

Combination vaccines for the treatment of grass pollen allergy consisting of genetically engineered hybrid molecules with increased immunogenicity

Birgit Linhart*, Beatrice Jahn-Schmid*, Petra Verdino[†], Walter Keller[†], Christof Ebner*, Dietrich Kraft*, and Rudolf Valenta*

*Department of Pathophysiology, Vienna General Hospital, AKH, University of Vienna, Austria; and [†]Institute of Chemistry, Karl-Franzens University of Graz, Austria

Corresponding author: Rudolf Valenta, Molecular Immunopathology Group, Department of Pathophysiology, Vienna General Hospital, AKH, Medical School, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: Rudolf.valenta@akh-wien.ac.at

ABSTRACT

Most of the 400 million grass pollen-allergic patients worldwide are co-sensitized to several unrelated grass pollen allergens. Based on frequent co-sensitization patterns determined in 200 grass pollen-allergic patients, three recombinant hybrid molecules were developed by polymerase chain reaction-based mending of cDNAs coding for the major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6) for vaccination against grass pollen allergy. The hybrids rP2-P6, rP6-P2, and rP5-P1 contained most of the epitopes of natural grass pollen extract and induced stronger lymphoproliferative responses in cultured mononuclear cells of grass pollen-allergic patients than did equimolar mixtures of the individual allergens. Immunization of mice with the hybrids yielded higher antibody titers than did immunization with the individual allergen components or grass pollen extract, which suggests that the individual components of the hybrids can serve as molecular scaffolds for each other to enhance their immunogenicity. Antibodies induced with the hybrids in mice inhibited the binding of grass pollen-allergic patients' immunoglobulin E to each of the individual allergens and grass pollen extract and may thus represent protective antibodies. The principle of increasing the immunogenicity of antigens by engineering hybrids thereof may be applied not only for the treatment of polysensitized allergic patients but also for general vaccine development.

Key words: allergy • recombinant allergen • specific immunotherapy

Type I allergy is an immunologically mediated hypersensitivity disease affecting almost 25% of the population (1). Allergic individuals are prone to mount IgE antibody responses against *per se* innocuous antigens (i.e., allergens); (2). The immediate symptoms of allergy (e.g., acute allergic rhinoconjunctivitis, asthma, anaphylactic shock) result from the allergen-induced crosslinking of effector cell-bound IgE and the release of biological mediators (e.g., histamine, leukotrienes) (3); whereas late symptoms (e.g., atopic dermatitis, chronic asthma) can be caused by IgE-mediated presentation of allergens to T cells resulting in T cell activation and release of proinflammatory cytokines (4, 5).

The only causative therapy approach for Type I allergy, allergen-specific immunotherapy, is based on the systemic administration of allergens to patients in order to induce allergen-specific “unresponsiveness” (6). A major disadvantage of specific immunotherapy is that the allergen extracts used for vaccination are composed of mixtures of allergenic and nonallergenic components. Therapeutic allergen extracts may lack important allergens, the ratio of the individual components in the extract may greatly vary and it has been demonstrated that individual allergens exhibited different immunogenicity (6–8). For the latter reasons, it is difficult to prepare vaccines for patients who are sensitized against several different allergen components (6).

Because of their worldwide distribution and heavy pollen production, grasses represent one of the most important allergen sources for almost 400 million allergic patients (1, 9, 10). Grass pollens contain several immunologically unrelated allergens against which allergic patients are frequently co-sensitized (11). These allergens have been classified in different groups, of which group 1, group 2, group 5, and group 6 represent the most important allergens because they are recognized by the majority of grass pollen-allergic patients and induce strong clinical reactions (11–16).

Here we present an approach for the design of combination vaccines for the treatment of grass pollen-allergic patients with complex sensitization patterns. This approach is based on the generation of hybrid molecules of those grass pollen allergens against which the majority of grass pollen-allergic patients is co-sensitized. cDNAs coding for these allergens, fused by “gene soeing” (17), are expressed as hybrid molecules in which each component acts as a molecular scaffold to increase the immunogenicity of the other component. Three hybrid molecules (rP2-P6, rP6-P2, rP5-P1), consisting of major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6) that are representative for the major grass pollen allergen groups (11–15), were engineered, expressed, and purified. Whether the relevant B cell and T cell epitopes are present in the hybrid molecules was demonstrated with specific antibody probes, by immunological competition experiments and lymphocyte proliferation experiments. Furthermore, mice were immunized to study the immunogenicity of the hybrid molecules and to analyze whether immunization induces IgG antibody responses, which can block the binding of grass pollen-allergic patients’ IgE antibodies to grass pollen allergens.

MATERIALS AND METHODS

Recombinant allergens, natural allergen extracts

Purified recombinant timothy grass pollen allergens Phl p 1 (26.1 kDa), Phl p 2 (10.7 kDa), Phl p 5 (28.5 kDa), and Phl p 6 (11.8 kDa), which had been expressed in *E. coli* (13–15), were obtained from BIOMAY (Vienna, Austria). Pollen from *Phleum pratense* was purchased from Allergon Pharmacia (Välinge, Sweden). Aqueous pollen extracts were prepared and checked for quantity and quality of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining as described (18–20). Total protein amounts were also measured by using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Characterization of allergic patients, allergen-specific rabbit antisera

Sera were obtained from 200 grass pollen-allergic patients. The diagnosis of grass pollen allergy was based on case history, skin-prick testing, and IgE serology (14). Total serum IgE levels and IgE levels specific for timothy grass pollen extract were determined by CAP RAST measurements (Pharmacia Diagnostics, Uppsala, Sweden). IgE reactivity profiles of the patients to natural and recombinant timothy grass pollen allergens were determined by Western blotting and ELISA, respectively (14).

Construction of plasmids expressing rP2-P6-, rP6-P2-, and rP5-P1 hybrid molecules

cDNAs coding for hybrid molecules consisting of the major timothy grass pollen allergens Phl p 2 and Phl p 6 were constructed by using polymerase chain reaction (PCR)-based “gene soeing” (17). To study whether the assembly of the allergens may affect the immunological properties of the resulting hybrid molecule, we constructed one hybrid, designated rP2-P6, containing Phl p 2 at the N-terminus and Phl p 6 at the C-terminus. The second hybrid molecule, rP6-P2, consisted of a N-terminal Phl p 6 and a C-terminal Phl p 2 unit. In a first PCR reaction, the cDNA coding for the complete rP6-P2 hybrid molecule was obtained by PCR using the primers P2a-5’ and P2a-3’ for Phl p 2 and P6a-5’ and P6a-3’ for Phl p 6 to create overlapping ends ([Table 1](#)). In a subsequent PCR reaction the two gel-purified PCR products (QIAGEN gel purification kit, Qiagen, Hilden, Germany) were used as templates. The cDNA coding for the complete rP6-P2 hybrid molecule was obtained by PCR using the primers P2a-5’ and P6a-3’ ([Table 1](#)). At the 3’ end of the Phl p 6 sequence, a hexahistidine tag was introduced followed by a stop codon and a BamHI restriction site. The resulting P2-P6 construct was inserted into the NdeI/BamHI site of plasmid pET17b (Novagen, Madison, WI).

The plasmid expressing a rP6-P2 hybrid molecule was constructed as described for the rP2-P6 hybrid molecule by using the primers P6b-5’ and P6b-3’ to amplify the cDNA coding for the Phl p 6 unit of the hybrid and the primers P2b-5’ and P2b-3’ to obtain a Phl p 2 encoding cDNA in the first PCR reaction ([Table 1](#)). The cDNA coding for the complete rP6-P2 hybrid molecule was obtained in a subsequent PCR reaction using the primers P6b-5’ and P2b-3’ ([Table 1](#)). The resulting P6-P2 construct was inserted into the NdeI/BamHI site of the pET17b vector.

The rP5-P1 expressing plasmid was constructed by generating cDNAs coding for Phl p 5 and Phl p 1 by using the primers P5-5’/P5-3’ and P1-5’/P1-3’, respectively ([Table 1](#)). The signal peptide of Phl p 5 was replaced by a NdeI restriction site containing the ATG start codon at the 5’ end of the coding region. We introduced a KpnI restriction site into the 3’ end of Phl p 5 by using a modified primer replacing the stop-codon. A cDNA coding for mature Phl p 1 containing a 5’ KpnI restriction site and a nucleotide stretch coding for a C-terminal hexahistidine-tag followed by a BamHI site was obtained by PCR with appropriately modified primers. The two PCR products coding for Phl p 5 and Phl p 1 were united via the KpnI site and the resulting fragment cloned into the NdeI/BamHI site of the pET17b expression vector.

Expression in *E. coli* and purification of recombinant hybrid molecules

All constructs were transformed into *E. coli* BL21 (DE3) and expressed in liquid culture. Cells were grown in LB-medium containing 100 mg/l ampicillin to an OD₆₀₀ of 0.8. The expression of

the recombinant proteins was induced by adding isopropyl- β -thiogalactopyranoside (IPTG) to a final concentration of 0.5mM. After 4 h at 37°C, cells were harvested by centrifugation.

Recombinant P2-P6 and P6-P2 were expressed as soluble proteins. Cells were resuspended in lysis buffer (50 mM NaH₂PO₄; 300 mM NaCl; 10 mM imidazole, pH 8.0) and lysed with an ultraturrax (Polytron, Kinematica, Bethlehem, PA). The lysate was centrifuged at 10,000 x g for 30 min at 4°C to remove the cellular debris. The supernatant was loaded onto a Ni-NTA column (QIAGEN), which was washed, and the bound proteins were eluted with 250 mM imidazole and dialyzed against water.

Recombinant P5-P1 was expressed in the inclusion body fraction. Cells were resuspended in 10 mM Tris and 0.1% Triton, pH 7.4, and was lysed by adding lysozyme (QIAGEN). After centrifugation at 14,000 x g for 20 min at 4°C, the pellet was washed 4 times with 5 mM Tris, pH 8.0; 0.05 M NaCl; 0.25% desoxicholate; 0.25 mM β -mercaptoethanol; and once with 10 mM Tris, pH 8.0, 3% isopropanol. Inclusion bodies were solubilized with 8 M urea; 100 mM NaH₂PO₄; and 10 mM Tris, pH 8.0. After centrifugation at 14,000 x g for 20 min, the supernatant was loaded on a Ni-NTA column. The proteins were renatured on the column by using a linear 6–1 M urea gradient in 500 mM NaCl, 20% glycerol, and 20 mM Tris, pH 7.4, over a period of 1.5 h (21). Proteins were eluted by addition of 250 mM imidazole and were dialyzed against PBS, pH 7.4.

The purity and integrity of the proteins was checked by SDS-PAGE and Coomassie blue staining (19, 20). Furthermore, nitrocellulose-blotted hybrid molecules were exposed to rabbit antisera, which had been raised against purified recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6) (14, 15) to demonstrate that the individual allergen components were exposed to antibodies.

Matrix-assisted laser desorption and ionization (MALDI)-time of flight (TOF) mass spectrometry and circular dichroism (CD) analysis of purified rP2-P6, rP6-P2, and rP5-P1

Laser desorption mass spectra were acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos, Manchester, UK) (piCHEM, Graz, Austria). CD measurements were performed in MilliQ water with protein concentrations ranging from 0.11–0.17 mg/ml (2.0×10^6 – 7.3×10^6 M). Protein mixes of the allergens were prepared by combining equimolar amounts of the single proteins to obtain a final protein concentration of 0.5 mg/ml, which was further diluted to a protein concentration of 0.17 mg/ml for the CD measurements. The investigations were performed on a Jasco (Tokyo, Japan) J-715 spectropolarimeter by using a 0.1 cm path-length cell equilibrated at 20°C. Spectra were recorded with 0.5-nm resolution at a scan speed of 100 nm/min and were averaged from three scans. The final spectra were baseline-corrected by subtracting the corresponding buffer spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity [Θ] at a given wavelength.

ELISA competition experiments

Sera from 20 representative grass pollen-allergic patients, diluted 1:5 in Tris-buffered saline (TBS), were preadsorbed overnight at 4°C either with rPhl p 1, rPhl p 5, rP5-P1, rPhl p 2, rPhl p 6, rP2-P6, rP6-P2, BSA (10 μ g/ml) or with timothy grass pollen extract (100 μ g/ml). ELISA

plates were coated with the individual recombinant allergens (rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6) (5 µg/ml) or timothy grass pollen extract (100 µg/ml) at 4°C overnight. IgE binding of the pretreated sera to ELISA plate-coupled allergens was determined by duplicate determinations as described (14). The percentage inhibition of IgE binding to each of the allergens was calculated as follows: percentage of inhibition of IgE binding = $100 - (OD_a/OD_b) \times 100$. OD_a and OD_b represent the optical density after preincubation with allergens and BSA, respectively.

T cell proliferation

Peripheral blood mononuclear cells (PBMC) were isolated from grass pollen-allergic patients containing Phl p 1-, Phl p 2-, Phl p 5-, and Phl p 6-specific IgE antibodies or from non-atopic persons mounting allergen-specific IgG antibodies by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of heparinized venous blood samples. IgE and IgG subclass responses specific for recombinant timothy grass pollen allergens were determined by ELISA in the plasma samples of the allergic and non-atopic persons. Cells were resuspended in AIM V medium (Life Technologies, Grand Island, NY). Mononuclear cells (2×10^5 cells/well in 96-well plates) were stimulated with different Ag doses (50 µg/ml, 12.5 µg/ml, 3.125 µg/ml, 0.78 µg/ml) of rP2-P6, rP6-P2, rP5-P1, with equimolar mixtures of rPhl p 2/rPhl p 6, and rPhl p 1/rPhl p 5 or with medium alone in triplicates. The proliferative responses were measured after 6 days by [^{3} H]thymidine incorporation and are expressed as stimulation indices (22).

Immunization of mice and measurement of specific antibody levels

Each of nine groups of eight female BALB/c mice (8 weeks old) (Charles River, Germany) were immunized subcutaneously with 2.5 µg rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, 5µg rP2-P6, rP6-P2, rP5-P1, 100 µg timothy grass pollen extract, or an equimolar mixture of the four recombinant allergens adsorbed to Al(OH)₃ (Alu-Gel-S, Serva, Ingelheim, Germany) (8). Animals were maintained in the animal care unit of the Dept. of Pathophysiology, University of Vienna, according to the local guidelines for animal care. Mice were immunized twice (day 1, day 28) and bled from the tail veins in 4-week intervals, and sera were stored at -20°C until analysis.

IgG₁ responses against rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, rP2-P6, rP6-P2, rP5-P1, and timothy grass pollen extract were measured by ELISA (8). Allergens (recombinant allergens, 5 µg/ml; extract, 50 µg/ml) were coated to NUNC Maxisorp plates (Roskilde, Denmark) and were incubated with 1:1000 diluted mouse-sera. Bound IgG₁ antibodies were detected with a 1:1000 diluted monoclonal rat anti-mouse IgG₁ (Pharmingen, San Diego, CA,) and a 1:2000 diluted HRP-labeled sheep anti-rat antiserum (Amersham, Buckinghamshire, UK).

ELISA for assessment of blocking antibody activity

The ability of the mouse anti-rP2-P6, anti-rP6-P2, and anti-rP5-P1 antibodies to inhibit the binding of grass pollen-allergic patients' IgE antibodies to rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, or timothy grass pollen extract was examined by ELISA competition experiments as described (8, 23). Recombinant Phl p 1, Phl p 2, Phl p 5, Phl p 6 (1 µg/ml), or timothy grass pollen extract (100 µg/ml) were coated to ELISA plates (NUNC) and were preincubated with mouse anti-rP2-P6, anti-rP6-P2, and anti-rP5-P1 antisera or, for control purposes, with the corresponding preimmune sera each diluted 1:50. Blocking of allergic patients IgE binding to plate-coupled timothy grass pollen extract (100 µg/ml) was shown with sera from timothy grass pollen extract-

immunized mice or a 1:1 mix of mouse anti-rP5-P1 and anti-rP6-P2 antisera each diluted 1:50. After washing, plates were incubated with 1:5 diluted human sera from grass pollen-allergic patients and bound human IgE was detected with an AP-labeled mouse monoclonal anti-human IgE Ab (Pharmingen). The percentage reduction of human IgE binding after preincubation with mouse antisera was determined according to the formula: percentage of inhibition of IgE binding = $100 - \text{OD}_I / \text{OD}_P \times 100$. OD_I and OD_P represent the extinctions after preincubation with the immune serum and the preimmune serum, respectively.

RESULTS

Genetic engineering and purification of recombinant hybrid molecules consisting of immunologically unrelated grass pollen allergens

Recombinant Phl p 1, Phl p 2, Phl p 5, and Phl p 6 represent the most important grass pollen allergens, which contain the majority of the epitopes present in natural grass pollen extracts (11–16). According to the rates of co-sensitization determined in a population of 200 grass pollen-allergic patients (rPhl p 1+rPhl p 5: 60%; rPhl p 2+rPhl p 6: 30%), we engineered hybrid molecules consisting of immunologically unrelated allergen components by PCR-based “gene-soeing”. With this method, cDNAs coding for a hybrid consisting of Phl p 2 (N-terminus) and Phl p 6 (C-terminus) (i.e., rP2-P6), one hybrid consisting of Phl p 6 (N-terminus) and Phl p 2 (C-terminus) (i.e., rP6-P2) and a hybrid made of Phl p 5 (N-terminus) and Phl p 1 (C-terminus) (i.e., rP5-P1), were inserted into expression plasmids. The hybrid molecules were expressed at high levels in *E. coli* (10–15 mg/l culture) and were purified to homogeneity by Nickel affinity chromatography. The rP2-P6 and rP6-P2 hybrids were expressed in the soluble cytoplasmic fraction of *E. coli*. The rP5-P1 hybrid formed inclusion bodies from which the protein could be isolated by using a denaturation step and a subsequent refolding protocol. *Figure 1* shows a Coomassie-stained SDS-PAGE comparing the purified hybrid molecules with the individual recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6. Although the rP2-P6 and rP6-P2 hybrid exhibited a slightly different migration pattern in SDS-PAGE ([Fig. 1](#)), mass spectroscopical analysis revealed an almost identical molecular mass (rP2-P6: 23935 Da; rP6-P2: 23910 Da), which was in agreement with the molecular weight calculated according to the deduced amino acid sequences of the proteins. Likewise, the molecular mass determined by mass spectroscopy for the rP5-P1 hybrid molecule (55972 Da) was in agreement with the molecular weight predicted for the protein.

Recombinant hybrid molecules contain a considerable amount of secondary structure

The CD spectrum of the equimolar mix of rPhl p 2 and rPhl p 6 indicates a mixed α - and β -secondary structure with a minimum at around 207 nm and a broad shoulder between 215 and 225 nm, which is in agreement with the fact that rPhl p 2 represents a protein with almost exclusive β -sheet structure (24), and rPhl p 6 is an almost exclusive α -helical protein (15). The rP2-P6 hybrid exhibits a spectrum comparable with that of the rPhl p 2/rPhl p 6 protein mix, with a slightly smaller minimum at 207 nm ([Fig. 2A](#)). The shape of the curve recorded for the rP6-P2 hybrid is almost superimposable to the curve of the mix and that of the rP2-P6 hybrid, but the signal increased by about 25% ([Fig. 2A](#)).

The CD spectrum recorded for the rPhl p 1/rPhl p 5 mix is dominated by minima at 208 nm and 222 nm typical for α -helices but also contains β -sheet secondary structures ([Fig. 2B](#)). The zero-

crossing was shifted significantly below 200 nm, indicating that an increasing portion of the proteins are in random coil conformation. Because rPhl p 5 represents an almost exclusive α -helical protein (25), it is likely that the presence of β -sheet elements and the shift are due to largely unfolded rPhl p 1. This assumption is supported by our finding that rPhl p 1 by itself is mainly unfolded (Ball, T., and Valenta, R., unpublished observations). The rP5-P1 hybrid exhibits a CD spectrum similar to the rPhl p 1/rPhl p 5 protein mix but with a reduced fold ([Fig. 2B](#)).

Hybrid molecules present the B cell epitopes of the individual allergen components

To test whether the hybrid molecules present the B cell epitopes of the individual components, we performed two types of experiments. First, we used rabbit-antisera raised against the individual recombinant allergens (rabbit anti-Phl p 1, anti-Phl p 2, anti-Phl p 5, and anti-Phl p 6 antibodies) to demonstrate binding to the hybrid molecules (data not shown). Second, we used immunological competition experiments to investigate whether the hybrid allergens can block the binding of grass pollen-allergic patients' ($n=20$) IgE antibodies to the individual allergen components ([Table 2](#)). We found that the rP2-P6, as well as the rP6-P2 hybrid, inhibited IgE binding to each of the components (rPhl p 2, rPhl p 6) equally well as the single recombinant allergens. Similar results were obtained for the rP5-P1 hybrid, which inhibited IgE binding of all 20 sera to rPhl p 1 and rPhl p 5, as efficient as the single components ([Table 2](#)). By using an equimolar mixture of the rP5-P1 and rP6-P2 hybrid molecules, we found that most of the timothy grass pollen extract-specific IgE could be adsorbed from sera of the 20 grass pollen-allergic patients ([Table 3](#)). The average inhibition of IgE binding to timothy grass pollen extract obtained with the two hybrid molecules was 64% and thus almost comparable with that achieved with timothy grass pollen extract (average inhibition 81%) ([Table 3](#)). That no epitopes were hidden in the hybrid molecules is remarkable considering that the hybrids contained only an intermolecular weight spacer of two amino acids.

Hybrid molecules resemble the T cell epitopes of the individual allergen components

To investigate whether the hybrid molecules rP2-P6, rP6-P2, and rP5-P1 contain the T cell epitopes of the individual allergen components, we performed *in vitro* proliferation experiments with PBMC from four grass pollen-allergic patients. As shown for one representative out of four grass pollen-allergic patients, we found that the hybrid allergens as well as equimolar mixtures of the individual allergens induced proliferation of PBMC ([Fig. 3A](#)). The rP5-P1 hybrid containing the two most important timothy grass pollen allergens (Phl p 1, Phl p 5) induced at all doses a stronger lymphoproliferative response than the mix of rPhl p 5 and rPhl p 1, which was even stronger than that induced by natural timothy grass pollen extract ([Fig. 3A](#); data not shown). The rPhl p 2/rPhl p 6 protein mix induced significant proliferative responses only at high concentrations (≥ 12.5 $\mu\text{g/ml}$) in the allergic patients. Again a much higher proliferative response was obtained with the hybrid molecules (rP2-P6; rP6-P2) at the highest concentration (50 $\mu\text{g/ml}$) ([Fig. 3A](#)). Using PBMC from non-allergic individuals who contained allergen-specific IgG antibodies but no specific IgE, proliferative responses could be induced by the hybrid molecules and, to a lower extent, by the individual allergen components ([Fig. 3B](#)). The latter finding is in agreement with previous studies demonstrating allergen-specific lymphoproliferative responses in non-atopic persons (26–28).

Immunization with recombinant hybrid molecules induces stronger and earlier IgG responses than immunization with individual allergens or allergen extract

To evaluate whether immunization with the hybrid allergens induces IgG antibodies that recognize the individual allergen components, groups of eight mice each were immunized with the hybrids, the individual allergens, or timothy grass pollen extract. [Figure 4](#) demonstrates that the average IgG₁ responses induced by the hybrid molecules to each of the individual allergens (rPhl p 1, rPhl p 2, rPhl p 5, or rPhl p 6) were higher than those obtained by immunization with the single-allergen components. IgG₁ antibody levels induced by the hybrids were already detectable 4 weeks after the first immunization and had increased further after 4 more weeks. Perhaps most interesting was the finding that the hybrid molecules induced higher IgG₁ levels to the individual allergen components than did timothy grass pollen extract or an equimolar mix of the four recombinant allergens ([Fig. 4](#)). The latter was particularly evident for Phl p 2, as well as for Phl p 6 and also for Phl p 1, which were poorly recognized by extract-immunized mice; whereas the hybrid molecules induced vigorous anti-Phl p 1-, anti-Phl p 2-, and anti-Phl p 6 antibody responses ([Fig. 4](#)). The higher induction of IgG anti-Phl p 5 and anti-Phl p 6 responses by allergen extract and the allergen mix compared with immunization with the isolated allergen may be explained by partial cross-reactivity between Phl p 5 and Phl p 6 (15).

Hybrid molecules induce antibodies that block the binding of allergic patients' IgE to grass pollen allergens

Next we examined whether mouse antibodies induced with the hybrid molecules can block the binding of grass pollen-allergic patients' IgE antibodies to purified grass pollen allergens ([Table 4](#)). ELISA competition experiments performed with sera from four representative grass pollen-allergic patients showed that antibodies induced by the hybrid molecules strongly inhibited IgE binding to the purified allergens. IgG antibodies induced with the rP2-P6 and the rP6-P2 hybrid molecule caused a 48–54% inhibition of IgE binding to Phl p 2 and a 54–67% inhibition of IgE binding to Phl p 6 ([Table 4A](#)). By contrast, the inhibition of IgE reactivity yielded by preincubation with antibodies induced with rPhl p 2 and rPhl p 6 alone was very low (0–15%) ([Table 4A](#)). Anti-P5-P1 antibodies caused a more than double inhibition of IgE binding to Phl p 5 (60% average inhibition) than antibodies raised against Phl p 5 alone (28%) ([Table 4B](#)). The inhibition of IgE binding to Phl p 1 yielded with the antibodies raised against the rP5-P1 hybrid (19% average inhibition) and Phl p 1 alone (30% average inhibition) were lower ([Table 4B](#)). Perhaps most important was the fact that mouse antibodies raised against the rP5-P1 and rP6-P2 hybrid inhibited allergic patients IgE binding to timothy grass pollen extract even stronger than extract-induced antibodies (average inhibition hybrids: 66%; average inhibition extract: 63%) ([Table 4C](#)).

DISCUSSION

Grass pollen allergy affects more than 400 million allergic patients worldwide. Specific immunotherapy, the only causal therapy approach for grass pollen allergy, uses allergen extracts prepared from grass pollen. As exemplified in [Figure 5](#), grass pollen allergen extracts consist of a variety of different allergens and non-allergenic components (6, 29, 30). Although it is possible to quantify the amount of certain allergens in therapeutic allergen extract preparations, it is not possible to influence the concentrations of the individual allergens in the vaccine preparation or to deplete non-allergenic components (31). Moreover, it has been demonstrated that different

grass pollen allergens exhibit different immunogenicity (Phl p 5>Phl p 1>Phl p 6>Phl p 2) in animals (8); accordingly it has been observed that immunotherapy based on grass pollen allergen extracts induces highly heterogeneous immune responses (32–36; Valenta, R. and Mothes, N., unpublished observations) ([Fig. 5a](#)). In this context it has been reported that extract-based immunotherapy can even induce IgE antibodies towards new allergens (32, 35, 37) and lead to clinically relevant new sensitizations (38). By using recombinant allergen molecules for diagnosis and treatment, it is—in principle—possible to tailor treatment according to the patient's sensitization profile and thus avoid the induction of irrelevant or even unwanted immune responses (29, 39). Treatment of allergy to complex allergen sources may, however, still be hampered by the different immunogenicity of the individual allergen molecules, which may give rise to heterogeneous immune responses even when these allergens are administered in equimolar mixtures (30, 32) ([Fig. 5b](#)).

Here we report the development of combination vaccines based on recombinant hybrid molecules consisting of four of the most important and frequently recognized grass pollen allergens (i.e., Phl p 1, Phl p 2, Phl p 5, Phl p 6) (40–42, 15). These combination vaccines have been developed based on the most frequent co-sensitization patterns of grass pollen-allergic patients and thus should be suited for patient-tailored treatment of the majority of grass pollen-allergic patients.

An unexpected finding of great importance for immunotherapy as well as vaccination in general was the strong increase of immunogenicity of unrelated allergens with poor immunogenicity (e.g., Phl p 2, Phl p 6) that was obtained by their production as hybrid molecules ([Fig. 5C](#)). Hybrid molecules consisting of immunologically unrelated allergens, (i.e., Phl p 2 and Phl p 6, as well as of Phl p 1 and Phl p 5), induced earlier and higher IgG antibody responses in mice and more vigorous lymphoproliferative responses in cultured mononuclear cells obtained from sensitized patients and individuals containing allergen-specific IgG antibodies than the individual allergens or equimolar mixtures thereof.

Several mutually nonexclusive explanations may be considered for this observation. First, it may be assumed that within the hybrid molecule each of the allergens can act as carrier molecule and thus enhance humoral as well as cellular immune responses to the linked component (43–48). The enhancement of immune responses in the case of the rP5-P1 hybrid molecule may be understood according to the principle of classical carrier effects. In this scenario, covalent linkage of a less immunogenic allergen (i.e., Phl p 1) with a highly immunogenic allergen (i.e., Phl p 5) containing a variety of potent T cell epitopes would explain the enhanced immunogenicity to the first allergen when it is presented in the context of the latter. However, we found that fusion of two different allergens with low capacity to induce antibody or lymphoproliferative responses (i.e., Phl p 2, Phl p 6) led to an almost equal enhancement of immune responses to each of the components. The increased immunogenicity observed for the rP2-P6 and the rP6-P2 hybrid molecules cannot be explained exclusively by the carrier effect alone because these hybrids did not contain additional potent T cell epitopes. It seems also unlikely that potent T cell epitopes or a molecular environment favoring vigorous T cell responses were created *de novo* (49) in these hybrid molecules because they did not contain relevant new sequences, and the order of the allergens had no apparent influence on the enhancement of immunogenicity.

Because our antigen preparations represented pure proteins, we consider T cell-independent carrier effects as were described for monophosphoryl lipid A (MLA) as a second, rather unlikely possibility for their increased immunogenicity (50).

Whether the hybrid molecules induce a different repertoire of co-stimulatory molecules on immune cells or are processed in a different manner compared with the isolated allergens remains to be elucidated (51, 52). However, the analysis of the secondary structure of the hybrid molecules and the equimolar allergen mixes by circular dichroism demonstrates similar folds. It therefore appears unlikely that changes in secondary structure or fold may have caused profound differences in the presentation of the hybrid molecules versus the isolated allergens.

Although we cannot provide a definitive answer for the mechanisms that are operative in the enhancement of immunogenicity of the hybrid molecules, we suggest fusion of low-immunogenic vaccine components against which protective immune responses are required as a generally applicable principle for immunotherapy and general vaccination. The principle of linking relevant antigens (e.g., allergens) by recombinant DNA technology may be applied whenever it is desired to induce strong protective immune responses or alternatively tolerization against independent and immunologically unrelated antigens (48, 53, 54) without using a foreign carrier molecule, which may give rise to unwanted immune responses. Another advantage of recombinant hybrid molecules is that they can be produced as defined molecular entities by standardized procedures, avoiding complicated chemical coupling and purification procedures. Our assumption, that the suggested strategy can be applied as a general principle, is supported by the finding that the immunogenicity of four different antigens could be increased by the hybrid approach.

The hybrids described in our study appear suitable for specific immunotherapy of grass pollen allergy because they contain most of the IgE epitopes and T cell epitopes present in natural grass pollen extract (14, 15, 55). Second, immunization of mice with the hybrid molecules induced strong and early induction of allergen-specific IgG antibody responses, which strongly blocked the binding of grass pollen-allergic patients' IgE antibodies to the individual allergens, as well as to grass pollen extract ([Table 4](#)). Moreover, we found that the hybrid molecules induced stronger lymphoproliferative responses in human allergen-specific mononuclear cells than the isolated allergen components.

The basic mechanisms underlying allergen-specific immunotherapy are not fully understood (56), and there is still considerable controversy regarding the importance of T cell and B cell epitopes for successful immunotherapy (57–59). Therefore, we have presently constructed hybrids consisting of allergen molecules, which closely resemble the immunological properties of natural grass pollen allergens. To reduce therapy-induced side effects resulting from the administration of active allergens, it will in principle be possible to construct hybrid molecules consisting of hypoallergenic allergen derivatives (57).

We suggest vaccination with recombinant hybrid molecules as a generally applicable strategy whenever the induction of simultaneous immune responses against unrelated antigens with different immunogenicity is desired.

ACKNOWLEDGMENTS

This study was supported by Grants Y078GEN and F01805, F01807 of the Austrian Science Fund, by the Austrian Academy of Sciences (CeMM-Center for Molecular Medicine), and by a grant from BIOMAY, Vienna, Austria.

REFERENCES

1. Wüthrich, B., Schindler, C., Leuenberger, P., and Ackermann-Lieblich, P. (1995) Prevalence of atopy and pollinosis in the adult population of Switzerland (SAPALDIA study). Swiss Study on Air Pollution and Lung Diseases in Adults. *Int. Arch. Allergy Immunol.* **106**, 149–156
2. Wills-Karp, M., Santeliz, J., and Karp, C.L. (2001) The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nature Rev. Immunol.* **1**, 69–75
3. Turner, H. and Kinet, J.P. (1999) Signalling through the high-affinity IgE receptor Fc epsilon RI. *Nature (London)* **402**, B24–30
4. Bieber, T. (1996) Fc epsilon RI on antigen-presenting cells. *Curr. Opin. Immunol.* **8**, 773–777
5. Stingl, G. and Maurer, D. (1997) IgE-mediated allergen presentation via Fc epsilon RI on antigen-presenting cells. *Int. Arch. Allergy Immunol.* **113**, 24–29
6. Bousquet, J., Lockey, R., and Malling, H.J. (1998) WHO position paper. Allergen immunotherapy: therapeutic vaccines for allergic diseases. *Allergy* **53**, 1–42
7. Vrtala, S., Mayer, P., Ferreira, F., Susani, M., Schon, A.H., Kraft, D., and Valenta, R. (1996) Induction of IgE antibodies in mice and rhesus monkeys with recombinant birch pollen allergens: different allergenicity of Bet v 1 and Bet v 2. *J. Allergy Clin. Immunol.* **98**, 913–921
8. Vrtala S., Ball T., Spitzauer S., Pandjaitan B., Suphioglu C., Knox B., Sperr W.R., Valent P., Kraft D., and Valenta R. (1998) Immunization with purified natural and recombinant allergens induces mouse IgG₁ antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J. Immunol.* **160**, 6137–6144
9. Freidhoff, L.R., Ehrlich-Kautzky, E., Grant, J.H., Meyers, D.A., and Marsh, D.G. (1986) A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). I. Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J. Allergy Clin. Immunol.* **78**, 1190–1201
10. Freidhoff, L.R., Ehrlich-Kautzky, E., Grant, J.H., Meyers, D.A., and Marsh, D.G. (1987) A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). II. Longitudinal variation of antibody levels in relation to

symptomatology and pollen exposure and correction of seasonally elevated antibody levels to basal values. *J. Allergy Clin. Immunol.* **80**, 646–655

11. Suphioglu, C. (2000) What are the important allergens in grass pollen that are linked to human allergic disease? *Clin. Exp. Allergy* **30**, 1335–1341
12. Laffer, S., Spitzauer, S., Susani, M., Pairleitner, H., Schweiger, C., Gronlund, H., Menz, G., Pauli, G., Ishii, T., Nolte, H., Ebner, C., Sehon, A.H., Kraft, D., Eichler, H.G., and Valenta, R. (1996) Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J. Allergy Clin. Immunol.* **98**, 652–658
13. Vrtala S., Susani M., Sperr W.R., Valent P., Laffer S., Dolecek C., Kraft D., and Valenta R. (1996) Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl p 1, Phl p 2, Phl p 5). *J Allergy Clin. Immunol.* **97**, 781–787
14. Niederberger, V., Laffer, S., Fröschl, R., Kraft, D., Rumpold, H., Kapiotis, S., Valenta, R., and Spitzauer, S. (1998) IgE antibodies to recombinant pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Bet v 2) account for a high percentage of grass pollen-specific IgE. *J. Allergy Clin. Immunol.* **101**, 258–264
15. Vrtala S., Fischer S., Grote M., Vangelista L., Pastore A., Sperr W.R., Valent P., Reichelt R., Kraft D., and Valenta R. (1999) Molecular, immunological, and structural characterization of Phl p 6, a major allergen and P-particle-associated protein from timothy grass (*Phleum pratense*) pollen. *J. Immunol.* **163**, 5489–5496
16. Rossi, R.E., Monasterolo, G., and Monasterolo, S. (2001) Measurement of IgE antibodies against purified grass-pollen allergens (Phl p 1,2,3,4,5,6,7,11, and 12) in sera of patients allergic to grass pollen. *Allergy* **56**, 1180–1185
17. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59
18. Vrtala, S., Grote, M., Duchene, M., van-Ree, R., Kraft, D., Scheiner, O., and Valenta, R. (1993) Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int. Arch. Allergy Immunol.* **102**, 160–169
19. Bradford, M.M. (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72**, 248–254
20. Fling, S.P., and Gregerson, D.S. (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. *Anal. Biochem.* **155**, 83–88
21. Holzinger, A., Phillips, K.S., and Weaver, T.E. (1996) Single-step purification/solubilization of recombinant proteins: application to surfactant protein B. *BioTechniques* **20**, 804–808

22. Ebner, C., Szepefalusi, Z., Ferreira, F., Jilek, A., Valenta, R., Parronchi, P., Maggi, E., Romagnani S., Scheiner, O., and Kraft, D. (1993) Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J. Immunol.* **150**, 1047–1054
23. Focke, M., Mahler, V., Ball, T., Sperr, W.R., Majlesi, Y., Valent, P., Kraft, D., and Valenta, R. (2001) Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J.* 10.1096/fj.01–0016fje
24. De Marino, S., Morelli, M.A., Fraternali, F., Tamborini, E., Musco, G., Vrtala, S., Dolecek, C., Arosio, P., Valenta, R., and Pastore, A. (1999) An immunoglobulin-like fold in a major plant allergen: the solution structure of Phl p 2 from timothy grass pollen. *Structure* **7**, 943–952
25. Flicker, S., Vrtala, S., Steinberger, P., Vangelista, L., Bufe, A., Petersen, A., Ghannadan, M., Sperr, W.R., Valent, P., Norderhaug, L., Bohle, B., Stockinger, H., Suphioglu, C., Ong, E.K., Kraft, D., and Valenta, R. (2000) A human monoclonal IgE antibody defines a highly allergenic fragment of the major timothy grass pollen allergen, Phl p 5: molecular, immunological, and structural characterization of the epitope-containing domain. *J. Immunol.* **165**, 3849–3859
26. Van Neerven, R.J., van de Pol, M.M., Wierenga, E.A., Aalberse, R.C., Jansen, H.M., and Kapsenberg, M.L. (1994) Peptide specificity and HLA restriction do not dictate lymphokine production by allergen-specific T lymphocyte clones. *Immunology* **82**, 351–356
27. Ebner, C., Schenk, S., Najafian, N., Siemann, U., Steiner, R., Fischer, G.W., Hoffmann, K., Szepefalusi, Z., Scheiner, O., and Kraft, D. (1995) Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J. Immunol.* **154**, 1932–1940
28. Hales, B.J., Shen, H.D., and Thomas, W.R. (2000) Cross-reactivity of T cell responses to *Dermatophagoides pteronyssinus* and *D. farinae*. Studies with group 1 and 7 allergens. *Clin. Exp. Allergy* **30**, 927–933
29. Valenta, R., Lidholm, J., Niederberger, V., Hayek, B., Kraft, D., and Grönlund, H. (1999) The recombinant allergen-based concept of component resolved diagnosis and immunotherapy (CRD and CRIT). *Clin. Exp. Allergy* **29**, 896–904
30. Fernández-Rivas, M., Brewczynski, P.Z., Kroon, A.M., and van Ree, R. (2000) A clinical trial with purified natural major allergens from grass pollen. *Regulatory Control and Standardization of Allergenic Extracts*. GIT Verlag: Darmstadt, Germany; pp. 131–138.
31. Larsen, J.N., Wikborg, T., Lombardero Vega, M., and Løwenstein, H. (1999) Manufacturing and standardizing allergen vaccines. *Allergens and Allergen Immunotherapy, Clinical Allergy and Immunology*, Marcel Dekker Inc.: New York, NY; pp. 297–320.
32. Van-Ree, R., Van-Leeuwen, W.A., Dieges, P.H., Van-Wijk, R.G., De-Jong, N., Brewczynski, P.Z., Kroon, A.M., Schilte, P.P., Tan, K.Y., Simon-Licht, I.F., Roberts, A.M.,

- Stapel, S.O., and Aalberse, R.C. (1997) Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3, and 5) during immunotherapy. *Clin. Exp. Allergy* **27**, 68–74
33. Van-Ree, R., Brewczynski, P.Z., Tan, K.Y., Mulder-Willems, H.J., Widjaja, P., Stapel, S.O., Aalberse, R.C., and Kroon, A.M. (1995) Grass pollen immunotherapy induces highly cross-reactive IgG antibodies to group V allergens from different grass species. *Allergy* **50**, 281–283
34. Gehlhar, K., Schlaak, M., Becker, W., and Bufe, A. (1999) Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG₄ to IgG₁ correlates with the clinical outcome. *Clin. Exp. Allergy* **29**, 497–506
35. Ball, T., Sperr, W.R., Valent, P., Lidholm, J., Spitzauer, S., Ebner, C., Kraft, D., and Valenta, R. (1999) Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur. J. Immunol.* **29**, 2026–2036
36. Ball, T., Fuchs, T., Sperr, W.R., Valent, P., Vangelista, L., Kraft, D., and Valenta, R. (1999) 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
37. Moverare, R., Elfman, L., Vesterinen, E., Metso, T., and Haahtela, T. (2002) Development of new IgE reactivities to allergic components in pollen extracts during specific immunotherapy studied with immunoblotting and the Pharmacia CAP System. *Allergy* **57**, 423–430.
38. Van-Ree, R., Antonicelli, L., Akkerdaas, J.H., Garritani, M.S., Aalberse, R.C., and Bonifazi, F. (1996) Possible induction of food allergy during mite immunotherapy. *Allergy* **51**, 108–113
39. Valenta, R., and Kraft, D. (1995) Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr. Opin. Immunol.* **7**, 751–756
40. Laffer, S., Valenta, R., Vrtala, S., Susani, M., van-Ree, R., Kraft, D., Scheiner, O., and Duchene, M. (1994) Complementary DNA cloning of the major allergen Phl p I from timothy grass (*Phleum pratense*); recombinant Phl p I inhibits IgE binding to group I allergens from eight different grass species. *J. Allergy Clin. Immunol.* **94**, 689–698
41. Dolecek, C., Vrtala, S., Laffer, S., Steinberger, P., Kraft, D., Scheiner, O., and Valenta, R. (1993) Molecular characterization of Phl p II, a major timothy grass (*Phleum pratense*) pollen allergen. *FEBS Lett.* **335**, 299–304
42. Vrtala, S., Sperr, W.R., Reimitzer, I., van-Ree, R., Laffer, S., Muller, W.D., Valent, P., Lechner, K., Rumpold, H., Kraft, D., Scheiner, O., and Valenta, R. (1993) cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl p V allergen. *J. Immunol.* **151**, 4773–4781

43. Paul, W.E., Siskind, G.W., and Benacerraf, B. (1966) Studies on the effect of the carrier molecule on antihapten antibody synthesis. II. Carrier specificity of anti-2,4-dinitrophenyl-poly-l-lysine antibodies. *J. Exp. Med.* **123**, 689–705
44. Paul, W.E., Katz, D.H., Goidl, E.A., and Benacerraf, B. (1970) Carrier function in anti-hapten immune responses. II. Specific properties of carrier cells capable of enhancing anti-hapten antibody responses. *J. Exp. Med.* **132**, 283–299
45. Katz, D.H., Paul, W.E., Goidl, E.A., and Benacerraf, B. (1970) Carrier function in anti-hapten immune responses. I. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. *J. Exp. Med.* **132**, 261–282
46. Mitchison, N.A. (1971) The carrier effect in the secondary response to hapten-protein conjugates. V. Use of antilymphocyte serum to deplete animals of helper cells. *Eur. J. Immunol.* **1**, 68–75
47. Boak, J.L., Mitchison, N.A., and Patisson, P.H. (1971) The carrier effect in the secondary response to hapten-protein conjugates. 3. The anatomical distribution of helper cells and antibody-forming-cell-precursors. *Eur. J. Immunol.* **1**, 63–65
48. Bullock, W.W., Katz, D.H., and Benacerraf, B. (1975) Induction of T lymphocyte responses to a small molecular weight antigen. III. T-T cell interactions to determinants linked together: suppression vs. enhancement. *J. Exp. Med.* **142**, 275–287
49. Leclerc, C., Martineau, P., Charbit, A., Lo-Man, R., Deriaud, E., and Hofnung, M. (1993) Immunodominance of a recombinant T-cell epitope depends on its molecular environment. *Mol. Immunol.* **30**, 1561–1572
50. Myers, K.R., Beining, P., Betts, M., Snippe, H., Inman, J., and Golding, B. (1995) Monophosphoryl lipid A behaves as a T-cell-independent type 1 carrier for hapten-specific antibody responses in mice. *Infect. Immun.* **63**, 168–174
51. George-Chandy, A., Eriksson, K., Lebens, M., Nordstrom, I., Schon, E., and Holmgren, J. (2001) Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. *Infect. Immun.* **69**, 5716–5725
52. Akdis, C.A., Blesken, T., Wymann, D., Akdis, M., and Blaser, K. (1998) Differential regulation of human T cell cytokine patterns and IgE and IgG₄ responses by conformational antigen variants. *Eur. J. Immunol.* **28**, 914–925
53. Bitoh, S., Takata, M., Maiti, P.K., Holford-Strevens, V., Kierek-Jaszczuk, D., and Schon, A.H. (1993) Antigen-specific suppressor factors of noncytotoxic CD8⁺ suppressor T cells downregulate antibody responses also to unrelated antigens when the latter are presented as covalently linked adducts with the specific antigen. *Cell. Immunol.* **150**, 168–193
54. Hoyne, G.F., Dallman, M.N., and Lamb, J.R. (1999) Linked suppression in peripheral T cell tolerance to the house dust mite derived allergen Der p 1. *Int. Arch. Allergy Immunol.* **118**, 122–124

55. Schenk, S., Breiteneder, H., Susani, M., Najafian, N., Laffer, S., Duchene, M., Valenta, R., Fischer, G., Scheiner, O., Kraft, D., and Ebner, C. (1995) T-cell epitopes of Phl p 1, major pollen allergen of timothy grass (*Phleum pratense*): evidence for crossreacting and non-crossreacting T-cell epitopes within grass group I allergens. *J. Allergy Clin. Immunol.* **96**, 986–996
56. Durham, S.R., and Till, S.J. (1998) Immunologic changes associated with allergen immunotherapy. *J. Allergy Clin. Immunol.* **102**, 157–164
57. Valenta, R., Vrtala, S., Focke-Tejkl, M., Bugajska-Schretter, A., Ball, T., Twardosz, A., Spitzauer, S., Gronlund, H., and Kraft, D. (1999) Genetically engineered and synthetic allergen derivatives: candidates for vaccination against type I allergy. *Biol. Chem.* **380**, 815–824
58. Valenta, R., Vrtala, S., Focke-Tejkl, M., Twardosz, A., Swoboda, I., Bugajska-Schretter, A., Spitzauer, S., and Kraft, D. (2001) Synthetic and genetically engineered allergen derivatives for specific immunotherapy of Type I allergy. *Inflammatory Mechanisms in Allergic Diseases, Clinical Allergy and Immunology*, Marcel Dekker Inc.: New York, NY; pp. 495–517
59. Valenta, R. (2002) The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* **2**, 446–453.

Received March 5, 2002. Accepted April 12, 2002.

Table 1

Primers used for the construction of the hybrid allergens

P2-P6:	Phl p 2:	P2a-5' : 5' GGA ATT CAT ATG GTG CCG AAG GTG ACG 3'
		P2a-3' : 5' CGT GGC CTT CCC CAT AAG CTT CTC TTC TGG CGC GTA GGT 3'
	Phl p 6:	P6a-5' : 5' AAG CTT ATG GGG AAG GCC ACG ACC 3'
		P6a-3' : 5' C GGG ATC CTA GTG GTG GTG GTG GTG GTG CGC GCC GGG CTT GAC AGC 3'
P6-P2:	Phl p 6:	P6b-5' : 5' GGA ATT CAT ATG GGG AAG GCC ACG ACC GAG 3'
		P6b-3' : 5' CAC CTT CGG CAC CAT AAG CTT CGC GCC GGG CTT GAC AGC 3'
	Phl p 2:	P2b-5' : 5' AAG CTT ATG GTG CCG AAG GTG ACG 3'
		P2b-3' : 5' C GGG ATC CTA GTG GTG GTG GTG GTG GTG CTC TTC TGG CGC GTA GGT 3'
P5-P1:	Phl p 1:	P1-5' : 5' CGG GGT ACC ATG ATC CCC AAG GTT CCC 3'
		P1-3' : 5' CGG GAT CCT CAG TGG TGG TGG TGG TGG TGC TTG GAC TCG TAG CTG GT 3'
	Phl p 5:	P5-5' : 5' GGA ATT CAT ATG GCC GAT CTC GGT TAC 3'
		P5-3' : 5' CGG GGT ACC GAC TTT GTA GCC ACC AGT 3'

Table 1. Primers used for the construction of hybrid molecules.

Table 2

Preadsorption of patients' sera with:	Percentage inhibition of IgE binding to									
	rPhl p 2			rPhl p 6			rPhl p 1		rPhl p 5	
	rPhl p2	rP2-P6	rP6-P2	rPhl p 6	rP2-P6	rP6-P2	rPhl p 1	rP5-P1	rPhl p 5	rP5-P1
Patient										
1	69	72	78	72	72	73	77	81	85	83
2	72	78	81	81	80	81	90	89	94	92
3	97	95	98	93	91	97	96	92	96	92
4	93	93	94	80	80	80	92	92	95	90
5	79	79	78	69	76	75	82	84	87	87
6	44	44	45	80	82	82	92	93	93	93
7	97	97	97	87	97	96	88	90	90	91
8	92	93	92	84	93	93	90	91	93	92
9	84	85	85	64	68	68	50	52	57	57
10	53	56	56	45	53	52	89	89	93	90
11	83	84	82	78	93	91	88	95	89	94
12	67	64	65	70	72	71	95	95	95	96
13	90	89	89	86	91	91	94	96	94	95
14	94	94	93	85	89	89	95	96	95	95
15	81	81	81	86	89	95	78	78	81	79
16	97	96	97	95	96	96	94	95	95	94
17	97	97	94	95	97	97	46	58	66	65
18	90	90	91	43	53	52	77	78	86	85
19	76	75	76	68	83	82	91	91	94	93
20	97	97	97	73	89	89	95	93	97	95
mean	83	83	84	77	82	83	85	86	89	88

Table 2. Hybrid molecules present the IgE epitopes of the individual allergen components. Sera from 20 grass pollen-allergic patients were pre-adsorbed with each of the hybrid molecules (rP2-P6, rP6-P2, rP5-P1) or the individual allergen components (rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6). The percentage inhibition of IgE binding to each of the individual allergens (rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6) is displayed for each patient. The mean percentage inhibition obtained for the 20 sera is shown at the bottom.

Table 3

**Percentage inhibition of IgE binding to timothy grass pollen
extract after preadsorption of patients' sera with:**

patient	timothy grass pollen extract	rP5-P1+rP6-P2
1	91	53
2	69	65
3	91	81
4	77	74
5	89	71
6	82	68
7	45	43
8	93	83
9	95	23
10	73	73
11	94	82
12	84	71
13	91	54
14	90	90
15	55	52
16	72	59
17	96	62
18	72	50
19	67	58
20	86	75
mean	81	64

Table 3. Inhibition of IgE binding to natural timothy grass pollen allergen extract with a mixture of two hybrid molecules. Sera from 20 grass pollen-allergic patients were pre-adsorbed with an equimolar mixture of two hybrid molecules (rP6-P2, rP5-P1) or timothy grass pollen extract. The percentage inhibition of IgE binding to timothy grass pollen extract is displayed for each patient. The mean percentage inhibition obtained for the 20 sera is shown at the bottom.

Table 4

Percentage inhibition of IgE binding to							
A		rPhl p 2	rPhl p 6	rPhl p 2	rPhl p 6	rPhl p 2	rPhl p 6
		with	mouse anti-rP2-P6	mouse anti-rP6-P2	mouse anti-rPhl p 2	mouse anti-rPhl p 6	
patient	1	56	62	48	63	0	5
	2	38	63	38	65	0	29
	3	56	39	53	65	0	18
	4	65	52	54	75	0	9
	mean	54	54	48	67	0	15

B		rPhl p 1	rPhl p 5	rPhl p 1	rPhl p 5
		with	mouse anti-rP5-P1	mouse anti-rPhl p 1	mouse anti-rPhl p 5
patient	1	16	59	33	23
	2	19	67	39	15
	3	14	69	17	40
	4	25	43	29	33
	mean	19	60	30	28

C		timothy grass pollen extract	
		with	
patient	1	mouse anti-timothy grass pollen extract	mouse anti-rP5-P1 + mouse anti-rP6-P2
	2		
	3		
	4		
	mean	63	66

Table 4. Mouse antisera raised against hybrid molecules inhibit the binding of grass pollen-allergic patients IgE to the individual allergens and grass pollen allergen extract. ELISA plate-bound allergens (**A**: rPhl p 2 and rPhl p 6; **B**: rPhl p 1 and rPhl p 5; **C**: timothy grass pollen extract) were preincubated with mouse antisera (mouse anti-rP2-P6, anti-rP6-P2, anti-rPhl p 2, anti-rPhl p 6, anti-rP5-P1, anti-rPhl p 1, anti-rPhl p 5, or a mix of anti-rP6-P2 and anti-rP5-P1 antisera). The percentages inhibition of IgE binding obtained for four grass pollen-allergic patients (1–4) are displayed.

Fig. 1

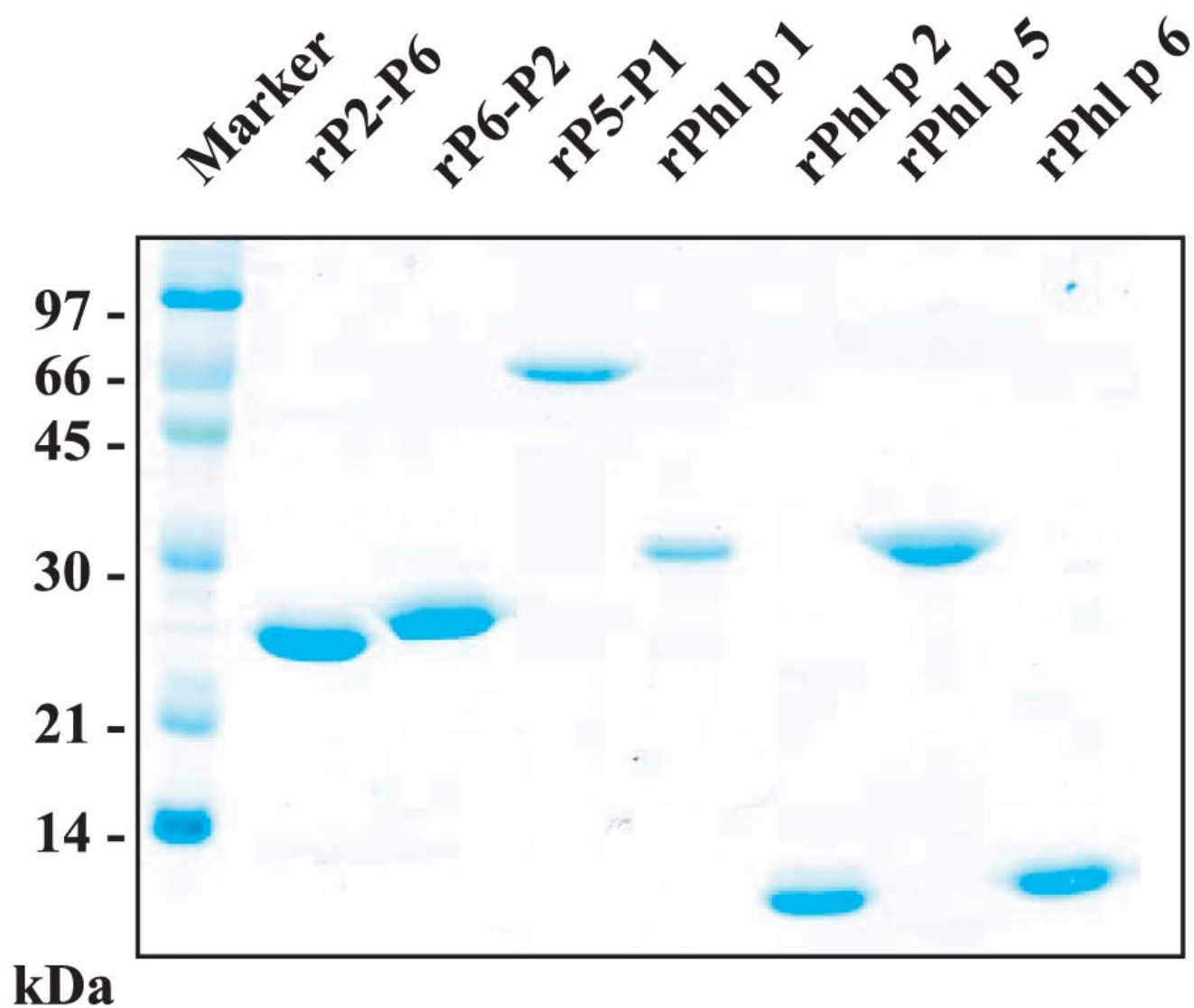


Figure 1. Coomassie brilliant blue-stained SDS-PAGE containing purified recombinant hybrid molecules rP2-P6, rP6-P2, and rP5-P1. Also, the recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6. Molecular weights (kDa) are indicated at the left margin.

Fig. 2

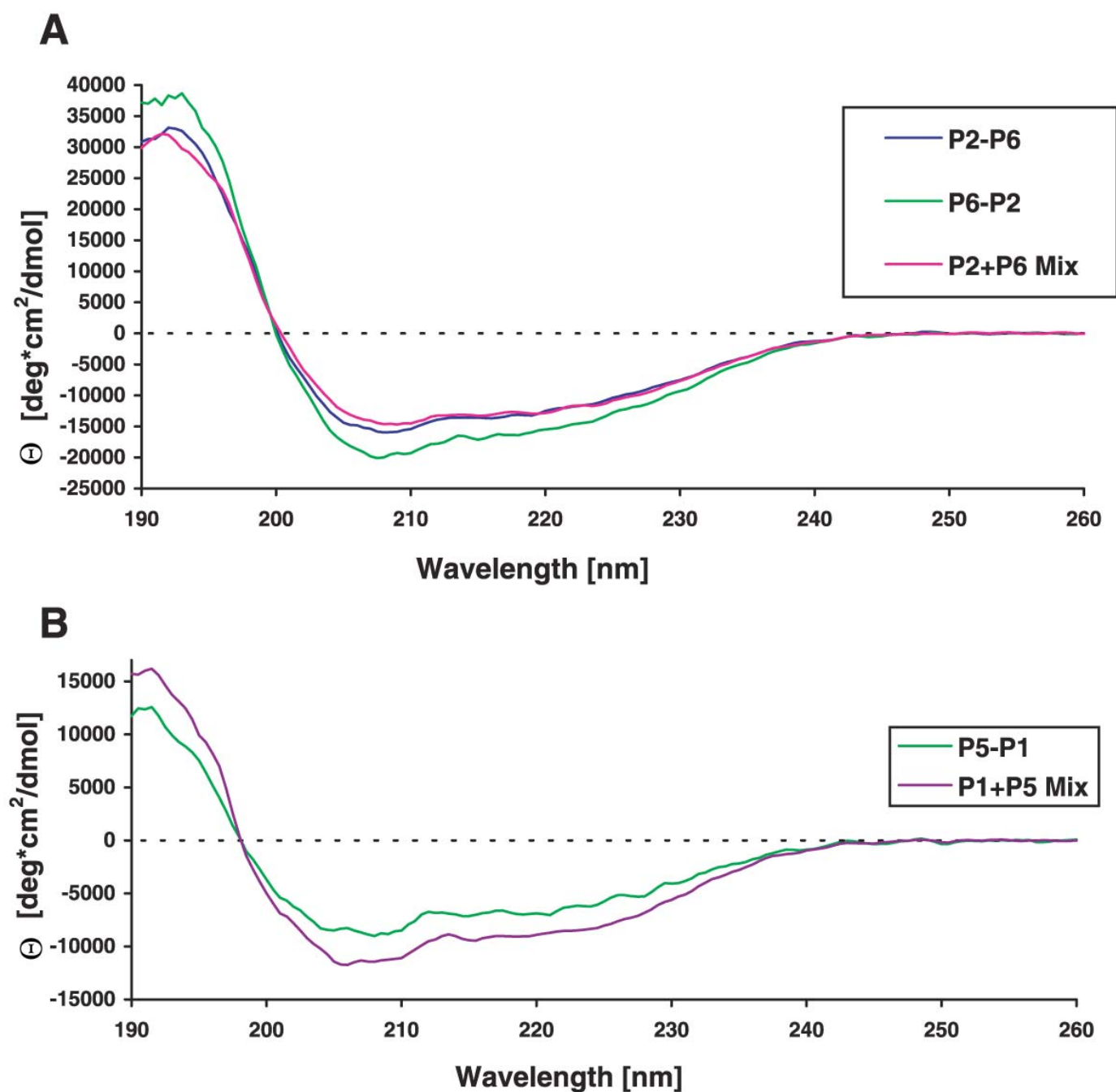


Figure 2. CD spectra of purified hybrid molecules and equimolar mixes of the individual allergens. Results are shown as the mean residue ellipticity (y-axis) at a given wavelength (x-axis) for each of the proteins or protein mixes [(A) rP2-P6, rP6-P2, mix rPhl p 2+rPhl p 6; (B) rP5-P1, mix rPhl p 1+rPhl p 5].

Fig. 3

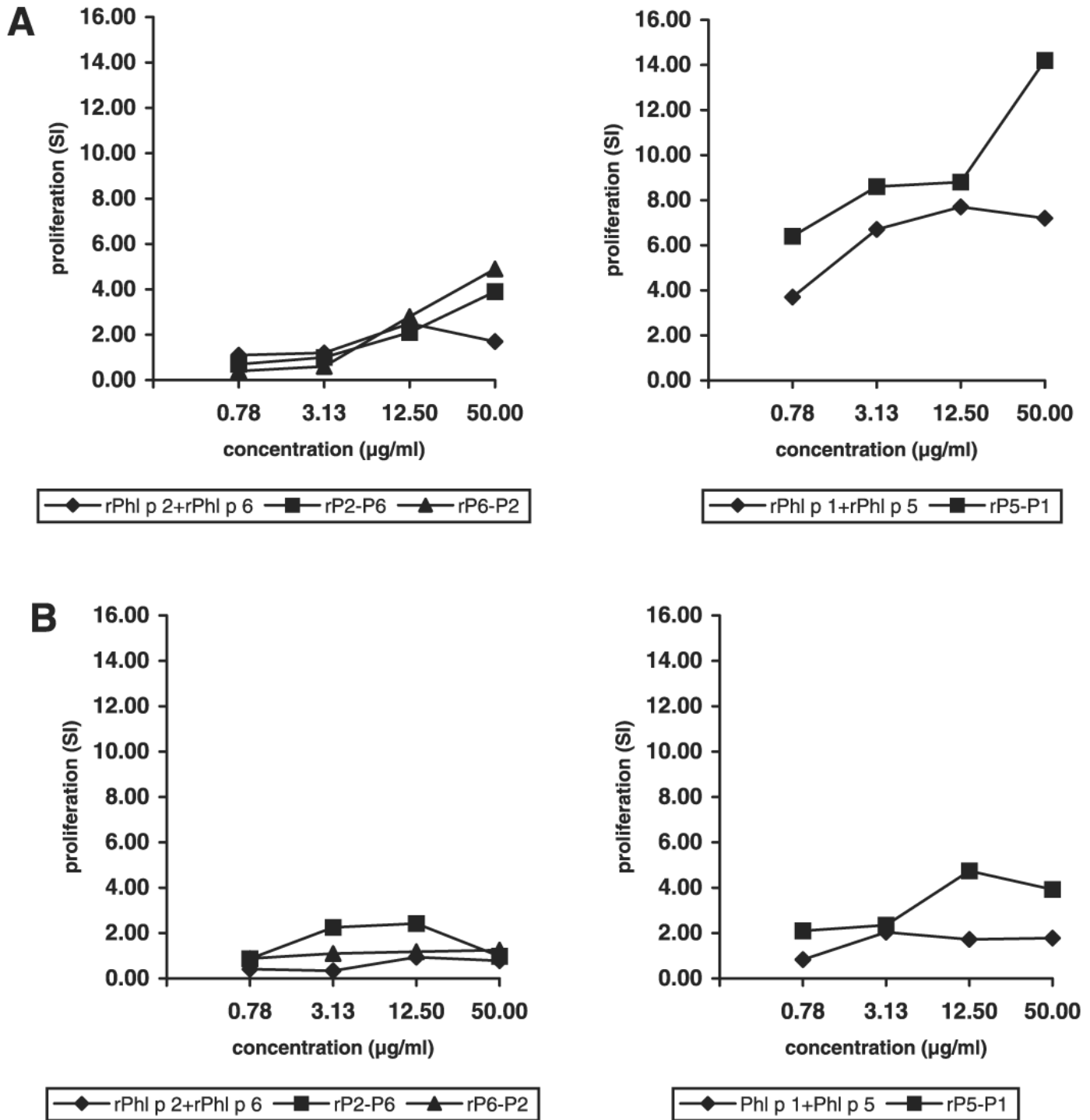


Figure 3. Hybrid molecules induce specific lymphoproliferative responses. PBMC from a grass pollen allergic patient (**A**) and from a non-atopic person (**B**) were incubated with different concentrations of antigens (rPhl p 2+rPhl p 6, rP2-P6, rP6-P2; rPhl p 1+rPhl p 5; rP5-P1) (x-axis). Stimulation indices (y-axis) are displayed for each of the antigen concentrations.

Fig. 4

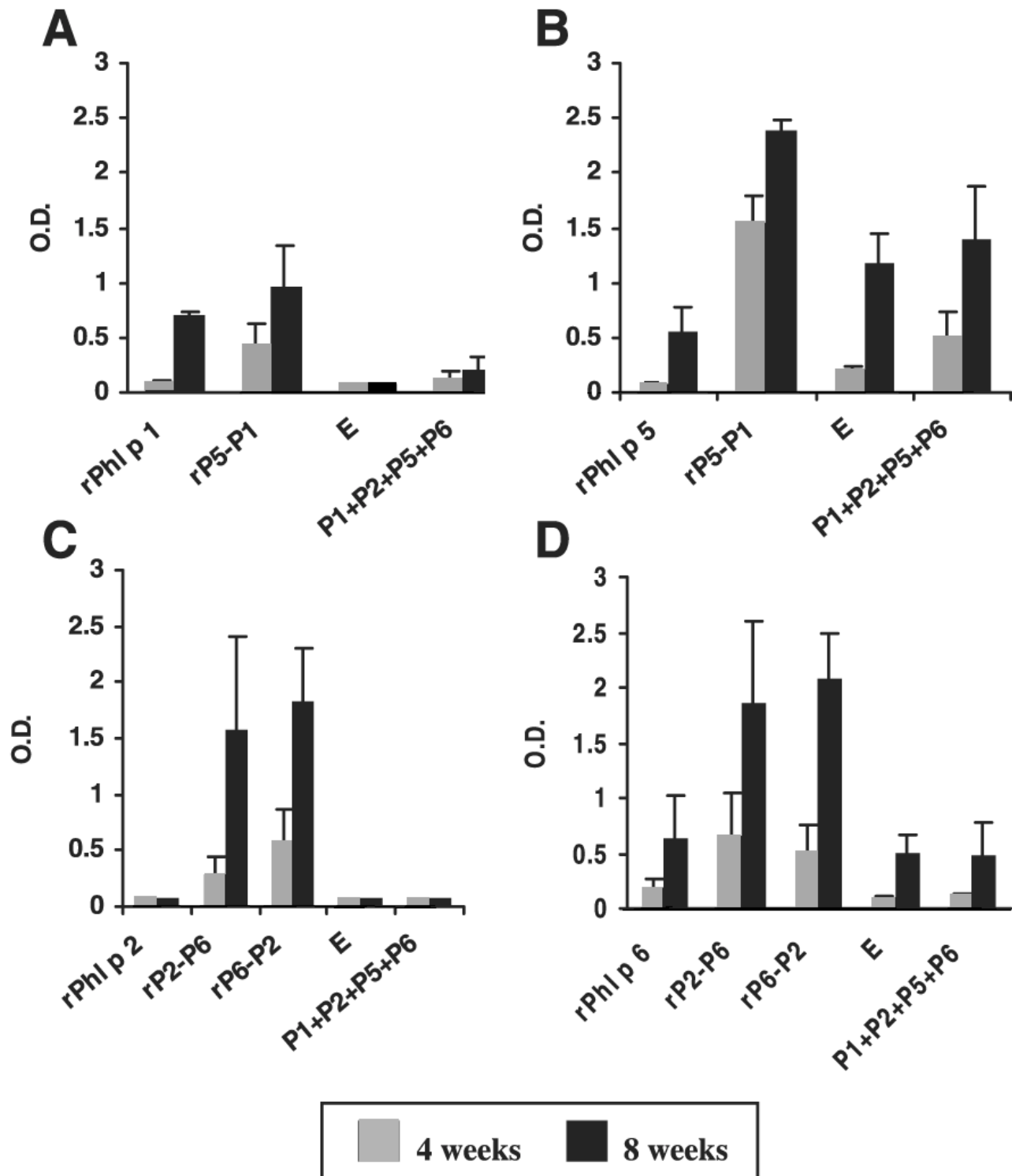


Figure 4. Recombinant hybrid molecules induce strong IgG antibody responses in mice. ELISA plate-bound purified recombinant allergens [(A) rPhl p 1; (B) rPhl p 5; (C) rPhl p 2; (D) rPhl p 6] were incubated with sera from mice (eight mice per group), which had been immunized with the hybrid molecules (rP2-P6, rP6-P2, rP5-P1) the individual recombinant allergens rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, or with timothy grass pollen extract as indicated on the x-axis. Mean IgG₁ levels of sera collected 4 and 8 weeks after immunization correspond to the OD values displayed on the y-axis.

Fig. 5

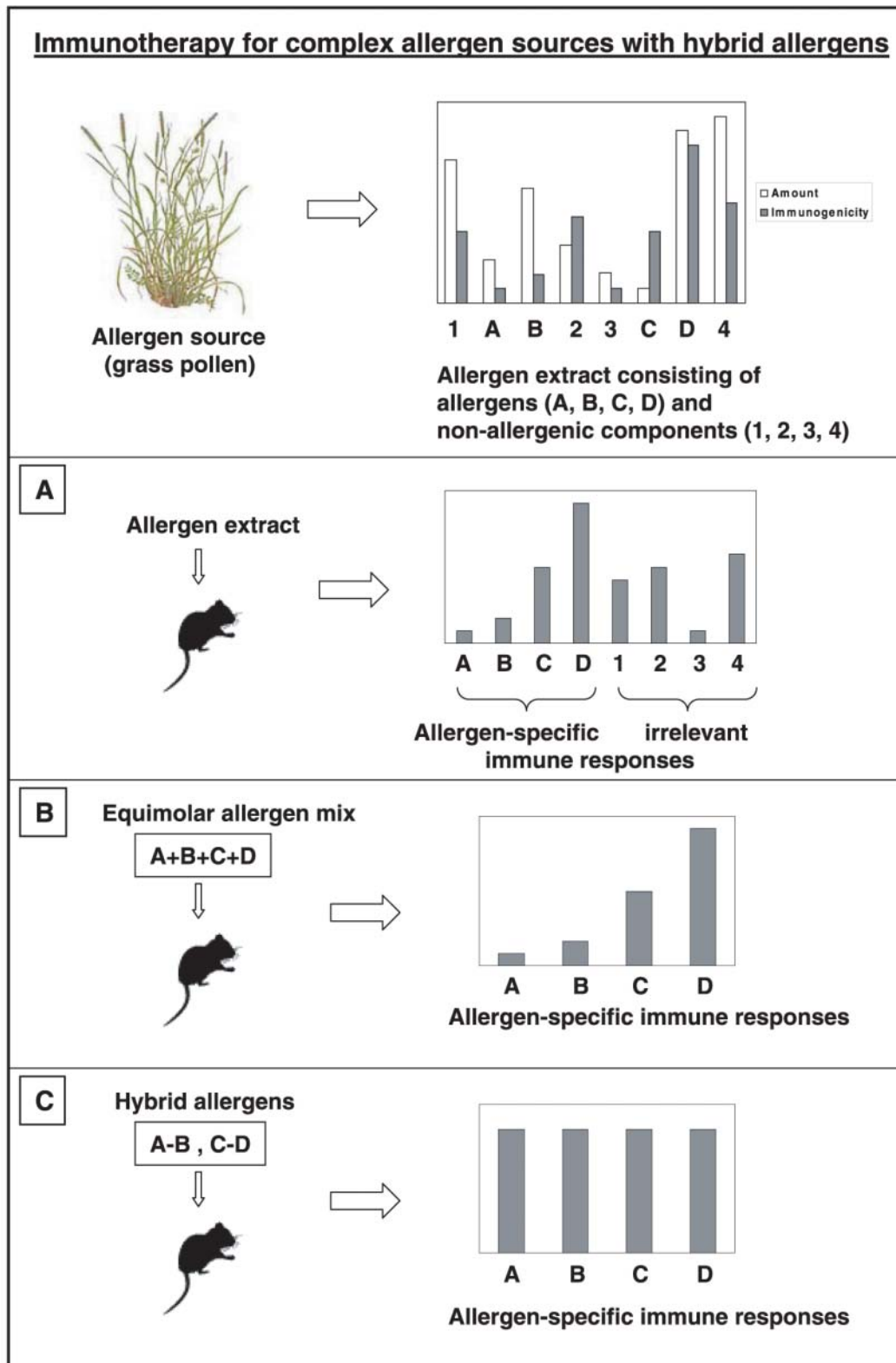


Figure 5. The advantage of using hybrid molecules for immunotherapy. Natural allergen sources (e.g., grass pollen) consist of varying amount of allergens (e.g., A–D) and non-allergenic components (e.g., 1–4) exhibiting different immunogenicity. **A)** Administration of allergen extracts causes immune responses of greatly varying magnitudes against allergens as well as against non-allergenic extract components. **B)** Administration of equimolar mixtures of purified allergens cannot overcome the problem of different immunogenicity of the individual allergens and also results in allergen-specific immune responses of different intensities. **C)** Immunization with hybrid molecules consisting of allergens with low immunogenicity (e.g., A–B) induces strong immune responses against each of the components.