Conversion of the Major Birch Pollen Allergen, Bet v 1, into Two Nonanaphylactic T Cell Epitope–containing Fragments

Candidates for a Novel Form of Specific Immunotherapy

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Abstract

A novel approach to reduce the anaphylactic activity of allergens is suggested. The strategy makes use of the presence of conformational immunoglobulin E (IgE) epitopes on one of the most common allergens. The three dimensional structure of the major birch pollen allergen, Bet v 1, was disrupted by expressing two parts of the Bet v 1 cDNA representing amino acids 1–74 and 75–160 in *Escherichia coli*. In contrast to the complete recombinant Bet v 1, the fragments showed almost no allergenicity and exhibited random coil conformation as analyzed by circular dichroism. Both nonanaphylactic fragments induced proliferation of human Bet v 1–specific T cell clones, indicating that they harbored all dominant T cell epitopes and therefore may be considered as a basis for the development of a safe and specific T cell immunotherapy. (*J. Clin. Invest.* 1997. 99:1673–1681.) Key words: major birch pollen allergen • Bet v 1 • recombinant allergen fragments • histamine release • skin testing

Introduction

Type I allergy represents a major health problem in industrialized countries where > 20% of the population suffers from type I allergic reactions (allergic rhinitis, conjunctivitis, allergic asthma, and anaphylactic shock) (1). Environmental proteins from pollen, mites, and animal dander belong to the major components which induce release of biological mediators (e.g., histamine) by cross-linking of effector cell (mast cell, basophil)-bound specific IgE antibodies. The production of specific IgE from B cells is stimulated by allergen-specific T helper cells which in their majority belong to the TH2 type (2). Therapy of type I allergic diseases is currently performed by pharmacological treatment and by specific immunotherapy. Specific immunotherapy was established early in this century (3)

and involves the systemic application of increasing doses of allergens for extended periods. Although specific immunotherapy is recognized as effective treatment, the occurrence of anaphylactic side effects represents one of the major disadvantages of this therapy. To reduce anaphylactic reactions, the use of T cell epitopes has recently been proposed for allergen-specific immunotherapy (4, 5).

Allergens harbor a great variety of different T cell epitopes (6–8) which may overlap with continuous IgE epitopes. To prevent cross-linking of effector cell (mast cell, basophil)-bound IgE and mediator release, T cell epitopes and IgE epitopes need to be dissected. Following the concept of converting a major allergen into a T cell vaccine, we have selected Bet v 1 (9), the major birch pollen allergen, as a model.

Bet v 1 was selected because epitope analysis indicated that it forms conformational IgE epitopes (10, 11). In addition, Bet v 1 represents one of the most common allergens which is recognized by 95% of tree pollen and food allergic individuals and almost 60% of them are sensitized exclusively against Bet v 1 (12). The cDNA coding for Bet v 1 has been isolated recently (9) and recombinant Bet v 1 was expressed in *Escherichia coli* (13, 14). Recombinant Bet v 1 has been shown to possess an IgE-binding capacity similar to that of natural Bet v 1 and shares IgE as well as T cell epitopes with Bet v 1 homologous proteins present in pollen from various trees and in plant-derived food (6, 15, 16). The biological activity of recombinant Bet v 1 has been demonstrated by histamine release experiments and by skin prick testing of allergic patients (17–19).

In this study we tested whether disruption of the Bet v 1 conformation may abolish allergenicity and simultaneously maintain relevant T cell determinants. The expression of two nonanaphylactic Bet v 1 fragments which harbor all relevant known T cell epitopes of the complete Bet v 1 molecule in *E. coli*, their purification, and structural and immunologic as well as their in vivo characterization as possible candidates for T cell–based specific immunotherapy are reported.

Methods

Sera from allergic patients, antibodies, protein extracts, plasmids, and E. coli strains. Sera from birch pollen allergic patients and control individuals were characterized by RAST and testing with recombinant allergens as described (13, 20). In addition, all patients were characterized by case history and skin prick test. The mouse mAb 14 with specificity for aa 40–66 of Bet v 1 is described (21). Natural birch pollen extract was prepared as described (22). Plasmid pET-17b containing the ampicillin resistance and a T7 promoter was obtained from Novagen, Inc. (Madison, WI). Recombinant Bet v 1 fragments were expressed in λDE3 lysogens of *E. coli* strain BL21 (F− *ompT* r_B-m_B-) (23).

Expression of Bet v 1 (aa 1–74, aa 75–160) fragments in E. coli. Recombinant Bet v 1 fragments (aa 1–74 and aa 75–160) were generated to maintain the epitopes (aa 44–60) of murine monoclonal antibodies which inhibited binding of allergic patients IgE to Bet v 1 (21) and to preserve major T cell epitopes which had been mapped using overlapping peptides synthesized according to the Bet v 1 sequence (6). The cDNAs coding for fragment aa 1–74 and 75–160 were obtained by PCR amplification of the Bet v 1 cDNA using the following oligonucleotide primers (Pharmacia, Uppsala, Sweden): Bet v 1 (aa 1–74): 5′ GGG AAT TCC *ATA TG*G GTG TTT TCA ATT AC 3′; 5′ CGG *GGT ACC* TTA CTC ATC AAC TCT GTC CTT 3′; Bet v 1 (aa 75–160): 5′ GGG AAT TCC *ATA TG*G TGG ACC ACA CAA ACT 3′; 5′ CGG *GGT ACC* TTA GTT GTA GGC ATC GGA 3′.

The EcoRI sites which were incorporated in the first primers are underlined, NdeI and KpnI sites are printed in italics. To improve subcloning efficiency, PCR products were first cut with EcoRI and KpnI, purified by preparative agarose gel electrophoresis, subcloned into EcoRI and KpnI sites of plasmid pET-17b (Novagen, Inc.), and transformed into *E. coli* BL 21 (DE3) (Novagen, Inc.) by electroporation. Inserts were then excised with NdeI/KpnI and subcloned again in plasmid pET-17b and transformed. Colonies expressing the correct fragments were identified by immunoscreening using mAb 14 for Bet v 1 aa 1–74 and a rabbit anti–Bet v 1 COOH-terminal antiserum for Bet v 1 aa 75–160. DNA from positive clones was isolated using Qiagen tips (Qiagen, Hilden, Germany) and both DNA strands were sequenced according to Sanger using a T7 polymerase sequencing kit (Pharmacia) and [^{35}S]dCTP (NEN, Stevenage, United Kingdom) (24). Recombinant Bet v 1 (aa 1–74) and Bet v 1 (aa 75–160) were expressed in *E. coli* BL 21 (DE 3) by induction with 0.5 mM isopropyl-β-thiogalactopyranoside at an OD600 of 0.5–0.8 in liquid culture for 5 h at 37°C.

Purification of recombinant Bet v 1 (aa 1–74) and Bet v 1 (aa 75–160). Recombinant Bet v 1 (aa 1–74) and recombinant Bet v 1 (aa 75–160) were expressed in inclusion bodies isolated as described (25). Inclusion bodies were solubilized with 8 M urea, 10 mM Tris, pH 8, 1 mM EDTA, 5 mM β-mercaptoethanol, diluted with 10 mM Tris, pH 8, to a concentration of 6 M urea and centrifuged for 15 min at 10,000 g to remove insoluble material. The supernatant, containing the recombinant protein, was dialyzed to a final concentration of 2 M urea. After centrifugation (15 min, 10,000 g), the supernatant was applied to a DEAE Sepharose (Pharmacia) column, and the protein was eluted with a 0–0.5 M NaCl concentration gradient. Fractions, containing the recombinant protein which was > 80% pure, were dialyzed against 6 M urea, 10 mM NaH$_2$PO$_4$, pH 4.8, and rechromatographed on an SP Sepharose column (Pharmacia). Fractions, containing recombinant Bet v 1 (aa 1–74) or recombinant Bet v 1 (aa 75–160) of > 95% purity, were dialyzed against 10 mM Tris, pH 7.5, and lyophilized until use.

Circular dichroism analysis of purified Bet v 1 fragments. Circular dichroism (CD)[1] spectra were recorded on a spectropolarimeter (model J-710; Jasco, Inc., Easton, MD) fitted with a thermostated cell holder and interfaced with a water bath (model RTE-110; NESLAB Instruments, Inc., Portsmouth, NH). The instrument was calibrated with a 0.10% aqueous solution of D-10-camphor-sulfonic acid. The results were expressed as the mean residue ellipticity at a given wavelength. Far ultraviolet CD spectra were recorded at 20°C in a 1-mm path-length quartz cuvette (Hellma, Jamaica, NY), at protein concentrations ranging from 4 to 53 μM, depending on the experiment and the protein under analysis. Spectra were recorded with 0.1 nm resolution and results were expressed as an average of 10 scans. Final spectra were corrected by subtracting the corresponding base line spectra, obtained for the buffer under identical conditions. All measurements were performed in 20 mM potassium phosphate, pH 7.0, containing 20 mM NaCl.

IgE-binding capacity of recombinant Bet v 1 and Bet v 1 fragments. Purified recombinant Bet v 1 and Bet v 1 fragments (aa 1–74, aa 75–160) were tested for IgE-binding capacity by Western blotting and in dot blot assays. For immunoblotting, ∼ 1 μg/cm purified protein was separated by SDS-PAGE (26) and blotted onto nitrocellulose according to Towbin (27). To avoid denaturation of the proteins, dot blot experiments were performed in parallel. 1 μg purified recombinant Bet v 1, 1 μg of each Bet v 1 fragment, and 1 μg of BSA and HSA (negative controls) were dotted on nitrocellulose strips.

Nitrocellulose strips containing Western blotted allergens or the dot blotted proteins were incubated with serum IgE from allergic individuals, nonallergic control individuals, and buffer without addition of serum as described (28). Bound IgE antibodies were detected with ^{125}I-labeled anti–human IgE antibodies and visualized by autoradiography using Kodak XOMAT films and intensifying screens (Kodak, Heidelberg, Germany) at −70°C.

Bet v 1–specific T cell clones, T cell proliferation assays. T cell lines were established by incubating PBMC of birch pollen allergic volunteers with Bet v 1 (10 μg/ml) (6). T cell blasts from Bet v 1–specific T cell lines were seeded in limiting dilution (0.3 cells/well) in 96-well round-bottom plates (Nunclone; Nunc, Roskilde, Denmark) together with 10^5 irradiated (5,000 rad) allogeneic PBMC as feeder cells, 1% vol/vol PHA (Gibco Laboratories, Grand Island, NY), and rIL-2 (Boehringer Mannheim, Mannheim, Germany) (4 U/well). The specificity of the T cell clones was determined by incubating 2–5 × 10^4 T cell blasts in triplicate cultures in 96-well plates together with 10^5 autologous irradiated PBMC with birch extract, purified nBet v 1, rBet v 1, and overlapping peptides spanning the Bet v 1 sequence for 48 h at 37°C, 5% CO$_2$ in a humidified atmosphere. Cells were then pulsed for 16 h with [^{3}H]thymidine and harvested, and [^{3}H] uptake was determined by scintillation counting. Proliferative responses were considered positive when the stimulation index (ratio between cpm obtained in cultures with T cell clones plus autologous irradiated PBMC plus antigen and cpm obtained in cultures containing T cell clones and PBMC alone) exceeded 10. Nine Bet v 1–specific T cell clones, recognizing eight relevant T cell epitopes (6), were selected for experiments with Bet v 1 fragments.

Histamine release experiments. Granulocytes were isolated from heparinized blood of birch pollen allergic individuals by dextran sedimentation (29). Cells were incubated with different concentrations (0.001–10 μg/ml) of purified recombinant Bet v 1, recombinant Bet v 1 fragments (aa 1–74, aa 75–160) separately and in an equimolar mixture, or anti–human IgE antibodies. Histamine released in the supernatant was measured by RIA (Immunotech, Marseille, France) (17). Total histamine was determined in cell lysates after freeze thawing. Results were obtained as mean values from triplicate determinations and expressed as a percentage of total histamine release.

Skin testing. Skin prick tests were performed on the individuals' forearms by placing 20 μl of each solution (18, 19). Recombinant Bet v 1 and recombinant Bet v 1 fragments were freshly dissolved in a 0.9% wt/vol sterile sodium chloride solution at concentrations of 100 and 10 μg/ml. As controls, birch pollen SQ (standard quality) extract, sodium chloride solution (negative control), and histamine hydrochloride (positive control) (ALK, Horsholm, Denmark) were used. Each drop was pricked with a fresh prick lancet (ALK) and results were recorded after 20 min with a ball point pen by transferring the wheal area with a tape to paper and by photography. The mean wheal diameter (Dm) was calculated by measuring the maximal longitudinal diameter (D) and the maximal transversal diameter (d) according to the formula: $[D + d]/2 = Dm$.

Results

Purification of recombinant Bet v 1 fragments to homogeneity. Recombinant Bet v 1 fragments were expressed using plasmid pET-17b (Novagen, Inc.) and *E. coli* BL21 (DE3) in liquid culture. The Coomassie blue–stained SDS-PAGE in Fig. 1 *A*

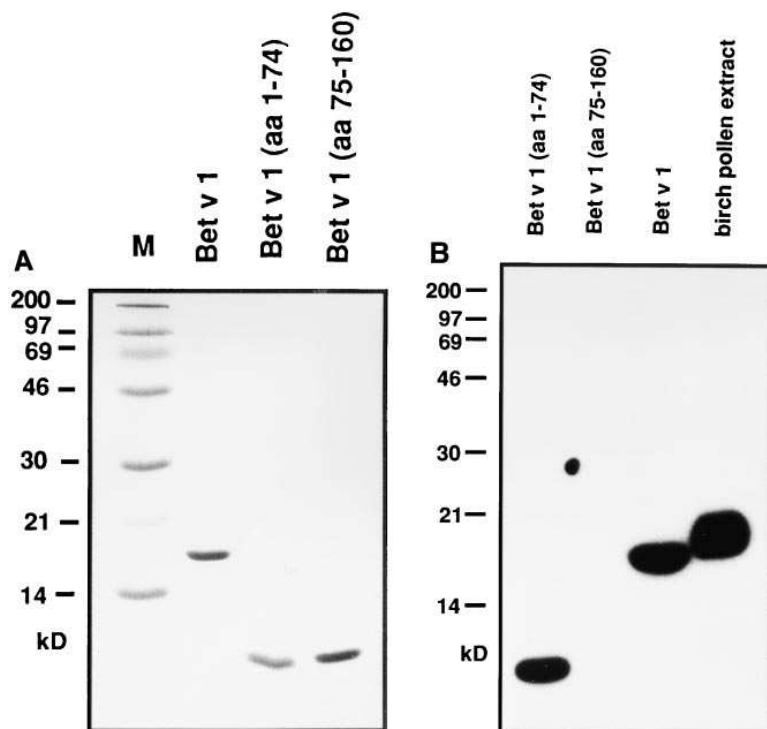1. *Abbreviation used in this paper:* CD, circular dichroism.

Figure 1. Purification of recombinant Bet v 1 fragments. In *A*, 2 μg each of purified recombinant Bet v 1 and the two Bet v 1 fragments (aa 1–74, aa 75–160) were analyzed by SDS-PAGE and Coomassie blue staining. In lane *M*, a molecular weight marker was loaded. *B* shows the reactivity of nitrocellulose blotted natural birch pollen extract, recombinant Bet v 1, and the two Bet v 1 fragments with a mouse monoclonal antibody recognizing aa 44–66 of Bet v 1. The bound monoclonal antibody was detected with a ^{125}I-labeled sheep anti–mouse antiserum and visualized by autoradiography.

shows that pure recombinant Bet v 1 fragments (aa 1–74, aa 75–160) were obtained by the purification protocol. The purified fragments were > 95% soluble as evaluated by subjecting the dissolved proteins to high speed centrifugation and subsequent analysis of the supernatant and pellet fraction (data not shown).

The immunoblot in Fig. 1 *B* shows that the binding site for a mouse monoclonal antibody located between aa 44–66 on the complete Bet v 1 molecule was preserved on the Bet v 1 fragment aa 1–74. The mouse monoclonal antibody was able to discriminate Bet v 1 fragment aa 1–74 and aa 75–160 indicating the presence of different B cell epitopes on the fragments.

CD analysis of recombinant Bet v 1 fragments (aa 1–74, aa 75–160). Far ultraviolet CD spectra of the purified recombinant Bet v 1 fragments aa 1–74 and aa 75–160 (Fig. 2 *A*) indi-

cate that the proteins contain predominantly random coil conformation. No variations in the CD spectra were observed upon mixing the two fragments at equimolar concentration (Fig. 2 *B*). Several spectra were recorded over a time range of 18 h from the equimolar solution preparation and no changes were observed in the dichroic signal (data not shown). From this it may be concluded that the two recombinant fragments do not show any tendency to fold, even in the presence of each other. In addition, no sign of aggregation was observed after analysis of different sample concentrations: Bet v 1 fragment aa 1–74 (13 and 53 μM), fragment aa 75–160 (5 and 23 μM), and two equimolar solutions (4 and 15 μM) (data not shown).

IgE-binding of allergic patients to recombinant Bet v 1 fragments is almost completely abolished. We compared the IgE-binding capacity of recombinant Bet v 1 and the two Bet v 1
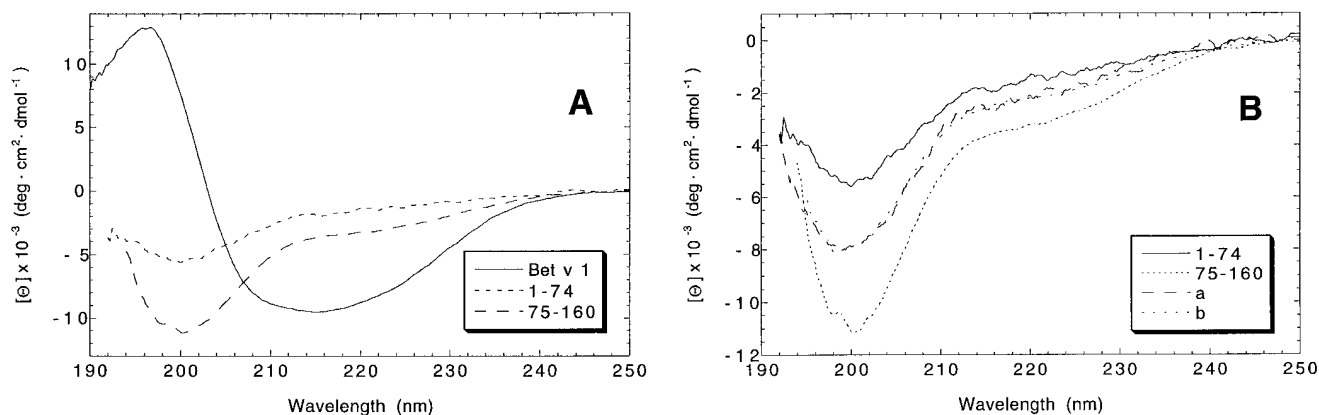

Figure 2. Far ultraviolet CD spectra of recombinant Bet v 1 and recombinant Bet v 1 fragments (aa 1–74, aa 75–160). (*A*) Complete recombinant Bet v 1 10 μM; fragment 1–74 14 μM; fragment 75–160 23 μM. (*B*) Fragments as in *A*; (*a*) fragments 1–74 and 75–160 in equimolar solution (4 μM); (*b*) fragments 1–74 and 75–160 in equimolar solution (15 μM). Spectra are expressed as mean residue ellipticity ([Θ]) at 20°C. Proteins were in 20 mM potassium phosphate, pH 7.0, containing 20 mM NaCl.
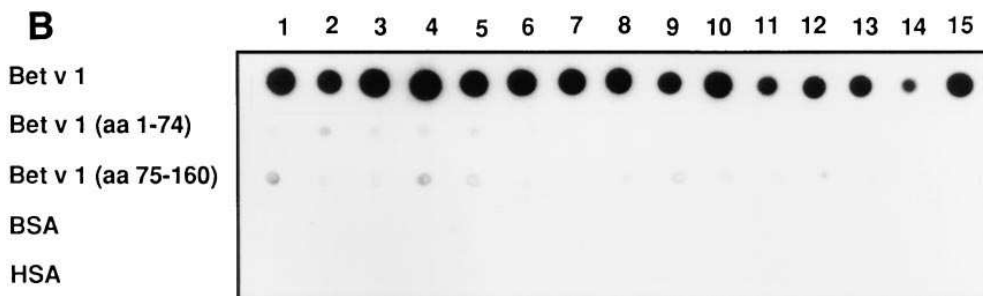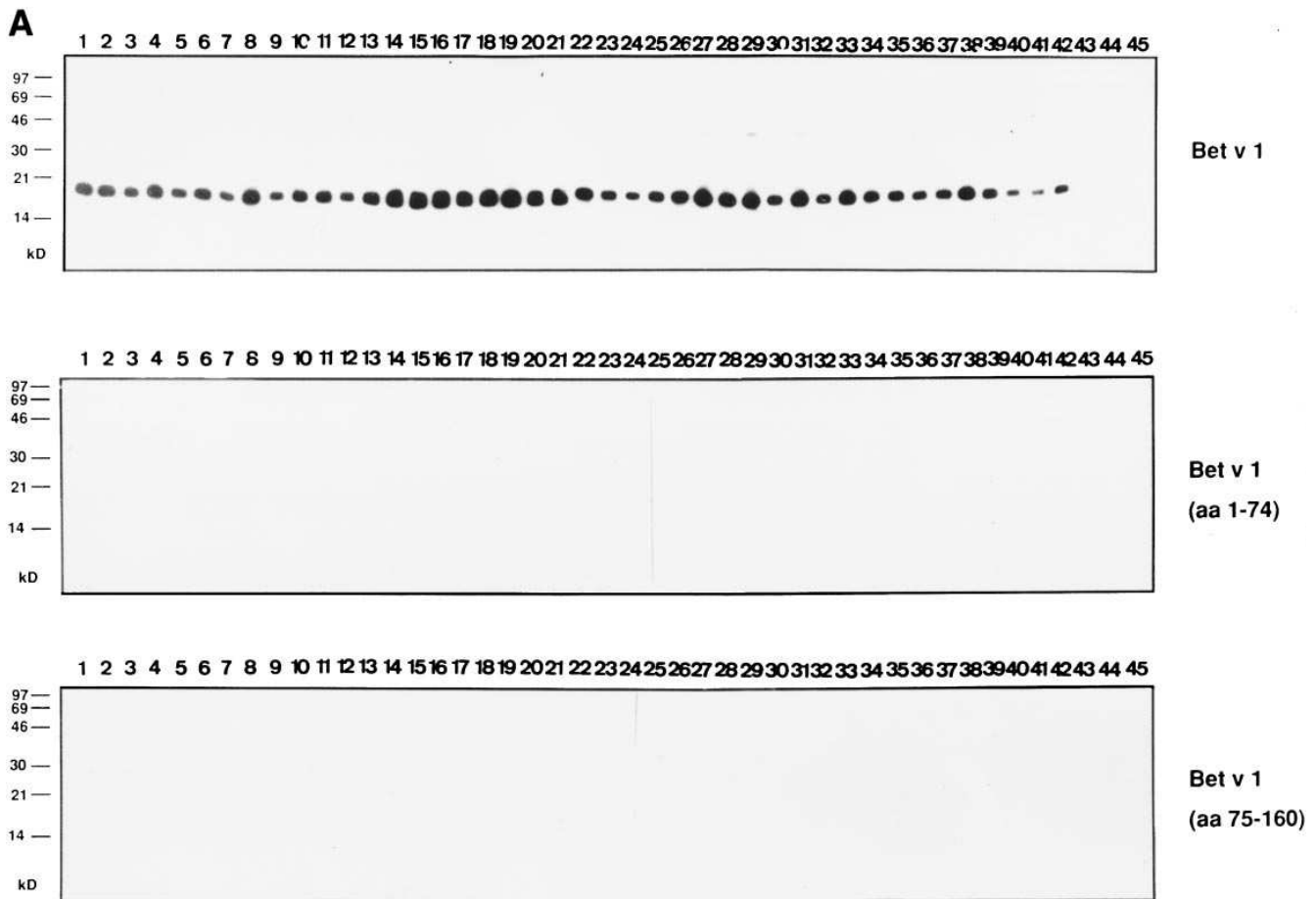
Figure 3. IgE-binding capacity of recombinant Bet v 1 and recombinant Bet v 1 fragments. *A* shows the IgE reactivity of sera from 42 birch pollen allergic individuals (lanes *1–42*), a grass pollen allergic patient without birch pollen allergy (lane *43*), a nonallergic individual (lane *44*), and buffer without addition of serum with the nitrocellulose blotted proteins. In *B*, sera from additional 15 birch pollen allergic individuals were tested for IgE reactivity with dot-blotted purified Bet v 1, purified Bet v 1 fragments (aa 1–74, aa 75–160), BSA, and HSA. Bound IgE was detected with ^{125}I-labeled anti–human IgE antibodies (Pharmacia) and visualized by autoradiography.

fragments (aa 1–74, aa 75–160) using Western blotting as well as nondenaturing dot blot assays. Sera from 42 birch pollen allergic patients displayed IgE reactivity to nitrocellulose blotted recombinant Bet v 1, whereas no IgE-binding to any of the two recombinant Bet v 1 fragments (aa 1–74, aa 75–160) could be detected (Fig. 3 *A*). Sera from a grass pollen allergic individual and a nonallergic person as well as buffer controls showed no IgE reactivity to Bet v 1 or the recombinant fragments (Fig. 3 *A*, lanes *43–45*).

Fig. 3 *B* shows the IgE reactivity of sera from 15 additional birch pollen allergic patients with dot blotted recombinant Bet v 1, the recombinant Bet v 1 fragments (aa 1–74, aa 75–160), and control proteins (BSA, HSA). All sera displayed intensive

IgE reactivity to the complete recombinant Bet v 1 (aa 1–160) whereas almost no IgE binding to the Bet v 1 fragments could be detected. The weak binding observed for sera 1–5, 8, 9, and 12 was estimated to represent < 1/1,000 of the reactivity to the complete Bet v 1 allergen by densitometry. No serum showed IgE binding to the control proteins (BSA, HSA).

Recombinant Bet v 1 fragments aa 1–74 and 75–160 harbor the relevant T cell epitopes of complete Bet v 1. The T cell epitopes of nine Bet v 1–specific T cell clones established from four different birch pollen allergic patients were determined by mapping with synthetic overlapping dodecapeptides. The ability of the two recombinant Bet v 1 fragments aa 1–74 and aa 75–160 to induce specific proliferation was investigated using T cell
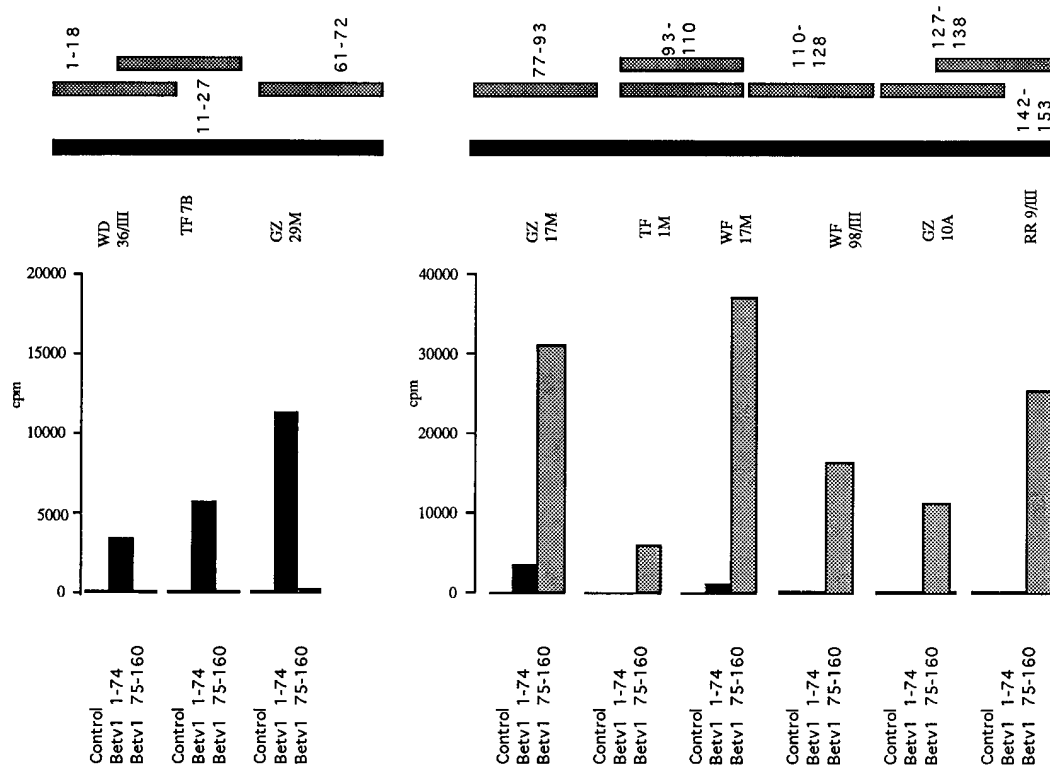
Figure 4. Proliferation of Bet v 1–specific T cell clones with recombinant Bet v 1 fragments. T cell clones from different donors (WD36/III, TF7B, GZ29M, GZ17M, TF1M, WF17M, WF98/III, GZ10A, and RR9/III) with specificity for Bet v 1 epitopes as indicated on the top of the figure were incubated with the recombinant Bet v 1 fragments aa 1–74 and 75–160. As a negative control, clones were tested with medium alone. Proliferation was determined by [³H]thymidine uptake and is displayed as counts per minute (cpm) on the *y*-axis. Values represent mean of duplicates and the standard deviation was always < 20%.

clones. T cell clones with specificity for T cell epitopes on the protein's NH_2-terminal portion showed proliferation after incubation with fragment aa 1–74 while those clones which were mapped to the COOH-terminal part proliferated in response to fragment aa 75–160. Using both recombinant Bet v 1 fragments or the complete Bet v 1 molecule (data not shown), significant proliferation could be induced in all nine T cell clones indicating that the combination of both fragments harbors all T cell epitopes of the complete Bet v 1 molecule. No significant proliferation was observed when the T cell clones were tested with medium and feeder cells without addition of antigen (Fig. 4, medium control).

Recombinant Bet v 1 fragments have ∼ 1,000-fold reduced capacity to induce histamine release from patients' basophils. Fig. 5 shows representative results obtained from the comparison of complete recombinant Bet v 1 and the two Bet v 1 fragments in basophil histamine release experiments in two donors. Granulocytes from two different Bet v 1 allergic patients (Fig. 5, *top* and *bottom*) were incubated with different concentrations (0.001–10 µg/ml) of complete recombinant Bet v 1 (aa

Table I. Recombinant Bet v 1 Fragments (aa 1–74; aa 75–160) Are Nonanaphylactic in Allergic Patients' Skin

Individual	Histamine	Birch	Bet v 1		aa 1–74		aa 75–160		aa 1–74 + aa 75–160	
			10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml	10 µg/ml	100 µg/ml
Birch pollen allergics										
HP	6.5	9.5	10	20	0	0	0	0	0	2
PH	8	8.5	7	9.5	0	0	0	0	0	0
SO	5.5	5.5	5	8	0	0	0	0	ND	ND
SS₁	5.5	8	6.5	9	0	0	0	0	ND	ND
SS₂	6	7	7	16	0	2	0	0	0	0
SF₁	8	8.5	8	23	0	0	0	0	ND	ND
SF₂	7.5	15	12.5	20	0	0	0	0	0	2
MD	5.5	9.5	7	12	0	0	0	0	ND	ND
Nonallergic controls										
TB	6	0	0	0	0	0	0	0	ND	ND
VR	8.5	0	0	0	0	0	0	0	ND	ND
CD	6.5	0	0	0	0	0	0	0	ND	ND
TL	9	0	0	0	0	0	0	0	ND	ND

Results displayed in the table reflect the mean wheal diameters in mm (Dm). The initials of the patients are displayed. Two of the birch pollen allergic patients were tested in two independent experiments (SS₁, SS₂; SF₁, SF₂).
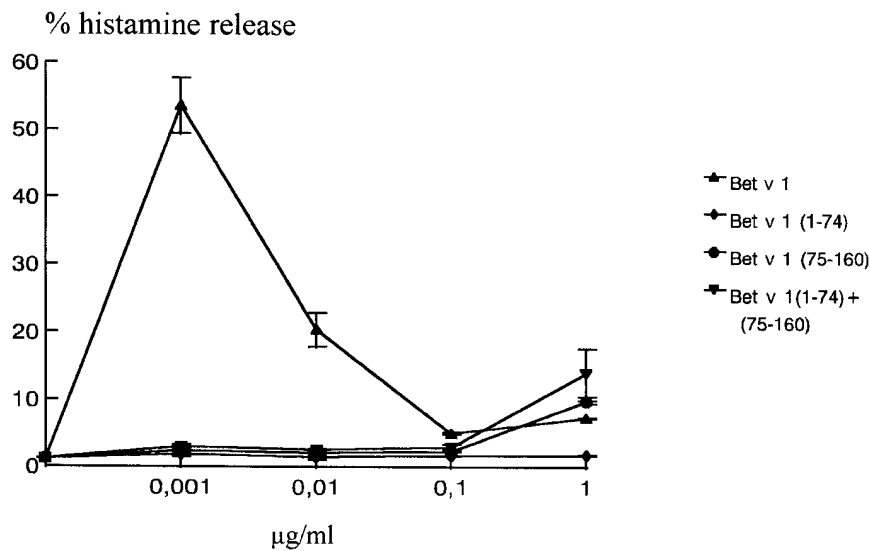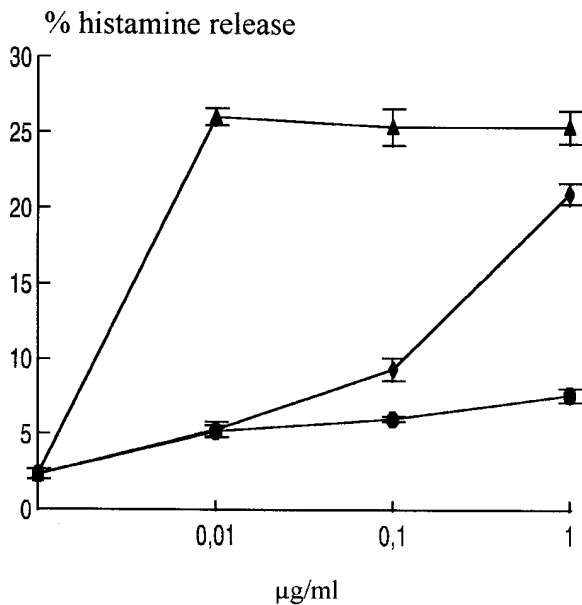
% histamine release

% histamine release

- Bet v 1
- Bet v 1 (1-74)
- Bet v 1 (75-160)
- Bet v 1(1-74)+ (75-160)

Figure 5. Capacity of recombinant Bet v 1 fragments to induce histamine release. Granulocytes from a birch pollen allergic individual (*top*) were incubated with different amounts of purified recombinant Bet v 1 and recombinant Bet v 1 fragments (aa 1–74, aa 75–160) to measure the dose dependency of histamine release. (*Bottom*) Granulocytes from a different birch pollen allergic patient were tested. In addition to recombinant Bet v 1 and the two fragments, an equimolar mixture of both fragments was used. Histamine released in the cell-free supernatant was measured by RIA (Immunotech) and is expressed as a percentage of total histamine release. Results represent means of triplicate determinations and deviations are indicated for each measurement.

1–160), the two Bet v 1 fragments (aa 1–74, aa 75–160), and anti–human IgE antibodies (positive control). Complete recombinant Bet v 1 induced maximal histamine release at a concentration of 0.01 μg/ml, whereas a comparable release with the Bet v 1 fragments was not observed up to a concentration of 10 μg/ml. An equimolar mixture of both Bet v 1 fragments did not induce significant histamine release compared with each of the fragments tested separately (Fig. 5, *bottom*) The results from the basophil release experiments correlate with the IgE-binding data, indicating an at least 1,000-fold reduced IgE binding capacity of the Bet v 1 fragments compared with the complete allergen.

Recombinant Bet v 1 fragments do not elicit anaphylactic skin reactions. Table I summarizes the results obtained by skin prick testing of six representative birch pollen allergic patients and four nonallergic individuals with complete recombinant Bet v 1 and recombinant Bet v 1 fragments (aa 1–74, aa

75–160). Individuals were pricked with 20 μl of the skin prick solution containing commercial birch pollen extract and using increasing concentrations of purified recombinant Bet v 1 or the Bet v 1 fragments (10 μg/ml, 100 μg/ml). Complete recombinant Bet v 1 elicited comparable wheal reactions to the natural birch pollen extract and histamine at a concentration of 10 μg/ml. The wheal reaction increased dose dependently with the Bet v 1 concentration. The two Bet v 1 fragments did not induce any wheal reaction up to concentrations of 100 μg/ml in any of the allergic patients or controls. Histamine induced wheal reactions in all four nonallergic control individuals, whereas neither birch extract nor any of the Bet v 1 preparations gave a wheal reaction in the nonallergic group, indicating that the protein preparations were not toxic. NaCl controls were negative in all individuals tested. To investigate the skin prick activity of an equimolar mixture of both Bet v 1 fragments two additional patients (Table I, *HP* and *PH*; Fig. 6) and
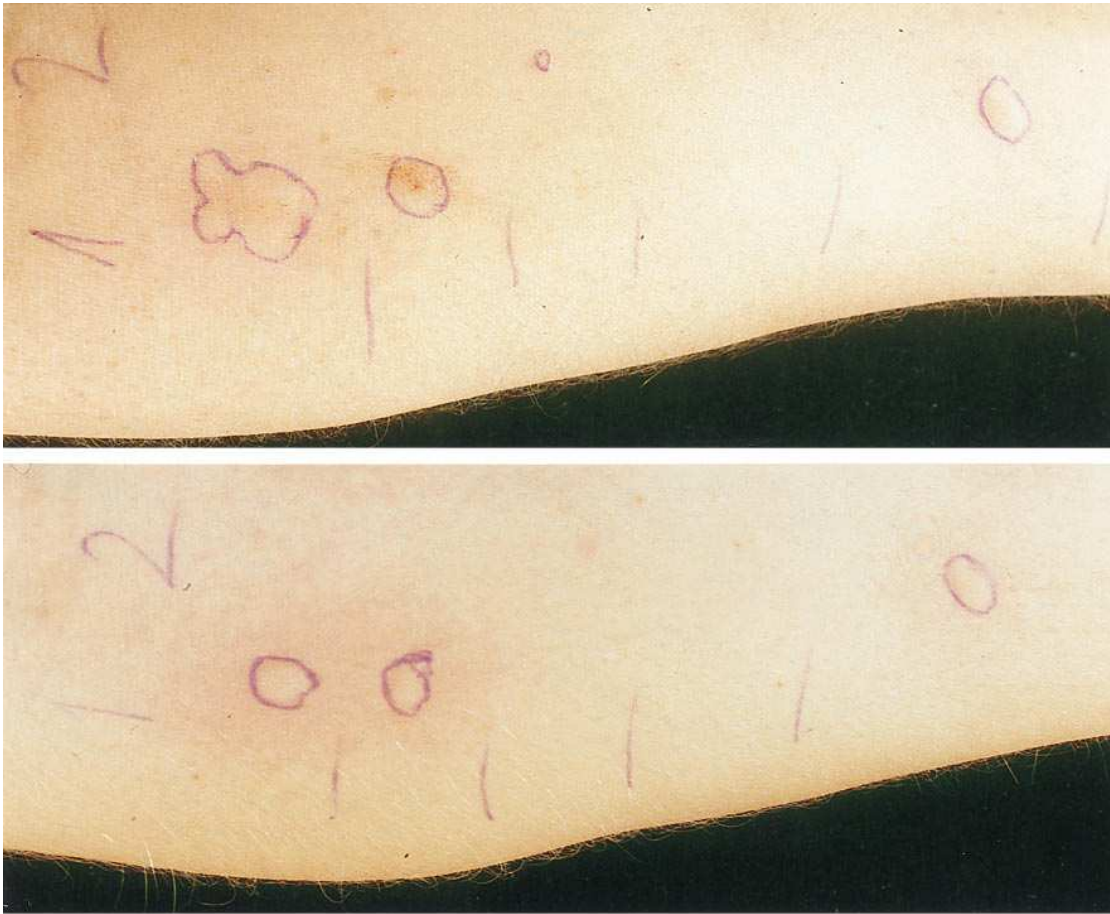
Figure 6. Lack of anaphylactic activity of recombinant Bet v 1 fragments. Two birch pollen allergic individuals (top depicts patient HP and the bottom depicts patient PH, both from Table I) were skin pricked on their forearms with different concentrations of recombinant Bet v 1, recombinant Bet v 1 fragments, an equimolar mixture of both fragments, and birch pollen extract.

two previously tested patients (Table I, SS_2 and SF_2) were tested also with 10 and 100 µg/ml of an equimolar mixture of both fragments. In none of the patients was a significant wheal reaction (one-third of the histamine wheal) observed up to 100 µg/ml of the equimolar mixture of both Bet v 1 fragments, confirming that the fragments are unable to reconstitute anaphylactic activity in vivo.

Discussion

The use of T cell epitopes for specific immunotherapy of type I allergy has been proposed as a possible alternative to current forms of immunotherapy which are based on natural allergen extracts (4, 5, 30). Anaphylactic side effects during immunotherapy are expected to be significantly reduced if non–IgE-binding T cell epitopes were used. As a novel approach for the construction of a T cell allergy vaccine, we have pursued the concept of disrupting conformational IgE epitopes of a relevant allergen by recombinant techniques. Birch pollen allergen, Bet v 1 (9, 12), was chosen as a model because it represents the major allergen for > 95% of tree pollen and plant food allergic patients and > 60% of these patients are sensitized exclusively to Bet v 1 (12, 13). In addition, Bet v 1 binds a high percentage of specific serum IgE antibodies and shares B

cell as well as T cell epitopes with homologous allergens present in tree pollen and plant-derived food (15, 16, 31–34).

In contrast to other allergens which possess continuous IgE epitopes (35), it has been shown that IgE-binding to Bet v 1 and homologous allergens (e.g., the major hazel pollen allergen, Cor a 1) depends on critical amino acid residues which are spread over the molecules. Therefore, it is conceivable that Bet v 1 assembles discontinuous (i.e., conformational) IgE epitopes. Isovariants of Bet v 1 were shown to have markedly reduced IgE-binding activity although T cell reactivity was retained (36, 37).

By high level expression of corresponding partial cDNA fragments derived from the most frequently recognized Bet v 1 isoform (9), two recombinant Bet v 1 fragments were generated in *E. coli*. Bet v 1 was divided in two portions representing aa 1–74 and aa 75–160 to maintain the binding site of monoclonal antibodies which were able to modulate IgE-binding to Bet v 1 (21) as well as to preserve the major T cell epitopes (6). The recombinant fragments were expressed separately in *E. coli* and purified to homogeneity. Both recombinant Bet v 1 fragments had almost completely lost their IgE-binding capacity and anaphylactic potential as evaluated by histamine release experiments and by skin prick testing of allergic individuals. All nine tested Bet v 1–specific human T cell clones showed strong proliferation with at least one of the two recombinant Bet v 1 fragments, indicating that all relevant T cell epitopes were represented by the two derivatives.

Comparison of the CD spectra of the whole recombinant Bet v 1 (aa 1–160) with the two recombinant Bet v 1 fragments (aa 1–74, aa 75–160) indicated that lack of IgE-binding capacity by the fragments parallels loss of nativelike structure. The CD spectra of the two fragments show features characteristic of a random coil structure which remains unchanged even after prolonged coincubation of equimolar mixtures of the fragments. This result agrees with what is expected on the basis of the secondary structure as determined by nuclear magnetic resonance (38). The fold of Bet v 1 is mainly stabilized by two antiparallel β sheets and three helices. Cutting Bet v 1 into two halves would undoubtedly disrupt contacts between secondary structure elements and strongly compromise the protein stability. This is consistent with the finding that neither fragment as single component or as equimolar mixture significantly bound IgE antibodies or displayed anaphylactic effects in vitro or in vivo. Hence, it is not likely that the fragments would reconstitute the Bet v 1 conformation after injection and cause anaphylactic effects.

Because of the lack of their anaphylactic activity due to disrupture of their tertiary fold, the two Bet v 1 fragments may be considered as safe tools for specific immunotherapy of tree pollen and associated allergies. Vaccination with nonanaphylactic allergen derivatives or peptides which target allergen-specific T helper cells might lead to disease improvement by (*a*) induction of tolerance in allergen-specific T cells (39–41); (*b*) induction of anergy (42, 43); (*c*) perhaps even the switching of allergen-specific TH2 clones into TH1-like clones which may then display a reduced and/or altered cytokine expression (44); or (*d*) induction of blocking antibodies which are able to recognize the unfolded fragments as well as natural Bet v 1 as exemplified by the mouse monoclonal antibody 14 (21). Although induction of T cell tolerance against a specific antigen has been demonstrated by injecting a single T cell epitope derived thereof (45, 46), the present approach comprises all

known relevant Bet v 1 T cell epitopes. Therefore, allergen fragments without or with markedly reduced allergenicity appear as possible candidates for the treatment of allergic patients by injecting high doses with reduced risk of anaphylactic side effects.

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