

Investigation of different recombinant isoforms of grass group-V allergens (timothy grass pollen) isolated by low-stringency cDNA hybridization

Antibody binding capacity and allergenic activity

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A cDNA library of timothy grass pollen was screened for homologous isoforms of major group-V allergens by low stringency hybridization with a Phl p 5 (*Phleum pratense*) probe. After restriction analysis of the 40 clones obtained, 17 were selected for cDNA sequencing. Of these clones, two were unrelated to group-V allergens, six showed high similarity but an incomplete open reading frame and nine had high similarity with a complete open reading frame. Comparison of deduced amino acids of ten complete cDNA clones confirmed the presence of two major isoforms, a and b. Within these two subgroups, only minor sequence variations were observed. Eight isoforms were expressed in *Escherichia coli* K12 and purified to homogeneity. Although the subgroups a and b could be distinguished by their molecular masses and by binding constants towards monoclonal antibodies, all isoforms turned out to be biochemically similar. Ribonuclease activity as a marker for the biological function of group-V allergens was shown to be in the same range for both subgroups.

Analysis of allergenic B-cell responses towards the isoforms in 26 grass pollen allergic patients revealed that the IgE reactivities to the different isoforms were identical for each individual. IgE reactivities and allergenic activities of three isovariants and an allergen of a different group were compared in a selected group of four grass pollen allergic patients by immunoblot, histamine-release and skin-prick tests. The IgE reactivity does not necessarily mirror the allergenic activity of the single molecule, and the variability of allergenic activity between the isovariants does not, in every case, depend on the structural differences of these allergens. We conclude that group-V isoallergens in grass pollen, although they can be structurally different, induce a similar B-cell response but can show variable allergenic activity. Thus, the most allergenic isoform of each important group of allergens should be sufficient for the diagnosis of type-I allergy. Whether the isoallergenic variation has any significant influence on the outcome of immunotherapy in allergic disease still has to be elucidated.

Keywords: grass pollen allergen; isoform; ribonuclease activity; IgE reactivity; allergenic activity.

Grass pollen allergy is one of the most relevant allergic diseases in Northern Europe and America [1] and can be treated with promising results by immunotherapy using pollen extracts [2, 3]. The immunological process underlying the change of allergic response during this treatment has not been clarified. It has been shown that there is an increase of so-called IgG-blocking antibodies in the course of immunotherapy [4] but their relevance for the success of treatment is still debated [5]. Furthermore, while the Th2 cytokine IL-4 was unchanged, an increase of the Th1 cytokine interferon γ in mucosal CD4-positive T cells was observed during immunotherapy [6, 7]. However, the presence of specific allergens in the grass pollen extract seems to be important for the immunologic switch during the therapy.

The extracts used for immunotherapy consist of a number of different molecules isolated from at least six different grass spe-

cies of which only a small part is responsible for the induction of allergic diseases [8]. Consequently, allergenic molecules in the extract mixture have been defined according to their biochemical and molecular differences [9]. All these allergens are qualified by the distinct feature that they are recognized by human IgE antibodies and have the ability to induce mediator release from IgE-sensitized mast cells or basophils, which finally leads to an allergic response [10]. Still, the immune response towards the different allergens varies individually, and it was shown that certain allergens are recognized by most allergic patients (major allergens) whereas others are not (minor allergens). Even at the level of specific IgE-binding epitopes of the allergens, variability in the immune response was observed [11].

Since recombinant allergens are now available, it is logical to propose that these highly purified and well characterized proteins should replace the mixture of defined and undefined molecules in an allergen extract used for diagnosis and therapy. In several studies [12] it was demonstrated that a number of purified recombinant allergen preparations exhibit the same biological and allergic activities as their equivalent natural counterparts. However, when cloning allergens, we and other authors [11, 13, 15] found isoforms in different groups of allergens charac-

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terized by single amino acid exchanges or genetic deletions. For the major birch pollen allergen Bet v1, a hypoallergenic isoform was found which showed no IgE-binding characteristics but induced a T-cell response which was comparable to that of the allergenic isoforms [16]. Whether the presence of these different variants in the pollen extract plays a role in the outcome of immunotherapy, bearing in mind the variability of immune responses, is an unsolved question.

In this paper, we describe the cloning and expression of a number of different isoforms of the major group-V allergen Phl p5 from timothy grass. Biochemical and structural differences among the isoforms are demonstrated. By analysis of the immune response of allergic patients towards the allergens, we investigate whether one isoform is representative for the diagnosis and possibly therapy of grass pollen allergy.

MATERIALS AND METHODS

cDNA library of timothy grass pollen and hybridization.

The cDNA library was constructed as described earlier [11]. For screening of the library, a 5'-cDNA fragment of pPHLP5B1912 [17] from nucleotides 76–419 was constructed by PCR. The PCR product was excised from a 1.2% agarose gel, purified, cloned into the pQE 10 vector (Qiagen) and *Escherichia coli* K12 M15 cells were transformed. For hybridization, the probes were prepared from purified plasmids (Plasmid purification kit, Quiagen). After restriction and purification from a 1.2% agarose gel, the DNA was labelled with digoxigenin using the DIG labelling kit (Boehringer) according to the instruction of the manufacturer. Hybridization was performed after plaque lifting of phages from the cDNA library onto nitrocellulose membrane and denaturation of DNA with 0.5 M NaOH. The further procedure was following the standard protocol (Stratagene) using decreased stringency [37°C, 50% formamide, 30 mM NaCl/3 mM sodium citrate (0.1% NaCl/Cit)] pH 7.0 for washing. DIG-d-UTP probes were detected with anti-DIG alkaline-phosphatase-labeled Fab fragments, using nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate, potassium salt as substrate.

Positive clones were plaque purified by several rounds of hybridization. pBluescript plasmids were excised *in vivo* from isolated phages by coinfection with the helper phage R408 (Stratagene) and plated. Plasmids from single colonies were analyzed after purification by restriction and consequent separation in a 1.2% agarose gel.

DNA sequencing. The double-stranded plasmid DNA was sequenced in both directions according to the dideoxy method of Sanger et al. [18] on an ABI 377 automatic sequencer (Perkin Elmer, Applied Biosystems) using a dideoxy-terminator kit (Perkin Elmer, Applied Biosystems). In most cases, sequencing was performed on restriction fragments subcloned into pBluescript plasmid and rarely by walking the cDNA sequence with sequence-specific primers.

Expression of fusion proteins. The open reading frame without signal sequence of selected cDNA clones was amplified by PCR using specific primers with appropriate restriction sites for subcloning into the pMal-c expression vector (New England Biolabs) and a suitable sequence at the 5' end coding for a cleavage site specifically recognized by factor-Xa protease. Primer sequences were the following. Phl p 5b sense, 5' ATAT GGA-TCC ATCGAGGGAAGG GCCGCCTACGCC 3'. Phl p 5b antisense, 5' ATAT AAGCTT CATGCACACATACCGTACAGT 3'. Phl p 5a sense, 5' ATAT GGATCC ATCGAGGGAAGG GCCG-ATCTCGGCTACGGC 3'. Phl p 5a antisense, 5' ATAT AAGCTT TCAGACTTTGTAGCCACCAGTAGCGGC 3'. (Italic se-

quences = restriction sites, bold sequences = cleavage site for factor Xa.) The cleavage site for factor Xa in the PCR primers guaranteed an unaltered N-terminus of the molecules after cleavage. The expression of Phl p 5 isoforms with the maltose-binding protein as fusion partner was induced by addition of isopropyl thio- β -D-galactoside (final concentration 0.3 mM) to the cultures. 3 hours after induction, the cells were pelleted by centrifugation and resuspended in the appropriate lysis buffer according to the protocol of the manufacturer (New England Biolabs). Cell extracts were prepared after incubation with lysozyme (13 mg/l culture volume).

Purification of recombinant isoforms. After separation of cell debris from the lysate by centrifugation, fusion proteins from the supernatant were affinity purified by amylose-resin columns (Pharmacia). Eluates containing the fusion protein were concentrated by lyophilization. Maltose-binding protein and the recombinant proteins were cleaved by factor Xa (New England Biolabs) using appropriate buffer conditions. Recombinant isoforms were separated from maltose-binding protein by ion-exchange chromatography using a DEAE column and FPLC (Pharmacia). Appropriate fractions were pooled, desalted with Centricon spin columns (Amicon) and lyophilized for further application.

Protein dot, SDS/PAGE and immunoblot analyzes. Proteins were either dotted directly onto nitrocellulose membrane (Schleicher & Schüll) or separated by SDS/PAGE (12%) according to Laemmli [19]. After SDS/PAGE, proteins were subsequently transferred onto a nitrocellulose membrane by semidry blotting for 30 min at 0.8 mA/cm on a Fastblot (Biotetra). Membranes were cut into strips and subsequently used for protein staining with india ink and immunological detection with monoclonal antibodies or sera in a procedure as previously described [20]. IgE-binding intensities were quantified by densitometric measurements (Cream™, Kem-En-Tec Software Systems).

Affinity determination by surface plasmon resonance experiments. Monoclonal antibody (mAb) Bo1 recognizes all isoforms of group-V allergens in grass pollen, whereas mAb BG6 serves as selective marker for group-5a allergens. We determined the binding constants of mAb Bo1 and mAb BG6 towards three selected recombinant isoforms by surface plasmon resonance experiments [21]. An anti-mouse IgG was immobilized on a Sensor Chip CM5 (Pharmacia Biosensor AB) and used for measurement of binding constants K_a and K_d on a Biacore 2000™ (Pharmacia Biosensor AB) by a sandwich-interaction analysis, first injecting the mAb, then the specific recombinant isoform in different known molar concentrations. Calculations of kinetic data were performed applying the BIAevaluation 2.1 software.

Ribonuclease activity assay. Ribonuclease activity was determined according to the method of Barna et al. [22] with modifications described earlier [17]. Maltose-binding protein, the fusion partner of the recombinant allergens, which was purified under identical conditions as the isoforms, served as a negative control.

Control subject and patient sera. Control serum samples were acquired from healthy persons who had no clinical history of allergy, IgE antibodies in the normal range, and negative RAST results. We collected sera from patients in the Out-patients Allergy Department at the Medical Clinic of Borstel, Germany. An atopic phenotype was confirmed by clinical history and diagnosis, based on a highly positive skin-prick test response to grass pollen allergens and specific IgE antibodies greater than 17.5 kU/l as measured in the CAP System (Pharmacia).

Histamine-release assay. Blood samples from selected patients (in 10 mM EDTA) were mixed with 0.25 vol 6% dextran

Iso A1	ADLGYGEATPAAPAAGYTPATPAAPAGAEPAKATTEEQKLIKINAGFKAALAAAGVPPADKYRTFVATFGAASNKFAE	87
A2	G.....E.A.....D.....K.....	76
A3	DA.....G.....Q.....P.....	86
Iso B1	..A..A.....E.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
B2	..A..A.....E.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
B3	..A..A.....a.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
B4	..A..A.....E.....D.V.a.V.....S..AG..FK.E.A.TSS.K----	67
B5	..A..A.....a.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
B6	..A..A.....E.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
B7	..A..A.....E.....D.V.K.V.....S..AG..FK.E.A.TSS.K----	67
Iso A1	GLSGEPKGAEESSSKAALTSKLDAAKYKAYKTAEAGATPEAKYDAYVATVSEALRIIAGTLEHVAVKPAEEVKV--IPAGEL	172
A2	S.....L.....	161
A3	L.....	171
Iso B1	-----ATAKAPG.VP.....SV...A.V.....F.SF.SLT...V...A.....VT..PGMAK.....	145
B2	-----ATAKAPG.VP.....SV...A.V.....F.SF.SLT...V...A.....VT..PGMAK.....	145
B3	-----ATAKAPG.VP.....SV...A.V.....F.SF.SLT...V...A.....VT..PGMAK.....	145
B4	-----ATAKAPG.VP.....SV...A.V.....F.SI.SLT...V...A.....VT..PGMAK.....	145
B5	-----AaAKAPG.VP.....SV...A.V.....F.SF.SLT...V...A.....VT..PGMAK.....	145
B6	-----ATAKAPG.VP.....SV...A.V.....F.SF.SLT...V...A.....VT..dPawpK.....	145
B7	-----ATAKAPG.VP.....SVs..A.V.....F.SF.SLT...V...A.....VT..PGMAK.....	145
Iso A1	QVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYESYKFIPALEAAVQAYAAATVATAPEVKYTVFETALK	259
A2	..F.....S.L.....A.....H.....	248
A3	E.....	258
Iso B1	..I.D.I.....AT...D.....K...E.....DT..C.S.....A.Q...A...A.T	232
B2	..I.D.I.....AT...D.....K...E.....DT..C.S.....A.Q...A...A.T	232
B3	..I.D.I.....AT...D.....K...E.....DT..C.S.....A.Q...A...A.T	232
B4	..I.D.I.....AT...D.....K...E.....DT..C.S.....p.....A.Q...A...A.T	232
B5	..I.D.I.....AT...D.....K...E.....DT..C.S.....A.ae...A...A.T	232
B6	..I.D.I.....AT...D.....K...E.....DT..C.S.....A.Q...A...A.T	232
B7	..I.D.I.....AT...D---.....K...E.....DT..C.S.....A.Q...A...A.T	232
Iso A1	KAITAMSEAQAAKAPAAATA-----TATAAVGAATGAATAATGGYKV	302 (Phl p 5.0103)
A2	TE.....	291 (Phl p 5.0104)
A3		301 (Phl p 5.0102)
Iso B1	V..VSQ..TG.ATVAAGA..T.T...S...V.T....	279 (Phl p 5.0202)
B2	V..VSQ..TG.ATVAAGA..T.a...S...V.T....	279 (Phl p 5.0203)
B3	V..VSQ..TG.ATVAAGA..T.a...S...V.T....	279 (Phl p 5.0204)
B4	V..VSQ..TG.ATVAAGA..T.a...S...V.T....	279 (Phl p 5.0205)
B5	V..VSQ..TG.ATVAAGA..T.a...S...V.T....	279 (Phl p 5.0201)
B6	V..VSQ..TG.ATVAAGA..T.T...S...V.T....	279 (Phl p 5.0206)
B7	V..VSQ..TG.ATVAAGA..T.a...S...V.T....	276 (Phl p 5.0207)

Fig. 1. Deduced amino acid sequence alignment of eight different group-V isoforms with the published sequences of Phl p 5a/Iso A3 [11] and Phl p 5b/Iso B5 [17]. Dots indicate identical amino acids, lines indicate gaps, capital letters indicate amino acid exchanges compared to Iso A1 and italic letters indicate amino acid exchanges of Iso B2-B7 molecules compared to Iso B1. The allergen names according to the official nomenclature [9] are given in brackets at the end of the sequences.

solution for 90 min sedimentation. The supernatant was centrifuged at $250\times g$, and cells were resuspended in 8 g NaCl, 1 g D-glucose, 0.2 g KCl, 0.05 g NaH_2PO_4 , 0.11 g CaCl_2 , 0.122 g MgCl_2 , 2.38 g HEPES/l adjusted to pH 7.4 (stimulation buffer). A cell sample was stained with May-Grünwald dye, and basophils were counted. 1×10^5 basophils/ml were stimulated with different dilutions of selected recombinant isovariants and bovine serum albumin as controls. Histamine concentrations were measured by RIA (Pharmacia) according to the instructions of the manufacturer. Maximum histamine release was determined in supernatants of disrupted basophils to be about $0.4\text{--}1\text{ }\mu\text{g}/10^5$ cells.

Skin-prick tests with recombinant isovariants. Skin-prick tests were done following the guidelines for clinical diagnosis of allergic diseases [23]. Selected isovariants were dissolved to a concentration of 4 mg/ml or 0.4 mg/ml and applied in a standard titrated skin-prick test using physiologic sodium chloride, 1.7 mg/ml or 0.17 mg/ml histamine dihydrochloride and 5000 BU or 500 BU timothy grass pollen extract (Allergopharma) as controls. Wheal areas and flaring were measured and compared by calculation of the surface area.

Computer analysis. Amino acid sequences were aligned applying PCGENE software (Intelligenetics).

RESULTS

Cloning of isoforms and comparison of deduced amino acid sequences. A λ -Zap cDNA library of timothy grass pollen [11]

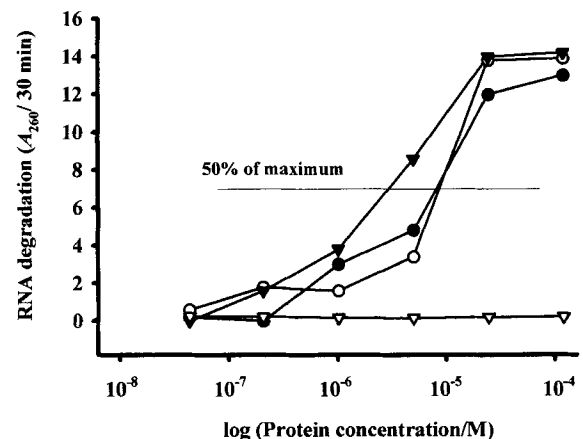


Fig. 2. Comparison of specific ribonuclease activity associated with the group-V isoforms. 1 μg RNA was incubated at 56°C with different concentrations of isoforms A2 (closed circles), B2 (closed triangles) and B7 (open circles) as well as maltose-binding protein as negative control (open triangles). After 30 min, RNA was measured in the supernatant at 260 nm.

was screened for homologous sequences by low stringency hybridization with a probe taken from the N-terminal part of the published sequence of Phl p 5b [17]. 40 clones were isolated, purified and characterized by restriction analysis and Southern

Table 1. Binding constants (K_A) of two different monoclonal antibodies directed against Phl p 5-isoforms. Association and dissociation parameters of isoforms to mAb Bo1 and BG6 at different concentrations of single recombinant molecules were measured by surface plasmon resonance experiments and K_A were calculated. Prominent values indicate specific affinities of the mAbs to isoforms.

Allergens	mAb Bo1	mAb BG6
	M^{-1}	
Phl p 5a (IsoA2)	1.23×10^8	1.92×10^8
Phl p 5b (IsoB2)	5.26×10^7	1.53×10^5
Phl p 5b (IsoB7)	4.90×10^7	2.10×10^5

blotting (data not shown). 17 of these clones were selected for cDNA sequencing because of a positive hybridization signal in the Southern blot and an insert greater than 1000 bp. Analysis of cDNA clones revealed that two were unrelated to group-V allergens, six showed high similarities but the open reading frames were incomplete and nine had high similarities and complete open reading frames. Fig. 1 shows an alignment of the complete deduced amino acid sequences of eight isoforms compared to the published sequences of Phl p 5a (Iso A3) [11] and Phl p 5b (Iso B5) [17]. Subgroups A and B can be distinguished by three substantial deletions and a large number of amino acid exchanges resulting in an overall sequence identity of 71% between the groups. Sequence identity increased when comparing the isoforms within the subgroups A and B up to identity 95% and identity 97.5% respectively. These data demonstrate the existence of two major subgroups in group-V allergens of timothy grass pollen consisting of isoforms with minor variations generated by a small number of mutations.

Ribonuclease activity of isoforms. To further analyze the biological properties of the clones, the isoforms were expressed in *E. coli* K12. The recombinant proteins were cleaved from the fusion partner maltose-binding protein and purified to homogeneity.

Group-V allergens in timothy grass pollen are known to have ribonuclease activity [17]. Therefore, we questioned whether quantitative differences of ribonuclease activities can be observed between the isoforms. Iso A2, Iso B2 and Iso B7 were selected and subjected to a ribonuclease activity assay. The allergens possess nucleolytic activity compared to the negative control (Fig. 2). Iso B2 exhibited slightly more nucleolytic activity than the two other molecules; molar concentrations of the three proteins at 50% of the maximum rate of RNA hydrolysis differed by a factor of 1.5. This indicates that structural differences, especially between subgroups A and B, do not significantly influence the functional properties of these molecules.

Immunological reactivities of isoforms. In order to analyze the influence of changes in the primary structures among the isoforms on antibody-binding characteristics, the binding constants of two group-V-specific monoclonal antibodies were determined and IgE reactivities of 26 grass pollen allergic patients analyzed towards selected isoforms. Under soluble conditions with a sandwich-like-assay format binding of mAb Bo1 and BG6 to Iso A2, Iso B2 and Iso B7 was measured with SPR and binding constants were calculated (Table 1). The affinity of mAb Bo1 towards the two group-B isoforms was reduced by over 50% compared with the affinity towards the group-A molecule. Affinities of Phl p-5a-specific mAb BG6 towards group-A isoforms are comparable with mAb Bo1, binding constants towards

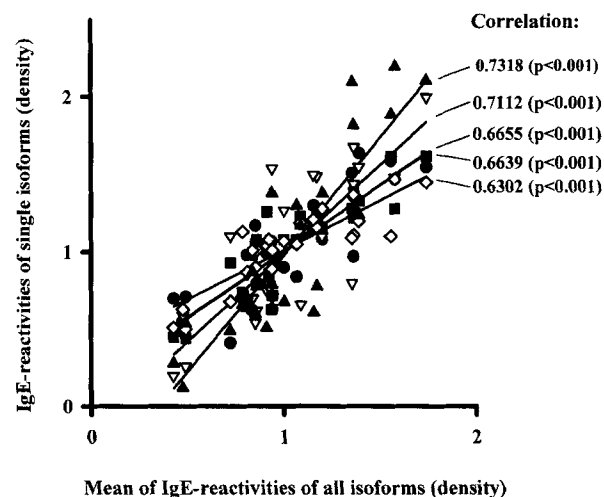


Fig. 3. Correlation of patient IgE reactivities of single versus mean IgE reactivities of all isoforms. IgE-binding intensities of sera from 26 grass-pollen-allergic patients were determined with the immunoblot technique. Results were quantified by densitometry and, for comparison, densities were calculated by division with mean density from the five isoforms and intensity values of each patient were correlated with the mean value of all patients reactivities. Closed triangles, Iso B7; open triangles, Iso B2; closed squares, Iso A2; closed circles, Iso B3; open diamonds, Iso B1.

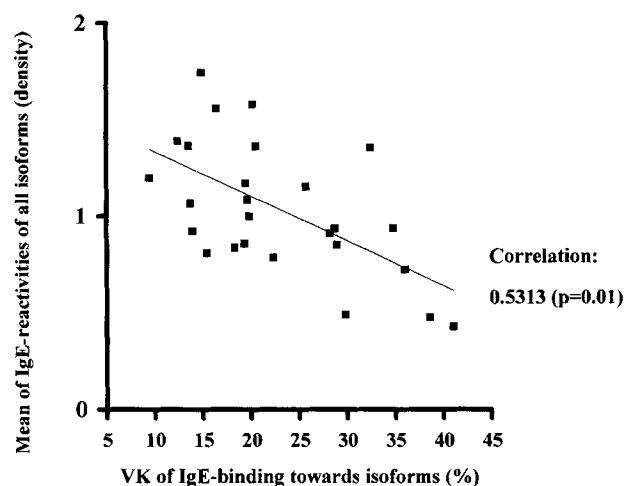


Fig. 4. Individual variation of IgE reactivities correlated to the mean IgE reactivities of all isoforms. The variation coefficient of single patients IgE reactivities towards the five isoforms Iso A2, B1, B2, B3 and B7 was calculated and correlation to the mean of all IgE reactivities is shown.

group-B molecules are in the range of non specificity. These results indicate that changes in the primary structure of the allergens can lead to changes in immunologic recognition by single antibodies. However, since the feature of the immune response of an allergic patient is a polyclonal antibody production towards one molecule, IgE reactivities of different grass-pollen-allergic patients were investigated to several isoforms. Iso A2, B1, B2, B3, and B7 were immunoblotted, strips were incubated with 26 sera of grass-pollen-sensitized patients, and IgE binding was detected. The density of each band was measured and calculated relative to the mean intensity of binding to the five isoforms. The individual patient means of all IgE-binding intensities were paired to each single value (Fig. 3); this results in positive correlations for each isoform with a significance of $P < 0.001$ ($n =$

Table 2. Serum IgE reactivity and allergenic activity of group-V isoforms compared to a group-I allergen in four grass-pollen-allergic patients. Recombinant allergens were used to determine serum IgE binding under denaturing conditions (Western blot) and native conditions (dot blot), histamine-releasing capacity of basophils and skin-prick-test reactivity. IgE binding in Western blot and dot blot was quantified by densitometry, histamine-releasing capacity by determination of molar protein concentration causing 50% of maximum histamine release and skin-prick-test reactivity by measurement of wheal areas. The results of every assay are presented as values relative to the mean. n.d., not determined.

Patient	Serum IgE reactivity		Histamine releasing capacity (Protein concentration)	Skin prick test (Wheal area)
	in Western blot (density)	in dot blot (density)		
Patient 1				
Phl p5 Iso A2	2.70	4.000	1.37	1.54
Phl P5 Iso B2	n.d.	1.340	0.16	—
Phl p5 Iso B7	1.89	1.480	0.16	1.54
Phl p1 Iso 1	0.87	3.680	—	0.26
Patient 2				
Phl p5 Iso A2	—	0.220	4.90	1.59
Phl p5 Iso B2	n.d.	0.007	3.92	2.01
Phl p5 Iso B7	—	0.014	0.98	1.83
Phl p1 Iso 1	0.76	0.012	—	1.02
Patient 3				
Phl p5 Iso A2	1.95	—	1.96	2.01
Phl p5 Iso B2	n.d.	—	1.57	—
Phl p5 Iso B7	0.63	—	0.98	1.81
Phl p1 Iso 1	—	1.590	—	0.86
Patient 4				
Phl p5 Iso A2	2.05	1.520	—	0.95
Phl p5 Iso B2	n.d.	0.362	—	—
Phl p5 Iso B7	1.14	0.540	—	0.50
Phl p1 Iso 1	—	1.260	—	—

26). The variability of IgE-binding intensities, as indicated by the variation coefficient with which patients recognize the isoforms, correlates negatively to the binding intensity (Fig. 4). This indicates that, with a low level of specific pool IgE antibodies, the variability of IgE-binding intensities increases. In summary, these results show that the pool of polyclonal IgE antibodies does not distinguish between the isoforms in case the specific IgE antibodies recognizes the allergen.

Allergenic activity of isoforms. In further experiments, we wanted to determine whether the immunoreactivity of the isoforms correlates to their allergenic activity. For this purpose, four grass-pollen-allergic patients were selected and the IgE reactivity, histamine-release-inducing capacity of basophils and *in vivo* skin reactivity of three isoforms of group-V allergens and one group-I allergen were determined (Table 2). It was shown (Table 2) that binding of an isoform under denaturing conditions does not necessarily lead to binding of the protein under native conditions, and *vice versa*. Furthermore, serological presence or absence of specific IgE antibodies did not guarantee similar biological reactivities in histamine release or the skin-prick test. Only Phl p 5 isoforms A2 and B7 were most reliable in terms of inducing a reaction in the selected grass-pollen-allergic patients ($n = 4$). The capacity to induce histamine release of the three Phl p 5 isoforms did not systematically correlate to sequential differences between group-Va and group-Vb molecules. Additionally, if a significant relative difference of histamine releas-

ing capacity between group Va and Vb in one patient was observed, skin-prick-test reactivity did not reflect this difference. In summary, these data gave no evidence for a systematic linkage between sequential variations of the isoforms and their allergenic activity.

DISCUSSION

The existence of the two major isoforms of group-V allergens in timothy grass pollen (Phl p 5) is well established [24, 25]. It is known that related grass species, which are also used for diagnosis and immunotherapy of grass pollen allergy, express highly similar molecules with more [26] or fewer [27] isoforms. Isoforms a and b of Phl p 5 have been cloned previously [11, 28], and it was shown that these molecules can exhibit ribonuclease activity [17].

In this paper, we describe the cloning and expression of recombinant isoforms belonging to these two subgroups. It was demonstrated that PCR-based screening and cloning of isoforms coincides with an average error incorporation rate of 1.5×10^{-4} , which leads to one nucleotide substitution in 451 base pairs after 30 cycles [15]. With low stringency cDNA hybridization, we thus used a reliable method for homology screening but, nevertheless, only Phl p 5 isoforms were identified, especially Phl p 5b, with minor sequence variations. This is in accordance with findings in Kentucky blue grass [20]. The only remarkable difference in the primary structure of our isoforms can be found between subgroups A and B. These results are contrary to expectations, since other authors [11, 13–15] reported the presence of multiple isoforms and isoforms in a number of different allergens and suggested the existence of multigene families. Biochemical studies of natural extracts [26, 29] in particular, and binding studies with different monoclonal antibodies [30], have indicated that the different groups of allergens mostly consist of a significant numbers of different isoallergens.

Since it was shown that Phl p 5b is an active ribonuclease, it is noteworthy that the nucleolytic activity is preserved in the Phl p 5a isoform and not further increased as has been observed with the C-terminal portion of Phl p 5b [31].

It is now of major interest to investigate whether the difference between homologous isoforms such as Phl p 5a and b has any diagnostic or therapeutic consequences for type-I allergic patients. The cloning of allergens and their isoforms from tree pollen has led to the observation that antigenic and allergenic recognition of isoforms can be variable in type-I allergic patients [14, 32]. In contrast, it was demonstrated with ragweed allergens that the homologous and immunologically crossreactive structures induced a more uniform reaction [33]. Most recently, a hypoallergenic isoform of the major birch pollen allergen Bet v1 was identified which was not detected by IgE antibodies but activated specific T-cell clones [16]. We have now presented evidence that the isoallergens of Phl p 5 in grass pollen, which show sequence variations and biochemical differences, are not distinguished immunologically in case isoforms are detected by the specific IgE antibodies. Differences in affinities of monoclonal antibodies are compensated by the fact that the immune response is polyclonal and that the group-V allergens bear at least two, if not more, IgE-binding epitopes [11, 34, 35].

With our methods, we were not able to isolate a hypoallergenic isoform among the group-V grass pollen allergens. In contrast, it was remarkable that, with Phl p 5 isoforms, no significant systematic association between their sequential differences and their allergenic activity could be observed, indicating that reasons other than these differences among isoforms must accord for hypoallergenicity. One aspect might be factors indepen-

dant of the specific immune response, such as the influence of interleukins on the activation of the allergic-response mechanism [36, 37]. When histamine release in our patients was performed in the presence of interleukin 3, releasability of basophils by Phl p 5 isoforms was increased by a factor of 10^3 to 10^6 (data not shown).

The most reliable assay for allergenic activity in our study was the skin-prick test. With this assay, the most potent isoforms of our collection were identified, Phl p5 Iso A2 and B7. These two forms induced a skin-prick-test response, even in patients who presented either no or very low amounts of specific serum IgE antibodies or who have negative histamine release. Therefore, failure to detect IgE antibodies in serum of grass-pollen-allergic patients or on intravascular basophils does not, in every case, exclude allergic responsiveness of mast cells in the skin. Skin-prick tests with selected recombinant isoforms and other groups of major grass pollen allergens should guarantee a safe diagnosis of grass-pollen-induced allergic disease as shown elsewhere with recombinant birch pollen allergens [38].

From the results presented in this paper, it seems unlikely that the absence of sequentially different grass pollen isoforms in the extract used for specific immunotherapy would influence the outcome of the treatment. In grass pollen allergy, it was demonstrated that partially purified timothy grass pollen extract is as effective in immunotherapy as an extract mixture of different grass pollen [39], showing that the different isoforms in related species are not required for successful therapy. This was confirmed with tree pollen extract which contained the important antigen components from one tree species [40]. In contrast, treatment with selected group-V and group-VI grass pollen allergens was less effective than with an extract containing almost all detectable allergens in one species [39]. It is thus more relevant to determine which group of grass pollen allergens is really essential for therapy. Using cells and sera of allergic patients undergoing a controlled standard immunotherapy with grass pollen extracts, we are about to finish a study where the immune response, the allergenic activity and ability of T-cell activation induced by a selected number of isoforms from the most important allergen groups in timothy grass pollen are compared. This data will be correlated to the successes of the treatment.

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