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Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy

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Abstract

Background—More than 50% of allergic patients have house dust mite (HDM) allergy. Group 1 and 2 allergens are the major HDM allergens.

Objective—We sought to produce and perform preclinical characterization of a recombinant hypoallergenic combination vaccine for specific immunotherapy of HDM allergy.

Methods—Synthetic genes coding for 2 hybrid proteins consisting of reassembled Der p 1 and Der p 2 fragments with (recombinant Der p 2 [rDer p 2/1C) and without (rDer p 2/1S) cysteines were expressed in *Escherichia coli* and purified to homogeneity by means of affinity chromatography. Protein fold was determined by using circular dichroism analysis, allergenic activity was determined by testing IgE reactivity and using basophil activation assays, and the presence of T-cell epitopes was determined based on lymphoproliferation in allergic patients. Mice and rabbits were immunized to study the molecules' ability to induce an allergic response and whether they induce allergen-specific IgG capable of inhibiting allergic patients' IgE binding to the allergens, respectively.

Results—rDer p 2/1C and rDer p 2/1S were expressed in large amounts in *E coli* as soluble and folded proteins. Because of the lack of disulfide bonds, rDer p 2/1S did not form aggregates and was obtained as a monomeric protein, whereas rDer p 2/1C did form aggregates. Both

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hypoallergens lacked relevant IgE reactivity and had reduced ability to induce allergic inflammation and allergic responses but induced similar T-cell proliferation as the wild-type allergens. Immunization with the hypoallergens (rDer p 2/1S > rDer p 2/1C) induced IgG antibodies in rabbits that inhibited the IgE reactivity of patients with HDM allergy to Der p 1 and Der p 2.

Conclusion—The preclinical characterization indicates that particularly rDer p 2/1S can be used as a safe hypoallergenic molecule for both tolerance and vaccination approaches to treat HDM allergy.

Keywords

Allergy; allergen; specific immunotherapy; recombinant hypoallergen; house dust mite allergy

Allergen-specific immunotherapy (SIT) represents the only allergen-specific and disease-modifying approach with long-lasting effects for the treatment of allergic patients.¹⁻⁵ However, SIT can induce side effects, ranging from mild and local to severe and life-threatening symptoms, such as anaphylactic shock.^{6,7} Severe side effects are frequently observed in patients with house dust mite (HDM) allergy,⁸ who constitute more than 50% of allergic patients and often have severe forms of respiratory allergy, such as asthma. The continuous exposure to HDM allergens further complicates the treatment of patients with HDM allergy. Additionally, the quality of natural HDM allergen extracts and vaccines based on these extracts is often poor.

Although 23 different HDM allergens have been characterized, several studies have indicated that group 1 and 2 allergens represent the clinically most important HDM allergens. In fact, it has been shown that a combination of the *Dermatophagoides pteronyssinus* allergens Der p 1 and Der p 2 allows diagnosis of more than 95% of patients with HDM allergy.⁹⁻¹² Therefore Der p 1 and Der p 2 represent important components for a vaccine for HDM allergy. Several clinical studies indicate that SIT with recombinant hypoallergenic allergen derivatives that have been engineered to reduce their allergenic activity and thus therapy-induced side effects holds promise to improve SIT.⁵ Several promising hypoallergenic derivatives of group 2 mite allergens have already been developed,¹³⁻¹⁹ but thus far, no hypoallergenic derivatives for group 1 mite allergens have been reported that fulfill all the criteria of the hypoallergens that have been successfully used in clinical trials. A recently reported recombinant Der p 1 (rDer p 1) hypoallergen induced almost no allergen-specific IgG antibodies on immunization of mice, and a Der p 1 variant expressed in rice was tested only in mice, but its allergenic activity and IgE reactivity in allergic patients has not been investigated.^{20,21} A hybrid molecule consisting of a truncated Der p 2 and Der p 1 was shown to exhibit reduced reactivity with patients' IgE, but it has not been unambiguously demonstrated that this molecule was able to induce Der p 1-specific IgG antibodies on immunization of animals.²²

In this study we report the construction, purification, and physicochemical, immunologic, and preclinical characterization of 2 recombinant hypoallergenic hybrid proteins, recombinant Der p 2 (rDer p 2)/1C and rDer p 2/1S, consisting of reassembled elements of Der p 1 and Der p 2. In particular, rDer p 2/1S, a hybrid molecule lacking all of the naturally

occurring cysteine residues of Der p 1 and Der p 2, could be expressed in large amounts in *E. coli*, lacked IgE reactivity and allergenic activity in patients with HDM allergy, and, on immunization of animals, showed low allergenicity and induced IgG antibodies, which blocked allergic patients' IgE binding to Der p 1 and Der p 2, fulfilling the preclinical requirements for a hypoallergenic vaccine for HDM allergy.

METHODS

Human sera, HDM allergens, and constructions and characterization of Der p 2/1 mosaic proteins

For more information on human sera and HDM allergens, see the Methods section in this article's Online Repository at www.jacionline.org.

Two synthetic genes coding for the Der p 2/1 mosaic proteins displayed in Fig 1 were synthesized by ATG Biosynthetics (Merzhausen, Germany) by using codons optimized for expression in *Escherichia coli*. The 2 recombinant mosaic proteins consisted of 3 Der p 1 fragments (1.1, amino acids 1-84; 1.2, amino acids 85-143; 1.3, amino acids 144-222) and 2 Der p 2 fragments (2.1, amino acid 1-53; 2.2, amino acids 54-129), which were reassembled in the following order: 1.3, 2.2, 1.2, 2.1, and 1.1 with (rDer p 2/1C) or without cysteines (rDer p 2/1S) and a C-terminal hexahistidine tag. The synthetic genes were cloned into the expression vector pET17b, and the DNA sequences were determined by means of sequencing (ATG Biosynthetics).

For more information on expression and purification of Der p 2/1 mosaic proteins, see the Methods section in this article's Online Repository.

For more information on SDS-PAGE under reducing and nonreducing conditions, see the Methods section in this article's Online Repository.

For more information on circular dichroism (CD) analysis, see the Methods section in this article's Online Repository.

IgE dot-blot assays

Aliquots ($v = 2 \mu\text{L}$) containing 0.2 μg of natural Der p 1 (nDer p 1), rDer p 2, the 2 mosaic proteins (Der p 2/1C or Der p 2/1S), or BSA (for control purposes) were dotted onto nitrocellulose membrane strips (Schleicher & Schuell, Dassel, Germany). Serum IgE reactivity of 32 patients with mite allergy and 2 control subjects (a nonallergic person and a buffer control) to the dot-blotted proteins were determined as previously described.¹⁹

Basophil activation assays

Basophil activation was assessed *in vitro* based on upregulation of CD203c expression. Heparinized blood samples from 10 patients with mite allergy were collected after informed consent was provided. Basophils were stimulated with various concentrations (0.04-400 nmol/L) of nDer p 1, rDer p 2, Der p 2/1C, or Der p 2/1S and, for control purposes, with an anti-IgE mAb (1 $\mu\text{g}/\text{mL}$; Immunotech, Vaudreuil-Dorion, Quebec, Canada) or PBS for 15 minutes (37°C). Expression of CD203c was determined as previously described.²³ Allergen-

induced upregulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFIstim) and unstimulated (MFIcontrol) cells and expressed as the stimulation index (ie, MFIstim/MFIcontrol).

Immunization of rabbits and mice

For more information on immunization of rabbits and mice, see the Methods section in this article's Online Repository.

Determination of IgG responses in rabbits

Rabbit IgG responses induced with the mosaic proteins were determined by using nondenaturing dot-blot assays. For this purpose, 0.2 µg of nDer p 1, Der p 1 peptides (Der p 1 P1, amino acids 1-30; P2, amino acids 52-84; P3, amino acids 85-115; P4, amino acids 99-135; P5 amino acids 145-175; P6, amino acids 155-187; P7, amino acids 175-208; P8, amino acids 188-222), rDer p 2, Der p 2 peptides (Der p 2 P1, amino acids 1-33; P2, amino acids 21-51; P3, amino acids 42-73; P4, amino acids 62-103; P5, amino acids 98-129; see Table E1 in this article's Online Repository at www.jacionline.org), the 2 mosaic proteins, and BSA (0.1 µg/µL; for control purposes) were dotted onto nitrocellulose strips (Schleicher & Schuell). The strips were then blocked with buffer A (40 mmol/L Na₂HPO₄, 6 mmol/L NaH₂PO₄, [pH 7.5], 0.5% Tween 20, 0.5% [wt/vol] BSA, and 0.05% [wt/vol] NaN₃) and exposed to rabbit sera obtained by means of immunization with nDer p 1, rDer p 2, or the 2 mosaic proteins or to the corresponding preimmune sera (dilution 1:10,000 in buffer A) overnight at 4°C. Bound rabbit IgG antibodies were detected with iodine 125-labeled donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, United Kingdom; dilution 1:1000 in buffer A) and visualized by means of autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany).

ELISA for allergen-specific IgG reactivity and IgE inhibition assays

Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with nDer p 1 or rDer p 2 (0.5 µg per well in PBS), washed twice with PBS with 0.05% (vol/vol) Tween 20 (PBST), and then blocked in blocking buffer (PBST and 1% [wt/vol] BSA) for 3 hours at room temperature. Binding of rabbit antibodies to wild-type allergens was measured by means of incubation with serial 10-fold dilutions of rabbit antibodies (1:100-1:10,000,000) made in PBST and 0.5% (wt/vol) BSA overnight at 4°C. After washing, bound rabbit IgG was detected with horseradish peroxidase-conjugated 1:2000 diluted goat anti-rabbit immunoglobulin (Amersham, GE Healthcare) and measured as previously described.²⁴

IgE inhibition ELISA assays were performed as previously described.²⁴ ELISA plates coated with nDer p 1 or rDer p 2 were preincubated with rabbit anti-nDer p 1, rDer p 2, Der p 2/1C, or rabbit anti-Der p 2/1S antisera (1:20) or the corresponding preimmune sera (1:20) overnight at 4°C. After washing, the plates were incubated with sera from patients with mite allergy (1:10). Bound human IgE antibodies were detected with horseradish peroxidase-coupled goat anti-human IgE antibodies (1:2500; KPL, Gaithersburg, Md). The percentage inhibition of IgE binding was calculated as follows:

$$100 - (\text{ODs}/\text{ODp}) \times 100.$$

ODs and *ODp* represent the extinctions after preincubation with the rabbit immune serum and preimmune serum, respectively.²⁴ When sera contained only low or no specific IgE reactivity to Der p 1 or Der p 2 (*OD* = 0.3), the inhibition experiments could not be analyzed.

Rat basophil leukemia cell degranulation assay

Serum samples were obtained from mice that had been immunized with aluminum hydroxide-adsorbed nDer p 1, rDer p 2, rDer p 2/1C, or rDer p 2/1S (*n* = 5) before immunization or 24 weeks after immunization. Aliquots of 2 μL of serum from each mouse were then exposed to rat basophil leukemia (RBL) cells (subline RBL-2H3) cultivated in 96-well tissue-culture plates (4×10^4 cells in 100 μL , Nunc) for 2 hours at 37°C. After addition of nDer p 1, rDer p 2, or the mosaic proteins, the release of β -hexosaminidase in the cultures was measured, and values are shown as median percentages $\pm$ SDs of total β -hexosaminidase release.¹⁹

T-cell proliferation assays

For more information on T-cell proliferation assays, see the Methods section in this article's Online Repository.

RESULTS

Purification and characterization of rDer p 2/1 mosaic proteins

With the aim to produce hypoallergenic combination vaccines for the major dust mite allergens Der p 1 and Der p 2, we have designed 2 mosaic proteins, each consisting of the entire sequence elements of Der p 1 and Der p 2 (Fig 1). Two previously defined hypoallergenic fragments of Der p 2 (Fig 1, 2.1 and 2.2)¹⁹ were shuffled with 3 Der p 1 fragments (Fig 1, 1.1, 1.2, and 1.3) in the form of 2 recombinant mosaic proteins designated Der p 2/1C and Der p 2/1S. Der p 2/1C contained the original amino acid sequences of Der p 1 and Der p 2, including 12 cysteine residues (dotted vertical lines), whereas in Der p 2/1S the cysteine residues were replaced by serine residues (Fig 1). The replacement of cysteine residues by serine residues was thought to reduce the potential aggregation behavior of the Der p 2/1S protein. The selection of the 3 Der p 1 fragments was based on IgE and T-cell epitope mapping studies (Chen et al, unpublished data), suggesting that amino acids from fragments 1.1 and 1.3 contribute to conformational IgE epitopes on Der p 1, although the isolated fragments lacked IgE reactivity. In the mosaic proteins fragment 1.3 was placed at the N-terminus, followed by the C-terminal Der p 2 fragment 2.2, the original Der p 1 core fragment 1.2, the N-terminal Der p 2 fragment 2.1, and, at the C-terminus, the original N-terminal Der p 1 fragment 1.1. This was done to achieve a maximal displacement and reorientation of Der p 1 fragments 1.1 and 1.3. Both mosaic proteins were produced as C-terminally His-tagged proteins to facilitate purification.

The 2 mosaic proteins were expressed in *E coli* (Fig 2, A, lanes 1) and purified to homogeneity (Fig 2, A, lanes 2). SDS-PAGE analysis under reducing and nonreducing conditions demonstrated that the 2 Der p 2/1 mosaic proteins migrated as monomeric proteins under reducing conditions (Fig 2, B, lanes r). However, under nonreducing conditions, Der p 2/1C occurred only in the form of high-molecular-weight aggregates, whereas Der p 2/1S appeared mainly as a monomeric protein (Fig 2, B, lanes nr).

Next we compared the fold of nDer p 1 and rDer p 2 with the mosaic proteins using CD analysis (Fig 3). nDer p 1 showed a high content of α -helical elements, which is characterized by a minimum at 208 nm and 222 nm (Fig 3). The rDer p 2 CD spectrum exhibited a minimum at 215 nm and a maximum at 197 nm, which is typical for β -sheet proteins (Fig 3). The CD spectra of Der p 2/1C and Der p 2/1S were very similar, exhibiting a broad minimum at 215 nm and a maximum at 195 nm, which is typical for proteins with a mixed α/β -fold (Fig 3). The CD spectrum of rDer p 1 resembled a denatured unfolded protein (data not shown).

rDer p 2/1 mosaic proteins show almost no detectable IgE reactivity or allergenic activity

The IgE-binding capacities of the 2 Der p 2/1 mosaic proteins were compared with nDer p 1 and rDer p 2 allergen in nondenaturing dot-blot and RAST-based IgE-binding assays by using sera from 32 patients with HDM allergy (Fig 4). Each of the allergic patients showed IgE reactivity to nDer p 1 and rDer p 2, whereas only 5 patients (ie, patients 1, 8, 18, 24, and 30) showed very weak IgE reactivity to Der p 2/1C. A barely visible reaction to rDer p 2/1S was observed for 2 patients (ie, patients 1 and 8; Fig 4). None of the other patients showed any detectable IgE reactivity to Der p 2/1C and Der p 2/1S. When serum from a nonallergic person or buffer without serum was used, no reactivities to any of the dotted proteins were found (Fig 4, lanes NC and BC). None of the patients showed any IgE reactivity to the control protein BSA (Fig 4).

The allergenic activity of the 2 Der p 2/1 mosaic proteins was compared with that of nDer p 1 and rDer p 2 wild-type allergens by determining upregulation of CD203c expression on basophils from patients with HDM allergy on antigen exposure (Fig 5). Both wild-type allergens nDer p 1 and rDer p 2 induced upregulation of CD203c expression in the tested patients with HDM allergy. No upregulations of CD203c expression were obtained with rDer p 2/1C or rDer p 2/1S up to a concentration of 40 nmol/L. A very weak upregulation was observed at the highest concentration of 400 nmol/L of rDer p 2/1C only in 5 patients (ie, patients 1, 5, 6, 7, and 18), whereas rDer p 2/1S did not induce basophil activation at any of the tested concentrations. Anti-human IgE antibodies were used as positive controls and induced upregulation of CD203c expression on basophils from all patients, whereas no upregulation was obtained with buffer alone (data not shown).

rDer p 2/1 mosaic proteins contain Der p 1– and Der p 2–specific T-cell epitopes

PBMCs from 9 patients with HDM allergy were stimulated with the Der p 2/1 mosaic proteins, nDer p 1, or rDer p 2. The stimulation indices in Table E2 in this article's Online Repository at www.jacionline.org show that both mosaic proteins induce

lymphoproliferative responses comparable with those induced by the wild-type allergens at 2 antigen concentrations (ie, 0.1 and 0.25 $\mu\text{mol/L}$).

Immunization of rabbits with the hypoallergenic Der p 2/1 mosaic proteins induced IgG antibodies that react with the wild-type allergens and inhibit allergic patients' IgE binding to both allergens

Both Der p 2/1 mosaic proteins induced IgG antibodies against themselves in rabbits and mice (data not shown), as well as Der p 1- and Der p 2-specific IgG antibodies (Fig 6). When rabbits were immunized with rDer p 1, no IgG antibodies could be induced that reacted with nDer p 1 (data not shown). None of the rabbit sera showed any reactivity to the BSA control. Interestingly, rabbit anti-sera raised against the rDer p 2/1 mosaic proteins reacted with 6 of the 8 Der p 1 peptides, whereas the rabbit anti-nDer p 1 antiserum reacted only with 2 peptides (ie, Der p 1 P3 and Der p 1 P6). Similar differences regarding the fine specificities of the antibodies were observed for the anti-rDer p 2 antiserum, which did not react with peptide Der p 2 P2, whereas this peptide was strongly recognized by the anti-sera raised against the mosaic proteins (Fig 6). Thus antibodies raised by immunization with mosaic antigens recognized more sequential epitopes than those obtained by means of immunization with the individual wild-type allergens.

Despite the partial differences in epitope specificities, the titers of anti-nDer p 1 and anti-rDer p 2 antibodies were almost identical in the anti-sera raised against the recombinant mosaic proteins and in the anti-sera raised against nDer p 1 and rDer p 2 (see Table E3 in this article's Online Repository at www.jacionline.org).

The inhibition of allergic patients' IgE binding to nDer p 1 or rDer p 2 by rabbit antibodies raised against the mosaic proteins is shown for 30 patients with HDM allergy in Table I. Almost no inhibition of allergic patients' IgE reactivity to Der p 1 was obtained with anti-rDer p 1 antibodies, whereas antibodies raised by immunization with nDer p 1 strongly inhibited IgE binding to Der p 1 (ie, 37.6% to 91.5%; mean, 81.3%). The inhibition of IgE binding to nDer p 1 achieved with rabbit anti-Der p 2/1S antibodies (ie, 39.7% to 69.2%; mean, 54.8%) was always better than that obtained with rabbit anti-Der p 2/1C (ie, 30.3% to 68.7%; mean, 45.3%). Anti-Der p 2 antibodies inhibited patients' IgE binding to rDer p 2 between 67.2% and 94.4% (mean, 83.6%). The inhibition of allergic patients' IgE binding to Der p 2 obtained with rabbit anti-Der p 2/1C (ie, 69.6% to 94.4%; mean, 80.6%) and with rabbit anti-Der p 2/1S (ie, 76.7% to 95.1%; mean, 85.7%) antibodies was comparable with that of rabbit anti-Der p 2 antibodies (Table I).

Reduced *in vivo* allergenicity of the mosaic proteins compared with the wild-type allergens Der p 1 and Der p 2

The 2 mosaic proteins were compared with nDer p 1 and rDer p 2 regarding their *in vivo* allergenicity. For this purpose, mice were immunized with the mosaic proteins nDer p 1 or rDer p 2, and the induction of Der p 1- or Der p 2-specific reagenic IgE antibodies was then studied by loading RBL cells with the sera, followed by allergen provocation (Fig 7). Very low or no release of β -hexosaminidase was obtained when RBL cells loaded with murine anti-Der p 2/1C or anti-Der p 2/1S IgE were exposed to nDer p 1 (Der p 2/1C: median,

0.0%; Der p 2/1S: median, 0.0%; Fig 7, A) or rDer p 2 (Der p 2/1C: median, 1.9%; Der p 2/1S: median, 2.6%; Fig 7, B). In contrast, immunization with nDer p 1 or rDer p 2 induced high levels of allergen-specific reagenic IgE, yielding a median Der p 1-specific degranulation of 54.5% or a median Der p 2-specific degranulation of 73.2% (Fig 7). No relevant release of β -hexosaminidase was obtained when RBL cells loaded with murine anti-Der p 2 IgE were exposed to nDer p 1 or *vice versa* (Fig 7) or when RBL cells loaded with murine anti-rDer p 2/1C or rDer p 2/1S IgE were exposed to the mosaic proteins themselves (data not shown).

DISCUSSION

Several clinical studies have demonstrated that SIT with recombinant birch and grass pollen allergens, as well as with recombinant hypoallergenic allergen derivatives, is clinically effective.^{5,25} One major mechanism of vaccination treatment is the induction of allergen-specific IgG antibodies, which block allergic patients' IgE binding and hence inhibit IgE-mediated effector cell degranulation, T-cell activation, and boosts of IgE production.^{3,5}

According to IgE reactivity data obtained for different populations and assessment of allergenic activity, group 1 and 2 allergens from HDM must be considered essential components of HDM allergy vaccines.^{9,10,26} Recombinant group 2 allergens resembling structurally and immunologically the natural allergens, as well as recombinant hypoallergenic derivatives of Der p 2 suitable for clinical trials, have been produced.^{13,17-19} However, fully IgE-reactive group 1 allergens have thus far only been expressed in eukaryotic cells but not in *E coli*.^{27,28} Recently engineered hybrid molecules consisting of Der p 1 and Der p 2 were either not stable or formed aggregates and therefore will not be suitable for SIT.²⁹ A hypoallergenic Der p 1-Der p 2 derivative was reported, but it has not been unambiguously shown that it induced IgG antibodies against Der p 1, and it is therefore not clear whether this vaccine will protect against sensitization to Der p 1.²²

The 2 recombinant hypoallergenic hybrid proteins described in our study address many of the hitherto unresolved problems regarding the construction of a hypoallergenic vaccine for HDM allergy. rDer p 2/1C and rDer p 2/1S combine all the primary sequence elements of Der p 1 and Der p 2 within 1 molecule, which can be expressed in large amounts in *E coli* and purified to homogeneity. Unexpectedly, both hybrid molecules showed a mixed α/β -fold, although the 2 Der p 2 fragments used for the rDer p 2/1 proteins were unfolded.¹⁹ Despite the folded nature of the hybrid molecules, both proteins show strongly reduced IgE reactivity and allergenic activity, as demonstrated by IgE reactivity testing and basophil activation studies in allergic patients. The possible disadvantage that the hybrid molecules cannot bind through IgE-Fc ϵ RI to antigen-presenting cells and downregulate through the tryptophan degradation pathway T-cell responses will likely be outweighed by the advantage that these molecules should not induce IgE-mediated side effects.³⁰ Furthermore, because all primary sequence elements of Der p 1 and Der p 2 and all T-cell epitopes have been preserved in the mosaic proteins, they can be used in SIT approaches aiming at the induction of T-cell tolerance. Finally, both proteins induce, on immunization, IgG antibodies that recognize both allergens and inhibit allergic patients' IgE binding to the wild-type allergens,

In summary, rDer p 2/1S shows strongly reduced allergenicity in a murine model, as well as strongly reduced allergenic activity and IgE reactivity. Furthermore, it contains all relevant T-cell epitopes needed for tolerance induction strategies and induces robust protective allergen-specific IgG responses as needed for vaccine SIT approaches. It therefore can be considered an extremely promising candidate molecule for clinical SIT trials in patients with HDM allergy.

Human sera and HDM allergens

The cDNA coding for rDer p 1 was obtained by means of reverse transcription from *D. pteronyssinus* RNA using RT-PCR (Invitrogen, Life Technologies, Carlsbad, Calif). The upstream primer (5'-GGAATTCATTAATATGACTAACGCCTGCAGT-3') used for amplification contained an *Xba*I restriction site, and the downstream primer (5'-GGAATTCCTTAGTGATGGTGATGGTGATGGAGAATGACAACATA-3') contained an

EcoRI site, as well as 6 His codons. The PCR product was subcloned into plasmid pET17b (Novagen, Merck, Darmstadt, Germany) and transformed into the *E. coli* strain XL-1 Blue (Stratagene, Agilent Technology, Santa Clara, Calif). Plasmid DNA was then isolated by using NucleoBond AX Kit-maxi-prep (Macherey-Nagel, Düren, Germany), and the sequence of the cDNA insert was confirmed by sequencing both DNA strands on an automated sequencing system (MWG, Ebersberg, Germany). The DNA sequence was identical to the sequence published by Chua et al.^{E4} rDer p 1 was expressed and purified as previously described.^{E3}

SDS-PAGE under reducing and nonreducing conditions

The purified Der p 2/1 mosaic proteins were mixed with SDS sample buffer with 5% vol/vol 2-mercaptoethanol (reducing conditions) or without 2-mercaptoethanol (nonreducing conditions) and were separated by using 14% SDS-PAGE.^{E5} Proteins were visualized with Coomassie brilliant blue staining (Bio-Rad, Richmond, Calif).

Expression and purification of Der p 2/1 mosaic proteins

Expression plasmids containing the Der p 2/1 constructs were transformed into *E. coli* strain BL21 (DE3; Novagen, Merck). Protein expression was performed in 250 mL of liquid culture by means of induction with 0.5 mmol/L isopropyl- β -D-1-thiogalactopyranoside at an OD₆₀₀ of 0.8 for 4 hours at 37°C, and cells were harvested by means of centrifugation at 4000g for 15 minutes at 4°C. The bacterial pellets obtained from 250 mL of liquid culture were resuspended in 10 mL of 25 mmol/L imidazole (pH 7.4) and 0.1% (vol/vol) Triton X-100. Cells were lysed by using 3 freeze/thaw cycles (−70°C/+50°C), DNA was degraded by means of incubation with 1 μ g of DNase I for 10 minutes at room temperature, and cell debris was removed by means of centrifugation (10,000g for 30 minutes at 4°C). Der p 2/1C and Der p 2/1S mosaic proteins were found in the pellet in the inclusion body fractions, which were solubilized with 6 mol/L guanidine hydrochloride, 100 mmol/L NaH₂PO₄, and 10 mmol/L Tris-Cl (pH 8) for 4 hours at room temperature. Insoluble debris was removed by means of centrifugation (10,000g for 15 minutes at 4°C), and the 2 mosaic proteins were purified under denaturing conditions over Ni-NTA resin affinity columns (Qiagen Hilden, Germany).

Fractions containing recombinant proteins of greater than 90% purity were dialyzed against 10 mmol/L NaH₂PO₄ (pH 4.7), and the final protein concentrations were determined by using the BCA Protein Assay Kit (Novagen, Merck).

CD analysis

The CD spectra of the proteins were measured on a JASCO (Tokyo, Japan) J-810 spectropolarimeter. CD measurements were performed with nDer p 1, rDer p 1, rDer p 2, Der p 2/1C, and Der p 2/1S at protein concentrations of 0.1 mg/mL in 10 mmol/L NaH₂PO₄ (pH 4.7) by using a rectangular quartz cuvette with a path length of 0.2 cm. Spectra were recorded from 190 to 260 nm with 0.5 nm resolution at a scan speed of 50 nm/min, and results were averaged from 3 scans. The final spectra were corrected by subtracting the baseline spectra obtained with the corresponding buffer (10 mmol/L NaH₂PO₄ [pH 4.7])

under identical conditions. Results are expressed as the mean residue ellipticity (Θ) at a given wavelength.

Immunization of rabbits and mice

Specific IgG antibodies were obtained by immunizing rabbits with each of the mosaic proteins, nDer p 1, rDer p 1, or rDer p 2 (200 μ g per injection) with complete Freund adjuvant and incomplete Freund adjuvant (Charles River, Kisslegg, Germany).

Eight-week-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany) and maintained according to the local animal care guidelines in the Animal Care Unit of the Department of Pathophysiology and Allergy Research of the Medical University of Vienna. Four groups consisting of 5 mice each were immunized subcutaneously monthly in the neck with 5 μ g of nDer p 1, rDer p 2, rDer p 2/1C, or rDer p 2/1S adsorbed to 200 μ L of AluGel-S (SERVA Electrophoresis, Heidelberg, Germany). Blood samples were collected on the day before each immunization and stored at -20°C .

T-cell proliferation assays

Heparinized venous blood samples were collected from patients with HDM allergy ($n = 9$) after informed consent was provided. PBMCs were isolated by means of Ficoll density gradient centrifugation (Amersham, GE Healthcare) and stimulated with 2 different concentrations of nDer p 1, rDer p 2, rDer p 2/1C, or rDer p 2/1S (0.2 or 0.05 $\mu\text{mol/L}$). Proliferation assays were performed as previously described, and results are displayed as stimulation indices (SIs).^{E6}

Extended Data

TABLE E1.

Characteristics of Der p 1 and Der p 2 peptides

Allergen peptide	Position (amino acids)	Length (amino acids)	Molecular weight (kDa)	Amino acid sequence
Der p 1 P1	1-30	30	3.22	TNAC SINGNAPAEIDLRQMRTVTPIRMQGG
Der p 1 P2	52-84	33	3.64	NQSLDLAEQELVDCASQHGCHGDTIPRGIEYIQ
Der p 1 P3	85-115	31	3.69	HNGVVQESYYRYVAREQSCRRPNAQRFGISN
Der p 1 P4	99-135	37	4.36	REQSCRRPNAQRFGISNYCQIYPPNVNKIREALAQTH
Der p 1 P5	145-175	32	3.79	CKDLDAFRHYDGRITIIQRDNGYQPNYHAVNIV
Der p 1 P6	155-187	34	3.89	GRTIIQRDNGYQPNYHAVNIVGY SNAQGVDYWIC
Der p 1 P7	175-208	35	3.98	VGYSNAQGVDYWIVRNSWDTNWGDNGYGYFAANIC
Der p 1 P8	188-222	36	4.23	CVRNSWDTNWGDNGYGYFAANIDLMMIEEYPYVVIL
Der p 2 P1	1-33	33	3.63	DQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGK

Allergen peptide	Position (amino acids)	Length (amino acids)	Molecular weight (kDa)	Amino acid sequence
Der p 2 P2	21-51	31	3.44	CHGSEPCIHRGKPFQLEAVFEANQNSKTAK
Der p 2 P3	42-73	32	3.36	EANQNSKTAKIEIKASIEGLEVDVPGIDPNAC
Der p 2 P4	62-103	42	4.79	EVDVPGIDPNACHYMKCPLVKGQYDIKYTWIVPKIAPKSEN
Der p 2 P5	98-129	32	3.31	APKSENVVVTVKVMGDNGVLACAIATHAKIRD

TABLE E2.

Induction of lymphoproliferative responses with nDer p 1, rDer p 2, and the rDer p 2/1C and rDer p 2/1S mosaic proteins

	0.1 μ mol/L Allergen				0.025 μ mol/L Allergen			
	nDer p 1	rDer p 2	rDer p 2/1C	rDer p 2/1S	nDer p 1	rDer p 2	rDer p 2/1C	rDer p 2/1S
Patient 2	6.9	4.9	6.4	3.8	4.7	2.7	7.2	2.1
Patient 5	3.1	3.2	4.7	3.3	3.4	4.3	5.0	2.1
Patient 6	3.3	2.5	2.6	4.1	3.3	2.9	2.4	2.0
Patient 7	2.1	3.0	1.8	1.6	2.1	3.7	2.6	2.4
Patient 10	4.2	2.6	1.9	3.0	4.8	3.9	1.7	2.5
Patient 12	2.5	2.3	3.3	2.7	2.9	2.2	2.7	3.8
Patient 13	2.5	2.7	3.0	5.0	2.9	2.8	2.9	4.2
Patient 18	3.8	4.6	5.3	6.7	5.4	4.1	4.2	5.2
Patient 23	2.2	2.9	1.6	1.6	2.3	3.8	1.2	2.4

PBMCs from patients with HDM allergy were stimulated with different concentrations of the proteins, and stimulation indices are displayed.

TABLE E3.

ELISA titrations of rabbit antisera

Dilution	nDer p 1			rDer p 2		
	Rabbit anti-nDer p 1	Rabbit anti-rDer p 2/1C	Rabbit anti-rDer p 2/1S	Rabbit anti-rDer p 2	Rabbit anti-rDer p 2/1C	Rabbit anti-rDer p 2/1S
1:100	1.156	1.188	1.024	1.268	1.307	1.081
1:1,000	1.288	1.382	1.280	1.721	1.673	1.591
1:10,000	1.261	1.023	1.169	1.168	1.054	1.263
1:100,000	0.571	0.443	0.658	0.477	0.386	0.560
1:1,000,000	0.126	0.095	0.128	0.105	0.078	0.099
1:10,000,000	0.066	0.036	0.037	0.037	0.039	0.033

Mean OD values corresponding to bound IgG antibodies are displayed.

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Abbreviations used

CD	Circular dichroism
HDM	House dust mite
MFI	Mean fluorescence intensity
nDer p 1	Natural Der p 1
PBST	PBS with 0.05% (vol/vol) Tween 20
RBI	Rat basophil leukemia
rDer p 1	Recombinant Der p 1
rDer p 2	Recombinant Der p 2
SIT	Allergen-specific immunotherapy

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Clinical implications: rDer p 2/1S is a recombinant hypoallergenic combination vaccine for safe SIT of HDM allergy.

FIG 1.

Construction of Der p 2/Der p 1 mosaic proteins. Three fragments of Der p 1 (1.1, 1.2, and 1.3) comprising amino acids 1 to 84, 85 to 143, and 144 to 222 and 2 fragments of Der p 2 (2.1 and 2.2) comprising amino acids 1 to 53 and 54 to 129 were reassembled in the order 1.3, 2.2, 1.2, 2.1, and 1.1. Der p 2/1C contains the original amino acid sequence of Der p 1 and Der p 2, including the 12 cysteine residues (*dashed vertical lines*), whereas in Der p 2/1S the cysteine residues were exchanged for serine residues. Both proteins contain a hexahistidine tag at the C-terminus.

FIG 2.

Expression and purification of rDer p 2/Der p 1 mosaic proteins. Coomassie-stained SDS-PAGE-containing protein extracts of BL21 (DE3) expressing Der p 2/1C and Der p 2/1S (*lanes 1*), purified Der p 2/1C and Der p 2/1S (*lanes 2*; **A**), and purified Der p 2/1C and Der p 2/1S separated under reducing (*lanes r*) and nonreducing (*lanes nr*) conditions (**B**) and molecular weight markers (*lanes M*) are shown.

FIG 3. Far-UV CD spectra of nDer p 1, rDer p 2, rDer p 2/1C, and rDer p 2/1S. The mean residue ellipticity (y -axis) at given wavelengths (x -axis) is shown for each protein.

FIG 4.

IgE reactivity of nDer p 1, rDer p 2, and rDer p 2/1 mosaic proteins. Dot-blotted nDer p 1, rDer p 2, the 2 Der p 2/1 mosaic proteins, and BSA were tested for IgE reactivity with sera from 32 patients with HDM allergy (*lanes 1-32*), serum from a nonallergic subject (*NC*), or buffer without serum (*BC*). Bound IgE was detected with iodine 125-labeled anti-human IgE antibodies and visualized by using autoradiography.

FIG 5.

Allergic activity of nDer p 1, rDer p 2, and rDer p 2/1 mosaic proteins. Basophils from 10 patients with HDM allergy were stimulated with various concentrations of nDer p 1, rDer p 2, rDer p 2/1C, and rDer p 2/1S (*x-axes*). Expression of CD203c was determined by using fluorescence-activated cell-sorting analysis, and stimulation indices (*SI*) are shown (*y-axes*).

FIG 6.

Reactivity and epitope specificity of IgG antibodies induced by immunization of rabbits with *nDer p 1*, *rDer p 2*, and *rDer p2/1* mosaic proteins. Sera obtained from rabbits before (*pre*) or after (*immune*) immunization with *nDer p 1*, *rDer p 2*, *rDer p 2/1C*, and *rDer p 2/1S* were tested for IgG reactivity to dot-blotted *nDer p 1*, *rDer p 2*, *rDer p 2/1* mosaic proteins, and BSA or with synthetic *Der p 1*- and *Der p 2*-derived peptides (*Der p 1* P1-P8 and *Der p 2* P1-P5). Bound IgG antibodies were detected with iodine 125-labeled donkey anti-rabbit IgG antibodies.

FIG 7.

Reduced allergenicity of the 2 rDer p 2/1 mosaic proteins. RBL cells were loaded with sera from mice obtained before (*preimmune*) or after (*immune*) immunization with nDer p 1, rDer p 2, or the 2 mosaic proteins (*x-axes*). nDer p 1-specific (**A**) and rDer p 2-specific (**B**) release of β -hexosaminidase are displayed as percentages of total β -hexosaminidase release (medians $\pm$ SDs for the 5 sera from each group of mice, *y-axes*).

TABLE I

Inhibition of allergic patients' IgE binding to Der p 1 and rDer p 2 with rabbit anti-nDer p 1, anti-rDer p 2, or anti-rDer p 2/1 antisera

	Inhibition to nDer p 1				Inhibition to rDer p 2		
	Rabbit anti-nDer p 1	Rabbit anti-rDer p 1	Rabbit anti-rDer p 2/1C	Rabbit anti-rDer p 2/1S	Rabbit anti-rDer p 2	Rabbit anti-rDer p 2/1C	Rabbit anti-rDer p 2/1S
Patient 1	88.9	0.0	54.8	60.2	91.3	88.7	92.0
Patient 2	91.5	0.0	37.5	48.6	83.3	83.0	87.8
Patient 3	90.6	0.0	61.7	61.9	94.4	94.5	95.1
Patient 4	66.8	0.0	41.8	55.8	71.5	72.6	78.4
Patient 5	NA	NA	NA	NA	80.1	75.5	84.0
Patient 6	68.1	0.0	31.4	39.7	85.3	83.7	88.3
Patient 7	75.2	6.6	43.5	53.8	83.2	78.6	84.3
Patient 8	81.2	0.0	46.5	60.2	92.4	91.2	93.6
Patient 9	83.8	0.0	46.7	55.0	85.5	82.0	86.8
Patient 10	69.9	0.0	31.0	40.5	82.7	76.9	84.7
Patient 11	63.2	0.0	55.5	64.4	81.3	77.7	86.3
Patient 12	67.8	4.0	44.2	54.8	82.2	78.6	84.3
Patient 13	69.4	0.0	35.3	49.1	79.6	79.2	83.3
Patient 14	81.3	3.8	37.4	49.5	84.4	72.7	85.3
Patient 15	NA	NA	NA	NA	78.7	76.9	83.3
Patient 17	NA	NA	NA	NA	84.0	83.8	87.4
Patient 18	86.8	0.0	49.6	50.8	87.0	86.3	91.1
Patient 19	NA	NA	NA	NA	71.7	72.0	76.7
Patient 20	37.6	0.2	30.3	69.2	77.3	72.2	83.4
Patient 21	79.4	0.0	47.3	64.4	84.6	81.5	86.1
Patient 22	NA	NA	NA	NA	84.4	82.2	84.6
Patient 24	89.8	0.0	61.3	65.2	91.0	87.0	88.8
Patient 25	74.3	2.6	45.3	52.2	85.2	81.0	86.6
Patient 26	76.7	2.4	42.6	48.8	79.6	76.7	82.8
Patient 27	NA	NA	NA	NA	84.3	81.2	87.0
Patient 28	NA	NA	NA	NA	67.2	69.6	78.3
Patient 29	85.3	0.0	53.1	58.5	91.6	90.3	94.2
Patient 30	84.0	4.2	68.7	63.9	87.1	86.7	87.8
Patient 31	82.3	7.1	45.3	53.8	81.6	80.1	83.6
Patient 32	NA	NA	NA	NA	78.1	78.7	82.5
Mean	81.3	0.0	45.3	54.8	83.6	80.6	85.7

Results are shown as percentages of IgE-binding inhibition.

NA, Could not be analyzed.