

Reduction of the *in vivo* allergenicity of Der p 2, the major house-dust mite allergen, by genetic engineering

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Abstract

The major allergen of the house-dust mite *Dermatophagoides pteronyssinus*, Der p 2, is recognized by approximately 90% of mite-allergic patients.

We have produced two recombinant fragments of Der p 2 comprising aa 1–53 and aa 54–129 and a hybrid molecule (aa 54–129 + 1–53), combining the two fragments in inverse order, by genetic engineering. The recombinant Der p 2 derivatives were expressed in *E. coli* and purified to homogeneity. rDer p 2 derivatives (fragments and hybrid) showed a considerably reduced beta sheet structure and IgE reactivity compared to the Der p 2 wild-type allergen. The allergenic activity of the Der p 2 derivatives was reduced more than tenfold as evaluated *in vitro* in basophil activation assays and *in vivo* by skin prick testing of mite-allergic patients. Immunization of mice and rabbits with rDer p 2 derivatives induced Der p 2-specific IgG antibodies, which inhibited the binding of allergic patients' IgE to Der p 2. Immunization of mice with rDer p 2 derivatives induced less allergenic IgE responses than immunization with rDer p 2. Thus the rDer p 2 derivatives exhibited less *in vivo* allergenic activity and allergenicity than the Der p 2 allergen but preserved immunogenicity and may hence represent candidates for specific immunotherapy of house-dust mite allergy.

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1. Introduction

House-dust mite (HDM) allergy belongs to the most common allergies worldwide which affects more than 50% of all allergic patients (Boulet et al., 1997).

Among the different mite allergens, group 2 allergens were identified as the major allergens, against which more than 80% of mite-allergic patients are sensitized (Ford et al., 1990; Heymann et al., 1989; Pittner et al., 2004). Group 2 mite allergens are present in high concentration in mite faeces (Park et al., 2000; Tovey et al., 1981), possess high-IgE-binding capacity and aller-

genic activity (Pittner et al., 2004; van der Zee et al., 1988). Group 2 allergens were first characterized in the house-dust mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* as 14,000–18,000 Da cross-reactive allergens (Heymann et al., 1989; Holck et al., 1986; Lind, 1985). Isolation and characterization of cDNA clones coding for Der p 2 (Chua et al., 1990) and Der f 2 (Trudinger et al., 1991) revealed that the allergens represent proteins of 129 amino acids with a molecular weight of 14,000 Da and without *N*-glycosylation sites (Thomas et al., 1989). Der p 2 and Der f 2 show a sequence identity of 87% (Chua et al., 1996) and both group 2 allergens contain three disulfide bonds and are composed of two anti-parallel β -sheets (Derewenda et al., 2002; Johannessen et al., 2005; Mueller et al., 1997). The T-cell epitopes of Der p 2 are spread over the protein

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(van Neerven et al., 1993; O'Brien et al., 1995; O'Hehir et al., 1993) and, besides some IgE-reactive recombinant fragments, the majority of IgE-epitopes appear to be conformational (Chua et al., 1991; Ichikawa et al., 2005; Takai et al., 1997b).

Allergen-specific immunotherapy represents one of the few causative approaches for treatment of house-dust mite allergy. Several immunotherapy studies performed with mite allergen extracts have demonstrated clinical efficacy of this treatment (Pichler et al., 1997; Varney et al., 2003). However, immunotherapy with HDM extracts is less efficient than immunotherapy with pollen extracts (Bousquet et al., 1998), severe systemic side effects have been observed (Akçakaya et al., 2000) and it has been reported that HDM immunotherapy induces new IgE reactivities and sensitisation to sea-foods via the cross-reactive HDM allergen, Der p 10, tropomyosin (van Ree et al., 1996).

In order to reduce side effects in the course of immunotherapy, several strategies have been applied to develop hypoallergenic group 2 allergen derivatives (Korematsu et al., 2000; Nakazawa et al., 2005; Smith and Chapman, 1996; Takai et al., 2000, 1999, 1997a). Der p 2 variants were developed with reduced IgE reactivity by site-directed mutagenesis of cysteine residues (Korematsu et al., 2000; Smith and Chapman, 1996; Takai et al., 1997a), through N- and C-terminal deletions (Takai et al., 1999), or by introducing other mutations (Nakazawa et al., 2005; Takai et al., 2000). It has been demonstrated that these group 2 allergen derivatives exhibited reduced IgE reactivity and allergenic activity as studied by histamine release experiments using basophils from HDM allergic patients.

Immunotherapy studies performed with genetically engineered derivatives of the major birch pollen allergen Bet v 1, as well as with recombinant grass pollen allergens have shown that the induction of allergen-specific IgG antibodies is an important mechanism underlying this treatment (Gafvelin et al., 2005; Jutel et al., 2005; Niederberger et al., 2004; Reisinger et al., 2005). Therapy-induced allergen-specific IgG antibodies block allergen-induced immediate type reactions (Niederberger et al., 2004; Reisinger et al., 2005), allergen-induced increases of IgE production (Niederberger et al., 2004) and IgE-facilitated allergen presentation to T-cells (van Neerven et al., 2006). However, vaccination with allergens and recombinant allergen derivatives may also induce allergen-specific IgE antibodies (Moverare et al., 2002).

In this study we aimed to construct genetically modified Der p 2 derivatives which are characterized by reduced allergenic activity, retained immunogenicity and which should induce less

allergen-specific IgE responses than the wild-type allergen (i.e., reduced allergenicity). Two recombinant fragments of the group 2 allergen of *D. pteronyssinus* (Der p 2) comprising aa 1–53 and aa 54–129, were produced to destroy conformational B-cell epitopes and to retain the major T-cell epitopes. Additionally, a recombinant Der p 2 hybrid molecule (aa 54–129 + 1–53), in which the two rDer p 2 fragments were recombined in inverse order by PCR-based gene-SOEing of the corresponding cDNAs, was constructed with the aim to combine the two candidate vaccines within one molecule. The recombinant Der p 2 derivatives were expressed in *E. coli*, purified to homogeneity and analyzed regarding their fold by circular dichroism. Furthermore, we studied the IgE reactivity of the derivatives with serum IgE from allergic patients and their allergenic activity by basophil histamine release experiments *in vitro* and by skin testing of allergic patients *in vivo*. Mice and rabbits were immunized to study whether rDer p 2 derivatives can induce IgG antibodies, which recognize the wild-type allergen and inhibit patients' IgE reactivity. Furthermore, the *in vivo* allergenicity (i.e., induction of Der p 2-specific IgE responses) of rDer p 2 derivatives was compared with the Der p 2 wild-type allergen to study their potential usefulness for prophylactic vaccination.

2. Materials and methods

2.1. Patient sera, plasmids and *E. coli* strains

Allergic patients from Austria (patients #1–20) and from Greece (patients #21–26) as well as non-allergic control individuals were characterized by case history, skin prick testing and serological analysis as described (Pittner et al., 2004). Plasmid pET17b, containing the ampicillin resistance and a T7 promoter, was obtained from Novagen Inc. (Madison, WI). Recombinant proteins were expressed in λ DE3 lysogens of *E. coli* strain BL21 (Novagen Inc.).

2.2. Expression in *E. coli* and purification of Der p 2, Der p 2 fragments and Der p 2 hybrid

cDNAs coding for His-tagged Der p 2, Der p 2 fragments (aa 1–53 and aa 54–129) and Der p 2 hybrid (aa 54–129 + 1–53) were generated by PCR amplification using primers (MWG, Ebersberg, Germany) as indicated in Table 1 and a Der p 2 cDNA which was obtained by reverse transcription from *D. pteronyssinus* RNA. Primers 1 and 4 were used for the ampli-

Table 1
PCR primers used for the amplification of cDNAs coding for Der p 2, rDer p 2 fragments and the rDer p 2 hybrid

Primer	Sequence
1 (F)	5'- GGAATTC CATATGGATCAAGTCGATGTC-3'
2 (R)	5'- GGAATTC CTTAGTGATGGTGATGGTGATGTTCAATTTTAGCGGT-3'
3 (F)	5'- GGAATTC CATATGATCAAAGCCTCAAT-3'
4 (R)	5'- GGAATTC CTTAGTGATGGTGATGGTGATGATCGCGGATTTTA-3'
5 (overlapping)	5'-CTTTGACATCGACTTGATCATCGCGGATTTTAGCAT-3'
6 (overlapping)	5'-CATGCTAAATCCGCGATGATCAAGTCGATGTCAAA-3'

Forward (F), reverse (R) and overlapping primers are indicated. The *EcoRI* sites are shown in bold letters and *NdeI* sites are underlined. Nucleotides coding for the His-tags are shown in italic letters.

fication of the rDer p 2 cDNA, primers 1 and 2 for the cDNA coding for rDer p 2 fragment 1 (aa 1–53) and primers 3 and 4 for the cDNA of the rDer p 2 fragment 2 (aa 54–129). rDer p 2 hybrid was generated by PCR-based gene-SOEing (Horton, 1995) using primers 2 and 3 and the two overlapping primers 5 and 6. Upstream primers contained an *NdeI* and *EcoRI* site and downstream primers contained an *EcoRI* site as well as six His codons. PCR products were cut with *NdeI/EcoRI*, gel-purified and subcloned into the *NdeI/EcoRI* sites of plasmid pET17b. The calcium chloride method was used for the transformation of the plasmids into *E. coli* strain XL-1 Blue (Sambrook et al., 1989). Plasmid DNA was isolated by NucleoBond AX Kit—maxi-prep (Macherey-Nagel, Düren, Germany) and the sequence of the cDNA inserts was confirmed by sequencing both DNA strands on an automated sequencing system (MWG, Ebersberg, Germany).

Recombinant proteins containing C-terminal hexahistidine-tails were expressed in *E. coli* strain BL21 (DE3) in liquid culture by induction with 0.5 mM isopropyl- β -thiogalactopyranoside (IPTG) at an OD₆₀₀ of 1 for 5 h at 37 °C. Cells were harvested by centrifugation at 4000 $\times$ g for 15 min at 4 °C.

The bacterial pellets obtained from 1 L liquid culture were resuspended in 10 ml 25 mM imidazol, pH 7.4, 0.1% (v/v) Triton X-100 and treated with 100 μ g lysozyme for 30 min at room temperature. Cells were lysed by three freeze/thawing cycles (–70 °C/+50 °C), DNA was degraded by incubation with 1 μ g DNase I for 10 min at room temperature and cell debris were removed by centrifugation at 10,000 $\times$ g for 30 min at 4 °C. rDer p 2 fragment 1 was found in the soluble fraction and purified under native conditions using Ni-NTA resin affinity columns (QIAGEN, Hilden, Germany).

rDer p 2, rDer p 2 fragment 2 and rDer p 2 hybrid were found in the pellet in the inclusion body fraction, which was solubilized with 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris–Cl, pH 8 for 60 min at room temperature. Insoluble residues were removed by centrifugation (10,000 $\times$ g, 15 min, 4 °C) and urea was removed by stepwise dialysis. After centrifugation (10,000 $\times$ g, 15 min, 4 °C), rDer p 2, rDer p 2 fragment 2 and rDer p 2 hybrid were purified under native conditions over Ni-NTA resin affinity columns (QIAGEN, Hilden, Germany).

Fractions, containing recombinant proteins of more than 90% purity were dialysed against 50 mM NaH₂PO₄ pH 7 and the final protein concentrations were determined by Micro-BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.3. Matrix-assisted laser desorption and ionization-time of flight (MALDI-ToF) mass spectrometry of rDer p 2 and rDer p 2 derivatives

Laser desorption mass spectra were acquired in a linear mode with a time of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.; piCHEM, Graz, Austria). Samples were dissolved in 10% acetonitrile, 0.1% trifluoroacetic acid and α -cyano-4 hydroxy-cinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

2.4. Circular dichroism (CD) analysis

The CD spectra of the purified recombinant proteins were measured on a JASCO (Tokyo, Japan) J-810 spectropolarimeter. CD measurements were performed with rDer p 2 (in 12.5 mM NaH₂PO₄ pH 7), with rDer p 2 fragment 1 (in 5 mM NaH₂PO₄ pH 7), rDer p 2 fragment 2 (in 20 mM NaH₂PO₄ pH 7) and with rDer p 2 hybrid (in 12.5 mM NaH₂PO₄ pH 7) at protein concentrations of 0.1 mg/ml 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 resulted from averaging of three scans. The final spectra were corrected by subtracting the baseline spectra obtained with the corresponding buffers under identical conditions. Results are expressed as the mean residue ellipticity (θ) at a given wavelength.

2.5. IgE reactivity of dot-blotted rDer p 2 and rDer p 2 derivatives

Purified recombinant Der p 2, the two rDer p 2 fragments, fragment 1 (aa 1–53) and fragment 2 (aa 54–129), and rDer p 2 hybrid were tested for IgE reactivity by RAST-based, non-denaturing dot blot assays. Two microlitres of the purified proteins (0.1 mg/ml) and, for control purposes, BSA were dotted onto nitrocellulose membrane strips (Schleicher & Schuell, Dassel, Germany).

Nitrocellulose strips containing the dot-blotted proteins were blocked in buffer A (40 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, pH 7.5, 0.5% Tween 20, 0.5% [w/v] BSA, 0.05% [w/v] NaN₃) and incubated with sera from mite-allergic patients, serum from a non-allergic person (dilutions 1:10), buffer A without serum and as positive control, with a 1:1000 diluted rabbit anti-rDer p 2 antiserum. Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies, bound rabbit antibodies with a ¹²⁵I-labeled donkey anti-rabbit Ig antiserum (Amersham, Buckinghamshire, UK) and visualized by autoradiography (Valenta et al., 1992).

2.6. Allergen-induced CD203c expression

Heparinized blood samples were obtained from allergic patients after informed consent was given. Blood samples (100 μ l) were incubated with various concentrations of rDer p 2, rDer p 2 fragments, rDer p 2 hybrid, a monoclonal anti-IgE antibody (Immunotech, Marseille, France), or PBS for 15 min (37 °C). CD 203c expression was determined as described (Hauswirth et al., 2002).

2.7. Skin prick testing

The skin prick study was approved by the institution's ethics committee. Skin prick tests were performed on the forearms of mite-allergic patients as described (Heiss et al., 1999). Patients were pricked with 20 μ l aliquots containing different concentrations (32, 16, 4 and 1 μ g/ml) of purified rDer p 2, rDer p 2 fragment 1 (aa 1–53), rDer p 2 fragment 2 (aa

54–129) and rDer p 2 hybrid. Histamine and NaCl (ALK, Horsholm, Denmark) were applied as positive and negative control.

2.8. Immunization of rabbits, immunization of mice, measurement of Der p 2-specific antibody responses

Rabbit antisera were obtained by immunizing rabbits three times in monthly intervals with 200 µg of each purified protein (rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2, rDer p 2 hybrid) once in Freund's complete and twice in Freund's incomplete adjuvant (Charles River, Kisslegg, Germany).

Eight-week-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany). The animals were maintained in the Animal Care Unit of the Institute of Pathophysiology of the Medical University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized with 5 µg of purified proteins (rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 or rDer p 2 hybrid), adsorbed to 200 µl of AluGel-S (SERVA Electrophoresis, Heidelberg, Germany) subcutaneously in the neck in 4 weeks intervals over a period of 20 weeks. Blood samples were collected 1 day before each immunization and stored at -20°C .

For determination of Der p 2-specific IgG antibody responses, ELISA plates (Greiner, Kremsmünster, Austria) were coated with rDer p 2 diluted in PBS ($c = 5 \mu\text{g/ml}$) over night at 4°C . The plates were washed twice with PBST (PBS; 0.05% [v/v] Tween 20) and blocked with blocking buffer (PBST, 1% [w/v] BSA) for 3 h at room temperature. Mouse sera were diluted 1:1000 in PBST, 0.5% (w/v) BSA and 100 µl of this dilution was added per well overnight at 4°C .

Plates were washed five times with PBST and bound IgG₁ antibodies were detected with a monoclonal rat anti-mouse IgG₁ antibody (BD Pharmingen, San Diego, CA, USA), followed by the addition of horseradish peroxidase-labeled goat anti-rat IgG antibodies (Amersham Bioscience, Uppsala, Sweden) as described (Vrtala et al., 1996).

2.9. Inhibition of human IgE binding to rDer p 2 by IgG antibodies as determined by ELISA inhibition assays

ELISA plates (Greiner, Austria) were coated with 100 µl purified rDer p 2, diluted with PBS to a concentration of 5 µg/ml, over night at 4°C . After washing twice with PBST and blocking with blocking buffer (PBST, 1% [w/v] BSA) for 3 h at room temperature, plates were incubated overnight at 4°C with anti-rDer p 2, anti-rDer p 2 fragment 1, anti-rDer p 2 fragment 2 or anti-rDer p 2 hybrid antisera or the corresponding preimmunesera. Mouse antisera were diluted 1:20 and rabbit antisera were diluted 1:100 in PBST, 0.5% (w/v) BSA. After washing, the plates were incubated with 1:10 diluted sera from mite-allergic patients overnight at 4°C and bound human IgE antibodies were detected with HRP-coupled goat anti-human IgE antibodies (KPL, Gaithersburg, MD) diluted 1:2500 in PBST, 0.5% (w/v) BSA as described (Swoboda et al., 2002). The percentage of inhibition of IgE binding was calculated as follows: $100 - (\text{OD}_s/\text{OD}_p) \times 100$, where OD_s and OD_p represent the extinction coefficients after prein-

cubation with the immune serum and the preimmune serum, respectively.

2.10. β -Hexosaminidase release assay from RBL-2H3 basophils

Rat basophil leukemia (RBL) cells (subline RBL-2H3) (Eccleston et al., 1973) were incubated on ELISA plates (Nunc, Roskilde, Denmark) (4×10^4 cells in 100 µl) in cell culture medium (100 ml RPMI 1649, 10% FCS, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 100 µM 2-mercaptoethanol, 1% Pen/Strep) overnight at 37°C , 5% CO_2 .

Cells were loaded with 2 µl serum aliquots from mice immunized with rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 or rDer p 2 hybrid for 2 h at 37°C , washed twice with 200 µl Tyrode/BSA buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl_2 , 1.8 mM CaCl_2 , 0.4 mM NaH_2PO_4 , 5.6 mM D-glucose, 12 mM NaHCO_3 , 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin, pH 7.2) (Sigma–Aldrich, Vienna, Austria) and then stimulated with rDer p 2 ($c = 0.3 \mu\text{g/ml}$). Total β -hexosaminidase release was induced by addition of 10 µl 10% (v/v) Triton X-100 (Merck, Darmstadt, Germany).

For measuring the release of β -hexosaminidase, 50 µl assay solution (80 µM 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate buffer, pH 4.5) was incubated with 50 µl supernatant for 1 h at 37°C , 5% CO_2 .

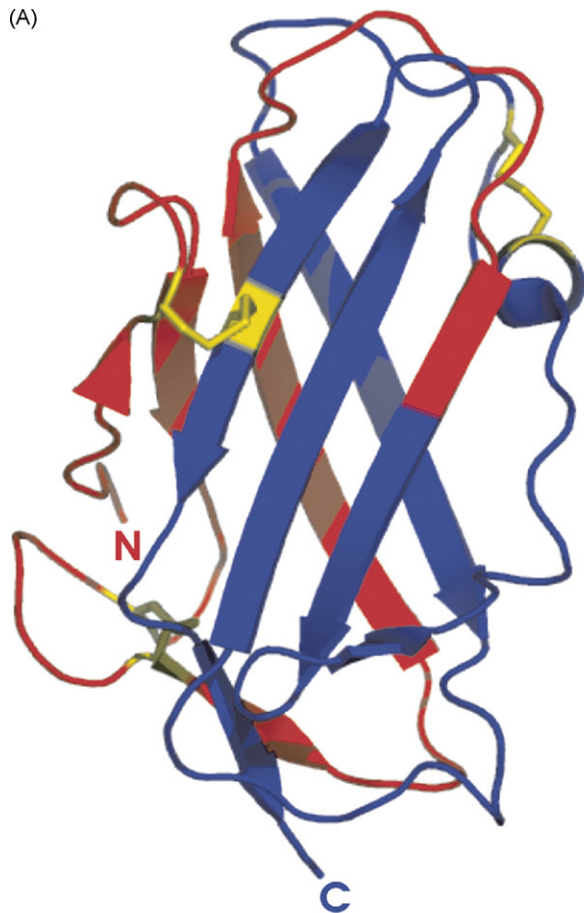
The reaction was stopped by adding 100 µl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex} : 360 nm and λ_{em} : 465 nm using a fluorescence microplate reader (Dynatech MR 7000, Dynatech Laboratories, Chantilly, VA, USA). Results are shown as mean percentages of total β -hexosaminidase release. Statistically significant differences of Der p 2-induced degranulation between RBL cells loaded with anti-Der p 2 mouse sera and RBL cells loaded with anti-Der p 2 derivative mouse sera were assessed with unpaired Mann–Whitney tests. SPSS statistical software system (SPSS Inc., Chicago, IL) was used for calculations. The reported p -values are the results of a two-sided test, corrected by Shaffer coefficient. A p -value smaller or equal to 5% is considered statistically significant.

3. Results

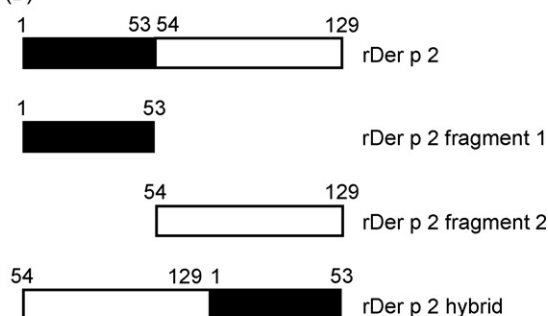
3.1. Expression, purification and physico-chemical characterization of rDer p 2, rDer p 2 fragments and rDer p 2 hybrid

Two fragments of Der p 2, comprising aa 1–53 and aa 54–129, as well as a hybrid molecule consisting of the two fragments in inverse order (aa 54–129 + 1–53) were expressed in *E. coli* with the aim to disrupt at least one of the two β -sheets of Der p 2 and the disulfide bond between C8 and C119 (Fig. 1A and B) (Derewenda et al., 2002; Mueller et al., 1997). The disruption of conformation was thought to destroy conformational IgE epitopes of Der p 2 (Chua et al., 1991; Takai et al., 1997b) but to preserve major T-cell epitopes (van Neerven et al., 1993;

(A)



(B)



(C)

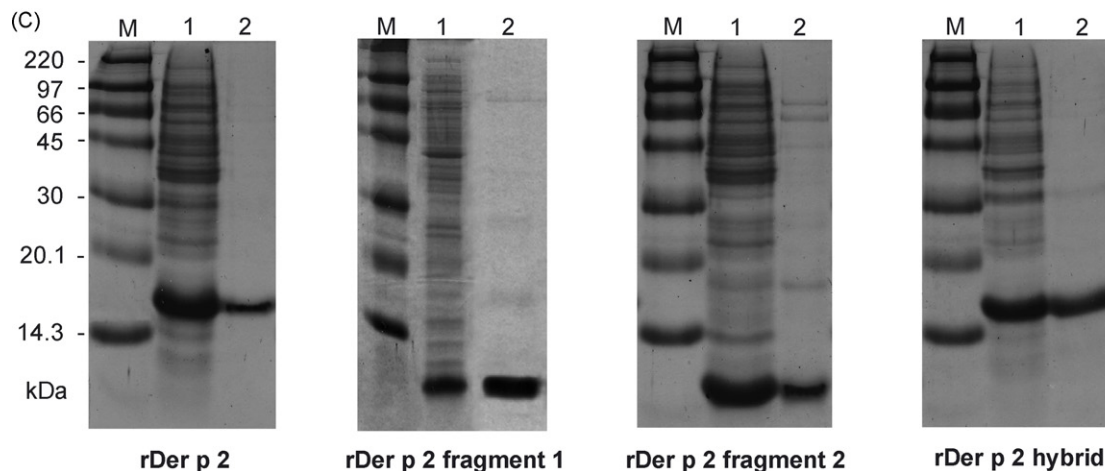


Fig. 1. Construction and expression of rDer p 2 derivatives. (A) The structure of Der p 2 (Derewenda et al., 2002) is shown as a ribbon diagram, with the sequences of Der p 2 fragment 1 (aa 1–53) and Der p 2 fragment 2 (aa 54–129) colored in red and blue, respectively. The side chains of the six cysteines forming three intramolecular disulfide bridges are drawn in stick representation and colored yellow. The image was generated with the program PyMOL (DeLano, 2002). (B) Two recombinant fragments of Der p 2 comprising aminoacids (aa) 1–53 and aa 54–129 (fragment 1 and fragment 2) were expressed in *E. coli*. A rDer p 2 hybrid molecule was obtained by tail-to-head fusion of the cDNAs coding for the two fragments (aa 54–129 + 1–53) by PCR-based gene-SOEing and subsequent expression in *E. coli*. (C) Coomassie-stained SDS-PAGE containing protein extracts of BL21 (DE3) expressing rDer p 2 and rDer p 2 derivatives as his-tagged proteins (lanes 1), purified rDer p 2, rDer p 2 fragments and rDer p 2 hybrid (lanes 2), and a molecular marker (lanes M).

O’Hehir et al., 1993; Verhoef et al., 1993). The rDer p 2 derivatives were overexpressed in *E. coli* (Fig. 1C, lanes 1). rDer p 2 fragment 1 was found in the soluble fraction, whereas the other proteins accumulated in the insoluble inclusion body frac-

tions but could be solubilized in urea. rDer p 2 and rDer p 2 derivatives were then purified by nickel affinity chromatography (Fig. 1C, lanes 2) yielding 20–30 mg protein/L *E. coli* culture. After refolding by dialysis, rDer p 2 and the rDer p 2 derivatives

remained soluble in physiological buffers at concentrations from 0.4 to 1 mg/ml. SDS-PAGE analysis indicated that the proteins were more than 90% pure and that they formed also dimers at the given concentrations (Fig. 1C, lanes 2).

Analysis of the proteins by MALDI-ToF mass spectrometry allowed to determine molecular masses of 15027.9, 6806.7, 9216.3 and 15001.8 Da for rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 and rDer p 2 hybrid, respectively, which are in

agreement with the theoretical masses of the proteins calculated from their amino acid sequences (Fig. 2A).

The far-ultraviolet CD spectrum of purified recombinant Der p 2 shows a negative band at 215 nm and a positive band at 197 nm, indicating a β -sheet conformation (Fig. 2B). In contrast, the CD spectra of the rDer p 2 derivatives indicate that these proteins are mainly random coiled. rDer p 2 fragment 1 showed a typical random coil conformation, identified by a negative band

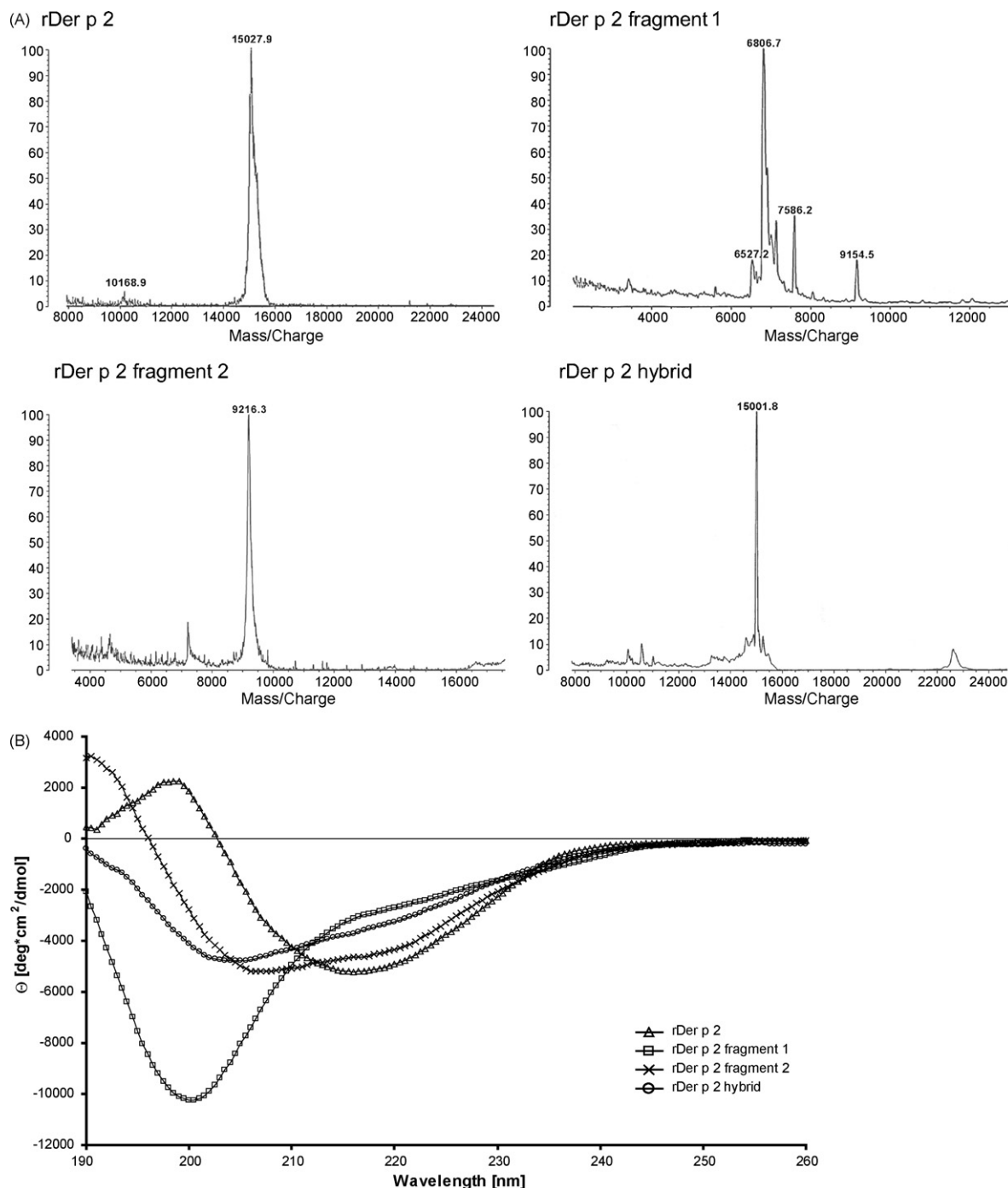


Fig. 2. Mass spectrometry analysis and far-ultraviolet CD spectra of purified rDer p 2 and rDer p 2 derivatives. (A) MALDI-ToF MS analysis was performed with purified rDer p 2, rDer p 2 fragments 1 and 2 and rDer p 2 hybrid. The x-axes show the mass/charge ratios and the signal intensities are displayed on the y-axes as percentages of the most intensive signals. (B) Results of the far-UV CD analysis of the proteins are expressed as mean residue ellipticities (y-axis) at given wavelengths (x-axis).

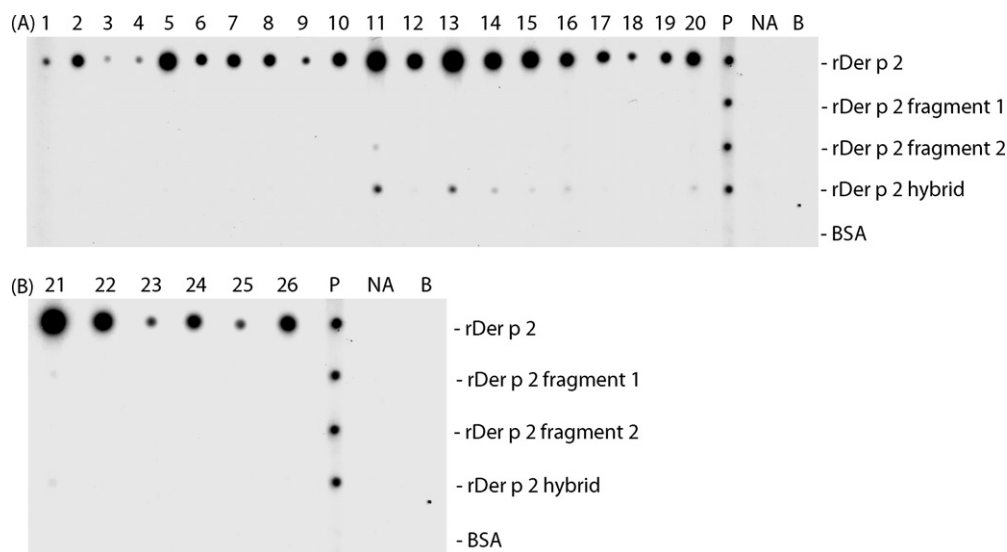


Fig. 3. IgE reactivity of recombinant Der p 2 and recombinant Der p 2 derivatives. Sera from mite-allergic patients (Austrian patients 1–20, A; Greek patients 21–26, B), a rabbit anti-Der p 2 antiserum (P), a non-allergic individual (lane NA) and buffer without serum (lane B) were tested for reactivity with dot-blotted recombinant Der p 2, rDer p 2 fragments, rDer p 2 hybrid and BSA. Bound human IgE and rabbit IgG were detected with ^{125} I-labeled anti-human IgE antibodies and a ^{125} I-labeled donkey anti-rabbit IgG antiserum, respectively and visualized by autoradiography.

at ~ 200 nm. Also rDer p 2 fragment 2 and rDer p 2 hybrid exhibited mainly random coil conformation with small amounts of residual β -sheet structures (Fig. 2B).

3.2. Reduced IgE reactivity of recombinant Der p 2 derivatives

The IgE reactivity of rDer p 2 wild-type allergen was compared with the two rDer p 2 fragments and rDer p 2 hybrid in non-denaturing dot blot assays (Fig. 3). Sera from 20 mite-allergic individuals from Austria (lanes 1–20) showed varying IgE reactivity to nitrocellulose-dotted rDer p 2, but almost no IgE reactivity to the rDer p 2 fragments could be detected. Some of the sera showed weak IgE reactivity with rDer p 2 hybrid (Fig. 3A). Similar results were obtained with sera from mite-allergic patients from Greece (lanes 21–26) (Fig. 3B). Again, almost no IgE reactivity with the rDer p 2 derivatives was found (Fig. 3B). A rabbit anti-rDer p 2 antiserum showed reactivity with rDer p 2 as well as with each of the rDer p 2 derivatives (P), demonstrating that the proteins had been transferred to the membrane. Serum from a non-allergic person (NA) as well as buffer without serum (B) showed no IgE reactivity to rDer p 2 or to rDer p 2 derivatives (Fig. 3, lanes NA, B). No IgE reactivity to the control protein, BSA, was found (Fig. 3).

3.3. Reduced allergenic activity of rDer p 2 derivatives as determined by *in vitro* CD 203c expression and *in vivo* skin prick testing

The upregulation of CD 203c has been described as a surrogate marker for allergen-induced basophil activation and degranulation (Hauswirth et al., 2002). We therefore compared the allergenic activity of recombinant Der p 2, rDer p 2 frag-

ments and rDer p 2 hybrid by measuring CD 203c upregulation on basophils from 12 house-dust mite-allergic patients (Fig. 4). Fig. 4A shows representative results from 3 patients. Incubation of basophils with 10 μ g/ml of rDer p 2 wild-type significantly upregulated CD 203c expression in each of the tested patients, whereas no upregulation was obtained with the same concentration of each of the fragments or with an equimolar mixture of the two fragments (Fig. 4A). Additionally, basophils from 12 patients were exposed to different concentrations (0.32 ng/ml to 5 μ g/ml in 1:5 dilution steps) of rDer p 2 and rDer p 2 hybrid. Fig. 4B shows the results from seven representative patients. Exposure of basophils with rDer p 2 hybrid resulted in an upregulation of CD 203c expression at concentrations between 8 and 5000 ng/ml, whereas rDer p 2 wild-type induced upregulation of CD 203c already at concentrations between 1.6 and 200 ng/ml. The rDer p 2 hybrid had a 5–100-fold reduced capacity to activate basophils compared to rDer p 2 in five out of seven tested patients. In the other two patients the allergenic activity of the rDer p 2 hybrid was less than that of the rDer p 2 wild-type in each of the concentrations tested (approximately 2-fold reduction) (Fig. 4B).

Anti-human IgE antibodies induced upregulation of CD 203c expression on basophils from all patients, whereas no upregulation was obtained with buffer alone (Fig. 4).

To compare the biological activity of rDer p 2 and rDer p 2 derivatives *in vivo*, skin prick tests were performed on the forearms of six mite-allergic patients from Greece. The results of the skin prick tests confirmed the reduced allergenic activity of the rDer p 2 derivatives observed *in vitro* (Table 2). The rDer p 2 fragments did not induce any wheal reaction up to a concentration of 32 μ g/ml except a very weak reaction with rDer p 2 fragment 1 in one patient. The skin reactions induced with the rDer p 2 hybrid were approximately 10-fold reduced compared to that induced with rDer p 2 wild-type (Table 2).

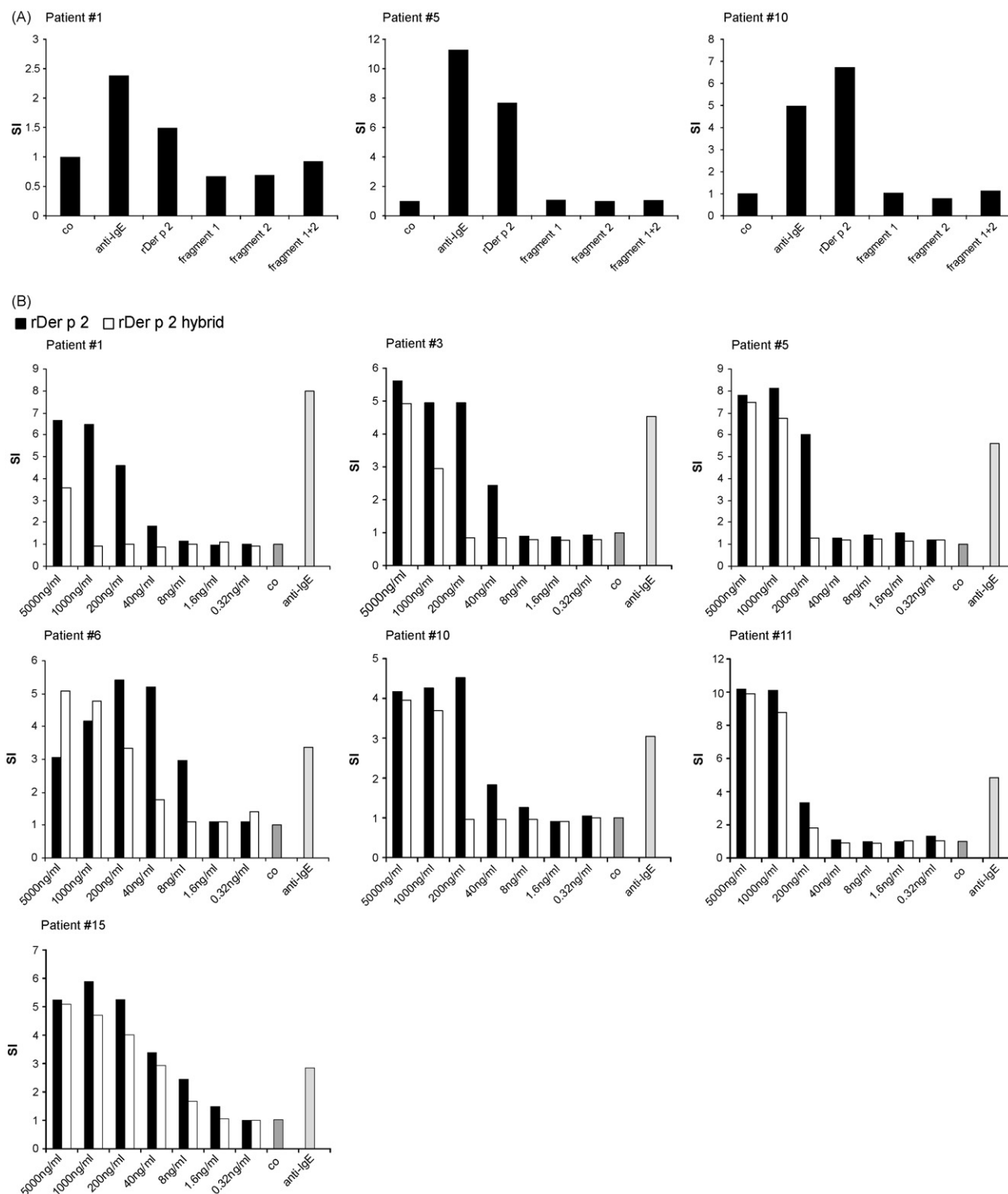


Fig. 4. Basophil activation by recombinant Der p 2 and rDer p 2 derivatives as measured by CD203c expression. (A) Blood samples from mite-allergic patients were exposed to 10 μ g/ml recombinant rDer p 2, each of the Der p 2 fragments, a mixture of the fragments, anti-IgE or buffer (co). The results of three representative patients (1, 5 and 10) are shown. (B) Blood samples from the same mite-allergic patients were exposed to several concentrations of rDer p 2 and rDer p 2 hybrid, anti-IgE or buffer (x-axes). The results of seven representative patients (1, 3, 5, 6, 10, 11 and 15) are shown. CD203c expression was determined by FACS analysis and is displayed as stimulation index (SI) (y-axes).

Table 2
Reduced *in vivo* allergenic activity of rDer p 2 derivatives as shown by skin testing of allergic patients

Patient #	Control		Dp	Concentration (μg/ml)	rDer p 2	rDer p 2 fragment 1	rDer p 2 fragment 2	rDer p 2 hybrid
	+	–						
21	4	0	5	1	3	0	0	0
				4	6	0	0	0
				16	7	0	0	3.25
				32	7	0	0	3.5
22	8	0	8.5	1	0	0	0	0
				4	6	0	0	0
				16	13.5	0	0	2.5
				32	9	0	0	4.5
23	3	0	nd	1	0	0	0	0
				4	0	0	0	0
				16	6.5	0	0	0
				32	7	0	0	0
24	7	0	8	1	4	0	0	0
				4	6	0	0	0
				16	8.5	0	0	2.5
				32	10	0	0	3.5
25	4.5	0	5.5	1	0	0	0	0
				4	0	0	0	0
				16	4.5	0	0	1
				32	5.5	1.5	0	2.5
26	4	0	6	1	0	0	0	0
				4	0	0	0	0
				16	5	0	0	0
				32	6	0	0	1.5

(+) Histamine; (–) NaCl; nd, not determined; Dp, *Dermatophagoides pteronyssinus* extract. The results are displayed as mean wheal diameters in mm.

3.4. rDer p 2 derivatives induce rDer p 2-specific IgG antibodies in mice similar as rDer p 2 wild-type allergen

Der p 2-specific IgG₁ levels were determined in serum samples obtained from mice after immunization with rDer p 2 and rDer p 2 derivatives (Fig. 5). rDer p 2 as well as the rDer p 2 derivatives induced Der p 2-specific IgG₁ responses in mice

which became detectable after the first immunization (week 4) (Fig. 5). After the second immunization (week 8), the IgG₁ responses induced with rDer p 2 fragment 1 and rDer p 2 hybrid were even higher than those induced with rDer p 2 (Fig. 5). After the last immunization (week 16), IgG₁ responses induced with the rDer p 2 derivatives were comparable to those induced with the rDer p 2 wild-type molecule (Fig. 5).

3.5. IgG₁ antibodies induced by immunization with rDer p 2 derivatives inhibit mite-allergic patients' IgE binding to rDer p 2 wild-type

Mouse IgG₁ antibodies induced by immunization with rDer p 2 and the rDer p 2 derivatives were investigated for their ability to inhibit mite-allergic patients' IgE binding to rDer p 2 in ELISA competition experiments.

The percentage of inhibition of allergic patients' IgE binding to rDer p 2 wild-type by mouse IgG antibodies is shown in Table 3A. The inhibition obtained with mouse anti-rDer p 2 antibodies was between 43 and 80% (mean 67%). Mouse anti-rDer p 2 hybrid antibodies, anti-rDer p 2 fragment 1 antibodies and anti-rDer p 2 fragment 2 antibodies inhibited serum IgE binding to rDer p 2 wild-type between 23 and 83% (mean 48%), between 23 and 82% (mean 30%) and between 46 and 80% (mean 56%), respectively (Table 3A). The inhibition with a mixture of anti-fragment 1 and anti-fragment 2 antibodies was between 32 and 80% (mean 52%) (Table 3A).

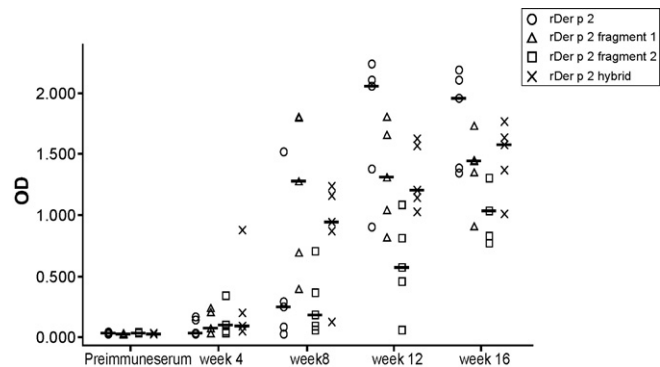


Fig. 5. Evolution of Der p 2-specific IgG₁ induced by immunization of mice with rDer p 2 and rDer p 2 derivatives. Groups of five mice each were immunized with purified rDer p 2 or rDer p 2 derivatives and Der p 2-specific IgG₁ antibodies were determined by ELISA at different time points (x-axis). The optical density values (OD 405 nm) displayed on the y-axis correspond to the levels of IgG₁ antibodies. The results are shown as scatter plots. Bars within the plots indicate the median values.

Table 3A

Inhibition of allergic patients' IgE binding to rDer p 2 by mouse anti-rDer p 2 and anti-rDer p 2 derivative antibodies

Patients #	Inhibition of IgE binding (OD _{pre} /OD _{immune}) (%)				
	rDer p 2 fragment 1	rDer p 2 fragment 2	Fragment 1 + fragment 2	rDer p 2 hybrid	rDer p 2
1	82 (0.864/0.155)	80 (0.748/0.150)	80 (0.778/0.155)	83 (0.902/0.156)	69 (0.547/0.167)
3	23 (0.214/0.165)	46 (0.219/0.119)	32 (0.227/0.155)	42 (0.226/0.131)	59 (0.224/0.093)
5	33 (0.927/0.621)	55 (0.901/0.408)	47 (0.918/0.484)	68 (0.924/0.297)	80 (0.929/0.188)
7	25 (0.724/0.540)	53 (0.713/0.335)	43 (0.739/0.421)	59 (0.716/0.293)	62 (0.754/0.285)
10	37 (0.368/0.233)	52 (0.388/0.186)	51 (0.399/0.194)	43 (0.374/0.214)	68 (0.406/0.131)
11	47 (3.349/1.772)	61 (3.295/1.270)	59 (3.404/1.379)	37 (3.158/1.985)	60 (3.716/1.278)
12	37 (2.037/1.131)	49 (2.041/0.835)	52 (2.037/0.860)	45 (1.999/1.355)	70 (2.119/0.894)
13	44 (2.051/1.338)	59 (2.015/1.058)	58 (2.052/1.083)	32 (1.991/1.541)	58 (2.125/1.211)
14	35 (1.337/0.842)	48 (1.353/0.688)	47 (1.412/0.673)	23 (1.332/0.732)	43 (1.330/0.394)
Mean	30	56	52	48	67

Table 3B

Inhibition of allergic patients' IgE binding to rDer p 2 by rabbit anti-rDer p 2 and anti-rDer p 2 derivative antibodies

Patients #	Inhibition of IgE binding (OD _{pre} /OD _{immune}) (%)				
	rDer p 2 fragment 1	rDer p 2 fragment 2	Fragment 1 + fragment 2	rDer p 2 hybrid	rDer p 2
1	31 (0.147/0.102)	48 (0.154/0.080)	44 (0.158/0.089)	33 (0.154/0.103)	51 (0.171/0.084)
3	43 (0.246/0.139)	64 (0.248/0.089)	63 (0.252/0.092)	65 (0.245/0.085)	64 (0.248/0.089)
5	63 (1.293/0.475)	91 (1.340/0.116)	89 (1.353/0.146)	87 (1.322/0.173)	90 (1.306/0.125)
7	55 (0.949/0.428)	79 (0.957/0.197)	77 (0.980/0.224)	79 (0.956/0.196)	78 (0.989/0.214)
10	65 (0.489/0.172)	89 (0.498/0.053)	88 (0.539/0.067)	87 (0.511/0.065)	89 (0.496/0.056)
11	42 (3.667/2.121)	93 (3.660/0.252)	89 (3.650/0.388)	81 (3.650/0.683)	92 (3.650/0.289)
12	46 (2.644/1.458)	88 (2.693/0.204)	83 (2.696/0.308)	79 (2.633/0.400)	88 (2.829/0.206)
13	45 (2.553/1.541)	92 (2.536/0.298)	89 (2.617/0.468)	85 (2.602/0.565)	93 (2.666/0.329)
14	40 (1.725/0.937)	88 (1.771/0.213)	82 (1.806/0.301)	78 (1.767/0.363)	88 (1.889/0.229)
Mean	48	81	78	75	81

In additional experiments, rabbits were immunized with purified rDer p 2 and the three rDer p 2 derivatives. The ability of rabbit antisera to inhibit IgE binding to rDer p 2 was also tested by ELISA inhibition assays with sera from the same 9 mite-allergic patients (Table 3B). Rabbit anti-rDer p 2 antibodies inhibited patients' IgE binding to rDer p 2 between 51 and 93% (mean 81%), whereas anti-rDer p 2 hybrid antibodies inhibited human IgE binding between 33 and 87% (mean 75%). The inhibition obtained with rabbit anti-rDer p 2 fragment 1 antibodies was between 31 and 65% (mean 48%) and the inhibition with rabbit anti-rDer p 2 fragment 2 antibodies was between 48 and 93% (mean 81%). Using a mixture of the anti-fragment 1 and anti-fragment 2 antibodies, the inhibition of patients' IgE binding to rDer p 2 wild-type was between 44 and 89% (mean 78%) (Table 3B).

3.6. Reduced allergenicity of rDer p 2 derivatives compared to rDer p 2

To investigate whether vaccination with rDer p 2 derivatives induces allergic immune responses to Der p 2 wild-type allergen, mice immunized with rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 or rDer p 2 hybrid were analyzed regarding the formation of IgE antibodies. Serum samples from the mice were used to load RBL cells with IgE and to quantify the allergenic immune response to rDer p 2 wild-type allergen by RBL degranulation

experiments. The release obtained with rDer p 2 wild-type allergen in RBLs loaded with mouse rDer p 2 derivative antibodies, was low (rDer p 2 fragment 1: between 0 and 16.6% (mean 6.4%), rDer p 2 fragment 2: between 0.2 and 28.6% (mean 13.2%), and rDer p 2 hybrid: between 4.7 and 37.1% (mean 18.3%)). RBLs, loaded with anti-rDer p 2 wild-type antibodies showed significantly stronger release after stimulation with rDer p 2 wild-type (between 35 and 39% (mean 37%)) (Fig. 6).

4. Discussion

Der p 2 is one of the most important house-dust mite allergens according to IgE reactivity and *in vivo* assessment of allergenic activity (Ford et al., 1990; Heymann et al., 1989; Pittner et al., 2004). Immunotherapeutic strategies for the treatment or prophylaxis of house-dust mite allergy must therefore incorporate components, which allow vaccination or tolerance induction against Der p 2. One possibility to reduce therapy-induced side effects mediated by allergen-specific IgE antibodies is the production of recombinant hypoallergenic allergen derivatives by genetic engineering (Valenta, 2002; Valenta and Niederberger, 2007).

For the reduction of IgE reactivity and allergenic activity of Der p 2 we have applied in a first step the fragmentation strategy. This strategy has been used to produce two hypoallergenic fragments of the major birch pollen allergen Bet v 1 (Vrtala

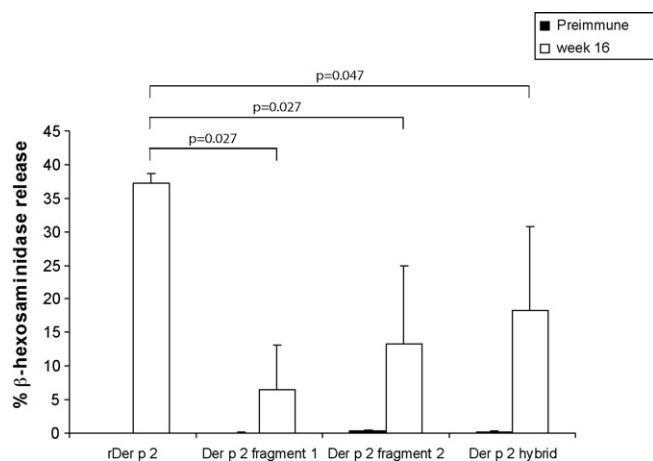


Fig. 6. Reduced allergenicity of rDer p 2 derivatives. Rat basophil leukemia (RBL) cells were loaded with sera from mice (preimmune bleeding, 16-week bleeding) immunized with rDer p 2 wild-type allergen or rDer p 2 derivatives (x-axis). rDer p 2-specific release of β -hexosaminidase is displayed as percentage of total β -hexosaminidase release (mean values $\pm$ S.D. for the five sera from each mouse group) (y-axis). Statistically significant differences ($p < 0.5$) between mouse-groups are indicated.

et al., 1997), which have already been administered to birch pollen allergic patients in a clinical trial (Niederberger et al., 2004).

The boundaries of the rDer p 2 fragments were chosen in order to disrupt the three-dimensional structure of Der p 2 and thus its conformational IgE epitopes (Chua et al., 1991; Takai et al., 1997b). The analysis of the Der p 2 fragments indeed demonstrated that their IgE reactivity and allergenic activity was strongly reduced. Next we combined the two fragments in an inverse order in the form of a hybrid molecule, which combined the C-terminal fragment at the N-terminus and the N-terminal fragment at the C-terminus. This hybrid molecule was engineered to incorporate the primary sequence of Der p 2 and thus the relevant T-cell epitopes (van Neerven et al., 1993; O'Brien et al., 1995; O'Hehir et al., 1993) within one molecule to ease the production of the potential vaccine and to retain the reduced allergenic activity. In fact, also the hybrid molecule exhibited a strongly reduced allergenic activity as shown by *in vitro* testing and *in vivo* skin prick testing of allergic patients. However, in two patients with IgE reactivity to dot-blotted rDer p 2 hybrid the reduction of allergenic activity of the hybrid versus the wild-type molecule was less pronounced.

Immunization experiments in mice and rabbits showed that all three rDer p 2 derivatives induced IgG antibodies, which inhibited IgE-binding from mite-allergic patients to Der p 2. Several immunotherapy studies provided evidence for the importance of blocking antibodies for successful immunotherapy and that these IgG antibodies are associated with clinical improvement (Gafvelin et al., 2005; Jutel et al., 2005; Niederberger et al., 2004; Reisinger et al., 2005). The molecules used in these clinical studies have been shown to induce blocking antibodies in *in vitro* and in animal experiments similar as the rDer p 2 derivatives. We therefore hope that the rDer p 2 derivatives will also induce protective antibodies and clinical improvement in patients.

Immunotherapy studies performed with natural allergens and recombinant allergen derivatives demonstrated that both of the active ingredients (natural allergens > hypoallergenic allergen derivatives) can induce IgE reactivities during vaccination (Moverare et al., 2002; Mothes et al., 2003; Niederberger et al., 2004).

We were therefore interested to study the recombinant Der p 2 derivatives and the Der p 2 wild-type allergen regarding the induction of IgE responses after vaccination.

We found that vaccination with Der p 2 induced significantly higher Der p 2-specific IgE responses in mice than immunization with the genetically modified Der p 2 derivatives. The reduced *in vivo* allergenicity of the Der p 2 derivatives may be due to their strongly altered three dimensional structure and may be considered as advantageous for therapeutic and especially prophylactic approaches for house-dust mite allergy.

The rDer p 2 fragments exhibited a more strongly reduced allergenic activity than the rDer p 2 hybrid. However, the Der p 2 hybrid incorporates the primary sequence information of Der p 2 within one molecule instead of the two fragments and can be easily produced in large amounts by recombinant expression in *E. coli*. Whether a mix of the two recombinant Der p 2 fragments or the rDer p 2 hybrid are better suited for either active vaccination or tolerance induction needs to be evaluated in clinical trials studying safety, immunogenicity and efficacy in patients.

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