Allergy multivaccines created by DNA shuffling of tree pollen allergens

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Background: The major allergens of trees belonging to the Fagales order are collectively known as the Bet v 1 family. Members of the Fagales order have distinct geographic distribution, and it is expected that depending on the exposure pattern of the individual, inclusion of other Bet v 1 family members might increase the efficacy of the treatment.
Objective: We aimed to generate molecules that are suitable for specific immunotherapy not only against birch pollen allergy but also against allergies caused by other cross-reactive tree pollens.
Methods: Fourteen genes of the Bet v 1 family were randomly recombined *in vitro* by means of DNA shuffling. This library of chimeric proteins was screened for molecules displaying low capacity to induce release of inflammatory mediators but with T-cell immunogenicity higher than that of the parental allergens.
Results: Two chimeric proteins were selected from the library of shuffled clones displaying low allergenicity and high immunogenicity, as determined in *in vitro* assays using human and animal cells and antibodies, as well as *in vivo* in animal models of allergy.
Conclusion: Our results show that it is possible to randomly recombine *in vitro* T- and B-cell epitopes of a family of related allergens and to select chimeric proteins that perfectly match the criteria presently thought to be relevant for improving allergen-specific immunotherapy.
Clinical implications: The hypoallergenic chimeras described here recombine epitopes of the major Fagales pollen allergens and thus can efficiently substitute a mixture of extracts used for treating patients with tree pollen–induced spring pollinosis worldwide. (J Allergy Clin Immunol 2007;120:374-80.)

Key words: *Gene shuffling, tree pollen allergens, Bet v 1 family, T-cell epitope, IgE epitope, IgE cross-reactivity, genetic immunization, hypoallergen, allergen chimera*

Allergen-specific immunotherapy (SIT) is the only treatment that is capable of altering the natural course of allergic diseases. It involves the subcutaneous injection of increasing doses of allergen to suppress symptoms on subsequent exposure to that allergen.[1] The benefit of SIT lasts for several years, and its efficacy in seasonal allergic rhinitis has been confirmed in many clinical studies using grass, ragweed, tree pollen extracts,[2] and, more recently, recombinant wild-type[3] and genetically modified[4] allergens. SIT has been shown to prevent the onset of new sensitizations to different allergens and in children prevents the progression of hay fever to asthma.[5,6] Despite its conspicuous efficacy with crude allergen extracts or with wild-type recombinant allergens, SIT bears the risk of sporadic IgE-mediated side effects, including life-threatening systemic anaphylaxis.

In the temperate climate zone, spring pollinosis is mainly caused by birch and related trees, all belonging to the Fagales order. The major allergens of Fagales trees are collectively known as the Bet v 1 family because of strong IgE cross-reactivity between Bet v 1 and homologous allergens in alder (Aln g 1), hazel (Cor a 1), hornbeam (Car b 1), and oak (Que a 1) pollen.[7-10] For patients with tree pollen allergy, SIT is in some cases performed with a mixture of tree pollen extracts, usually birch, hazel, and alder.[11] Members of the Fagales order have distinct geographic distribution, and it is expected that depending on the exposure patterns of the patients, inclusion of other Bet v 1 family members might increase the efficacy of the treatment, particularly for those individuals living in birch-free areas.[12] To generate molecules that would be suitable for SIT not only against birch pollen allergy but also against allergies caused by other cross-reactive tree pollen of the Fagales order, we recombined *in vitro* genes coding for the Bet v 1 family of pollen allergens. Two chimeric proteins were selected, displaying low capacity to induce release of inflammatory mediators but high T-cell

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Supported by "Fonds zur Förderung der Wissenschaftlichen Forschung, FWF" Vienna, Austria, grants P16456-B05, NFN S88 (S8811, S8813), and FSB18 (F1802, F1807).

Disclosure of potential conflict of interest: L. Vogel and S. Vieths have received grants/research support from the European Union (EuroPrevall). S. Vieths is also an associate of the Institute for Product Quality in Berlin, Germany, and has received grants/research support from the German Research Society, the Research Fund of the German Food Industry, and Monsanto.

Received for publication February 16, 2007; revised April 20, 2007; accepted for publication May 16, 2007.

Available online July 12, 2007.

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0091-6749/$32.00

doi:10.1016/j.jaci.2007.05.021

Abbreviations used
ELISpot: Enzyme-linked immunosorbent spot
RBL: Rat basophilic leukemia
SIT: Allergen-specific immunotherapy
TCL: T-cell line
TPC: Tree pollen chimera

immunogenicity. The hypoallergenic chimeras described here recombine epitopes of the major allergens from different trees and thus might efficiently substitute mixture of extracts used for treating tree pollen allergy, thereby increasing the efficacy of the treatment and reducing the risks of side effects for the patient.

METHODS

Patients and sera

Patients with Fagales pollen allergy from Austria were selected on the basis of typical case history, positive *in vivo* skin prick test responses, and *in vitro* IgE detection (CAP System; Phadia, Uppsala, Sweden). Patients with IgE values of greater than class 3 were selected. For screening of the tree pollen shuffled library, a serum pool comprising 42 patients was assembled. Experiments with blood samples from patients with pollen allergy were approved by the Ethics Committee of the Medical University and the General Hospital of Vienna (no. EK028/2006). Informed written consent was obtained from all subjects included in the study.

Recombinant allergens

Recombinant parental allergens used as references (rBet v 1a, rCor a 1/11, and rAln g 1) were purchased from Biomay (Vienna, Austria).

Bacterial strains and plasmids

Vector pMW175 was used for general cloning procedures and for the *Escherichia coli*–based expression of recombinant nonfusion proteins. The *E coli* strain BL21 (DE3) pLysS (Stratagene, La Jolla, Calif) was used for expression of recombinant proteins. For DNA immunization, the plasmids pCMV–tree pollen chimera (TPC) 7 and pCMV-TPC9 were constructed as previously described.[13] Forward (5′-CACCGAATTCATGGGTGTTTTCAATTACGA-3′) and reverse (5′-GATCTCTAGATTAGTTGTAGGCATCAGAGT-3′) primers introduced flanking *Eco*RI or *Xba*I sites, respectively, for ligation into the *Eco*RI/*Xba*I sites of the mammalian expression vector pCI (Promega, Madison, Wis). For DNA immunization, plasmids were purified with EndoFree Plasmid Giga kits (Qiagen, Hilden, Germany) and dissolved in pyrogen-free PBS. Endotoxin content was less than 3 EU/mL, as determined by using the Limulus amoebocyte lysate assay (Associates of Cape Cod, East Faulmouth, Mass).

DNA shuffling

Allergens with National Center for Biotechnology Information accession numbers S50892, X15877, X77200, X77265, X77266, X77267, X77268, X77269, X77271, X77273, X70999, X71000, X70997, and X70998 were used for gene-family recombination. The 14 selected genes comprised 9 Bet v 1 isoforms from birch pollen, 4 Cor a 1 isoforms from hazel pollen, and Aln g 1 from alder pollen. Bet v 1 isoforms and Aln g 1 genes were amplified by using 5′-GGGCGCCATGGGTGTTTTCAATT-3′ as the forward primer and 5′-CCTTTGAATTCTTAGTTGTAGGCAT-3′ as the reverse primer. Cor a 1 genes were amplified with 5′-GGGCGCCATGGGTGTTTTCAATT-3′ as the forward primer and 5′-CCTTTGAATTCTTAGTTGTATTCAGCA-3′ as the reverse primer. Flanking *Nco*I and *Eco*RI sites were used for cloning into the respective vectors. DNA shuffling of Bet v 1 and homologous genes was performed according to the protocol described by Zhao and Arnold.[14]

High-throughput expression of chimeric proteins

Chimeric proteins were expressed as nonfusion proteins in *E coli* in 96-deep-well (1.2 mL) plates (Corning Costar, Acton, Mass). Single colonies were grown in 200 μL of LB medium supplemented with ampicillin at 37°C for 8 hours. Afterward, 300 μL of fresh medium and 1 mmol/L of isopropyl-β-D-thiogalactopyranoside were added for protein expression. Incubation continued for 16 hours at 16°C. Ten-microliter aliquots of bacterial suspension were dot blotted onto nitrocellulose (Whatman, Brentford, United Kingdom). Cells were lysed by overlaying the membrane twice (5 minutes each) with a paper filter soaked in 0.5 mol/L NaOH and 1.5 mol/L NaCl, followed by neutralization (2 times for 5 minutes each) with 1 mol/L Tris-HCl, pH 7.5. Membranes were processed as described below for IgG and IgE detection.

SDS-PAGE and dot blots

E coli lysates and purified proteins were analyzed by means of SDS-PAGE with 15% gels. Proteins were visualized by means of silver staining (Invitrogen, Carlsbad, Calif). For dot-blot analysis, proteins were applied on nitrocellulose membranes (Whatman, Brentford, United Kingdom). Polyclonal rabbit anti-rBet v 1a IgG antibodies were affinity purified with recombinant Protein G (Pierce, Rockford, Ill). Antibodies were used in a dilution of 1:50,000 in 25 mmol/L Tris-HCl (pH 7.5), 0.5% (vol/vol) Tween-20, 5% (wt/vol) BSA, and 0.05% (wt/vol) sodium azide. Rabbit IgG was detected with alkaline phosphatase–conjugated goat anti-rabbit IgG Fc (Jackson Immunoresearch, West Grove, Pa). Sera from individuals with tree pollen allergy were diluted 1:10 in 50 mmol/L sodium phosphate buffer (pH 7.4), 0.5% (vol/vol) Tween-20, 5% (wt/vol) BSA, and 0.05% (wt/vol) sodium azide. Bound IgE antibodies were detected by using iodine 125–labeled rabbit anti-human IgE (MALT Allergy System; IBL, Hamburg, Germany).

Expression and purification of chimeric proteins

Expression plasmids were transformed into *E coli*, and cells were grown at 37°C to an OD_{600} of 0.8. Cultures were then cooled to 16°C, and protein expression was induced by addition of 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside. After incubation for 18 hours, cells were harvested by means of low-speed centrifugation and resuspended in PBS containing 10% (vol/vol) glycerol.[15] The chimeric proteins TPC7 and TPC9 were expressed as nonfusion proteins and purified from soluble bacterial lysates by means of immunoaffinity chromatography with BIP1,[16] an mAb raised against natural Bet v 1. Recombinant proteins were dialyzed against 10 mmol/L sodium phosphate buffer (pH 7.4), quantified by means of amino acid analysis, and stored at 4°C.

Physicochemical characterization of recombinant allergens and chimeras

Amino acid composition of allergens was determined by using the Pico Tag method (Waters, Milford, Mass). Circular dichroism spectra and thermal denaturation spectra of the proteins were recorded in 5 mM sodium phosphate (pH 7.4) with a JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan). Baselines were corrected, and results were presented as mean residue molar ellipticity $[\Theta]_{MRW}$ at a given wavelength. Mass spectra were acquired by using electrospray