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Recombinant allergen fragments as candidate preparations for allergen immunotherapy

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Background: Lately, renewed interest has arisen in the new forms of allergen immunotherapy because they may offer alternatives for drug treatment.

Objective: The purpose of this study was to develop a well-characterized preparation of the main respiratory cow dander allergen, Bos d 2, with attenuated allergenic activity.

Methods: The immunologic characteristics of Bos d 2 preparations were studied by indirect IgE ELISA, ELISA inhibition, Western blotting, histamine release, skin prick tests, and the proliferation tests of allergen-specific T-cell clones.

Results: The complete recombinant Bos d 2 was observed to bind effectively, IgE of cow-allergic patients in indirect ELISA. In other experiments, the IgE-binding capacity of recombinant Bos d 2 proved to be lower compared with native Bos d 2. When the two overlapping recombinant fragments of Bos d 2 (corresponding amino acids 1-131 and 81-172, respectively) covering the whole molecule were compared with the complete recombinant Bos d 2 with several methods, only a low level of residual reactivity was observed. For example, recombinant fragments could not bind antibody at all in ELISA inhibition tests retaining, however, some reactivity in skin prick tests. In contrast, the fragments were able to stimulate vigorously Bos d 2-specific T-cell clones.

Conclusion: The approach we have taken may offer a simple and reproducible way to produce hypoallergenic preparations for immunotherapy, circumventing simultaneously some of the problems of other experimental methods such as individual T-cell epitope recognition in peptide-based immunotherapy. (*J Allergy Clin Immunol* 1997;100:721-7.)

Key words: recombinant allergen, fragment, cow, allergic asthma, immunotherapy

Allergen immunotherapy has recently aroused increasing interest. Partly this is due to the better understanding of the mechanisms of cellular regulatory events in allergy, especially of the role of T-helper cell subsets^{1,2} and partly because of the great progress in allergen research. In addition, there is an increasing amount of data showing that immunotherapy, by repeated subcutaneous injections, is effective.³ Furthermore, the aim of immunotherapy, that is, the downregulation of inflammatory processes at the level of a shock organ, is in good concordance with the current concept of pathogenetic mechanisms of allergic diseases.⁴

The efforts to develop more efficient modalities of immunotherapy have been hampered considerably by the poorly standardized composition of allergen extracts, often containing a mixture of allergenic and nonallergenic proteins. However, this problem has been disappearing during recent years in parallel with the increasing number of allergens defined by sequential data.⁵ Most of the credit of this progress is due to the methods of modern molecular biology. These methods allow targeted modification of allergenic molecules so that new approaches, such as the use of selected T-cell epitope-containing sequences, can be adapted for immunotherapy.⁶

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Abbreviations used

APC:	Antigen-presenting cell
Bos d 2:	Bos domesticus allergen 2
GST:	Glutathione-S-transferase
HR:	Histamine release
mAb:	Monoclonal antibody
nBos d 2:	Native Bos d 2
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PHA:	Phytohemagglutinin
rBos d 2:	Recombinant Bos d 2
rIL-2:	Recombinant IL-2
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SPT:	Skin prick test

The development of safer and more tolerable preparations for immunotherapy is one of the aims of allergen research. Since most of the side effects of immunotherapy in the immediate type of allergy are the result of IgE-mediated reactions, it is reasonable to aspire to decrease allergenicity (capacity to bind IgE) of the preparation while maintaining its reactivity with the other components of the immune system, such as T cells. We have adapted this approach for the main bovine dander allergen, Bos d 2,⁷ which recently has been shown to belong to a group of proteins called lipocalins.⁸ We show in this report that by deleting a segment from either amino or carboxy terminal end of Bos d 2, its IgE-binding capacity could be significantly diminished. This treatment did not affect the capacity of the fragments to induce T-cell proliferation. We expect that this simple technique for developing low-allergenic preparations for immunotherapy could also be adapted for other allergens.

METHODS

Antibodies

Thirteen patients with clinically diagnosed cow asthma were included in the study (eight men, five women). Sera were collected from 12 patients. The mean age of the group was 42 years (range 25 to 58 years). The bovine origin of the asthma was confirmed at the Pulmonary Clinic of Kuopio University Hospital as described elsewhere.⁹ For a person to be classified as being asthmatic to cow-derived material, inhalation test with skin prick test and/or RAST had to be positive.

The production and purification of the three monoclonal antibodies (mAbs 1 through 3) against Bos d 2 has been described elsewhere.^{9,10}

Allergen preparations

Bos d 2 was purified from commercial raw material with affinity chromatography and gel filtration as described previously.⁹

The production of recombinant Bos d 2 has been described in detail elsewhere.⁸ In brief, rBos d 2 and its fragments were

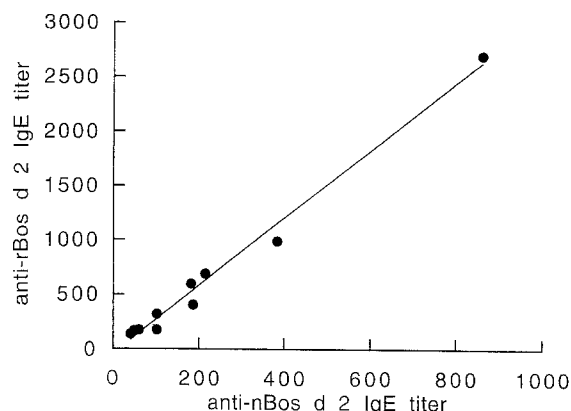


FIG. 1. Correlation of anti-rBos d 2 and anti-nBos d 2 IgE titers of cow-asthmatic farmers ($r = 0.98$, $p = 0.003$). Regression curve is indicated.

produced as follows. The cloning vector for expression was pGEX2T, which expresses sequences fused to the carboxy terminus of glutathione-S-transferase from *Schistosoma japonicum*.¹¹ The numbering of the amino acids was started at the first methionine of the open reading frame.⁸ For the construction of the rBos d 2 (1-131) expression plasmid, *Stu*I and *Eco*RI restriction sites in the pGEX2T-POT clone were used according to a standard procedure.¹² The generation of the rBos d 2 (81-172) insert was carried out by polymerase chain reaction. The Bos d 2-specific primers including 5' and 3' cloning sites for the pGEX2T expression vector were used in PCR reaction with the clone Pot12⁸ to produce a DNA fragment encoding the carboxy terminal fragment (corresponding amino acids 81-172) of the Bos d 2 sequence. The recombinant expression vectors were transformed into *Escherichia coli* strain TG10B. Fusion protein expression and purification were performed with the GST Gene Fusion System according to the manufacturer's procedures (Pharmacia Biotech).

For ELISA inhibition experiments and SPTs, the sodium dodecyl sulfate polyacrylamide gel electrophoresis gels of recombinant protein preparations were analyzed densitometrically by a flatbed scanner and the NIH Image program, and the proportions of recombinant proteins were calculated to determine "effective" protein concentrations. Preparations used for SPTs were dialyzed against 0.15 mol/L NaCl/0.01 mol/L bicarbonate buffer, pH 9.2. For histamine release tests, recombinant proteins were gel-filtrated similarly as described above.

All the protein concentrations were determined by the method of Bradford¹³ with the use of bovine serum albumin as a standard. Sterile-filtered preparations were stored either at 4°C or frozen at -70°C.

Measurement of specific IgE

The levels of specific IgE antibodies against nBos d 2, rBos d 2, fragments, or GST were measured with ELISA similarly as described before.⁹ Serum samples were tested in duplicate at several dilutions beginning from the dilution of 1:10. Reagent blank absorbance values were subtracted from the results. Background absorbances obtained from anti-GST IgE measurements were subtracted from the results of recombinant preparations. Final results were then determined as titers from the dilution curve, with the absorbance of 0.3 as a cutoff.

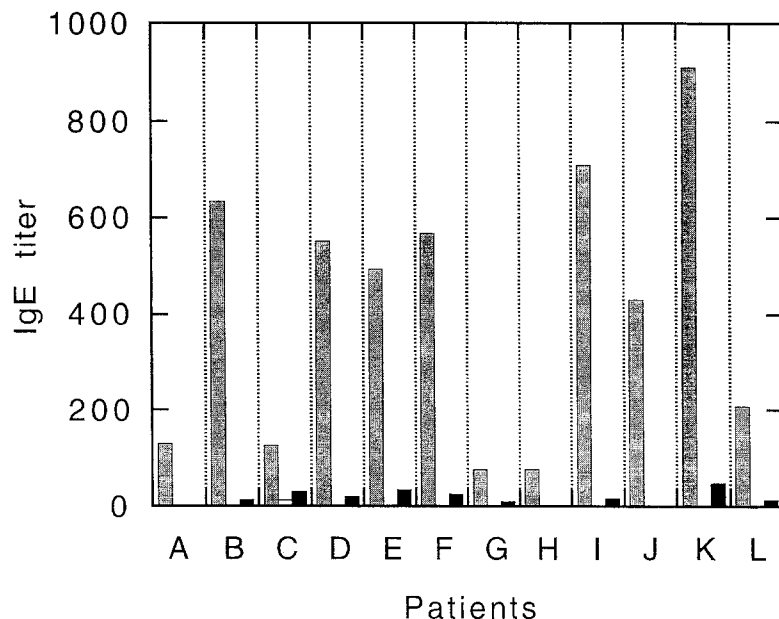


FIG. 2. IgE titers against rBos d 2 (gray columns), rBos d 2 (1-131) (open columns), and rBos d 2 (81-172) (black columns) of cow-asthmatic farmers. Letters indicate patients.

ELISA inhibition

The capacity of nBos d 2, rBos d 2, fragments, and GST to inhibit the binding of mAbs 1 and 3 to the solid-phase rBos d 2 or nBos d 2 and on the other hand, patient IgE to the solid-phase nBos d 2, was examined by a similar ELISA technique as described previously.¹⁴ First, several dilutions of each preparation (0.001 to 10 µg/ml) were allowed to react with a predetermined dilution of an antibody in a test tube for 30 minutes at 37° C. The inhibited antibody as well as the uninhibited control antibody then were transferred into the wells of a microwell plate. In IgE inhibitions, the ELISA procedure was completed as described above. In the inhibitions of mAbs, the bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako A/S, Glostrup, Denmark; P161). Finally, the amount of each preparation that could cause 50% inhibition of the maximal absorbances obtained in the wells without an inhibitor was determined.

Western blotting

The IgE reactivity of recombinant proteins was detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described before,^{9,15} with modifications. The antigens separated in SDS-PAGE were transferred to a Hybond-ECL nitrocellulose membrane (0.45 µm, Amersham International, Buckinghamshire, England). The membrane strips were incubated overnight at room temperature in sera (diluted 1:10) from the cow-asthmatic subjects, washed in Tween-PBS, and then incubated with peroxidase-labeled rabbit anti-human IgE (Dako P295). The reactive bands were visualized with ECL detection reagents (Amersham). The analyses were performed in the same way with mAbs (diluted 1:10), with the peroxidase-labeled conjugate being rabbit anti-mouse immunoglobulins (Dako P161).

Histamine release

Release of histamine from peripheral blood leukocytes was studied as described by Cromwell et al.,¹⁶ using nBos d 2, rBos d 2, rBos d 2 (1-131), and GST as antigens. Supernatants were stored at -70° C until the measurement of histamine. Histamine was measured by a high-performance liquid chromatography method as described by Yamatodani et al.¹⁷

Skin prick tests

Skin prick tests were performed according to Nordic recommendations¹⁸ on the back of cow-asthmatic patients in duplicate with nBos d 2, rBos d 2, fragments, and GST. Six 10-fold dilutions of each preparation were used. The strongest concentration of nBos d 2 was 100 µg/ml. The strongest concentration of other preparations was 200 µg/ml. Histamine (10 mg/ml) and diluent PBS were included as positive and negative controls. After 15 minutes, the wheals were marked and documented with a strip of tape. The diameters were calculated with the formula $(d_{\max} + d_{\min})/2 = d_{\text{mean}}$.

T-cell clones

For the cloning of antigen-specific T cells, peripheral blood mononuclear cells, separated from the heparinized peripheral blood of cow-asthmatic farmers by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation as described elsewhere,⁹ were cultivated in 24-well plates (Corning) at a density of 1.5×10^6 cells/well with nBos d 2 (50 µg/ml) for 5 days in a humidified 5% CO₂ incubator at 37° C. Growth medium was RPMI 1640 (Gibco) supplemented with 2 mmol/L L-glutamine, 20 µmol/L 2-mercaptoethanol, sodium pyruvate (Gibco), nonessential aminoacids (Gibco), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5% human AB serum (Finnish Red Cross). After the initial stimulation, the cells were cultivated for 3 days with human recombinant IL-2 (rIL-2; CLB, Amsterdam, Netherlands) at a suboptimal concentration of 5

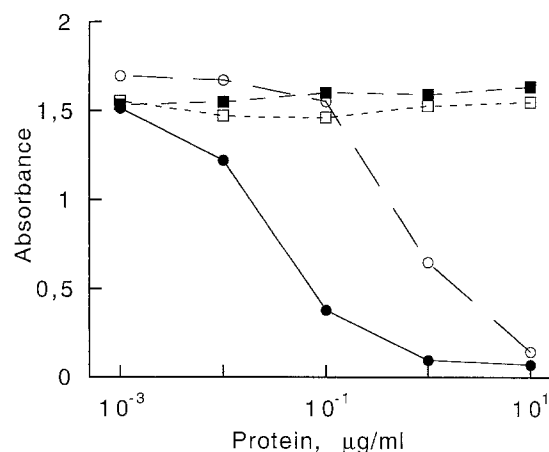


FIG. 3. Inhibition of the binding of mAb3 to rBos d 2 by nBos d 2 (filled circles), rBos d 2 (open circles), rBos d 2 (1-131) (filled squares), and rBos d 2 (81-172) (open squares).

IU/ml and for additional 4 days at a concentration of 25 IU/ml. Blasts were then separated by density gradient centrifugation and restimulated (10^6 cells/well) with nBos d 2 (50 µg/ml) and irradiated autologous PBMCs (60 Gy, 10^6 cells/well) as antigen-presenting cells in 24-well plates. Blasts were further cultivated with increasing concentrations rIL-2 as described above. Established T-cell lines (10^6 cells/well) were expanded with phytohemagglutinin (11.3 µg/ml; PHA), rIL-2 (25 IU/ml) and irradiated allogeneic PBMCs (10^6 cells/well) as feeder cells. Antigen-reactive T-cell lines were used for cloning according to the limiting dilution method, as reported previously.¹⁹ T cells were seeded in the wells of round-bottomed 96-well microtiter plates (Corning) at a concentration of 0.3 or 1 cells/well with feeder cells (300 000 cells/well), PHA (11.3 µg/ml), and rIL-2 (25 IU/ml). Cultures were then refed weekly with feeder cells (200 000 cells/well) and rIL-2 (25 IU/ml). When the growth of the clones became visible (days 14 to 20), 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) with the use of the CD4FITC+CD8PE+CD3PerCP reagent (Becton Dickinson).

Antigen specificity of the clones was tested with lymphocyte proliferation assay as described elsewhere.^{9,19} T cells were stimulated in triplicate in the wells of round-bottomed 96-well microtiter plates (50 000 cells/well) with test antigens (nBos d 2, rBos d 2, fragments, or GST) at predetermined optimal concentrations and APCs (100 000 cells/well). Plates were incubated for 3 days in an incubator, and the cultures were pulsed for 16 hours 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

IgE measurements by ELISA

IgE responses of asthmatic farmers were measured by ELISA against native and recombinant Bos d 2. As shown in Fig. 1, there was a good correlation between

TABLE I. Amounts of cow allergen preparations able to cause the 20% release of histamine in HR assay

Patient	Preparations (µg/ml)			GST
	nBos d 2	rBos d 2	rBos d 2 (1-131)	
B	2.2×10^{-2}	4.1	0.3	—
D	8.6×10^{-5}	0.3	0.8	—
K	5.1×10^{-6}	9.3×10^{-4}	0.1	0.18

IgE titers against the two preparations (Spearman rank correlation; $r = 0.98$, $p = 0.003$).

The IgE titers of individual farmers to rBos d 2 and its fragments are shown in Fig. 2. Only one patient (C) had IgE antibodies reactive to the amino terminal fragment of rBos d 2 (rBos d 2 [1-131]), the titer being 10% of the titer against the complete rBos d 2. The carboxy terminal fragment of rBos d 2 (rBos d 2 [81-172]) turned out to be more reactive. Only three of the asthmatic patients did not exhibit any reactivity against rBos d 2 (81-172), whereas the other nine patients had some reactive IgE antibodies to this fragment. However, the mean level of anti-rBos d 2 (81-172) IgE titers was 6% (range 0% to 25%) of the titers against the whole rBos d 2 molecule. The IgE titers against the complete rBos d 2 correlated moderately with the titers against the carboxy terminal fragment of rBos d 2, but the correlation was not significant (Spearman rank correlation; $r = 0.55$, $p > 0.05$).

Antibody responses by Western blotting

IgE responses of farmers were further examined by the immunoblotting technique after the separation of protein components in SDS-PAGE. All the 12 sera reacted with the complete rBos d 2, whereas none of the sera reacted with rBos d 2 (1-131) and only three with rBos d 2 (81-172) (data not shown). These three sera (patients E, F, and K) had high anti-rBos d 2 (81-172) IgE titers in ELISA.

The reactivity of rBos d 2 and its fragments was also studied with three anti-Bos d 2 mAbs. All the mAbs recognized rBos d 2 well but failed to recognize any of the fragments (data not shown).

Inhibition of ELISA

To study the IgE-binding capacity of Bos d 2 and its fragments in the fluid phase, a series of inhibition experiments was performed with anti-Bos d 2-specific mAbs (mAb1 and mAb3) and five randomly selected patient sera (patients B, D, F, I, and K). In experiments with mAbs, recombinant or native Bos d 2 was attached to the solid phase; rBos d 2, nBos d 2, and recombinant fragments were used as inhibitors. Regardless of the solid-phase antigen or mAb, the results were similar (Fig. 3). At the concentrations used, the fragments of rBos d 2 did not inhibit antigen-antibody reactions at all. In all four experimental situations, the most efficient inhibitor was nBos d 2; 19 to 26 times

TABLE II. Proliferative responses of Bos d 2-specific T-cell clones against rBos d 2 and its fragments

Clone	Stimulation index/preparation			GST
	rBos d 2	rBos d 2 (1-131)	rBos d 2 (81-172)	
KE 17	37	53	< 2	< 1
KE 3-2	337	2	368	< 1
KE 9-10	370	< 1	350	< 1
FV 1-1	63	51	1	< 1
FV 1-9	322	1	250	< 1
FV 5-12	8	3	33	< 1

Results are indicated as stimulation indexes.

more of rBos d 2 was needed to cause the same level of inhibition (50% of the maximum reaction without inhibitor). The results of IgE inhibition experiments were comparable to those made with mAbs. The fragments did not cause any inhibition (data not shown), and rBos d 2 was a weaker inhibitor than nBos d 2. On an average, 60 times more (range 34 to 111) of rBos d 2 was needed to cause the same level of inhibition as nBos d 2.

In all experiments with mAbs, nBos d 2 completely inhibited the reaction between the solid-phase antigen (rBos d 2 or nBos d 2) and the antibody (Fig. 3). However, inhibition was not complete when human IgE was used as an antibody. The maximal inhibition (plateau) was obtained in all cases with the second-highest Bos d 2 concentration (1 µg/ml), and residual reactivity varied between 4% and 25% (mean 13%), depending on the serum.

Histamine release

The histamine-releasing capacity of rBos d 2 and its amino terminal fragment was examined with the peripheral blood leukocytes of three randomly selected asthmatic patients (B, D, and K). The complete rBos d 2 was clearly the less efficient histamine releaser than nBos d 2 (Table I); quantitatively, thousands of times more of rBos d 2 was needed to release the same amount of histamine. The poor IgE binder, rBos d 2 (1-131), also turned out to be capable of releasing histamine, although in two cases it was less efficient than the complete rBos d 2. In one case (patient B), the amount of rBos d 2 (1-131) that induced the 20% release of histamine was approximately 10 times lower than that of the complete rBos d 2. For this patient, the complete rBos d 2 also turned out to be an especially weak inhibitor in the ELISA inhibition test (it was needed 111 times more than nBos d 2; see above) and his IgE reacted weakly against rBos d 2 in the immunoblotting (data not shown).

Skin tests

Seven cow-asthmatic patients were subjected to SPTs. Fig. 4 shows the results of six patients with skin reactivity

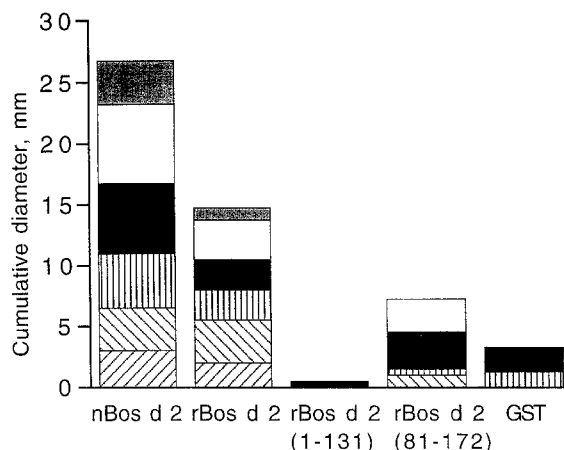


FIG. 4. Cumulative diameters of skin prick test reactions of six cow-asthmatic patients against different preparations at the highest protein concentrations used (nBos d 2 100 µg/ml, recombinant proteins 200 µg/ml). Different patterns in columns indicate different patients.

against the complete rBos d 2. The six patients gave strong positive reactions (diameter ≥ 3 mm; mean 4.5 mm) with nBos d 2 at 100 µg/ml. In contrast, rBos d 2 induced only two clearly positive reactions (> 3 mm) at the highest protein concentration used (200 µg/ml). Three of the four remaining patients had skin reactivity between ≥ 2 to 3 mm. One patient showed only a weak reaction (1 mm). As shown in Fig. 4, the fragments were less effective than rBos d 2 in inducing skin reactivity. The residual reactivity observed was mainly induced by the carboxy terminal end of the molecule, the cumulative diameter of the reaction being about 50% (7.2 mm) of that induced by the complete rBos d 2 (14.7 mm). In part, the residual reactivity of rBos d 2 (81-172) could have been caused by GST (cumulative diameter of 3.4 mm).

Responses of T-cell clones

Six rBos d 2-reactive T-cell clones from two cow-asthmatic patients (B and D) were tested with the fragments of rBos d 2. All these clones were CD4⁺ by FACS analysis. As shown in Table II, the clones responded to either amino or carboxy-terminal fragment of the molecule, with stimulation indices being at the same level between the complete molecule and the fragments.

DISCUSSION

One of the aims of allergen research in recent years has been the development of effective recombinant allergen preparations for immunotherapy at the same time minimizing the IgE-mediated side effects. Ideally, the construction of such preparations would start with detailed mapping of B-cell and T-cell epitopes of the allergen. This is not always feasible because great efforts are needed for such tasks. We have chosen another

approach based on the observation that allergen fragments seem to bind poorly to antibody.

The high and significant correlation between the IgE levels against the recombinant and native Bos d 2 proved that rBos d 2, produced as a fusion protein in *E. coli*, exhibits its IgE-binding epitopes in an immunologically functional manner in indirect ELISA. The carboxy terminal part of the molecule seemed to be critical for IgE binding because most of the human humoral reactivity against rBos d 2 was directed to this part of the molecule when the binding of IgE to the fragments was analyzed by ELISA or Western blot. The good proliferative responses of T-cell clones indicated that rBos d 2 and its fragments exhibited their T-cell epitopes properly.

Despite the fact that rBos d 2 bound efficiently IgE when it was attached to the solid phase, other experiments showed that the IgE-binding capacity of rBos d 2 was not equal to that of nBos d 2. For example, approximately 20 times more of rBos d 2 than nBos d 2 was needed in mAb-based ELISA inhibitions and approximately 60 times more in ELISA inhibitions with human IgE to achieve the same level of inhibition. The results of histamine release assays as well as SPTs were concordant with these findings. There are similar data suggesting that in some cases, recombinant allergens do not bind IgE as efficiently as their native equivalents.²⁰⁻²² This phenomenon has been attributed to either differences in glycosylation between the native and recombinant allergens, to isoallergens, or to the conformational modifications of recombinant molecules. It is known that carbohydrate groups may contain IgE-binding epitopes.²³ Recombinant allergens produced in bacteria do not have these groups, but this explanation does not apply to rBos d 2 because nBos d 2 is not a glycoprotein (unpublished data) and its amino acid sequence does not contain sites for glycosylation.⁸ The second possibility is that rBos d 2 represents just one of the isoallergens of Bos d 2. This we cannot rule out at the current stage of our cow allergen studies. However, this hypothesis does not explain the difference in the IgE-binding capacity of rBos d 2 between the solid and fluid phases compared with nBos d 2. The third possibility is that rBos d 2 does not take fully natural conformation, which is known to be important for IgE binding.^{20, 24} The weakness of IgE binding was prominent when rBos d 2 was in the fluid phase, not in the solid phase, and we find it interesting that this kind of a differential behavior of a recombinant allergen has been described previously.²² As to the conformational changes of rBos d 2 and its reduced IgE-binding capacity, one possibility is that the reduced IgE binding is due to the fusion part of the molecule, as has been suggested previously with the major birch pollen allergen.²¹

The important finding in this study was that in practically all analyses, the fragments of rBos d 2 exhibited less allergenic activity than the complete rBos d 2. There is scattered information suggesting that several allergens lose their allergenic properties if they are in the form of fragments.^{6, 24-26} It can be supposed that the diminished

IgE binding of the fragments is due to the conformational changes in their structure compared with the respective parts of the complete molecule. In the case that the epitopes are not destroyed by fragmentation, conformational changes may also cause the physical separation of epitopes within the fragment or between the fragments so that the cross-linking of IgE receptors on mast cells is not possible.

Our observation may have significant consequences because it may offer a simple way to produce hypoallergenic preparations for immunotherapy. The search for developing such preparations has been quite intense lately. One approach has been the use of shorter or longer peptides containing immunodominant T-cell epitopes.²⁷⁻²⁹ Even though this approach is attractive, its main problem is that individuals exhibit considerable variation in their responses against the epitopes of allergens³⁰; therefore the minimum requirement for practical immunotherapeutic use of epitope-containing peptides would be an individual mixture of them. However, the representative mapping of the epitopes to which an allergic individual's T cells react is a considerable task. To circumvent this problem, Rogers and coworkers have suggested the use of recombinant protein constructs that contain noncontiguous T-cell-reactive regions.⁶ An alternative possibility might be offered by the finding that isoallergens may possess different capacities in binding IgE.³¹ In this case, isoallergens of low IgE-binding capacity could be used for immunotherapy.

Taken together, our results indicate that the fragments of rBos d 2 that have been produced in *E. coli* are less allergenic than the complete rBos d 2 and that these fragments are potent stimulators of specific T-cell clones. Although exact mechanisms of allergen immunotherapy are not known, on the basis of the current knowledge on the regulation of T cells and the immune system, it can be anticipated that beneficial results in the therapy of allergic diseases could be obtained by targeting immunomodulatory effects to T cells.³² Because the production of defined recombinant proteins is within the reach of any competent immunologic laboratory and because allergen fragments in general may have a lowered IgE-binding capacity, this technology may offer a reproducible, simple, and more universal way to develop a wide range of hypoallergenic preparations of different allergens than the methods described so far. An important aspect is also that by the use of overlapping fragments instead of peptides, the problem of individual T-cell epitope recognition can be solved.

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REFERENCES

1. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone, I: definition according to profiles of lymphokine activities and secreted protein. *J Immunol* 1986;136:2348-57.

2. Romagnani S. Regulation of the development of type 2 T-helper cells in allergy. *Curr Opin Immunol* 1994;6:838-46.
3. Ohman JL Jr. Clinical and immunologic responses to immunotherapy. New York: Marcel Dekker, Inc.; 1991. p. 209-32.
4. Mapp CE, Saetta M, Maestrelli P, Distefano A, Chitano P, Boschetto P, et al. Mechanisms and pathology of occupational asthma. *Eur Respir J* 1994;7:544-54.
5. Liebers V, Sander I, van Kampen V, Raulf-Heimsoth M, Rozynek P, Baur X. Overview on denominated allergens. *Clin Exp Allergy* 1996;26:494-516.
6. Rogers BL, Bond JF, Craig SJ, Nault AK, Segal DB, Morgenstern JP, et al. Potential therapeutic recombinant proteins comprised of peptides containing recombined T cell epitopes. *Mol Immunol* 1994;31:955-66.
7. Ylönen J, Mäntyjärvi R, Taivainen A, Virtanen T. Comparison of the antigenic and allergenic properties of three types of bovine epithelial material. *Int Arch Allergy Immunol* 1992;99:112-7.
8. Mäntyjärvi R, Parkkinen S, Rytkönen M, Pentikäinen J, Pelkonen J, Rautiainen J, et al. Complementary DNA cloning of the predominant allergen of bovine dander: a new member in the lipocalin family. *J Allergy Clin Immunol* 1996;97:1297-303.
9. Virtanen T, Zeiler T, Rautiainen J, Taivainen A, Pentikäinen J, Rytkönen M, et al. Immune reactivity of cow-asthmatic dairy farmers to the major allergen of cow (BDA20) and to other cow-derived proteins: the use of purified BDA20 increases the performance of diagnostic tests in respiratory cow allergy. *Clin Exp Allergy* 1996;26:188-96.
10. Ylönen J, Virtanen T, Rytkönen M, Mäntyjärvi R. Quantification of a major bovine allergen by a two-site immunometric assay based on mAbs. *Allergy* 1994;49:707-12.
11. Smith DB, Johnson KS. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 1988;67:31-40.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Vol. 2. New York: Cold Spring Harbor Laboratory Press; 1989.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
14. Ylönen J, Virtanen T, Horsmanheimo L, Parkkinen S, Pelkonen J, Mäntyjärvi R. Affinity purification of the major bovine allergen by a novel monoclonal antibody. *J Allergy Clin Immunol* 1994;93:851-8.
15. Ylönen J, Mäntyjärvi R, Taivainen A, Virtanen T. IgG and IgE antibody responses to cow dander and urine in farmers with cow-induced asthma. *Clin Exp Allergy* 1992;22:83-90.
16. Cromwell O, Durham SR, Shaw RJ, MacKay JA, Kay AB. Provocation tests and measurements of mediators from mast cells and basophils in asthma and allergic rhinitis. In: Weir DM, editor. *Handbook of experimental immunology*. Oxford: Blackwell Scientific Publications; 1986. p. 127.1-127.51.
17. Yamatodani A, Fukuda H, Wada H, Iwaeda T, Watanabe T. High-performance liquid chromatographic determination of plasma and brain histamine without previous purification of biological samples: cation-exchange chromatography coupled with post-column derivatization fluorimetry. *J Chromatogr* 1985;344:115-23.
18. Frandsen P, Backman A, Cato Arntzen F, Sjöholm I, Busk M, Larhammar H. Registration of allergen preparations: Nordic guidelines. NLN Publication, No. 23. 2nd ed. Uppsala: Nordic Council on Medicines; 1989. p. 1-34.
19. Virtanen T, Maggi E, Manetti R, Piccinni MP, Sampognaro S, Parronchi P, et al. No relationship between skin-infiltrating Th2-like cells and allergen-specific IgE response in atopic dermatitis. *J Allergy Clin Immunol* 1995;96:411-20.
20. Dudler T, Chen WQ, Wang S, Schneider T, Annand RR, Dempsey RO, et al. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A2. *Biochim Biophys Acta* 1992;1165:201-10.
21. Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, et al. Purification and characterization of recombinant Bet v I, the major birch pollen allergen: immunological equivalence to natural Bet v I. *J Biol Chem* 1993;268:19574-80.
22. Slunt JB, Rogers BL, Chapman MD. IgE antibodies to recombinant forms of Fel d 1: dichotomy between fluid-phase and solid-phase binding studies. *J Allergy Clin Immunol* 1995;95:1221-8.
23. Batanero E, Villalba M, Rodriguez R. Glycosylation site of the major allergen from olive tree pollen: allergenic implications of the carbohydrate moiety. *Mol Immunol* 1994;31:31-7.
24. Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995;50:384-91.
25. Bond JF, Brauer AW, Segal DB, Nault AK, Rogers BL, Kuo MC. Native and recombinant Fel d I as probes into the relationship of allergen structure to human IgE immunoreactivity. *Mol Immunol* 1993;30:1529-41.
26. King TP, Kochoumian L, Lu G. Murine T and B cell responses to natural and recombinant hornet venom allergen Dol m 5.02 and its recombinant fragments. *J Immunol* 1995;154:577-84.
27. Briner TJ, Kuo MC, Keating KM, Rogers BL, Greenstein JL. Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d I. *Proc Natl Acad Sci U S A* 1993;90:7608-12.
28. Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T-cell and antibody responses to house dust mite allergen by inhalation of the dominant T-cell epitope in naive and sensitized mice. *J Exp Med* 1993;178:1783-8.
29. Oshiba A, Hamelmann E, Bradley KL, Loader JE, Renz H, Larsen GL, et al. Pretreatment with allergen prevents immediate hypersensitivity and airway hyperresponsiveness. *Am J Respir Crit Care Med* 1996;153:102-9.
30. Ebner C, Schenk S, Szepfalusi Z, Hoffmann K, Ferreira F, Willheim M, et al. Multiple T cell specificities for Bet v I, the major birch pollen allergen, within single individuals: studies using specific T cell clones and overlapping peptides. *Eur J Immunol* 1993;23:1523-7.
31. Ferreira F, Hirtenlehner K, Jilek A, Godnikvar J, Breiteneder H, Grimm R, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;183:599-609.
32. Secrist H, Dekruyff RH, Umetsu DT. Interleukin 4 production by CD4(+) T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J Exp Med* 1995;181:1081-9.