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BRIEF COMMUNICATION

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Model of Walnut Allergy in CC027/GeniUnc Mice Recapitulates Key Features of Human Disease

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Tree nut allergies affect 1% of the United States population, are often severe in nature and rarely outgrown. Despite the severity and prevalence, there are no FDA-approved treatments for tree nut allergy. Development of a therapeutic would be expedited by having a mouse model that mimics the human disease. We utilized the CC027/GeniUnc mouse strain, which was previously identified as an orally reactive model of peanut allergy, to develop a model of walnut allergy. Mice were sensitized with walnut and cholera toxin for 4 weeks and subsequently challenged by oral gavage. Blood samples were collected to measure serum IgE. Walnut-sensitized mice produced high levels of walnut-IgE and were cross-sensitized to pecan. Oral challenges with walnut resulted in severe anaphylaxis and accompanying allergic symptoms. Importantly, pecan challenges also led to severe allergic reactions, indicating cross-reactivity to pecan. Overall, this novel mouse model reproduces key characteristics of human walnut allergy, which provides a platform to develop novel therapies and better understand sensitization mechanisms.

INTRODUCTION

Food allergy is an IgE-mediated disease affecting an estimated 4% of children and 10% of adults [1,2]. Tree nut allergies have grown in prevalence in the past decade, now affecting 1% of the United States population, and persist throughout life for 90% of individuals [3,4]. Walnuts, pecans, cashews, pistachios, almonds, Brazil nuts, pine nuts, hazelnuts, and macadamia nuts are tree nuts that are often consumed in the United States. Tree nut allergies are especially severe and account for 18-40% of fatalities from food allergy, with even trace amounts causing severe anaphylaxis [5]. Due to the potential severity of accidental exposures, allergic individuals must maintain strict avoidance of the eliciting food. Together, these measures lead to decreased quality of life in allergic

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Abbreviations: OIT, Oral immunotherapy.

Keywords: Food allergy, walnut allergy, mouse model, IgE, anaphylaxis

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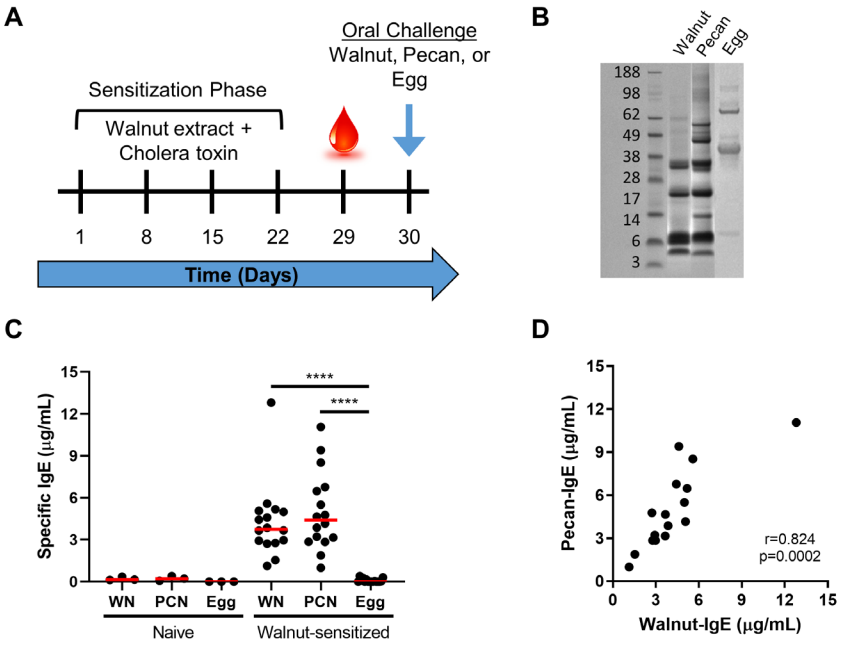

Figure 1. Walnut sensitization in CC027/GeniUnc mice. (**A**) Experimental scheme for sensitization of CC027/GeniUnc mice and subsequent oral challenges. (**B**) SDS-PAGE gel for walnut, pecan, and egg extracts. (**C**) Walnut-, pecan-, and egg-IgE quantified in serum from naïve and walnut-sensitized mice. (**D**) Correlation between walnut- and pecan-IgE levels in walnut-sensitized mice. *WN*, walnut; *PCN*, pecan. **** P<0.0001

individuals [6].

The majority of patients allergic to a tree nut are allergic to multiple tree nuts, with walnut, hazelnut, cashew, and almond most commonly causing allergic reactions [7]. Patients allergic to certain tree nuts experience cross-reactions to other tree nuts, due to the high homology between specific nut allergens [7]. For example, walnut-allergic patients are often cross-reactive to pecan. Indeed, the correlation between walnut- and pecan-IgE was determined to be 0.96 [8]. Walnut and pecan are both members of the Juglandaceae family, and their 2S albumin allergens Jug r 1 and Car i 1, respectively, have 88% sequence identity [5]. Similarly, cashew-allergic patients are often cross-reactive to pistachio, with a correlation between cashew- and pistachio-IgE of 0.95 [8]. Cashew and pistachio are both members of the Anacardiacea family, and their 2S albumin allergens, Ana o 3 and Pis v 1, respectively, have 66% sequence identity [5]. In general, patients allergic to one tree nut are often advised to avoid all tree nuts, due to this cross-sensitization and cross-reactivity.

After a successful Phase 3 trial, a peanut oral immunotherapy (OIT) drug has been approved by the FDA; however, there are no FDA-approved therapies for tree nut allergies [9]. An OIT study with 73 walnut-allergic subjects demonstrated that 49 of 55 subjects in the OIT group were desensitized to walnut [10]. Furthermore, all 46 subjects who were co-allergic to pecan were also desensitized to pecan, demonstrating cross-desensitization after single tree nut immunotherapy. While these results are promising, OIT has several limitations including adverse side effects, requirement for daily dosing, and a lack of tolerance induction. Therefore, future studies investigating novel therapies are necessary.

Murine models provide the ability to test novel therapies and better understand sensitization mechanisms. Several peanut allergy mouse models exist, including our previously reported model where CC027/GeniUnc mice are orally sensitized and react upon oral peanut challenge [11]. Few tree nut allergy mouse models exist, and these rely on injection of antigen to induce reactions [12-15]. However, no orally reactive mouse models of tree nut allergy exist. Here, we sought to develop a model of walnut allergy in the CC027/GeniUnc mouse strain that was orally reactive and had similar characteristics of human walnut allergy, including allergen-specific IgE production and cross-reactivity to pecan.

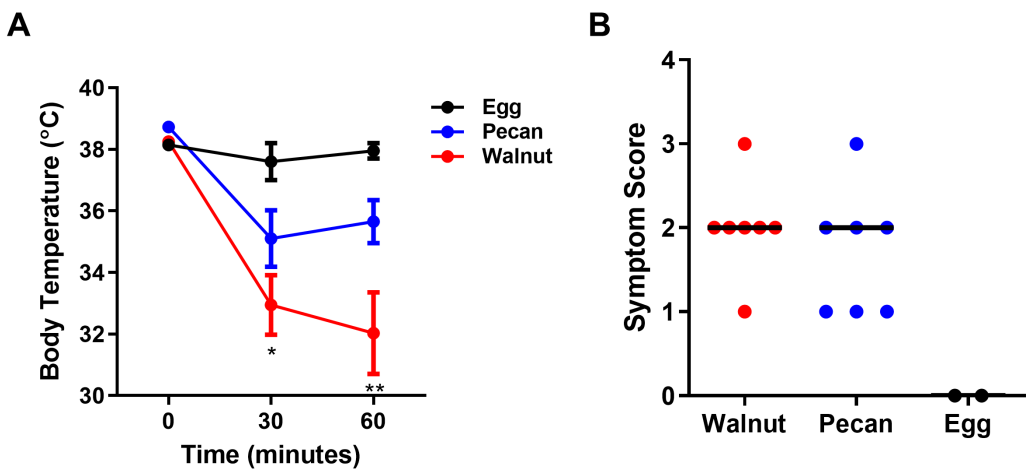

Figure 2. Oral challenge outcomes for mice challenged to walnut, pecan, or egg. (**A**) Body temperatures recorded post-oral challenge. Statistical comparisons are between walnut and egg at 30 min and walnut and both groups at 60 min. (**B**) Symptom scores recorded 30 minutes post-oral challenge. * P<0.05, ** P<0.01

MATERIALS AND METHODS

Mice

Female CC027/GeniUnc mice aged 4-6 weeks were obtained from the UNC Systems Genetics Core. Mice were raised on standard mouse chow free of any tree nut and egg ingredients and kept on a 12:12-hour light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill under protocol 15-185.

Sensitization and Oral Food Challenges

Mice were sensitized to walnut by administration of walnut extract (2 mg for 3 weeks and 5 mg for the final week) plus cholera toxin (10 µg) via oral gavage once weekly for 4 weeks (Figure 1A). The following week, serum was collected by submandibular bleed to measure immunoglobulin production. Mice were challenged via oral gavage to walnut (5 mg), pecan (7 mg) or egg (5 mg) protein extract. Core body temperatures were recorded every 30 minutes following challenge with a rectal thermometer (Physitemp, Clifton, NJ). Symptom scores were recorded 30 minutes post-challenge according to the following scale: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around eyes and mouth, diarrhea, pilar erecti, reduced activity, increased respiratory rate; 3, wheezing, labored respiration, cyanosis around mouth, feet, tail; 4, no activity after prodding, tremor or convulsion; 5, death.

Walnut, Pecan, and Egg Extractions

Extractions were similar to previously described methods [16]. Specifically, proteins were extracted from roasted, defatted walnut flour (Holmquist Hazelnut Orchards, Lynden, WA), defatted pecan flour (Ambient Temperature Extraction Alternatives, Edmond, OK) or egg white powder (Deb El Foods, Elizabethport, NJ) in PBS. Protein concentrations were measured by BCA assay (Pierce, Waltham, MA) and extracts were determined to contain all major allergens by SDS-PAGE gel (Figure 1B).

ELISAs

Walnut-, pecan- or egg-specific IgE was quantified via ELISA as described previously [11]. Briefly, 96-well plates were coated with 20 µg/mL walnut, pecan, or egg extract (for samples) or HSA-DNP (for standard curves) and blocked with 2% BSA in PBS-0.5% Tween. Serum samples were diluted 1:100, and standard curves ranging from 0.002-2 µg/mL of IgE anti-DNP (Accurate Chemicals, Westbury, NY) were generated via 1:2 serial dilutions. The following antibodies were used in succession for detection: sheep IgG anti-mouse IgE (0.5 µg/mL, The Binding Site, Birmingham, UK), biotinylated donkey anti-sheep IgG (0.5 µg/mL, Accurate Chemicals), and NeutrAvidin-HRP (0.5 µg/mL, Pierce Biotechnology, Waltham, MA). Plates were developed using TMB (SeraCare, Milford, MA), stopped using 1% HCl (SeraCare), and read at 450 nm using a microplate spectrophotometer (BioTek Instruments, Winooski, VT).

Statistical Analysis

GraphPad Prism version 8.3.0 (San Diego, CA) was used to perform all statistical analyses, including Mann-Whitney, Spearman correlation, and two-way ANOVA.

RESULTS

In order to induce sensitization, walnut extract and cholera toxin were administered to CC027/GeniUnc mice via oral gavage according to the schematic in Figure 1A. To determine whether sensitization occurred, walnut-IgE was quantified in serum. In naïve mice, walnut-IgE was undetectable, whereas walnut-sensitized mice produced high levels of walnut-IgE (Figure 1C). Pecan and walnut extracts contain 2S albumin, legumin, and vicilin proteins, which are highly homologous and approximately equal molecular weight between the two nuts (Figure 1B). Accordingly, mice also produced similarly high levels of pecan-IgE (Figure 1C), indicating cross-sensitization to pecan. Pecan-IgE was highly correlated with walnut-IgE (Figure 1D), further demonstrating that sensitization to walnut alone led to cross-sensitization to pecan. Egg-IgE was also quantified to determine whether there was any cross-sensitization to a non-homologous food allergen. Mice did not produce any egg-IgE, indicating that sensitization was specific to the tree nuts, walnut and pecan (Figure 1C). Together, these results indicate that walnut sensitization in this model leads to cross-sensitization to pecan, but not egg.

Following sensitization, mice underwent oral challenges to walnut to confirm they were allergic. In mice, anaphylaxis is indicated by severe hypothermia accompanied by allergic symptoms. Mice experienced anaphylaxis after the walnut challenge, as demonstrated by their 5°C body temperature decrease post-challenge (Figure 2A). The vast majority of mice challenged to walnut had symptom scores of 2 or greater (Figure 2B), consistent with their severe decreases in body temperature. Based on the cross-sensitization observed between walnut and pecan, oral challenges to pecan were performed to investigate cross-reactivity. Mice also experienced anaphylaxis after the pecan challenge, with an average body temperature decrease of 3°C and a median symptom score of 2 (Figure 2A, 2B), demonstrating cross-reactivity to pecan. Egg challenges were also performed in a subset of mice to confirm the lack of sensitization. Mice did not react upon egg challenge and experienced no symptoms (Figure 2A, 2B). Overall, walnut-sensitized mice reacted severely to oral walnut challenge and were cross-reactive to oral pecan challenge, which mimics the cross-reactivity often observed in walnut-allergic human subjects.

DISCUSSION

Cross-sensitization to multiple tree nuts has been well-documented in humans, with especially high cross-reactivity between walnut and pecan, and cashew and pistachio [7,8]. Mouse models of food allergy are an important tool to develop novel therapies. Previously, we took advantage of the genetic diversity within the Collaborative Cross mouse strains to identify CC027/GeniUnc mice as genetically susceptible to developing peanut allergy [11]. Here, we aimed to improve upon the limited existing tree nut allergy models by developing a model of walnut allergy where mice are sensitized and challenged orally and are also cross-reactive to pecan. Specifically, mice sensitized to walnut produce high levels of both walnut- and pecan-IgE and react upon challenge to both nuts. These characteristics mimic key features of human walnut allergy. Furthermore, mice are not cross-sensitized to egg, as evidenced by no egg-IgE production and no reaction upon egg challenge, indicating the specificity of sensitization in this model.

Due to the similarities to human walnut allergy, this mouse model provides a platform to develop and test novel therapeutic approaches for tree nut allergy. This will be especially useful considering there are no treatments available for tree nut allergies. Additionally, this model will afford an opportunity to study the pathophysiology of food allergy and better understand the underlying mechanisms of tree nut sensitization. Since each Collaborative Cross strain is a genetic mosaic of the eight founder strains, future studies may investigate influences of genetic variants driving sensitization and anaphylaxis to tree nuts [17,18]. In conclusion, this novel mouse model of tree nut allergy demonstrates key features of human allergy and serves as a useful tool for future studies.

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