A hypoallergenic cat vaccine based on Fel d 1–derived peptides fused to hepatitis B PreS

Katarzyna Niespodziana, MSc,[a] Margarete Focke-Tejkl, PhD,[b] Birgit Linhart, PhD,[a] Vera Civaj,[a] Katharina Blatt,[c] Peter Valent, MD,[c] Marianne van Hage, MD,[d] Hans Grönlund, PhD,[d] and Rudolf Valenta, MD[a,b] *Vienna, Austria, and Stockholm, Sweden*

Background: Allergen-specific immunotherapy is clinically effective for the treatment of cat allergy but shows a high rate of side effects.

Objective: We sought to engineer recombinant fusion proteins for cat immunotherapy that allow reducing both IgE-mediated and T cell–mediated side effects.

Methods: Fusion proteins consisting of the hepatitis B virus–derived PreS domain and 2 nonallergenic Fel d 1–derived peptides were expressed in *Escherichia coli* and purified. IgE reactivity and allergenic activity of Fel d 1 and the fusion proteins were compared by using IgE-binding assays and basophil activation tests in patients with cat allergy. Mice and rabbits were immunized subcutaneously with Fel d 1 and the fusion proteins to investigate the allergenicity of the vaccines and the development of Fel d 1–specific IgG antibodies.

Results: The recombinant fusion proteins showed no relevant IgE reactivity and exhibited more than 1000-fold reduced allergenic activity in basophil activation tests. On immunization of mice and rabbits, the fusion proteins induced Fel d 1–specific IgG antibodies that inhibited the binding of allergic patients' IgE to the allergen without allergic sensitization to Fel d 1.

Conclusion: The described recombinant fusion proteins exhibit strongly reduced IgE-mediated allergenic activity, contain less than 40% of the Fel d 1 sequence, and thus lack many of the specific T-cell epitopes. Therefore they should represent safe vaccines for the treatment of cat allergy. (J Allergy Clin Immunol 2011;127:1562-70.)

Key words: *Recombinant allergen, cat allergy, Fel d 1, peptide, hypoallergenic vaccine, immunotherapy*

The domestic cat *(Felis domesticus)* is one of the most common causes of IgE-mediated allergic diseases.[1-3] The severity of symptoms ranges from relatively mild rhinitis to life-threatening asthmatic responses. Allergen-specific immunotherapy (SIT) is the only disease-modifying treatment for IgE-mediated allergies that leads to long-lasting relief of symptoms.[4,5] Several studies have demonstrated the clinical efficacy of SIT for cat-induced asthma. In these patients SIT is associated with the induction of IgG antibodies specific for the major cat allergen Fel d 1 and reduced cutaneous and respiratory symptoms.[6-10] However, SIT with cat allergen extracts is often associated with a high rate of severe side effects that limit its broad applicability.[11] Because the majority of patients with cat allergy are almost exclusively sensitized to the major allergen Fel d 1, several strategies have been developed for the reduction of side effects during SIT.[3] One approach toward side effect–free SIT for cat allergy is based on the use of Fel d 1–derived T cell epitope–containing peptides without IgE reactivity.[12] The administration of these peptides has been thought to induce T-cell tolerance. Several clinical trials have been performed with Fel d 1–derived peptides. In these studies IgE-mediated immediate-type side effects could be eliminated, but a considerable number of patients experienced T cell–dependent late-phase side effects that preceded the suppression of chronic inflammation.[13-18] Another recently studied approach involves the coadministration of an anti-IgE antibody in the course of SIT to reduce IgE-mediated side effects.[19]

Here we report the construction of a novel type of vaccine for the treatment of cat allergy that should eliminate both IgE-mediated and T cell–mediated side effects. This approach is based on the selection of allergen-derived peptides that lack IgE reactivity and IgE-mediated allergenic activity and exhibit reduced T-cell reactivity. Coupled to a non–allergen-related carrier, they should then lead to a vaccine that induces allergen-specific IgG with T-cell help from carrier-derived epitopes.[20]

Recombinant fusion proteins consisting of the PreS domain of hepatitis B virus (HBV) containing nonallergenic Fel d 1 peptides at the N- and C-termini were expressed in *Escherichia coli* and purified. The PreS domain is a part of the large surface protein, which together with the middle and small surface proteins comprises the HBV envelope containing important antigenic sites for both B and T cells in the PreS sequence.[21-23] We report

From [a]the Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna; [b]the Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Medical University of Vienna; [c]the Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna; and [d]the Clinical Immunology and Allergy Unit, Department of Medicine, Karolinska Institutet and University Hospital, Stockholm.

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Reprint requests: Rudolf Valenta, MD, Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. E-mail: rudolf.valenta@meduniwien.ac.at.

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Abbreviations used
HBV: Hepatitis B virus
HRP: Horseradish peroxidase
RBL: Rat basophil leukemia
SI: Stimulation index
SIT: Allergen-specific immunotherapy

the engineering, expression, purification, and immunologic characterization of 3 different fusion molecules for vaccination against cat allergy. Furthermore, we demonstrate more than 1000-fold reduced allergenic activity of the fusion proteins and their ability to induce, on immunization, IgG antibodies that inhibit IgE binding of patients with cat allergy to Fel d 1.

METHODS

Allergic patients, recombinant allergens, synthetic peptides and antibodies

Patients with cat allergy (n = 23) and nonallergic control subjects (n = 4) were characterized by case history, skin prick test responses, and measurements of specific IgE antibodies.[24] Blood and serum samples were used after informed consent was obtained with approval of the local ethics committee. rFel d 1 was expressed in *E coli* and purified.[25] The rFel d 1–derived peptides P1 (EICPAVKRDVDLFLTGTPDEYVEQVAQYKALPVV) and P5 (MTTISSSKDCMGEAVQNTVEDLKLNTLGR) were synthesized.[26] Peptide-specific rabbit antibodies were obtained by immunizing rabbits with the keyhole limpet hemocyanin–coupled peptides (Charles River, Kissleg, Germany). For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

Expression and purification of recombinant PreS fusion proteins

The amino acid sequence of the PreS region of the HBV subtype adw was obtained from the National Center for Biotechnology Information database (GenBank: AAT28735.1). Genes (codon use optimized for *E coli* expression) coding for the PreS protein containing Fel d 1–derived peptides fused to the N- and C-termini (Fig 1) were synthesized (ATG:biosynthetics, Merzhausen, Germany) and inserted into the *Nde*I/*Xho*I sites of either pET-17b (PreS protein) or pET-27b (PreS fusion proteins; Novagen, Darmstadt, Germany). The DNA sequences were confirmed by means of restriction enzyme analysis of midi-prep DNA (Promega, Madison, Wish) with *Nde*I/*Xho*I (Roche, Mannheim, Germany) and by means of automated sequencing of both DNA strands.

Recombinant PreS and PreS fusion proteins were expressed in *E coli* strain BL21 (DE3; Stratagene, La Jolla, Calif). The protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mmol/L for 4 hours at 37°C. Cells were harvested by means of centrifugation at 3500 rpm for 10 minutes. The inclusion bodies containing the recombinant proteins were purified as follows. Cells were resuspended in 25 mmol/L imidazole and 0.1% Triton X-100 (pH 7.4; Buffer A), lysed with Ultra-Turrax (Janke & Kunkel-IKA Labortechnik, Staufen, Germany), and incubated for 30 minutes with 10 μg/mL DNaseI (Roche) at room temperature. After addition of 6% Triton X-100, 60 mmol/L EDTA, and 1.5 mol/L NaCl pH 7.0 (Buffer B), the pellets were collected by means of centrifugation at 18,000 rpm for 20 minutes at 4°C and washed again with Buffer B and subsequently with Buffer A. The inclusion bodies were solubilized in 6 mol/L guanidine hydrochloride, 100 mmol/L NaH_2PO_4, and 10 mmol/L Tris-HCl (pH 8.0) overnight at room temperature. The lysates were cleared by means of centrifugation and incubated with 2 mL of a previously equilibrated Ni-NTA resin for 2 hours (Qiagen, Hilden, Germany). The suspensions were then loaded onto a column, washed with 2 column volumes of washing buffer (8 mol/L urea, 100 mmol/L NaH_2PO_4, and 10 mmol/L Tris-HCl [pH 6.1]), and eluted with the same buffer (pH 3.5). Urea was removed by means of dialysis against 10 mmol/L NaH_2PO_4 (pH 4.8). The purity of recombinant proteins was analyzed by means of SDS-PAGE. The identity of the fusion proteins was confirmed with rabbit anti-P1, anti-P5, anti–rFel d 1, and anti-PreS anti-sera diluted 1:1000 by means of immunoblotting. Bound rabbit IgG was detected with iodine 125–labeled donkey anti-rabbit IgG antibodies (GE Healthcare, Buckinghamshire, United Kingdom) diluted 1:2000 and visualized by means of autoradiography. Protein concentrations were determined by using the BCA Protein Assay Kit (Novagen).

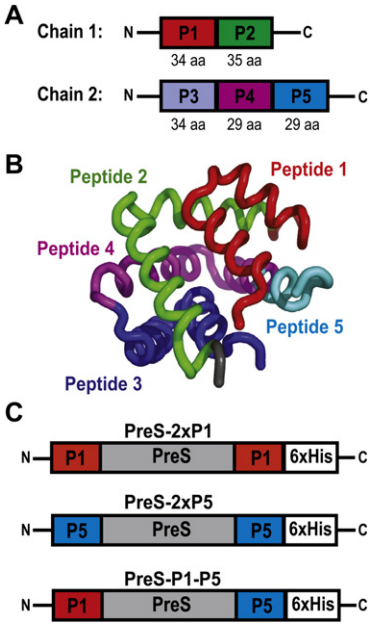

FIG 1. Construction of PreS fusion proteins. **A**, Chains 1 and 2 of Fel d 1 with peptides indicated. *aa*, Amino acid. **B**, Ribbon representation of Fel d 1 with peptides highlighted in different colors. **C**, PreS fusion proteins containing peptide 1 (*P1*; PreS-2xP1) or peptide 5 (*P5*; PreS-2xP5) at the N- and C-termini, as well as PreS-P1-P5 with an N-terminal P1 and C-terminal P5 peptide. Hexahistidine tags (6xHis) are at the C-terminal end.

IgE reactivity and allergenic activity of Fel d 1 and PreS fusion proteins

Allergic patients' IgE reactivity to recombinant PreS, PreS-2xP1, PreS-2xP5, PreS-P1-P5, and human serum albumin was determined with sera from 23 patients with cat allergy and 4 nonallergic subjects by means of ELISA with antigens bound to the solid phase or in the liquid phase (see the Methods section in this article's Online Repository).[27] *In vitro* basophil activation tests were performed with heparinized peripheral blood from 7 patients with cat allergy after informed consent was given (Table I, nos. 4, 7, 13, 14, 17, 18, and 23). Blood samples (100 μL) were incubated with serial dilutions of rFel d 1, PreS fusion proteins (0.01-0.00001 mg/mL), a monoclonal anti-IgE antibody (1 μg/mL; Immunotech, Marseille, France), or PBS for 15 minutes at 37°C. CD203c expression on basophils was measured by means of flow cytometry with the phycoerythrin-conjugated mAb 97A6 (CD203c). Allergen-induced upregulation of CD203c on basophils was expressed as the stimulation index, as described previously.[28]

Peptide-specific lymphoproliferation was assessed in PBMCs from patients with cat allergy (see the Methods section in this article's Online Repository).

Inhibition of allergic patients' IgE binding to rFel d 1 and CD203c expression on basophils by rabbit IgG antibodies

The inhibition of allergic patients' IgE binding to rFel d 1 by rabbit IgG antibodies raised against rFel d 1, PreS-2xP1, PreS-2xP5, and PreS-P1-P5 was measured by using the ELISA competition assay.[29-31] ELISA plates were