Mol. Nutr. Food Res. 2004, *48*, 363 – 369 DOI 10.1002/mnfr.200400011

Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry

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Cow's milk allergy (CMA) has become a common disease in early childhood, its prevalence ranging from 1.6% to 2.8% among children younger than 2 years of age. The role of different cow's milk protein (CMP) in the pathogenesis of CMA is still controversial. Even if the proteins most frequently and most intensively recognized by immunoglobulin E (IgE) seem to be the most abundant in milk (caseins and β-lactoglobulin), with an although great variability all milk proteins appear to be potential allergens, even those that are present in trace amounts (*i.e.*, lactoferrin, IgG, and BSA). In this work proteomics techniques have been applied for CMP allergens analysis. Allergens have been identified by immunoblotting following resolution of CMP components by two-dimensional electrophoresis. Sera from 20 milk-allergic subjects, as proven by oral provocation test, CAP-RAST and skin prick test, have been used for cow's milk major allergen identification. Cow's milk proteins and their isoforms were identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)-mass spectrometry. In our group of patients, the prevalence of CMP allergens, *i.e.*, the total number of subjects sensitized to CMP divided by the total number of the subjects enrolled in the study, was: 55% α_{s1}-casein, 90% α_{s2}-casein, 15% β-casein, 50% κ-casein, 45% β-lactoglobulin, 45% BSA, 95% IgG-heavy chain, 50% lactoferrin, and 0% α-lactalbumin.

Keywords: Allergen identification / Cow's milk allergens / Food allergy / Immunoblotting / Proteomics

Received: April 27, 2004; revised: June 22, 2004; accepted: June 24, 2004

1 Introduction

Cow's milk allergy (CMA) has become a common disease in early childhood, its prevalence ranging from 1.6% to 2.8% in children younger than 2 years of age [1]. The symptoms commonly arise in skin and in the respiratory and gastrointestinal tracts. Different immunopathogenic mechanisms may be involved in the variable clinical presentations, but immunoglobulin E (IgE)-mediated reactions account for about 60% of milk-induced allergic disorders [2]. Although most infants with IgE-mediated CMA outgrow their sensitivity by the third year of life, 15% retain their sensitivity into the second decade [3].

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Abbreviations: CMA, cow's milk allergy; **CMP**, cow's milk protein; **IgE**, immunoglobulin E; **RAST**, radio-allergosorbent test; **SPT**, skin prick test

The role of different cow's milk proteins (CMPs) in the pathogenesis of CMA is still controversial. Even if the proteins most frequently and most intensively recognized by IgE seem to be the most abundant in milk (casein and β-lactoglobulin), with an although great variability all milk proteins appear to be potential allergens, even those that are present in trace amounts (*i.e.*, lactoferrin, IgG, and BSA). Moreover, a change in the prevalence of sensitization to different proteins has been observed during the last few years with a striking decrease of whey proteins and an increase of caseins in terms of importance as milk allergens [4]. This change in the pattern of allergenicity of the different milk proteins could be due both to modification in technologic processes applied to milk or to improvement in the analytical methods to detect and identify the IgE-reactive CMPs as analyzed by 1-D SDS-PAGE immunoblotting and radioallergosorbent test (RAST) [1].

Given a complex mixture of proteins as for CMP, two general features characterize current proteomics studies: the methods chosen for "display" of proteins and for identifica-

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tion of proteins. At the display level, two general approaches are common in current proteomics investigations. For display of proteins, 2-D polyacrylamide gel electrophoresis (2-DE) is the most commonly used method. Display based on 2-DE gels preserves the natural units of protein function, *i. e.*, the diverse protein variants that represent the phenotype of a given protein. In contrast, display based on LC methods involves display of peptides usually following tryptic digestion of the complete protein mixture. This means that even for a single protein the LC methods necessarily analyze a superposition of phenotypic variants. Most current proteomics identifications rely on determination of proteins by MS of peptides. This is clearly a bottom-up approach to identifying the phenotypic variants [5]. A global proteomic approach, exploiting the full power of 2-DE and mass spectrometric allergen identification, has not yet applied to identify cow's milk IgE-reactive protein isoforms in CMA patients. The aim of this work was to separate and identify by 2-DE and peptide mass spectrometry cow's milk proteins reactive to IgE in children with documented IgE-mediated CMA.

2 Materials and methods

2.1 Patients

Twenty pediatric patients (median age 4 months, range 1–14 months; 12 male and 8 female) affected by atopic dermatitis referred to the Food Allergy Division of the Regina Margherita Hospital of Torino were selected on the basis of documented allergic reactions to cow's milk proteins. The patients were studied by undergoing a protocol that included: (i) skin prick test (SPT) with fresh whole cow's milk performed by using the prick-by-prick technique; (ii) measurement of cow's milk protein-specific IgE antibodies by usig the CAP-RAST System (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) for α-lactalbumin, β-lactoglobulin, and caseins; (iii) open oral challenge with fresh whole milk following a milk-free diet period of 2–4 weeks.

2.2 Preparation of allergen extract

Bovine milk was skimmed by centrifugation at 2000 × *g* for 30 min at 4°C. Protein concentration was determined by 2-D Quant-Kit (Amersham Biosciences).

2.3 1-D electrophoresis

Skimmed milk was diluted in NuPage LDS Sample Buffer (Invitrogen) in order to achieve a final concentration of 15 μg/cm gel and heated for 10 min at 70°C. Proteins were then separated according to their molecular weights on NuPAGE ZOOM gels 12% (Invitrogen), using MOPS-SDS as running buffer, 200 V costant. Gels were either stained with colloidal Coomassie blue or electroblotted onto 0.2 μm nitrocellulose (Sigma) in a semi-wet blot module (Invitrogen) and reversibly stained with Red Ponceau.

2.4 2-D electrophoresis

Skimmed milk was diluted with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% BioLyte (Bio-Rad), 65 mM DTT, traces of Bromo Phenol Blue (BBF)) and applied to 7 cm ReadyIPG strips (Bio-Rad) 3–10 NL and 3–6; total protein loads were 20 and 40 μg, respectively. The strips were focused on Protean IEF Cell (Bio-Rad) with a rapid linear voltage slope until 30 000 Vh were reached. Prior to SDS-PAGE, the strips were equilibrated in NuPAGE sample buffer (Invitrogen) containing 50 mM DTT in the first step and 65 mM iodoacetamide in the second one. SDS-Page were performed as described in Section 2.3.

2.5 Immunoblotting

Immunolabeling was performed with individual patient's sera on monodimensional immunoblots as a first screening. All sera were further analyzed by 2-D immunoblots. Control serum was from a patient affected by atopic dermatitis, with undetectable level of CMP-specific IgE. Membranes were blocked with TBS, pH 7.4/0.3% Tween and incubated overnight with patients and control sera diluted 1 : 5 in TBS/0.05% Tween/0.05% gelatin (Bio-Rad). 2-D immunoblots were rinsed with TBS/0.03% Tween and incubated with alkaline phosphatase-conjugated goat anti-human-IgE antibody (KPL) diluted 1 : 1000 in TBS/0.05% Tween/0.05% gelatin (Bio-Rad) for 1 h. After the washing steps, immunoblots were developed with phosphatase substrate BCIP/NBT (Bio-Rad).

2.6 Immunoblot inhibition analysis

Patients' sera positive to the 18 kDa band were pooled, diluted 1 : 10, and incubated for 1 h 30′ with pure β-lactoglobulin prior to immunodetection. Pure β-lactoglobulin, three times crystallized, was prepared by the method of the Aschaffenburg and Drewry [6]. Blot, incubation, and immunodetection were performed as described above. The inhibition test was performed on 1-D blots with 200, 20 or 2 μg pure β-lactoglobulin and subsequently on 2-D blot selecting the highest amount among the three mentioned above.

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2.7 Image analysis and IgE-reactive spot detection on 2-D maps

Coomassie-stained gels, Ponceau-stained and subsequently immunolabeled 2-D blots were acquired on a GS-800 densitometer (Bio-Rad) and the signal attribution to the Coomassie-stained spots was obtained by virtual overlapping the three images by means of graphic software (Adobe Photoshop 5.5).

2.8 MS analysis

IgE-reactive spots were excised from 2-D maps and in-gel digested with trypsin (Promega, Madison, WI, USA) as described by Hellman *et al.* [7]. For MALDI-TOF-MS, 0.5 µL of each peptide mixture was applied to a target disk and allowed to dry. Subsequently, 0.5 µL matrix solution (1% w/v α-cyano-4-hydroxycinnamic acid in 30% acetonitrile, 0.1% TFA) were applied to the dried sample and again allowed to dry. Spectra were obtained using a Bruker Reflex III MALDI-TOF spectrometer (Bremen, Germany). To interpret the MS spectra of protein digests, ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) and MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) software were used.

3 Results

Twenty patients, all positive to the open oral challenge, were recruited for the *in vitro* study. Table 1 reports the results of SPT, the open oral challenge, and the CAP-RAST values expressed in KU/L for total caseins, α-lactalbumin, and β-lactoglobulin. All patients were positive at the SPT, but five of them (No. 2, 3, 8, 12, and 13) had negative RAST results. The IgE 1-D immunoblot screening for cow's milk proteins in the 20 patients confirmed the SPT and oral challenge results (Fig. 1). To identify the cow's milk protein allergens and their isoforms, a 2-DE separation of defatted cow's milk was performed. Figure 2A shows the 2-DE map of cow's milk protein in the pH range 3–10. This map allowed the identification of the most basic allergens (IgG and lactoferrin). To achieve a better separation of the acidic cow's milk proteins, a second 2-DE map in the pH range 3–6 was obtained (Fig. 2B). Individual spots for each casein fraction and related isoforms, as well as β-lactoglobulin variants A and B, are here clearly visible and therefore were identified by MALDI-TOF-mass spectrometry by peptide mass fingerprinting (PMF). The results of MALDI-TOF identifications are shown in Table 2. Identification of the IgE-immunoreactive proteins for each patient was performed by matching the image of the membrane stained with Red Ponceau after Western blotting of the 2-DE cow's milk proteins gel to the image of the same membrane after immunodetection. Figure 3 shows the 2-D immunoblots from the 20 patients to give an overview of all identified allergens. To confirm the identification of the 18 kDa immunoreactive spot at ~p*I* 5.5, a blotting inhibition with pure ß-lactoglobulin was performed because some β-casein fragments might comigrate with β-lactoglobulin [8]. Aliquots of sera 1, 4, 6, 8, 9, 10, 12, 14, and 17 with such specific immunoreactivity were pooled and incubated with different concentrations of pure β-lactoglobulin. Figure 4 depicts the dose-dependent inhibition determined by 1-D SDS-PAGE immunoblotting (A, B, and C) and the complete inhibition on the 2-D immu-

Table 1. Results of clinical tests at recruitment

Patient	SPT	Oral challenge	RAST caseins kU/L (class)	RAST α-lac. kU/L (class)	RAST β-lac. kU/L (class)
1	+	+	63.6 (5)	15.4 (3)	9.74 (3)
2	+	+	0.34 (0)	0.34 (0)	0.34 (0)
3	+	+	0.34 (0)	0.34 (0)	0.58 (1)
4	+	+	0.63 (1)	0.34 (0)	1.52 (2)
5	+	+	3.86 (3)	0.34 (0)	1.25 (2)
6	+	+	16.8 (3)	32.2 (4)	2.2 (2)
7	+	+	<0.35 (0)	1.96 (2)	0.48 (1)
8	+	+	0.34 (0)	0.34 (0)	0.34 (0)
9	+	+	29.6 (4)	6.78 (3)	4.24 (3)
10	+	+	2.5 (2)	0.34 (0)	0.91 (2)
11	+	+	7.66 (3)	0.73 (2)	13.0 (3)
12	+	+	0.34 (0)	0.34 (0)	0.34 (0)
13	+	+	0.34 (0)	0.34 (0)	0.34 (0)
14	+	+	10.3 (3)	5.04 (3)	6.12 (3)
15	+	+	64.2 (5)	23.7 (4)	2.08 (2)
16	+	+	4.45 (3)	<0.35 (0)	<0.35 (0)
17	+	+	43.6 (4)	25.0 (4)	11.9 (3)
18	+	+	7.25 (3)	5.74 (3)	0.9 (2)
19	+	+	0.37 (1)	0.34 (0)	1.23 (2)
20	+	+	1.65 (2)	0.58 (1)	2.7 (2)
N.C.			<0.35 (0)	<0.35 (0)	<0.35 (0)

<0.35 kU/L = class 0; 0.35–0.69 kU/L = class 1; 0.70–3.49 kU/L = class 2; 3.5–17.49 kU/L = class 3; 17.5–49 kU/L= class 4; 50–99 kU/L = class 5; >100 kU/L = class 6.
N.C., negative control

Table 2. CMPs identified by MALDI-TOF-MS

Letter spot marker on Fig. 2	Protein name	No. of peptides matched	% Coverage	Protein mass/p*I*
a	α_{s1} cas	7	43	24 529/5.0
b	α_{s2} cas	7	35	26 019/8.5
c	β cas	4	15	50 134/6.1
d	κ cas	5	22	21 270/6.3
e	BSA	16	72	69 294/5.8
f	β-lac	9	76	17 167/4.7
g	α-lac	3	32	14 156/4.8
h	IgG	6	32	35 849/6.1
i	LTF	28	44	78 057/8.7

PMF software: MS-Fit

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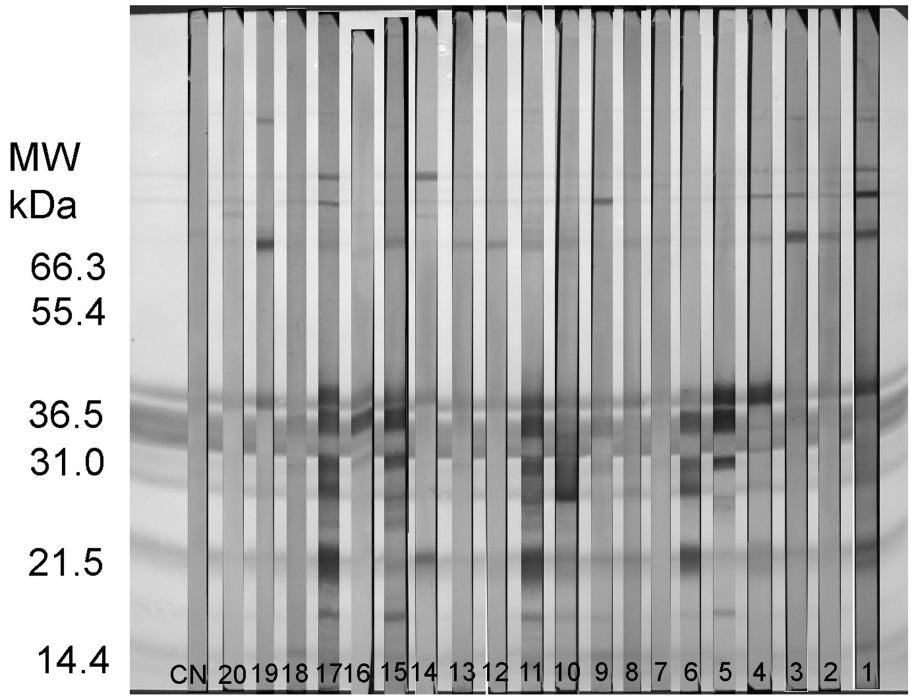
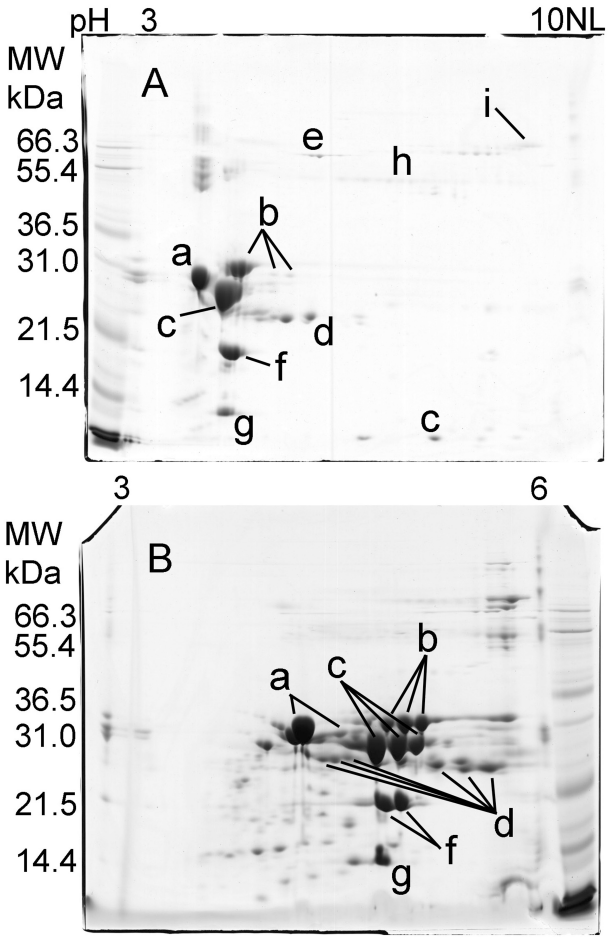


Figure 1. 1-D SDS-PAGE immunoblotting screening of the 20 patients' sera for cow's milk protein-binding specific IgE. The immunoblotting result for each patient is superimposed on the 1-D SDS-PAGE separation of cow's milk proteins.



noblot obtained pre-incubating the pooled serum with 200 μg β-lactoglobulin (D).

Table 3 summarizes the results of the identification of the IgE-immunoreactive spots for the 2-DE maps for the 20 patients. All subjects show multiple sensitization (median polysensitization to 4.3 allergens, range 2–7 allergens). All patients except one were sensitized to IgG heavy chain (95%), while 9/20 (45%) were sensitized to BSA and 10/20 (50%) to lactoferrin. It is still not assessed if sensitization to these three minor component of cow's milk – not specifically expressed in milk – can be correlated to cow's milk allergy. All patients were sensitized to one or more casein fractions, the prevalence of sensitization being: β-casein 3/20 (15%), κ-casein 10/20 (50%), α_{s1}-casein 11/20 (55%), and α_{s2}-casein 18/20 (90%). When a patient was sensitized to only one casein fraction, sensitization to α_{s2}-casein occurred in five cases and to α_{s1}-casein in two cases. Sensitization to β- and κ-casein was always associated to α-casein sensitization. Specific IgE to β-lactoglobulin were detected in 9/20 (45%) patients, in agreement with the most recent reports [3]. As shown in Table 3, specific IgE to

Figure 2. 2-DE separation of cow's milk proteins. (A) First dimension: pH range 3–10 NL; second dimension: 12% homogeneous gel. (B) First dimension: pH range 3–6; second dimension: 12% homogeneous gel. Labelled spots were identified by MALDI-TOF-mass spectrometry. a, α_{s1}-casein spots; b, α_{s2}-casein spots; c, β-casein spots; d, κ-casein spots; e, BSA spots; f, β-lactoglobulin spots; g, α-lactalbumin spots; h, IgG spots; i, lactoferrin spots.

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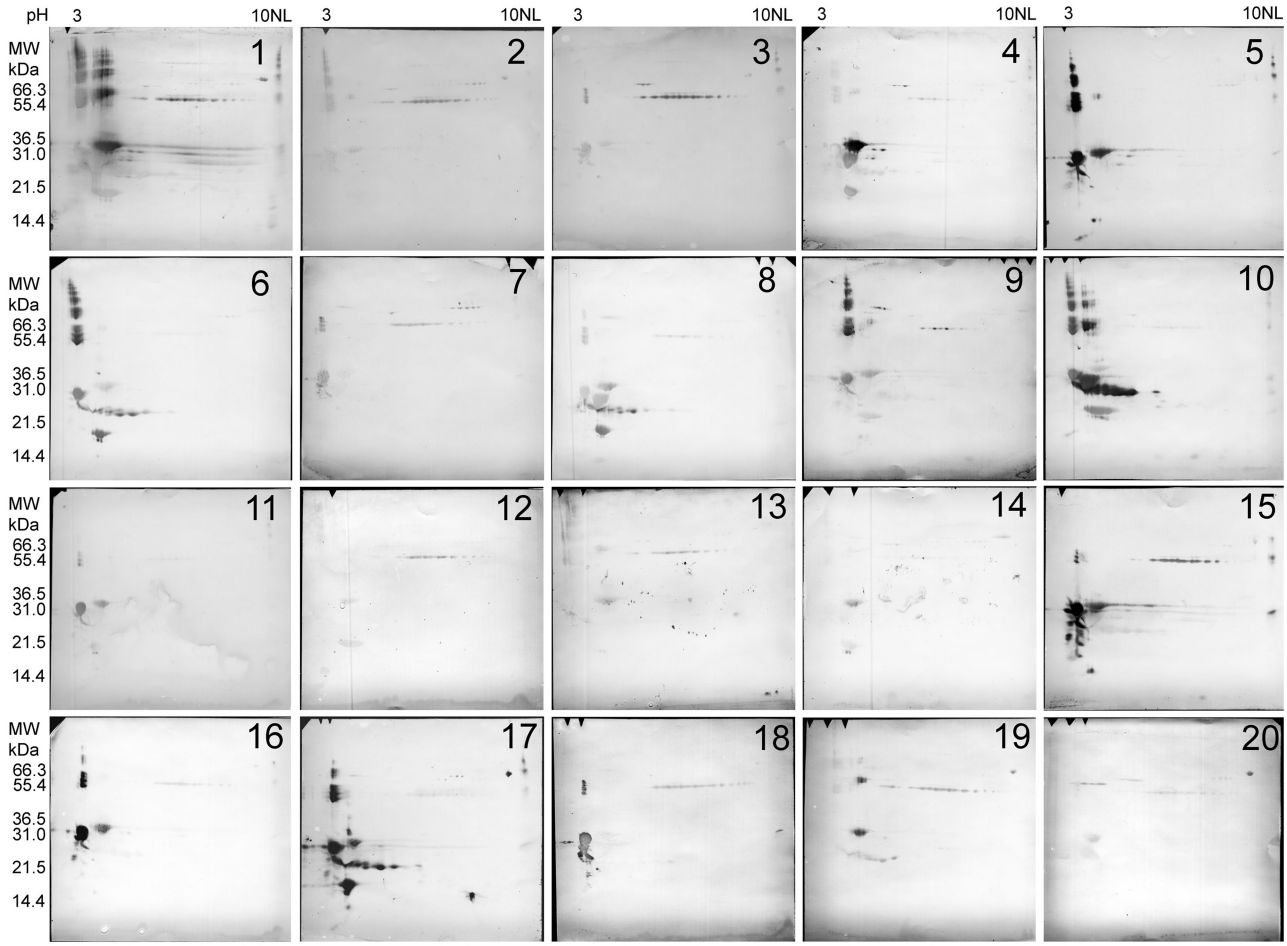

Figure 3. 2-D Immunolabeling of sera from the 20 patients.

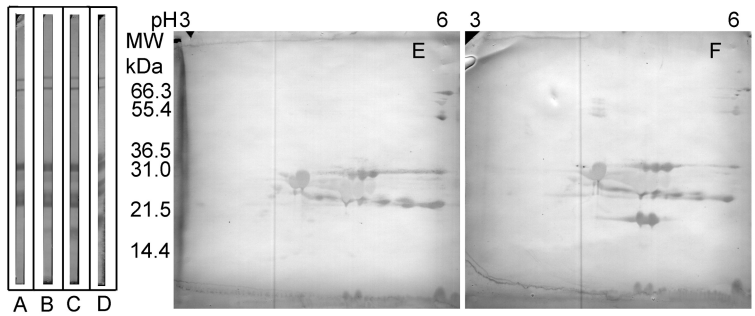

Figure 4. Blotting inhibition of cow's milk protein by pure β-lactoglobulin. 1-D immunoblot: (A) inhibition with 200 μg β-lactoglobulin; (B) inhibition with 20 μg β-lactoglobulin, (C) inhibition with 2 μg β-lactoglobulin, (D) no inhibition. 2-D immunoblot: (E) inhibition with 200 μg β-lactoglobulin, (F) no inhibition.

α-lactalbumin were not detected in any patient by 2-D immunoblotting. This is in disagreement with the RAST results where, at least seven patients reported positive scores for α-lactalbumin. In our group of patients, the prevalence of cow's milk protein allergens was: 55% α_{s1}-casein, 90% α_{s2}-casein, 15% β-casein, 50% κ-casein, 45% β-lactoglobulin, 45% BSA, 95% IgG heavy chain, 50% lactoferrin, and 0% α-lactalbumin.

4 Discussion

There has been considerable interest in milk proteomics in recent years because the occurrence of co- and post-translational protein modifications means that many gene products are present in milk as multiple protein forms. From a structural proteome perspective, identification of both the high- and the low-abundance cow's milk proteins by 2-DE

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Table 3. IgE immunoreactivities of the 20 patients' sera on the basis of the 2-DE immunodetection

Patient	α_{s1} cas a	α_{s2} cas b	β cas c	κ cas d	BSA e	β-lac f	α-lac g	IgG h	LTF i
1		++		+	++	+		++	++
2		+			+			++	+
3	+	+			++			++	+
4		++	+	+	++	+		+	+
5	++	++							
6	+	+		++		++		+	
7	+				+			+	
8		+		+		++		+	
9	+	+		+		+		++	
10	+	+	++	++	+	+		+	
11	+	+						+	
12		+				+		+	
13		+			+			++	
14		+			+	+		+	+
15	++	+	+	+				++	+
16	++	+		+				+	+
17	++	+		++		++		+	++
18	++							+	
19		+		+				+	++
20		+			+			+	++
N.C.									

+ = weak reaction; ++ = strong reaction; α_{s1} cas = α_{s1}-casein; α_{s2} cas = α_{s2}-casein; β cas = β-casein; κ cas = κ-casein; BSA = bovine serum albumin; β-lac = β-lactoglobulin; α-lac = α-lactalbumin; IgG = immunoglobulin G heavy chain; LTF = lactoferrin; N.C., negative control. a, α_1-casein spots; b, α_{s2}-casein spots; c, β-casein spots; d, κ-casein spots; e, BSA spots; f, β-lactoglobulin spots; g, α-lactalbumin spots; h, IgG spots; i, lactoferrin spots

and mass spectrometry [9–11] and the specific ability of the proteomic approach in analysing single isoforms arising from genetic variants and post-translational modifications for the analysis of bovine κ-casein microheterogeneity [12] have previously been reported. The spatial and temporal distributions of isoforms can play a critical role in determining functionality whether in the context of development, maintenance of homeostasis or progression of allergic disease. From a health perspective, there have been a number of reports on proteomic characterization of milk fat globule membrane-associated proteins (MFGMPs) [13, 14], otherwise difficult to study due to their hydrophobic nature. The MFGMPs, mostly post-translationally modified in glycoproteins, are thought to act as specific viral and bacterial ligands that prevent (or contribute to the prevention of) the attachment of pathogenic organisms to the intestinal mucosa of the infant [15]. The ability of proteomic tools to separate single isoforms of milk proteins has also been utilized to correlate specific genetic variants of milk proteins with some disease states, such as for insulin-dependent diabetes [16].

From the perspective of adverse reactions to cow's milk proteins, casein fractions are well-known allergenic proteins of cow's milk [17], but prevalence of sensitization to each casein fraction is still controversial [18]. Identification of some α_{s1}-casein linear epitopes have been correlated with persistent cow's milk allergy [17] and even single amino acid substitutions have been shown, for a few epitopes, to reduce drastically their IgE binding capacity [3]. In this work, using 20 cow's milk allergic patients' sera, as confirmed by SPT and open oral challenge, we have been able to identify by a proteomics approach specific IgE-binding cow's milk proteins and their isoforms.

The 2-D immunoblot results are not in as good agreement with the RAST scores as they are with the SPT and open oral challenge results. The most evident discrepancy concerns the α-lactalbumin result: while seven patients were classified at least as class 2 in the RAST test for this allergen, in the immunoblot experiments identification of α-lactalbumin as IgE-immunoreactive protein failed in all subjects. This discrepancy could be due to the presence of exclusively conformational epitopes of α-lactalbumin, although IgE binding linear epitopes have already been described [20], or to undesidered cross-reactions with false positive results in the RAST analysis. On the contrary, patients No. 2, 3, 8, 12, 13, and 19 with negative RAST results for total caseins were sensitized to α_{s2}-casein by 2-D immunoblot: this could be explained by the lack of detection of some α_{s2}-casein epitopes in the RAST analysis, giving rise to some false negative results. The very high prevalence of sensitization to IgG heavy chain, bovine serum albumin, and lactoferrin (95%, 45%, and 50%, respectively) in our group of patients indicates the need for clinical tests also for these allergens.

The discrepancies we observed between the results of RAST and those of 2-D immunoblotting in identifying the CMPs that are recognized by the IgEs of subjects allergic to cow's milk cast doubt on the complete reliability of RAST for diagnostic purposes. In particular, from our results RAST cannot be considered suitable for studies aimed at identifying the prevalence in the population of reactivity to individual cow's milk allergens. A precise knowledge of these different allergens gained through applying 2-D techniques may lay foundations on which to clarify the clinical role of sensitization, and may also enable the type of allergen to be correlated with the severity of symptoms. It should also be possible to clarify the correlation between sensitization to different proteins and the onset of different responses to challenge (late or early symptoms) or the acquisition of tolerance. In the light of these results it is to be hoped that the diagnostic criteria, and above all the diagnostic methods currently in use, will be revised. The proteomic approach we applied in our study will be useful for the identification of naturally occurring hypoallergenic isoforms among cow's milk major allergens.

This work was partially supported by the Foundation of Biotechnology of Torino, with thanks to the Business Unit Nutrition (B. U. N.) of Nestlé-Italiana, S. p. A.

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