

IgE Cross-reactivity with Caseins from Different Species in Humans Allergic to Cow's Milk

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Fifty-eight sera from humans allergic to cow's milk proteins were analysed for the specificity of their IgE response to the whole casein fraction of milk from different ruminant and non-ruminant species (e.g. cow, sheep, goat, rabbit and rat). IgE-specific responses were determined by an enzyme allergosorbent test using the purified casein fractions as immobilized antigen and an anti-human IgE monoclonal antibody labelled with acetylcholinesterase. Co-and/or cross-sensitizations to caseins of the different ruminant species occurred extensively, though IgE responses to ovine and caprine casein appeared to be lower than that obtained with bovine casein. Cross-reactivity is suggested by the significant reactivity of rat and rabbit casein toward human IgE. In terms of specificity and intensity, the IgE response to caseins demonstrates a great variability. Structural homologies in caseins of such different species, that can share common epitopes for the IgE of some patients, suggest that prevention of cow's milk allergy cannot be achieved by using milk from other species as substitutes.

Keywords: Allergy, specific IgE antibodies, cow's milk, goat's milk, ewe's milk, casein, EIA

INTRODUCTION

Allergy to bovine milk proteins appears to be a common pathology in infancy. Apart from the dramatic and extreme symptoms eventually encountered (anaphylactic shock), one of the major problems remains the adaptation of the diet of the sensitized children. Different ways have been recommended to avoid the allergic reactions. For instance, the use of milk from species other than cow has been one of these suggested alternatives. It has been shown that the use of ass's milk could be a substitute for cow's milk (Lacono *et al.*, 1992). The use of milk from species more related to the cow, like goat, has also been discussed (Coveney &

Darnton-Hill, 1985; Park, 1994). However comparison of milk from the different ruminant species reveals great homologies in both protein composition and structure, particularly for the four caseins (α_{s1} -, α_{s2} -, β -, κ -caseins) that are found in cow's, sheep's and goat's milk, representing about 80% of milk proteins. Moreover important sequence conservation (between 80 and 90%) is observed for each of these caseins (Soulier *et al.*, 1975; Richardson & Mercier, 1979; Boissard & Petrisant, 1985, Brignon *et al.*, 1989; Provot *et al.*, 1989; Roberts *et al.*, 1992; Persuy *et al.*, 1992; Bouniol, 1993). This could lead patients allergic to cow's milk to express adverse reactions to milk from other ruminant species. The aim of this study was to investigate IgE specificity against caprine whole casein (CWCN) and ovine whole casein (OWCN) in sera from patients presenting allergic symptoms to cow's milk casein, in order to check possible IgE cross-reactions between these related species. Complementary information was also obtained by testing the same sera with whole casein coming from more divergent species such as rodents (rat and rabbit).

MATERIAL AND METHODS

Reagents

Unless otherwise stated, all reagents were of analytical grade and obtained from Sigma (St Louis, USA).

Human serum albumin (HSA) was obtained from Centre National de Transfusion Sanguine (Les Ulis, France); free fatty acids were removed on active charcoal at pH 3 (Chen, 1967). After dialysis against water, purified albumin was freeze-dried.

Human sera (from 92 patients) were obtained from the sera banks of hospital paediatricians. They corresponded to children (median age 11 months) who presented clinical symptoms of cow's milk allergy and positive Rast® tests to milk. These sera were tested for specific anti-whole bovine casein IgE determination by using the original enzyme immunoassay (EIA) developed in our laboratory (Wal *et al.*, 1995). Approximately 60% (58 sera) of the tested sera indeed had an IgE response to bovine whole casein (BWCN) and were selected for the study. Out of the 58 sera, 45 had also IgE response to β -lactoglobulin, and 42, 28 and 25 also react to α -lactalbumin, bovine serum albumin (BSA) and lactoferrin, respectively. Eight control sera were obtained from non-allergic human volunteers.

Buffers and reagents used for EIA were prepared as follows: coating buffer: 0.025 M-EDTA (disodium salt), pH 9.3 (Campbell *et al.*, 1987); 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; washing buffer: 0.01 M-potassium phosphate buffer, pH 7.4, containing 0.05% Tween-20; Ellman's medium : 7.5×10^{-4} M-acetylthiocholine and 5×10^{-4} M-dithio-bis-nitrobenzoate in 0.1 M-potassium phosphate buffer, pH 7.4 (Ellman *et al.*, 1961).

Solid-phase enzyme immunoassay was performed on 96-well microtitre plates (Nunc, Denmark) using automatic Titertek microtitration equipment (washer, dispenser and reader) from Labsystem (Helsinki, Finland).

Preparation of the Whole Casein Fraction

Whole casein was isolated by isoelectric precipitation at pH 4.6 of skimmed raw milk from a single cow homozygous at the four casein loci, e.g. α_{s1} -casein variant B; β -casein variant A1; α_{s2} -casein variant A, and κ -casein variant A. The precipitate was recovered by centrifugation, washed twice with water at pH 4.6, redissolved at pH 7 and precipitated again at pH 4.6. The precipitate was dialysed at 4°C against water adjusted at pH 7.4, lyophilized and stored at -20°C. Whole casein from goat and sheep was obtained by the same procedure but was isolated from pooled milk samples for sheep and from a milk sample from one goat heterozygous for α_{s1} -casein (Variant B/F). Potential contamination of whole casein by the whey proteins and particularly by β -lactoglobulin was checked by RP-HPLC using a Waters 600E system, a Waters 486 detector (Milford, USA) and a 300 Å C4-Vydac column

(250×4.6 mm; Shandon, France). The samples were dissolved (1 mg ml⁻¹) in 8 M-urea containing 0.1% dithiothreitol. Each sample was five-fold diluted in solvent A and was injected (200 µl corresponding to 40 µg). Elution was performed using a 40-min linear gradient from 25 to 45% of solvent B (B: acetonitrile/trifluoro acetic acid (TFA) 0.04%) in solvent A (A: H₂O/TFA 0.1%) at a flow rate of 1 ml min⁻¹ and was monitored at 220 nm.

Rabbit and rat whole casein were a kind gift from Dr Houdebine (INRA, Jouy en Josas, France).

Preparation of Enzyme Conjugate

Acetylcholinesterase (AChE, EC 3.1.1.7) was purified from the electric organs of electric eel *Electrophorus electricus* by affinity chromatography according to Massoulié & Bon (1976). The tetrameric form of AChE (G₄ form) was used for labelling monoclonal antibodies (MoAbs) (Pradelles *et al.*, 1989; Grassi *et al.*, 1988).

Briefly, anti-human IgE MoABs (MoAb BS17) were treated with pepsin in acidic medium to obtain F(ab')₂ fragments. Reduction of these fragments by β-mercaptoethylamine led to the production of Fab' fragments which were subsequently covalently coupled to AChE pretreated with *N*-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) as previously described (Grassi *et al.*, 1989).

Enzyme Immunoassays

Coating of the microtitre plates with each of the purified caseins was performed at a concentration of 5 µg ml⁻¹ in a 25 mM-EDTA solution, pH 9.3 (Campbell *et al.*, 1987). After 24 h incubation, plates were washed and saturated with EIA buffer.

EIA experiments were carried out to screen the IgE response of the allergic patients sera to the different coated caseins. These assays corresponded to direct tests where serum samples were diluted and incubated (50 µl/well) on coated immunoplates for 24 h at 20°C. After washing, IgE bound onto immunoplates was revealed by addition of 50 µl of BS17-AChE conjugate (3 Ellman units ml⁻¹). After a further incubation for 18 h at 20°C, plates were washed and 200 µl of Ellman's reagent were added for revelation of AChE activity. The quantification of specific IgE was performed by comparison with an immunometric assay involving the same enzyme conjugate (BS17-AChE) and another coated anti-human IgE MoAb (LE27) ensuring the capture of the IgE. Standard curves were obtained using known standards of human IgE allowing quantification of specific IgE as previously described (Grassi *et al.*, 1986; Wal *et al.*, 1995). Quantification was expressed in IU ml⁻¹ (International Unit per ml). One unit corresponds to 2.4 ng of IgE. In such conditions, the detection limit of anti-casein specific IgE was lower than 0.1 IU ml⁻¹ with good reproducibility and precision. No interference occurs with specific anti-casein IgG (Wal *et al.*, 1995).

Results obtained with the whole casein preparation from ruminant species were analysed using statistical methods. Due to the high variability and the non-Gaussian distribution of the specific IgE levels, comparison of the data were performed with non-parametric tests (Friedman test and Spearman rank-correlation test).

RESULTS

Purified Allergens

Figure 1 shows the RP-HPLC chromatograms of the whole purified casein of the different ruminant species. Whole casein appears as a multiplicity of peaks corresponding to the elution of the different components (κ-; α₂-; α_{s1}-; β-casein, with respect to the elution time). In order to check contamination by whey proteins, each of these whole casein samples has been spiked with the corresponding β-lactoglobulin. In any case, β-lactoglobulin eluted later than the components of the whole casein and no peak could be detected in the initial chromatograms at the corresponding time.

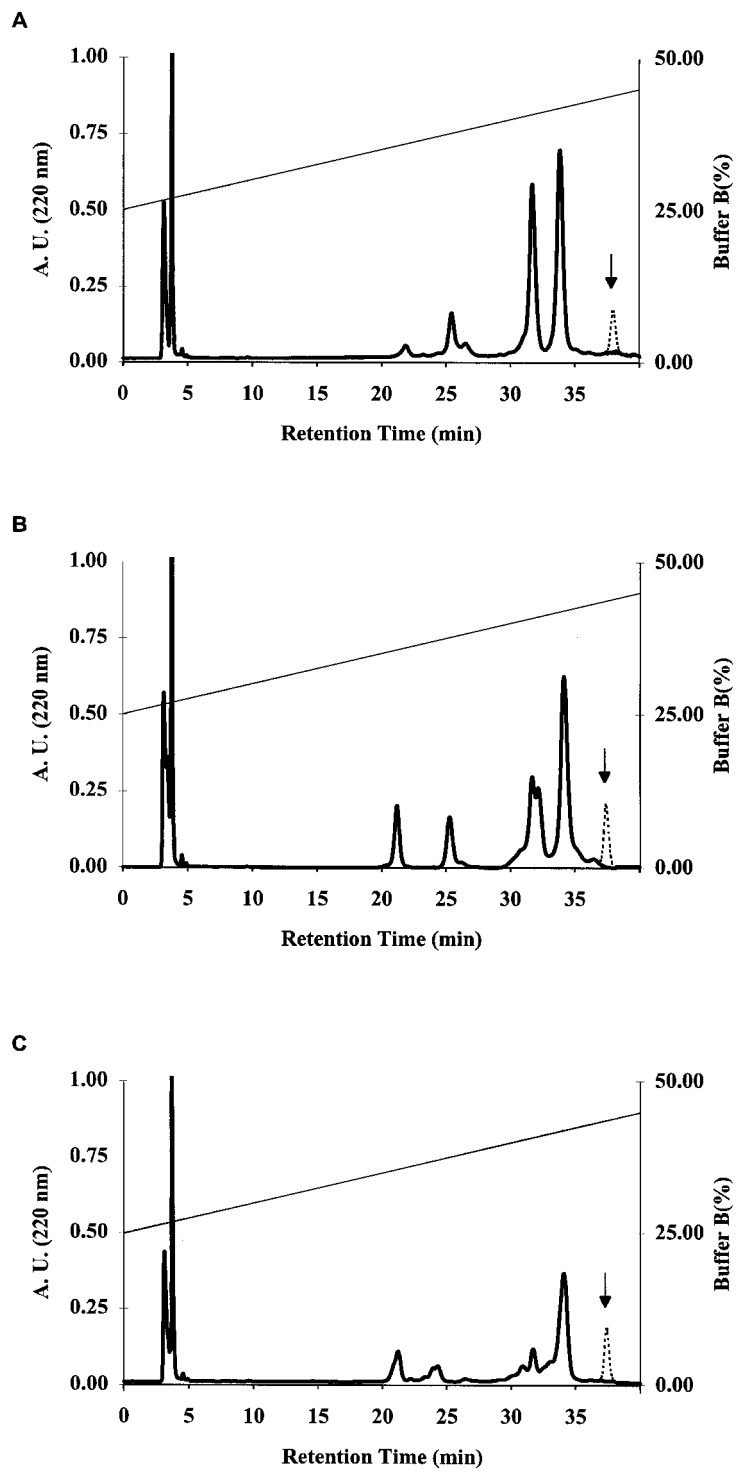


FIG. 1. RP-HPLC chromatograms of purified whole caseins: bovine (a); ovine (b); caprine (c). The arrows show the elution of standard β -Lg co-injected with the whole casein samples. No detectable peak could be observed in the initial chromatograms at this retention time.

TABLE 1. Distribution (number of sera) of IgE response to ovine and caprine whole casein in 58 sera from patients allergic to bovine casein (expressed as percent of IgE response to cow's milk whole casein fraction)

	0-25%	25-50%	50-75%	75-125%	< 125%
Ovine casein	8	9	22	16	3
Caprine casein	15	10	19	10	4

IgE Specificity Towards Casein from Ruminant Species

Fifty-eight sera having an IgE response against BWCN were selected from patients presenting allergic symptoms to cow's milk. A large range of anti-BWCN IgE concentrations comprising between 0.3 to 1000 IU ml⁻¹ was observed in these sera. In 22 sera the specific anti-BWCN IgE concentration ranged between 0.3 and 5 IU ml⁻¹ while in 16 others, it was between 5 to 20 IU ml⁻¹. A higher IgE response against BWCN was observed in 14 sera with specific IgE concentrations ranging between 20 and 100 IU ml⁻¹. The six last sera had very high specific IgE levels to BWCN (>100 IU ml⁻¹).

Among the 58 sera, a very high frequency (>90%) of IgE immunoreactivity was observed against whole casein obtained either from sheep (57 positive sera out of 58) or goat (54 positive sera out of 58). The IgE response exhibited a great variability in intensity and specificity.

Comparison of IgE titres obtained with the caseins from the different ruminant species demonstrated that a large majority of the sera displayed lower IgE immunoreactivity with CWCN and OWCN than with BWCN (Table 1). IgE titres appeared almost two-fold higher for BWCN than for CWCN and OWCN in 25 and 17 sera, respectively. 16 sera presented IgE responses to OWCN similar to those obtained with BWCN (ranging from 75 to 125% of IgE response to cow's milk casein). These figures were reduced (10 sera *versus* 16) when CWCN was concerned instead of OWCN. In 3 and 4 sera, the IgE response to OWCN and to CWCN, respectively, was much higher (>125%) than the IgE response to BWCN.

Mean and median IgE responses to ruminant caseins were calculated on the total population of the 58 sera (Table 2). Mean anti-BWCN IgE appeared two-fold higher than that of anti-CWCN or anti-OWCN IgE. Mean IgE levels directed against CWCN proved to be the weakest response. When medians are considered, IgE levels are lower than mean values. This can be attributed to the great variability of IgE response and the existence of a minority of sera exhibiting very high specific IgE titres against casein. Moreover, differences between the IgE responses were similar when comparing median or mean values.

According to the non-parametric test of Friedman, the IgE response to BWCN was confirmed to be significantly the highest one ($P < 0.01$). In the same test, the IgE response against CWCN also proved to be significantly lower than the anti-OWCN one ($P < 0.05$).

TABLE 2. Statistical analysis of human IgE responses to whole casein from milk of different ruminant species in 58 sera from patients allergic to bovine casein: Mean and median specific IgE responses

IgE (IU ml ⁻¹)	Mean	Medium
Bovine casein	43.2	8.1
Ovine casein	26.8	5.2
Caprine casein	21.4	4.5

Moreover, the Spearmann rank correlation test revealed that IgE responses to BWCN and OWCN or CWCN are highly correlated ($r=0.84$ and 0.79 , respectively). The correlations obtained are highly significant ($p<0.01$) and the best is obtained for IgE responses against OWCN and CWCN ($r=0.96$).

However, analysis of the individual response demonstrates a great variability, some particular specificities being noticed. As shown in Table 3, some specific sera exhibit a marked predominant IgE response against BWCN (S16, S87 and S89). The example of serum S89 appears particularly demonstrative since no IgE response was detected against CWCN and OWCN. Conversely, in some cases, the intensity of the IgE response for the CWCN or OWCN proved to be equal to the IgE response observed with the BWCN (S3 and S83 for instance). Moreover in one case (serum S25), the IgE detection reached a four-fold more intense response for CWCN or OWCN compared with the bovine one.

Interestingly, a large majority of sera (43 sera) exhibited a very similar response to the CWCN and OWCN. This is probably accounted for by some conserved epitopes located along the casein molecules in the different ruminant species. Nevertheless in a minority of sera, an important discrepancy can be observed between anti-OWCN and anti-CWCN IgE responses. This difference ranges between two and four-fold, OWCN being better recognized for 12 sera (e.g. S32 or S39) while CWCN presents a higher IgE response for three sera (S2 for example).

IgE Specificity Towards Casein from Non-ruminant Species

To better analyse the immunoreactivity observed with ruminant species, the same enzyme immunoassay was performed by using the whole casein fraction of rat and rabbit milk as coated antigen. An important decrease in IgE immunoreactivity was observed but numerous sera still provided positive response (33 sera out of 58 and 34 out of 58 for rabbit and rat casein, respectively). In most sera, anti-rat and anti-rabbit casein IgE responses appeared very low ($<10\%$ of intensity) when compared with the anti-bovine casein one. Only 12 and 20 sera presented an anti-rabbit and anti-rat casein IgE response, respectively, corresponding to at least 10 percent of the IgE response to bovine casein. Surprisingly, as shown in Table 4, some sera presented IgE levels to rodent casein just above IgE levels to BWCN suggesting

TABLE 3. Analysis of the specific IgE binding to ovine and caprine immobilized casein (expressed as the percentage of binding to the whole bovine immobilized casein) in some sera. Separation was made between sera presenting similar IgE response to OWCN and CWCN and sera (S32, S39 and S2) providing a more specific IgE response either to OWCN or to CWCN

Serum no.	IgE binding to immobilized whole casein (%)		
	Bovine	Ovine	Caprine
S89	100	0	0
S16	100	15	14
S87	100	16	17
S3	100	81	83
S83	100	95	98
S25	100	430	350
S32	100	51	11
S39	100	43	20
S2	100	26	85

TABLE 4. Analysis of the specific IgE binding to rabbit and rat immobilized casein (expressed as the percentage of binding to the whole bovine casein) in some characteristic sera

Serum no.	IgE binding to immobilized whole casein (%)		
	Bovine	Rabbit	Rat
S3	100	35	37
S6	100	26	26
S60	100	22	42
S67	100	45	76

very particular specificities. Serum S67 with anti-rat and anti-rabbit casein IgE corresponding to 76 and 45% of the anti-BWCN casein appeared particularly demonstrative.

Fifty per cent of the sera (29 out of 58) exhibited IgE against each of the different casein fractions tested whatever their origin, ruminant or rodent species.

DISCUSSION

Cow's milk is a frequent food allergen. Hypersensitivity with IgE mediation occurs in a large part of cow's milk allergy (Ventura *et al.*, 1988; Host, 1994). Using substitutes for cow's milk such as milk from other ruminants or non-ruminant species has been proposed as a way to circumvent this allergic phenomenon. However, IgE cross-reaction between milk proteins from cow and others species might preclude this prevention.

Numerous studies have focused on the allergenicity of cow's milk casein (Wahn & Gangster, 1982; Otani *et al.*, 1989; Stöger & Wüthrich, 1993; Docena *et al.*, 1996). We have also evidenced the presence of specific IgE to BWCN and to its different components for more of 50% of sera in a population of 92 allergic patients to bovine milk (Bernard *et al.*, 1998). In the present study, we have studied the allergenicity of whole casein from different species. IgE responses observed were specific for whole casein and could not be accounted by whey proteins since RP-HPLC experiments demonstrated the absence of detectable contamination of the different prepared whole caseins by β -lactoglobulin, the main component of whey proteins.

More than 90% of the 58 studied sera exhibiting IgE against BWCN presented IgE to whole casein from the two other ruminant species (caprine and ovine). In a similar way, Baldo (1984) had detected IgE to CWCN and OWCN in allergic patients to bovine milk. Two hypothesis were then suggested. The first one was based on the ingestion of milk from different ruminants, leading to co-sensitization of the patient to milk and particularly to whole casein fraction from different ruminants. In a second possibility, IgE responses to OWCN or CWCN could be explained by the cross-reactivity between caseins from these ruminants. Risk of cross-reactivity was suggested by Goldman and Heiner (1977) and confirmed by Gjesing *et al.* (1986). These last authors showed by radioimmuno-electrophoresis experiments, that the IgE of children allergic to bovine milk recognized caprine milk proteins although these children had never ingested such milk.

Indeed whole casein represents 80% of proteins in the milk of these three ruminants. All the four caseins are present in the ruminant species with varying proportions especially for caprine α_{s1} -casein which is present in milk with quantitative and qualitative variations (Grosclaude *et al.*, 1987; Brignon *et al.*, 1990; Mercier *et al.*, 1991). Moreover the structure of each casein is well conserved for the different ruminants, presenting more than 80% homology. These similarities corroborate the risk of cross-reactivity. This hypothesis has

been confirmed for a fraction of the casein by Spuergerin *et al.* (1997). These authors demonstrated: (1) a higher IgE response to bovine α_{s1} -casein than to the goat and sheep counterparts; (2) a cross-reaction between these caseins. In our study, the levels of IgE response were also statistically higher to the BWCN than to the other counterparts. We may suppose that part of the polyclonal anti-BWCN IgE population may recognize whole casein from the other ruminants. Moreover this cross-reactivity can be analysed by using statistical correlation of ranks estimated between IgE levels of OWCN, CWCN and BWCN, which appeared strong and significant. The strongest one was obtained with IgE levels directed against OWCN and CWCN which display the greatest homology in terms of structural features. Correlation values then appeared to be in good agreement with sequence homologies found with caseins from the ruminant species (Table 5). Conversely, some specific cases could be remarked upon, with IgE responses more than two-fold higher for caprine and ovine casein than for cow. In this case, very particular specificities could be suggested due to better conformation and presentation of the epitopes in the whole casein. Higher proportions of one casein, like β -casein, in the whole casein or point substitutions of amino acid residues may then contribute to a better recognition by specific IgE. However, we should note that all these substitutions are widely spread along the molecule and no particular region can thus be suspected for immunoreactivity as already evidenced by the high variability of IgE response among the population. On the other hand, different IgE levels between OWCN and CWCN could be explained by the slight differences of casein composition and the occurrence of quantitative and qualitative expression of α_{s1} -casein in goat (Grosclaude *et al.*, 1987; Brignon *et al.*, 1989, 1990). Actually, the CWCN we have purified for this study presented intermediate levels of α_{s1} -casein with two iso forms (including an α_{s1} -casein in which the sequence 59–95 is deleted). These two facts could partly explain the decrease of the IgE recognition of whole caprine casein *versus* bovine and ovine ones.

Moreover, the existence of IgE response to rabbit or rat casein confirmed the occurrence of cross-reactivity between caseins from different species. Composition and structure of caseins in rat and rabbit milk appear more divergent but remain important (Blackburn *et al.*, 1982; Hobbs & Rosen, 1982; Nakhasi *et al.*, 1984; Schaerer *et al.*, 1988; Devinoy *et al.*, 1988; Bosze *et al.*, 1993; Dawson *et al.*, 1993). Particularly sequence homologies ranging from 30 to 50% are observed for the rat or rabbit caseins by comparison to the bovine caseins (Table 5). Indeed, in spite of these structural divergences of casein, more than 30% of sera positive to bovine caseins present IgE immunorecognition of the caseins from these two species. This fact suggested that cross-reactivity occurs between rat, rabbit and bovine caseins which obviously could be stronger between caseins from more closely related species (ruminants).

In conclusion, we stress the heterogeneity of IgE response to casein among a large population of patients allergic to milk. Some of them had similar IgE responses to whole caseins from the three ruminant species while others are more specific to bovine whole casein and even sometimes to ovine or caprine casein. A relationship appears between the IgE

TABLE 5. Sequence homology between caseins from different species (comparison expressed as percent of homology). The Symbol (–) means that no significant homology is observed between bovine α_{s2} -casein and a component of whole rat casein (γ -casein) (Hobbs & Rosen, 1982).

Protein	Sheep/cow	Goat/cow	Sheep/goat	Rat/cow	Rabbit/cow
α_{s1} -casein	89	87	97	31	45
α_{s2} -casein	89	88	98	–	< 50
β -casein	90	90	99	38	34
κ -casein	84	85	95	49	< 50

response and the degree of homology in the casein structure. However, depending upon the sera, the region responsible for allergenicity does not seem to be only located either in the portions of the molecule that remained stable, or in the regions that have diverged through evolution. Allergenic structures appear to be widely spread within the casein molecules.

With respect to the high frequency of IgE against casein from different ruminants, the use of these constituents as substitutes for cow's milk appears very likely not to be helpful in cases of allergy. Confirmation of the clinical or pathological significance of such *in vitro* immunological cross-reactions by *in vivo* studies such as skin tests and provocation tests have not been discussed in this work. However, our results agree with other previous observations (Dean *et al.*, 1993). Moreover, the risk of cross-reactivity has also been confirmed *in vivo* by Juntunen & Ali-Yrkkö (1983) who demonstrated by Rast® tests, skin prick tests and provocation tests that 22 patients were allergic to both cow's and goat's milk out of 30 patients allergic to cow's milk. On the other hand, specific allergenicity of milk products from sheep and goat was also described by Wüthrich & Johansson (1995). In their study, eight patients developed allergic reactions after eating cheese from either cow's, goat's or sheep's milk, while two patients allergic to sheep and goat's milk products appeared to be tolerant to cheese from cow's milk. This suggests a very particular specificity of IgE responses as we observed with some sera in this paper. Another similar clinical case study was reported by Calvani & Alessandri (1998). Cow's, sheep's and goat's milk can thus exhibit an allergenic potential due to cross-reactivity resulting from high sequence homologies and/or to species specific structural features of their casein molecules.

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