

An Adjuvant-Free Mouse Model of Tree Nut Allergy Using Hazelnut as a Model Tree Nut

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Key Words

Food allergy · Systemic anaphylaxis · Immunoglobulin E · Cytokine · Adjuvant

Abstract

Background: Tree nut allergy, a major group of food allergy, is often linked to fatal or near-fatal systemic anaphylaxis. Currently, an adjuvant-free mouse model to study tree nut hypersensitivity is unavailable. Here we tested the hypothesis that transdermal exposure to hazelnut, a model tree nut, without the use of an adjuvant is sufficient to sensitize mice for immediate hypersensitivity reaction to oral hazelnut challenge. **Methods:** BALB/c mice were repeatedly exposed to hazelnut protein via the transdermal route and systemic allergic and anaphylactic responses were studied. **Results:** Transdermal exposure to hazelnut protein elicited robust systemic IgE response in a dose-dependent manner with immunological memory. Oral challenge of transdermally sensitized mice with hazelnut protein resulted in immediate (30 min after the challenge) clinical signs of systemic anaphylaxis as measured by significant clinical scores and drop in rectal temperature. Clinical hypersensitivity reaction was associated with severe pathological changes in the small intestine.

Hazelnut-allergic but not control mice exhibited in vivo activation of GATA-3 and hazelnut-driven recall IL-4, IL-5 and IL-13 response by splenocytes, thus elucidating the underlying mechanism of hazelnut allergy development in this model. **Conclusions:** These data suggest that (1) transdermal exposure to hazelnut protein is sufficient to activate the key immune pathways necessary for sensitizing mice for clinical immediate hypersensitivity reactions and (2) this mouse model may be useful for further basic and applied studies on tree nut allergy, especially because it does not depend on an adjuvant for eliciting immediate hypersensitivity reactions to nut protein. Copyright © 2007 S. Karger AG, Basel

Introduction

Immediate hypersensitivity response to food, commonly called food allergy, afflicts 6–8% of children and ~2–4% of adults in Westernized countries, including Europe and the USA [1, 2]. Tree nut allergies in particular affect ~0.5% of adults in the USA [1]. There is growing concern and indication that the prevalence of food allergy might be increasing consistent with asthma and

other allergic diseases for reasons that are not completely clear [1, 3, 4]. Although most food allergies typically start in infancy and tend to be outgrown before adulthood, some food allergies such as tree nut allergies are considered to last for a lifetime as chances of outgrowing such sensitizations are minimal [1]. Furthermore, peanuts, tree nuts (such as hazelnuts and walnuts) and sesame seeds are the major food types that often cause systemic anaphylaxis with fatal or near-fatal consequences [1, 2, 5, 6]. Thus, tree nut allergy is a clinically significant and very serious immune-mediated disorder that remains incompletely understood at present.

Scientific evidence based on double-blind placebo-controlled oral hazelnut challenge tests clearly confirms that hazelnut can cause immediate hypersensitivity reaction in humans, often with systemic anaphylaxis [7–9]. Hazelnut allergy is more common in Europe than in the USA. One reason often cited for this geographic difference is cross-reactivity between birch pollen, hazel pollen allergens and hazelnut proteins [10, 11]. However, whether hazelnut proteins themselves can act as primary sensitizers for clinical disease is not known. We have previously demonstrated that hazelnut proteins themselves can elicit IgE responses in mice [12]. However, whether exposure to hazelnut protein itself is sufficient to sensitize the host for clinical disease including systemic anaphylaxis is not known.

In the past several years, a number of interesting mouse models – often using an adjuvant such as cholera toxin in the experimental protocols – to study food allergy have been reported. These include peanut, milk and egg allergies [13–15]. Currently, an adjuvant-free mouse model of tree nut allergy is unavailable.

The specific mechanism of sensitization to food proteins is not completely understood. Exposure to peanut protein via skin has been suggested as a possible route of sensitization in the development of peanut allergy [16]. Here we tested the hypothesis that transdermal exposure to hazelnut, a model tree nut, is sufficient to sensitize mice for immediate hypersensitivity reaction to oral hazelnut challenge. Our data demonstrate that transdermal exposure to hazelnut protein, without the use of an adjuvant, is sufficient to sensitize mice for clinical disease, including systemic anaphylaxis associated with severe damage to the small intestine. This is because transdermal exposure to hazelnut protein leads to robust activation of type 2 transcription factor and type 2 cytokines resulting in IgE response. We also suggest that this adjuvant-free model may be useful for further mechanistic and applied studies on tree nut allergy.

Materials and Methods

The following materials were used: hazelnut/filbert protein extract (Greer Labs, Lenoir, N.C., USA), hazelnut extract was tested for protein content (Lowry's method); lipopolysaccharide content of this material was tested and found to be <0.5 pg/mg protein as measured by limulus amebocyte lysate assay (Cambrex Bio Science Walkersville Inc., Walkersville, Md., USA); biotin-conjugated rat anti-mouse IgG1, IgG2a and IgE antibodies; paired antibodies and recombinant standards for mouse IL-4, IL-5, IL-13 and IFN- γ (BD Pharmingen, San Diego, Calif., USA); *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo., USA); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, Pa., USA).

Transdermal Sensitization and Bleeding

BALB/c female mice were purchased from The Jackson Lab (Bar Harbor, Me., USA). Only adult animals (6–8 weeks old) were used in the study. All animal procedures used were in accordance with the Michigan State University policies. Transdermal exposure experiments were performed using the method described by us before [12]. Groups of mice were exposed to saline or hazelnut protein extract (5, 50 and 500 μ g/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 was covered with nonlatex nonocclusive bandage for 3 days. Mice were rested for 4 days. Then the cycle of exposure to saline or hazelnut was continued for 6 weeks. Blood samples were collected by the saphenous vein and plasma was used in the antibody analysis.

Measurement of Hazelnut-Specific IgG1, IgE, IgG2a Antibodies and Total IgE Levels in the Plasma

We have previously described optimization of enzyme-linked immunosorbent assay (ELISA) for food-specific IgG1, IgG2a and IgE antibody analyses [17, 18]. The ELISA procedure used in this study was essentially as described by us before using pooled plasma samples [17, 18]. Total plasma IgE levels were measured using paired antibodies and isotype standard purchased from BD Pharmingen.

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

Groups of hazelnut-sensitized versus saline-exposed mice were orally challenged with hazelnut protein (13 mg/mouse) or 500 μ l of saline per mouse on day 10 following the 6th exposure, using mouse feeding needles (22 gauge; Popper and Sons Inc., New Hyde Park, N.Y., USA). Mice were then observed for signs of systemic anaphylaxis during the next 30 min. Clinical scoring (on a scale of 0–5) was performed by 3–4 individuals in a blinded manner according to the method described previously [15]. Scores were as follows: 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; 5 = death.

Rectal temperature was measured using a temperature probe (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA) before and 30 min after oral challenge.

Histopathologic Analysis

Histological changes in the gastrointestinal tract were evaluated 1 h after an oral challenge. Mice transdermally sensitized with hazelnut protein or saline were orally challenged with hazelnut on day 10 after the 6th transdermal exposure. Mice were sacrificed 1 h after oral challenge by CO₂ euthanasia. Tissues from the gastrointestinal tract were removed from the abdominal cavity for histological analysis. The gastrointestinal tract was divided into sections (esophagus, stomach, duodenum, jejunum, ileum and colon) and fixed in 10% formalin in PBS for 4 days. Fixed tissues were embedded in paraffin, cut into 3- to 5- μ m-thick sections and mounted on glass slides. Slides were then stained with hematoxylin-eosin and analyzed for histological changes by light microscopy.

Spleen Cell Culture and Cytokine Analyses

Spleen cells were harvested and standard cell cultures were set up essentially as described [19]. Briefly, spleen cells were cultured (7.5×10^6 /ml) in the absence and presence of hazelnut protein extract (100 and 500 μ g/ml). Cell culture in the presence of medium alone served as a negative control and phorbol 12-myristate 13-acetate (1 μ g/ml) plus ionomycin (1 μ g/ml) as a positive control. Cell culture supernatants were harvested at various time points for use in cytokine analyses using preoptimized ultrasensitive assays (assay sensitivity: IL-4, 0.13 pg/ml; IL-5, 2.8 pg/ml; IL-13, 2.8 pg/ml; IFN- γ , 0.72 pg/ml).

GATA-3 Western Blot Analysis

Spleens from hazelnut-sensitized and saline-exposed mice were harvested 1 h after oral challenge. Total protein was isolated and subjected to 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 16 h at 4°C. The blot was blocked (4% nonfat dry milk) for 2 h at room temperature. The blot was incubated for 1 h at room temperature with antibody to GATA-3 (HG3-31, sc-268) at a dilution of 1/1,000 (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA). Then it was washed in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 30 min with shaking. The blot was then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse antibody (1/3,000 final dilution; Pierce Biotechnology, Rockford, Ill., USA) followed by washing in TBST for 30 min with shaking. The signal was detected with a chemiluminescence detection kit (Super Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology).

Western Blot and N-Terminal Amino Acid Sequence Analysis

Hazelnut protein extract was subjected to 12% SDS-PAGE and then electrophoretically transferred onto a PVDF membrane (Bio-Rad) for 12 h at 4°C. The blot was blocked (4% nonfat dry milk) and then incubated with protein G-treated (Pharmacia) plasma samples. Following washing with TBST, protein-bound IgE was detected using biotin-labeled rat anti-mouse IgE followed by streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch). The blot was developed with a chemiluminescence detection kit (Super Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology). The IgE-binding protein was isolated and used in sequence analysis. N-terminal amino acid sequencing was performed at the macromolecular structure facility (Michigan State University) on a Perkin Elmer/Applied Biosystems 494cLC Procise Protein Sequencer which utilizes Edman Degradation Chemistry.

Statistical Analysis

Student's t test and one-way ANOVA were used to evaluate significance using the Analyse-It™ software program (Analyse-It Software Ltd., Leeds, UK). The statistical significance level was set at 0.05.

Results

Transdermal Exposure to Hazelnut Results in Dose-Dependent Induction of IgE Response with Immunological Memory

We conducted dose-response and time course analyses of IgE antibody responses during transdermal exposure over a 6-week period. As evident in figure 1a, a dose of 5 μ g/mouse did not elicit detectable hazelnut-specific IgE response, but IgE response was clearly evident with the 50 and 500 μ g/mouse doses. Further analysis of antibody titers revealed progressively higher memory IgE antibody responses. Furthermore, analysis of total plasma IgE levels further confirmed elevated, dose- and time-dependent induction of allergic antibody response (fig. 1b). Since the dose of 500 μ g/mouse elicited the best IgE response, all follow-up studies used this dose for transdermal exposure. Hazelnut also elicited a robust IgG1 antibody response and a modest IgG2a antibody response (data not shown).

Transdermal Exposure to Hazelnut Is Sufficient to Sensitize BALB/c Mice for Systemic Anaphylaxis in Response to Oral Challenge with Hazelnut

In these experiments, mice were transdermally exposed to a dose of 500 μ g/mouse of hazelnut or saline 6 times over a 6-week period and then used in the oral challenge studies. Following oral challenge with hazelnut, only hazelnut- but not saline-exposed control mice exhibited significant signs of systemic anaphylaxis that were scored as described in Materials and Methods (fig. 2a). All mice with symptoms of systemic anaphylaxis with scores of 4 also exhibited signs of labored respiration. This data from all symptom scorers was highly consistent and reproducible. Opposite challenge confirmed the necessity of prior transdermal exposure to hazelnut to sensitize mice for systemic anaphylaxis in response to oral challenge. Furthermore, only hazelnut-sensitized but not control mice exhibited a significant drop in rectal temperature after 30 min (fig. 2b).

We then conducted histopathologic analysis. During tissue collection, severe edema of the small intestine was noted in hazelnut-sensitized mice following oral hazelnut challenge. In treated mice there was marked thickening of

Fig. 1. Dose-response and time course analyses of IgE antibody response to transdermal hazelnut exposure in BALB/c mice. Groups of mice (4 mice/group) were transdermally exposed to indicated doses of hazelnut protein extract (0, 5, 50 or 500 $\mu\text{g}/\text{mouse}$). **a** Specific IgE antibody levels were measured before (Pre) and after 1st, 2nd, 3rd, 4th, 5th and 6th exposure (1R–6R). Data are shown as geometric means $\pm$ SE. **b** Total IgE levels were measured before (3 days before 1st exposure, Pre) and 3 days after 3rd (3R) and 6th (6R) exposure. Data are shown as means $\pm$ SE. AVOVA test: bars with different letters are significantly different ($p < 0.05$).

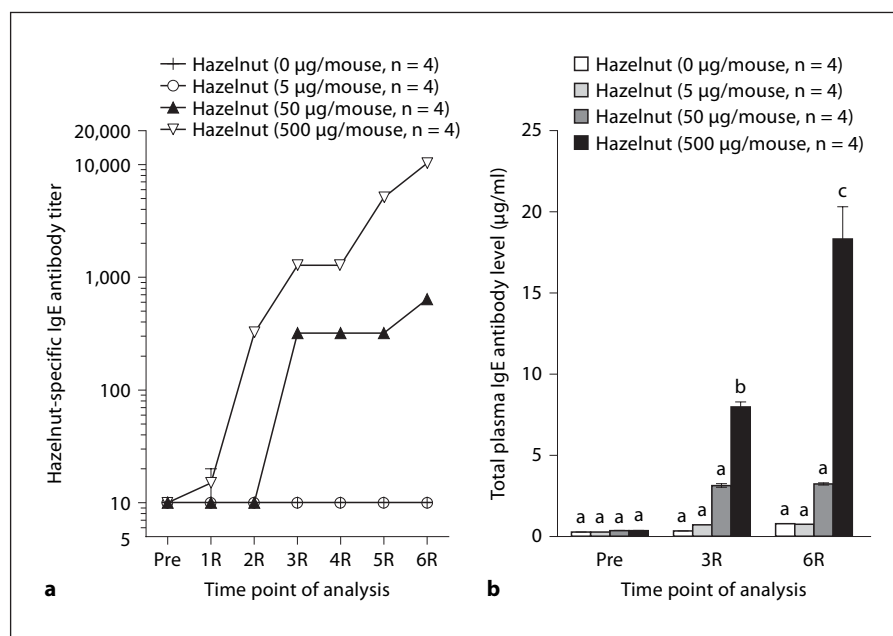
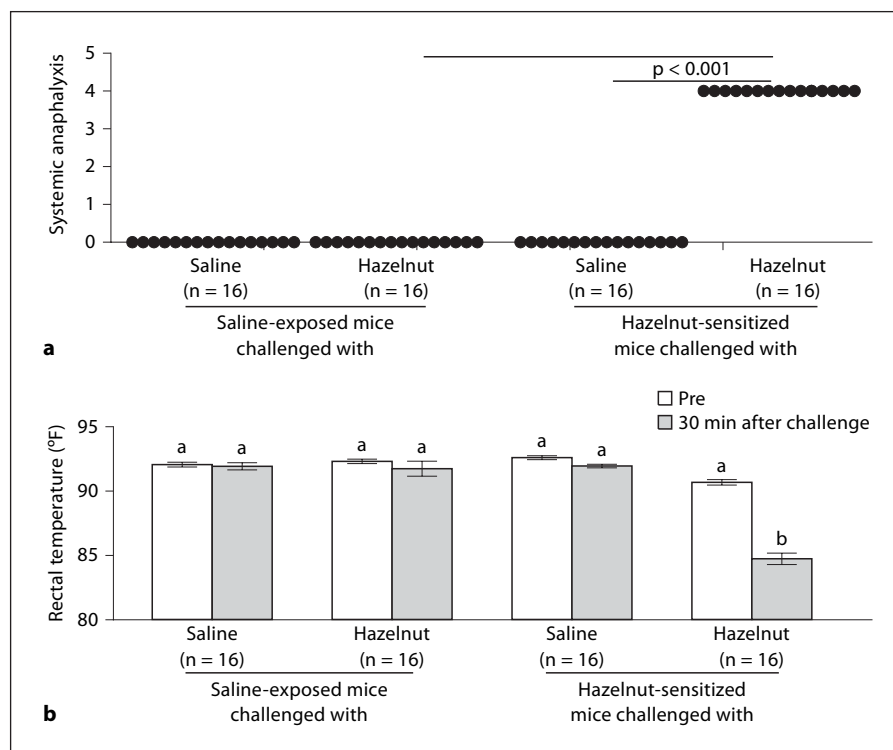


Fig. 2. Transdermal exposure to hazelnut is sufficient to sensitize BALB/c mice for systemic anaphylaxis following oral challenge with hazelnut. Groups of BALB/c mice (16 mice/group) were sensitized with hazelnut (500 $\mu\text{g}/\text{mouse}$) or saline by transdermal exposure as described in Materials and Methods. After 6 cycles of exposure, IgE induction was confirmed and then orally challenged with hazelnut (13 mg/mouse) or saline and examined for indicators of systemic anaphylaxis. Data are shown as means $\pm$ SE. **a** Clinical symptoms are shown as a scatter plot with each symbol representing 1 mouse. Kruskal-Wallis test results: hazelnut-sensitized mice with hazelnut challenge, $p < 0.05$ vs. all other groups. **b** Changes in rectal temperature. ANOVA test results: bars with different letters are significantly different ($p < 0.01$).



the duodenal, jejunal and ileal mucosa due to diffuse interstitial edema and vascular congestion in the lamina propria (fig. 3). Exfoliation of villar epithelial cells often accompanied these peracute lesions. Inflammatory cell infiltration was not a concurrent histopathologic feature in

these sections of small intestine. Whereas remarkable histopathology was absent in the colon or stomach, only minimal to mild intramural edema was evident in the esophagus. However, there was no major morphologic alteration to the surface squamous epithelium in the esophagus.

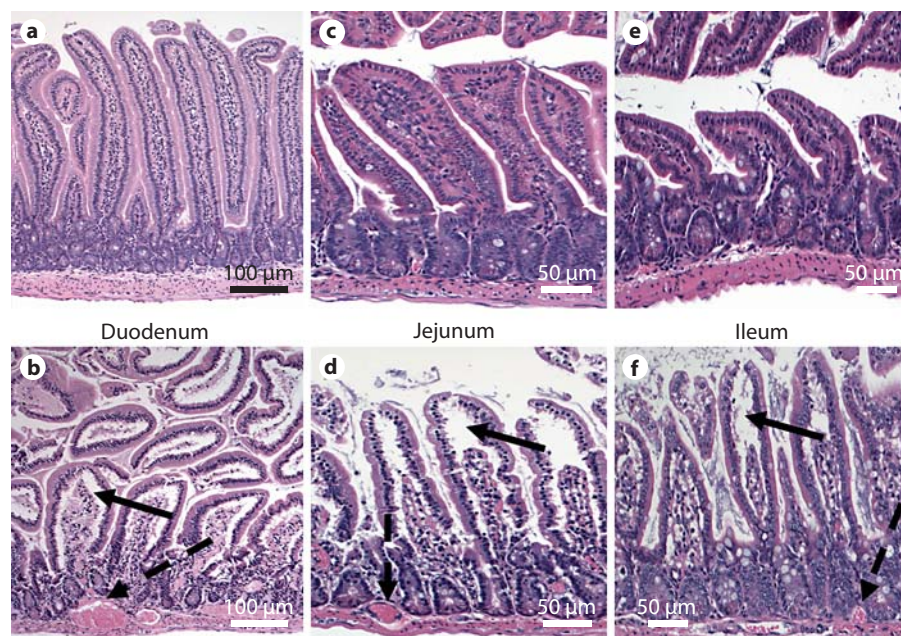


Fig. 3. Severe intestinal damage is associated with clinical signs of systemic anaphylaxis in this model. Groups of BALB/c mice ($n = 16$) were sensitized with hazelnut ($500 \mu\text{g}/\text{mouse}$) or saline by transdermal exposure as described in Materials and Methods. After 6 cycles of exposure, IgE induction was confirmed and mice were challenged orally with hazelnut ($13 \text{ mg}/\text{mouse}$). One hour later, sections of the gastrointestinal tract were taken and fixed for 4 days in 10% neutral buffered formalin. Fixed tissues were em-

bedded in paraffin, cut into 3- to 5- μm -thick sections and mounted on glass slides and stained with hematoxylin-eosin. Slides were analyzed with a light microscope for histological changes. Large solid arrows indicate edema, broken arrows indicate vascular congestion. **a, b** Duodenum from a saline- vs. hazelnut-sensitized mouse. **c, d** Jejunum from a saline- vs. hazelnut-sensitized mouse. **e, f** Ileum from a saline- vs. hazelnut-sensitized mouse.

Hazelnut-Sensitized Mice Exhibit in vivo Activation of GATA-3 and Recall Type 2 Cytokine Responses

Hazelnut significantly activated recall type 2 cytokine (IL-4, IL-5 and IL-13) responses in spleen cells from hazelnut-but not saline-exposed mice in a dose-dependent manner (fig. 4a-f). Hazelnut also elicited a modest specific IFN- γ response [day 3: culture medium, $10.6 \pm 0.4 \text{ ng}/\text{ml}$; hazelnut ($500 \text{ ug}/\text{ml}$) culture, $13.9 \pm 1 \text{ ng}/\text{ml}$, t-test, $p < 0.05$). We examined in vivo GATA-3 protein expression by Western blot analysis and found markedly higher GATA-3 protein in the spleen of hazelnut-sensitized mice compared to saline control mice (fig. 4g).

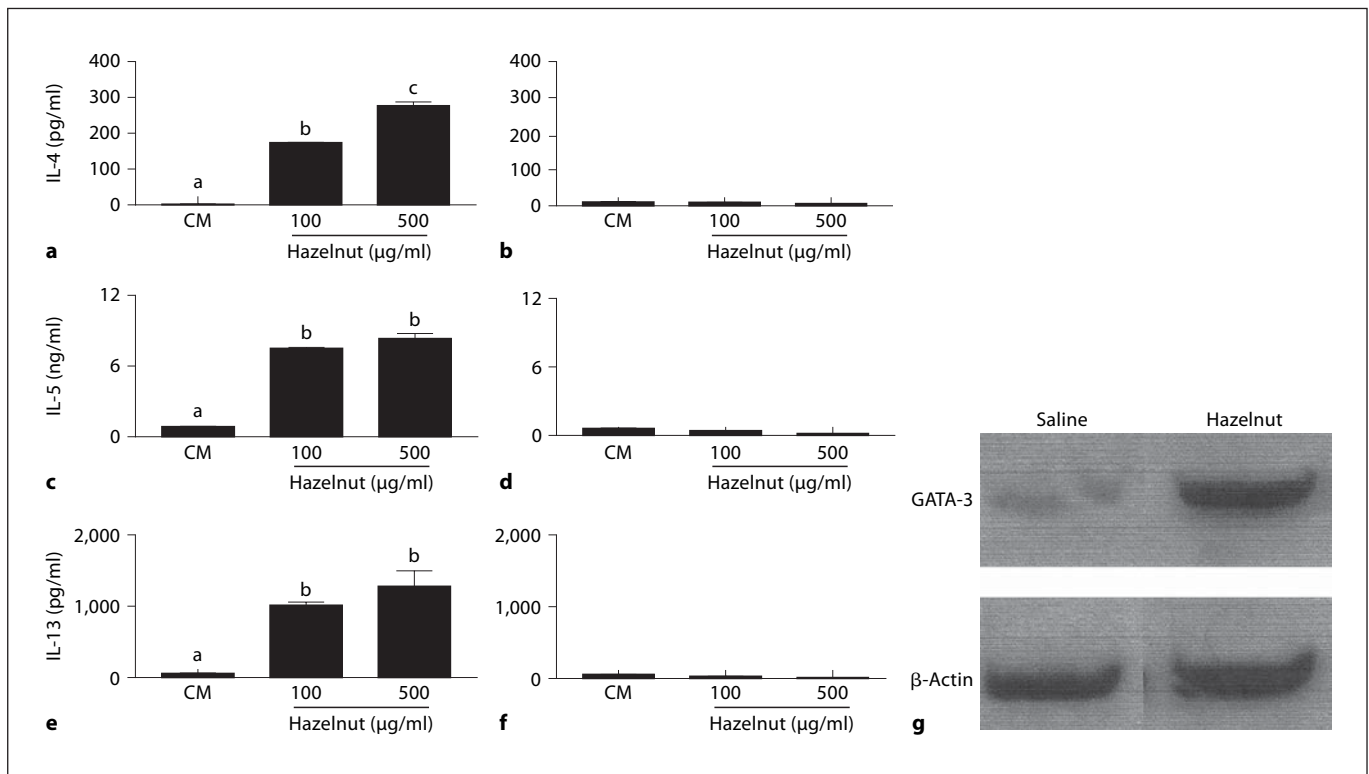
Identification of Hazelnut Allergens

We performed Western blot analysis using control plasma versus hazelnut-sensitized plasma and identified 1 major allergen (49 kDa) and 1 minor allergen ($\sim 37 \text{ kDa}$; fig. 5). Subsequently, N-terminal amino acid sequence analysis identified the major allergen as Cor a 9, an 11 s globulin (N-terminal sequence: GFEETICSLRLRENI). The minor allergen could not be sequenced.

Discussion

There are 3 important and novel findings from this study. (1) Repeated transdermal exposure of BALB/c mice to hazelnut protein (in the absence of an adjuvant) is sufficient to sensitize mice for clinical immediate hypersensitivity reaction including clinical signs of systemic anaphylaxis and hypothermia in response to oral challenge with hazelnut protein. (2) Systemic anaphylaxis was associated with severe damage in the small intestine. (3) The mechanism underlying the transdermal sensitization involves activation of GATA-3 transcription factor and type 2 cytokine responses (IL-4, IL-5 and IL-13), thus leading to systemic IgE antibody response and clinical sensitization.

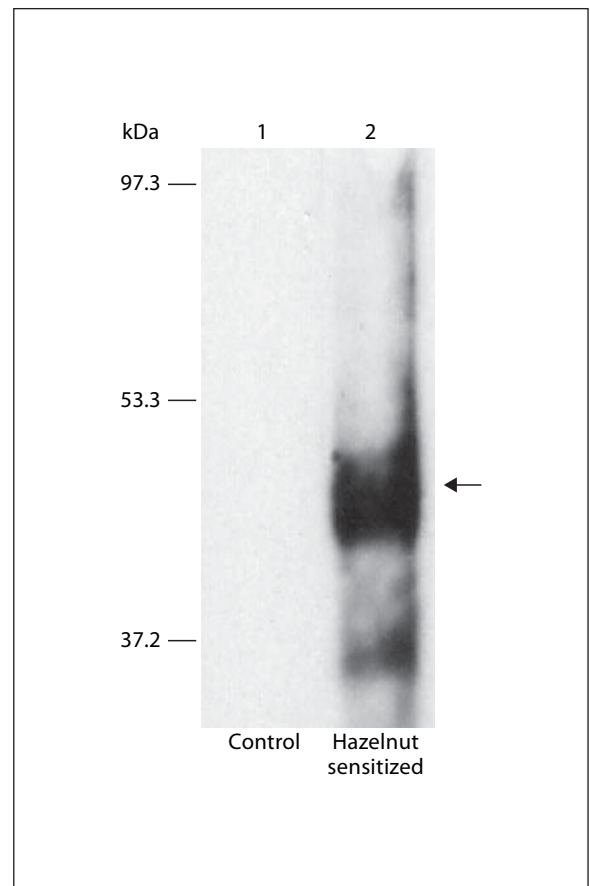
In this study, the BALB/c strain was chosen to develop a tree nut allergy model because (1) this strain has been widely used in allergy studies earlier although it was not clear whether BALB/c mice develop immediate hypersensitivity reactions to hazelnut [18, 20-22] and (2) we have previously reported IgE antibody response to hazel-



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Fig. 4. Hazelnut-sensitized but not saline control mice exhibit memory type 2 cytokine (IL-4, IL-5 and IL-13) responses and in vivo activation of GATA-3. Spleen cells were isolated from groups of BALB/c mice sensitized to hazelnut (**a, c, e**) or saline (**b, d, f**; 4–5 mice/group) and cultured with the indicated dose of hazelnut protein or culture medium (CM) alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown are average peak cytokine responses $\pm$ SE of duplicate analyses of data. Significance was determined by ANOVA test. Bars with different letters are significantly different ($p < 0.05$). **g** Groups of BALB/c mice (5 mice/group) were transdermally exposed to hazelnut (500 µg/mouse) or saline as described in Materials and Methods. Three days following a booster exposure with hazelnut or saline, spleen was harvested and used in Western blot analysis for GATA-3 and β -actin protein expression (25 µg of protein/lane).

Fig. 5. Western blot analysis and identification of the major hazelnut allergen. Hazelnut protein extract was subjected to SDS-PAGE followed by Western blot analysis using protein G-treated plasma from hazelnut-sensitized mice (lane 2) and control mice (lane 1). Bound IgE was detected using anti-IgE antibody. The major allergen (49 kDa) is indicated by an arrow.



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nut in BALB/c mice; here, we reconfirm these findings and extend our analysis to the dose-response, time course analysis including both specific IgE antibody and total IgE analyses [12]. More importantly, here we have developed a disease model focused on systemic anaphylaxis and hypersensitivity reactions in the gut. In addition, we have also studied *in vivo* GATA-3 activation and type 2 cytokine response as a possible mechanism leading to clinical allergic sensitization via induction of IgE antibodies.

We are not aware of previous studies in humans or animals examining immune consequences of transdermal exposure to any tree nuts or hazelnut. However, one study reported elevated spleen cell IL-5 cytokine response following epicutaneous exposure to purified ovalbumin protein [23]. But they did not study IL-4/IL-13 or the role of GATA-3. Another study, also using ovalbumin antigen, reported an epicutaneous method involving tape stripping after applying to skin, associated with elevated IL-4/IL-5 response by spleen cells [24, 25]. They also did not study GATA-3. More recently, Strid et al. [26] demonstrated delayed hypersensitivity response to peanut via skin exposure in the ear using the tape-stripping method. They also noted that mice suffered from signs of anaphylaxis. Thus, opposed to these previous reports, our model demonstrates in more detail systemic immune response following a transdermal route of exposure to a model tree nut, in an immediate hypersensitivity reaction model without using the tape-stripping sensitization method. In our model, symptoms of skin irritation or dermatitis were not noticed.

A previous interesting study reported that oral administration of hazelnut in BALB/c mice did not lead to sensitization unless antiulcer drugs were administered [27]. Notably, this study demonstrated that antiulcer drugs facilitate induction of hazelnut-specific IgG1 antibodies with immediate hypersensitivity skin reactions, although IgE antibodies were not detectable, thus demonstrating a role for antiulcer drugs in allergic response to hazelnut proteins. In contrast to this study, without the use of antiulcer drugs or any other adjuvant, here, we describe a mouse model of transdermal sensitization to hazelnut proteins that exhibits classical features of human hazelnut allergy such as (1) robust specific IgE antibody response associated with type 2 transcription factor and type 2 cytokine activation and (2) immediate hypersensitivity reaction including systemic anaphylaxis associated with severe intestinal damage in response to oral hazelnut challenge.

Previous efforts to develop models of food-induced systemic anaphylaxis have used a number of interesting and valuable approaches. Li et al. [15] used oral sensitization of C3H/HeJ mice with peanut plus cholera toxin, Snider et al. [28] used hen egg lysozyme and OVA with cholera toxin, while Li et al. [14] used a similar approach to develop a model of systemic anaphylaxis to milk. Hsieh et al. [23] used an ovalbumin/epicutaneous exposure method to develop similar models. Thus, in contrast to these studies, our efforts demonstrate that an adjuvant-free model could be developed for a model tree nut (such as hazelnut) that is implicated in potentially fatal human systemic anaphylaxis. Furthermore, our model provides an opportunity to study the mechanisms of tree nut allergy in the absence of any adjuvant-related effects.

The question whether exposure to allergenic foods via skin might lead to food-allergic sensitization in humans has been discussed recently [16, 29–31]. Thus, there is epidemiological evidence implicating exposure to peanut via skin in infants associated with peanut allergy [16]. Although for tree nuts or hazelnuts in particular there is no evidence as yet for transdermal sensitization in humans, our study demonstrates this possibility using a mouse model. It is noteworthy that the major allergen identified in our model (Cor a 9) has also been reported to cause systemic anaphylaxis in human hazelnut-allergic subjects [32].

In summary, these data argue that (1) transdermal exposure to hazelnut protein is sufficient to activate the key immune pathways necessary for sensitizing mice for clinical immediate hypersensitivity reactions and (2) this mouse model may be useful for further basic and applied studies on tree nut allergy especially because it does not depend on an adjuvant for eliciting immediate hypersensitivity reactions to nut protein.

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