

Humoral and Cell-Mediated Immune Responses to Fractions of Birch Pollen Extract

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IgE and IgG antibodies (ab) and lymphocyte transformation (LT) were studied in untreated and hyposensitized birch pollen allergic subjects and in non-atopic controls using whole extract and fractions obtained by gel filtration of birch pollen extract. All the allergic subjects had positive IgE ab, IgG ab and LT responses to the whole extract. Both the untreated and the hyposensitized subjects had peak IgE ab and LT responses against the allergenic fractions of the extract, while negative responses were obtained in the non-atopic controls. Only hyposensitized subjects had developed high IgG ab responses to the allergenic fractions. Most of the treated and untreated subjects showed IgG ab and LT responses to the high molecular weight fractions with low allergenic activity. Significantly higher IgE ab responses to these fractions were observed in the treated subjects than in the untreated ones, indicating potentiation of IgE ab responses against some antigens during immunotherapy. Some of the allergic subjects also responded to the fractions of low molecular size (mol.wt. 2000-5000) with low allergenic activity. Both IgE ab, IgG ab and LT responses to these fractions were observed.

Key words: allergens; antibodies, IgE, IgG; antigens; hyposensitization therapy; immunity, human; immunoenzyme techniques; lymphocyte transformation; pollen, birch; radioallergosorbent test.

Crude pollen extracts contain several antigens, both allergenic and non-allergenic in man (1, 4, 14, 16). Immune response to these antigens is determined by genetic factors, by dose and structure of the antigen and by route of immunization (2, 15, 16). Depending on the genetic responsiveness of the individual, hyposensitization therapy using crude extracts may thus result in formation of reagins of a new specificity, which are not detected in sera of untreated subjects (16, 22, 24).

Belin & Strannegård (6) demonstrated that in rabbits transmucosal immunization resulted in formation of reaginic antibodies only against allergenic antigens of birch pollen, while both the allergenic and the non-

allergenic antigens induced immediate hypersensitivity when administered parenterally. In the present work immune response against fractionated antigens of birch pollen was studied in man. IgE and IgG antibodies and lymphocyte transformation by the various fractions of birch pollen were determined in hyposensitized and untreated allergic subjects and in non-atopic controls.

MATERIAL AND METHODS

Patients and blood samples

Ten hay fever patients with birch pollen allergy were selected for the study. Diagnosis

was confirmed by a positive skin prick test and by a positive nasal provocation test (26). Five of the patients had received specific immunotherapy with birch pollen extract (Allpyral® birch, Dome Laboratories Ltd., Slough, England) for 2 years at the time of testing (26). The extract used for skin and nasal testing and for the immunological studies was prepared by the author from the same batch of pollen (see below) as that used for the hyposensitization treatment. We have recently shown that non-atopics only rarely develop immune responses to the whole extract of birch pollen (25). Therefore, only two of the non-atopics with negative allergy history, negative skin prick tests and negative immune responses to birch pollen served as controls. Blood samples for lymphocyte cultures and for antibody determination were obtained and stored as described elsewhere (25).

Birch pollen extract and its fractionation by gel filtration

The pollen preparations in this study were all of the same origin: *Betula pendula* (verrucosa) lot 464, kindly provided by Dome Laboratories Ltd., England. Preparation of birch pollen extract (1:10 w/v) and its fractionation by gel filtration have been described (27). In brief, one gram of pollen was extracted in 10 ml of phosphate buffered saline at +4°C for 24 h, centrifuged for 20 min at 22,000 g, and the sterilized supernatant (0.45 µm filter) was stored in small portions at -20°C until used. Fractionation of the extract by gel filtration was performed at +4°C on a column (K 25/100) of Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with 10mM phosphate buffer, pH 7.5 containing 0.15 M NaCl in elution. The sample of 5 ml (1:10 w/v extract) was applied. Fractions of 4 ml were collected and sterilized by a 0.45 µm filter, and stored in small portions at -20°C until used.

Heat-denatured birch pollen extract

To destroy all other antigenic determinants of

birch pollen, except the main allergenic antigen (4, 27) the extract was heat-treated (100°C for 15 min), and thereafter the aggregated proteins were removed by centrifugation. The protein contents of the native (1:10 w/v) and the heat-denatured extracts were 7.9 mg/ml and 7.5 mg/ml, respectively. Crossed immunoelectrophoresis and rocket immunoelectrophoresis (27) revealed less than 10% reduction in the amount of the main (thermostable) antigen after heat-treatment of the birch pollen extract. The allergenic activity of the heat-treated extract in prick skin tests was slightly diminished.

Lymphocyte cultures using whole blood

Lymphocyte stimulation was carried out in whole blood microcultures (25). Two ml of blood was mixed with 10 ml of RPMI (Grand Island Biological Co., Grand Island, New York) containing 100 units/ml of penicillin and 100 µg/ml of streptomycin. Thereafter, 150 µl of blood-RPMI suspension was distributed into each well of a microtiter plate (IS-MRC-96, Linbro Chemical Co., New Haven, Connecticut). Four 10-fold dilutions of birch pollen extract (1–1000 µg/ml) or of pooled fractions (0.1–100 µg/ml) in 25 µl of RPMI were then added into the desired wells. Control cultures received 25 µl of RPMI. All cultures were prepared in quadruplicate. The plates were incubated in humidified air and 5% CO₂ at 37°C. After 96 h incubation, 0.25 µCi of ¹²⁵I-labelled 5-iodo-2'-deoxyuridine (¹²⁵IUdR, sp. act. 90–110 mCi/mg; The Radiochemical Centre, Amersham, England) was added into each well together with 5-fluoro-2'-deoxyuridine (FUdR, Fluka, Buchs, Switzerland) to a final concentration of 10⁻⁶ M. After 18 h the cultures were harvested with a multiple cell-culture harvester (Skatron, Lierbyen, Norway) and the radioactivities were measured by a gamma counter (LKB-Wallac, Turku, Finland). The responses were expressed as counts per minute (c.p.m.) or as net counts, i.e., c.p.m. of the stimulated cultures—c.p.m. of the control

cultures. The criterion for a positive LT response was the same as reported earlier (25), i.e. a net incorporation of ^{125}I UDR greater than or equal to 200 c.p.m.

Specific IgE antibodies

Specific IgE ab against birch pollen extract and its fractions were measured by the RAST method (28). Coupling of the allergens to the paper discs activated by cyanogen bromide (BrCN) was performed exactly as described by Ceska et al. (9) using allergen dilutions corresponding to 15 $\mu\text{g}/\text{ml}$ of protein. After incubation with 50 μl of patient serum (3 h, 20°C) and washing with RAST buffer (Phadebas RAST, Pharmacia AB, Uppsala, Sweden) 50 μl of ^{125}I -labelled rabbit anti-human-IgE (supplied by Pharmacia AB) was added for a second incubation (18 h, 20°C). The results are expressed as radioactivity (c.p.m.) bound to the discs coupled with the allergens. The limit for a positive IgE ab response was determined by using an AB-negative serum pool (The Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) as a reference. Counts three times higher than those obtained with the reference serum were regarded as a positive response, i.e. the limit was 600 c.p.m.

Specific IgG antibodies

Specific IgG ab against the various fractions and against the whole extract were measured by enzyme-linked immunosorbent assay (ELISA, 10, 25). Polystyrene cuvettes were coated with either 200 μl of birch pollen extract (1:1000 w/v) or 200 μl of pooled fractions in a dilution corresponding to 15 $\mu\text{g}/\text{ml}$ of protein, by incubation at 37°C overnight. Thereafter, the cuvettes were incubated with PBS containing 5% of normal sheep serum (PBS-NSS) at 37°C for 3 h and washed with PBS. 1:40 dilutions of patients' sera were made in 20% PBS-NSS. 150 μl of the dilution was incubated in the antigen-coated wells at 4°C overnight. After washing, sheep anti-human-IgG conjugated with alkaline

phosphatase (ALP type VII, Sigma, St. Louis, Missouri) was added and the cuvettes were incubated at 4°C overnight. Before adding the substrate (*p*-nitrophenylphosphate, 2 mg/ml, Sigma) washings with 0.9% NaCl containing 0.05% of Tween 20 were again performed. The absorbance was measured at 405 nm using a semiautomated nine-channel photometer (Finnpipette analyser FP-9, Finnpipette-Labsystems, Helsinki, Finland) combined with a Hewlett-Packard 9815A calculator for recording of the results. The results are expressed as the mean absorbances (OD_{405}) of triplicate measurements. A positive IgG ab response was defined by using an AB-negative serum pool (The Finnish Red Cross Blood Transfusion Service) as a reference. Absorbances (OD_{405}) three times higher than those obtained with the reference serum were regarded as positive. Thus OD-values 0.45 or greater than that were taken as a positive response.

Protein determinations

Protein determinations were performed by the Lowry method using bovine serum albumin as a reference, as described in a previous paper (27).

RESULTS

Gel filtration of birch pollen extract

Gel filtration on Sephadex G-100 column of birch pollen extract (1:10 w/v) was performed as described in a previous paper (27). Protein contents of pooled fractions of peak A, B, C and D are shown in Fig. 1.

Immune response to fractions of birch pollen extract in atopic and in non-atopic individuals

Individual responses of 5 untreated and 5 hyposensitized subjects are seen in Fig. 1. The highest IgE and lymphocyte responses were generally against the allergenic fractions (peak B and peak C) in both hyposensitized and in untreated subjects. None of the untreated

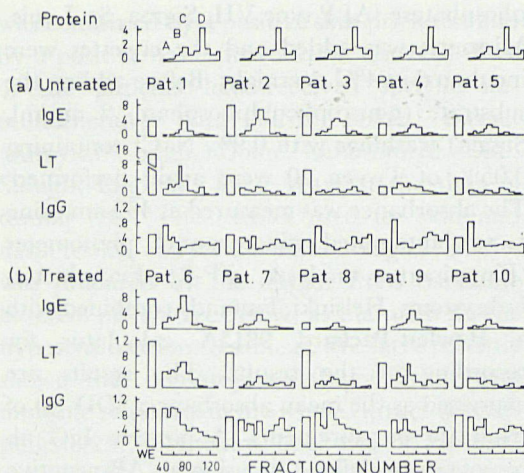


Fig. 1. IgE ab, IgG ab and LT responses of untreated and hyposensitized subjects to the whole birch pollen extract (WE), and to the various pooled fractions after gel filtration on Sephadex G-100 column of the extract. A, B, C and D refer to the different protein peaks after gel filtration (1 unit = 0.1 mg/ml of protein).

The results are expressed as follows: The amount of IgE ab bound to paper discs coupled with the allergens (RAST; 1 unit = 1000 c.p.m.); IgG ab bound to polystyrene cuvettes coated with the allergens (ELISA; absorbancy at 405 nm = OD_{405}); and LT as net c.p.m. of the stimulated lymphocyte cultures in the presence of the allergens (1 unit = 100 c.p.m.).

The limits for positive results were the following: IgE: 600 c.p.m.; IgG 0.45 (absorbancy); LT: 200 c.p.m.

subjects and only 1 out of the 5 treated ones had developed both IgE and IgG antibodies and lymphocyte transformation (LT) responses to the high molecular weight fractions of peak A with low allergenic activity. However, all but one of the untreated and all the treated subjects had IgG ab, and three of the treated ones had also positive LT and IgE responses to these antigens. On the other hand, only one out of the 10 allergic subjects showed both IgE ab, IgG ab and LT responses to the fractions of low molecular weight (mol.wt. = 5000) in peak D. Nevertheless, 4 out of 10 allergic subjects had IgE ab, 7 had IgG ab and 4 had positive LT responses to these fractions.

Mean results of the different groups are presented in Table 1. IgE and lymphocyte responses to the whole extract and to the different fractions were generally similar in the

untreated and in the hyposensitized group. However, the mean IgE ab response to the fractions of peak A (expressed as a percentage of the values obtained with the whole extract) was significantly higher in the treated than in the untreated subjects ($P < 0.01$). Furthermore, significantly higher IgG responses to the whole extract and to the allergenic fractions of peak B and peak C were found in the hyposensitized than in the untreated patients ($P < 0.01$, $P < 0.01$, $P < 0.001$, respectively). In contrast, proportionally higher IgG responses to the fractions of peak A were found in the untreated patients. Non-atopic individuals with negative immune responses to the whole extract showed negative IgE ab, IgG ab and LT responses also to the allergenic fractions of birch pollen extract. Very weak IgG ab and LT responses were observed against the fractions of peak A, while no evidence for a specific IgE response against any of the fractions was obtained in these subjects.

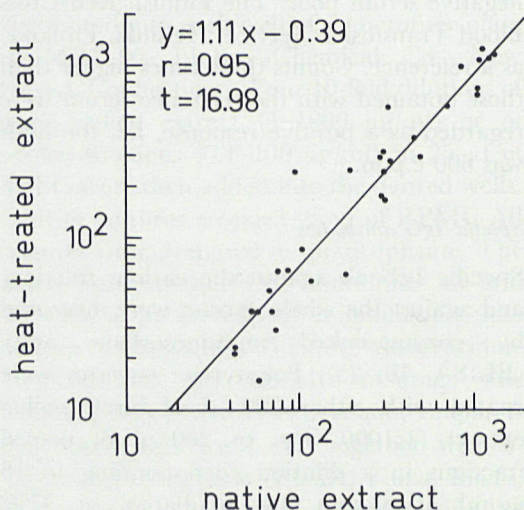


Fig. 2. The effect of heat-denaturation of birch pollen extract on lymphocyte stimulation by birch pollen antigens. Correlation between the uptake of $^{125}\text{IUdR}$ (c.p.m., log-transformed data) in the lymphocyte cultures with three doses (120, 12 and 1.2 $\mu\text{g/ml}$) of native (x-axis) and heat-denatured (100°C for 15 min) (y-axis) birch pollen extracts.

Eleven patients with different lymphocyte responses were tested.

Table 1
Mean IgE ab, IgG ab and LT responses of atopic and non-atopic subjects to birch pollen extract and its main protein fractions^a

	Untreated (n=5)			Hypsensitized (n=5)			Non-atopics (n=2)		
	IgE c.p.m. (%)	LT c.p.m. (%)	IgG OD ₄₀₅ (%)	IgE c.p.m. (%)	LT c.p.m. (%)	IgG OD ₄₀₅ (%)	IgE c.p.m. (%)	LT c.p.m. (%)	IgG OD ₄₀₅ (%)
Whole extract	4992 (100)	770 (100)	.41 (100)	4716 (100)	1082 (100)	.70 (100) ^c	175 (100)	286 (100)	.24 (100)
Fractions ^b									
A 37-51	388 (8.1)	226 (41.0)	.71 (179)	769 (15.2) ^c	370 (39.4)	.81 (121)	155 (88.6)	287 (100)	.29 (121)
B 58-64	1424 (27.0)	230 (39.1)	.31 (74.5)	1345 (26.3)	343 (32.1)	.77 (115) ^c	140 (80.0)	150 (52.4)	.21 (87.5)
C 69-77	4443 (89.5)	295 (50.0)	.14 (33.7)	4203 (87.7)	474 (45.7)	.57 (82.7) ^d	165 (94.3)	164 (57.3)	.11 (45.8)
D 97-104	793 (17.2)	232 (38.1)	.39 (99.2)	591 (12.7)	271 (27.8)	.48 (73.1)	171 (97.7)	189 (66.1)	.18 (75)
114-123	160 (3.5)	101 (18.9)	.26 (66.9)	198 (4.8)	208 (22.6)	.38 (62.9)	153 (87.4)	150 (52.4)	.21 (87.5)

^aThe methods used were RAST (IgE), ELISA (IgG) and lymphocyte transformation. The results are expressed as mean of the absolute values and as mean percentage of the values obtained with the whole extract.

^bPooled fractions of Sephadex G-100 gel filtration of birch pollen extract (1:10 w/v). A, B, C, and D refer to different protein peaks (27). See also Fig. 1.

^c $P < 0.01$, when compared with the untreated subjects (t -test).

^d $P < 0.001$, when compared with the untreated subjects (t -test).

Lymphocyte transformation induced by native and heat-denatured birch pollen extract

The results of lymphocyte cultures with either heat-treated (100°C for 15 min) or native birch pollen extract are presented in Fig. 2. The pollen sensitive patients were tested with three concentrations (120 µg/ml, 12 µg/ml, 1.2 µg/ml) of heat-treated and native extract. A linear correlation ($r = 0.95$) between these extracts was found. Only slight decrease in lymphocyte transformation was found after heat-treatment, indicating that the antigens stimulating the lymphocytes are thermostable.

DISCUSSION

In the present work IgE and IgG antibodies and lymphocyte transformation by fractions of birch pollen extract were studied in hyposensitized and in untreated allergic subjects and in non-atopic controls. The exact antigenic composition of the commercial allergen extract used for the hyposensitization treatment was not known. However, it was prepared from the same batch of pollen as the extract used for the immunological tests.

Both the untreated subjects and the hyposensitized ones had developed the highest IgE and lymphocyte responses to the main allergenic fractions of birch pollen extract. As expected, IgG ab responses to the allergenic fractions were significantly higher in the hyposensitized group than in the untreated one. On the other hand, even the untreated patients showed rather high binding of IgG ab to the fractions of large molecular size, composed mainly of non-allergenic proteins and of carbohydrates (5, 27). Also non-atopic individuals, with negative IgE ab responses to any of the fractions, had weak LT and IgG ab responses to these high molecular weight fractions. These results are in keeping with those observed in rabbits. Rabbits immunized intranasally produced hemagglutinating antibodies to the non-allergenic fractions, but reaginic antibodies only to the allergenic fractions of birch pollen (6).

Although the highest IgE ab responses both in the untreated subjects and in the hyposensitized ones were against the allergenic fractions of birch pollen, a significantly higher proportion of IgE ab to the high molecular weight fractions with low allergenic activity was observed in the hyposensitized group. There are reports of increased levels of serum IgE ab to allergens after immunotherapy (3, 7), but the heterogeneity of these antibodies has rarely been studied (16, 22, 24). Belin & Strannegård (6) studied the effect of transmucosal and parenteral administration of whole birch pollen on reaginic antibody production in rabbits and obtained results similar to those of the present study. The higher IgE ab responses against the fractions with low allergenic activity might be explained by immunization to new antigens during the hyposensitization therapy (22). Using parenteral immunization with high doses of antigens the genetic requirements for IgE antibody responses may be overcome (13, 15, 16, 17), thus allowing responses against antigens different from those observed after natural exposure to environmental allergens. However, recently, Østerballe et al. (19) using crossed radioimmunoelectrophoresis could not show development of IgE ab against new timothy antigens after immunotherapy with whole timothy extract. Therefore, it is more likely that our results reflect preferential potentiation of IgE ab responses against the high molecular weight antigens with low allergenic activity rather than immunization to new antigens during immunotherapy.

A positive lymphocyte transformation was obtained with the whole extract and with the allergenic fractions of birch pollen in all birch pollen allergic subjects. The main stimulating antigens were resistant to heating (100°C for 15 min, Fig. 2). Thus they correspond to the main heat-stable allergens of birch pollen (4, 5, 27). This is consistent with our previous findings of a positive correlation between lymphocyte transformation by the whole birch pollen extract and RAST in allergic subjects (25). However, also the low molecular weight

fractions containing mainly non-allergenic antigens were stimulatory in some of the patients. Positive lymphocyte transformation in allergic subjects by both the allergenic and non-allergenic fractions of *Holcus lanatus* pollen has been reported by Romagnani et al. (23). On the other hand, allergenic fractions of *D. pteronyssinus* extract separated by isoelectrofocusing were the most stimulatory ones in lymphocyte transformation (21). These findings suggest that the bulk of the lymphocyte-stimulating capacity of the whole allergenic extracts is associated with the allergenic antigens, while specific responses to the non-allergenic antigens are only occasionally seen.

In a previous study a fraction of a low molecular size (mol.wt. approx. 5000) was separated by gel filtration on Sephadex G-100 of birch pollen extract (27). The present results show development of IgE ab, IgG ab and lymphocyte responses to this fraction in some of the allergic subjects. Similarly, humoral and cell-mediated immune responses have been observed to the low molecular weight antigen of ragweed pollen (Ra5; 8, 18). However, haptenic material from timothy pollen (antigen D) inhibited lymphocyte transformation (11, 12), while some low molecular weight timothy antigens stimulated lymphocytes from sensitive patients (12). A probable explanation of the different antigenicity and allergenicity of the small molecular weight substances is their different number of antigenic/allergenic determinants (2, 14, 16, 24). Our data indicate the presence of both antigenic and allergenic determinants in the low molecular weight antigens of birch pollen. Allergenic cross-reactivity with peak C allergens suggests that these antigens may be break-down products of the main allergens of birch pollen (27). The biological significance of these allergens can be studied after a more complete purification has been achieved.

The results of this study are consistent with the findings that most individuals with pollen allergy develop both IgE ab, IgG ab and lymphocyte transformation responses to the different pollen allergens (8, 20, 25).

Preferential potentiation of IgE ab responses against the high molecular weight fractions with low allergenic activity and of IgG ab responses to the allergenic fractions was observed in the hyposensitized group. This suggests different regulatory immune mechanisms after natural exposure and after parenteral immunization by pollen antigens. Consequently, the use of whole extracts of allergens in immunotherapy might lead to sensitization to some antigens, in place of "desensitization". Thus it should be controlled that allergen extracts made either for diagnosis or treatment of allergic diseases contain all the major and the minor allergens present in the original allergenic material, but only minimal amounts of non-allergenic antigens.

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