

A hypoallergenic hybrid molecule with increased immunogenicity consisting of derivatives of the major grass pollen allergens, Phl p 2 and Phl p 6*

Birgit Linhart^{1,**}, Nadine Mothes-Luksch^{1,a},
Susanne Vrtala¹, Michael Kneidinger², Peter
Valent² and Rudolf Valenta¹

¹Division of Immunopathology, Department of Pathophysiology, Center of Physiology, Pathophysiology and Immunology, Medical University of Vienna, A-1090 Vienna, Austria

²Department of Internal Medicine I, Division of Hematology and Hemostaseology, Vienna General Hospital, Medical University of Vienna, A-1090 Vienna, Austria

** Corresponding author

e-mail: birgit.linhart@meduniwien.ac.at

Abstract

Allergen-specific immunotherapy is currently based on the administration of allergen extracts containing natural allergens. However, its broad application is limited by the poor quality of these extracts. Based on recombinant allergens, well-defined allergy vaccines for allergen-specific immunotherapy can be produced. Furthermore, they can be modified to reduce their allergenic activity and to avoid IgE-mediated side effects. Here, we demonstrate that the immunogenicity of two grass pollen-derived hypoallergenic allergen derivatives could be increased by engineering them as a single hybrid molecule. We used a hypoallergenic Phl p 2 mosaic, generated by fragmentation of the Phl p 2 sequence and reassembly of the resulting peptides in an altered order, and a truncated Phl p 6 allergen, to produce a hybrid protein. The hybrid retained the reduction of IgE reactivity and allergenic activity of its components as shown by ELISA and basophil activation assays. Immunization with the hybrid molecule demonstrated the increased immunogenicity of this molecule, leading to higher levels of allergen-specific IgG antibodies compared to the single components. These antibodies could inhibit patients' IgE binding to the wild-type allergens. Thus, the described strategy allows the development of safer and more efficacious vaccines for the treatment of grass pollen allergy.

Keywords: allergy; allergy vaccines; recombinant allergens; specific immunotherapy.

Introduction

More than 20% of the population in industrialized countries suffer from type I allergy with an increasing prevalence (Eder et al., 2006). Allergic patients are characterized by the formation of IgE antibodies to environmental antigens (allergens), mainly from pollen, mites, animal dander, and molds (Kay, 1997; Geha et al., 2003). The immediate allergic symptoms, allergic rhinitis, conjunctivitis, allergic asthma, and anaphylactic shock, are mediated by the allergen-induced cross-linking of IgE antibodies bound to the high affinity receptor of IgE (FcεRI) on effector cells (e.g., basophils, mast cells) leading to the release of inflammatory mediators, cytokines, and proteases (Turner and Kinet, 1999; Bischoff, 2007). In addition, IgE-facilitated antigen presentation may contribute to T-cell activation (van Neerven et al., 2006). Allergen-specific immunotherapy represents the only antigen-specific and disease-modifying treatment of allergy. It is based on the administration of increasing amounts of allergens to allergic patients and has been proven to be effective in several clinical studies (Bousquet et al., 1998; Durham et al., 1999; Möller et al., 2002). However, the poor quality of allergen extracts, which represent the active components, limits the broad applicability and efficacy of allergen-specific immunotherapy. Allergen extracts are crude mixtures of varying amounts of poorly defined allergenic and non-allergenic materials. Important allergens are sometimes lacking and the concentrations of allergens may show great variations in extracts made by different manufacturers and from one batch to another (Focke et al., 2008). Secondly, allergen extracts contain allergens, which may cause severe side effects, particularly when the allergens are injected without adjuvant (Valenta and Niederberger, 2007). Finally, only poor immune responses can be achieved, if certain allergens in a given extract are either underrepresented or poorly immunogenic (Mothes et al., 2003).

Due to the progress made in the field of molecular allergen characterization, most of the clinically relevant allergens have been identified. According to the DNA template, they can be produced as recombinant allergens with high purity, in defined concentrations and consistent quality (Valenta and Kraft, 2002). These recombinant wild-type allergens mimic the properties of their natural counterparts and can be applied in diagnostic tests to analyze and monitor the patients' sensitization profiles and precisely select patients for allergen-specific immunotherapy (Valenta et al., 1999; Hiller et al., 2002; Kazemi-Shirazi et al., 2002). They can also be used as active components of therapeutic allergy vaccines, and their effectiveness has recently been shown in clinical trials (Niederberger et al., 2004; Jutel et al., 2005; Pauli

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^aPresent address: Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Vienna General Hospital, Medical University of Vienna, A-1090 Vienna, Austria.

et al., 2008; Purohit et al., 2008). Furthermore, modified variants of these allergens with reduced allergenic activity have been generated to avoid IgE-mediated side effects in the course of immunotherapy. Advantages of these hypoallergenic derivatives are the strongly reduced allergenic activity, the presence of most of the allergen-derived T-cell epitopes, and their ability to induce protective antibody responses, that interfere with IgE-mediated effects (Valenta, 2002). This has been demonstrated in an immunotherapy study conducted in birch pollen allergic patients with genetically modified derivatives of the major birch pollen allergen Bet v 1 (Niederberger et al., 2004; Gafvelin et al., 2005; Reisinger et al., 2005; Pree et al., 2007). While the application of recombinant wild-type allergens and hypoallergenic molecules addresses the questions of vaccine composition and safety, we recently developed a strategy to overcome the problem of poor immunogenicity and demonstrated this for wild-type allergens (Linhart et al., 2002, 2005; Linhart and Valenta, 2004). In this study, we show that the latter approaches, the generation of hypoallergenic allergen derivatives and the hybrid technology, can be combined in the form of a hypoallergenic hybrid molecule with increased immunogenicity. This was exemplified for the hypoallergenic derivatives of the two major grass pollen allergens Phl p 2 and Phl p 6, namely a Phl p 2 mosaic molecule (Mothes and Valenta, unpublished data) and the deletion mutant Phl p 6 (aa 31–110) (Vrtala et al., 2007). We report the construction, molecular and immunological characterization of a hybrid molecule consisting of these two hypoallergenic derivatives and

demonstrate its ability to induce an increased and protective IgG antibody response upon vaccination.

Results

Construction and characterization of the recombinant hybrid allergen hP6-2

For the generation of the recombinant hybrid allergen hP6-2, an approach consisting of several steps was chosen (Figure 1A). In a first step, Phl p 2- and Phl p 6-derived derivatives were generated, based on previously performed IgE-epitope mapping experiments, and evaluated in terms of patients' IgE-binding and their ability to induce a Phl p 2- and Phl p 6-specific antibody response, respectively. In the case of the grass pollen allergen Phl p 6, a fragment comprising aa 31–110 with a strongly reduced allergenic activity was identified. The Phl p 6-derivative induced a protective IgG antibody response to the Phl p 6 allergen in an animal model (Vrtala et al., 2007). A total of three non-allergenic Phl p 2-derived peptides covering the whole Phl p 2 sequence were reassembled in an altered order (Figure 1A), resulting in a hypoallergenic 'mosaic' molecule (Mothes and Valenta, unpublished data). The cDNAs encoding these two hypoallergenic molecules were fused by PCR resulting in a hybrid molecule, termed hP6-2 (Figure 1A–C). This hybrid allergen could be expressed in large amounts in *E. coli*, and purified from the inclusion body fraction on a DEAE-sepharose column. Figure 2A shows a Coomas-

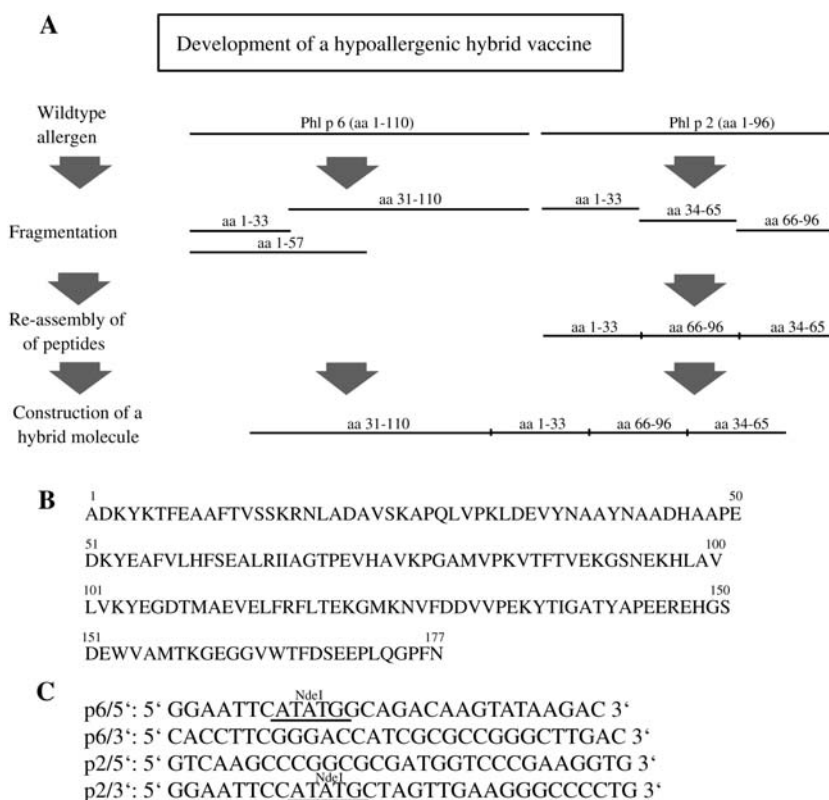


Figure 1 Construction of the Phl p 2-Phl p 6 hybrid hypoallergen.

(A) The hybrid molecule was generated by the reassembly of Phl p 2- and Phl p 6-derived fragments as illustrated. (B) Amino acid sequence of the resulting hybrid hypoallergen hP6-2. (C) Sequences of the PCR primers used for the construction of hP6-2.

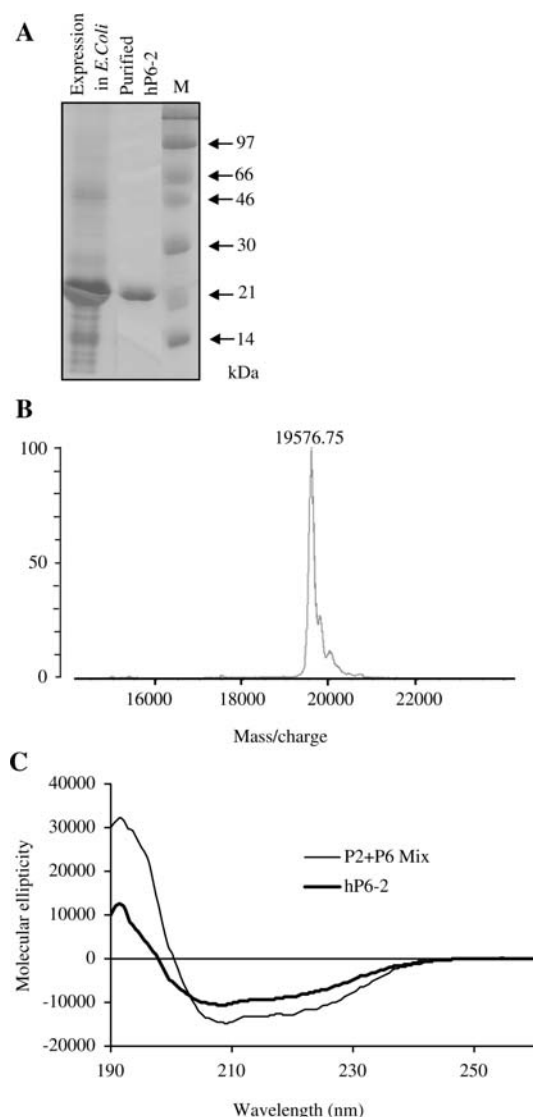


Figure 2 Biochemical and structural analysis of the hP6-2 hybrid molecule.

(A) Coomassie Brilliant Blue-stained SDS-PAGE containing extract from *E. coli*, expressing the hP6-2 protein and purified hP6-2. Lane M shows the molecular mass marker. (B) Mass spectrometry of the hP6-2 hybrid. The x-axis shows the mass/charge ratio, and signal intensity is displayed on the y-axis as a percentage of the most intensive signal obtained in the investigated mass range. (C) CD spectra of the purified hybrid molecule and a mixture of rPhl p 2 and rPhl p 6 (P2+P6). Results are shown as the mean residue ellipticity (y-axis) at a given wavelength (x-axis).

sie-stained SDS-PAGE of the *E. coli*-expressed and purified hP6-2 protein. The molecular mass of the hP6-2 hybrid was determined by mass spectrometry (19 576.75 Da), which was in agreement with the predicted molecular weight without the N-terminal methionine (Figure 2B).

For the estimation of the secondary structure, the CD spectrum of the hP6-2 hybrid was compared to a mixture of rPhl p 2 and rPhl p 6. The far-UV CD spectrum of the equimolar mix of rPhl p 2 and rPhl p 6 showed a minimum at 209 nm and a broad shoulder between 215 and 225 nm, indicating a mixed α - and β -secondary structure. This was in agreement with the findings that rPhl p

2 represented an almost exclusive β -sheet structure (De Marino et al., 1999), while rPhl p 6 was described as an α -helical protein, forming a four-helical up-and-down bundle (Vrtala et al., 2007). The far-UV CD spectrum recorded for the purified hP6-2 hybrid molecule was characterized by a weaker signal, with a minimum at around 208 nm and a maximum at 191 nm (Figure 2C). The secondary structure prediction using the program CDSSTR (Whitmore and Wallace, 2004) was in good agreement with the measurement, as it also indicated a mixed α - and β -secondary structure, with 17% α -helix, 27% β -strand, 25% turns and an increased portion of 31% random coiled structures. This might be due to the fact that the isolated rPhl p 6-derived fragment (aa 31–110), representing helices α 2 to α 4 from Phl p 6, had lost its α -helical structure (Vrtala et al., 2007) and was dominated by a random coil secondary structure.

Low IgE reactivity and allergenic activity of hP6-2

We previously described a recombinant hybrid allergen consisting of Phl p 6 and Phl p 2 (designated P6-2; Linhart et al., 2002), which contained an amount of secondary structure comparable to its single components. Accordingly, the P6-2 hybrid was recognized by IgE antibodies from grass pollen allergic patients equally well as the isolated allergens Phl p 2 and Phl p 6. We compared the IgE reactivity of the hP6-2 hybrid consisting of two hypoallergenic derivatives of Phl p 2 and Phl p 6 to that of the P6-2 hybrid by ELISA. Recombinant Phl p 2- and Phl p 6-specific IgE antibodies in the patients' sera were also determined to assess their sensitization profile to these allergens. The ELISA plate-bound molecules were exposed to sera from 50 grass pollen allergic patients. We observed a strong reduction of IgE reactivity of the hP6-2 hybrid compared to the P6-2 hybrid (mean reduction: 85%; see Table 1).

The effect of reduced IgE reactivity of the hP6-2 hybrid on IgE-dependent effector cell activation was examined by the analysis of CD203c expression on human basophils isolated from grass pollen allergic patients who were sensitized against at least one of the two allergens. CD203c has previously been described as an activation marker on human basophils, which is up-regulated upon allergen-induced crosslinking of receptor-bound IgE (Hauswirth et al., 2002). Exposure of basophils isolated from blood samples of two grass pollen allergic blood donors to recombinant Phl p 2, rPhl p 6, and the P6-2 hybrid resulted in an up-regulation of CD203c, whereas the hP6-2 hybrid allergen failed to up-regulate CD203c up to a concentration of 0.2 μ g/ml (Figure 3).

Although the allergenic activity of the hP62 molecule was reduced, the T-cell epitopes of the wild-type Phl p 2 and Phl p 6 allergens have been preserved as shown by *in vitro* proliferation experiments with PBMCs isolated from two Phl p 2- and Phl p 6-allergic patients. The hP62 hybrid molecule and a mixture of Phl p 2 and Phl p 6 induced T-cell proliferation in PBMCs of both patients [stimulation indices (SIs) of patient 1 (5, 2.5, 1.25 μ g/ml): 2.3, 3.9, 2.1 Phl p 2+Phl p 6 mix; 1.3, 3.8, 2.1 hP62; patient 2: 1.2, 1.4, 1.7 Phl p 2+Phl p 6 mix; 1.6, 2.1, 2.4 hP62].

Table 1 Recognition of rPhl p 2, rPhl p 6, and the hybrid molecules P6-2 and hP6-2 by 50 grass pollen allergic patients' IgE.

Patient	rPhl p 2	rPhl p 6	P6-2	hP6-2
1	1.08	0.04	1.57	0.05
2	0.16	0.42	1.13	0.08
3	0.08	0.22	0.44	0.04
4	0.02	1.72	2.06	0.25
5	0.02	0.11	0.14	0.03
6	0.92	0.29	0.72	0.04
7	0.4	0.08	0.88	0.03
8	0.03	0.45	0.68	0.07
9	0.4	3.26	3.26	1.4
10	0.08	0.64	0.88	0.03
11	1.16	2.78	3.02	1.22
12	0.35	0.23	0.96	0.3
13	0.43	1.39	2.54	0.14
14	0.11	0.36	0.82	0.22
15	0.18	0.22	0.65	0.08
16	0.05	0.33	0.49	0.02
17	0.08	0.36	0.29	0.03
18	0.43	0.3	0.81	0.04
19	0.25	0.14	0.55	0.29
20	0.62	0.57	0.95	0.15
21	1.24	2.12	2.66	0.63
22	0.32	0.93	1.8	0.21
23	0.01	0.08	0.09	0.03
24	0.27	0.02	0.61	0.05
25	0.02	0.09	0.06	0.07
26	0.02	0.21	0.33	0.03
27	0.03	0.58	0.71	0.18
28	0.09	0.75	1.11	0.12
29	0.02	0.66	1.16	0.03
30	0.36	0.37	0.54	0.07
31	0.03	0.32	0.49	0.04
32	0.57	1.1	2.39	0.21
33	0.36	0.32	1.36	0.03
34	0.02	1.37	2.17	0.03
35	0.19	0.49	1.54	0.16
36	0.72	1.16	2.46	0.25
37	0.71	0.54	1.9	0.07
38	0.43	0.28	0.98	0.13
39	0.36	1.76	3.22	0.51
40	0.07	0.25	0.49	0.04
41	0.27	0.02	0.37	0.12
42	1.13	1.51	2.79	0.27
43	1.07	0.47	1.83	0.2
44	0.21	0.02	0.42	0.03
45	0.14	0.11	0.48	0.03
46	0.03	0.57	0.71	0.47
47	0.02	1.33	0.93	0.29
48	0.24	0.02	0.69	0.19
49	0.55	0.69	1.7	0.13
50	0.44	0.14	1.0	0.04
Mean	0.34	0.64	1.2	0.18

Optical density values corresponding to bound IgE antibodies are shown in the table.

Comparison of allergen-specific IgG levels in rabbit antisera induced by immunization with the hP6-2 hybrid, with those induced by immunization with Phl p 2- and Phl p 6-derived hypoallergenic components, or the recombinant Phl p 6 and Phl p 2 allergens

IgG antibodies specific for the hP6-2 hybrid molecule were generated by immunizing a rabbit. Rabbit anti-hP6-2 IgG antibodies were able to recognize the rPhl p 2 and rPhl p 6 wild-type allergens in ELISA experiments (Figure 4). We had previously observed that the fusion of allergen

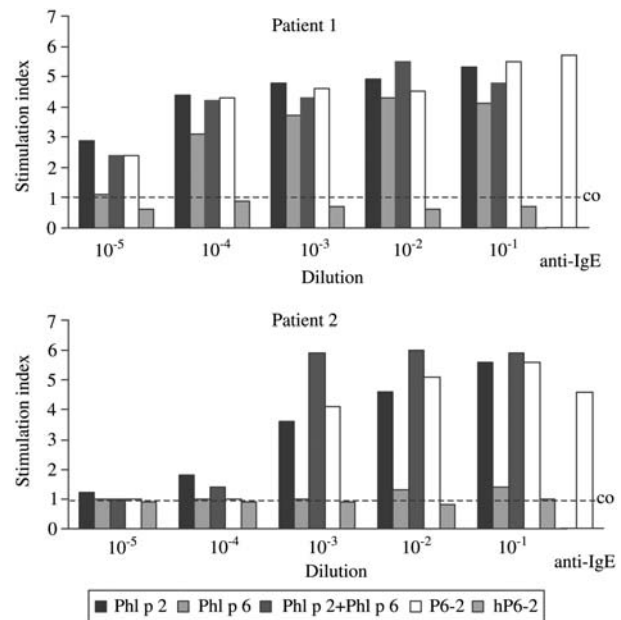


Figure 3 CD203c expression on blood basophils isolated from two grass pollen allergic patients after stimulation with rPhl p 2, rPhl p 6, a mixture and a fusion protein thereof (Phl p 2+Phl p 6: P6-2), and the hP6-2 hybrid. Heparinized blood samples were incubated with serial dilutions (10⁻¹ to 10⁻⁵) of the recombinant allergens or allergen derivatives, an anti-IgE antibody (x-axis) or PBS (negative control: co) and analyzed for up-regulation of CD203c by flow cytometry. The stimulation indices are displayed on the y-axis.

molecules as hybrid allergens could enhance the immune responses to the individual allergens (Linhart et al., 2002, 2005). We therefore compared the Phl p 2- and Phl p 6-specific antibody levels in sera of rabbits, which had been immunized with the wild-type allergens (rPhl p 2, rPhl p 6), the two hypoallergenic components [P2M and Phl p 6 (aa 31–110)] with those induced by immunization with hP6-2. Immunization with the hP6-2 hybrid induced higher levels of specific IgG antibodies than immunization with the hypoallergenic components alone. In particular, Phl p 2-specific antibody responses could be strongly increased (Figure 4).

Immunization with rPhl p 6 has recently been shown to induce higher levels of Phl p 6-specific antibodies than immunization with the Phl p 6-derived fragment (aa 31–110) (Vrtala et al., 2007). Using hP6-2 for immunization, we could obtain comparable levels of Phl p 6-specific IgG antibodies as with immunization using rPhl p 6 (Figure 4). Phl p 2 is known to be an antigen with low immunogenicity in animal models as well as in humans (Vrtala et al., 1998; Linhart et al., 2002, 2005; Mothes et al., 2003). In this context, it was interesting to see that immunization with the hP6-2 hybrid even lead to much higher Phl p 2-specific IgG antibody levels compared to immunization with the wild-type Phl p 2 allergen (Figure 4).

Immunization with the hybrid allergen induces a protective IgG response blocking allergic patients' IgE binding to the wild-type allergens

We investigated whether IgG antibodies induced with the hypoallergenic hP6-2 hybrid could inhibit grass pollen

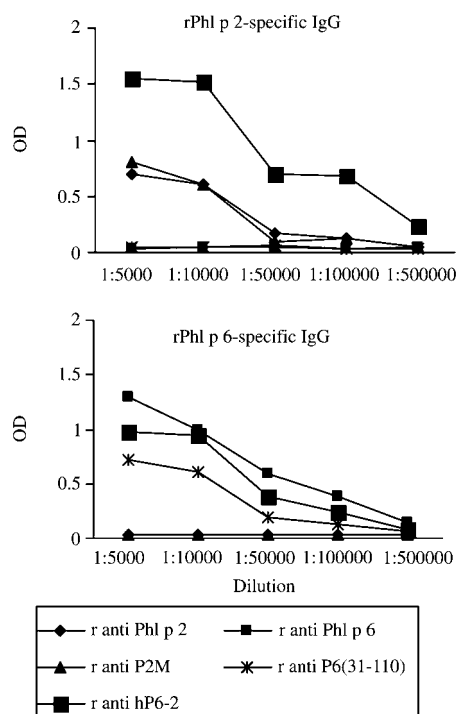


Figure 4 Titration of antisera.

Antisera raised by immunization of rabbits with rPhl p 2, rPhl p 6, the hP6-2 hybrid molecule or its components, the rPhl p 6 fragment [rP6(31–110)] and the Phl p 2-derived mosaic allergen (P2M) were tested for reactivity with rPhl p 2 and rPhl p 6, measured by ELISA. Optical density (OD) values corresponding to the amounts of bound antibodies are displayed for each serum dilution.

allergic patients' IgE binding to rPhl p 2 and rPhl p 6 by ELISA inhibition experiments. Preincubation of rPhl p 2 with rabbit anti-hP6-2 inhibited recognition of Phl p 2 by IgE antibodies from allergic patients ($n=13$) more efficiently than preincubation with rabbit anti-Phl p 2 antisera (mean inhibition with anti-Phl p 2: 41.6%; with anti-hP6-2: 85.2%) (Table 2). The ability of anti-Phl p 6 IgG antibodies to interfere with patients' IgE binding to rPhl p 6 was comparable to that of anti-hP6-2 IgG, leading to an average inhibition of 69.4% and 64.2%, respectively (Table 2).

Discussion

Here, we have demonstrated that the fusion of two hypoallergenic components made from two major grass pollen allergens, Phl p 2 and Phl p 6, results in a hypoallergenic hybrid protein, which may have several advantages over grass pollen extract- and recombinant allergen-based allergy vaccines. Allergen extracts, consisting of aqueous solutions of allergenic and non-allergenic material, do not meet the contemporary pharmaceutical standards in terms of composition, dosage, consistency, and purity (Valenta and Niederberger, 2007; Focke et al., 2008). In contrast, the recombinant hybrid protein can easily be produced in large amounts in *E. coli*, yielding a protein with defined molecular, biochemical, and immunological characteristics, which ensures a batch-to-batch consistency. In principle, the problems related to crude allergen extracts could also be overcome

Table 2 Percent inhibition of patients' IgE binding to rPhl p 2 and rPhl p 6 by Phl p 2-, Phl p 6-, and hP6-2 hybrid-specific IgG antibodies.

Patient	Phl p 2 coated		Phl p 6 coated	
	r anti-Phl p 2	r anti-hP6-2	r anti-Phl p 6	r anti-hP6-2
1	35	90	73	84
2	14	97	83	71
3	24	90	93	20
4	56	96	92	92
5	38	95	96	82
6	84	95	84	81
7	37	65	50	58
8	35	95	85	71
9	56	94	44	60
10	39	50	76	82
11	42	89	22	33
12	35	66	70	57
13	33	97	34	43
Mean	41.6	85.2	69.4	64.2

by the use of recombinant allergens for allergy vaccination. Vaccines consisting of recombinant wild-type allergens are based on pure molecules and can be tailored according to the patient's sensitization profile (Valenta et al., 1999). However, recombinant wild-type allergens, as well as their natural counterparts present in allergen extracts, represent biologically active materials and may lead to IgE-mediated side effects (urticaria, allergic asthma, anaphylactic shock). One possibility to reduce therapy-induced side effects mediated by allergen-specific IgE antibodies is the production of recombinant allergen derivatives that exhibit reduced allergenic activity by genetic engineering (Linhart and Valenta, 2005). Such hypoallergenic recombinant proteins derived from the major birch pollen allergen, Bet v 1, have already been administered to birch pollen allergic patients in a clinical trial (Niederberger et al., 2004). While birch pollen contains only one major allergen (i.e., Bet v 1) (Niederberger et al., 1998b), grass pollen represents a more complex allergen source consisting of a variety of different allergens (Andersson and Lidholm, 2003). For two major grass pollen allergens, Phl p 2 and Phl p 6, hypoallergenic derivatives have already been constructed, namely a Phl p 2-derived mosaic molecule and a Phl p 6-derived fragment comprising aa 31–110 (Vrtala et al., 2007; Mothes and Valenta, unpublished data). These molecules exhibited a strongly reduced IgE-binding capacity and allergenic activity and, in principle, could be used as compounds of a grass pollen vaccine. In this study, we aimed to improve the Phl p 2- and Phl p 6-based allergy vaccines. An increase of immunogenicity has recently been reported for wild-type allergens, which were expressed as hybrid molecules together with other allergens (Linhart et al., 2002, 2005; Linhart and Valenta, 2004). Here, we applied the hybrid approach to the Phl p 2- and Phl p 6-derived hypoallergenic constructs and obtained a hypoallergenic hybrid molecule. We could show that the hybrid retained the low IgE reactivity and lack of allergenic activity of its single components, while the hybrid induced stronger Phl p 2- and Phl p 6-specific IgG antibody responses upon vaccination. These IgG antibodies seemed to be of a protective nature, as they

could inhibit grass pollen allergic patients' IgE binding to the wild-type allergens Phl p 2 and Phl p 6.

The described approach may be particularly applicable to the development of a composite grass pollen vaccine for several reasons. First, grass pollens contain a number of proteins, which display allergenic activity. Up to now, 11 different groups of grass pollen allergens have been identified and characterized from one or more species (Andersson and Lidholm, 2003). An essential prerequisite for the construction of such a vaccine is the selection of the relevant grass pollen allergens, which should be included in the hybrid. The analysis of the frequencies of sensitizations to single allergens as well as IgE competition experiments in different populations indicated that a panel of recombinant timothy grass pollen allergens, Phl p 1, Phl p 2, Phl p 5, and Phl p 6, contained the majority of IgE epitopes present in grass pollens (Niederberger et al., 1998a; Linhart et al., 2005). A hybrid molecule consisting of these four allergens has been shown to be sufficient for the diagnosis of grass pollen allergy in a clinical study (Metz-Favre et al., 2007), and a mixture of these allergens was recently used in a clinical immunotherapy trial (Jutel et al., 2005). The selection of these allergens as essential components of a grass pollen vaccine was also confirmed by a skin prick test study, performed in grass pollen allergic patients, which evaluated *in vivo* the allergenic activity of the individual grass pollen allergens (Westritschnig and Valenta, unpublished data). Secondly, beside the Phl p 2- and Phl p 6-derived hypoallergenic variants, derivatives of the major grass pollen allergens Phl p 1 and Phl p 5 will also be available soon for the construction of a hypoallergenic hybrid vaccine. Thirdly, the use of allergen extracts for immunization results in heterogeneous allergen-specific antibody responses, due to differences in the concentration and/or immunogenicity among different allergen molecules present in the extract, which cannot be eliminated by the use of recombinant allergens or hypoallergenic derivatives thereof (Mothes et al., 2003). A hybrid vaccine consisting of derivatives of the major grass pollen allergens should be able to induce strong, allergen-specific antibody responses, and, when adsorbed to aluminum hydroxide, should be suitable for injection therapy. Encouraging first injection immunotherapy trials with recombinant wild-type allergens and allergen derivatives have indeed induced strong increases of allergen-specific serum antibodies in actively treated patients (Niederberger et al., 2004; Jutel et al., 2005; Klimek et al., 2005; Pauli et al., 2006, 2008; Purohit et al., 2008). Therapy-induced, allergen-specific IgG antibodies have been shown to antagonize immune and inflammatory responses caused by IgE recognition of allergens (Wachholz et al., 2003; Niederberger et al., 2004; Nouri-Aria et al., 2004; Reisinger et al., 2005; van Neerven et al., 2006; Pree et al., 2007). We expect that vaccination of grass pollen allergic patients with such a hybrid molecule will induce a strong allergen-specific IgG antibody response, which can block allergen-induced effector cell degranulation and IgE-mediated immediate inflammation. We also expect that these IgG antibodies will reduce proliferation and cytokine secretion in allergen-specific T-cells by interfering with IgE-facilitated allergen presentation, as has been observed for immunotherapy with natural allergens (Wachholz et al., 2003).

We believe that our approach is also applicable to other molecules and may lead to the preparation of hypoallergenic hybrid vaccines not only for the treatment of grass pollen allergy, but also for other allergen sources.

Materials and methods

Allergic patients, recombinant allergens

Grass pollen allergic patients were characterized by case history and skin-prick testing. Serum IgE antibodies specific for grass pollen extract, Phl p 2, and Phl p 6 were determined by CAP RAST (Phadia, Uppsala, Sweden), dot blot, and ELISA analysis as described previously (Niederberger et al., 1998a). Purified recombinant timothy grass pollen allergens Phl p 2 (rPhl p 2) (Dolecek et al., 1993) and Phl p 6 (rPhl p 6) (Vrtala et al., 1999) were obtained from BIOMAY (Vienna, Austria).

Construction, expression, and purification of the hP6-2 hybrid molecule

The cDNAs encoding the Phl p 2 mosaic allergen (P2M) (Mothes and Valenta, unpublished data) as shown in Figure 1, and the Phl p 6-derived fragment comprising aa 31–110 (Vrtala et al., 2007) served as templates for the PCR. In a first PCR reaction, primers p2/5' and p2/3' (Figure 1C) were used to amplify the Phl p 2 mosaic, and primers p6/5' and p6/3' (Figure 1C) for the amplification of Phl p 6 aa 31–110. The resulting overlapping PCR products together with primers p6/5' and p2/3' were used in a subsequent PCR reaction to generate the cDNA encoding the hybrid molecule, designated hP6-2. The PCR product was ligated into the expression vector pET17b (Novagen, Madison, WI, USA) via the NdeI restriction sites. The DNA sequence of the construct was confirmed by sequence analysis.

The hP6-2 hybrid molecule was expressed in *Escherichia coli* BL21(DE3) (Stratagene, East Kew, Australia) grown in LB-medium supplemented with 100 mg/l ampicillin at 37°C to an OD₆₀₀=0.7. Expression of the recombinant hP6-2 was induced by addition of 0.5 mM isopropyl-β-thiogalactopyranoside. Cells were harvested by centrifugation after overnight expression. Cells were lysed by several cycles of freezing and thawing. The hP6-2 protein was found in the insoluble inclusion body fraction. The pellet was washed 3× in 1% Triton, 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and another 2× in 50% EtOH, 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and dissolved in 8 M urea, 20 mM Tris pH 8.0, 1 mM EDTA, 2 mM β-mercaptoethanol. The sample was diluted to 6 M urea and loaded onto a DEAE Sepharose column (DEAE Sepharose Fast Flow, Pharmacia Biotech, Uppsala, Sweden). For the refolding of the protein, a 6 M–1 M urea gradient was applied for 90 min (1 ml/min). The hP6-2 protein was eluted in 400 mM NaCl, 1 M urea, 20 mM Tris, 1 mM EDTA, 2 mM β-mercaptoethanol, 20% glycerol, pH 8.0, and dialyzed against distilled water.

Purity of the recombinant protein was judged by Coomassie Brilliant Blue staining of a 14% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and the protein concentration was determined with a Micro BCA kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry and circular dichroism (CD) analysis of the purified hP6-2 protein

A laser desorption mass spectrum of the hP6-2 protein was acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos; piCHEM, Graz, Austria).

For the CD measurements, a mixture of Phl p 2 and Phl p 6 was prepared by mixing equimolar amounts of the single proteins to obtain a final protein concentration of 0.17 mg/ml. The hP6-2 hybrid was diluted to a final concentration of 0.1 mg/ml. Far UV CD spectra of the proteins were measured on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) 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 resulted from averaging 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 $[\theta]$ at a given wavelength.

Proliferation of PBMCs from allergic patients

Peripheral blood mononuclear cells (PBMCs) were isolated from two grass pollen allergic patients by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of heparinized venous blood samples. Phl p 2- and Phl p 6-specific IgE-antibodies of the allergic donors were determined by ELISA. Cells (2×10^6 cells/well) were cultured in triplicates in 96-well plates (Nunc-clone, Denmark) in serum-free Ultra Culture medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 50 μ M β -ME (Sigma), and 0.1 mg/ml gentamicin (Sigma) at 37°C in a humidified atmosphere containing 5% CO₂. Mononuclear cells were stimulated in triplicate with dilutions (5 μ g/ml, 2.5 μ g/ml, 1.25 μ g/ml) of hP62, or an equimolar Phl p 2 and Phl p 6 mixture, or with medium only. The proliferative responses were measured after 6 days by [³H]thymidine incorporation and are expressed as stimulation indices.

Immunization of rabbits

A rabbit was immunized on day 0 with 200 μ g hP6-2 using Complete Freund's Adjuvant, followed by two immunizations with 200 μ g of the immunogen in Incomplete Freund's Adjuvant on days 28 and 50 (Charles River Laboratories, Kisslegg, Germany). Antisera against rPhl p 2, rPhl p 6, and rPhl p 6(aa 31–110) were generated as described (Vrtala et al., 1999, 2007; Mothes and Valenta, unpublished data).

ELISA experiments

Allergen-specific IgE antibodies in sera from grass pollen allergic patients were determined by ELISA as previously described (Linhart et al., 2002).

The ability of hP6-2-induced rabbit IgG antibodies to inhibit the binding of allergic patients' IgE to rPhl p 2 and rPhl p 6 was examined by an ELISA competition assay. ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with rPhl p 2 or rPhl p 6 (1 μ g/ml) and preincubated with 1:100 dilutions of the anti-rPhl p 2, anti-rPhl p 6, or anti-rhP6-2 rabbit antisera, and for control purposes, with the corresponding preimmune sera. After washing, plates were incubated with 1:10 diluted sera from Phl p 2- and Phl p 6-sensitized grass pollen allergic patients. Bound IgE antibodies were detected with horseradish peroxidase (HRP)-coupled goat anti-human IgE antibodies (KPL, Gaithersburg, MD, USA), diluted 1:2500. The percentage of inhibition of IgE binding achieved by preincubation with the rabbit antisera was calculated as follows: percentage of inhibition of IgE binding = $100 - OD_i / OD_p \times 100$. OD_i and OD_p represent the extinctions after preincubation with the rabbits' immune sera and the corresponding preimmune sera, respectively. For the titration of rabbit antisera, ELISA-plate bound rPhl p 2 and rPhl p 6 (5 μ g/ml) was incubated with dilutions (1:5000–1:500 000) of rabbit antisera. Allergen-specific IgG antibodies were detected using a HRP-labeled donkey anti-rabbit IgG antiserum (Amersham, Buckinghamshire, UK).

Analysis of CD203c expression on basophils

Heparinized peripheral blood samples were obtained from two grass pollen allergic patients after informed consent was obtained. Blood samples (100 μ l) were incubated with serial dilutions of recombinant allergens or allergen derivatives (rPhl p 2, rPhl p 6: 10; 1; 0.1; 0.01; 0.001 μ g/ml; P6-2 and hP6-2: 20; 2; 0.2; 0.02; 0.002 μ g/ml, anti-IgE antibody 1 μ g/ml), or phosphate buffered saline (PBS) for 15 min (37°C). Cells were washed in PBS containing 20 mM EDTA, and incubated with 10 μ l of PE-conjugated CD203c mAb 97A6 (Immunotech, Marseille, France) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with 2 ml FACS™ Lysing Solution (Becton Dickinson, San Jose, CA, USA). Cells were washed, resuspended in PBS, and analyzed by two-color flow cytometry on a FACScan (Becton Dickinson). Allergen-induced up-regulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells, and expressed as stimulation index (MFI_{stim}:MFI_{control}) (Hauswirth et al., 2002).

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