

“Allergen Engineering”: Variants of the Timothy Grass Pollen Allergen Phl p 5b with Reduced IgE-Binding Capacity but Conserved T Cell Reactivity¹

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One problem of conventional allergen-specific immunotherapy is the risk of anaphylactic reactions. A new approach to make immunotherapy safer and more efficient might be the application of engineered allergens with reduced IgE-binding capacity but retained T cell reactivity. Using overlapping dodeca-peptides, the dominant T cell epitopes of the timothy grass pollen allergen Phl p 5b were identified. By site-directed mutagenesis outside these regions, point and deletion mutants were generated. Allergen variants were analyzed for IgE-binding capacity with sera of different grass pollen allergic patients by Western blotting, Dot blotting, and EAST inhibition test, and for histamine releasing capacity with peripheral blood basophils from different patients. The deletion mutants revealed significantly reduced IgE reactivity and histamine releasing capacity, compared with the wild-type Phl p 5b. Furthermore, in vivo skin prick tests showed that the deletion mutants had a significantly lower potency to induce cutaneous reactions than the wild-type Phl p 5b. On the other hand, T cell clones and T cell lines from different allergic patients showed comparable proliferation after stimulation with allergen variants and wild-type Phl p 5b. Considering their reduced anaphylactogenic potential together with their conserved T cell reactivity, the engineered allergens could be important tools for efficient and safe allergen-specific immunotherapy. *The Journal of Immunology*, 1999, 162: 2406–2414.

More than 20% of the western population suffer from symptoms of type I allergy. Substances inducing this type of allergy are often inhalant allergens from house dust mite, animal dander, or grass and tree pollen. Until now, conventional immunotherapy is performed by s.c. injection of high doses of complete natural allergen extracts as introduced 1911 by Noon (1). These large amounts of complete allergens can lead to anaphylactic side reactions, posing a risk for treated patients. Furthermore, the mechanism of this empirically found immunotherapy is only partly understood, and, especially for type I allergy to house dust mite, animal dander, and pollen, it is not always successful. Consequently, new concepts for successful immunotherapy of type I allergy are greatly required. One promising new approach for therapy is the usage of T cell peptides instead of complete allergens. Application of high doses of specific T cell peptides lead to T cell anergy or a change in cytokine production in vitro and in mouse models (2–5).

The advantage of the application of T cell peptides is that these small peptides usually have no or reduced IgE-binding epitopes. Thus, in contrast to complete allergens, they are not able to cross-link FcεRI receptor-bound IgE on basophils or mast cells, and, therefore, are not able to induce negative side effects like histamine

release or even release of IL-4 from basophils (6). A drawback of the application of T cell peptides is the existence of multiple T cell epitopes on one allergen, which are recognized individually by different patients (7–10).

Different solutions to overcome this problem have been suggested. First, Rogers et al. (11) proposed the combination of dominant T cell epitopes in one recombinant polypeptide (recombinotope). Second, for the birch pollen allergen Bet v 1, a non-IgE-reactive (hypoallergenic) isoform with retained T cell reactivity was described (12). For grass pollen allergens, such isoforms could not be found (13, 14). Third, recently the destruction of conformational IgE-binding epitopes of the house dust mite allergens Der p 2 and Der f 2 by site directed mutagenesis of cysteine residues was described (15, 16). We took up the latter idea for Phl p 5b, the group V grass pollen allergen of timothy grass (*Phleum pratense*). A total of 90% of the grass pollen allergic patients react to this major grass pollen allergen, which is composed of two isoforms, Phl p 5a and Phl p 5b (17, 18). The isoform Phl p 5b was cloned from a cDNA library of timothy grass pollen, and the protein was shown to have ribonuclease activity (19). By epitope mapping of the homologous group V allergen of velvet grass pollen Hol l 5 using overlapping recombinant fragments, at least four continuous IgE-binding epitopes were identified (G. Schramm et al., manuscript in preparation). In addition to the continuous epitopes, our results indicated the presence of discontinuous epitopes, the exact amino acid composition of which cannot be defined by fragmentation experiments. Both allergens, Hol l 5 and Phl p 5b, display such high homology (14) that a similar distribution of IgE-binding epitopes was assumed. The dominant T cell epitopes on Phl p 5b were determined using a series of overlapping synthetic peptides. By stepwise site-directed mutagenesis, we inserted point and deletion mutations within the putative IgE-binding regions but outside the identified T cell epitopes to reduce the IgE reactivity but not the T cell reactivity. Reduced IgE reactivity was investigated

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in vitro by different methods like Western blot, Dot blot, EAST inhibition test, and histamine release assay and in vivo by skin prick testing. T cell reactivity was tested with T cell clones (TCC)³ and T cell lines (TCL) from different grass pollen allergic patients.

Materials and Methods

Site-directed mutagenesis of Phl p 5b

Mutants of Phl p 5b were generated by PCR cloning using sequence-specific primers with individual mismatches (mutagenesis primer). For positions of mutations, see Fig. 1. All PCR reactions were done on the thermocycler (Perkin-Elmer, Norwalk, CT) using 1 ng DNA template, 1 μ M primers, 200 μ M deoxynucleoside triphosphate, 1 U TaqPlus Long Polymerase (Stratagene, La Jolla, CA), and buffer #7 from Opti-Prime PCR Optimization Kit (Stratagene) plus 1% DMSO under the following conditions: 1 min at 96°C, 30 s at 58°C, and 1 min at 72°C for 30 cycles. PCR products were cloned after restriction with the appropriate restriction enzyme (MBI Fermentas, Vilnius, Lithuania) into the expression vector pMAL-c2 (New England Biolabs, Beverly, MA) and transformed into *Escherichia coli* JM109 cells. Transformants were screened by plasmid preparation followed by restriction analysis and automated double-stranded sequencing on ABI 377 (Perkin-Elmer).

Point mutant PM1. The plasmid pB1912 harboring the cDNA coding for Phl p 5b (19), accession number Z27083, was used as template. Two PCR reactions were performed using the primer pairs 5'-terminal primer 5b sense (5'-ATATGGATCCATCGAGGGAAGGCGCGATGCCGGCTACG CC-3')/mutagenesis primer MP1 antisense (5'-GAACGCTAGCGCCG CAGGGACGCTGGC-3'), and 3'-terminal primer 5b antisense (5'-AT ATAAGCTTTCTCTGAAGGAAGGCAACCC-3')/mutagenesis primer MP1 sense (5'-GCGCTAGCGTTCAAGACCTTCGAG-3'). Compared with the original sequence, the two overlapping mutagenesis primers contain six exchanges (bold) within their shared region, leading to two amino acid exchanges D⁴⁹ → L (GAC → CTA) and K⁵⁰ → A (AAG → GCG), and, simultaneously, a new *NheI* restriction site is generated. The resulting PCR products were ligated at the *NheI* site and introduced into the *BamHI/HindIII*-restricted pMAL-c2 expression vector (restriction sites are underlined).

Point mutant PM3. PCR was performed with the 3'-terminal 5b antisense primer and the 5'-terminal mutagenesis primer MPCys (5'-ATATGGATCC ATCGAGGCTAGGGCCGATGCCGGCTACGCCCCGGCCACCCCG GCTGCATGCGGAGCG-3') containing three exchanges (bold) leading to an amino acid exchange A¹³ → C (GCC → TGC) introducing a diagnostic *SphI* restriction site for screening of the transformants (restriction sites are underlined).

Deletion mutants DM1 and DM2. Mutagenesis-PCR was performed with an internal sequence-specific primer, MP2 sense (5'-ATATGCTAGCCG GCGAGCTGCAGACATCG-3') or MP3 sense (5'-ATATGCTAGCCG GCGGCGCTACGACACCTACAAG-3'), and the 3'-terminal 5b antisense primer, leading to two shorter 3'-terminal fragments and generating new *NheI* restriction sites (underlined). From plasmid pPM1 containing the cDNA coding for the point mutant PM1, the original larger 3'-terminal *NheI/HindIII*-fragment was removed. This fragment was replaced by the new shorter PCR-derived fragments described above, leading to the deletion mutants DM1 and DM2.

Deletion mutant DM3. The same mutagenesis protocol was used as described for DM1 and DM2. From plasmid pB1912 the *KspI/HindIII* fragment was removed, and this original 3'-terminal larger fragment was replaced by a shorter PCR-derived fragment, which was obtained using the sequence-specific internal mutagenesis primer MP4 sense with a *KspI* restriction site (5'-ATATCCGCGGCGCGCCTACGACACCTACAAG-3') and the 3'-terminal 5b antisense primer.

Deletion mutant DM4. Plasmid pDM3 containing the cDNA coding for the deletion mutant DM3 was used as template for two PCR reactions with the primer pairs 5'-terminal primer 5b sense/mutagenesis primer MP5 antisense (5'-ATATATGGGTCCCGGCGCCTTGCGCG-3') and mutagenesis primer MP5 sense (5'-ATATATGGGACCCCGAGGC CAAGTTCGAC-3')/3'-terminal primer 5b antisense. The two resulting PCR products were ligated at their joined *EcoO109* site (underlined) and cloned into the *BamHI/HindIII*-restricted pMAL-c2-vector.

Expression and purification of recombinant proteins

The recombinant Phl p 5b wild-type and the variants were expressed as fusion proteins with maltose-binding protein (MBP) in *E. coli* JM109

(MBP expression system; New England Biolabs). The fusion protein was induced and purified according to the manufacturer's manual. Briefly, *E. coli* JM109 cells, harboring the appropriate plasmids, were grown as 1-L cultures to A₆₀₀ = 0.8. Expression of MBP fusion proteins was induced with 0.3 mM isopropyl thiogalactoside. After incubation for an additional 4 h at 37°C, cells were harvested by centrifugation. The cell pellet was resuspended in 30 ml column buffer (20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA). After breaking the cells by passing twice through a French press and subsequent centrifugation at 17,000 × g, MBP fusion proteins were purified from the supernatant by affinity chromatography using an amylose resin column with an average yield of 30 mg fusion protein/L culture. Factor Xa cleavage was conducted at a w/w ratio of 0.5% of the amount of fusion protein, and the reaction mixture was incubated for at least 24 h at room temperature. After desalting using Sephadex G-25 (Pharmacia, Uppsala, Sweden), recombinant proteins were further purified by DEAE anion exchange chromatography. A 10-ml column was eluted with a linear NaCl gradient from 0 to 0.5 M in 20 mM Tris/HCl, pH 8.0. Purity of the proteins was controlled by SDS-PAGE followed by Coomassie staining.

Analysis of IgE reactivity

For Western blotting, 3 pmol/cm purified recombinant proteins were separated by SDS-PAGE (12%) according to Laemmli under reducing conditions if not stated otherwise (20). Proteins were visualized by Coomassie staining or transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by semidry blotting for 30 min at 0.8 mA/cm² (21).

For Dot blotting, different concentrations (200–3 nM) of recombinant proteins were dotted on nitrocellulose membrane using the Minifold I Apparatus (Schleicher & Schuell).

Nitrocellulose membranes were incubated in 0.1 M Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 for blocking free binding sites. Immunological detection of IgE reactivity was performed with patients' sera diluted 1:20 in Tris-buffered saline, 0.05% Tween 20. Alkaline phosphatase-conjugated monoclonal mouse anti-human IgE (1:3000) (Allergopharma, Reinbek, Germany) was used as secondary Ab. Ab binding was visualized with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromogen/substrate mixture (0.033%/0.017% in 0.1 M Tris-buffered saline (pH 9.5) (22).

Purification of nPhl p 5b

The first step of purification was achieved by affinity chromatography of *P. pratense* extract with the mAb 1D11 (23) immobilized on protein G Sepharose (Pharmacia) as described (24). Monoclonal Ab 1D11 recognizes both Phl p 5 isoforms, Phl p 5a and Phl p 5b. Purified Phl p 5 isoforms were subjected to a second affinity chromatography with the mAb BG6 (17), which is specific for the Phl p 5a isoform. The flow-through fraction, containing Phl p 5b, was subjected to size exclusion HPLC using a Superdex 75 column (Pharmacia). Fractions containing Phl p 5b were collected, dialyzed against bidist H₂O, and freeze dried. Purity was checked by SDS-PAGE.

EAST inhibition test

Microtiter plates (Greiner, Deisenhofen, Germany) were coated with nPhl p 5b (0.1 μ g/well). Plates were washed with PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T plus 1% (w/v) BSA. Before loading to the microtiter plate, patients' sera (diluted 1:4) were preincubated with serial dilutions of recombinant proteins as inhibitors (10⁻⁶ M to 3 × 10⁻¹¹ M) for 1 h. IgE-binding was detected with a monoclonal anti-human IgE Ab, alkaline phosphatase conjugate (Allergopharma) and incubated with the substrate *p*-nitrophenylphosphate (PNPP; Sigma, Deisenhofen, Germany). Optical density was measured at 405 nm on the Multiscan MCC340 (ICN, Meckenheim, Germany). The allergenic activity was calculated as relative potency (p_{rel}) on the basis of the 25% inhibition values and the slope of the regression curve according to the method described by the U.S. Food and Drug Administration Bureau of Biologics (25).

Histamine release assay

Granulocytes from grass pollen allergic patients were isolated from EDTA-blood samples by dextran sedimentation, and cell number were adjusted to 10⁵ basophils per ml. Cells were incubated with serial dilutions of purified recombinant proteins (10 μ M to 1 pM), and histamine release was measured in the cell-free supernatant by RIA (Pharmacia). Stimulation with phorbol myristate/ionomycin or anti-IgE was used as positive control. Total histamine was determined after repeated freeze-thawing of the cells.

³ Abbreviations used in this paper: TCC, T cell clone; TCL, T cell line; MBP, maltose-binding protein; SPT, skin prick test.

Peptide synthesis

Peptides were synthesized by solid phase synthesis on polyethylene pins (Multipin, Chiron Mimotopes Peptide Systems, Clayton, Victoria, Australia) with F-moc-amino acids. The C-terminal amino acid coupled to a Lys-Pro anchor group allowed the cleavage of the peptides from the pins with a diketopiperazine-C-terminus. A total of 85 12-mer peptides, overlapping by three amino acids, spanned the amino acid sequence of rPhl p 5b.

T cell epitope mapping

For epitope mapping, the TCC were incubated with peptide pools (10 µg/ml), each pool containing 5 peptides at concentrations of 2 µg/ml. Single peptides were investigated from those pools, which gave stimulation indices ≥3.

Allergen-specific TCL

Allergen-specific TCL were raised from PBMC of 25 grass pollen allergic patients with a clinical history of hayfever and EAST classes ≥3 (EAST-RV; Allergopharma) using *P. pratense* extract. The isolation of PBMC was performed with Lymphoprep media (density = 1077; Life Technologies, Eggenstein, Germany) in Leucosep tubes (Greiner) according to the instructions of the manufacturers. All T cell cultures and stimulation experiments were performed in serum-free Ultraculture medium (Bioproducts, Heidelberg, Germany) supplemented with 2 mM Glutamax I (Life Technologies), antibiotic-antimycotic solution (Sigma) and 20 µM mercaptoethanol (Life Technologies). T cell lines were raised by seeding 1 × 10⁵ PBMC and 10 µg of Phl p 5 or rPhl p 5b/ml in a 96-well round-bottom culture plate in a total volume of 100 µl/well. After 5–7 days, the cultures were fed by adding 100 µl medium with 25 U/ml IL-2 (PBH, Hannover, Germany). Three to four days later, half of the medium was exchanged against fresh medium containing 25 U IL-2/ml and incubated for another 4 days. Fourteen days after set up of these cultures, a proliferation assay was performed to detect allergen-specific T cells. Cultures with stimulation indices of at least 3 were pooled (in general from 12–16 wells) and represent a TCL. Often, more than one TCL was collected from such a culture plate. The TCL were cloned immediately and expanded.

Allergen-specific TCC

Selected Phl p 5-specific TCL were cloned by seeding 0.3 and 0.6 T cells/20 µl medium under stimulation with 5 µg Phl p 5 or rPhl p 5b/ml, 50 U IL-2/ml, 5 U IL-4/ml (Biosource, Ratingen, Germany) and 1 × 10⁴ irradiated (30 Gy) autologous PBMC/well in Terasaki culture plates (Greiner). The plates were checked for growing clones after 7–10 days. Growing clones were expanded in 96-well culture plates under stimulation with 5 × 10⁴ irradiated autologous PBMC/well, allergen, and cytokines in the same concentrations as described above. The clones were cultured by changing half of the medium every 3–4 days with fresh medium supplemented with 25 U IL-2/ml. Fourteen days after feeding with autologous PBMC and allergen, cells were assayed for allergen-induced proliferation.

Proliferation assays

For the proliferation assays, T cells were seeded at 2 × 10⁴ cells/well in a 96-well culture plate in triplicate under stimulation with the allergen (0.3 µM) and 5 × 10⁴ irradiated (30 Gy) autologous PBMC/well. After 48 h incubation at 5% CO₂, 37°C, and humidified atmosphere 1 µCi, [³H]thymidine (Amersham, Braunschweig, Germany) was added to each well, and incubation continued for 16 h. Cells were harvested on microbeta filter mats using a 96-well cell harvester (Wallac ADL, Freiburg, Germany). The stimulation index was calculated as the quotient of cpm of the unstimulated control (T cells and irradiated autologous PBMC) and allergen stimulation.

Skin prick test

Skin prick test (SPT) was done according to the guidelines described by Dreborg (26). Purified nPhl p 5b, rPhl p 5b, and selected variants DM2 and DM4 were applied at different concentrations (3 × 10⁻⁶ M to 3 × 10⁻⁸ M). Commercially available test solutions served as controls: *P. pratense* extract (5000 BU), histamine dihydrochloride (Allergopharma) and 0.9% sodium chloride. Wheal diameters were measured, and calculated wheal areas (mm²) were compared.

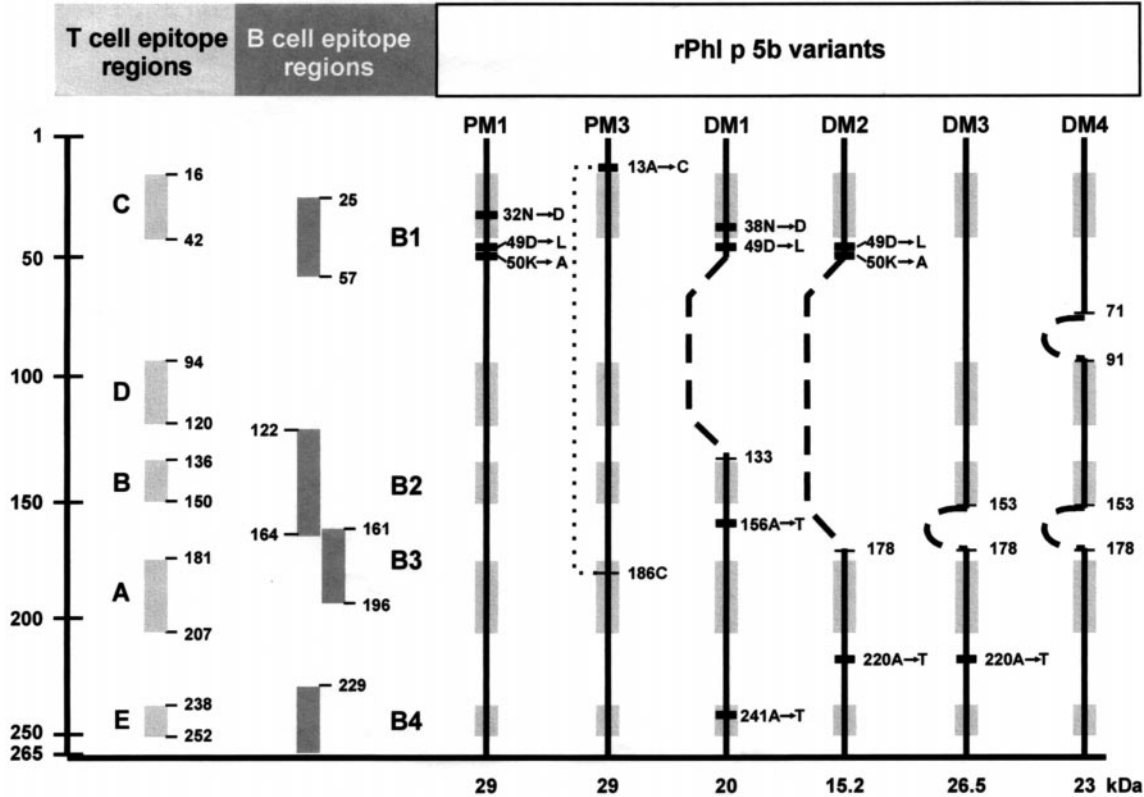


FIGURE 1. Schematic map of the identified T cell epitope regions (light gray) and putative IgE-binding epitope regions (dark gray) of rPhl p 5b and the allergen variants generated by site-directed mutagenesis. PM, point mutants; DM, deletion mutants. Point mutations are indicated by arrows; dotted line shows the putative disulfide bond in PM3; dashed lines indicate deleted stretches.

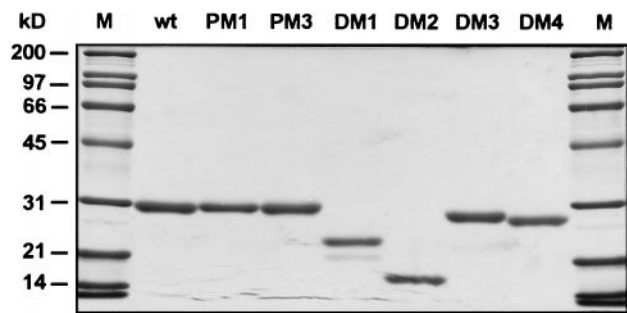


FIGURE 2. Coomassie-stained reducing SDS-PAGE of purified recombinant Phl p 5b wild-type and variants. M, broad range m.w. marker; wt, rPhl p 5b wild-type; PM1 and PM3, point mutants of rPhl p 5b; DM1, DM2, DM3, and DM4, deletion mutants of rPhl p 5b.

Results

Generation of allergen variants of Phl p 5b

Dominant T cell epitopes on the timothy grass pollen allergen Phl p 5b were determined by epitope mapping using 85 dodeca peptides with an increment of 3. Five immunodominant regions were identified based on 111 TCC from 25 grass pollen allergic patients, designated A to E according to the frequency of their recognition by TCC (Fig. 1).

Recently, Hol l 5, one of the major group V grass pollen allergens, was cloned from velvet grass pollen (14). Detailed B cell epitope mapping of this allergen using overlapping recombinant fragments revealed at least four continuous IgE-binding epitopes on small fragments (G. Schramm et al., manuscript in preparation). IgE reactivity was also detected on larger fragments. Further fragmentation of these large fragments abrogated IgE reactivity, indicating the presence of discontinuous epitopes. The discovery of the exact location of such discontinuous epitopes is not possible by fragmentation of the molecule. Thus, the identified continuous IgE-binding epitopes served as basis for allergen engineering (Fig. 1, B1-B4). Both allergens, Hol l 5 and Phl p 5b, display a high overall similarity of 78%, thus, a similar distribution of IgE-binding epitopes could be assumed. Based on the knowledge about T cell epitopes and putative continuous IgE-binding epitopes on Phl p 5b, we generated six different allergen variants carrying mutations within the putative IgE-binding epitopes but outside the determined T cell epitopes. In Fig. 1 the location of the point and deletion mutations are shown. The point mutant PM1 exhibits two amino acid exchanges within the N-terminal IgE-binding epitope (D⁴⁹ → L, K⁵⁰ → A). The aim was to destroy the most important of the putative continuous epitopes (B1) and therefore obtain a reduction of IgE reactivity. The point mutant PM3 displays a new cysteine residue (A¹³ → C) in addition to the wild-type cysteine residue C¹⁸⁶. It was assumed that the formation of an intramolecular disulfide bond between these cysteines should change the con-

formation of the molecule drastically and consequently reduce its IgE-binding properties. The other mutants are deletion mutants lacking larger parts of the molecule: DM1 lacks the stretch between T cell epitope region B and C, including epitope region D, whereas in DM2 the deletion also includes T cell epitope region B. In DM3, a short stretch between T cell epitope regions A and B is deleted, and DM4 carries an additional deletion between D and C. Some of the mutants carry additional amino acid exchanges from PCR cloning: PM1, N³² → D; DM1, N³⁸ → D, A¹⁵⁶ → T, A²⁴¹ → T; DM3, A²²⁰ → T; DM4, A²²⁰ → T. Recombinant proteins were expressed as MBP fusion proteins in *E. coli* and purified by affinity chromatography. MBP was cleaved from proteins using factor Xa and subsequently separated by DEAE chromatography. Purity was checked by SDS-PAGE (Fig. 2).

IgE binding capacity of the Phl p 5b variants

The IgE-binding capacity of the recombinant variants was analyzed by three different methods: Western blot, Dot blot, and EAST inhibition test.

Table I contains the IgE reactivity of 20 individual patients' sera investigated by Western blotting. The data clearly show that introduction of two point mutations in PM1 did not affect the IgE-binding capacity of this variant. Due to the presence of multiple putative IgE-binding epitopes on the molecule, the destruction of only one of them does not seem to be sufficient to reduce the overall IgE reactivity significantly. Accordingly, we did not observe a reduction of IgE reactivity for PM3, although under non-reducing conditions we observed a mobility shift of this mutant in SDS-PAGE compared with the wild-type Phl p 5b (data not shown) indicating that the conformation of the molecule has changed. In contrast, the deletion mutants revealed a clear reduction of IgE-binding. Besides the fact that different patients' sera exhibited individual binding intensity to the different variants, we observed the most reduced overall IgE reactivity to the deletion mutants DM2 and DM4.

For Western blotting, proteins were unfolded by loading with SDS and separated on polyacrylamid gels. Although there might be some refolding after blotting on nitrocellulose membrane, this procedure might lead to a certain degree of denaturation of the proteins. To get a more native situation than by Western blotting, IgE reactivity of a pool serum and 10 individual sera were analyzed by Dot blotting. Fig. 3 shows the results for the pool serum and three of the individual sera. However, this assay confirmed the results obtained by Western blots, namely PM1 and PM3, showing no reduction of IgE reactivity, whereas all deletion mutants displayed reduced binding to IgE. Deletion mutants DM2 and DM4 revealed strongly reduced IgE-binding capacity, while IgE binding to DM3, the mutant with the smallest deletion, was only marginally reduced. As seen by Western blotting, we observed individual IgE reactivity of different patients' sera to the different variants.

Table I. IgE reactivity of 20 different patients' sera with Phl p 5b wild-type (wt) and variants (PM1-DM4) analyzed by Western blotting

Variants	Patients' Sera																			
	W	KH	BS	DB	SF	II,3	II,4	II,5	II,16	II,17	II,19	II,20	A2	A6	A11	A15	A22	A29	A31	A33
wt	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PM1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PM3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
DM1	+	+	++	++	++	+	+	+	++	++	+	+	++	++	++	+	+	+	++	+
DM2	—	+	—	+	—	—	—	—	—	+	—	+	+	+	—	+	—	+	+	—
DM3	++	++	+	++	++	+	+	++	+	++	+	+	++	++	++	+	+	++	++	++
DM4	—	+	—	+	—	—	—	—	—	+	—	+	+	—	—	+	—	+	+	—

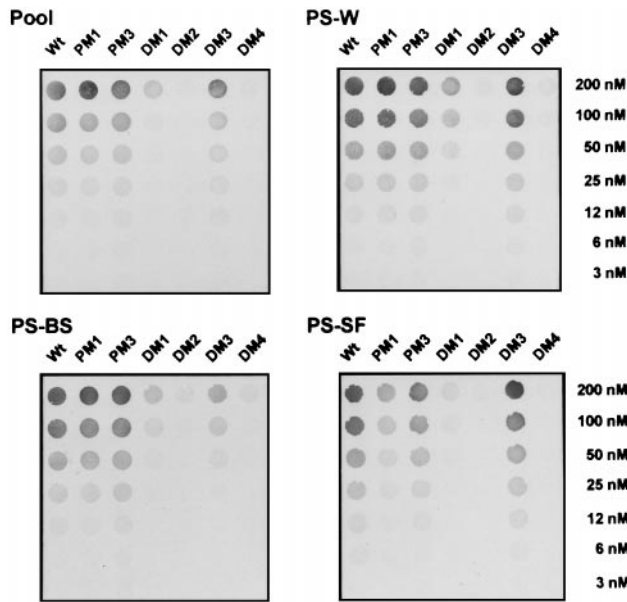


FIGURE 3. IgE reactivity of a pool serum and three different individual patients' sera with rPhl p 5b wild-type and variants analyzed by Dot blotting. Pool, pool serum, $n = 11$; PS-W, PS-BB, and PS-SF, individual patients' sera; wt, rPhl p 5b wild-type; PM1 and PM3, point mutants of rPhl p 5b; DM1, DM2, DM3, and DM4, deletion mutants of rPhl p 5b. Recombinant proteins were dotted at different concentrations (200 nM to 3 nM).

While serum BB showed weaker binding to DM3 than serum W and SF, the latter exhibited less reactivity to DM2 and DM4.

Further analysis of IgE reactivity was done by EAST inhibition test. In this case, proteins are not artificially coated to a solid phase, but react with the IgE Abs in solution. Natural Phl p 5b was coated to the microtiter plate and Phl p 5b wild-type and variants were used at different concentrations as inhibitors of the IgE-binding of pool serum (Fig. 4) and 10 individual sera. With this assay we obtained comparable results to the Dot blot. Wild-type Phl p 5b and point mutants were able to inhibit IgE-binding to the coated allergen completely (Fig. 4, open symbols), but the deletion mutants exhibited a clear reduction of their inhibition capacity (Fig. 4, filled symbols). Table II shows the results of EAST inhibition test of all investigated sera. Again we found individual differences between the different sera: e.g., IgE reactivity of the sera BB and SF was reduced to a larger extent than IgE reactivity of the serum DB.

Histamine release assay

The functional activity of the variants was investigated by histamine release assay with human basophils. Therefore, peripheral

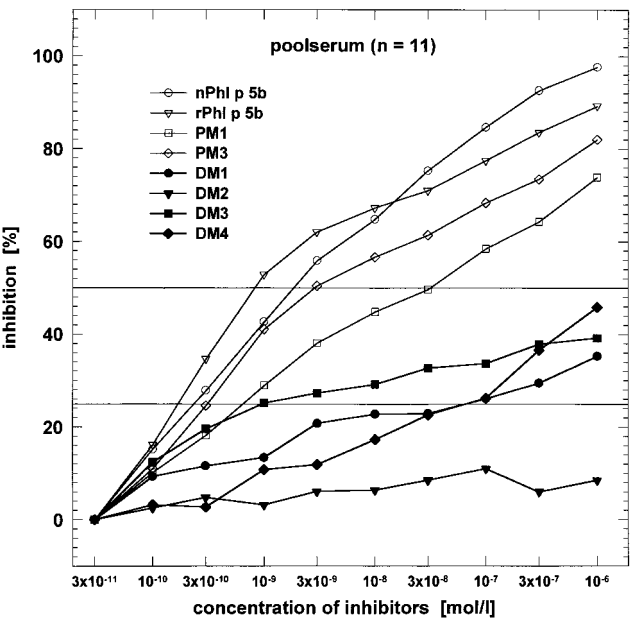


FIGURE 4. Inhibition of IgE reactivity of a pool serum ($n = 11$) to solid phase nPhl p 5b by soluble nPhl p5b, rPhl p 5b wild-type, and variants analyzed by EAST inhibition test. PM1 and PM3, point mutants of rPhl p 5b; DM1, DM2, DM3, and DM4, deletion mutants of rPhl p 5b.

blood basophils of eight different patients were stimulated with wild-type Phl p 5b and variants. Fig. 5 shows paradigmatically the results of four of the investigated patients. While the wild-type Phl p 5b and point mutants PM1 and PM3 induced comparable release of histamine, the deletion mutants revealed a strongly reduced (DM1, DM3) or completely abolished (DM2, DM4) histamine releasing capacity. These results correlate very well with the results found in the other test systems. For selected patients, the histamine release assay was repeated at different times, during and out of pollen season, with comparable results (data not shown).

Skin prick test

The ability of the Phl p 5b wild-type and two selected deletion variants DM2 and DM4 to elicit cutaneous reactions *in vivo* was evaluated for five grass pollen allergic patients (Table III). Proteins were applied at three different concentrations from 3.4×10^{-6} M to 3.4×10^{-8} M. For all patients, we observed a dramatically reduced reactivity to the deletion mutants. As seen before in the *in vitro* assays, a strongly individual reactivity of the different patients was found. Patient DB, exhibiting a relatively strong reactivity in EAST inhibition test and histamine release (not shown),

Table II. IgE reactivity of a poolserum ($n = 11$) and 10 individual patients' sera with nPhl p 5b, rPhl p 5b wild-type and the variants (PM1-DM4) analyzed by EAST inhibition test

Inhibitor	P_{rel}^a										
	Pool	PS-W	PS-KH	PS-BB	PS-DB	PS-SF	PS-II,3	PS-II,12	PS-II,17	PS-II,19	PS-II,20
nPhl p 5b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
rPhl p 5b	1.362	0.363	0.887	0.766	0.851	1.773	0.436	0.483	0.797	0.182	0.779
PM1	0.308	0.215	0.274	0.408	0.419	0.082	0.880	0.706	0.622	0.004	0.379
PM3	0.789	0.684	0.506	0.846	0.793	3.055	0.843	0.361	1.561	0.619	0.643
DM1	0.006	0.000	0.000	0.000	0.088	0.000	0.028	0.009	0.000	0.000	0.000
DM2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DM3	0.191	0.064	0.232	0.000	0.276	0.672	0.000	0.005	0.002	0.000	0.000
DM4	0.008	0.001	0.004	0.000	0.010	0.014	0.000	0.001	0.000	0.000	0.000

^a Relative potency (p_{rel}) is calculated from 25% inhibition values of nPhl p 5b with respect to the reference nPhl p 5b (25).

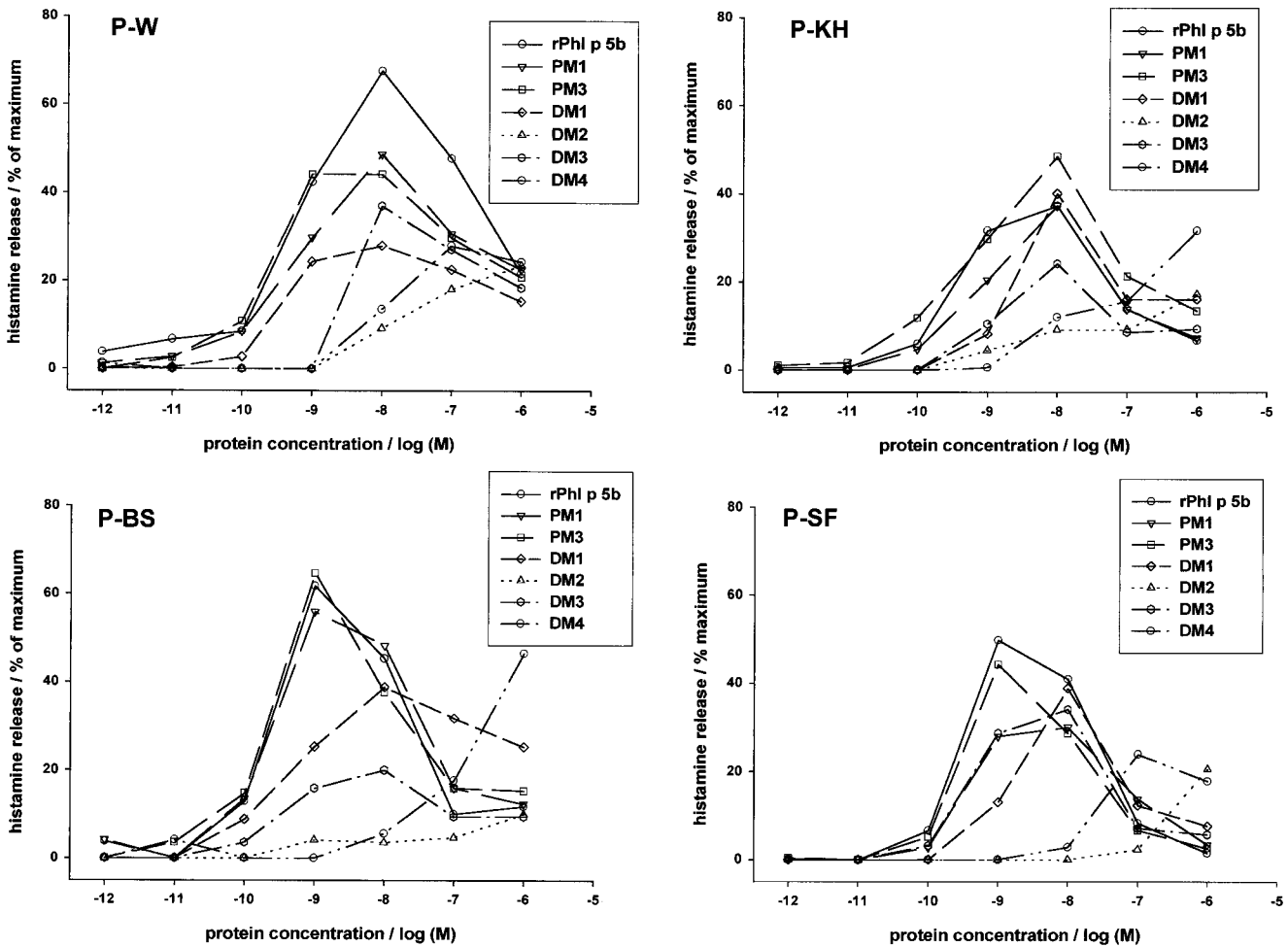


FIGURE 5. Histamine releasing capacity of rPhl p 5b wild-type and variants from basophils of four different patients. wt, rPhl p 5b; PM1 and PM3, point mutants of rPhl p 5b; DM1, DM2, DM3, and DM4, deletion mutants of rPhl p 5b.

revealed a 10-fold reduced skin reaction to the deletion mutants in SPT. In contrast W, BS, and SF revealed 100-fold reduced skin reactions to DM2 and DM4 compared with the wild-type Phl p 5b. Nonallergic subjects and a grass pollen allergic patient not reactive to group V grass pollen allergens showed no skin reactions to wild-type and variant Phl p 5b indicating the high specificity of the SPT.

T cell reactivity of TCC and TCL with Phl p 5b and allergen variants

Table IV shows the proliferation of 13 TCC, derived from 11 different patients, after stimulation with the different allergen vari-

ants. T cell reactivity was conserved throughout all variants, with some exceptions: TCC specific for epitope D failed to proliferate after stimulation with the mutants DM1 and DM2 because these mutants lack the epitope D, as well as TCC specific for epitope B failed to recognize mutant DM2 due the absence of this epitope. Point mutations derived by PCR led to nonreactivity of particular TCC. For example, TCC 3.2.2A12 failed to recognize PM1 due to the mutation N³² → D affecting the epitope C, and TCC specific for epitope E failed to recognize DM1 due to the mutation A²⁴¹ → T affecting the epitope E. Table V shows the reactivity of four TCL. For all TCL, we observed conserved proliferation indices

Table III. SPT of five grass pollen allergic patients, a non-allergic control (con 1), and a grass pollen allergic patient not reactive to group V grass pollen allergens (con 2) with nPhl p5b, rPhl p 5b, and the deletion mutants DM2 and DM4.

	nPhl p 5b (mol/L)			rPhl p 5b (mol/L)			DM2 (mol/L)			DM4 (mol/L)			g6 ^a	His ^a	NaCl ^a
	3.4 × 10 ⁻⁶	3.4 × 10 ⁻⁷	3.4 × 10 ⁻⁸	3.4 × 10 ⁻⁶	3.4 × 10 ⁻⁷	3.4 × 10 ⁻⁸	3.4 × 10 ⁻⁶	3.4 × 10 ⁻⁷	3.4 × 10 ⁻⁸	3.4 × 10 ⁻⁶	3.4 × 10 ⁻⁷	3.4 × 10 ⁻⁸			
W	87	20	8	86	20	11	10	2	neg	14	6	neg	105	16	neg
KH	57	13	5	165	13	5	24	5	neg	28	2	neg	113	13	neg
DB	57	22	14	20	20	6	11	6	2	28	2	neg	105	20	neg
SF	33	16	5	8	8	5	4	neg	neg	6	2	neg	21	11	neg
BS	82	11	3	31	22	2	31	neg	neg	5	neg	4	81	7	neg
con 1	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	17	neg
con 2	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	23	11	neg

^a g6, commercially available timothy grass pollen extract; His, histamine-positive control; NaCl, NaCl-negative control. Values are wheal areas in mm².

Table IV. Proliferative response of human Phl p 5-specific TCC to nPhl p 5b, rPhl p 5b, and rPhl p 5b variants^a

TCC	Epitope	nPhl p 5b	rPhl p 5b	PM1	PM3	DM1	DM2	DM3	DM4
3.2.2A12	31–42 (C)	19.4	15.9	0.5	15.3	5.5	16.0	18.7	14.6
10.3.21E7	33–45 (C)	6.6	8.7	10.6	3.5	11.1	11.3	7.8	9.6
16.1.27A3	94–105 (D)	3.0	3.3	3.9	3.0	1.3	0.8	3.3	6.0
19.1.10C6	106–120 (D)	30.1	32.4	35.2	32.7	1.2	1.1	39.1	39.3
AHE 2	94–108 (D)	10.5	7.7	3.4	4.1	0.6	0.5	4.2	3.6
DW 8	136–150 (B)	12.0	11.3	10.8	10.4	11.2	1.4	6.1	9.9
JR 7	136–150 (B)	23.3	10.0	10.2	18.3	8.7	1.6	15.3	19.7
UZH 2	136–150 (B)	21.3	12.7	9.3	12.7	8.0	0.7	6.0	5.0
17.3.11C2	181–195 (A)	19.2	22.7	21.9	18.5	22.2	29.5	25.8	24.6
17.3.1D8	181–195 (A)	13.2	14.8	15.6	14.1	15.8	18.8	17.6	16.7
KS 3	181–195 (A)	14.1	18.3	10.3	9.8	21.1	32.2	17.5	22.4
20.2.3.C6	238–252 (E)	ND	32.3	29.0	30.7	1.8	25.8	33.4	33.2
20.1.9B6	238–252 (E)	ND	58.6	55.1	45.6	1.6	73.3	66.5	62.1

^a Values are stimulation indices. Boldface values indicate negative proliferative response.

after stimulation with the different allergen variants indicating conserved T cell reactivity of the mutants.

Discussion

In this work, we describe the construction of variants of the timothy grass pollen allergen Phl p 5b with reduced IgE reactivity in vitro and in vivo but conserved T cell reactivity. We chose Phl p 5b as a model allergen from grass pollen because it is a well-characterized major allergen to which 90% of the grass pollen allergic patients are reactive (17).

The aim of this study is to define allergen variants that may be used in the future as alternative reagents for a successful immunotherapy replacing the commonly used allergen extracts. Extracts usually contain the complete allergens, e.g., molecules with several IgE-binding epitopes (27, 28), which are able to cross-link FcεRI-bound IgE on mast cells and basophils. Cross-linking does not only induce the release of histamine but also the release of IL-4 from basophils (6), thereby maintaining a Th2 cytokine milieu and supporting the allergic reaction. Because the application of high doses of T cell peptides from different allergens like Fel d 1, Der p 1, and Bet v 1 is known to result in the unresponsiveness or anergy of allergen-specific T cells in the murine model (2, 29, 30), this therapy concept is discussed as a novel approach for successful immunotherapy. T cell peptides have no or only reduced IgE reactivity, thus, in contrast to the complete allergens, they are not able to induce anaphylactic side reactions. However, patients display individual reactivity to multiple T cell epitopes, especially shown for grass and tree pollen allergens (7–10) making the application of one or two peptides harboring the major T cell epitopes suitable for some but not all patients. This might be the reason for the observation that although successful modulation of the immune re-

sponse after treatment with T cell peptides could be achieved in the mouse model, treatment of type I allergy to cat dander in human subjects was of little success (31–33). For immunotherapy, the use of tailor-made peptide mixtures or single allergens that lack IgE-binding epitopes but carry as many T cell epitopes as possible is desirable. If such modified allergens cannot be obtained from natural sources as described for the birch pollen allergen Bet v 1 (12), it may be possible to use recombinant DNA technology to get such non-IgE-reactive allergen variants, as described for the house dust mite allergens Der p 2 and Der f 2 (15, 16). These allergens have been shown to possess only conformational IgE-binding epitopes dependent on the existence of disulfide bonds. IgE reactivity was successfully destroyed by site-directed mutagenesis of one relevant cysteine residue. We used the group V grass pollen allergen Phl p 5b as model for such an approach. Epitope mapping studies of different group V grass pollen allergens (27, 28; G. Schramm et al., manuscript in preparation) revealed the presence of both discontinuous and several continuous IgE-binding epitopes. Phl p 5b was shown to possess a very stable C-terminal part of the molecule exhibiting all properties of the complete allergen as ribonuclease activity, IgE reactivity, and histamine releasing capacity (34). Thus, it is expected that exchange of one or few amino acids does not lead to a complete loss of IgE reactivity. Nevertheless, we started with the attempt to destroy the putative N-terminal continuous IgE-binding epitope identified on Hol l 5 by two point mutations. We expected not a complete, but a clearly reduced IgE reactivity of about 30% of the investigated patients' sera, which are reactive with this epitope. However, point mutant PM1 did not show any significant reduction of IgE reactivity. Possible explanations for this result could be: 1) amino acid exchanges were inappropriate for elimination of the IgE-binding epitope; and 2) the presence of multiple IgE-binding

Table V. Proliferative response of human Phl p 5-specific TCL to nPhl p 5b, rPhl p 5b, and rPhl p 5b variants^a

TCL	nPhl p 5b	rPhl p 5b	PM1	PM3	DM1	DM2	DM3	DM4
P1	22.4	9.5	7.9	8.0	6.9	4.1	11.7	12.7
P2	3.9	2.8	2.9	2.1	2.3	2.7	5.9	2.3
P3	4.5	2.5	5.5	2.5	2.7	3.8	6.5	2.3
P4	59.3	20.8	12.5	5.9	10.7	36.9	53.3	49.8
P5	8.5	15.5	16.3	12.7	11.6	8.9	16.2	17.4
P6	20.5	18.9	16.8	14.5	15.6	14.6	17.5	18.8
P7	5.3	4.8	4.7	3.2	4.2	2.1	5.1	5.8
P8	36.2	28.8	25.9	22.3	16.2	15.6	28.2	31.6
P9	12.3	9.5	9.0	6.6	5.8	4.8	9.2	9.4
P10	25.4	18.4	18.3	16.5	15.9	14.6	18.4	19.2

^a Values are stimulation indices.

epitopes on this molecule. Thus, regarding the overall IgE reactivity, destruction of only one of the IgE epitopes might be not detectable. The second approach was to destroy the discontinuous epitopes by changing the conformation of the complete molecule 1) by inserting a disulfide bond, and 2) by deleting regions of the molecule. Interestingly, the cysteine mutant PM3 did not show any change in IgE reactivity compared with the wild-type Phl p 5b, although we assumed the formation of a disulfide bond because the mutant experienced a mobility shift in nonreducing SDS-PAGE compared with the wild-type. Change of the conformation seems not to influence the epitopic structure of the molecule, underlining the importance of continuous IgE epitopes for this allergen. In contrast to the results observed for the point mutants, all deletion mutants revealed a significantly reduced IgE reactivity.

For investigation of the IgE reactivity, we used three different methods: Western blotting, a procedure useful for first investigations before purification of the proteins from bacterial lysates, Dot blotting, and EAST inhibition assay, where proteins retain their native folding pattern. Although Western blotting is often thought to provide artificial results due to unfolding and denaturation of the proteins, we observed comparable behavior of all mutants in the respective test systems. Concerning the deletion mutants DM1 and DM3, different patients' sera revealed individual IgE reactivity, some of them reacting stronger to DM1 than to DM3, and vice versa. This individual reactivity was obtained in all applied test systems and correlates with the finding that different patients react individually with the different IgE binding epitopes on the group V allergens (G. Schramm et al., manuscript in preparation). To assess the functional capacity of the variants, histamine release experiments were performed. For the deletion mutants, we observed again a significantly reduced histamine releasing capacity during and out of pollen season. To test the *in vivo* activity of DM2 and DM4, we performed skin prick tests with five different patients. Both mutants revealed clear reduction of their potency to induce skin reactions, 10- to 100-fold depending on the patient. As seen in the *in vitro* test systems, we observed highly individual reactivity of the different patients. For example, DB retained a relatively high reactivity in all *in vitro* assays and in SPT (10-fold reduction), whereas other patients as W or SF, exhibiting nearly no reactivity *in vitro*, showed 100-fold reduction of reactivity in SPT. The good correlation of results using a combination of different test systems allowed us to convincingly demonstrate the reduction of the IgE reactivity of the allergen variants.

Of note, compared with DM3, the deletion mutant DM4 displays an additional deletion (amino acid 71–91). Although this deletion lies outside the putative continuous IgE-binding epitopes depicted in Fig. 1, DM4 exhibits significantly less IgE reactivity than DM3. Considering that we have used results of an epitope mapping performed on the homologous allergen Hol l 5 as basis for allergen engineering, on Phl p 5 and Hol l 5 not all of the IgE-binding epitopes might necessarily be the same. Phl p 5b may exhibit an additional continuous IgE-binding epitope in region 71–91, which was not found on Hol l 5. On the other hand, in addition to the continuous epitopes, Hol l 5 has been found to contain discontinuous epitopes. These were detected only on large fragments of the molecule and were destroyed by further fragmentation. Thus, it seems not unlikely that region 71–91 might crucially contribute to the formation of such a discontinuous IgE-binding epitope.

T cell reactivity of the mutants was analyzed by stimulation of Phl p 5-specific TCC and TCL to ensure that the total Phl p 5-specific T cell repertoire was retained. We observed conserved T cell reactivity of TCC as well as TCL. Mutants lacking individual epitopes were not able to induce proliferation of T cells specific for

these epitopes. These results were reproducible and served as internal control of our test system.

In general, the IgE-binding capacity was reduced in dependence of the length of the deletion, best shown for the deletion mutant DM2 lacking the largest region. For optimal conservation of T cell reactivity, most of the protein should be retained as in the case of the deletion mutant DM3. However, this mutant still exhibited a relatively strong IgE reactivity. A compromise was found in the case of the deletion mutant DM4, where we observed a nearly complete reduction of IgE-binding capacity together with an overall conserved T cell reactivity due to very small deletions. These results make DM4 the best allergen variant for our purpose.

Our results suggest that allergen engineering by recombinant DNA technology is a suitable method to generate non-IgE-reactive, but T cell-reactive variants of a variety of different allergens. Even allergens, such as the grass pollen allergen Phl p 5b, exhibiting stable discontinuous and several continuous IgE binding epitopes spread over the entire molecule and recognized individually by different patients, can be modified to a dramatically reduction of IgE reactivity. Such attenuated allergen variants may be valuable tools for improved immunotherapy of type I allergy caused by allergens of many different sources.

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