

B cell epitopes of the major timothy grass pollen allergen, Phl p 1, revealed by gene fragmentation as candidates for immunotherapy

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ABSTRACT Group 1 grass pollen allergens are recognized by IgE antibodies of almost 40% of allergic individuals and therefore belong to the most important elicitors of Type I allergy worldwide. We have previously isolated the cDNA coding for the group 1 allergen from timothy grass, Phl p 1, and demonstrated that recombinant Phl p 1 contains most of the B cell as well as T cell epitopes of group 1 allergens from a variety of grass and corn species. Here we determine continuous B cell epitopes of Phl p 1 by gene fragmentation. IgE antibodies of grass pollen allergic patients identified five continuous epitope-containing areas that on an average bound 40% of Phl p 1-specific IgE antibodies and were stably recognized in the course of disease. In contrast to untreated patients, patients undergoing grass pollen immunotherapy started to mount IgG₄ antibodies to the recombinant IgE-defined fragments in the course of immunotherapy. The protective role of these IgG₄ antibodies is demonstrated by observations that 1) increases in rPhl p 1 fragment-specific IgG₄ were in parallel with decreases in Phl p 1-specific IgE, and 2) preincubation of rPhl p 1 with patients sera containing rPhl p 1 fragment-specific IgG₄ blocked histamine release from basophils of an untreated grass pollen allergic patient. We propose to use recombinant Phl p 1 fragments for active immunotherapy in order to induce protective IgG responses against IgE epitopes in grass pollen allergic patients. This concept may be applied for the development of allergy vaccines whenever the primary sequence or structure of an allergen is available.—Ball, T., Fuchs, T., Sperr, W. R., Valent, P., Vangelista, L., Kraft, D., Valenta, R. B cell epitopes of the major timothy grass pollen allergen, Phl p 1, revealed by gene fragmentation as candidates for immunotherapy. *FASEB J.* 13, 1277–1290 (1999)

Key Words: Type I allergy • allergen gene fragmentation • immunotherapy • blocking antibodies

TYPE I ALLERGY, a genetically determined immunodisorder that is based on the production of immunoglobulin E (IgE)² antibodies against *per se* innocuous antigens (i.e., allergens), represents a health problem for more than 20% of the population in industrialized countries (1, 2). Cross-linking of effector cell-bound IgE antibodies by allergens results in the release of biological mediators (e.g., histamine, leukotriene), leading to symptoms like allergic rhinitis, conjunctivitis, dermatitis, allergic asthma, and, in severe cases, anaphylactic shock (3). At least 40% of Type I allergic patients are sensitized against grass pollen allergens, which therefore belong to the group of most frequent elicitors of allergic symptoms (4). Group 1 grass pollen allergens are recognized by more than 95% of patients with grass pollen allergy and hence constitute the major allergenic components of monocot pollens (5). They represent glycoproteins with a molecular mass of ~32 kDa and occur as cross-reactive allergens in most grass and corn species (6–8). Group 1 allergens are localized in the cytoplasm of the pollen grain but are released rapidly when pollen is hydrated, as occurs on mucosal surfaces (9, 10). Notably, this allergen family shares significant sequence homology with expansins (11), a group of proteins that possess cell wall loosening activity and thus may facilitate invasion of the pollen tube into the maternal tissues (12), and perhaps the release of allergens on the mucosa of patients.

The cDNAs coding for group 1 allergens from rye grass (Lol p 1) (13, 14), timothy grass (Phl p 1) (15), velvet grass (Hol l 1) (16), bermuda grass (Cyn d 1) (17), canary grass (Pha a 1) (18), rice (Ory s 1) (19), and maize (Zea m 1) (20) share high homology. Sequence similarity of these allergens explains why

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² Abbreviations: ELISA, enzyme-linked immunoassay; Ig, immunoglobulin; PCR, polymerase chain reaction.

grass pollen allergic patients cross-react with pollens from various grass and corn species. The cDNA coding for Phl p 1, the major allergen of timothy grass and one of the most widespread grass species, was expressed in *Escherichia coli* as recombinant allergen (21). Recombinant Phl p 1 bound IgE of more than 90% of grass pollen allergic patients (22) and its biological activity has been demonstrated by histamine release and skin test experiments (22; S. Heiss and R. Valenta, unpublished results). It contained most of the B and T cell epitopes of group 1 allergens from a variety of grass species (8, 23, 24).

Here we use Phl p 1 as a model antigen to investigate the interaction of IgE and IgG antibodies with a prominent allergen at the molecular level. An expression cDNA library was constructed from the randomly fragmented Phl p 1 cDNA and recombinant Phl p 1 fragments were generated by polymerase chain reaction (PCR). Sera from grass pollen allergic patients with and without grass pollen immunotherapy were used to define rPhl p 1 fragments containing IgE and IgG₄ epitopes and to monitor antibody recognition during natural allergen exposure and in the course of grass pollen immunotherapy. IgE and IgG₄ binding sites of Phl p 1 were compared and their amino acid sequences were aligned with homologous regions in group 1 allergens of other grass species. The percentage of Phl p 1-specific IgE directed against continuous Phl p 1 epitopes was studied by competition experiments. In light of our finding that serum containing immunotherapy-induced, Phl p 1 fragment-specific IgG₄ antibodies prevented Phl p 1-triggered histamine release from basophils, we suggest using rPhl p 1 fragments as candidates for grass pollen immunotherapy to focus IgG antibodies to IgE epitopes.

MATERIALS AND METHODS

Characterization of allergic patients and sera

Patients allergic to grass pollen and nonallergic control individuals were characterized by case history, skin prick test, and RAST (Pharmacia, Uppsala, Sweden) (25). In addition, sera were tested by IgE immunoblotting for reactivity to natural timothy grass pollen proteins and with recombinant Phl p 1, as described (8). Grass pollen-specific immunotherapy was performed using aluminum hydroxide-adsorbed timothy grass pollen extracts (Allergovit; Allergopharma, Joachim Ganzer KG, Reinbek, Germany; ALK depot SQ; ALK, Horsholm, Denmark), according to the manufacturer's instructions. Serum samples were collected before immunotherapy and twice during each year of immunotherapy (spring, autumn) from patients showing clinical improvement.

Characterization of recombinant Phl p 1 and measurement of rPhl p 1-specific antibody responses

The major allergen Phl p 1 was isolated by IgE immunoscreening of a timothy grass pollen cDNA library (15). Almost

95% of grass pollen allergic patients display IgE reactivity to recombinant Phl p 1 (22). Purified recombinant Phl p 1 induced T cell proliferation (24) and specific histamine release from basophils of grass pollen allergic patients (21). Phl p 1-specific IgE and IgG subclass (IgG₁-IgG₄) reactivity to rPhl p 1 was evaluated by enzyme-linked immunoassay (ELISA) measurements as described (21).

Multiple sequence alignment and structural prediction of Phl p 1

A search between Phl p 1 sequence and protein databases was made using the FASTA program of the GCG package (26). Multiple sequence alignment was produced with CLUSTAL W (27) and, if necessary, edited by hand. The GDE sequence editor (S. Smith, Harvard University, Cambridge, Mass.) and COLORMASK (J. Thompson, EMBL, Heidelberg, Germany) were used to color conserved residues with related properties (27). Protein secondary structure and solvent accessibility predictions were made using the PHD program on the EMBL PredictProtein server (28, 29).

Isolation and characterization of IgE and IgG₄ epitopes of rPhl p 1

An epitope expression cDNA library was constructed from the randomly fragmented Phl p 1 cDNA, and IgE binding phage clones were isolated by immunoscreening with serum IgE from a grass pollen allergic individual (30). To determine IgG₄-reactive rPhl p 1 fragments, the random fragment expression cDNA library was screened with serum IgG₄ of 20 grass pollen allergic patients. Ten of these patients had received grass pollen immunotherapy and 10 were untreated. Sera were diluted 1:20 in buffer A (50 mmol/l sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% bovine serum albumin, 0.05% NaN₃), and bound IgG₄ antibodies were detected using a mouse anti-human IgG₄ antibody (Pharmingen, San Diego, Calif.) and a 1:500 in buffer A diluted ¹²⁵I-labeled sheep anti-mouse Ig antiserum (Amersham, Buckinghamshire, U.K.). The cDNA clones encoding IgE and IgG₄ epitopes were amplified from phage DNA by using PCR using λ gt11 forward (5' CCG GAT CCC GGT TTC CAT ATG GCG ATT GGT GGC 3') and reversed (5' CGC GGA TCC CCG TGA CAC CAG ACC AAC TGG TAA TG 3') primers. Both primers contained *Bam*HI restriction sites (underlined), which allowed subcloning of the PCR products into plasmid pUC18 (Boehringer Mannheim, Germany). Plasmids were transformed into *E. coli* XL-1 Blue using the calcium chloride method and plasmid DNA was isolated using Quiagen tips (Quiagen, Hilden, Germany) (31). The sequence of subcloned fragments was determined by DNA sequence analysis according to Sanger (32) using the λ gt11 primers described above, ³⁵S-dCTP (NEN, Stevenage, U.K.), and a T7 polymerase sequencing kit (Pharmacia).

Recombinant Phl p 1 fragments

Recombinant fragments were generated by PCR amplification using phage DNA encoding Phl p 1 as template and primers (MWG, Ebersberg, Germany), as indicated in Table 1. PCR fragments were cut with *Eco*RI, gel purified, and cloned into λ gt11 arms (Pharmacia). The phage DNA was then *in vitro* packaged using *in vitro* packaging extracts (Promega, Madison, Wis.). IgE and IgG₄ binding phage clones were isolated by immunoscreening with serum IgE and IgG₄ from grass pollen allergic patients and characterized by sequence analysis of the inserted cDNA as described above.

TABLE 1. Primers used to construct recombinant Phl p 1 fragments

Fragment A: (Phl p 1 aa 24–112):	N1: 5'G AGA <u>GAA TTC</u> ATC CCC AAG GTT CCC CCG GGT CCG 3' 1R: 5'GTC <u>GAA TTC</u> TAC CAC GGG CTC GCC AGA GCA GGC 3'
Fragment B: (Phl p 1 aa 24–150):	N1: 5'G AGA <u>GAA TTC</u> ATC CCC AAG GTT CCC CCG GGT CCG 3' 2R: 5'GTC <u>GAA TTC</u> GGC GCT GCG CAG CTT CTG CTC ATC 3'
Fragment C: (Phl p 1 aa 85–150):	1F: 5'G AGA <u>GAA TTC</u> ATC TTC AAG TCC GGA CGC GGC TGC 3' 2R: 5'GTC <u>GAA TTC</u> GGC GCT GCG CAG CTT CTG CTC ATC 3'
Fragment D: (Phl p 1 aa 85–228):	1F: 5'G AGA <u>GAA TTC</u> ATC TTC AAG TCC GGA CGC GGC TGC 3' 3R: 5'GTC <u>GAA TTC</u> GAG CTT GTC GGG AGT GTC GAT CCT 3'
Fragment E: (Phl p 1 aa 149–263):	2F: 5'G AGA <u>GAA TTC</u> AGC GCC GGC GAG CTG GAG CTC CAG 3' 4R: 5'GTC <u>GAA TTC</u> CTT GGA CTC GTA GCT GGT GTC GGC 3'
Fragment F: (Phl p 1 aa 203–263):	3F: 5'G AGA <u>GAA TTC</u> GAG AAG GGC AAG GAC AAG TGG ATC 3' 2R: 5'GTC <u>GAA TTC</u> GGC GCT GCG CAG CTT CTG CTC ATC 3'

IgE and IgG₄ binding to nitrocellulose-dotted phage clones expressing rPhl p1 fragments

Two microliter aliquots of phage lysates ($>10^5$ pfu/ μ l) expressing β -galactosidase-fused Phl p 1 fragments and, for control purposes, β -galactosidase alone, were used to infect *E. coli* Y1090. The synthesis of recombinant β -galactosidase-fused Phl p 1 fragments was induced by overlay with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM IPTG. Nitrocellulose filters containing the recombinant Phl p 1 fragments and *E. coli*/phage proteins were probed with sera from Phl p 1 allergic patients diluted 1:5 for IgE and 1:20 for IgG₄ detection. Bound IgE and IgG₄ antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (Pharmacia, RAST) or a sandwich consisting of a mouse monoclonal anti-human IgG₄ antibody (Pharmingen), followed by ¹²⁵I-labeled sheep anti-mouse Ig antibodies (Amersham).

Inhibition of IgE binding to complete rPhl p 1 with rPhl p 1 fragments

Sera from 13 Phl p 1 allergic patients were diluted 1:5 in buffer A and preincubated with a combination of nitrocellulose-immobilized, recombinant small rPhl p 1 fragments (c: NT, 34, 35, 36, 43, 50, 80, 95, 97, 98, 103, 113, 114, H₇, H₁₆, H₁₈) (Fig. 1), large rPhl p 1 fragments (f: A–F) (Table 1), or, for control purposes, with immobilized *E. coli*/phage proteins (λ) as described (33). Preincubated sera were exposed to nitrocellulose strips containing 1.2 μ g purified rPhl p 1; bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (RAST, Pharmacia) and visualized by autoradiography. To quantify IgE binding to rPhl p 1, bound ¹²⁵I-labeled anti-human IgE was measured by γ -counting (Wallac, LKB, Turku, Finland). The percentage inhibition of IgE binding was calculated as follows: inhibition = $100 - (100 \times \text{cpm c or f})/\text{cpm } \lambda$. Cpm (counts per minute) c, f, or λ correspond to the amount of rPhl p 1-bound IgE antibodies after preadsorption of sera with a mix of fragments c, f, or *E. coli*/phage proteins (λ) (Table 2).

Basophil histamine release experiments

A heparinized blood sample from a grass pollen allergic patient was obtained by venipuncture after informed consent was given. Granulocytes were isolated by dextran sedimentation (34). Purified rPhl p 1 was dissolved in histamine release buffer (Immunotech, Marseille, France) at different concentrations (1 μ g/ml, 0.1 μ g/ml) after pre-exposure to an equal volume of patients' sera obtained before or after immunotherapy for 30 min at 37°C. Granulocytes were then exposed to the preincubated rPhl p 1 and histamine released into the cell-free supernatants was determined by radioimmunoassay

(Immunotech). Histamine release was measured in triplicate and expressed as percentage of total histamine determined after cell lysis, as described (34).

RESULTS

Characterization of IgE-reactive rPhl p 1 fragments

The cDNA coding for Phl p 1 contains an open reading frame of 789 nucleotides that codes for a protein of 263 amino acids with a deduced molecular mass of 26.1 kDa. The first 23 amino acids represent a hydrophobic leader peptide that is absent in the mature allergen. To identify continuous (i.e., sequential) IgE binding fragments of Phl p 1, a random fragment expression library was constructed from DNA fragments of less than 400 bp obtained by DNase I digestion of the complete Phl p 1 cDNA (30). The Phl p 1 epitope expression cDNA library was screened with serum IgE of 11 grass pollen allergic patients containing IgE against rPhl p 1. The amino acid sequences of 22 IgE-reactive Phl p 1 epitope clones were aligned with the Phl p 1-deduced amino acid sequence in Fig. 1; they define five major IgE-reactive portions of Phl p 1. Three clones mapped to the NH₂ terminus of Phl p 1 (NT, 113, 45; Fig. 1), eight clones clustered at the carboxyl terminus (109, 85, 86, 41, 108, 28, 42, 52), and nine clones defined an IgE-reactive domain in the center of the allergen (43, 34, 80, 114, 95, 50, 97, 103, 98; Fig. 1) that was previously found to represent a major IgE binding hapten (30). Two additional IgE-reactive fragments (H7: aa 144–162; 64: aa 173–199; Fig. 1) in the center of the protein were obtained. When compared with the amino acid sequences of group 1 allergens of other species (Fig. 2), we found that IgE-reactive Phl p 1 fragments comprised regions that are highly conserved among group 1 allergens. Additional, larger (>60 amino acids) IgE-reactive fragments covering the complete Phl p 1 sequence (fragment A–F) were obtained by PCR amplification using the primers described in Table 1.

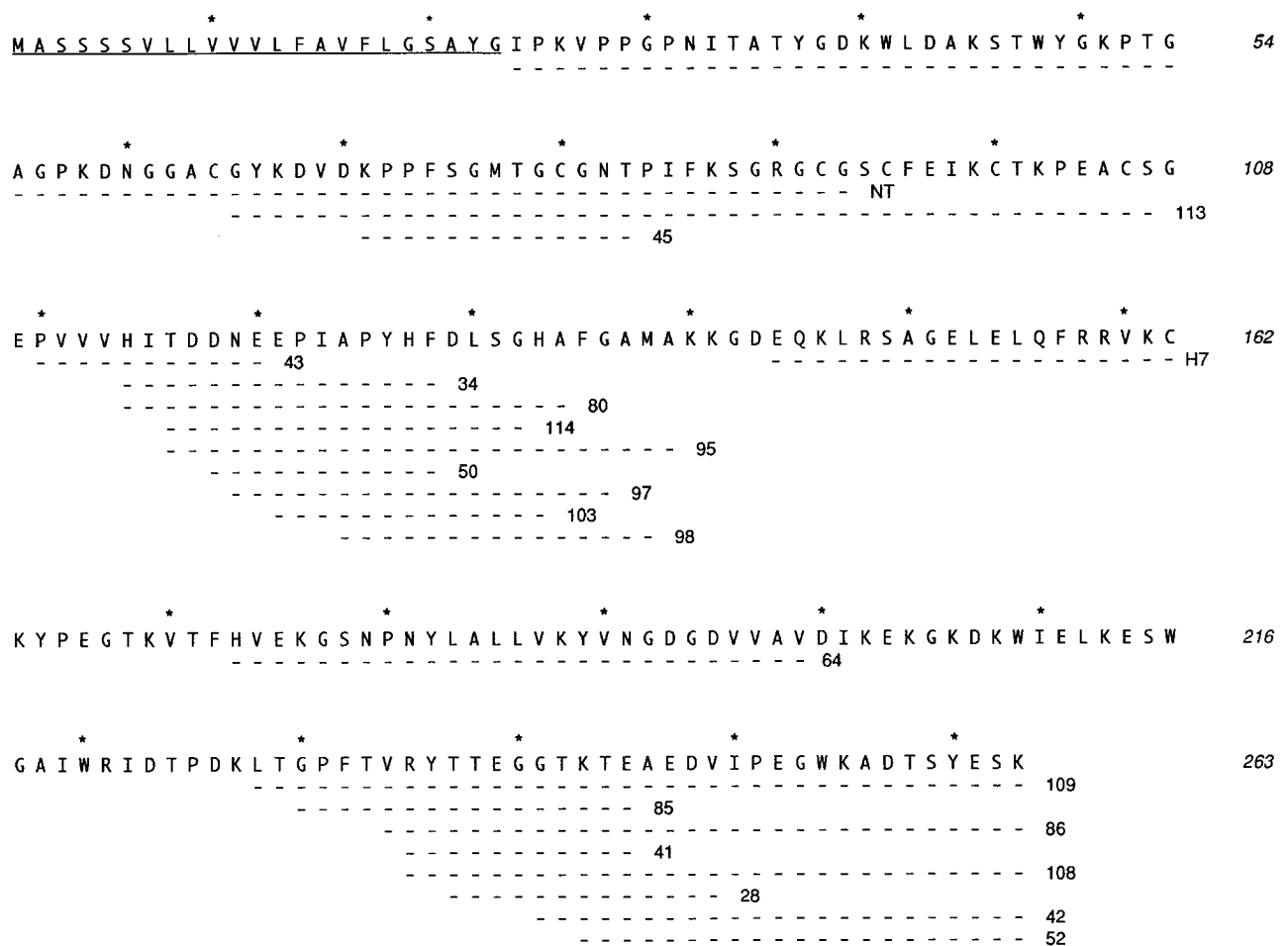


Figure 1. Localization of IgE-reactive recombinant Phl p 1 fragments. The deduced amino acid sequence of Phl p 1 is displayed in the single-letter code. The hydrophobic leader peptide absent from the mature allergen is underlined. IgE-reactive fragments are indicated by dashes and designated (NT, H7, 28–113). Amino acids are numbered in the right margin.

rPhl p 1 fragments bind a high percentage of Phl p 1-specific IgE

To estimate the percentage of Phl p 1-specific IgE directed against the rPhl p 1 fragments, RAST-based

IgE inhibition experiments were performed. Sera from 13 Phl p 1-reactive allergic patients were preadsorbed with a mixture of the smaller rPhl p 1 fragments described in Fig. 1, a mixture of the larger rPhl p 1 fragments A-F summarized in Table 1, or,

TABLE 2. Inhibition of IgE binding to rPhl p 1 with recombinant Phl p 1 fragments

	#1		#2		#3		#4		#5		#6		#7	
	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %
λ	504.5	0	296	0	677	0	1540	0	372	0	411	0	436	0
c	449.5	11	112	62	266.5	61	1540	0	117	69	290	29	351	20
f	18	96	17	94	93.5	86	326.5	79	17.5	95	17	96	137	69
	#8		#9		#10		#11		#12		#13			
	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %	IgE binding cpm	Inhibition %		
λ	53	0	389	0	716	0	1888.5	0	1034.5	0	482	0		
c	36	32	131.5	66	432.5	40	1133	40	635.5	39	268	44		
f	6.5	88	21.5	94	64	91	70	96	48.5	95	89	82		

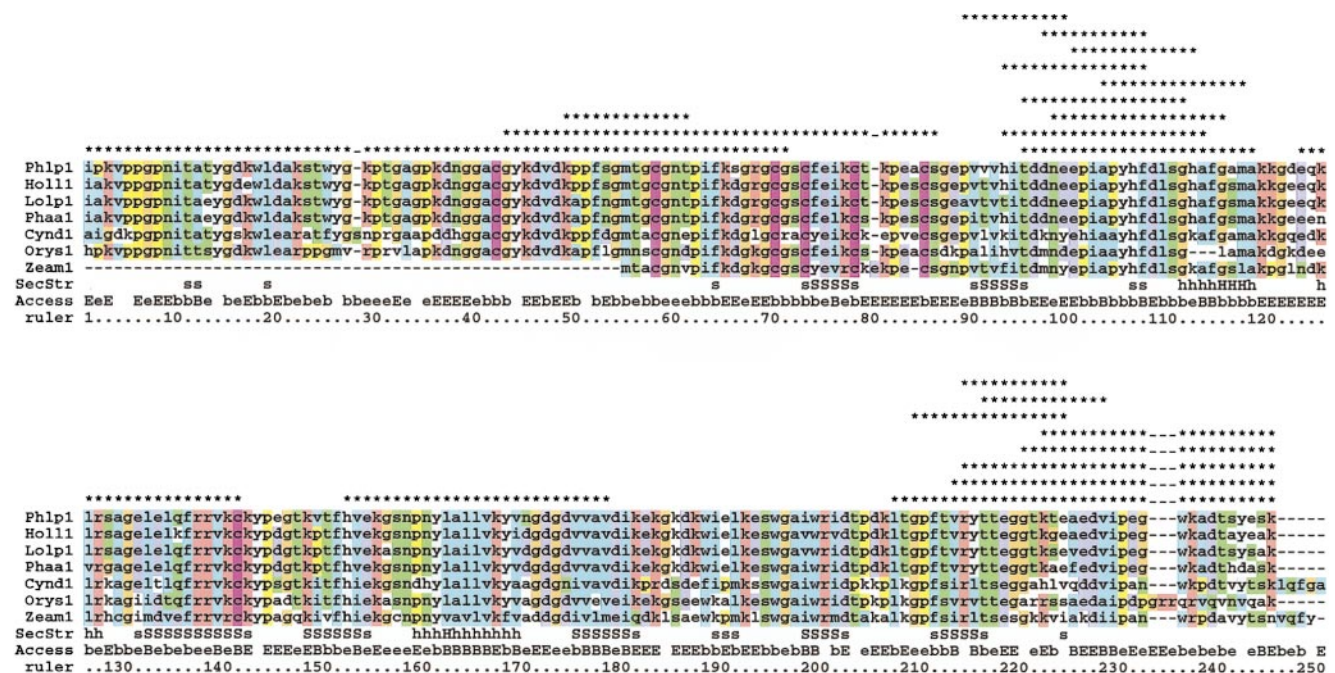


Figure 2. Multiple sequence alignment and secondary structure prediction of group 1 grass pollen allergens. The deduced amino acid sequence of Phl p 1, displayed in the single-letter code, has been aligned with group 1 allergens from other grass species (Hol 1, Lol 1, Pha 1, Cyn 1, Ory 1, Zea 1); hyphens in the sequences indicate gaps. The sequences are colored to illustrate conservation of features in addition to amino acid identity. All glycines and prolines are orange and yellow, respectively. Other residues are colored according to conservation of their physicochemical properties (purple: acidic; blue: hydrophobic; light blue: hydrophobic tendency; red: basic; green: hydrophilic; white: unconserved). SecStr gives the predicted secondary structure according to the PHD program. In the secondary structure prediction, uppercase letters are used for positions in which accuracy exceeds 82%; the overall accuracy of the prediction is 72%. H and S indicate α helices and β sheets, respectively. Access indicates the solvent accessibility (E: exposed; B: buried) of amino acids, where uppercase letters represent a more than 69% accuracy of prediction. The numbering of amino acids is displayed in the ruler (last line) and the IgE epitope-containing fragments are indicated on top by asterisks.

for control purposes, *E. coli*/phage proteins (Table 2). Results obtained showed that preincubation of all but one serum (Table 2: #4) with smaller Phl p 1 fragments yielded a significant inhibition of IgE binding to rPhl p 1 ranging from 11 to 69% (average inhibition 40%) (Table 2: c). The degree of inhibition of IgE binding to rPhl p 1 obtained after preincubation of sera with the larger Phl p 1 fragments was much greater and ranged from 69 to 96% (average inhibition 89%) (Table 2: f). These results indicate that certain Phl p 1 allergic patients (e.g., Table 2: #4) recognize preferentially larger Phl p 1 fragments and perhaps conformational IgE epitopes, whereas most of the patients also mount IgE responses to shorter rPhl p 1 fragments (i.e., continuous epitopes).

Grass pollen allergic patients without immunotherapy fail to mount IgG responses to rPhl p 1 fragments

Whereas we occasionally found sera from untreated grass pollen allergic patients that showed IgG-subclass reactivity to complete rPhl p 1, we were unable to detect IgG₁₋₄ reactivity to rPhl p 1 fragments in

these sera (data not shown). Assuming that sera from grass pollen allergic patients without immunotherapy may contain IgG antibodies to rPhl p 1 fragments other than those defined by IgE antibodies, we screened the Phl p 1 random fragment expression library with 10 such sera, but without success. The lack of IgG₄ reactivity of sera from grass pollen allergic patients without immunotherapy to smaller and larger rPhl p 1 fragments is exemplified in Fig. 3A, B. Three of the five sera displayed IgE reactivity to an immunodominant IgE binding region already defined, but none of the sera exhibited IgG₄ reactivity to the small rPhl p 1 fragments (Fig. 3A). Exposure of the same five sera to the larger rPhl p 1 fragments revealed that all sera mounted IgE antibody responses to at least one fragment but failed to show IgG₄ reactivity to any of the large fragments, although some of these sera had displayed IgG₄ reactivity to complete rPhl p 1 (Fig. 3B). These results indicate that sera from untreated grass pollen allergic patients either contain low levels of Phl p 1-specific IgG₄ antibodies and/or the Phl p 1-specific IgG₄ antibodies are of low affinity and/or directed against epitopes other than continuous IgE epitopes (e.g., conformational epitopes).

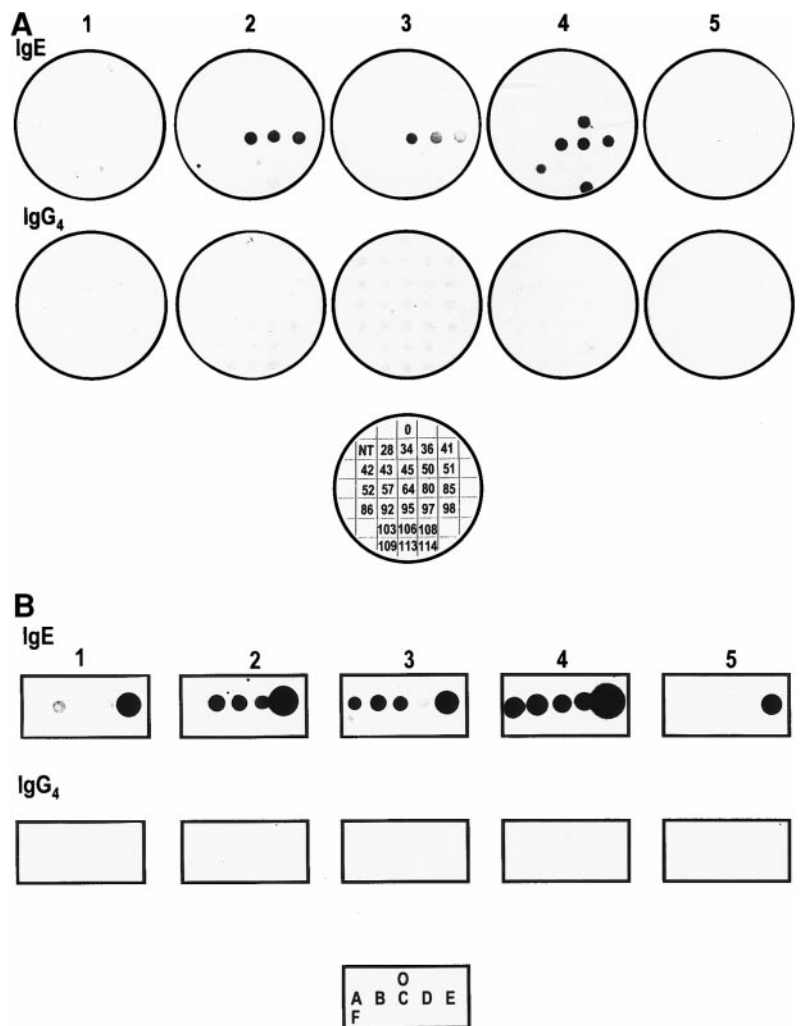


Figure 3. IgE and IgG₄ reactivity of sera from five patients allergic to grass pollen (1–5) who had not received immunotherapy to nitrocellulose-dotted small (A: NT, 28–114) and large (B: A–F) rPhl p 1 fragments as well as to *E. coli*/phage proteins (A, B: 0).

rPhl p 1 fragments defined by serum IgG₄ of grass pollen allergic patients who had received immunotherapy overlap with the IgE-reactive domains

To obtain IgG₄-reactive rPhl p 1 fragments, we screened the Phl p 1 random fragment expression library with serum IgG₄ of 7 grass pollen allergic patients who had received immunotherapy. The cDNA and amino acid sequences of 13 IgG₄-reactive clones were determined and aligned with the Phl p 1 amino acid sequence (Fig. 4). Although obtained independently from the IgE-reactive fragments, we found that the IgG₄-defined Phl p 1 fragments overlapped with the IgE-reactive domains. The amino- as well as carboxyl-terminal portion of Phl p 1 contained IgG₄ epitopes and most of the IgG₄-reactive clones mapped to the immunodominant central region of Phl p 1 previously defined by IgE antibodies (Fig. 4) (30).

IgE and IgG₄ binding capacity of IgG₄-defined rPhl p 1 fragments

Next we wanted to study whether the IgG₄-defined Phl p 1 fragments are also recognized by IgE anti-

bodies. Twenty-three IgG₄-defined clones, of which 13 had been sequenced (Fig. 4), were tested for their capacity to bind IgE and IgG₄ of sera from seven patients who had received grass pollen immunotherapy (Fig. 5). Sera from all seven patients displayed IgE reactivity of varying intensity to several of the IgG₄-defined rPhl p 1 fragments (Fig. 5: IgE). Four of the seven sera (#2, #3, #4, #6; Fig. 5: IgG₄) showed patterns of specific IgG₄ reactivity to the epitope clones that were different from each other. When we compared the IgE and IgG₄ reactivity patterns to the epitope clones in the same patients, we found that 1) certain sera (e.g., #1, #7) displayed IgE reactivity but no IgG₄ reactivity to Phl p 1 fragments (Fig. 5), 2) IgE and IgG₄ reactivities of different intensity to the same epitope clones were observed within the same serum samples (Fig. 5), and 3) certain sera displayed IgG₄ but not IgE reactivity to the same epitope clones (e.g., FI 61 reacted with serum IgG₄ but not with IgE of patient 4) (Fig. 5). These experiments indicate that IgE and IgG₄ antibodies of patients with immunotherapy frequently recognize the same epitopes, but IgE and IgG₄ antibodies may also bind to epitopes that are different from each other.

* * * * *
MASSSSVLLVVVLFAVFLGSAYGIPKVP P GPNITATYGDKWLD AKSTWY GKPTG 54
 * * * * *
 A GPKDNGGACGYKD VDKPPFSGMTGCGNTPIFKSGRGCGSCFEIKCTKPEACSG 108
 - - - - - CS29
 - - - - -
 - - - - -
 - - - - -
 - - - - -
 * * * * *
 EPVVVHITDDNEEPIAPYHFDLSGHAFGAMAKKGDEQKLRSAGELELQFRRVKC 162
 - - - - - SB79 H16
 - - - - - Fl60
 - - - - - Fl65
 - - - - - CS32
 - - - - - Fl56
 - - - - - CS30
 - - - - - H18
 - - - - - Fl61
 - - - - - H17
 - - - - - CS31
 * * * * *
 KYPEGTKVTFHVEKGSNP NYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW 216
 * * * * *
 GAIWRIDTPDKLTGPFTVRYTTTEGGTKTEAEDVIPEGWKADTSYESK 263
 - - - - - Fl63

Figure 4. Localization of IgG₄-reactive recombinant Phl p 1 fragments. The deduced amino acid sequence of Phl p 1 is displayed in the single-letter code. The hydrophobic leader peptide that is absent from the mature allergen is underlined. IgG₄-reactive fragments are indicated by dashes and are designated with numbers and initials corresponding to the patient's sera used for their identification (CS, H, SB, FI). The amino acids are numbered at the right margin.

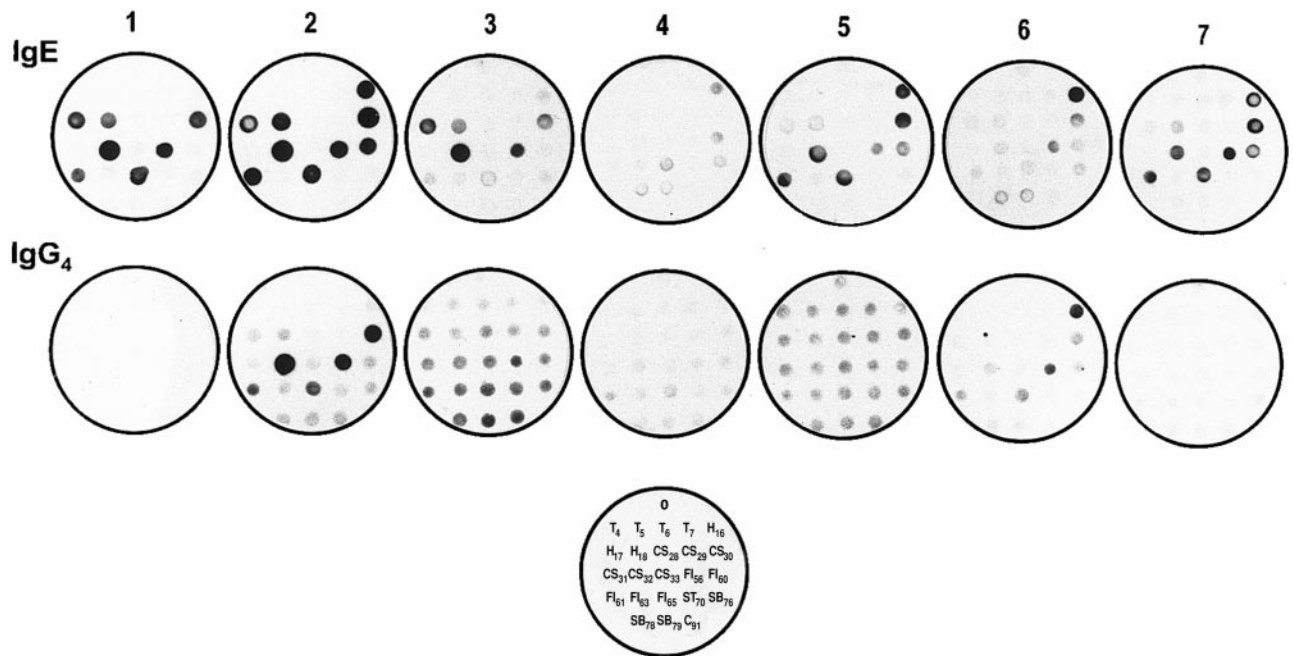


Figure 5. IgE and IgG₄ binding capacity of recombinant IgG₄-defined Phl p 1 fragments. Recombinant IgG₄-defined Phl p 1 fragments (T, H, CS, FI, ST, SB, C) and β -galactosidase (0) were immobilized to nitrocellulose and exposed to serum IgE and IgG₄ of seven Phl p 1 allergic patients.

Lack of evidence for IgE epitope spreading during natural allergen exposure

For several autoimmune diseases it has been reported that patients expand their B cell as well as T cell reactivity to new antigens and/or epitopes during the course of their disease, a phenomenon termed 'epitope spreading' (35). We therefore investigated whether grass pollen allergic patients start to mount IgE antibody responses to new allergens or epitopes in the natural course of their disease. We tested serum samples collected from 10 adult grass pollen allergic patients over a period of 4 years for the presence of IgE antibodies against recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl 12 (timothy grass profilin). Although we observed changes in allergen-specific IgE titers (e.g., rises after seasonal allergen exposure), no patient started to mount IgE antibody responses against allergens that were not recognized before (data not shown). When tested for IgE reactivity to recombinant Phl p 1 fragments, we found that grass pollen allergic patients ($n=4$) without immunotherapy (e.g., patient HM: Fig. 6A; patient SC: Fig. 6B) displayed IgE reactivity to the same epitopes when monitored for 1 or 2 years. Although not proof, these data suggest that adult grass pollen allergic patients display no tendency for IgE epitope spreading in the natural course of their disease.

Grass pollen immunotherapy induces *de novo* IgE and IgG₄ responses against rPhl p 1 fragments

To examine whether immunotherapy can influence IgE and IgG₄ antibody responses to complete rPhl p 1 and rPhl p 1 fragments, serum samples were collected from grass pollen allergic patients before and during the course of grass pollen immunother-

apy at intervals of ~6 months. Sera were tested in parallel for IgE and IgG₄ reactivity to complete rPhl p 1 by ELISA and for IgE and IgG₄ reactivity to rPhl p 1 fragments. As exemplified for three patients (Figs. 7, 8, and 9), we found that rPhl p 1-specific IgE and IgG₄ levels showed opposite tendencies: decreases of rPhl p 1-specific IgE were mostly accompanied by rises in rPhl p 1-specific IgG₄ (Figs. 7A, 8A, 9A). rPhl p 1-specific IgG₄ levels were low or undetectable before immunotherapy and increased during immunotherapy. It is therefore likely that the IgG₄ rises observed during therapy were induced by the treatment. When serum samples were tested for IgE and IgG₄ reactivity to the rPhl p 1 epitope clones, we found that patients who did not mount detectable IgE or IgG₄ anti-rPhl p 1 fragment reactivity in the beginning of immunotherapy started to display IgE and IgG₄ reactivity to the epitopes in the course of treatment. The induction of rPhl p 1 fragment-reactive IgG₄ was always in parallel with the rPhl p 1-specific IgG₄ levels, whereas IgE anti-rPhl p 1 fragment reactivity was not accompanied by rises in rPhl p 1-specific IgE. Comparing the patterns of rPhl p 1 fragments defined by the IgE and IgG₄ antibodies in the course of immunotherapy, we found that in the same patients, therapy-induced IgE and IgG₄ antibodies bound mainly to the same fragments. We noted that in the course of immunotherapy, patients often mounted IgE to rPhl p 1 fragments earlier than IgG₄ responses (e.g., Fig. 7B: sample spring 91, autumn 91, autumn 92, spring 93; Fig. 8B: sample autumn 91, autumn 92, spring 93, autumn 93).

Serum containing immunotherapy-induced Phl p 1-specific IgG antibodies inhibits rPhl p 1-dependent basophil degranulation

To investigate whether immunotherapy-induced, rPhl p 1 fragment-specific IgG₄ antibodies have

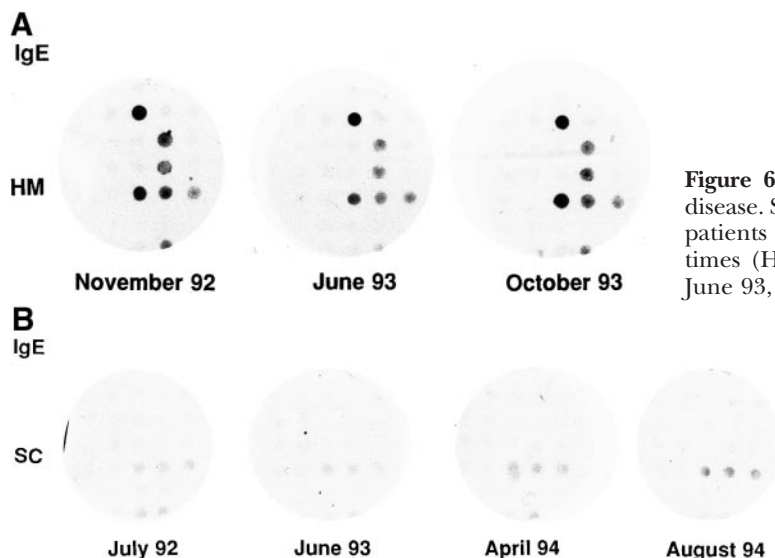


Figure 6. IgE epitope recognition during natural course of disease. Serum samples were collected from two Phl p 1 allergic patients (panel A: HM; panel B: SC), collected at different times (HM: November 92, June 93, October 93; SC: July 92, June 93, April 94, August 94).

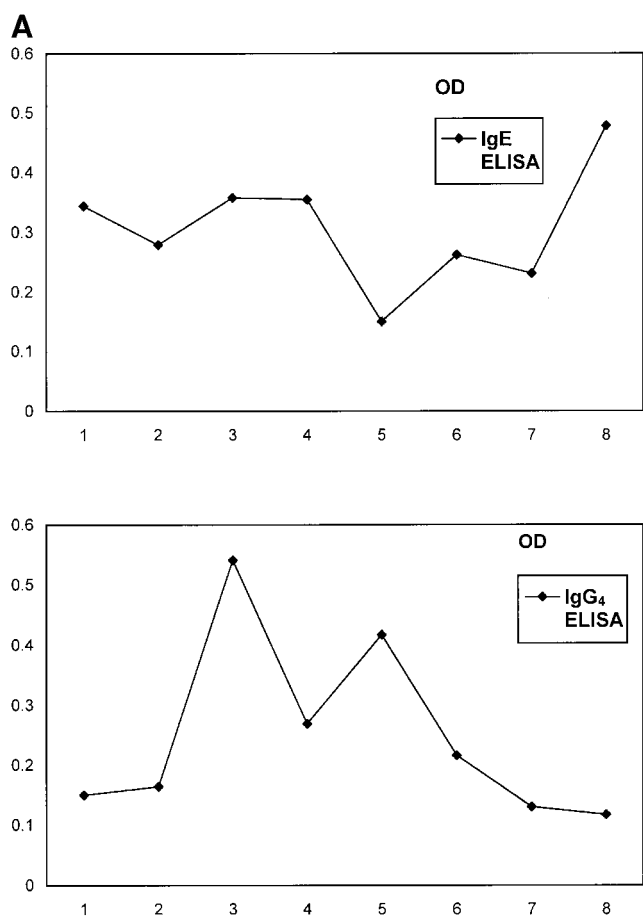
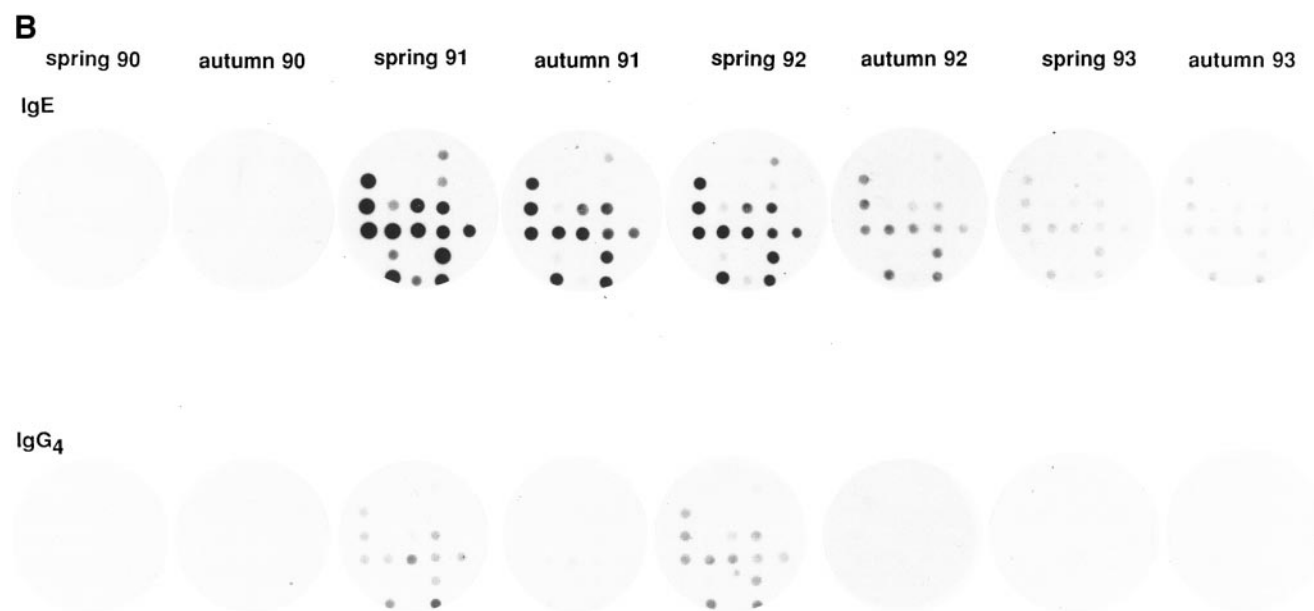


Figure 7. Monitoring of rPhl p 1-specific IgE and IgG₄ antibody levels in a grass pollen allergic patient (A) before and during immunotherapy by ELISA. The optical densities corresponding to the amounts of bound IgE and IgG₄ antibodies are displayed on the y axes. The sample numbers on the x axis correspond to the time points of serum collection (1: spring 90; 2: autumn 90; 3: spring 91; 4: autumn 91; 5: spring 92; 6: autumn 92; 7: spring 93; 8: autumn 93). The same serum samples were also exposed to nitrocellulose-dotted recombinant Phl p 1 fragments (B).



biological activity and protect against Phl p 1-induced allergic reactions, we performed basophil histamine release experiments. We preincubated purified rPhl p 1 with serum obtained from a grass pollen allergic patient before immunotherapy (serum sample: spring 90; Fig. 8A, B) or with serum obtained from the same patient during immunotherapy, when strong IgG₄ anti-rPhl p 1 and anti-rPhl p 1

fragment reactivity could be detected (Fig. 8A, B: spring 92). Different concentrations of preincubated rPhl p 1 were incubated with basophils of a Phl p 1 allergic patient and the histamine released in the culture supernatants was measured. Results showed that preincubation of rPhl p 1 with serum containing rPhl p 1-specific IgG₄ significantly inhibited the rPhl p 1-induced histamine release (~30% at concentra-

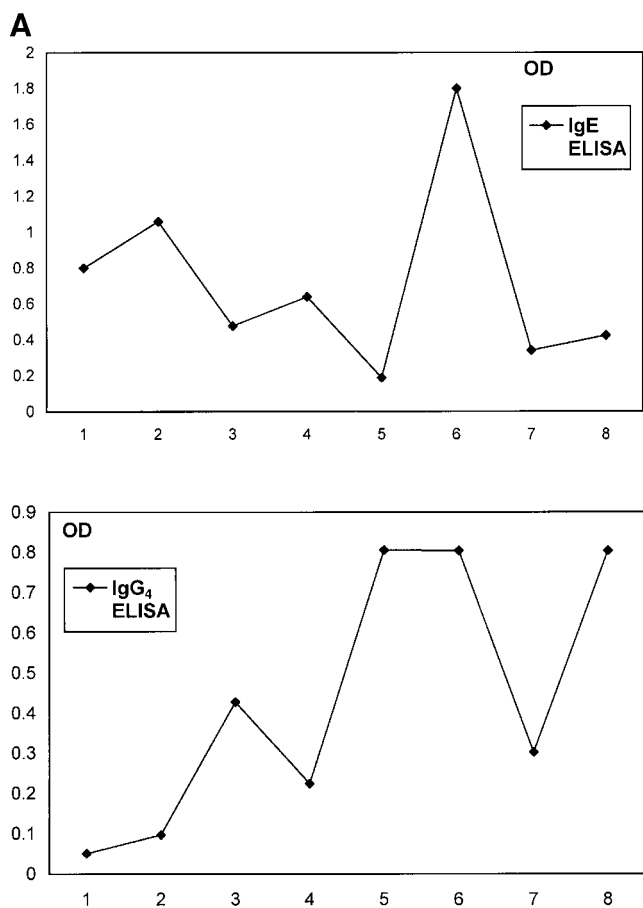
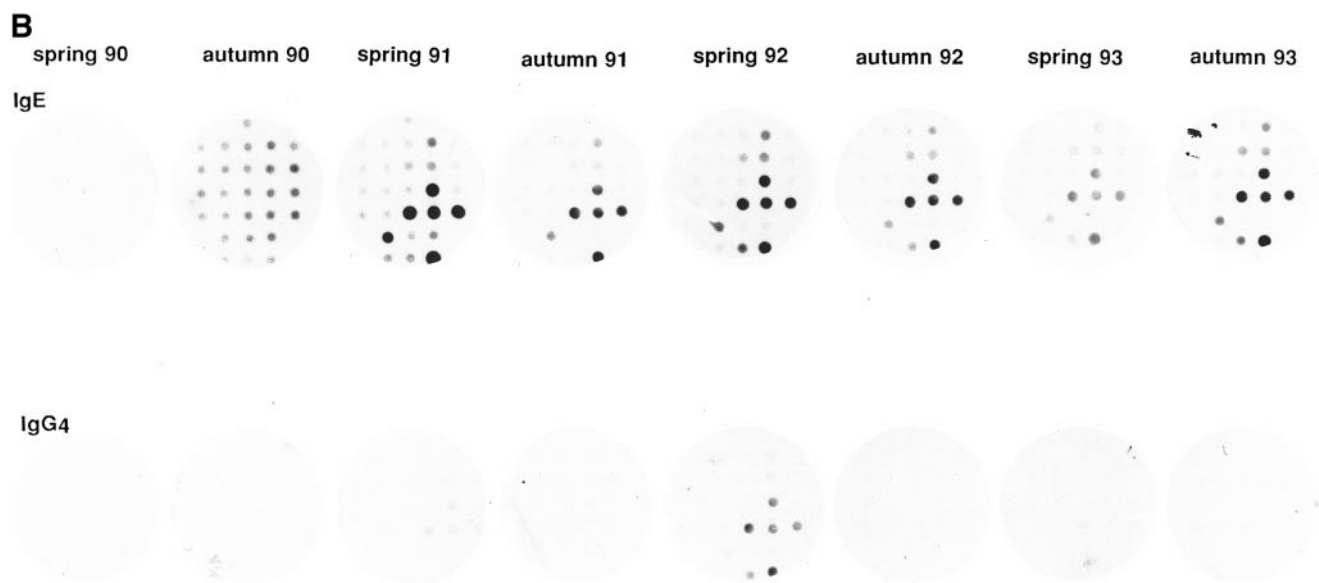


Figure 8. Monitoring of rPhl p 1-specific IgE and IgG₄ antibody levels in a grass pollen allergic patient (A) before and during immunotherapy by ELISA. The optical densities corresponding to the amounts of bound IgE and IgG₄ antibodies are displayed on the y axes. The sample numbers on the x axis correspond to the time points of serum collection (1: spring 90; 2: autumn 90; 3: spring 91; 4: autumn 91; 5: spring 92; 6: autumn 92; 7: spring 93; 8: autumn 93). The same serum samples were also exposed to nitrocellulose-dotted recombinant Phl p 1 fragments (B).



tions of 0.1 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$) (**Fig. 10**). Almost identical results were obtained with sera (serum collected before and after immunotherapy) from a second patient (data not shown).

DISCUSSION

In the present study we used the cDNA of one of the most relevant environmental allergens, the major

timothy grass pollen allergen, Phl p 1 (15), to generate recombinant allergen fragments. By screening of an expression cDNA library prepared from the randomly fragmented Phl p 1 cDNA (30) and of larger rPhl p 1 fragments obtained by PCR, we identified five continuous IgE epitope-containing areas on Phl p 1. Our contention that these IgE epitope-containing areas are representative of group 1 allergens from other grass species and therefore

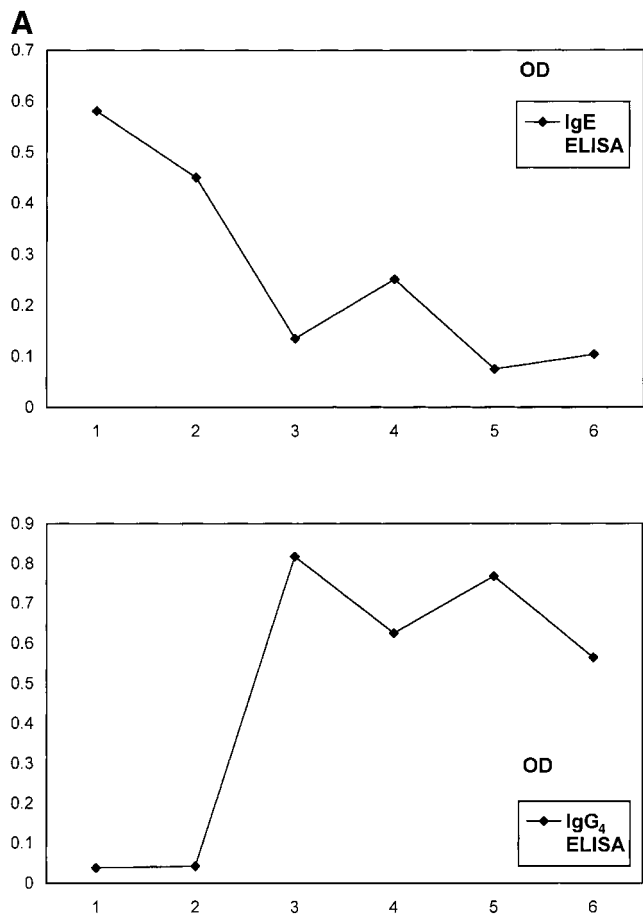
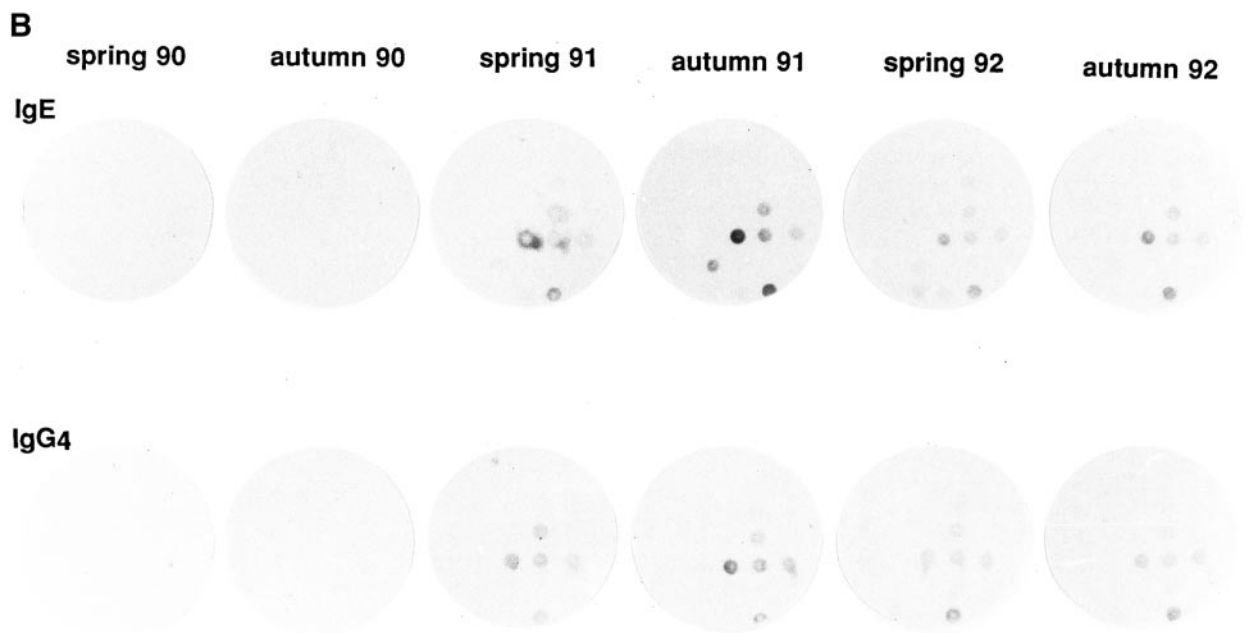


Figure 9. Monitoring of rPhl p 1-specific IgE and IgG₄ antibody levels in a grass pollen allergic patient (A) before and during immunotherapy by ELISA. The optical densities corresponding to the amounts of bound IgE and IgG₄ antibodies are displayed on the y axes. The sample numbers on the x axis correspond to the time points of serum collection (1: spring 90; 2: autumn 90; 3: spring 91; 4: autumn 91; 5: spring 92; 6: autumn 92). The same serum samples were also exposed to nitrocellulose-dotted recombinant Phl p 1 fragments (B).



relevant for grass pollen allergy in general is supported 1) by the finding that the amino acid sequences of these regions are highly conserved among group 1 allergens from various grasses and corn, and 2) by the fact that several of these regions were reported to represent prominent IgE epitopes of group 1 allergens from other grass species [*Lolium*

perenne (36, 37), *Holcus lanatus* (16)]. Evidence for the possible clinical relevance of the continuous IgE epitope-containing areas defined in our study comes from the demonstration that a mixture of rPhl p 1 fragments bound a high percentage (average 40%) of Phl p 1-specific IgE, and grass pollen allergic patients displayed a stable IgE recognition pattern of

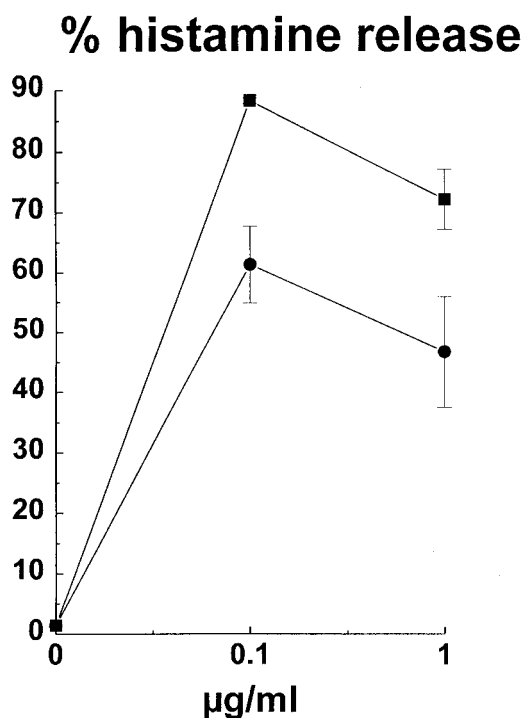


Figure 10. Preincubation of rPhl p 1 with serum containing immunotherapy-induced IgG antibodies inhibits histamine release. Different concentrations of rPhl p 1 (0.1 µg/ml, 1 µg/ml; x axis) were preincubated with serum from a patient obtained before immunotherapy (squares) and with serum containing high levels of therapy-induced, Phl p 1-specific IgG4 (dots) collected after 1 year of specific immunotherapy. The percentage of histamine released is displayed on the y axis. Results represent the percentage values of released histamine and are expressed as the mean $\pm$ SD of triplicates.

rPhl p 1 fragments without signs of epitope spreading in the natural course of their disease. The incomplete inhibition of IgE binding to rPhl p 1 by the rPhl p 1 fragments together with the stronger IgE binding capacity of the larger Phl p 1 fragments suggests, however, that a considerable proportion of Phl p 1-specific IgE is also directed to conformational Phl p 1 epitopes.

We could not detect IgG₄ antibodies directed to rPhl p 1 fragments in grass pollen allergic patients who had not received immunotherapy, even though several of the sera tested contained ELISA-detectable IgG₄ against complete rPhl p 1. This finding agrees with other reports describing that allergic patients do not mount significant levels of allergen-specific IgG (38) and/or that their IgG antibodies are directed against epitopes different from the IgE binding sites (39–41). That allergen-specific IgG antibodies can be directed against epitopes other than IgE would explain controversial reports regarding the protective role of IgG antibodies in atopy. The first evidence for a protective role of blocking antibodies in atopy came from the classical demonstration that allergic rhinitis could be cured by transfusing to an allergic recipient the blood obtained from a patient

who had received specific immunotherapy (42). Later on, the blocking antibodies were identified as belonging to the IgG and IgG₄ subclass, respectively, (43–49). Although several studies demonstrated that immunotherapy induces IgG antibodies (50, 51), lack of correlation between the induction of IgG antibodies and clinical improvement in immunotherapy-treated patients was reported (52).

When we screened rPhl p 1 fragments with sera from patients who were treated successfully by grass pollen immunotherapy, we were able to define several continuous IgG₄ epitope-containing fragments that overlapped with the IgE-defined fragments. The analysis of sera obtained from grass pollen allergic patients before and during immunotherapy revealed that patients started to mount IgE and IgG₄ responses to rPhl p 1 fragments during immunotherapy that were not recognized before the therapy. It is equally possible that the IgE and IgG₄ recognition of the rPhl p 1 fragments results from the amplification of an already existing antibody response of low magnitude or affinity or, perhaps more likely, from a therapy-induced new immune response against B cell epitopes not recognized before therapy. The latter hypothesis that immunotherapy can induce a *de novo* allergen-specific immune response does not necessarily contradict studies reporting a higher occurrence of Th1-like cells after immunotherapy (53–55). A therapy-induced increase of allergen-specific Th1 cells can result equally well from switching of already existing Th2 cells or from the *de novo* formation of Th1 cells in response to allergen administration.

Whether IgE or IgG₄ antibodies are induced against rPhl p 1 fragments may depend on the amount and/or condition of antigen (denatured, folded), and thus the mode of antigen presentation and patterns of cytokines produced. It is of note that several studies suggest that high allergen doses as well as the administration of denatured allergens, as is probably the case for the aluminum-hydroxide-adsorbed grass pollen extracts used in this study, will lead to endocytosis rather than to immunoglobulin-receptor mediated antigen presentation, and through activation of Th1 cells induce IgG production (56–58). That the increase of Phl p 1-specific IgG₄ monitored during therapy was in parallel with a decrease of ELISA-detectable Phl p 1-specific IgE levels indicated that the therapy-induced IgG₄ antibodies compete with the binding sites for IgE on Phl p 1. Whether the therapy-induced IgG₄ antibodies have a protective role was investigated by basophil histamine release experiments. The demonstration that preincubation of rPhl p 1 with serum from a treated patient containing high levels of Phl p 1-fragment specific IgG₄ strongly inhibited rPhl p 1-induced histamine release indeed suggested that therapy-induced serum factors,

presumably IgG₄ antibodies, may have protective functions.

In summary, our findings indicate that in allergen-specific immunotherapy, it may be important to focus protective IgG responses directly to IgE epitopes (59). This could be achieved by the administration of recombinant allergens (60), hypoallergenic derivatives of recombinant allergens (61–65), or allergen fragments that contain IgE epitopes (30) or represent portions of IgE epitopes (63, 66). The recombinant Phl p 1 fragments described in our study define relevant IgE binding sites of one of the most important grass pollen allergens and thus may represent candidates for immunotherapy of grass pollen allergy. **[F]**

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