

The effects of grass pollen allergoid immunotherapy on clinical and immunological parameters in children with allergic rhinitis

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Allergoid immunotherapy is a new form of allergen immunotherapy allowing safe administration of high allergen doses. There is limited information on the effects of allergoid immunotherapy in children with allergic rhinitis. To investigate the immunological and clinical effects of allergoid immunotherapy in children with allergic rhinitis due to grass pollen allergy. Children with allergic rhinitis were assigned to allergoid immunotherapy ($n = 27$) or control ($n = 26$, no immunotherapy) groups. Children in the immunotherapy group received seven injections of grass pollen allergoid immunotherapy before grass pollen season and continued to receive maintenance immunotherapy for 27 months. All patients were offered a pharmacotherapy regimen to be used on demand during the pollen seasons. Clinical and laboratory parameters were compared between the immunotherapy and control groups. The rhinoconjunctivitis symptom-medication score and asthma symptom score were lower in the immunotherapy group after 1 yr of maintenance immunotherapy ($p < 0.01$ for both). Skin test reactivity and nasal reactivity as determined by nasal provocation testing for grass pollen were significantly decreased after 1 yr of immunotherapy ($p < 0.001$ for both). The seasonal increase in bronchial reactivity and nasal lavage eosinophil cationic protein levels were prevented after the first year of immunotherapy ($p < 0.05$ for both). The seasonal increase in immunoglobulin (Ig)E decreased ($p < 0.05$) and grass-specific IgG, IgG₁ and IgG₄ increased significantly already at the end of the seven-injection build-up therapy ($p < 0.001$, for all). Interleukin (IL)-4 levels in the culture supernatants showed a steady decline from baseline at first and second year of immunotherapy ($p < 0.001$) but remained unchanged in the control group. Allergoid immunotherapy is an effective method in the treatment of grass pollen-induced allergic rhinitis in children and prevents the seasonal increase in bronchial hyper-reactivity. Changes in specific IgE and IgG levels and decreased IL-4 production in peripheral blood mononuclear cell culture supernatants may account for the observed clinical effects.

Key words: allergic rhinitis; allergoid immunotherapy; bronchial reactivity; children; grass; eosinophil cationic protein; interleukin-4

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Allergen-specific immunotherapy is an effective treatment for allergic rhinitis caused by pollen allergy (1–6) and has a profound potential to

change the natural course of the respiratory allergies (7). It is generally well appreciated that the clinical effectiveness can only be obtained

with the administration of high quality allergenic extracts at relatively high doses (8, 9). However, high-dose treatment also increases the risk of adverse events including anaphylaxis. Therefore, various approaches such as modifying the route of administration (10) or the dosage and schedule of application (4, 5), and using chemically modified allergens (allergoids) have been undertaken to achieve higher doses without increasing the risk of adverse events (11–14).

Despite its well-known clinical effects, the advancements in our knowledge of the clinical efficacy of specific immunotherapy have far exceeded our understanding of the immunological mechanisms underlying the clinical response. Moderate reductions in immunoglobulin (Ig)E and increases in allergen-specific IgG₄ (15); reductions in mucosal recruitment of eosinophils and basophils (16); decreased T-cell responsiveness to allergen (17); a Th2 to Th1 deviation (17); and induction of T cells expressing the IL-10⁺CD4⁺CD25⁺ phenotype (18) have been proposed as the underlying mechanisms of immunotherapy. Most of the information on the immunological mechanisms of immunotherapy has been derived from conventional immunotherapy studies and there are limited data on the effects of allergoid immunotherapy. A shift of the cytokine profile toward Th1 profile during treatment with ovalbumin (OVA) allergoid was shown in mice (19). In humans, effective allergoid pre-seasonal immunotherapy was associated with significant reductions in interleukin (IL)-5 and increases in interferon- γ (IFN)- γ production in nasal secretions (5).

One of the most promising aspects of immunotherapy is its potential to reduce bronchial hyper-reactivity and thus prevent the emergence of asthma in patients with allergic rhinitis (7). Similar to the immunological effects, almost the whole body of information in this field is derived from clinical studies of conventional immunotherapy (7).

As allergoid immunotherapy allows higher doses to be administered (11–14), there is reason to believe that the immunological and clinical effects of allergoid immunotherapy may start earlier or be more pronounced compared with conventional immunotherapy. Therefore, we studied the immunological and clinical effects of allergoid immunotherapy in a group of children with allergic rhinitis due exclusively to grass pollen allergy. For this purpose, in a prospective, controlled, open, parallel group study, we compared the efficacy of allergoid immunotherapy to pharmacotherapy with respect to the immunolo-

gical and clinical changes including bronchial hyper-reactivity.

Materials and methods

Patients

Children aged 6–18 yr who had symptoms of moderate–severe allergic rhinitis according to the allergic rhinitis and its impact on asthma (ARIA) guidelines (1) for the last two grass pollen seasons with a home location suitable for regular visits to the Hacettepe Children's Hospital, Ankara were invited to participate in the study. Children with allergen sensitivities other than grass pollen and children with perennial symptoms or systemic disease were excluded from the study. Of the 97 children thus contacted, 39 patients who accepted the invitation were recruited for their first evaluation in December 2002.

All patients were offered a choice of allergen-specific immunotherapy (2). Of these, 27 children desired to be in the immunotherapy group and the remaining 12 were assigned to the control pharmacotherapy-only group. Because of a smaller number of individuals in the control group, 18 additional children with moderate–severe allergic rhinitis were recruited into the control group the following year. Seven patients in the immunotherapy group and 10 patients in the control group reported to have had a wheezing episode and used a bronchodilator at least once before.

Immunotherapy group. Patients started to receive allergoid immunotherapy in February 2003 and continued to receive maintenance immunotherapy for a total duration of 27 months. The immunotherapy injections comprised a mixture of standardized grass pollen allergoid (Allergovit®; Allergopharma Joachim Ganzer KG, Hamburg, Germany) and included *Dactylis glomerata*, *Festuca elatior*, *Holcus lanatus*, *Phleum pratense*, *Poa pratensis* and *Lolium perenne*. The study compound Allergovit is an aluminum hydroxide-adsorbed depot allergen preparation produced by modification of the allergen by formaldehyde. The starting dose of immunotherapy was 100 therapeutic units (TU). During the build-up phase, the dose was doubled every week until a maintenance dose of 6000 TU was reached within 7 wk before the beginning of the pollen season. This maintenance dose was administered throughout the study except during grass pollen seasons when the maintenance dose was decreased to 4000 TU. The major allergen equivalent (Phleum group 5 allergens) in the last measurable production step (before allergoidization)

was 20 µg Phl p group 5 equivalent per maintenance dose. During the grass pollen season, patients were offered the same symptomatic drug treatment regimen as the control group to be used as needed (see below).

Control group. The patients in the control and immunotherapy groups were offered a pharmacotherapy regimen to be used on-demand during the pollen seasons as follows: oxymetazoline nasal spray for nasal blockage for a maximum of five consecutive days; loratadine for sneezing, runny nose and nasal itching; and olopatadine for symptoms of conjunctivitis. The patients were instructed to start nasal steroid treatment with budesonide nasal spray once or twice per day until alleviation of nasal blockage when the nasal blockage persisted despite decongestant treatment.

Study design

The study was performed as a prospective, controlled, open trial. The personnel performing the immunological and clinical tests were unaware of the treatment arm of the patients.

The time course of the study and the clinical and laboratory investigations performed are outlined in Fig. 1. The comparisons before the start of the immunotherapy included only eight patients in the control group. With the recruitment of new patients in the control group, this number increased to 26 the next year (Fig. 1).

Study measurements and procedures

Symptom and medication scoring. All patients kept a symptom and medication diary during the

pollen seasons for 2 consecutive years in 2003 and 2004. Nasal symptoms (itching, sneezing, discharge, and obstruction) and ocular symptoms (redness, itching, and lacrimation) were recorded separately on a scale of 4 with 0 being no symptoms and 3 most severe symptoms. A single rhinoconjunctivitis score was calculated from the summation of nasal and ocular symptoms. Pulmonary symptoms (coughing, wheezing, and dyspnea) were also graded on a scale of 4. The highest possible daily rhinoconjunctivitis score was 21 and pulmonary score was 9. Weekly scores were obtained by adding up daily scores for each given week.

For the medications, the patients received a score of 1 for every day that they had to use a rescue medication, i.e. 1 for antihistamine and 1 for decongestant. They received a score of 1 for every dose nasal corticosteroid they used.

Skin-prick testing. Skin-prick testing was performed on the child's upper back with a commercially available grass-mix allergen (Allergopharma Joachim Ganzer KG) and individual grass, tree and weed pollens, *Dermatophagoides Pyteronyssynius*, *Dermatophagoides Farinae*, cat, dog and mold antigens by the same nurse upon first presentation and then at 1 and 2 yr of immunotherapy. All skin-prick tests were carried out outside the pollen season and evaluated by the same physician blinded to the study procedures. Wheal reactions 3 mm greater than the negative control were considered positive.

Bronchial reactivity. Bronchial reactivity was measured using a standard methacholine (Mch) provocation test as previously described (7). Mch

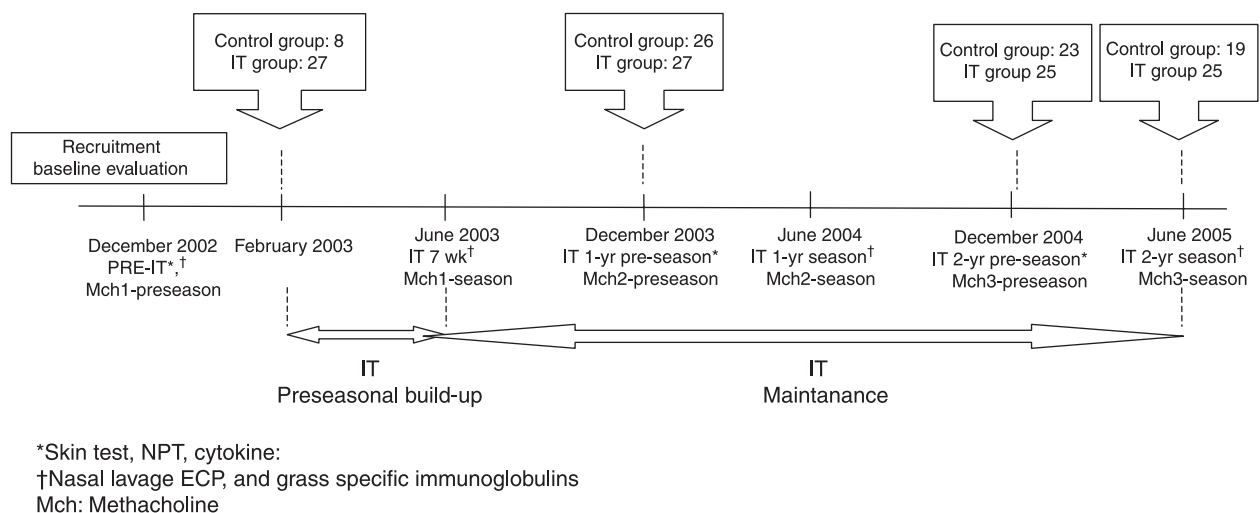


Fig. 1. Study design.

was inhaled in doubling concentrations starting with 0.06 mg/ml up to a maximum concentration of 8 mg/ml. The results were expressed as the concentration of the Mch solution causing a 20% fall in forced expiratory volume in 1 s (PC20). In the patients who had a negative response with 8 mg/ml, Mch PC20 was recorded as 16 mg/ml.

Titrated intranasal provocation testing. Specific nasal provocation test was performed according to Klimek et al. (5) using active anterior rhinomanometry using a nasal volume flow (cm^3/s) with a transnasal pressure difference of 150 Pa (V150) as target parameter (Rhinomanometer 2000; Allergopharma). After adaptation to the test surrounding, rhinomanometry was performed and symptom scores were evaluated to obtain baseline values. The allergen extract mixture used for nasal provocation testing consisted of the same antigens used for immunotherapy. Initial administration of the diluent was followed by the application of the allergen extract (Allergopharma) in increasing doses (100, 500, 1000, 5000, 10 000 SBU/ml). To do this, one puff of solution was instilled into the head of the inferior turbinate using a metered pump spray in both nostrils. The following scoring systems, previously described by Bousquet and coworkers (20), were used: more than five consecutive sneezes = 3; rhinorrhea = 1–3; nasal blocking = 1–3; itching of the nose = 1. The test was terminated when a positive response was obtained or when the highest antigen dose was reached. The test was considered positive when nasal flow decreased by 40% and/or symptom score reached a value of >5 . The result was expressed as the concentration of allergen extract producing a positive response.

Nasal lavage. Nasal lavage was obtained for the analysis of eosinophil cationic protein (ECP) as previously described (21). Briefly, subjects first exhaled forcefully through the nose to clear excess mucus. 0.1–0.3 ml of preservative-free pre-warmed (37°C) phosphate-buffered saline (150 mmol/l NaCl, 50 mmol/l NaH_2PO_4) was then instilled into each nostril sequentially. To collect the nasal washings, the fluid was allowed to decant passively into a sterile plastic tube containing 5 mmol/l ethylenediaminetetraacetic acid and 0.5 mmol/l phenylmethanesulfonyl fluoride solution (Fluka, Steinheim, Switzerland). During this procedure the tube was submerged to the rim in crushed ice. The procedure was repeated, in alternating nostrils, until a total of 3.0 ml of liquid was collected. The individual variation in the recovered vs. intro-

duced volume (the mean recovery of lavage fluid was $58 \pm 14\%$) prompted us to base our studies on constant recovered volumes rather than volume introduced. This method increases consistency of comparisons between individuals (21). The sample was centrifuged at 400 g for 10 min at 4°C . Three glass beads (diameter, 2 mm) were then put into the collected supernatant and vigorously vortexed for 10 min at maximal velocity so as to disrupt mucus clumps. The sample was then filtered through a 0.22- μm pore membrane and aliquots were stored at -80°C until they were assayed for ECP.

Cell preparation and culture. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density-gradient centrifugation on Histopaque 1077 (Sigma, St Louis, MO, USA) and cultured (1×10^6 cells/ml) in complete medium consisting of RPMI 1640 (Sigma) supplemented with penicillin, streptomycin (100 U/ml and 100 $\mu\text{g}/\text{ml}$ respectively) (Sigma), and 10% heat-inactivated fetal bovine serum (Sigma) at 37°C in 5% CO_2 humidified atmosphere in the presence or absence of grass mix protein (1 $\mu\text{g}/\text{ml}$). Supernatants were collected at 24 h for interleukin (IL)-4, IL-5, and IL-10 and at 72 h for interferon (IFN)- γ and transforming growth factor (TGF)- β and stored at -80°C until analysis.

Cytokine analysis. IL-4, IL-5, IL-10, IFN- γ , and TGF- β were analyzed in PBMC culture supernatants using commercial ELISA (Biosource, Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. The detection limit was 0.27 pg/ml for IL-4, 3.0 pg/ml for IL-5, 1 pg/ml for IL-10, 4 pg/ml for IFN- γ , and 15.6 pg/ml for TGF- β .

IgE, IgG, and ECP. Grass pollen-specific IgE (ImmunoCAP; Pharmacia, Uppsala, Sweden); grass pollen-specific IgG (Pharmacia); ECP (Pharmacia) were measured according to the instructions of the manufacturer. Grass pollen-specific IgG₁ and IgG₄ measurements were performed at Allergopharma Laboratories by personnel unaware of patients' treatment arm (Allergopharma).

Pollen counts. A Burkhard spore trap was used to collect pollen grains as previously described. All the study patients were living within 30 km of the spore trap.

Ethics. All study procedures were performed in accordance with a protocol previously approved

by the Ethics Committee of Hacettepe University. All parents provided written informed consent for the study procedures and the children gave assent.

Statistical analyses

Statistical analysis was performed with SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). The data were tested for assumptions of normalcy, and differences between the groups were compared using Mann–Whitney *U*-test. Multiple comparisons within the group were carried out using ANOVA on ranks for related samples, and pairwise comparisons within the group by Wilcoxon matched-pairs signed rank test. Bonferroni's correction was applied for the statistical significance of pair-wise comparisons. For symptom and medication scores, comparisons between the groups were carried out at weeks when grass pollen counts peaked in the atmosphere. Spearman's rank correlation was used for correlation studies.

Results

Of the initial 39 patients who were recruited for the study, 27 accepted immunotherapy and the remaining 12 preferred pharmacotherapy. Before the beginning of the pollen season, four patients in the control group withdrew their consent leaving eight patients in the control group. Therefore, comparisons for the early effects of the immunotherapy were carried out with only eight patients in the control group. The next year, 18 more patients were recruited into the control group and after this point the comparisons were made with 26 patients in the control group. Because of the drop-outs in both groups, 2-yr evaluations were performed with 25 patients in the immunotherapy group and 23 patients in the control group (Fig. 1).

There was no difference in the demographics and laboratory parameters between the two groups except that the intranasal provocation testing (NPT) dose was higher in the control group (Table 1). The values are given for 26 patients in the control group. The initial control group (in 2002) which is composed of eight patients had lower Mch PC20 [median (interquartile range)] values [2.9 mg/ml (1.6–13.0)] than the immunotherapy group [16 mg/ml (5.2–16.0), $p = 0.013$].

Reactions due to immunotherapy injections were generally local and mild. One patient had urticaria and one symptoms of rhinoconjunctivitis which responded to antihistamine; and one

Table 1. Characteristics of the study population

	Immunotherapy (n = 27)	Controls (n = 26)	p-value
Gender			
Male	17	14	>0.05*
Female	10	12	
Age (yr)	12 (10.5–15.5)	11.2 (8.8–14)	>0.05‡
Duration of rhinitis (yr)†	4.3 (3.0–5.5)	2.7 (2.2–4.0)	>0.05‡
Phleum-specific IgE (kU _A /l)†	45.2 (19.9–100.0)	23.3 (7.2–91.1)	>0.05‡
Phleum-specific IgG ₄ (μg/ml)†	0.35 (0.04–0.65)	0.24 (0.04–0.73)	>0.05‡
Phleum-specific IgG ₁ (μg/ml)†	0.04 (0.04–0.69)	0.04 (0.04–2.60)	>0.05‡
Wheal diameter (mm)†§	11.0 (9.0–16.0)	11.5 (8.0–16.6)	>0.05‡
Mch PC20†	16.0 (6.2–16.0)	11.7 (2.4–16.0)	>0.05‡
NPT dose (SBU/ml)†	500 (500, 1000)	1000 (1000, 5000)	<0.05‡

Mch, methacholine; NPT, intranasal provocation testing.

*By chi-square test.

†Median (interquartile range).

‡By Mann–Whitney *U*-test.

§Epidermal test with grass mix.

patient who had an anaphylactic reaction responded promptly to adrenalin administration.

The effect of immunotherapy on symptom and medication scores

Symptom and medication scores were compared between the immunotherapy and control groups. Comparisons were performed during the pollen season immediately after the build-up phase of immunotherapy is completed and at 1 yr of immunotherapy. During the second year of immunotherapy, in 2005 pollen season, the compliance of patients to keep daily records of the symptoms and medications have dropped significantly. Therefore, no comparisons were carried out for the second year of immunotherapy.

The difference in rhinoconjunctivitis symptom, rhinoconjunctivitis symptom-medication score and asthma symptom scores between the immunotherapy and control groups were not detectable after 7 wk of build-up treatment but became highly significant after 1 yr of maintenance immunotherapy ($p < 0.01$ for all of them) (Fig. 2a,b). The results of the medication scores were similar except that a significant effect became detectable already towards the end of the first pollen season immediately after the build-up phase ($p = 0.02$), and this effect increased further after 1 yr of immunotherapy ($p < 0.01$).

Skin reactivity

Skin testing for each group was performed in December of every year, i.e. outside the pollen

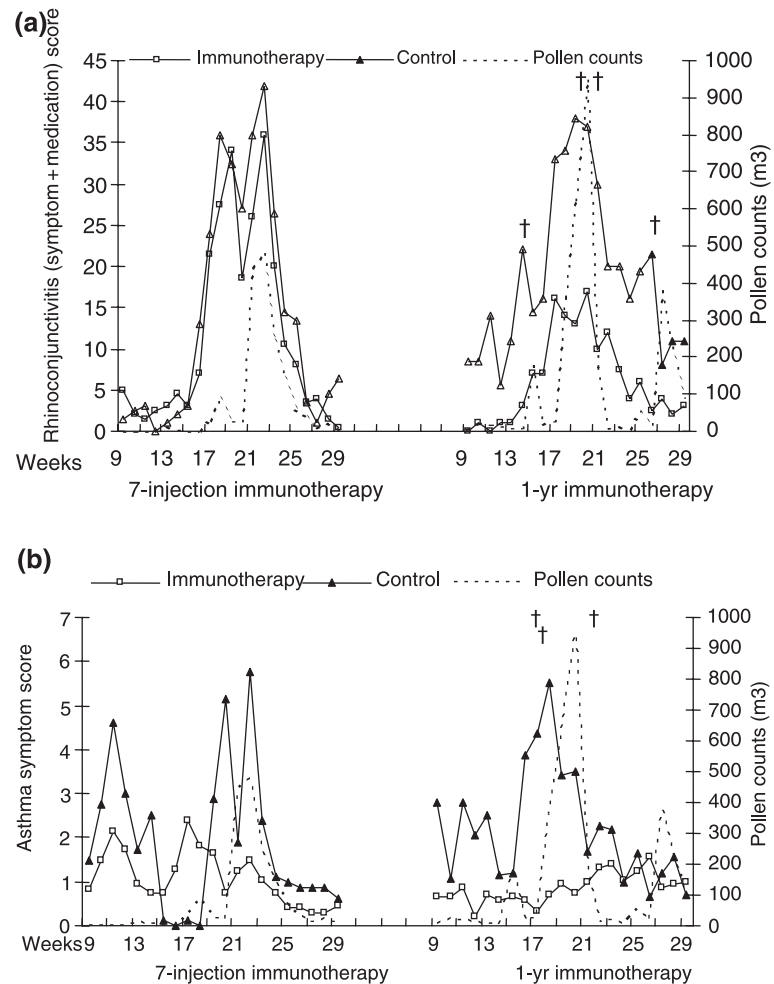


Fig. 2. Mean weekly: (a) rhinoconjunctivitis medication + symptom (combination) score; (b) asthma symptom score and mean weekly pollen counts during grass pollen season; p-value is for the comparison between the two groups ($p < 0.01$). Weeks are calculated with reference to the beginning of the year.

season. Comparison of the wheal diameter produced by the epidermal application of a grass pollen mix revealed that there was a significant difference in the wheal diameters among three visits in the immunotherapy group ($p < 0.001$, ANOVA on ranks). The wheal diameter was significantly smaller in the immunotherapy group after one year of treatment compared to the pre-immunotherapy values; 11 mm (9–16) at pre-immunotherapy vs. 8.5 mm (6–11) at 1 yr immunotherapy; $p < 0.001$, Wilcoxon with Bonferroni's correction) but not in the control group [10.5 mm (9.2–12.7) study entry vs. 12.5 mm (8.0–17.1) at 1 yr]. No further changes were observed in skin reactivity in either group at 2 yr (data not shown).

Bronchial reactivity

Mch PC20 determinations were carried out every year once before and once during the pollen season.

Seven injections of immunotherapy failed to prevent the seasonal decrease in Mch PC20 at the end of the build-up phase [16 mg/ml (5.2–16.0) pre-immunotherapy, preseason, in December 2002 vs. 5.3 (1.8–16.0) season in June 2003, $p = 0.005$, Wilcoxon]. At the first year of immunotherapy, however, the seasonal increase in bronchial reactivity was effectively prevented by immunotherapy (16.0 mg/ml (11.7–16.0) pre-season in December 2003 vs. 16.0 (7.7–16.0) season in June 2004, $p > 0.05$, Wilcoxon]. The preventive effect on the seasonal increase in bronchial reactivity continued into the second year of immunotherapy [16.0 mg/ml (6.2–16.0) pre-season in December 2004 vs. 16.0 (4.5–16.0) season in June 2005, $p > 0.05$, Wilcoxon].

In the control group, on the other hand, a consistent increase in bronchial reactivity was noted both at the first [Mch PC20 11.7 mg/ml (2.3–16.0) pre-season in December 2003 vs. 1.8 (0.6–10.0), season in June 2004 $p = 0.012$, Wilcoxon] and second year of the study [12.0 mg/ml (6.5–16.0) pre-season in December 2004 vs. 1.5

(0.8–16.0) season in June 2005 $p = 0.012$, Wilcoxon].

Titration NPT

Intranasal provocation testing was performed every year outside the pollen season and the results obtained after immunotherapy are compared with values obtained before the commencement of immunotherapy in December 2002.

Comparison of allergen concentration required to provoke a positive response revealed that there was a significant difference among three visits in the immunotherapy group ($p < 0.001$, ANOVA on ranks). The allergen concentration required to provoke a positive response was significantly higher after one year of immunotherapy [(5000 SBU/ml (1000–20 000)] compared with pre-immunotherapy [500 SBU/ml (500–1000), $p < 0.001$, Wilcoxon with Bonferroni's correction). This difference remained the same through the second year of immunotherapy (data not shown).

The allergen dose provoking a positive NPT response at the beginning of the study remained unchanged at the first and second year of the study (data not shown) in the control group.

ECP levels in nasal lavage fluid

Eosinophil cationic protein levels in nasal lavage were measured first in December 2002 to obtain baseline values. Afterwards, the measurement was done within the pollen season every year and the results were compared with baseline values.

Comparison of nasal lavage ECP levels revealed that there was a significant difference in the ECP levels among four visits in the immunotherapy group ($p = 0.003$, ANOVA on ranks). Seven injections of immunotherapy failed to prevent the increase in nasal lavage ECP levels observed during the pollination season at the end of the build-up phase [8.6 $\mu\text{g/ml}$ (2.8–25.3) baseline vs. 28.8 $\mu\text{g/ml}$ (4.8–84.1), season in June 2003 $p = 0.004$, Wilcoxon], but nasal ECP levels measured during the pollen season at 1 yr [6.7 $\mu\text{g/ml}$ (3.5–14.3), season in June 2004] and at 2 yr [8.5 $\mu\text{g/ml}$ (1.5, 18.1), season in June 2005] were not different from the baseline ($p > 0.05$ for both) showing that the seasonal increase in ECP is prevented by immunotherapy after 1 yr.

In the control group, on the other hand, nasal lavage ECP levels increased consistently from 4.8 $\mu\text{g/ml}$ (2.6–13.3) at baseline to 7.8 $\mu\text{g/ml}$ (5.2–31.3), season in June 2004, at 1 yr ($p =$

0.036 with Bonferroni's correction), and to 33.8 $\mu\text{g/ml}$ (7.4–77.8), season in June 2005, at 2 yr ($p = 0.012$ with Bonferroni's correction).

Grass-specific Ig

Grass-specific Ig levels were measured first in December 2002 to obtain baseline values. Afterwards, the measurement was done within the pollen season every year and the results are compared with baseline values.

Allergen-specific IgE

After seven injections of build-up phase of immunotherapy, the seasonal fold-increase in grass-specific IgE with respect to baseline was 1.87 (1.42–3.76) in the control group and 1.06 (1.00–1.48) in the immunotherapy group ($p = 0.003$ for the difference in the fold increase between the two groups) suggesting that the increase in the allergen-specific IgE was blocked by immunotherapy already after the build-up phase. In the control group, the grass-specific IgE values increased by 2.37-fold (1.16–2.88) and 1.02-fold (0.8–1.14) after 1 and 2 yr respectively. In the immunotherapy group, on the other hand, the ratios of grass-specific IgE to pre-immunotherapy values were 1.00 (0.78–1.69) at 1 yr and 0.74 (0.57–1.03) at 2 yr ($p = 0.008$ and $p = 0.012$ for the difference between the immunotherapy and control groups, respectively).

Allergen-specific IgG, IgG₁ and IgG₄

Results are summarized in Table 2. Grass-specific IgG, IgG₁ and IgG₄ increased very significantly at the end of the seven-injection build-up therapy in the immunotherapy group. A further increase was noted in IgG, IgG₁ and IgG₄ at 1 yr of immunotherapy. There was no increase in grass-specific IgG, IgG₁ and IgG₄ in the control group.

Allergen-specific IgE/IgG ratio

As expected grass-specific IgE/IgG ratio decreased in the immunotherapy group, while it increased in the control group during the study period (Fig. 3). There was a positive correlation between symptom-medication score and IgE/IgG ratio [$p = < 0.001$, $r = 0.6$] at the time of pollen peak.

Cytokine concentrations in PBMC culture supernatants

Cytokines were measured three times in the course of the study. All experiments were

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Table 2. Phleum-specific immunoglobulin (Ig)G, IgG₁ and IgG₄ results in the immunotherapy and control groups

	Baseline	Seven injection build-up	p-value†	1 yr immunotherapy	p-value‡	2 yr immunotherapy	p-value§
Phleum-specific IgG (mgA/l)*							
Immunotherapy	2.52 (1.9–3.4)	12.5 (7.7–21.0)	<0.001	14.30 (12.60–24.40)	0.001	16.40 (11.00–25.00)	>0.05
Control	2.66 (1.9–3.3)	2.63 (2.38–2.87)	>0.05	2.72 (1.90–3.43)	>0.05	2.16 (0.00–2.80)	>0.05
Phleum-specific IgG ₁ (μg/ml)*							
Immunotherapy	0.04 (0.04–0.69)	40 (25–120)	<0.001	97 (73–159)	0.006	NA	
Control	0.04 (0.04–0.04)	0.47 (0.04–3.97)	>0.05	0.04 (0.04–1.32)	>0.05		
Phleum-specific IgG ₄ (μg/ml)*							
Immunotherapy	0.35 (0.04–0.65)	24 (8–102)	<0.001	96 (45–246)	<0.001	NA	
Control	0.04 (0.04–0.34)	0.04 (0.04–0.49)	>0.05	0.04 (0.04–0.60)	>0.05		

For purposes of clarity, the time point in the control group corresponding to the seven injections, 1 yr immunotherapy, and 2 yr immunotherapy evaluations in the immunotherapy group are also given as seven-injection build-up, 1 yr immunotherapy, and 2 yr immunotherapy.

Not available.

*Median (interquartile range).

†Baseline vs. seven-injection build-up (by Wilcoxon with Bonferroni's correction).

‡Seven injection build-up vs. 1 yr immunotherapy (by Wilcoxon with Bonferroni's correction).

§1 yr immunotherapy vs 2 yr immunotherapy (by Wilcoxon with Bonferroni's correction).

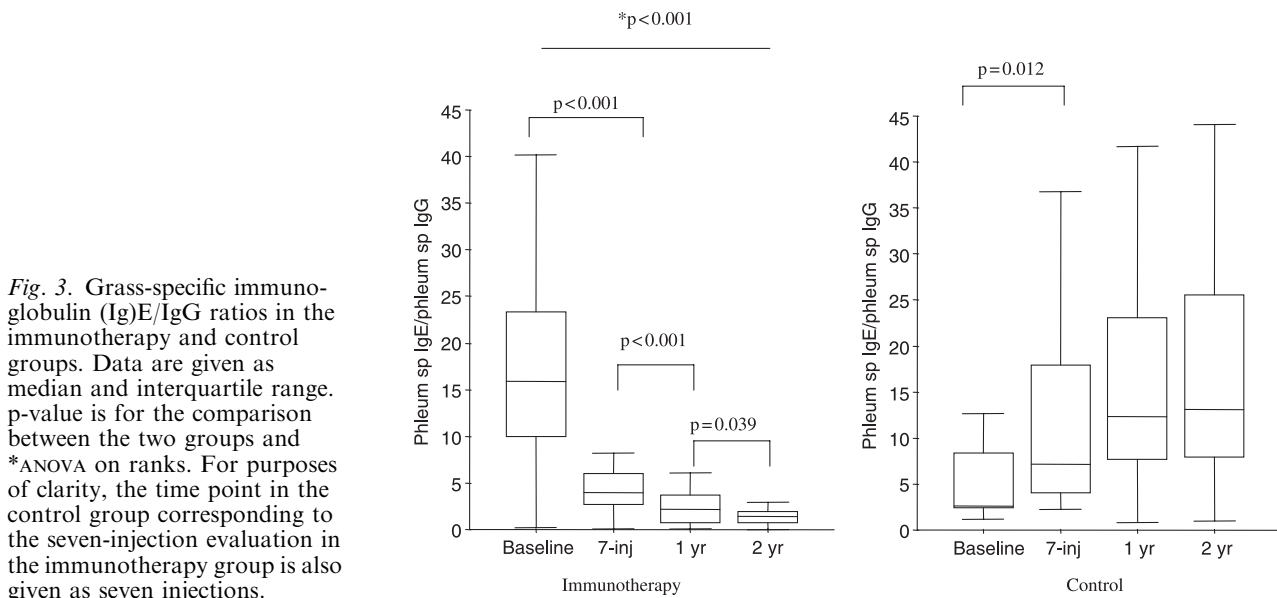


Fig. 3. Grass-specific immunoglobulin (Ig)E/IgG ratios in the immunotherapy and control groups. Data are given as median and interquartile range. p-value is for the comparison between the two groups and *ANOVA on ranks. For purposes of clarity, the time point in the control group corresponding to the seven-injection evaluation in the immunotherapy group is also given as seven injections.

performed outside the grass pollen season in the month of December every year. The results obtained after 1 and 2 yr of immunotherapy were compared with baseline values.

IL-4. Many of IL-4 measurements were below the detection limits of the assay used. However, the percentage of individuals with IL-4 levels below the detection limit (0.27 pg/ml) of the assay increased steadily from 29% to 74% at 1 yr and to 81% at 2 yr in the immunotherapy group. In the control group, this remained essentially unchanged with 88% of individuals having unmeasurable levels at baseline, 81% at 1 yr and 65% at 2 yr. Accordingly, when the median

levels were compared, IL-4 levels in the culture supernatants showed a steady decline from baseline at first and second year of immunotherapy but it remained essentially unchanged in the control group (Fig. 4a).

IFN-γ and IL-5. The amount of IFN-γ and IL-5 produced after antigen stimulation were in general below the detection limits of the assay used and failed to show any difference at one and two years of immunotherapy both in the study and control groups (data not shown).

IL-10 and TGF-β. Both IL-10 and TGF-β showed significant increases after one and two

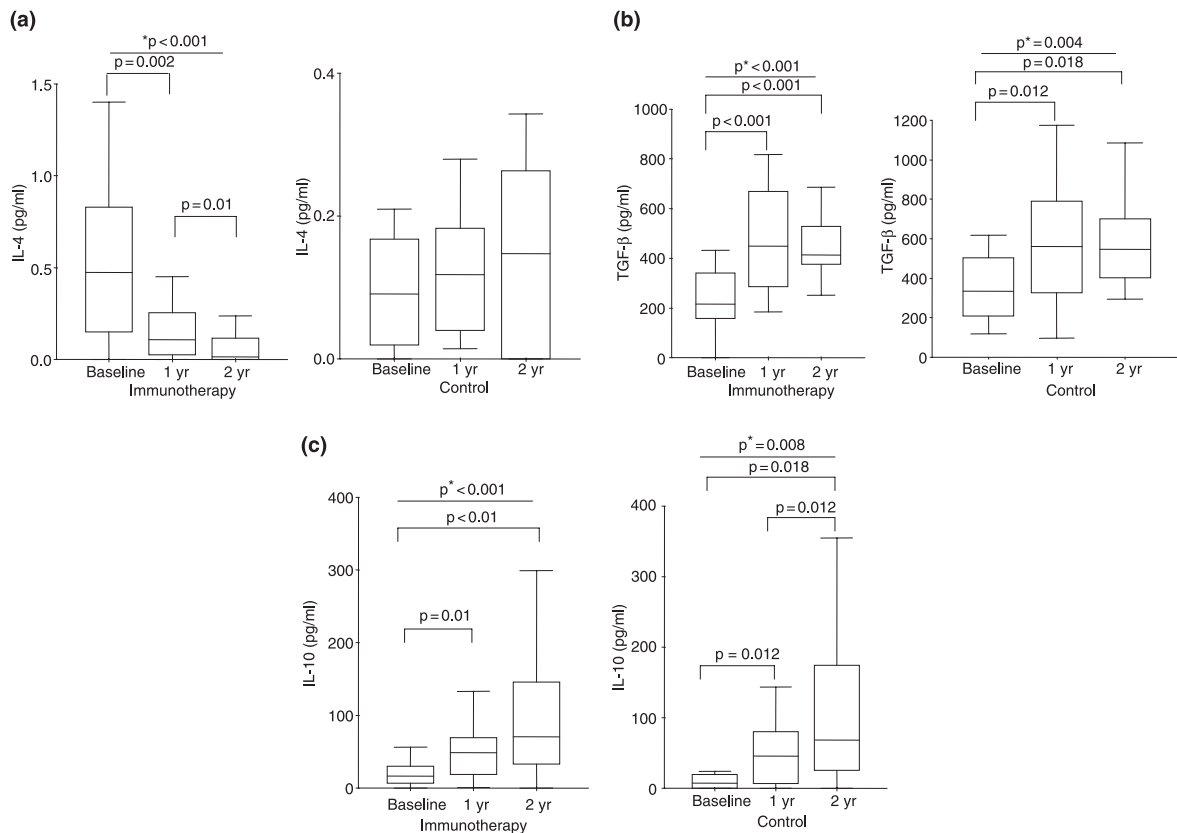


Fig. 4. (a) Interleukin (IL)-4; (b) transforming growth factor- β ; (c) IL-10 levels after grass pollen stimulation in peripheral blood mononuclear cell culture supernatants. p-value is for the comparison between the two groups and *ANOVA on ranks.

years of immunotherapy in the immunotherapy group. However, the same significant increase was also observed in the control group suggesting that the observed differences were not due to the effects of immunotherapy (Fig. 4b,c).

Discussion

Allergoid immunotherapy is a relatively new form of allergen immunotherapy allowing safe administration of high doses of allergen (4–6, 11–14). Even though immunotherapy is expected to be more efficacious when started at a younger age and early in the course of the disease (22), almost the whole body of the information concerning the efficacy of allergoid immunotherapy has been derived from studies on adults (4–6, 11–14) and there is very limited information on the effects of allergoid immunotherapy in children (23). Our study confirms the previous observations in adults that allergoid immunotherapy is an effective method of treatment also in children with allergic rhinitis due to grass pollen allergy and extends them further by showing that the allergoid immunotherapy results in a reduction in the

medication scores already after seven injections of pre-seasonal build-up allergoid immunotherapy.

In contrast to the early decrease in the medication score which was observed already after seven injections of build-up immunotherapy, the effect of allergoid immunotherapy on nasal symptom scores became evident only after 1 yr of maintenance immunotherapy, an effect similar to conventional immunotherapy (3). Even though the clinical effects appear at comparable times, the interval required to reach the monthly once maintenance dose is much shorter in allergoid immunotherapy compared with conventional immunotherapy (6). This may have significant implications not only with respect to the comfort of the individual patient but also to the economy of healthcare resources.

Similar to nasal symptom scores, the effect of allergoid immunotherapy on asthma-related findings became evident after 1 yr of allergoid immunotherapy. Our study allowed us to investigate the effect of immunotherapy on asthma in two different lanes. First, immunotherapy alleviated asthma symptoms after 1 yr suggesting that the allergoid immunotherapy can be used in children who already have asthma due to grass

pollen allergy supporting previous studies with conventional immunotherapy (7). Secondly, and maybe more importantly, the seasonal increase in bronchial hyper-reactivity was almost completely prevented in the immunotherapy group but not in the control group already after 1 yr. The potential for prevention of bronchial hyper-reactivity, and thus maybe asthma is a very exciting feature of immunotherapy. It has been reported for conventional immunotherapy (7) but has not been investigated for allergoid immunotherapy before. Rhinitis frequently precedes the onset of asthma (24) and patients with allergic rhinitis who also have bronchial hyper-reactivity are more likely to develop asthma (25). Therefore, intervention at early stages of disease and methods to produce early clinical effects would be highly desirable. In the biggest study to date, Möller et al. (7) have shown that the effect of immunotherapy on bronchial hyper-reactivity becomes significant after 2 yr of conventional immunotherapy. Our study, while supporting the results of the Preventive Allergy Treatment (PAT) study, also suggests that this effect may start earlier with allergoid immunotherapy and thus may confer a small advantage over the conventional method.

Our observations on the effect of immunotherapy on nasal hyper-reactivity are in keeping with bronchial hyper-reactivity and show that immunotherapy significantly increases the dose of allergen required to elicit a positive response. The first nasal provocation after baseline was performed after 1 yr and before the pollen season. However, since the improvement in nasal medication score was evident already after seven injections of build-up immunotherapy, it is also possible that improvement in nasal hyper-reactivity may have actually occurred earlier (5).

In addition to clinical parameters and clinical tests, our study provides some evidence about the immunological effects of immunotherapy both at the local (nasal) and systemic level. Locally, the prevention of the increase in nasal ECP levels paralleled the improvement in nasal symptom scores and was almost totally abolished after one year of immunotherapy. The prevention of increase in nasal ECP levels has been demonstrated for conventional (26) but not for allergoid immunotherapy previously. Our results suggest that allergoid immunotherapy is at least as effective as the conventional method in this regard.

Systemically, we observed highly significant changes in grass specific IgE, IgG, and its subclasses IgG₁ and IgG₄ that may be relevant with respect to the clinical effects. The seasonal

increase in IgE was significantly diminished while IgG and IgG₁ and IgG₄ dramatically increased in the immunotherapy but not in the control group. Immunotherapy with inhalant allergens is associated with increases in serum allergen-specific IgG₁, IgG₄ levels (4, 14). IgG antibodies have been proposed to act as blocking antibodies by competing with IgE for allergen binding to mast cells, basophils, and other IgE receptor-expressing cells (27, 28). IgG₄ induced by means of immunotherapy blocks allergen-induced IgE-dependent histamine release by basophils and suppresses allergen-specific T-cell responses *in vitro* by inhibiting binding of IgE-allergen complexes to antigen-presenting cells (27). There are contradictory reports on the role of IgG on the efficacy of the immunotherapy (27–29) and the issue whether there is a cause and effect relationship between the increase in IgG levels and the symptomatic improvement conferred by immunotherapy or if this is an indicator of exposure to the immunotherapy agent remains unresolved. However, if lower IgE and higher IgG concentrations are independently associated with symptomatic improvement, there is reason to believe that a measure taking both values into account may be more relevant with respect to the linkage between the immunological and clinical changes. In fact, the presence of a high and significant correlation between rhinoconjunctivitis symptom-medication scores and IgE/IgG ratio ($r = 0.6$, $p < 0.001$) showing that the IgE/IgG ratio accounts for 36% of the variance in symptom-medication scores may support this concept.

Another attractive hypothesis for the immunological basis of specific immunotherapy is its effects on the cytokine profile of the inflammatory cells. Decreased production of IL-4 has been documented in patients with a favorable clinical response to immunotherapy (17). Recent reports indicate a shift from a more Th2-like type of immune response to Th1 after successful allergen-specific immunotherapy (17). Fu et al. (30) showed that both IL-4 expressing CD4⁺ and CD8⁺ T cells significantly decreased after immunotherapy in the asthmatic children. In contrast, the frequency of IFN- γ expressing CD4⁺ and CD8⁺ T cells did not significantly differ between untreated and immunotherapy-treated groups in the same study. Accordingly, we also observed a significant decline in the IL-4 levels in the grass antigen stimulated PBMC culture supernatants. The fact that significant changes were observed for IL-4 and not for IFN- γ and that the change in IL-4 was confined to the immunotherapy and not the control group

suggests that the effect is cytokine specific and not merely a play of chance.

In this study, we also attempted to study the effect of immunotherapy on IL-10 and TGF- β , two cytokines that are generally believed to be predominantly of regulatory T-cell origin. Our study clearly shows an elevation of IL-10 and TGF- β production by PBMC after the first and second years of immunotherapy. Interestingly, however, we observed the same crescendo pattern of IL-10 and TGF- β production in the control group. Clearly, therefore, the increase in these regulatory cytokines cannot be attributed to immunotherapy itself. Our findings seem to contradict the results of some previous studies investigating the effect of immunotherapy on IL-10 production. Jutel et al. (31) have shown that T-cell suppression observed in the course of venom immunotherapy is induced by IL-10 and TGF- β . It was suggested that inhibition of IgE and enhancement IgG₄ production induced by increased IL-10 may actually be one of the main mechanisms underlying the clinical effects of immunotherapy. Similarly, Francis et al. (18) have shown that patients undergoing immunotherapy produced significantly more IL-10 than atopic control subjects. In yet another study (32), however, Savolainen et al. have shown that even though immunotherapy was associated with a decreased production of IL-10 after 1 yr, the clearest increase of IL-10 mRNA expression from baseline to 1 yr of the treatment was seen in patients who did not benefit from immunotherapy and there was no significant increase compared with the control group in the immunotherapy group with the greatest reduction of symptoms. Since age was reported to influence the IL-10 production by PBMC (33), it is possible that age may be one of the factors underlying the discrepant results and thus account for the different results of our study performed in children. Alternatively, the differences in the type of allergen and the type of immunotherapy (venom vs. pollen and conventional vs. allergoid) might be important in determination of the IL-10 response. However, precise factors underlying the discrepant results among these studies remain to be elucidated.

Our study has some important limitations including the lack of a placebo group, and non-randomized and open trial design. The injection of placebo is generally unacceptable by the parents in our population and was considered to be unethical. In addition, because these patients were considered to be good candidates for a positive response to immunotherapy, we felt that it would be inappropriate to withhold

immunotherapy from patients who desired to receive this therapy. Since many of the patients wanted to be in the immunotherapy group an imbalance in the number of patients between the two groups was created. Even though these flaws in the study design might have influenced our results and a placebo-effect that is observed in many immunotherapy studies (34) cannot be excluded with certainty, the consistency of laboratory and clinical investigations and the clear-cut differences observed between the immunotherapy and control groups makes the placebo effect to be a less likely explanation in our study.

To conclude, our study showed that allergoid immunotherapy is an effective method in the treatment of grass pollen-induced allergic rhinitis in children. In addition to its effects on the clinical outcome of allergic rhinitis it has a potential to prevent bronchial hyper-reactivity and thus maybe asthma in children with allergic rhinitis. Changes in specific IgE and IgG levels and decreased IL-4 production in PBMC culture supernatants may account for the clinical effect.

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