



# Molecular basis of IgE cross-reactivity between human $\beta$ -casein and bovine $\beta$ -casein, a major allergen of milk

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## Abstract

Twenty patients allergic to cow's milk proteins and with high levels of specific IgE directed against bovine whole casein were selected to evaluate reactivity of their IgE antibodies with human  $\beta$ -casein. Highly purified human and bovine  $\beta$ -caseins were prepared by selective precipitations and FPLC separation. Their identity and purity were assessed by HPLC, analysis of amino acid composition, sequencing of the five N-terminal amino acid residues and immunochemical tests. Direct and indirect ELISAs were performed using human and bovine  $\beta$ -casein coated into microtiter plates and monoclonal anti-human IgE antibody AChE labelled for revelation. Seven sera contained specific IgE directed against human  $\beta$ -casein. Inhibition studies using native human and bovine  $\beta$ -caseins as well as bovine  $\beta$ -casein-derived peptides demonstrated that, depending on the sera, one or several common epitopes located in different parts of the molecule were shared by the two homologous proteins. © 2000 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Whole casein accounts for 80% of the total protein in cow's milk (27 g/l) and acts as a major allergen in cow's milk allergy (Stöger and Wütrich, 1993; Docena et al., 1996; Bernard et al., 1998). It comprises four different caseins,  $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -casein, with  $\beta$ -casein representing 38% of the whole casein fraction (approximately 10 g/l).

In human milk, the proportion of total protein accounted for by the casein fraction depends on the stage of lactation (Kunz and Lönnerdal, 1990a, 1990b, 1992; Sanchez-Hidalgo et al., 1998) and increases from

early lactation (casein vs. whey proteins ratio: 10:90), to mature milk (40:60), and to late lactation (50:50) (Kunz and Lönnerdal, 1990a, 1990b, 1992). The casein fraction is essentially composed of  $\beta$ -casein, at an average concentration of 5 g/l of milk (Chtourou et al., 1985; Kroening et al., 1998).

The amino acid sequence of human  $\beta$ -casein has been established by protein sequencing using recurring Edman degradation and by deduction from the nucleotide sequence (Greenberg et al., 1984; Lönnerdal et al., 1990). Comparison of the sequences of bovine and human  $\beta$ -caseins requires alignment of the N-terminal extremity of the human protein with residue 10 of bovine  $\beta$ -casein. Marked homology is then observed between the two proteins, which share 47% of identical residues.

The allergenicity of bovine  $\beta$ -casein has already been demonstrated since 92% of sera from patients allergic

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to whole casein have IgE directed against bovine  $\beta$ -casein (Bernard et al., 1998).

Actually, Cantisani et al. (1997) have already reported the presence of specific IgE for human milk whey proteins in the blood sera of atopic infants. These specific IgE cross-reacted with the corresponding cow's milk proteins (CMP) which very likely explained why specific IgE for CMP could be found in the blood sera of exclusively breast-fed infants. Our aim was to confirm these observations on the casein fraction of milk and especially on  $\beta$ -casein which is a major component of milk of most mammals and to identify which part of the molecule is involved in this cross-reactivity and might therefore be responsible for an allergenic risk. We have investigated the occurrence of specific IgE for human  $\beta$ -casein in patients allergic to the casein fraction of CMP, and identified molecular domains comprising common epitopes between the bovine and human  $\beta$ -caseins.

## 2. Materials and methods

### 2.1. Human sera

Twenty serum samples from patients allergic to cow's milk proteins were obtained in banks of sera from pediatrics and/or allergy departments of different hospitals in France. They were selected according to their positive RAST<sup>R</sup> tests to milk proteins, and particularly to casein.

The children/adult repartition among these sera was as follows: five were obtained from 2 to 3 years old children, 10 from 5 to 12 years old children and five from adults, i.e. more than 15 years. In this study, there were no sera from infants less than 2-year old.

Specific IgE to whole casein fraction, and particularly to bovine  $\beta$ -casein were determined according to Wal et al. (1995). The concentrations of serum anti-bovine  $\beta$ -casein IgE ranged from 2.7 to 330 IU/ml. Eight control sera were obtained from non-allergic human volunteers.

### 2.2. Purification of milk proteins and derived peptides

Bovine  $\beta$ -casein was prepared from the milk of a cow homozygous for variant A1 of  $\beta$ -casein according to Bernard et al. (1998). Different  $\beta$ -casein-derived peptides were obtained after enzymatic hydrolysis. Peptides f(1–52) and f(53–139) were generated by mild controlled  $\alpha$ -chymotryptic hydrolysis, while peptides f(106–209) were produced by classical extensive enzymatic digestion with plasmin. These peptides were purified by anion-exchange chromatography (Mono Q FPLC, Pharmacia), preparative HPLC, or both. Peptide purity was checked by analytical HPLC. Pure pep-

tides were characterized by amino acid analysis (see below).

Human  $\beta$ -Casein was prepared from breast milk collected during the first months of lactation from a female volunteer who was on a low milk product diet.  $\beta$ -casein was prepared according to the method of Kunz and Lönnerdal (1990a, 1990b)). Milk was defatted and freeze-dried after collection. Just before processing, milk samples were reconstituted with water before acidification to pH 4.3 using 1 M HCl. Calcium chloride was then progressively added to a final concentration of 60 mM  $\text{Ca}^{2+}$  in acidified milk. After ultracentrifugation at 189,000  $\times g$  for 1 h at 4°C, whole casein was recovered in the supernatant and further purified using a mono Q anion-exchange system as previously described (Kunz and Lönnerdal, 1990b). The late eluted major peaks were collected, dialysed against water and freeze-dried.

### 2.3. Characterization of the purified fractions

The purified peaks were studied by amino acid analysis using an automatic analyzer (Biotronik Lc 500) after acid hydrolysis (110°C, 24 h, 5–7 N HCl, under vacuum) and by sequencing of the five N-terminal amino acid residues by recurring Edman degradation using Tar's procedure.

### 2.4. Purity of the $\beta$ -caseins

Purity of the bovine and human  $\beta$ -caseins was further assessed by RP-HPLC (Waters, Milford, USA) using a C4-Vydac 250  $\times$  4.6 mm ID column (Shandon, France). Elution was performed with a 40-min linear gradient from 30 to 50% of buffer B (acetonitrile 0.04% TFA) in buffer A ( $\text{H}_2\text{O}$  0.01% TFA) at a flow rate of 1 ml/min. Detection was monitored at 220 nm. The purity of the prepared caseins was also extensively investigated by immunochemical studies using specific anti- $\beta$ -lactoglobulin, anti  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein antisera as previously described (Wal et al., 1995; Bernard et al., 1998).

### 2.5. Purity of the fragments from bovine $\beta$ -casein

The purity of the isolated fragments was checked in a second HPLC system using a C18 Deltapak column (150  $\times$  3.9 mm). Elution was performed with a 60-min linear gradient from 0 to 100% of buffer B (60% of acetonitrile in 50 mM ammonium acetate pH 6.0) in solvent A (25 mM ammonium acetate pH 6.0) at a flow rate of 0.8 ml/min and monitored at 220 nm.

### 2.6. Specific direct IgE ELISA

96-well microplates (Nunc, Denmark) were coated

with 200  $\mu$ l of purified protein (5  $\mu$ g/ml) in 0.025 M EDTA carbonate buffer pH 9.3 as described by Campbell et al. (1987). The plates were incubated for 18 h at 4°C and further saturated for 18 h at +4°C with 300  $\mu$ l of EIA buffer (0.1 M potassium phosphate buffer pH 7.4 containing 0.1% HSA, 0.4 M NaCl, 0.001 M EDTA and 0.01% sodium azide). Just before use, the plates were washed five times in an automatic plate washer (Titertek from Labsystem, Helsinki, Finland) with 0.01 M potassium phosphate buffer pH 7.4 containing 0.05% Tween 20.

These experiments were performed as previously described (Bernard et al., 1998). Briefly, 50  $\mu$ l of diluted sera from allergic patients were dispensed per well. After incubation for 24 h at room temperature, followed by extensive washing, 50  $\mu$ l of a solution of an anti-human IgE mouse monoclonal antibody (mAb) (i.e., clone BS17) labelled with acetylcholinesterase (AChE) were added per well. After a further 18 h incubation at 20°C, the plates were extensively washed before revealing bound AChE by addition of 200  $\mu$ l/well of Ellman reagent (Ellman et al., 1961). The enzymatic reaction was monitored at 414 nm using a Titertek plate reader.

Eight sera from non-allergic patients were used as negative controls. Non-specific binding was also evaluated by dispensing positive sera into HSA-coated wells. Specific IgE were quantified (IU/ml, one IU corresponding to 2.4 ng) by comparison with concentration–response curves obtained with a total IgE assay performed with known standards of human IgE (Pharmacia Prist test kit). The corresponding immunometric assays of total and specific IgE were performed under identical conditions. An anti-human IgE mAb (clone LE27), complementary to the BS17-AChE tracer, was used as capture antibody for the total IgE assay, instead of the immobilized allergen used for specific IgE determination. The detection limit of specific IgE, calculated as the concentration corresponding to the average non-specific binding plus three standard deviations was ca. 0.1 IU/ml.

### 2.7. Indirect IgE ELISA

According to the intensity of the IgE response to human  $\beta$ -casein, two types of indirect ELISA studies were performed. Initially, inhibition tests were performed for all sera on bovine  $\beta$ -casein-coated plates using human and bovine  $\beta$ -caseins as inhibitors. For sera giving a strong signal, inhibitions were then performed on human  $\beta$ -casein-coated plates in the presence of potential inhibitors (human and bovine  $\beta$ -caseins, and peptides derived from bovine  $\beta$ -casein).

In both cases, 50  $\mu$ l of immune or control sera were first incubated for 6 h with mixing at 20°C with 50  $\mu$ l of increasing concentrations of inhibitors (ranging

from 0.001 to 40 nmol/ml) in EIA buffer. HSA was used as a control to evaluate the non-specific binding at high inhibitor concentrations. Aliquots (50  $\mu$ l) of the mixture were then added to the antigen-coated wells. The different steps of the assay were then performed as described above.

### 3. Results

Using previously described methods, we purified human and bovine  $\beta$ -caseins, whose amino acid compositions and amino acid sequences (determined on the five amino terminal residues) matched those of bovine and human  $\beta$ -caseins as described by Ribadeau-Dumas et al. (1972) and Lönnerdal et al. (1990), respectively (data not shown).

Each casein eluted as a single homogeneous peak in HPLC. Moreover, FPLC and HPLC of the human  $\beta$ -casein detected no contamination either by human

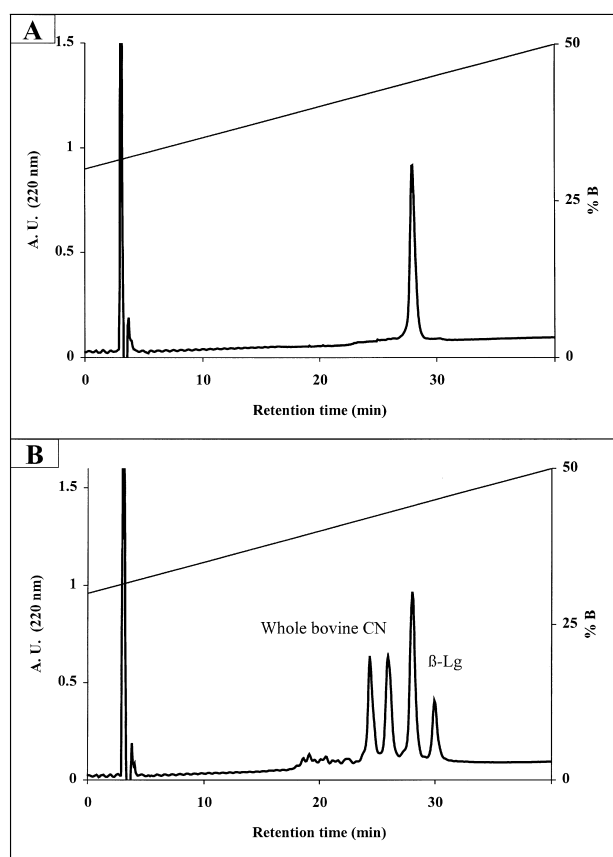


Fig. 1. RP-HPLC analysis of purified human  $\beta$ -casein: human  $\beta$ -casein alone (A); human  $\beta$ -casein co-injected with bovine whole casein and  $\beta$ -lactoglobulin (B). Retention times are the following: 18–23 min: mixture of isoforms of bovine  $\kappa$ - and  $\alpha$ s2-caseins that have not been further separated; 24 min: bovine  $\alpha$ s1-casein; 26 min: bovine  $\beta$ -casein; 28 min: human  $\beta$ -casein; 30 min: bovine  $\beta$ -lactoglobulin.

wey proteins, such as human  $\alpha$ -lactalbumin, or by bovine wey proteins and caseins. Comparison with purified human  $\beta$ -casein spiked with other bovine proteins is shown in Fig. 1. Any contamination of the purified  $\beta$ -casein by other caseins or bovine  $\beta$ -lactoglobulin, derived from the mother's diet through excretion into breastmilk, was extensively investigated by immunochemical studies. The absence of contamination by major bovine milk proteins was confirmed by ELISA inhibition, using specific polyclonal antibodies raised against  $\beta$ -lactoglobulin and each of the different bovine caseins.

We checked for possible recognition of purified human  $\beta$ -casein by specific IgE from patients allergic to bovine proteins. Twenty patients with IgE responses to bovine  $\beta$ -casein ranging from 2.7 to 330 IU/ml were selected. No false-positive response was observed with these sera using HSA-coated plates as a control. No significant binding was detected with either bovine or human  $\beta$ -casein using control sera from non-allergic volunteers.

Specific IgE to human  $\beta$ -casein were determined in the sera of seven patients, e.g. two females 4 and 19 years old and 5 males ranging from 2 to 11 years old. The results are illustrated in Fig. 2 using one representative serum. These sera gave a clearly positive response with coated human  $\beta$ -casein, with parallel titration curves for both bovine- and human-casein-coated plates. The specific IgE concentrations of the seven reactive sera ranged from 2 to 55 IU/ml. In four out of seven of these sera, anti-human  $\beta$ -casein IgE appeared as a minor fraction (5–17%) when compared to anti-bovine  $\beta$ -casein IgE (Table 1). The response was greater for two sera (S130 and S131), and represented 58 and 113% of the anti-bovine  $\beta$ -casein response, respectively. The distribution of the specific

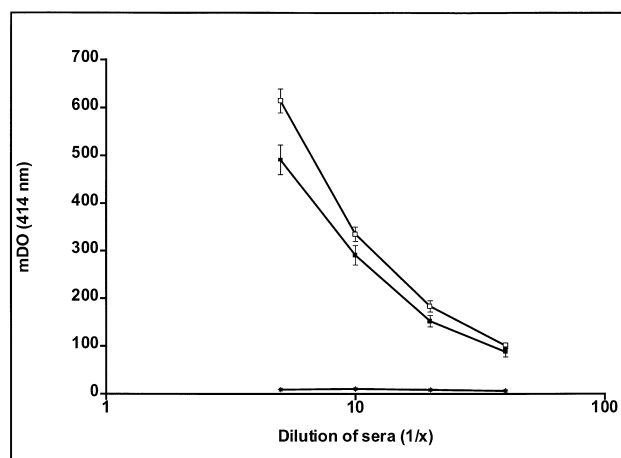


Fig. 2. Direct ELISA of specific IgE from one representative serum using human  $\beta$ -casein ( $\square$ ), bovine  $\beta$ -casein ( $\blacksquare$ ) and human serum albumin ( $*$ ) as immobilized proteins. Each value is mean with standard deviation represented by vertical bars for  $n = 3$ .

IgE response to human vs. bovine  $\beta$ -casein in the tested population of 20 allergic patients to bovine casein appears widely spread as shown in Fig. 3. Using the specific IgE concentrations in the seven positive sera to human  $\beta$ -casein, the Spearman's test provides a correlation coefficient of 0.21 which appears to be not significant.

To further verify the specificity of this IgE-positive response to human  $\beta$ -casein, we performed competitive inhibition ELISA. With bovine  $\beta$ -casein as coated antigen, human  $\beta$ -casein was a poor inhibitor of IgE binding compared to bovine  $\beta$ -casein. High concentrations of human protein were required to achieve inhibition, which always remained partial.

Conversely, using human  $\beta$ -casein-coated plates, bovine protein appeared as efficient as its human counterpart in inhibiting IgE binding. As observed in Fig. 4, the inhibition curves obtained with the two proteins were superimposable. We also used different fragments of bovine  $\beta$ -casein to investigate the recognized part of the protein further. Enzymatic digestion of bovine  $\beta$ -casein yielded three peptides, i.e. f(53–139), f(106–209) and f(1–52), which were purified by HPLC (purity > 95%) and characterized by amino acid analysis. Different peptides appear efficient to inhibit IgE binding to human  $\beta$ -casein. As shown in Fig. 4A, the f(1–52) bovine fragment totally displaced IgE, but was less efficiently recognized than the whole protein. This may reflect partial structural modification induced during enzymatic processing. Another interesting feature appeared, for other sera, in Fig. 4B. Three different bovine peptides partially inhibited IgE binding to coated human  $\beta$ -casein. Fragments f(53–139) and f(106–209) gave curves similar to those obtained with the native caseins for concentrations ranging from 0 to 0.1 nmol/ml. For concentrations of fragments f(53–139) and f(106–209) higher than 0.1 nmol/ml, a plateau of ca. 65% inhibition was reached while increasing amounts of native human and bovine casein gave a

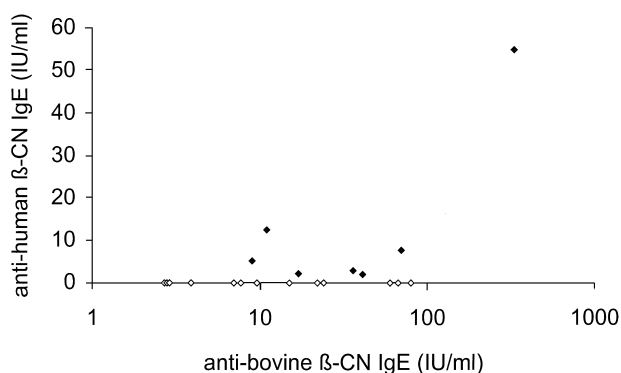


Fig. 3. Distribution of specific anti-human vs. anti-bovine  $\beta$ -casein IgE in the sera of a population of 20 allergic patients to bovine casein.

Table 1

Specific anti-bovine and anti-human  $\beta$ -casein IgE in sera from seven patients allergic to cow's milk

| Serum No. | Specific IgE (I.U./ml) <sup>a</sup> |                   | Ratio of anti-human $\beta$ -CN/anti-bovine $\beta$ -CN IgE (%) |
|-----------|-------------------------------------|-------------------|-----------------------------------------------------------------|
|           | Bovine $\beta$ -CN                  | Human $\beta$ -CN |                                                                 |
| S59       | 330 $\pm$ 35.2                      | 55 $\pm$ 8.9      | 17 $\pm$ 2.5                                                    |
| S81       | 41 $\pm$ 1.5                        | 2 $\pm$ 0.2       | 5 $\pm$ 0.8                                                     |
| S101      | 36 $\pm$ 2.5                        | 2.9 $\pm$ 0.8     | 8 $\pm$ 1.5                                                     |
| S130      | 9 $\pm$ 3.2                         | 5.2 $\pm$ 2.5     | 58 $\pm$ 13                                                     |
| S131      | 11 $\pm$ 1.2                        | 12.5 $\pm$ 1.0    | 113 $\pm$ 3.4                                                   |
| S146      | 17 $\pm$ 3.8                        | 2.2 $\pm$ 0.4     | 13 $\pm$ 2.1                                                    |
| S153      | 70 $\pm$ 3.5                        | 7.7 $\pm$ 1.5     | 11 $\pm$ 1.7                                                    |

<sup>a</sup> Each value is mean  $\pm$  standard deviation for eight measurements.

total inhibition. The very similar inhibition curves obtained with these two peptides suggest that the immunoreactivity could be due to the common part of the sequences, i.e. 106–139. The inhibition observed using increasing concentrations of fragment f(1–52) is weak as shown by the slope of the curve on Fig. 4B and is limited to ca. 35% at concentration as high as 4 nmol/ml. Interestingly, inhibition appeared complete with f(1–52) plus f(106–209), this mixture tending to mimic the result obtained with the total protein.

#### 4. Discussion

We have detected IgE against human  $\beta$ -casein in sera from patients allergic to cow's milk. Seven patients out of a population of 20 allergic patients to bovine  $\beta$ -casein had anti-human  $\beta$ -casein IgE. These serum IgE levels varied greatly among the seven patients and were not correlated to the anti-bovine  $\beta$ -casein IgE levels.

As we detected no contamination of human  $\beta$ -casein by trace amounts of bovine milk proteins, particularly by  $\beta$ -casein, these results can very likely be explained by immunological cross-reaction between the two  $\beta$ -caseins. This tallies with the results of Cantisani et al. (1997) who observed IgE cross-reactivity between milk proteins from cow and human. However, these authors worked on sera obtained from exclusively breast-fed infants and suspected the presence of specific IgE primarily directed against human milk proteins and cross-reacting with the homologous cow's milk protein. In the present study, using sera from patients allergic to cow's milk, IgE binding to human  $\beta$ -casein was very efficiently blocked by its bovine counterpart, whereas the human  $\beta$ -casein only slightly inhibited IgE binding to bovine  $\beta$ -casein. This suggests that only a fraction of the population of the polyclonal IgE antibodies directed against bovine  $\beta$ -casein reacts with human  $\beta$ -casein, possibly with a lower apparent affinity. This fraction is generally small and variable with the patient.

Previous observations using SDS-PAGE experiments (Cantisani et al., 1997) clearly showed cross-reactions for human IgE, and we have demonstrated that native proteins also give good inhibition results, showing that the recognized epitopes could belong to conformational as well as linear sites. Variable results have been derived from studies analyzing the cross-reactivity of human and bovine  $\beta$ -caseins using polyclonal and monoclonal antibodies obtained from different animal models. Simickova et al. (1991) failed to find any reactivity of different polyclonal antisera raised against

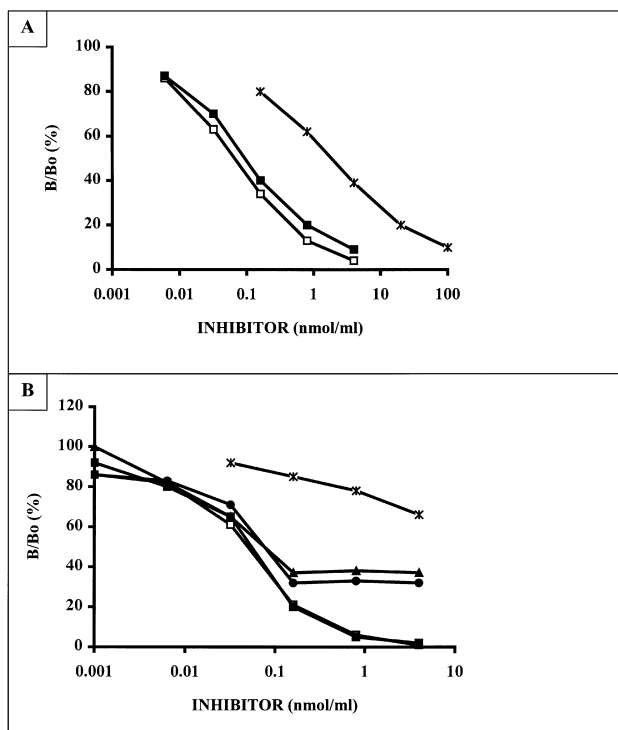


Fig. 4. Indirect ELISA showing inhibition of the binding of specific anti- $\beta$ -casein ( $\beta$ -CN) IgE to immobilized human  $\beta$ -CN by bovine (■) and human (□)  $\beta$ -CNs and derived  $\beta$ -CN peptides (f(53–139): ●; f(106–209): ▲; f(1–52): \*); with serum S59 (A); with serum S153 (B).

human casein with bovine  $\beta$ -casein. Earl and McIlhinney (1985) noted cross-reaction between an anti-human casein monoclonal antibody and bovine  $\beta$ -casein. Otani et al. (1988) described a common epitope located on peptide 110–144 of bovine  $\beta$ -casein using polyclonal antibodies.

The 50% identity between human and bovine  $\beta$ -caseins corresponds to conserved regions widely spread all along the molecules as can be seen in Fig. 5 where the sequences of bovine and human  $\beta$ -caseins are aligned according to Ribadeau-Dumas et al. (1972) and Lönnerdal et al. (1990), respectively. However, two domains appeared more interesting. One contains the major sites of phosphorylation, i.e. fragments 5–14 and 14–23 in human and bovine  $\beta$ -casein, respectively. The other particular conserved region (i.e., fragments 118–132 and 127–141 of bovine and human  $\beta$ -casein, respectively) includes a predicted  $\alpha$ -helical segment, i.e. NLHLP, at positions 123–127 and 132–136 in human and bovine  $\beta$ -casein, respectively.

Interestingly, as shown in Fig. 4 for two sera, IgE binding to human  $\beta$ -casein was inhibited by bovine  $\beta$ -casein fragments containing these sequences. This was particularly striking with serum S153 which was partially inhibited by the sequence f(1–52) containing the phosphorylation site and by the two peptides f(53–139) and f(106–209) comprising the sequence 106–139, which corresponds to the helical segment previously observed. These results tend to demonstrate that common regions shared by the two  $\beta$ -caseins are responsible for the observed cross-reactivity.

It must be emphasized that the ELISA test used is a two-site immunometric assay involving reagents in excess (i.e., immobilized allergen and tracer antibody) and allowing the quantitative determination of specific human IgE without any interference due to specific serum IgGs (Wal et al., 1995). It is well known that

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1      10      20      30      40      50
RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQTDELQDKIH
-*****-TI-----EY*-V--VKH-D---G---H---Y

60      70      80      90      100
PFAQTQSLVYFPFGPIPNS*LPQNIPLTQTTPVVVFFLQPEVMGVSKVKE
-SF-P-P-I---VE---YGF-----L--A-PA--L*-VP---I-E-P-A-D

110     120     130     140     150
AMAPKKHKMPFKYPVEPFTESQSLTLTDVENLHLPPLLQSWMHQPHQP
TVYT-GRV--VL-S-TI--FDP-IPK---L-----PL-Q-VP--

160     170     180     190     200
LPPTVMFPFQSVLSLSQSKVLPVPQKAVPYQRDMPIQAFLLYQEPVLGP
I-Q-LAL---PLW-VP-P----I--QV-----AV-V--L--N--LL-N-

209
VRGFFPIIV
THQIY-VTQPLAPVHNPISV

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Fig. 5. Alignment of sequences of bovine and human  $\beta$ -caseins, according to Ribadeau-Dumas et al. (1972) and Lönnerdal et al. (1990). Symbol (\*) indicates insertions for the best alignment. Symbol (-) indicates homologies. Sequences in bold characters indicate IgE cross-reacting epitopes.

the affinity of IgE for the coated allergen or for the same allergen in solution may be different. Slight structural modifications induced by interactions of the allergen with the hydrophobic solid phase during the coating process may affect the immunoreactivity and/or mask epitope(s) for IgE recognition. In the case of  $\beta$ -casein and  $\beta$ -casein derived peptides, no such interference seemed to occur since a total inhibition of the IgE binding to immobilized  $\beta$ -casein was always observed using increasing concentrations of soluble antigens. Common epitopes between human and bovine  $\beta$ -caseins did not appear to be affected by such possible alterations since cross-reactions have been observed with serum IgE of ca. 30% of allergic patients tested. As expected, the two common domains that are particularly conserved in human and bovine  $\beta$ -casein molecules have been shown to be responsible for such cross-reactivity.

Confirmation of the actual involvement of these conserved sequences and particularly of the major site of phosphorylation is presently under investigation using shorter native or modified peptides (Bernard et al., 2000).

To our knowledge, no case of anaphylactic shock triggered by human casein in allergic patients to cow's milk proteins has been reported. The response levels and the likely low IgE affinity to human casein observed in our study only reflect immunological cross-reactivity and could not be related to an eventual induction of specific anti-human casein IgE synthesis.

As already stated by Maynard et al. (1999), immunological cross-reactions with IgE binding are often observed between more or less related proteins. They may involve low affinity specific IgE and cannot always be related to clinical symptoms and thus cannot lead to relevant conclusions on the allergenic risk in groups at risk such as nursing women and their infants. The clinical or pathological significance of such in vitro immunological cross-reactions should be confirmed.

However, available antibodies exhibiting a restricted specificity make it possible to locate the particular antigenic/allergenic site on the molecule. These properties of IgE cross-reactivity are therefore of great interest for a study of the relationship between structure, IgE binding capacity and allergenicity in  $\beta$ -caseins.

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