# Allergens, IgE, mediators, inflammatory mechanisms

## Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: Diagnostic value for birch pollen and associated allergies

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**Objective:** *This study assesses the value of two recombinant birch allergens for diagnosis of patients sensitized to birch pollen with or without associated food allergy.*
**Methods:** *Fifty-one patients with positive skin test responses to Betulaceae and seven nonallergic control subjects were investigated; specific IgE antibodies were evaluated by specific immunoassay and blot immunodetection.*
**Results:** *Among 51 patients, 47 reacted to rBet v 1 and 10 to rBet v 2. Seven patients reacted to both recombinant allergens. In skin prick tests we found a correlation between the wheal produced by the commercial birch extract and the wheal produced by rBet v 1. Among 47 patients with positive test responses to rBet v 1, 83% had IgE binding to the Bet v 1 protein as determined by immunoblotting. Among 10 patients sensitized to rBet v 2, six had IgE binding to Bet v 2. Eleven patients with negative results, as determined by immunoblotting, had low levels of birch IgE in the sera (<10 kU/L) and low concentrations of IgE to rBet v 1 or rBet v 2 in ELISA. The nonallergic control subjects* (n = 7) *did not react to rBet v 1 or rBet v 2 in skin prick tests, nor did they have detectable amounts of specific IgE to rBet v 1 or rBet v 2. Histamine release tests confirmed sensitization to Bet v 1 in two patients with discordant results; for Bet v 2, one patient had positive results only at a high concentration, and one had results that remained negative. Thirty-four patients had birch pollinosis, and all reacted to rBet v 1. Patients who were monosensitized to birch never reacted to rBet v 2. Sensitization to rBet v 2 was only found in patients who reacted to other pollens (mainly grass). Twenty-nine patients demonstrated allergy to apples, cherries, or hazelnuts; and all reacted to rBet v 1. Among 11 patients with allergy to Umbelliferae, only three reacted to rBet v 2.*
**Conclusions:** *Use of the two recombinant allergens (rBet v 1 and rBet v 2) always permits the diagnosis of birch sensitization. Sensitization to rBet v 1 is specific for birch and Rosaceae allergies, whereas sensitization to birch profilin, Bet v 2, is encountered in multisensitized subjects and is not always related to Umbelliferae allergy. (J Allergy Clin Immunol 1996;97: 1100-9.)*

***Key words:*** *Birch pollinosis, food allergy, recombinant birch allergen (rBet v 1), recombinant birch profilin (rBet v 2), skin tests*

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Diagnosis of birch allergy is based on clinical history and the detection of specific IgE antibodies by both cutaneous tests and specific IgE determinations with RAST or immunoblotting techniques. Both in vivo and in vitro techniques are influenced by the inherent heterogeneity of allergen extracts. Recently, two important allergens for birch pollen were cloned and sequenced.[1, 2] Previously, in birch-sensitized populations, rBet v 1 and rBet v 2 had been shown to be as efficient as their natural counterparts in both IgE antibody binding[3] and histamine release from basophils.[4] The conclusion of these studies was that recombinant allergens allowed in vitro diagnosis of birch sensitization. However, the most common and relevant way to monitor allergy is to demonstrate immediate reactions by skin testing. From this point of view, each new recombinant allergen should be assessed in an appropriately sensitized population. In this clinical study we evaluated the potential of rBet v 1 and rBet v 2 for eliciting immediate type I skin test reactions in Betulaceae-sensitized subjects in comparison with routinely used commercial birch extracts. We correlated skin test reactivity with the presence of the corresponding specific IgE antibodies, as detected by immunoblotting and ELISA with natural and recombinant allergens. In addition, we attempted to determine whether patients had different patterns of reactions to recombinant allergens according to clinical history, associated sensitization to grass and weed pollens, and manifestations of fruit or vegetable food allergy.

*Abbreviations used*

| | |
|---|---|
| BSA: | Bovine serum albumin |
| PBS: | Phosphate-buffered saline |
| PST: | PBS containing 10% newborn calf serum and 1% Tween-20 |
| SDS-PAGE: | Sodium dodecylsulfate–polyacrylamide gel electrophoresis |

## METHODS

### Subjects

The study comprised 51 patients with either rhinitis or respiratory complaints who were referred to our clinic between December 24, 1993 and June 15, 1994 and who had a positive skin prick test response to a mixed Betulaceae pollen extract containing 25% alder, 25% birch, 25% hornbeam, and 25% hazelnut pollen (Stallergènes Laboratories, Fresnes, France), seven healthy control subjects with no clinical history of allergy and no specific IgE to a panel of aeroallergens (Matrix; Abbott Laboratories, Chicago, Ill.) and eight atopic control subjects (3 monosensitized to cat allergens, 5 to mite allergens). Furthermore, a positive skin prick test response to a birch pollen extract (Stallergènes Laboratories) was required as an unquestionable criterion for birch sensitization. No patients received antihistaminic medication at the time of the study.

The 51 patients were tested with a panel of common aeroallergens, including grass and weed pollens. They were classified with respect to age, sex, symptoms, chronology of symptoms, and allergen sensitization (Table I). Thirty-five patients had birch pollen–related symptoms (i.e., early spring rhinoconjunctivitis with or without asthma); among them, 16 patients had both birch- and grass-related symptoms (during May and June) and 19 patients had birch pollinosis exclusively. Ten patients of 51 had no symptoms in response to birch and had grass and/or weed pollinosis (symptoms occurring in May and June and/or August). Five of 51 patients had perennial symptoms (asthma or rhinitis), and one patient had exercise-induced angioedema. Twenty-nine patients had associated fruit allergy (Rosaceae and/or hazelnuts) with essentially oral-pharyngeal pruritus and lip edema; 11 patients had food allergy to vegetables from the Umbelliferae family (10 to celery, 5 to carrots). Fruit or vegetable allergy was confirmed by either cutaneous tests with raw material or presence of specific IgE.

Ethical approval for skin testing with recombinant allergens in human subjects was obtained from the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale of Lyon. A full explanation of the procedure was given to all participants before testing, and written consent was obtained.

### Skin testing

For skin prick tests, 30 μl of each solution was placed on the patient's forearm. Thereafter, the skin was pricked with a Prick-Lancette (Dome Hollister Stier Laboratories, Puteaux, France). The test sites were placed 3 cm apart to avoid false-positive results. Birch pollen extract was provided by Stallergènes Laboratories and was used at a concentration recommended by the manufacturer (100 IR). From an initial solution of recombinant birch allergens (1 mg protein/ml), serial dilutions (from 50 μg to 3 μg/ml) were made in sterile physiologic saline solution. These dilutions were selected according to the concentration of rBet v 1 and rBet v 2 allergens, which elicited significant histamine release by sensitized basophils.[5] It has been shown that the thresholds of positivity for histamine release tests and intradermal reactions are in the same range[6]; and it is assumed that the sensitivity of prick tests is $10^2$ to $10^3$ times lower than that of intradermal tests. A negative control test was performed with saline solution, and a positive control test was done with histamine at 1 mg/ml. The prick test was considered positive when the mean diameter ([D + d]/2) of the wheal induced by the allergen was at least 75% of the mean diameter of the wheal induced by a histamine control.

**TABLE I.** Numbers, sex, age, type of pollinosis, and food allergy of the population with positive skin prick test responses to the mixed Betulaceae extract ($n = 51$)

| Clinical symptoms | No. of patients | Sex ratio (F:M) | Mean age (yr) | Food allergy | | | |
|---|---|---|---|---|---|---|---|
| | | | | Rosaceae | Umbelliferae | Rosaceae & Umbelliferae | No food allergy |
| Birch pollinosis* | 19 | 0.73 | 39.7 | 7 | 0 | 5 | 7 |
| Birch pollinosis associated with grass or weed pollinosis* | 16 | 0.45 | 30.4 | 9 | 0 | 3 | |
| Grass or weed pollinosis* | 10 | 1 | 32.5 | 2 | 2 | 1 | 5 |
| Other complaints† | 6 | 2 | 29.6 | 2 | 0 | 0 | 4 |
| Healthy control subjects | 7 | 2.5 | 23.5 | 0 | 0 | 0 | 7 |

*The diagnosis of pollinosis is based on the seasonal occurrence of symptoms.
†Perennial symptoms: asthma and/or rhinitis ($n = 5$), exercise-induced angioedema ($n = 1$).

## Patients' sera

Serum samples for serologic analysis were obtained immediately before testing and stored at −20° C until use.

## Allergenic extracts

*Expression and purification of recombinant birch pollen allergens (rBet v 1 and rBet v 2).* The complementary DNA coding for the major birch pollen allergen, Bet v 1,[1] was expressed in *Escherichia coli* JM 105 by using plasmid pKK223-3, as previously described.[3] *E. coli* were induced in liquid culture with isopropyl-B-thiogalactoside at a final concentration of 2 mmol/L. *E. coli* cells were then harvested by centrifugation, and after several freeze-thaw cycles, recombinant Bet v 1 was purified by chromatofocusing and high-performance liquid chromatography as previously described.[7] The purified recombinant allergen, checked for purity by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent silver staining, was shown to specifically bind IgE from sera of patients allergic to Bet v 1 and to release histamine from basophils of sensitized patients in a specific and dose-dependent manner. The cDNA coding for birch profilin, Bet v 2[2] was amplified by polymerase chain reaction with the original cDNA as a template. The polymerase chain reaction product was then subcloned in plasmid pET and expressed at high efficiency in *E. coli* DE3 (BL21) under the control of T7 RNA polymerase (Susani et al. Unpublished data). Milligram amounts of rBet v 2 could be purified from 100 ml of liquid culture by using poly-L-proline affinity chromatography as previously described.[5] Both recombinant allergens were tested for lack of cell toxicity by means of histamine release assays, and the protein preparations were checked for the absence of nucleic acids by dotting onto ethidium bromide plates. Proteins were stored lyophilized at −20° C until use. For skin testing, the proteins were freshly dissolved at a concentration of 1 mg/ml in physiologic saline solution and further diluted as described.

*Birch pollen extract.* Pollen from *Betula alba* (Sigma Chemical Co., St. Louis, Mo.) was extracted by overnight agitation in distilled water with a magnetic stirrer. The supernatant obtained after centrifugation at 4000 *g* for 30 minutes (referred to as crude extract) was dialyzed against 10 mmol/L Tris/HCl, pH 6.8 and concentrated in a 60% (wt/vol) polyethylene glycol 35,000 solution made in 10 mmol/L Tris/HCl, pH 6.8.

## Detection of specific antibodies

*Determination of birch-specific IgE antibodies.* Specific birch IgE antibody levels were estimated by immunoassay with the CAP system (Pharmacia, Uppsala, Sweden). Results were expressed in kilounits per liter.

*Detection of specific IgE antibodies to Bet v 1 and Bet v 2 on birch pollen extract immunoblots.* Birch proteins from birch *(Betula alba)* crude extract were separated on 12% SDS-PAGE[8] and then transferred from polyacrylamide gel to a nitrocellulose membrane (0.45 μm; Schleicher and Schuell, Dassel, Germany) as previously described.[9] The membrane was incubated for 30 minutes in phosphate-buffered saline (PBS) containing 1% Tween-20 and 5% non-fat milk to block free protein binding sites. Half-diluted sera in PBS containing 10% newborn calf serum and 1% Tween-20 (PST) were incubated overnight at +4° C, under agitation. After three washes with PST, the nitrocellulose sheet was incubated sequentially for 2 hours with a rabbit anti-human IgE (ε-specific; Dako, Copenhagen, Denmark) diluted 1:500, and for 1 hour with a peroxidase-labeled anti-rabbit IgG (Vector; Biosys, Compiègne, France) diluted 1:5000. After two washes with PST and two washes with PBS containing 1% Tween-20, the nitrocellulose membrane was immersed in electrochemoluminescence detection reagents (ECL Western Blotting detection reagents; Amersham, Buckinghamshire, U.K.) for 1 minute and applied close to a film (Hyperfilm Betamax; Amersham) for 20 seconds.

*Detection of IgE antibodies specific for rBet v 1 and rBet v 2 by immunoblotting.* Thirty micrograms of rBet v 1 and rBet v 2, respectively, were separated per 15 cm (2 μg/cm) by SDS-PAGE (12.5%)[8] and then transferred to nitrocellulose by electroblotting.[10] Nitrocellulose sheets were then cut and incubated with 1:10 diluted patients' sera, and bound IgE antibodies were detected with a iodine 125–labeled anti-human IgE antibody (Pharmacia).

*Detection of IgE and IgG antibodies specific for rBet v 1 and rBet v 2 by ELISA.* For semi-quantitative measurements of allergen-specific IgE and IgG subclass reactivity, 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with 2 μg of recombinant allergens per well dissolved in 0.1 mmol/L sodium carbonate buffer, pH 9.6, overnight at 4° C. The plates were washed twice with PBS containing 0.05% Tween 20, blocked for 2.5 hours with PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 at room temperature, and incubated overnight at 4° C with sera. Patients' sera were diluted 1:10 in PBS containing 0.5% BSA and 0.05% Tween-20 for the detection of specific IgE and 1:100 for detection of $IgG_1$, $IgG_2$, $IgG_3$, and $IgG_4$. The plates were washed five times with PBS containing 0.05% Tween-20, and bound immunoglobulins were detected with monoclonal mouse anti-human Ig antibodies (PharMingen, San Diego, Calif.), diluted 1:1000 in PBS containing 0.5% BSA and 0.05% Tween-20 overnight at 4° C. The plates were then washed five times with PBS containing 0.05% Tween-20 and incubated for 30 minutes at 37° C and 30 minutes at 4° C with a horseradish peroxidase–coupled sheep anti-mouse antiserum (Amersham), diluted 1:2000 in PBS containing 0.5% BSA and 0.05% Tween-20. Plates were subsequently washed five times with PBS containing 0.05% Tween-20, and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma Chemical Co.) solution was added. Plates were incubated in the dark for 30 minutes at room temperature and the color reaction was stopped by addition of 100 μl/well 0.32% NaF. Absorbances (optical density 450 to 405 nm) were determined with an ELISA reader (Dynatech, Denkendorf, Germany)

*Histamine release tests.* The challenge of whole blood with birch pollen extract, rBet v 1, rBet v 2, and anti-IgE as a positive control was performed in a dose-response fashion according to the method described by Tanizaki et al.[11] Venous blood (10 ml) was drawn into a plastic syringe containing 1 ml of heparin. Two hundred fifty microliters of different concentrations of allergens (dose range: $10^{-4}$ to 10 μg/ml) or anti-IgE (dose range: $10^{-4}$ to $10^{-3}$ dilution [ε-specific, Dako]) was added to the test tubes containing 500 μl of whole blood diluted 1:4 in Tris buffer (10 mmol/L Tris, 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.23 mmol/L $MgCl_2$, 1.8 mmol/L $Cl_2Ca$, 5.5 mmol/L glucose; pH 7.3). The mixed solution was incubated for 30 minutes at 37° C. The reaction was stopped, and the cells were separated by cold (4° C) centrifugation at 375 *g* for 5 minutes. Two hundred microliters of the cell-free supernatant was used for histamine quantification in a radioimmunoassay with acylated histamine monoclonal antibodies (Immunotech, Marseille, France) as described previously.[12] Total histamine was measured after cell lysis by repeated thawing and freezing. All experiments were performed in duplicate.

## RESULTS

### Results of skin tests with commercial birch extracts and recombinant birch allergens

Among 51 patients with either rhinitis or respiratory complaints who were sensitized to a commercial Betulaceae extract, 50 reacted to at least one of the two recombinant allergens; 47 (92%) reacted to rBet v 1 (27 at 3 μg/ml; 20 at 10 μg/ml), and 10 (19.6%) reacted to rBet v 2 (3 at 3 μg/ml; 7 at 10 μg/ml). Seven patients reacted to both recombinant allergens. No positive test results were found among the seven control patients or among the eight atopic patients.

Three of the 51 patients who reacted to mixed Betulaceae extracts were considered to have negative responses to a birch pollen extract (Stallergènes 100 IR) according to our criteria. Among them, two reacted to rBet v 1 at 10 μg/ml. The third patient did not react to either rBet v 1 or rBet v 2.

For 27 patients who reacted to rBet v 1 at the lowest concentration (3 μg/ml), we found a correlation between the wheal produced by the prick test with commercial birch extract and the wheal produced by rBet v 1 ($n = 27, r = 0.45, p < 0.05$) (Fig. 1). A significant correlation was also found for the 20 patients who reacted only to rBet v 1 at a concentration of 10 μg/ml ($n = 20, r = 0.45, p < 0.05$). There was also a good correlation between the erythema induced by the commercial birch extract and rBet v 1 ($r = 0.6, p < 0.01$) in patients who reacted to rBet v 1 at the lowest concentration.

### Correlation of skin prick tests and detection of specific antibodies

All sera contained specific IgE against birch allergens (Table II).

The presence of IgE antibodies to Bet v 1 and Bet v 2 was determined on crude birch extract blot (Fig. 2). Detection of IgE antibodies to rBet v 1 and rBet v 2 was performed by immunoblotting, specific immunoassay, and in some cases by histamine release tests.

*Concerning Bet v 1.* Among the 47 patients who had a positive skin prick test response to rBet v 1, we found 39 patients who displayed IgE binding to

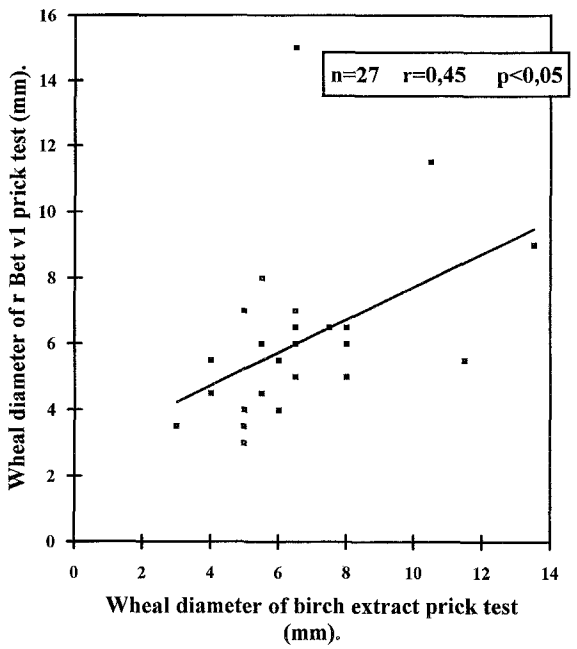


**FIG. 1.** Correlation of wheal diameters obtained by prick tests with birch extract (Stallergene, 100 IR) and prick tests with rBet v 1 (3 μg/ml) in 27 patients.

rBet v 1 on immunoblotting with crude extract (83%). The serum samples from eight patients who had a positive skin prick test response to Bet v 1 without IgE binding to Bet v 1 on immunoblotting (crude extract) were all characterized by low concentrations of IgE against birch pollen in CAP RAST (<10 kU/L) and against rBet v 1 in ELISA. The mean values of specific IgE to rBet v 1 were significantly different in these two groups of patients ($p < 0.05$). These eight patients also had lower concentrations of $IgG_4$ against rBet v 1 in ELISA (results not shown). Six of these eight patients had detectable IgE binding to rBet v 1 on blots; for the two remaining patients with discordant results, histamine release tests were performed and showed a bell-shaped dose-response curve (i.e., release of half of the maximum histamine release: $10^{-3}$ and $10^{-4}$ μg/ml) (Fig. 3, *A*).

*Concerning Bet v 2.* Among the 10 patients who had positive skin prick test response to rBet v 2, we found six who displayed IgE binding to Bet v 2 on immunoblotting with crude extract. Again, all four patients with discordant results had low serum levels of IgE against birch pollen (<10 kU/L) and low serum concentrations of IgE and $IgG_4$ against rBet v 2 in ELISA. After detection of rBet v 2 on immunoblotting, only two patients had negative results. In these two patients histamine release was negative in one and only positive for elevated concentrations (1 μg/ml) in the other (Fig. 3, *B*). Histamine release tests were also performed in two control subjects, and results remained negative for both rBet v 1 and rBet v 2, even at the highest concentrations used. In the serum of one patient with no skin reactivity to rBet v 2, we found specific IgE to rBet v 2.

## Comparison of pattern of skin reactivity with clinical history

*Different patterns of skin reactivity to recombinant allergens according to clinical classification.* Taking into account clinical aspects, it should be pointed out that patients with birch pollinosis monosensitized to birch were always sensitized to rBet v 1 and never reacted to rBet v 2 (Table III). Moreover, when birch pollinosis was associated with another type of pollinosis, patients were always sensitized to rBet v 1 and occasionally also to rBet v 2. Sensitization to rBet v 2 is significantly more frequent in patients with birch pollinosis associated with grass or weed pollinosis ($p < 0.05$). Three patients who had positive skin test responses to birch but who were monosensitized to rBet v 2 had no clinical symptoms during the birch pollen season but had typical grass or weed pollinosis. Sixty-three percent of patients with clinical birch allergy reacted to the lowest concentration of rBet v 1 (3 μg/ml) as compared with 30% in the group of patients without birch pollinosis.

*Concerning food-associated allergy.* Twenty-nine patients had allergy to Rosaceae and/or hazelnuts. Nineteen reacted to hazelnuts, 24 to apple, and 17 to several fruits from the Rosaceae family (Table IV). All of these patients reacted to rBet v 1. The thresholds of rBet v 1 skin test response were not different from those of patients with birch pollinosis without food allergy. No patient monosensitized to rBet v 2 had clinical symptoms of food allergy to nuts or members of the Rosaceae family.

The chronology of appearance of fruit allergy is not always precisely known, but in most cases it begins when pollinosis has already started. However, in one of our patients, food allergy to apple preceded birch pollinosis by 9 years; this patient was monosensitized to rBet v 1 in skin tests. Another patient had exercise-induced anaphylaxis and food allergy to apple and hazelnuts; he had no birch pollinosis but reacted to rBet v 1 in skin tests. So it appears that all patients who had food allergy to apple or nuts reacted to rBet v 1 in skin tests, independently of the presence of birch pollinosis.

Among 11 patients with allergy to members of the Umbelliferae family, all reacted to rBet v 1 and three of them reacted to both rBet v 1 and rBet v 2; these three patients also had grass and mugwort

**TABLE II.** Comparative results of skin prick tests with recombinant birch allergens, birch CAP immunoassay, and immunodetection of specific IgE to Bet v 1 and Bet v 2 in 51 patients sensitized to Betulaceae

| Patient No. | Skin prick test results | | Birch CAP immunoassay (kU/L) | ELISA | | Birch pollen immunoblot | | Recombinant birch immunoblot | |
|---|---|---|---|---|---|---|---|---|---|
| | rBet v 1 | rBet v 2 | | IgE rBet v 1 | IgE rBet v 2 | Bet v 1 | Bet v 2 | rBet v 1 | rBet v 2 |
| 1 | + | − | 4.6 | 0.181 | 0.059 | − | − | + | − |
| 2 | + | − | 15.6 | 0.32 | 0.042 | + | − | + | − |
| 3 | − | + | 5.5 | 0.13 | 0.055 | − | + | − | + |
| 4 | + | − | 22.3 | 0.298 | 0.037 | + | − | + | − |
| 5 | + | − | 17.1 | 0.328 | 0.027 | + | − | + | − |
| 6 | + | − | 81.5 | 2.093 | 0.024 | + | − | + | − |
| 7 | + | − | >100 | 0.628 | 0.051 | + | − | + | − |
| 8 | − | − | ND | 0.173 | 0.061 | ND | ND | − | − |
| 9 | + | − | 10 | 0.35 | 0.047 | + | − | + | − |
| 10 | + | − | >100 | 0.834 | 0.03 | + | 1 band | + | ± |
| 11 | + | − | >100 | 2.201 | 0.03 | + | − | + | − |
| 12 | + | − | >100 | 1.525 | 0.02 | + | − | + | − |
| 13 | + | − | 44 | 0.648 | 0.023 | + | − | + | − |
| 14 | + | + | 34.2 | 0.48 | 0.034 | + | + | + | − |
| 15 | + | − | 30.6 | 0.502 | 0.053 | + | − | + | − |
| 16 | + | + | 76.6 | 2.5 | 0.061 | + | + | + | + |
| 17 | + | − | 5.7 | 0.15 | 0.047 | − | − | + | − |
| 18 | + | − | 10.3 | 0.171 | 0.038 | + | − | + | − |
| 19 | + | − | 7.6 | 0.318 | 0.095 | + | − | + | − |
| 20 | + | − | >100 | 2.5 | 0.034 | + | − | + | ± |
| 21 | + | − | 8.8 | 0.411 | 0.033 | + | − | + | − |
| 22 | + | − | 1.05 | 0.088 | 0.031 | + | − | + | − |
| 23 | + | + | 9.5 | 0.112 | 0.11 | + | + | + | + |
| 24 | + | − | 4.2 | 0.168 | 0.055 | − | − | + | − |
| 25 | + | − | 23.8 | 0.497 | 0.045 | + | − | + | − |
| 26 | + | − | 7.1 | 0.128 | 0.029 | + | − | + | − |
| 27 | + | − | 70.7 | 2.5 | 0.032 | + | − | + | − |
| 28 | + | − | 29.3 | 0.332 | 0.031 | + | − | + | − |
| 29 | + | − | 11 | 0.226 | 0.033 | + | − | + | − |
| 30 | + | − | 38.7 | 0.282 | 0.04 | + | − | + | − |
| 31 | + | − | 1.57 | 0.257 | 0.058 | − | − | + | − |
| 32 | + | − | 87.1 | 0.971 | 0.061 | + | − | + | − |
| 33 | + | + | 3.8 | 0.137 | 0.032 | − | − | + | − |
| 34 | + | − | 22.4 | 0.2 | 0.031 | + | − | + | − |
| 35 | + | − | 19.1 | 0.222 | 0.026 | + | − | + | − |
| 36 | + | − | 99.5 | 1.723 | 0.033 | + | − | + | − |
| 37 | − | + | 2.9 | 0.166 | 0.073 | − | − | − | + |
| 38 | + | − | >100 | 0.17 | 0.17 | + | − | + | − |
| 39 | + | + | 6.6 | 0.137 | 0.071 | + | − | + | + |
| 40 | + | + | 62.3 | 0.665 | 0.212 | + | + | + | + |
| 41 | + | − | 1.65 | 0.117 | 0.033 | − | − | − | − |
| 42 | + | − | 84.1 | 1.099 | 0.035 | + | − | + | − |
| 43 | + | − | >100 | 1.52 | 0.045 | + | − | + | − |
| 44 | + | − | 6.8 | 0.115 | 0.043 | + | − | + | − |
| 45 | − | + | 7.6 | 0.125 | 0.048 | − | + | − | + |
| 46 | + | − | 6.1 | 0.234 | 0.043 | + | − | + | − |
| 47 | + | − | >100 | 0.61 | 0.056 | + | + | + | + |
| 48 | + | − | 2.47 | 0.104 | 0.069 | − | − | − | − |
| 49 | + | − | 16.1 | 0.403 | 0.046 | + | − | + | − |
| 50 | + | + | 6.4 | 0.129 | 0.062 | + | − | + | − |
| 51 | + | − | 0.38 | ND | ND | − | − | ND | ND |

*ND,* Not done.

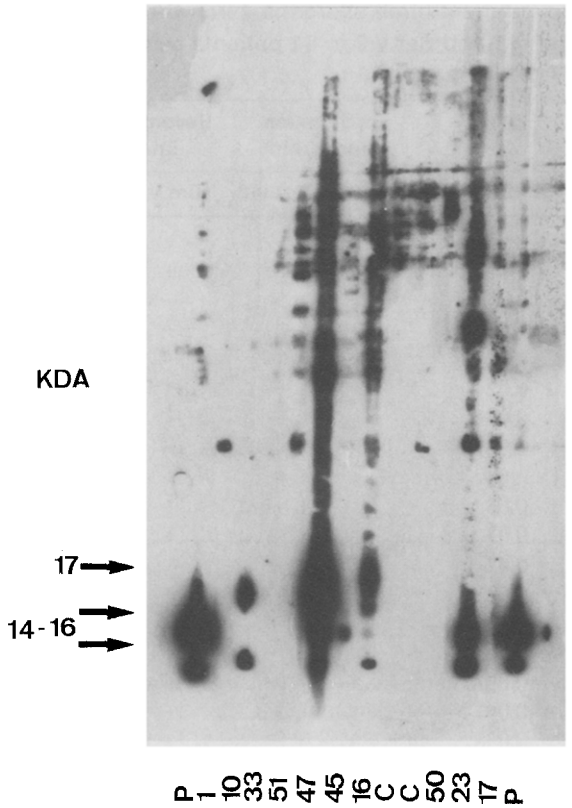


**FIG. 2.** Natural birch pollen extract immunoblot in electrochemoluminescence. Immunodetection of blots from birch pollen was performed with anti-profilin rabbit antibodies *(P)*, sera from patients sensitized to birch pollen, and sera from two control subjects *(C)*. Binding to Bet v 1 and Bet v 2 are shown in 17 kd and 14 to 16 kd, respectively.

pollinosis. Among the patients with birch pollinosis, food allergy to members of the Umbelliferae family was always associated with food allergy to members of the Rosaceae family.

## DISCUSSION

Replacement of traditional diagnostic procedures by new techniques should be evaluated according to three major criteria: innocuousness, sensitivity, and specificity. All 48 patients with positive skin test responses to a commercial birch pollen extract reacted to at least one of the two recombinant allergens. These results demonstrate that the sensitivity in establishing birch sensitization is 100% when both recombinant birch allergens, rBet v 1 and rBet v 2, are used. Moreover, skin tests with recombinant birch pollen allergens verified birch sensitization in two patients who had positive responses to a mixed Betulaceae extract but negative responses to the commercial birch pollen extract. In seven nonatopic and eight atopic control subjects with negative birch skin test results, there was no positive reaction to recombinant allergens, even at the highest concentrations, confirming the specificity of the recombinant allergen skin tests. In control subjects, as well as in patients, no adverse reaction was observed at any concentration.

The diagnostic value of recombinant birch allergens, assessed in our study by skin tests, can be compared with the results obtained previously in in vitro studies. In 1991 Valenta et al.[3] found among 100 subjects with a clinical birch pollinosis 97% sensitized to rBet v 1, 9% sensitized to rBet v 2, and 6% sensitized to both allergens. In this study we found 92%, 19.6%, and 7%, respectively. The higher incidence of sensitization to rBet v 2 in our population may be related to the different geographic origins of the two populations.

The evaluation of the clinical relevance of recombinant allergens was further assessed by detecting specific IgE antibodies in the sera. In the majority of patients, positive skin test responses to rBet v 1 and rBet v 2 correlated with detectable amounts of specific IgE. However, in some patients specific IgE for natural or recombinant birch pollen allergens could not be detected on blots or by immunoassay. In these cases, as far as Bet v 1 is concerned, sensitization was proven by positive histamine release test results. For Bet v 2, we observed some discrepancies: in one patient with a positive response to rBet v 2, no specific Bet v 2 IgE could be demonstrated, either serologically or by cellular tests. In one patient a negative skin test response to rBet v 2 was observed, despite the presence of specific Bet v 2 IgE. It cannot be excluded that a positive prick test result could be obtained with a higher concentration of rBet v 2 in this case. Nevertheless, as a whole, our results point out that for recombinant allergens, as well as for natural allergen extracts in general, skin testing is the most sensitive means of establishing sensitization.

Skin testing with a well characterized allergenic molecule may be of interest because in some instances sensitization to a specific allergenic molecule is responsible for a defined clinical syndrome. Our study allows us to correlate sensitization to a specific birch allergen with the clinical symptoms. Isolated symptoms of birch pollinosis are always associated with sensitization to Bet v 1 alone. Sensitization to Bet v 2, when isolated, always corresponded to another pollinosis. How-

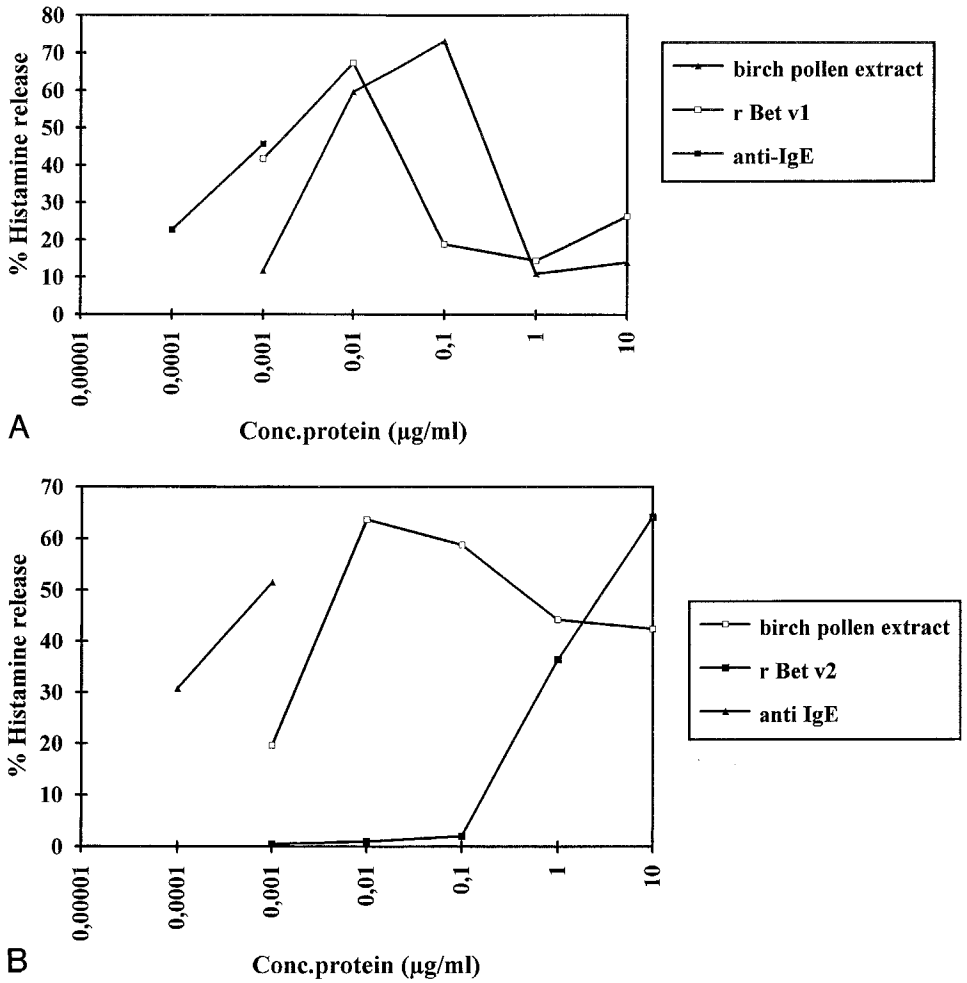


**FIG. 3.** Histamine release curves for rBet v 1 **(A)** and rBet v 2 **(B)** in two patients with positive skin test results but without detectable specific IgE antibodies. **A**, Patient no. 41; **B**, patient no. 50.

ever, sensitization to rBet v 1 was encountered in patients with clinical birch pollinosis and also in nine patients sensitized to birch without clinical expression of this allergy. Ebner et al.[13] showed that Bet v 1 shared common epitopes with a 17 kd protein in apple extracts. They were able to shown that among 63 patients who had birch sensitization and apple intolerance, all had sera that reacted to Bet v 1 on immunoblotting. We were able to confirm these results in 24 patients by using in vivo test. Thus it appears that a positive cutaneous test response to rBet v 1 is a hallmark of the birch-apple syndrome. One of our patients had exercise-induced angioedema after apple ingestion without any pollinosis: he was monosensitized to Bet v 1 as determined by skin prick tests. Another patient complained of apple-induced oral allergy syndrome 9 years before the onset of birch pollinosis; this patient was also monosensitized to rBet v 1. Higher levels of anti-rBet v 1 IgE were found in the patients allergic to birch pollen who also had food-associated allergy (apple, Rosaceae fruits, and/or hazelnuts) ($n = 35, p < 0.01$), when compared with the group without food allergy, confirming results of previous studies.[14, 15] For anti-rBet v 1 $IgG_4$, there was no significant difference among patients with or without food-associated allergy. Therefore use of rBet v 1 in skin prick tests permits not only detection of birch sensitization but also identification of sensitization to Bet v 1–related proteins responsible for intolerance to foods in patients allergic to tree pollen.

Less than 20% of the birch-sensitized population has specific IgE to Bet v 2, indicating that Bet v 2 is a minor allergen. This confirms previous findings by Jarolim et al.[16] In no case was sensitization to Bet v 2 alone found to be associated with clinical symptoms of allergy to birch. In a previous

**TABLE III.** Results of skin prick tests with mixed Betulaceae extract, commercial birch pollen extract, and recombinant birch allergens, according to clinical symptoms

| Clinical symptoms | No. of patients | Positive skin prick tests results | | | | | |
|---|---|---|---|---|---|---|---|
| | | Betulaceae | Birch pollen | rBet v 1 (10 μg/ml) | rBet v 1 (3 μg/ml) | rBet v 2 (10 μg/ml) | rBet v 2 (3 μg/ml) |
| Birch pollinosis | 19 | 19 | 18 | 7 | 12 | 0 | 0 |
| Birch pollinosis & other pollinoses | 16 | 16 | 16 | 6 | 10 | 3 | 1 |
| Grass or weed pollinosis | 10 | 10 | 9 | 2 | 4 | 4 | 2 |
| Other complaints* | 6 | 6 | 5 | 5 | 1 | 0 | 0 |
| Healthy control subjects | 7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 58 | 51 | 48 | 20 | 27 | 7 | 3 |

*Perennial symptoms: asthma and/or rhinitis ($n = 5$), exercise-induced angioedema ($n = 1$).

**TABLE IV.** Results of skin prick tests with rBet v 1 and rBet v 2 according to associated food allergies

| Food allergy | No. of patients | Positive skin prick test results | | |
|---|---|---|---|---|
| | | rBet v 1+ | rBet v 2+ | rBet v 1 + rBet v 2 |
| Negative | 20 | 15 | 3 | 1 |
| Rosaceae | 20 | 17 | 0 | 3 |
| Umbelliferae | 2 | 0 | 0 | 2 |
| Rosaceae & Umbelliferae | 9 | 8 | 0 | 1 |

study Valenta et al.[3] found only three patients sensitized to Bet v 2 alone among 100 patients allergic to birch. On the other hand, sensitization to Bet v 2 is responsible for positive cutaneous reactions to birch in some patients with grass or weed pollinosis exclusively. In this case it can be speculated that sensitization to birch profilin is a consequence of a primary sensitization induced by cross-reactive profilins present in grass or weed pollens. Because profilin has been characterized in various members of the Umbelliferae and Rosaceae families,[17-20], it may be a candidate for cross-sensitization between pollens and fruit and vegetable allergens. Our results demonstrate that in no case was sensitization to profilin alone associated with food allergy. Moreover, among 11 patients with allergy to Umbelliferae, only three reacted to rBet v 2. Sensitization to Bet v 1 is always present when birch sensitization is associated with Umbelliferae food allergy. This can be related to the recent finding of a recombinant allergen in celery, Api g 1, which demonstrates a 65% similarity to Bet v 1.[20] This suggests that allergens belonging to the Bet v 1–related family are involved in Umbelliferae and in Rosaceae food allergies in patients allergic to birch.

Although many allergens have been cloned and produced as recombinant allergens, only a few have been validated by skin testing in allergic individuals. This has been done for rAsp f 1a[21] and recently for Der p 2.[22] As in our study, these two recombinant allergens demonstrate their capacity to elicit cutaneous reactions in the corresponding sensitized population. In these studies the percentage of positive skin test results obtained with the recombinant allergen is lower than that obtained with the crude extract. Because of the very dominant position of Bet v 1 in birch sensitization, testing with either rBet v 1 or the crude birch extract produces the same results in patients with birch pollinosis. More interesting is the use of rBet v 1 for the diagnosis of apple allergy. In fact, our results show that rBet v 1 can replace the rather unstable apple extract[23] in the diagnosis of the apple-birch syndrome.

In conclusion, this study has proven the efficiency of two recombinant birch allergens used in skin testing for assessing birch allergy. It also shows the usefulness of rBet v 1 for identifying food cross-sensitization induced by Bet v 1–related proteins.

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