

Mutant derivatives of the main respiratory allergen of cow are less allergenic than the intact molecule

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Summary

Background Allergen immunotherapy offers an alternative for drug treatment in the management of allergic diseases. Because immunotherapy often induces side-effects, less allergenic preparations would be beneficial.

Objective The purpose of this study was to examine whether the allergenicity of a cow-derived lipocalin allergen, Bos d 2, could be diminished by substituting or deleting carboxy-terminal amino acids including the cysteine which forms a disulphide bond with a cysteine inside the molecule.

Methods Four recombinant mutants of Bos d 2 were created by substituting or deleting the four most carboxy-terminal amino acids. The immunological characteristics of the mutant preparations were compared with the unmodified rBos d 2 by Western blotting, ELISA inhibition, skin prick tests, and the proliferative responses of allergen-specific T-cell clones.

Results In Western blot, one of the two monoclonal antibodies showed reduced binding to the preparations without the terminal cysteine. In contrast, the other monoclonal antibody, human IgE and rabbit immune serum bound equally well to all the preparations. ELISA inhibition analyses revealed, however, that the preparations without the terminal cysteine bound antibody less efficiently. They were needed 15–38 times more than the unmodified rBos d 2 to cause the same level of inhibition. Surprisingly, one of the mutants with the terminal cysteine but a mutated adjacent amino acid turned out to be the weakest in inducing skin reactivity. All the preparations stimulated well allergen-specific T-cell clones.

Conclusions The results show that the allergenicity of a lipocalin allergen, Bos d 2, can be diminished by modifying the carboxy-terminal end of the molecule. Modifications in the area which encompasses a disulphide bond impaired the antibody binding without affecting the T-cell stimulatory capacity. It was also shown that *in vivo* tests are necessary for determining the allergenicity of a modified allergen.

Keywords: allergic asthma, cow, disulphide bond, immunotherapy, recombinant allergen, site-directed mutagenesis

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Introduction

The development of safe and tolerable preparations for allergen immunotherapy is one of the aims of allergen research. Because most of the side effects of allergen

immunotherapy are the result of IgE-mediated reactions it is conceivable to try to decrease the IgE-binding capacity of allergens. Until recent years, the progress in developing such preparations was relatively slow because the prerequisites were lacking. Firstly, allergen preparations used and available were not pure; they were mainly poorly characterized extracts containing both allergenic and nonallergenic proteins [1].

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Secondly, possibilities for targeted approaches to decrease the allergenicity (i.e. IgE-binding capacity) of allergens were limited because methods available were mainly crude chemical or physical treatments [2]. Thirdly, it has become increasingly evident that the IgE-binding capacity of allergens is dependent on conformation of the molecule [3–5]. Therefore, interfering with important structural features of allergenic molecules, such as areas affected by intramolecular disulphide bonds, could be anticipated to result in reduced allergenicity and thus in better tolerance in immunotherapy.

Modern methods of molecular biology have changed the situation, and the number of allergens characterized at the molecular level is constantly increasing [6]. These methods, besides allowing the production of defined allergenic molecules in large quantities, make possible the systematic development of molecules with desired properties [4].

In our previous study we showed that the allergenicity of Bos d 2, the main respiratory allergen of cow, was greatly reduced when the allergen was in fragments [5]. Because Bos d 2 contains an intramolecular disulphide bond another possibility for reducing the IgE-binding capacity of Bos d 2 could be the elimination of the bond. We report here that the substitution or deletion of amino acids, including a cysteine, in the carboxy-terminal end of the rBos d 2 results in a significant reduction of the allergenicity of the molecule. This finding suggests that an intact three-dimensional structure of the carboxy-terminal end of Bos d 2 is important for antibody binding.

Materials and methods

Antibodies

Twelve patients with clinically diagnosed cow asthma were included in the study (five men, seven women). The bovine origin of the asthma was confirmed at the pulmonary clinic of Kuopio University Hospital as described elsewhere [7]. For a person to be classified as being asthmatic to cow-derived material, inhalation test, skin prick test and RAST had to be positive. Sera from five of these patients were randomly selected for further analyses.

The production and purification of the two monoclonal antibodies (MoAbs 1 and 3) against Bos d 2 has been described elsewhere [7,8]. To obtain rabbit immune serum against Bos d 2, the raw extract of bovine epithelium was first run in the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described before [7,9]. The 20-kDa band was then cut out from the gel and the protein was subjected to an electrophoretic elution in a dialysis tube. The eluted protein was dialysed, concentrated by lyophilization and its purity was verified by SDS-PAGE. Rabbits were immunized with the protein as described previously [9].

Allergen preparations

Natural Bos d 2 contains a disulphide bond between the carboxy-terminal cysteine (C170) and a cysteine inside the molecule (C79) (crystallographic analysis; J. Rouvinen, University of Joensuu, personal communication).

The four mutant derivatives of rBos d 2 were generated by the site-directed mutagenesis (Table 1). One of the preparations contained a mutation at C170 (C170 → S170) and two of them at the both sides of C170 (N169 → A169 and P171 → A171). In the fourth preparation, the last four amino acids were deleted. The mutagenic oligonucleotide primers used in polymerase chain reaction (PCR) are also listed in Table 1 for the 3'-end of the sequence of Bos d 2 cDNA [10]. The primer for 5'-end of cDNA was the same as used previously in generating the intact rBos d 2 insert [10]. The PCR products were purified and then cloned to the cloning site of the pGEX2T expression vector according to the standard procedure [11] using *Bam*HI and *Eco*RI restriction sites included in the 5'-end of the primers. DNA sequence of the mutant inserts in pGEX2T were verified by ALF automatic DNA sequencer using an Auto Read Sequencing kit (Pharmacia Biotech, Sollentuna, Sweden). The recombinant (r) Bos d 2 and its mutant derivatives were produced in *Escherichia coli* strain TG10B as fused proteins with glutathione S-transferase (GST) as described previously [10].

For ELISA inhibition experiments and skin prick tests (SPT), the SDS-PAGE gels of recombinant protein preparations were analysed densitometrically by a flat-bed scanner using the NIH Image program, and the proportions of recombinant proteins were calculated to determine 'effective' protein concentrations. All the preparations used in SPTs were dialysed against 0.15 mol/L NaCl/0.01 mol/L bicarbonate buffer, pH 9.2.

Natural (n) Bos d 2 was purified from commercial raw material with affinity chromatography and gel filtration as described previously [7].

All the protein concentrations were determined by the method of Bradford [12] using bovine serum albumin as a standard. Sterile-filtered preparations were stored either at 4 °C or frozen at –70 °C.

Western blotting

The IgE reactivity of recombinant preparations and the GST control were examined by SDS-PAGE and immunoblotting with minor modifications as described before [7,9]. The antigens separated in SDS-PAGE were transferred to a Hybond-ECL nitrocellulose membrane (0.45 µm, Amersham International, Amersham, UK). The membrane strips were incubated overnight at room temperature in sera (diluted 1:10) from the cow-asthmatic subjects, washed

autologous PBMCs (6000 rad) as antigen-presenting cells (APCs). Blasts were then further cultivated with increasing concentrations rIL-2 as described above. The established T-cell lines were expanded with phytohaemagglutinin (PHA), rIL-2 and irradiated autologous PBMCs as feeder cells. Antigen-reactive T-cell lines were used for cloning according to the limiting dilution method as reported previously [5]. T cells were seeded in the wells of round-bottomed 96-well microtitre plates at a concentration of 0.3 or 1 cell/well with irradiated allogeneic PBMCs as feeder cells, PHA and rIL-2. Cultures were then refed weekly with feeder cells and rIL-2. When the growth of the clones became visible after 14–20 days, the cultures were expanded with PHA, feeder cells and rIL-2.

The phenotype of T-cell clones was examined by flow cytometry on a FACScan machine (Becton Dickinson, San José, CA, USA) using the CD4FITC + CD8PE + CD3PerCP reagent (Becton Dickinson).

Antigen specificity of the clones was tested with lymphocyte proliferation assay as described elsewhere [5]. T cells were stimulated in triplicate in the wells of round-bottomed 96-well microtitre plates (50 000 cells/well) with test antigens (nBos d 2, mutants or GST) at predetermined optimal concentrations and APCs (100 000 cells/well). Plates were incubated for 3 days in an incubator, and then the cultures were pulsed for 16 h with 0.5 mCi of ^3H -thymidine (specific activity 2.0 Ci/mmol; Amersham International plc) per well. Radionuclide uptake was measured with scintillation counting and the results expressed as a stimulation index (SI: ratio between the mean counts per minute obtained in cultures stimulated with APC plus antigen and the mean counts per minute obtained in cultures with APC alone).

Results

Antibody binding by Western blotting

The antibody-binding capacity of rBos d 2 and its derivatives was studied by immunoblotting with MoAbs,

patient sera, and rabbit immune serum. Figure 1 shows that MoAb3 appeared to bind to all preparations similarly. In contrast, MoAb1 exhibited variable binding. It bound weakly to rBos d 2/C170S, not at all to rBos d 2/del and largely in a uniform manner to other preparations. The IgE-binding capacity of the preparations was also studied with five patient sera, an example of the results is included in Fig. 1. In all cases, IgE seemed to bind to the derivatives of rBos d 2 with the same intensity as to the unmodified molecule. Rabbit immune serum behaved accordingly, showing reactivity against all the allergen preparations (data not shown). Three of the patient sera, rabbit immune serum and MoAbs showed slight binding to the GST fusion part (26 kDa) as shown for the MoAbs in Fig. 1.

ELISA inhibition

The capacity of fluid-phase rBos d 2 and its derivatives to inhibit the binding of MoAb3 (Fig. 2) and the IgE of two randomly selected patient sera to the solid-phase nBos d 2 was studied in ELISA. As demonstrated in Table 2, rBos d 2/C170S and rBos d 2/del had lost a great part of their antibody-binding capacity. In contrast, the other two derivatives of rBos d 2 with intact cysteine bound antibody quite efficiently; they were needed only 2–3 times more to cause the same level of inhibition than the complete rBos d 2. GST did not inhibit the reactions.

Skin prick tests

For testing *in vivo* responses to the preparations, nine cow-asthmatic patients were subjected to skin prick tests. All the patients showed clearly positive reactions (> 3 mm) against nBos d 2 (mean 6.5 mm at 250 $\mu\text{g}/\text{mL}$) and the cow dander preparation of ALK A/S, but three of the patients failed to react against rBos d 2. One of the six rBos d 2-reactive patients had a skin prick test result of 2.5 mm whereas the other five patients exhibited reactions ≥ 3 mm at the highest protein concentration used. The cumulative skin prick test results of the six patients against the rBos d 2 (five for rBos d

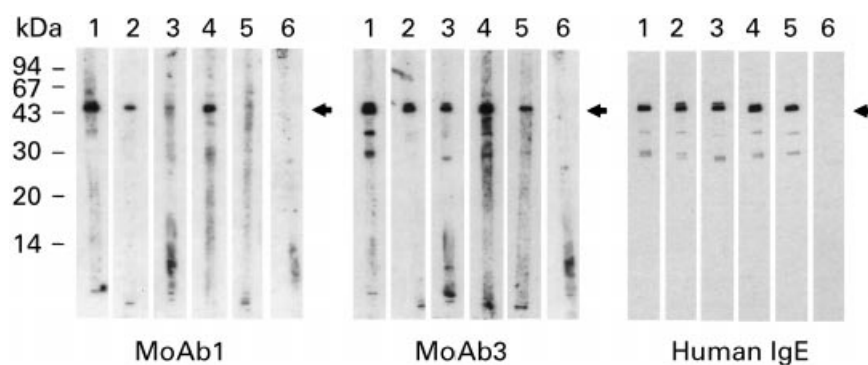


Fig. 1. The binding of MoAbs 1, MoAb 3 and human IgE to rBos d 2 and its mutant derivatives (indicated by arrows) in Western blotting. The fusion preparations are as follows: rBos d 2 (lane 1), rBos d 2/N169A (lane 2), rBos d 2/C170S (lane 3), rBos d 2/P171A (lane 4), rBos d 2/del (lane 5) and GST (lane 6).

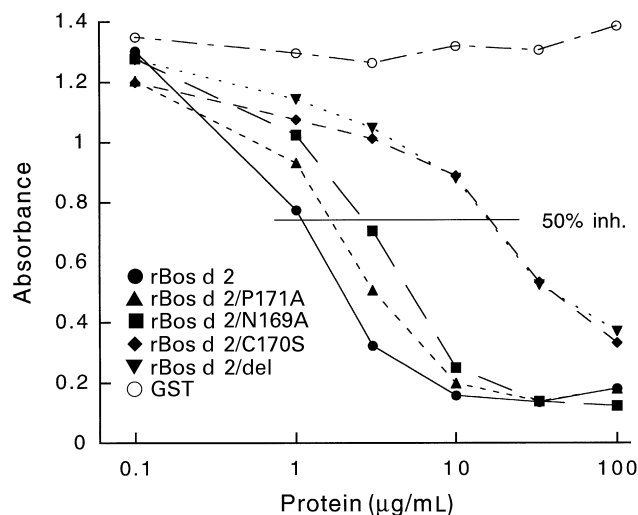


Fig. 2. Inhibition of the binding of IgE from patient A (Table 2) to nBos d 2 by rBos d 2 (filled circles), rBos d 2/P171A (triangles), rBos d 2/N169A (squares), rBos d 2/C170S (rhombs), rBos d 2/del (inverted triangles) and GST (open circles). The 50% inhibition cut-off level is indicated by a line.

2/P171 A) and its derivatives are shown in Fig. 3. The least allergenic of the preparations was rBos d 2/N169 A, the size of the weal it induced being 24% (5.1 mm) of that induced by the complete rBos d 2 (21.7 mm). The difference was statistically significant ($P = 0.03$, Wilcoxon-Signed Rank Test). Other preparations were more allergenic than rBos d 2/N169 A ($P > 0.05$). For example, the size of the weal rBos d 2/del induced was 57% (12.4 mm) compared with that induced by the unmodified allergen. In two cases, GST induced some skin reactivity (Fig. 3).

Responses of allergen-specific T-cell clones

Five rBos d 2-reactive T-cell clones of a cow-asthmatic patient were tested with the preparations (Table 3). All the clones proved to be CD4-positive in the flow-cytometric analysis. As shown in the table, the clones responded well to all the preparations.

Table 2. Relative and absolute amounts of the mutant preparations compared with intact rBos d 2 able to cause the 50% inhibition in ELISA. Absolute amounts in parentheses. GST did not inhibit the reactions.

	Antibodies		
	MoAb3	Patient A IgE	Patient B IgE
rBos d 2	1.0 (1.3 µg/mL)	1.0 (1.1 µg/mL)	1.0 (3.3 µg/mL)
rBos d 2/N169A	3.1 (4.0 µg/mL)	2.5 (2.7 µg/mL)	2.5 (8.3 µg/mL)
rBos d 2/C170S	32 (41 µg/mL)	15 (16 µg/mL)	24 (79 µg/mL)
rBos d 2/P171A	1.6 (2.1 µg/mL)	1.5 (1.7 µg/mL)	1.6 (5.4 µg/mL)
rBos d 2/del	38 (49 µg/mL)	15 (16 µg/mL)	24 (80 µg/mL)

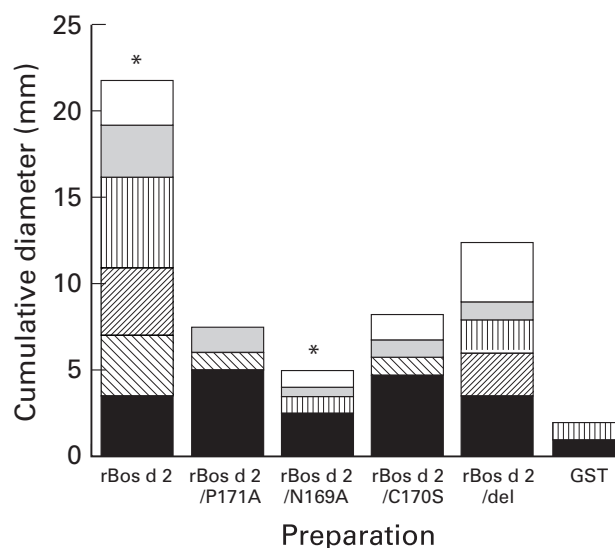


Fig. 3. Cumulative diameters of skin prick test reactions of six cow-asthmatic patients (five for rBos d 2/P171A) against different preparations at the highest protein concentrations used (250 µg/mL; rBos d 2/P171A 208 µg/mL). Different patterns in columns indicate different patients. * $P = 0.03$ (Wilcoxon Signed Rank Test).

Discussion

For the successful immunotherapy of allergy, the reduction of allergenicity (IgE-binding capacity) of an allergen preparation is a rational aim because most of the side effects of the treatment are due to the immediate type of reactions. Several approaches have been adapted to achieve the goal. A common factor for all these approaches has been the aspiration to target the immunomodulatory effects to T cells which have the central role in the regulation of immune system [15].

The use of immunodominant T-cell epitope-containing peptides for immunotherapy has raised considerable interest. Even though the functional elimination of allergen-specific T cells is the primary aim of this approach, a beneficial decrease of IgE-mediated reactions is also achieved [16,17]. The drawback of the method is that a detailed and laborious

Table 3. Proliferative responses of Bos d 2-specific T-cell clones of a cow-asthmatic patient against nBos d 2 and its mutant derivatives. Results are indicated as stimulation indices.

Clone	Stimulation index/preparation					GST
	nBos d 2	rBos d 2/N169A	rBos d 2/C170S	rBos d 2/P171A	rBos d 2/del	
T-144	590	665	982	945	471	< 1
T-149	262	480	828	676	254	< 1
T-152	100	193	355	291	118	< 1
T-190	138	160	318	265	99	< 1
T-296	96	116	218	160	82	< 1

mapping of the epitopes is necessary. In addition, the individual variation of T-cell recognition of epitopes further complicates the situation [4]. One possibility could be the use of recombinant constructs that contain non-contiguous T-cell-reactive areas [18] or epitope-rich regions of allergenic molecules [19].

An alternative for the peptide-based strategy could be the use of complete or fragmented allergen molecules with reduced allergenicity. Ferreira and coworkers reported that part of the iso-allergens of the main allergen of birch bound poorly to IgE but vigorously stimulated T cells [20]. We and others have observed that allergens in the form of fragments have a strongly reduced IgE-binding capacity but maintained the capacity to stimulate T cells [5,21].

The antibody-binding capacity of B-cell epitopes is often strongly dependent on the conformation of antigen [4]. This is not the case for T-cell epitopes which are recognized as linear peptides. Therefore, an ideal allergen preparation for immunotherapy could be the one with lost or distorted three-dimensional structure combined with minimal sequential changes so that all the potential T-cell epitopes are present. One possibility to try to distort the three-dimensional structure of a molecule is to interfere with the important structural areas of the molecule, such as those containing intramolecular disulphide bonds. This approach was presumed to be possible for Bos d 2 because lipocalin proteins typically contain a disulphide bond between the carboxy-terminal cysteine and the cysteine inside the molecule [22,23]. It has recently been shown by crystallography (J. Rouvinen, University of Joensuu, personal communication) that this is also the case with nBos d 2. In this study, we examined the effect of modifying the carboxy-terminal end of rBos d 2 which contains a cysteine potentially forming an intramolecular disulphide bond.

The immunoblotting analyses revealed that in most cases, the amino acid mutations introduced at the carboxy-terminal end of the molecule did not much influence the antibody-binding capacity of the preparations. The exception was MoAb1 which failed to react with rBos d 2/del and reacted

only weakly with rBos d 2/C170S. The finding indicates that C170 has an important role for the integrity of the epitope of MoAb1.

The binding of antibody was also studied in the fluid phase by ELISA inhibition because proteins may bind antibody differentially depending on whether they are in the solid or fluid phase [5]. It was observed that MoAb3 bound clearly less efficiently to the cysteine-deficient rBos d 2 preparations than to the preparations with cysteine (Table 2) although no difference between preparations was observed in immunoblotting with this antibody. Therefore, it seems that the integrity of the carboxy-terminal end of Bos d 2 is also important for the formation of the epitope of MoAb3 as it seems to be the case for MoAb1 according to the immunoblotting analysis. The results with two patient sera were similar to that of MoAb3. These findings are of interest because they suggest that the carboxy terminus of Bos d 2 actively takes part in forming the immunodominant B-cell epitope area in Bos d 2. In accordance with this view is our previous observation that most cow-asthmatic patients showed some IgE reactivity to the carboxy-terminal fragment of rBos d 2 in the indirect ELISA whereas the amino-terminal fragment was virtually silent [5].

The skin prick test results showed that the biological response to an allergen can be difficult to predict by *in vitro* tests. Cumulative skin prick test reaction which sums up individual reactions was the weakest with rBos d 2/N169A, and the cysteine-mutated or the carboxy terminus-deleted preparations induced larger skin reactions even though they remained smaller than that induced by intact rBos d 2. The result suggests that the epitopes in rBos d 2/C170S and rBos d 2/del responsible for the cross-linking of Fcε receptors on basophils and mast cells are able to bind IgE in sufficient amounts *in vivo* to launch the release of mediators. In contrast, rBos d 2/N169A which bound IgE relatively well *in vitro* turned out to be inefficient in cross-linking Fcε receptors and causing immediate skin reaction. It appears that the change of a single amino acid at a critical position can induce major changes in the molecule and

result in such a conformation that the cross-linking of Fcε receptors is inefficient. The intact conformation of a molecule is known to be important for IgE binding [4,24].

It is obvious that if a molecule contains a disulphide bond its significance for the structure may be varying. In fact, as reported by Smith and Chapman, breaking the three disulphide bonds of Der p 2 one by one, reduced its antibody-binding capacity in various amounts [25]. The most effective reduction in allergenicity was obtained when the bond inside the molecule between cysteines separated by a few amino acids was broken. In that case, IgE binding was reduced by more than 100-fold and skin reactivity by 10–100-fold. On the other hand, in the study with Der f 2, the removal of a disulphide bond between the amino- and carboxy-terminal ends of the molecule impaired more IgE binding than the removal of the bonds inside the molecule [3]. In this latter study, it was also observed that mutations around the disulphide bond-forming cysteines, without removing them, reduced IgE binding.

Several animal-derived main allergens have turned out to belong to the group of proteins called lipocalins. These include the major urinary proteins of mouse and rat, Mus m 1 [26,27] and Rat n 1 [27,28], horse allergen Equ c 1 [29], bovine allergen Bos d 2 [10], the canine allergens Can f 1 and Can f 2 [30], and the food allergen β-lactoglobulin [31]. In addition, a cockroach allergen, Bla g 4, is known to be a lipocalin (calycin) [32]. The situation is very interesting and may offer a way to approach the basic question of the molecular determinants of allergenicity. However, very little is known about the immunological properties of these proteins. It has been reported previously that the major continuous IgE-binding epitope of β-lactoglobulin is situated in the terminal half of the molecule [33]. This is an interesting observation because our current and previous [5] results suggest that the carboxy terminus in Bos d 2 is important for IgE binding. In another study with overlapping peptides, rabbit and murine sera detected in β-lactoglobulin 7–8 regions with antibody-binding capacity. Further analyses with phage-display library peptides revealed that the three antibody-binding regions detected comprised a subset of the previously identified 7–8 regions. One of these three regions was situated at the carboxy terminus of the molecule [34].

Taken together, our results corroborate that the cysteines C79 and C170 form a disulphide bond also in *E. coli*-produced rBos d 2 as is the case with nBos d 2. This was especially clear in the ELISA analyses and skin prick tests which showed that interfering with the area in rBos d 2 which encompasses the carboxy-terminal cysteine resulted in the decreased antibody-binding capacity of the molecule. The information provided by this study is important because we could show that minute changes in the allergen in an important structural point can cause major effects for

antibody binding. It was also observed that it is necessary to verify the allergenicity of a modified allergen preparation by *in vivo* tests because the plain IgE binding does not sufficiently reflect the biological activity of an allergen. The modifications introduced did not impair the capacity of Bos d 2 mutants to induce T-cell proliferation which is to be considered important for immunotherapeutic effects [35]. Because lipocalins typically contain a disulphide bond between the carboxy-terminal cysteine and the cysteine inside the molecule [22,23], the modification of the sequence around the terminal cysteine in these proteins may be an alternative for reducing their allergenicity.

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