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Human IgE Binding to the Glycosidic Moiety of Bovine κ-Casein

ROSA PIZZANO,*,† MARIA A. NICOLAI,†,‡ CARLA MANZO,† MATTEO GIANNATTASIO,§ AND FRANCESCO ADDEO†,‡

CNR, Istituto di Scienze dell'Alimentazione, via Roma 52 A-C, I-83100 Avellino, Italy; Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, via Università 100, Parco Gussone, I-80055 Portici, Italy; and Dipartimento di Arboricultura, Botanica e Patologia Vegetale, Università degli Studi di Napoli Federico II, via Università 100, Parco Gussone, I-80055 Portici, Italy

IgE ability for recognizing milk proteins was assayed in the serum of an adult atopic patient who outgrew cow milk allergy in early childhood. A number of protein species included in casein from bovine milk were detected by human IgE in immunoblotting experiments. Comparing these results with those obtained from an analysis using antibody preparations specifically directed toward the different casein fractions, IgE-reactive bands were identified as isoforms of κ-casein. IgE-reactive protein was not present in neither bovine cheese, regardless of cheese-making technology and time ripening, nor milk from any other dairy animal, such as ewe, goat, and water buffalo. Chemical deglycosylation of protein bands immobilized onto nitrocellulose proved that the glycosidic moiety of bovine κ-casein was principally involved in IgE recognition.

KEYWORDS: ***N*-Acetylneuraminic acid; κ-casein; milk allergy; IgE cross-reaction; CCD**

INTRODUCTION

All major milk proteins have been recognized as potential food allergens. A lot of effort has been put into defining the IgE linear epitope structures of the four casein components, α_{s1}-casein (*1*−*3*), β- and κ-casein (*4*), and α_{s2}-casein (*5*), as well as the two major whey proteins, β-lactoglobulin (*6*−8) and α-lactalbumin (*8*−9), although some discrepancies are evident, likely due to the different methods and human sera used in epitope mapping.

Sensitivity to different milk proteins has been frequently observed in patients affected by cow milk allergy (*10*). However, milk proteins are encoded by different genes and have no common primary structure stretch that could be related to their allergenic potential, apart from the phosphopeptide region of α_{s1}-, α_{s2}-, and β-casein. Indeed, the cluster sequence of phosphorylated serine residues included in these casein regions has been found to be responsible for the production of IgE cross-reacting with α_{s1}-, α_{s2}-, and β-casein (*11*). One of the major allergenic sites of β-lactoglobulin has been found to share similar conformational features as one of those identified in α-lactalbumin (*12*), thus suggesting that IgE cross-sensitization could arise also owing to similar characteristic conformations of the allergenic sites. To explain the occurrence of cross-reacting IgE in the sera of patients sensitive to milk proteins, in most cases a co-sensitization to the different proteins during the digestive process of milk has been generally invoked, but the relative mechanisms have not yet been clearly understood. It is worth noting that milk proteins tend to form macromolecular aggregates held together by noncovalent interactions, often being stabilized by intermolecular disulfide bonds. Apart from self-assembly of casein components in micellar structures, following the model originally developed by Waugh and Talbot (*13*), polymerization of whey proteins (*14*) as well as association among major whey proteins (*15*) and whey proteins and casein micelles (*16*) have been described. All these reactions may occur simultaneously in milk, with different intermediates being formed, depending on environmental conditions such as temperature, pH, individual protein content, and mineral concentration. Therefore, IgE recognizing different milk proteins could have been developed in response to peptides generated by protein aggregates processing.

To identify protein structures and/or substructures responsible for IgE cross-sensitization in milk allergy, we selected individual human sera displaying a widespread IgE specificity spectrum toward milk proteins. In this paper capacity binding of the IgE fraction of one of these sera was studied in detail to recognize its actual target/s among milk proteins. It is worth noting that the serum donor was an adult atopic patient who had outgrown cow milk allergy in early childhood.

MATERIALS AND METHODS

Samples. Whole casein, FPLC-enriched casein fractions by ion-exchange chromatography, RP-HPLC-purified κ-casein, and

* To whom correspondence should be addressed. Phone: +39 825 299561. Fax: +39 825 781585. E-mail: rpizzano@isa.cnr.it
† CNR.
‡ Dipartimento di Scienza degli Alimenti.
§ Dipartimento di Arboricultura.

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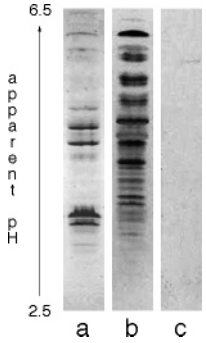

Figure 1. PAGIF analysis of whole bovine casein: (a) Coomassie-stained profile; (b) IgE immunodetection using serum P; (c) IgE immunodetection using a control human serum.

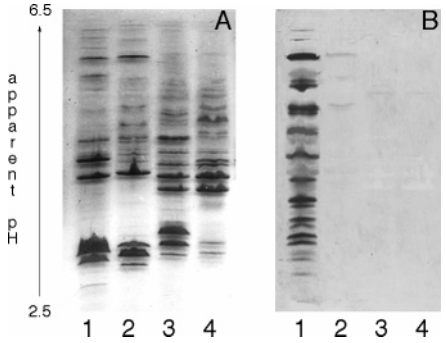

Figure 2. PAGIF analysis of whole casein from cow (lane 1), water buffalo (lane 2), sheep (lane 3), and goat (lane 4) milks: (A) Coomassie-stained gel; (B) IgE immunodetection using serum P.

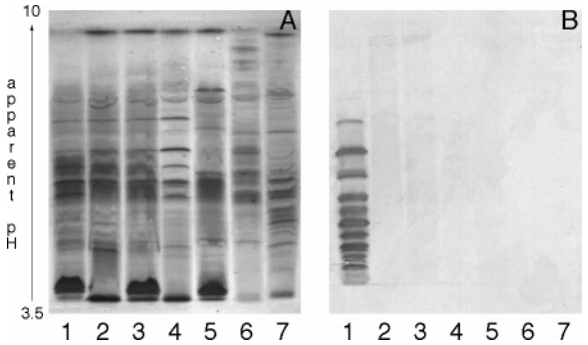

Figure 3. PAGIF analysis of whole bovine casein (lane 1), bovine cheese samples of Stracchino (lane 2), Mozzarella (lane 3), Parmigiano Reggiano (lane 4), water buffalo Mozzarella cheese (lane 5), caprine cheese (lane 6), and ovine Pecorino Romano cheese (lane 7): (A) Coomassie-stained gel; (B) IgE immunodetection using serum P.

cheese samples were prepared according to the procedures previously described (*17*).

Antisera. Human sera were obtained from a hospital bank of sera. Antisera specifically directed against each of the casein fractions were produced by Primm (Milano, Italy) by immunizing rabbits with ovoalbumin-coupled synthetic peptides, each reproducing a sequence stretch properly chosen within the primary structure of each casein component, as previously reported (*18*).

Electrophoresis and Immunoblotting. Electrophoretic separations were obtained by polyacrylamide gel isoelectric focusing (PAGIF) using a PhastSystem Apparatus (Pharmacia, Uppsala, Sweden). The protein profiles were transferred from the gel onto nitrocellulose paper by capillary diffusion. Gel and samples preparation, run conditions, Coomassie Brilliant Blue G-250 staining, transfer, and immunodetection with rabbit antisera were as already reported (*17*). Human sera as immunostaining reagents were used at a 1:3 dilution in phosphate-buffered saline (PBS) containing 10% v/v heat-inactivated horse serum. Human IgE binding was revealed using 1:60 diluted peroxidase-labeled goat anti-human IgE polyclonal antibodies (Sigma, St. Louis, MO).

In Situ Deglycosylation of Proteins. Mild periodate oxidation was performed in order to remove sugar chains from proteins bound to nitrocellulose sheets by capillary diffusion. The procedure was based on the method developed for deglycosylation of proteins fixed by Western blot (*19*). Following protein transfer, nitrocellulose was incubated in 0.5% v/v Tween 20/PBS for at least 1 h. After a rinse with 50 mM sodium acetate buffer, pH 4.5, the nitrocellulose paper was incubated in the same buffer containing 50 mM sodium periodate for 1 h in the dark. Periodate was washed off with the sodium acetate buffer, and 50 mM sodium borohydride in PBS was added. After 30 min, nitrocellulose was equilibrated in PBS and used for immunostaining according to the usual procedure. All steps were carried out at room temperature with approximately 10 mL of solution being used for a 4 cm × 4 cm membrane.

RESULTS

Performance of Serum P toward Milk and Cheese Proteins. Serum from an adult atopic patient who had outgrown cow milk allergy in early childhood (serum P) was tested, regarding its IgE binding capacity toward cow milk proteins in comparison to serum from a healthy person. For this reason casein and whey proteins were separately used in immunoblotting experiments. In **Figure 1** the IgE binding specificity displayed by the two human sera toward whole bovine casein is shown. Along the isoelectric focusing profile of the casein sample in the pH range from 2.5 to 6.5 two groups of bands were mainly stained by Coomassie Brilliant Blue G-250, corresponding to the most abundant α_{s1}-casein and β-casein family (lane a). Due to the high-resolution power of PAGIF, the well-known microheterogeneity of casein fractions was detected (*20*). IgE contained in serum P recognized a series of electrophoretic bands along the profile of the whole bovine casein (lane b), whereas serum from a healthy person was ineffective (lane c). The pattern immunostained by serum P did not match with the nonspecifically Coomassie-stained profile since a number of IgE-reactive minor protein bands were detected. On the contrary, the IgE fraction from serum P was not able to recognize any acid whey protein in similar immunoblotting experiments (not shown).

Despite the strict homology existing among caseins from the different species of dairy interest (*21*), no component along the electrophoretic profiles of ovine, caprine, and water buffalo whole casein was recognized by IgE from serum P, as shown in **Figure 2**. As previously reported (*18*), epitope structures may not be easily accessible to antibodies in native caseins, but they can be attained in peptides generated by limited proteolysis of caseins. To verify if any IgE epitope of native caseins became accessible in partially hydrolyzed cheese casein, commercial samples were analyzed in immunoblotting experiments. As shown in **Figure 3**, no immunoreactivity was found in profiles of ovine, caprine, and water buffalo cheese samples (lanes 5−7). This result means that no common casein epitope was involved in IgE development in the sensitized patient. In addition, analysis of different bovine cheese samples pointed out that IgE responsiveness of bovine casein was definitively lost after cheese making, irrespective of the cheese variety (**Figure 3**, lanes 2−4)

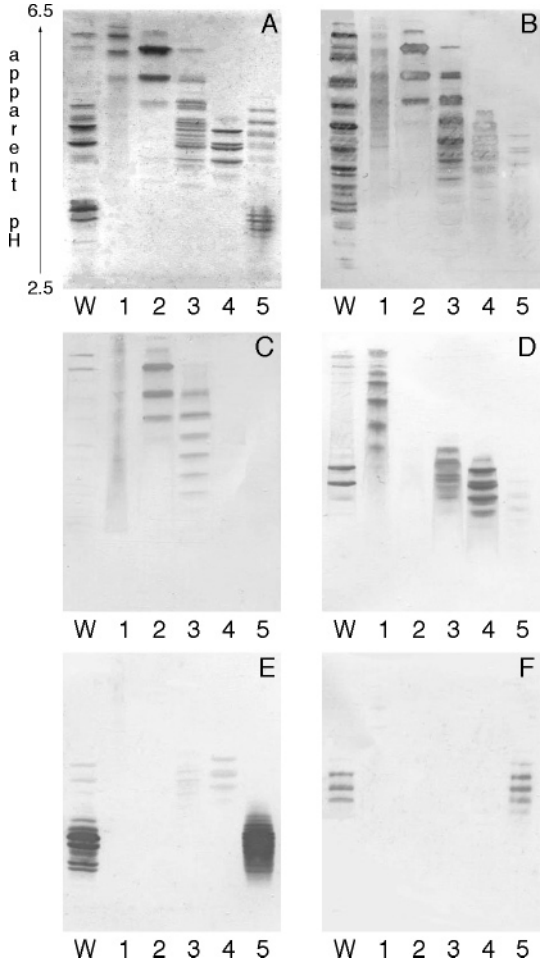

Figure 4. PAGIF analysis of whole bovine casein (lane W) and casein fractions separated by FPLC anion-exchange chromatography (lanes 1–5): (A) Coomassie-stained gel; (B) IgE immunodetection using serum P. For comparison, immunodetection using anti-peptide antibodies recognizing specifically κ-casein, β-casein, α_{s1}-casein, and α_{s2}-casein was reported, respectively, in panels C, D, E, and F.

and ripening length (Parmigiano Reggiano cheese from 1 day to 24 months, not shown). These results indicated that casein IgE sensitivity was missing during milk clotting by chymosin (Rennin, EC 3.4.23.4), as confirmed by assaying a bovine casein sample hydrolyzed with recombinant chymosin (not shown).

Identification of the Epitope Recognized by IgE from Serum P. To identify which bovine casein component was responsible for binding to IgE from serum P, casein fractions separated by FPLC (**Figure 4**, lanes 2−5) from a whole bovine casein sample (**Figure 4**, lane 1) were analyzed in immunoblotting experiments by using anti-peptide antibodies with each recognizing a specific casein component (anti-κ-casein in **Figure 4C**, anti-β-casein in **Figure 4D**, anti-α_{s1}-casein in **Figure 4E**, and anti-α_{s2}-casein in **Figure 4F**). The results obtained through using serum P and the Coomassie-stained gel are reported in **Figure 4B** and **Figure 4A**, respectively. Regarding the profiles relative to FPLC-enriched casein fractions, the immunoblotting print developed using serum P was quite similar to that obtained from using anti-κ-casein, apart from staining intensity. Some differences in immunostaining were found in the whole casein profile (cfr. lanes 1 in **Figures 4B** and **4C**), where anti-κ-casein was able to detect only the most abundant κ-casein components, corresponding to the major κ-casein genetic variants A and B (*22*). However, taking into account the electrophoretic pattern of each casein component as resulting from specific antibody staining, any contribution to IgE response from caseins other than κ-casein could be clearly excluded.

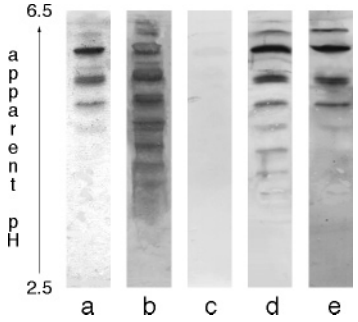

Figure 5. Immunoreactivity of PAGIF profiles of RP-HPLC-purified κ-casein after chemical deglycosylation of protein bands bound to nitrocellulose: the native (lane b) and deglycosylated κ-casein profile (lane c), as immunostained by serum P; the native (lane d) and deglycosylated (lane e) κ-casein profile, as immunostained by anti-κ-casein antibodies. In lane a the Coomassie-stained gel profile of RP-HPLC purified κ-casein is shown.

Microheterogeneity of bovine κ-casein has been recently described following a proteomic approach (*22*). Apart from genetic variants, posttranslation modifications account for the existence of many different κ-casein isoforms. Two phosphorylation sites and up to six potential O-glycosylation sites have been identified. A variety of glycoforms for each genetic variant has been found, depending on the number, location, and content of protein-bound carbohydrate residues. To establish the role played by the sugar chain in IgE recognition of κ-casein, experiments shown in **Figure 5** were performed using a RP-HPLC-purified κ-casein sample. Its Coomassie Brilliant Blue G-250 stained profile is shown in lane a. After blotting, the protein bands included in either the native (lane b) or the in situ chemically deglycosylated κ-casein profile (lane c) were immunostained using serum P as a reagent. In a parallel experiment, native (lane d) and chemically in situ deglycosylated (lane e) RP-HPLC-purified κ-casein were probed with the anti-κ-casein antibody preparation in order to establish that the in situ deglycosylation procedure did not impair neither protein structure nor its binding to nitrocellulose. The results obtained clearly indicate that protein detection by IgE from serum P was fundamentally based on the specific recognition of the glycosidic moieties linked to κ-casein. In fact, human IgE occurring in the serum sample was not able to further detect any band in the deglycosylated sample, varying with the anti-peptide antibody preparation recognizing κ-casein, regardless of the glycosylation state.

DISCUSSION

Although some indications about the allergenic character of the glycosidic moiety of κ-casein had been previously provided, the next studies based on the epitope mapping approach could not evaluate the impact of posttranslational modifications on the potential allergenicity of κ-casein (*4*). According to the analysis previously reported in **Figure 5**, O-linked glycosylation motifs in the glycopeptide domain of bovine κ-casein proved to be fundamentally responsible for IgE binding, despite a widespread IgE reactivity spectrum shown by the serum toward cow milk proteins.

At present, a statistical evaluation of immunodominance of the κ-casein glycosidic epitopes in milk allergy is not possible. In compliance with previous reports, in preliminary screening of sera from milk allergic patients we observed an apparent

multiple IgE reactivity toward different milk components in several individual human sera, but specificity and intensity of the IgE response varied greatly between the sera and the protein components. Therefore, in principle, the IgE binding capacity of serum P, as defined in this work, cannot be generalized to the other sera, even displaying similar reactivity spectra.

Identification of the IgE-recognized components enables us to fully justify the absolute absence of immunoreactivity in different cheese samples (results reported in **Figure 3**). In fact, κ-casein is the sole glycosylated casein component in casein from the four animal species. During the primary stage of cheese making, κ-casein is cleaved by chymosin at the peptide bond Phe105-Met106, yielding the whey-soluble C-terminal 106−169 fragment, known as casein glycopeptide. The latter retains all carbohydrates originally present in κ-casein, so that its release in whey makes cheese devoid of any glycosylated component (*22*).

It is a more difficult task to completely explain the IgE unreactivity toward ovine, caprine, and water buffalo κ-casein (results reported in **Figure 2**). Characterization of κ-casein glycoforms from ovine and caprine milk has been recently reported with no data about this being currently available with regard to the water buffalo milk. An overall lower level of κ-casein glycosylation was observed in ovine (30%) (*23*) and caprine milk (36%) (*24*) with respect to the bovine milk (at least 60%) (*25*). Regarding the monosaccharides included in κ-caseins glycoforms, apart from galactose and *N*-acetylgalactosamine, both common to bovine κ-casein, *N*-glycolylneuraminic acid was identified in ovine casein instead of *N*-acetylneuraminic acid, which was observed in bovine milk (*23*, *26*). Both the *N*-acetyl- and *N*-glycolylneuraminic acid were detected in caprine milk, but no tetrasaccharide structure was noted (*24*). Other features of the oligosaccharide structures of ovine and caprine κ-casein glycoforms, such as attachment of the *N*-acetylgalactosamine residue to threonine residues of the protein chain and occurrence of sialic acid as a terminal unit, were common to those already found in bovine κ-casein glycoforms.

Besides the glycosidic moiety, additional involvement of any amino acid residue included in the bovine κ-casein sequence might contribute to determining IgE specificity toward bovine κ-casein glycoforms. However, taking into consideration the difference between sugar chains in κ-casein glycoforms of milk from different species, IgE epitope could also consist only in a *N*-acetylneuraminic acid as a terminal unit of a tetrasaccharide chain. On the other hand, an IgG antibody preparation raised against a lactosylated peptide proved to be capable of distinguishing between free galactose and glucose, its C-4 epimer, as previously reported (*27*).

Immunoblotting analysis in **Figure 4** provided a rationale for understanding multiple reactivity shown by the patient's serum IgE. Results clearly showed that the human IgE fraction did not recognize different casein fractions but exclusively the different glycosylated κ-casein components (*22*), each at its own isoelectric point, coeluting with the different FPLC-enriched casein fractions.

As mentioned above, the IgE reactivity pattern of the selected human serum toward milk proteins did not reflect the real sensitivity of the serum donor. In fact, the patient had experienced a severe adverse reaction to milk during early childhood but had achieved tolerance to it over time. By contrast, the patient presented clinical allergic manifestations against different foods (mainly maize, apple, spinach, and lettuce).

Cow milk allergy has been extensively studied due to the importance of milk in the human diet, especially in infants (*28*). Although in most cases clinical reactions in milk allergic children disappear within the first 3 years of life, milk allergy plays an important role in the pathogenesis of other atopic diseases (*29*). Immunologic changes associated with the development of milk tolerance has been evaluated in sera from patients at the time of their clinical reactivity (*30*), but no data is currently available concerning the residual content of IgE arisen against milk proteins in sera from patients after tolerance to milk was acquired. Indeed, we cannot know whether sensitization to the sialylated components of κ-casein had been one of the events determining milk allergy of the patient during infancy. However, sialic acid residues are included in protein glyconjugates of both animal and plant cells (*31*) and could therefore possibly act as cross-reactive carbohydrate determinants (CCD) (*19*, *32*). According to this hypothesis, patient sensibility toward the sialylated κ-casein might have played a role in the development of any other adverse reactions, classified as atopic manifestations, on exposure to different sialylated proteins.

The relevance of the glycosidic moiety of bovine κ-casein as a source of allergenicity remains to be evaluated. Work is in progress to determine the prevalence of IgE reactive to κ-casein glycoforms in sera from cow milk allergic patients and association of IgE recognition of κ-casein glycoforms with the natural history of cow milk allergy (*28*).

Protein hydrolysates are currently used as milk substitutes to either avoid clinical reactions in cow milk allergic patients or prevent sensitization to milk proteins in allergy-risk subjects (*29*). However, even extensively hydrolyzed milk formulas have occasionally been observed to elicit allergic reactions in sensitized patients (*33*). Possible recognition of the κ-casein glycosidic domain as an additional immunodominant epitope in milk proteins may be also of importance for evaluating the residual allergenic potential of marketed milk formulas and developing safer milk-substituting products.

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