

A Single Oral Sensitization to Peanut without Adjuvant Leads to Anaphylaxis in Mice

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Key Words

Food allergy • Peanut • Murine model • Oral exposure • Sensitization • Anaphylaxis • Skin tests • Specific IgE

Abstract

Background: A model of peanut food allergy has been developed in mice using a simple sensitization protocol leading to a quantitatively measurable allergic response.

Methods: C3H/HeJ mice received a single intragastric administration of whole peanut (80 mg) without adjuvant. Two weeks later, intraperitoneal challenge with peanut extract led to a severe anaphylaxis. **Results:** Anaphylactic reaction was evidenced by vascular leakage, severe clinical symptoms, a drop in body temperature, a decrease in breathing rate and also by increased concentrations of serum mouse mast cell protease-1. Sensitization to peanut was demonstrated by positive skin tests (ear swelling test and intradermal skin testing) and increased peanut-specific IgE levels.

Conclusions: Thus, we obtained a model of severe peanut hypersensitivity within 2 weeks following single oral exposure without adjuvant. This model may be useful for further basic and applied studies on peanut allergy.

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Introduction

Peanut food allergy is a major public health problem because of its severity, persistence and prevalence, which is estimated to be between 0.5 and 1% [1–3]. To date, strict dietary avoidance is the only way to manage peanut allergy, which implies constant dietary vigilance leading to severe effects on the quality of life of affected patients and their families and, in consequence, careful label reading of manufactured products [4]. Despite this, peanut is responsible for the greatest number of fatal food-induced allergic reactions [5–7]. These accidents often occur following unintentional ingestion because peanut is a common hidden allergen in food products. To produce a safe product, the manufacturer therefore has to ensure that any residual allergen is below levels which might provoke reactions, even in highly sensitive individuals. It constitutes an important challenge for food industries to guarantee safe products in order to reduce the risk of serious reactions in highly sensitized individuals [8]. For the understanding of immunological mechanisms and exploration of new therapeutic approaches to food allergy, clinical studies are the gold standard tests, but for obvious ethical reasons, it is not possible to implement them on a

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routine basis. Consequently, previous efforts have focused on developing mouse models of peanut-induced allergy using a number of interesting and valuable approaches to better understand immunopathogenic mechanisms [9–14] and to develop appropriate immunotherapeutic approaches to food allergies [15–18]. In order to study peanut allergy, these models often include in their experimental protocols several oral exposures to allergen coadministered with an adjuvant such as cholera toxin (CT). Currently, the development of an adjuvant-free mouse model of peanut allergy and its time course characterization using a single oral exposure for sensitization has not yet been reported.

The aim of our study was to design a food peanut allergy model using a simple protocol with the following criteria: (1) sensitization through the oral route without adjuvant in order to mimic the natural human exposure to peanut, (2) demonstration and follow-up of clinical allergic reactions upon a challenge with peanut and (3) induction of production of specific IgE recognizing the same allergens as those of peanut-allergic patients.

Materials and Methods

Mice

Six-week-old female C3H/HeJ mice were purchased from Charles River Laboratory (Lyon, France). The animals were maintained on peanut-free chow (Harlan Teklad, Gannat, France) under specific pathogen-free conditions on a 12-hour light/dark cycle in a room maintained at a mean temperature of $21 \pm 2^\circ\text{C}$ with a relative humidity of $50 \pm 20\%$. Drinking water and food pellets were provided ad libitum. Animals were handled in accordance with the French State Council guidelines for the use and care of laboratory animals (decree No. 87-848, 19 October 1987 and decree No. 2001-464, 29 May 2001) and the study was approved by the institutional Animal Care and Use Committee.

Antigens and Reagents

Raw whole peanuts and crude peanut extract (CPE) were provided by Allerbio (Varennnes-en-Argonne, France). Antibodies were purchased from Serotec Ltd. (Kidlington, UK) and Southern Biotech (Birmingham, Ala., USA). Compound 48/80 was obtained from Sigma (St. Louis, Mo., USA). Major recombinant peanut allergens rAra h 1, rAra h 2 and rAra h 3 were produced and purified in our laboratory [19].

Protocol for Peanut Sensitization and Challenge

Six groups of C3H/HeJ mice were sensitized by a single intragastric (i.g.) gavage of 80 mg/mouse of freshly homogenized whole peanut. At each time point (days 4, 7, 11, 14, 18 and 21 after sensitization), a group of mice was challenged by intraperitoneal (i.p.) injection of 30 mg CPE/mouse. Naive mice never exposed to peanut were used as negative controls. Just before each peanut challenge, individual blood samples from each group of mice were

obtained from the retroorbital venous plexus under isoflurane anesthesia, centrifuged and the sera were stored at -20°C until use. Skin tests (ear swelling test and intradermal skin testing) were then performed. After allergenic challenge, anaphylaxis was assessed by determining vascular leakage, monitoring clinical symptoms, rectal temperature, breathing rate and measuring a specific marker of mast degranulation, mouse mast cell protease-1 (mmcp-1), in serum.

Ear Swelling Test

CPE (10 μl , 5 mg/ml) was intradermally injected into the dorsal aspect of a mouse ear. Ear thickness was measured with a Digi-matic micrometer (Mitutoyo, Kawasaki, Japan). Ear swelling response was determined as the incremental increase in thickness above baseline control values. Compound 48/80 (5 mg/ml; Sigma), a potent histamine-releasing agent from mast cells, and PBS were used as positive and negative controls, respectively. Ear swelling response to CPE was determined 40 min after the injection.

Intradermal Skin Testing

Before testing, the abdominal skin was shaved. Evan's blue dye (100 μl , 0.25%) was intravenously injected. Five minutes later, CPE (10 μl , 2.5 mg/ml) was intradermally injected under isoflurane anesthesia. Compound 48/80 (30 $\mu\text{g}/\text{ml}$) and PBS were used as positive and negative controls, respectively. To avoid solution confluence, a 1.5-cm distance was kept between the sites of injections. A blue wheal with a diameter >0.3 cm appearing within 5 min after the injection of allergen was considered positive.

Measurement of Serum Peanut-Specific IgE Levels

Peanut-specific antibodies were assayed by ELISA. Plates (Maxi-Sorp; Nunc Immunoplate, Roskilde, Denmark) were coated overnight with 0.5 $\mu\text{g}/\text{ml}$ of CPE diluted in carbonate buffer (pH 9.6). Plates were blocked and incubated with diluted samples at 37°C for 2 h. Bound specific IgE was detected by horseradish peroxidase-labeled goat anti-mouse IgE (1:5,000; Serotec) and developed with tetramethyl benzidine substrate (Pierce, Rockford, Ill., USA). Plates were read at 450 nm with an automated microplate reader (Biorad, Hercules, Calif., USA). A pool of serum from peanut-sensitized and challenged mice was used as positive control. An index was calculated for IgE: $(\text{OD sample} - \text{OD buffer only})/(\text{OD positive control} - \text{OD buffer only})$, where OD is the optical density.

SDS-PAGE and Immunoblotting

CPE and recombinant peanut allergens were separated by SDS-PAGE under reducing conditions using 12% polyacrylamide NuPage gels (Invitrogen, Carlsbad, Calif., USA). After protein transfer and blocking in TBST (Tris 0.1 M, pH 7.5; 0.15 M NaCl; 0.1% Tween) containing 1% gelatin (Sigma), membranes were incubated with diluted pooled sera from 4 mice. Bound specific IgE were detected by horseradish peroxidase-labeled goat anti-mouse IgE (1:10,000; Serotec), using ECL plus Western blotting kit (Amersham Biosciences, Little Chalfont, UK).

Determination of Vascular Leakage

Before i.p. challenge, 2–3 mice from each group received intravenously 100 μl of 0.25% Evan's blue dye. Footpads were examined for signs of vascular leakage (visible blue color) within 30 min following i.p. injection.

Clinical Scoring System

Anaphylactic symptoms were evaluated by 2 independent investigators within 0–45 min after i.p. challenge using a scoring system established from anaphylactic symptoms induced by compound 48/80 following i.p. injection in naive mice. Disease severity was graded using the following progressive score: 0 = no symptoms; 1 = reduced activity; 2 = partial immobility; 3 = prostration, total immobility, pilar erection; 4 = edema around the mouth and the eyes, puffiness around the eyes; 5 = no activity after prodding, convulsion, death.

Measurement of Rectal Temperature

Rectal temperature was measured before and 60 min after the i.p. challenge using a thermal probe (Anritsu Meter Co. Ltd., Tokyo, Japan).

Measurement of Breathing Rate

Immediate airway responsiveness was assessed in conscious unrestrained mice after assessment of clinical symptoms using a barometric plethysmography method (EMKA Technologies, Paris, France). The breathing rate was evaluated to determine the severity of anaphylaxis.

Measurement of Serum mmcp-1

Blood was collected 90 min after the i.p. challenge and serum levels of mmcp-1 were measured with an ELISA kit (Moredun Scientific Ltd., Midlothian, UK) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Monitoring of Peanut Sensitization Status in C3H/HeJ Mice

Positive Ear Swelling and Intradermal Skin Tests in Peanut-Sensitized C3H/HeJ Mice

C3H/HeJ mice received a single i.g. administration of freshly ground whole peanut (80 mg/animal). Ear swelling responses following intradermal injection with CPE were measured on days 4, 7, 11, 14, 18 and 21 after sensitization. Positive skin tests indicated by increases in ear thickness in peanut-sensitized mice were observed at 4, 7, 11, 14, 18 and 21 days after sensitization in contrast to naive mice (fig. 1a). In these sensitized mice, the ear swelling response to CPE was similar to that observed with the 48/80 compound. To complete skin investigation of sensitization status, we also performed intradermal skin testing on all mice tested for ear thickness. Positive skin responses were observed in all peanut-sensitized mice compared to naive mice treated similarly (fig. 1b).

Increased Peanut-Specific Serum Antibodies in Peanut-Sensitized C3H/HeJ Mice

In order to better characterize the sensitization response, we next measured the levels of peanut-specific IgE in sensitized mice (fig. 1c). A significant increase in specific IgE was observed from day 14.

Peanut-Specific IgE Recognized the Recombinant Allergens Ara h 1, Ara h 2 and Ara h 3

As shown in figure 2, murine IgE from peanut-sensitized C3H/HeJ mice sera from day 14 recognized the major allergens present in peanut extract (lane 5). Peanut-specific IgE also bound to recombinant forms of the main peanut allergens rAra h 3 (lane 2), rAra h 2 (lane 3) and rAra h 1 (lane 4). Similar results were obtained with sera collected on days 18 and 21 after sensitization (data not shown).

Anaphylaxis Induced by i.p. Peanut Challenge at Day 14 in Peanut-Sensitized C3H/HeJ Mice

We next tested the anaphylactic response in peanut-sensitized mice. Unlike humans, rodent intestinal food absorption follows a circadian rhythm that prevents delivery of stomach contents to the duodenum during the light phase [20]. Further, while it is possible to override these changes in food bioavailability using 12–24-h food restriction, we did not use this approach because it creates a significant body weight reduction (2–4 g/mouse) [21]. In order to minimize variations of allergen bioavailability, we chose to use the i.p. route for the challenge. In contrast to noninjected naive mice (fig. 3a), a general anaphylactic response was triggered in i.p. CPE-challenged mice at day 14 after sensitization, causing a marked vascular leakage shown by blue color in mouse footpads (fig. 3c). CPE-induced vascular leakage was also observed in mice on days 18 and 21 after sensitization (data not shown). A slight bluish coloring of the skin due to intravascular Evan's blue dye in cutaneous blood vessels appeared in naive mice (fig. 3b). Figure 3d shows no significant increase in clinical score following i.p. challenge in peanut-sensitized animals for up to 11 days (score 0), but a dramatic response reaching an average clinical score of 4 occurred starting on day 14. The decrease in body temperature followed a slightly different time course (fig. 3e). Indeed, a significant decrease in body temperature was observed as early as 7 days after sensitization, reaching a maximum between days 14 and 18, and then diminishing on day 21, although remaining significant compared to naive mice. To further investigate anaphylactic response when most severe symptoms were record-

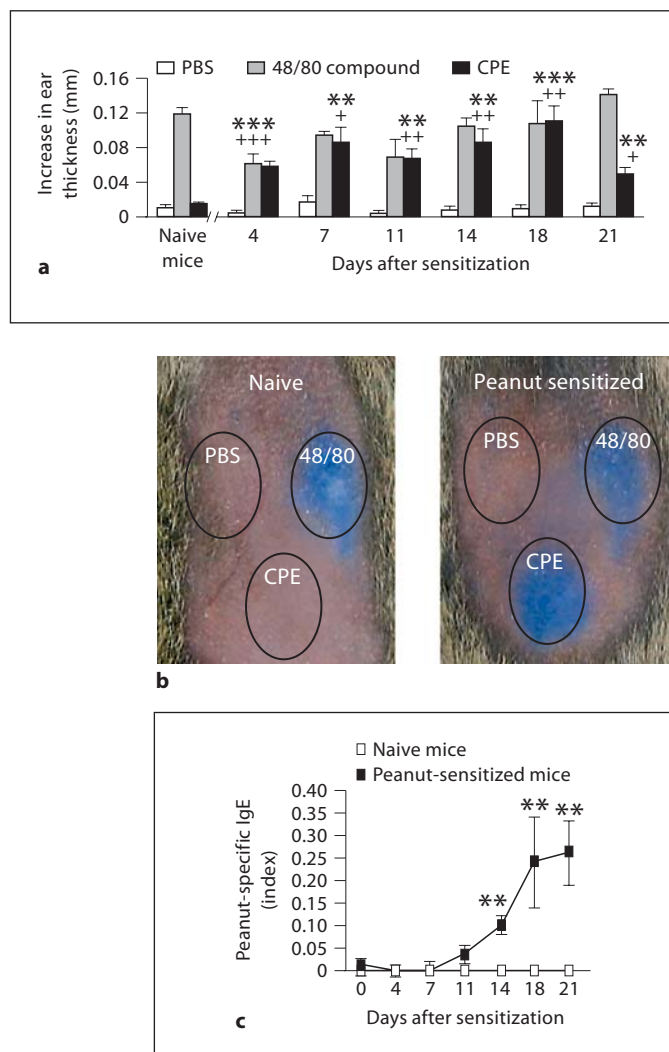


Fig. 1. Monitoring of peanut sensitization status in C3H/HeJ mice. **a** Ear swelling tests were performed at the times indicated. Forty minutes after intradermal injection of PBS, compound 48/80 or CPE, increase in ear thickness was measured in naive and peanut-sensitized mice. Results are expressed as means $\pm$ SEM of 8 mice per group from 2 sets of experiments. $^+ p < 0.05$, $^{++} p < 0.01$, $^{+++} p < 0.001$, CPE versus PBS treatment for each group. $^{**} p < 0.01$, $^{***} p < 0.001$, peanut-sensitized versus naive mice treated with CPE. **b** Immediate cutaneous hypersensitivity reaction. Skin tests were performed 4, 7, 11, 14, 18 and 21 days after sensitization. After Evan's blue dye injection, immediate cutaneous hypersensitivity was induced by intradermal injections of CPE (2.5 mg/ml), positive control (48/80 compound) and negative control (PBS). Intradermal skin testing results from day 14 shown here are representative of each group. Similar results were obtained at days 4, 7, 11, 18 and 21. **c** Peanut-specific IgE in C3H/HeJ mice. Sera peanut-specific IgE levels were determined by ELISA. Data are given as means $\pm$ SEM of 8 mice per group from 2 sets of experiments. $^{**} p < 0.01$ compared with day 0.

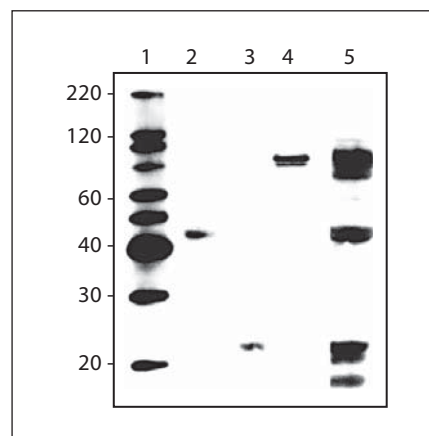


Fig. 2. IgE immunoblots with peanut-sensitized C3H/HeJ mice serum. Pool of peanut-sensitized mice ($n = 12$) sera were tested for their immunoreactivity with recombinant peanut allergens by immunoblotting at days 14, 18 and 21 after sensitization. A representative immunoblot obtained with sera from day 14 is shown here. Lanes 1–5 represent molecular weight standards (in kDa, MagicMark™ XP; Invitrogen), rAra h 3, rAra h 2, rAra h 1 and CPE, respectively.

ed, we determined the effect of i.p. CPE challenge on the breathing rate. i.p. challenge caused a 1.5-fold reduction in breathing rate of peanut-sensitized mice tested at days 14, 18 and 21 after sensitization, but no change was observed in naive mice after the CPE challenge (fig. 3f). Moreover, CPE challenge-induced anaphylaxis was associated with a significant increase in serum mmcp-1, a specific biologic marker of mast cell degranulation, in peanut-sensitized compared to naive mice 14 days after sensitization (fig. 3g).

Discussion

In this paper, we generated a murine model of severe peanut anaphylaxis in C3H/HeJ within 2 weeks. In peanut-sensitized and challenged mice, peanut-induced severe anaphylaxis at day 14 was demonstrated through different methods. First, vascular leakage gave a positive response [18, 22]. Second, clinical reactions yielded a severe response reaching a clinical score of 4 [9, 15–18, 22, 23]. Third, clinical symptoms were associated with a drop in body temperature symptoms indicating a sign of disease severity [15, 18, 23]. Fourth, the profound decrease in breathing rate observed evidences a severe sign of anaphylactic shock in mice [17]. Finally, after peanut chal-

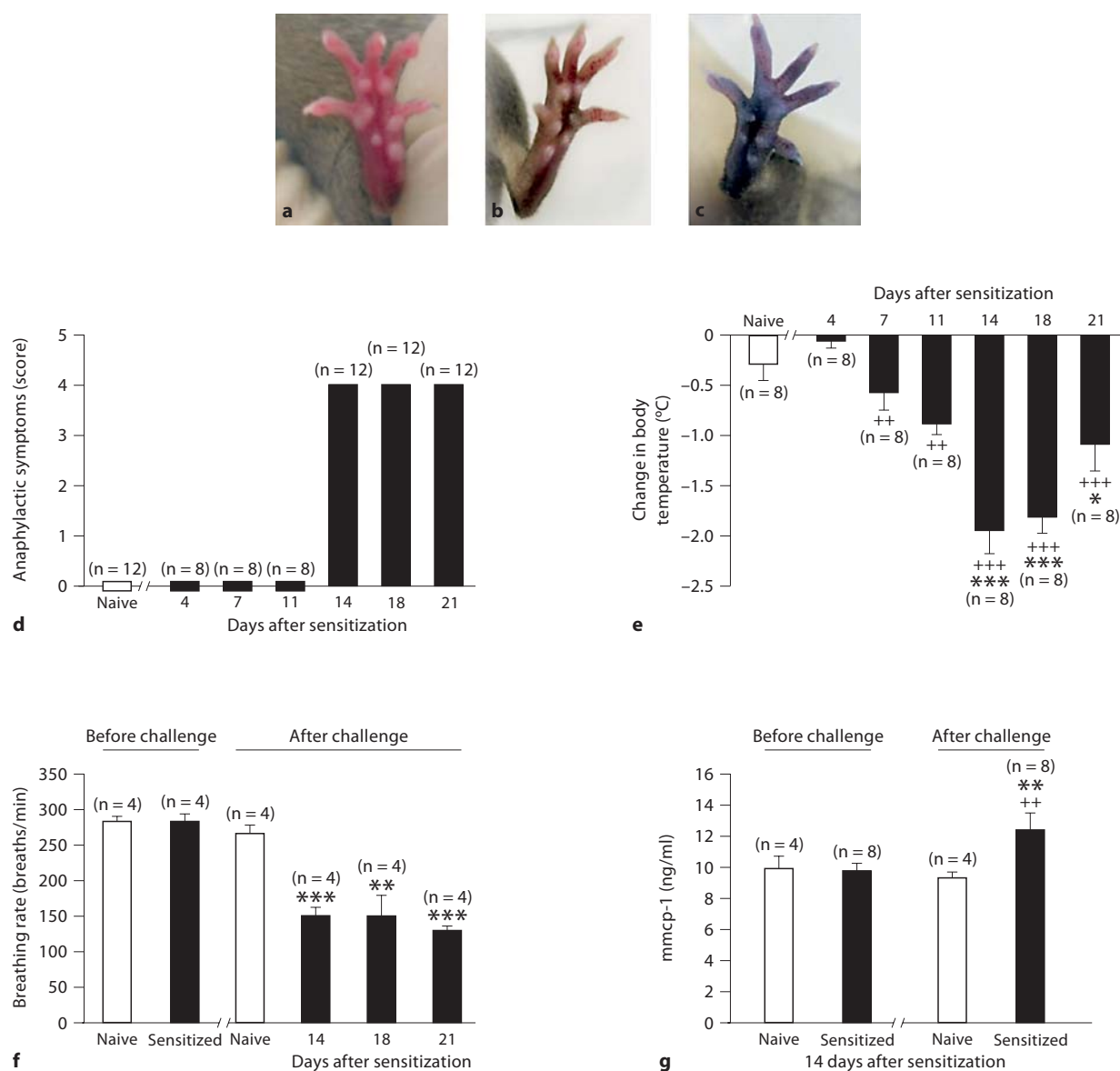


Fig. 3. Assessment of anaphylaxis after i.p. challenge in naive and peanut-sensitized C3H/HeJ mice. **a–c** Peanut allergen-induced vascular leakage in C3H/HeJ mice. On days 4, 7, 11, 14, 18 and 21 after single i.g. administration of peanut, peanut-sensitized mice were injected with Evan's blue dye and peanut as described in Materials and Methods. Photographs of footpads from day 14: naive mice not injected with Evan's blue dye (**a**), naive (**b**) and peanut-sensitized (**c**) mice injected with Evan's blue dye/peanut. **d–g** At the times indicated, naive and peanut-sensitized C3H/HeJ mice were challenged i.p. with 30 mg CPE. **d** Anaphylactic symptoms were scored at each time point. Data are the combined results of

2–3 sets of experiments. **e** Change in body temperatures was determined after i.p. challenge. Data are given as means $\pm$ SEM of 8 mice per group from 2 sets of experiments. **f** Breathing frequency was immediately assessed following evaluation of anaphylactic symptoms after the i.p. challenge. Results are expressed as means $\pm$ SEM of 4 mice per group. **g** Serum mmcp-1 levels 90 min after injection were determined by ELISA. Results are expressed as means $\pm$ SEM of 4–8 mice per group. ++ $p < 0.01$, +++ $p < 0.001$, significant differences before and after challenge within each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, peanut-sensitized versus naive mice (after challenge).

lenge, the significant release of serous mmcp-1, more stable than histamine that is used in most food allergy animal studies [9, 10, 15–18, 22, 23], demonstrated the involvement of mast cells in the symptoms of peanut-induced anaphylaxis. The positivity of *in vivo* skin testing to peanut allergy and *in vitro* tests with increased peanut-specific IgE demonstrates an IgE-dependent sensitization to peanut in C3H/HeJ mice [9, 10, 15–18]. The main interest of this model is that it presents objective and quantifiable parameters to monitor the appearance of anaphylaxis to peanut.

Previous efforts to develop mouse models of peanut allergy and food anaphylaxis have used a number of interesting and valuable approaches for understanding immunopathological mechanisms and for exploring new therapeutic approaches [9–18]. In contrast to these studies using several oral exposures of peanut coadministered with CT, we demonstrate that a single *i.g.* administration of peanut without adjuvant is effective and generates a murine model of severe peanut anaphylaxis in C3H/HeJ mice within 2 weeks. The strength of our model is the relevant route of natural exposure as in humans. A single *i.g.* administration of peanut was able to induce sensitization without the use of CT in animals that were never previously exposed to peanut. This shows that CT is not essential for the development of a murine model of peanut anaphylaxis. Moreover, no use of adjuvant allowed focusing on the true intrinsic peanut allergenicity. Indeed, the use of adjuvant should be taken into consideration with caution in the model of food allergy, because it is possible that it confers on nonallergic proteins the ability to cause sensitization, thus creating false positives [24]. However, little data is available to substantiate this hypothesis. In contrast to previous studies [9, 22], our mouse model used the *i.p.* route to induce anaphylaxis. It is clear that this route of administration does not take into account the induction of mucosal immune responses after normal encounters with the allergen after *i.g.* challenge. Although it might appear that the oral route is most appropriate for the induction of anaphylaxis, in our model the application of *i.g.* peanut challenge was less sensitive than parenteral administration. Indeed, in contrast to the *i.p.* route applied in our study, the use of the *i.g.* route for peanut challenge induced a less consistent anaphylactic response with clinical symptom scores ranging between 2 and 3, a slightly significant global change in body temperature ($-0.7 \pm 0.15^{\circ}\text{C}$, $p < 0.01$) and in breathing rate (315 ± 5.54 before versus 273 ± 9.01 breaths/min after *i.g.* challenge, $p < 0.01$) in peanut-sensitized and challenged mice (data not shown). Under

the same conditions of *i.g.* challenge, no anaphylactic reaction was observed in naive mice. Finally, another advantage of this model was based on the short period of time required to obtain anaphylaxis, since a single *i.g.* administration of peanut is sufficient to induce peanut anaphylaxis in 2 weeks.

Our model provides new insights into the natural history of peanut allergy, albeit in rodents. Unlike previously published reports, we explored the sensitization status kinetically by testing ear swelling responses, intradermal skin reactions and specific IgE to peanut at different time points. We observed positive skin responses (ear swelling test and intradermal skin testing) from 4 days after sensitization, while circulating peanut-specific IgE antibodies were weak and not detected until day 14. Since positive skin tests are an indication of mast cell degranulation caused by binding of peanut to cell-bound specific IgE, the difference in time course between skin sensitization and circulating IgE levels could suggest that at the initial stage, specific IgE are preferentially bound to mast cells. Similar observations of positive skin tests associated with undetectable circulating IgE were also reported in humans, indicating that skin tests are more reliable in confirming allergy [25]. Interestingly, anaphylaxis occurred only when IgE levels in serum were significantly increased, suggesting a link between the level of circulating specific IgE and disease severity [9, 22, 26, 27]. Moreover, it is important to highlight the fact that specific IgE in mice sensitized through our protocol recognized the 3 major recombinant peanut allergens (rAra h 1, rAra h 2, rAra h 3) similar to what is observed in human allergy [19, 28].

In summary, our paper reports an adjuvant-free model of peanut allergy using a simple and short sensitization peanut protocol leading to anaphylactic reactions within 2 weeks. We describe that a single *i.g.* exposure of C3H/HeJ mice to freshly ground whole peanut without CT induced a peanut sensitization status, indicated by positive skin tests (intradermal skin testing and ear swelling response) and increased peanut-specific IgE recognizing the same major allergens as in peanut-allergic patients. Moreover, 14 days after oral exposure, C3H/HeJ mice developed a severe anaphylactic reaction characterized by clinical symptoms and increased serum levels of mmcp-1 upon peanut *i.p.* challenge. This model may be particularly useful for further basic and applied studies on peanut allergy, since it does not depend on an adjuvant for eliciting peanut hypersensitivity reactions to peanut protein.

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