

Development of an Adjuvant-Free Cashew Nut Allergy Mouse Model

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Key Words

Adjuvant · Cashew nut allergy · Cytokines · Food allergy · IgE · Mouse model · Systemic anaphylaxis · Th2

Abstract

Background: Cashew nut allergy is an emerging food allergy with a high risk of systemic anaphylaxis. Currently, an adjuvant-free animal model to study cashew nut allergy is not available. **Methods:** BALB/c mice were exposed to cashew nut protein using a transdermal sensitization protocol that does not use adjuvant. Systemic IgE antibody response, systemic anaphylaxis to oral challenge and allergen-driven, spleen-cell, type-2 cytokine responses were studied. **Results:** Transdermal exposure to cashew nut resulted in a significant dose-dependent allergic response. Oral challenge of sensitized mice with cashew resulted in severe signs of systemic anaphylaxis and a significant hypothermia. Spleen cell culture with cashew nut protein resulted in allergen-driven IL-4, IL-5 and IL-13 responses only in sensitized but not in saline control mice. **Conclusions:** These data demonstrate that (i) transdermal exposure to cashew nut protein elicits a robust IgE response leading to clinical sensitization of mice for systemic anaphylaxis to oral cashew nut challenge; (ii) cashew nut is a potent activator of type-2 cytokines, thus explaining the mechanism of cashew allergy, and (iii) this mouse model may be useful for further basic and preclinical studies on cashew nut allergy.

Introduction

Immediate hypersensitivity responses to food, commonly called food allergy, afflict 6–8% of children and ~2–4% of adults in Westernized countries including Europe and the United States [1, 2]. Tree nut allergies in particular affect ~0.5% of adults in the USA and 0.2% of children in the Isle of Wight cohort of the UK [3]. Cashew nut allergy is the second most commonly reported tree nut allergy, after walnut, in the USA [4]. There is growing concern that cashew nut allergy is increasingly recognized in clinical practice in recent years in pediatric populations for reasons that are not well understood [5–8]. Furthermore, it is also reported that cashew nut causes more severe reactions than peanut in children [6]. Thus, cashew nut allergy is an emerging, clinically significant and very dangerous food allergy.

Global prevalence of cashew nut allergy is unknown. In the UK, approximately 0.08% of 4-year-old children are estimated to be allergic to cashew nut [3, 9]. Hourihane et al. [10] first reported clinical features of cashew nut allergy. They concluded that cashew nut allergy represents a threat to life, and is at least as severe as that of peanut and other tree nut allergies [10]. Many reports of cashew nut allergy have been published in the past several years [11–13]. More recently, elaborate studies involving a large number of subjects highlight the clinical severity of cashew nut allergy [5–7]. Thus, there is growing

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scientific evidence that cashew nuts cause immediate hypersensitivity reactions in humans, often with severe signs of systemic anaphylaxis.

Cashew nut allergy is often associated with allergies to other foods such as pistachio nuts, almonds, hazelnuts, walnuts, pine nuts, pecans and Brazil nuts; it may or may not be associated with peanut allergy [7, 11, 12]. Ferdman et al. [13] reported a case of pectin anaphylaxis and possible association with cashew nut allergy. There are suggestions that cross-reactivity due to shared B-cell epitopes among tree nuts may trigger cashew nut allergy [12]. However, whether cashew nut protein itself can act as a primary sensitizer leading to a clinical disease such as systemic anaphylaxis is not known.

Currently, there is no animal model available to study cashew nut allergy. Here we tested the hypothesis that cashew nut protein, without the use of adjuvant, would elicit allergic and anaphylactic reactions in mice similar to those in humans. Our results demonstrate that: (i) transdermal exposure to cashew nut protein results in a robust IgE response leading to clinical sensitization of mice for systemic anaphylaxis to oral cashew nut protein challenge, and (ii) cashew nut protein is a potent activator of IL-4, IL-5 and IL-13, thus explaining the mechanism of allergic response. We also suggest that this adjuvant-free mouse model may be useful for further basic and preclinical studies on cashew nut allergy.

Material and Methods

The materials were purchased from the sources indicated in parentheses: cashew nut protein extract (Greer Labs, Lenoir, N.C., USA); protein content was measured by the Lowry-Folin assay; LPS content of this material was tested and found to be <0.5 pg/mg of protein as measured by the LAL assay (Cambrex Bio, Walkersville, Md., USA); biotin-conjugated rat anti-mouse IgG1 and IgE antibodies; paired antibodies and recombinant standards for mouse IL-4, IL-5 (BD PharMingen, San Diego, Calif., USA); IL-13 (R&D Systems, Minneapolis, Minn., USA); *p*-nitro phenyl phosphate (Sigma, St. Louis, Mo., USA), and streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, Pa., USA).

Transdermal Sensitization and Bleeding

BALB/c female mice were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). Only adult animals (6–8 weeks age) were used in this study. All animal procedures used were in accordance with the Michigan State University policies. Transdermal exposure experiments were performed using a method described earlier [14]. Groups of mice ($n = 10$) were exposed to saline or cashew nut protein extract (0.05, 0.5 and 1 mg/100 μ l in saline per mouse per application); each mouse had the reagent applied to the skin of the back that had the hair clipped off and

covered with a non-latex non-occlusive bandage for 1 day. Mice were rested for 4 days. Then the cycle of exposure to saline or nut protein was continued for 4 weeks. Hair was clipped on the back, above the lumbar vertebrae. Animals received a total of 4 non-occlusive bandages. Allergen was prepared in sterile normal saline in a volume of 100 μ l; the solution was applied on the clipped area using a sterile tip. Animals were not re-clipped during the 4-week immunization protocol. We have been using this protocol for over 3 years; we have not observed any visible macroscopic injuries or inflammation of the skin. Blood samples were collected from the saphenous vein and plasma was used in the antibody analysis.

Measurement of Food-Specific Antibodies and Total Plasma IgE Levels

We have previously described optimization of enzyme-linked immunosorbent assay (ELISA) for food specific IgG1 and IgE antibody analyses [15]. The ELISA procedure used in this study was essentially as described by us [15]. Total plasma IgE levels were measured using an ELISA optimized with paired antibodies and isotype standard purchased from BD PharMingen.

Induction of Systemic Anaphylaxis, Clinical Scoring and Measurement of Rectal Temperature

Groups of cashew-nut-sensitized versus saline-exposed mice were orally challenged with cashew nut protein (15 mg/mouse) or saline (500 μ l per mouse) on day 6 following the 4th exposure using mouse feeding needles (22 gauge; Popper and Sons, New York, N.Y., USA). Mice were then observed for signs of systemic anaphylaxis during the next 60 min. Clinical scoring (on a scale from 0 to 5) was performed by 2 individuals according to the method described previously [16]: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsion, and 5, death. Rectal temperature was measured using a temperature probe (Physitemp Instruments, Clifton, N.J., USA) before and 30 min after oral challenge.

Spleen Cell Culture and Cytokine Analyses

Spleen cells were harvested and standard cell cultures were set up essentially as described [17]. Briefly, spleen cells were cultured (7.5 million/ml) in the absence and presence of cashew nut protein extract (100 and 500 μ g/ml). Cell culture supernatants were harvested for use in cytokine analyses using pre-optimized ultrasensitive assays (assay sensitivity: IL-4: 3.1, IL-5: 7.8 and IL-13: 31.2 pg/ml).

Statistical Analyses

The Wilcoxon two-sample test was used to compare all treatments for clinical score data. All antibody level data were log transformed and subsequently analyzed using ANOVA; similarly, *t* test inference on differences between treatments for rectal temperatures was adjusted for pretreatment temperatures using ANCOVA. SAS software was used for all statistical analyses (SAS Institute, Cary, N.C., USA). The statistical significance level was set to 0.05.

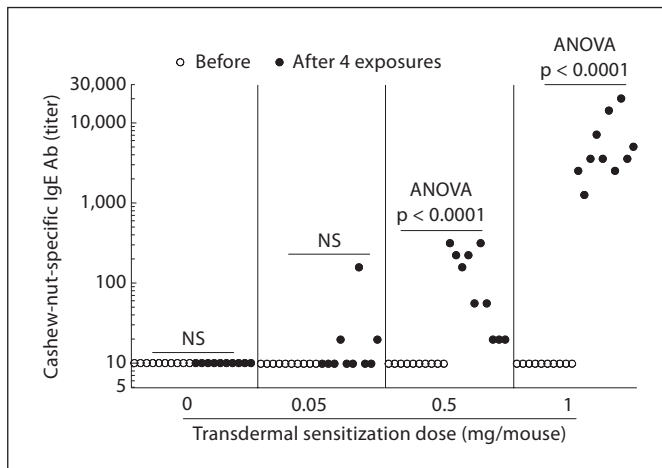


Fig. 1. Specific IgE antibody response to transdermal exposure of BALB/c mice to cashew nut protein. Ten mice per group were transdermally exposed to the indicated doses of cashew nut protein (0, 0.05, 0.5 or 1 mg/mouse). Specific IgE antibody titers were measured before exposure and after the 4th exposure. Data shown represent IgE antibody titers (geometric mean $\pm$ SE).

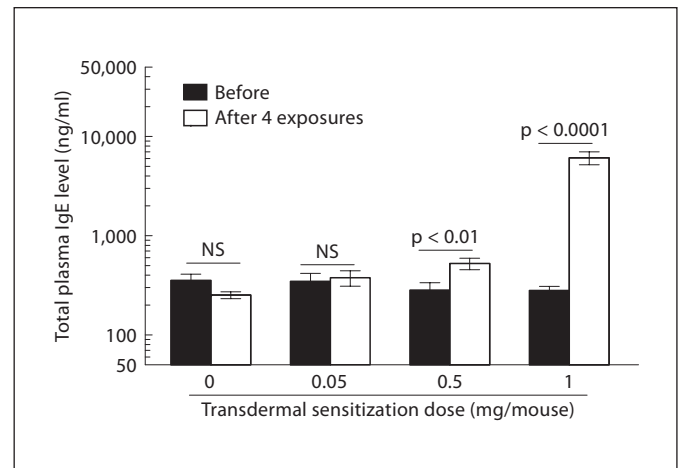


Fig. 2. Total plasma IgE levels before and following transdermal exposure of BALB/c mice to cashew nut protein. Ten mice per group were transdermally exposed to cashew nut protein or saline as described in figure 1. Total plasma IgE levels were measured before and 4 days after the 4th exposure. Means $\pm$ SE.

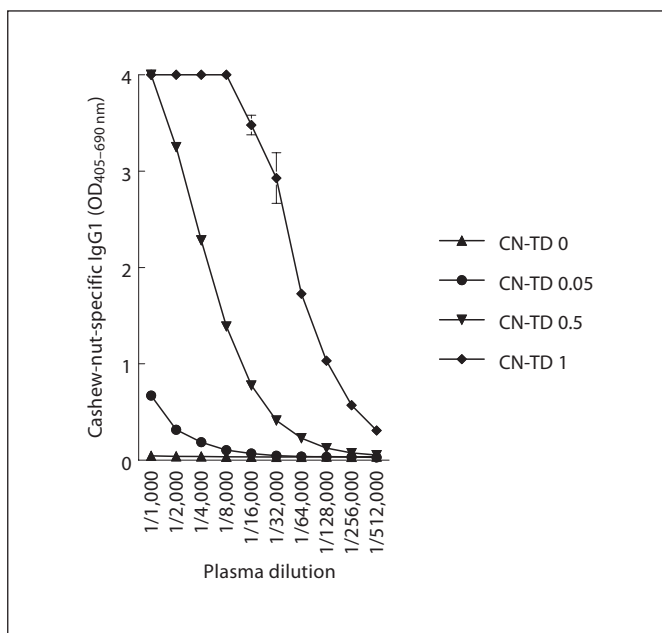


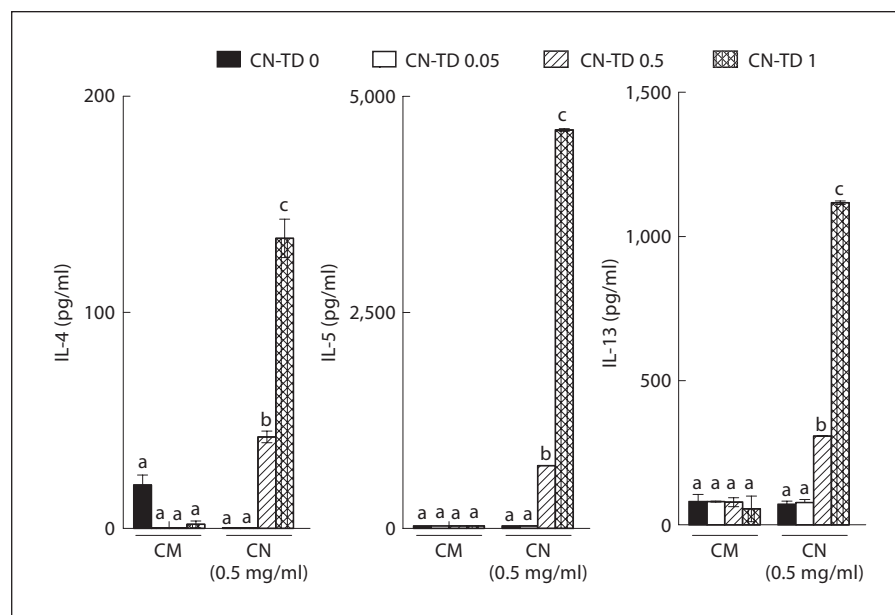
Fig. 3. Specific IgG1 antibody response to transdermal exposure of BALB/c mice to cashew nut protein. Ten mice per group were transdermally exposed to the indicated doses of cashew nut protein (0, 0.05, 0.5 or 1 mg/mouse). Specific IgG1 antibody levels were measured before exposure and after the 4th exposure. Data shown represent IgG1 antibody levels after the 4th exposure (means $\pm$ SE); there was no significant cashew-nut-specific IgG1 antibody in the plasma before exposure. CN = Cashew nut; TD = transdermal.

Results

Transdermal Exposure to Cashew Nut Protein Results in Marked IgE Antibody Response

We performed dose-response and time-course experiments and analyzed antibody responses to transdermal exposure to cashew nut protein. As evident, whereas a dose of 0.05 mg/mouse did not elicit significant cashew-nut-specific IgE antibody titers, a dose of 0.5 and 1 mg/mouse did after the 4th exposure (fig. 1). Analysis of plasma samples collected before exposure and after the 2nd and 4th exposures revealed a progressively higher memory IgE antibody response (data not shown). Enhanced total plasma IgE levels further confirmed induction of an allergic response (fig. 2). In order to test the specificity of the allergic response to cashew nut, we screened the plasma from sensitized mice with a panel of other allergenic foods (peanut, ovalbumin and sesame). There was no significant IgE binding to these food proteins. Cashew nut also elicited a robust dose-dependent IgG1 antibody response (fig. 3).

Fig. 5. Cashew-nut-allergic mice exhibit memory type-2 cytokine (IL-4, IL-5 and IL-13) responses: dose-response analysis. Spleen cells were isolated from the various groups of BALB/c mice (as described in figure 4) and cultured with cashew nut (CN) protein (0.5 mg/ml) or culture medium (CM) alone. Cell culture supernatants were harvested on days 1 and 3 and analyzed for cytokines using optimized ELISA. Data shown are from day 3, which exhibited significant cytokine responses (average $\pm$ SE of duplicate analyses). Bars with different letters are significantly different ($p < 0.05$; ANOVA). TD = Transdermal.



ing ours, have shown that transdermal exposure to other allergenic food proteins, e.g. ovalbumin, hazelnut and sesame seed, can result in both immune activation and clinical sensitization for immediate hypersensitivity reaction including systemic anaphylaxis [18, 19, 22]. Strid et al. [23] demonstrated a delayed hypersensitivity response to peanut via skin exposure in the ear using the tape-stripping method. They reported that mice also suffered from signs of anaphylaxis [23]. Thus, using cashew nuts, we further add evidence to the emerging concept that transdermal exposure to food proteins can activate the necessary systemic immune response pathways leading to clinical sensitization for severe oral food-allergic reactions such as systemic anaphylaxis in mice.

Previous efforts to develop a mouse model of food allergy may be divided into two types: (i) the adjuvant-based approach to develop a mouse model, and (ii) the adjuvant-free approach to develop a mouse model. Thus, mouse models of peanut allergy and cow's milk allergy are based on using adjuvant such as cholera toxin and alum [16, 24–26]. In contrast, mouse models of hazelnut allergy and sesame seed allergy have used a novel adjuvant-free method involving exposure to food proteins via the skin [18, 19, 22]. Here, we demonstrate for the first time that it is possible to use the adjuvant-free approach to develop a mouse model of cashew nut allergy. This is significant because an adjuvant-free mouse model of cashew nut allergy provides an opportunity to study the mechanism of disease in the absence of any adjuvant-re-

lated effects. Furthermore, this model that is independent of adjuvant is similar to the human disorder that also occurs in the absence of adjuvant.

It is largely unclear whether exposure to allergenic foods via the skin might lead to food-allergic sensitization in humans; however, this possibility has been discussed in the recent literature [27, 28]. There is epidemiological evidence implicating that peanut exposure via the skin in infants is associated with peanut allergy [28]. However, for cashew nut in particular, there is no evidence as yet for transdermal sensitization in humans. Our data demonstrate that it is clearly possible in BALB/c mouse. Therefore, these data warrant investigation of such a possibility in humans.

A number of human cashew-nut-allergic patients have been reported to exhibit allergies to other foods including peanut, pistachio nuts, walnuts, almonds, hazelnuts and pecans [7, 11, 12]. These and other studies suggest that there is potential for immune cross-reactivity among the tree nuts [12]. There is also a report of potential cross-reactivity between cashew and pectin [13]. Thus, it was unclear whether cashew nut allergy occurred in nature due to primary sensitization to cashew nut itself or secondary to immune cross-reactivity to other allergenic foods. Using a mouse model, we directly demonstrate that cashew nuts have intrinsic allergenic potential. Therefore, we suggest that cashew nut allergy could potentially result not only from immune cross-reactivity with other foods, but also from primary sensitization to cashew nut itself.

In summary, these data demonstrate that: (i) transdermal exposure to cashew nut protein, in the absence of adjuvant, elicits a robust IgE response leading to clinical sensitization of mice for systemic anaphylaxis to oral cashew nut protein challenge; (ii) cashew nut protein is a potent activator of type-2 cytokines, thus explaining the mechanism of cashew nut allergy, and (iii) this adjuvant-free mouse model may be useful for further basic and preclinical studies on cashew nut allergy.

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References

- 1 Sampson HA: Update on food allergy. *J Allergy Clin Immunol* 2004;113:805–819.
- 2 Bernhisel-Broadbent J, Dintzis HM, Dintzis RZ, Sampson HA: Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice. *J Allergy Clin Immunol* 1994;93:1047–1059.
- 3 Tariq SM, Stevens M, Matthews S, Ridout S, Twiselton R, Hide DW: Cohort study of peanut and tree nut sensitisation by age of 4 years. *BMJ* 1996;313:514–517.
- 4 Sicherer SH, Furlong TJ, Munoz-Furlong A, Burks AW, Sampson HA: A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J Allergy Clin Immunol* 2001;108:128–132.
- 5 Davoren M, Peake J: Cashew nut allergy is associated with a high risk of anaphylaxis. *Arch Dis Child* 2005;90:1084–1085.
- 6 Clark AT, Anagnostou K, Ewan PW: Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children. *Allergy* 2007;62:913–916.
- 7 Rance F, Bidat E, Bourrier T, Sabouraud D: Cashew allergy: observations of 42 children without associated peanut allergy. *Allergy* 2003;58:1311–1314.
- 8 Barre A, Sordet C, Culerrier R, Rance F, Didier A, Rouge P: Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Mol Immunol* 2008;45:1231–1240.
- 9 Li H, Zhang H: Hypersensitivity of cashew nut. *Chin Med Sci J* 1997;12:189–192.
- 10 Hourihane JO, Harris H, Langton-Hewer S, Kilburn SA, Warner JO: Clinical features of cashew allergy. *Allergy* 2001;56:252–253.
- 11 Quercia O, Rafanelli S, Marsigli L, Foschi FG, Stefanini GF: Unexpected anaphylaxis to cashew nut. *Allergy* 1999;54:895–897.
- 12 Goetz DW, Whisman BA, Goetz AD: Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific IgE serologic surveys. *Ann Allergy Asthma Immunol* 2005;95:45–52.
- 13 Ferdman RM, Ong PY, Church JA: Pectin anaphylaxis and possible association with cashew allergy. *Ann Allergy Asthma Immunol* 2006;97:759–760.
- 14 Wang LF, Lin JY, Hsieh KH, Lin RH: Epicutaneous exposure of protein antigen induces a predominant th2-like response with high IgE production in mice. *J Immunol* 1996;156:4077–4082.
- 15 Birmingham N, Payankaulam S, Thanavornakul S, Stefura B, HayGlass K, Gangur V: An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay. *J Immunol Methods* 2003;275:89–98.
- 16 Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA: A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;106:150–158.
- 17 Rempel JD, Lewkowich IP, HayGlass KT: Endogenous IL-12 synthesis is not required to prevent hyperexpression of type 2 cytokine and antibody responses. *Eur J Immunol* 2000;30:347–355.
- 18 Birmingham NP, Parvataneni S, Hassan HM, Harkema J, Samineni S, Navuluri L, Kelly CJ, Gangur V: An adjuvant-free mouse model of tree nut allergy using hazelnut as a model tree nut. *Int Arch Allergy Immunol* 2007;144:203–210.
- 19 Navuluri L, Parvataneni S, Hassan H, Birmingham NP, Kelly C, Gangur V: Allergic and anaphylactic response to sesame seeds in mice: identification of Ses i 3 and basic subunit of 11s globulins as allergens. *Int Arch Allergy Immunol* 2006;140:270–276.
- 20 Tsuchiya T, Nishimura Y, Nishiuma T, Kotani Y, Funada Y, Yoshimura S, Yokoyama M: Airway remodeling of murine chronic antigen exposure model. *J Asthma* 2003;40:935–944.
- 21 Yang X, HayGlass KT: IFN-gamma, but not IL-4, synthesis by antigen-primed murine T cells is IL-2 dependent. Differential requirements in de novo and established responses. *J Immunol* 1993;150:4354–4363.
- 22 Hsieh KY, Tsai CC, Wu CH, Lin RH: Epicutaneous exposure to protein antigen and food allergy. *Clin Exp Allergy* 2003;33:1067–1075.
- 23 Strid J, Hourihane J, Kimber I, Callard R, Strobel S: Epicutaneous exposure to peanut protein prevents oral tolerance and enhances allergic sensitization. *Clin Exp Allergy* 2005;35:757–766.
- 24 Adel-Patient K, Bernard H, Ah-Leung S, Creminon C, Wal JM: Peanut- and cow's milk-specific IgE, Th2 cells and local anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin. *Allergy* 2005;60:658–664.
- 25 Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA: A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* 1999;103:206–214.
- 26 Snider DP, Marshall JS, Perdue MH, Liang H: Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. *J Immunol* 1994;153:647–657.
- 27 Hayday K, Shannon S: Are early exposures linked with childhood peanut allergy? *J Fam Pract* 2003;52:516–517.
- 28 Lack G, Fox D, Northstone K, Golding J: Factors associated with the development of peanut allergy in childhood. *N Engl J Med* 2003;348:977–985.