

## Assessment of the immunoglobulin E-mediated immune response to milk-specific proteins in allergic patients using microarrays

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### Clinical and Experimental Allergy

#### Summary

**Background** Cow's milk allergy (CMA) is one of the most widespread human allergies, especially in young children. Although CMA is intensively studied, little is known about the recognition patterns of milk allergens in allergic patients, and the determination these patterns is a prerequisite for the development of efficient diagnostic and prognostic tools. Several factors present difficulties for such a determination, because (i) milk contains a large number of potential allergens; (ii) the majority of these allergens consist of complex suspensions rather than solutions; (iii) the major allergens, such as caseins, cannot be highly purified in large amounts; and (iv) most of the time, very small amount of young patients' sera are readily available.

**Methods** To overcome these difficulties, we developed a sensitive microarray assay that, in combination with near-infrared fluorescence detection, was used to study the immune response to milk and purified native milk proteins.

**Results** This new assay allowed us to assess the binding ability of IgE to milk allergens from a large number of young patients using reduced amounts of clinical material. The data show that bovine lactoferrin can be classed as a strong milk allergen. We confirmed that bovine caseins are the main allergens in milk and that  $\alpha_{S1}$ -casein is more allergenic than  $\alpha_{S2}$ -,  $\beta$ - and  $\kappa$ -caseins, which were recognized with almost a similar frequency by the sera of patients.

**Conclusion** Microarray methods, in combination with near-infrared fluorescence detection, can be useful for the *in vitro* diagnosis of food allergies.

**Keywords** antigen microarrays, casein, food allergy, IgE immune response, lactoferrin, milk

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#### Introduction

Food allergies are considered to be the sixth problem of human health by the World Health Organization (WHO) in the classification of human pathologies. The number of people exhibiting symptoms of allergic reactions to food and the severity of these symptoms have continued to increase in recent years. In the United States, it is estimated that between 4.5% and 8% of children under the age of 2 are subject to food allergies. In France, the number of children with allergies has reached about 4%. From the top six listed food allergies, cow's milk allergy (CMA) holds the third position with about 9% of the total number of diagnosed cases [1]. Although approximately 80% of the patients allergic to cow's milk outgrow their allergy by the age of 3, about 20% of them remain allergic

at older ages. Milk allergy symptoms vary from one patient to another and often include gastrointestinal symptoms, such as nausea and diarrhoea, dermatologic symptoms, asthma, rhinitis and systemic anaphylaxis. Promising results have been obtained in allergen-specific immunotherapy [2, 3], which might be considered to be the only specific and curative approach in the treatment of IgE-mediated allergic diseases, and the desensitization to CMA by immunotherapy may also be taken into consideration. Specific immunotherapy implies knowledge of the specific pattern of sensitization of the patient, especially in the case of milk, which contains several allergens.

The abundance of milk-specific proteins and their relationship with allergic reactions have been studied intensively. The most abundant proteins present in milk are  $\beta$ -lactoglobulin (BLG, Bos d5),  $\alpha$ -lactalbumin (ALA,

Bos d4) and a mixture of caseins known as Bos d8. Bos d8 or 'casein' is often considered to be a unique allergen by allergists while this protein fraction of milk, accounting for 78% of the total protein content of bovine milk, consists of four distinct proteins:  $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ - and  $\kappa$ -caseins ( $\alpha_{S1}$ CAS,  $\alpha_{S2}$ CAS,  $\beta$ CAS and  $\kappa$ CAS), in the proportions of 40 : 10 : 35 : 12. BLG (Bos d5) and ALA (Bos d4) represent only about 7.5% and 3.5% of bovine milk proteins, respectively. Bovine caseins are poorly folded proteins ranging from 169 to 209 amino acids in length (19–25.2 kDa), which are rich in proline and phosphorylated at serine (from 2 to 11 phosphorylated residues according to the protein). Owing to the presence of these phosphorylated residues, they bind  $\text{Ca}^{2+}$  cations. The caseins are not glycosylated, with the exception of  $\kappa$ CAS, which is *O*-glycosylated at a unique position. BLG is a 162 amino acid residue globular belonging to the lipocalin family that shares common secondary and tertiary structures ( $\beta$ -barrel) and the ability to bind small hydrophobic molecules [4]. The structure of BLG is stabilized by two disulphide bonds. ALA is also a globular protein 123 amino acids long sharing 40% homology with lysozyme but devoid of hydrolytic activity. It contains a high-affinity  $\text{Ca}^{2+}$ -binding site and possesses four disulphide bonds that increase the stability of the native structure to various treatments [5]. Bovine milk contains less abundant proteins such as serum albumin [bovine serum albumin (BSA), Bos d6], lactoferrin (LF), lactoperoxidase, immunoglobulins and traces of many other proteins.

BLG (Bos d5) appeared to be the most important milk allergen recognized by IgE antibodies from the serum of young patients [6]. However, other data indicate that milk caseins Bos d8 possess comparable allergic determinants [7, 8]. It has been shown that other milk proteins present in smaller amounts are also recognized by IgE from patients with CMA [8]. These include BSA (Bos d6), LF and IgG-heavy chain (Bos d7). Recently, contrary to previous observations, no response against ALA (Bos d4), another milk allergen, has been detected using the 2D PAGE immunoblotting method [8]. Despite the progress in the diagnosis of CMA, little is known about the specific recognition patterns of patient IgE. An important factor to consider is whether the recognition patterns of IgE differ from patient to patient, and whether these differences are related to the symptoms of the patient. The differences in recognition patterns can largely be attributed to the fact that the commonly used assay for detecting specific IgE, the ImmunoCAP System<sup>®</sup> (CAP/RAST) developed by Phadia (Uppsala, Sweden), only recognizes IgE response to total milk proteins, ALA and BLG, and not to individual caseins or other milk allergens. BSA and LF ImmunoCAP Systems<sup>®</sup> have been developed but are not yet currently used in the diagnostic of CMA.

Most of the patients with CMA are young children and hence, the amount of sera available is very limited for the

comparative studies and for studies that define patterns of allergen sensitization (CMA is usually caused by different allergen sensitizations). Miniaturized protein arrays open up new avenues in medicine because a minute spot area with immobilized samples provides greater sensitivity for the detection of molecular interactions compared with other binding assays [9]. This high-throughput and multiplexed protein array technology is proving to be attractive for the development of new immunoassays and diagnostic tools of antigen-caused pathologies (see [10] for a review). This technology has been used successfully in the detection of low-abundance allergen-specific IgE in the sera of patients suffering from various allergies [11–14]. Therefore, we were interested in applying this technology to CMA studies in order to circumvent the methodological limitations described previously, and to evaluate the IgE-mediated immune response to milk allergens in the sera of young patients.

In this study, we describe a protein microarray method based on near-infrared fluorescence detection, which was applied to determine the recognition patterns of milk allergens from young patients suffering from CMA. We showed that, for the studied cohort of French patients, the reactivity of the caseins, which were the major allergens, could be expressed as  $\alpha_{S1} > \alpha_{S2} \approx \kappa \approx \beta$  in terms of the immune response. Additionally, some patients developed a strong IgE response against LF.

## Materials and methods

### Sera

Forty sera samples were obtained from patients with CMA presenting various symptoms. All of them had 72 kIU/L < [IgE] < 18 591 kIU/L total IgE and 3 kIU/L < [IgE] < 190 kIU/L milk protein-specific IgE. Six sera samples used as negative controls were obtained from non-milk-allergic patients with 66 kIU/L < [IgE] < 13 242 kIU/L total IgE. Milk protein-specific IgE concentrations were determined with the Phadia ImmunoCAP System<sup>®</sup>.

### Preparation of allergens

Lyophilized native bovine  $\alpha_{S1}$ CAS,  $\alpha_{S2}$ CAS,  $\beta$ CAS and  $\kappa$ CAS were a kind gift from Dr Carl Holt [Hannah Research Institute (HRI), Ayr, UK]. These proteins were purified from bulk milk from the HRI herd of Holstein–Frisian cows. Native bovine BLG (variant A) was purified from cow's milk according to Maillart and Ribadeau Dumas [15]. Native bovine ALA was purified from whey using two-step chromatography as described previously [16]. LF, BSA, human serum albumin (HSA) and chicken egg white lysozyme were purchased from Sigma (St Quentin-Fallavier, France). Lyophilized proteins were diluted in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L  $\text{KH}_2\text{PO}_4$ ,

8 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and their concentrations were adjusted to 1 mg/mL by titration with the BCA assay kit (Sigma). The purity of proteins was analysed by SDS-PAGE.

#### *Fabrication of protein microarrays*

The following proteins and their complex solutions were used to prepare microarrays: the second WHO international reference preparation of human IgE (NIBSC, Potters Bar, UK) at 10 kIU/mL, bovine milk proteins (ALA, BLG, LF, BSA,  $\alpha_{S1}$ CAS,  $\alpha_{S2}$ CAS,  $\beta$ CAS,  $\kappa$ CAS), raw milk, commercial ultra-high temperature (UHT)-treated milk (Lactel, Laval, France) and commercial fat-free lyophilized milk (Delisse, Issy-les-Moulineaux, France). HSA and lysozyme were added as controls. Because the constituents of the human serum might interfere with the binding of the secondary antibody to human IgE, or with the binding of the tertiary antibody to mouse anti-IgE, serially diluted human standard IgE spots (1 : 2, 1 : 5, 1 : 10, 1 : 50, 1 : 100, 1 : 250 dilutions) were included in each arrayed slide and used as an internal control. All allergen solutions were adjusted to 1 mg/mL initial concentration (for milk samples, 1 mg/mL total protein concentration) and six human standard IgE dilutions were prepared in 96-plate immunological plates for printing on a FAST<sup>®</sup>-slide nitrocellulose membrane (Whatman, Maidstone, UK). The porous nitrocellulose structure provides an elevated retention capacity for the proteins, which is a crucial advantage over the functionalized plane surfaces in the detection of low-abundance binding in biological fluids [17]. An aliquot (0.5 nL) of each protein dilution was printed five times in the same spot with a four steel pin-equipped GMS 417 arrayer (Affymetrix Inc., Santa Clara, CA, USA) at 18 °C and 60% humidity. The fabricated microarrays were used immediately or sealed in a slide box and stored at 4 °C until use.

#### *Binding and fluorescence detection of immunoglobulin E on microarrays*

Membranes with immobilized proteins were incubated in blocking solution of PBS containing 0.1% Tween-20 and 2% polyvinyl alcohol (v/v) (Sigma) for 1 h at 4 °C. After discarding the blocking solution, serum samples of patients (1 : 50 diluted in PBS containing 0.1% Tween-20) were added to each microarray and incubated overnight. Excess non-bound serum was removed by threefold washing with PBS/0.1% Tween-20 at 18 °C, and then the slides were incubated with a mouse monoclonal anti-human IgE antibody (IgG<sub>2 $\beta$</sub> ) (Fitzgerald, Concord, MA, USA) diluted to 1 : 1000 (4  $\mu$ g/mL) for 1 h. After three washings for 5 min in PBS/0.1% Tween-20, the slides were incubated with Alexa Fluor 680<sup>®</sup>-conjugated goat anti-mouse IgG (Invitrogen, Breda, the Netherlands) diluted to

1 : 10 000 (200 ng/mL) for 1 h. After three washings for 5 min in PBS/0.1% Tween-20, the slides were air dried and scanned at 700 nm with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The use of near-infrared fluorescent dyes for the detection of bound antigen-antibody complexes on a nitrocellulose membrane provides a higher signal/noise ratio, which increases the detection sensitivity without the need for additional signal amplification [18]. Operations of binding and washing of microarrays were performed automatically in a 160  $\mu$ L volume of a 12-chamber Protein Array Workstation (Perkin Elmer, Wellesley, MA, USA). This allowed multiplexed assays with different serum probes under the same conditions.

#### *Signal analysis*

The intensity of fluorescence signals was analysed using GenePix Pro4 software (Axon Instruments/MDC, Downingtown, PA, USA). The fluorescence signal of each spot was calculated as the median fluorescence intensity subtracted from the local background median intensity. The standard deviation was calculated by comparing the fluorescence intensity data from three spots of each printed sample. The signal intensity of each bound protein or allergen was averaged, and the data obtained were placed in Excel tables or used to draw histograms.

As the maximal fluorescence intensity value varied from slide to slide (Fig. 1), the internal calibration curve spotted on each slide was used to normalize the measured fluorescence intensity values. For each array, the fluorescence intensity value obtained with the 1 : 10 dilution (1000 IU/mL) of the human IgE standard, situated at the top of the linear region of the internal calibration curve (just below saturation), was considered to be the 100% response. The measured value of every spot was converted and expressed as a percentage of this critical value. The response to an allergen was measured as follows: negative (–)  $\leq$  5%; uncertain 6%  $\leq$  +/–  $\leq$  10%; or positive 11%  $\leq$  +  $\leq$  24%; 25%  $\leq$  ++  $\leq$  49%; 50%  $\leq$  +++  $\leq$  100%; and ++++ when > 100%.

## **Results**

#### *Suitability of a calibration series and normalization of fluorescence values*

The produced microarrays with immobilized potential milk allergens and dilutions of the second WHO international IgE standard were probed with the sera of 40 patients with CMA, and six patients control sera asymptomatic for CMA. The bound IgE were revealed by a near-infrared fluorescent dye (Alexa Fluor 680<sup>®</sup>)-conjugated antibody.

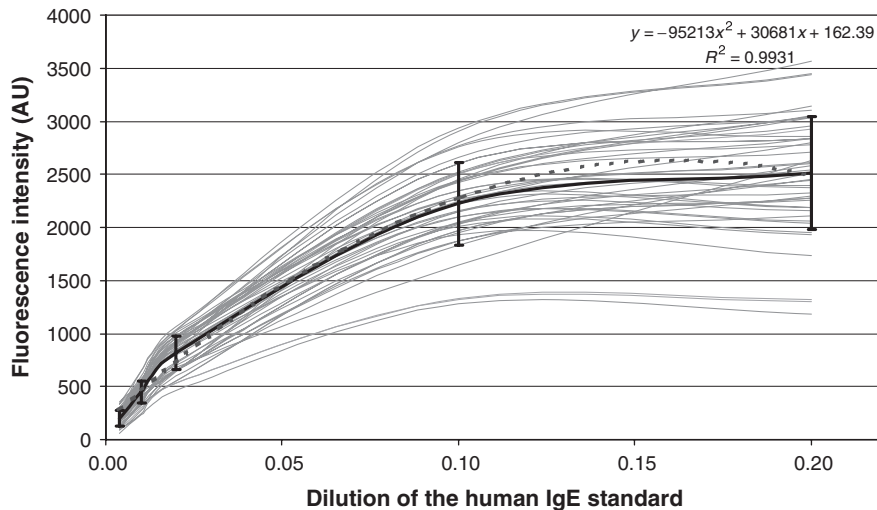


Fig. 1. Detection of IgE on microarrays. (a) Five dilutions (1 : 5, 1 : 10, 1 : 50, 1 : 100 and 1 : 250) of the second international reference preparation of human IgE international standard were spotted on FAST slides. The arrays were successively incubated with patient sera, a mouse monoclonal IgG anti-human IgE antibody and an Alexa Fluor 680<sup>®</sup>-conjugated anti-mouse IgG antibody. The fluorescence intensity of the spots (average of three spots) is shown in arbitrary units (AU) as a function of the human IgE standard dilution (thin grey lines). The mean curve (thick black line) and its regression curve ( $y = -95\,213x^2 + 30\,681x + 162.39$ ,  $R^2 = 0.9931$ ) (thick dotted black line) are also plotted.

The fluorescence intensity of the spots generated with the dilutions of the second WHO international IgE standard increased linearly in the range of 1 : 250 to 1 : 10 dilutions of the IgE standard (Fig. 1). At higher IgE concentrations, the fluorescence intensity increased more slowly or even, on some slides, slightly decreased, indicating that saturation was reached. Inter-slides variations in the global fluorescence intensity were observed (Fig. 1). On the mean standard generated curve, the fluorescence intensity values reached a maximum almost at 1 : 6 dilution with a detection limit of IgE  $> 2 \times 10^{-4}$  IU per spot. From the fluorescence intensity values obtained on the 46 slides at the IgE standard dilution 1 : 10, the coefficient of variation (CV) for the inter-spots was 10.5%, and the CV for inter-slides was 13%.

As the maximal fluorescence intensity value varied from slide to slide, it appeared necessary to use the internal calibration curve spotted on each slide to normalize the measured fluorescence intensity values.

#### Mean immunoglobulin E response intensity of patients with and without declared cow's milk allergy

The results in Fig. 2 show the mean normalized intensity of the responses against milk allergens of 40 sera from patients with declared CMA, and six negative sera of asymptomatic individuals for CMA (negative control C<sup>-</sup>). For each allergen studied, the average of the normalized responses of the six negative sera was calculated and considered to be the background given by the negative controls. This background did not exceed 4%.

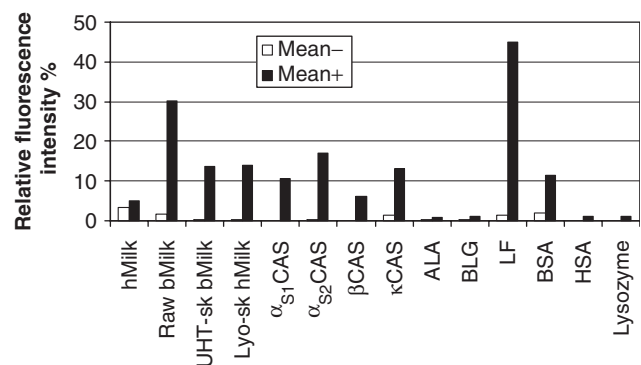


Fig. 2. Averaged response of patients with or without CMA towards milk allergens. The mean normalized fluorescence intensities of the responses of 40 sera from patients with CMA (CMA<sup>+</sup>) and of six sera from patients without CMA (CMA<sup>-</sup>) are represented. hMilk, human milk; bMilk, bovine milk; lyo sk milk, lyophilized skim milk; CMA, cow's milk allergy; ALA,  $\alpha$ -lactalbumin; BLG,  $\beta$ -lactoglobulin; LF, lactoferrin; BSA, bovine serum albumin; HSA, human serum albumin.

The recognition intensity of total milk proteins was as follows: raw milk >UHT skim milk >lyophilized skim milk. The intensities of the responses elicited by caseins and LF were the strongest. A lower reactivity of BSA was also observed. No reaction was observed against the control proteins HSA and egg white lysozyme, confirming the specificity of the method. Surprisingly, none of the sera appeared to contain IgE reactive against BLG and ALA. In order to check whether the absence of IgE reactivity was due to a problem of presentation of BLG and ALA to the specific IgE in the experimental conditions used (e.g. IgE with a very low affinity), or whether the sera

were really devoid of BLG- and ALA-specific IgE, 21 sera ( $3 < [\text{IgE}] < 190$  kIU/L milk protein-specific IgE) were assayed by ELISA (data not shown). Seventy percent contained BLG-specific IgE ( $7 < [\text{IgE}] < 75$  kIU/L) and 32% contained ALA-specific IgE ( $28 < [\text{IgE}] < 230$  kIU/L). This result shows that, under the experimental conditions used, specific IgE for BLG and ALA were not detectable, even at a high concentration.

#### Individual immunoglobulin E reactivity

The allergenic recognition pattern of each patient was analysed. As a consequence of their absence of reactivity, BLG and ALA were not taken into consideration in the prevalence studies. Some examples of individual recognition patterns of milk and purified milk allergens are reported in Table 1. A large diversity in the responses was observed, each patient presenting their own pattern of allergen recognition.

In the set of patients studied, 85% were clearly positive for at least milk and/or one of the purified milk allergens. Fifteen percent showed a negative response similar to the individuals used as negative controls, or had an uncertain (+/-) response against raw milk, without any clear positive response against any of the purified milk allergen. The strength of the IgE reactivity varied considerably from patient to patient. In general, less reactive sera had a low content of milk protein-specific IgE.

#### Immunoglobulin E reactivity against bovine and human milk

Among the patients who tested positive for at least milk and/or one of the purified milk allergens, 90% showed a clear positive response to raw milk and 10% showed an uncertain/inconclusive (+/-) response (Fig. 3). No patient generated a totally negative response to raw milk. The proportion of positive IgE responses vs. UHT skim milk and lyophilized skim milk was lower, 50% and 52%, respectively, whereas the uncertain/inconclusive (+/-) response numbers were 29% and 19%, respectively.

Three patients (8.5%) reacted positively against human milk and two patients (6%) showed uncertain (+/-) reactions. All five sera reacted with  $\alpha_{S2}$ CAS, suggesting that cross-reactivity between human milk components and bovine  $\alpha_{S2}$ CAS may occur. Three samples also presented a positive response to bovine  $\beta$ CAS.

#### Specific recognition of purified milk allergens

The study of the recognition by IgE of the pure bovine caseins showed that  $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ - and  $\kappa$ -caseins were recognized by 50%, 31%, 28% and 31% patient's sera who tested positive for at least milk and/or for one of the purified milk allergen, respectively (Fig. 3). LF-specific IgE were found in 41% of the sera (Fig. 3). About a quarter of these LF-positive sera generated very strong fluorescence signals (++++). Twenty-four percent of the patients

**Table 1.** Individual responses of patients with or without CMA towards milk allergens

Serum number	C $\ominus$	1	4	7	48
MP-IgE (kIU/L)		80	14	61	62
hMilk	-	-	++	-	-
Raw bMilk	-	+++	++	++	+
UHT-sk bMilk	-	+++	+	++	+/-
Lyo-sk bMilk	-	+++	+	+	+/-
$\beta$ CAS	-	++	+/-	-	-
$\alpha_{S1}$ CAS	-	++	+	+	+/-
$\alpha_{S2}$ CAS	-	++++	+	+/-	+/-
$\kappa$ CAS	-	++	++	+/-	+/-
ALA	-	-	-	-	-
BLG	-	-	-	-	-
LF	-	++++	++	-	+/-
BSA	-	-	-	+++	-
HSA	-	-	-	-	-
Lysozyme	-	-	-	-	-

Examples of the relative intensity of the responses towards milk allergens of four sera from patients with CMA (columns 1, 4, 7 and 48) and the mean relative intensity of the response of six sera from patients without CMA (column C $\ominus$ ) are reported. The milk protein-specific IgE (MP-IgE) concentration (kIU/L) is indicated. The response to an allergen was as follows: negative (-)  $\leq 5\%$ , uncertain  $6\% \leq +/- \leq 10\%$  or positive:  $11\% \leq + \leq 24\%$ ,  $25\% \leq ++ \leq 49\%$ ,  $50\% \leq +++ \leq 100\%$  and ++++ when  $> 100\%$ ; results are shown after normalization of the fluorescence values.

hMilk, human milk; bMilk, bovine milk; UHT-sk Milk, ultra-high temperature-treated skim milk; lyo-sk Milk, lyophilized skim milk; BSA, bovine serum albumin; ALA,  $\alpha$ -lactalbumin; LF, lactoferrin; HSA, human serum albumin; CMA, cow's milk allergy.

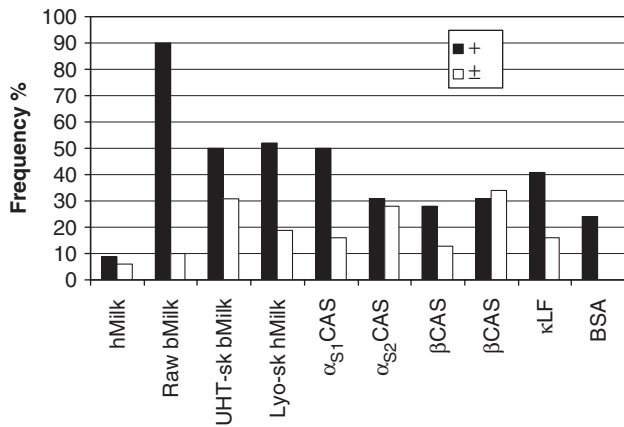


Fig. 3. Frequency of milk allergen recognition. The percentages of positive (+: black bars) (calculated from the addition of + to ++++ responses), uncertain (+/-: white bars) responses are reported for each allergen. hMilk, human milk; bMilk, bovine milk; lyo sk milk, lyophilized skim milk; BSA, bovine serum albumin.

presented a positive response against BSA. Among them, 20% of the patients generated very strong positive signals.

## Discussion

Protein microarrays formulated to assess the binding of milk allergens to specific IgE antibodies in the sera of patients suffering from CMA allowed the detection of an immune response to at least one of the allergens tested, in the majority of the sera from the patients studied. The assay allows the detection of  $\text{IgE} > 2 \times 10^{-4}$  IU per spot, compared with a detection limit of about 0.8 IU and  $\sim 4 \times 10^{-4}$  IU in the assays described by Lebrun et al. [14] and Deinhofer et al. [19]. This array-based immunoassay did not give clear responses only in the case of a few patients. This may be due to the possible limits of detection of low levels of specific IgE and due to the fact that specific IgE for BLG and ALA were not detected by the system. Further experiments are needed to explain the absence of BLG- and ALA-specific IgE detection by microarrays.

The binding of IgE to total raw milk proteins was stronger than their binding to proteins from UHT skim milk or lyophilized skim milk. These differences may be due to the effects of milk processing and skimming on the accessibility of epitopes. Indeed, in raw milk, proteins are mainly associated in micelles and some are also associated with fat globules. Transformations, such as homogenization and heating, cause the dissociation/fragmentation of micelles and the alteration of the tertiary and secondary structures of proteins, thus affecting the structures of conformational epitopes. The results of our study do not confirm the observations that pasteurized and homogenized/pasteurized milks elicit a stronger reaction than raw milk when tested in skin prick tests (SPTs) [20]. This

discrepancy may be due to the different approaches used to assess allergenicity. While an *in vitro* method was used in our study, an *in vivo* method was used in [20]. Our data allow us to assume that milk processing, and possibly milk fat content, might also have an influence on the recognition of spotted milk allergens by serum antibodies.

A specific reaction of a few patients against human milk was observed. This may be due to cross-reactivity between human milk proteins and bovine  $\alpha_{s2}$ CAS (which is unlikely because human milk does not contain  $\alpha_{s2}$ CAS [21]) and/or to cross-reactivity between human and bovine  $\beta$ CAS, which have been shown to share common IgE epitopes [22].

In agreement with Natale et al. [8], BSA and LF were identified as milk allergens. Consequently, the reactivity of IgE against BSA and LF should be taken into consideration in the diagnosis of CMA, especially in therapeutic approaches. An additional reason to focus attention on this protein is its presence in meat products. It is quite likely that patients with CMA can develop cross-reactivity to bovine meat products simply due to the ubiquity of BSA in milk and in meat.

In our study, LF-specific IgE generated strong fluorescence signals, suggesting that most of the sensitized patients to LF have developed a strong specific IgE production against the protein. However, the clinical significance of the presence of LF-specific IgE remains to be demonstrated by positive oral challenges to LF ingestion and positive SPTs to LF. We propose to include it in the official IUIS allergen nomenclature as Bos d9. Little is known about LF antigenicity. LF is a globular protein containing three iron-binding sites and five potential glycosylation sites with a complex known tertiary structure [23]. Like BLG and ALA, LF may contain both conformational and linear epitopes. Therefore, the study of the influence of LF structure on its recognition by IgE and the localization of linear epitopes would be a future challenge.

The comparison of prevalence data for cow's milk allergens obtained in this study, and also by Natale et al. [8] and by Bernard et al. [24] shows discrepancies (see Table 2). If the prevalence observed for  $\alpha_{s1}$ CAS was almost identical in the three studies, it differs for the three other caseins: from 10% to 90% for  $\alpha_{s2}$ CAS; from 15% to 40% for  $\beta$ CAS; and from 10% to 45% for  $\kappa$ CAS. LF prevalence was similar and BSA prevalence ranged from 24% to 45%. To explain the differences observed, it is important to point out that different methods, which are not entirely equivalent, were used in these studies. We spotted the allergens on a nitrocellulose membrane under native conditions for proteins whereas Bernard et al. [24] used the ELISA method. Natale et al. [8] used 2D electrophoresis gels for immunoblot analysis. Under such conditions, milk proteins were diluted in a denaturing buffer and globular proteins such as LF and BSA were extensively

**Table 2.** Comparison of milk allergen prevalence in three studies

	$\alpha$ S1CAS (%)	$\alpha$ S2CAS (%)	$\beta$ CAS (%)	$\kappa$ CAS (%)	LF (%)	BSA (%)	Method
This study	50	31	28	31	41	24	Protein microarray
Natale <i>et al.</i> [8]	55	90	15	45	50	45	2D-electrophoresis blotting
Bernard <i>et al.</i> [24]	40	10	40	10	ND	ND	ELISA

For each allergen, the prevalence is indicated.

BSA, bovine serum albumin; LF, lactoferrin; ND, non-defined.

denatured/unfolded. Denatured forms of those milk allergens, in which linear epitopes are more accessible and conformational epitopes are altered, are likely to be recognized differently when compared with native forms.

It is worth noting that in this study, our prevalence data are slightly under-estimated as the immune responses in the range 6–10% (+/–) were not taken into consideration (they were considered to be uncertain positive responses). Using the ELISA method, we found that around 80% of these (+/–) responses considered to be uncertain corresponded to real positive responses (data not shown). A comparison of the performance of antigen microarray vs. ELISA immunoassay evaluated with a few sera indicated that the sensitivity of  $\alpha$ S<sub>1</sub>CAS- and  $\kappa$ CAS-specific IgE detection (0.8 and 1.2 kIU/L, respectively) was almost similar in both cases, and the sensitivity of  $\beta$ CAS- and  $\alpha$ S<sub>2</sub>CAS-specific IgE detection was lower in microarrays (6.5 and 9 kIU/L, respectively) than in ELISA (2.9 and 2.4 kIU/L, respectively). A more precise evaluation of the negative/positive response thresholds would require comparative experiments on a much larger cohort of patients with and without CMA.

Another explanation for the differences in our results with those observed by Natale *et al.* [8] is that the sera used came from patients in distinct geographic areas, Italy vs. northwest France, with a significant diversity in the pattern of food consumption that may be responsible for variations in the sensitization patterns.

No strong correlation could be established between the sensitization profile or the strength of the IgE response and the clinical symptoms elicited by the patients. Nevertheless, in the majority of the cases, patient with severe symptoms presented stronger responses than people associated with atopic dermatitis or urticaria. Only patients with severe symptoms presented positive reactions to LF. These observations need to be confirmed on a larger cohort of patient to be more pertinent statistically.

The microarray immunoassay method used in this study is comparable to the sensitivity of ELISA and is characterized by a low consumption of serum. These characteristics of the antigen arrays, in combination with sensitive near-infrared fluorescence detection, are promising advantages for the development of a high-performance diagnostic tool for milk allergies and for the evaluation of sensitization patterns required for further immunotherapy.

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