

A Combination Vaccine for Allergy and Rhinovirus Infections Based on Rhinovirus-Derived Surface Protein VP1 and a Nonallergenic Peptide of the Major Timothy Grass Pollen Allergen Phl p 1¹

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Allergens and rhinovirus infections are among the most common elicitors of respiratory diseases. We report the construction of a recombinant combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived VP1, the surface protein which is critically involved in infection of respiratory cells, and a nonallergenic peptide of the major grass pollen allergen Phl p 1. Recombinant hybrid molecules consisting of VP1 and a Phl p 1-derived peptide of 31 aa were expressed in *Escherichia coli*. The hybrid molecules did not react with IgE Abs from grass pollen allergic patients and lacked allergenic activity when exposed to basophils from allergic patients. Upon immunization of mice and rabbits, the hybrids did not sensitize against Phl p 1 but induced protective IgG Abs that cross-reacted with group 1 allergens from different grass species and blocked allergic patients' IgE reactivity to Phl p 1 as well as Phl p 1-induced basophil degranulation. Moreover, hybrid-induced IgG Abs inhibited rhinovirus infection of cultured human epithelial cells. The principle of fusing nonallergenic allergen-derived peptides onto viral carrier proteins may be used for the engineering of safe allergy vaccines which also protect against viral infections. *The Journal of Immunology*, 2009, 182: 6298–6306.

According to the World Health Organization, asthma belongs to one of the most severe and disabling diseases (1). Allergens and respiratory viruses are among the most common environmental factors implicated in the pathogenesis of asthma (2). More than 25% of the population suffers from IgE-mediated allergies and ~30% of patients suffering from persistent allergic rhinitis also suffer from asthma (3). The link between upper and lower airway diseases is also underlined by the fact that patients suffering from untreated allergic rhinoconjunctivitis frequently develop asthma bronchiale (4). In this context, it has been shown that allergen exposure via the nasal and respiratory mucosa induces strong rises of allergen-specific IgE levels, which are responsible for increased allergen sensitivity in the target organs of allergy (5). In addition, several studies highlight the importance of rhinovirus infections in the context of allergic asthma (6, 7).

Human rhinoviruses (HRV)³ have been identified in the late 1950s and early 1960s as the cause of the common cold in the upper respiratory tract. With the use of PCR-based technology, HRV have been identified in 60–90% of acute exacerbations of asthma in children and adults (6). Rhinoviruses are not only a major cause of asthma exacerbations (8) but allergic individuals suffer also more often and prolonged from rhinovirus infections in the lower respiratory tract (9). Furthermore, there is evidence for a deficient innate immune response (i.e., a deficient-type III IFN- λ production) to rhinovirus infections in asthmatic individuals (10).

To date, no effective vaccines or antiviral therapies have been approved for either the prevention or the treatment of HRV infection. However, for allergic diseases, allergen-specific immunotherapy is available as an allergen-specific and disease-modifying form of allergy treatment (11). It is effective for the treatment of allergic asthma and prevents the progression of allergic rhinoconjunctivitis to allergic asthma (12). Due to the rapid progress made in the field of allergen characterization, several new forms of allergen-specific immunotherapy have been developed and entered in clinical trials in allergic patients (reviewed in Refs. 11, 13, and 14).

According to the sequences of major allergens, synthetic peptides containing T cell epitopes without IgE reactivity have been identified with the aim to induce tolerance in allergen-specific T

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³ Abbreviations used in this paper: HRV, human rhinovirus; KLH, keyhole limpet hemocyanin; HSA, human serum albumin; MFI, mean fluorescence intensity; SI, stimulation index; TCID₅₀, 50% tissue culture infectious dose; RBL, rat basophil leukemia cell.

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cells (15). DNA-based vaccination has been shown to redirect allergic immune responses in experimental animal studies and CpG-coupled allergens have been used to modulate the immune responses and were used for immunotherapy of ragweed allergic patients (16–18). Furthermore, recombinant allergens and genetically engineered allergen derivatives with reduced allergenic activity have been used for allergy vaccination of allergic patients (reviewed in Ref. 19).

In this study, we report the development of a novel type of vaccine for the combined treatment of allergen- and rhinovirus-induced asthma. The active ingredient of this vaccine is a fusion protein consisting of the rhinovirus-derived VP1 surface protein (20, 21) and a hypoallergenic peptide selected from the major grass pollen allergen Phl p 1 (22). VP1 contains the motifs required for HRV binding to the target cells and is recognized by HRV-neutralizing Abs (20, 21). The Phl p 1 peptide is located at the Phl p 1 C terminus which contains the majority of IgE epitopes recognized by patients' IgE but lacks allergenic activity (22, 23). The fusion protein was thus expected to induce IgG Abs which block allergic patients' IgE recognition of the Phl p 1 allergen and to inhibit HRV infection.

In this study, we report the construction, expression, and purification of the VP1 fusion protein. We demonstrate the lack of allergenic activity of the fusion protein using allergic patients' IgE and their blood basophils. Furthermore, we show that IgG Abs obtained after immunization of animals with the fusion protein inhibit allergic patients' IgE binding to the Phl p 1 allergen, allergen-induced basophil degranulation, and protect against HRV infection of cultured human cells.

Materials and Methods

Allergic patients, allergen extracts, recombinant allergens, synthetic peptides, and peptide conjugates

Sera were obtained from patients allergic to grass pollen as confirmed by case history, skin prick testing, and measurement of specific IgE Abs and are numbered consistently in the manuscript (22). Pollen from different grass and corn species (*Phleum pratense*: Timothy grass; *Secale cereale*: rye; *Poa pratensis*: Kentucky bluegrass; *Phragmites australis*: Australian reed; *Triticum sativum*: cultivated wheat; *Lolium perenne*: rye grass; *Avena sativa*: cultivated oat; *Anthoxanthum*: sweet vernal grass) were purchased from Allergon. Natural grass pollen extracts were prepared as described previously (24). Purified recombinant rPhl p 1 was obtained from Biomay.

The Phl p 1-derived peptide P5 CVRYTTEGGTKTEAEDVIPEG WKADTAYESK was synthesized and coupled to keyhole limpet hemocyanin (KLH) as described previously (22).

Construction of vector pVP1

Virus stocks of strains HRV89 and 14 were obtained from the collection at the Institute of Virology, Medical University of Vienna. Viral RNA was prepared from cell culture supernatants using the QIAamp viral RNA kit (Qiagen) and RNase inhibitor (Boehringer Mannheim) was added to a final concentration of 0.01 U/ μ l. The VP1 cDNA was amplified by RT-PCR using a SuperScript One-Step RT-PCR kit from Invitrogen using the following primers: 5'-CGGAATTCATTAAATATGAACCCAGTTGAAAATATATAGATAGTGTTATTA-3' and 5'-CGATTAATTCAGTGGTGGTG GTGGTGGTGGACGTTTGTAAACGGTAA-3'. The restriction sites (*Eco*RI, *Ase*I) are underlined. The VP1 cDNA was subcloned into the *Nde*I and *Eco*RI sites of the plasmid pET 17b and transformed into *Escherichia coli* BL21 DE3 from Novagen (Merck Bioscience) for protein expression. The DNA sequence of the construct was confirmed by nucleotide sequencing (MWG) (25).

To allow the insertion of cDNAs coding for unrelated peptides at the 5' end of the VP1-encoding cDNA, the plasmid construct was modified by changing the CATAAT site which resulted from the subcloning of the *Ase*I ends of the cDNA into the *Nde*I site of the vector into an *Afl*III site (CTT AAG) by site-directed mutagenesis using a Quick Change Site Mutagenesis kit (Stratagene) and the primers *Afl*III forward, 5'-CTTTAAGAAGGA GATATACTTAAGATGAACCCAGTTG-3' and *Afl*III reverse, 5'-CAA CTGGGTTTCATCTTAAGTATATCTCTCTTAAAG-3'. Next, the primers *Age*I forward, 5'-CCTGATGTTTTACCGGTACAAACGTCC

ACCAC-3' and *Age*I reverse, 5'-GTGGTGGACGTTTGTACCGGTAAA AACATCAGG-3' were used to introduce an *Age*I site before the last three codons of the VP1-encoding cDNA to allow the insertion of cDNAs coding for peptides at the 3' end. The resulting plasmid was designated pVP1 (Fig. 1a and supplemental Fig. 1⁴).

Construction of plasmids expressing a VP1-P5 and a VP1-2xP5 hybrid protein

A recombinant hybrid protein (VP1-P5) consisting of VP1 and the Phl p 1-derived peptide P5 fused to the VP1 N terminus was obtained by PCR amplification of a P5-encoding cDNA using forward 5'-CGCGCTTAAG ATGGTCCGCTACACCACCGAGGGC-3' and reverse 5'-CGCG CTTAAGCTTGGACTCGTAGGCGGTGTCGGC-3' primers and the Phl p 1-encoding cDNA as a template.

The P5-encoding cDNA was inserted into the *Afl*III restriction site of the pVP1 vector. The resulting construct was further modified by insertion of a P5-encoding cDNA into the *Age*I site at the 3' end of the VP1-encoding cDNA to yield a hybrid consisting of VP1 with a N-terminal and a C-terminal P5 peptide designated VP1-2xP5.

Expression and purification of VP1, VP1-P5, and VP1-2xP5

Recombinant VP1, VP1-P5, and VP1-2xP5 were expressed in *E. coli* BL21(DE3) and purified from the inclusion body fraction after solubilization in 6 M guanidinium hydrochloride, 100 mM NaH₂PO₄, and 10 mM Tris (pH 8) using a Ni-NTA affinity matrix (Qiagen). Elution was performed at pH 3.5. Protein preparations were dialyzed against H₂O_{dd} and checked for purity by SDS-PAGE and Coomassie blue staining. Membranes containing purified VP1, VP1-P5, and VP1-2xP5 were exposed to a monoclonal mouse anti-His tag Ab (Dianova). Bound Abs were detected with alkaline phosphatase-coupled rabbit anti-mouse Abs (BD Pharmingen).

Allergic patients' IgE reactivity to the fusion proteins and basophil activation

Allergic patients' IgE reactivity to Phl p 1, VP1-P5, VP1-2xP5, or human serum albumin (HSA) was measured by ELISA as described elsewhere (26). Sera from three nonallergic individuals were tested as negative controls. The coating of the test Ags to the ELISA plates was confirmed with specific Ab probes. The allergenic activity of the VP1 fusion proteins was compared with that of the Phl p 1 allergen by in vitro basophil activation tests. For this purpose, peripheral blood was obtained from three grass pollen allergic patients after informed consent was obtained. Basophil activation in heparinized whole blood samples and flow cytometric measurement of CD203c expression was performed as previously described (27). In brief, blood aliquots (100 μ l) were incubated with serial dilutions of rPhl p 1, VP1-2xP5 (0.05–50 pM), anti-IgE Ab (1 μ g/ml, E-124-2-8 D ϵ ; Immunotech), or buffer alone (PBS). Allergen-induced up-regulation of CD203c as determined by flow cytometry with PE-conjugated mAb 97A6 (CD203c) was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells and was expressed as stimulation index (SI = MFI_{stim}:MFI_{control}) (27).

Immunization of mice and rabbits, reactivity of mouse and rabbit Abs with VP1 and Phl p 1, and demonstration of reagenic activity of mouse Abs

Groups of five mice each were immunized three times s.c. with 5 μ g of P5, rPhl p 1, VP1, VP1-P5, or VP1-2xP5 adsorbed to aluminum hydroxide in 3-wk intervals and bled from the tail veins. Animals were maintained in the animal care unit of the Department of Pathophysiology (Medical University of Vienna) according to the local guidelines for animal care (28). The mouse immunization experiments were repeated three times. Rabbit Abs specific for VP1, KLH-P5, VP1-P5, and VP1-2xP5 were obtained by immunizing rabbits (Charles River). ELISA plates (Nunc Maxisorb) were coated with 5 μ g/ml of the Ags and incubated with mouse sera diluted 1/500 as previously described (28, 29). Bound mouse IgG1, IgG2a, or IgG2b were detected with monoclonal rat anti-mouse IgG1, IgG2a, or IgG2b Abs (BD Pharmingen) diluted 1/1000, respectively, and then with goat anti-rat IgG HRP-coupled Abs (Amersham Biosciences) diluted 1/2000. Bound rabbit IgG Abs were detected with 1/2000 diluted donkey anti-rabbit IgG HRP-coupled Abs (Amersham Biosciences). OD was measured at 405 and 490 nm in an ELISA reader (Dynatech).

The induction of Phl p 1-specific IgE Abs with allergenic activity was determined in sera from mice that had been immunized with Phl p 1, VP1-P5,

⁴ The online version of this article contains supplemental material.

VP1-2xP5, or VP1 using RBL-2H3 cells. Rat basophil leukemia cells (RBLs) were loaded with mouse sera and timothy grass pollen extract containing natural Phl p 1 allergen ($\sim 5 \mu\text{g/ml}$) or VP1 as described elsewhere (30).

Proliferation of mouse spleen cells and cytokine analysis

Spleen cells were prepared from mice that had been immunized four times with P5, VP1, VP1-P5, KLH-P5, or PBS 10 days after the last immunization. Spleen cell cultures from each mouse were stimulated with P5 ($0.26 \mu\text{g}/100 \mu\text{l}$), VP1 ($2 \mu\text{g}/100 \mu\text{l}$), Con A ($2 \mu\text{g}/100 \mu\text{l}$) (positive control), or medium alone and after 4 days [^3H]thymidine uptake was measured and displayed as SI as described previously (30). Cytokines were measured after stimulation of spleen cells with Phl p 1, KLH, VP1, or Con A ($15 \mu\text{g}/50 \mu\text{l}$ each). IL-4, IL-5, IL-10, TGF- β , and IFN- γ levels were measured by xMAP Luminex fluorescent bead-based technology. The assay was performed according to the manufacturer's introduction (R&D Systems) and fluorescence signal was read on a Luminex 100 System (Luminex). Cytokine measurements were done for splenocytes from each mouse for a particular cytokine. From these values, the assay cutoff level for the given cytokine was subtracted and this value was normalized to the proliferation results (SI) according to the formula: (mean cytokine level - cytokine cutoff)/SI = factor. The mean values of the factors were calculated for each group of mice and each cytokine to allow a comparison among the groups.

Statistics

Differences in Ab and cytokine levels induced by immunization of mice were determined by the Mann-Whitney *U* test using SPSS software. A value of $p < 0.05$ was considered as significant.

Cross-reactivity of anti-VP1-2xP5 Abs with natural group 1 grass pollen allergens

Grass pollen extracts from eight grass species were separated on a 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane. Identically prepared membranes were incubated with rabbit anti-VP1-2xP5 Abs or the corresponding preimmune Ig overnight at 4°C and bound IgG Abs were detected with ^{125}I -labeled donkey anti-rabbit IgG as previously described (22).

Inhibition of allergic patients' IgE binding to Phl p 1 and of Phl p 1-induced basophil degranulation with vaccine-induced IgG Abs

The inhibition of allergic patients' IgE reactivity to Phl p 1 by IgG Abs which had been raised by immunization of rabbits with the rPhl p 1 allergen, VP1-P5, VP1-2xP5, and a KLH-coupled peptide P5 (22) was measured by ELISA as described elsewhere (28, 29). In brief, ELISA plates were coated with $1 \mu\text{g/ml}$ rPhl p 1, washed, and blocked. Then plate-bound Phl p 1 was preincubated with 1/250 dilutions of specific rabbit Ig or, for control purposes, of the preimmune Ig. After washing, plates were incubated with 1/3 diluted sera from allergic patients with a RAST class of >3 to timothy grass pollen allergens and bound IgE Abs were detected with 1/1000 diluted alkaline phosphatase-coupled mouse monoclonal anti-human IgE Abs (BD Pharmingen). The OD corresponding to bound IgE was measured at 405 and 450 nm. The percentage of inhibition of IgE binding achieved by preincubation with the anti-peptide antisera was calculated as previously described (28).

The inhibition of Phl p 1-induced basophil degranulation by vaccine-induced IgG Abs was investigated as follows: RBL-2H3 cells transfected with human high-affinity IgE receptor (Fc ϵ R1) (31) were loaded with serum IgE from Phl p 1-allergic patients and then exposed to 100 ng/ml Phl p 1 that had been preincubated with increasing concentrations (0, 2.5, 5, or 10% v/v) of rabbit Ig raised against Phl p 1 or VP1-2xP5 or the corresponding preimmune Ig. The release of β -hexosaminidase was measured as described previously (31) and is expressed as percentage of total cellular β -hexosaminidase.

HRV neutralization test

The virus stock titer used in the experiment was determined by 50% tissue culture infectious dose (TCID_{50}) titration on HeLa cells according to the Spearman-Kärber method (32).

For the neutralization tests, $300\text{-}\mu\text{l}$ aliquots containing a 100 TCID_{50} of HRV89 or 14 were preincubated for 2 h at 37°C with $300\text{-}\mu\text{l}$ aliquots (undiluted, 1/2–1/32) of the anti-VP1-P5 or anti-VP1-2xP5 antiserum before addition to HeLa cells. Infection of HeLa cells with 100 TCID_{50} of HRV89 or 14 was performed for control purposes. For control purposes, cells were incubated with medium alone or immune serum without virus. The cytotoxic effect of the virus was visualized with crystal violet after 3 days (33).

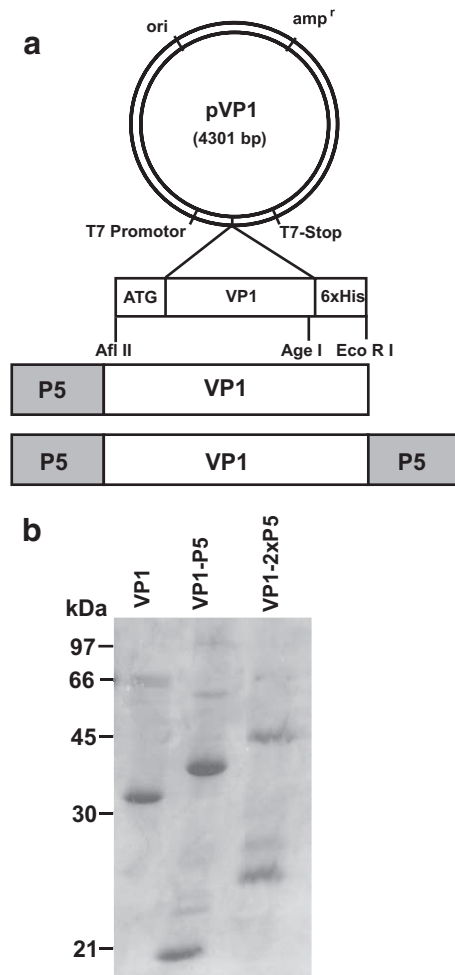


FIGURE 1. Representation of pVP1, expression and purification of VP1, VP1-P5, and VP1-2xP5. *a*, Construction of an expression plasmid (pVP1) containing the cDNA coding for VP1. The VP1-encoding cDNA was inserted into the multiple cloning site of plasmid pET17b and an *Afl*III and *Age*I restriction sites were introduced at the 5' and 3' end of the VP1-encoding cDNA, respectively. *b*, SDS-PAGE containing purified VP1, VP1-P5, and VP1-2xP5. Molecular masses in kDa are indicated.

Results

Expression and purification of VP1 and of a fusion protein consisting of VP1 and one or two grass pollen allergen peptides

Plasmid pVP1 (Fig. 1*a*) was constructed by replacing the *Nde*I/*Eco*RI fragment of the multiple cloning site of pET-17b with the cDNA sequence encoding the complete VP1 protein of human rhinovirus strain 89. The *Nde*I and *Eco*RI restriction sites (of pET-17b) were used for insertion of the VP1 cDNA containing *Ase*I and *Eco*RI sites at the 5' and 3' end, respectively. An *Afl*III (5' end) and an *Age*I (3' end) restriction site were generated at the ends of the VP1-encoding cDNA by mutagenesis to allow fusion with foreign cDNAs. The *Age*I restriction site was placed between the 3' end of the VP1 cDNA and a cDNA coding for a C-terminal hexahistidine tag (Fig. 1*a* and supplemental Fig. 1). Plasmid VP1 thus allows the expression of complete VP1 with a C-terminal hexahistidine tag. In addition, allergen-derived peptides can be fused to the N and/or C terminus of VP1.

Recombinant VP1 was expressed in *E. coli* with a C-terminal hexahistidine tag and purified by nickel affinity chromatography in a single-step procedure yielding $\sim 5 \text{ mg}$ of protein/L of culture. Recombinant VP1 migrates at $\sim 34 \text{ kDa}$ in SDS-PAGE (Fig. 1*b*). Next, we expressed and purified two fusion proteins consisting of

Table I. IgE reactivity to HSA, Phl p 1, VP1-P5, and VP1-2xP5^a

Patient	IgE Reactivity of Allergic Patients' Sera			
	HSA	Phl p 1	VP1-P5	VP1-2xP5
1	0.056	0.235	0.077	0.087
2	0.046	0.586	0.061	0.066
3	0.049	0.9	0.059	0.057
4	0.07	1.031	0.061	0.059
5	0.016	0.889	n.d.	0.178
6	0.044	0.308	0.044	0.051
7	0.054	0.605	0.05	0.046
8	0.047	0.476	0.052	0.055
9	0.045	0.151	0.045	0.045
10	0.061	0.288	0.048	0.047
11	0.048	1.879	0.059	0.061
12	0.059	1.638	n.d.	0.092
13	0.051	2.098	0.048	0.046
14	0.064	0.166	0.044	0.046
15	0.05	0.697	0.059	0.048
16	0.058	1.864	0.079	0.069
17	0.053	1.39	0.053	0.048
18	0.046	1.062	0.045	0.045
19	0.045	0.845	0.059	0.056
20	0.008	0.4	n.d.	0.099
21	0.048	0.397	0.058	0.057
22	0.045	0.24	n.d.	0.02
23	0.051	1.117	0.056	0.058
Mean	0.048	0.837	0.056	0.062
24	0.048	0.052	0.051	0.047
25	0.042	0.043	0.05	0.044
26	0.043	0.048	0.048	0.044

^a Serum IgE reactivity to the purified Ags was determined by ELISA and is displayed as mean OD values of duplicate measurements for sera from 23 grass pollen allergic patients. Mean OD values for the 23 sera and each antigen are indicated in bold.

VP1 and the nonallergenic peptide (P5) derived from the major Timothy grass pollen allergen Phl p 1. In the fusion protein VP1-P5, the peptide is fused to the N terminus of VP1 and in the fusion protein VP1-2xP5 one peptide is attached to the VP1 N and one to the C terminus. Recombinant VP1-P5 and VP1-2xP5 exhibited a molecular mass of ~37 and 41 kDa, respectively, in SDS-PAGE (Fig. 1b). Bands below 30 kDa in the recombinant protein preparations reacted with an anti-His tag Ab and therefore represent degradation products of the purified recombinant proteins.

VP1-P5 and VP1-2xP5 are nonallergenic fusion proteins

Recombinant VP1-P5 and VP1-2xP5 were compared with the complete recombinant Phl p 1 allergen regarding IgE reactivity using sera from 23 grass pollen allergic patients by ELISA (Table I). Each of the grass pollen allergic patients showed IgE reactivity to rPhl p 1 (mean OD, 0.837). However, no relevant IgE reactivity to VP1-P5 or VP1-2xP5 was found. Likewise, no relevant IgE

binding was observed when three nonallergic individuals were tested for IgE reactivity to the fusion proteins (Table I). No IgE reactivity to HSA (mean OD, 0.048) could be detected (Table I).

The lack of allergenic activity of VP1-2xP5 was confirmed by experiments using basophils from grass pollen allergic patients (Fig. 2). rPhl p 1 and anti-IgE induced the up-regulation of CD203c on basophils from each of the three grass pollen allergic patients at concentrations of 0.05 pM, whereas VP1-2xP5 did not induce any response up to a concentration of 50 pM (Fig. 2).

VP1-P5 and VP1-2xP5 induce a VP1- and grass pollen-specific Th1-like immune response with lowered Th2 activation

To determine the immunogenicity of VP1 and its ability to act as a carrier for allergen-derived peptides, groups of mice were immunized with the following Ags: P5, rPhl p 1, VP1, VP1-P5, and VP1-2xP5. VP1- and Phl p 1-specific IgG1 Ab levels were determined by ELISA (Fig. 3). VP1-specific IgG1 Abs were detected already 3 wk after immunization in mice that had received VP1 or VP1- containing allergen-derived peptides, but not in mice that had been immunized with P5 or Phl p 1 (Fig. 3a).

The fusion of the Phl p 1-derived peptide (P5) to VP1 strongly increased the immunogenicity of P5 because no relevant Phl p 1-specific IgG1 responses were found in mice immunized with P5 alone, whereas VP1-P5 and VP1-2xP5-immunized mice showed Phl p 1-specific IgG1 responses after 3 wk, which continued to increase after 6 and 9 wk (Fig. 3b). The Phl p 1-specific IgG1 responses in the latter two groups of mice were even of comparable magnitude to the IgG1 responses induced by immunization with the complete Phl p 1 allergen. Immunization with VP1 alone did not induce any Phl p 1-specific immune response (Fig. 3b).

VP1-P5 and VP1-2xP5 induced IgG2a and IgG2b responses to Phl p 1. No Phl p 1-specific IgG2 responses were found in P5- and VP1-immunized mice (Fig. 3b). Interestingly, VP1-specific IgG2a and IgG2b responses were significantly stronger ($p < 0.05$) in the VP1-immunized mice as compared with the mice having received VP1-P5 or VP1-2xP5, indicating that VP1 contributes to the Th1 component of the immune responses whereas the allergen-derived peptides seemed to reduce this activity (Fig. 3a). To determine the specificity of T cell responses, spleen cells from immunized mice were exposed to P5, VP1, or to culture medium alone (Fig. 4, x-axes). Spleen cells from VP1-immunized mice showed proliferation (SI > 6) to VP1 but no relevant proliferation to P5 or to medium alone. Spleen cells from VP1-P5-immunized mice that had developed IgG responses against Phl p 1 did not show relevant proliferation to P5 but responded strongly (SI > 6) to VP1, indicating that the Phl p 1-specific IgG responses had received VP1-specific T cell help (Fig. 4). P5-immunized mice did not show any relevant proliferation similar to cells from a nonimmunized mouse (Fig. 4). Spleen cells from VP1-P5-immunized mice did not release relevant levels of IL-5, whereas a stronger secretion of IL-5 was found in culture supernatants from

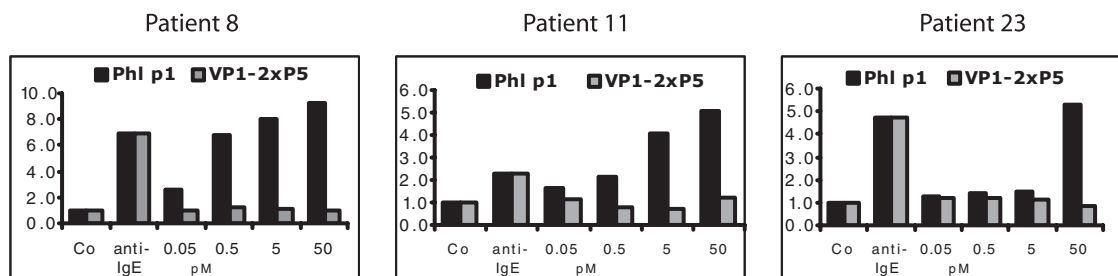


FIGURE 2. Activation of human basophils. Blood of three grass pollen allergic patients (patients 8, 11, and 23) were stimulated with PBS (Co), anti-IgE, serial dilutions (0.05, 0.5, 5, and 50 pM) of Phl p 1 (■) or VP1-2xP5 (▒) (x-axes). CD203c expression is displayed as SI (y-axes).

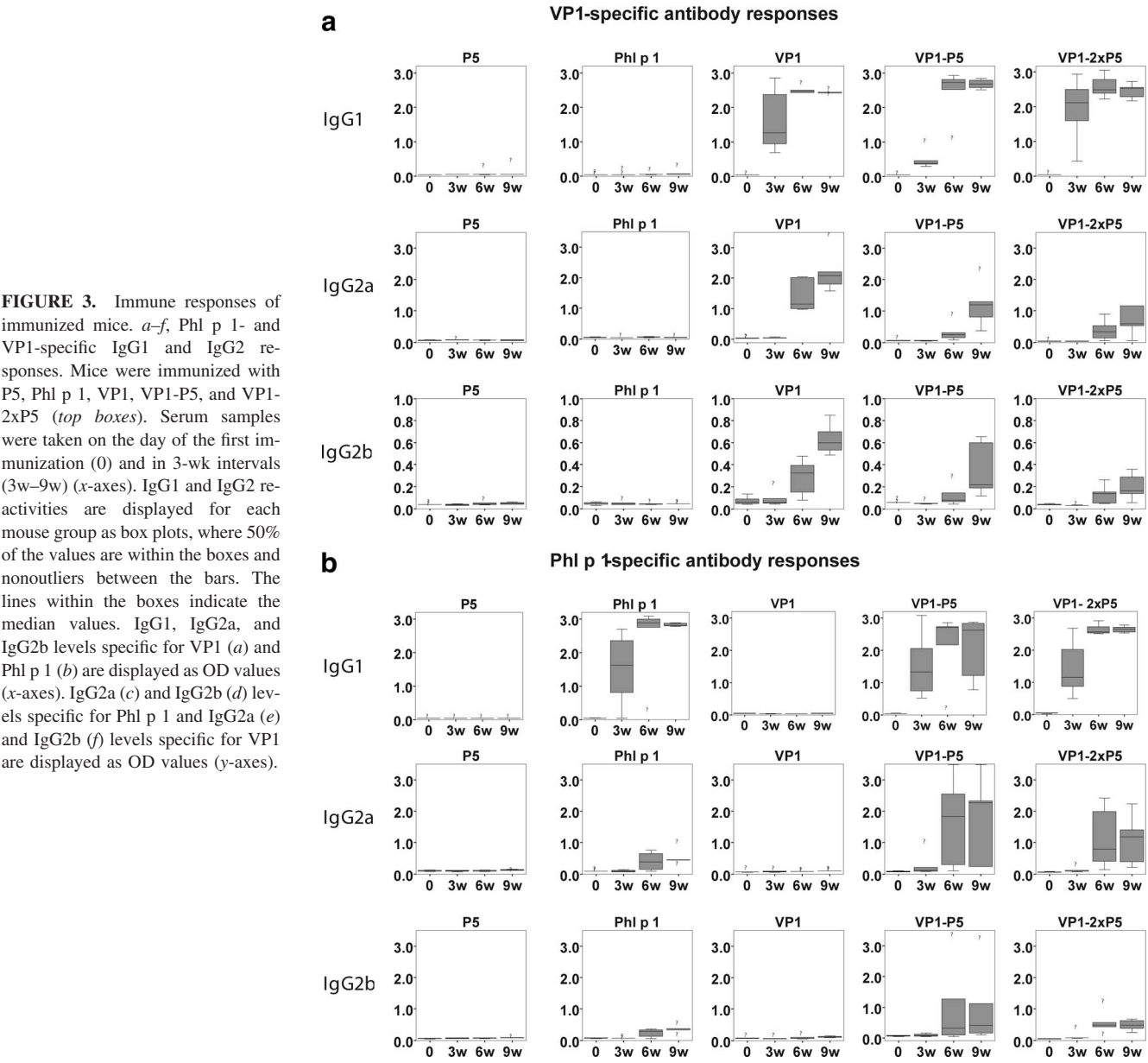


FIGURE 3. Immune responses of immunized mice. *a–f*, Phl p 1- and VP1-specific IgG1 and IgG2 responses. Mice were immunized with P5, Phl p 1, VP1, VP1-P5, and VP1-2xP5 (*top boxes*). Serum samples were taken on the day of the first immunization (0) and in 3-wk intervals (3w–9w) (*x-axes*). IgG1 and IgG2 reactivities are displayed for each mouse group as box plots, where 50% of the values are within the boxes and nonoutliers between the bars. The lines within the boxes indicate the median values. IgG1, IgG2a, and IgG2b levels specific for VP1 (*a*) and Phl p 1 (*b*) are displayed as OD values (*x-axes*). IgG2a (*c*) and IgG2b (*d*) levels specific for Phl p 1 and IgG2a (*e*) and IgG2b (*f*) levels specific for VP1 are displayed as OD values (*y-axes*).

spleen cells of mice that had been immunized with KLH-P5 (Fig. 4). No relevant differences regarding the production of IL-10, IL-4, and TGF- β were found between the different groups (Fig. 4).

VP1-2xP5 does not induce an allergenic immune response against Phl p 1 or against VP1

To study whether immunization with the various Ags (Phl p 1, VP1, VP1-P5, VP1-2xP5) induces reagenic IgE Abs, RBLs were

loaded with sera from immunized mice and exposed to pollen-derived Phl p 1 or VP1 (Fig. 5). Immunization with Phl p 1 but not with VP1 or the VP1-P5 constructs induced reagenic Abs which lead to degranulation of RBLs in response to timothy grass pollen-derived Phl p 1 (Fig. 5, Timothy grass pollen extract). Interestingly, neither immunization with VP1, VP1-P5, nor VP1-2xP5 induced IgE Abs which caused degranulation of RBLs in responses to the VP1 protein (Fig. 5, VP1).

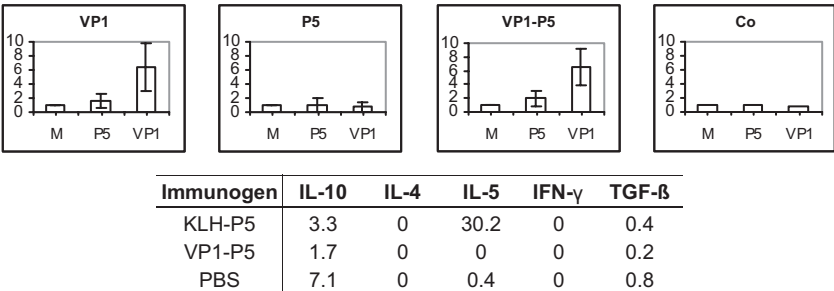


FIGURE 4. T cell responses of immunized mice. The lymphoproliferative responses (*y-axes*: SI $\pm$ SD) of immunized mice (*top of the boxes*: immunogens) and a nonimmunized (Co) mouse stimulated with medium alone (M), P5, or VP1 (*x-axes*) are displayed. The table shows the levels of IL-10, IL-4, IL-5, IFN- γ , and TGF- β in Phl p 1-stimulated spleen cell cultures after normalization to the proliferations results (i.e., cytokine level/SI) in groups of mice immunized with different immunogens (KLH-P5, VP1-P5) or buffer (PBS).

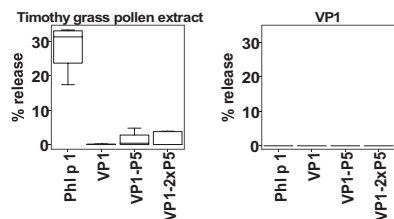


FIGURE 5. Reduced allergenicity of VP1-P5 and VP1-2xP5 vs Phl p 1. RBLs were loaded with sera from immunized mice (Immunogens: *bottom* of the boxes) and incubated either with natural Phl p 1 (Timothy grass pollen extract) or VP1 (*top* of the boxes). The β -hexosaminidase releases are displayed as percentages of total releases for each mouse group as box plots, where 50% of the values are within the boxes and nonoutliers between the bars. The lines within the boxes indicate the median values.

Anti-VP1-2xP5 Abs cross-react with natural group 1 pollen allergens from several grass species

To study whether IgG Abs induced with the hybrid proteins cross-react with natural group 1 allergens from several grass species, immunoblot experiments were performed. Fig. 6a shows that rabbit anti-VP1-2xP5 Abs strongly bound to natural Phl p 1 in *P. pratense* pollen and to natural group 1 allergens in pollen from several other grass species including *P. pratensis*, *L. perenne*, and *Anthoxanthum odoratum*. A distinct but weaker reactivity to group 1 allergens from *P. australis*, *A. sativa*, *T. aestivum*, and *S. cereale* were found (Fig. 6a). No IgG reactivity to the pollen allergens was found when an identically prepared blot was exposed to the rabbit's preimmune serum (Fig. 6b).

VP1-2xP5-specific Abs inhibit allergic patients' IgE binding to Phl p 1 and Phl p 1-induced basophil degranulation more efficiently than Abs raised against the complete allergen

Serum IgE Abs from 18 grass pollen allergic patients were allowed to bind to Phl p 1 which had been preincubated with anti-Phl p 1, anti-KLH-P5, anti-VP1-P5, or anti-VP1-2xP5 Abs (Table II). Abs raised against the complete Phl p 1 allergen showed a rather weak inhibition of IgE reactivity ranging from 0 to 45%. A stronger

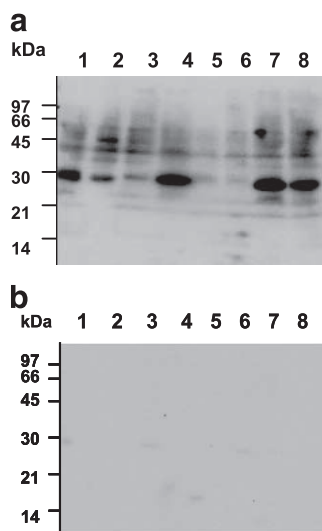


FIGURE 6. Cross-reactivity of anti-VP1-2xP5 Abs with natural group 1 grass pollen allergens. Nitrocellulose-blotted pollen extracts from eight grasses (nos. 1–8: *P. pratense*, *P. australis*, *A. sativa*, *P. pratensis*, *T. aestivum*, *S. cereale*, *L. perenne*, *A. odoratum*) were incubated with rabbit anti-VP1-2xP5 Abs (*a*) or the corresponding preimmune serum (*b*). Molecular masses are displayed on the left margin in kDa.

Table II. Inhibition of grass pollen allergic patients' IgE binding to Phl p 1 with IgG Abs^a

Patient	Inhibition of IgE binding (%)			
	Anti-Phl p 1	Anti-KLH-P5	Anti-VP1-P5	Anti-VP1-2xP5
1	1	13	13	31
2	0	51	19	66
3	27	49	36	65
4	16	46	40	71
5	14	55	39	57
6	13	35	24	55
7	18	37	26	61
8	29	55	24	72
9	17	51	41	64
10	9	46	31	65
11	0	64	50	67
12	0	55	28	88
13	14	55	50	91
14	0	7	32	29
15	11	56	58	58
16	45	70	68	74
17	28	58	55	72
18	24	41	50	36
Mean	15	47	39	63

^a The inhibitions of allergic patients' (nos. 1–18) IgE binding by anti-Phl p 1, anti-KLH-P5, anti-VP1-P5, or anti-VP1-2xP5 Abs to ELISA plate-bound Phl p 1 are expressed as percentages of inhibition using the preimmune Abs as reference. Mean inhibitions for the group of 18 patients are displayed in the bottom line.

inhibition of IgE reactivity was obtained with Abs raised against VP1-P5 (13–68%; mean inhibition 39%) and Abs raised against KLH-coupled P5 (7–70%; mean inhibition 47%; Table II). The strongest inhibition of allergic patients' IgE reactivity was obtained with Abs raised against the VP1-2xP5 construct ranging from 29 to 91%, with a mean inhibition of 63% (Table II).

The much stronger inhibition of patients' IgE reactivity of Phl p 1 by anti-VP1-2xP5 Abs compared with anti-Phl p 1 Abs may be explained by the higher titer of the anti-VP1-2xP5 IgG compared with the anti-Phl p 1 IgG (Table III).

Similar results were obtained when anti-VP1-2xP5 Abs were studied for their capacity to inhibit Phl p 1-induced degranulation of RBLs that had been loaded with IgE from four grass pollen allergic patients (Fig. 7). The anti-VP1-2xP5 antiserum started to inhibit Phl p 1-induced basophil degranulation already at a concentration of 2.5% and caused a >50% inhibition of degranulation at a concentration of 10% (Fig. 7). The Phl p 1-specific Abs caused a much weaker inhibition of Phl p 1-induced degranulation, which became detectable only at a concentration of 10%.

VP1-specific Abs inhibit HRV89 infection of HeLa cells

Next, we were interested to investigate whether VP1-specific IgG Abs can inhibit human rhinovirus infection of HeLa cells. Results

Table III. Titration of rabbit anti-Phl p 1 and anti-VP1-2xP5 IgG Abs^a

Serum Dilution	IgG Reactivity to HSA, Phl p 1, and VP1-2xP5					
	Anti-Phl p 1			Anti-VP1-2xP5		
	HSA	Phl p 1	VP1-2xP5	HSA	Phl p 1	VP1-2xP5
1/10,000	0.003	0.493	0.116	0.000	0.914	1.438
1/20,000	0.000	0.454	0.063	0.000	0.835	1.476
1/50,000	0.000	0.075	0.026	0.044	0.332	0.903
1/100,000	0.000	0.117	0.020	0.018	0.281	0.885

^a Different serum dilutions of rabbit anti-Phl p 1 and rabbit anti-VP1-2xP5 were tested for reactivity to HSA, Phl p 1, or VP1-2xP5. OD values correspond to bound IgG Abs.

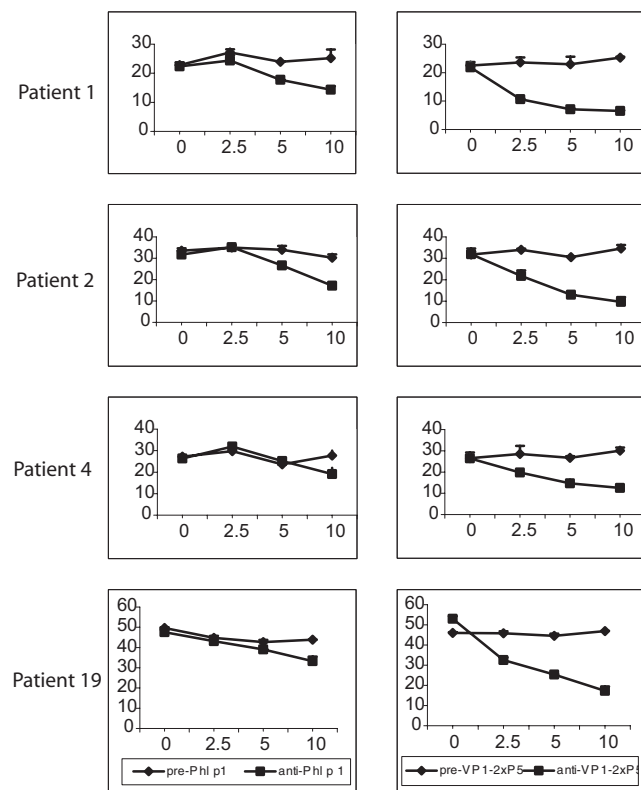


FIGURE 7. Inhibition of IgE-mediated basophil degranulation by VP1-2xP5-specific IgG Abs. RBLs transfected with the human FcεRI were loaded with IgE from different grass pollen allergic patients (*left margin*, nos. 1–4 and 19) and exposed to Phl p 1 along with increasing concentrations of rabbit anti-Phl p 1 or anti-VP1-2xP5 antiserum or the preimmune serum in the presence of Phl p 1 (X-axes: percentages, v/v, of rabbit antisera). β-Hexosaminidase releases are displayed as percentages of total β-hexosaminidase contents of the cells (y-axes).

from a representative experiment performed with a HRV89 strain are shown in Fig. 8. When cells were infected with 100 TCID₅₀ of HRV89, a complete cytopathic effect was observed (Fig. 8, *row C*, well 1). Addition of VP1-P5- as well as VP1-2xP5-specific Abs prevented HRV-induced cell death up to a dilution of the VP1-specific antiserum of 1/32 (Fig. 8, *rows A and B*). Cells incubated in medium without virus were fully alive (Fig. 8, *row C*, wells 2 and 3). Addition of antisera to the noninfected cells showed no relevant effects (Fig. 8, *row C*, wells 4–6). Similar results with

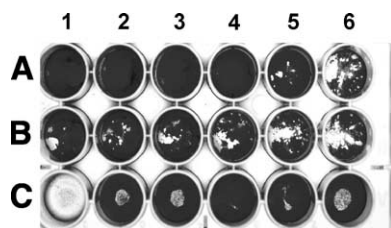


FIGURE 8. VP1-specific Abs inhibit HRV89 infection of HeLa cells. HeLa cells were seeded in a tissue culture plate at equal density per well. In rows A and B, cells were infected with the 100-fold dose of HRV89 that had left 50% of cells intact (100 TCID₅₀). Viral infection was performed in the presence of serial dilutions of anti-VP1-P5 Abs (undiluted, 1/2–1/32 wells 1–6) (*row A*) or serial dilutions of anti-VP1-2xP5 Abs (*row B*). Well 1 in *row C* was incubated with 100 TCID₅₀, wells 2 and 3 with medium alone, and wells 4–6 with the Abs alone (well 4, anti-VP1; well 5, anti-VP1-P5; well 6, anti-VP1-2xP5). Intact cells were stained with a violet dye.

anti-VP1 Abs were obtained when another rhinovirus strain (HRV14) was used instead of HRV89 (data not shown).

Discussion

Allergens and rhinovirus infections are among the most common triggers of asthma. To develop a vaccine which would confer protective immunity against rhinovirus infections as well as against allergies, we constructed an expression plasmid that allows the expression and purification of chimeric proteins consisting of rhinovirus-derived VP1 and allergen-derived peptides. VP1 was selected because it is the HRV-derived surface protein that is critically involved in rhinovirus infection of human cells, shows a high degree of sequence homology among various rhinovirus strains, and is recognized by Abs neutralizing rhinovirus infections (34, 35).

The VP1-based allergy vaccine was designed to minimize the risk of inducing IgE- and T cell-mediated side effects during therapy. For the reduction of IgE-mediated side effects, allergen-derived peptides without IgE reactivity, as represented by the Phl p 1-derived peptide can be selected according to B cell epitope mapping data, prediction of surface-exposed areas using computer programs, or according to the three-dimensional allergen structures (22, 29). When coupled to a foreign carrier molecule and used for immunization, these peptides induce robust allergen-specific IgG responses, which block IgE recognition of the allergen and IgE-mediated allergic inflammation. The peptides can be further selected to minimize the presence of frequently recognized allergen T cell epitopes to reduce T cell-mediated side effects as have been described in clinical trial performed with non-IgE-reactive T cell epitope peptides (36). The carrier principle is based on classical work performed by Benacerraf and colleagues (37–39) who studied the mechanisms underlying Ab production against small haptens that have been coupled to carriers. Their work has demonstrated that Ab responses can be obtained against haptens when they are coupled to unrelated carrier molecules that are recognized by T lymphocytes.

The VP1-based vaccine for the major timothy grass pollen allergen exemplifies the possible beneficial features of a combined vaccine for allergy and rhinovirus infections that is based on small allergen-derived peptides that are coupled to carrier molecules derived from viruses.

Recombinant VP1-P5 and rVP1-2xP5 lack IgE reactivity and allergenic activity. Therefore, the vaccine should induce little or no IgE-mediated side effects in allergic patients. Immunization of mice and rabbits showed that the proteins induced allergen-specific IgG responses which inhibited allergic patients' IgE binding to the complete Phl p 1 allergen and Phl p 1-induced immediate allergic inflammation as demonstrated by the inhibition of basophil degranulation. Interestingly, VP1-2xP5-induced IgG Abs inhibited allergic patients' IgE recognition of Phl p 1 much stronger than those IgG Abs induced by immunization with the complete Phl p 1 allergen, suggesting that the VP1-based vaccine should be more effective than currently existing vaccines containing the Phl p 1 allergen. IgG Abs induced with the VP1-based vaccine cross-reacted with group 1 allergens from several common grasses, indicating that the vaccine should be also useful to treat allergies to various grasses. A National Center for Biotechnology Information BLAST search with the P5 amino acid sequence demonstrated in fact a close relation of the timothy grass-derived peptide with homologous peptides from *L. perenne*, *P. pratensis*, *T. aestivum*, and *A. odoratum* which also reacted strongly with the VP1-2xP5-induced IgG Abs. Vaccination of one healthy volunteer has shown that the VP1-based vaccine also induces allergen-specific and rhinovirus-specific immune responses in humans (data not shown).

Unfortunately, the efficacy of prophylactic or therapeutic application of our vaccine in a purely mouse-based model could not be studied because the murine IgE response is directed against different IgE epitopes than that of Phl p 1-allergic patients. However, the VP1-based allergy vaccine has induced in animals IgG Abs that blocked allergic patients' IgE recognition of the allergen. Thus, similar data have been obtained for the VP1-based vaccine as for other hypoallergenic allergen derivatives that have already been successfully applied in patients in clinical trials (40, 41).

Grass pollen also include other important allergens besides the group 1 allergen represented by Phl p 1, but it has been shown that a mixture of only four allergens, i.e., Phl p 1, Phl p 2, Phl p 5, and Phl p 6, was sufficient to treat grass pollen allergy (42). At present, we have identified hypoallergenic peptides from the latter three allergens and it should therefore be feasible to construct a VP1-based grass pollen vaccine using the approach described in this study.

As another possibly advantageous feature of the VP1-based vaccine, we noted that the VP1 portion of the hybrid vaccine seemed to drive the immune response toward Th1 because we observed higher Phl p 1-specific IgG2 Ab levels and lower levels of IL-5 in VP1-P5- and VP1-2xP5- immunized mice compared with Phl p 1-immunized mice.

The VP1-based grass pollen vaccine induced mainly VP1-specific T cell responses but no allergen-specific T cell responses could be detected. We therefore assume that the T cell help for the allergen-specific IgG response induced by the vaccine comes from VP1-specific T cells. It is therefore likely that the VP-based allergy vaccines will induce little or no side effects through stimulation of allergen-specific T cells and thus be superior to recombinant hypoallergenic allergen derivatives which still can elicit T cell activation (43) and also induced late-phase side effects when injected into patients (44).

Furthermore, it may be expected that the VP1-based vaccine does not induce allergen-specific T cell responses or allergic sensitization and therefore should be suited for prophylactic applications. Support for this assumption comes from our finding that the VP1-based vaccines did not induce reagenic Phl p 1-specific IgE Abs upon immunization, whereas the complete natural Phl p 1 allergen primed strong reagenic Phl p 1-specific IgE responses.

Finally, we found that the VP1-based vaccine gave rise to VP1-specific Abs which inhibited the rhinovirus infection of human HeLa cells. In fact, we have demonstrated that Abs induced with HRV89-derived VP1 not only protected against HRV89 infections but also against infections caused by a distantly related rhinovirus strain such as HRV14 (data not shown).

VP1-based allergy vaccines may therefore be useful for therapy and prevention of asthma caused by allergens and rhinovirus infections.

Disclosures

R.V. is a consultant for Phadia (Uppsala, Sweden) and for Biomay (Vienna, Austria).

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 TCC AAC CAA AAA CAT GAT TTA AAT ATT GTG TGC CGC ATT TAC CAC AAG
 S N Q K H D L N I V C R I Y H K
 GCC AAA CAT ATA AAA GCA TGG TGT CCT CGC CCA CCA AGG GCT GTT GCC
 A K H I K A W C P R P P R A V A
 TAT CAA CAC ACA CAC TCA ACC AAT TAC ATA CCA TCC AAT GGT GAG GCC
 Y Q H T H S T N Y I P S N G E A
 ACA ACT CAG ATT AAA ACC AGA CCT GAT GTT TTT ACC GGT ACA AAC GTC
 T T Q I K T R P D V F T^{Age I} G T N V
 CAC CAC CAC CAC CACCAC tgagaattctgcagatatccatcacactggcgccgctcgag
 H H H H H H stop EcoR I EcoR V NotI
 cagatccggctgctaacaagcccgaaaggaagctgagttggctgctgccaccgctgagcaataactag 3'

Supplemental FIGURE 1