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

Johanna M. Smeekens\textsuperscript{a,b,*}, Kelly A. Orgel\textsuperscript{a,b}, Janelle Kesselring\textsuperscript{a,b}, Ken Bagley\textsuperscript{c}, and Michael D. Kulis\textsuperscript{a,b}

\textsuperscript{a}Department of Pediatrics, School of Medicine, University of North Carolina, Chapel Hill, NC; \textsuperscript{b}UNC Food Allergy Initiative, School of Medicine, University of North Carolina, Chapel Hill, NC; \textsuperscript{c}Profectus Biosciences, Baltimore, MD

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 individuals.
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 Anacardiaceae 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 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
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).

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
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.

Funding: This work was funded in part by NIH NIAID SBIR 1 R43 AI134225-01 and American Research Foundation for Nut Allergies. JMS is funded by a T32 Allergy/Immunology Training Grant (AI007062) through Duke University and University of North Carolina at Chapel Hill. The Systems Genetics Core Facility is supported in part by the P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center.

REFERENCES

1. Gupta RS, Warren CM, Smith BM, Jiang J, Blumenstock JA, Davis MM, et al. Prevalence and Severity of Food Allergies Among US Adults. JAMA New Open. 2019;2(1):e185630. Epub 2019/01/16. doi: 10.1001/jamanetworkopen.2018.5630.
2. Branum AM, Lukacs SL. Food allergy among children in the United States. Pediatrics. 2009;124(6):1549-55. Epub 2009/11/18. doi: 10.1542/peds.2009-1210.
3. Sicherer SH, Muñoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. J Allergy Clin Immunol. 2010 Jun;125(6):1322-6. doi: 10.1016/j.jaci.2010.03.029.

4. Fleischer DM. The natural history of peanut and tree nut allergy. Curr Allergy Asthma Rep. 2007 Jun;7(3):175-81. doi: 10.1007/s11882-007-0018-y.

5. Smeekens JM, Bagley K, Kulis M. Tree nut allergies: Allergen homology, cross-reactivity, and implications for therapy. Clin Exp Allergy. 2018;48(7):762-72. Epub 2018/04/28. doi: 10.1111/cea.13163.

6. Avery NJ, King RM, Knight S, Hourihane JO. Assessment of quality of life in children with peanut allergy. Pediatr Allergy Immunol. 2003 Oct;14(5):378-82. doi: 10.1034/j.1399-3038.2003.00072.x.

7. Elizur A, Appel MY, Nachshon L, Levy MB, Epstein-Rigbi N, Golobov K, Goldberg MR. NUT Co Reactivity - Acquiring Knowledge for Elimination Recommendations (NUT CRACKER) study. Allergy. 2018 Mar;73(3):593-601. doi: 10.1111/all.13353.

8. Maloney JM, Rudengren M, Ahlstedt S, Bock SA, Sampson HA. The use of serum-specific IgE measurements for the diagnosis of peanut, tree nut, and seed allergy. J Allergy Clin Immunol. 2008 Jul;122(1):145-51. doi: 10.1016/j.jaci.2008.04.014.

9. PALISADE Group of Clinical Investigators, Vickery BP, Vereda A, Casale TB, Beyer K, du Toit G, et al. AR101 Oral Immunotherapy for Peanut Allergy. N Engl J Med. 2018 Nov 22;379(21):1991-2001. doi: 10.1056/NEJMoa1812856. Epub 2018 Nov 18.

10. Elizur A, Appel MY, Nachshon L, Levy MB, Epstein-Rigbi N, Pontoppidan B, et al. Walnut oral immunotherapy for desensitization of walnut and additional tree nut allergies (Nut CRACKER): a single-centre, prospective cohort study. Lancet Child Adolesc Health. 2019;3(5):312-21. Epub 2019/03/31. doi: 10.1016/S2352-4642(19)30029-X.

11. Orgel K, Smeekens JM, Ye P, Fotsch L, Guo R, Miller DR, et al. Genetic diversity between mouse strains allows identification of the CC027/GeniUnc strain as an orally reactive model of peanut allergy. J Allergy Clin Immunol. 2019;143(3):1027-37 e7. Epub 2018/10/22. doi: 10.1016/j.jaci.2018.10.009.

12. Kulis M, Burks AW. Effects of a pre-existing food allergy on the oral introduction of food proteins: findings from a murine model. Allergy. 2015;70(1):120-3. Epub 2014/08/27. doi: 10.1111/all.12519.

13. Kulis M, Li Y, Lane H, Pons L, Burks W. Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. J Allergy Clin Immunol. 2011;127(1):81-8. Epub 2010/11/26. doi: 10.1016/j.jaci.2010.09.014.

14. Kulis M, Macqueen I, Li Y, Guo R, Zhong XP, Burks AW. Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. J Allergy Clin Immunol. 2012;130(3):716-23. doi: 10.1016/j.jaci.2012.05.044.

15. Kulis M, Pons L, Burks AW. In vivo and T cell cross-reactivity between walnut, cashew and peanut. Int Arch Allergy Immunol. 2009;148(2):109-17. doi: 10.1159/000155741. Epub 2008 Sep 19.