

T Cell Epitope-Containing Hypoallergenic Recombinant Fragments of the Major Birch Pollen Allergen, Bet v 1, Induce Blocking Antibodies¹

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Allergen-specific immunotherapy represents one of the few curative approaches toward type I allergy. Up to 25% of allergic patients are sensitized against the major birch pollen allergen, Bet v 1. By genetic engineering we produced two recombinant (r) Bet v 1 fragments comprising aa 1–74 and aa 75–160 of Bet v 1, which, due to a loss of their native-like fold, failed to bind IgE Abs and had reduced allergenic activity. Here we show that both fragments covering the full Bet v 1 sequence induced human lymphoproliferative responses similar to rBet v 1 wild type. The C-terminal rBet v 1 fragment induced higher lymphoproliferative responses than the N-terminal fragment and represented a Th1-stimulating segment with high IFN- γ production, whereas the N-terminal fragment induced higher IL-4, IL-5, and IL-13 secretion. Immunization of mice and rabbits with rBet v 1 fragments induced IgG Abs, which cross-reacted with complete Bet v 1 and Bet v 1-related plant allergens and strongly inhibited the IgE binding of allergic patients to these allergens. Thus, our results demonstrate that hypoallergenic T cell epitope-containing rBet v 1 fragments, despite lacking IgE epitopes, can induce Abs in vivo that prevent the IgE binding of allergic patients to the wild-type allergen. The overall demonstration of the immunogenic features of the hypoallergenic rBet v 1 fragments will now enable clinical studies for safer and more efficient specific immunotherapy. *The Journal of Immunology*, 2000, 165: 6653–6659.

Type I allergy represents a genetically determined immunodisorder that affects almost 25% of the population (1). The symptoms of type I allergy (i.e., allergic rhinoconjunctivitis, asthma, atopic dermatitis) are caused by the formation of IgE Abs against harmless Ags. Cross-linking of effector cell (e.g., mast cell, basophil)-bound IgE Abs by allergens causes immediate symptoms via the release of biological mediators (e.g., histamine, leukotrienes) (1–3), whereas IgE-mediated allergen presentation to T cells greatly enhances T cell activation, release of proinflammatory cytokines, and thus late reactions (4–5). Although immediate as well as late symptoms can be ameliorated by pharmacological treatment, specific immunotherapy represents a curative approach toward type I allergy (6–8). Specific immunotherapy is based on the administration of increasing doses of the disease-eliciting allergens to induce a state of unresponsiveness toward the applied allergens. Several clinical studies document the clinical efficacy of specific immunotherapy, but the mechanisms underlying immunotherapy are not precisely known (6). One major disadvantage of specific immunotherapy is that the administration

of allergenic material may cause severe and life-threatening anaphylactic side effects. To prevent spreading of allergens during injection immunotherapy and thus to reduce the rate of severe systemic anaphylactic reactions, allergen extracts were adsorbed to adjuvants (9–10). Furthermore, chemically modified allergen extracts (e.g., allergoids) with low IgE binding capacity have been developed to reduce anaphylactic side effects during specific immunotherapy (11–12).

During the last several years several groups have produced recombinant allergens by recombinant DNA technology, which equal the natural allergens regarding biochemical, biological, immunological, and structural properties (13, 14). Recombinant allergens can now be used for new forms of component-resolved diagnosis, which may represent the basis for immunotherapy tailored according to the sensitization profile of the patient (15). Moreover, recombinant DNA technology, as well as synthetic peptide chemistry, is currently applied to generate allergen derivatives for immunotherapy with reduced anaphylactic side effects (16). Here we report the characterization of recombinant hypoallergenic fragments of the major birch pollen allergen, Bet v 1, as candidate molecules for specific immunotherapy. Bet v 1 is recognized by >95% of birch pollen allergic patients, and 60% of these patients react exclusively with this allergen (17, 18). The cDNA coding for Bet v 1 was isolated (19), and recombinant Bet v 1, which has immunological and biological properties comparable to natural Bet v 1 (18, 20) and shares epitopes with homologous proteins present in the pollen of trees of the order *Fagales* and in plant-derived food was produced in *Escherichia coli* (21–24). When analyzed by circular dichroism spectroscopy, the bacterially expressed rBet v 1 wild-type molecule was folded and consisted of mixed α helical and β -sheet conformation (25, 26). The three-dimensional structure of rBet v 1 was determined by x-ray crystallography and nuclear magnetic resonance analysis (27).

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The fact that natural and recombinant Bet v 1 fragments failed to bind IgE Abs indicated that IgE epitopes of Bet v 1 belong to the conformational (discontinuous) type (26). When we engineered two rBet v 1 fragments comprising aa 1–74 and aa 75–160, we found that the fragments, despite comprising the full Bet v 1 sequence, lacked IgE binding capacity due to a loss of their fold but induced proliferation of rBet v 1-specific T cell clones (26). Moreover, rBet v 1 fragments had a >100-fold reduced capacity to induce basophil histamine release and immediate type skin reactions in birch pollen allergic patients (26, 28, 29). Both rBet v 1 fragments also failed to induce eosinophil activation and late reactions in skin blisters of birch pollen-allergic patients (30).

Here we investigated whether the hypoallergenic rBet v 1 fragments can induce proliferation in PBMC of birch pollen-allergic patients comparable to that induced by the complete rBet v 1 wild-type molecule and thus contain the relevant Bet v 1-specific T cell epitopes. We were also interested to compare the types and levels of cytokines released from PBMCs after incubation with rBet v 1 and rBet v 1 fragments. Furthermore, we investigated whether the hypoallergenic rBet v 1 fragments, despite loss of their conformational IgE epitopes, could induce IgG Abs in mice and rabbits, which recognize the folded wild-type allergen and Bet v 1-homologous allergens from other plants. Finally, we determined whether the rBet v 1 fragment-induced Abs can inhibit the recognition of complete rBet v 1 by IgE Abs from birch pollen-allergic patients. We discuss the possible advantages of using hypoallergenic T cell epitope-containing fragments of Bet v 1 for specific immunotherapy.

Materials and Methods

Patients' sera, plasmid vectors, E. coli strains, and recombinant allergens

Birch pollen-allergic patients were characterized by case history and skin prick testing. In addition, sera were screened for the presence of IgE Abs to birch pollen extract by radio allergosorbent test (RAST) (Pharmacia, Uppsala, Sweden) and by IgE immunoblotting as described (21).

Plasmid pET 17b and *E. coli* strain BL 21 (DE3) ($F^- ompT r_B^- m_B^-$ (DE3)) were purchased from Novagen (Madison, WI). Folded rBet v 1a, which had been expressed in *E. coli* and purified as described (31), was obtained from Biomay (Linz, Austria). rBet v 1 fragments, comprising aa 1–74 and aa 75–160, were generated by PCR, using the rBet v 1a cDNA as a template, subcloned into plasmid pET-17b, expressed in *E. coli* strain BL 21 (DE3), and purified as described (26).

Natural allergen extracts

Pollen from birch (*Betula alba*), alder (*Alnus glutinosa*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*), and oak (*Quercus alba*) were purchased from Allergon AB (Välinge, Sweden). Golden delicious apples were purchased from a local market (Vienna, Austria). Pollen extracts were prepared by homogenizing 1 g tissue in 10 ml H_2O_{dd} containing 5 mM PMSF using an ultraturrax (IKA, Heidelberg, Germany) and extracting at 4°C for 2 h. Extracts were then centrifuged for 30 min at $20,000 \times g$ to remove insoluble particles. Supernatants were lyophilized and stored at -20°C until use. Apple protein extracts were obtained by homogenizing fruits in SDS-sample buffer with an ultraturrax as described (24). Extracts were boiled for 5 min and centrifuged for 20 min at $10,000 \times g$ to remove insoluble materials. All protein extracts were checked for protein quantity and quality by SDS-PAGE (32) and Coomassie blue staining (Bio-Rad, Richmond, CA) (33).

T cell proliferation and measurement of cytokines

T cell experiments were performed using PBMC of Bet v 1-sensitized individuals who had not received immunotherapy. PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were suspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 50 μM 2-ME (Life Technologies, Grand Island, NY), and 10% heat-inactivated FCS (34, 35). PBMC (10^5 cells/well in 96-well flat-bottom plates; Costar, Cambridge, U.K.) were stimulated with different Ag doses (20, 2, and 0.2 $\mu\text{g/ml}$) of rBet v 1 monomer and rBet v 1 fragments

in triplicate. Proliferative responses were measured by [^3H]thymidine incorporation after 5 days (34). IL-4 was measured from 24-h supernatants and IFN- γ , IL-5, IL-10, and IL-13 were measured from 5-day supernatants by sandwich ELISA (35, 36). The sensitivities of the ELISAs were as follows: IFN- γ = 10 pg/ml (mAbs and standards were obtained from Dr. S. S. Alkan, Novartis, Basel, Switzerland); IL-4 = 20 pg/ml (mAbs and standards were obtained from Dr. C. H. Heusser, Novartis); IL-5 = 50 pg/ml, IL-10 = 50 pg/ml, IL-13 = 100 pg/ml (moAbs and standards were obtained from PharMingen, San Diego, CA). Results are shown as mean $\pm$ SD. Student's *t* test for paired samples was used for statistical analysis to compare results between paired stimulation conditions. When data were analyzed with a nonparametric test (Mann Whitney *U* test) no change of statistical significance was found between the groups.

Immunization of mice and rabbits and measurement of mouse IgG subclass responses

Eight-week-old female BALB/c mice were purchased from Charles River Breeding Laboratories (Kislegg, Germany). Animals were maintained in the animal care unit of the Department of Pathophysiology of the University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized monthly either with 5 μg of purified rBet v 1, rBet v 1 aa 1–74, or rBet v 1 aa 75–160 adsorbed to 200 μl of CFA (Sigma, St. Louis, MO). Blood samples were taken shortly before each of the four immunizations and stored at -20°C until use. IgE and IgG subclass (IgG1, IgG2a, IgG2b, and IgG3) responses to complete rBet v 1 were determined by ELISA as described (37, 38).

Rabbits were immunized three times with 200 μg of purified rBet v 1, rBet v 1 aa 1–74, or rBet v 1 aa 75–160 adsorbed to CFA over a period of 3 mo (Charles River Breeding Laboratories). Rabbit sera were tested for IgG response to complete rBet v 1 by immunoblotting as described (39).

Analysis of mouse and rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs for reactivity to Bet v 1 and Bet v 1-related allergens

Approximately 1 $\mu\text{g/cm}$ of purified rBet v 1 or 100 $\mu\text{g/cm}$ of natural tree pollen or apple extracts were separated by 14% SDS-PAGE (32) and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) (40). Nitrocellulose strips were blocked in buffer A (50 mM sodium phosphate, pH 7.5, 0.5% w/v BSA, 0.5% v/v Tween 20, 0.05% $\text{Na}_2\text{S}_2\text{O}_3$) two times for 5 min and once for 30 min and incubated overnight at 4°C with sera from mice or rabbits diluted 1:1000 in buffer A. Nitrocellulose strips were then washed three times in buffer A. Bound mouse and rabbit Abs were detected with a ^{125}I -labeled sheep anti-mouse Ig antiserum (Amersham, Buckinghamshire, U.K.) and an ^{125}I -labeled donkey anti-rabbit Ig antiserum (Amersham), both diluted in buffer A 1:1000, respectively, and visualized by autoradiography using Kodak XOMAT films and intensifying screens (Kodak, Heidelberg, Germany).

Inhibition of allergic patients IgE binding to rBet v 1 by anti-rBet v 1- or anti-rBet v 1 fragment-specific Abs as determined by ELISA competition

ELISA plates (Greiner, Kremsmünster, Austria) were coated with 1 $\mu\text{g/ml}$ purified rBet v 1 or 20 $\mu\text{g/ml}$ of pollen protein extracts overnight at 4°C. Plates were washed two times with PBS, 0.05% v/v Tween 20, blocked for 3 h at room temperature with PBS, 1% w/v BSA, 0.05% v/v Tween 20, and incubated overnight at 4°C with mouse or rabbit anti-rBet v 1, anti-rBet v 1 aa 1–74, or anti-rBet v 1 aa 75–160 Abs and, for control purposes, with the corresponding preimmune sera. Mouse preimmune and immune sera were diluted 1:20, rabbit preimmune and immune sera were diluted 1:100 in PBS, 0.5% w/v BSA, and 0.05% v/v Tween 20. Plates were washed five times with PBS and 0.05% v/v Tween 20 and incubated overnight at 4°C with sera from birch pollen-allergic patients, diluted 1:5 in PBS, 0.5% w/v BSA, and 0.05% v/v Tween 20. Plates were again washed five times with PBS, 0.05% v/v Tween 20 and bound IgE was detected with alkaline phosphatase-coupled mouse monoclonal anti-human IgE Abs (PharMingen), diluted 1:1000 in PBS, 0.5% w/v BSA, 0.05% v/v Tween 20 for 1 h at 37°C and 1 h at 4°C. After washing five times with PBS and 0.05% v/v Tween 20, plates were incubated in the dark with alkaline phosphatase substrate (Sigma) and absorbance was determined with an ELISA reader (Dynatech, Denkendorf, Germany). The percentage of reduction of human IgE binding after preincubation with mouse and rabbit immune sera was determined according to the formula: % inhibition of IgE binding = $100 - \text{OD}_i/\text{OD}_p \times 100$, where OD_i and OD_p represent extinctions after preincubation with immune serum and preimmune serum, respectively.

Results

Hypoallergenic rBet v 1 fragments induce lymphoproliferative responses and cytokine release from PBMC of birch pollen-allergic patients

To investigate whether rBet v 1 fragments contain the relevant Bet v 1-specific T cell epitopes, we performed cultures for proliferation and cytokine production with PBMC of birch pollen-allergic patients. Fig. 1A shows that rBet v 1 fragments as well as complete rBet v 1 induced a dose-dependent proliferation of PBMC from birch pollen-allergic patients. In all of the seven experiments performed with PBMC from five birch pollen-allergic individuals, the C-terminal rBet v 1 fragment aa 75–160 induced significantly higher PBMC stimulations than the N-terminal fragment ($p < 0.001$) (Fig. 1, A and B). rBet v 1 fragments as well as complete rBet v 1 induced dose-dependent release of IL-4, IL-5, IL-10, IL-13, and IFN- γ (Fig. 1A). Similar as in the proliferative responses, the C-terminal rBet v 1 fragment aa 75–160 induced significantly

higher IFN- γ and IL-10 secretion compared with the N-terminal fragment ($p < 0.001$) (Fig. 1, A and B). The overall IL-4, IL-5, and IL-13 production was slightly higher for rBet v 1 fragment aa 1–74 ($p < 0.05$) compared with complete rBet v 1 or the C-terminal fragment (Fig. 1B). This divergence in cytokine profiles between the two fragments is clearly reflected by the significantly higher ratio of IL-4/IFN- γ or IL-13/IFN- γ secretion induced by rBet v 1 or rBet v 1 fragment aa 1–74 ($p < 0.05$ for IL-4/IFN- γ ratio; $p < 0.01$ for IL-13/IFN- γ ratio) (Fig. 2).

Induction of Bet v 1-reactive IgG subclass responses in mice with rBet v 1 fragments

Groups of five mice each were immunized with rBet v 1 fragment aa 1–74 and rBet v 1 fragment aa 75–160, respectively. Table I displays the mean IgE and IgG subclass reactivities to rBet v 1 determined in serum samples obtained from each group before (0), after 12 wk (III) and 16 wk (IV) of immunization. We found that both rBet v 1 fragments induced IgE, IgG1, IgG2a, and IgG2b, but not IgG3 subclass responses to complete rBet v 1 (Table I). The IgE anti-rBet v 1 responses induced with the rBet v 1 fragments were rather low and could be detected only at serum dilutions of 1:10. IgG1 anti-rBet v 1 immunoreactivity was found at serum dilutions of 1:1000 and IgG2a/2b anti-rBet v 1 Abs could be detected at serum dilutions of 1:100 (Table I). In both serum samples obtained 12 and 16 wk after immunization the IgG1 anti-rBet v 1 Ab levels induced by the rBet v 1 fragment aa 1–74 were much lower (12 wk, 0.67; 16 wk, 1.36) than those induced by rBet v 1 fragment aa 75–160 (12 wk, 2.92; 16 wk, 2.88) (Table I). Likewise, we found that the IgG2a and IgG2b levels induced by the C-terminal fragment were higher than those induced by the N-terminal fragment (Table I). These findings, together with our observation that the anti-rBet v 1 aa 1–74 Ab levels induced by the first fragment were lower than the anti-rBet v 1 aa 75–160 Ab levels induced by the second fragment (data not shown) indicated a higher immunogenicity of rBet v 1 aa 75–160.

Hypoallergenic rBet v 1 fragments induce IgG Abs in rabbits and mice that cross-react with the Bet v 1 wild-type allergen and Bet v 1-homologous allergens

Rabbits and mice were immunized with rBet v 1 fragments, and serum samples were tested for cross-reactivity with rBet v 1 and Bet v 1-related plant allergens. Immunization of rabbits with purified rBet v 1 aa 1–74 or aa 75–160 gave rise to antisera that reacted with rBet v 1 wild type up to serum dilutions of 1:100,000. Rabbit antisera raised against rBet v 1 aa 1–74 (Fig. 3A, panel 1, lane 1) and against rBet v 1 aa 75–160 (Fig. 3A, panel 2, lane 1)

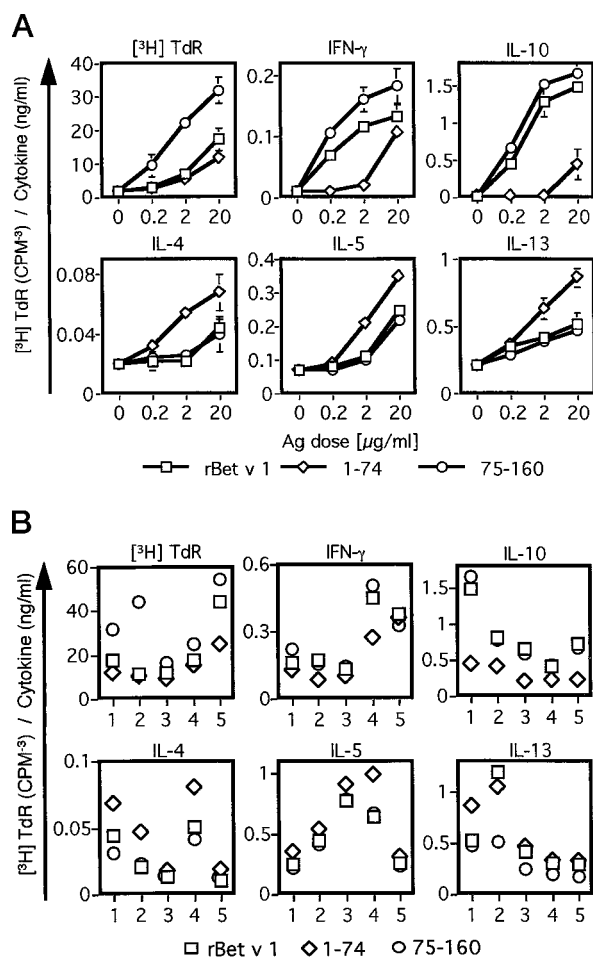


FIGURE 1. A and B, Induction of proliferation and cytokine production in PBMC from birch pollen-allergic patients with rBet v 1 and rBet v 1 fragments. PBMCs from five donors were stimulated with different doses of complete rBet v 1, rBet v 1 fragment aa 1–74, and rBet v 1 fragment aa 75–160. Supernatants were harvested 24 h later for IL-4 and 5 d later for IL-5, IL-10, IL-13, and IFN- γ . Thymidine incorporation was measured in parallel cultures at day 5. Results demonstrate means of two separate experiments in triplicate from a birch pollen-allergic patient (A). B, Thymidine incorporation and cytokine production of five patients with 20 $\mu\text{g/ml}$ of rBet v 1 and the two fragments. Background thymidine incorporation in unstimulated cultures was <3153 cpm; IFN- γ was <0.023 ng/ml; IL-10 was <0.05 ng/ml; IL-4 was <0.021 ng/ml; IL-5 was <0.08 ng/ml; and IL-13 was <0.14 ng/ml.

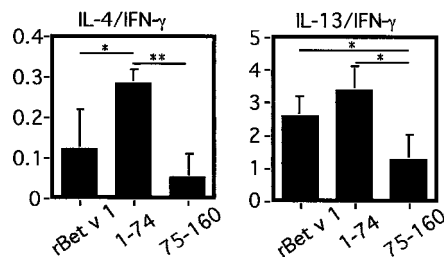


FIGURE 2. IL-4/IFN- γ and IL-13/IFN- γ ratios induced by rBet v 1 and rBet v 1 fragments. PBMC from five birch pollen-allergic patients were stimulated with 20 $\mu\text{g/ml}$ of complete rBet v 1, rBet v 1 fragment aa 1–74, and rBet v 1 fragment aa 75–160. Supernatants were harvested 24 h later for IL-4 and 5 days later for IL-13 and IFN- γ . Results shown are mean $\pm$ SD of triplicate experiments from five birch pollen-allergic patients. *, $p < 0.05$; **, $p < 0.01$.

Table I. Mean IgE and IgG subclass responses to rBet v 1 were determined for groups of five mice each immunized with rBet v 1 aa 1–74 or rBet v 1 aa 75–160^a

	rBet v 1 aa 1–74			rBet v 1 aa 75–160		
	0	III	IV	0	III	IV
IgE (1:10)	0.09 ± 0.01	0.14 ± 0.05	0.22 ± 0.22	0.11 ± 0.01	0.26 ± 0.14	0.32 ± 0.29
IgG1 (1:1000)	0.06 ± 0.01	0.67 ± 1.04	1.36 ± 1.07	0.05 ± 0.01	2.92 ± 0.14	2.88 ± 0.16
IgG2a (1:100)	0.08 ± 0.004	1.23 ± 1.06	1.42 ± 0.75	0.08 ± 0.01	2.20 ± 1.06	1.55 ± 1.03
IgG2b (1:100)	0.05 ± 0.003	0.69 ± 0.84	0.87 ± 1.22	0.05 ± 0.004	1.27 ± 0.88	0.85 ± 0.44
IgG3 (1:100)	0.05 ± 0.003	0.11 ± 0.03	0.07 ± 0.02	0.06 ± 0.01	0.18 ± 0.10	0.09 ± 0.03

^a The mean OD ± SD corresponding to the amount of bound Abs are displayed for IgE, IgG1, IgG2a, IgG2b, and IgG3 for serum samples obtained in each group shortly before (0), 12 wk (III), and 16 wk (IV) after the first immunization.

reacted with nitrocellulose-blotted rBet v 1, natural Bet v 1, and Bet v 1-related allergens present in pollens of alder, hazel, hornbeam, and oak, as well as in apples (Fig. 3A). The rabbits' preimmune sera showed no reactivity to the nitrocellulose-blotted allergens (Fig. 3A, lane P). The intensity of reactivity to Bet v 1-homologous allergens was in parallel to the degree of sequence homology. For example, the major allergen from alder pollen, Aln g 1, (41), which has a higher degree of sequence identity to Bet v 1 (81.1%) than the major hazel pollen allergen Cor a 1 (42) (72.3%), reacted stronger with the rabbit anti-rBet v 1 fragment Abs than Cor a 1 (Fig. 3A). Sera from all immunized mice contained IgG Abs to complete rBet v 1 wild type, however, with a somewhat lower titer (1:1000) than the rabbit antisera. As exemplified in Fig. 3B, mouse anti-rBet v 1 aa 1–74 (panel 1, lane I) and mouse anti-rBet v 1 aa 75–160 (panel 2, lane I) reacted with nitrocellulose-blotted rBet v 1, natural Bet v 1, and Bet v 1-related allergens, exhibiting a high degree of sequence identity with Bet v 1 (e.g., alder, Aln g 1 81.1%; hornbeam, Car b 1 73%) (Fig. 3B). No (apple, Mal d 1, 56.3%) or weak responses (hazel, Cor a 1, 72.3%, oak) to allergens with low sequence identity to Bet v 1 were found (data not shown). No reactivity was observed when blotted allergens were incubated with the corresponding mouse preimmune sera (Fig. 3B, lane P).

rBet v 1 fragment-induced mouse and rabbit Abs inhibit IgE binding of allergic patients to complete rBet v 1 wild type

Next we investigated whether rBet v 1 fragment-induced IgG Abs can inhibit the IgE binding of patients to the complete Bet v 1 wild-type allergen. Preincubation of ELISA plate-coupled rBet v 1

with a mouse serum raised against rBet v 1 fragment aa 1–74 inhibited the IgE binding of allergic patients (Table II, patients 1–4) to Bet v 1 between 45 and 83% (59.5% mean inhibition) (Table II). A mouse serum raised against rBet v 1 fragment aa 75–160 inhibited IgE binding to rBet v 1 of the same patients between 73 and 78% (74.8% mean inhibition).

In another series of experiments we analyzed rabbit anti-rBet v 1 fragment aa 1–74 and anti-rBet v 1 fragment aa 75–160 for their ability to block IgE binding of a representative number ($n = 70$) of birch pollen-allergic patients' sera to Bet v 1. In one series of experiments performed with 35 sera from Bet v 1-allergic patients, we found that rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs inhibited human IgE binding to rBet v 1 between 29 and 89% (62.1% mean inhibition) and between 22 and 76% (56.8% mean inhibition), respectively (data not shown). The mean inhibition obtained with a mixture of both antisera was slightly higher (64.1% mean inhibition) than that found for each antiserum (data not shown). Next, we compared the inhibitory capacity of the anti-fragment antisera with that of an antiserum raised against complete rBet v 1 in 35 additional Bet v 1-allergic patients (Table III). Rabbit anti-rBet v 1 aa 1–74 and anti-rBet v 1 aa 75–160 Abs inhibited human IgE binding to rBet v 1 between 33 and 88% (67% mean inhibition) and between 10 and 81% (54% mean inhibition), respectively (Table III). Rabbit anti-rBet v 1 Abs blocked human IgE binding to rBet v 1 between 17 and 86% (52% mean inhibition) (Table III). We also noted that the degree of inhibition was not associated with the levels of Bet v 1-specific IgE Abs present in the sera from allergic patients (data not shown).

FIGURE 3. A and B, Cross-reactivity of mouse and rabbit anti-rBet v 1 fragment Abs with Bet v 1 and Bet v 1-homologous allergens. Nitrocellulose-blotted rBet v 1 and natural allergen extracts were exposed to rabbit sera (A; rBet v 1, birch, alder, hazel, hornbeam, oak, apple) or mouse sera (B; rBet v 1, birch, alder, hornbeam) raised against purified rBet v 1 fragments (panel 1, aa 1–74; panel 2, aa 75–160) (lane I) or to the corresponding preimmune sera (lane P).

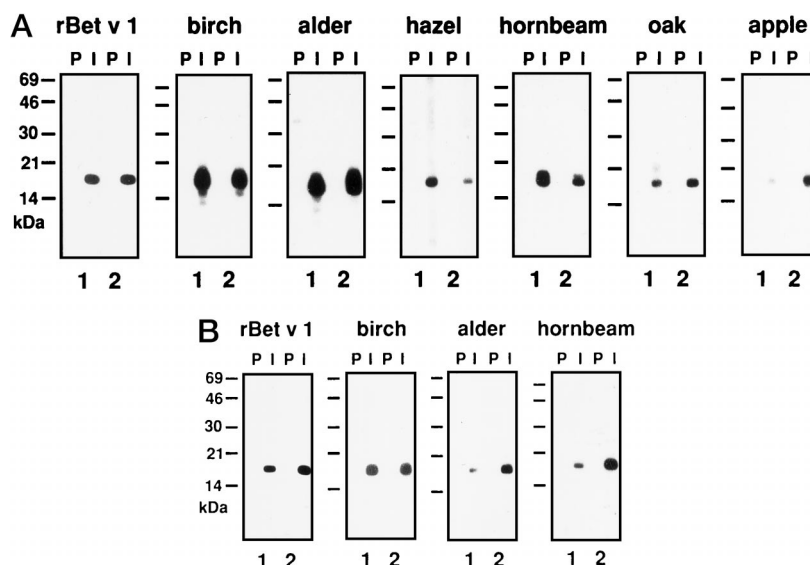


Table II. *Mouse anti-rBet v 1 fragment antisera inhibit allergic patient's IgE binding to Bet v 1^a*

Patient	% Inhibition of IgE Binding			
	1	2	3	4
Mouse anti-rBet v 1 aa 1–74	51	60	45	83
Mouse anti-rBet v 1 aa 75–160	74	78	74	73

^a The percentage inhibition of serum IgE binding of four patients to ELISA plate-bound Bet v 1 after preincubation with a mouse serum raised against rBet v 1 fragment aa 1–74 and against rBet v 1 fragment aa 75–160 is displayed.

rBet v 1 fragment-induced rabbit Abs inhibit IgE binding of allergic patients to natural Bet v 1 and Bet v 1-related Fagales pollen allergens

Natural pollen extracts represent allergen mixtures that also contain isoallergenic variants of certain allergens (43). Therefore, we were interested to investigate whether rabbit anti-rBet v 1 fragment antisera can also inhibit IgE binding of allergic patients to birch pollen extract and to pollen extracts from botanically related trees (alder, hornbeam, and oak) containing Bet v 1-homologous allergens. As exemplified in Table IV, we found that rabbit anti-rBet v 1 fragment antisera strongly inhibited IgE binding to birch pollen extract (Table IV, rabbit anti-rBet v 1 fragment aa 1–74, 64 and 54%; rabbit anti-rBet v 1 fragment aa 75–160, 58 and 49%). An albeit lower but substantial inhibition of IgE binding, ranging between 10 and 56%, to pollen extracts from alder, hornbeam, and oak, was observed (Table IV).

Discussion

More than 95% of birch pollen-allergic patients are sensitized against the major birch pollen allergen, Bet v 1 (17, 18). Recently we produced by genetic engineering two recombinant fragments of Bet v 1 comprising aa 1–74 and aa 75–160 (26). The recombinant Bet v 1 fragments had lost their IgE binding capacity due to lack of their conformation and thus exhibited a >100-fold reduced allergenic activity when tested for their ability to elicit immediate skin reactions in patients (26, 28, 29). Although T cell epitopes of several Bet v 1-specific T cell clones seemed to be present on the rBet v 1 fragments (26), no information was available as to whether the derivatives would be capable of inducing significant lymphoproliferative responses in birch pollen-allergic patients. In this study, we show that the recombinant Bet v 1 fragments contain the relevant Bet v 1-specific T cell epitopes by comparing their ability with that of complete rBet v 1 to induce proliferation of PBMC from birch pollen-allergic patients. The strong lymphoproliferative responses induced by the rBet v 1 fragments can be explained by the fact that both fragments together resemble the T cell epitope repertoire of complete rBet v 1 and the fact that they were engineered by selecting the breakpoint between the fragments out-

side known Bet v 1-specific T cell epitopes (26, 44). Our finding that rBet v 1 fragments were potent inducers of T cell responses are in line with a previous study demonstrating that changes in antigenic conformation to a non-IgE binding structure increases the T cell stimulation capacity and induces higher proliferation and IFN- γ production leading to decreased IL-4/IFN- γ and IgE/IgG ratios (45).

We consider the presence of relevant T cell epitopes on the rBet v 1 fragments as important for their potential use for immunotherapy of birch pollen allergy as there is evidence that induction of Th1 responses or T cell tolerance is associated with a successful outcome of immunotherapy (34, 35, 46–49). IL-10-induced peripheral T cell anergy and reactivation by microenvironmental cytokines were shown to be critical steps in specific immunotherapy (35, 49). In this context, induction of higher IL-10 levels by the second rBet v 1 fragment suggests that this particular fragment may also exert tolerogenic activity. Interestingly, the same fragment induced stronger IFN- γ secretion in PBMC of all patients tested and, in a murine model of nasal tolerance induction, was found to suppress Bet v 1-specific IgE production and airway hyperresponsiveness (50).

Although each of the recombinant Bet v 1 fragments had lost IgE binding capacity due to lack of structural fold, they were able to induce Bet v 1-specific Abs in mice and rabbits. The fact that the fragments failed to induce high IgE levels could be as much a feature of the adjuvant used as of the Ag. However, we consider it important that the fragment-induced Abs recognized the complete, folded wild-type allergen as well as Bet v 1-homologous allergens from other plants and inhibited IgE binding of allergic patients to the wild-type allergens. This finding indicates that rBet v 1 fragments contain sufficient sequence motifs to induce blocking Ab responses against the complete wild-type allergen *in vivo*. Because both rBet v 1 fragments represent unfolded molecules (26), it is unlikely that they induced IgG Abs to conformational (i.e., discontinuous) Bet v 1 IgE-defined epitopes. Therefore, their ability to block human IgE recognizing conformational Bet v 1 epitopes may be explained in two ways. One possibility is that the fragment-induced Abs bound in close proximity to the IgE-defined epitopes and thus exhibited steric hindrance of human IgE binding. A second, not mutually exclusive, possibility is that the fragment-induced Abs recognized continuous portions within discontinuous IgE epitopes and thus were able to prevent IgE binding.

Recently, increasing evidence has accumulated that the induction of blocking Abs of the IgG isotype may be important for the successful outcome of immunotherapy in patients. It has been shown that allergen-specific IgG Abs can inhibit the IgE-mediated release of biological mediators from effector cells and thus may prevent immediate symptoms (37, 51–53). Although not yet proven, it is also possible that blocking Abs may capture allergens during natural allergen exposure and thus prevent allergen-induced

Table III. *Rabbit anti-rBet v 1 fragment and anti-rBet v 1 antisera inhibit allergic patient's binding to rBet v 1 wild type^a*

Patient	% Inhibition of IgE Binding																																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Rabbit anti-rBet v 1 aa 1–74	78	76	71	45	63	70	52	75	81	84	47	73	72	61	40	64	39	66	40	80	85	87	69	88	83	64	69	75	33	86	75	80	66	67	38
Rabbit anti-rBet v 1 aa 75–160	45	62	45	10	32	55	47	66	57	62	43	57	62	22	49	42	21	53	33	69	71	80	58	71	76	66	54	74	28	81	66	73	54	65	38
Rabbit anti-rBet v 1	62	83	71	41	76	63	70	58	57	75	58	41	44	17	54	69	26	44	23	67	79	86	46	80	53	23	21	32	26	51	47	43	49	43	29

^a The percentage inhibition of IgE binding after preincubation of rBet v 1 with rabbit anti-rBet v 1 aa 1–74, rabbit anti-rBet v 1 aa 75–160, and with a rabbit anti-rBet v 1 antiserum is displayed for sera from 35 birch pollen-allergic patients (1–35).

Table IV. Rabbit anti-rBet v 1 fragment antisera inhibit patient's IgE binding to natural tree pollen extracts^a

Patient	% Inhibition of IgE Binding							
	Birch		Alder		Hornbeam		Oak	
	A	B	A	B	A	B	A	B
Rabbit anti-rBet v 1 aa 1–74	64	54	10	12	27	30	20	11
Rabbit anti-rBet v 1 aa 75–160	58	49	56	40	16	12	35	15

^a ELISA plate-coated pollen extracts from birch, alder, hornbeam, and oak were preincubated with rabbit anti-rBet v 1 aa 1–74 and aa 75–160. The percentage of inhibition of IgE binding is displayed for two allergic patients (A, B).

risers of IgE production in patients. Experimental support for the beneficial role of blocking Abs regarding late allergic symptoms comes from two other recent observations that 1) sera containing therapy-induced allergen-specific IgG Abs were found to suppress IgE-mediated presentation of allergens to T cells and thus to reduce T cell proliferation and cytokine release (54); and 2) hypoallergenic rBet v 1 derivatives lacking the capacity to induce cross-linking of effector cell-bound IgE Abs also failed to activate eosinophils, suggesting that allergen-induced IgE-mediated activation of eosinophils is operative in vivo and might be suppressed by blocking Abs (30).

Our observation that hypoallergenic rBet v 1 fragments induced strong lymphoproliferative responses and, despite lacking IgE epitopes, could induce blocking Ab responses in vivo suggests that both molecules might represent candidate molecules for immunotherapy of birch pollen allergy. Using recombinant birch pollen allergens for component-resolved diagnosis (15), patients who are not sensitized to Bet v 1 or who are sensitized to birch pollen allergens other than Bet v 1 can be excluded from treatment. Different regimens of immunotherapy may be considered for the rBet v 1 fragments (e.g., rush immunotherapy, sublingual immunotherapy, and tolerance induction) but we suggest to administer the molecules bound to adjuvants by conventional injection immunotherapy because it was shown that administration of unbound peptides, even when they contain exclusively T cell epitopes and lack allergenic activity, can elicit systemic side effects (i.e., asthma) (55).

We anticipate several advantages from an immunotherapy protocol with hypoallergenic rBet v 1 fragments. Selection of patients by component-resolved diagnosis will allow component-resolved immunotherapy with the disease-eliciting allergen and thus prevent de novo sensitization against other components present in natural allergen-extracts (15, 53). As rBet v 1 fragments exhibited a >100-fold reduced allergenic activity, we expect fewer side effects and think that higher doses can be administered. The latter will likely favor the rise of a Bet v 1-specific Th1 immune response, which is accompanied by the induction of high levels of Bet v 1-specific blocking Abs belonging to the IgG isotype. The fact that the rBet v 1 fragment-induced Abs cross-reacted with Bet v 1-homologous allergens from other plants even gives rise to the hope that also birch pollen-related allergies may be treated with the new vaccine. In this context it has been shown that conventional immunotherapy with birch pollen extract was effective for the treatment of allergies to other *Fagales* pollens (e.g., alder, hazel) (56) and birch pollen-related plant food allergies (e.g., apple) (57). Clinical trials with the rBet v 1 fragment-based vaccine will now have to follow. Such trials will allow the study of mechanisms underlying injection immunotherapy at a component level and will determine whether a rBet v 1 fragment-based vaccine is clinically effective for the treatment of birch pollen allergy.

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