

Efficacy of birch-pollen immunotherapy on cross-reactive food allergy confirmed by skin tests and double-blind food challenges

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Summary

Background The effect of birch-pollen immunotherapy (IT) on cross-reactive food allergies is controversial.

Objective The aim of this study was to investigate the effect of birch-pollen IT on apple allergy and to evaluate recombinant allergens and double-blind placebo-controlled food challenges (DBPCFCs) as monitoring tools.

Methods Twenty-five adult birch-pollen- and apple-allergic patients were randomly divided into two groups, either receiving birch-pollen IT or symptomatic drugs only. IgE and IgG4 antibodies against birch pollen, apple, natural Bet v 1 and Mal d 1 were measured. In addition, skin prick tests (SPT) were performed using recombinant Bet v 1 (rBet v 1) and Mal d 1 (rMal d 1). Clinical outcome was evaluated by DBPCFC. CD4⁺CD25⁺ regulatory T cells (Tregs) were isolated from peripheral blood and tested in functional assays.

Results Birch-pollen IT resulted in a significant decrease of SPT reactivity for rBet v 1 (30-fold) and rMal d 1 (10-fold) already after 3 months. IgG4 antibodies were potently induced against Bet v 1, displaying cross-reactivity to Mal d 1. Visual analogue scale scores decreased >10-fold in 9/13 patients of the IT group, with three patients converting to negative. In the control group, no decrease was observed. Birch-pollen IT did not lead to detectable changes in the number or function of the CD4⁺CD25⁺ Tregs.

Conclusions This trial supports the claims that birch-pollen IT also decreases allergy to foods containing Bet v 1-homologous allergens. Recombinant allergens and DBPCFCs have proven to be useful tools for monitoring the effect of birch-pollen IT on linked food allergies.

Keywords allergy, Bet v 1, birch, cross-reactivity, DBPCFC, human regulatory T cells, immunotherapy, Mal d 1, recombinant allergens

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Introduction

Up to 70% of patients with birch-pollen allergy develop an IgE-mediated food allergy to fresh fruits of the Rosaceae family, nuts or vegetables [1, 2]. This food allergy is characterized by immediate itching in mouth and throat, sometimes associated with mild to moderate angioedema, and is also known as the oral allergy syndrome (OAS) [3, 4]. The combination of pollen and fruit allergy is explained by cross-reactive IgE antibodies that recognize homologous allergens in birch pollen and fruits [5–7]. The primary sensitizer is the major birch-pollen allergen, Bet v 1. Its fruit homologues are generally believed to lack the capacity to sensitize directly,

because they are labile (sensitive to processing) and pepsin sensitive, i.e. easily digested in the stomach. Consequently, the spectrum of IgE specificities is broadest against the pollen allergen. Selective recognition of epitopes on fruit or vegetable homologues of Bet v 1 is extremely rare [8].

Immunotherapy (IT) for the treatment of birch-pollen allergy has been a well-accepted and successful therapy for many years. IT for the treatment of inhalant allergies was described in 1900 by Curtis [9] and is now widely used as an effective treatment of pollen, animal, mite and also insect venom allergy [10]. The mechanism of allergen-specific IT is slowly being unravelled. It has been described that IT leads to a shift in specific IgE and IgG4 concentrations; IgE concentrations tend to rise initially after IT and subsequently decrease over time, whereas IgG4 concentrations increase during IT. Since IL-10 stimulates the production of IgG4, IL-10-producing T cells may be involved in the induction of tolerance by IT [11]. IL-10 can be produced by different T cell subsets, such as type 1 regulatory T cells (Tr1), Th3 cells or

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CD4⁺CD25⁺ regulatory T cells (Tregs) [12, 13]. Recent publications have speculated on the possibility that CD4⁺CD25⁺ Tregs are involved in the process of tolerance induction, following successful IT treatment [14, 15].

In view of the cross-reactivity between birch pollen and fruits and/or nuts, IT with birch-pollen allergens might be expected to improve associated food allergies. It is a long-standing debate whether IT for inhalant allergies indeed has beneficial effects on accompanying food allergies [16–19]. Some studies reported improvement [16, 17, 19], while another study found no beneficial effect of tree pollen IT on apple allergy [18]. In a case report of one patient, Kelso et al. [17] showed improvement of apple allergy after tree mix IT by open challenge. In another study 56% of patients treated with tree mix IT reported improvement as evaluated by a questionnaire [19]. Asero [16] reported clinical improvement in 84% of the patients as judged by the open apple challenge. A study by Moller [18] could not demonstrate a beneficial effect of birch-pollen IT on apple allergy in children that were evaluated by a questionnaire. Considering these conflicting results there is a need for well-controlled studies.

The aim of this study was to investigate whether support can be found for cross-desensitization for apple during birch-pollen IT. To focus on the structures that are at the basis of birch–apple cross-reactivity, humoral (IgE and IgG4) and cellular (Treg) immune reactivity was monitored both *in vitro* and by skin prick test (SPT) using purified natural and recombinant Bet v 1 (rBet v 1) and Mal d 1 (rMal d 1). Previous studies had never used the gold standard for food allergy diagnosis, the double-blind placebo-controlled food challenge (DBPCFC), to determine the clinical outcome of treatment. In our study the DBPCFC was for the first time applied to monitor the effect of birch-pollen IT on cross-reactive apple allergy.

Methods

Patients

Twenty-five adult patients (nine males and 16 females, mean age 35.6 years) with a history of birch-pollen and apple allergy were recruited from the outpatient department of Allergology of the University Medical Center Utrecht in the period between August and October 2001. This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All patients gave written informed consent before enrolment in the study. Inclusion criteria were a positive skin test ($>7\text{ mm}^2$) to birch-pollen extract (ALK-ABELLÓ, Nieuwegein, the Netherlands) and fresh apple (Golden Delicious) and specific serum IgE concentrations of $>0.7\text{ kU/L}$ to birch pollen and apple (CAP-FEIA, Pharmacia & Upjohn, Woerden, the Netherlands). We randomized 25 patients into two groups: one group to receive birch-pollen IT ($n = 13$) and the control group to receive only symptomatic treatment ($n = 12$). Both patient groups were similar with respect to sex, mean age, birch-pollen SPT reactivity, IgE concentrations for natural Bet v 1 (nBet v 1) and natural Mal d 1 (nMal d 1) and the severity of symptoms after eating apple (Table 1).

Two patients of the control group (Table 1, patients 24 and 25) were excluded because they were not willing to participate

in the study without receiving IT, so in the control group 10 patients were left. Patient characteristics of these two persons did not differ from the other patients in the control group.

Double-blind placebo-controlled food challenge

Clinical outcome was investigated by DBPCFC before start of IT in November 2001 ($t = 0$) and after 1 year ($t = 12$ months). On the day of the challenge only a light breakfast was allowed at least 2 h before the first test meal. The challenges were performed in a clinical research setting equipped for resuscitation and monitoring of vital signs. During the challenge, the patient was permanently accompanied by an investigator or a nurse. The skin and oral cavity were inspected before the challenge for pre-existing lesions and during the challenge when symptoms like itching or a feeling of swelling occurred. Oral allergy symptoms were scored using visual analogue scales (VAS) ranged 0–100 mm. An interval of 15 min was allowed between each test meal when no reaction occurred, or longer if (subjective) symptoms were still present. A challenge was regarded positive when subjective symptoms were reported for at least three times after active doses or when objective symptoms were observed [20]. Patients were kept for observation for at least 1 h after resolution of symptoms. The challenge meals were prepared within 5 min before administration and contained 4, 10, 40 or 120 g of fresh shredded apple (Golden Delicious). The meals were completed with a mixture of yogurt, orange juice, apple juice, applesauce, oatmeal flakes and dry rasped coconut. The placebo doses consisted of the same ingredients except fresh apple. Apple juice and sauce did not contain any IgE-reactive Mal d 1 allergen due to processing steps and were added for optimal blinding of taste. All patients were nose clipped while eating the test meals. The increasing apple doses ($n = 4$) were randomly interspersed with placebos ($n = 4$).

IT protocol

The patients of the IT group received a standardized aluminium hydroxide adsorbed birch-pollen extract, Alutard SQ (ALK-ABELLÓ). All patients were given IT according to a modified cluster schedule, between November 2001 and January 2002, followed by a monthly maintenance injection of 100 000 standard quality units (SQ-U) for 1 year with a dose reduction of 50% during the pollen season. All patients were pre-treated with an antihistamine at least 1 h before injection, since this reduces side-effects and has been reported to possibly enhance efficacy [21].

Allergens

nMal d 1 and Bet v 1 were purified by monoclonal antibody affinity purification [22]. rMal d 1 and rBet v 1 were produced in *Escherichia coli* and purified as described elsewhere [23].

SPT reactivity

Patients were evaluated at 3 monthly intervals during 1 year by SPT. SPT was performed on the flexor aspect of the forearm with a standardized prick needle [24] (ALK-ABELLÓ, Hørsholm, Denmark). The reaction was measured after 15 min by copying the weal reaction with transparent

Table 1. Patient characteristics

Patient number	Pollen sensitivity	Apple-related symptoms (CH)	SPT apple (mm ²)	SPT birch (mm ²)	RAST apple (kU/mL)	RAST birch (kU/mL)
1	B G	OAS RC CU	52	52	5.8	12.3
2	B G	OAS	42	27	2.9	5.8
3	B	OAS RC CU	11	31	4.8	11.4
4	B	OAS	30	51	1.3	3.4
5	B G	OAS RC	15	53	0.7	1.9
6	B G	OAS	49	29	4.8	11.7
7	B	OAS RC	22	47	8.2	23.8
8	B G	OAS RC	48	67	6.8	14.1
9	B G	OAS RC	61	64	0.8	0.7
10	B	OAS RC	28	46	1.6	5.4
11	B G	OAS	24	37	0.8	8.3
12	B G	OAS RC	21	27	0.7	1.4
13	B	OAS RC CU	55	15	2.2	2.9
14	B G	OAS	15	38	2.7	5.8
15	B G	OAS	17	35	7.0	13.1
16	B G	OAS RC	43	26	7.2	27.3
17	B G	OAS RC	29	34	9.9	17.8
18	B G	OAS RC	49	23	3.7	11.6
19	B G	OAS RC CU	42	37	5.3	26.1
20	B G	OAS RC	20	36	1.8	14.5
21	B	OAS RC	64	29	18.8	32.3
22	B G	OAS	25	12	0.7	5.8
23	B	OAS RC CU	25	32	2.1	3.4
24*	B	OAS	30	45	1.1	3.4
25*	B G	OAS	53	23	5.7	4.9

Patient numbers 1–13 IT treated, patient numbers 14–25 control group. Explanation of apple-related symptoms: OAS, oral allergy syndrome; RC, rhinoconjunctivitis; CU, contact urticaria; IT, immunotherapy.

*Patient excluded after randomization.

adhesive tape onto a record sheet. The skin weal areas were determined by computer scanning [25]. SPTs were regarded positive when the area was at least 7 mm². Histamine dihydrochloride (10 mg/mL) was used as a positive control, and the glycerol diluent of the SPT extracts was used as a negative control (ALK-ABELLÓ). We used the recombinant allergens and commercial extracts in serial dilutions; birch-pollen extract (0.1–10 000 Bioequivalent allergy units (BAU/mL; ALK-ABELLÓ) and the recombinant allergens, rBet v 1 (0.0002–20 µg/mL) and rMal d 1 (0.02–100 µg/mL) (Biomay, Vienna, Austria)). Reactivity to apple was tested by prick-to-prick method using fresh apple (Golden Delicious).

IgE and IgG4 RAST

Specific IgE titres against nBet v 1, nMal d 1, apple and birch-pollen extracts were determined every 3 months. RAST was performed as described previously. In brief, 100 µg purified protein or 4 mg extract (dry weight) was coupled to 100 mg CNBr-activated sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). Per test, 50 µL serum was added to 0.5 mg purified protein or pollen extract sepharose or 1.5 mg food extract sepharose in a final volume of 300 µL PBS/0.3% (w/v) BSA/0.1% (v/v) Tween 20 (PBS-AT). Immuno-detection was performed with ¹²⁵I-sheep anti-human IgE (Sanquin, Amsterdam, the Netherlands). To calculate RAST results in international units (IU) per millilitre an in-house standard of mouse/human chimeric IgE antibodies against Der p 2 was

used. These antibodies were calibrated against the WHO international reference for IgE and tested in different dilutions for binding to sepharose-coupled rDer p 2 to generate a standard curve. Patients were designated as sensitized if specific IgE concentrations were >0.7 IU/mL. IgG4 antibodies against birch pollen and apple were measured using the same RAST protocol adapted for detection of IgG4. To this end, 1.25 µL of serum was used instead of 50 µL. Detection was performed with radiolabelled monoclonal antibody against human IgG4 (Sanquin).

Allergen-binding radioimmuno assay (RIA)

IgG4 antibodies against nBet v 1 and nMal d 1 were measured using an antigen-binding RIA with radiolabelled purified allergens [27]. Serum (1.25 µL/test) was incubated overnight with 0.5 mg anti-γ₄-sepharose and radiolabelled allergen (~10 000 c.p.m.) in a final volume of 350 µL PBS-AT. Radioactivity, bound to the solid phase was measured on a gamma counter. Results were expressed in arbitrary units using a standard curve of a rabbit polyclonal antiserum against Bet v 1.

Isolation of peripheral blood mononuclear cell (PBMC) and purification of CD4⁺ T cell subsets

PBMC were isolated from heparinized venous blood from the IT and control group 1 year after IT (*t* = 12) and age-matched healthy controls (without atopic history) using Ficoll–

Isopaque centrifugation (Amersham Biosciences, Uppsala, Sweden). Three subsets, namely $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ Tregs were isolated from PBMC using magnetic-activated cell sorting (MACS). $CD4^+$ T cells were negatively selected with a $CD4^+$ T cell isolation kit obtained from Miltenyi (Bergisch Gladbach, Germany). To separate the $CD4^+CD25^-$ from the $CD4^+CD25^+$ T cells, the $CD4^+$ T cells were incubated with anti- $CD25$ microbeads (Miltenyi) and separated using a positive selection column. The various T cell subsets were tested in proliferation and suppression assays, as described previously [28, 29]. Briefly, for proliferation assays, the proliferative response of $CD4^+$ T cells depleted of the $CD25^+$ subset ($CD4^+CD25^-$ T cells) and $CD4^+CD25^+$ Tregs (1×10^5 T cells/well) was compared. Irradiated autologous PBMCs, depleted of $CD4^+$ T cells, were used as antigen-presenting cells (APC). For suppression assays, $CD4^+CD25^-$ T cells were stimulated in the absence (ratio 1:0, total amount of T cells/well 1×10^5) or presence (ratio 1:1, total amount of T cells/well 2×10^5) of autologous $CD4^+CD25^+$ Tregs. The following stimuli were used: 0.04 $\mu\text{g/mL}$ anti- $CD3$ mAb (Pelicluster $CD3$ culture supernatant, Sanquin), birch-pollen extract (50 $\mu\text{g/mL}$), rBet v 1 (5 $\mu\text{g/mL}$), rMal d 1 (5 $\mu\text{g/mL}$) and 50 $\mu\text{g/mL}$ purified cow's milk antigen (consisting of casein, α -lactalbumin and β -lactoglobulin) (NIZO, Ede, the Netherlands). Cells were cultured for 3 days with anti- $CD3$ mAb or 5 days with specific antigen. During the last 18 h of the culture period, [^3H]-TdR was added at 1 μCi per well (Amersham, Aylesbury, UK). Proliferative responses of the $CD4^+CD25^+$ T cells alone and in combination with the $CD4^+CD25^-$ T cells were compared with the proliferation to the $CD4^+CD25^-$ T cells alone (set at 100%).

Flow cytometry

To determine the percentage of $CD4^+CD25^+$ Tregs in peripheral blood of the patients and healthy controls, PBMC were stained with $CD4$ -PE-Cy5 and $CD25$ -FITC (both from DAKO, Glostrup, Denmark). The phenotype was assessed using a combination of the following antibodies: $CD152$ -PE, $CD69$ -PE, $CD45RO$ -PE (all from BD Biosciences, San Jose, CA, USA). To stain the cells for $CD152$ (CTLA-4), a Fix and Perm kit of Caltag (Burlingame, CA, USA) was used, according to the manufacturer's recommendations. Viable cells were gated based on their forward/sideward scatter profile, and analysed using CellQuest software.

Analysis of cytokine production

The production of cytokines was measured in the supernatant of the various T cell populations upon mitogenic and antigen-specific stimulation using ELISAs specific for IL-10, IL-13, IL-5 and IFN- γ (Sanquin) according to the manufacturer's instructions. The detection limit was 1.2 pg/mL for IL-10, 0.5 pg/mL for IL-13, 5 pg/mL for IL-5 and 2 pg/mL for IFN- γ .

Statistics

The Mann-Whitney test was used for statistical evaluation of the results of SPT, IgE, IgG4 and DBPCFC before and after 1 year between the two different patient groups. Non-parametric paired analysis (Wilcoxon's test) was performed to examine differences in the suppressive capacity of $CD4^+CD25^+$ T cells. The differences associated with P -values of less than 0.05 were considered significant.

Results

Evaluation of the clinical effect of IT with DBPCFC

All patients included in this study were challenged with fresh apple in a DBPCFC before the start of IT and after 1 year. All patients reacted during the initial challenges, confirming their apple allergy. At $t = 0$ no significant differences between both groups were found. Symptoms started within 5–10 min after ingestion of the challenge meal, and were recognized by the patients as the typical symptoms of OAS: itching or feeling of tightness in the oral cavity or tingling of the lips. Patient reactivity was expressed using VAS scores. After 1 year of IT, both groups were tested with DBPCFC. In the IT group VAS scores were significantly decreased at $t = 12$ ($P < 0.001$, Fig. 1) compared with those at the start of the study. This resulted in an increase of the amount of apple tolerated of a factor of 24.

Nine of the 13 patients treated with IT improved significantly, whereas four patients showed VAS scores similar to $t = 0$. In three of the nine patients who showed improvement, the provocation was completely negative after 1 year of birch-pollen IT, suggesting they had overcome their apple allergy.

In the control group, nine out of 10 patients showed unchanged or even increased VAS scores relative to the year

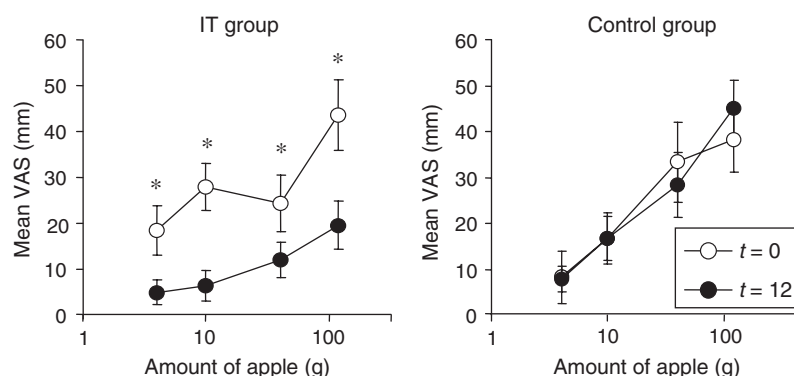


Fig. 1. Clinical effect of immunotherapy (IT) on apple allergy measured by visual analogue scale scores. Results are shown as mean values $\pm$ SEM for $n = 13$ and 9 patients treated with (left panel) or without (right panel) IT, respectively. * $P < 0.05$ (Mann-Whitney test).

before, and no significant differences were found between $t = 0$ and 12. One patient in the control group was not willing to undergo a challenge for the second time. No placebo reactions were observed in both groups.

SPT reactivity to birch pollen, rBet v 1, apple and rMal d 1 during IT

Patients were evaluated at 3 monthly intervals by SPT with birch-pollen extract, rBet v 1 and rMal d 1. For optimal accuracy, these tests were performed in serial dilutions. SPT to apple was performed at $t = 0$ and 12. Birch-pollen IT resulted in a significant decrease of SPT reactivity to rBet v 1 and birch pollen by a factor of 30 ($P < 0.001$) after 3 months of IT and drops further after 1 year IT by a factor of 38, which was not observed in the control group (Fig. 2). A 10-fold decrease ($P < 0.05$) was found in SPT reactivity to rMal d 1 after 3 months IT and drops further after 1 year by a factor of 20. (Figs 2e and f). Reactivity to fresh apple was also decreased significantly (Figs 2g and h).

Specific IgE and IgG4 reactivity to birch pollen, nBet v 1, apple and nMal d 1 during IT

Specific IgE concentrations for nBet v 1 and nMal d 1 increased already before start of the pollen season in the IT group. In both groups the IgE concentrations for birch pollen, nBet v 1, apple and nMal d 1 showed an increase during the pollen season ($t = 6$). After 1 year of IT, the IgE concentrations for all allergens were back to concentrations comparable with those at the start of IT in both groups (Figs 3a, c, e and g).

The concentration of IgG4 for birch pollen and nBet v 1 was strongly induced in the IT group after 3 months and remained high during the total study period of 1 year ($P < 0.05$, Figs 3b, d, f and h). The IgG4 concentration for apple and nMal d 1 was also significantly elevated after 3 months in the IT group as compared with the control group ($P < 0.05$). Together, these data indicate that IT leads to the induction of both cross-reactive IgE as well as IgG4.

Effect of IT on CD4⁺CD25⁺ Tregs

After 1 year of IT treatment no differences were observed in the percentage of CD4⁺CD25⁺ Tregs in peripheral blood between IT patients, control patients and healthy controls ($9.0 \pm 0.7\%$, $n = 15$ vs. $9.16 \pm 1.6\%$, $n = 10$ vs. $8.1 \pm 0.6\%$, $n = 6$, respectively). The phenotype of the CD4⁺CD25⁺ Tregs isolated from both patient groups was comparable with that of healthy controls for the expression of CD69, CD45RO and CD152 (CTLA-4) (data not shown).

CD4⁺CD25⁺ Tregs from both patient groups and from healthy controls displayed significantly lower proliferation than CD4⁺CD25⁻ T cells in response to anti-CD3 mAb stimulation or birch-pollen extract, confirming the anergic state of the Tregs (Figs 4a and b). When assessing the T cell-suppressive capacity of the CD4⁺CD25⁺ Tregs (in a 1 : 1 ratio with CD4⁺CD25⁻ T cells), significant suppression was observed when anti-CD3 mAb was used as a stimulus (mean % inhibition $56 \pm 16.4\%$, $58 \pm 14.5\%$ and $56 \pm 6.2\%$ for IT patients, control patients and healthy controls, respectively). However, in response to birch-pollen extract rBet v 1 or rMal

d 1 in the patient groups, no CD4⁺CD25⁺ Treg-mediated suppression was observed. Also, in the healthy controls no suppression of birch-pollen-specific proliferation was found. The lack of suppression appeared to be specifically related to birch-pollen-related allergens since in both patient groups and healthy controls significant suppression of a control antigen (cow's milk antigen) by CD4⁺CD25⁺ Tregs was observed (data not shown).

For the cytokine production, addition of CD4⁺CD25⁺ Tregs to CD4⁺CD25⁻ T cells diminished the IFN- γ production after anti-CD3 stimulation in the IT patients, control patients and healthy controls. However, as for the proliferation, the IL-13, IL-5 and IFN- γ production after allergen-specific stimulation was not affected by CD4⁺CD25⁺ Tregs in any of the groups. The amount of IL-10 measured in the supernatant of the culture system was very low (5–30 pg/mL) and showed no correlation with the inhibition of proliferation by CD4⁺CD25⁺ Tregs (data not shown).

Discussion

The current management of food allergy is based on the avoidance of the foods involved. Due to the cross-reactivity of the major birch-pollen allergen (Bet v 1) and many plant food allergens, patients are often allergic not only to birch, but also to various fruits, nuts and vegetables. Thus extensive elimination diets are not uncommon, which might have a social impact and may lead to vitamin deficiencies, when inadequately supervised. Therefore, there is a need for a curative treatment. This study focused at a possible role of birch-pollen IT as a curative treatment for cross-reactive food allergies.

Birch-pollen IT induced a very rapid and significant decrease in SPT reactivity for birch-pollen extract, rBet v 1 and also for apple and rMal d 1 after 3 months. Such a rapid decrease in SPT reactivity has been reported for other allergens (cat) as well [30]. At the same time, specific IgE concentrations for nBet v 1 and nMal d 1 increased, indicating the induction of a cross-reactive IgE response as a result of the IT. After 1 year IT, SPT reactivity was still inhibited to a similar extent as observed after 3 months. In contrast, IgE concentrations for all allergens were back to concentrations close to those at the start of IT, which has also been reported for other allergens [31, 32]. These observations illustrate that the overall positive correlation between specific IgE and skin reactivity is lost during IT. It is still unclear which mechanism underlies this simultaneous increase of specific IgE titres and inhibition of skin reactivity. Induction of specific IgG4 antibodies is a possible factor in the inhibition of SPT reactivity, because these antibodies were indeed observed after 3 months. Whether IgG4 antibodies may be considered as blocking antibodies or just as an index of altered T cell function remains to be determined. At least the early increase is in agreement with the literature on other allergens, such as grass pollen and insect venom [33, 34].

High specific IgE titres against house dust mite accompanied by poor skin reactivity was also reported by van den Biggelaar et al. [35] in helminth-infected subjects. The authors clearly showed that SPT reactivity was inversely correlated with helminth-induced IL-10. An explanation for the

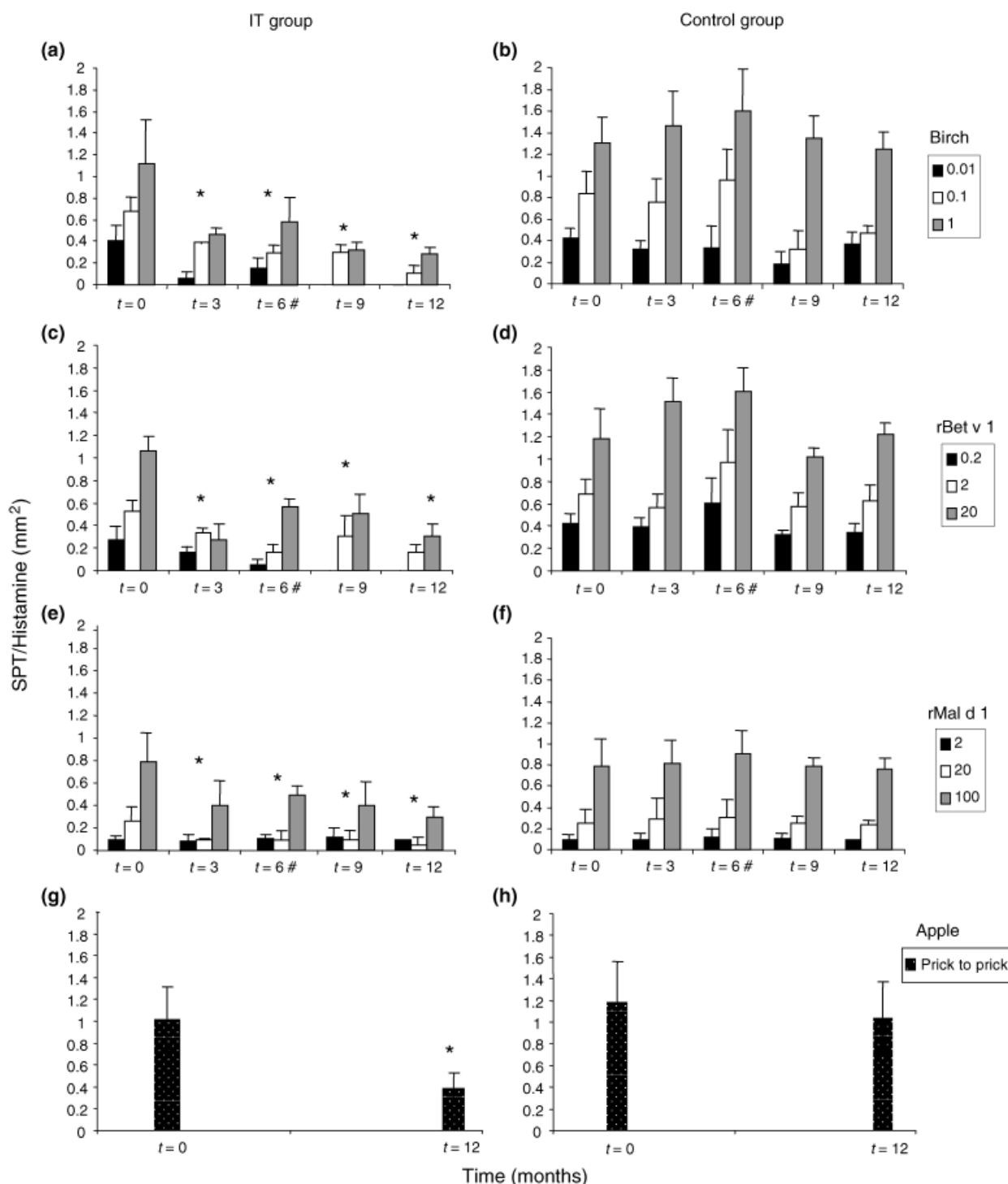


Fig. 2. Skin prick test (SPT) reactivity to birch pollen, rBet v 1, apple and rMal d 1 in patients with birch-pollen immunotherapy (IT) (a, c, e, g) and the control group (b, d, f, h). Results are shown as mean values $\pm$ SEM for $n = 13$ and 10 patients treated with and without IT, respectively. Significant differences compared with $t = 0$ are shown in the figures with an asterisk (*). Birch pollen season #.

unexpectedly low number of IgE-positive subjects with a concordant skin test, as observed during chronic helminth infections and allergen-specific IT, could be the result of direct inhibition of skin mast cells by IL-10 [36] or a decrease in the number of mast cells under the influence of this inflammatory cytokine [37].

The IgG4 response without IgE, induced by exposure to the antigen, has been called a 'modified Th2 response', as class

switching to IgG4, similar to switching to IgE, is IL-4 dependent [38]. This modified Th2 response is often associated with high concentrations of IL-10, which may induce Tregs. Evidence for the latter suggestion came from a mouse model where induction of tolerance led to IL-10-secreting dendritic cells and induction of Tregs [39], which suggests a key role for Tregs in the development of tolerance induction.

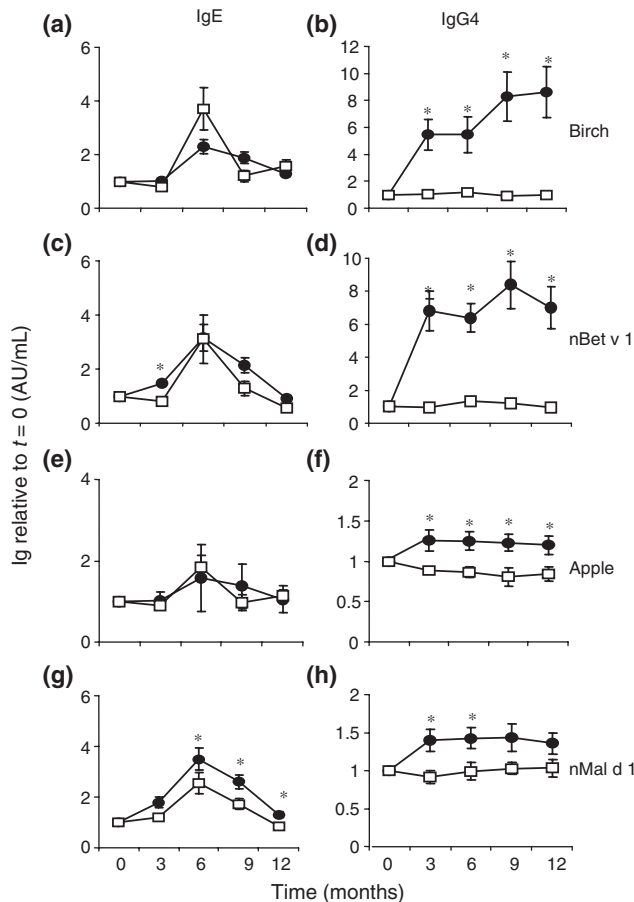


Fig. 3. Effect of birch-pollen immunotherapy (IT) on birch pollen, rBet v 1, apple and nMal d 1 specific IgE and IgG4 titres. IgE and IgG4 levels were measured via RAST and radioimmuno assay. Results are shown as mean values $\pm$ SEM for $n = 13$ and 10 patients treated with (IT, $\bullet$) and without (CON, $\square$) IT, respectively. * $P < 0.05$ (Mann-Whitney test), $t = 6$ pollen season.

Therefore, the effect of IT on the number and function of the regulatory $CD4^+CD25^+$ T cell population was examined in both patient groups. The data in this study show that their number was not enhanced in peripheral blood of patients receiving IT. For short-term birch-pollen IT it was shown that IT could modulate antigen-specific T cells at local sites (in the nose) but not in the periphery [40]. Therefore, although their presence in the local tissues (lung, nose, lymph nodes) was not investigated in this study, the $CD4^+CD25^+$ Tregs may be enhanced in their number at local sites as a result of IT. The proliferative T cell response to anti-CD3 mAb and cow's milk antigen was clearly suppressed by addition of the $CD4^+CD25^+$ Tregs in both patient groups. Suppression was not observed when crude birch-pollen extract or the recombinant allergens (rBet v 1 and rMal d 1) were used. Previously, we demonstrated that in cow's milk-allergic individuals and in healthy controls the cow's milk-specific T cell response can be suppressed by $CD4^+CD25^+$ Tregs [41]. The lack of suppression by $CD4^+CD25^+$ Tregs towards birch pollen observed in this study may reflect differences in either recognition or presentation of the allergen. These differences are not related to the allergic status of an individual, since suppression was also absent in the healthy controls. Akdis et al. [11] previously proposed that the $CD4^+CD25^+$ Tregs are the source of the produced IL-10 in a bee venom-specific IT trial. However, whether the suppression of an allergen-specific T cell response by $CD4^+CD25^+$ Tregs is mediated via IL-10 remains unclear [42]. Our data and the results from others do not demonstrate an increase of IL-10 production in culture supernatant after successful birch-pollen IT [43]. Still, analysis of the IL-10-producing capacity of cells with intracellular staining after IT could perhaps have resulted in higher values in the IT group, as this has recently been shown in a study with grass pollen IT [44]. Together, these findings indicate that birch-pollen IT did not result in an induction of the suppressive function of the $CD4^+CD25^+$ Tregs towards the T cell response towards

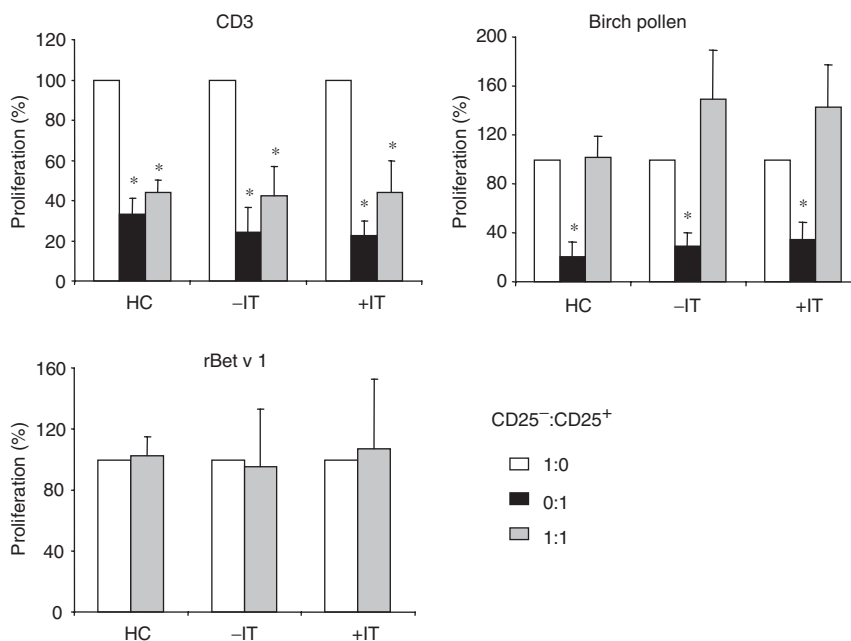


Fig. 4. $CD4^+CD25^+$ Tregs derived from birch-pollen-allergic donors and healthy controls are not suppressive after birch-pollen stimulation. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated from peripheral blood mononuclear cell from healthy controls (HC) and birch-pollen-allergic donors with immunotherapy (+IT) and without birch-pollen IT (-IT). $CD4^+CD25^-$ (1:0 ratio, white bars) and $CD4^+CD25^+$ T cells (0:1 ratio, black bars) were stimulated with irradiated autologous antigen-presenting cells and the indicated antigens (CD3, birch pollen, rBet v 1). $CD4^+CD25^-$ T cells were also cultured in presence of $CD4^+CD25^+$ T cells (1:1 ratio, grey bars) with the same antigens. The mean proliferation $\pm$ SEM for six independent experiments of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells in combination with $CD4^+CD25^-$ T cells is shown as percentages compared with the proliferation of the $CD4^+CD25^-$ T cell subset alone (set at 100%). Significant differences compared with the proliferation of the $CD4^+CD25^-$ T cells are shown in the figures with an asterisk (*).

birch pollen, rBet v 1 or rMal d 1 and was also not associated with higher concentrations of IL-10 in the supernatant.

So far, the limited number of studies investigating the clinical effects of pollen IT on food allergy were not optimally controlled and gave conflicting results [16–19].

For another inhalant-food allergen cross-reactive syndrome, namely mite-snail allergy, a harmful effect of IT has been described [45]. So possible negative effects of IT with inhalant allergens on the related food allergy cannot be excluded. This might be related to the allergen involved. In order to focus on the active molecules of this cross-reactive allergic syndrome, we chose to use purified natural and recombinant allergens as reagents. These molecules proved to be very sensitive and stable tools for measuring changes in humoral immune responses and in SPT reactivity. For assessment of clinical food allergy, the DBPCFC is generally regarded as the most reliable technique. Earlier studies were limited to recording clinical histories or open food challenges. Our study is the first clinical trial that has performed DBPCFCs to monitor clinical reactivity to apple. To avoid breakdown of the labile allergen Mal d 1, each active dose was prepared within 5 min prior to administration. This protocol facilitated the use of labile food allergens in DBPCFC. Of course real proof for the clinical effect has to come from a trial in which not only the evaluation is blinded as we did, but also the IT treatment is placebo controlled. Such study is now under preparation. Despite the lack of a placebo treatment group, the challenge data appear to support a clinical effect of the treatment. Challenge data of the control group did not significantly differ at $t=0$ and 12, indicating a good reproducibility of the method. A recent study performed by our group had already demonstrated that threshold dose in peanut- and hazelnut-allergic patients determined by DBPCFC do not significantly change over time [46].

In conclusion, this clinical trial provides support for earlier reports claiming that birch-pollen IT has a beneficial effect on cross-reactive food allergies. Furthermore, our study has established the use of recombinant allergens and DBPCFC as tools for monitoring cross-desensitization in future double-blind placebo-controlled studies.

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