

Reduced in vivo allergenicity of Bet v 1d isoform, a natural component of birch pollen

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Background: The major allergen of birch pollen, Bet v 1, is present in structurally slightly different isoforms. It has been postulated that certain isoforms show a distinct ability to bind birch pollen-specific IgE, although the T-cell response remains similar.

Objective: We verified the hypothesis of a distinct allergenicity but similar T-cell immunogenicity of 2 isoforms in birch pollen-allergic subjects by in vivo tests and an in vitro assay for T-cell stimulation.

Methods: Forty-eight birch pollen-allergic, 11 grass pollen-allergic, and 10 nonatopic control individuals were tested with 10-fold increasing concentrations (0.01 to 10.0 µg/mL) of recombinant (r) Bet v 1a and rBet v 1d by skin prick test (SPT), intradermal test (IDT), and conjunctival provocation test (CPT). An allergen-specific proliferation assay was performed on 21 patients with the 2 recombinant and the natural birch pollen allergens.

Results: In each test system only birch pollen-allergic subjects but no controls reacted to the recombinant allergens. A positive in vivo response to 10 µg/mL of rBet v 1a was observed in 21 of 48 by SPT, in 48 of 48 by IDT, and in 33 of 48 by CPT. In contrast, the IDT response to 10 µg/mL of rBet v 1d was reduced by a factor of 100 because it was equivalent to the response to 0.1 µg/mL of rBet v 1a. rBet v 1d failed to elicit a positive reaction in SPT and CPT. The proliferative response of T cells was similar for both recombinant isoforms because 8 of 21 individuals reacted to rBet v 1a and 6 of 21 to rBet v 1d. Only 1 subject had a positive reaction to rBet v 1d alone.

Conclusion: The natural isoforms rBet v 1a and rBet v 1d differ in their ability to bind IgE but are similar in their immunogenicity for T cells. Thus rBet v 1d might be a promising candidate for use in immunotherapy of birch pollen-allergic individuals. (*J Allergy Clin Immunol* 1999;104:1239-43.)

Key words: Type I allergy, recombinant Bet v 1, recombinant isoforms, skin prick test, intradermal test, conjunctival provocation test

Abbreviations used

CPT: Conjunctival provocation test
IDT: Intradermal test
r: Recombinant
SI: Stimulation index
SPT: Skin prick test

The major birch pollen allergen (Bet v 1) is one of the main causes of respiratory allergy in Europe. More than 95% of all tree pollen-allergic individuals exhibit IgE antibodies to Bet v 1 and more than 60% of these are sensitized to this protein only.¹ This clearly shows the predominant role of Bet v 1 in the development of type I allergy to birch pollen.

Bet v 1, the key allergen used in our study, has been cloned, sequenced,² produced as recombinant protein,³ and crystallized.⁴ This birch pollen allergen displays a considerable degree of heterogeneity, and pollen preparations consist of a mixture of closely related isoforms, as shown by mass spectrometry and complementary DNA cloning.⁵ Although these isoforms display high amino acid sequence identity, differing only in a few exchanges of amino acids, their immunologic properties may be very different.^{3,6} In vitro experiments, immunoblots, histamine-release assays, and T-cell proliferation tests confirmed that Bet v 1 isoforms show remarkable variations in their immunologic behavior.⁷ So far, more than 11 isoforms of Bet v 1 have been identified.^{4,5} It has been established that each birch tree expresses a specific subset of isoforms.⁴ The different isoforms have been named Bet v 1a, 1b, 1c, etc. Bet v 1a, formerly termed Bet v 1, seems to represent at least 50% of all major birch pollen allergens.⁵ The molecular mass of intact Bet v 1a is 17,438.6 kd and that of Bet v 1d is 17,413.7 kd.⁷

Today, allergen extracts from natural sources are used for serologic assays and skin tests to diagnose type I allergy. Usually, these extracts contain a mixture of many different proteins, only a few of which show allergenic properties. Many ill-defined mixtures are also used for immunotherapy, although there is a risk of sensitizing patients to additional proteins. By applying recombinant complementary DNA technology, it is now possible to produce highly purified allergens of stable quality and in sufficient quantity for reliable diagnosis.

In the current study we evaluated the question of

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TABLE I. Characterization of patients and control groups

		Age (y) (mean/range)	Male/female ratio	Total IgE (kU/mL) (mean/range)	CAP nBet v 1 (mean/range)	CAP rBet v 1 (mean/range)
Birch pollen-allergic subjects	(n = 48)	28.19/18-37	19:29	404.33/32-2803	3.98/2-6	3.96/2-6
Grass pollen-allergic subjects	(n = 11)	31.00/27-47	4:7	227.73/13-1120	0	0
Nonallergic subjects	(n = 10)	38.50/27-53	2:8	27.30/2-71	0	0

whether recombinant (r) Bet v 1a and rBet v 1d display different patterns of reactivity by skin prick test (SPT), intradermal test (IDT), and conjunctival provocation test (CPT). In addition, a T-cell proliferation assay was performed in a group of birch pollen-allergic individuals to demonstrate the immunogenicity of the isoforms.

MATERIAL AND METHODS

Study subjects

All study subjects were enrolled at the allergy outpatient clinic, Inselspital, Bern, Switzerland. None of these individuals had undergone specific immunotherapy, and all were asymptomatic and not receiving any medication at the time of investigation. The study protocol was approved by the ethical committee of the University of Bern, and all individuals gave informed written consent before participation in the study.

Forty-eight patients with a mean age of 28.2 years had a birch pollen allergy established by case history and a positive SPT to birch pollen extract. All patients had a CAP radioallergosorbent class ≥ 2 (≥ 0.7 kU/L) to birch pollen allergen. Eleven grass pollen-allergic subjects (mean age 31.0 years) with a positive SPT to grass pollen and a negative SPT and a negative CAP to birch pollen (class = 0) served as the first control group. The second control group consisted of 10 healthy individuals (mean age 38.5 years) with no history of respiratory allergy, negative SPT to common inhalants, and a negative CAP to birch pollen (Table I).

Recombinant birch pollen

rBet v 1a and rBet v 1d were purchased from ABS, Advanced Biological Systems, Salzburg, Austria. rBet v 1a was prepared as described previously,⁸ and the isoform rBet v 1d was prepared according to the same protocols. Purified recombinant allergens were stored lyophilized at -20°C until use. Spectroscopic methods^{9,10} were used to show that reconstituted Bet v 1a and Bet v 1d were structurally similar after lyophilization (data not shown). Both allergens were dissolved in 0.9% saline solution at concentrations of 10, 1, 0.1, and 0.01 $\mu\text{g/mL}$ for IDT and for CPT.

Skin tests

Allergen solutions used for skin tests were prepared from lyophilized samples immediately before use and handled as described.¹¹ SPT was performed according to the guidelines previously described¹² and placed on the volar surface of the forearm. For SPT the recombinant pollen allergens were used only at the highest concentrations (10 $\mu\text{g/mL}$). The skin reactions were recorded after 15 minutes. A wheal diameter of 3 mm or greater surrounded by erythema was considered positive.

IDT was performed by injecting the test solutions intradermally until a wheal of approximately 4 mm diameter was obtained. Saline solution diluent and histamine dihydrochloride (0.01%) were used as negative and positive controls, respectively, and rBet v 1a and rBet v 1d were used at the 4 concentrations described. The surface of the wheal was calculated according to the formula $(D_1 + D_2/2)^2$,

where D_1 and D_2 represent the mutual perpendicular diameters of the wheal produced by the antigen preparation, measured in millimeters.¹¹ A wheal size >5 mm in diameter with erythema was considered a positive reaction.¹³

CPT

A bilateral CPT was performed according to the method of Möller et al¹⁴ with use of an Eppendorf pipette with a sterile tip. After the negative control test with isotonic saline solution diluent, 15 μL of either rBet v 1a or rBet v 1d at increasing concentrations (0.01 to 10 $\mu\text{g/mL}$) were applied to the conjunctival sac every 10 minutes, alternating right and left eye. Postchallenge evaluation with regard to hyperemia, itching, and tear formation was performed 10 minutes after each administration. A symptom score was used as proposed by Abelson et al.¹⁵ Our score included the assessment of redness, tear formation, and itching. A positive response was defined as a score of at least 4 points. All tests were performed on the same day by the same investigator (A. O.).

Serologic tests

Specific IgE antibodies to birch pollen, rBet v 1, and total serum IgE levels were measured with the Pharmacia CAP FEIA system according to the manufacturer's instructions (Pharmacia Diagnostics, Uppsala, Sweden). Results were expressed on a scale from class 1 to 6. IgE antibody values (≥ 0.7 kU/L = class 2) were regarded as positive.

Proliferation assays

Mononuclear cells were obtained by density centrifugation over Ficoll-Hypaque of heparinized blood from 21 birch pollen allergic-subjects. A total of 2×10^6 cells were cultured in 0.2 mL of RPMI 1640 supplemented with either 10% human AB serum or 10% autologous plasma. Antigens of rBet v 1a or rBet v 1d were added in the final concentrations of 0.5, 0.05, and 0.005 $\mu\text{g/mL}$. Tetanus toxoid (Serum and Vaccine Institute, Bern) at 1 and 10 $\mu\text{g/mL}$ served as control antigen. Proliferation was measured after 6 days of culture at 37°C in 5% carbon dioxide by tritiated thymidine incorporation after addition of 1 mL of tritiated thymidine for 12 hours.¹⁶ A cutoff value of the stimulation index of >2 was considered positive. The data are shown as stimulation index values (SI), owing to the variability of the spontaneous proliferation of the PBMCs, which was between 450 and 2200 counts/min (see Fig 2).

Statistical analysis

For statistical testing, IDT, CPT, and lymphocyte transformation test were analyzed at different doses. All confidence intervals were calculated with respect to a 95% confidence probability. For the global testing of a difference in the median, the multivariate Friedman test was applied. Depending on the paired situation, the Wilcoxon signed-rank test was used. For testing the proportion of values above the cutoff values of $\text{SI} = 2$ and $\text{SI} = 3$ in the lymphocyte transformation test, the McNemar-test was used. Owing to the multiple testing, the significance level was corrected by Bonferroni.

RESULTS

Table I summarizes the data of the birch pollen-allergic and the 2 control-group subjects. The mean total IgE in the birch pollen-allergic, grass pollen-allergic, and nonallergic individuals was 404, 227, and 27 kU/L, respectively. All birch pollen-allergic subjects exhibited specific IgE in comparable strength to both birch pollen and rBet v 1 (mean CAP class 4). No serologic IgE to birch pollen or to rBet v 1 was detected in the 2 control groups (CAP class 0).

Skin tests

With use of recombinant Bet v 1a and Bet v 1d, 21 of the 48 birch pollen-allergic subjects had a positive SPT for rBet v 1a but none for rBet v 1d at its highest concentrations. None of the control subjects had a positive SPT to the recombinant allergens.

By IDT, all birch pollen-allergic subjects demonstrated positive skin reactions to rBet v 1a at 10 µg/mL, 43 at 1 µg/mL, 36 at 0.1 µg/mL and 26 at 0.01 µg/mL (Fig 1). Thirty-six individuals had positive skin results by IDT to rBet v 1d at 10 µg/mL, 11 at 1 µg/mL, 5 at 0.1 µg/mL, and none at 0.01 µg/mL (Fig 1). The differences between rBet v 1a and rBet v 1d were highly significant for each concentration.

Thus the same number of patients ($n = 36$) reacted to 10 µg/mL Bet v 1d as to 0.1 µg/mL of Bet v 1a, and the average wheal size was similar (Fig 1). None of the grass pollen-allergic or nonallergic individuals had positive skin reactions to either rBet v 1a or rBet v 1d in the different concentrations tested.

CPTs

In birch pollen-allergic subjects a positive symptom score ≥ 4 points to rBet v 1a was recorded in 33 of 48 at 10 µg/mL, in 10 of 48 at 1 µg/mL, and in none at the 0.1 and 0.01 µg/mL concentrations. As opposed to this, no positive results were obtained even at the highest concentration of 10 µg/mL with rBet v 1d. In birch pollen-allergic subjects symptoms usually occurred within 1 minute after allergen challenge, whereas all test solutions failed to elicit subjective or objective responses in either control group.

Proliferative response to Bet v 1a and Bet v 1d

With concentrations between 0.005 and 0.5 µg/mL of Bet v 1a and Bet v 1d and a cutoff value of SI >2 , only 8 of the 21 patients tested showed an enhanced proliferative response to rBet v 1a (Fig 2). In 5 of these 8 individuals the response was elicited by Bet v 1d as well, and only 1 individual reacted to rBet v 1d alone. With a cutoff value of SI >3 , 6 reacted to Bet v 1a and 3 to Bet v 1d. All patients reacted to the control antigen tetanus toxoid. The proliferation to rBet v 1a led to higher stimulation index values than to rBet v 1d and appeared to be more consistent than the proliferation to rBet v 1d. Thus we were unable to find a statistical difference between the

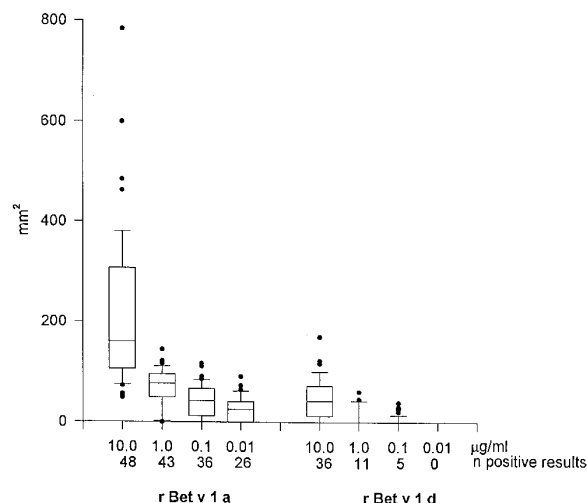


FIG 1. Comparison of Bet v 1a and Bet v 1d IDT reactivity in 48 birch pollen-allergic individuals at different concentrations. Data are shown as box plots of wheal size in square millimeters including median and 25th and 75th percentiles. Error bars above and below box indicate 90th and the 10th percentiles. On x axis number of skin test-positive individuals is indicated as well. Mean wheal size in birch pollen-allergic subjects to rBet v 1a at 10 µg/mL was 212.1 mm², at 1 µg/mL 69.5 mm², at 0.1 µg/mL 43.3 mm², and at 0.01 µg/mL 24.4 mm², whereas mean wheal size to rBet v 1d at 10 µg/mL was 48.0 mm², at 1 µg/mL 9.4 mm², at 0.1 µg/mL 3.0 mm², and 0.01 µg/mL did not elicit positive result at all.

proliferation to Bet v 1a or Bet v 1d with use of either SI >2 or >3 for all concentrations as a cutoff value.

Six birch pollen-negative control subjects have been tested and confirmed as negative.

DISCUSSION

In this study we evaluated the IgE and T-cell reactivity to 2 well-known isoforms of Bet v 1, namely, Bet v 1a and Bet v 1d. These isoforms have been shown to differ only in 7 amino acids.⁵ In spite of this small difference, the reactivity with IgE seems to differ drastically.^{3,6} On the other hand, the proliferative response of T cells in vitro to these isoforms is similar.

IgE-mediated reactivity to the recombinant material was found only in clearly sensitized persons with a history of seasonal rhinoconjunctivitis, positive SPT to natural pollen allergens, and positive CAP. It was consistently negative in both control groups. This rules out the possibility that either of the 2 preparations tested elicits an unspecific reaction.

rBet v 1a appears to be a major isoform of Bet v 1 because all 48 patients reacted with the rBet v 1a. However, this reactivity was only found with intradermal testing. With use of SPT, only 21 of 48 patients reacted with rBet v 1a at the highest concentration. In contrast, none of the patients reacted with the isoform Bet v 1d in SPT or in CPT, and only 36 of 48 patients reacted to the highest concentration of rBet v 1d in IDT.

Our data suggest that IgE binding to Bet v 1a and Bet

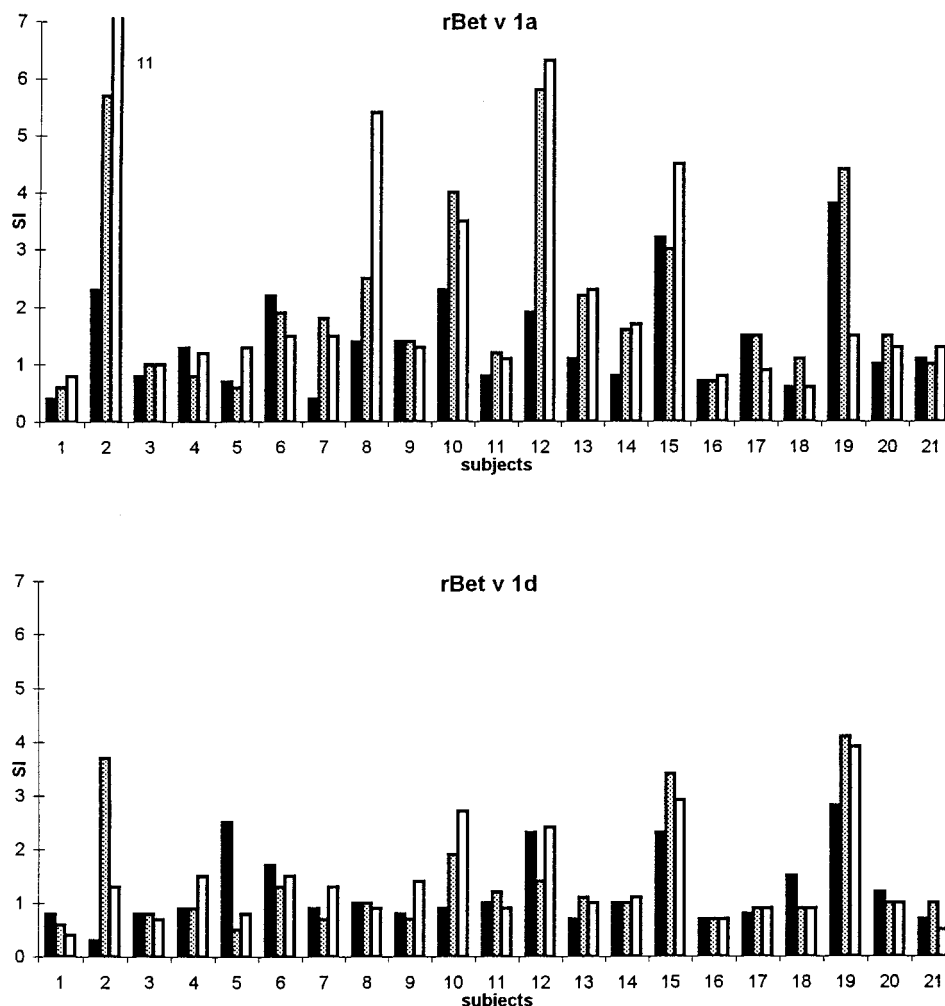


FIG 2. Proliferative response of PBMCs from 21 birch pollen-allergic individuals with use of autologous plasma. Data are shown as SI on y axis of cultures in triplicate with use of 0.5, 0.05, and 0.005 $\mu\text{g/mL}$ of either rBet v 1a or rBet v 1d. SDs ranged from 0.004 to 1.233 with a median of 0.227. The spontaneous proliferation (without antigen) was between 450 and 2200 counts/min. All proliferation assays were performed both in AB serum and in autologous plasma, but only data of tests with autologous plasma are presented because results did not show statistical difference.

v 1d differs quantitatively: rBet v 1d is actually able to bind IgE and to elicit the typical skin response but only at high concentrations and intradermal testing. Judging by the concentration-dependent reactivity of both isoforms, it might be calculated that rBet v 1d is about 100-fold less potent than the structurally closely related rBet v 1a. Both the number of reactive patients and the wheal sizes were quite similar when 10 $\mu\text{g/mL}$ of rBet v 1d was compared with 0.1 $\mu\text{g/mL}$ of rBet v 1a. Significantly, this quantitatively lower binding of rBet v 1d to IgE may indeed have clinical implications because Bet v 1d failed to induce a response in the provocation test (CPT).

Only a minority of the patients demonstrated a proliferative response to the recombinant allergens (8 and 6/21), whereas all tested persons had a proliferative response to tetanus toxoid. The reason for this unexpectedly low response to the allergens in proliferation assays is unclear but unlikely to be the result of a lack of

immunogenicity of the proteins because they are biologically active in skin test. Moreover, natural pollen extract also failed to induce strong proliferative responses in the majority of the subjects (data not shown). However, in patients with a positive response the proliferation to rBet v 1a and rBet v 1d was rather similar both in quantity and strength (Fig 2). In our view, the fact that only a minority of patients had a positive proliferative response is a result of a low T-cell precursor frequency outside of season and perhaps also to the use of a suboptimal allergen concentration.

These data endorse the concept that in comparison to rBet v 1a the isoform rBet v 1d is an immunologically active compound with suitable immunogenicity for T cells but strongly reduced allergenicity for IgE. This concept has recently been further strengthened by the finding that modulation of IgE reactivity of allergens by site-directed mutagenesis in analogy to Bet v 1d also reduces

allergenicity without affecting immunogenicity.¹⁰ Because high allergen concentrations seem to be advantageous for the clinical efficacy of specific immunotherapy,¹⁷ rBet v 1d might be an appropriate candidate for such treatment, permitting the use of relatively high allergen concentrations. Such a study could also reveal whether alteration of an immunologic response to a single, albeit immunodominant, allergenic protein can effectively suppress clinical symptoms elicited by natural pollen exposure.

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