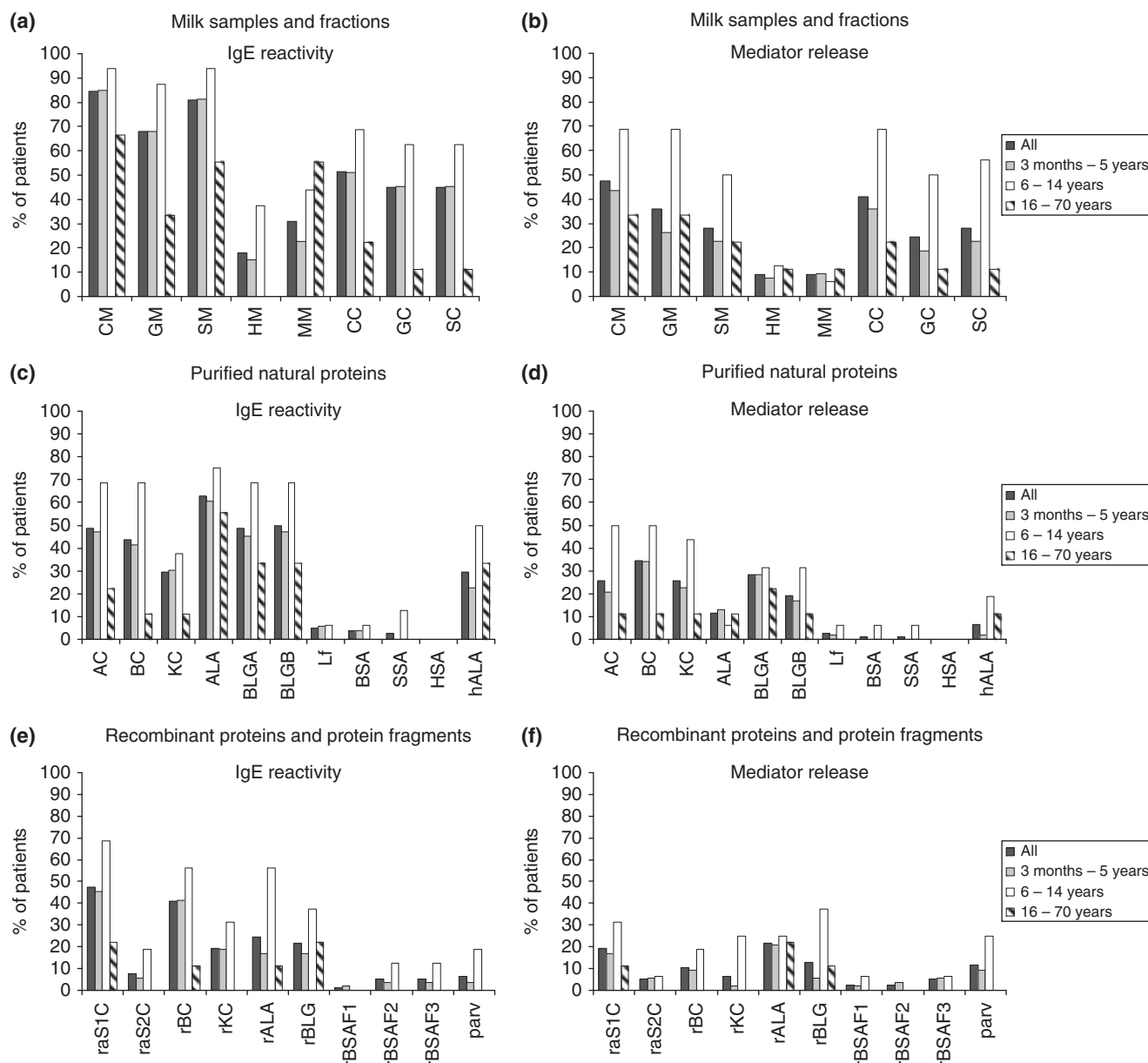


Fig. 2. Frequencies of IgE recognition and basophil degranulation. The percentages of sera exhibiting IgE reactivity and inducing mediator release are displayed for all investigated patients ($n = 78$; black bars) and for the three age groups (Group 1: 3 months–5 years; $n = 53$; grey bars; Group 2: 6–14 years; $n = 16$; white bars; Group 3: 16–70 years; $n = 9$; hatched bars). (a, b) Milk samples: CM, GM, SM, HM, MM, CC, GC, SC. (c, d) Purified natural cow's milk allergens: AC, BC, KC, ALA, BLGA, BLGB, Lf, BSA, SSA, HSA, hALA, and (e, f) recombinant allergens/allergen fragments: raS1C, raS2C, rBC, rKC, rALA, rBLG, rBSAF1, rBSAF2, rBSAF3, parv.

frequently recognized (30.8% and 17.9%, respectively) (Fig. 2a). The casein fractions of CM, GM, and SM reacted with IgE from approximately 50% of the tested sera (Fig. 2a).

Among the natural cow's milk proteins (Fig. 2c), ALA reacted with 62.8% of the sera and thus was the most frequently detected natural cow's milk allergen followed by BLG and AC which were recognized by approximately 50% of the tested sera. BC (43.6%) and KC (29.5%) were also frequently recognized, whereas Lf, BSA, and SSA

showed low IgE-binding frequencies (5.1%, 3.8%, 2.6%, Fig. 2c). Interestingly, natural human ALA reacted almost with 30% of the sera. In summary, 65 of the sera (83.3%) showed IgE reactivity with at least one of the natural cow's milk allergens.

Comparing the IgE-binding frequencies of natural and recombinant cow's milk allergens some interesting differences were noted (Fig. 2c and e, black bars). While the recombinant caseins showed comparable IgE-binding frequencies as the natural counterparts, rALA and also

rBLG reacted with less than half of the sera which had reacted with their natural counterparts. Recombinant BSA fragments reacted with a similar low percentage of sera as purified natural BSA (Fig. 2c and e). Overall, 49 (62.8%) sera showed IgE reactivity to at least one of the recombinant allergens.

A comparison of the IgE recognition frequencies in the different age groups showed that the frequencies of recognition were highest in group B (age 6–14 years; Fig. 2, white bars) followed by group A (age 3 months–5 years; grey bars), and group C (age 16–70 years; Fig. 2, hatched bars). These results reflected the levels of cow's milk-specific IgE determined by ImmunoCAP being highest in group B followed by A and C (Table 1).

Natural cow's milk allergen preparations are contaminated with unrelated milk allergens

In order to analyse whether discrepancies in the IgE recognition frequencies between the recombinant forms of ALA and BLG and their natural counterparts are due to contaminations in the natural allergen preparations, we tested the latter with specific antibody probes. For this purpose, rabbit antibodies had been raised against each of the purified recombinant allergens which showed highly specific reactivity to the protein backbones but did not recognize carbohydrates or other post-translational modifications. Each of the anti-sera reacted with the corresponding natural allergens (Table 2). However, most of the casein-specific antibodies recognized natural AC, BC, and KC preparations which suggest that the natural caseins are not pure. Cross-reactivity between AC, BC, and KC is unlikely because no relevant sequence homologies were found when their amino acid sequences were aligned (data not shown). In the KC fraction we even detected BLG (Table 2).

Table 2. Reactivity of natural cow's milk proteins with specific antibody probes

Natural proteins	Rabbit antisera						
	raS1C	raS2C	rBC	rKC	rALA	rBLG	rLF
AC	1.3	1.3	0.3	0.4	0.0	0.0	0.0
BC	0.4	1.2	1.1	0.8	0.0	0.0	0.0
KC	1.1	1.2	0.1	1.4	0.1	0.4	0.0
ALA	0.0	0.0	0.0	0.0	1.5	0.0	0.0
BLGA	0.0	0.0	0.0	0.0	0.0	1.5	0.0
BLGB	0.0	0.0	0.0	0.0	0.0	1.8	0.0
Lf	0.0	0.0	0.0	0.0	0.0	0.0	0.5
BSA	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Antibody reactivities are presented as OD values, which are calculated as the OD values obtained with the immune sera minus the OD values obtained with the corresponding pre-immune sera. Elevated values are given in bold and contaminations are highlighted in grey.

Natural ALA, BLG, Lf, and BSA preparations did not react with anti-sera raised against other milk allergens and hence appeared to be pure. Yet, the biggest discrepancies of IgE reactivity between natural and recombinant components were observed in case of ALA and BLG, both representing glycoproteins containing two and one glycosylation sites, respectively (data not shown).

Mono- and oligo-sensitization to cow's milk allergen components in cow's milk-allergic patients

As impurities were detected in natural casein preparations and as IgE reactivities to carbohydrates present in natural allergen preparations should be excluded, mono- or oligo-sensitization to cow's milk allergen components was assessed with purified recombinant allergens. It was found that 16 (32.7%) out of the 49 patients who had reacted with recombinant allergens reacted only with one allergen. The other 33 sera (67.3%) reacted with at least two allergens (two allergens: $n = 13$; three allergens: $n = 9$; four allergens: $n = 1$; five allergens: $n = 5$; six allergens: $n = 3$; seven allergens: $n = 2$). There was no obvious difference regarding mono- or oligo-sensitization in the three age groups.

In the mono-sensitized group ($n = 16$), six patients exhibited > 10 kUa/L cow's milk-specific IgE in the ImmunoCAP test. Far more, i.e. 27 patients exhibited > 10 kUa/L cow's milk-specific IgE in the oligo-sensitized group ($n = 33$) indicating that higher cow's milk-specific IgE levels are associated with oligo-sensitization.

Comparison between immunoglobulin E reactivity and allergenic activity of cow's milk allergens

In order to determine the biological relevance of IgE reactivity to cow's milk allergens, RBL cells which had been transfected with the α -chain of the human Fc ϵ receptor were loaded with sera and challenged with the allergens (Fig. 2b, d, and f). Basophil degranulation was observed for 47.4% ($n = 37$) of tested patients sera ($n = 78$) with cow's milk extract, 37.2% ($n = 29$) of the sera showed degranulation with at least one natural and 34.6% ($n = 27$) of the sera with at least one recombinant allergen. Generally, fewer sera gave positive results in the basophil degranulation assay than in the IgE reactivity test. Eighty-five percent of sera showed IgE reactivity to cow's milk extract in the microarray but only 47.4% induced basophil degranulation (Fig. 2b). The differences between IgE reactivity and basophil degranulation were less pronounced for the natural caseins (e.g. AC: IgE reactivity, 48.7%; basophil degranulation, 25.6%; BC: IgE reactivity, 43.6%; basophil degranulation, 34.6%; KC: IgE reactivity, 29.5%; basophil degranulation, 25.6%), whereas striking differences were noted for natural ALA and BLG (ALA: IgE reactivity, 62.8%; basophil degranulation, 11.5%; BLGA:

IgE reactivity, 48.7%; basophil degranulation, 28.2%; BLGB: IgE reactivity, 50%; basophil degranulation, 19.2%) indicating that ALA and BLG have a lower allergenic activity than the caseins.

When the recombinant proteins were compared regarding their frequency of IgE reactivity and allergenic activity, we found for rALA comparable results (rALA: IgE reactivity, 24.4%; basophil degranulation, 21.8%) and for rBLG a small difference (rBLG: IgE reactivity, 21.8%; basophil degranulation, 12.8%) (Fig. 2f). In contrast, recombinant caseins induced much less frequently basophil degranulation than IgE reactivities were observed (Fig. 2f).

Thus, the association of IgE reactivity with basophil degranulation was poor. However, it was noted that sera from group B with the highest levels of cow's milk-specific IgE (Table 2) which also contained more IgE positive sera than the other two groups induced more frequently basophil degranulation than sera from groups A and C (Fig. 2b, d and f).

Identification of patients without and with severe symptoms based on microarray and basophil degranulation results

In the next step, we analysed whether there are any associations between clinical symptoms, IgE reactivity and basophil degranulation data (Fig. 3). For this purpose, patients were grouped in individuals (i) without clinical reaction, (ii) with solely oral allergy syndrome, (iii) with only gastrointestinal symptoms, (iv) with gastrointestinal symptoms and other symptoms, (v) with only skin symptoms, (vi) with only respiratory symptoms, (vii) with skin and respiratory symptoms, and (viii) with severe systemic reactions.

The most striking result was that persons containing IgE antibodies against cow's milk extract and allergens without clinical symptoms showed no relevant basophil degranulation with any of the tested components (Fig. 3). By contrast, sera from most cow's milk-allergic patients who

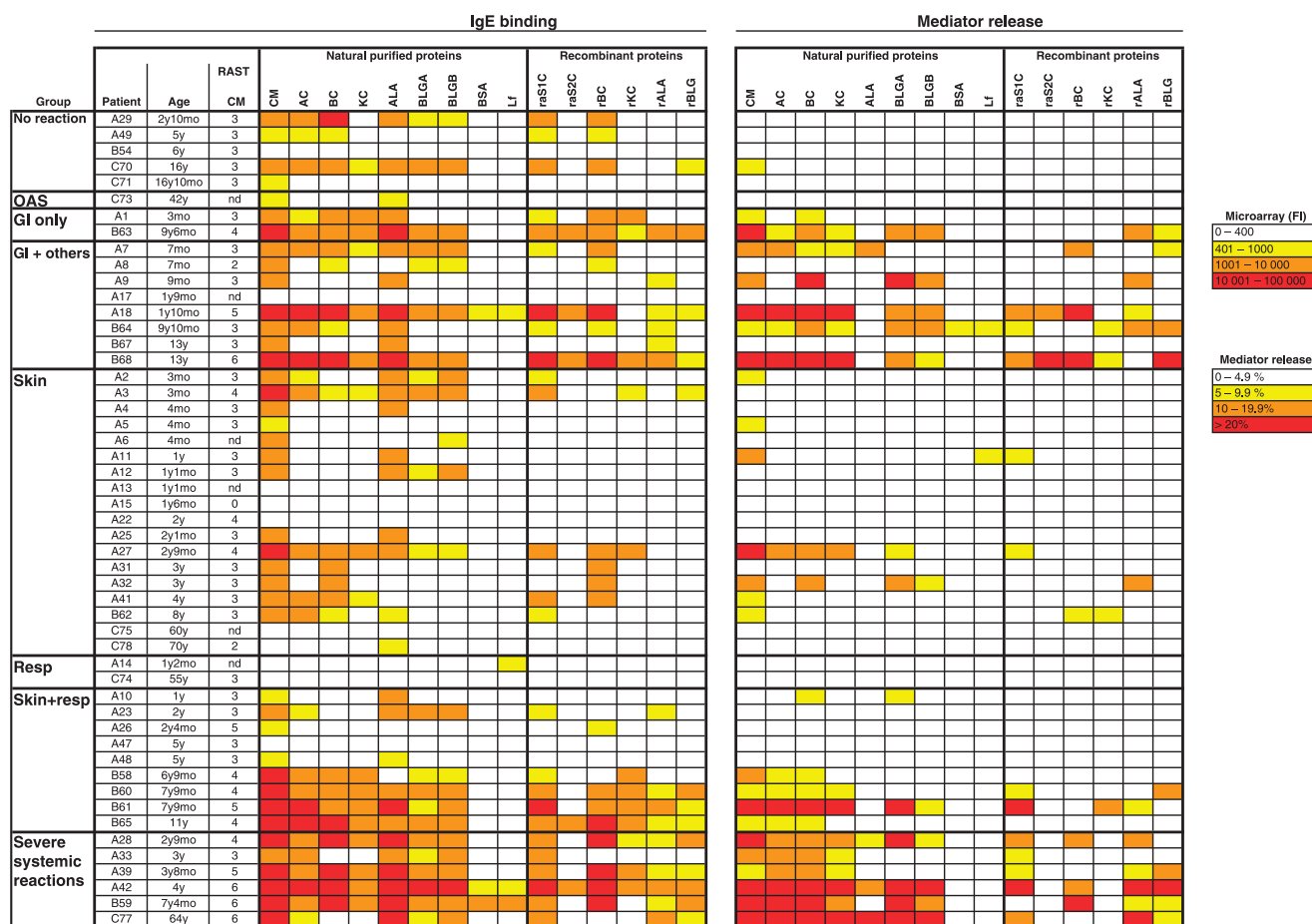


Fig. 3. Associations of milk-related symptoms with IgE reactivities and basophil degranulation. Milk-allergic patients were grouped according to milk-related symptoms as follows: no reaction; OAS, oral allergy syndrome; GI only, gastrointestinal reactions; GI+others, gastrointestinal symptoms and/or skin and/or respiratory symptoms; Skin, skin symptoms; Resp, respiratory symptoms; Skin+resp, skin symptoms and respiratory symptoms; severe systemic reactions. Displayed items are: Age, RAST class to cow's milk (CM). The intensities of IgE reactivities to CM, purified natural cow's milk allergens (AC, BC, KC, ALA, BLGA, BLGB, BSA, LI), and recombinant cow's milk allergens (raS1C, raS2C, rBC, rKC, rALA, rBLG), and the intensities of mediator release are indicated in different colours.

suffered from severe systemic reactions and from those who suffered from gastrointestinal symptoms induced basophil degranulation with many of the tested components. It appeared that patients with severe systemic reactions also showed stronger IgE reactivity to more components than the other groups (Fig. 3). However, IgE reactivity testing did not allow distinguishing persons without symptoms from patients with severe and gastrointestinal symptoms.

Immunoglobulin E reactivity and basophil activation tests with purified cow's milk allergens may identify patients who grew out cow's milk allergy

For nine of our patients, it was shown that they had outgrown cow's milk allergy and for 11 patients, it was known that they were still suffering from cow's milk allergy. Figure 4 shows the IgE reactivity profiles and basophil degranulation results from these patients. Patients with persistent cow's milk allergy showed more frequent and more intense IgE reactivity to natural as well as recombinant cow's milk allergens than patients who had outgrown their milk allergy. Likewise, patients with persistent cow's milk allergy showed more often and more pronounced basophil degranulation than patients who had outgrown milk allergy. The analysis of cow's milk-specific IgG antibody levels showed that there was no statistically significant difference regarding allergen-specific IgG levels in patients with persistent and transient cow's milk allergy (data not shown).

Discussion

In this study, we analysed sera from cow's milk-sensitized persons for IgE reactivity to natural and recombinant milk allergen preparations. The allergens were microarrayed on

a chip to allow testing of small serum volumes. Protein and peptide microarrays have become powerful tools for the diagnosis of food allergies in the last years [13–17]. Based on IgE reactivity profiles established with natural cow's milk allergens, ALA > BLG > AC > BC > KC represented the most frequently recognized natural cow's milk allergens, whereas raS1C > rBC > rALA > rBLG > rKC > raS2C represented the most frequently recognized recombinant proteins in our protein microarray. The high IgE-binding activity to the casein was also shown in another milk protein microarray tested with sera from 40 cow's milk-allergic patients [18]. Recombinant caseins appeared to exhibit similar IgE-binding properties as the natural caseins and offered the additional advantage of purity. 'Purified natural caseins' were contaminated with unrelated milk allergens and therefore did not seem suitable for component-resolved diagnosis of cow's milk allergy. A considerable discrepancy in IgE reactivity was noted for ALA and BLG, where the recombinant allergens reacted only with approximately half of the sera that reacted with the natural allergen. However, only approximately half of the sera which showed IgE reactivity to purified natural ALA and BLG showed allergenic activity in the basophil degranulation assays, indicating that the natural allergens may contain IgE-reactive structures, such as carbohydrates or other post-translational modifications, with low allergenic activity [19]. By contrast, approximately the same percentage of sera which had reacted with the recombinant ALA and BLG proteins also gave positive results in the basophil degranulation assay, indicating that these allergen preparations perhaps resemble more closely clinically relevant structures.

A finding of potential relevance for future allergen-specific therapeutic and prophylactic strategies is that the described milk allergen array allowed establishing the patients' sensitization profiles to individual components.

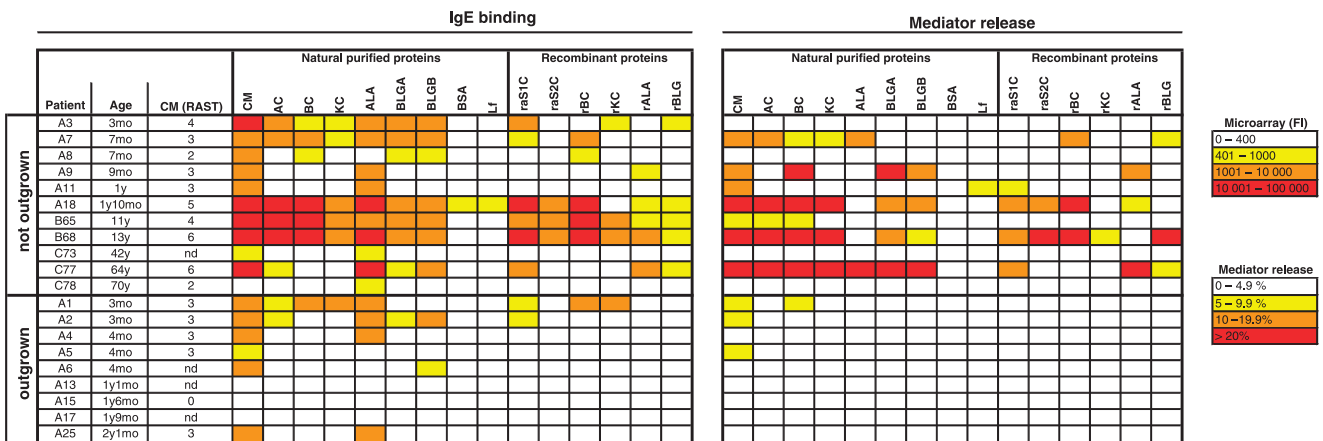


Fig. 4. IgE reactivities and inductions of basophil activation in patients with persistent and transient cow's milk allergy. IgE reactivities and basophil degranulation in response to cow's milk (CM), purified natural cow's milk allergens (AC, BC, KC, ALA, BLGA, BLGB, BSA, Lf), and recombinant cow's milk allergens (raS1C, raS2C, rBC, rKC, rALA, rBLG) are shown for patients with persistent (not outgrown) or transient (outgrown) cow's milk allergy. The intensities of IgE reactivity and basophil activation are displayed in different colours.

Using the microarray, we identified a considerable percentage of patients who appeared to be mono-sensitized towards one major allergen and could discriminate these patients from oligo-sensitized patients. Individuals growing out cow's milk allergy appeared to react with fewer allergen components than those who did not grow out cow's milk allergy suggesting that the microarray may provide additional information over quantitative IgE measurements.

The combination of IgE reactivity testing to microarrayed allergens and the testing for allergenic activity using RBL cells expressing the human FcεRI turned out to provide additional useful diagnostic information. In fact, several studies described that basophil activation tests provide additional information for the diagnosis of food allergy [20–22]. Furthermore another study showed that combining a protein microarray with a cell-based test may enhance the detection of allergic sensitization [23]. While IgE serology to cow's milk and cow's milk allergens was positive for many individuals with cow's milk sensitization but also for individuals without clinical symptoms, the RBL assay may identify the subgroup of individuals without clinical symptoms and thus seems to provide additional information over quantitative allergen-specific IgE measurements. Also patients with only skin symptoms gave weak or no reaction in the basophil degranulation test, whereas all patients with severe systemic and the majority of patients with gastrointestinal symptoms induced basophil degranulation. Accordingly, a recent study showed that basophil activation tests are capable of distinguishing sensitized but clinically tolerant from clinically allergic patients with peanut or egg allergy [24].

Moreover, it appeared that the basophil degranulation assay could distinguish between cow's milk-allergic patients who outgrow their allergy from those who did not because those who grew out milk allergy displayed no basophil activation capacity. In this context, a recent study showed that children who are likely to outgrow their cow's milk allergy show reduced basophil activation with heat-denatured milk [25]. Since the basophil degranulation assays are performed in the absence of allergen-specific IgG because cells are washed after loading with IgE it is unlikely that the reduction of IgE reactivity and degranulation is due to the presence of blocking allergen-specific IgG antibodies. This assumption is also corroborated by the finding that there was no significant difference regarding allergen-specific IgG levels between children who grew out milk allergy and those who did not. It thus seems that the children who grew out milk allergy have avoided milk consumption leading to real reduction of milk-specific IgE.

The golden standard for the diagnosis of clinically relevant cow's milk sensitization is certainly the controlled food challenge test, but it must be kept in mind that this test cannot always be easily used, particularly not

in small infants and in patients who mounted life-threatening reactions upon exposure to cow's milk.

The component-resolved microarray test combined with an assessment of the allergenic activity of the individual components using a basophil activation assay may therefore provide interesting additional diagnostic information. Component-resolved diagnosis of cow's milk allergy may also represent a first step towards the development of new immunotherapeutic strategies for cow's milk allergy similar to those which have been developed based on recombinant allergens for respiratory allergies [26,27].

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