

Hazelnut Allergy: Evidence that Hazelnut Can Directly Elicit Specific IgE Antibody Response via Activating Type 2 Cytokines in Mice

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Key Words

Hazelnut · Food · Allergy · Type 2 cytokines · IgE ·
Mouse model

Abstract

Background: Hazelnut is one of the major tree nuts that causes potentially fatal food allergy, with underlying mechanisms that are unclear at present. One suggestion is that hazelnut allergy results from immune crossreactivity of IgE antibodies produced against certain aeroallergens. We tested the hypothesis that hazelnut is intrinsically capable of eliciting an allergic response using a mouse model. **Methods:** Groups of mice were injected intraperitoneally with hazelnut/filbert protein extract with or without alum as an adjuvant, and hazelnut-specific antibody (IgE, IgG1) responses were examined using optimized enzyme-linked immunosorbent assay. Hazelnut-specific type 2 and type 1 cytokine responses were evaluated by ex vivo antigen-mediated activation of spleen cells. **Results:** Hazelnut elicited robust IgE and IgG1 antibody responses. Timecourse and dose-response analyses further provided evidence for memory type 2-dependent antibody responses to hazelnuts. Hazelnut-specific IgE response in two strains of mice with different MHC haplotypes and IgE response to hazelnut

without the use of alum adjuvant asserted that hazelnut is intrinsically an allergenic food. The type 2 cytokine analyses revealed that hazelnut sensitization results from activation of IL-4 and IL-5, thus providing a mechanistic basis for hazelnut-specific IgE response. **Conclusion:** Our data argue that hazelnut – a widely consumed food – is intrinsically an allergenic food capable of directly eliciting hazelnut-binding specific IgE antibodies via activation of type 2 cytokines in mice.

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Introduction

Immediate hypersensitivity to food, commonly called food allergy, afflicts 6–8% children and 2% adults in Westernized countries such as the United States [1, 2]. The incidence of these diseases is believed to be increasing, consistent with asthma and other allergic diseases for reasons that are not clear [2–4]. Typically, food allergies start in infancy, and some food allergies such as tree nut allergies tend to be chronic, often lasting for lifetime [2]. Peanuts and tree nuts (e.g., hazelnuts and almonds) are the major food types that often cause systemic anaphylaxis with fatal or near fatal consequences [1, 2].

Scientific evidence based on double-blind placebo-controlled oral hazelnut challenge tests clearly confirms that hazelnut can cause immediate hypersensitivity in humans [5–7]. Hazelnut allergy is more common in Europe than in the United States. One reason often cited for this geographic difference is crossreactivity between birch pollen (and hazel pollen) allergens and nut proteins [8, 9]. Given this crossreactivity, it is assumed that many cases of hazelnut allergy are actually secondary allergies and that one or more of the aeroallergens are the primary sensitizer. Nevertheless, it is not clear whether this is always the case, particularly in the United States where birch pollen allergy is relatively rare. Therefore, it is important to know whether hazelnut proteins can act as primary sensitizers. Thus, it is unclear at present whether hazelnut by itself is capable of directly eliciting specific IgE antibodies.

In this study, we tested the hypothesis that hazelnut by itself is capable of eliciting IgE response via activating prototypic type 2 cytokines (IL-4 and IL-5) in a mouse model. Our results provide the evidence that hazelnut is capable of eliciting both hallmarks of an allergic response, i.e. hazelnut-binding allergenic (IgE) antibodies as well as type 2 cytokine response (IL-4 and IL-5), in mice.

Materials and Methods

The following materials were purchased: hazelnut/filbert protein extract (Greer Labs, Lenoir, N.C., USA); hazelnut extract was tested for protein content by Lowry's method; biotin-conjugated rat anti-mouse IgG1, IgG2a and IgE antibody; paired antibodies and recombinant standards for mouse IL-4, IL-5 and IFN- γ (BD PharMingen, San Diego, Calif., USA); *p*-nitro phenyl phosphate (Sigma, St. Louis, Mo., USA), streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, Pa., USA), and Con-A (Sigma).

Mice Immunization and Bleeding

All mice were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). Only adult animals (6–7 weeks old) were used in the study. All animal procedures used were in accordance with the Michigan State University policies. A group of adult mice ($n = 4$) received intraperitoneal (i.p.) injection of 100 μ g of hazelnut protein extract plus alum (2.5 mg) as an adjuvant. Another group ($n = 4$) received 1,000 μ g of hazelnut protein extract plus alum. A third control group of mice ($n = 6$) received saline plus alum alone. All mice were bled 3 days before injections (preimmune bleeding), on day 11 after first injection (primary response). Animals received booster injections and were bled on indicated days during secondary and tertiary immune responses. Experiments where hazelnut alone was used involved 4 mice per group, per dose and per strain.

Epicutaneous exposure experiments were performed using a modified method described for ovalbumin [10]. Groups of mice ($n = 8$) were exposed to saline or hazelnut extract ($n = 12$; 500 μ g/100 μ l in saline per mouse and per application); the reagent was applied on the back skin of each mouse, where hair had been clipped off, and covered with non-latex bandage for 3 days. Mice were rested for 4 days. Then, the cycle of exposure to saline or hazelnut was continued for 6 weeks. Weekly blood samples were collected and plasma was used in the antibody analysis.

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

We have previously described optimization of enzyme-linked immunosorbent assay (ELISA) for food-specific IgG1, IgG2a and IgE antibody analyses [11, 12]. The ELISA procedure used in this study was essentially as described by us before using pooled plasma samples [11, 12].

Spleen Cell Culture and Cytokine Analyses

Spleen cells were harvested and standard cell cultures were set up essentially as described [13]. Briefly, spleen cells were cultured (7.5 million/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 Con-A (2 μ g/ml) served 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, IFN- γ 0.72 pg/ml).

Statistical Analysis

Student's *t* test was used to evaluate significance using the Analyse-ItTM software program (Analyse-It software Ltd., Leeds, UK). The statistical significance level was set at 0.05.

Results

Characterization of Hazelnut-Specific Antibody Responses in C57BL/6 Mice

Mice were evaluated for the appearance of hazelnut-binding type 2-dependent specific IgG1 and IgE antibodies in the plasma during primary (day 11), secondary (days 7 and 15) and tertiary (day 40) immune responses. Preimmune serum did not have detectable hazelnut-specific IgE antibodies (mean titer <40) or significant amounts of hazelnut-binding IgG1 antibodies (mean titer <800). Following injection with hazelnut extract (100 μ g), elevated levels of both IgE and IgG1 antibodies appeared (primary response day 11, mean IgE titer 160, mean IgG1 titer 1,024,000; secondary response day 15, mean IgE titer 452 $\pm$ 160, mean IgG1 titer 32,768,000). Next, we examined the dose response and timecourse of type 2-dependent antibody responses over a 3-month period. As evident from the results (fig. 2a), low dose of hazelnut (100 μ g) consistently elicited higher primary as well as higher memory

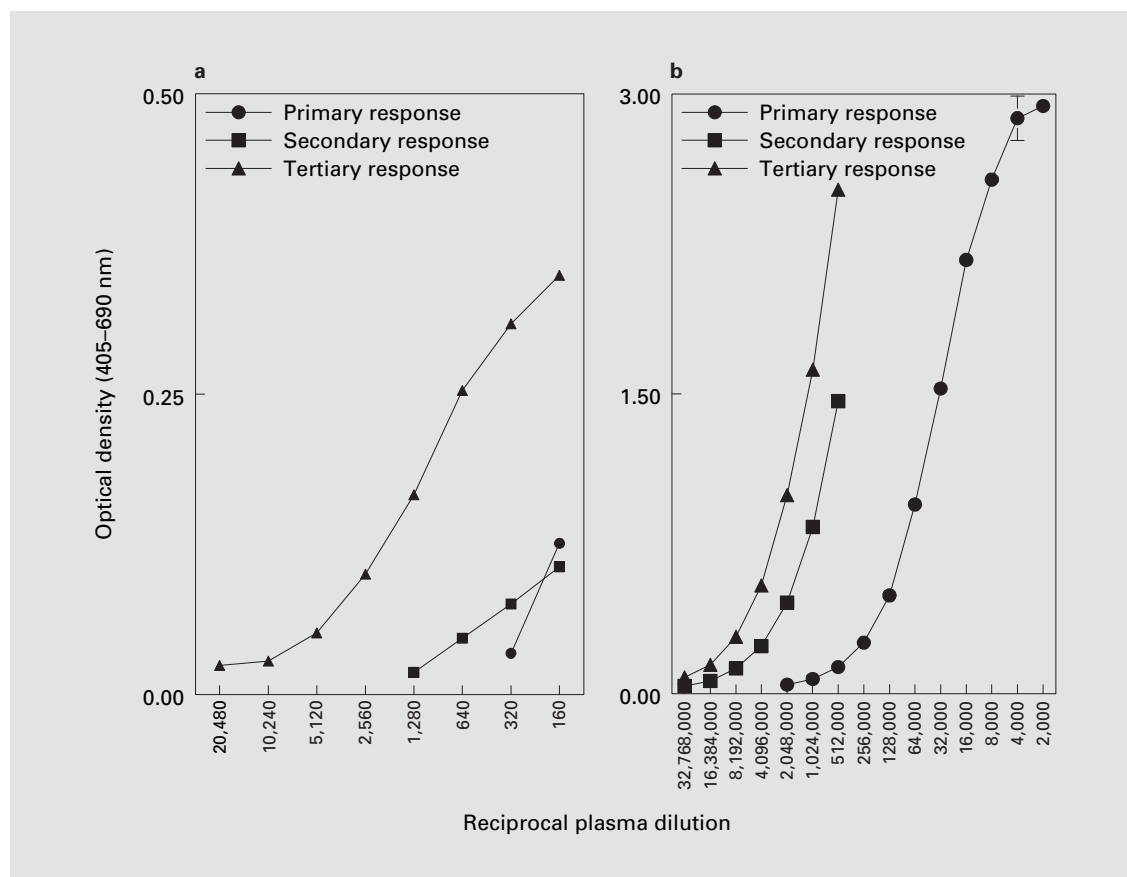


Fig. 1. Immune response to hazelnut in C57BL/6 mice. Groups of mice ($n = 4$) were injected with hazelnut protein extracts plus alum, and hazelnut-binding specific IgE (**a**) and IgG1 (**b**) antibodies were measured. Primary response = Antibody levels on day 11 after first injection; secondary response = antibody levels on day 7 after the second injection; tertiary response = antibody levels on day 40 after third injection. Data shown are optical density (mean $\pm$ SE). At some points, error bars are not visible.

IgE antibody responses relative to the high dose of 1,000 μ g per mouse. In contrast, although there was a memory IgG1 response to hazelnut, the intensity of the response was comparable at both doses tested (fig. 2b). These data provide direct evidence that hazelnut is capable of eliciting type 2-dependent antibody responses in mice.

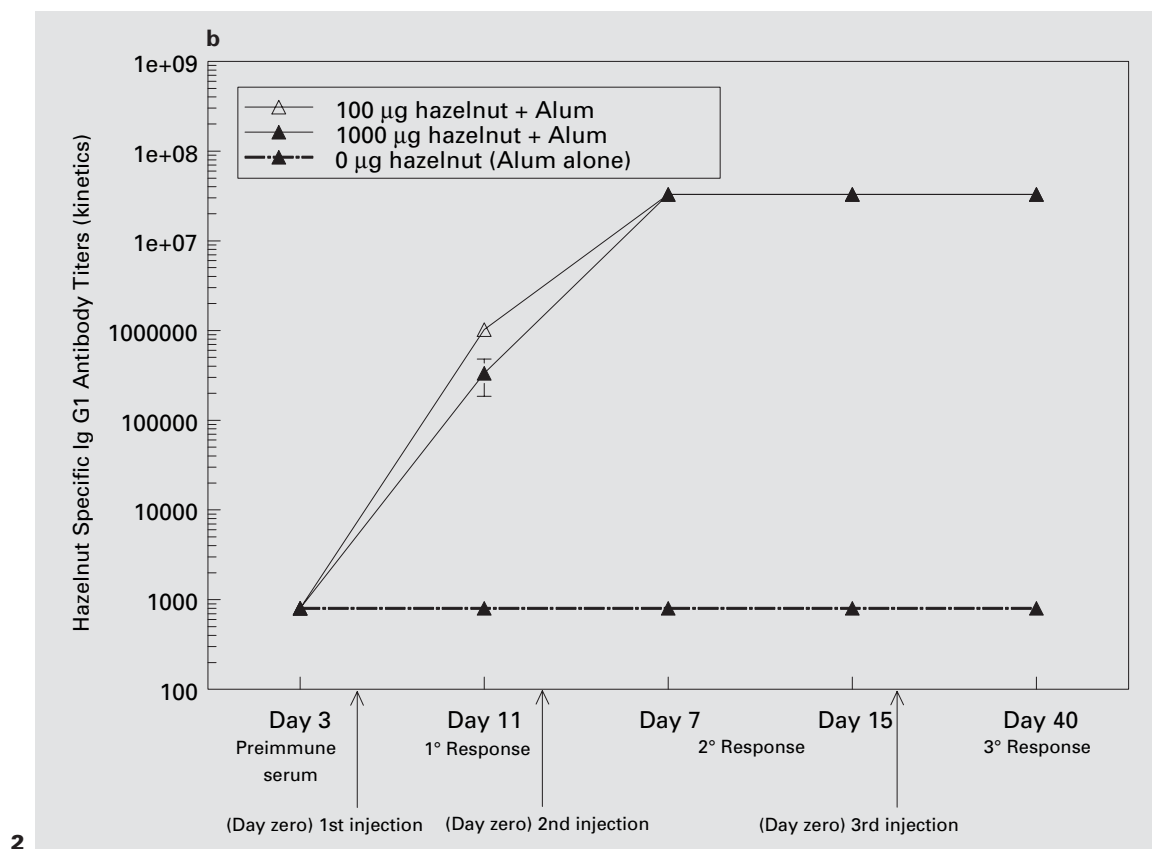
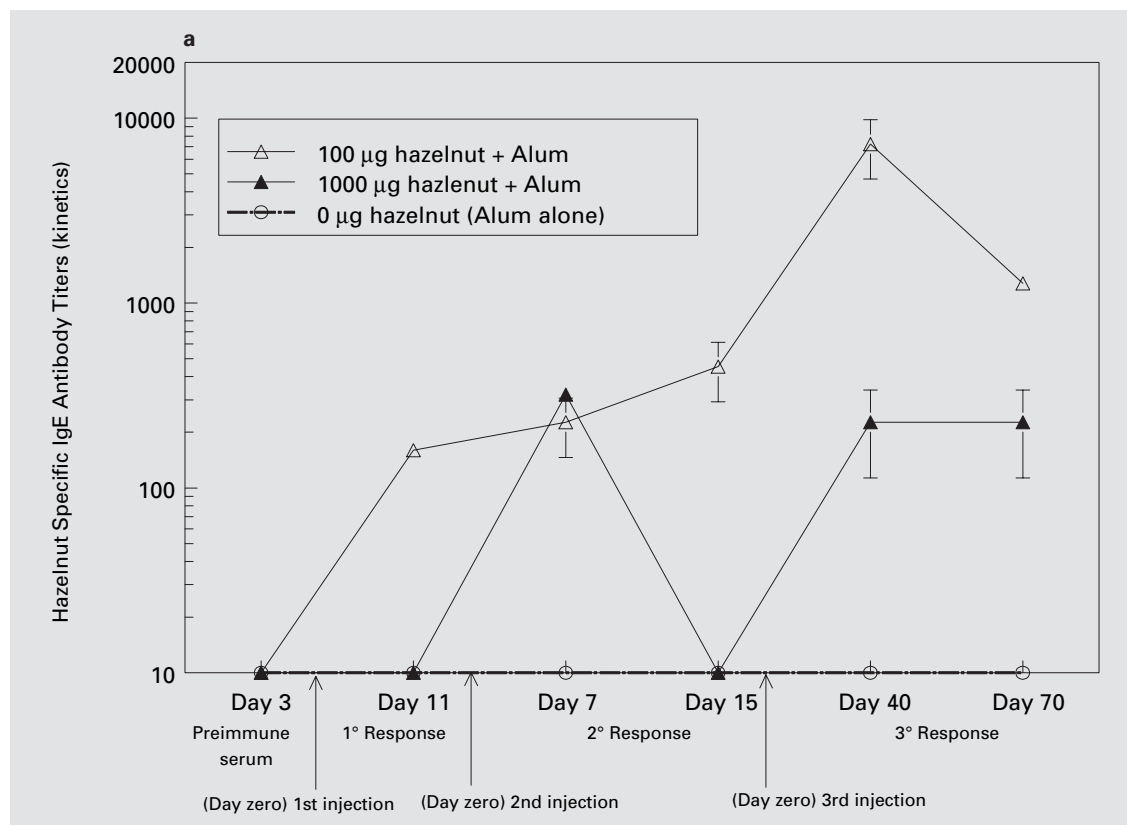
IgE Antibody Response to Hazelnut: Role of Host Strain, Alum Adjuvant and Route of Exposure

In order to test whether hazelnut is intrinsically an allergenic food, immune response to hazelnut was examined in two inbred strains of mice with different MHC haplotypes (C57BL/6 H-2^b and Balb/c H-2^d). As evident, hazelnut elicited a robust primary as well as memory IgE antibody response in both strains, although Balb/c exhib-

ited higher IgE titers (fig. 3a). Hazelnut-specific IgG1 and IgG2a antibody titers are shown in figure 3b, c. Furthermore, we tested whether hazelnut can elicit IgE antibody response in the absence of alum adjuvant. Results (table 1) demonstrated that hazelnut in the absence of an adjuvant is capable of eliciting IgE antibody response in Balb/c but not C57BL/6 mouse when injected by the i.p. route. We then found that exposure to hazelnut in Balb/c mice by the physiologically relevant epicutaneous route also elicits robust IgE antibody response (fig. 3d), confirming that hazelnut is intrinsically an allergenic food.

Characterization of Hazelnut-Specific Type 2 Cytokine Responses in Mice

In order to study the mechanism of IgE and IgG1 response to hazelnuts, we examined type 2 cytokine re-



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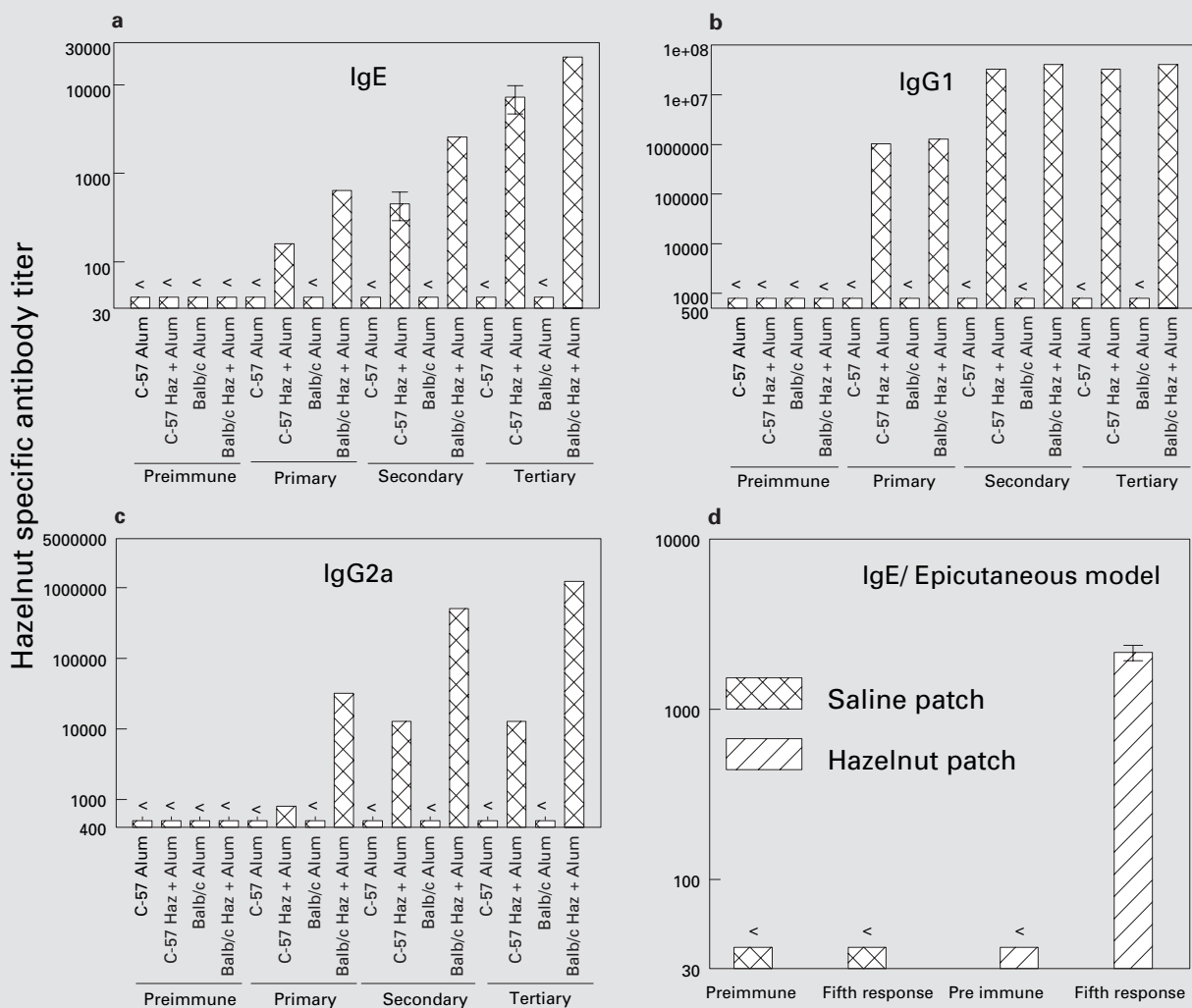


Fig. 3. Hazelnut-specific antibody responses in mice strains with differing MHC haplotype. **a–c** Groups of C57BL/6 H-2^b and Balb/c H-2^d mice (n = 4) were injected with hazelnut protein extracts plus alum or alum alone, and hazelnut-binding specific IgE, IgG1 and IgG2a responses were measured before and after three injections of hazelnut extracts. Data shown are geometric mean $\pm$ SE of specific antibody titers. **d** Groups of Balb/c mice were epicutaneously exposed to hazelnut (n = 12) or saline (n = 8), and systemic IgE antibody responses were analyzed. Data shown are geometric mean $\pm$ SE of specific IgE antibody titers in preimmune plasma versus plasma collected after 5 weeks of epicutaneous sensitization. Where titers were identical, error bars are not shown.

Fig. 2. Dose-response and timecourse analyses of type 2-dependent allergenic versus antigenic response to hazelnuts in C57BL/6 mice. Groups of mice (n = 4) were injected with hazelnut protein extracts plus alum, and hazelnut-binding specific allergenic antibody IgE (**a**) and antigenic IgG1 (**b**) responses were measured over a 3-month period with three injections of hazelnut extracts. Data shown are geometric mean $\pm$ SE of specific antibody titers. Where titers were identical, error bars are not shown.

responses in hazelnut-sensitized mice. As evident, hazelnut significantly activated the prototypic type 2 cytokine (IL-4 and IL-5) responses in spleen cells from hazelnut plus alum-sensitized C57BL/6 mice (fig. 4a, b). Cytokine response from control mice with alum alone is shown in figure 4c, d. Similar responses were also evident in Balb/c mice sensitized with hazelnut alone in the absence

Fig. 4. Hazelnut-specific type 2 cytokine (IL-4 and IL-5) responses in mice. Groups of C57BL/6 mice (n = 4) were sensitized with hazelnut (100 µg per mouse) plus alum or alum alone. After 3 days following a booster injection with hazelnut or saline, spleen cells were harvested and cultured with an indicated dose of hazelnut (HZ) or culture medium (CM) alone. Cell culture supernatants were harvested at different time points over 3 days and analyzed for cytokines using optimized ELISA. Data shown are peak cytokine response (average ± SE of duplicate analyses of data) from hazelnut plus alum-sensitized mice (**a**, **b**) and mice injected with alum alone (**c**, **d**). Student's *t* test was used: for IL-4, hazelnut 100 and 500 µg/ml versus culture medium alone, *p* < 0.05 (**a**); for IL-5, hazelnut 100 and 500 µg/ml versus culture medium alone, *p* < 0.05 (**b**). Con-A was used as a positive control (at 24 h IL-4 10.11 ± 0.42 pg/ml, IL-5 658.88 ± 8.13 pg/ml).

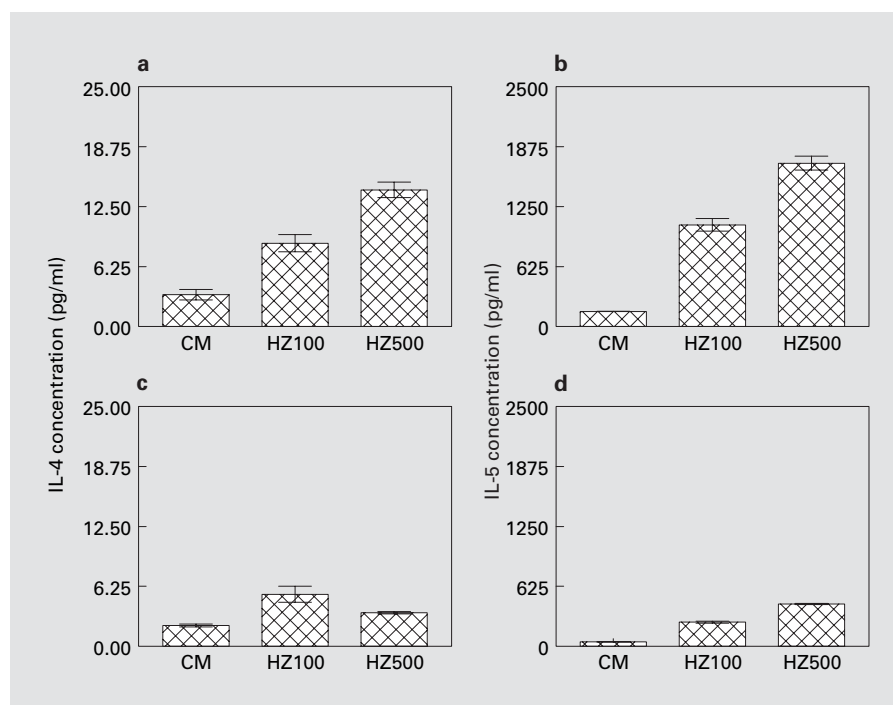


Table 1. Hazelnut-specific IgE antibody response in mice without the use of alum adjuvant

Mouse strain	Time point	Dose of hazelnut, µg/per mouse		
		0	100	1,000
<i>Balb/c</i> (n = 4/group/dose)	Preimmune	≤20	≤20	≤20
	Post	≤20	2,560	160
<i>C-57BL/6</i> (n = 4/group/dose)	Preimmune	≤20	≤20	≤20
	Post	≤20	≤20	≤20

Groups of mice were injected i.p. with hazelnut at indicated doses 6 times over a 4-week period, and antibody response was analyzed at multiple time points. Preimmune = Antibody levels in preimmune plasma; Post = peak antibody levels in the plasma following 6 injections of hazelnut protein extract.

of alum (data not shown). Hazelnut elicited a modest IFN-γ response in the C57BL/6 model of hazelnut plus alum sensitization: culture medium: 51.8 ± 4 pg/ml; hazelnut 100 µg/ml: 159 ± 2 pg/ml; hazelnut 500 µg/ml: 251 ± 35 pg/ml (12 h); culture medium: 87 ± 6 pg/ml; hazelnut 100 µg/ml: 252 ± 2 pg/ml; hazelnut 500 µg/ml: 404 ± 23 pg/ml (48 h).

Discussion

There are three important findings drawn from this study: (1) hazelnut can directly elicit hazelnut-binding IgE antibodies in mice; (2) this ability is an intrinsic property since IgE response was demonstrable in two different strains of mice with different MHC haplotype, as well as in the absence of alum adjuvant in Balb/c mice by the i.p. and epicutaneous route, and (3) hazelnut is capable of activating prototypic type 2 cytokine (IL-4 and IL-5) responses, thus explaining the mechanism underlying the observed IgE and IgG1 responses.

In this study, we chose C57BL/6 and Balb/c mice to examine allergenic immune response to hazelnuts. Previously, these strains have been widely used in the allergy studies although it was not clear whether they respond to hazelnut with specific IgE antibodies and type 2 cytokine responses [12, 14–16].

We chose to examine IgG1 and IgE responses to hazelnut as readouts of type 2 responses in this study. This is because both IgG1 and IgE isotypes of antibody production are dependent on the prototypic type 2 cytokine IL-4, although dependence of IgG1 is not complete [17–19]. Interestingly, hazelnut also elicited type 1 cytokine (IFN-γ)-dependent IgG2a antibody isotype.

In the present study, we found that hazelnut elicited very high levels of specific IgG1 antibodies with titers in

several millions. In mice, IgG1 antibodies participate in systemic anaphylaxis [20]. However, at present, it remains unclear whether hazelnut-specific IgG antibodies have any disease relevance in humans.

We chose to inject hazelnut to mice, because earlier studies in mice using other allergens (such as ovalbumin) have shown that i.p. injection is a more reliable and sensitive method for inducing IgE antibodies compared with oral exposure [21]. Since our objective was to test whether hazelnut elicits IgE antibodies in mice, we decided to choose the i.p. route over the oral exposure. In our laboratory, oral sensitization to hazelnut has not succeeded so far using very young (day 8 of birth) C57BL/6 mice (Birmingham et al., unpublished data). A very recent study (published subsequent to the submission of our manuscript) reported that oral administration of hazelnut in Balb/c mice did not lead to sensitization unless antiulcer drugs were administered [22]. 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. In contrast to this study, besides the i.p. route, using the physiologically relevant epicutaneous route of exposure to hazelnut, we found robust specific IgE antibody response confirming the intrinsic allergenic nature of hazelnuts.

There are previous studies examining antibody response to hazelnut in rabbits [23, 24], which reported that hazelnut elicited IgG antibodies. However, they did not study IgE or type 2 cytokine responses to hazelnuts.

It is noteworthy that hazelnut is an allergenic food (not an allergen per se) as it represents a source of allergens of which some have been characterized: Cor a 1 (a Bet v 1 homologue) [8], Cor a 2 profilin [8], 11S globulin [25], 2S albumin, lipid transfer protein and proteins with 47, 35 and 32 kDa, identified as allergens from hazelnut [7]. It is known that some of them are present in the pollen as well as in the seed explaining immune crossreactivity. It should be noted that results from our study do not provide evidence against the immune crossreactivity hypothesis of hazelnut sensitization. Rather, we have demonstrated that hazelnut by itself is also capable of eliciting specific IgE antibody responses in mice.

In summary, data from this study argue that hazelnut is capable of directly eliciting hazelnut-binding allergenic (IgE) antibodies via activating type 2 cytokine responses in mice.

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