METHODS

Subjects
Exclusion criteria were general maladies, severe skin inflammation, pregnancy and/or lactancy, autoimmune diseases, contraindication for adrenalin, long-term use of systemic corticosteroids, intake of immunosuppressive or psychoactive drugs, a positive SPT response to hypoallergenic Bet v 1 fragments, simultaneous participation in another clinical study, and risk of noncompliance with study procedures and restrictions. Use of antihistamines within the previous 3 days and use of systemic short-term and topical corticosteroids in the tested area within the previous 14 days were not allowed. Participants signed informed consent forms before inclusion in the study.

Study materials, SPTs, and APTs
SPTs were conducted with commercial birch pollen extract (Stallergenes, Antony, France), purified rBet v 1, and 2 hypoallergenic rBet v 1 fragments (F1 and F2 each or as the equimolar mix F1 + F2), which together comprise the complete Bet v 1 sequence.\(^1\) Concentrations were 20 and 40 \(\mu\)g/mL (rBet v 1, each of the fragments), as well as an equimolar mix of F1 + F2 containing 10 and 20 \(\mu\)g/mL of each fragment. Aliquots of 20 \(\mu\)L were applied in duplicates on the left and right side of the forearm of the subjects at a distance of more than 2 cm between individual application points. As controls, 1 \(\mu\)g/mL histamine hydrochloride (positive) and sodium chloride solution (negative, Stallergenes) were used. After 20 minutes, wheal-and-flare reactions were photodocumented, pen marked, and transferred with scotch tape to a paper. The mean wheal area of duplicate tests was calculated by using digital planimetry. Only wheals of more than 4 mm in diameter were regarded as positive reactions.

For APTs, birch pollen extract (Stallergenes), as well as rBet v 1 (160 \(\mu\)g), rBet v 1 fragment 1 (160 \(\mu\)g), rBet v 1 fragment 2 (160 \(\mu\)g), and an equimolar rBet v 1 fragment mix (80 \(\mu\)g of each rBet v 1 fragment), were applied for 48 hours in patch test chambers (12 mm in diameter; Finn Chambers on Scanpor, Large, Epitest Ltd Oy) onto nonlesional skin on the backs of the subjects. After 48 hours in patch test chambers (12 mm in diameter; Finn Chambers on Scanpor, Large, Epitest Ltd Oy) onto nonlesional skin on the backs of the subjects. After 48 hours, patches were removed, and reactions were evaluated and photodocumented. Grading of positive APT reactions were done on the left and right side of the forearm of the subjects at a distance of more than 2 cm between individual application points. As controls, 1 \(\mu\)g/mL histamine hydrochloride (positive) and sodium chloride solution (negative, Stallergenes) were used. After 20 minutes, wheal-and-flare reactions were photodocumented, pen marked, and transferred with scotch tape to a paper. The mean wheal area of duplicate tests was calculated by using digital planimetry. Only wheals of more than 4 mm in diameter were regarded as positive reactions.

Total and specific IgE levels
Nitrocellulose strips (Whatman Protran nitrocellulose membrane; Sigma-Aldrich, St Louis, Mo) containing 2-\(\mu\)L aliquots (ie, 1 \(\mu\)g of rBet v 1, rBet v 1 fragment 1, rBet v 1 fragment 2, an equimolar mix of the rBet v 1 fragments 1 and 2 [ie, 0.5 \(\mu\)g each], and BSA) were incubated overnight with sera from the 30 study participants (1:10 in gold buffer) or with buffer without addition of serum. IgE reactivity was detected, as previously described.\(^3\) Signals obtained from the dot blots were quantified by means of densitometry with National Institutes of Health ImageJ software analysis, as described previously.\(^4\)

Lymphocyte proliferation assays and detection of secreted cytokines
PBMCs were isolated from heparinized blood samples by means of ficoll density gradient separation (Amersham, GE Healthcare, Buckinghamshire, United Kingdom). PBMCs were stimulated with 5 \(\mu\)g/well of rBet v 1 or an equimolar mix of hypoallergenic rBet v 1 fragments (F1 + F2) in triplicates at a density of 2 \(\times\) 10^5 cells/well in 96-well round-bottom plates (Thermo Fischer Scientific, Roskilde, Denmark). Cells were cultured in 200 \(\mu\)L Ultra Culture Medium (Lonza, Verviers, Belgium) supplemented as previously described.\(^5\) Wells containing 4 units of human IL-2 each (Roche Diagnostics GmbH, Mannheim, Germany) or medium alone were used as positive and negative controls, respectively. Cells were incubated for 6 days in a humidified atmosphere containing 5% CO_2 at 37°C. Proliferation was measured based on thymidine uptake (0.5 \(\mu\)Ci/well; PerkineElmer, Boston, Mass) after 16 hours of culture. Radioactivity was measured in counts per minute. The results were displayed as the stimulation index, which was calculated as the quotient of counts per minute in stimulated and unstimulated cultures. A response was considered positive when the stimulation index was greater than 1.

Supernatants from equally prepared PBMC cultures were harvested at day 6, and IL-1, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, INF-\(\gamma\), TNF-\(\alpha\), C-IGF, GM-CSF, monocytic chemoattractant protein 1, and macrophage inflammatory protein 1 levels were measured with the Bio-Plex Pro human cytokine 17-plex immunoassay (Bio-Rad Laboratories, Hercules, Calif). In addition, IL-22 levels were measured in each sample by using the FlowCytomix Human IL-22 Simplex Kit (eBioscience, San Diego, Calif). Cytokine values were log transformed before statistical analysis because their distributions were asymmetric.

Flow cytometric analysis of CLA\(^+\) and CCR4\(^+\) T cells
PBMCs were isolated as described above and stained with carboxyfluorescin diacetate succinimidyl (Invitrogen, Oslo, Norway; 1 mL of 5 \(\mu\)mol/L carboxyfluorescin diacetate succinimidyl ester solution per 10 \(\times\) 10^6 cells) for 10 minutes at 37°C. The labeling reaction was stopped by adding FBS (GIBCO, Invitrogen, Carlsbad, Calif) for 5 minutes. Labeled cells were washed with medium and cultured in triplicates (2 \(\times\) 10^5 cells/well) with rBet v 1 (5 \(\mu\)g/well), equimolar mix of hypoallergenic rBet v 1 fragments (F1 + F2), medium alone (negative control; Lonza, Verviers, Belgium), or 3 \(\mu\)L/well of Dynabeads containing anti-CD3 and anti-CD28 (positive control, Invitrogen) for 7 days at 37°C. At day 7, cells were centrifuged and incubated in 50 \(\mu\)L of solution containing 7-amino-actinomycin D (3 \(\mu\)L/well; BioLegend, San Diego, Calif) plus 10 \(\mu\)L/well of biotin mouse anti-human CD194 (Anti-CCR4) or biotin rat anti-CLA (BD Biosciences, San Jose, Calif) diluted in fluorescence-activated cell sorting (FACS) buffer (PBS, 0.01% w/vol Na_3HPO_4 and 1% w/vol BSA) for 20 minutes on ice. For control purposes, a biotin rat IgM isotype (CLA isotype) and a mouse IgG1 isotype (CCR4 isotype, BD Bioscience) were used. Cells were then centrifuged and stained with 1 \(\mu\)L/well of streptavidin-phycocyanin-cyanine 7 (PC7-Streptavidin, BD Biosciences) diluted in FACS buffer for 20 minutes on ice. After centrifugation, cells were resuspended in FACS buffer and measured with a Cytomics FC 500 flow cytometer ( Beckman Coulter, Fullerton, Calif). Data analysis was done with FlowJo Version 7.2.5 (TreeStar, Ashland, Ore). The gating strategy was based on forward and side scatter. 7-Amino-actinomycin D (BioLegend) was used for dead-cell exclusion. Mean percentages of CLA\(^+\) and CCR4\(^+\) T cells were calculated. The results were presented as stimulation indices calculated as the quotient of mean percentages of CD3\(^+\) T cells expressing CLA or CCR4 in stimulated and medium-only cultures.

Basophil activation by means of flow cytometry: CD203c and CD63 assay
Peripheral blood was obtained from a donor in heparinized tubes after informed consent was provided. Blood aliquots (100 \(\mu\)L) were incubated (triplicates) for 15 minutes at 37°C with 10-\(\mu\)L supernatants from PBMC cultures stimulated with rBet v 1, rBet v 1 F1 + F2, and medium and then washed in PBS containing 20 mmol/L EDTA (Gibco). Anti-IgE mAb E-124.2.8 (1 \(\mu\)g/mL) or PBS were used as controls. Thereafter, cells were incubated with 5 \(\mu\)L of phycoerythrin-conjugated CD203c mAb 9A3 (BD Biosciences) or 5 \(\mu\)L of fluorescein isothiocyanate–labeled CD63 mAb CLB-gr112 for 15 minutes at room temperature. After erythrocyte lysis with FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, Calif), cells were washed, resuspended in PBS, and analyzed by means of 2-color flow cytometry on a FACScan (Becton Dickinson Biosciences) with FlowJo software (Tree Star). Anti-IgE–induced upregulation of CD203c/CD63 was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI\(_{stim}\)) and unstimulated (MFI\(_{control}\)) cells and expressed as the stimulation index (MFI\(_{stim}\) / MFI\(_{control}\)).\(^6\)
REFERENCES

E1. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. J Clin Invest 1997;99:1673-81.

E2. Turjanmaa K, Darsow U, Niggemann B, Rance F, Vanto T, Werfel T. EAACI/GA2LEN position paper: present status of the atopy patch test. Allergy 2006;61:1377-84.

E3. Campana R, Vrtala S, Maderegger M, Jertschin P, Stegfellner G, Swoboda I, et al. Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rational sequence reassembly. J Allergy Clin Immunol 2010;126:1024-31.

E4. Resch Y, Weghofer M, Seiberler S, Horak F, Scheiblhofer S, Linhart B, et al. Molecular characterization of Der p 10: a diagnostic marker for broad sensitization in house dust mite allergy. Clin Exp Allergy 2011;41:1468-77.

E5. Vrtala S, Fohr M, Campana R, Baumgartner C, Valent P, Valenta R. Genetic engineering of trimers of hypoallergenic fragments of the major birch pollen allergen, Bet v 1, for allergy vaccination. Vaccine 2011;29:2140-8.

E6. Hauswirth AW, Natter S, Ghanadan M, Majlesi Y, Schernthaner GH, Sperr WR, et al. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. J Allergy Clin Immunol 2002;110:102-9.
FIG E1. IgE reactivity to rBet v 1 and rBet v 1 fragments. Dot-blotted purified recombinant antigens (rBet v 1, rBet v 1 fragments F1 and F2, an rBet v 1 fragment mix [F1 + F2], and BSA) were incubated with sera from the study subjects from groups 1 to 4 (1-30) or with buffer alone as a negative control (NC). Bound IgE antibodies were detected with iodine 125–labeled anti-human IgE antibodies and visualized by means of autoradiography.
FIG E2. Immediate-type skin reactions to rBet v 1 and rBet v 1 fragments in subjects with residual IgE reactivity to the F1 + F2 mix in RAST-based dot blotting assay. SPTs were performed with birch pollen extract (1), rBet v 1 (2 and 4), and the mix of rBet v 1 fragments F1 + F2 (4 and 5). SPTs were performed with antigen concentrations of 20 μg/mL (2 and 4) or 40 μg/mL (3 and 5).
FIG E3. Delayed-type skin reactions to rBet v 1 and rBet v 1 fragments in selected subjects. Shown are APT reactions to rBet v 1 and the rBet v 1 fragment mix (F1+F2) in patients from study groups 1 to 4 (group 1, subjects 26 and 19; group 2, subjects 13 and 11; group 3, subject 4; and group 4, subject 16). APTs were performed with 160 μg of rBet v 1 and a mix containing 80 μg of each rBet v 1 fragment (F1+F2).
**FIG E4.** Cytokine levels measured in PBMC cultures on stimulation with rBet v 1 shortly before SPTs and APTs. Shown are cytokine levels (in picograms per milliliter) determined for triplicate cultures as box-and-whisker plots showing minimum, quartiles, median, and maximum values (y-axes) for APT-positive patients with birch pollen allergy with positive APT reactions (A), patients with birch pollen allergy with negative APT reactions (B), and subjects without birch pollen allergy with negative APT reactions (C; x-axes). Statistically significant differences ($P<.05$) are indicated. G-CSF, Granulocyte colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1; MIP-1b, macrophage inflammatory protein 1.
FIG E5. Cytokine levels measured in PBMC cultures on stimulation with the F1+F2 mix shortly before SPTs and APTs. Shown are cytokine levels (in picograms per milliliter) determined for triplicate cultures as box-and-whisker plots showing minimum, quartiles, median, and maximum values (y-axes) for APT-positive patients with birch pollen allergy with positive APT reactions (A), patients with birch pollen allergy with negative APT reactions (B), and subjects without birch pollen allergy with negative APT reactions (C; x-axes). Statistically significant differences (P < .05) are indicated. G-CSF, Granulocyte colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1; MIP-1b, macrophage inflammatory protein 1.
### TABLE E1. Clinical and serologic characterization of subjects

| Group | Subject | Sex/age (y) | Allergies | Symptoms | Total IgE (kU/L) | IgE CAP (kUA/L) | IgE reactivity |
|-------|---------|-------------|-----------|----------|-----------------|----------------|---------------|
| 1     | F/36    | F/36        | b, a, g, pf, mo, mi | AD, RC, OAS, AS | 934             | 3.52           | 5.5           |
| 2     | F/32    | F/32        | a, a         | AD, RC    | 55               | 0.72           | 0.76          |
| 3     | M/29    | M/29        | b, a, g, pf, mi | AD, RC, OAS | 84.2            | 8              | 5.76          |
| 4     | M/26    | M/26        | b, a, g, mo, mi | AD, RC    | 590             | 11.38          | 12.82         |
| 5     | F/26    | F/26        | b, a, g, mo, mi | AD, RC    | 522             | 37.4           | 41.2          |
| 6     | M/30    | M/30        | b, a, g, pf, npf, mo, mi, w | AD, RC, OAS | 4,788           | 100.2          | 93.4          |
| 7     | M/24    | M/24        | b, a, g, pf, npf, mi, w | AD, RC, OAS | 240             | 31.2           | 6.62          |
| 8     | F/22    | F/22        | b, a, g, pf, mi | AD, RC, OAS | 248             | 7.1            | 6.78          |
| 9     | F/19    | F/19        | b, a, g, pf, npf, mo, mi | AD, RC, OAS | 742             | 46.8           | 49            |
| 10    | M/31    | M/31        | b, a, g, pf, npf, mo, mi, w | AD, RC, OAS | 3,198           | 122.4          | 125.6         |
| 11    | M/22    | M/22        | b, a, g, pf, npf, mo, mi, w | AD, RC, OAS | 446             | 8.26           | 7             |
| 12    | F/44    | F/44        | b, a, g, pf, npf, mi | AD, RC, AS | 13,840          | 52.8           | 30.2          |
| 13    | F/26    | F/26        | a             | AD, RC, OAS | 29              | 13.24          | 13.6          |
| 14    | F/28    | F/28        | g, mi        | AD, RC    | 388             | 36.4           | 39            |
| 15    | M/35    | M/35        | b, a, mo, mi | AD, RC    | 17.96           | 0.7            | 0.7           |
| 16    | M/25    | M/25        | b, a, g, pf, mo, mi | RC, OAS | 90.8            | 6.6            | 6.44          |
| 17    | M/34    | M/34        | b, a, g, pf, npf, mo, mi, w | RC, OAS | 44.6            | 4.76           | 3.18          |
| 18    | F/41    | F/41        | b, a, g, pf, mi | RC, OAS | 392             | 47.4           | 61            |
| 19    | M/47    | M/47        | b, a, g      | RC, OAS | 42              | 12             | 13.78         |
| 20    | F/25    | F/25        | b, a, g, pf, mi | RC, OAS | 412             | 36             | 39.4          |
| 21    | F/30    | F/30        | g, w         | RC        | 39              | <0.35          | <0.35         |
| 22    | M/33    | M/33        | g, pf, mi, mo | RC       | 52.6            | <0.35          | <0.35         |
| 23    | M/32    | M/32        | mi           | RC        | 2,552           | <0.35          | <0.35         |
| 24    | M/31    | M/31        | mi           | RC        | 450             | <0.35          | <0.35         |
| 25    | F/28    | F/28        | g            | RC        | 1,708           | <0.35          | <0.35         |
| 26    | F/27    | F/27        | No           | RC        | <2             | <0.35          | <0.35         |
| 27    | F/30    | F/30        | No           | RC        | 47.8           | <0.35          | <0.35         |
| 28    | F/33    | F/33        | No           | RC        | <2             | <0.35          | <0.35         |
| 29    | M/23    | M/23        | No           | RC        | 21.8           | <0.35          | <0.35         |
| 30    | F/36    | F/36        | No           | RC        | 7.1             | <0.35          | <0.35         |

**Allergies:** a, Animals; b, birch; g, grass; mo, mites; mo, molds; npf, non-plant-derived food; pf, plant food; w, weeds.

**Symptoms:** AD, Atopic dermatitis; AS, asthma; OAS, oral allergy syndrome; RC, rhinoconjunctivitis.