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

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Summary

We dissected the T cell activation potency and the immunoglobulin (Ig) E–binding properties (allergenicity) of nine isoforms of Bet v 1 (Bet v 1a-Bet v 1l), the major birch pollen allergen. Immunoblot experiments showed that Bet v 1 isoforms differ in their ability to bind IgE from birch pollen–allergic patients. All patients tested displayed similar IgE-binding patterns toward each particular isoform. Based on these experiments, we grouped Bet v 1 isoforms in three classes: molecules with high IgE-binding activity (isoforms a, e, and j), intermediate IgE-binding (isoforms b, c, and f), and low/no IgE-binding activity (isoforms d, g, and l). Bet v 1a, a recombinant isoform selected from a cDNA expression library using IgE immunoscreening, exhibited the highest IgE-binding activity. Isoforms a, b, d, e, and l were chosen as representatives from the three classes for experimentation. The potency of each isoallergen to activate T lymphocytes from birch pollen–allergic patients was assayed using peripheral blood mononuclear cells, allergen-specific T cell lines, and peptide-mapped allergen-specific T cell clones. Among the patients, some displayed a broad range of T cell–recognition patterns for Bet v 1 isoforms whereas others seemed to be restricted to particular isoforms. In spite of this variability, the highest scores for T cell proliferative responses were observed with isoform d (low IgE binder), followed by b, l, e, and a. In vivo (skin prick) tests showed that the potency of isoforms d and l to induce typical urticarial type I reactions in Bet v 1–allergic individuals was significantly lower than for isoforms a, b, and e. Taken together, our results indicate that hypoallergenic Bet v 1 isoforms are potent activators of allergen-specific T lymphocytes, and Bet v 1 isoforms with high in vitro IgE-binding activity and in vivo allergenicity can display low T cell antigenicity. Based on these findings, we propose a novel approach for immunotherapy of type I allergies: a treatment with high doses of hypoallergenic isoforms or recombinant variants of atopic allergens. We proceed on the assumption that this measure would modulate the quality of the T helper cell response to allergens in vivo. The therapy form would additionally implicate a reduced risk of anaphylactic side effects.

In the last few years, cDNA cloning has provided a number of complete deduced amino acid sequences of atopic allergens (1–4; for review see reference 5). The availability of nucleotide sequences and deduced amino acid sequences of allergenic proteins made it possible to reveal biological functions and to characterize the structures within these molecules that interact with the human immune system (T and B cell epitopes). Bet v 1, the model allergen used in this study, is the major allergen of birch (*Betula verrucosa*) pollen (6). More than 95% of birch pollen–allergic individuals display IgE binding to Bet v 1, and >60% react exclusively with it (7). Bet v 1 belongs to a group of pathogenesis-related proteins with homologies to plant disease resistance response gene products (3).

T cell epitopes are linear peptide fragments, and particular amino acids within these peptides were shown to be crucial for immunological recognition (MHC binding, TCR binding) (8, 9). Several studies showed that atopic al-

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lergens harbor multiple T cell epitopes scattered over the complete amino acid sequence (10–19). Moreover, it has been demonstrated that allergic and nonallergic individuals display a polyclonal T cell response to the epitopes of one allergenic protein. Some T cell epitopes seemed to represent "major epitopes," as almost every sensitized individual recognized these sequences. Identification of B cell epitopes, on the other hand, has only been partly successful. This is obviously because B cell epitopes represent—with a few exceptions (20, 21)—conformational structures and are therefore difficult to identify. In the case of our model allergen Bet v 1, fragments obtained by chemical or enzymatic treatment of the molecule were not reactive with IgE antibodies (6), and only complete clones were reactive with IgE, indicating the relevance of structural features in antibody binding to this protein (22).

Allergenic pollen proteins consist of a mixture of closely related isoforms, as was shown in most cases by cDNA cloning. Isoforms have been described for Amb a 1, the predominant allergen from short ragweed (*Ambrosia artemisiifolia*) pollen (23), for Poa p 9, a group of basic isoallergens from Kentucky bluegrass (*Poa pratensis*) pollen (24), for Car b 1, the major allergen from hornbeam (*Carpinus betulus*) pollen (25), for Cor a 1, the major allergen from hazel (*Corylus avellana*) pollen (26), and for Bet v 1 (27). The existence of isoforms of Bet v 1 in birch pollen was confirmed at the protein level by mass spectrometry (27).

In this study we have addressed the subject of isoallergens and their interactions with the immune system of pollen-allergic individuals. Previous studies described differences in the antibody (IgE)-binding properties of pollen isoallergens (26, 28, 29) and invariably focused on isoallergen molecules with high IgE-binding activity, because this property is considered essential for allergenicity of a protein. Strong IgE-binding isoforms, however, might not necessarily represent potent activators of T cells. To determine whether high IgE-binding activity of an individual isoallergen correlates to high T cell antigenicity, isoforms of Bet v 1 were tested for both properties. To define exactly the role of each isoallergen in the activation of T lymphocytes from allergic patients, we performed assays using PBMC, allergen-specific T cell lines (TCL),[1] and allergen-specific T cell clones (TCC). It can be foreseen that this knowledge will be important for the development of adequate reagents for immunotherapy of pollen-allergic patients. Based on the results presented here, we suggest a new concept for immunotherapy of type I allergic diseases.

Materials and Methods

Birch Pollen Extract. Protein extract from birch (*B. verrucosa*) pollen (Allergon AB, Engelhom, Sweden) was prepared as previously described (30).

SDS-PAGE and Immunoblots. Birch pollen protein extract and *Escherichia coli* lysates of recombinant Bet v 1 isoforms were analyzed by SDS-PAGE according to the method of Laemmli (31), using 15% acrylamide gels. Proteins were visualized by staining with Coomassie brilliant blue R-250. As the level of expression varied for each Bet v 1 isoform, Coomassie-stained gels were analyzed on an imaging densitometer (model GS-670; Bio-Rad Laboratories, Richmond, CA), and peak areas were measured. In this way, it was possible to calculate the amount of lysate to achieve equivalent amounts for each isoform in immunoblot experiments.

For immunoblot analysis, proteins were separated by 15% SDS-PAGE and electroblotted (32) onto nitrocellulose membranes. IgE immunoblots were performed as described previously (7). Bound IgE was detected using ^{125}I-rabbit anti–human IgE (Pharmacia, Uppsala, Sweden). *E. coli* lysates harboring the plasmid without insert were used as a control. In all experiments, reagents and cell lysates were from identical batches and were used in the same concentrations. Autoradiography was performed at −70°C for 12–48 h with intensifying screens.

Purification of Natural Bet v 1. Natural Bet v 1 (nBet v 1) was purified from aqueous extract of birch (*B. verrucosa*) pollen (Allergon AB) by reversed-phase HPLC (C_8 Hypersil WP 300, 10 μm, 8 × 250 mm) using a linear gradient of 2-propanol at room temperature (solvent A: aqueous 0.1% TFA; solvent B: aqueous 90% 2-propanol/0.1% TFA; gradient 0–80% B within 50 min; flow rate 1.8 ml/min). UV absorbance was monitored at 280 nm. Fractions (2 ml) were collected, dried in vacuo, and resuspended in water. Aliquots were analyzed by SDS-PAGE and immunoblots using mAbs and allergic patients' sera (7, 30).

Cloning of Recombinant Bet v 1 Isoforms as Nonfusion Proteins in pMW175/172. Expression plasmids containing cDNA inserts coding for Bet v 1 isoforms were constructed in the vector pMW175 or pMW172 (33), which are based on the original pET vectors of Studier et al. (34). These vectors direct the synthesis of a nonfusion protein under the control of the T7 promoter. In this system, T7 polymerase is under control of the lacUV5 promoter, leading to inducible production of the recombinant protein. The nucleotide sequences and derived amino acid sequences of nine isoforms of Bet v 1 have been described elsewhere (Bet v 1a, reference 3; Bet v 1b–g, j and l, reference 27). The 5′ primers B-1 (5′GGGCCATGGGTGTTTTCAATTACGA3′) and B-3 (5′GGGCATATGGGTGTTTTCAATTACGA3′) and the 3′ primer B-2 (5′CCCGAATTCTTAGTTGTAGGCATCAGAGTGTGCCAA3′) were used in a PCR with cDNAs coding for Bet v 1 isoforms to produce DNA fragments without 5′ and 3′ nontranslated sequences and with appropriate recognition sequences at their 5′ and 3′ ends. The primer B-1 encoded six NH_2-terminal amino acids (identical in all Bet v 1 isoforms) and had an NcoI site at its 5′ end. The primer B-3 was similar to B-1 except that it had an NdeI site at its 5′ end. The primer B-2 encoded eight COOH-terminal amino acids (identical in all isoforms) and had an EcoRI site at its 5′ end. The resulting NcoI–EcoRI coding sequences (Bet v 1a–f, j, and l) were inserted into NcoI–EcoRI–digested pMW175 vector and the NdeI–EcoRI coding sequence (Bet v 1g) inserted into the NdeI–EcoRI–digested pMW172 vector. These expression constructs were designated pMW175/Bet v 1a–f, j, and l and pMW172/Bet v 1g, respectively. All PCR-derived inserts were sequenced according to the dideoxy chain termination method (35).

Expression and Preparation of E. coli Cell Lysates of rBet v 1 Isoforms. For expression of the pMW175/Bet v 1a–f, j, l, and pMW172/Bet v 1g plasmids, competent *E. coli* strain BL21(DE3) was transformed and selected on plates containing 100 mg/liter

[1]*Abbreviations used in this paper:* MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; nBet v 1, natural Bet v 1; SIT, specific immunotherapy; SPT, skin prick test; TCC, T cell clones; TCL, T cell lines.

ampicillin (34). A single transformant colony was picked and grown to an OD_{600} of 1.0. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1.0 mM, and incubation was continued for 6 h at 37°C. After expression, cells were harvested by centrifugation, and pellets were resuspended in 50 mM Tris-HCl, pH 7.5, containing 220 mM NaCl (buffer A). The cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37°C. This step was repeated twice. Isoforms a and e were recovered in the supernatant after centrifugation at 30,000 *g* for 25 min at 4°C, which was then used for immunoblot analysis. Isoforms b–d, f, g, j, and l were recovered in the pellet by low-speed centrifugation. Insoluble inclusion bodies were solubilized in buffer A containing 6 M urea and dialyzed at 4°C against buffer A. After dialysis, the extracts were used for immunoblot experiments. In a previous publication (22), we have shown by quantitative ELISA inhibition experiments that denaturation of Bet v 1 in 6 M urea and renaturation by dialysis does not affect IgE binding.

Purification of rBet v 1 Isoforms. rBet v 1 isoform a was purified from crude *E. coli* lysates by a combination of chromatofocusing on a PBE-94 (Pharmacia) exchanger column and reversed-phase HPLC, as previously described (22). rBet v 1 isoforms b, d, e, and l were purified using the method described for rBet v 1a (22), with few modifications. Briefly, bacterial lysates were prepared in 25 mM imidazole/HCl buffer containing 6 M urea (buffer C). The extracts were loaded onto a PBE-94 exchanger column equilibrated with buffer C, at room temperature. Bound proteins were eluted with aqueous 12.5% (vol/vol) Polybuffer 74/HCl (Pharmacia), pH 4.0, containing 6 M urea, as previously described (36). Fractions containing rBet v 1 isoforms, as determined by dot blot immunoassays using mAbs, were pooled and subjected to reversed-phase HPLC, as described previously (22).

Protein concentrations of all purified allergen preparations were determined by the micro-Kjeldahl method, using glycine as standard (37).

NH_2-terminal Sequence Analysis. Birch pollen extract and *E. coli* lysates of Bet v 1 isoforms were separated by 15% SDS-PAGE and blotted onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Bands corresponding to nBet v 1 (a mixture of isoforms) or rBet v 1 isoforms were excised and proteins eluted by incubation with 40% (vol/vol) acetonitrile and 30% (vol/vol) TFA for 1 h at room temperature. Samples were vacuum dried, resuspended in water, and sequenced with a protein sequencing system (G1005AQ; Hewlett-Packard Co., Palo Alto, CA). Protein samples were loaded directly onto the hydrophobic part of the biphasic sequencer column and subjected to Edman degradation using the manufacturer's Routine 3.0 chemistry.

Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. 0.5 μl of purified isoforms was mixed with 2.5 μl protein calibration standard (cytochrome *c*, myoglobin, and BSA) and 6 μl of sinapinic acid containing citric acid as chelator. After vacuum crystallization, samples were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis using a MALDI-TOF system (HP G2025A; Hewlett-Packard Co.) equipped with a nitrogen laser.

Allergen-specific TCL. Allergen-specific TCL were obtained by a technique described previously (14). Briefly, 0.5×10^6 PBMC were stimulated with individual optimal concentrations (5–20 μg/ml) of purified nBet v 1 in Ultra Culture Medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 2 mM/liter glutamine and 2×10^{-5} M 2-ME in 24-well flat-bottomed culture plates (model 3524; Costar Corp., Cambridge, MA) for 5 d. Subsequently, human rIL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany) was added, and the culture was continued for an additional 7 d.

Allergen-specific T Cell Clones. T cell blasts from nBet v 1–specific TCL were seeded in limiting dilution (0.3 cells/well) in 96-well round-bottom plates (Nunclone; Nunc, Roskilde, Denmark) in the presence of 10^5 irradiated (5,000 rad) allogeneic PBMC as feeder cells, 1% PHA vol/vol (Gibco Laboratories, Grand Island, NY), and rIL-2 (4 U/well) in the above-described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2. The specificity of TCC was assessed as previously described (14). Briefly, 2×10^4 T cell blasts were incubated in triplicate cultures in 96-well plates in the presence of 10^5 autologous irradiated PBMC with one of the following ingredients: whole birch pollen extract, purified nBet v 1, rBet v 1a, or overlapping Bet v 1a peptides. Microtiter plates were then incubated for 48 h at 37°C in a 5% CO_2 humidified atmosphere. After pulsing for 16 h with [^{3}H]TdR, cells were harvested, and the incorporated radioactivity was measured by scintillation counting. When the stimulation index (ratio between cpm obtained in cultures with TCC plus autologous irradiated PBMC plus antigen and cpm obtained in cultures containing TCC and PBMC alone) was >10, responses were considered positive.

Proliferation Assays. PBMC and nBet v 1–specific TCL and TCC were tested with nBet v 1, with two recombinant Bet v 1 isoforms displaying high IgE-binding capacity (rBet v 1a and rBet v 1e; see Fig. 2), two recombinant isoforms diplaying low IgE-binding properties (rBet v 1d and rBet v 1l; see Fig. 2), and an isoform displaying intermediary levels of IgE binding (rBet v 1b). All proliferation assays were performed in triplicate; incubation periods before addition of [^{3}H]TdR were 6 d for PBMC and 48 h for TCL and TCC, respectively. In all experiments, identical concentrations of allergen (1 μg/well = 5 μg/ml) were used.

Epitope Mapping. T cell epitope mapping was performed in TCC reactive with rBet v 1a, using 50 peptides (Cambridge Research Biochemicals Limited, Cambridge, UK) overlapping for three amino acids and spanning the whole sequence of the protein (14).

Analysis of the Phenotype of TCC. FITC-labeled mAb anti-Leu 4/CD3, anti-Leu 3a/CD4, anti-Leu 2a/CD8, anti-TCR-1 a/b WT31, and anti-TCR g/d I (Becton Dickinson & Co., Mountain View, CA) were used to analyze the phenotype of the TCC. For flow cytometry analysis, TCC and irradiated feeder cells alone were harvested and washed, and 10^5 cells were incubated for 30 min on ice with the appropriate dilution of mAb or isotype-matched control Abs. After washing three times with PBS containing 0.3% BSA and 0.3% NaN_3, the cells were stained with propidium bromide to allow exclusion of dead cells and analyzed on a FACScan® (Becton Dickinson & Co.).

Skin Testing with Purified rBet v 1 Isoforms. Skin prick test (SPT) was performed according to guidelines described (38). For SPT, 100 ng/25 μl of each isoform (39) and commercially available test solutions (birch pollen extract, grass pollen extract, histamine, 0.9% sodium chloride) were applied. For comparison, computer-aided measurements of wheal areas were performed. Skin testing of birch pollen–allergic individuals with recombinant Bet v 1 molecules was approved by the Ethics Committee of the University of Vienna, Austria.

Results

Expression of Bet v 1 Isoforms, IgE-binding Properties, and Purification of Bet v 1 Isoforms. Nonfusion forms of full-length cDNAs coding for nine Bet v 1 isoforms were ex-

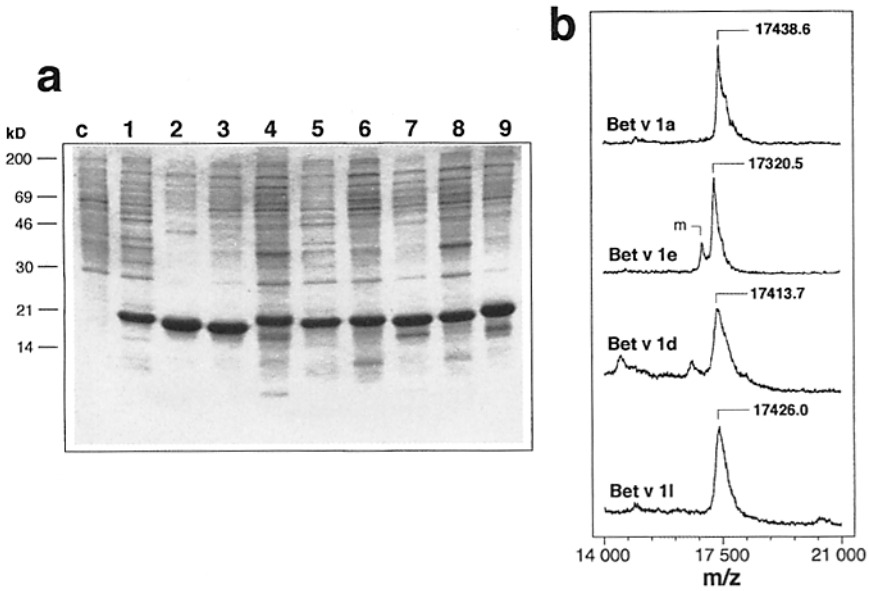

Figure 1. Expression of Bet v 1 isoforms in *E. coli* and MALDI-TOF mass spectrometry analysis of purified isoforms. (*a*) Coomassie-stained 15% SDS-PAGE of lysates of *E. coli* BL21(DE3) host strain containing Bet v 1 isoforms expression plasmids. (Lane *c*) Host strain lysate containing the expression vector without an insert; (lanes *1–9*) Bet v 1 isoforms a–g, j, and l, respectively. (*b*) MALDI-TOF mass spectra of purified recombinant Bet v 1 isoforms a, e, d, and l. *m*, myoglobin (16,944.0 daltons) was added to the Bet v 1e preparation as an internal molecular mass standard.

pressed in *E. coli* (Fig. 1 *a*). High levels of expression (between 20 and 100 mg protein per liter bacterial culture) were obtained. NH_2-terminal sequence analysis of all recombinant isoforms showed that the initiating methionine was removed.

To evaluate the ability of recombinant Bet v 1 isoforms to bind IgE from allergic patients, immunoblot experiments were performed. In total, sera from 30 patients were tested. Fig. 2 shows the IgE-binding patterns of eight representative allergic individuals with each isoform. In general, isoforms a–c, e–f, and j displayed good IgE-binding reactivity. Among these, isoforms a, e, and j were prominent in this respect (Fig. 2). Most interestingly, sera of all patients tested failed to recognize or showed remarkably low IgE binding to isoforms d, g, and l (Fig. 2). Taking rBet v 1a as a reference (100% IgE binding), the relative percentage of IgE binding for the other isoforms was calculated (see Table 2).

Five isoforms were selected for detailed experiments concerning activation of T lymphocytes from allergic individuals. For this purpose, the chosen isoforms (isoforms a, b, d, e, and l) were purified to homogeneity, as determined by SDS-PAGE (data not shown). The purified isoallergen preparations were further analyzed by MALDI-TOF mass spectrometry (Fig. 1 *b*). The molecular masses of intact Bet v 1 isoforms a, d, e, and l were measured as 17,438.6, 17,413.7, 17,320.5, and 17,426.0, respectively. The theoretical molecular masses obtained, based on the cDNA sequences, were 17,439.62 (Bet v 1a), 17,417.68 (Bet v 1d), 17,316.55 (Bet v 1e), and 17,421.73 (Bet v 1l). Thus, the experimental molecular mass measurements are in excellent agreement with the expected mass values. The measured mass values are also consistent with the removal of the initiating methionine.

For an estimation of the relative amounts of the different Bet v 1 isoforms in the pollen washout, NH_2-terminal sequence analysis of purified nBet v 1 was performed. The

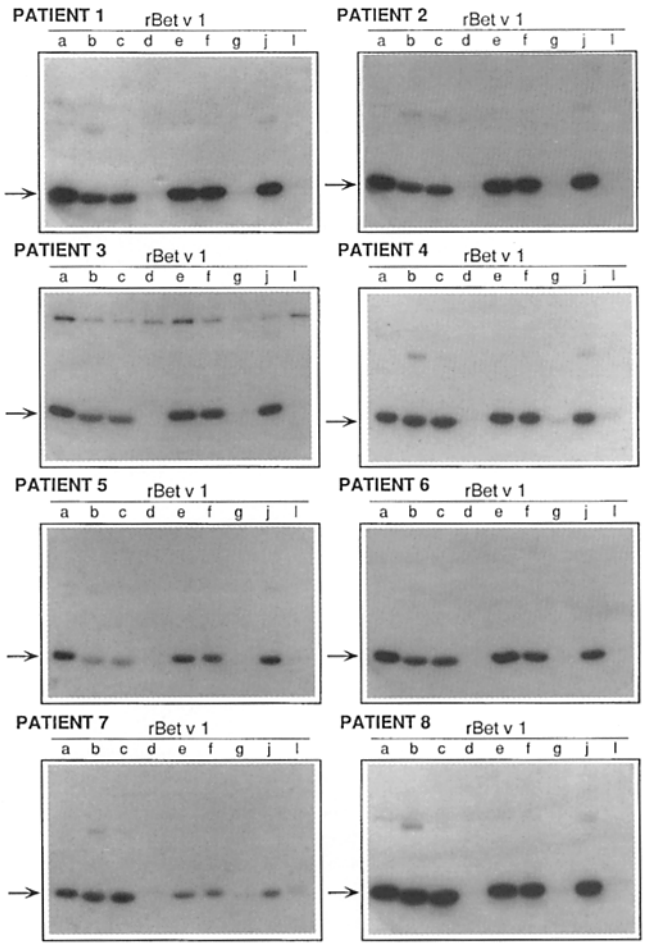

Figure 2. IgE immunoblot of recombinant Bet v 1 isoforms. Sera from birch pollen–allergic patients 1–8 were tested for IgE binding to recombinant Bet v 1 isoforms expressed in *E. coli* BL21. Bacterial lysates containing the expression vector without an insert were used as controls (not shown).

Table 1. *Proliferative Responses of Human Bet v 1-Specific TCC to nBet v 1 and rBet v 1 Isoforms Presented by Autologous PBMC*

Patient	TCC	Control	nBet v 1	rBet v 1a	rBet v 1e	rBet v 1b	rBet v 1d	rBet v 1l
1	XPC2	0.1 ± 0	50.5 ± 3	**0.1 ± 1**	2.2 ± 1	5.6 ± 2	12.2 ± 2	16.9 ± 2
	XPC3	0.2 ± 0	21.3 ± 4	49.7 ± 5	63.0 ± 3	11.9 ± 3	39.4 ± 2	47.2 ± 6
	XPC8	0.1 ± 0	2.1 ± 0	8.1 ± 0	5.2 ± 1	**1.1 ± 0**	13.0 ± 2	15.3 ± 3
	XPC26	0.2 ± 0	21.1 ± 3	32.4 ± 4	11.5 ± 0	43.6 ± 6	7.6 ± 1	39.2 ± 17
	XPC31	0.2 ± 0	23.5 ± 1	19.6 ± 5	21.9 ± 1	**0.2 ± 0**	20.6 ± 1	25.1 ± 5
	XPC33	0.3 ± 0	33.4 ± 2	18.8 ± 4	4.1 ± 0	38.9 ± 5	34.4 ± 0	39.2 ± 1
2	XPF1	0.1 ± 0	74.9 ± 0	44.6 ± 2	99.2 ± 11	73.1 ± 5	95.6 ± 5	107.8 ± 1
	XPF7	0.2 ± 0	95.0 ± 7	7.7 ± 0	105.2 ± 10	66.0 ± 3	165.6 ± 21	**1.0 ± 0**
	XPF10	0.2 ± 0	1.8 ± 0	21.0 ± 0	9.6 ± 0	7.3 ± 0	9.3 ± 1	15.8 ± 2
	XPF5II	0.1 ± 0	7.8 ± 1	**0.1 ± 0**	**0.1 ± 0**	10.5 ± 0	10.2 ± 0	**0.1 ± 0**
	XPF10II	0.7 ± 0	8.2 ± 0	**4.1 ± 0**	7.4 ± 0	9.2 ± 0	**3.3 ± 0**	9.8 ± 1
	XPF62II	0.1 ± 0	15.6 ± 2	**0.1 ± 0**	**0.1 ± 0**	3.4 ± 1	8.0 ± 0	**0.1 ± 0**
	XPF77II	0.1 ± 0	1.3 ± 0	5.2 ± 1	14.8 ± 0	16.0 ± 6	10.0 ± 0	14.1 ± 0
	XPF113II	0.1 ± 0	6.1 ± 1	**0.1 ± 0**	**0.1 ± 0**	**0.1 ± 0**	**0.1 ± 0**	**0.1 ± 0**
3	XPH8	0.4 ± 0	48.0 ± 14	10.5 ± 1	52.6 ± 2	66.5 ± 2	67.0 ± 2	88.3 ± 4
	XPH23	0.3 ± 0	5.2 ± 0	**1.2 ± 0**	**2.1 ± 0**	**0.1 ± 0**	12.7 ± 1	20.0 ± 3
4	XPD18	0.1 ± 0	**0.7 ± 0**	26.3 ± 2	**0.2 ± 0**	102.0 ± 39	11.9 ± 6	**0.4 ± 0**
	XPD22	0.1 ± 0	46.0 ± 20	28.9 ± 5	**0.2 ± 0**	147.1 ± 24	10.9 ± 1	**0.3 ± 0**
	XPD24	0.9 ± 0	98.2 ± 5	70.7 ± 11	77.0 ± 15	85.1 ± 6	86.9 ± 2	77.4 ± 8
	XPD25	0.1 ± 0	78.4 ± 2	24.4 ± 1	81.8 ± 9	65.7 ± 4	87.2 ± 6	64.2 ± 2
	XPD27	1.1 ± 1	77.8 ± 16	14.7 ± 2	**0.2 ± 0**	128.5 ± 3	**4.1 ± 2**	**0.2 ± 0**
5	XPWF17	0.1 ± 0	145.3 ± 12	83.8 ± 3	11.2 ± 2	168.6 ± 21	167.7 ± 11	167.3 ± 2
	XPWF24	0.1 ± 0	40.3 ± 2	25.4 ± 3	25.0 ± 1	42.4 ± 2	42.8 ± 2	46.8 ± 1
	XPWF29	0.1 ± 0	45.8 ± 10	14.8 ± 2	53.5 ± 3	**0.3 ± 0**	93.7 ± 5	122.6 ± 6
	XPWF39II	0.1 ± 0	29.2 ± 1	**5.4 ± 2**	19.2 ± 1	17.1 ± 3	32.2 ± 4	19.7 ± 6
6	XPR4	2.3 ± 1	208.8 ± 51	152.6 ± 12	201.3 ± 7	240.5 ± 5	224.5 ± 2	204.2 ± 18
	XPR4II	5.5 ± 1	201.1 ± 12	**5.4 ± 2**	**6.3 ± 2**	82.2 ± 6	160.0 ± 20	**4.2 ± 1**
	XPR6	0.2 ± 0	24.3 ± 3	9.5 ± 1	17.8 ± 1	25.4 ± 2	20.4 ± 3	17.5 ± 1
	XPR9	0.8 ± 0	79.6 ± 2	66.4 ± 4	34.3 ± 2	86.6 ± 3	111.7 ± 2	50.6 ± 3
	XPR11	2.8 ± 1	108.7 ± 9	**2.8 ± 0**	**2.1 ± 1**	81.7 ± 9	161.9 ± 3	**0.9 ± 0**
	XPR16	0.5 ± 0	23.8 ± 2	**0.7 ± 0**	**0.7 ± 0**	13.2 ± 4	47.6 ± 1	**0.3 ± 0**
	XPR29	3.9 ± 1	75.4 ± 21	**2.5 ± 0**	**2.8 ± 1**	44.4 ± 8	123.4 ± 14	**1.6 ± 0**
	XPR44	2.4 ± 0	173.3 ± 7	**3.5 ± 1**	**2.1 ± 1**	92.9 ± 31	170.1 ± 7	**1.9 ± 0**
	XPR51	0.2 ± 0	217.0 ± 13	**0.3 ± 0**	215.4 ± 11	287.3 ± 6	84.4 ± 6	**1.3 ± 1**
	XPR86	2.4 ± 1	95.0 ± 38	**2.6 ± 1**	**1.1 ± 0**	11.5 ± 0	73.9 ± 5	**0.8 ± 0**
7	XPZ10	0.7 ± 0	7.5 ± 0	6.6 ± 1	6.4 ± 1	6.5 ± 1	6.1 ± 0	6.1 ± 0
	XPZ11	0.7 ± 0	9.6 ± 1	**0.5 ± 0**	9.2 ± 1	11.3 ± 0	6.5 ± 1	**3.7 ± 1**
	XPZ31	0.1 ± 0	58.7 ± 17	3.3 ± 0	30.6 ± 1	7.1 ± 2	37.7 ± 0	42.5 ± 3
	XPZ53	0.1 ± 0	8.2 ± 3	9.4 ± 4	8.5 ± 3	8.4 ± 3	8.7 ± 3	8.7 ± 3
8	XPGZ10	0.1 ± 0	12.5 ± 4	3.1 ± 1	26.8 ± 8	**0.1 ± 0**	25.8 ± 8	37.3 ± 10
	XPGZ16	0.1 ± 0	110.3 ± 30	85.4 ± 23	114.9 ± 31	77.8 ± 21	102.3 ± 28	89.8 ± 27
	XPGZ17	0.1 ± 0	18.0 ± 5	12.8 ± 4	18.0 ± 5	**0.2 ± 0**	27.7 ± 8	44.8 ± 13
	XPGZ17II	0.5 ± 0	45.7 ± 13	**0.2 ± 0**	**0.6 ± 0**	12.2 ± 3	7.0 ± 2	43.2 ± 16
	XPGZ29	0.1 ± 2	31.9 ± 9	20.4 ± 6	25.6 ± 7	28.1 ± 8	31.9 ± 9	31.4 ± 9
	XPGZ62	5.7 ± 0	53.3 ± 15	**0.4 ± 0**	33.4 ± 9	49.7 ± 14	43.3 ± 12	**7.3 ± 2**
	XPGZ64	0.6 ± 0	48.7 ± 14	**0.1 ± 0**	14.8 ± 4	54.3 ± 15	25.9 ± 7	**1.5 ± 0**
	XPGZ70	2.6 ± 1	33.8 ± 9	**0.2 ± 0**	13.6 ± 4	26.3 ± 8	18.8 ± 5	**2.3 ± 1**
	KPGZ72	4.1 ± 1	33.9 ± 9	**0.2 ± 0**	28.2 ± 8	25.8 ± 7	33.3 ± 9	**6.1 ± 2**

Control: TCC + irradiated autologous PBMC, no antigen. Values are cpm $\times 10^3 \pm$ SD. Boldface values indicate negative proliferative responses.

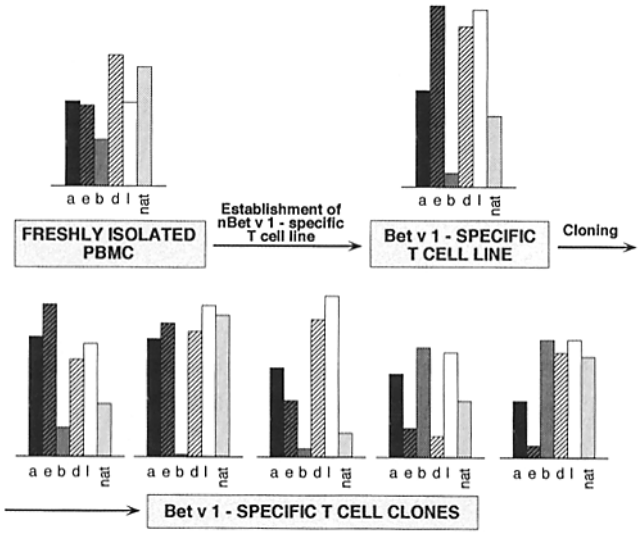

Figure 3. Experimental strategy and results of T lymphocyte stimulation experiments. As an example, T cell reactivity patterns of patient 7 are shown (see Fig. 2 and Table 1). Bars are stimulation indices obtained with rBet v 1 isoforms a, b, d, e, and l and with pollen Bet v 1 (*nat*).

results clearly indicated that the naturally occurring Bet v 1 isoforms have their initiating methionine removed and start with the glycine residue. A single sequence was determined until residue 7, where serine, threonine, and isoleucine were detected at a ratio of ~2:2:1 (data not shown). In position 9, threonine and alanine were detected at a ratio of 6:1. From this experiment and from our previous analysis of proteolytic digests of nBet v 1 by mass spectrometry (24), we estimated that isoforms a and b represent ~35% of pollen Bet v 1, whereas isoforms d and f seem to represent ~10% each. Together, isoforms a–c, e, f, and j represent up to 80% of total Bet v 1 released from birch pollen upon hydration.

Polyclonal T Cell Reactivity with Bet v 1 Isoforms. Responses of freshly isolated PBMC to five isoforms (a, b, d, e, and l) were analyzed in 15 birch pollen–allergic patients. Individual reactivity patterns could be observed reflecting the polyclonal nature of T cell recognition. Bet v 1–specific TCL were established using purified natural Bet v 1, a mixture of isoallergens. In general, results obtained in proliferation assays using nBet v 1–specific TCL reflected the reactivity pattern toward each isoform seen in PBMC. However, reactivity patterns to Bet v 1 isoforms differed among individuals. A schematic representation of the experimental approach is shown in Fig. 3.

Bet v 1–specific TCC. 48 TCC were tested for reactivity with 5 recombinant Bet v 1 isoforms (a, b, d, e, and l). All TCC revealed the Th phenotype ($CD3^+CD4^+CD8^-$) and expressed TCR-α/β. Results of T cell proliferation are summarized in Table 1. Different patterns of cross-reactivity between the isoforms could be observed. Based on a stimulation index >10, 47 of 48 (98%) TCC proliferated in response to stimulation with purified nBet v 1, 28 of 48 (58%) reacted with rBet v 1a, 34 of 48 (71%) reacted with

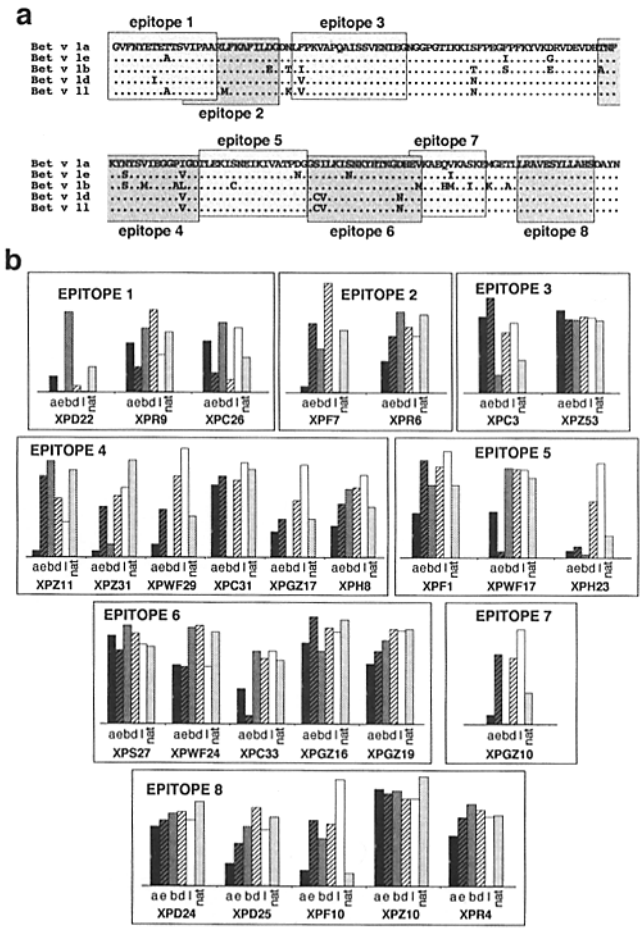

Figure 4. Epitope specificities and proliferative responses of 27 Bet v 1–specific TCC. (*a*) Amino acid sequence alignment of five Bet v 1 isoforms. Highlighted boxes indicate T cell epitopes identified using overlapping peptides synthesized according to the Bet v 1a sequence. (*b*) Bars show stimulation indices of 27 TCC (grouped according to the epitope recognized) with rBet v 1 isoforms a, b, d, e, and l and with pollen Bet v 1 (*nat*).

rBet v 1e, 41 of 48 (85%) reacted with rBet v 1b, 45 of 48 (94%) reacted with rBet v 1d, and 29 of 48 (60%) responded to rBet v 1l. Interestingly, only 39% of TCC reacted with all isoforms investigated. One TCC (XPF113II, patient 2, Fig. 1) failed to recognize any of the selected recombinant molecules in spite of reactvity with nBet v 1.

27 TCC displaying reactivity with rBet v 1a were analyzed for epitope specificity using overlapping peptides. In total, eight T cell epitopes recognized by different TCC were identified (Fig. 4). Positions and frequencies of T cell epitopes were in accordance with our previously published results (14, 15, 40). In several cases, amino acid differences between different isoforms of Bet v 1 caused decreased reactivity or nonreactivity in particular TCC recognizing the corresponding isoform sequence. For instance, crucial amino acid differences in the sequence representing epitope 4 and epitope 7 in isoform B in general led to decreased recognition of this isoform by TCC reactive for these epitopes. On the other hand, epitopes that were well conserved through-

Table 2. *Human IgE and T Cell Reactivity Patterns of Bet v 1 Isoforms a, e, b, d, and l*

	T cell reactivity (rel.%)	IgE binding (rel. %)	Skin prick test wheal areas (mm^2)
nBet v 1	100	100	ND
rBet v 1a	41	100	70
rBet v 1e	67	83	50
rBet v 1b	92	60	19
rBet v 1d	100	5	7
rBet v 1l	87	3	10

T cell reactivity values are relative percentages of added stimulation indices (48 TCC) taking nBet v 1 as reference. Relative percentages of IgE binding in immunoblots (sera from 30 patients) were calculated taking nBet v 1 and rBet v 1a as references. Values of wheal areas in skin prick tests are the average for five patients. ND, not determined.

out the sequences of these five isoforms seemed to be relatively well recognized by all TCC reactive with these peptides, for example, epitopes 3 and 8. In several cases, because of individual factors, no uniformity within reactivity patterns toward the same epitope was evident. For example, reactivity of TCC XPR6 was not affected by an amino acid exchange (Leu → Met) in epitope 2, position 18 of Bet v 1l, whereas the same exchange led to nonreactivity in response to Bet v 1l in TCC XPF7. In other TCC, a discrepancy between the capacity of isoforms to induce T cell proliferation could be observed, in spite of apparently identical epitope sequences (e.g., TCC XPD22, epitope 1, isoforms a and b). This phenomenon was reproducible. A possible explanation could be a different treatment of the isoforms during antigen processing due to diverse cleavage sites, leading to the creation of distinct peptides with variable antigenicity. Table 2 shows the relative potency of Bet v 1 isoforms a, b, d, e, and l in inducing proliferation of Bet v 1–specific TCC, taking nBet v 1 as a reference (100% T cell reactivity).

SPT. The capability of the recombinant Bet v 1 isoforms to elicit cutaneous reactions was evaluated for five birch pollen–allergic patients. Using Bet v 1d, two patients showed a negative SPT and three patients showed extremely weak skin reactivity. Similar results were obtained with Bet v 1l. As expected from in vitro results, isoforms a and e consistently produced stongly positive wheal and flare reactions in SPT, whereas isoform b showed intermediate skin reactivity (Fig. 5). In none of the individuals could late-phase reactions of the skin be observed. The calculated wheal areas reflecting the allergenicity of Bet v 1 isoforms are compared in Table 2.

Discussion

In this study, we compared isoforms of an important atopic allergen with respect to their T cell activation potency and IgE-binding capacity (allergenicity). Nine isoforms of Bet v 1 (27), the major allergen of birch pollen (6, 7), were cloned and produced as recombinant nonfusion proteins in *E. coli* (Fig. 1). Although these isoforms showed high sequence similarity, they revealed striking differences in their IgE-binding properties; that is, some isoforms displayed high binding activity, whereas others showed extremely low reactivity. Interestingly, this pattern was similar for all 30 patients tested. Based on our experiments, we grouped Bet v 1 isoforms into three classes: molecules with high IgE-binding activity (isoforms a, e, and j), intermediate IgE–binders (isoforms b, c, and f), and proteins with low/no IgE-binding capacity (isoforms d, g, and l) (Fig. 2 and Table 2). Representatives of each group were selected for further experiments with T lymphocytes. Bet v 1 isoforms a and e were chosen as representatives of the high IgE-binding group. Moreover, Bet v 1a seems to be the most abundant isoallergen in birch pollen. Isoforms d and l were chosen as members of the low IgE-binding group. Bet v 1b was selected as an intermediate IgE-binding isoform.

Each isoallergen was tested in vitro for its ability to activate allergen-specific T lymphocytes derived from the peripheral blood of birch pollen–allergic patients. To ensure that the total repertoire of T cell–specificities for pollen Bet v 1 was evaluated, proliferative responses to individual isoforms were investigated using PBMC and Bet v 1–specific TCL. TCC were isolated and reflected the patterns seen in experiments with PBMC and TCL (Fig. 3). Some patients seemed to be biased toward particular isoforms. All TCC (10 of 10) isolated from patient 6 recognized isoforms b and d (Table 1), whereas only 3 of 10 TCC reacted with isoforms a and l and 4 of 10 with isoform e. Other patients displayed a broad range of recognition of Bet v 1 isoforms; that is, their TCC recognized epitopes on all five isoforms tested (e.g., patients 1, 2, and 7; Table 1). Despite this variability among individuals, however, isoform Bet v 1d showed high T cell antigenicity throughout our experiments. A total of 48 Th clones from eight allergic patients were analyzed, which covered all important T cell epitopes on the Bet v 1 molecule (14, 15, 40; Fig. 4). Considering each isoform individually, the highest scores for T cell proliferative responses were observed with isoform d, followed by b, l, e, and a (Table 2). Surprisingly, Bet v 1a, the isoform with the highest IgE-binding activity, had the lowest score for T cell proliferation. Scores obtained for the low IgE-binding isoform d were significantly higher than scores obtained for isoforms with high IgE-binding activity and equal to those found with nBet v 1 as the stimulant.

To demonstrate the clinical relevance of these findings, in vivo tests (SPT) were performed. Indeed, the potency of isoforms d and l to induce typical urticarial skin reactions (wheal and flare) in allergic subjects was dramatically reduced compared with isoforms a, b, and e and commercially available test solutions (Fig. 5 and Table 2). This shows that data obtained from our in vitro experiments correlate well with the in vivo IgE-binding activity of the isoforms and demonstrates their functional properties lead-

Figure 5. SPT with recombinant Bet v 1 isoforms and commercially available extracts. Solid and dashed lines indicate wheal and flare (erythema) areas, respectively. *A*, rBet v 1a; *E*, rBet v 1e; *B*, rBet v 1b; *D*, rBet v 1d; *L*, rBet v 1l; *Bi*, birch pollen extract; *Gr*, grass pollen extract; *Hi*, histamine positive control; *Na*, NaCl-negative control.

ing to allergic effector mechanisms. To summarize, the following conclusions can be drawn: (*a*) hypoallergenic Bet v 1 isoforms d and l are potent activators of allergen-specific T lymphocytes from birch pollen–allergic patients; and (*b*) Bet v 1a, the most abundant isoform in birch pollen, shows the highest allergenicity and low T cell antigenicity.

Specific immunotherapy (SIT) is an efficient treatment of patients suffering from pollinosis (41–43). The benefit for the patient is dependent on the dose of allergen applied, and the therapeutic success correlates with the concentration of allergen given during the maintenance period, which is reached after a period of continuously increasing the dose. On the other hand, the risk of anaphylactic side effects also increases with the amount of allergen applied per injection. Therefore, it can be stated that the risk of side effects is the crucial obstacle to reach a satisfying clinical therapy success for some patients, and that SIT represents a risk for all patients treated by this method (44). In the last few years it has become evident that T lymphocytes are key players in the induction and maintenance of type I allergy (45–47). Therefore, the allergen-specific T cell should be the target of therapeutic strategies. There is evidence that successful SIT modulates the T cell response to allergens, leading to a decrease of clinical symptoms (48). Furthermore, it has been shown in mice that administration of peptides representing T cell epitopes induces tolerance to the whole allergenic molecule (49, 50). The idea behind this therapy concept is that, by treating allergic patients only with T cell–stimulating sequences, a modulation of the allergic immune response at the level of the Th cell could be achieved. In addition, IgE-mediated anaphylactic side effects would be avoided, because these T cell–stimulating peptides are different from the B cell epitopes (14, 17), and therefore no anaphylactic effector mechanisms could be triggered. One problem concerning this therapeutic approach, however, is the manifold T cell epitopes harbored by different allergens that are differentially recognized by individual allergic patients (10–19).

Taking all these aspects into consideration, we propose a novel approach in SIT of type I allergy: the use of full-length molecules with no or very low IgE-binding activity, but containing all T cell–reactive domains. Such molecules may occur naturally, as shown here to be the case for the major allergen Bet v 1. Bet v 1 binds IgE in >95% of all birch pollen–allergic individuals (>65% react exclusively with this molecule; 7): for these patients, a combination of isoforms d and l could be used for SIT. Alternatively, suitable molecules may be produced by means of genetic engi-

neering. For example, single amino acid substitutions obtained by site-directed mutagenesis can dramatically change IgE-binding activity of allergens without altering their ability to stimulate allergen-specific TCC (Ferreira, F., unpublished results). This approach for desensitization combines the advantage of low risk of IgE-mediated side effects with the possibility of down regulation of a broad repertoire of T cell specificities. High antigen doses lead to a modulation of the quality of the T lymphocyte response to the allergen (51, 52). It can be presumed that this T cell–targeted therapeutic strategy will function via a reduction of the secretion of IL-4 by lymphocytes of the Th2 type and lead to an improvement of the clinical efficacy of SIT.

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