Allergenicity and antigenicity of wild-type and mutant, monomeric, and dimeric carrot major allergen Dau c 1: Destruction of conformation, not oligomerization, is the roadmap to save allergen vaccines

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Background: Carrot allergy is caused by primary sensitization to birch pollen. Continuous carrot exposure results in additional Dau c 1–specific allergic responses. Thus, immunotherapy with birch pollen may not improve the food allergy.
Objective: Evaluation of mutation and oligomerization of the major carrot allergen, Dau c 1, in regard to alteration of antibody binding capacities, structure, and the ability to induce blocking IgG antibodies.
Methods: Measurement of IgE reactivities to monomers, dimers of wild-type and mutant Dau c 1.0104 and Dau c 1.0201, and Dau c 1.0104 trimer, their ability to induce blocking antibodies in mice, and their allergenic potency by histamine release.
Results: The reactivity of human IgE to the mutant dimer was reduced on average by 81%. Sera of immunized Balb/c mice showed specific IgG similar to the human IgE antibody response; Dau c 1.01 was more antigenic than Dau c 1.02. Both wild-type and mutant Dau c 1 variants induced cross-reacting IgG, which blocked binding of human IgE. The mutants were more antigenic than the wild-type forms, and the dimers induced higher IgG responses in mice than the monomers. The results of the histamine release experiments corroborated the findings of the antibody binding studies.
Conclusion: Destruction of native conformation rather than oligomerization is the appropriate strategy to reduce the allergenicity of Bet v 1–homologous food allergens.
Clinical implications: The dimer composed of mutants of Dau c 1.0104 and Dau c 1.0201 is a promising candidate vaccine for treatment of carrot allergy because of its high immunogenicity and drastically reduced allergenicity. (J Allergy Clin Immunol 2007;119:944-51.)

Key words: *Dau c 1, isoforms, carrot allergen, allergen vaccine, mutation, secondary structure, hypoallergenic variant, monomer, dimer, trimer*

Abbreviations used
CD: Circular dichroism
EAST: Enzyme-linked allergosorbent test
SIT: Specific immunotherapy

The major allergen Dau c 1 of carrot (*Daucus carota*) is a homologue of the major birch pollen allergen Bet v 1. Two isoforms sharing only approximately 50% amino acid sequence identity and 6 variants of Dau c 1 have been identified so far.[1-4] Clinical observations and cross-reactivity data[2,4-7] suggest that carrot allergy is mainly a result of primary sensitization to birch pollen allergens; continuous exposure to carrots, however, results in recognition of discrete epitopes on the major allergen Dau c 1.[2,6] This effect is not limited to IgE-binding epitopes; T-cell epitope mapping showed that even though Bet v 1 is the primary sensitizing allergen in allergies to foods containing Bet v 1 homologues, the immune response to allergens such as Cor a 1 and Dau c 1 is at least in part Bet v 1–independent.[8] This implies that immunotherapy with birch pollen allergens may not improve carrot allergy, and food allergies to birch pollen–related foods must be treated as a separate disease. Consistent with this view, studies on the effect of birch pollen immunotherapy on apple allergy revealed controversial results,[9-11] despite the fact that Mal d 1 shares more than 60% amino acid sequence identity with Bet v 1 whereas the identity of Dau c 1 with Bet v 1 is below 40%. Novel strategies for allergen-specific immunotherapy (SIT) aim at the reduction of the IgE antibody-binding capacity of the allergen and preservation of their tolerogenic properties. Two strategies have been published that reduced IgE antibody binding capacity of Bet v 1 and its homologues: first, amino acid substitutions at position 112 with a proline[12-14] and multiple mutations at other positions,[15,16] and second, oligomerization.[17-21] To evaluate the contributions of mutation and oligomerization to the alteration of the antibody binding capacities, structure, and the ability to induce blocking IgG antibodies in mice with wild-type and mutant monomeric Dau 1.01 and Dau c 1.02, dimers of wild-type and mutant monomeric Dau 1.01 and Dau c 1.02 and the Dau c 1.01 trimer were studied.

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METHODS

Subjects

The diagnosis of carrot allergy in 24 patients had been confirmed by double-blind placebo-controlled food challenges[6] and/or by clinical history of carrot allergy,[2] and elevated carrot-specific serum IgE was determined by CAP-FEIA (Phadia, Uppsala, Sweden; see this article's Table E1 in the Online Repository at www.jacionline.org).

Generation and production of recombinant wild-type and mutant Dau c 1 monomers and dimers, and removal of LPS

N-terminally His-tagged Dau c 1.0104 (GenBank Z81362[4]) and Dau c 1.0201 (GenBank AF456481) were produced as described.[2] The dimer of wild-type Dau c 1.01 and Dau c 1.02 was generated by fusion PCR. Briefly, Dau c 1.01 and Dau 1.02 constructs, carrying sequences that overlapped with the respective 5′ or 3′ ends of the other allergen, were generated by PCR using Dauc1.01(+) 5′-ATGGGTGCCCAGAGCCATTCACTCGAGATC-3′, Dau1.01fus(-) 5′-CAGTCTTTTGGACACCCATATTAGCAATGAGGTAGG C-3′, Dau1.02fus(+) GCCTACCTCATTGCTAATATGGGTGTCCAAAAGACTG-3′, and Dau1.02(-) 5′-TTAGTTTGCTAGGAGGTAAGCCTCAACAGC-3′ as primers. These 2 constructs were fused by using Dau1.01(+) as forward primer and Dau1.02(-) as reverse primer. The C-terminally His-tagged Dau c 1.01 trimer was generated analogously to the Bet v 1 trimer.[17] 112P mutants of wild-type monomers and dimer were generated by site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, LaJolla, Calif). All Dau c 1 forms were purified by immobilized metal affinity chromatography. For the immunization of Balb/c mice, endotoxin was removed (EndoTrap; Profos, Regensburg, Germany).

Comparison of human IgE antibody reactivity to wild-type and mutant Dau c 1 dimers by enzyme-linked allergosorbent test

Five micrograms of each allergen were coupled to cyanogen bromide-activated paper disks (Schleicher & Schuell, Dassel, Germany).[22] For detection, the enzyme-linked allergosorbent test (EAST) kit (Allergopharma, Reinbek, Germany) was used according to the manufacturer's instructions.

Histamine release assay

The Histamine Enzyme Immunoassay Kit (Immunotech, Marseille, France) was performed according to the manufacturer's instructions by using heparinized blood from 2 subjects with carrot allergy (PEI-61, PEI-62) and 1 without (PEI-232). The stimulation concentrations ranged from 1 ng/mL to 1 μg/mL for the recombinant allergens, and from 10 ng/mL to 10 μg/mL for the carrot extract.

Immunization of Balb/c mice and measurement of IgG antibody response

Female mice 5 to 6 weeks old (BALB/c; Harlan-Winkelmann, Borchen, Germany) were immunized 6 times intraperitoneally with 0.01 μg/dose, 0.1 μg/dose, and 1.0 μg/dose of (1) the mixture of wild-type Dau c 1.01 and Dau c 1.02 monomers, (2) the dimer of wild-type Dau c 1.01 and Dau c 1.02, (3) the mixture of mutant Dau c 1.01 and Dau c 1.02 monomers, and (4) the dimer of mutant Dau c 1.01 and Dau c 1.02, respectively, using alum as adjuvant. At week 12, sera were collected and stored at −20°C. To determine the IgG response, ELISA plates (Maxisorb; Nunc, Wiesbaden, Germany) were coated over night at 4°C with antigen solution (0.25 μg/mL in 50 mmol/L sodium carbonate buffer, pH 9.6) and blocked. Dilution series (1:4, starting with 1:100) of the different mouse serum pools were added. After incubation with alkaline phosphatase–labeled goat antimouse IgG (1:5000; Sigma, Taufkirchen, Germany), p-nitrophenyl phosphate substrate was used to visualize antibody binding, and absorbance was read at 405 nm.

Inhibition ELISA

ELISA plates were coated overnight at 4°C with 0.2 μg/100 μL and blocked. Dilution series of the mouse serum pools were added, followed by human serum pools containing 5 sera each. Biotinylated goat antihuman IgE (KPL, Gaithersburg, Md), NeutrAvidin-HRP (Pierce, Bonn, Germany), and 3,3′,5,5′-tetramethylbenzidine plus H_2O_2 were used for detection. Absorbance was read at 450 nm; the inhibition was calculated in percentages.

Circular dichroism spectroscopy

Purified antigens were dialyzed against 10 mmol/L KH_2PO_4/K_2HPO_4 buffer, pH 7.4, and protein concentrations were adjusted to 5.2 μmol/L. Circular dichroism (CD) spectroscopy was performed on a J-810 S spectropolarimeter (Jasco, Groß-Umstadt, Germany). The mean residue ellipticity ($[\theta]_{m.r.w.}$) was calculated from the measured ellipticity [θ].[23]

RESULTS

Folding of wild-type, mutant, monomers, and dimers of Dau c 1 isoforms

The effect of oligomerization and/or mutations at an amino acid position (position 112) critical for the correct folding was studied by CD spectrometry. The comparison of wild-type and mutant monomers demonstrated that mutations at position 112 destroyed the secondary structures of mutants (Fig 1, *A*). Dimerization of wild-type Dau c 1.01 and Dau c 1.02 did not destroy the secondary structure of Dau c 1 (Fig 1, *A and B*). Even the mutant Dau c 1.01/02 dimer seemed to be partially folded. The Dau c 1.01 monomer and trimer exhibited identical CD spectra (Fig 1, *C*), demonstrating that the trimer had likely preserved the native conformation of the monomer.

Murine IgG antibody response to wild-type, mutant, monomers, and dimers of Dau c 1 isoforms

Mice were immunized with 1.0 μg/dose, 0.1 μg/dose, and 0.01 μg/dose of (1) the mixture of wild-type Dau c 1.01 and Dau c 1.02 monomers, (2) the dimer of wild-type Dau c 1.01 and Dau c 1.02, (3) the mixture of mutant Dau c 1.01 and Dau c 1.02 monomers, and (4) the dimer of mutant Dau c 1.01 and Dau c 1.02, respectively. Because the immunization with the 0.1 μg/dose always resulted in an antibody response very similar to those that were a result of the immunization with 1.0 μg/dose, Fig 2 shows only the antibody responses to 1.0 μg/dose and 0.01 μg/dose.

The immunization with 1 μg/dose of the mixture of monomeric Dau c 1.01 and Dau c 1.02 wild-types induced antibodies to all test antigens (Fig 2, *A*). The strongest antibody response was directed toward the dimer of the 2 Dau c 1 isoforms, whereas the SP112 mutant of Dau c 1.01 gave rise to the lowest antibody reactivity. The