

Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome

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Summary

Background Although allergen immunotherapy has been established as a treatment of type I allergy back in 1911, until now the underlying mechanisms have not been fully understood, nor are there any parameters which would allow one to monitor an ongoing treatment or to assess therapeutic success in the meantime.

Objective We wanted to define allergen-specific parameters that change due to treatment in correlation with the clinical outcome.

Methods We conducted a controlled study with grass pollen-allergic children and compared allergen-specific antibody titres before and 1 year after the onset of immunotherapy in contrast with untreated allergic and healthy children. Two recombinant forms of the major allergen group V of *Phleum pratense* (Phl p 5) served as model allergens.

Results No change in IgE levels and no significant reduction of skin prick test (SPT) reactivity were seen. On the other hand, a significant reduction of symptom scores in the treated group and a significant rise in allergen-specific IgG1, IgG2 and IgG4 due to the treatment could be observed, but in neither case could we establish a correlation between the increasing amounts of the single antibody classes and the reduction of symptom scores. But most interestingly, when comparing the ratio of IgG4 to IgG1 with the symptom scores, we found significant correlations. Nevertheless, treated allergic patients still differ considerably from healthy controls as nonatopics have hardly any measurable allergen-specific IgG antibodies and no IgE antibodies at all.

Conclusion The ratio of IgG4 to IgG1 can serve as a valuable parameter that allows us to assess the success of immunotherapy already 1 year after the onset. The increase of specific IgG1 in relation to IgG4 during treatment reflects a possible influence of this subclass on the induction of tolerance towards allergens.

Keywords: allergen immunotherapy, IgG subclasses, grass pollen allergy, recombinant allergens, IgE

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Introduction

Allergen immunotherapy is a widely accepted treatment of type I allergy which has several times proven to be an effective measure to relieve patients from allergy symptoms. Although this form of therapy has been established in 1911 [1], until now the mechanism which leads to

desensitization is still not clarified. Evidence has been presented that allergen immunotherapy induces changes on the T-cell level, e.g. the induction of T suppressor cells [2], or changes in the cytokine pattern which indicates a switch from a more TH2-like type of immune response to TH1 after successful treatment [3–7]. Further mechanisms are discussed regarding the B cells. Here, a rising amount of allergen-specific IgG antibodies could 'block' allergens before they reach the mast cell-bound IgE and prevent degranulation [8,9]. In addition, IgG (especially IgG4)

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could play a role in co-crosslinking mast cell IgE (FcεRI, high-affinity receptor I for IgE) and IgG (FcγRIIb, low affinity receptor IIb for IgG) receptors, and could thereby inhibit the signal transduction of FcεRI leading to mediator release [10,11].

Despite being quite effective, allergen immunotherapy is expensive, might be dangerous and usually lasts for 3–5 years. There are no parameters which allow the monitoring of an ongoing treatment or the assessment of the success in the meantime. Until now, several allergen-specific changes during immunotherapy have been noted:

1. There is a significant rise in IgG4 antibodies, which correlates with a decrease in symptom score after 5 years of treatment [12]. IgG4 is also thought to have a protective effect in bee-keepers who are frequently stung but do not develop allergic reactions [13].
2. The IgG1 antibody titres rise at the beginning of treatment but tend to decline, whereas IgG4 remains at high levels [14–17].
3. There seems to be a correlation between IgG4/IgG1 and IgE which becomes evident after more than 2 years of treatment [12,18].

Most of these studies measured antibody titres against crude extract, which contains a lot of antigenic proteins besides the relevant allergens. Therefore in our study, we determined the changes in all allergen-specific antibody classes directed against purified recombinant forms of a major allergen group from timothy grass (*Phleum pratense*, Phl p 5). Group V allergens belong to the major allergens of grass pollen, recognized by more than 50% of sensitized patients [19]. Timothy grass pollen allergens (*Ph. pratense*) were used as model proteins. The group V allergens are represented by two subgroups, Phl p 5a and 5b, which differ significantly in their protein sequences. In a controlled clinical study sera were collected from patients with grass pollen-allergic disease before and 1 year after the onset of an allergen immunotherapy. Antibody titres were determined and changes were then correlated to the symptom score to work out parameters that might be responsible for declining allergic symptoms in the patients and which would allow to assess the outcome of the treatment.

Material and methods

Isolation, expression and purification of recombinant Phl p 5

The isolation, expression and purification of the recombinant Phl p 5 isoallergens has been described elsewhere in detail [20].

Briefly, the isoforms were isolated from a Timothy grass pollen cDNA library (complementary DNA) by low stringency hybridization with an N-terminal fragment of Phl p

5b serving as probe. The isolated clones were analysed by restriction analysis and sequencing. Clones which contained recombinant group V allergens from timothy grass were subcloned into the pMalc2 expression vector (New England Biolabs, Beverly, MA, USA), using maltose-binding protein (MBP) as fusion partner for the allergen. Proteins were expressed after induction with IPTG (isopropyl thiogalactoside). The fusion proteins were separated from total protein after cell lysis with lysozyme by affinity chromatography using an amylose resin column. MBP was cleaved from the allergen by incubation of the fusion proteins with the protease factor Xa (New England Biolabs) and separated by DEAE ion exchange chromatography.

Patients

Twenty patients, 12 boys and eight girls, who were selected from an outpatient population of originally 25, finished the study (see Table 1). For comparison of antibody titres, a group of 10 healthy controls was included. Patients were selected according to a history of severe summer hay fever and poor symptom control in previous seasons despite regular anti-allergic treatment with antihistamines and sodium cromoglycates. Inclusion criteria were doctors diagnosis of seasonal symptoms in terms of rhinoconjunctivitis, allergic asthma or both. The diagnosis of asthma was given according to history, clinical assessment and lung function tests. Sensitization towards grass pollen allergens measured by skin prick test (SPT) was mandatory. Patients with additional SPT reactivities to three or more other allergen groups (i.e. animal dander, mites, moulds), with chronic asthma, with previous immunotherapy or other systemic diseases such as diabetes, leukaemia, immunodeficiency, etc. were excluded from the study.

Study design

After selection patients, or parents of the patients, gave written informed consent. The study was approved by the ethical committee of the Ärztekammer Schleswig Holstein in Northern Germany. We used a non-placebo randomized controlled study design, covering seven centres. Treatment of the patients started in September 1995 and the study was continued over 1 year until December 1996. All selected patients were randomly divided into two groups: the treatment group with immunotherapy ($n = 11$) and the non-treatment group without immunotherapy ($n = 9$). Before and 1 year after the onset of the treatment, a titrated prick test was performed and blood samples were drawn. After the first test, the first group started standard subcutaneous immunotherapy. Treatment was carried out according to German Guidelines as published by the Deutsche Gesellschaft für Allergie und Immunitätsforschung

(DGAI, München, Germany). All patients were treated with extracts of the same company (Novo-Helisen DepotTM, Allergopharma, Reinbek, Germany). The induction phase was performed with once per week injections of depot-preparation over 8–11 weeks, increasing to a maintenance treatment with 1 ml of extract (5000 TE units/mL). Adjustments in the schedule were made on an individual basis. If patients developed a delayed local reaction greater than 10×10 cm, the same dose was repeated at the next attendance. Injections were postponed if the patients were unwell. Maintenance injections were reduced by 80% during the pollen season. The maintenance dose was given once per month.

Skin prick test

Titration SPTs were performed according to the standards of clinical diagnosis of allergies [21]. SPT solutions were purchased from Allergopharma (Reinbek, Germany). They were performed with three different concentrations of Timothy grass pollen extract containing 50, 500 and 5000 BU/mL; histamine dihydrochloride solutions at 1% and 0.1% and a 0.9% sodium chloride solution served as controls. All solutions contained 50% glycerol and 0.4% phenol as preservatives. They were tested in duplicate on the volar surface of the forearms. After 10 min two diameters of the wheal reactions were measured. The area was calculated in mm² ((medium diameter/2)² × (π)). Values of histamine and

Timothy grass pollen reactions were correlated to the concentrations of solutions used and regressions were determined. The angle (here called angle-α) between the two slopes (histamine and allergen) of one SPT was taken as a marker for the individual reactivity of one patient. The difference between angle-α before and after the treatment phase served as a relative value for a change in SPT reactivity: a decreasing sensitivity towards allergens led to a declining inclination of the regression curve of timothy grass, resulting in a growing angle-α.

Symptom scores

During the course of the study allergic symptoms of all study subjects were measured in three different ways. Scores were calculated according to Walker *et al.* [22]:

1. Analogue score: Once every 3 months the patients were asked about the development of their hay fever or asthma during this period. The values ranged from -3 (much worse) to +3 (much better). Later on these values were transformed to numbers between 0 and 3 (+3 = 0, +2 and +1 = 1, 0 and -1 = 2 and -3 = 3).
2. Questionnaire: Every patient kept a diary to take down daily symptoms during the pollen season affecting lung (breathlessness, cough, wheeze, tightness), nose (sneezing, blocking, running, bleeding), eyes (itching, redness, streaming, swelling), mouth and throat (itching, drying). The values ranged from 0 (no symptoms) to 3 (very

Table 1. Comparison of age, sex, diagnosis and serum parameters of the three study groups.

	Group 1: healthy controls (n=10)	Group 2: allergics (untreated) (n=9)	Group 3: allergics (treated) (n=11)	P value
Age (mean ± SD)	10.2 ± 3.91	10.22 ± 3.01	10.82 ± 4.76	0.85
Sex (male: female)	8/2	4/5	8/3	0.71* 0.21†
Diagnosis				
Rhinoconjunctivitis	0	1	4	
Asthma	0	3	1	
Rhinoconjunctivitis and asthma	0	5	6	
Serum parameters				
CAP-total IgE (IU/mL) (mean ± SD)	76.9 ± 47.7	421.9 ± 334.7	552.6 ± 572.4	0.005* 0.74†
CAP Timothy grass pollen-specific IgE (% of standard)	2.1 ± 6.1	57.1 ± 38.2	62.7 ± 38.5	0.001* 0.55†

*P for group 1 vs group 3; †P for group 2 vs group 3.

severe symptoms). Additionally the symptoms were multiplied by the time the complaints lasted (1 = less than 1 week, 2 = 1–4 weeks, 3 = more than 4 weeks). Daily medication was also written down and the score-value was calculated including the medication: 0 = no medication; 1 = sodium cromoglycate, locally if needed; 2 = sodium cromoglycate permanently or antihistamine if needed; and 3 = antihistamine permanently and inhaled β 2-mimetics or local and inhaled cortisone permanently. This estimation was transformed to values between 0 and 3.

3. Visual Analogue Score: Once in every 4 weeks patients recorded the degree of their complaints on an open scale ranging from 'no symptoms' to 'severe symptoms'. Afterwards we transformed these values to scores ranging from 0 to 3.

All scores for each patient were added up and the mean served as total symptom score for the pollen season.

CAP tests

Blood samples were collected before the beginning of the treatment time and 1 year later. Sera were kept frozen until the end of the study and then tested altogether in one experiment for each antibody class. The measurements of Timothy grass pollen-specific IgE and IgG4 and of total IgE were done at Pharmacias (Pharmacia & Upjohn, Freiburg, Germany). Patients' antibodies in the sera were detected by CAP-RAST-FEIA (fluorescence immunoassay). For the quantification of specific IgG4 and IgE control sera served as standards. Total IgE was quantified as units/ml.

Measurement of group V-specific antibodies by ELISA

For ELISA 5 μ g/ml purified recombinant allergen (= 1 μ g/well) were coated onto microtiter plates (MaxiSorb, Nunc, Roskilde, Denmark) over night. Plates were blocked with 5% defatted milk in NaCl/Tris, pH 7.4 for 2 h, followed by incubation with patients' sera (diluted 1:3 in 2% defatted milk in TBS, pH 7.4) for 2 h at 37°C. After washing with 0.05% Tween in NaCl/Tris pH 7.4, the plates were incubated with the respective antibodies for 2 h at 37°C. The antibodies were either alkaline phosphatase-coupled (AP) or were incubated with a second alkaline phosphatase-conjugated antimouse IgG antibody for additional 2 h at 37°C. The dilutions were: 1:2000 for antihuman IgE (Allergopharma, Reinbek, Germany), AP-antihuman IgG1, antihuman IgG2, AP-antihuman IgG3, α -human IgA and antihuman IgM (Dianova, Hamburg, Germany) and 1:5000 for antihuman IgG Fc, antihuman IgG4 and AP-anti-mouse IgG (Dianova, Hamburg, Germany). After washing, bound conjugate was detected using 4-nitrophenyl phosphate substrate (Merck, Darmstadt, Germany). Reaction was

measured at 405 nm on an ELISA reader (MR 7000, Dynatech, Billingham, UK).

Statistical analysis

Statistical analysis was carried out using the statistical program SPSS (SPSS GmbH Software, Munich, Germany). Paired data were analysed by Wilcoxon's two-tailed test, unpaired data by the Mann-Whitney *U*-test. Correlations were carried out according to the Spearman's rank correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

Figures show the interquartiles, whisker caps indicate the 10 and 90%iles, and lines represent the medians of the data.

Results

Protein sequences of the group V isoforms

Grass pollen extracts used for diagnosis and therapy of allergic diseases consist of a number of different proteins of which only a small part are known to be allergens. To assure that only allergen-specific antibodies were detected we used highly purified recombinant allergens for the ELISA assays. The amino acid sequences of the two Phl p 5 isoforms we used in this study are shown in Fig. 1. The upper sequence belongs to subgroup Phl p 5a, the lower one to Phl p 5b. The sequence identity is 73.6%. As shown in earlier studies [18] the two subgroups represent the most important molecules of grass group V allergens.

Patients

Twenty of originally 25 allergic patients completed the study. One patient from the untreated group was excluded during the first weeks because he moved away. Two patients from the untreated and two from the treated group withdrew for unknown reasons. The composition of the control and the actual study groups is shown in Table 1. Age, sex, diagnosis and serum IgE (total and Timothy grass pollen-specific) are given. As the data show, the two allergic groups do only differ insignificantly in their basic values and can therefore be compared with each other in all following investigations.

Symptom scores

Figure 2 shows the symptom scores during the grass pollen season 1996. The scores of the patients in the treated group were significantly reduced compared to those in the untreated group ($P = 0.02$). No patient in the healthy control group had allergic symptoms (data not shown).

Prick tests

Titration SPT reactivity towards specific allergens in some but not all immunotherapy studies has been shown to be reduced in the course of treatment. In our study after 1 year of treatment, the susceptibility of the patients to Timothy grass pollen extracts was slightly but not significantly reduced compared to the untreated group (data not shown).

Timothy grass pollen-specific IgE and IgG4, measured by CAP test

Regarding the allergen-specific IgE, the allergic patients had significantly more antibodies compared to the normal controls ($P < 0.001$ for both groups). Between the allergic groups there were no differences, neither before the onset of immunotherapy nor 1 year later (Fig. 3). It shows that the IgE level is not influenced during the first year of treatment.

Allergen-specific IgG4 usually increases during immunotherapy. In our case the antibody titres of allergen-specific IgG4 before treatment were significantly higher in the allergic groups compared to the healthy controls ($P < 0.05$ for each group). No differences were found between the allergic groups. One year later, the difference between the normal controls and the untreated allergic patients was no longer detectable, but the IgG4 titres of the treated group had increased significantly compared to the two control groups ($P < 0.001$ for both groups), Fig. 4. This clearly documents that the patients in the therapy group were actually responding to the treatment.

Specific IgG antibodies against Phl p 5 allergens, measured by ELISA

In accordance with the results obtained by CAP-test for Timothy grass pollen-specific IgG4, the titres of IgG1, IgG2 and IgG4 directed against the two Phl p 5 allergens raised significantly in the group of treated patients, compared to the values measured before the beginning of immunotherapy, which was also reflected by the rising titres of total IgG (Fig. 5). In opposition to this, the total IgG titres of the untreated patients showed a tendency to decline, which was significant for both allergens (data not shown).

Neither in the healthy, nor in the allergic control group, were there detectable levels of IgG1 or IgG2 at any time, and only small quantities of Phl p 5 specific IgG4 antibodies were present in the untreated allergic group.

In addition, the level of Phl p 5b-specific IgG was measured in the healthy control group. In either subclass they had significantly less IgG compared with the allergics at the beginning of the study (Fig. 6). Taken together, during immunotherapy one can observe a general increase of IgG antibodies in grass pollen-allergic patients. Without treatment the subclasses are rarely detectable. Interestingly, allergic patients have a significantly higher IgG titre compared to the healthy controls.

Correlation of IgG1 and IgG4 with symptom scores

It is well established that allergen-specific IgG1 and IgG4 antibodies arise from immunotherapy, but none of the previous studies could prove substantially that these parameters are linked to the outcome of the treatment. In our

Phl p 5a	ADLGYPATPAAPAAGYTPATPAAPAGAEPACKATTEEQKLIKINAGFKAA	52
Phl p 5bA.....-A.....D.V.....	38
Phl p 5a	LAAAAGVPPADKYRTFVATFGAASNKAFEAELSGEPKGAESSKAA-LTSK	103
Phl p 5b	V...S..A...FK..E.A.-TS.S.....AT.....PG.VP.	75
Phl p 5a	LDAAYKLAYKTAEGATPEAKYDAYVATVSEALRIIAGTLEVHAVKPAEE--	155
Phl p 5bSV...A.V.....F.SF..SLT...V...A.....VT..PG	127
Phl p 5a	-VKVIPAGELQVIEKVDAAFKVAATAANA-APANDKFTVFEAAFNDIAKAST	203
Phl p 5b	MA.-.....I.D.I.....-T...D.....K...E..	177
Phl p 5a	GGAYESYKFI PALEAAVKQAYAATVATAPEVKYTVFETALKKAITAMSEAQK	255
Phl p 5b	...DT..C..S.....A..Q...A...A..T.....V..	229
Phl p 5a	AAKPAA-AATATA---TAAVGAATGAATAATGGYKV	287 (Phl p 5.0103)
Phl p 5b	VSQ..TG...VA.GAA.T.A...S...V.A.....	265 (Phl p 5.0204)

Fig. 1. Alignment of the deduced amino acid sequences of Phl p 5a and Phl p 5b. The dots indicate identical amino acids, lines indicate gaps and capital letters in the Phl p 5b sequence mark amino acid substitutions. The allergen names according to the official nomenclature are given in brackets at the end of the sequences.

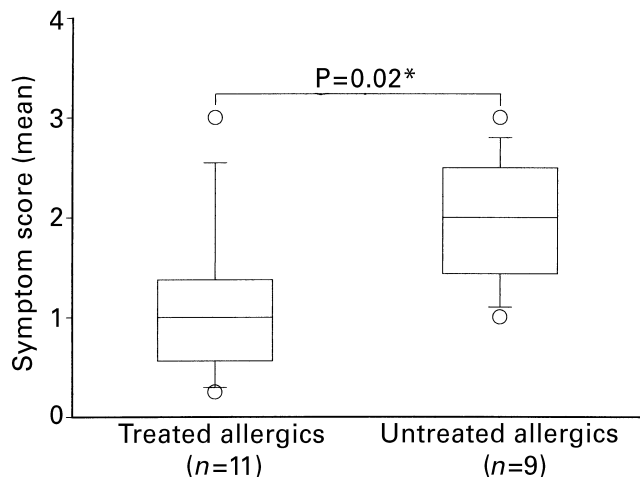


Fig. 2. Symptom scores of the treated and untreated allergic patients in the pollen season 1 year after the onset of allergen immunotherapy with grass pollen extracts. The symptom scores range from 0 (no symptoms) to 3 (severe symptoms).

study IgG1 and IgG4 also rose significantly. When related to the symptoms, IgG1 showed a tendency to an inverse correlation and IgG to a positive correlation with the symptom scores, but neither correlation was significant (data not shown). Therefore we introduced a ratio of IgG4 to IgG1 which is calculated by division of the two parameters measured 1 year after the onset of immunotherapy. The resulting quotient was correlated to the symptom score of each patient and each of the two isoforms (Fig. 7). The correlations were significant in any case ($r^2 = 0.82$, $P = 0.002$ for Phl p 5a; $r^2 = 0.78$, $P = 0.005$ for Phl p 5b).

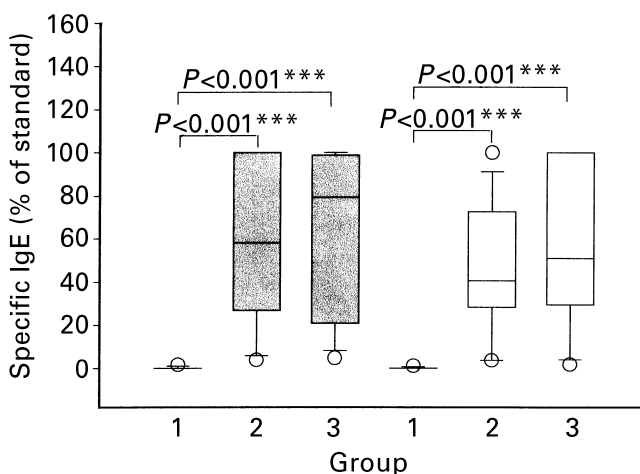


Fig. 3. Comparison of Timothy grass pollen-specific IgE, measured by CAP-test, for the three study groups. 1 = healthy controls, 2 = untreated allergics, 3 = treated allergic patients. Filled bars indicate the values before therapy, open bars indicate values obtained 1 year after the onset of therapy.

This shows that the increase of IgG1 in relation to an increase of IgG4 is a diagnostic marker for the success of allergen immunotherapy. It also demonstrates the significance of group V allergens in this treatment, since the correlation between the change of subclasses and the symptoms of patients can easily be analysed with the two isoforms of Phl p 5.

Discussion

Allergen immunotherapy can act on different levels and has been shown to reduce IgE production [23]. We asked to what extent these effects may be due to the generation of new allergen-specific antibody isotypes. In this paper we show that after 1 year of grass pollen immunotherapy the levels of group V-specific IgG1, IgG2 and IgG4 had risen significantly, whereas the levels of IgE remained unchanged.

In agreement with Hasegawa *et al.* [24] we couldn't find any influence of immunotherapy on IgE levels. This is in contrast to other studies [25–27], which show a rise of IgE at beginning of immunotherapy followed by a slow decline. Possibly, changes could have been observed, if we had looked after 2 or more years of treatment.

Some studies report the occurrence of new IgE specificities during the treatment [28,29]. In our study there was only one grass pollen-allergic patient who had no detectable IgE against group V allergens at the beginning, and this fact was still unchanged 1 year later. This means that even the application of allergens via immunotherapy does not necessarily lead to new sensitizations.

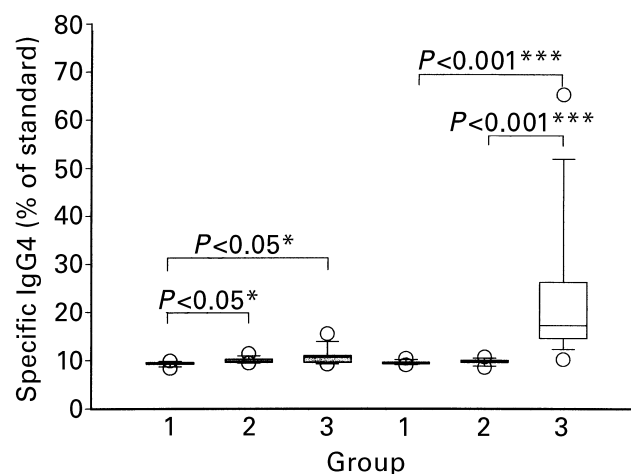


Fig. 4. Comparison of Timothy grass pollen-specific IgG4, measured by CAP-test, for the three study groups. 1 = healthy controls, 2 = untreated allergics, 3 = treated allergic patients. Filled bars indicate the values before therapy, open bars indicate values obtained 1 year after the onset of therapy.

Furthermore, it must be pointed out, that the above mentioned studies could not prove whether the new IgE idiotypes resulted from the injections or from natural exposure. But besides these data, the predictive value of specific IgE amounts in serum is questionable. The patients in our study showed decreasing symptoms despite unchanged IgE titres. Therefore it has to be pointed out that the existence of certain levels of specific IgE does not provide any information about the actual condition of a patient.

In contrast with IgE, IgG1 and IgG4 titres seem to be closely linked to clinical improvement in patients under immunotherapy. The mechanism that leads to the production of specific IgG1 and IgG4 during immunotherapy is still not clarified, as both antibody subclasses are regulated by opposing factors, that strongly exclude each other under *in vitro* conditions. The balance of TH1 and TH2 cytokines

seems to be crucial for the isotype switch in B cells. IgG4 and IgE are produced in the presence of TH2 cytokines like IL-4 and IL-13 and downregulated by IFN- γ and IL-2 [30–33]. IgG1 expression on the other hand is promoted by IFN- γ [34]. As we observed simultaneous increases in IgG1 and IgG4 and no change in IgE, a cytokine milieu shifting from TH2 to TH1 under the influence of immunotherapy can not be the only explanation for these changes in antibody production. Nevertheless, the fact that IgG1 and IgG4 rise together during immunotherapy has been described by many authors. Usually IgG1 rises first, followed by IgG4, that remains at high levels whereas IgG1 starts to decline after some time [14–17]. But no consistent results exist, that show, which antibody class is responsible for a therapeutical success. Until now, the measurement of IgG4 has served as 'golden standard' to assess the effectiveness of immunotherapy, but our results clearly show, that IgG4 alone can only tell that the immune system is responding to the treatment, but it can not predict the outcome. Looking at the antibody isotypes separately indicated, that IgG4 is positively correlated to high symptom scores, which is in accordance with other authors who find a positive association between IgG4 titres and treatment failure [35,36], whereas IgG1 shows a tendency to correlate with declining symptoms (neither correlations significant in our study, data not shown). But the ratio of IgG4 to IgG1 strongly correlates with the course of complaints.

In 1991 Pastorello *et al.* [37] were the first to correlate the ratio of IgG4 and IgG1 to clinical parameters. In contrast to our study, they measured antibody titres directed against a crude *Phleum pratense* extract, which contains a lot of antigenic proteins besides the relevant allergens. Since our assay was performed using purified recombinant group V allergens we could show that IgG antibodies, which are

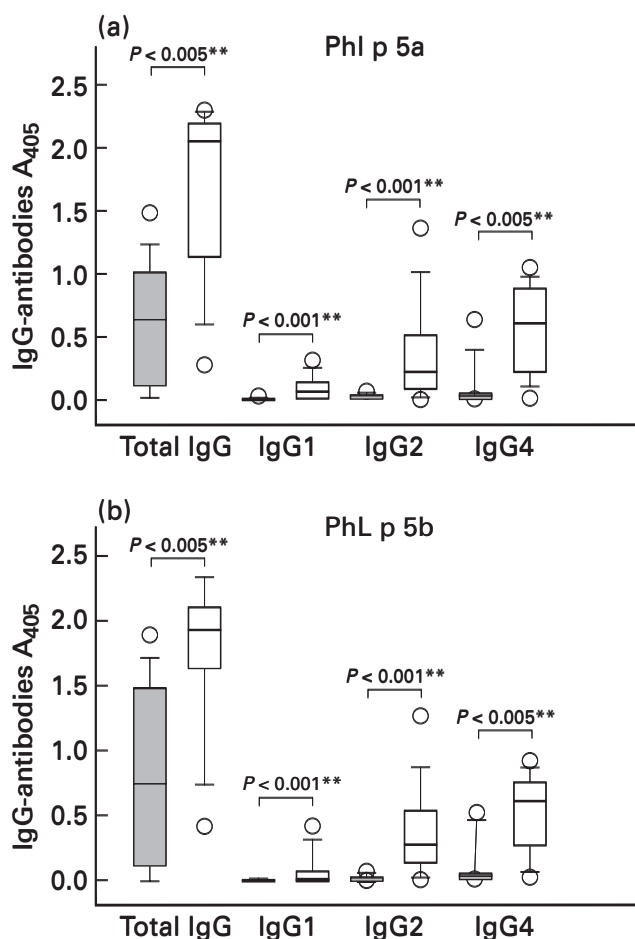


Fig. 5. Comparison of (a) Phl p 5a- and (b) Phl p 5b-specific total IgG, IgG1, IgG2, and IgG4 levels of the treated allergic patients. Filled bars indicate values obtained before therapy, open bars indicate the levels 1 year after the onset of therapy

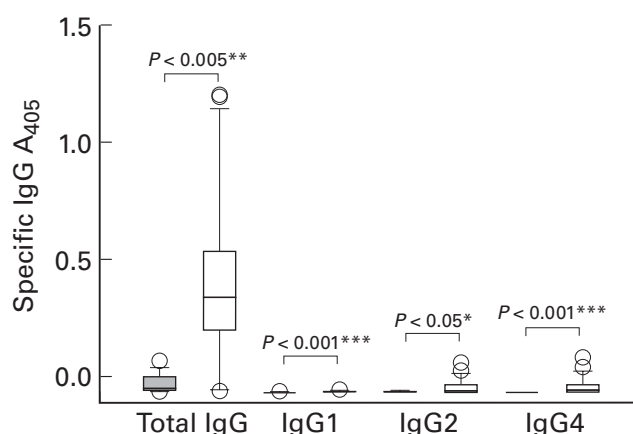


Fig. 6. Levels of Phl p 5b-specific total IgG, IgG1, IgG2, and IgG4 of healthy controls (filled bars, $n = 9$) in comparison to allergic patients (open bars, $n = 19$) before the beginning of the study.

allergen-specific, are relevant for the observed clinical changes. Pastorello *et al.* [37] found significant correlations between high ratios and high symptom scores but a decrease in skin prick reactivity. The positive correlation between IgG4/IgG1 and the symptom score is in accordance with our results, but the association with a decrease in skin prick reactivity remains to be explained. It seems possible, that for the sensitization of local mast cells in the skin IgG4 is the relevant antibody subclass, whereas in the reduction of overall symptoms both antibodies are involved.

The way these antibodies participate in the process of desensitization is still not clarified, but some facts are obvious. IgG1 is functionally bivalent, it can form immune complexes with antigens and bind to Fc receptors on lymphocytes and activate complement. IgG4 on the other hand can only bind one antigen at a time and binds to Fc receptors on mast cells and basophils without sensitizing

them [38]. In addition to this, IgG4 can inhibit the IgG1-mediated complement activation and protect the organism against complement-induced damages [39]. Therefore the hypothetical mechanism of immunotherapy could be the following: First, a production of IgG1 is induced, which leads to the formation of immune complexes. They can bind via IgG1 to the Fc γ -receptors on T suppressor cells and thereby induce their proliferation [23]. The induction of these T cell subtype due to immunotherapy has been demonstrated [2,40,41]. Thus, IgG1 antibodies might be most important in establishing tolerance towards challenging allergens. The IgG4 antibodies on the other hand can bind to mast cells and basophils. Daeron [10,11] could show that crosslinking of Fc ϵ RI and Fc γ RIIb receptors by allergens can downregulate the inflammatory response in these cells. In addition to this, IgG4 can counteract the complement activating capacity of IgG1.

Taken together these mechanisms can explain the positive effects of immunotherapy and are in concordance with our results which show that a certain IgG1 level in relation to IgG4 is essential for a good clinical outcome. Furthermore it has to be pointed out, that the ratio of IgG4/IgG1 already allows the assessment of the therapeutical success after 1 year of treatment and can therefore serve as valuable parameter for monitoring an ongoing immunotherapy.

In addition, our results obtained with the non-allergic controls show, that the difference between an atopic and a healthy state is not simply reflected by the antibody classes produced against common allergens. It has been demonstrated that in non-atopic subjects the immune response is balanced towards a TH1 milieu which favours the production of IgG1 antibodies, whereas in atopic individuals the TH2 cytokines drive the immune response towards the production of IgE and IgG4 [42–44]. In contrast to this, our results clearly show that the healthy state is not characterized by IgG antibodies against Phl p 5 isoallergens but in fact by hardly any measurable specific immune response at all. The children in the healthy control group had little allergen-specific IgG, but in neither case there was enough IgG to determine the subclasses. This was also true for a group of adult healthy controls we additionally tested (data not shown). Our results are in concordance with other studies that failed to detect allergen-specific IgG or IgG1 in nonatopics [45,46]. Therefore, looking at IgG subclasses, being nonatopic reflects a state of non-responsiveness rather than a TH1-driven ongoing immune response.

In summary, this report shows that the study of IgG subclasses in allergen immunotherapy of allergic patients is an important task to understand the regulation of immunological changes during this treatment. Further research will clarify the level on which the IgG subclass regulation is controlled and whether this knowledge may help to improve the effect of this therapy concept.

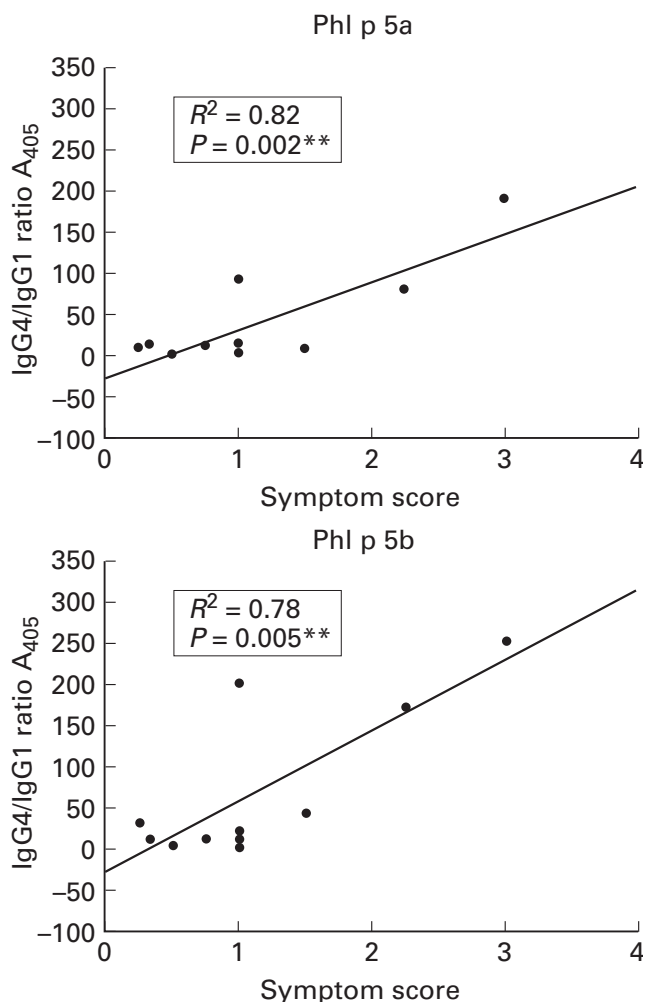


Fig. 7. This shows the regression line, calculated for the correlation between the IgG4/IgG1 ratios and the symptom scores.

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References

- Noon L, Cantar BC. Prophylactic inoculation against hay fever. *Lancet* 1911; 1:1572.
- Rocklin RE, Sheffer A, Greineder DK, Melmon KL. Generation of antigen-specific suppressor cells during allergy desensitization. *N Engl J Med* 1980; 302:1213–18.
- Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA+ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. *J Allergy Clin Immunol* 1997; 99:254–60.
- Hakansson L, Heinrich C, Rak S, Venge P. Priming of eosinophil adhesion in patients with birch pollen allergy during pollen season: effect of immunotherapy. *J Allergy Clin Immunol* 1997; 99:551–62.
- Ito H, Suzuki M, Mamiya S, Takagi I, Baba S. Study on changes in the level of serum-IL-4 and soluble CD23 (sCD23) with immunotherapy in nasal allergy patients. *Acta Otolaryngol (Stockh)* 1996; 525:98–104.
- O'Brien RM, Byron KA, Vargios GA, Thomas WR. House dust mite immunotherapy results in a decrease in Der p 2-specific IFN- γ and IL-4 expression by circulating T lymphocytes. *Clin Exp Allergy* 1997; 27:46–51.
- Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med* 1993; 178:2123–30.
- van Ree R, Aalberse RC. Rabbit IgG directed to a synthetic C-terminal peptide of the major grass pollen allergen Lol, p. 1 inhibits human basophil histamine release induced by natural Lol, p. 1. *Int Arch Allergy Immunol* 1995; 106:205–57.
- Batard T, Weyer A, Laroze A et al. Isotypic analysis of grass pollen-specific antibodies in human plasma. 4. Biological activity of allergen-specific and autoanti-IgE antibody fractions on basophil histamine release. *Clin Exp Allergy* 1996; 26:1308–15.
- Daeron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J Clin Invest* 1995; 95:577–85.
- Daeron M. Negative regulation of mast cell activation by receptors for IgG. *Int Arch Allergy Immunol* 1997; 113:138–41.
- Ohashi Y, Nakai H, Okamoto H et al. Significant correlation between symptom score and IgG4 antibody titer following long-term immunotherapy for perennial allergic rhinitis. *Ann Otol Rhinol Laryngol* 1997; 106:483–9.
- de Garcia-Robaina JC, la Torre-Morin F, Vazquez-Moncholi C, Fierro J, Bonnet-Moreno C. The natural history of *Apis*-specific IgG and IgG4 in beekeepers. *Clin Exp Allergy* 1997; 27:418–23.
- Devey ME, Wilson DV, Wheeler AW. The IgG subclasses of antibodies to grass pollen allergens produced in hay fever patients during hyposensitization. *Clin Allergy* 1976; 6:227–36.
- Nakagawa T. IgG subclass antibodies in response to house dust mite immuno-therapy. *N Engl Reg Allergy Proc* 1987; 8:423–8.
- McHugh SM, Lavelle B, Kemeny DM, Patel S, Ewan PW. A placebo-controlled trial of immunotherapy with two extracts of *Dermatophagoides pteronyssinus* in allergic rhinitis, comparing clinical outcome with changes in antigen-specific IgE, IgG and IgG subclasses. *J Allergy Clin Immunol* 1990; 86:521–31.
- Sanchez Armengol A, Castillo Gomez J, Ortega Ruiz F, Segado Soriano A. Blocking antibodies in *Dermatophagoides* allergy. *Allergol Immunopathol (Madr)* 1995; 23:271–6.
- Peng Z, Naclerio RM, Norman PS, Adkinson NF. Quantitative IgE- and IgG-subclass responses during and after long-term ragweed immunotherapy. *J Allergy Clin Immunol* 1989; 89:519–29.
- Matthiesen F, Löwenstein H. Group V allergens in grass pollen. II. Investigation of group V allergens in pollens from 10 grasses. *Clin Exp Allergy* 1991; 21:309–20.
- Gehlhar K, Petersen A, Schramm G, Becker W-M, Schlaak M, Bufe A. Investigation of different recombinant isoforms of grass group-V allergens (timothy grass pollen) isolated by low stringency cDNA hybridization. Antibody binding capacity and allergenic activity. *Eur J Biochem* 1997; 247:217–23.
- Dreborg S. Skin-prick test. *Allergy* 1985; 40:55–9.
- Walker SM, Varney VA, Gaga M, Jacobsen MR, Durham SR. Grass pollen immunotherapy: efficacy and safety during a 4-year follow-up study. *Allergy* 1995; 50:405–13.
- Djurup R. The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen-specific immunotherapy. *Allergy* 1985; 39:433–41.
- Hasegawa M, Yasueda H, Maeda Y et al. Changes in antigen-specific IgG, IgG4, and IgE antibodies in patients receiving immunotherapy with house-dust extract. *Arerugi* 1993; 42:809–16.
- Fennerty AG, Jones KP, Davies BH, Fifield R, Edwards J. Immunological changes associated with a successful outcome of pollen immunotherapy. *Allergy* 1988; 43:415–9.
- Pienkowski MM, Norman PS, Lichtenstein LM. Suppression of late-phase skin reaction by immunotherapy with ragweed extract. *J Allergy Clin Immunol* 1985; 76:729–34.
- Søndergaard I, Poulsen LK, Østerballe O, Weeke B. Evidence

- of a common regulation of IgE and IgG-subclass antibodies in humans during immunotherapy. *Allergy* 1992; 47:467–70.
- 28 Roberts AM, van Ree R, Cardy SM, Beavan LJ, Walker MR. Recombinant pollen allergens from *Dactylis glomerata*: preliminary evidence that human IgE cross-reactivity between Dac g II and Lol p I/II is increased following grass pollen immunotherapy. *Immunology* 1992; 76:389–96.
- 29 van Ree R, Hoffmann DR, van Dijk W et al. Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. *Clin Exp Allergy* 1997; 27: 68–74.
- 30 Ishizaka A, Koh K, Shibata R et al. Regulation of IgE and IgG4 synthesis in patients with hyper IgE syndrome. *Immunology* 1990; 70:414–16.
- 31 King CL, Nutman TB. IgE and IgG subclass regulation by IL-4 and IFN- γ in human helminth infections. *J Immunol* 1993; 151:458–65.
- 32 Punnonen J, Aversa G, Cocks BG et al. Interleukin 13 induces IL-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730–4.
- 33 Nonoyama S, Farrington ML, Ochs HD. Effect of IL-2 on human immunoglobulin production by anti-CD40-activated human B cells: Synergistic effect with IL-10 and antagonistic effect with IL-4. *Clin Immunol Immunopathol* 1994; 72:373–9.
- 34 Milburn HJ, Poulter LW, Dilmec A, Cochrane GM, Kemeny DM. Corticosteroids restore the balance between locally produced Th1 and Th2 cytokines and immunoglobulin isotypes to normal in sarcoid lung. *Clin Exp Immunol* 1997; 108:105–13.
- 35 Djurup R, Østerballe O. IgG subclass antibody response in grass pollen-allergic patients undergoing specific immunotherapy: prognostic value of serum IgG subclass antibody levels early in immunotherapy. *Allergy* 1984; 39:433–41.
- 36 Djurup R, Malling HJ. High IgG4 antibody level is associated with failure of immunotherapy with inhalant allergens. *Clin Allergy* 1987; 17:459–68.
- 37 Pastorello EA, Pravettoni V, Inocarvaia C et al. Clinical and immunological effects of immunotherapy with alum-absorbed grass allergoid in grass-pollen-induced hay fever. *Allergy* 1992; 47:281–90.
- 38 Poulsen LK, Skov PS, Mosbech H, Weeke B. Role of IgG4 in histamine release from human basophil leukocytes. I. Sensitization of cells from normal donors. *Int Arch Allergy Appl Immunol* 1988; 86:383–90.
- 39 Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983; 139:722–6.
- 40 Pesce AJ, Freisheim JM, Litw A, Michael JG. Modulation of the immune response to allergens: phospholipase A degradation products suppress IgG and IgE response in mice. *Int Arch Allergy Appl Immunol* 1990; 92:88–93.
- 41 Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998; 102:157–64.
- 42 Romagnani S. Regulation and deregulation of human IgE synthesis. *Immunol Today* 1990; 11:316–21.
- 43 Shimojo N, Kohno Y, Katsuki T et al. Diminished interferon-gamma (IFN- γ) production by bacterial antigen-specific T cells in atopic patients. *Clin Exp Immunol* 1996; 106:62–6.
- 44 Piccini M-P, Beloni L, Giannarini L et al. Abnormal production of T helper 2 cytokines interleukin-4 and interleukin-5 by T cells from newborn with atopic parents. *Eur J Immunol* 1996; 26:2293–8.
- 45 Smith AM, Yamaguchi H, Platts-Mills TA, Fu SM. Prevalence of IgG, Der anti p 2 antibodies in children from high and low antigen exposure groups: relationship of IgG and subclass antibody responses to exposure and allergic symptoms. *Clin Immunol Immunopathol* 1998; 86:102–9.
- 46 Härfast B, van Hage-Hamsten M, Lilja G. Birch pollen allergens fail to evoke IgG1 responses in non-atopic individuals. *Immunol Letters* 1995; 45:223–4.