ORIGINAL ARTICLE

Immunotherapy With a *Phleum pratense* Allergen Extract Induces an Immune Response to a Grass-Mix Allergen Extract

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Abstract

Background: Grass pollen allergy is one of the most common allergies worldwide, and patients often show sensitization to an array of phylogenetically related species.
Objective: To determine the effect of specific immunotherapy (SIT) with *Phleum pratense* extract on induction of the immune response to a mixture composed of 5 grass pollen extracts.
Methods: Forty-six adult patients suffering from rhinitis and sensitized to a mix of grass pollen allergen extracts were randomized 3:1 to receive a short course of SIT with *P pratense* or to an open control group without SIT. At baseline and after 3-4 months, we evaluated levels of specific immunoglobulin (Ig) E and IgG4, as well as the immediate and delayed cutaneous responses to the grass mix and *P pratense*. IgG4 to *Lolium perenne* was also determined.
Results: Levels of IgE and IgG4 to grass mix and *P pratense* increased significantly during treatment ($P<.001$). However, this increase was only significantly higher in the SIT group than in the control group for IgG4 ($P<.001$). The levels of IgG4 to Phl p 5 and Lol p 5 were highly correlated ($r=0.99$, $P<.001$). The immediate and delayed cutaneous responses were significantly diminished to both extracts after SIT ($P<.001$).
Conclusions: Patients with rhinoconjunctivitis diagnosed using skin prick testing with a grass mix allergen extract and treated with a short course of SIT based on a single species *P pratense* allergen extract are able to develop an immune response that targets not only the immunizing species, but also the grass mix allergen extract.

Key words: Allergen. Grass pollen. Cross-reactivity. Specific immunotherapy (SIT).

Resumen

Antecedentes: La alergia al polen de gramíneas es una de las alergias más comunes en el mundo y los pacientes muestran a menudo sensibilizaciones a especies filogenéticamente relacionadas.
Objetivo: Determinar el efecto de la inmunoterapia específica (ITE), con un extracto de polen de *Phleum pratense* en la inducción de la respuesta inmune a una mezcla de pólenes de cinco gramíneas.
Métodos: Cuarenta y seis pacientes adultos con rinitis sensibilizados a una mezcla de polen de gramíneas fueron aleatorizados 3:1 para recibir ITE con un extracto de polen *P pratense* o pertenecer a un grupo control sin ITE. Al inicio y después de 3-4 meses, se evaluaron la IgE e IgG4 específicas y la respuesta cutánea inmediata y tardía a la mezcla de gramíneas y a *P pratense*. También se midieron los niveles de IgG4 a *Lolium perenne*.
Resultados: La respuesta de IgE e IgG4 a la mezcla de gramíneas y a *P pratense* aumentó de forma estadísticamente significativa durante el tratamiento ($P<0,001$). Sin embargo, tan sólo para la IgG4 este aumento fue significativamente superior en los pacientes tratados en comparación con el grupo control ($P<0,001$). La correlación entre los niveles de IgG4 a Phl p 5 y Lol p 5 fue elevada (r de Pearson $=0,99$, $P<0,001$). Las respuestas cutáneas inmediata y tardía se redujeron significativamente después del tratamiento ($P< 0,001$).
Conclusiones: Los pacientes con rinoconjuntivitis diagnosticados con un extracto mezcla de gramíneas tratados con ITE con *P pratense*, son capaces de desarrollar una respuesta inmunológica dirigida no sólo a la especie inmunizante sino también a la mezcla de gramíneas.

Palabras clave: Alérgeno. Polen de gramíneas. Reactividad cruzada. Inmunoterapia específica (ITE).

Introduction

Specific immunotherapy (SIT) is the practice of administering an allergen to a sensitized patient in order to ameliorate symptoms and induce sustained, long-term clinical and immunological tolerance to the causative allergen. SIT has documented efficacy in a large number of well-designed studies with selected products [1-5]. The indication for SIT is based on the assumption that sensitization to an allergen plays an important role in eliciting symptoms. The selection of the allergen extract is based on the results of an exhaustive study of the patient's clinical history (potential exposure to allergens in a particular environment) and the demonstration of specific sensitization based on diagnostic tests. The standard panel of allergens for diagnostic purposes contains extracts from different sources. Patients are often not sensitized exclusively to a single species, but to a whole array of taxonomically related species and, frequently, to unrelated ones. Responses to taxonomically unrelated species in diagnostic tests may be due to true sensitizations to several agents, although in many patients they are due to the presence of cross-reactive panallergens—profilins [6], polcalcins [7], and lipid transfer proteins [8]—in the extracts. Another source of cross-reactivity comes from the presence of homologous allergens with high degrees of sequence identity in taxonomically related species [9]. *Phleum pratense* group 1 allergen (Phl p 1) shows a sequence identity of between 91% and 95% with group 1 allergens from other members of the Poaceae family [10], while the sequence identity for group 5 allergens is between 55% and 85% [11]. Consequently, both diagnostic tests and SIT are often performed with mixtures of extracts from several taxonomically related species, for example, from the pollen of temperate grasses. However, high sequence identity in major allergens could indicate that only one of the related species would be effective in the treatment of allergy in patients sensitized to all the related species. In the present study, patients diagnosed with allergy to grass pollens according to routine practice (clinical history and positive skin test result to a grass mix) were treated with an allergen extract of *P pratense*. We evaluated the effect of this extract on immediate and delayed cutaneous sensitivity. We also assessed levels of immunoglobulin (Ig) E and IgG4 to allergen extracts from a mix of grass pollen allergen extracts and *P pratense*, and IgG4 to group 5 grass allergens (Phl p 5 and Lol p 5).

Materials and Methods

Study Design

The study was a multicenter, open-label, controlled, randomized, parallel group trial approved by the Ethics Committees of the participating hospitals and by the Spanish Agency for Medicines and Health Care Products. Patients who gave their informed consent and fulfilled the inclusion criteria were randomized in a 3:1 ratio to a group receiving SIT with subcutaneous injections of a biologically standardized *P pratense* allergen extract (Pangramin Depot, ALK-Abelló, S.A., Madrid, Spain) or to a control group not receiving SIT. Patients were enrolled before the 2006 pollen season. SIT involved a 4-visit cluster up-dosing phase followed by 1 fortnightly dose and monthly maintenance doses of 2 μg of Phl p 5 major allergen (800 STU) for 2-3 months. The inclusion criteria were age between 18 and 55 years, clinical history of rhinoconjunctivitis with or without concomitant asthma to grass pollen, positive skin test results to a mix of grass pollen allergen extracts (*Dactylis glomerata, Festuca pratensis, Lolium perenne, P pratense*, and *Poa pratensis*), a negative pregnancy test result and commitment to use suitable contraception during the trial, and intention to complete the protocol. The exclusion criteria were a forced expiratory volume in 1 second (FEV_1) less than 80% of predicted, severe asthma, atopic dermatitis and/or contraindications to SIT according to the position paper of the European Academy of Allergy and Clinical Immunology (EAACI) [12], and previous SIT with grass allergens.

Evaluations

At baseline and 3-4 months after the beginning of treatment, patients were tested for immediate and delayed cutaneous reactivity to the grass pollen mix and to *P pratense* allergen extracts, and blood was taken for analysis of specific Ig. To evaluate the immediate response, patients underwent a skin prick test (ALK-Lancet, ALK-Abelló A/S, Hørsholm, Denmark) in duplicate with four 5-fold dilutions of the grass allergen mix and *P pratense* (100, 20, 4, and 0.8 μg/mL of grass group 5 or Phl p 5 allergen, respectively). Histamine 10 mg/mL and saline (ALK-Abelló, S.A Madrid, Spain) were used as controls. The results were read after 15 minutes and the contour of the wheal was outlined and transferred to a piece of paper for scanning and calculation of the area (SigmaScan Pro 5.0, SPSS Inc, Chicago, Illinois, USA).

The delayed cutaneous responses were evaluated using intradermal injection of 0.02 mL of the grass mix and *P pratense* solutions containing 0.1 μg/mL of grass group 5 or Phl p 5 allergens, respectively. The size of the indurations was read 6 hours after the injection and expressed as the mean diameter (largest + perpendicular) [13].

IgE and IgG4 specific to the grass mix and *P pratense* were analyzed by means of the CAP system (Phadia AB, Uppsala, Sweden) using the gx1 (grass mix) and *P pratense* allergens. In addition, IgG4 to Phl p 5 and Lol p 5 were analyzed using a monoclonal antibody–based enzyme-linked immunosorbent assay [14].

Statistical Analysis

Changes in immediate cutaneous response were evaluated using the parallel line assay [15,16] (AIASA CRS PLA, Alk-Abelló, S.A.) after a logarithmic transformation, according to the recommendation of the EAACI Subcommittee on Allergen Standardization and Skin Tests [17]. The cutaneous tolerance index (CTI) is the factor by which the concentration of an allergen extract has to be multiplied in order to achieve the same response after treatment as before. CTI values higher than 1 indicate a reduction in skin sensitivity to the allergen. Data from the delayed cutaneous response were analyzed using the *t* test. Concentrations of specific Ig were also analyzed using the *t* test, although after logarithmic transformation. Paired *t* tests

Table 1. Baseline Characteristics

		Intervention (n=33)	Control (n=13)	Total (N=46)	*P* Value[a]
Sex, No. (%)	Male	13 (39.4)	6 (46.2)	19 (41.3)	.746
	Female	20 (60.6)	7 (53.8)	27 (58.7)	
Asthma, No. (%)	Absent	13 (39.4)	7 (53.8)	20 (43.5)	.547
	Mild intermittent	16 (48.5)	5 (38.5)	21 (45.7)	
	Mild persistent	3 (9.1)	0 (0.0)	3 (6.5)	
	Moderate	1 (3.0)	1 (7.7)	2 (4.3)	
	Severe	0 (0.0)	0 (0.0)	0 (0.0)	
Rhinitis, No. (%)	Absent	0 (0.0)	0 (0.0)	0 (0.0)	.096
	Mild intermittent	11 (33.3)	2 (15.4)	13 (28.3)	
	Mild persistent	12 (36.4)	2 (15.4)	14 (30.4)	
	Moderate-severe intermittent	5 (15.2)	6 (46.2)	11 (23.9)	
	Moderate-severe persistent	5 (15.2)	3 (23.1)	8 (17.4)	
Conjunctivitis, No. (%)	Absent	3 (9.1)	0 (0.0)	3 (6.5)	.204
	Mild	18 (54.5)	4 (30.8)	22 (47.8)	
	Moderate	11 (33.3)	8 (61.5)	19 (41.3)	
	Severe	1 (3.0)	1 (7.7)	2 (4.3)	
Age, y (mean [SD])		31.4 (7.3)	29.5 (6.1)	30.9 (7.0)	.417

[a]Group comparison: χ^2 test, except age (*t* test).

were used to analyze within-group comparisons and the Pearson correlation was calculated after logarithmic transformation.

Results

Forty-six patients were randomized, 13 to the control group and 33 to the intervention group. There were no statistically significant differences between the groups in demographic and clinical data (Table 1). Three patients in the intervention group withdrew from the study before initiating SIT and 5 withdrew before completing treatment, for reasons unrelated to treatment except in 1 case (anaphylaxis, see below). Thirty patients received 317 SIT doses of the *P pratense* allergen extract and 25 patients who completed the treatment received a mean of 11.4 doses and a mean accumulated dose of 9.45 µg of Phl p 5 after an average of 3.6 months.

Immediate Cutaneous Response

SIT with *P pratense* significantly reduced the immediate cutaneous response to the grass mix in

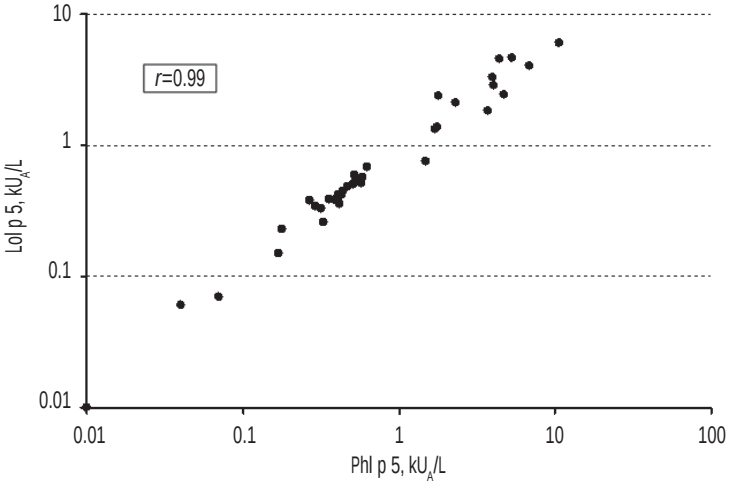

Figure. Correlation of IgG4 levels to Phl p 5 and Lol p 5 allergens after SIT. *r* indicates the Pearson correlation coefficient, *P*<.001.

the intervention group from baseline to the end of treatment (CTI, 2.93; 95% confidence interval [CI], 2.03-4.23; *P*<.01) (Figure). There were no significant changes in the control group. The behavior of the skin response to the *P pratense* allergen extract was similar (CTI, 3.45; 95% CI, 2.22-5.37; *P*<.01). As a consequence of these changes, the intervention group had a significantly lower cutaneous response after SIT than the control group, both to the grass mix (CTI, 2.56; 95% CI, 1.16-5.88; *P*<.05) and to *P pratense* (CTI 4.55; 95% CI, 2.17-11.1; *P*<.01).

Delayed Cutaneous Response

At baseline, 86% of the patients experienced a late-phase skin reaction to the intradermal test. Patients in the treatment group experienced a significantly reduced delayed cutaneous response after SIT compared with baseline, both to the grass mix and *P pratense* (*P*<.001 for both extracts). Patients in the control group did not show a significant reduction in the delayed skin reaction (Table 2) and there were no differences between the size

Table 2. Delayed Cutaneous Response After Intradermal Testing With a *Phleum pratense* Extract and a Grass-Mix Extract[a]

	Intervention (n=25)			Control (n=12)			Comparison		
	Before SIT	After SIT	*P* Value[a]	Before SIT	After SIT	*P* Value[b]	*P* Value[c]	*P* Value[d]	*P* Value[c]
Phleum pratense	38.4 (27.0)	16.9 (18.6)	<.001	36.8 (28.6)	33.1 (27.0)	.558	.837	.021	.013
Grass mix	34.5 (28.5)	15.9 (17.5)	<.001	33.9 (29.6)	30.5 (24.5)	.631	.843	.015	.050

Abbreviation: SIT, specific immunotherapy.
[a]Values are expressed in mm as mean (SD). All *P* values correspond to the *t* test.
[b]Paired (before SIT–after SIT).
[c]Differences between groups before SIT.
[d]Differences between groups after SIT.
[e]Differences between groups in the change in delayed response.

Table 3. Values of Immunoglobulin E and G4 to *Phleum pratense* and Grass Mix[a] (*Dactylis glomerata, Festuca pratensis, Lolium perenne, Phleum pratense,* and *Poa pratensis*)

	Intervention (n=25)			Control (n=13)			Comparison		
	Before SIT	After SIT	Change[b]	Before SIT	After SIT	Change[b]	Before SIT[c]	After SIT[c]	Change[d]
IgE grass mix (kU_A/L)	11.3 (5.6-22.7)	35.3 (18.4-67.7)	<.001	13.2 (5.6-30.7)	27.7 (10.1-75.5)	.012	0.793	.682	.224
IgE *Phleum pratense* (kU_A/L)	15.5 (8.1-29.7)	44.9 (23.3-86.3)	<.001	18.2 (7.7-43.2)	36.3 (13.2-99.4)	.010	0.777	.721	.237
IgG4 grass mix (mgA/L)	0.17 (0.10-0.29)	1.16 (0.63-2.12)	<.001	0.12 (0.06-0.23)	0.17 (0.10-0.32)	.036	0.449	<.001	<.001
IgG4 *Phleum pratense* (mgA/L)	0.36 (0.25-0.50)	1.64 (1.01-2.68)	<.001	0.27 (0.19-0.38)	0.37 (0.27-0.50)	.047	0.320	<.001	<.001

Abbreviation: Ig, immunoglobulin; SIT, specific immunotherapy.
[a]Values are expressed as geometric mean (95% confidence interval)
[b]*P* values correspond to paired *t* test.
[c]*P* values correspond to differences between groups (*t* test).
[d]*P* values correspond to differences between groups in the change in Ig values (*t* test).

of the late-phase reaction elicited by the grass mix and *P pratense*, and the size of the reduction in the skin reaction to both extracts.

Specific Immunoglobulins

The IgE level to the grass mix and *P pratense* allergen extracts increased slightly but significantly in the intervention group after SIT and also in the control group ($P<.001$ in both groups), with no differences in the degree of change between the groups. The IgG4 values to both allergen extracts were also higher in both groups, but significantly ($P<.001$) higher in the intervention group (Table 3). The IgE response to grass mix and *P pratense* was similar, although in the intervention group, levels of IgG4 to *P pratense* increased more than those of IgG4 to the grass mix ($P<.001$). The IgG4 level for Phl p 5 and Lol p 5 after SIT showed a correlation of ($r=0.99$, $P<.001$, Figure).

Adverse Events

Thirty-seven adverse events were reported, all in the intervention group: 15 were related to SIT and appeared in 9 patients after 10 doses, all during the up-dosing phase. Two of the reactions were nonspecific, 7 were local, 4 affected the upper airway and 1 the lower airway, and 1 was an anaphylactic reaction consisting of asthma and pruritus of the ear canal and palate immediately after injection of 0.4 mL (vial 3, 1 µg Phl p 5). Although the pruritus resolved rapidly after treatment with epinephrine and oral corticosteroids, it led the patient to withdraw from the study. Only 3 of the remaining adverse reactions (2 moderate rhinoconjunctivitis and 1 nasal congestion) required treatment with oral antihistamines.

Discussion

Grass pollen allergy, one of the most common types of allergy worldwide, is caused by a number of different grass species that coexist geographically, especially in temperate areas, giving rise to multiple sensitizations in allergic individuals. In addition, the homogeneity of grass allergens in phylogenetically related species can generate high levels of cross-reacting antibodies, which bind to common epitopes within homologous allergens [18,19]. Several studies report high levels of cross-reactivity, both in terms of IgE as measured using radioallergosorbent test inhibition [20], antisera raised in animals [21], and monoclonal antibodies [22], and in terms of T-cell reactivity [23] between the allergens present in different grass species, especially those species belonging to the same subfamily, eg, Pooideae. Therefore, some authors have proposed simplification of in vitro diagnosis and SIT by using only 1 species [24-26] instead of mixtures of related species, because of the inherent benefits in the development of better defined and controlled products. In properly conducted clinical trials, SIT with a single allergen extract (*P pratense*) has demonstrated its efficacy both subcutaneously [3] and sublingually [27,28].

The aim of this study was to determine whether the immune response to SIT targets not only the immunizing allergen extract, but also other related grass species. Allergic patients with a clinical history of seasonal rhinitis due to grass pollen sensitization and diagnosed by skin prick testing and specific IgE to a mix of grass pollen extracts were randomized to a control group (symptomatic drugs only) or to receive treatment with SIT with only 1 grass species, *P pratense*. The patients live in the center of Spain, an area in which *P pratense* is practically absent. Therefore, exposure to grass pollens is primarily due to other grass species.

We used a grass-mix allergen extract that is widely used in our setting for diagnosis and SIT. Although the grass mix contains the immunizing allergen *P pratense*, this species is responsible for only 20% of the allergen content of the extract. In these circumstances, we found that a short course of SIT with *P pratense* induced changes in serum IgE and IgG4 levels, both in the immediate and delayed cutaneous reactions to the grass-mix allergen extract, and in the reaction to the *P pratense* allergen extract.

We recorded specific IgE and IgG4 levels because of their importance in establishing an immune response in successful immunotherapy. IgG4 blocking antibodies can participate in the inhibition of the allergen-induced release of inflammatory mediators from basophils and mast cells, and in the prevention of IgE-facilitated antigen presentation to T cells, thus reducing IgE production and immediate and late inflammatory responses [29-31]. As for skin reactions to allergens, it is well known that SIT diminishes the immediate [32,33] and delayed [5] cutaneous response.

As expected, levels of specific IgE and IgG4 to *P pratense* and grass mix increased after the short course of SIT. The increase in the control group could be ascribed to the exposure to pollen during the season and/or to the effect of the intradermal injection of the 2 allergen extracts. Significant differences between groups after SIT and in the change from baseline to end of treatment values were only seen in IgG4. In order to distinguish whether the IgG4 response was to the *P pratense* allergens present in the allergen mix or to other allergens, we established a correlation between IgG4 levels to the major allergens of *P pratense* and *L perenne*. The positive and high correlation found shows that the IgG4 induced by SIT was able to bind Phl p 5 and Lol p 5 in an almost identical way, indicating that the response induced by SIT with one grass (*P pratense*) is recognized by allergens from a related grass species. The cutaneous response was significantly diminished in both the immediate and delayed reactions, thus resulting in significant differences between the intervention and control groups after treatment.

Few side effects were observed during SIT, and their nature and onset were as expected. All adverse events appeared during the up-dosing phase, and the only severe event was an anaphylactic reaction (asthma and pruritus of the ear canal and palate), which resolved rapidly after treatment with epinephrine and oral corticosteroids. The possibility that subcutaneous SIT triggers this kind of reaction, which can be controlled easily in properly equipped centers, necessitates measures such as a 30-minute observation period in the center after each injection.

In conclusion, patients with seasonal allergic rhinitis

confirmed by positive results after testing with a 5-grass mix (including *P pratense*) respond to SIT based on a single grass allergen extract SIT with a modification of the immune response to the grass mix allergens, even in the absence of natural exposure to the allergen in the vaccine. SIT based on a single species could eliminate the need for allergen mixtures in favor of better-defined products.

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