Abstract. Background: Allergic reactions to cow’s milk are common in small children. One of the main protein allergens found in cow’s milk is β-lactoglobulin (β-Lg). Reindeer and bovine milk both contain related β-Lg proteins, but the allergenicity of reindeer β-Lg has not previously been studied. The purpose of this study was to analyze the immunological cross-reactivity of IgE antibodies from children with cow’s milk allergy to reindeer and bovine β-Lg.

Methods: Sera from 17 children and a serum pool of 4 patients with elevated cow’s milk-specific IgE were investigated. β-Lg from bovine and reindeer milk was isolated in native form and an enzyme-linked immunosorbent inhibition assay was developed. Bovine β-Lg was used as a capturing antigen and the inhibiting effects of reindeer and bovine β-Lg on the IgE binding were measured.

Results: Cross-reactivity patterns of bovine milk β-Lg specific IgE to reindeer β-Lg varied among patients. In general, reindeer β-Lg showed significantly lower inhibition (mean 43%) of IgE binding to the capturing antigen than did bovine β-Lg (mean 89%). In some patients, even high concentrations of reindeer β-Lg only partly eliminated the IgE binding to bovine β-Lg.

Conclusions: The partial cross-reactivity of human anti-bovine IgE with reindeer β-Lg suggests that it lacks important bovine epitopes and those that are recognized are only weakly bound.

Key words: Cow’s milk allergy. Reindeer milk. β-lactoglobulin. ELISA inhibition.
Introduction

Allergy to cow’s milk affects approximately 2.5% of children during the first 3 years of life [1], and several cow’s milk substitutes have been investigated for feeding babies with cow’s milk allergy. Soy-based [2] and hydrolyzed cow’s milk-based [3] formulas have been studied extensively, but it has been shown that these products are also potentially allergenic in children with cow’s milk allergy. Goat’s milk also shows some cross-allergenicity [4] whereas camel’s milk proteins have not been shown to be recognized by IgE in sera from cow’s milk allergic patients [5]. Ass’s and mare’s milk might be good substitutes for cow’s milk, since ass’s milk is well tolerated by cow’s milk allergic infants [6] and only a low percentage of IgE from children with cow’s milk allergy recognized mare’s milk proteins [7]. The allergenicity of reindeer’s milk in patients with cow’s milk allergy has not been previously studied. To gain detailed information of the allergenic properties of reindeer’s milk it is essential to study the allergenic potential of the main allergens. The molecular mimicry between mammalian allergens. The molecular mimicry between ß-lactoglobulin belongs to the lipocalin protein family, to several cow’s milk proteins [10]. However, the main milk proteins by weight, ß-Lg and caseins, are usually the major cause of cow’s milk allergies. These proteins pose a major allergenic risk to young children, considering the frequency of exposure [11].

The allergenic properties of cow’s milk ß-Lg have been intensively studied, and several immunoreactive epitopes have been identified [12-15]. In addition, the IgE-mediated responses towards bovine ß-Lg have been shown to be heterogenic among cow’s milk allergy patients [14]. ß-lactoglobulin belongs to the lipocalin protein family, the members of which share a similar 3-dimensional structure [16]. Lipocalins are the most important group of mammalian allergens. The molecular mimicry between endogenous lipocalins and exogenous lipocalin allergens has been suggested as an explanation for the allergenicity of lipocalins [17]. However, more studies are needed to fully understand their allergenic properties.

Reindeer’s milk ß-Lg has been isolated [18] and some of its chemical properties characterized. Its amino acid composition is very similar to that of bovine ß-Lg [18, 19]. Both proteins contain 162 amino acids, only 9 of which are different. In this study, we isolated reindeer and bovine ß-Lgs in their native form and used the proteins in enzyme-linked immunosorbent assay (ELISA) inhibition tests. The in vitro cross-reactivity of IgE from cow’s milk allergic patients’ sera with immobilized bovine ß-Lg and soluble reindeer ß-Lg was measured.

Materials and Methods

Serum samples from 17 Finnish children (median age, 3.8 years; range, 0.5-11.2 years; 9 male and 8 female) with a clinical diagnosis of cow’s milk allergy were collected from Oulu University Hospital (Oulu, Finland), and 1 serum pool sample of 4 Swedish cow’s milk allergic patients (median age, 28.5 years; range, 19-44 years; 1 male and 3 female) was received from Sahlgrenska University Hospital (Gothenburgh, Sweden). Six serum samples collected from Oulu University Hospital from Finnish children (median age, 3.8 years; range, 0.3-23 years; 1 male and 5 female) without clinical cow’s milk allergy were used as negative controls. The levels of cow’s milk-specific IgE of all patient samples were analyzed by the Department of Pediatrics, Oulu University Hospital by fluorescent-enzyme immunoassay with the CAP System (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) according to the manufacturer’s recommendations. Values of allergen-specific IgE below 0.35 kU/L were considered negative and values above 0.35 kU/L were considered positive.

Reindeer’s milk (Rangifer tarandus) was obtained from the Reindeer Research Station (Mauri Nieminen PhD, Kaamanen, Finland). The milk from 6 reindeers was pooled and stored at –20°C. Cow’s milk, obtained from a local farmer, was used for ß-Lg isolation immediately after milking.

Isolation of ß-Lg

Native ß-Lg was isolated from pooled reindeer’s milk by a method [18] slightly modified of the method of de IgE fijada al antígeno de captura que aquel registrado en bLG bovina (siendo del 89%). En algunos casos, incluso los niveles más altos de bLG de reno tan sólo eliminaron de manera parcial la IgE unida a bLG. Conclusiones: La reactividad cruzada parcial de IgE humana antibovina y de bLG de reno sugiere que existe una falta relevante de epitopos bovinos y que aquellos detectados, están débilmente ligados.

Palabras clave: Alergia a la leche de vaca. Leche de reno. ß-Lactoglobulina. ELISA por inhibición.
Jongh et al. [20] for bovine milk β-Lg. Caseins from reindeer’s milk were precipitated at pH 3.2, and ion exchange was carried out with 0.01 M Tris-HCl at pH 6.2. Bovine milk β-Lg was isolated according to the original method described by de Jongh et al. By using these methods we avoided most denaturing conditions, such as low pH, high ionic strengths and high temperatures. Both bovine and reindeer β-Lg were isolated in an analytical scale. The purity and molecular mass of the isolated β-Lgs were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the β-Lgs were identified by immunoblotting with polyclonal antisera to bovine β-Lg [21]. The isoelectric points of the isolated reindeer and bovine milk β-Lgs were investigated with isoelectric focusing (IEF) as previously described [21].

### Protein Concentration Determination

Protein concentrations of the isolated β-Lgs were determined by 2 methods. In the first method, 2 mL of isolated bovine β-Lg diluted in 20 mM phosphate-buffer saline, pH 6.7 (Christ BETA 2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), was lyophilized, weighed and then dissolved in distilled water to make a series of reference solutions (0.015–2 mg/mL) in order to draw a β-Lg standard curve. In the second method isolated bovine and reindeer β-Lgs were diluted in distilled water (1:10-1:160), and the absorbencies were measured at 215 nm and 225 nm (UV-1601, Shimadzu Corporation, Kyoto, Japan). Distilled water was used as a blank. Protein concentrations were then estimated using the method of Waddell [22].

### Characterization of β-Lg

SDS-PAGE of isolated proteins was performed on a 15% polyacrylamide gel in reducing conditions at pH 8.3 [21]. Gels were run on a Bio-Rad Mini Protean II (Bio-Rad, Hercules, California, USA) at 100 V (stacking gel), then 200 V (running gel). β-Lg samples were dissolved in the sample buffer (100 μL for 4 min) under reducing conditions (100 mM DTT) and were loaded into wells (1.0-2.5 μg/well). The Fermentas (MBI Fermentas, Vilnius, Lithuania) protein molecular mass marker was used. The gel was stained with Coomassie Brilliant Blue R-250 (Serva, Feinbiochemica, Heidelberg, Germany).

Electrofocusing was carried out in pH gradient gels (PhastGel IEF 4-6.5, Amersham Biosciences, Sweden) using a Phast apparatus (Amersham Pharmacia Biotech, Little Chalfont, England) [21]. Commercial bovine β-Lg A/B (Sigma Chemical Co, St Louis, Missouri, USA) was used as a reference. The IEF runs were carried out according to the manufacturer’s instructions. The focused proteins were precipitated with 10% trichloroacetic acid and stained with Coomassie Brilliant Blue R-250 in a 0.1% (wt/vol) copper sulphate, 10% acetic acid, 30% methanol solution.

The β-Lgs run by SDS-PAGE were also electroblotted onto nitrocellulose transfer membrane (Trans-Blot, Bio-Rad) as described in a previous paper [21]. The nitrocellulose membrane was blocked with tris-buffered saline (TBS) with 1% Tween 20. To detect the electroblotted β-Lgs, polyclonal rabbit antisera to bovine β-Lg A/B (1:3000) in TBS with 0.05% Tween 20 and goat anti-rabbit IgG horseradish conjugate (GAR-HRP, Bio-Rad) (1:3000) in TBS with 0.05% Tween 20 were used. Staining of the blot was performed with a horseradish peroxidase (HRP) conjugate substrate kit (Bio-Rad) according to the manufacturer’s instructions, followed by scanning of the blotted membrane with an Epson scanner (Epson Perfection 1260 Photo, Seiko Epson Corp, Nagano, Japan).

### ELISA Inhibition Tests

Inhibition experiments were performed using a newly developed ELISA-inhibition method [23]. Microtiter wells (Nunc MaxiSorp, Nalge Nunc Int, Roskilde, Denmark) were coated with bovine β-Lg using 100 μL of a 10 μg/mL solution in PBS by overnight incubation at room temperature. All cow’s milk allergy and control sera were diluted 1:2 in PBS. Inhibitor solutions of bovine and reindeer β-Lgs were each diluted to 200 μg/mL in PBS. Then, 55 μL of β-Lg solution was mixed with 55 μL of the serum sample and was left for 1 hour at room temperature, the final inhibitor concentration being 100 μg/mL. Later in the study, when sufficient volumes of patient sera were available, several inhibitor concentrations (0.1-100 μg/mL) were used to determine the inhibition curves of IgE binding to coated bovine β-Lg. Respective sera mixed with PBS in the absence of the inhibitors were used as controls for the maximal IgE binding to coated bovine β-Lg. All samples were made in duplicate.

The bovine β-Lg coated plates were washed 3 times with PBS with 0.05% Tween 20, and 50 μL of the serum-inhibitor mixture was added to the wells. The plates were incubated for 1 hour at room temperature and the unbound IgE was removed by 3 washes with PBS with 0.05% Tween. Monoclonal mouse anti-human IgE (Clone GE-1, Product No I6510, Sigma) diluted (1:10000) in PBS with 0.05% Tween and then Fc specific biotin-conjugated goat anti-mouse IgG (Product No. B9904, Sigma) diluted (1:50000) in PBS with 0.05% Tween was added to the wells. Each of the antibodies (50 μL/well) was incubated at room temperature for 1 hour, followed by 3 washes with PBS with 0.05% Tween. To visualize the bound IgE,
horseradish peroxidase-conjugated streptavidin (Strept ABCComplex/HRP, K0377, DakoCytomation, Glostrup, Denmark), diluted 1:10000 in PBS with 0.05% Tween, was added (100 µL/well), and the samples were then incubated for 30 minutes in the dark. After a washing step, 100 µL of the substrate for HRP 3.3’,5.5’-tetramethylbenzidine (Bio Rad) was added to each well and the plates incubated for 15 to 30 minutes in the dark. The reaction was stopped with 1M H2SO4 (100 µL/well) and the absorbance was measured at 450 nm with Labsystems Multiscan MS (Labsystems, Vantaa, Finland). In cases where the total anti-β-Lg–IgE binding of a patient’s serum (without inhibition) gave an optical density at 450 nm (OD450) over 0.2, the inhibition of binding caused by either reindeer or bovine β-Lg was presented and calculated as follows [12]:

\[
\text{percent inhibition} = \frac{\text{noninhibited OD}_{450} - \text{inhibited OD}_{450}}{\text{noninhibited OD}_{450}} \times 100
\]

Statistics

Statistical analyses were performed with the SPSS program (SPSS Inc, Chicago, Illinois, USA). As the distributions of both allergen-specific IgE concentration and ELISA inhibition test results were nonnormal; nonparametric statistical tests were applied. Correlation between the patient’s age and either the levels of cow’s milk-specific IgE or its binding inhibition by β-Lg were determined with the Spearman rank correlation test. The Mann-Whitney test was used for estimation of the effect of the patient’s gender. Inhibition percentages of bovine and reindeer β-Lg bands and controls our concentration determinations. Figure 1B shows that when the 2 isolated proteins were analyzed by immunoblotting, the respective bovine and reindeer bands (lanes 1 and 2) were recognized by the rabbit antisera to bovine β-Lg, as shown above and also in previous results [21]. The isoelectric points of the isolated β-Lgs are presented in Figure 2. Only 1 protein band of reindeer’s milk β-Lg was detected (lanes 5, 6), whereas bovine β-Lg showed 2 protein bands (lanes 3 and 4) corresponding to the A and B chains of the protein [21]. Our data indicate a lower isoelectric point for reindeer β-Lg (pI 4.9) compared to those of bovine β-Lg A/B (pI 5.1/5.3), in agreement with previous reports [18, 19]. ELISA inhibition experiments with constant amounts of inhibitor (100 µg/mL, 5 µg/well) showed that preincubation of cow’s milk allergic patient sera with bovine β-Lg inhibited IgE binding to a coated bovine β-Lg (10 µg/mL, 1 µg/well) by 73% to 98% (mean 89%),

Ethical Considerations

The ethics committee of Oulu University Hospital (Oulu, Finland) approved these studies on human sera collected at Oulu University Hospital and written informed consent was obtained from each patient or patient’s parent. The ethics committee of the Medical Faculty of Gothenburg University (Gothenburg, Sweden) granted permission for these experiments on serum samples collected in collaboration with Gothenburg University.

Results

Seventeen patient sera and the pooled patient sera contained IgE antibodies specific to cow’s milk proteins (median IgE level, 4.8 kU/L, range, 0.6-100 kU/L) as judged by the IgE response to cow’s milk fluorescent-enzyme immunoassay. Ten out of these 17 patient sera and the pooled sera contained significant amounts of IgE specific to bovine β-Lg, defined as an ELISA OD450 value greater than 0.2 units (median OD450, 1.409; range, 0.286-2.864). Sera from the 6 control patients did not demonstrate measurable amounts of cow’s milk-specific IgE (< 0.35 kU/L) or bovine β-Lg specific IgE (OD450 < 0.2), and were used as negative controls.

Characterization of the proteins isolated from bovine and reindeer’s milk by SDS-PAGE, immunoblotting and IEF is illustrated in Figures 1A, 1B, and 2. Figure 1A shows that the molecular masses of the bovine (lanes 2 and 4) and reindeer β-Lg proteins (lanes 3 and 5) are almost identical when analyzed by a reduced SDS-PAGE, and this is consistent with earlier data [18, 19]. Visual inspection of the β-Lg bands in Figure 1A also indicates similar intensities for bovine and reindeer β-Lg bands and confirms our concentration determinations. Figure 1B shows that when the 2 isolated proteins were analyzed by immunoblotting, the respective bovine and reindeer bands (lanes 1 and 2) were recognized by the rabbit antisera to bovine β-Lg, as shown above and also in previous results [21]. The isoelectric points of the isolated β-Lgs are presented in Figure 2. Only 1 protein band of reindeer’s milk β-Lg was detected (lanes 5, 6), whereas bovine β-Lg showed 2 protein bands (lanes 3 and 4) corresponding to the A and B chains of the protein [21]. Our data indicate a lower isoelectric point for reindeer β-Lg (pI 4.9) compared to those of bovine β-Lg A/B (pI 5.1/5.3), in agreement with previous reports [18, 19]. ELISA inhibition experiments with constant amounts of inhibitor (100 µg/mL, 5 µg/well) showed that preincubation of cow’s milk allergic patient sera with bovine β-Lg inhibited IgE binding to a coated bovine β-Lg (10 µg/mL, 1 µg/well) by 73% to 98% (mean 89%)

![Figure 1](image-url)
IgE Cross Reactivity Between Reindeer and Bovine Milk β-Lactoglobulin

Gender had no effect on the inhibition ($P = .421$; Mann-Whitney), and no significant correlations were detected between percentage inhibition and the age of the child (bovine β-Lg, $\rho = -.176, P = .0176$; Spearman) or the serum concentration of cow’s milk-specific IgE (bovine, $\rho = 0.406, P = .244$; reindeer β-Lg, $\rho = 0.433, P = .244$).

Inhibition studies with increasing inhibitor concentrations (0-100 μg/mL) of bovine and reindeer β-Lg showed that a higher concentration of reindeer β-Lg was needed for a comparable inhibition of IgE binding to coated bovine β-Lg (Figure 3 A and B). In all cases, inhibition was observed with soluble bovine β-Lg, which showed clear competition between the soluble and captured proteins, whereas soluble reindeer β-Lg inhibited IgE binding significantly less. In the ELISA inhibition test carried out with patient serum 1 the soluble bovine β-Lg concentration of 0.44 μg/mL (0.022 µg/well) was able to inhibit 50% of IgE binding to captured bovine β-Lg, whereas the maximum concentration used in the tests, 100 μg/mL, was required for a similar inhibition with reindeer β-Lg (Figure 3A). Moreover, with patient serum 2 (Figure 3B), even the highest concentration of reindeer β-Lg, 100 μg/mL, induced only 8.1% IgE binding inhibition. The same concentration of bovine β-Lg inhibited 92.2% of IgE binding and a bovine β-Lg concentration of only 2.8 μg/mL (0.14 µg/well) was needed for 50% inhibition in the case of patient serum 2. The bovine β-Lg concentrations needed for 50% inhibition and the levels of bound bovine β-Lg specific IgE (OD450 values 2.147 and 1.409 for patients 1 and 2 respectively) do not correlate with serum levels of cow’s milk-specific IgE (>100, and 19.6 kU/L for patients 1 and 2 respectively), indicating different sensitizing patterns to cow’s milk proteins. Since only very small volumes of sera were available, it was not possible to determine inhibition curves for all of our cow’s milk allergic patients.

Figure 2. Isoelectric points of isolated bovine and reindeer β-Lg. Bovine β-Lg, lanes 3 and 4; reindeer β-Lg, lanes 5 and 6; and commercial bovine β-Lg, A/B lane 2.

whereas reindeer β-Lg inhibited the IgE binding by only 7% to 95% (mean 43%). The pooled serum sample confirmed these results, since 92% of the total IgE binding was inhibited by bovine β-Lg, but only 22% was inhibited by reindeer β-Lg. Reindeer β-Lg inhibited IgE binding by 50% or more in only 40% of individual serum samples. In only 1 patient serum sample did reindeer β-Lg inhibit IgE binding to bovine β-Lg almost as intensively as bovine β-Lg. Altogether, preincubation of cow’s milk allergic patient sera with reindeer β-Lg decreased the IgE binding to coated bovine β-Lg significantly less than preincubation with bovine β-Lg ($P = .005$; Wilcoxon signed rank test).

Figure 3. Percentage inhibition of anti-bovine β-Lg IgE binding to bound bovine β-Lg. Soluble inhibitors bovine β-Lg (●) or reindeer β (○) solutions added to sera from patients 1 (A) and 2 (B).
Discussion

This is the first study to investigate the immunological cross-reactivity of IgE from cow’s milk allergic patients with reindeer’s milk ß-Lg. Our findings indicate that reindeer ß-Lg shows only a partial capability to inhibit the binding of ß-Lg-specific IgE in sera of patients with cow’s milk allergy. The results suggest that some bovine IgE epitopes are either absent, different or present in smaller amounts in reindeer ß-Lg. Reindeer ß-Lg fits the definition of an incomplete allergen of Aalberse [24]: one able to cause only a partial inhibition of IgE binding to the complete allergen.

Our results suggest that reindeer’s milk might be tolerated by patients with low levels of cow’s milk-specific IgE or patients evolving cow’s milk tolerance as the binding affinity of reindeer ß-Lg for cow’s milk allergic patient IgE is poor. It should be emphasized, however, that tolerance of reindeer milk ß-Lg is likely to be variable in subjects with cow’s milk allergy. In our study, reindeer ß-Lg inhibited IgE binding to bovine ß-Lg almost as intensively as bovine ß-Lg in only 1 patient. This result probably predicts intolerance to reindeer ß-Lg in this individual. In the majority of patients with cow’s milk allergy, reindeer milk ß-Lg inhibited IgE binding by considerably less than 50% of that induced by a similar concentration of bovine ß-Lg. Our result showing a low IgE binding inhibition capacity of reindeer milk ß-Lg, even at the highest concentration (100 µg/mL), is interesting when compared to an earlier study investigating the IgE binding abilities of heat-modified (74°C or 90°C for 60 minutes) bovine milk ß-Lg [25]. IgE binding to heated bovine ß-Lg was significantly decreased, but pronounced inhibition was attained by using higher concentrations (30 µg/mL) of heated protein.

Since reindeer and bovine milk ß-Lgs were used as antigens in their native form in the inhibition assays, our data mainly reflect IgE reactivity with the conformational epitopes, although some of the linear epitopes are also reactive in native proteins. The divergent IgE reactivity between reindeer and bovine ß-Lg suggests differences in their folding patterns and 3-dimensional structures, at least on the protein surfaces. This is interesting considering the high amino acid composition homology of reindeer and bovine ß-Lgs [19]. However, we have noticed that reindeer ß-Lg has a lower solubility than the bovine protein, and the surface charges of the proteins differ (unpublished data). Differences in surface residues may alter the allergenic properties of ß-Lg proteins. The full amino acid sequence and 3-dimensional structure of reindeer ß-Lg would be required to make structural comparisons with bovine ß-Lg.

It is currently unknown why mammalian lipocalins are significant allergens [17]. Molecular mimicry of human proteins may be an explanation. Human glycodelin, a protein predominantly expressed in genital organs, has a close resemblance to bovine ß-Lg, but the extent of homology between reindeer ß-Lg and glycodelin is not known at present. Presumably, careful epitope mapping of bovine and reindeer ß-Lg will help reveal the structural features of lipocalins that determine their allergenicity.

It is assumed that a milk substitute whose composition more closely approaches that of human breast milk would be ideal for cow’s milk allergic patients. Reindeer’s milk is rich in fats and proteins and poor in lactose [26]. On average, the major reindeer’s milk constituents are fats (15.5%), proteins (9.9%) and lactose (2.5%). The corresponding values in human milk are 3.6%, 1.4% and 6.7%, and in cow’s milk 3.6%, 3.3% and 4.9% [27]. Caseins have been shown to be the predominant proteins in reindeer’s milk. The whey protein fraction of reindeer’s milk is about 19%; slightly more than in cow’s milk (15%) but still significantly less than in human milk (55%) [28, 29]. The high fat composition of reindeer’s milk [26] is a factor that might decrease its clinical allergenicity. Reindeer’s milk has been used by the native people of Lapland for centuries as the primary milk substitute after weaning, and the recent development of a reindeer milking machine has increased its availability [26]. The safety of reindeer’s milk in children has been established by tradition and its availability would make clinical trials feasible.

Our data shows clear antigenic differences between reindeer and bovine milk ß-Lg, suggesting that important epitopes are either different, present in reduced numbers or absent in reindeer’s milk. Tolerance studies of reindeer’s milk proteins in children with cow’s milk allergy are needed to see whether reindeer’s milk could be recommended as a cow’s milk substitute for cow’s milk allergic patients, or whether reindeer’s milk-based formula could be used as a less allergenic substitute in infants at risk of developing milk allergy. More detailed comparison of the 2 lipocalin allergens, which share a similar amino acid composition but are antigenically different, may provide clues to why lipocalins are such important allergens.

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