An Adjuvant-Free Mouse Model of Transdermal Sensitization and Oral Elicitation of Anaphylaxis to Shellfish

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Shellfish allergy · Systemic anaphylaxis · Adjuvant-free mouse model · Immediate hypersensitivity · Food allergen

Abstract
Background: Shellfish (SF) allergy is a leading cause of systemic anaphylaxis in humans. An adjuvant-free mouse model to evaluate allergenicity and oral anaphylaxis to SF is currently unavailable. Here, we tested the hypothesis that transdermal exposure (TDE) to SF protein extract (SFPE) not only elicits a systemic allergic immune response but also will clinically sensitize mice for oral anaphylaxis. Methods: Adult BALB/c female mice (6–8 weeks of age) were exposed to saline or SFPE once a week for 4 weeks using a transdermal sensitization method. Systemic SF-specific IgE, IgG1 and IgG2a and total (t)IgE responses were measured using ELISA. Systemic anaphylaxis upon oral SFPE administration was assessed according to clinical symptoms and the hypothermia shock response (HSR). Using individual mouse data, the correlation between the readouts of allergenicity was determined using Pearson’s analysis. Spleen-cell IL-4 and IFN-γ responses were determined using primary cell culture and ELISA. Results: TDE to SFPE resulted in marked systemic specific (s)IgE, tIgE, IgG1 and IgG2a responses. Oral challenge with SFPE in sensitized mice (but not controls) elicited systemic anaphylactic clinical reactions and HSR. A strong correlation was observed between sIgE, tIgE and HSR. Spleen cells isolated from allergic mice (but not controls) exhibited memory IL-4 and IFN-γ cytokine responses. Conclusion: We report a novel adjuvant-free mouse model of SF allergy with robust quantifiable and correlated readouts of allergenicity that may be used in basic biomedical, preclinical and applied food/nutrition research on SF allergy.

Introduction
Allergic and anaphylactic responses to food proteins are the leading cause of near-fatal/fatal hypersensitivity reactions [1]. Of the major allergenic foods, shellfish (SF), fish, peanuts and tree nuts are more likely to trigger life-threatening systemic anaphylactic reactions [1–6]. Furthermore, these 4 types of food allergy have also been identified as persistent, i.e. where individuals sensitized to these foods rarely outgrow their clinical reactivity [1–6]. The prevalence of SF allergy has risen to significant levels in many westernized countries including the USA (1.3% among children and up to 2% among adults) [7, 8], Europe (0.6%) and Canada (1.6%) [9, 10]. SF allergy is a very common food allergy in Western countries, but appears to also have become more prevalent among both children and adults in parts of Asia. For example, in Singapore and the Philippines, the reported prevalence among teenagers is as high as 5.2% [4, 11, 12]. It is also a
significant problem for people working in the seafood industry [1–6]. Thus, SF allergy is a growing and significant global public health problem.

The mechanism of sensitization to SF proteins, although unknown, is generally thought to be similar to other food allergies, i.e. occurring upon oral exposure to SF and due to a breach in the innate tendency to develop oral tolerance to SF proteins [1]. There is growing evidence that nonoral routes of exposure to food allergens (for example, via the skin) may be involved in some food allergy sensitization. Previous studies elegantly show the importance of nonoral routes of exposure such as the skin and aerosol routes to seafood allergens in occupational settings [13–15]. In 1 study, the prevalence of occupational protein contact dermatitis was 3–11% among seafood workers [15], and in another, reactions to skin exposure (e.g. rash) accounted for 78–81% of all health problems, followed by aerosol exposure resulting in asthma symptoms (7–10%) [14]. Another linked transdermal exposure (TDE) to peanut protein in the risk for peanut allergy among children [16]. In mouse models, TDE to hazelnut, milk, cashew nut and sesame seed proteins (without adjuvant) can clinically sensitize mice for anaphylaxis [17–23]. Although TDE to SF protein is possible for people who work in the seafood industry, it is unclear whether clinically relevant sensitization can occur via this route [2–6, 11].

Mouse models of SF allergy that closely simulate the human disorder are a valuable tool for basic biomedical, preclinical and applied food/nutrition research into this allergy [24]. However, there are relatively few efforts reported in the literature on the development of animal models. Three reported mouse models of shrimp tropomyosin allergy and 1 of mud crab allergy share the following features: (1) the use of adjuvants (cholera toxin/complete Freund’s adjuvant) and (2) the use of either systemic (intraperitoneal injections) or oral exposure to induce sensitization [25–28]. These models are highly valuable and useful for studying SF allergy mechanisms in the context of coexposure of allergens with powerful adjuvants. However, they have the limitation of not distinguishing the intrinsic immune effects of the SF allergens from the costimulatory effects of the adjuvants [24]. There are no previous reports on the use of TDE to evaluate allergic response to SF protein, which is the focus of this study. The hypothermia shock response (HSR) is a very useful quantifiable physiological response associated with systemic anaphylaxis used in murine models of food anaphylaxis including hazelnut and sesame allergy [17, 18, 24]. In view of these data in the literature, here we studied the utility of this readout in an SF allergy mouse model.

We tested the hypothesis that TDE to SF protein extract (SFPE) results in not only a robust systemic allergic immune response but will also clinically sensitize mice for oral anaphylaxis. Our data suggest that TDE to SFPE results in systemic IgE responses and clinical sensitization for oral anaphylaxis. We also demonstrate, for the first time, robust correlations between the 3 quantifiable readouts, i.e. specific (s)IgE, total (t)IgE and HSR, for SF allergenicity.

Materials and Methods

Chemicals and Reagents

We purchased biotin-conjugated rat anti-mouse IgG1-, IgG2a- and IgE-paired antibodies and standards as well as paired antibodies and recombinant standards for mouse IL-4 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 standardized, mixed SFPE (consisting of clam, crab, oyster, scallop and shrimp; Greer Labs, Lenoir, N.C., USA).

Mice

Adult BALB/c female mice (6–8 weeks of age) from The Jackson Laboratory, Bar Harbor, Me., USA, were used in this study. All animal procedures were in accordance with Michigan State University policies.

Transdermal Sensitization and Bleeding

Groups of mice were exposed to saline or SFPE (1 mg/mouse/exposure) using a transdermal sensitization protocol described by us previously [14–20]. Briefly, the fur on the back was clipped and then SFPE or saline was applied to the skin and covered by a latex-free bandage. Mice received 1 exposure/week for 4 weeks. Blood was collected from the saphenous vein and plasma was used in the antibody analysis.

Measurement of SF-Specific IgE, IgG1 and IgG2a Antibodies and tIgE Level

SF-specific IgG1, IgG2a, IgE and tIgE ELISAs were optimized, using the general ELISA-based method described by us previously [1–23, 29]. For the plasma tIgE measurement, ELISA plates were coated with a capture anti-mouse IgE antibody per the supplier’s protocol (BD Pharmingen). For the measurement of SF-specific IgE, IgG1 and IgG2a antibody levels in the plasma, ELISA plates were coated with SF protein (Greer Labs) at 5 mg/ml and 20 and 20 μg/ml, respectively, as per the method previously described [29].

Oral Challenge, Clinical Symptom Scoring Analysis and Measurement of Rectal Temperature

Groups of sensitized versus saline-exposed mice were orally challenged with SFPE (15 mg/mouse) using mouse-feeding needles (22-gauge, Popper and Sons Inc., New Hyde Park, N.Y., USA).
The mice were observed for immediate hypersensitivity reactions during the next 30 min. The clinical symptoms of anaphylaxis were scored by veterinarians according to the previous method [17–23]. Rectal temperature (RT) was measured before and at 30 min after oral challenge (OC) and changes in temperature were calculated and used in the analysis.

**Spleen-Cell Culture and Cytokine Analyses**
A standard spleen-cell culture was set up essentially as we have described before [17–24]. Briefly, spleen cells were cultured (7.5 million/ml) in the absence and presence of SFPE (0 and 0.5 mg/ml), and the culture supernatants harvested on day 3 were used in cytokine analyses with optimized ultrasensitive ELISA.

**Statistical Analysis**
Student’s t test, one-way ANOVA and Pearson correlation analyses were conducted using the SlideWrite program and online software services (http://www.socscistatistics.com/tests/pearson/). The statistical significance level was set at p = 0.05.

**Results**

**Systemic Allergic Response upon TDE to SFPE**
Groups of adult BALB/c female mice received TDE to saline or SFPE. Blood samples collected before and after the fourth TDE were used for the measurement of SF-specific IgE antibodies by optimized ELISA. Saline-exposed mice demonstrated no detectable sIgE antibodies. Robust sIgE was observed in the SFPE-exposed mice after the fourth TDE. Using individual mouse plasma samples, SF-specific IgE titers were determined using the published method [29]. Robust sIgE titers were observed in 100% of the SFPE-exposed mice but not in the saline-exposed mice after 4 TDEs (fig. 1a). The plasma IgE levels were measured in these samples. A significant elevation of IgE was observed after the fourth TDE in the mice exposed to SFPE but not in those exposed to saline (fig. 1b).

**Systemic IgG1 and IgG2a Antibody Responses upon TDE to SFPE in BALB/c Mice**
Blood samples collected before and after the fourth TDE were used in the measurement of SF-specific IgG1 and IgG2a antibodies by optimized ELISA. Saline-exposed mice demonstrated no detectable IgG1 (fig. 2a) or IgG2a antibodies (fig. 2c). Significant IgG1 (fig. 2b) and IgG2a (fig. 2d) were observed in SFPE-exposed mice after the fourth TDE. Furthermore, we determined the titers of IgG1 and IgG2a antibodies and the ratio of the IgG1/IgG2a titers. The SF-allergic mice displayed a very high IgG1/IgG2a titer ratio (fig. 2e).

**Oral Elicitation of Systemic Anaphylaxis to SFPE in Transdermally Sensitized BALB/c Mice**
During optimization of this novel transdermal sensitization method, we found that a minimum of 4 TDEs to the allergen is necessary for obtaining robust readouts of oral anaphylaxis in a majority of mice. Consequently, 4 TDEs were used in the experiments. Upon confirmation of systemic SF-specific IgE responses, both saline-exposed and SFPE-exposed mice were orally challenged with SFPE.
(15 mg/mouse). Significant symptom scores were observed among SFPE-sensitized mice but not in saline-exposed mice (fig. 3). The majority (70%) of the SFPE-sensitized mice exhibited scores of \( \geq 4 \), and no saline-exposed mice exhibited clinical symptoms of anaphylaxis.

The severity of oral SFPE-induced systemic anaphylaxis was quantified by the HSR by measuring the RT. No significant change in RT was observed in saline-exposed, nonallergic mice upon OC with SFPE (fig. 4a). In contrast, SFPE-sensitized mice exhibited a significant HSR (fig. 4b). Furthermore, the drop in RT (\( \Delta \text{RT} \)) at 30 min was also significantly different between the 2 groups (fig. 4c).

**Pearson Correlation Analysis of the Four Readouts of SF Allergenicity**

The data on sIgE, tIgE, symptom scores and the drop in RT obtained for each individual mouse were used in
the correlation analysis, initially performed on the SF-allergic mice. A highly significant correlation was observed between tIgE levels in the plasma and SF-specific IgE antibody titers (fig. 5a). The OC-induced drop in RT, which is a precisely quantifiable objective amount, showed a highly significant positive correlation with both sIgE and tIgE data (fig. 5b, c). Correlations between clinical symptom scores and other readouts on allergic mice were modest (r = 0.40–0.44) but not significant (data not shown). Therefore, we performed a correlation analysis of the four readouts for the entire colony of allergic and nonallergic mice (table 1). Highly significant and robust positive correlations were observed between the four readouts.
relations were observed between tIgE, sIgE and the drop in RT, and modest but significant correlations were observed between clinical symptom scores and tIgE, sIgE and the drop in RT.

**Spleen Cells Recall IL-4 and IFN-γ Cytokine Responses in Allergic and Nonallergic Mice**

To evaluate recall SFPE-induced Th1 (IFN-γ) and Th2 (IL-4) cytokine responses, spleen cells were isolated and cultured with SFPE as described in the Methods section. Cell culture supernatants were analyzed for respective cytokines using ELISA. SFPE elicited recall IL-4 (fig. 6a) and IFN-γ (fig. 6b) responses in SF-allergic but not in nonallergic mice.

**Discussion**

The exact mechanism of sensitization to SF allergens is incompletely understood at present. Here, we hypothesized that the transdermal route of exposure to SF is sufficient to clinically sensitize mice for oral SF-induced allergic responses. There are several novel findings from this study: (1) TDE to SFPE without use of adjuvants elicits a robust sIgE response and elevates tIgE in Balb/c female mice, (2) TDE to SFPE also elicits immunogenic responses as measured by IgG1 and IgG2a responses, (3) mice with systemic allergic antibody responses exhibit clinical symptoms of systemic anaphylaxis upon OC with SFPE, with a majority exhibiting near-fatal anaphylactic symptoms, (4) OC with SFPE induces an HSR after 30 min in SF-allergic mice, but not in saline-exposed control mice, (5) sIgE, tIgE and the drop in RT exhibit highly significant positive correlations and clinical symptom score data have a modest and significant correlation with other readouts of SF allergy and (6) TDE to SFPE elicits systemic IL-4 and IFN-γ responses in the spleen. These data, for the first time, provide a novel adjuvant-free mouse model of SF allergy with 3 robust quantifiable and highly correlated readouts: tIgE, sIgE and HSR.

There are 3 previous elegant studies that reported on the development of shrimp allergy and 1 on mud crab allergy using mouse models with BALB/c and C3H/HeJ mice [25–28]. Thus, Capobianco et al. [27] reported a shrimp tropomyosin allergy mouse model using C3H/HeJ mice with oral exposure to purified shrimp tropomyosin along with cholera toxin adjuvant; their readouts of allergenicity included sIgE (OD values; no titers), spleen-cell IL-4 production and systemic anaphylactic symptom scores upon OC. Lam et al. [25] and Leung et al. [26] reported another shrimp tropomyosin allergy model using recombinant protein and BALB/c mice using intragastric exposure with cholera toxin adjuvant [25, Table 1. Pearson correlation analysis of 4 readouts of shellfish allergenicity

<table>
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<tr>
<th>Allergenicity readouts</th>
<th>Correlation between readouts in this model</th>
<th>n</th>
<th>r</th>
<th>R²</th>
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The plasma tIgE levels and sIgE (SF-specific) antibody titers were recorded after 4 TDEs to SFPE.

ᵃ Drop in RT at 30 min after SFPE OC.

ᵇ Clinical symptom score of systemic analysis as evaluated by veterinarians.

![Fig. 6. a, b SF-induced memory IL-4 and IFN-γ cytokine responses by spleen cells from nonallergic and allergic mice. Groups of mice were transdermally exposed to saline (n = 5) or SFPE (1 mg/mouse; n = 5) once a week for 4 weeks. Spleens were collected, and single-cell suspensions were made and then used in primary cell culture, as described in the text. Cell supernatants were collected on day 3 and used in cytokine protein measurement using optimized ELISA. a IL-4 levels in nonallergic and SF-allergic mice after spleen-cell culture with 0.5 mg/ml SFPE. b IFN-γ levels in nonallergic and SF-allergic mice after spleen-cell culture with 0.5 mg/ml SFPE. Student’s t test: p < 0.05.](image-url)
Readouts of allergenicity included sIgE responses (OD values; no titers), spleen-cell IL-4 responses, oral allergen-induced symptom scores and histopathological changes in the gut. A mouse model of mud crab allergy was developed using BALB/c mice and intraperitoneal injection with purified allergens along with complete Freund’s adjuvant [28]. Notably, these above studies did not evaluate quantifiable oral reactions such as HSR. Furthermore, they did not study the correlations of readouts of allergenicity (e.g. between sIgE, tIgE responses and clinical score). In our study, we demonstrate, for the first time, not only quantifiable readouts such as oral allergen-induced HSR, but also robust correlations between IgE responses (both sIgE and tIgE) and HSR. Our data also show a modest and significant correlation between clinical symptom scores and other allergenicity readouts. Clinical scoring requires trained personnel such as veterinarians, but even then, it tends to be subjective to some extent. Instead, HSR, which is objective and highly quantifiable, provides a reliable and robust readout of oral SF-induced allergic reactions in mice and a highly significant correlation with both sIgE and tIgE responses. Two potential uses of the hypothermia readout are: (1) to evaluate the quantitative relationship between the extent of food processing of SF proteins versus the corresponding change in SF anaphylactic potencies and (2) to quantify the effectiveness of novel oral immunotherapy protocols for SF anaphylaxis using this model as a preclinical testing tool.

We found a strong correlation between sIgE and tIgE responses in this mouse model, suggesting that the elevation of circulating tIgE was primarily due to SFPE-induced sIgE antibody responses. Technically, measuring food sIgE is more expensive because of the need for more plasma samples, the use of titrations of the samples to deduce the titers and the need for a large quantity of food protein for coating in ELISA. We previously published a novel ELISA-based method for measuring food-specific IgE antibodies in mice [29]. A critical part of this method involves the use of food protein in coating ELISA plates at an optimized concentration of 5,000 μg/ml. This would require 25 mg of purified protein for coating per plate, making it relatively expensive. Furthermore, the ELISA-based assay for food-specific IgE responses must be optimized for each type of food (as described by us before) due to the background activity and the secondary antibody compatibility issue [29]. In contrast, measuring tIgE is relatively easy, and our findings of a highly significant correlation between sIgE and tIgE suggest that this measurement may be a suitable alternative when there are limitations of sample amounts and budget restrictions for experiments.

We chose to use IL-4 and IFN-γ because IL-4 is a prototypic Th2 cytokine and IFN-γ is a prototypic Th1 cytokine [30]. In general, it is well-known that during allergic responses, the levels of Th2 cytokine tend to increase while the Th1 cytokine levels tend to decrease [31, 32]. We found that the levels of both IFN-γ and IL-4 were significantly increased. This finding is very similar to a previous report on the IFN-γ/IL-4 cytokine responses in a shrimp allergy mouse model [27]; similar to in our model, the levels of both cytokines were significantly elevated (IFN-γ: approx. 5,000 pg/ml and IL-4: approx. 4 pg/ml) [27]. So the significant elevation of both these cytokines may be a distinct feature of the SF allergy mouse model.

As opposed to previous models of shrimp and mud crab allergy that used purified/recombinant proteins, we chose to use standardized mixed SFPE that is used in clinical testing for humans [25–28]. The rationale underlying our approach was to simulate the natural TDE to SF proteins in an occupational setting such as the SF industry where exposure is likely to be to multiple types of SF proteins.

Animal models are highly valuable for the preclinical evaluation of novel, therapeutic and prophylactic methods for treating SF allergy. A given model used for this purpose must therefore simulate the human situation as closely as possible [24]. While no animal model would be expected to be identical to the entire spectrum of human SF allergies, which is highly complex, further refinement of existing models is desirable. Our approach of not using adjuvants provided a unique opportunity to study adverse immune reactions to SF protein in the natural context of human TDE such as in the seafood industry [13–15, 27, 33]. Furthermore, an adjuvant-free model, such as the one described here, is not only useful for studying the mechanistic and genetic aspects of SF allergy development, it can be used to evaluate the long-term consequences of disease (e.g. persistence of the disease) in the absence of the effects of adjuvants. For example, human SF allergy is rarely outgrown, for reasons that are not well understood at present. It would be valuable to find out the natural course of SF allergy in this model and identify the underlying immune mechanisms to explain this (e.g. the molecular basis of the persistent memory Th2/IgE response). In addition, the effect of food processing and food matrix on SF allergenicity could also be evaluated using the adjuvant-free mouse model described in this study. For example, one could quantitatively evaluate the impact of thermal processing, irradiation and high-pressure processing on SF allergenicity.
In summary, we report a novel, adjuvant-free mouse model of SF allergy and provide the evidence for robust and highly correlated quantifiable allergenicity readouts (sIgE, tIgE and HSR). This model may be used in the future to study basic biomedical, preclinical and applied food/nutrition research into SF allergy [24].

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