

IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa**

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IgE and IgG antibody responses to birch pollen antigens were studied by means of immunoblotting experiments testing 58 sera from patients with Type I allergy to birch pollen. 56/58 patients showed IgE antibodies reactive with Bet v I, a 17 kilodalton (kD) pollen protein. 2D-electrophoresis/immunoblot revealed a heterogeneity of that protein. Ten spots (pH 4.9-5.9) could be detected, presumably representing differentially glycosylated isoallergens. In 33/58 patients, there was no evidence of IgE antibodies directed against allergens other than Bet v I. However, in 25/58 of patients' sera, 11 minor allergens (13, 15, 18, 27, 29, 32, 36, 39, 44, 57, and 68 kD) with individual incidences from 1.7% to 17.2%, were identified. All proteins were also recognized by the patients' IgG antibodies: in the case of Bet v I recognition was weak, whereas the IgG response to the minor allergens was pronounced. Sera from healthy individuals showed similar IgG antibody responses, but no IgG to the 15, 27, and 29 kD proteins. Our results suggest that IgG directed against minor allergens may function as trapping antibodies in healthy individuals. Too low or lacking amounts of anti-Bet v I IgG may facilitate an allergic reaction.

Key words: birch pollen; IgG-response; minor allergens; two-dimensional electrophoresis.

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CLINICAL ASPECTS

The present study investigates the significance of birch pollen (BP) minor allergens for patients in central Europe. IgE-binding patterns to different pollen proteins (shown by immunoblotting) varied significantly. For this reason, immunotherapy with extracts lacking IgE-binding components might be unsuccessful in certain patients. Therefore, hyposensitization should be conducted with extracts composed according to IgE-binding patterns of the individual patient. On testing sera of healthy and non-hyposensitized allergic individuals, minor BP allergens evoked a much higher IgG response than the major allergen Bet v I. Possibly, antibodies of IgG isotype act as trapping antibodies; insufficient trapping of the major BP allergen may result in failure to inhibit an allergic reaction.

In northern and central Europe, Type I reactions due to exposure to birch pollen (BP) can be observed in about 22% of allergic individuals

suffering from pollinosis (14). Various attempts have been made to identify the responsible allergens in aqueous extracts. In 1972, Belin (4, 5) described a major allergen as a

thermostable component of aqueous pollen extracts, with an MW of 20 kD. In the same year, Ceska et al. (7) found allergenic components from BP to span a pH range of 4.5–6.0. In 1979, Viander et al. (27) described an MW range of 10 to 17 kD for a thermostable major allergen. In 1981, Apold et al. (2) observed allergenic activity in BP extracts with MW ranging from 15 to 60 kD, and a "major" allergen component of 29 kD with an isoelectric point of 5.18. Identity of this protein with a 10 kD glycoprotein (BV⁴⁵) was claimed by Vik et al. (28, 29). Using anion exchange chromatography and gel filtration, Ipsen & Løwenstein (17) purified a major allergen with MW 17 kD, heterogeneous in isoelectrofocussing (pI 5.25, 5.5, 5.0). This protein was further characterized by Ipsen & Larsen (16) in immunoblotting (IB) experiments as a 16.8 kD protein and designated as Bet v I according to WHO-guidelines (21). In 1987, Florvaag et al. (11) applied two-dimensional electrophoresis combined with IB on BP extracts. Allergenic activity in a serum pool from BP-allergics was found in MW of 12.5, 17, and 29 kD, pH range 4.8–5.4.

The question whether the 10, 12.5, 17, 20 or 29 kD proteins represent distinct allergens rather than a form of isoallergens (oligomers, fragments), remains so far unanswered, probably due to technical difficulties. These include impurities in preparations derived from gel filtration, interactions of non-protein material in pollen extracts with charged matrices in ion-exchange chromatography, and coprecipitations in crossed radioimmunoelectrophoresis (CRIE). Moreover, the possibility should be considered that IgE antibodies, which are found in only few patients, may be diluted beyond the sensitivity of the assay employed when pooled sera are used for CRIE or IB experiments.

We therefore decided to use IB as an alternative approach in studying the IgE response in patients with Type I allergy against BP, since this method allows the investigation of numerous individual sera reacting with allergens separated according to their MW by means of SDS-PAGE. Furthermore, other immunoglobulin isotypes can be studied on the same sheet

of nitrocellulose and the pattern of reactivity directly compared.

MATERIAL AND METHODS

Sera

Sera from 58 BP-allergic patients, 25 male (age 10 to 48), 33 female (age 7 to 54) were selected for this study according to case history, skin prick test, and radioallergosorbent test (RAST) (24). None of the patients had received hypo-sensitization therapy before collection of serum.

All patients showed reactivity to other allergens than BP, in skin prick tests or RAST: 49/58 to other tree pollen (hazel, alder, beech), 25/58 to grass/rye pollen, 21/58 also to other allergens (e.g., mite, cat and horse dander, guinea pig epithelia).

Three serum pools (A, B, and C) with distinct binding patterns to BP extract were used (18).

Sera from 18 healthy persons (HP), 11 male (age 23 to 60), 7 female (age 21 to 45), with negative case history of any Type I allergy and negative skin prick test and RAST to birch pollen extracts served as controls. 11 sera were pooled and used as negative control serum pool (N).

Chemical and general reagents

Tris, glycine acetic acid, methanol, glycerol, ammoniumcarbonate, sodiumazide, chloramine-T, dinatriumhydrogenphosphate, natriumdihydrogenphosphate, sodiummetabisulfite, Coomassie Brilliant Blue G250 and R250, EDTA, boric acid, and borax were obtained from Merck (Darmstadt, FRG). Tween 20 (poly oxyethylene-sorbitanmonolaurate), Nonidet P-40 (NP40), SDS (sodiumdodecylsulfate), N, N-methylene-bis-acrylamide and acrylamide were purchased from Sigma (Munich, FRG). Na¹²⁵I, hyperfilm MP (RPN6) was obtained from Amersham International (Buckinghamshire, UK) and ammoniumpersulfate, 2-mercaptoethanol, N,N,N,N-tetramethylethylene-

diamine (TEMED) from Bio-Rad Laboratories (Richmond, CA, USA). ^{125}I -rabbit anti-human IgE was taken from Phadebas RAST 100-kit from Pharmacia (Uppsala, Sweden). ϵ -Aminocaproic acid (EACA) was obtained from Fluka AG (Buchs, Switzerland), bovine serum albumin (BSA), normal goat serum (NGS), gelatine from Janssen (Olen, Belgium) and dithiothreitol from Serva Feinbiochemical (Heidelberg, FRG). BP SQ (standardized quality)-extract was obtained from ALK (Copenhagen, Denmark).

Chemicals and reagents for 2-dimensional electrophoresis (2DE)

Cyclohexylaminosulfonic acid (CHES), acrylamide and N,N-methylene-bis-acrylamide was obtained from Bio-Rad Laboratories, ampholines from LKB-Instruments (Bromma, Sweden), urea from BDH (Poole, UK), nitrocellulose from Schleicher & Schüll (Dassel, FRG), arginine and lysine from Merck.

Extraction methods

BP-extraction. Pollen from *Betula verrucosa* (white birch) were obtained from Allergon AB (Engelholm, Sweden) and were extracted essentially according to Ipsen & Løwenstein (17) with modifications (19); briefly, 10% pollen (w/v) in 0.125 M ammoniumhydrogencarbonate, 0.015 M sodium azide, 20mM EDTA, 5mM EACA, 1mM phenylmethylsulfonylfluoride (PMSF), pH 7.1, under continuous shaking for 20 h at 4°C. Particles were removed by centrifugation (24,000 *g* for 1 h at 4°C).

The supernatant was extensively (48 h) dialysed against a 20-fold volume of 20 mM EDTA, 5mM EACA, pH 7.4 for 24 h and again centrifuged at 500 *g* for 15 min. The supernatant was dialysed against distilled water, adjusted to pH 8, lyophilised and stored at -20°C. Before use, extracts were dissolved in distilled water.

SDS-extraction. Pollen were heated as 0.8% (w/v) suspension for 10 min at 95°C in an

electrophoresis sample buffer (20), containing 1% (v/v) NP40 and then centrifuged at 500 *g* for 10 min. The supernatant was used for the experiments described.

The protein concentration of extracts was determined by the method of Bradford (6).

SDS-PAGE

Samples were diluted 3:1 in a 4-fold concentrated Laemmli sample buffer (0.0625 M Tris, 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.025% (w/v) bromophenolblue) and heated at 95°C for 5 min. SDS-PAGE was performed on a vertical slab gel apparatus (Hoefer, San Francisco, CA, USA). 12% homogeneous and 10–16% gradient gels, respectively, (160 × 180 × 5mm) with 5% stacking gels were used. Electrophoresis was carried out according to Laemmli (20) in a discontinuous buffer system (0.025 M Tris, 0.2 M glycine), containing 0.1% (w/v) SDS. For IB experiments, a one-well comb was used. BP extracts were applied in a concentration of 200 µg/cm gel. MW were estimated, using pre-stained protein standards (14.3–200 kD) (Amersham International and Bethesda Research Laboratories, MD, USA).

Staining of the gels was done for 2 h with Coomassie Brilliant Blue G250 0.25% (w/v), in 50% (v/v) methanol, 7.5% (v/v) acetic acid, destaining in 10% (v/v) methanol, 15% (v/v) acetic acid, 3% (v/v) glycerol.

Two-dimensional electrophoresis

2DE was performed on the ISO-DALT electrophoresis system of Anderson & Anderson (1) (Electro Nucleonics, Incorp., Oak Ridge, TN, USA) as described previously (8).

Specimen preparation. 300 µg of BP extract was dissociated in 100 µl sample buffer, containing 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 50 mM cyclohexylaminosulfonic acid (CHES, Bio-Rad), 10% (w/v) glycerol at pH 9.5 with heating for 5 min at 95°C. 10 µl of this preparation was loaded on one ISO-gel.

Electrophoresis. The first or ISO-dimension is isoelectric focusing in polyacrylamide (35 g/l) under denaturing conditions with 9 mol urea, 20 g surfactant NP40, 19 g 3.5–10 ampholytes and 5 g 5–7 ampholytes (Ampholines, LKB-Instr.), 1.6 g arginine and 1.0 g lysine/l. Isoelectric focusing was performed at 14,000 Vh after prefocusing for 1 h at 200 V with 0.85 g/l phosphoric acid and 1.0 mol/l sodiumhydroxide as anolyte. ISO-gels were immediately fused onto 2D slab gels without equilibration. The second dimension, SDS-PAGE in a 10 to 23 % polyacrylamide gradient slab gel was performed in a DALT tank with a buffer containing 3.5 mmol/l SDS, 24 mmol/l Tris, 0.2 mol/l glycine. Proteins were transferred on NC-sheets for 2.5 h at 1.2 A, as described previously (8).

Prestained MW markers from BRL were run simultaneously on the SDS-PAGE for determination of MW. The pH gradient of isoelectric focusing gels was determined by cutting a pair from each patch into 15 equal parts. Each part was put into 0.5 ml degased distilled water. After incubation for at least 1 h on a shaker, the pH was measured (9).

Immunoblot

SDS-PAGE was performed as mentioned above, the separated proteins were transferred to nitrocellulose (NC) by use of a Hoefer blotting apparatus, at 150 mA for 2 h from homogeneous gels, at 150 mA for 4 h from gradient gels onto NC filters (pore size 0.2 μ m, Schleicher & Schüll, Dassel, FRG). Transfer was performed in a Tris-glycine medium (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3) (25). NC-sheets were cut into 5 mm strips. Per strip, 1 ml of the following reagents was used: For IgE-detection buffer A (50 mM natriumphosphate buffer, pH 7.5, 0.5 % w/v Tween 20, according to Batteiger et al. (3), 0.5 % (w/v) BSA, 0.05 % (w/v) sodiumazide), buffer B (buffer A + 1 % (v/v) NGS), buffer C (buffer A + 20 mM sodiumazide, 0.4 % w/v gelatine). Strips were processed by saturation for 30 min at room temperature (RT) in buffer A. They were then incubated overnight at 4 °C with

patients' serum, diluted 1:4 in buffer B and washed in buffer A for 30 min. Bound IgE was detected by ^{125} I-rabbit anti-human IgE (Pharmacia Int.) (300,000 cpm/ml by incubation in buffer C overnight at RT). In the case of 2DE - NC, sheets were incubated with patients' sera diluted 1:10 in buffer B. ^{125} I-rabbit anti-human IgE was used in a concentration of 7.5×10^6 cpm / NC-sheet in buffer C.

For IgG detection buffer D (100 mM boric acid, 25 mM borax, 75 mM NaCl, 0.5 % Tween 20, pH 8.2), buffer E (buffer D + 3 % powdered milk). Strips were processed by saturation 30 min at RT in buffer E, then incubated overnight at 4 °C with patients' serum, diluted 1:20 in buffer E, and washed for 30 min in buffer D. Bound IgG was detected by affinity purified rabbit anti-human IgG (Fc) (Jackson, MD, USA), which was diluted in buffer E 1:2000. Incubation was performed at RT for 1 h. Strips were washed in buffer D for 30 min and incubated for 1 h at RT with ^{125} I-goat anti-rabbit Ig (300,000 cpm/ml buffer E). A goat anti-rabbit Ig antibody (Kirkegaard & Perry, London, UK) was ^{125} I-labelled according to the method of McConahey (22).

Control strips were processed either with normal human serum pool (N) or buffer E or buffer B (B), respectively. Autoradiography was performed at -70 °C for 72 h, using intensifying screens (X-omatic regular, Kodak) in exposure cassettes (X-omatic, Kodak).

RESULTS

Characterization of BP extract

The protein composition of BP extract was characterized by SDS-PAGE (Fig. 1) and compared with a commercial extract (SQ) and with SDS-treated pollen material (SDS extract). The BP and SQ extracts showed a higher content of the 13, 17, 27, 29, and 36 kD proteins than the SDS extract.

To ascertain that there was no loss of allergenic material during the extraction procedure, BP extract was compared with SDS extract also in IB experiments, using three serum pools (A,

B, and C, see Material and Methods). IgE-binding to various proteins was more pronounced in the BP extract than in the SDS extract. No evidence was obtained that SDS extract contained any IgE-binding bands not found with BP extract (Fig. 2).

IgE response to birch pollen proteins in sera from patients and healthy individuals

Sera from 58 patients and 18 healthy individuals were tested for the presence of IgE antibodies against birch pollen antigens, using BP extract in IB experiments (Fig. 3).

Fifty-six (96.6%) patients' sera contained IgE antibodies against the 17 kD protein (Bet v I), which in 33 (56.9%) patients represented the only antigen recognized by IgE. 23 (39.7%) patients' sera contained in addition to the anti-Bet v I IgE, IgE-antibodies against other proteins with MW of 13, 15, 18, 27, 29, 32, 36, 39, 44, 57, and 68 kD, with individual incidences ranging from 1.7–17.2% (Fig. 4). In one serum, IgE antibodies reacted exclusively with a 13 kD protein (Fig. 3, panel "IgE", lane 19). A second serum showed IgE reactivity exclusively with a 13 and a 36 kD protein.

Sixteen (88.9%) sera from normals showed no IgE reactivity, whereas IgE antibodies

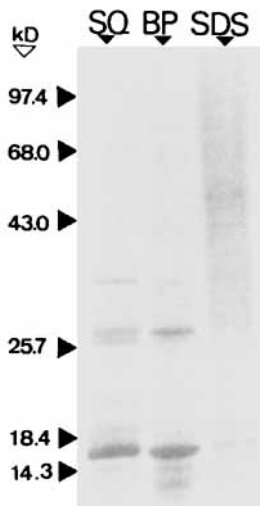


Fig. 1. SDS-PAGE of birch pollen extracts: 240 µg of three extracts (SQ, BP, SDS) (see Material and Methods) were separated on a 10–16% linear polyacrylamide gradient gel and stained by Coomassie Brilliant Blue

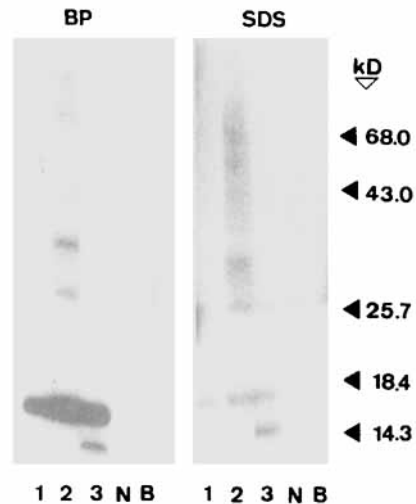


Fig. 2. IgE-binding proteins in birch pollen extracts, BP and SDS. Birch pollen extracts were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and incubated with three RAST-positive serum pools, pool A (lane 1), pool B (lane 2), pool C (lane 3), a RAST-negative pool (lane N), and buffer (lane B). IgE-binding was detected by ^{125} I-rabbit anti-human IgE (300,000 cpm/strip).

against 36, 44, and 68 kD proteins were found in two sera (11.2%).

IgG response to birch pollen proteins in sera from patients and healthy individuals.

All proteins mentioned above were recognized in patients' sera by IgG antibodies (Fig. 3). Reactivity with Bet v I, the 17 kD major allergen of BP, was weak or lacking. In contrast, IgG antibodies of patients reacted strongly with proteins at MW of 13, 15, 18, 27, 29, 32, 36, 39, 44, 57, and 68 kD, with incidences from 8.6 to 93.1% (Fig. 4).

Sera from healthy persons contained IgG antibodies against 13, 17, 18, 32, 36, 39, 44, 57, and 68 kD proteins, but were devoid of IgG antibodies against 15, 27, and 29 kD proteins (Fig. 5).

Analysis of the 13 and 17 kD allergens by means of 2-DE/Immunoblot

Bet v I and the 13 kD minor allergen were further characterized by means of

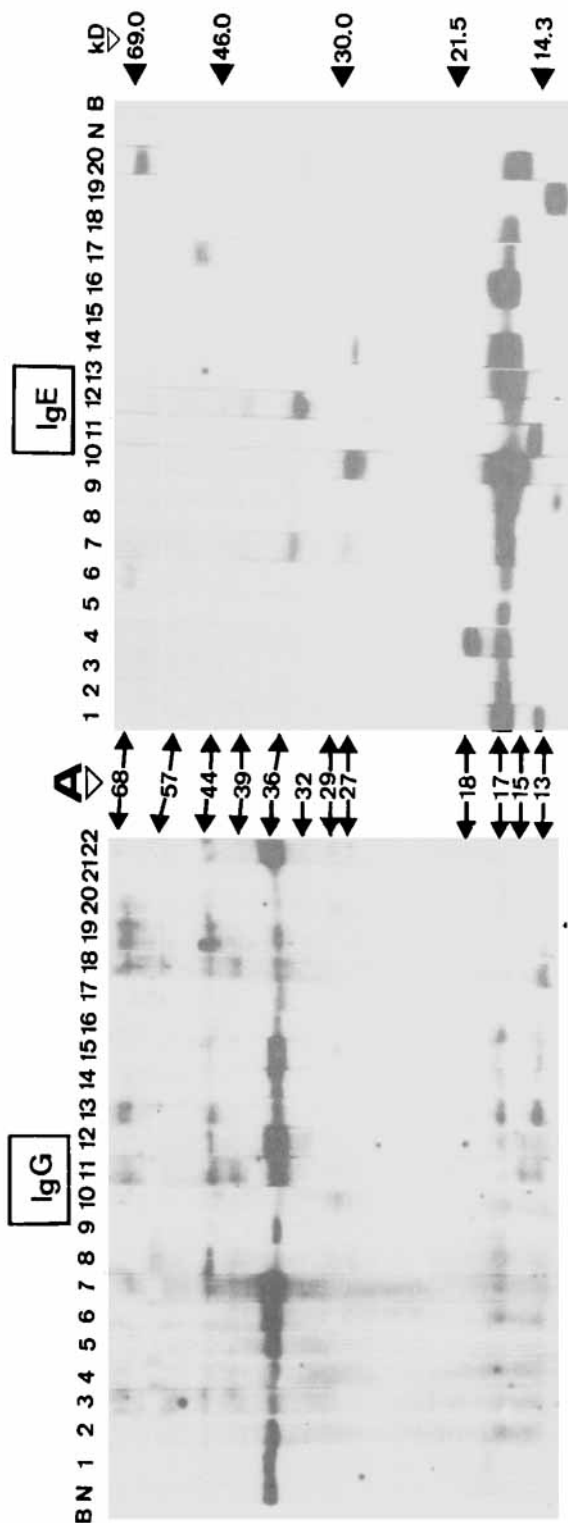


Fig. 3. IgE and IgG-binding to birch pollen proteins. BP extract was separated on a 12% polyacrylamide gel, transferred to nitro-cellulose and examined for IgG- and IgE-binding. Panel "IgG": in lanes 1-9 strips were incubated with individual sera (1:20) from healthy persons, in lanes 10-22 with individual sera (1:20) from BP allergic patients. Bound IgG was detected by rabbit anti-human IgG (Fc) and visualized by ^{125}I -goat anti-rabbit Ig (300,000 cpm/strip).

Panel "IgE": in lane 1-20, strips were incubated with individual sera (1:4) from BP-allergic patients. Bound IgE was detected by ^{125}I -anti-human IgE (300,000 cpm/strip).

Strips "N" were incubated with a human serum pool from 11 healthy persons' sera, strips "B" with buffer (negative control). Lane "A": molecular weights (kD) estimates of antigens binding IgE and/or IgG.

2DE/immunoblot (Fig. 6). By probing with a serum containing anti-BP IgE antibodies directed exclusively against the 17 kD and 13 kD allergens (compare Fig. 3, panel "IgE", lane 1) Bet v I was detected as a cluster of at least 10 IgE-binding protein spots in a pH range from 4.9 to 5.9 (2 spots at pH 5.9, 2 at pH 5.8, 2 at pH 5.7, 2 at pH 5.4, 1 at pH 5.2, 1 at pH 4.9). Most intensive IgE-binding of Bet v I is detected at pH 5.9 and 5.4.

The 13 kD minor allergen was resolved into 4 IgE-binding spots at pH 4.4, 4.6, 4.7, and 6.0. Identical reactivity with the 13 kD allergen was observed by testing with an individual serum, containing 13 kD monospecific IgE (compare Fig. 3, panel "IgE", lane 19).

DISCUSSION

To develop better reagents for diagnosis and therapy of Type I allergies it is important to gain more information about the responsible allergens. In the BP system, various attempts have been made to characterize those allergens, and it is generally agreed that a 17 kD protein, designated as Bet v I, represents the major allergen (21). In addition, other molecules from pollen of *Betula verrucosa* with different MW were shown to be active as allergens, as judged

by RAST inhibition and skin prick tests, thus indicating the existence of other allergenic components (2, 17, 27, 30). However, in these preparations the potential to inhibit RAST or provoke positive skin prick tests was weak compared with that of Bet v I, and a possible contamination of these allergens with Bet v I was always probable. By probing a serum pool, IgE antibodies directed against up to six distinct allergens were detected in CRIE (17). Since this technique is highly dependent on the quality of the precipitating antibody, the possibility of coprecipitation of antigens could not be excluded, and, therefore, the independency of these so-called minor allergens was not unambiguously established.

The IB technique provides an alternative approach to study the antibody response to distinct allergens in crude preparations. Using this method, molecules are separated according to their MW and no precipitation step is required before probing with patients' sera. This technique is a useful tool for further characterization of allergen extracts (16), and was recently applied by Hemmens et al. (13), thus giving the opportunity to test sera individually and compare IgE-binding profiles of two patient collectives (Scandinavian and Australian) on different birch species.

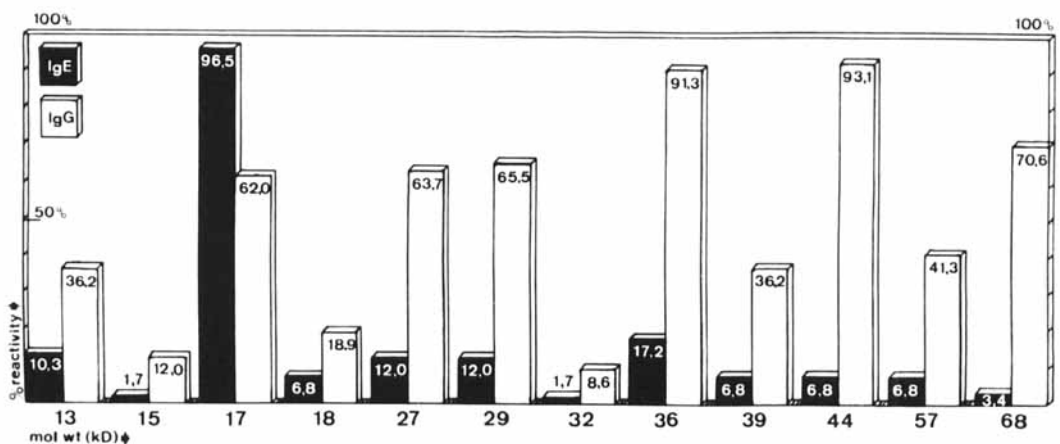


Fig. 4. Incidences of IgE and IgG antibodies to various antigens in allergic patients. IgE- and IgG-binding of birch pollen proteins was tested by immunoblotting using BP extract. On the y-axis, percentages ($n = 58 = 100\%$) are given of antibodies found to antigens of different MW (kD) shown on the x-axis. The y-axis represents the percentage of patients ($n = 58 = 100\%$), reacting with different antigens shown on the x-axis. Black columns: IgE reactivity, white columns: IgG reactivity.

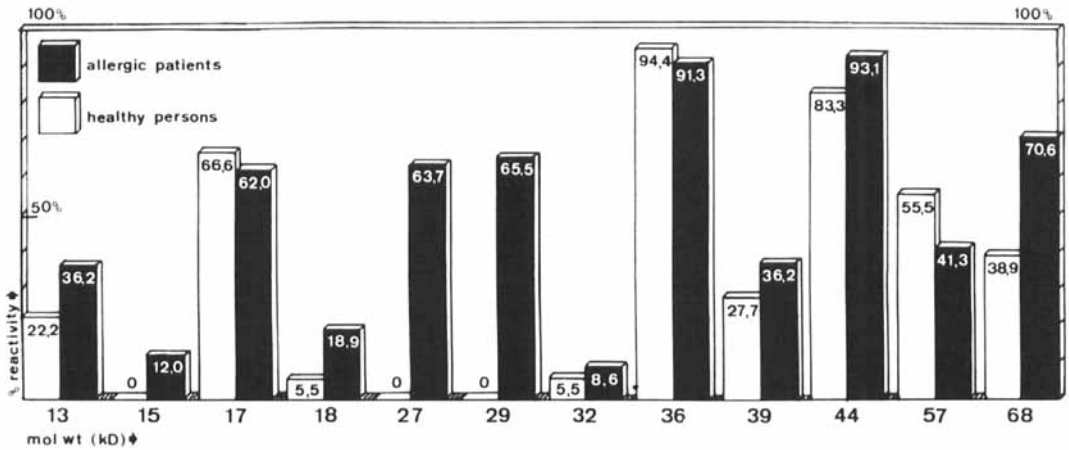


Fig. 5. Comparison of incidences of IgG antibodies to birch pollen antigens in patients ($n = 58 = 100\%$) and healthy persons ($n = 18 = 100\%$). IgG-binding to birch pollen proteins was tested by immunoblotting using BP extract (see Material and Methods and Fig. 3). On the y-axis, percentages are given of IgG antibodies bound to antigens of different MW (kD) shown on the x-axis. White columns: healthy persons, black columns: BP-allergic patients.

We tested sera from healthy individuals and patients with Type I allergy to BP by IB, focusing on comparisons of allergics' IgE and IgG, and allergic and healthy individuals' IgG response. For IB experiments, a BP extract was prepared in the presence of protease inhibitors to prevent enzymatic degradation of proteins during long-term extraction due to a release of enzymes from the pollen grain wall (19).

BP was compared with a commercial extract of standardized quality (SQ). Protein profiles and the capacity to bind IgE antibodies were similar in these extracts, as shown by IB experiments. To ascertain that there was no loss of allergenic material during the extraction procedure, a SDS extract was prepared by boiling birch pollen in Laemmli sample buffer containing NP40 (SDS extract). This extract contained several proteins with MW ranging from 46 to 100 kD, which were not observed in SQ and BP preparations (Fig. 1). We consider them to represent membrane proteins. IB experiments showed no evidence of an IgE-binding capacity of these proteins. As the highest concentration of IgE-binding proteins was observed in the BP preparation (Fig. 2), this extract was used throughout the study.

To reveal the number of allergens characterized according to their MW, individual IgE and

IgG antibody-binding patterns of 58 patients' sera were compared. Regarding IgE-binding patterns, a total of 12 allergenic proteins were

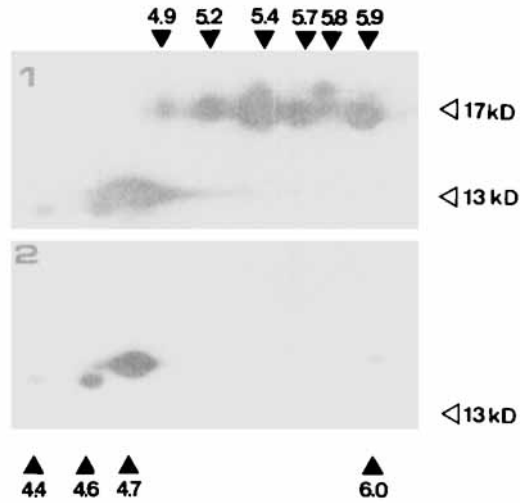


Fig. 6. IgE-binding to birch pollen proteins separated by 2DE. BP extract was separated in the first dimension in a pH gradient (pH 3–10), in the second dimension in an SDS-PAGE (10–23% gradient gel), and blotted onto nitrocellulose-sheets. Sheet 1 was incubated with a patient's serum (1:10) corresponding to lane 1, panel "IgE" in Fig. 3, sheet 2 with a patient's serum (1:10) corresponding to lane 19, panel "IgE" in Fig. 3. Bound IgE was detected with ^{125}I -rabbit anti-human IgE (7.5×10^6 cpm/2DE-NC-sheet). MW of allergens: Δ = kD. Isoelectric points: $\blacktriangle$.

found. Earlier work (5, 11, 13, 16, 17, 27) showed that a protein of approximately 17 kD represents the major allergen of BP. In our immunoblot experiments this protein is recognized by 96.5% of the investigated birch pollen allergic patients, which is in agreement with Ipsen et al. (16), who found IgE-binding to the major allergen in all sera tested in IB. Hemmens et al. (13) observed binding of IgE to that protein in only half of the 26 patients' sera in IB testing. This result might be due to minimal loading of gels determined for IB (approximately 10 µg/ NC strip by Hemmens et al., 50 µg by Ipsen & Larsen and 100µg in the present study). Seventeen allergenic molecules were described (13), of which 11 (see below) are confirmed in the present study. Although similar electrophoresis, blot and detection systems were applied, resolution was not sufficient (13) and therefore MW estimation of bands differing by only one kD should be reconsidered. This might minimize the number of allergens described.

In addition to Bet v I, we found a 13 kD allergen, being recognized by several patients. This allergen could be one of the two thermostable proteins described by Belin (4, 5). These thermostable proteins were further characterized by Viander et al. (27) as Ag7 (17 kD) and Ag8 (10 kD), which were seen to be antigenically cross-reacting in CRIE. In 1982, Vik et al. (28) again found evidence for the existence of an allergen of about 10 KD (BV⁴⁵), which was claimed to be a major allergen. In an attempt to purify Ag23 (Bet v I), Ipsen & Løwenstein found, in addition to Bet v I (17 kD), a protein with an MW of 14 kD (17). This protein showed no IgE-binding in CRIE, using a serum pool of 20 BP-allergic patients. Our studies indicate that only 10.3% of patients are recognizing this low MW protein. Thus, in the studies of Ipsen & Løwenstein, IgE antibodies might have been diluted too much for detection of the 13 kD allergen in CRIE when using a serum pool. The existence of BP minor allergens of MW < 17 kD was verified in 1987 by Hemmens et al. (13) and Florvaag et al. (11), by applying IB techniques. Florvaag performed 2DE combined with IB, and found allergenic

activity in 12.5, 17, and 29 kD (pH 4.8–5.4), probing a serum pool for IgE-binding. We applied 2DE/IB regarding IgE-binding patterns of individual patients. Bet v I was found to consist of 10 spots in pH range 4.9–5.9, which confirms results of earlier workers (2, 7, 11, 17, 27). The observed heterogeneity of Bet v I indicates an existence of isoallergens due to differences in glycosylation of or to multiple gene loci coding for this protein.

The 13 kD protein was detected as four IgE-binding spots in a pH range of 4.4–6.0 (Fig. 6). Our observation that two sera showed IgE antibodies directed exclusively to the 13 kD protein, without IgE-binding to Bet v I, indicates that the 13 kD protein represents an independent allergenic entity.

In addition to Bet v I and the 13 kD allergen, a variety of 10 IgE-binding proteins was observed (Fig. 4). The 27 and 29 kD proteins may correspond to proteins already published (2, 11, 13, 17, 27), and the 13, 15, 18, 32, 36, 39, 44, and 57 kD proteins may correspond to allergens already described by Hemmens et al. (13). IgE-binding to allergens with a higher MW than 70 kD was not observed in any of the 58 sera tested. Incidences of IgE-binding to BP minor allergens varied between 1.7 and 17.2% in our study and 11% in that of Hemmens et al. (13)). Illustrating these data with absolute numbers (1 to 10 of 58 patients in our study, 2 to 3 of 26 patients in Hemmens et al.'s, reacted via IgE with one distinct band) elucidates the relative small chance of detecting minor allergens. This fact, in addition to differences in patient collectives might explain the variation in allergen profiles.

A marked difference was observed when the IgG response of BP-allergic patients was compared with that of healthy persons. In the latter, IgG antibodies against 9 proteins of 13, 17, 18, 32, 36, 39, 44, 57, and 68 kD were observed. A similar incidence of IgG antibodies against these proteins was observed in patients' sera. However, patients' sera contained, in addition, IgG antibodies against the 15, 27, and 29 kD proteins. These findings indicate that these antigens are differently processed by the immune systems of patients and normal individuals.

Reactivity with a 36 kD protein was sometimes seen also in control strips, which indicates a reaction of the ^{125}I -goat anti-rabbit antibody with that protein, probably due to a lectin-like interaction.

Comparing the incidence of IgG and IgE antibodies against the various proteins in patients, IgG responses against Bet v I were difficult to estimate. Only weak IgG-binding was observed (66.6%). 33.4% of patients showed no IgG-reactivity to Bet v I at all. This observation is in accordance with findings from Viander (27) who, in sera from patients prior to hyposensitization, described only a weak IgG-binding to the "thermostable antigens" in CRIE. Incidences of IgG antibodies against minor allergens exceeded those of IgE and were similar to those of the control group.

The pathophysiological significance of IgG antibodies is speculative, although the induction of specific IgG antibodies during immunotherapy has been observed for various antigens (10, 12, 15, 23, 26). In healthy persons, IgG antibodies may be induced due to normal exposure to BP proteins. It is tempting to speculate that these antibodies prevent IgG-IgE switching by an antigen-trapping mechanism. In the case of Bet v I, IgG antibody levels might be lacking or too low for sufficient trapping, thus facilitating the induction of IgE antibodies, particularly in patients with genetic susceptibility to atopic diseases.

Further study is needed to find whether IgG antibodies in healthy persons or patients after hyposensitization can function as "blocking antibodies": by being directed against the same epitopes as are specific IgE they may diminish Type I reactions to these allergens.

Our study shows that BP allergens, originally defined by such techniques as CRIE or chromatography, could also be identified by the IB technique. Using the IB technique as a routine method to detect IgE antibodies against other pollen allergens has proven that IgE binding can readily be monitored by this technique. Considering that the IB method involves a denaturation/renaturation step these findings indicate stable IgE-binding epitopes. Apparently, pollen proteins are at least partially disinte-

grated during their passage through the mucosal barrier, and delicate conformational epitopes may be destroyed and thus be of minor importance for IgE antibody binding.

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