

Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations

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Background: Complementary DNAs coding for the major timothy grass pollen (*Phleum pratense*) allergens Phl p 1, Phl p 2, and Phl p 5 and birch profilin were isolated, expressed as recombinant nonfusion proteins in *Escherichia coli*, and purified.

Objective: In this study the *in vitro* IgE-binding capacity of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin and their IgE recognition frequencies were investigated by using sera from different populations.

Methods: One hundred eighty-three sera from patients allergic to grass pollen were obtained from different populations in Europe, Japan, and Canada. The sera were selected according to clinical criteria, skin testing, and RAST (CAP system; Pharmacia, Uppsala, Sweden) and then tested for IgE reactivity with natural and purified recombinant timothy grass pollen allergens by ELISA and Western blot.

Results: Most (94.5%) of the patients allergic to grass pollen could be diagnosed with a combination of recombinant Phl p 1, Phl p 2, Phl p 5, and profilin by means of ELISA. Sera that did not react with the recombinant allergens contained low levels of timothy grass pollen-specific IgE. Although considerable variability in IgE recognition frequency of the recombinant allergens was observed in certain populations, a good correlation was found between natural timothy CAP results and the combination of recombinant allergens in all 183 tested sera ($r = 0.87$).

Conclusions: Despite considerable variability in the IgE recognition frequency, purified recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5) and profilin permitted successful *in vitro* diagnosis of grass pollen allergy in 94.5% of allergic individuals from different populations. The addition of other recombinant allergens (e.g., recombinant Phl p 4) would only slightly improve the *in vitro* test sensitivity. (*J Allergy Clin Immunol* 1996;98:652-8.)

Key words: Population study, recombinant timothy grass pollen allergens, Phl p 1, Phl p 2, Phl p 5, profilin, *in vitro* diagnosis, grass pollen allergy

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Abbreviations used

BSA:	Bovine serum albumin
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate–polyacryl- amide gel electrophoresis

Up to 40% of patients with type I allergy display IgE reactivity to grass pollen allergens.¹ In previous studies the isolation of complementary DNAs coding for three major timothy grass (*Phleum pratense*) allergens, Phl p 1, Phl p 2, and Phl p 5 was reported.²⁻⁴ The recombinant timothy grass pollen allergens were expressed in *Escherichia coli* as β -galactosidase fusion proteins and shown to bind IgE antibodies from patients allergic to grass pollen.^{5,6} Purified recombinant timothy grass pollen allergens induced specific and dose-dependent histamine release from basophils of patients allergic to grass pollen,⁷ and T-cell clones with specificity for recombinant Phl p 1 were established.⁸ The IgE-binding capacity of recombinant timothy grass pollen allergens was investigated by IgE absorption studies, indicating that a combination of recombinant Phl p 1, Phl p 2, and Phl p 5 can bind a high proportion of grass pollen-specific IgE.⁶ It was further demonstrated that recombinant Phl p 1 and Phl p 5 share most IgE epitopes with naturally occurring isoallergenic variants.^{4,9}

Assuming that patients from different countries are sensitized to different grass pollen species, this study raises the question of whether the given panel of recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5) and recombinant birch profilin (Bet v 2),^{10,11} which share IgE epitopes with timothy grass profilin,¹² can be used for in vitro allergy diagnosis in different populations. For this purpose, 183 sera from individuals allergic to grass pollen from different parts of Europe, Canada, and Japan were tested with recombinant Phl p 1, Phl p 2, Phl p 5, profilin, and natural timothy grass pollen extract by using ELISA, immunoblotting, and the CAP assay (Pharmacia, Uppsala, Sweden).

METHODS

Natural timothy grass pollen extracts and recombinant allergens

Timothy grass (*Phleum pratense*) pollen was purchased from Allergon, Valinge, Sweden. Protein extracts were prepared by shaking 2 gm of pollen in 50 ml of distilled water for 2 hours at 4°C. The extract was centrifuged at 20,000 g for 30 minutes to remove insol-

uble particles.¹³ The supernatant was then divided into aliquots, frozen in liquid nitrogen, and lyophilized. Lyophilized extracts were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)¹⁴ and Coomassie Brilliant Blue (BioRad, Richmond, Calif.) staining¹⁵ for quality and estimation of protein content and were stored at –20°C until use.

Purified recombinant major timothy grass pollen allergens, Phl p 1, Phl p 2, and Phl p 5 were obtained by expression of the corresponding cDNAs in *E. coli* BL21 (DE3) by using a T7 polymerase-based expression system as described previously.⁷ Recombinant allergens that were more than 95% pure and soluble were prepared by conventional protein chemical techniques and stored in lyophilized form until use.⁷ Instead of purified recombinant timothy grass profilin (Phl p 11),¹² recombinant birch profilin (Bet v 2) was expressed in *E. coli* and purified as described previously.^{10,11,16}

Sera from patients allergic to grass pollen

Sera from patients allergic to grass pollen were obtained from different parts of Europe, comprising Austria ($n = 29$), Denmark ($n = 26$), France ($n = 38$), Sweden ($n = 35$), and Switzerland ($n = 20$), as well as from Japan ($n = 20$) and Canada ($n = 15$). Sera were selected as being grass pollen-positive according to a positive case history (at least one of the symptoms: rhinitis, conjunctivitis, or asthma during grass pollen season) indicative of seasonal grass pollen allergy and positive skin prick test results with grass pollen extracts.

Measurement of total serum IgE and timothy grass pollen-specific IgE (CAP, IgE immunoblots)

Total serum IgE was measured by radioimmunoassay (RIACT; Pharmacia) with the same serum samples as for immunoblotting, timothy grass pollen CAP (Pharmacia), and ELISA. Timothy grass pollen-specific IgE was determined with the CAP system. For immunoblot detection, natural timothy grass pollen extract was separated by denaturing SDS-PAGE at a concentration of approximately 200 μ g/cm preparative gel, according to the method of Fling and Gregerson,¹⁴ and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany).¹⁷ Nitrocellulose sheets were then cut into strips of approximately 0.6 cm in width, which were incubated two times for 5 minutes and once for 30 minutes in buffer A (50 mmol/L sodium phosphate [pH 7.5], 0.5% vol/vol Tween-20, 0.5% wt/vol bovine serum albumin [BSA], and 0.05% wt/vol NaN₃). Strips were then incubated with sera diluted 1:10 in buffer A overnight at 4°C, washed as for blocking, and incubated with iodine 125-labeled anti-human IgE (Pharmacia) overnight at room temperature. Strips were then washed as described previously, dried, and exposed to Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany) for 24 to 48 hours. Sera that showed no reactivity to

recombinant allergens in the ELISA were also tested with nitrocellulose-blotted recombinant allergens by using the radioactive detection system described previously.

IgE reactivity to recombinant allergens determined by ELISA

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 100 μ l (2 ng/ μ l) of purified recombinant Phl p 1, Phl p 2, Phl p 5, and Bet v 2 nonfusion proteins, respectively, overnight at 4° C. Plates were washed twice with 200 μ l of phosphate-buffered saline (PBS), 0.05% (vol/vol) Tween-20 per well and were incubated with 200 μ l of PBS, 0.05% (vol/vol) Tween-20, 1% (wt/vol) BSA for 2.5 hours at room temperature to block the nonspecific binding sites. Patients' sera were diluted 1:10 in PBS, 0.05% (vol/vol) Tween-20, 0.5% (wt/vol) BSA and were added. After incubation at 4° C overnight, plates were washed five times with 200 μ l of PBS, 0.05% (vol/vol) Tween-20. A monoclonal mouse anti-human IgE antibody (Pharmingen, San Diego, Calif.), diluted 1:1000 in PBS, 0.05% (vol/vol) Tween-20, 0.5% (wt/vol) BSA was added. After overnight incubation at 4° C, plates were washed as described above. Plates were incubated with a sheep anti-mouse antibody coupled to peroxidase (Amersham, Buckinghamshire, U.K.), diluted 1:2000 in PBS, 0.05% (vol/vol) Tween-20, 0.5% BSA for 30 minutes at 37° C and for 30 minutes at 4° C. Plates were washed as described above. 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) solution (Amersham) was added. After incubation for 15 to 30 minutes at room temperature, the reaction was stopped by addition of 0.32% NaF. The optical density was determined with an ELISA reader (Dynatech, Denkerdorf, Germany).

Statistical analysis of the data

The following parameters were correlated by using the EXCEL 5.0 MSDOS program: total IgE levels (in kilounits per liter); RAST timothy grass pollen (in kilounits per liter); levels of IgE specific for recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin determined as optical densities by ELISA. For values above or below a certain limit, the exact value was analyzed.

RESULTS

Determination of total IgE levels and timothy grass pollen-specific IgE by CAP

Total IgE levels between 20 kU/L and greater than 2000 kU/L could be measured in the different sera. No correlation of total IgE levels and timothy grass pollen-specific IgE levels was found ($r = 0.44$). Sera with high total IgE levels but low timothy grass pollen-specific IgE levels were identified, as well as sera with high total IgE and high levels of timothy grass pollen-specific IgE. Several sera from patients with positive case history and

positive skin prick test results showed low levels of timothy grass pollen-specific IgE. Tables containing the total IgE levels and timothy grass RAST determinations are available on request from the corresponding author.

IgE reactivity with nitrocellulose-blotted timothy grass pollen allergens

Sera were analyzed for IgE reactivity to nitrocellulose-blotted natural timothy grass pollen allergens to identify IgE-binding components that migrate at molecular weights, which are different from those of the recombinant allergens included in the study. Among these components, group 4 grass pollen allergens migrated at molecular weights between 50 and 68 kd,¹⁸ and unidentified components migrated at approximately 20 kd and at less than 10 kd. Thirty percent of all sera reacted with bands between 50 and 68 kd, presumably representing group 4 allergens. Fifty-seven percent of the sera reacted with components at approximately 20 kd, and 5% bound to bands smaller than 10 kd. Sera that showed little or no IgE reactivity to nitrocellulose-blotted natural timothy grass pollen allergens also had low timothy grass pollen-specific IgE levels as measured by CAP assay. This indicates that most of the IgE epitopes were not severely affected by the SDS-PAGE procedure and subsequent Western blotting.

Determination of the IgE reactivity profiles in different populations with purified recombinant timothy grass pollen allergens and recombinant birch profilin

The analysis of the patients' IgE reactivity profiles with the recombinant allergens identified Phl p 1 and Phl p 5 as the most frequently recognized allergens in all populations tested. The ELISAs indicated that, on average, Phl p 1 and Phl p 5 had induced high levels of specific IgE, whereas most sera contained much less Phl p 2- and profilin-specific IgE. In accordance with this observation, the levels of timothy grass pollen-specific IgE, as determined by CAP assay, correlated significantly with the levels of Phl p 1-specific ($r = 0.84$) and Phl p 5-specific ($r = 0.74$) IgE. Little or no correlation was observed with the Phl p 2-specific ($r = 0.64$) and profilin-specific ($r = 0.24$) IgE levels. Phl p 1 and Phl p 5 were hence not only identified as major allergens, which are recognized by IgE antibodies of a high percentage of patients allergic to grass pollen, but also bound a high percentage of grass pollen-specific IgE antibodies.

TABLE I. Frequency of IgE binding to natural and recombinant allergens in different population groups and in all sera

	Austria (%)	Denmark (%)	France (%)	Sweden (%)	Switzerland (%)	Canada (%)	Japan (%)	Total (%)
rPhl p 1	100	96	95	86	70	93	65	88
rPhl p 2	69	77	58	34	30	20	10	46
rPhl p 5	93	92	68	63	60	67	45	71
rBet v 2	14	12	8	11	15	13	5	11
Phl p 4*	45	58	58	51	45	13	20	30
20 kd*	90	81	50	49	50	33	45	57
<10 kd*	0	0	11	6	20	0	0	5

r, Recombinant; rBet v 2, recombinant birch profilin.

*IgE reactivity to natural nitrocellulose-blotted timothy grass pollen allergens.

Sera from different populations show different IgE reactivity profiles

A remarkable variability in IgE recognition frequency of the recombinant allergens was observed in the different populations. Table I summarizes the IgE recognition frequency of the recombinant allergens and selected natural allergens in the different populations tested. The percentage of sera reactive with recombinant Phl p 1 ranged from 65% to 100%; for Phl p 2, from 10% to 77%; for Phl p 5, between 45% and 93%; and for profilin, between 5% and 15%. The cDNAs coding for the recombinant allergens were isolated with serum IgE from Austrian patients, and in fact, the highest recognition frequencies were observed in the Austrian population. However, in most populations recombinant Phl p 1 and Phl p 5 were detected by a comparably high percentage of sera. Recombinant Phl p 2 was even more frequently detected in the Danish group. The most different detection frequencies were found for recombinant Phl p 2, ranging from 10% to 77%.

Specificity of the in vitro IgE reactivity to purified recombinant allergens: Comparison of total IgE levels and IgE specific for recombinant allergens

The reactivity to grass pollen extracts and to individual recombinant allergens was not always associated with elevated total serum IgE levels. For example, a Swedish serum sample (no. 17) had a high total IgE level of more than 2000 kU/L and displayed specificity only for recombinant Phl p 1 and weak reactivity to recombinant Phl p 2. No reactivity with recombinant Phl p 5 or profilin was observed. On the other hand, a Danish serum sample (no. 23) with a similar high total IgE value reacted specifically with recombinant Phl p 5. Tables containing detailed information regarding

the IgE levels specific for the recombinant allergens are available on request from the corresponding author.

In fact, total IgE levels were found to be neither correlated with timothy grass pollen-specific IgE ($r = 0.44$) nor with the IgE reactivity to a combination of all recombinant allergens ($r = 0.51$). In contrast, an excellent correlation of the timothy grass pollen-specific IgE levels and the IgE directed against a combination of recombinant allergens was found ($r = 0.87$).

A combination of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin allowed in vitro allergy diagnosis in different populations in a comparable manner

Despite the variability of recognition frequencies in the different populations, a combination of the recombinant allergens permitted in vitro diagnosis of grass pollen allergy in 94.5% of 183 patients with an ELISA. Those sera that could not be identified with the combination of recombinant allergens in the ELISA had timothy grass pollen CAP levels of less than 3.88 kU/L (Table II).

Analysis of sera that contained no ELISA-detectable IgE against recombinant allergens

The analysis of those sera that were negative with all recombinant allergens in the ELISA revealed that the timothy grass pollen-specific IgE levels were very low (Table II). Only two of the negative sera bound to nitrocellulose-blotted Phl p 4 at 54 to 68 kd. The addition of a recombinant Phl p 4 would hence increase the test sensitivity from 94.5% to 95.6%. Four patients with negative ELISA results displayed IgE reactivity to nitrocellulose-blotted recombinant allergens when the more sensitive detection system, based on

TABLE II. Analysis of sera without ELISA-detectable IgE against recombinant allergens

Patient	Total IgE (kU/L)	RAST timothy (kU/L)	ELISA				Immunoblot					
			rPhl p 1 OD	rPhl p 2 OD	rPhl p 5 OD	rBet v 2 OD	54-68 kd	20 kd	<10 kd	rPhl p 1	rPhl p 2	rPhl p 5
France												
2	182	0.43	0.02	0.01	0.01	0.00	—	—	—	—	—	—
3	255	0.75	0.04	0.02	0.01	0.02	—	—	—	—	—	±
Japan												
4	38.2	1.62	0.04	0.01	0.02	0.03	—	—	—	ND	ND	ND
7	1083	1.28	0.01	0.01	0.02	0.01	+	—	—	—	—	—
15	199	1.99	0.01	0.00	0.04	0.01	—	—	—	ND	ND	ND
Sweden												
4	255	1.32	0.04	0.01	0.00	0.00	+	—	—	—	—	—
Switzerland												
9	194	1.69	0.03	0.02	0.02	0.00	—	—	—	—	+	—
10	61.6	2.78	0.03	0.03	0.04	0.00	—	—	—	±	±	+
15	212	3.88	0.03	0.03	0.03	0.01	—	—	—	+	+	—
20	213	<0.35	0.01	0.01	0.02	0.00	—	—	—	—	—	—

OD, Optical density; ND, not done.

¹²⁵I-labeled anti-human IgE antibodies, was used (Table II). Those four patients who had finally negative results with the combination of recombinant allergens had timothy grass pollen CAP levels of less than 1.99 kU/L.

DISCUSSION

In this study we have tested 183 sera from patients allergic to grass pollen from different populations by using a panel of purified recombinant timothy grass pollen allergens, Phl p 1,² Phl p 2,³ Phl p 5,⁴ and recombinant birch profilin (Bet v 2),^{10,11} which share IgE epitopes with timothy grass profilin.¹² Sera were obtained from different parts of Europe, Canada, and Japan. Although in a previous study it could be demonstrated that a panel of a few recombinant timothy grass pollen allergens expressed as β -galactosidase fusion proteins could be used for successful in vitro diagnosis of grass pollen allergy in a group of Austrian patients,⁶ nothing was known about possible differences in the IgE recognition patterns in sera from different populations. Assuming that because of geographic differences patients from different countries were exposed and sensitized to different grass species, we questioned whether the sensitization to different grass species might affect the IgE reactivity to a given panel of recombinant timothy grass pollen allergens, which were isolated with Austrian sera.

Although remarkable differences in IgE recognition frequency to certain recombinant allergens were noted in the different populations (e.g., 77%

of the Danish sera were positive with Phl p 2, whereas only 10% were positive in the Japanese group), the combination of the four recombinant allergens was comparably useful to diagnose grass pollen allergy in all tested populations. One explanation for the different recognition frequencies might be that because of the different content of certain allergen groups in different grass species, patients were sensitized differently. Alternatively, it is possible that, depending on geographic differences, certain grass species (e.g., timothy grass) express different amounts of allergens.

It was further noted that certain recombinant grass pollen allergens, such as Phl p 5 and Phl p 1, bound high levels of timothy grass pollen-specific IgE in all study groups, whereas rather low levels of IgE were directed against allergens such as Phl p 2 and Bet v 2. This indicates that certain allergens possess a high capacity to induce IgE antibodies, whereas others are apparently less allergenic. The observation is corroborated by the good correlation of Phl p 1- and Phl p 5-specific IgE with the IgE levels measured against total timothy grass pollen allergens. In fact, immunization of mice with different recombinant allergens (among them: Phl p 1, Phl p 2, Phl p 5, and Bet v 2) had also indicated that Phl p 5 and Phl p 1 induced high levels of IgE, whereas the other allergens were less immunogenic.¹⁹ A similar observation was also made when the allergenicity of the major birch pollen allergen, Bet v 1,²⁰ was compared with that of birch profilin (Bet v 2) in mice and rhesus monkeys.²¹ As in different populations of patients

allergic to tree pollen,^{22, 23} Bet v 1 induced much higher levels of IgE than Bet v 2. It is hence suggested that allergens be characterized not only in terms of the percentage of reactive patients as major, intermediate, or minor allergens but also with attention to the levels of IgE, which are directed against certain components. Allergens might therefore be characterized as high, medium, and low IgE binders or inducers according to the percentage of specific IgE directed against the total extract ($\geq 50\%$ = high, $\geq 25\%$ = medium, $< 25\%$ = low).

The analysis of the sera (10 of 183) from patients who were not diagnosed by means of ELISA with the panel of recombinant allergens revealed that all negative sera contained very low levels (< 3.88 kU/L) of timothy grass pollen-specific IgE, and six of the 10 sera showed no IgE reactivity to nitrocellulose-blotted timothy grass pollen extracts. It is interesting to note that positive immunoblot results were obtained even in patients with very low timothy grass pollen levels as determined by CAP assay. Overall, there was no evidence for loss of IgE epitopes caused by SDS-PAGE and immunoblotting. It is conceivable to assume that those sera that were not detected with the recombinant allergens and showed low reactivity with timothy grass pollen extracts were incorporated in the study because of positive skin prick test results, which are much more sensitive than serologic assays. In fact, four of the 10 ELISA-negative sera did contain IgE specific for recombinant allergens when tested with a more sensitive assay based on ¹²⁵I-labeled anti-human IgE antibodies.

This study indicated that the addition of, for example, a recombinant group 4 allergen would increase the percentage of diagnosed sera only from 94.5% to 95.6% and hence would have rather marginal effects on the sensitivity of a combined test.

In conclusion, recombinant timothy grass pollen allergens, in combination with recombinant Bet v 2, allowed in vitro diagnosis of grass pollen allergy in different populations of patients in a comparable way. Grass pollen allergy was successfully diagnosed in almost 95% of the patients; and their qualitative, as well as quantitative, IgE reactivity pattern (allergogram) could be determined. According to the notion that a rather limited panel of epitopes was necessary to diagnose grass pollen allergy, it is conceivable to assume that recombinant timothy grass pollen allergens may contribute to a component-based allergy diagnosis system, which can be applied in different populations,

regardless of the original source of sensitizing grass pollen species, and perhaps later for a component-based form of hyposensitization treatment.

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