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Original Article

Measurement of IgE and IgG4 antibodies against purified grass pollen allergens (Phl p 1, Phl p 2, Phl p 5 and Bet v 2) and natural extracts after short-term grass immunotherapy

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Abstract

We wanted to define allergen-specific antibodies that change due to specific immunotherapy. We conducted a study with grass pollen-allergic patients and compared allergen-specific IgE and IgG4 before and 5 months after the onset of immunotherapy. Twenty-seven patients were treated with a mixture of two grass species: *Phleum pratense* and *Dactilis glomerata*. Sera of patients were tested for IgE and IgG4 against four recombinant allergens (RA): rPhl p 1, 2, 5 and rBet v 2. Specific IgE and IgG4 to timothy and olive pollen were also evaluated. No change in total and specific IgE levels to RA was seen, except to rPhl p 5. We found a decrease in specific IgE levels to olive after immunotherapy. Ten of 10 patients with specific IgE against a single recombinant allergen or two RA showed the same pattern of sensitization before and after 5 months of immunotherapy and the administration of 4000 U/mL allergen extract. Interestingly, we found a significant increase in specific IgG4 to rBet v 2 and olive after grass immunotherapy. These results indicate that application of two grass species in immunotherapy may be sufficient to induce an IgE and IgG4 response to RA, grass and olive extracts. The observations in the present study indicate that monitoring of antibodies against RA is necessary to evaluate patients' pattern of sensitization and emphasize the need of component-resolved immunotherapy.

Key words: grass pollen allergy, IgE, IgG4, immunotherapy, olive pollen, recombinant allergens.

Introduction

In atopic individuals, persistent exposure to allergens, through either natural exposure or immunotherapy, leads to synthesis of both allergen-specific IgE and IgG4 antibodies.[1–3] The proposed practice parameters for allergen immunotherapy,[4] established by representatives from both the American Academy of Allergy, Asthma and Immunology and the American College of Allergy, Asthma and Immunology, do state that omission of clinically relevant allergens from therapeutic mixtures may decrease the effectiveness of immunotherapy, suggesting a more inclusive approach to the treatment of grass pollen allergy. The rationale behind this approach is to cover the spectrum of grass pollen allergens as completely as possible. Previous studies[2] have indicated that the grass pollens elicit a complex array of antibody specificities in atopics and that the profile of antibodies specific to the pollen extract and pure allergens differs, suggesting that single grass allergen may be inadequate for replacing grass pollen extracts for immunotherapy. However, it was only recently that Van Ree *et al*.[5] and Ball *et al*.[6] showed that allergen extract administered with an adjuvant may induce immune reactions against extract components not recognized before treatment.

Moreover, it was previously demonstrated that application of just one grass species in immunotherapy may be

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Table 1 Demographic and serological characterization of individuals allergic to grass pollen before (November) and after (April) immunotherapy

Patient	Demographic data				Total	Specific IgE						Specific IgG4						ECP
no.	Sex	Age (years)	Allergies	Symptoms	IgE (kU/L)	Timothy (kUA/L)	rPhl p1 (kUA/L)	rPhl p2 (kUA/L)	rPhl p5 (kUA/L)	rBetv2 (kUA/L)	Olive tree (kUA/L)	Timothy (mg/L)	rPhl p1 (mg/L)	rPhl p2 (mg/L)	rPhl p5 (mg/L)	rBetv2 (mg/L)	Olive tree (mg/L)	(μg/mL)
1	F	50	g6, t9, w6, w9, t3, d1	r, c, as														
Before					225	>100	14.5	<0.35	73	<0.35	4.22	1.4	0.22	<0.15	0.65	<0.15	0.56	10.3
After					280	>100	31.3	<0.35	>100	<0.35	4.17	12.5	1.73	<0.15	3.88	0.25	1.84	9.5
2	F	27	g6, t9, t3, k82, w21	r, c, as														
Before					1365	>100	78.2	45.6	72	16.2	23.2	2.78	1.12	0.88	0.66	0.23	0.47	4.7
After					1267	>100	>100	65.1	>100	14.7	20.3	17.7	4.43	3.72	4.34	1.3	3.09	8
3	F	29	g6, k82, t3, t9, w21	r, c, as														
Before					249	>100	63.1	6.31	42.2	3.94	12.9	0.66	0.2	<0.15	0.3	0.2	0.21	9.4
After					299	>100	55.9	9.14	71.9	4.54	16.8	13.1	1.87	0.79	4.39	0.49	1.77	21.9
4	F	16	g6, d1, t3, t9	r, c, as														
Before					113	23.5	3.56	1.8	13.9	<0.35	1.96	1.29	<0.15	<0.15	0.46	<0.15	0.26	16.9
After					110	20.4	2.28	1.2	8.86	<0.35	1.16	0.73	<0.15	<0.15	<0.15	<0.15	<0.15	8.92
5	M	27	g6, t9, w6, w9	r, c, as														
Before					122	52.7	18.3	<0.35	18.1	<0.35	4.08	0.38	<0.15	<0.15	<0.15	<0.15	<0.15	7.66
After					117	45.5	17.7	<0.35	16	<0.35	2.03	8.01	1.36	0.23	2.67	<0.15	1.28	4.98
6	M	30	g6, k82, t9, t3, w21, w6, w9	r, c														
Before					52	21.4	22.5	<0.35	0.54	2	2.5	0.55	0.24	<0.15	<0.15	<0.15	0.21	9.35
After					90	14.5	15.3	<0.35	0.5	1.94	2.06	4.53	0.83	<0.15	1.05	<0.15	0.28	7.62
7	F	16	g6, t3, t9, w6, w9, w21	r, c, as														
Before					119	59.3	14.4	4.97	29.9	1.66	3.5	0.24	<0.15	<0.15	<0.15	<0.15	<0.15	5.6
After					157	63.5	15.1	6.49	33.8	2.67	3.11	4.26	1.1	0.33	1.45	<0.15	0.24	2.5
8	M	10	g6, d1, k82, t3, t9, w21, w6, w9	r, c, as														
Before					676	>100	30.2	7.48	70.2	12.3	19.6	1.42	0.35	<0.15	0.32	0.22	0.3	14.1
After					664	>100	32.4	8.56	89.7	12.1	14.6	7.81	0.91	1.45	2.9	0.45	1.14	14.3
9	M	17	w6, g6, t9	r, c, as														
Before					369	43.7	4.29	8.26	10.3	<0.35	5.77	0.81	<0.15	<0.15	<0.15	<0.15	0.88	28.1
After					294	42.8	3.48	8.49	9.74	<0.35	3.65	6.46	0.61	1.52	0.54	<0.15	1.02	58.7
10	F	24	w9, g6, d1, t9	r, c, as														
Before					322	16	4.08	1.03	7.3	0.7	1.74	9.26	0.7	< 0.15	3.19	1.44	1.99	6.7
After					307	13.6	3.69	1.19	7.06	0.59	1.03	10.9	0.86	< 0.15	3.77	2.63	2.55	5
11	M	16	w9, w6, t3, g6, d1, k82, t9, w21	r, c, as														
Before					884	>100	76.6	25.9	> 100	2.78	22.3	0.51	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	6.3
After					831	>100	71.5	24.9	> 100	2.24	12.8	7.07	1.54	0.72	1.92	0.2	0.56	10.7
12	M	22	g6, t9	r, c, as														
Before					823	46.6	47.5	< 0.15	5.31	< 0.35	1.28	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	3.67
After					863	41.7	45.8	< 0.15	5.36	< 0.35	0.89	< 0.15	2.31	0.51	1.93	0.39	0.4	6.97
13	F	8	g6, t9	r, c, as														
Before					54	10.2	1.74	3.63	5.52	< 0.35	0.86	0.39	< 0.15	0.24	< 0.15	< 0.15	0.35	9.1
After					64	16.2	2.09	5.31	9.97	< 0.35	0.48	9.56	1.34	1.73	3.06	< 0.15	0.61	4.1
14	F	18	t9, w21, w6, g6, k82, t3	r, c, as														
Before					1065	>100	> 100	< 0.15	> 100	29.2	47.4	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	5.9
After					1263	>100	> 100	< 0.35	> 100	24.4	30.8	0.94	< 0.15	< 0.15	< 0.15	< 0.15	< 0.15	12.3
15	F	19	g6, t3, t9	r, c														
Before					101	18.8	5.64	< 0.35	14.7	< 0.35	0.97	0.36	< 0.15	< 0.15	0.2	< 0.15	0.45	19.7
After					93	19.2	6.2	< 0.35	13.9	< 0.35	0.7	9.97	2.62	0.33	3.48	0.74	1.53	22.4
16	F	15	t9, w6, w21, g6, k82, t3	r, c, as														
Before					791	>100	>100	43.6	86.1	4.23	15.5	0.33	< 0.15	< 0.15	< 0.15	0.45	0.22	4.77
After					2000	98.9	>100	51.7	>100	4.77	< 0.35	35	5.08	2.41	5.25	1.24	1.54	2.43

Table 1 *Continued*

Patient no.	Sex	Demographic data Age (years)	Allergies	Symptoms	Total IgE (kU/L)	Specific IgE Timothy (kUA/L)	rPhl p1 (kUA/L)	rPhl p2 (kUA/L)	rPhl p5 (kUA/L)	rBetv2 (kUA/L)	Olive tree (kUA/L)	Specific IgG4 Timothy (mg/L)	rPhl p1 (mg/L)	rPhl p2 (mg/L)	rPhl p5 (mg/L)	rBetv2 (mg/L)	Olive tree (mg/L)	ECP (μg/mL)
17	M	11	g6, d1, t9	r, c, as														
Before					54	6.84	1.59	1.13	3.29	<0.35	0.46	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15	16.8
After					56	5.54	1.61	1.1	3.08	<0.35	< 0.35	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15	4
18	F	70	w9, g6, t9, w21	r, c, as														
Before					101	3.19	2.72	0.5	<0.35	<0.35	< 0.35	<0.15	0.24	<0.15	<0.15	<0.15	<0.15	7.7
After					100	6.34	6.11	0.53	<0.35	<0.35	0.76	0.75	0.51	0.54	<0.15	<0.15	0.22	6.1
19	F	48	g6, d1, t9	r, c														
Before					493	12.6	11.3	< 0.35	<0.35	<0.35	2.65	1.47	<0.15	<0.15	<0.15	0.94	1.35	34.7
After					475	9.71	10.7	< 0.35	<0.35	<0.35	2.68	1.31	<0.15	<0.15	0.52	1.03	0.76	7.7
20	F	17	t9, w21, w6, w9, g6, k82, t3	r, c														
Before					291	>100	25.5	29.8	66.4	9	13	8.36	1.28	<0.15	1.76	<0.15	0.83	4.1
After					377	>100	39.1	27.6	>100	8.29	10.6	1.16	<0.15	1.79	<0.15	<0.15	<0.15	4.5
21	F	43	g6, t9, w9	r, c, as														
Before					206	73.3	10.8	8.34	35.8	<0.35	2.75	5.09	3.53	<0.15	<0.15	<0.15	<0.15	8.1
After					195	95.2	12.9	4.14	44.6	<0.35	1.99	3.94	1.09	<0.15	2.34	<0.15	0.57	8.6
22	F	12	t9, w21, w6, w9, g6, k82, t3	r, c, as														
Before					137	64.6	11.6	2.85	21.3	4.09	3.21	1.82	0.27	0.58	<0.157	<0.15	<0.15	6.78
After					103	44	7.82	2.54	18.3	3.31	2.46	<0.15	0.37	<0.15	<0.15	<0.15	<0.15	11
23	F	20	g6, d1	r, c														
Before					38	7.7	8.56	<0.35	<0.35	<0.35	<0.35	0.25	0.25	0.96	<0.15	<0.15	<0.15	10.4
After					62	5.81	6.16	<0.35	<0.35	<0.35	<0.35	0.77	0.65	0.53	<0.15	<0.15	<0.15	3.3
24	F	22	t3, t9, w21, g6, d1, k82, w6, w9	r, c, as														
Before					540	86.4	28.9	<0.35	52.7	2.11	7.22	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15	19.2
After					755	95.5	45	<0.35	67.5	3.7	6.32	5.66	4.36	<0.15	0.41	0.59	0.51	17
25	F	27	g6, d1, t9	r														
Before					123	31.1	8.37	6.18	14.3	<0.35	1.28	0.24	<0.15	<0.15	<0.15	<0.15	<0.15	3
After					201	62	17.6	12.6	35.5	<0.35	0.9	9.84	4.95	3.64	3.59	<0.15	0.64	2.1
26	M	53	g6, d1	r, c, as														
Before					111	17.2	0.96	4.83	3.26	<0.35	1.5	0.46	<0.15	<0.15	<0.15	<0.15	<0.15	23.1
After					124	21	0.91	5.45	4.53	<0.35	1.01	5.91	1.24	1.05	1.04	<0.15	0.49	12.8
27	F	14	w9, g6, t9, w6	r														
Before					1020	>100	85.2	41.7	91.1	<0.35	8.03	1.19	0.21	0.31	0.49	<0.15	0.22	6.43
After					950	>100	83.2	36	71.5	<0.35	4.46	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15	4.32

g6, timothy pollen; t3, birch pollen; t9, olive tree; w6, mugwort; w9, plantain; w21, pellitory of the wall; d1, mites; k82, latex; ECP, eosinophil cationic protein; r, rhinitis; c, conjunctivitis; as, asthma.

sufficient to induce an IgG response that covers other relevant Gramineae species as well.[7] In other studies, it was demonstrated that in a high percentage of patients allergic to grass pollen, a mixture of only four recombinant allergens (RA) accounted for more than 50% of IgE to nine different natural grass pollen extracts[8] and the majority of grass pollen allergic patients is cosensitized to rPhl p 1, rPhl p 2 and rPhl p 5.[9] In contrast, a fraction of patients allergic to grass pollen are lacking a specific IgE against one to three RA (e.g. rPhl p 1+, rPhl p 2–, rPhl p 5–). These subjects may be studied to evaluate whether they start to mount *de novo* IgE as well as IgG4 responses against RA that were negative before grass pollen immunotherapy.

In the present study we investigated a possible active induction of new IgE and IgG specificities against RA and other allergen extracts by grass pollen immunotherapy.

METHODS

Patients

Twenty-seven patients (eight males and 19 females; mean age 25.2 years), who were selected from an outpatient population, finished the study. The demographic and serologic characterization of these 27 patients is shown in Table 1.

Of 27 patients, 19 (70.37%) had symptoms of seasonal asthma and rhinitis; eight (29.63%) suffering from rhinitis during the pollen season.

Sensitization towards grass pollen allergens was measured by the skin prick test (SPT). All patients had additional SPT reactivities to one or more other allergen groups (i.e. tree, weeds, mites).

Study design

Treatment of the patients started in November 1998 and the study was continued until April 1999. Two serum samples were taken at the start of therapy (October/November) and preseasonally (April) in the first year of therapy. All patients were symptomless before and after immunotherapy.

All patients were treated with extracts obtained from the same company (Mix 2 grass: *Dactilis glomerata* and *Phleum pratense* conjugated with sodium-alginate; Conjuvac®; Stallergenes Italia, Saronno, Italy). Information regarding the concentration of allergen molecules in the extract in no longer available from the manufacturer. The induction phase was performed with once weekly injections of a depot preparation over 10 weeks, increasing to a maintenance treatment with 0.8 mL extract (1000 U/mL). The maintenance dose was given once every 3 weeks. The total dose administered to each patient was 4000 U/mL.

CAP tests

Blood samples were collected before the beginning of the treatment time and 5 months later. Sera were kept frozen until the end of the study and were then tested all together in one session for each antibody class. Total and specific IgE to timothy, mugwort, birch, olive tree, wall pellitory, mites, cat dander, *Alternaria tenuis*, *Cladosporium herbarum*, latex, rPhl p 1, rPhl p 2, rPhl p 5, rBet v 2, rBet v 1 were detected by an ImmunoCAP kit, which uses allergens covalently attached to a proprietary sponge, a solid, phase allergosorbent (Pharmacia & Upjohn, Uppsala, Sweden). Specific IgE levels were considered positive at a level of 0.35 kUA/L or higher (≥ class 1). Specific IgG4 to the same allergens was measured by the Pharmacia CAP System Specific IgG4 FEIA.

Assessment of eosinophil cationic protein

Eosinophil cationic protein (ECP) assessment was performed by radioimmunoassay (Pharmacia & Upjohn) according to the manufacturer's instructions. The normal range of ECP is 2.3–13.0 μg/L; total variation coefficients of the kit vary from 6.6 to 12%.

Assessment of myeloperoxidase

Myeloperoxidase (MPO) was measured in the serum by radioimmunoassay (Pharmacia & Upjohn) according to the manufacturer's instructions. The normal range of MPO is 170–470 μg/L.

Statistical analysis

Paired data were analyzed by Wilcoxon's two-tailed test. $P < 0.05$ was considered statistically significant.

RESULTS

The majority of grass pollen allergic patients (17 subjects; 63%) was cosensitized to rPhl p 1, rPhl p 2 and rPhl p 5. Sera from seven patients (30%) reacted to rPhl p 1 and rPhl p 5, but not rPhl p 2. Two sera (7.4%) reacted to rPhl p 1 but not to rPhl p 2 and rPhl p 5; one serum sample (3.7%) reacted to rPhl p 1 and rPhl p 2, but not to rPhl p 5. No change in total IgE levels, ECP and MPO levels was seen before and after immunotherapy (Fig. 1). This confirms the absence of allergen exposure of treated patients.

Similarly, no change in specific IgE levels to timothy, rPhl p 1, rPhl p 2, rBet v 2 and rBet v 1, was found. In contrast, a significant ($P = 0.042$) rise in allergen-specific IgE to rPhl p 5 was observed after treatment. A significant ($P = 0.003$) reduction in specific IgE to olive pollen due the treatment was found (Fig. 2). Interestingly, 10 of 10 patients with specific IgE towards a single recombinant allergen or two RA showed the same patterns of sensitization before and after immunotherapy (Table 1). We did not find significant variations of specific IgE to other allergens (data not shown).

Timothy grass pollen- and olive tree pollen-specific IgG4 directed against the four RA timothy and olive extracts was significantly raised in treated patients compared with values measured before the beginning of immunotherapy (Fig. 3). Taken together, these results indicate that after immunotherapy one can observe a general increase of IgG4 antibodies against pollen extracts and RA in grass pollen-allergic patients. Interestingly, we found a significant increase in levels of specific IgG4 to rBet v 2 and olive, but not to other aeroallergens (data not shown) after immunotherapy with *D. glomerata* and *P. pratense* extracts.

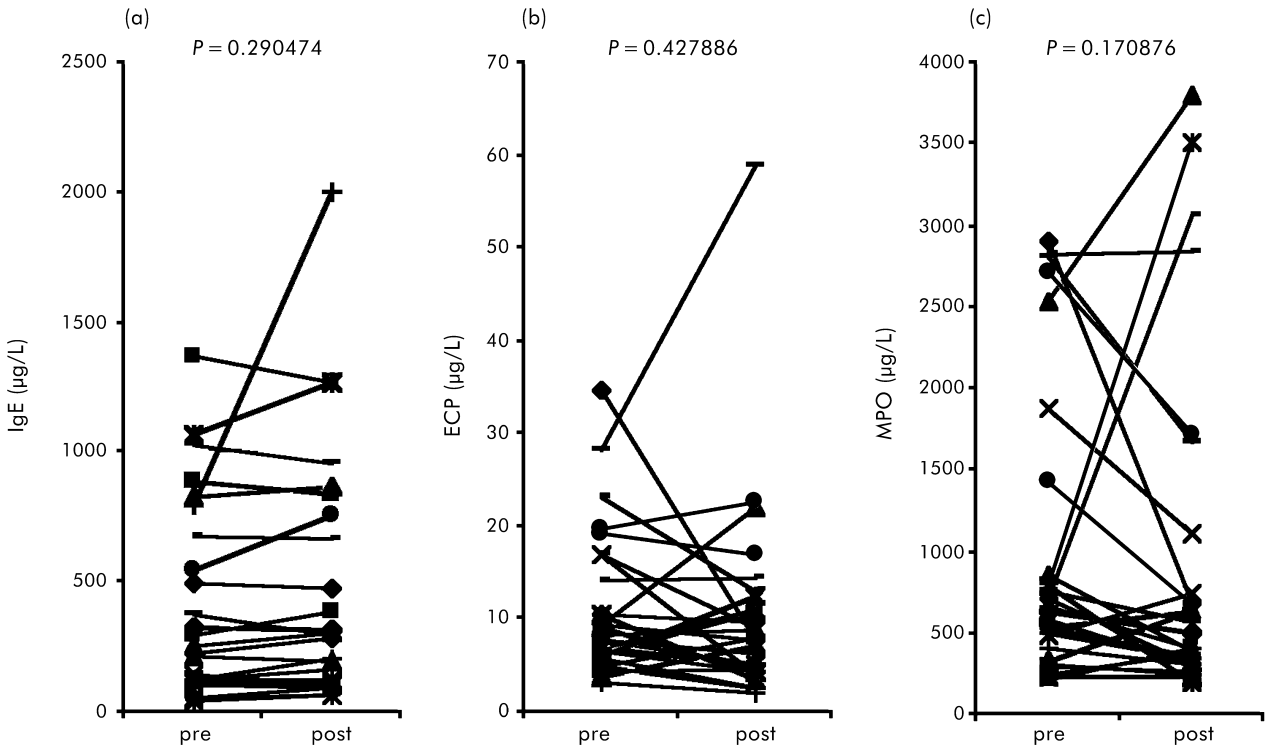

Fig. 1 Levels of (a) total IgE, (b) eosinophilic cationic protein (ECP) and (c) myeloperoxidase (MPO) before (November) and after (April) short-term immunotherapy.

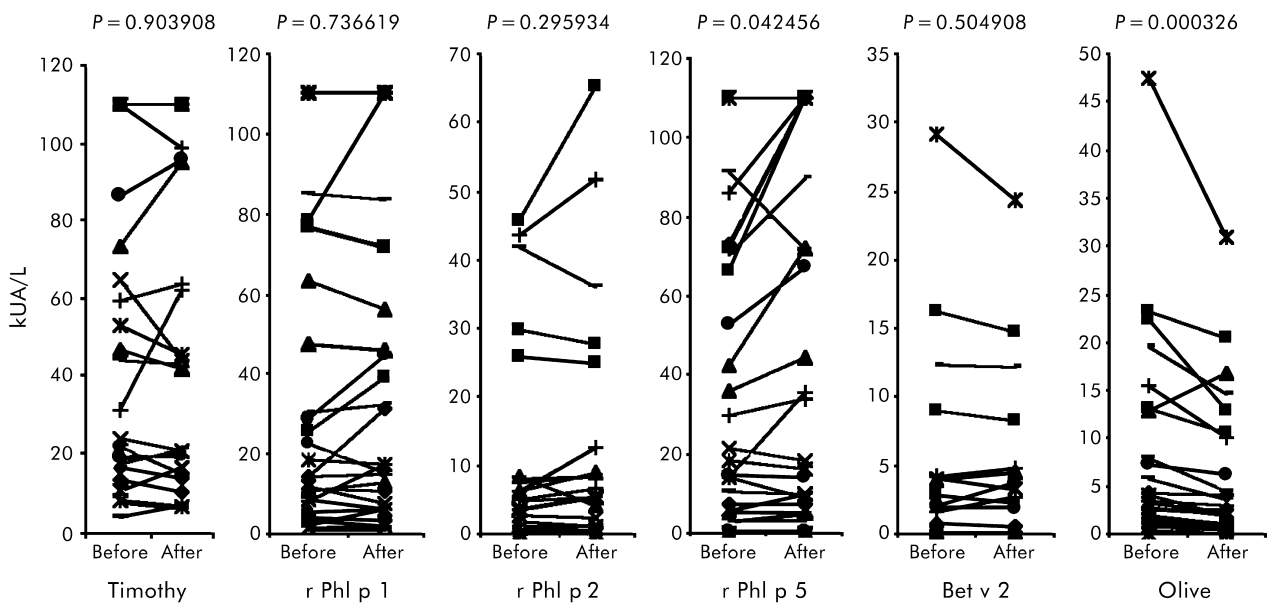

Fig. 2 Comparison of natural extract and recombinant allergen-specific IgE levels of treated patients obtained before (November) and after (April) immunotherapy.

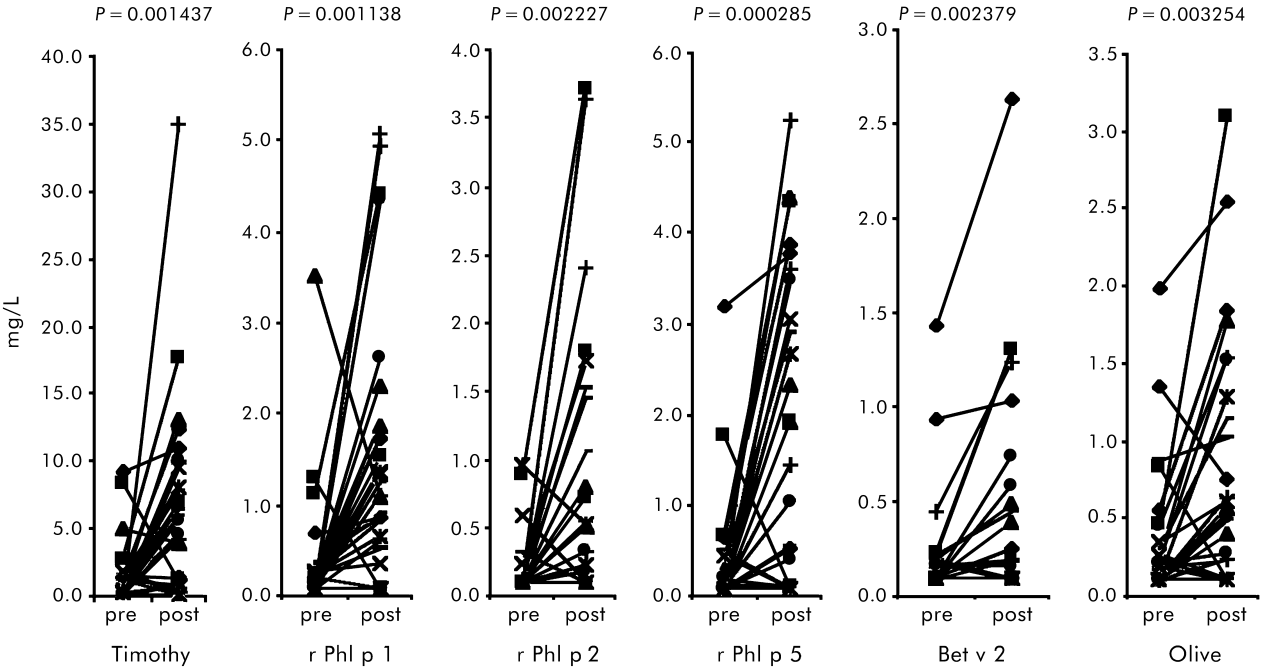

Fig. 3 Comparison of natural extract and recombinant allergen-specific IgG4 levels of treated patients obtained before (November) and after (April) immunotherapy.

Discussion

In the present study, we have shown that levels of rPhl p 1-, rPhl p 2-, rPhl p 5- and rBet v 2-specific IgG4 rose significantly after 5 months of grass pollen immunotherapy. In contrast, the levels of IgE against RA, except to rPhl p 5, remained unchanged. Therefore, in partial agreement with Gehlhar *et al.*,[10] we did not find any influence of immunotherapy on IgE levels. Interestingly, the patterns of IgE production to RA remained stable. In fact, 10 patients failed to mount IgE to RA not recognized before therapy. This may reflect the fact that the patients were treated for a brief period. In contrast, it may be argued that IgE production to individual RA may be under genetic control and, therefore, varying patterns of IgE against RA may represent stable phenotypes that are not conditioned by allergens administered. Van Ree *et al.*[5] have reported that a conversion from negative to positive was observed in 8% of patients. This phenomenon was also observed in sample taken > 1 year after the start of the therapy.[5] It could be argued that the appearance of IgE with specificities not detected before immunotherapy may be due to natural exposure. In a previous study, we have documented that 127 patients with a mean time of exposure to grass pollen of 26.6 years were lacking specific IgE towards particular RA. In some individuals, it is possible that changes in specific IgE during immunotherapy are unlikely to be a result of natural exposure.

Some studies have reported the occurence of new IgE specificities during immunotherapy.[5–11] Gehlhar *et al.* reported on a grass pollen-allergic patient who had no detectable IgE against group 5 allergens at the beginning and whose status was still unchanged 1 year later. This means that the application of allergens via immunotherapy does not necessarily lead to new sensitizations.[10] Recently, Valenta demonstrated a mutual boosting effect of pollen and latex sensitization *in vivo* that may be also operative in polysensitized plant-allergic patients.[12]

Moreover, we cannot exclude specific IgG4 interference on specific IgE binding. Depletion for IgG antibodies was not performed in the present study.

We found that a mixture of *D. glomerata* and *P. pratense* extracts triggered a significant increase in specific IgG4 antibodies to rBet v 2 and to olive. This could be due to the allergic patients' immune system that recognized profilin from *Phleum*.[13] Van Ree *et al*. had previously found that a major allergen isolated in *Lolium perenne* pollen (Lol p XI) showed a high degree of homology with the major olive pollen allergen Ole e 1.[14] Otherwise, Daschner *et al*. found a correlation between specific IgE

to profilin and specific IgE to *L. perenne*.[15] The same authors suggested that the sensitization with elevated specific IgE to olive pollen is an indicator of specific IgE to rBet v 2.[15]

Therefore, the appearance of IgG4 to olive not detected before immunotherapy, as observed in the present study, indicates that profilins are probably responsible for the cross-reactivities between different biologic sources.[16–18]

In a recent study, we found seasonal variation of IgE levels to RA and natural allergens,[19] as demonstrated in previous studies.[20] This could partially explain the observed off-season decrease in olive-specific IgE. However, the lack of reliable information about the contents of relevant allergens in the extract used for immunotherapy emphasizes the need for allergen extracts with definite molecular compositions. Moreover, IgE and IgG responses in allergic patients may also evolve in a non-sequential way and possess different affinities of fine specificities. Finally, perhaps some allergens may be less efficiently adsorbed in depot materials, resulting in variable induction of IgG or downregulation of IgE.

The main implications from the results of the present study are that: (i) 10 patients with a particular pattern of sensitization to RA before immunotherapy maintained the same pattern of sensitization after subcutaneous administration of 4000 U/mL grass extracts 5 months later; and (ii) the appearance of IgG4 with specificities not detected before immunotherapy and directed to timothy, rPhl p 1, rPhl p 2, rPhl p 5 and, interestingly, rBet v 2 and olive was observed after immunotherapy.

In conclusion, given that induction of IgE antibodies against new allergens in patients under immunotherapy was reported,[5,6] innovative forms of component-resolved immunotherapy[18–21] will be welcome.

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