ABSTRACT

Purpose: Tolerance to shrimp has been reported in some patients with a history of shrimp allergy. The predictors of the natural resolution of shrimp allergy have not been widely explored. This study aimed to investigate the role of specific IgE (sIgE) and specific IgG4 (sIgG4) to shrimp extracts and the cross-reactive shrimp allergens tropomyosin (TM), arginine kinase (AK) and myosin light-chain (MLC), as markers of persistent or resolved shrimp allergy (PSA or RSA).

Methods: Seventeen patients with a 10-year history of allergy to *Penaeus monodon* (*Pm*) and/or *Macrobrachium rosenbergii* (*Mr*) were recruited. Oral shrimp challenges identified 10 patients with PSA and 7 patients with RSA. Sera from these patients were evaluated for sIgE and sIgG4 to *Mr* and *Pm* extracts as well as to TM, AK and MLC.

Results: The levels of sIgE to *Mr* and *Pm* extracts were lower in the RSA than in the PSA groups (*P* = 0.05 and *P* = 0.008, respectively), but sIgG4 or sIgG4:sIgE ratio did not show statistical significance. The sIgE to AK and MLC, but not TM, were lower in the RSA group than in the PSA group (*P* = 0.009 and *P* = 0.0008, respectively). There was no difference in sIgG4 to TM, AK and MLC between both groups. The ratio of sIgG4:sIgE to MLC, but not TM or AK, was higher in the RSA than in the PSA group (*P* = 0.02). A higher diversity of sIgE to shrimp components was found in the PSA group than in the RSA group (*P* = 0.006).

Conclusions: Specific bioassays can be used to identify patients with RSA. Oral shrimp challenges in these patients may provide a higher rate of passing the challenges and finally reintroducing shrimp in their diet.

Keywords: Food hypersensitivity; crustacea; shrimp; immune tolerance; biomarkers; immunoglobulin E; tropomyosin; arginine kinase; myosin light-chains

INTRODUCTION

Shrimp is one of the most common causes of allergic reactions in children and adults. It is also the leading cause of food-induced anaphylaxis, especially in Asian countries. At least 5 shrimp allergens have been identified in many different populations worldwide. Shrimp allergens can be grouped as cross-reactive or pan-allergens and non-cross-reactive allergens. The major cross-reactive shrimp allergen is tropomyosin (TM), a 38 kDa protein found in
Muscle and non-muscle cells. The second cross-reactive allergen is arginine kinase (AK), a 40 kDa phosphotransferase that plays a critical role in energy metabolism. Other important cross-reactive allergens include myosin light-chain (MLC) and sarcoplasmic calcium-binding protein (SCBP), 20 kDa muscle proteins with calcium-binding function. In addition, shrimp hemocyanin was first identified as a non-cross-reactive allergen. The hemocyanin is a hetero-hexamer of 77 kDa monomers and functions as an oxygen carrier.

Although the natural resolution of food allergy has been observed in children suffering from egg, milk and soy, it was thought that only a minority of children with shrimp allergy outgrow their food allergies. Moreover, adult-onset shrimp allergy is also considered a lifelong condition. Patients with shrimp allergy are generally instructed to strictly restrict the ingestion of shrimp due to the risk of severe allergic reactions. Patients who had food allergies and their families reported anxiety, depression and impaired quality of life. Therefore, identifying patients who outgrow food allergy may improve the quality of life in patients and their families. Recently, our group has examined the natural resolution of shrimp allergy among non-anaphylactic shrimp-allergic patients 10 years after the first diagnosis and reported that 46% of the patients had resolved shrimp allergy (RSA), while 54% had persistent shrimp allergy (PSA). Interestingly, patients with RSA had significantly smaller shrimp skin test responses than did patients with PSA at both diagnosis and follow-up, suggesting an important role of specific IgE (sIgE) as a potential biomarker for RSA.

Both sIgE and specific IgG4 (sIgG4) may play a role in the resolution of food allergy. Many studies reported that patients with resolved food allergies had lower titers of serum sIgE to the corresponding food allergens. However, the role of sIgG4 in predicting resolved food allergies was controversial. Nevertheless, the reactivity of sIgE and sIgG4 to shrimp cross-reactive allergens has not been explored in PSA and RSA patients. Therefore, we aimed to examine the interaction of sIgE and sIgG4 to known shrimp allergens in PSA and RSA patients. We also investigated the potential biomarker identifying the status of shrimp allergy in these patients.

MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board (COA No. 017/2559, EC1). It is a prospective study that followed patients diagnosed with shrimp allergy to Penaeus monodon (Pm), Macrobrachium rosenbergii (Mr), or both from our previous study conducted during the period 2005-2006. The current oral food challenges (OFCs) to shrimp were conducted during follow-up in the period of 2015–2016. The sera of patients were collected on the same day of current challenges for further analysis. Due to the ethical concern, patients who had a previous history of anaphylaxis or had a recent allergic reaction to shrimp in the past 6 months were not included. We excluded patients with underlying diseases such as cardiovascular, hepatobiliary and renal diseases, severe systemic infection, and pregnancy. Patients with allergic diseases, such as allergic rhinitis, urticaria, atopic dermatitis, and asthma, were stable for more than 7 days before OFC. The peak expiratory flow rates were more than 70% of predicted value on the day of OFC. Of the 46 patients who met the inclusion criteria, 9 refused to participate and 20 could not be contacted. The remaining 17 patients were recruited. Written informed consent was obtained from all participants.

Disclosure

There are no financial or other issues that might lead to conflicts of interest.
**OFC**

The methods and results of OFCs with shrimp were described in our previous report. In brief, a 3-step challenge protocol was completed with multiple doses in 2 of the steps. Each step was performed 15 minutes apart. The first step consisted of raw lyophilized shrimp in a capsule with a cumulative provocation dose (PD) of 7.5 g. The second step was to identify oral-mucosal reactions by swabbing cooked shrimp onto the inner lips, placing it into the mouth without chewing for 5 minutes, and then removed. The third step was open feeding of cooked shrimp with a cumulative PD of 15 g. A positive reaction to 1 or more steps of the OFC was considered a positive challenge. Patients who previously had a positive reaction to both shrimp species were challenged 2–4 weeks later with other shrimp species. Anaphylaxis was diagnosed according to the standard criteria. Patients with a negative shrimp challenge were designated RSA and a positive challenge was assigned to PSA. Patients who were previously diagnosed as having allergies to both shrimp species would need to have negative OFC to both shrimp species in order to be categorized as RSA. The sera of these patients were collected on the same day of OFC while obtaining the intravenous access and were stored at −20°C until further analysis. Sera from healthy subjects who had no allergic diseases were used as controls.

**Expression and characterization of recombinant TM, AK and MLC**

Recombinant TM was expressed from transformed *Escherichia coli* cells containing plasmid pET-28b ligated with TM cDNA (Mac r1.0101, nucleotide accession# GU369816) as previously reported. The TM was purified from lysis of transformed *E. coli* cells before IgE reactivity was confirmed with the sera of shrimp-allergic patients.

Recombinant AK and MLC were also expressed from plasmid pET-28b-AK or pET-28b-MLC transformed *E. coli* cells. The cDNA of AK (nucleotide accession# DQ975203) and MLC (nucleotide accession# EU449515) of pacific white leg shrimp *Litopenaeus vannamei* (Lv) were synthesized (GenScript, Piscataway, NJ, USA). Both recombinant AK and MLC were also purified from cell lysis of transformed *E. coli* cells before IgE reactivity was confirmed by immunoblot using the sera of shrimp-allergic patients.

**Indirect binding of serum IgE and IgG4 to shrimp extract and cross-reactive allergens**

Shrimp extract of *Pm* and *Mr* was prepared as previous described. To measure serum IgE and IgG4 to allergen in the *Pm* and *Mr* extract by indirect binding ELISA, shrimp extract was diluted in phosphate-buffered saline (PBS) and coated individually at 500 ng per well on 96-well Maxisorb plates (Nunc™, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 4°C overnight. Moreover, to measure serum IgE and IgG4 binding to recombinant TM, AK and MLC, each recombinant allergen was also diluted in PBS and coated individually at 500 ng per well on 96-well Maxisorb plates (Nunc™, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 4°C overnight. The coated 96-well plate was washed with PBS-A (PBS+3% non-fat dried milk). Sera of patients and controls were diluted at 8:1 in PBS-A. Diluted sera were incubated with coated allergens in PBS-A for 2 hours at room temperature. The plate was washed before either diluted HRP-labelled goat IgG anti-human IgE or diluted HRP-labelled rabbit IgG anti-human IgG4 antibodies was added into the designated wells. Substrate (3,3′,5,5′-tetramethylbenzidine or TMB, Thermo Fisher Scientific) was added to each well before absorbance at OD₆₅₀nm was measured.
**Statistical analysis**

Biomarkers were compared between PSA and RSA groups using the Mann-Whitney U test since the data were not normally distributed. GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis. A P value ≤ 0.05 was considered statistically significant.

**RESULTS**

Seventeen patients who were previously diagnosed as having Mr allergy, Pm allergy, or both were recruited. Three of these patients had an isolated allergy to Mr (Mr1–3), another 3 had an isolated allergy to Pm (Pm1–3) and 11 had an allergy to both shrimp species (B1–11). The patients’ sex, age, as well as results and symptoms upon OFC, are shown in **Table**.

Upon re-evaluation, 10 patients were categorized as having PSA and 7 as RSA. Patients were further grouped based on their previous reactions to shrimp species. Of 10 PSA patients, 2 were previously allergic to Pm (Pm1–Pm2), while 8 were allergic to both Mr and Pm (B1–B8). Two patients who were previously allergic to both shrimp species (B7–8) refused to receive the second challenge after having an allergic reaction to the first challenge. Four patients (B1, B2, B4 and B5) were positive to both Mr and Pm, while 3 (B3, B6 and B7) were positive to Mr, and B8 was positive to Pm (**Table**).

Patients with dual shrimp allergy were categorized as having RSA only when they had a negative challenge to both shrimp species. Among 7 RSA patients, 3 were previously allergic to Mr (Mr1–Mr3), 1 to Pm (Pm3), and another 3 to both Mr and Pm (B9–B11). Upon re-evaluation, 1 patient (Pm3) was negative to Pm, 3 (Mr1–M3) were negative to Mr, and 3 (B9–B11) were negative to both Pm and Mr (**Table**). A total of 26 challenges were done; 6/13 (46%) challenges to Pm, and 6/13 (46%) challenges to Mr were negative.

| Shrimp allergy status | Sex | Current age (yr) | Results of Mr OFC | Symptoms upon Mr OFC | Results of Pm OFC | Symptoms upon Pm OFC |
|-----------------------|-----|-----------------|-------------------|---------------------|-----------------|---------------------|
| Persistent shrimp allergy |
| Pm1 | Male | 26 | NP | NP | Positive | LI |
| Pm2 | Female | 18 | NP | NP | Positive | TI |
| B1 | Male | 23 | Positive | LI, TI | Positive | TI |
| B2 | Male | 18 | Positive | U, N/V, AP, A | Positive | U |
| B3 | Female | 18 | Positive | LI, TI | Negative | No |
| B4 | Female | 21 | Positive | TI, U, LSI | Positive | AG, R, A |
| B5 | Male | 18 | Positive | F | Positive | TI, LI |
| B6 | Male | 24 | Positive | R | Negative | No |
| B7 | Male | 18 | Positive | TI, LI, U, N/V, AP, A | NP | NP |
| B8 | Male | 20 | NP | NP | Positive | AP |
| Resolved shrimp allergy |
| Pm3 | Male | 19 | NP | NP | Negative | No |
| Mr1 | Male | 19 | Negative | No | NP | NP |
| Mr2 | Male | 19 | Negative | No | NP | NP |
| Mr3 | Female | 25 | Negative | No | NP | NP |
| B9 | Male | 23 | Negative | No | Negative | No |
| B10 | Female | 24 | Negative | No | Negative | No |
| B11 | Female | 24 | Negative | No | Negative | No |

A, anaphylaxis; AG, angioedema; AP, abdominal pain; B, both Mr and Pm shrimp allergy; F, flushing; LI, lip itching; LSI, lip swelling and itching; Mr, Macrobrachium rosenbergii; NP, not performed; N/V, nausea and/or vomiting; OFC, oral food challenge; Pm, Penaeus monodon; R, rhinitis; TI, throat itching; U, urticaria.

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Binding of sIgE and sIgG4 to allergens of shrimp Mr and Pm extracts as well as sIgG4:sIgE ratios in PSA and RSA groups

After determining the levels of sIgE and sIgG4 binding to allergens in Mr or Pm extract, patients were categorized as PSA or RSA groups according to the result of the OFC to each shrimp species (Fig. 1). For Mr shrimp allergy, patients Mr1–M3 and B9–B11 were categorized as RSA, while patients B1–B7 were categorized as PSA. For Pm shrimp allergy, patients Pm3, B3, B6 and B9–B11 were categorized as RSA, and patients Pm1–Pm2, B1, B2, B4, B5 and B8 were categorized as PSA.

When the levels of sIgE binding to allergens in Mr and Pm extracts were determined, RSA patients had significantly lower sIgE to allergens in Mr (Fig. 1A) and Pm (Fig. 1B) extract than PSA patients ($P = 0.05$ and $P = 0.008$, respectively).

In contrast, the levels of sIgG4 to allergens in Mr (Fig. 1C) and Pm (Fig. 1D) extracts of RSA patients were not significantly different from those of PSA patients ($P = 0.68$ and $P = 0.45$, respectively).

![Fig. 1. The levels of sIgE, sIgG4 and the ratios of sIgG4:sIgE to allergens in Mr and Pm shrimp extracts in PSA and RSA groups. The dotted lines are presented as OD$_{650}$ nm values of bound antibodies from non-allergic donors. Each plotted symbol represents duplicate data from each patient. sIgE, specific IgE; sIgG4, specific IgG4; Mr, Macrobrachium rosenbergii; Pm, Penaeus monodon; PSA, persistent shrimp allergy; RSA, resolved shrimp allergy.](https://e-aair.org)
respectively). The ratios of sIgG4:sIgE to allergens in Mr (Fig. 1E) and Pm (Fig. 1F) extracts in RSA patients were not significantly different from those of PSA patients ($P = 0.94$ and $P = 0.24$, respectively).

**Binding of sIgE and sIgG4 to 3 shrimp cross-reactive allergens as well as sIgG4:sIgE ratios in PSA and RSA groups**

To examine the role of allergen components in the natural resolution of shrimp allergy, the levels of sIgE and sIgG4 to the cross-reactive shrimp allergens (TM, AK and MLC) were determined in PSA and RSA groups. The sIgE (Fig. 2A), sIgG4 (Fig. 2B) and sIgG4:sIgE ratio (Fig. 2C) to TM in RSA and PSA groups were not significantly different ($P = 0.08$, $P = 0.49$ and $P = 0.81$, respectively). The level of sIgE (Fig. 2D), but not sIgG4 (Fig. 2E) or sIgG4:sIgE ratio (Fig. 2F), to AK was significantly lower in the RSA group than in the PSA group ($P = 0.009$, $P = 0.55$ and $P = 0.13$, respectively). The sIgE (Fig. 2G) to MLC was significantly lower in the RSA group than in the PSA group ($P = 0.0008$). However, the sIgG4 (Fig. 2H) to MLC was not significantly different between the RSA and PSA groups ($P = 0.81$). In contrast, the sIgG4:sIgE ratio to MLC was significantly higher in the RSA group than in the PSA group ($P = 0.02$, Fig. 2I).

![Fig. 2. The levels of sIgE, sIgG4 and the ratios of sIgG4:sIgE to shrimp cross-reactive allergens in PSA and RSA groups. Each plotted symbol represents duplicate data of one patient.](https://e-aair.org)
The number of allergen components bound to patients’ sIgE was higher in the PSA group than RSA group \((P = 0.006, \text{Fig. 3A})\). In contrast, the number of allergen components bound to the patients’ sIgG4 was not significantly different in either group \((P = 0.56, \text{Fig. 3B})\).

**DISCUSSION**

Development of oral tolerance involves suppression of Th2 cells, generation of Treg cells, suppression of effector T cells, decreased production of IgE, increased production of IgG4 by B cells, induction of IL-10–producing DCs and suppression of basophil, eosinophil and mast cell activation.\(^1\) Growing evidence suggests that the balance between antigen-sIgE and sIgG4 is important for acquiring food tolerance.\(^2\) In oral immunotherapy (OIT), sIgG4 increases, while sIgE initially increases, but then declines after several months of therapy.\(^2,3\) Since sIgG4 may increase with allergen exposure, many studies have explored the sIgG4:IgE ratio as a marker for food tolerance. In the LEAP study, the early introduction of peanuts decreased the development of peanut allergy in high-risk children and was associated with increased peanut-sIgG4 and peanut-sIgG4:IgE ratio.\(^4\) A proposed mechanism of desensitization through sIgG4 involves the competitive binding of sIgE to the same allergen and blocking allergen-triggered activation of effector cells.\(^4,14,24,25\) Thus, determining differences in sIgE and sIgG4 to shrimp allergens may help us to identify markers of RSA.

Although it was proposed that patients with resolved food allergy would have lower food-specific IgE and higher food-specific IgG4 or IgG4:IgE ratio than patients with persistent food allergy, the data from recent studies were conflicting. In a cohort study by Wood et al.,\(^5\) patients with resolved milk allergy had lower levels of milk sIgE compared to those with persistent milk allergy. However, the milk to sIgG4 or milk to IgG4:IgE ratio were not predictive of resolution. Another cohort study by Sicherer et al.\(^6\) demonstrated that both sIgE and sIgG4 to egg were lower in patients with resolved egg allergy compared to those with persistent egg allergy. Santos et al.\(^7\) studied peanut-allergic (PA), peanut-sensitized but nonallergic (PS) and non-peanut-sensitized nonallergic (NA) children, and discovered that PS children had higher levels of peanut-sIgG4 as well as peanut-specific IgG4:IgE ratios compared to PA children. Our study supported the previous study\(^8\) that RSA patients had significantly lower sIgE to shrimp extracts compared to PSA patients. However, sIgG4 or the sIgG4:sIgE ratio to shrimp extracts did not predict RSA or PSA.
Patients with food allergy may be sensitized to multiple allergens in a single food source. Therefore, evaluating the levels of sIgE, sIgG4 and the ratio of sIgG4:sIgE to allergen components may allow more detailed profiling of the allergen-sIgE and allergen-sIgG4 repertoires. In children with cow’s milk protein allergy (CMPA), the β-lactoglobulin (BLG)-sIgG4 level and BLG-sIgG4:CM-sIgE ratio, but not casein-sIgG4 level or casein-sIgG4:CM-sIgE ratio, were higher in CMPA patients at the tolerance phase compared to the symptomatic phase. In a study of egg-allergic children, baked-egg tolerant patients had significantly lower levels of egg-, ovalbumin- and ovomucoid-sIgE compared to baked-egg-reactive patients. Another study showed that a high IgG4:IgE ratio to ovomucoid and ovalbumin was the marker of tolerance to the baked egg in egg-allergic children.

In this study, we further investigated the levels of sIgE and sIgG4 against 3 cross-reactive allergens and revealed that the levels of sIgE to AK and MLC, but not to TM, were significantly lower in the RSA group. The ratio of sIgG4:sIgE to MLC, but not to TM or AK, was also significantly higher in the RSA group.

Ayuso et al. compared sIgE to shrimp TM, AK, MLC and SCBP between children and adults who had a history of IgE-mediated shrimp allergy. Compared to adults with shrimp allergy, children with shrimp allergy had greater frequency of sIgE and individual epitope recognition as well as increased intensity of IgE binding to all 4 allergen components. The authors observed that sIgE to TM, but not to the other 3 components, was frequently found in both children and adults, and postulated that TM might be associated with PSA. Our study could not confirm this hypothesis since 6/10 PSA patients had an undetectable level of sIgE to TM. In contrast, most PSA patients had sIgE to AK and MLC, suggesting that these components were important predictors of PSA. Of interest, the number of allergen components bound to patients’ sIgE was higher in the PSA group. This finding was supported by the HealthNuts study which showed that the diversity of sIgE to multiple egg allergens (Gal d 1, 2, 3 and 5) predicted persistent egg allergy at 4 years of age.

The strength of our study was that we followed patients who had confirmed shrimp allergy by OFC and identified the PSA or RSA patients by OFC after 10 years. Our long-term follow-up study facilitated the recruitment of real shrimp-allergic patients and finally confirmed that 46% of them could outgrow shrimp allergy. Our study was unlike a cross-sectional one that could only identify shrimp-allergic or non-allergic patients. However, the limitations of our study were the small number of PSA and RSA patients. The serum samples of patients at the diagnosis in 2005–2006 were not obtained for sIgE and sIgG4 to shrimp extracts and shrimp allergen components; therefore, they could not be compared to the current biomarker data. Also, a single serum dilution (1:10) was used because the sIgE levels varied among patients. The 1:10 dilution was predetermined based on immunoblot and ELISA at various dilutions.

In conclusion, patients with RSA had significantly lower sIgE to shrimp extracts, AK and MLC as well as significantly higher sIgG4:sIgE ratios to MLC compared to PSA patients. PSA patients had more diverse sIgE binding to shrimp allergen components than RSA patients. These specific bioassays can be used to identify patients with RSA. Performing oral shrimp challenge in these patients may provide a higher rate of passing the challenge and finally reintroducing shrimp in their diet.

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