A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination

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Background: Allergy vaccines based on natural allergen extracts contain greatly varying amounts of individual allergens with different immunogenicity.
Objective: To develop a novel type of allergy vaccine for complex allergen sources that combines defined amounts of the major allergens in the form of single hybrid molecules.
Methods: A hybrid molecule was engineered by PCR-based mending and expression of the cDNAs coding for the 4 major grass pollen allergens and compared with its single components by circular dichroism analysis, T-cell proliferation, ELISA competition, and histamine release assays. Immune responses to the hybrid molecule were studied in BALB/c mice and rat basophil leukemia assays.
Results: The hybrid contained most of the B-cell epitopes of grass pollen and could be used to diagnose allergy in 98% (n = 652) of patients allergic to grass pollen. Immunization of mice and rabbits with the hybrid induced stronger and earlier IgG antibody responses than equimolar mixtures of the components, which can be explained by the induction of stronger T-cell responses by the hybrid versus the individual components. IgG antibodies induced by vaccination with the hybrid blocked immediate allergic reactions, as demonstrated by rat basophil degranulation assays in a murine model of grass pollen allergy.
Conclusion: We demonstrate for grass pollen allergy that recombinant hybrid molecules covering the spectrum of the disease-eliciting epitopes of complex allergen sources can be engineered. (J Allergy Clin Immunol 2005;115:1010-6.)

Key words: *Allergy, recombinant allergens, allergy vaccine*

The prevalence of type I allergy, an IgE-mediated hypersensitivity disease, has continuously increased from 5% to 30% during the last few decades.[1] In untreated patients with allergy, the disease progresses from mild (eg, hay fever) to severe and life-threatening manifestations such as asthma.[2] The symptoms of allergy are caused by IgE recognition of harmless environmental antigens and the subsequent activation of cells of the innate and adaptive immune system, leading to severe inflammation.[3] Although the symptoms of allergic inflammation can be mitigated by pharmacological treatment, allergen-specific vaccination represents the only causative form of treatment that prevents the progression of the disease.[4,5]

Allergen-specific vaccination is the practice of administering gradually increasing doses of the allergen-containing vaccines to patients with allergy with the aim to vaccinate against the disease-eliciting allergens.[6] Clinical efficacy of vaccination is documented by clinical studies, and several underlying mechanisms (eg, induction of protective antibody responses, modulation of T-cell responses, and tolerance induction) have been proposed.[6,7] However a major disadvantage of today's allergy vaccination is the application of crude allergen extracts containing greatly varying amounts of the individual allergens with different immunogenicity.[6]

It has recently been demonstrated that molecules consisting of several copies of the same allergen,[8] of 2 homologous allergens,[9] or even of immunologically unrelated allergens[10] can be engineered for allergy vaccination. Here we present an approach for vaccination to complex allergen sources that is based on the identification of the major disease-eliciting allergens and the assembly of their most important epitopes as recombinant hybrid molecules. The feasibility of this approach is demonstrated for allergy to grass pollen, a complex allergen source containing several unrelated major allergens, against which more than 400 million patients with allergy are sensitized.[11]

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Abbreviations used
CD: Circular dichroism
RBL: Rat basophil leukemia

METHODS

Allergic patients, allergen extracts, recombinant allergens

Sera were obtained from patients allergic to grass pollen with confirmed diagnosis of grass pollen allergy (case history, skin prick testing, and measurement of specific IgE antibodies; Pharmacia Diagnostics, Uppsala, Sweden). Pollens from different grass and corn species (*Phleum pratense,* timothy grass; *Zea mays,* maize; *Secale cereale,* cultivated rye; *Avena sativa,* cultivated oat; *Anthoxanthum odoratum,* sweet vernal grass; *Poa pratensis,* Kentucky bluegrass; *Phragmites australis,* Australian reed; *Triticum sativum,* cultivated wheat; *Lolium perenne,* rye grass) were purchased from Allergon (Välinge, Sweden). Aqueous pollen extracts were prepared and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Total protein amounts were determined by Micro BCA Protein Assay (Pierce, Rockford, Ill). Purified recombinant grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6) were obtained from Biomay (Vienna, Austria).

Construction of the plasmid expressing the hybrid molecule

We constructed the cDNA coding for the hybrid molecule by PCR-based recombination of the cDNAs coding for the major grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6.[12] In a first step, cDNAs for Phl p 1 and Phl p 5 were separately obtained by PCR by using the primer pair 5′-CGG *GGTACC*ATGATCCCCAAGGTTCCC-3′ and 5′-CGGGATCCTCAGTGGTGGTGGTGGTGGTGCTTGGACTC-GTAGCTGGT-3′ for Phl p 1 and the primer pair 5′-GGAATT*CAT-ATG*GCCGATCTCGGTTAC-3′ and 5′-CGGGGTACCGACTTT-GTAGCCACCAGT-3′ for Phl p 5. A Kpn I restriction site (italics) was introduced at the 5′ end of the Phl p 1 sequence and a hexahistidine tag followed by a stop codon and a Bam HI restriction site at the 3′ end. The cDNA coding for a signal peptide at the 5′ end of Phl p 5 was replaced by a Nde I site (italics) and the stop codon at the 3′ end by a Kpn I restriction site. The 2 PCR products were inserted into the Nde I/Bam HI site of the pET17b vector (Novagen, Madison, Wis), yielding a construct capable of expressing a Phl p 5–Phl p 1 fusion protein.

In parallel, the cDNAs coding for Phl p 2 and Phl p 6 were fused by PCR. In a first PCR reaction, the primers 5′-AAGCTTATGGTGCCGAAGGTGACG-3′ and 5′-GGGAATTC-*CATATG*CTCTTCTGGCGCGTAGG-3′ (Phl p 2) and the primers 5′-GGAATTCATATGGGGAAGGCCACGACCGAG-3′ and 5′-CACCTTCGGCACCATAAGCTTCGCGCCGGGCTTGACAGC-3′ (Phl p 6) were used to create PCR products with overlapping ends. Both PCR products were gel purified and fused by a subsequent PCR reaction by using the primers 5′-GGAATT*CATATG*GGG-AAGGCCACGACCGAG-3′ and 5′-GGGAATTC*CATATG*CTCTT-CTGGCGCGTAGG-3′ to obtain a cDNA coding for a Phl p 6–Phl p 2 fusion protein. A Nde I restriction site was inserted into the 5′ end of the Phl p 6 cDNA to obtain a start codon (ATG), and the stop codon in the 3′ end of the initially obtained Phl p 2 cDNA was replaced by another Nde I site. The resulting PCR product was inserted into the Nde I site of the pET17b construct containing the Phl p 5–Phl p 1 cDNA to yield a construct expressing the hybrid molecule consisting of the 4 allergens in the order Phl p 6–Phl p 2–Phl p 5–Phl p 1.

Expression and purification of the hybrid molecule

The hybrid molecule was expressed in *Escherichia coli* BL21 (DE3). Cells were grown in Luria Bertani-medium containing 100 μg/mL ampicillin to an OD_{600} of 0.8. Protein expression was induced by addition of isopropyl-β-thiogalactopyranoside to a final concentration of 0.5 mmol/L growing for 5 hours at 37°C. The hybrid was mainly detected in the inclusion body fraction. Inclusion bodies were solubilized in 8 mol/L urea, 100 mmol/L NaH_2PO_4, and 10 mmol/L TRIS, pH 8.0. The homogenate was centrifuged at 14,000*g* for 30 minutes, and the resulting supernatant was loaded on a Ni-NTA column (Quiagen, Hilden, Germany). The column-bound protein was refolded on the Ni-NTA column by using a linear 6 mol/L–1 mol/L urea gradient containing 500 mmol/L NaCl, 20% glycerol, and 20 mmol/L TRIS, pH 7.4, over a period of 1.5 hours.[13] The soluble protein was eluted by addition of 250 mmol/L imidazole and dialyzed against H_2O. The purified hybrid molecule remained soluble in water at a concentration of 1 mg/mL.

Circular dichroism analysis of the purified hybrid molecule

Circular dichroism (CD) measurements were performed in MilliQ water (Millipore, Billerica, Mass) with a hybrid protein concentration of 0.17 mg/mL (2.1×10^{-6} mol/L). An equimolar protein mix of the individual allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6 was prepared to obtain a final protein concentration of 0.5 mg/mL, which was further diluted to a protein concentration of 0.17 mg/mL (3.0×10^{-6} mol/L) for the CD measurements. The term *equimolar* used here and in the consecutive immunological experiments means that the concentration of the individual allergens in the mix was exactly adjusted according to their molar representation in the hybrid. The investigations were performed on a Jasco J-715 spectropolarimeter (Jasco Inc, Easton, Md) by using a 0.1-cm pathlength cell equilibrated at 20°C. Spectra were recorded with 0.5-nm resolution at a scan speed of 100 nm/min and resulted from averaging 3 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 (ø) at a given wavelength.[14]

ELISA experiments

Allergic patients' serum IgE reactivity to ELISA plate-bound allergens and ELISA competition experiments measuring the percentage of allergen-specific IgE that can be preadsorbed from patients' sera with allergens were conducted as described.[15] The ability of rabbit anti-hybrid IgG and rabbit anti-timothy grass pollen extract IgG to inhibit IgE binding of patients allergic to grass pollen to timothy grass pollen allergens was investigated by ELISA competition experiments.[16]

Basophil histamine release experiments

Heparinized blood samples were obtained by venipuncture from patients allergic to grass pollen with sensitization to the timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6. Granulocytes were isolated by dextran sedimentation. Granulocytes were exposed to different concentrations (10^{-8} μg/mL, 10^{-7} μg/mL, 10^{-6} μg/mL, 10^{-5} μg/mL, 10^{-4} μg/mL, 10^{-3} μg/mL, 10^{-2} μg/mL, 10^{-1} μg/mL) of the purified hybrid molecule or an equimolar mix of rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6 dissolved in histamine release buffer (Immunotech, Marseille, France). Histamine release into the cell-free supernatants was determined by radioimmunoassay (Immunotech).

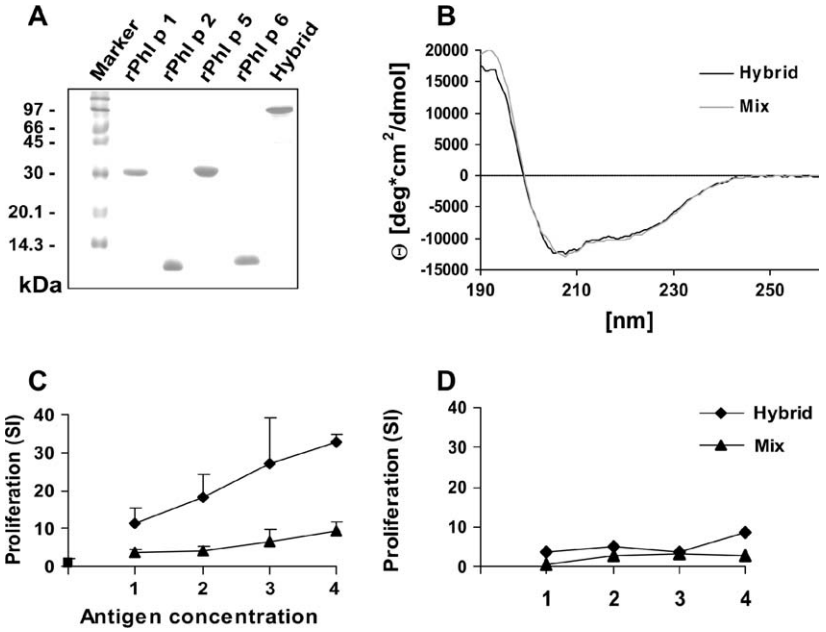

FIG 1. A recombinant hybrid molecule representing the allergy-eliciting components of grass pollen. **A,** Coomassie-stained SDS-PAGE containing recombinant timothy grass pollen allergens *(lanes: rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6)* and the hybrid *(lane: Hybrid)*. Molecular weights *(lane: Marker)* are indicated on the *left* side. **B,** CD spectrum of the hybrid molecule and of an equimolar mix of the 4 recombinant timothy grass pollen allergens. Mean residue ellipticity *(y-axis)* is displayed for different wave lengths *(x-axis)*. Lympho-proliferative responses of PBMC from 4 grass pollen allergic patients **(C)** and a nonatopic donor **(D)** to the hybrid and to an equimolar mix of the 4 allergens. Mean stimulation indices *(y-axis)* are displayed for 4 antigen concentrations. Hybrid and allergen mix: 0.8 μg/mL (1), 3.1 μg/mL (2), 12.5 μg/mL (3), 50 μg/mL (4).

Measurements were performed in triplicate and are expressed as percentages of total histamine determined after cell lysis.[17]

T-cell proliferation assay

We isolated PBMCs from patients allergic to grass pollen with Phl p 1–specific, Phl p 2–specific, Phl p 5–specific, and Phl p 6–specific IgE antibodies or from nonatopic donors by Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. PBMCs were resuspended in AIM V medium (Life Technologies, Grand Island, NY) to a final concentration of 2×10^5 cells/well and stimulated with increasing antigen doses (0.78 μg/mL, 3.125 μg/mL, 12.5 μg/mL, 50 μg/mL) of the hybrid, with an equimolar mixture of the individual recombinant allergens (rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6), or with medium alone in triplicates. After 6 days, proliferative responses were measured by [^{3}H] thymidine incorporation and are expressed as stimulation indices.[18]

Murine model for grass pollen allergy

Female 8-week-old BALB/c mice (Charles River, Sulzfeld, Germany), kept in the animal care unit of the Department of Pathophysiology, University of Vienna, according to the local guidelines for animal care, were immunized subcutaneously (day 1 and day 30) with 100 μg timothy grass pollen extract adsorbed to $Al(OH)_3$ (Alu-Gel-S; Serva, Ingelheim, Germany). Allergic sensitization to timothy grass pollen extract was confirmed by the measurement of grass pollen allergen–specific IgE antibodies in sera and by the demonstration of type I hypersensitivity reactions by using intradermal skin testing (2.5 μg/mL timothy grass pollen extract or an equimolar mixture of rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6).[19] For a quantification of immediate-type reactions, rat basophil leukemia (RBL) cell mediator release assays were performed. RBL-2H3 cells were cultivated in 96-well tissue culture plates (4×10^4 cells/well) for 24 hours at 37°C by using 7% CO_2. Passive sensitization was performed by incubation with 1:30 diluted murine sera with or without (negative control) timothy grass pollen allergen-specific IgE for 2 hours. Cells were washed twice with Tyrode's buffer (Sigma, St Louis, Mo) (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 0.4 mmol/L NaH_2PO_4, 5.6 mmol/L D-glucose, 12 mmol/L $NaHCO_3$, 10 mmol/L HEPES, and 0.1% BSA, pH 7.2) to remove unbound antibodies. Degranulation of RBL cells was induced by adding 1.5 μg/mL timothy grass pollen extract or 0.3 μg/mL of an equimolar mixture of the recombinant allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6). The protective activity of rabbit anti-hybrid IgG or rabbit anti-timothy grass pollen extract IgG was measured by adding these antibodies or, for control purposes, the corresponding preimmune IgG to the allergens immediately before exposure to the RBL cells. After 30 minutes, β-hexosaminidase release was analyzed.[20] Results are expressed as percentages of total β-hexosaminidase released after addition of 1% Triton X-100 (Merck, Darmstadt, Germany).

RESULTS

Characteristics of the recombinant hybrid molecule

We engineered a hybrid molecule consisting of the most important grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6.[21-24] The cDNAs coding for the 4 major allergens were fused by PCR-based recombination, and

TABLE I. Inhibition of IgE binding to purified allergens and grass pollen extract after preadsorption of patients' sera with allergens, extract, or the hybrid

	Percentage inhibition of IgE binding to									
	rPhl p 1		rPhl p 2		rPhl p 5		rPhl p 6		Extract	
Pre-adsorption with	rPhl p 1	Hybrid	rPhl p 2	Hybrid	rPhl p 5	Hybrid	rPhl p 6	Hybrid	Extract	Hybrid
mean (n = 20)	79	72	72	70	71	74	65	69	85	68

the resulting cDNA construct coding for a 79-kd hybrid molecule was inserted into an expression plasmid. The strategy of PCR-based mending of the allergen-encoding cDNAs allowed us to produce the hybrid molecule with insertion of only 2 foreign amino acids between each antigen in the order Phl p 6–Phl p 2–Phl p 5–Phl p 1. The recombinant hybrid molecule was expressed at high levels in *Escherichia coli* (10 mg/l culture) and could be purified to homogeneity by Nickel affinity chromatography. Fig 1, *A*, shows the individual recombinant timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 and the purified recombinant hybrid molecule in a Coomassie-stained SDS-PAGE.

By using CD analysis, we could demonstrate that the hybrid molecule contained a considerable amount of folded α/β-secondary structure. The fold of the hybrid molecule was almost identical to that of an equimolar mix of the isolated allergen components, of which Phl p 5 and Phl p 6 indeed represent predominantly α-helical proteins, whereas Phl p 2 and Phl p 1 consist mainly of a β-sheet structure (Fig 1, *B*).[24-26]

The hybrid molecule accounts for the major B-cell and T-cell epitopes

We investigated the presence of the B-cell epitopes of the individual components in the hybrid molecule by using antibody probes (antisera as well as mAbs) specific for the individual grass pollen allergens (data not shown) and by IgE inhibition tests. Immunological competition experiments showed that the hybrid molecule blocked the binding of IgE antibodies of patients allergic to grass pollen (n = 20) to the single grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6) and to grass pollen extract as efficient as the individual allergens and the extract (Table I). These results demonstrate that the hybrid molecule contained most of the IgE epitopes present in natural grass pollen extract. Furthermore, we found that the hybrid induced histamine release from basophils of patients allergic to grass pollen in a manner comparable with an equimolar mixture of the individual allergens, demonstrating that the covalent linking of several allergens did not increase their allergenic potential (Fig 2).

Lymphoproliferative responses were studied by using cultured PBMCs from patients allergic to grass pollen. The results showed that the hybrid molecule induced much stronger T-cell proliferation than an equimolar mix of the single allergens at all tested concentrations (Fig 1, *C*). Interestingly, we noted that the hybrid induced much higher release of IL-10 and IFN-γ and a reduced release of IL-5 in PBMCs of patients allergic to grass pollen (data not shown). As has been described in previous studies, PBMCs from a nonallergic individual who mounted allergen-specific IgG antibodies proliferated also in the presence of the hybrid molecule or of an allergen mix, but to a lower extent than those from the patients with allergy (Fig 1, *D*).[27]

Immunization with the hybrid molecule induces stronger and earlier IgG responses than immunization with grass pollen extract or with the individual allergens

We investigated whether the hybrid molecule induces allergen-specific IgG responses *in vivo*. Groups of 8 mice were immunized with the hybrid molecule, grass pollen extract, or the individual allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6). Blood samples taken 4 and 8 weeks after immunization were analyzed for IgG antibodies to the immunogens. We found that immunization with the hybrid induced earlier and stronger IgG responses to grass pollen extract and to each of the individual allergens than immunization with total extract or the single allergens (Fig 3, *A and B*). The increase in immunogenicity was especially pronounced for those allergens, which induced very low IgG responses as isolated components (ie, Phl p 2, Phl p 1; Fig 3, *B*). This result indicates strongly enhanced immunogenicity of the individual allergens when presented in the context of the hybrid molecule.

Blocking activity of hybrid-induced IgG antibodies

Next, we studied whether immunization with a hybrid molecule made from major allergens of 1 allergen source (ie, timothy grass pollen) would induce IgG antibodies that cross-reacted with other botanically related allergen sources (ie, other grass species, corn). That the IgG antibodies induced by immunization with the hybrid indeed cross-reacted with major allergens from 9 of the most important grass and corn species was demonstrated by cross-reactivity of rabbit anti-hybrid IgG (Fig 3, *C*). No reactivity of preimmune immunoglobulin with the natural pollen allergens was found in the same experiment.

Assuming that IgG antibodies that compete with the binding of IgE of allergic patients to allergens may have protective activity, we analyzed the extent to which anti-hybrid IgG can inhibit binding of IgE antibodies of patients allergic to grass pollen to grass pollen allergens. As studied for 6 patients, we found that rabbit antihybrid IgG inhibited IgE reactivity of patients with allergy even

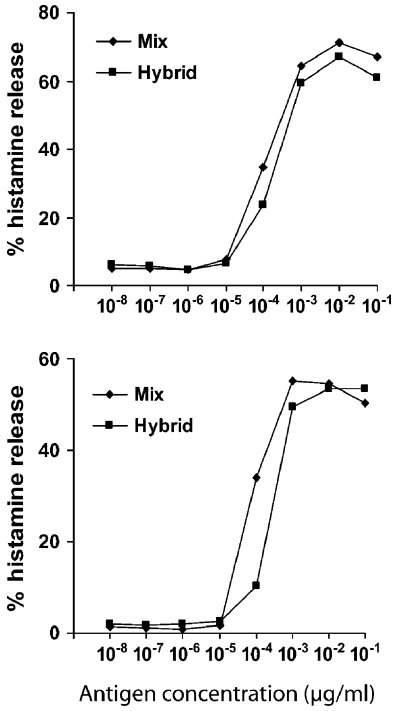

FIG 2. Biological activity of the hybrid versus an equimolar mix of its components. Basophils from 2 patients allergic to grass pollen were exposed to increasing concentrations of the hybrid molecule or an equimolar mixture of the single components *(x-axis)*. The percentages of histamine release are shown on the *y-axis*.

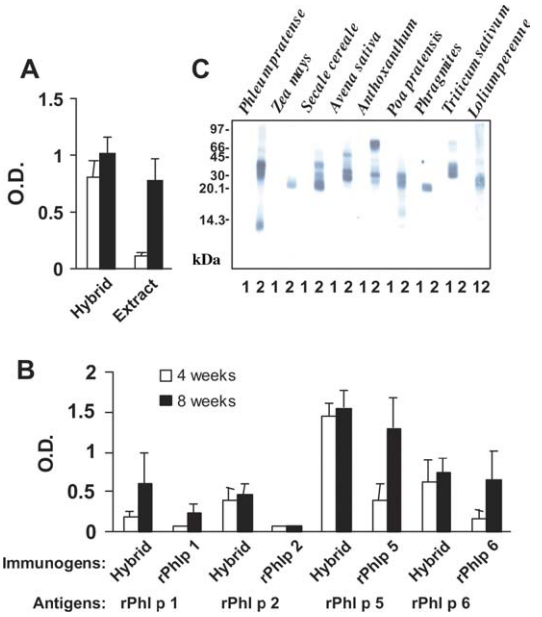

FIG 3. Increased immunogenicity of the hybrid. **A,** Mean IgG_1 response of 8 mice immunized with the hybrid or grass pollen extract *(x-axis)* displayed as OD values *(y-axis)* 4 *(white bars)* and 8 weeks *(black bars)* after immunization. **B,** Mean IgG_1 responses of 8 mice (OD, *y-axis*) to purified allergens *(Antigen)* induced with different immunogens *(x-axis)* 4 weeks and 8 weeks after immunization. **C,** Cross-reactivity of rabbit IgG induced by hybrid immunization to pollen extracts from different grasses and corn *(lanes 2)*. Preimmune sera *(lanes 1)*. Molecular weights displayed on the *left* margin.

more strongly (average, 61%) than rabbit anti-grass pollen extract IgG (average, 52%).

IgG antibodies raised against the hybrid block immediate-type reactions in a murine model of grass pollen allergy

The biological significance of the blocking antibodies was studied in a murine model of grass pollen allergy. BALB/c mice were sensitized to grass pollen extract and, as exemplified in Fig 4, *A*, exhibited immediate-type cutaneous hypersensitivity reactions to natural grass pollen extract as well as to the mix of recombinant major timothy grass pollen allergens. Whether hybrid molecule-specific rabbit IgG can inhibit allergen-induced immediate reactions was analyzed and quantified in detail by using rat basophil leukemia cells loaded with grass pollen allergen-specific IgE antibodies from the sensitized mice (Fig 4, *A*). We demonstrate that hybrid molecule- as well as grass pollen extract-specific rabbit IgG almost completely inhibited the release of β-hexosaminidase from loaded basophils. A concentration of only 2% vol/vol added serum blocked mediator release caused by grass pollen allergen extract or purified major grass pollen allergens. The addition of preimmune sera to a concentration of 10% vol/vol had no influence on allergen induced hexosaminidase release, and the addition of serum alone did not cause basophil degranulation (Fig 4, *B and C*).

DISCUSSION

Almost 25% of the population has type I allergy. Further development and improvement of allergen-specific immunotherapy, the only curative therapy approach, has been impeded by the lack of pure allergens. With the application of recombinant DNA technology in the field of allergen characterization, pure recombinant allergens have become available for improvement of allergy diagnosis and for the formulation of defined allergy vaccines.[28] To reduce the allergenic activity of allergy vaccines and thus therapy-related side effects, hypoallergenic allergen derivatives have been engineered (see reviews[7,28]). However, several other major problems related to the use of crude allergen extracts remained. In fact, allergen extracts represent mixtures of varying amounts of allergens and nonallergenic components. Moreover, they contain different concentrations of the individual allergens, of which each may exhibit different immunogenicity. Therefore, highly heterogenous immune responses have been observed in patients undergoing specific immunotherapy based on allergen extracts.[29] In particular, it has been noted for grass pollen allergy, one of the most frequent allergy forms, affecting about 400 million patients, that certain allergens elicit very poor or no protective IgG response.[29]

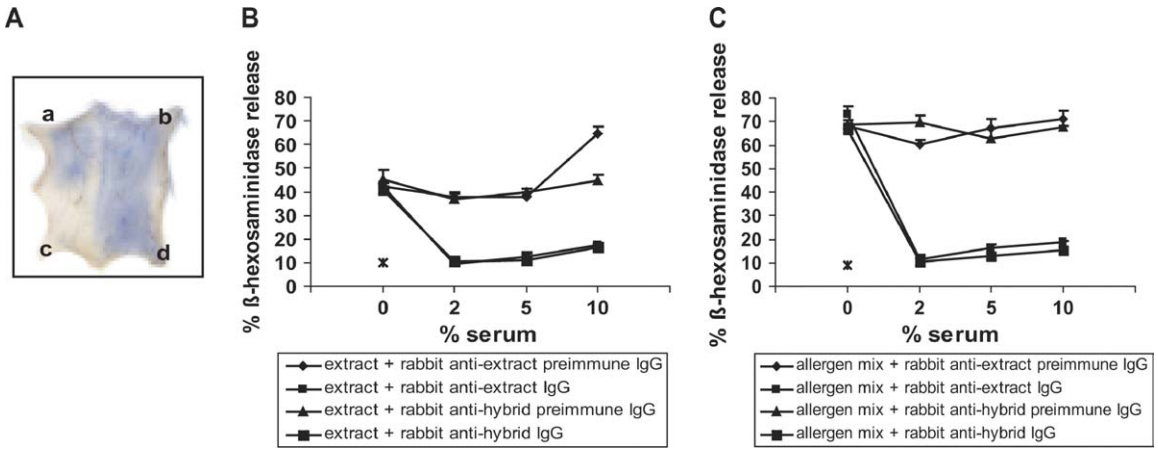

FIG 4. IgG antibodies induced by hybrid immunization block immediate type reactions in a murine model of grass pollen allergy. **A,** Grass pollen extract–sensitized mice exhibit immediate-type skin reactions to grass pollen extract *(a)* and to a mix of purified grass pollen allergens *(b)*. Negative control (*c,* PBS); positive control (*d,* 48/80). Dose-dependent inhibition of β-hexosaminidase release *(y-axis)* from RBL cells containing grass pollen-specific IgE by addition of increasing doses of antiextract or antihybrid IgG mixed with grass pollen extract **(B)** or an allergen mix **(C)**. Results from the addition of the corresponding preimmune IgG mixed with pollen extract or the allergen mix are also shown. *Spontaneous release of unloaded cells.

Here we present an approach to address the latter problem by assembling the disease-eliciting epitopes of a complex allergen source within a single hybrid molecule. We fused the 4 major grass pollen allergens by genetic engineering and compared the recombinant hybrid molecule with the individual allergens in structural and immunological experiments. One key finding of our experiments was that it was possible to engineer a recombinant hybrid molecule containing most of the relevant T-cell and B-cell epitopes present in natural grass pollen. The somewhat reduced inhibition of IgE reactivity to natural grass pollen allergens by the hybrid compared with natural allergen extract may be explained by the fact that certain grass pollen allergens/isoallergens/epitopes were not incorporated in this molecule.

The unambiguous diagnosis of type I allergy is in fact based on the determination of allergen-specific IgE antibodies.[30] Because of the preservation of grass pollen-specific IgE epitopes on the recombinant hybrid molecule, it could be used to identify 642 out of 652 grass pollen allergic patients by IgE serology. The 10 hybrid-negative patients reacted with profilin, a highly cross-reactive allergen present in numerous allergen sources,[31] but failed to exhibit sensitization to genuine and major grass pollen allergens. The hybrid molecule can thus be used for allergy diagnosis to discriminate patients with a genuine grass pollen allergy from those patients who, because of immunological cross-reactivity, appear wrongly as grass pollen–sensitized in extract-based diagnostic tests.

The assembly of the individual allergen components in the form of a hybrid molecule did not increase their allergenic activity as demonstrated by histamine release experiments by using basophils from patients allergic to grass pollen. Because of the almost complete representation of the disease-eliciting epitopes in the hybrid and because its allergenic activity was not increased, it may be assumed that the hybrid can replace complex allergen extracts in allergen-specific immunotherapy.

Modulation of allergen-specific T-cell responses and the induction of allergen-specific IgG antibodies, which by simple competition or via Fcγ-receptor cross-linking inhibit IgE-mediated mast cell and T-cell activation, have been suggested as potential mechanisms underlying allergy vaccination.[7,28,32-39] We therefore studied humoral and cellular immune responses specific for the hybrid molecule. Our results demonstrate that the hybrid induced much stronger T-cell activation than the individual allergen components. It is thus expected that it will be superior to a cocktail of the individual allergen molecules and can be used in smaller doses to achieve therapeutic immunomodulatory effects at the T-cell level. Regarding the induction of blocking antibody responses, we found that the hybrid induced much stronger and earlier induction of IgG responses than a mix of the individual allergens or grass pollen extract. These IgG antibodies were of protective nature because they inhibited the binding of IgE antibodies of patients with allergy to the wild-type allergens and, as demonstrated and quantified in a murine model of grass pollen allergy, blocked immediate allergic reactions.

We have thus exemplified that recombinant hybrid molecules resembling the epitope spectrum of complex allergen sources can be engineered. The hybrid molecule approach can also be used for the generation of derivatives of a single allergen to reduce the rate of anaphylactic side effects in the course of allergen-specific vaccination.[6] In this context, we have also shown that reshuffling of allergen fragments leads to modified molecules with strongly reduced allergenic activity (Stumvoll et al, unpublished data, January 2005). Furthermore, it will be possible

to engineer hybrid molecules consisting of hypoallergenic allergen derivatives.[40] Because of the strongly enhanced immunogenicity, these molecules might induce strong protective IgG antibody responses on active vaccination.

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