

Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy

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Whether the modulation of antibody responses can contribute to the improvement of clinical symptoms in patients receiving allergen immunotherapy represents a controversial issue. We have used purified [seven recombinant (r) and one natural] timothy grass pollen allergens as well as recombinant B cell epitope-containing fragments of the major timothy grass pollen allergen, Phl p 1, to investigate humoral immune responses in eight allergic patients receiving grass pollen-specific immunotherapy. We found that the administration of aluminium hydroxide-adsorbed grass pollen extract induced complex changes in allergen/epitope-specific antibody responses: increases in IgG subclass (IgG1, IgG2, IgG4) responses against allergens recognized before the therapy were observed. All eight patients started to mount IgE and IgG4 responses to continuous Phl p 1 epitopes not recognized before the therapy and a *de novo* induction of IgE antibodies against new allergens was found in one patient. Evidence for a protective role of IgG antibodies specific for continuous Phl p 1 epitopes was provided by the demonstration that preincubation of rPhl p 1 with human serum containing therapy-induced Phl p 1-specific IgG inhibited rPhl p 1-induced histamine release from basophils of a grass pollen-allergic patient. Our finding that immunotherapy induced antibody responses against previously not recognized B cell epitopes indicates the vaccination character of this treatment. The fact that patients started to mount *de novo* IgE as well as protective IgG responses against epitopes may explain the unpredictability of specific immunotherapy performed with allergen extracts and emphasizes the need for novel forms of component-resolved immunotherapy.

Key words: Allergy / Immunotherapy / Vaccine / Epitope / Antibody

Received	1/2/99
Accepted	16/3/99

1 Introduction

Atopy, a genetically determined immune disorder that affects almost 20 % of the population, is characterized by the tendency of a given individual to mount IgE responses against otherwise harmless antigens (*i.e.* exogenous or endogenous allergens) [1–5]. While the symptoms of atopy can be treated by pharmacotherapy, allergen-specific immunotherapy represents the only causative therapeutic approach [6]. Allergen-specific

immunotherapy is based on the administration of increasing doses of allergen-containing extracts to allergic individuals in order to suppress the reaction to the disease-eliciting allergens. Although immunotherapy has been utilized since 1911 [7], the underlying immunological mechanisms are a matter of great debate [8]. After the first classical report that allergic symptoms in a ragweed-allergic individual were cured by transfusion of blood from a patient who had received successful ragweed immunotherapy [9], heat-stable antibodies [10, 11], later classified as “blocking allergen-specific IgG antibodies” were implicated in the abrogation of IgE-mediated effects [12–15]. It was subsequently demonstrated that prolonged immunotherapy induced IgG1 and IgG4 responses to allergen

[19270]

extracts [16–18], although some studies reported that clinical improvement was not always correlated with the induction of an IgG response [19, 20]. Other immunological changes that were reported to be associated with immunotherapy include: (1) reduction of mediator concentrations [21], (2) decrease in the number of mast cells [22, 23] and eosinophils [24, 25] in the target tissues of atopy, and (3) generation of suppressor cells [26] which were thought to modulate allergen-specific IgE production.

Support for an important role of allergen-specific CD4-positive T cells during immunotherapy came from several observations. They include the demonstration that allergen-specific T cells occurring after immunotherapy exhibited a decrease in IL-4 production, while IFN- γ secretion remained unchanged [27, 28] or even increased [29]. When the skin and noses of patients who had received immunotherapy were investigated after allergen provocation, an inhibition of T lymphocyte and eosinophil recruitment as well as a reduction of eosinophil activation was found together with an increase of Th1-type cells [23]. Direct analysis of circulating allergen-specific T cells indicated a reduction of the overall allergen-specific production of inflammatory cytokines, including IL-4 and IFN- γ [30]. These findings were interpreted as induction of T cell tolerance/anergy, a deletion of allergen-reactive lymphocytes and/or a switch from an allergen-specific Th2 towards a Th1 T cell phenotype.

All previous studies on the immunological mechanisms underlying immunotherapy were, however, hampered by the fact that patients received complex mixtures of allergens and non-allergenic moieties and that the subsequent analysis of parameters was performed with likewise complex allergen extracts. Recent experiments performed with purified natural and recombinant allergens have demonstrated that prolonged immunization of mice can induce IgG antibodies recognizing epitopes defined by human IgE antibodies, with the capacity to block human IgE binding to the allergens and inhibit allergen-induced basophil histamine release [31]. Here we studied the humoral immune responses in patients receiving timothy grass pollen-specific immunotherapy, using purified recombinant and natural timothy grass pollen allergens as well as recombinant fragments containing continuous B cell epitopes of the major timothy grass pollen allergen, Phl p 1 [32]. The protective role of therapy-induced anti-Phl p 1 and anti-Phl p 1 epitope-specific IgG antibodies was investigated by basophil histamine release experiments. The observed alterations of the therapy-induced allergen- and epitope-specific antibody responses strongly suggest that allergen-specific immunotherapy induces *de novo* immune responses to allergens and epitopes in the administered extract and thus exhibits features of a vaccine.

2 Results

2.1 IgE responses to purified recombinant and natural timothy grass pollen allergens in the course of immunotherapy; evidence for a *de novo* sensitization to new allergens/epitopes

Quantitative IgE measurements require an assay where allergens are immobilized in great excess to exclude interference of other isotypes with the IgE determination. We therefore measured allergen-specific IgE antibody levels in sera from eight grass pollen-allergic patients obtained before, during and after timothy grass pollen-specific immunotherapy with the Pharmacia CAP system, using immuno CAP tests containing an excess of recombinant or natural allergens. The results obtained showed a great variety of changes in allergen-specific IgE levels (Table 1): certain patients (e.g. patients no. 5, 6, 8) displayed increases of allergen-specific IgE levels (e.g. rPhl p 1, rPhl p 5) followed by decreases after 1 year of maintenance treatment (Table 1). On the other hand, patient no. 3 showed a continuous rise in allergen-specific IgE levels (e.g. rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 6) during the therapy (Table 1). Patient no. 1 exhibited a mixed pattern of IgE reactivity: rPhl p 1-specific IgE decreased after the initial injections and increased after the maintenance treatment, whereas rPhl p 11-specific IgE levels increased throughout the treatment. Results obtained with total extracts were often, but not always, in parallel with those gained with purified allergens (Table 1): timothy grass pollen-specific IgE increased continuously in patient no. 3, whereas anti-rPhl p 5-specific IgE increased during the initial treatment and decreased after the maintenance treatment. In most patients the allergen-specific IgE levels exhibited only subtle changes and there was no evidence for a change in the spectrum of recognized allergens. However, in patient no. 5 we were able to detect the induction of IgE responses to two allergens which were not recognized before treatment. While no Phl p 4-specific IgE could be measured before therapy, a continuous increase of Phl p 4-specific IgE was found after initial and maintenance treatment in patient no. 5. This patient started to mount an IgE response to Phl p 6 after the maintenance treatment. Our assumption that the IgE responses to Phl p 4 and Phl p 6 result from therapy-induced *de novo* sensitization is supported by at least two arguments: first, IgE levels to all other allergens decreased in this patient after maintenance treatment, while nPhl p 4- and rPhl p 6-specific IgE increased or became detectable. Second, *de novo* IgE responses are uncommon in the natural course of allergic disease, as indicated by our monitoring of ten grass pollen-allergic patients over a period of, on average, 2 years (data not shown).

Table 1. Determination of total and allergen-specific IgE before and in the course of grass pollen immunotherapy^{a)}

		no. 1			no. 2			no. 3			no. 4		
		I	II	III	I	II	III	I	II	III	I	II	III
Total IgE	kU/l	133	37.5	40.7	24.2	29.6	–	169	100	124	41.6	25.8	32.2
Timothy grass	kUA/l	43.7	19.3	22.7	14.7	10.8	–	18.1	23.7	29.3	25.7	21	20
rPhl p 1	kUA/l	9.24	3.78	5	4.72	4.69	–	7.65	8.95	11.1	13.8	10.5	10.9
rPhl p 2	A	0.254	0.284	0.241	0.119	0.119	–	0.103	0.104	0.115	0.124	0.130	0.126
nPhl p 4	kUA/l	< 0.35	0.38	< 0.35	< 0.35	< 0.35	–	2.32	2.66	2.79	0.65	0.44	0.99
rPhl p 5	A	0.335	0.444	0.408	0.352	0.374	–	0.507	0.745	0.664	0.567	0.403	0.338
rPhl p 6	kUA/l	1.58	2.35	2.20	2.53	3.57	–	5.03	7.17	7.45	8.50	6.10	4.05
rPhl p 7	kUA/l	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	–	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35
rPhl p 11	kUA/l	3.42	5.85	6.63	4.86	5.90	–	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35
rPhl p 12	kUA/l	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	–	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35

		no. 5			no. 6			no. 7			no. 8		
		I	II	III	I	II	III	I	II	III	I	II	III
Total IgE	kU/l	106	148	77	217	246	262	31.8	–	54.9	71.3	92.5	61.1
Timothy grass	kUA/l	13.6	19	11.3	72	98.7	86.6	17.3	–	27.5	25.7	33	27.4
rPhl p 1	kUA/l	7.95	12.2	6.25	27	33	30	3.62	–	5.73	14.6	17.6	15.5
rPhl p 2	A	0.285	0.299	0.231	0.483	0.838	0.683	0.138	–	0.187	0.106	0.088	0.1
nPhl p 4	kUA/l	< 0.35	1.09	1.83	12.8	15.5	6.25	ND	–	ND	4.64	7.02	5.21
rPhl p 5	A	0.079	0.123	0.107	1.899	2.364	1.899	0.318	–	0.375	0.497	0.542	0.392
rPhl p 6	kUA/l	< 0.35	< 0.35	0.66	2.28	7.64	6.33	ND	–	ND	3.01	7.03	3.62
rPhl p 7	kUA/l	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	ND	–	ND	< 0.35	< 0.35	< 0.35
rPhl p 11	kUA/l	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	ND	–	ND	3.86	5.13	4.08
rPhl p 12	kUA/l	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	< 0.35	ND	–	ND	< 0.35	< 0.35	< 0.35

a) Sera from eight grass pollen-allergic patients (nos. 1–8) collected before (I) and during the course of immunotherapy (II, III) were analyzed for total IgE levels, timothy grass pollen-specific IgE and IgE to eight purified timothy grass pollen allergens (rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11 and rPhl p 12) by CAP RAST measurements or ELISA. Total IgE levels are displayed in kU/l, allergen-specific IgE levels in kUA/l or A (absorbance) values. Values below 0.35 kUA/l indicate lack of detectable specific IgE and dashes indicate lack of serum samples. ND = not determined.

In summary, the component-based analysis of allergen-specific IgE levels reveals that immunotherapy can cause subtle but complex changes in allergen-specific IgE levels as well as a *de novo* induction of IgE responses against previously not recognized allergens/epitopes. The significance of the therapy-induced sensitization (e.g. to Phl p 4) is supported by our finding that in the course of immunotherapy patient no. 5 also developed IgE reactivity to unrelated allergen sources containing allergens that cross-react with Phl p 4 [33]. Before treatment no IgE specific for ragweed, mugwort, birch or olive pollen could be detected, whereas after the period

of dose increase the following specific IgE levels were measured: ragweed: 0.77 kU antigen/l (kUA/l); mugwort: 0.48 kUA/l; birch: 0.38 kUA/l; olive: 0.97 kUA/l.

2.2 ELISA measurements of IgG1–4 subclass responses to purified rPhl p 1 indicate induction of Phl p 1-specific IgG antibodies

To investigate alterations of IgG1–4 subclass responses towards the major timothy grass pollen allergen Phl p 1 during the course of grass pollen immunotherapy, sera

were obtained before and during treatment. We found an increase of rPhl p 1-specific IgG1, IgG2 and IgG4 responses after 1 year of treatment in all sera analyzed (Fig. 1). rPhl p 1-specific IgG3 levels remained almost unchanged (Fig. 1). The changes of Phl p 1-specific IgG subclass responses indicate the induction of a Th2- (IgG4) as well as, to a lower extent, of a Th1-like (IgG2) immune response [34].

2.3 Timothy grass pollen-specific immunotherapy induces IgE and IgG4 responses to continuous Phl p 1 epitopes that were not recognized before therapy

To examine at the molecular level the epitope recognition pattern of patients in the course of immunotherapy, serum samples were tested for the development of IgE and IgG4 binding to 26 different recombinant Phl p 1 fragments. The recombinant Phl p 1 fragments (Fig. 2: NT, 28–114) which comprise different portions of the Phl p 1 molecule were expressed as β -galactosidase (β -gal) fusion proteins and, together with β -gal alone (Fig. 2: 0), were immobilized to nitrocellulose filters. Filters were exposed to sera collected before and after timothy grass pollen-specific immunotherapy. We found an increase (Fig. 3A) or *de novo* induction of IgE responses to continuous Phl p 1 epitopes (Fig. 3B, C) after the phase of dose increase (3 months) and after 1 year of maintenance treatment. The fact that the IgE levels specific for complete rPhl p 1 decreased or showed little change indicates that the epitope-specific IgE responses do not result from the stimulation of an already existing but rather from a *de novo* induced Phl p 1-specific IgE response (Fig. 3A–C).

None of the eight patients exhibited rPhl p 1 fragment-specific IgG4 responses before immunotherapy. However, after 1 year of maintenance treatment we found pronounced IgG4 responses to continuous Phl p 1 epitopes which, by contrast to the IgE reactivity, were associated with increases in IgG4 levels to the complete rPhl p 1. IgE reactivity to continuous Phl p 1 epitopes could be detected before epitope-specific IgG4 responses, *i.e.* patients mounted epitope-specific IgE responses already after the period of dose increase. Notably, IgE and IgG4 antibodies bound to the same but also to different continuous Phl p 1 epitopes. IgG4 antibodies of the patient in Fig. 3A bound to fragments recognized by IgE (fragments NT, 80, 95, 97, 98, 114) as well as to fragments which did not bind IgE (fragments 42, 52, 108, 109, 113). Likewise, IgG4 but not IgE of the patient in Fig. 3B reacted strongly with fragment 108, whereas fragments 42, 52, 86, 95, 97, 98, 109, 114 bound IgG4 as well as IgE antibodies. We also found that patients

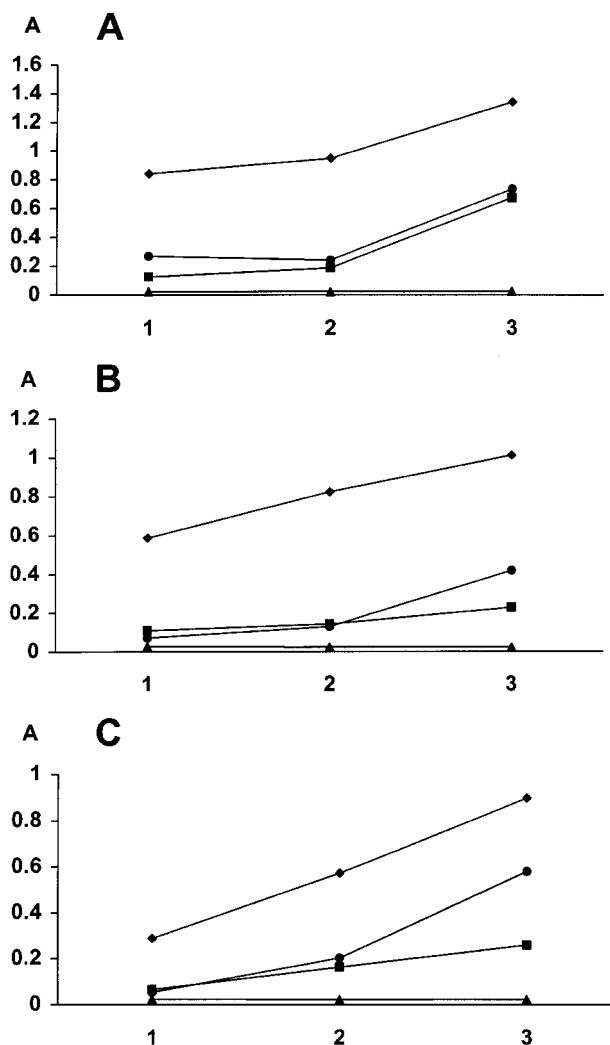


Figure 1. Monitoring of rPhl p 1-specific IgG subclass responses in three representative grass pollen-allergic patients (A: no. 4, B: no. 8, 1C: no. 3) by ELISA before and during the course of immunotherapy. The absorbance values are displayed on the y-axis and correspond to the amounts of bound IgG1 (◆), IgG2 (■), IgG3 (▲) and IgG4 (●) antibodies. The sample numbers on the x-axis correspond to the time points of serum collection [1: before immunotherapy (I); 2: after the dose increase phase (3 months) (II); 3: after 1 year of immunotherapy (III)].

started to mount IgE responses to fragments which were not recognized by IgG4 antibodies; *e.g.* fragments 45 and 50 were exclusively recognized by IgE but not IgG4 antibodies of the patient in Fig. 3C.

In summary, our data indicate that timothy grass pollen-specific immunotherapy induces *de novo* IgE and IgG4 antibody responses to the same but also to different Phl p 1 epitopes.

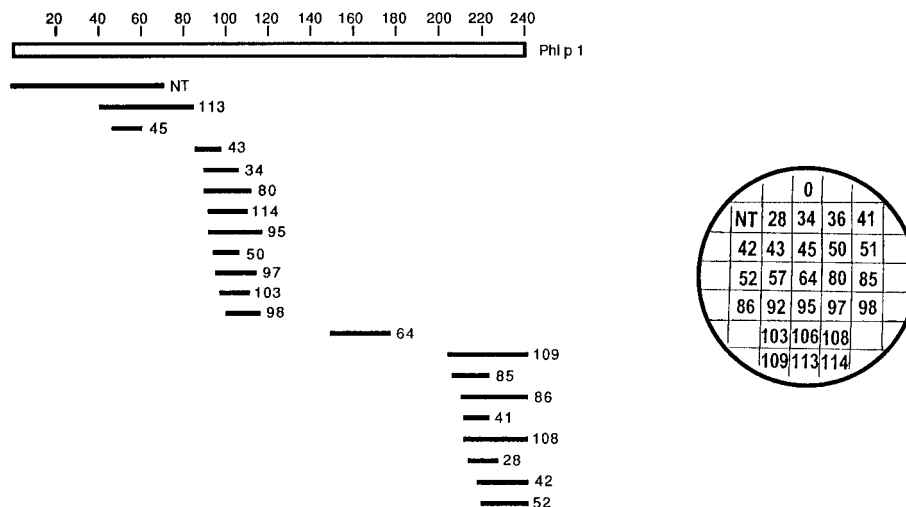


Figure 2. Localization of IgE-reactive recombinant Phl p 1 fragments. The full length of Phl p 1 (240 amino acids) is represented by the top line (N terminus: left end; C terminus: right end). The localization and length of the recombinant IgE-reactive fragments (NT, 28–114) is shown below by bars. The order of phage expressing β -gal-fused rPhl p 1 fragments (NT, 28–114) or β -gal alone (0) that were immobilized to a nitrocellulose filter is shown in the right hand part of the figure.

2.4 Sera containing immunotherapy-induced IgG antibodies specific for continuous Phl p 1 epitopes inhibit rPhl p 1-induced basophil histamine release

To evaluate whether immunotherapy-induced IgG antibodies (e.g. IgG1, IgG2, IgG4) are capable of inhibiting allergen-induced effector reactions and thus may represent protective antibodies, histamine release experiments were performed. Purified rPhl p 1 that was preincubated with sera from two patients after successful immunotherapy induced a much lower histamine release from basophils of a Phl p 1-allergic individual compared to rPhl p 1 that was preincubated with the same patients' sera obtained before immunotherapy (Fig. 4; data not shown). Since the serum obtained after immunotherapy contained increased levels of rPhl p 1-specific IgG1, IgG2 and IgG4 antibodies as well as rPhl p 1 fragment-specific IgG4 while rPhl p 1-specific IgE levels remained almost unchanged (Fig. 3 B), the reduction of rPhl p 1-induced histamine release may result from the presence of blocking IgG antibodies.

3 Discussion

In the present study we analyzed the IgE and IgG subclass recognition of purified recombinant and natural timothy grass pollen allergens as well as of recombinant B cell epitope-containing fragments of the major timothy grass pollen allergen, Phl p 1, before and in the course

of specific immunotherapy. Several of our observations gained at the B cell epitope level suggest that specific immunotherapy has vaccination character: we found that patients had little or no IgG subclass reactivity to the major timothy grass pollen allergen, Phl p 1, before treatment but mounted increasing levels of Phl p 1-specific IgG1, IgG4 and IgG2 in the course of immunotherapy. Our contention that the Phl p 1-specific IgG subclass reactivity represents a *de novo* induced immune response rather than the amplification of an already existing reactivity is supported by the finding that in all patients investigated, the therapy-induced Phl p 1-specific IgG4 antibodies bound to continuous Phl p 1 epitopes that were not recognized before therapy. Likewise, we found that immunotherapy induced IgE antibodies to continuous Phl p 1 epitopes which could not be detected before treatment. The fact that immunotherapy induced rPhl p 1 epitope-specific IgE responses at an earlier stage (*i.e.* after the period of dose increase) than the epitope-specific IgG4 reactivity (*i.e.* after the maintenance treatment) may be explained by preferential induction of IgE rather than IgG by the low dose of allergen administered at the beginning of the treatment. Our assumption that high allergen doses are required for the induction of IgG antibodies is supported by earlier experiments which demonstrated that the administration of low allergen doses induces a Th2 response that is accompanied by IgE production, whereas higher doses of denatured allergens induce preferentially Th1 responses and blocking IgG antibodies [28, 35, 36].

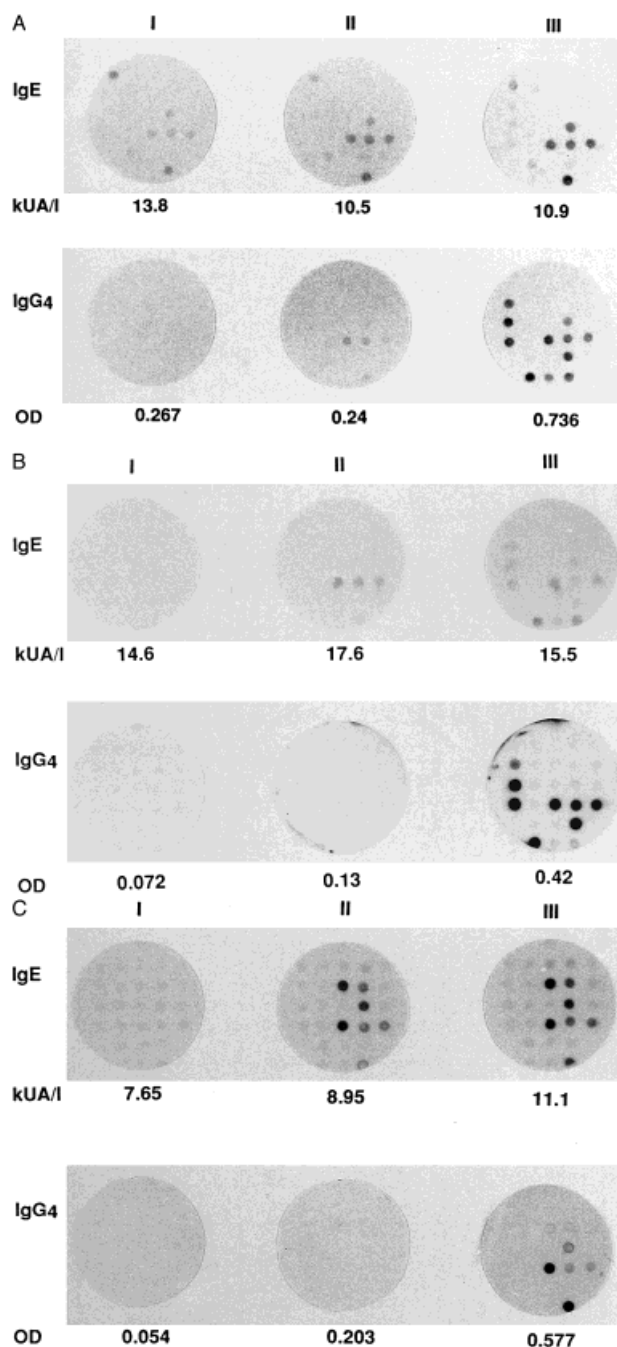


Figure 3. A–C. Induction of IgE and IgG4 antibody responses to recombinant Phl p 1 fragments by immunotherapy. Serum samples from the three patients described in Fig. 1 A–C were exposed to nitrocellulose-dotted rPhl p 1 fragments (Fig. 2) and bound IgE and IgG4 antibodies were detected. The levels of rPhl p 1-specific IgE and IgG4 in each serum sample are shown in kUA/l and optical density, respectively.

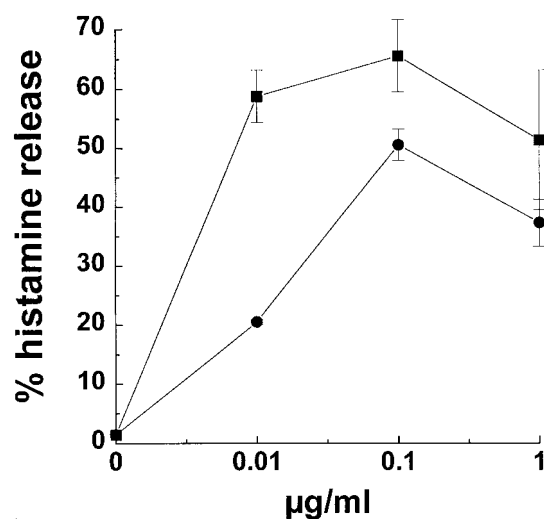


Figure 4. Preincubation of rPhl p 1 with serum containing therapy-induced rPhl p 1 and rPhl p 1 epitope-specific IgG inhibits allergen-induced histamine release. Different concentrations of rPhl p 1 as indicated on the x-axis were preincubated with serum from a patient taken before (■) and after 1 year of immunotherapy (●) and exposed to basophils of a Phl p 1-allergic patient. Results displayed on the y-axis represent the percentage values of released histamine and are expressed as the mean $\pm$ SD of triplicate determinations.

The concept that specific immunotherapy induces a new humoral immune response does not contradict previous findings obtained at the T cell level: the reported shift to allergen-specific Th1 clones in the course of immunotherapy [37] may equally well result from the switching of existing Th2 clones or from a *de novo* induction of an allergen-specific Th1 immune response. The latter assumption would fit our observation that in the course of therapy, patients started to mount allergen-specific IgG2 subclass responses which indeed would be indicative of a Th1 immune response [34].

While the magnitude of pre-existing IgE levels did not change significantly during the course of treatment, we found that all patients became sensitized *de novo* against continuous epitopes of the major timothy grass pollen allergen, Phl p 1. One out of eight patients even became sensitized to two new allergens, Phl p 4 and Phl p 6.

The induction of IgE anti-Phl p 6 immunoreactivity will add a small contribution to the overall IgE anti-grass pollen allergen immunoreactivity and thus will likely remain clinically unnoticed. By contrast, the therapy-induced anti-Phl p 4 IgE response may have also clinical relevance as it expanded the patients' sensitization spectrum to tree and weed pollens which reportedly contain

Phl p 4-homologous allergens [33]. Nevertheless, all patients showed an overall clinical improvement which notably correlated with the induction of IgG4 responses to continuous epitopes of the major timothy grass pollen allergen, Phl p 1. A protective role of the therapy-induced IgG antibodies is indicated by our finding that preincubation of rPhl p 1 with serum containing Phl p 1 and Phl p 1 epitope-specific IgG antibodies inhibited the rPhl p 1-induced basophil histamine release.

Based on our analysis we believe that the partly opposite and unpredictable effects of immunotherapy performed with complex allergen extracts may, at least in part, be explained both by the fact that, at the moment, patients cannot be treated according to their particular sensitization patterns and by the inherent difficulties in injecting doses of allergens sufficient to induce high titers of blocking antibodies but without causing anaphylactic side effects. Both these problems may be overcome in the future by the use of recombinant allergens for diagnosis and treatment [38, 39]. First, recombinant allergens will allow a component-resolved form of diagnosis which should permit the selection of exactly those allergens for specific immunotherapy against which a patient is sensitized. A thorough selection of allergens for treatment will thus exclude the possibility that patients become sensitized against new components. Second, genetic engineering makes it possible to convert many important allergens into hypoallergenic derivatives which can be administered at higher doses with reduced risk of anaphylactic side effects and a hopefully greater potential to induce blocking IgG antibodies rather than IgE responses [39, 40]. Clinical studies performed with hypoallergenic allergens [41–46] selected according to the patients' reactivity profile will, however, be required to prove this hypothesis. Such studies will not only allow a reinvestigation of the immunological mechanisms underlying specific immunotherapy at a component and epitope level but, perhaps, also help to develop safer and more efficacious forms of specific immunotherapy, *i.e.* vaccination protocols.

4 Materials and methods

4.1 Characterization of grass pollen-allergic individuals, blood sampling, timothy grass pollen-specific immunotherapy

Eight grass pollen-allergic patients (mean age 26.5 years) were characterized by case history, skin prick testing and radioimmunosorbent assay (RAST) (Pharmacia, Uppsala, Sweden). Serum samples were collected before immunotherapy (September: I), after a 3-month period of dose increase with weekly injections (December/January: II) and

after 1 year treatment with monthly injections with the maintenance dose (September/October: III). Specific immunotherapy was performed using aluminium hydroxide-adsorbed grass pollen extract (six grass pollen + rye) according to a conventional immunotherapy protocol [37]. The clinical success of immunotherapy was evaluated by subjective case history, by symptom- and medication score calendars [37].

4.2 Determination of total and allergen-specific IgE

Total and allergen-specific IgE levels were determined by CAP RAST measurements (Pharmacia, Uppsala, Sweden). Timothy grass pollen and Phl p 1 [32, 47] immunoCAP tests were purchased from Pharmacia. Experimental CAP tests containing purified recombinant timothy grass pollen allergens rPhl p 6 (Vrtala and Valenta, unpublished), rPhl p 7 [48], rPhl p 11 (Lidholm, unpublished data), rPhl p 12 [49, 50] and natural Phl p 4 ([33]; Lidholm, unpublished data) were prepared by Pharmacia & Upjohn AB Diagnostics. Recombinant *Escherichia coli*-expressed Phl p 11 represents the Lol p 11-homologous allergen from timothy grass pollen [51]. Natural Phl p 4 [33] was purified from timothy grass pollen extracts (Lidholm, unpublished). Levels of rPhl p 1, rPhl p 2 and rPhl p 5-specific IgE were determined by ELISA as described [47]. Results are displayed as means of duplicate determinations.

4.3 ELISA measurements of IgG subclass reactivity to purified rPhl p 1

IgG1, IgG2, IgG3 and IgG4 reactivity to rPhl p 1 was measured by ELISA [47]. ELISA plates were coated with 5 µg rPhl p 1/ml. Sera were diluted 1:20. Bound IgG1–4 antibodies were detected with mouse anti-human IgG1–4 mAb (Pharmingen, San Diego, CA) and visualized with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse anti-serum (Amersham, Amersham, GB) and ABTS solution [60 mM citric acid, 77 mM Na₂HPO₄ · 2H₂O, 1.7 mM ABTS (Sigma, St. Louis, MO), 3 mM H₂O₂] as developer. Results are displayed as mean of duplicate determinations.

4.4 Detection of IgE and IgG4 responses to rPhl p 1 fragments

4.4.1 Characterization of recombinant Phl p 1 fragments

Recombinant B cell epitope-containing fragments of the major timothy grass pollen allergen, Phl p 1, were obtained by IgE immunoscreening of an expression library constructed from randomly fragmented Phl p 1-encoding cDNA ([41]; Ball and Valenta, unpublished data). The sequences of all but five of the rPhl p 1 fragments (clones NT, 28, 34, 41, 42, 43, 45, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109,

113, 114) were verified by DNA sequence analysis of the epitope-encoding λ clones. cDNA were obtained by PCR (λ gt11 forward primer: 5' CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GGC 3'; λ gt11 reverse primer: 5' CGC GGA TCC CGT TGA CAC CAG ACC AAC TGG TAA TG 3') from the IgE-reactive phage clones. The PCR products were subcloned via Bam H I restriction sites (underlined in the primers) into plasmid pUC18 (Boehringer Mannheim, Mannheim, Germany) and sequenced according to Sanger [52] using the λ gt11 primers described above, [³⁵S]dCTP (NEN, Stevenage, GB) and a T7 polymerase sequencing kit (Pharmacia). The remaining five clones (36, 51, 57, 92, 106) contained inserts of less than 200 base pairs. By oligonucleotide hybridization [53], clone 36 was mapped to the clone 98-defined region, clone 51 mapped to the clone 64-defined portion and clone 92 was mapped to the Phl p 1 C terminus.

4.4.2 Expression and antibody reactivity of recombinant Phl p 1 fragments

Two-microliter aliquots of phage lysates ($>10^5$ PFU/ml) expressing β -galactosidase-fused Phl p 1 IgE epitopes, and for control purposes β -galactosidase alone, were dotted onto LB amp plates containing a lawn of *E. coli* Y1090. Expression of recombinant Phl p 1 fragments was induced by overlay with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) presoaked in 10 mM isopropyl β -D-thiogalactopyranoside (IPTG). Nitrocellulose filters containing the recombinant Phl p 1 fragments and *E. coli*/phage proteins were incubated with serum from Phl p 1-allergic patients, diluted 1:5 for IgE- and 1:20 for IgG4 detection. Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies and bound IgG4 antibodies with a mouse anti-human IgG4 antibody (Pharmingen), followed by ¹²⁵I-labeled sheep anti-mouse Ig antibodies (Amersham), respectively.

4.5 Isolation of basophils and histamine release experiments

Heparinized blood was obtained from a grass pollen-allergic patient by venipuncture after informed consent was given. Granulocytes were isolated by dextran sedimentation as described [54]. The proportion of basophils in the dextran-isolated cell preparation amounted to 4 %. Enriched basophils were exposed to different concentrations (1 μ g/ml, 0.1 μ g/ml, 0.01 μ g/ml) of purified rPhl p 1 that had been preincubated for 15 min at 37 °C with equal volumes of patients' sera obtained before or after immunotherapy. Histamine released in the cell-free supernatants was determined in triplicates by radioimmunoassay (Immunotech, Marseille, France) and is expressed as percentage of total histamine determined after cell lysis [54].

Acknowledgements: We would like to acknowledge the excellent technical help of Ute Siemann. This work was supported by grant Y078GEN of the Austrian Science Fund, by the ICP program of the Austrian Ministry of Research and Transports and by a grant from Pharmacia & Upjohn AB, Uppsala, Sweden.

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