Single–tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts

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Background: Allergic reactions to tree nuts are often severe and are outgrown in less than 10% of diagnosed patients.
Objectives: To determine whether treatment of underlying tree nut sensitization will prevent allergic reactions to cross-reacting tree nuts and to determine the effects of single–tree nut immunotherapy on true multi–tree nut sensitization.
Methods: Cross-reactivity model: Cashew-sensitized mice underwent immunotherapy with cashew and were subsequently challenged with cashew and pistachio. Multisensitization model: Cashew plus walnut-sensitized mice were treated with cashew alone, walnut alone, or both cashew and walnut and then underwent challenges to cashew and walnut. Challenges were assessed on the basis of symptoms, changes in body temperature, and mouse mast cell protease-1 release.
Results: In the cross-reactivity model, cashew immunotherapy completely prevented allergic reactions on challenges with cashew or the cross-reactive pistachio. In the multisensitization model, mice with cashew plus walnut allergy were significantly protected from anaphylactic reactions on cashew challenge in both the cashew-alone and walnut-alone immunotherapy groups. Results from the walnut challenge demonstrated significantly decreased allergic responses in the walnut immunotherapy group, whereas mice in the cashew immunotherapy group experienced significantly lower symptoms. In the cross-reactivity model, immunotherapy effectively decreased IL-4 and IL-5 production and increased IL-12 relative to placebo while also inducing a 5-fold increase in specific IgG_1.
Conclusion: Single–tree nut immunotherapy can effectively decrease allergic responses in both the cross-reactivity and multisensitization mouse models. Further studies are needed to determine which single–tree nut immunotherapies will be most effective for specific multi–tree nut allergy profiles. (J Allergy Clin Immunol 2011;127:81-8.)

Key words: *Food allergy, tree nut allergy, immunotherapy, cross-reactivity, cashew, walnut, pistachio*

Allergies to tree nuts affect approximately 0.5% of children under 18 years old and 0.9% of adults in the United States.[1] Unlike egg and milk allergies that are outgrown by a vast majority of allergic children, nut allergies are outgrown by only an estimated 9% of patients.[2] Among tree nuts, walnut, cashew, almond, pecan, and pistachio appear to be most problematic, which may be a result of these being the most consumed.[3,4] Sensitization to multiple tree nuts is common once diagnosis to a tree nut or peanut is made, with greater than 80% of patients having IgE for several nuts by age 14 years.[5] Tree nuts can induce severe, and even fatal, reactions.[6] Recently, a study from the United Kingdom (UK) provided evidence that cashew may induce allergic reactions more severe than peanut,[7] which is often thought of as the most severe form of food allergy.

The major allergenic proteins in tree nuts, like peanut and other seeds, have been identified as seed storage proteins. The vicilin (7S globulin), legumin (11S globulin), and 2S albumins from cashew, walnut, and pistachio, along with other nuts, have been sequenced and demonstrated to be allergenic.[3,8-10] Cross-reactivity among the allergenic proteins in tree nuts has been demonstrated *in vitro*.[9,11-13] In particular, combinations that are highly cross-reactive are cashew and pistachio (Anacardiaceae family), and walnut and pecan (Juglandaceae family).[11] For instance, correlation coefficients of 0.95 and 0.96 were found for cashew and pistachio, and walnut and pecan serum IgE levels, respectively.[14] Clinically, cashew and pistachio cross-reactivity appears to be relevant. A recent report stated that in 22 patients with documented reactions on first known exposure to pistachio, all 22 had IgE to cashew.[8] The cross-reactivity among tree nut allergens typically leads to the recommendation to patients with nut allergy that all nuts should be avoided.

Currently it is not possible to determine whether patients with allergies to multiple nuts have been initially sensitized to several nuts, or whether 1 sensitization has occurred and the other sensitizations are a result of cross-reactivity. Both possibilities are conceivable; from a therapeutic perspective, it is worth investigating immunotherapy for both scenarios. We are particularly interested in determining whether a single-nut immunotherapy approach can induce tolerance to cross-reacting nuts and/or downregulate hypersensitivities for truly multisensitized patients. This idea may have broad-reaching implications for both pediatric and adult populations with allergies to tree nuts, fish, or shellfish, in which patients typically have multiple triggers from an allergenic food group because of the homology among the offending protein allergens.

Our approach to study cross-reactivity and multisensitization to tree nuts *in vivo* is to use a mouse model in which we control the

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Abbreviations used
HRP: Horseradish peroxidase
MMCP-1: Mouse mast cell protease 1
UK: United Kingdom

sensitizing antigens and can easily administer multiple food challenges.[15] In this report, we addressed 2 hypotheses by using our tree nut mouse models: (1) specific immunotherapy for cashew allergy will effectively prevent allergic reactions to the highly cross-reactive pistachio, and (2) single–tree nut immunotherapy with either of 2 sensitizing tree nuts (cashew and walnut) will lead to decreased allergic reactions to both nuts.

METHODS

Food extract preparations

Cashew, walnut, pistachio, peanut, and egg extracts were prepared as previously reported.[15] Walnut seeds were extracted in a phosphate buffer with 1 mol/L NaCl to increase protein solubility.[16] Briefly, ground seeds were mixed in a 1:5 (wt:vol) ratio in 2x PBS and homogenized for 30 minutes while maintaining an alkaline pH (8.5). Supernatants were clarified by centrifugation at 30,000*g* for 30 minutes at 4°C. Ammonium sulfate precipitation at 90% saturation was used to concentrate the proteins. After dialysis into PBS, all extracts were filter-sterilized, and protein concentration was determined via bicinchoninic acid assay (Pierce, Rockford, Ill).

Mouse model of immunotherapy for tree nut cross-reactivity

Five-week-old female C3H/HeJ mice purchased from Jackson Laboratory (Bar Harbor, Me) were maintained on tree nut–free, peanut-free, and egg-free food (PMI Nutrition International, St Louis, Mo) under pathogen-free conditions following standard guidelines for care and use. The sensitization, immunotherapy, and challenge protocols were based on our previous work.[17] Mice were divided into 5 groups: (1) naive, (2) cashew-sensitized treated with cashew immunotherapy, (3) cashew-sensitized treated with placebo (PBS), (4) egg-sensitized treated with cashew immunotherapy, and (5) egg-sensitized treated with placebo. All groups consisted of 5 mice, except groups 4 and 5 contained 3 mice each. Mice were sensitized intraperitoneally with 0.5 mg appropriate food extract plus 2 mg alum (Imject Alum; Thermo Scientific, Rockford, Ill) with 1 injection during weeks 1, 2, and 4. The immunotherapy regimen began in week 7 and lasted 4 weeks with 3 intraperitoneal injections given per week (ie, Monday, Wednesday, and Friday). The doses followed a build-up and maintenance schedule intended to mimic clinical allergy immunotherapy. The doses were as follows: week 7, mice received 0.1 mg cashew extract or PBS (placebo); week 8, 0.25 mg cashew or PBS; weeks 9 and 10, 0.5 mg cashew or PBS. Submandibular facial sampling was used to collect sera from individual mice during week 6 (postsensitization) and week 13 (postimmunotherapy) for measuring antibody levels. Mice were challenged at week 14 via intraperitoneal injection of 1.0 mg of the food used for sensitization (cashew or egg). An additional challenge was performed at week 17 by using 1.0 mg pistachio extract in mice that were sensitized to cashew (ie, groups 2 and 3). Anaphylactic reactions were assessed by measuring core body temperatures with a rectal probe at 15-minute intervals for 45 minutes postinjection. Symptoms were scored after 30 minutes using a pre-established scale: 0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eyes and mouth with reduced activity; 3, labored respiration and/or cyanosis around the mouth and tail; 4, no activity after prodding, or tremor and convulsion; and 5, death.[18] Blood was collected at 45 minutes to assess mouse mast cell protease 1 (MMCP-1) levels in select mice by using an ELISA kit (Moredun Scientific, Midlothian, Scotland). All procedures were approved by the Institutional Animal Care and Use Committee at Duke University.

Mouse model of immunotherapy for multi–tree nut sensitization

Five-week-old female C3H/HeJ mice were used and maintained as discussed. Mice were divided into 5 groups of 5 mice each. Groups 1 to 4 were sensitized to cashew plus walnut and treated as follows: (1) placebo, (2) walnut plus cashew immunotherapy, (3) cashew immunotherapy alone, and (4) walnut immunotherapy alone. Group 5 was a naive control. Mice were sensitized intraperitoneally during weeks 1, 2, and 4 with 0.5 mg tree nut extract plus 2 mg alum as previously described.[15] Immunotherapy began in week 7 and was administered as discussed. Cashew immunotherapy alone and walnut immunotherapy alone were given in identical doses as described. The walnut plus cashew immunotherapy group received 3 injections of 0.1 mg cashew and 0.1 mg walnut extracts during week 7; 3 injections of 0.25 mg cashew and 0.25 mg walnut during week 8; and 3 injections per week for weeks 9 and 10 of 0.5 mg cashew and 0.5 mg walnut extracts. The cashew and walnut extracts were injected within 15 minutes of each other for each immunotherapy dose given. Mice were bled at week 12 for antibody level measurements. Mice were challenged at week 13 with 1.0 mg cashew extract and at week 15 with 1.0 mg walnut. Allergic reactions were assessed as discussed.

IgE, IgG_1, and IgG_{2a} measurements

Specific IgE, IgG_1, and IgG_{2a} were measured by ELISA using a reference curve. Plates were coated with appropriate food extracts (20 μg/mL) in carbonate-bicarbonate buffer at pH 9.6. Detection of IgE was performed with sheep antimouse IgE (0.5 μg/mL; Binding Site, Birmingham, UK), followed by biotinylated donkey antisheep IgG (0.5 μg/mL; Accurate Chemical, Westbury, NY) and neutravidin–horseradish peroxidase (HRP; 0.2 μg/mL; Pierce). IgG_1 was detected by HRP-conjugated goat antimouse IgG_1 (Southern Biotech, Birmingham, Ala) used at 1:40,000. IgG_{2a} was detected by HRP-conjugated goat antimouse IgG_{2a} (Southern Biotech). The HRP activity was measured with color development of 3,3',5,5'-tetramethylbenzidine substrate (KPL, Gaithersburg, Md). Inhibition ELISA experiments for cashew-sensitized mouse IgE were conducted with log-fold increases in protein extracts from 1 ng/mL to 1 mg/mL for pistachio, walnut, peanut, and egg.

Western blotting

Protein extracts, under denatured and reduced conditions, were separated on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, Calif) and then transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, Mass). Blots were blocked by using 2% BSA in PBS containing 0.05% Tween-20. Sera from cashew-sensitized mice were pooled and diluted in 2% BSA in PBS containing 0.05% Tween-20 before incubation with the blot. IgG_1 was detected with HRP-conjugated goat antimouse IgG_1 (Southern Biotech). The blots were developed with a chemiluminescence detection kit (Super Signal West Pico Chemiluminescent Substrate; Pierce).

Proliferation assay and quantification of secreted cytokines

Splenocytes were isolated from individual mice at least 2 weeks after completion of challenges. Cells were plated at a density of 2.5×10^6/mL in complete medium (RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine). Antigens, also in complete medium, were used at 100 μg/mL for cashew, pistachio, walnut, peanut, and egg. All cultures were carried out in a 37°C, 5% CO_2, humidified incubator. Proliferation assays were carried out in triplicate in 96-well round-bottom plates with cells pulsed with 0.5 μCi [^{3}H]-thymidine/well at 78 hours, harvested 18 hours later, and counted for β-radioactivity (Wallac, Turku, Finland). Cultures for cytokine assays were performed in 24-well plates with supernatant collection at 96 hours. Cytokines secreted into the culture medium were quantified by ELISA, using antibodies from Biolegend (San Diego, Calif) for IL-4, IL-5, and IFN-γ[15] or R&D Systems (Minneapolis, Minn) for IL-12 p40.