

Evaluation of immunotherapy-induced changes in specific IgE, IgG and IgG subclasses in birch pollen allergic patients by means of immunoblotting

Correlation with clinical response

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Sera from 27 birch pollen-allergic patients who had undergone hyposensitization treatment for 22–41 months were studied by immunoblotting before and after therapy, whereby the levels of IgE, IgG and IgG1–4 antibodies directed against the major allergen *Bet v* I and minor allergens of birch pollen were monitored. The clinical benefit of immunotherapy (IT) was evaluated using a symptom specific questionnaire. In patients with good clinical response (responders, $n = 18$), as defined by improvement of symptoms, anti-*Bet v* I IgE antibodies were found to decrease in 10/18 patients (55.5 %), whereas in 6/18 (33.3 %) no change and in two cases (11.2 %) an increase of specific IgE was observed. In the group of patients with unsatisfactory clinical outcome (non-responders, $n = 9$), 3/9 patients (33.3 %) showed a decrease, 3/9 (33.3 %) no change and 3/9 (33.3 %) an increase in levels of IgE antibodies directed against *Bet v* I. In the case of minor allergens, 5/18 responders (27.7 %) and 8/9 non-responders (88.8 %) showed specific IgE before IT. In the responder group, no increase of specific IgE could be observed after IT. In non-responders, however, an increase of IgE directed against minor allergens was seen in 3/9 patients (33.3 %). In all patients, regardless of therapeutical success, IT-induced elevated levels of specific IgG, IgG1 and in particular IgG4 directed against *Bet v* I were found. Regarding minor allergens, a heterogeneous pattern of IgG responses without significant correlation to clinical benefit was observed. Our results indicate that changes in IgG reactivity patterns against *Bet v* I and minor allergens, as shown by the immunoblot technique, did not correlate with good or bad clinical outcome.

Key words: birch pollen allergy; immunotherapy; immunoblot; specific IgE, IgG, IgG1, IgG4 response; clinical benefit.

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CLINICAL ASPECTS

Previous studies have shown that more than 95 % of the patients with Type I allergy to birch pollen exhibit IgE antibodies against the major allergen *Bet v* I, and about 60 % of these patients react exclusively with this allergen. Therefore, birch pollinosis seems to be an excellent model to study changes in levels of specific antibodies as a consequence of hyposensitization. To achieve this, the immunoblot technique was applied. An increase of IgG, IgG1 and IgG4 antibodies to *Bet v* I and minor allergens was associated with good as well as with unsatisfactory clinical outcome. Therefore, other reasons than the commonly presented hypothesis of inducing “blocking” antibodies by immunotherapy (IT) seem to be responsible for the well-known effects of hyposensitization.

Hyposensitization, or specific immunotherapy (IT), has been successfully used for the treatment of patients with severe pollinosis and other Type I allergies. Although the efficacy of this kind of therapy has been shown for many allergens, the underlying mechanisms are still a matter of controversy (2, 8). Blocking antibodies, decrease of specific IgE by restoration/generation of antigen-induced suppressor T-cell function (43), changes in cell sensitivity or activation (2, 8), and auto-anti-idiotypic antibodies (3, 4) could be of importance for the success of IT. Furthermore, IgE-binding factors which can suppress IgE synthesis in an isotype-specific fashion should be taken into account regarding the outcome of hyposensitization (14, 19, 25). The aim of our study was to test the anti-birch reactivity of sera obtained before and after IT using the immunoblot technique. This technique was chosen because it provides a useful tool to show reactions between antibodies and distinct allergens with known molecular weights with high sensitivity. After determining the changes in allergen-specific IgE, IgG and IgG subclasses, clinical outcome was compared with results of *in vitro* and *in vivo* tests in order to show possible correlation of laboratory parameters with clinical benefit of IT.

MATERIAL AND METHODS

Twenty-seven patients (19 females and 8 males, age ranging from 12–68 years, median 40 years) were included in this study. All patients had a history of birch pollen allergy, positive skin prick tests, and revealed serum RAST classes of 2.4–4. Before IT, all patients complained of conjunctivitis and rhinitis. 5/27 complained of nasal congestion changing with rhinitis, 14/27 of dry cough and 12/27 of *asthma bronchiale* (most of the mentioned symptoms were subjectively assessed as severe). The most severe symptoms were observed between the end of March and the middle of May.

Subjective symptom ratings were used to measure the severity of the allergic disease and to assess the benefit of IT (27). By means of a questionnaire the patients were asked to score the symptoms conjunctivitis, rhinitis, nasal con-

gestion, dry cough and *asthma bronchiale* on a three grade scale – absent, moderate or severe – corresponding to the numerical values 0, 1 and 2, respectively. By adding the score for each of the three symptoms, a sum of between 0 and 10 was obtained in each case. Each patient was asked for his consumption of anti-allergic medicine before, during and after IT and was requested to describe his most disturbing ailments. By means of these subjective ratings, two groups – responders and non-responders – were formed. Responders were defined by decrease of allergic symptoms by at least 30% according to the score rating.

Immunotherapy was carried out with Alutard SQ extracts (ALK, Copenhagen, Denmark) with one weekly injection starting at a dose of 20 SQ units. The dose was then increased, and after 13 weeks all patients received a maintenance dose of 60,000 SQ units. Maintenance doses were given monthly and were reached before the pollen season, during which the dosage was reduced to 1/2. Duration of IT (usually 24–36 months in Austria) ranged from 22–41 months, median 31 months. Two patients stopped IT after 22 months due to very good success respectively failure of treatment. None of the patients had received IT with any tree pollen extract before. One patient had successfully been hyposensitized against house dust mite (1975–1978) and animal dander (1979–1981). Since birch pollen-allergic patients usually show a very good response to IT (proven benefit in 70–80% of the treated patients according to own observations and literature (18, 36, 45)), special efforts were made to follow up on the group of patients with IT-failure, thus including an adequate number of non-responders.

Skin prick tests (SPT) were performed on the volar aspect of the forearm, using Soluprick SQ birch pollen extract (ALK, Copenhagen, Denmark) in a concentration of 10 HEP. As positive reference, histaminedihydrochloride (10 mg/ml) was used as proposed by the standardization committee at the XV Nordic Congress in Allergology, Turku, Finland, 1984 (28). A reaction was recorded as 3+ (+++) when the wheal was equal to the histamine control. Each additional + referred to a doubling of wheal

size. Two + (++) were given for a wheal half of the positive control. One + (+) was recorded when the wheal was smaller than half of the positive control but the diameter extended 4 mm.

RAST was performed using t3 (birch) paper disks and ^{125}I -labeled affinity chromatography purified anti-human IgE (Pharmacia, Uppsala, Sweden) following the instructions of the supplier. All sera were tested in a single run. RAST activity was described in PRU/ml, and changes were expressed in % in relation to the activity of the sera obtained before IT was started.

Birch pollen extracts and SDS-PAGE were prepared as previously described (22).

Immunoblotting

For the detection of allergen-specific IgE antibodies, nitrocellulose (nc) strips were incubated with patients' sera diluted 1:4 in buffer (50 mM sodiumphosphate, 0.5 % (v/v) Tween 20, 0.5 % (w/v) bovine serum albumin, 0.05 % (w/v) sodiumazide, pH 7.5), at 4°C, overnight. After washing with buffer for 30 min, strips were incubated with ^{125}I -labeled rabbit anti-human IgE (Pharmacia, Uppsala, Sweden) diluted in buffer in order to achieve about 200,000 cpm/strip and supplemented with 1 % Gelatine (Janssen, Olen, Belgium), at room temperature, overnight. Thereafter, the strips were washed and subjected to autoradiography (-70°C for 48 h), using high performance autoradiography films (Hyperfilm-MP, Amersham International, UK) in exposure cassettes (x-Omatic, Kodak-Austria, Vienna, Austria).

For IgG and IgG subclass blots patients' sera were diluted 1:20 in buffer. IgG bound to nc strips was detected with an affinity purified rabbit anti-human IgG antibody (Jackson, West Grove, PA, USA) diluted 1:1000 and ^{125}I -labeled donkey anti-rabbit Ig (Amersham International UK) diluted in buffer in order to achieve 200,000 cpm/strip. In each of these antibody dilutions, nc strips were incubated for 1h, each step at room temperature. Finally, the strips were washed and subjected to autoradiography (-70°C for 48 h).

To detect IgG subclasses bound to nc strips, the following monoclonal mouse antibodies were used: anti-human IgG1 - Clone HP-6012 (24, 40), obtained from Oxoid (Oxoid, Hampshire, UK); anti-human IgG2 - Clone HP-6014 (24, 40), obtained from Bio Makor (Bio Makor, Rehovot, Israel); anti-human IgG3 - Clone HP-6047 (40), obtained from Caltag (Caltag Laboratories Inc., San Francisco, CA, USA); anti-human IgG4 - Clone HP-6025 (24, 40), obtained from Bio Makor. Each monoclonal antibody was diluted 1:1000 in buffer. Nc strips were incubated with the monoclonal antibody for 1h, at room temperature. Thereafter, ^{125}I -labeled sheep anti-mouse Ig (Amersham International UK) - 200,000 cpm/strip - was used (1h, room temperature). Then the strips were washed and subjected to autoradiography (-70°C for 96 h). Each pair of sera (before and after IT) was tested in the same run to guarantee identical conditions. Changes in levels of antibodies directed against single allergens could be shown by visual evaluation of the different density of bands in immunoblots of sera obtained before and after IT.

RESULTS

Results of RAST, SPT and symptom scores are summarized in Table 1.

Immunoblotting

Changes in IgE directed against Bet v I. In responders, 10/18 patients (55.5 %) showed a decreased IgE reactivity to *Bet v I* after termination of treatment. 6/18 patients (33.3 %) showed no change, and two cases (11.2 %) showed an increased anti-*Bet v I* reactivity. In non-responders, 3/9 patients (33.3 %) showed a decrease, 3/9 (33.3 %) no change, and 3/9 (33.3 %) an increase in reactivity to *Bet v I*. Representative IgE-binding patterns are shown in Fig. 1.

Changes in IgE directed against minor allergens. Before IT, only 5/18 responders (27.7 %) exhibited specific IgE directed against some minor allergens. In contrast, the majority of non-responders

ders (8/9 patients, 88.8%) showed manifold IgE-binding patterns to minor allergens of birch. In the responder group, no increase of specific IgE could be observed. In non-responders, an increase of IgE directed against minor allergens in 3/9 patients (33.3%) was seen, but only one patient exhibited a marked induction (Pat. III; Fig. 1). Generally, IT had only slight effects on the reactivity to minor allergens. Representative IgE-binding patterns are shown in Fig. 1.

Changes in IgG and IgG subclasses directed against Bet v I. Before IT, IgG and IgG subclass reactivity was weak or absent in all patients with a few exceptions (4/27 patients, 14.8%; for example Pat. I, Fig. 4). After termination of treat-

ment, both responders and non-responders exhibited an increased anti-Bet v I IgG reactivity (Fig. 2). The IT-induced subclass response was restricted to IgG1 (Fig. 3) and IgG4 (Fig. 4) in each patient, whereas the IgG2 and IgG3 reactivity remained unchanged (not shown).

Changes in IgG and IgG subclasses directed against minor allergens. Patients' sera already exhibited a heterogeneous pattern of IgG (Fig. 2) and IgG1 responses (Fig. 3) to minor allergens before the therapy was started. Generally, IT had only slight effects on the IgG and IgG1 reactivity to them. Before IT only 13/27 patients (48.1%) showed IgG4-binding to various minor allergens. After termination of treatment, IgG4-binding to minor allergens was seen in 13/18

Table 1

Summary of age, sex, duration of immunotherapy (= IT), RAST-changes (expressed in % in relation to the activity of the sera obtained before IT was started), changes in skin prick tests (SPT) and in symptom score before and after IT

Pat. No.	Age (years)	Sex	IT (months)	% RAST-change	SPT (before/after IT)	Symptom score (before/after IT)
1	25	m	35	-44	+ + + / neg	7/3
2	46	f	31	0	+ + + / + +	6/3
3	40	f	27	-17	+ + + / + +	6/3
4	50	f	39	0	+ + + / +	4/1
5	49	f	30	-65	+ + + / + +	*8/4
6	40	f	26	0	+ + + / +	*8/4
7	63	f	26	-9	+ + + / + +	5/2
8	68	f	30	-23	+ + + / + +	4/1
9	12	f	30	-40	+ + + / + +	7/2
10	19	f	35	-65	+ + / neg	*5/1
11	47	f	41	0	+ + + / + +	6/3
12	67	f	41	-72	+ + + / +	6/3
13	35	f	39	-43	+ + / neg	4/1
14	40	f	27	-17	+ + + / + +	6/3
15	33	m	31	-10	+ + + / neg	*8/4
16	33	m	36	-6	+ + + / + +	4/2
17	49	f	22	-15	+ + + / +	*9/3
18	46	m	29	-10	+ + + / + +	*8/5
I	49	m	36	-24	+ + + / + + +	4/4
II	29	f	33	-8	+ + + + / + + +	*10/9*
III	28	m	27	+ 11	+ + + / + + +	5/5
IV	33	m	35	-6	+ + + / + + +	*6/6*
V	49	f	24	0	+ + + / + + +	*8/6*
VI	49	f	41	-8	+ + + / + + +	*6/6*
VII	30	f	22	0	+ + + + / + + +	*8/8*
VIII	37	m	41	0	+ + + + / + + +	*8/8*
IX	53	f	24	0	+ + + / + + +	6/6

Pat. Nos. 1-18: responders; I-IX: non-responders. All patients were suffering from rhinoconjunctivitis and/or (*) pollen asthma.

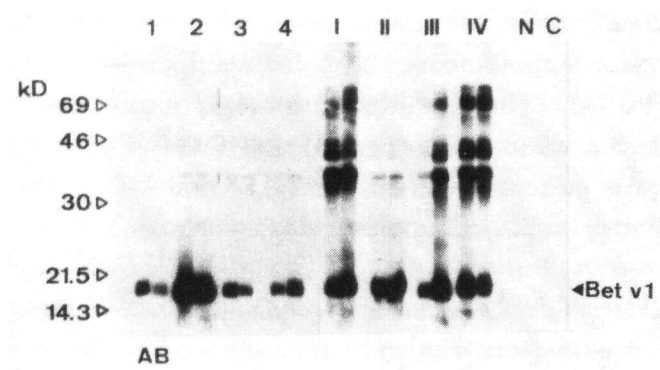


Fig. 1. Representative IgE antibody patterns against birch pollen allergens: reactivity of sera from 8 patients (1–4 responders, I–IV non-responders) before (A) and after (B) immunotherapy. Pat. Nos. correspond to Table 1. N = normal human serum pool, C = buffer control. Black arrow indicates position of *Bet v 1*, the major allergen of birch.

responders (72.2 %) and all non-reponders. Representative IgG4-binding patterns are shown in Fig. 4.

DISCUSSION

Previous studies have shown that more than 95 % of the patients with Type I allergy to birch pollen exhibit IgE antibodies to *Bet v 1*, the major allergen of *Betula verrucosa* (17), and about 60 % of these patients react exclusively with this allergen (22, 23). Therefore, the anti-birch reactivity of sera from birch pollen-allergic patients

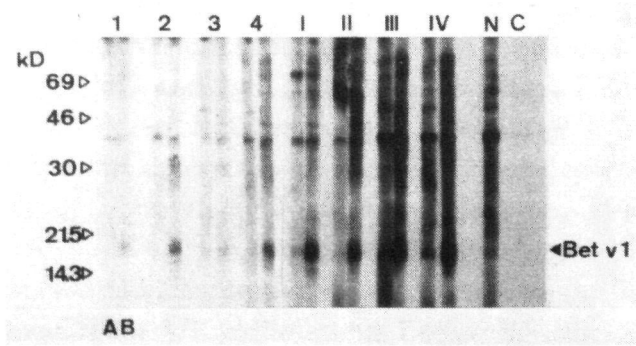


Fig. 2. Representative IgG antibody patterns against birch pollen allergens: reactivity of sera from 8 patients (1–4 responders, I–IV non-responders) before (A) and after (B) immunotherapy. Pat. Nos. correspond to Table 1. (For key to symbols see Fig. 1.)

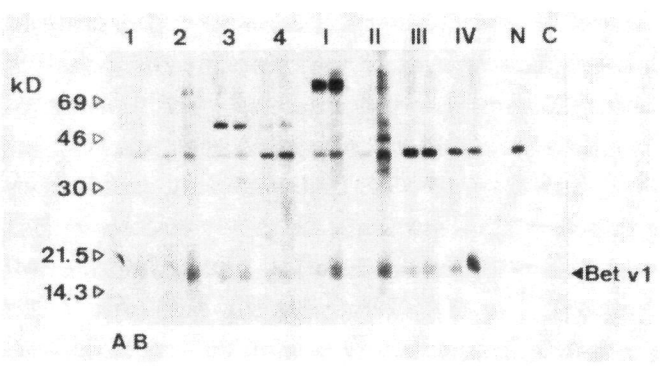


Fig. 3. Representative IgG1 antibody patterns against birch pollen allergens: reactivity of sera from 8 patients (1–4 responders, I–IV non-responders) before (A) and after (B) immunotherapy. Pat. Nos. correspond to Table 1. (For key to symbols see Fig. 1.)

seems to be an excellent model to study antibody reponses during hyposensitization. It should be stressed that birch pollen-allergic patients usually show a very good response to IT, according to own observations and literature (18, 36, 45). Therefore, special efforts were necessary to follow up on the group of patients with IT-failure, thus including an adequate number of non-responders.

The changes of allergen-specific antibodies described in this paper refer to results of immunoblots. Most of the earlier IT studies using CRIE, RAST and ELISA-related techniques describe an early increase of allergen-specific IgE after onset of hyposensitization. This is followed by declining IgE values which do not always fall below pretreatment levels (29,

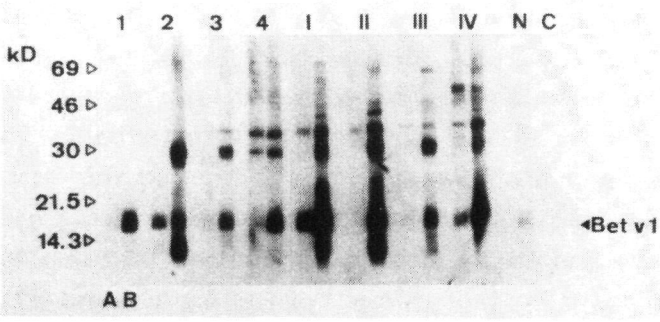


Fig. 4. Representative IgG4 antibody patterns against birch pollen allergens: reactivity of sera from 8 patients (1–4 responders, I–IV non-responders) before (A) and after (B) immunotherapy. Pat. Nos. correspond to Table 1. (For key to symbols see Fig. 1.)

30). However, since it is known that cross-linking of mast cell- and basophil-bound IgE by allergens leads to an activation of these cells and to mediator release (20), one would assume that IT might be successful because of reduced IgE levels. Since this effect only occurs after several months of treatment, it does not provide an explanation for the early efficacy of IT. For example, Möller et al. (33) found decreased conjunctival sensitivity paralleled by increased birch-specific IgE levels during the first months of IT. In a study by Fennerty et al., patients with successful IT (responders) revealed IgE levels significantly higher than both non-responders and controls at the time of treatment outcome assessment (10). In our study, approximately 40% of the patients with satisfactory therapeutical result showed an unchanged or increased anti-*Bet v I* IgE reactivity. There are several explanations for these observations. For example, it could be postulated that there is no correlation of titer of allergen-specific IgE levels in serum with the sensitivity against the same allergen in the shock organ (33). This concept is supported by a study by Somville et al. (42) who claimed that a precise quantitative evaluation of specific IgE is useless for clinical purposes. Also Fennerty et al. stressed that it is not clear to what extent the sensitivity of the respective target organ is dependent on levels of circulating allergen-specific IgE (11). Another possible explanation, as suggested by Malling et al. (29), could be that IT-induced specific IgE does not lead to an increased number of specific IgE molecules bound to mast cells and basophils, possibly due to a low affinity of IT-induced IgE to Fc-receptors. Furthermore, if the early IT-induced polyclonal boost of IgE led to an increase in mast cell- and basophil-bound IgE, some yet unknown IT-induced subcellular mechanisms could account for the decrease in mediator releasability. In accordance with other publications (37), we did not find evidence that a decrease of specific IgE alone was responsible for the success of IT.

It was interesting to observe that in contrast to the responders, the majority of non-responders showed not only IgE-binding to *Bet v I* but also to a variety of minor birch allergens. Poul-

sen et al. suggested the preparation of patient-adapted allergen mixtures which could be of value in patients in whom the immune response is directed against minor allergens (38). But in the majority of patients reacting exclusively with the major allergen, the use of an individual patient-adapted allergen preparation seems to be unnecessary, since treatment of tree pollen-allergic patients with birch pollen extract alone or patient-tailored extracts of birch, alder and hazel pollen were equally effective (18, 36, 45). In our study, induction of IgG4 antibodies directed against minor allergens pointed to the fact that extracts used for IT contained immunogenic amounts of minor allergens. The question whether the efficacy of IT can be improved by use of patient-adapted allergen preparations should be the subject of further studies.

For several years it has been postulated that allergen-specific IgG present in serum and secretions may bind to allergens before they can react with mast cell-bound IgE. It has been published that such "blocking" antibodies are important for the success of IT with hymenoptera venoms (12, 15, 44). Other authors were not able to show correlation of hymenoptera venom-specific IgG with the success of IT (26, 31). Studying airborne allergens, we observed that each patient showed a stronger IgG reactivity - predominantly directed against *Bet v I* - after termination of treatment, regardless of treatment outcome. Therefore, in inhalant allergy we have evidence that mechanisms other than the commonly presented hypothesis of inducing blocking antibodies as a consequence of IT are responsible for the well-known effects of hyposensitization. This point of view is supported by others (8).

The role of IgG subclasses in response to IT is still a matter of controversy. Some authors suggest that increased levels of certain allergen-specific IgG subclasses (IgG1, IgG4) correlate with clinical benefit of IT (34, 35). Other authors found symptoms deteriorated simultaneously with high levels of IgG1 and/or IgG4 antibodies (5, 6, 7, 32).

In accordance with other publications (1, 5, 6, 7, 16, 32), we found an IT-induced IgG response restricted to the subclasses 1 and 4. No

striking differences between responders and non-responders could be seen regarding IgG1 and IgG4 reactivity patterns (Figs. 3 and 4).

Malling et al. observed correlation of an increase of specific IgG1 with failure of IT as well as the number of systemic side effects (32). The deleterious role of IgG1 antibodies may be based on the formation of immune complexes with the allergen. This could lead to activation of complement resulting in formation of anaphylatoxins and, consequently, in histamine release from mast cells. The possibility of a pathogenetic role of IgG1 antibodies is supported by Ito et al. (21) who observed a close correlation of late asthmatic responses with high levels of IgG1 antibodies to house dust mite. However, Djurup claimed that only IgG1 antibodies possess the capacity for cellular interactions necessary for initiating immune-regulation, possibly resulting in formation of tolerogenic complexes (6).

A number of investigators have described a basophil-sensitizing capacity of IgG4 (9), although this is not unequivocally accepted by others (1, 39). Djurup et al. observed correlation of high IgG4 levels with failure of IT (7). On the other hand, based on the knowledge that IT leads to increased levels of IgG4 antibodies, it was speculated that IgG4 could act as blocking antibodies (34, 35). Fling et al. observed correlation of increased allergen-specific IgG4 antibodies with suppression of late cutaneous responses, but no association with changes in symptom medication score (13). We and others (41) could find no indications that the IgG4 subclass represents the sole blocking antibodies, or that increased levels of allergen-specific IgG4 after at least 22 months of treatment are correlated with failure of IT.

In summary, changes in allergen-specific IgG and IgG subclasses do not appear obviously to be associated with treatment outcome. Therefore the described changes in allergen-specific antibody reactivity and the clinical improvement induced by IT are inconclusive consequences of the treatment.

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