Original Article

Molecular Diagnosis of Shrimp Allergy: Efficiency of Several Allergens to Predict Clinical Reactivity

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What is already known about this topic? Tropomyosin is considered responsible for most shrimp allergenic activity and for the cross-reactivity among crustaceans and other arthropods and other invertebrates. Other crustacean allergens have been identified, but their clinical relevance and role in cross-reactivity has not been fully defined.

What does this article add to our knowledge? Among subjects who report shrimp-allergic symptoms, sensitization to tropomyosin and sarcoplasmic calcium-binding protein is associated with clinical reactivity to shrimp. Arginine kinase and hemocyanin appear to be cross-reacting allergens between shrimp and arthropods, but still some arginine-kinase epitopes may be clinically significant. Myosin light chain may also be associated with clinical reactivity to shrimp.

How does this study impact current management guidelines? Detection of specific IgE to these allergenic components and some of their epitopes could provide more specificity for shrimp allergy diagnosis.

BACKGROUND: The diagnosis of shellfish allergy remains a challenge for clinicians. Several shellfish allergens have been characterized and their IgE epitopes identified. However, the clinical relevance of this sensitization is still not clear.

OBJECTIVE: The objective of this study was to identify allergens and epitopes associated with clinical reactivity to shrimp.

METHODS: Shrimp-sensitized subjects were recruited and grouped based on the history of shrimp-allergic reactions and challenge outcome. IgE reactivity to recombinant crustacean allergens, and IgE and IgG4 reactivity to peptides were determined. Subjects sensitized to dust mites and/or cockroach without shrimp sensitization or reported allergic reactions, as well as nonatopic individuals, were used as controls.

RESULTS: A total of 86 subjects were recruited with a skin prick test to shrimp; 74 reported shrimp-allergic reactions, 58 were allergic (38 positive double-blind placebo-controlled food challenge and 20 recent anaphylaxis), and 16 were tolerant. All subjects without a history of reactions had negative challenges. The individuals with a positive challenge more frequently recognized tropomyosin and sarcoplasmic calcium-binding proteins than those found tolerant by the challenge. Especially a sarcoplasmic-calcium-binding-protein positive test is very likely to result in a positive challenge, though the frequency of recognition is low. Subjects with dust mite and/or cockroach allergy not sensitized to shrimp recognized arginine kinase and hemocyanin. Several epitopes of these allergens may be important in predicting clinical reactivity.

CONCLUSION: Tropomyosin and sarcoplasmic-calcium-binding-protein sensitization is associated with clinical reactivity to shrimp. Myosin light chain testing may help in the diagnosis of clinical reactivity. Arginine kinase and hemocyanin appear to be cross-reacting allergens between shrimp and arthropods. Detection of IgE to these allergens and some of their epitopes may be better diagnostic tools in the routine workup of shrimp allergy. © 2015 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2015;■:■-■)

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Abbreviations used
AK- Arginine kinase
AUC- Area under the curve
DBPCFC- Double-blind placebo-controlled food challenge
FABP- Fatty-acid-binding protein
FDR- False discovery rate
HDM- House dust mite
LH+- Positive likelihood ratio
LH−- Negative likelihood ratio
MLC- Myosin light chain
SCP- Sarcoplasmic calcium-binding protein
SE- Sensitivity
sIgE- Specific IgE
SP- Specificity
SPT- Skin prick test

Key words: *Component-resolved diagnosis; Shellfish allergy; Epitope; Microarray; DBPCFC; Tropomyosin; Arginine kinase; Myosin light chain; Sarcoplasmic calcium-binding protein; Hemocyanin; Troponin C; Fatty-acid-binding protein*

Shellfish allergy is a common long-lasting disorder, with Crustacea species, especially shrimp, being the most frequent offender in children and adults. Reported prevalence rates range from 1.3% to 5.2% depending on the country and dietary habits. Shellfish allergy is often associated with severe reactions, including life-threatening anaphylaxis. Despite the high prevalence and severity of reactions, there is still no cure and accurate diagnosis, and strict avoidance is the only recommendation.[1-5]

Clinical history, skin prick test (SPT), and serum-specific IgE (sIgE) are aids in diagnosis. The SPT and sIgE test identify sensitization, but do not establish clinical relevance; thus, a double-blind placebo-controlled food challenge (DBPCFC) remains the gold standard, despite its potential risk.[6] Component-resolved diagnosis and epitope mapping have been applied to a wide range of allergens to elucidate distinct sensitization profiles that more accurately reflect clinical reactivity, avoiding the need for a challenge.[7,8]

Tropomyosin is considered to be responsible for most shrimp-allergic reactions.[9] Nevertheless, other shrimp proteins have been characterized (arginine kinase [AK],[10-12] myosin light chain [MLC],[13] sarcoplasmic calcium-binding protein [SCP],[14,15] hemocyanin,[16-18] troponin C,[17,19] and triosephosphate isomerase[19]) and novel potential allergens identified (fatty-acid-binding protein [FABP],[17] alpha-actinin,[20] beta-actin,[20] and ubiquitin[20]).

The clinical relevance of sensitization to some of them still needs to be fully determined.[1,2] The relevance of tropomyosin sensitization has been studied in subjects with house dust mite (HDM) allergy sensitized to shrimp, finding tropomyosin-sIgE more efficient to diagnose shrimp allergy than the whole shrimp extract.[21,22] The use of the 6 allergens of the North Sea shrimp seemed to improve the diagnosis and treatment of shrimp allergy in a European cohort.[19] Recently, AK and SCP have been found to be minor allergens but clinically relevant in an Italian study.[18]

Cross-reactivity issues also limit correct diagnosis.[1,2,6] Tropomyosin sensitization accounts for the extensive cross-reactivity observed among crustaceans and other arthropods and other invertebrates.[1,2,23,24] However, the role of cross-reactivity with the other allergens has not been fully defined. Some HDM allergens potentially cross-react to homologous allergens in crustaceans,[25] such as HDM-AK (Der p 20),[26] and high molecular weight allergens in both shrimp and HDM, such as hemocyanin.[18,27] Recently, ubiquitin, alpha-actinin, and AK shrimp allergens have been found to be responsible for mite-seafood cross-reactivity.[20] Also cockroach Bla g 8 shows 62.6% of similarity and 48.5% of identity to shrimp MLC.[13,28]

The IgE epitopes of 4 shrimp allergens (Lit v 1-4) were identified.[29] Later, some of those epitopes were identified as clinically relevant in a pilot study.[30]

Hereby we sought to determine whether sensitization to certain allergens and their epitopes correlated more closely with clinical reactivity.

METHODS

Patient selection

Subjects were recruited from the Allergy Clinic at the Mount Sinai Medical Center, New York, USA; the Division of Clinical Immunology and Allergy, University of São Paulo School of Medicine, São Paulo, Brazil; the Allergy Clinic of Hospital Infantil Universitario Niño Jesús and Allergy Department of Fundación Jimenez-Diaz, Madrid, Spain. The inclusion criterion was a positive shrimp SPT, including subjects with shrimp-allergy-related symptoms and also subjects with respiratory allergy, eczema, or food allergies that were skin-prick tested for shrimp-sIgE because it is included in the routine diagnosis panel. DBPCFCs were performed in all individuals except those who reported an anaphylactic episode within the previous 3 months. Subjects were grouped based on whether histories of immediate shrimp-allergic reactions were reported and challenge results. Two control groups were included, one with subjects allergic to HDM and/or cockroach with no sensitization to shrimp or reported histories of allergic reactions after ingestion, and another one with nonatopic individuals. The respective hospital local ethical committees approved the study and informed consent was obtained.

Specific IgE detection and challenge

Serum sIgE to shrimp (f24, a mixture species: *Pandalus borealis, Penaeus monodon, Metapenaeopsis barbata*, and *Metapenaeus joyneri*), HDM (d1: *Dermatophagoides pteronyssinus,* DP), and cockroach (i6: *Blatella germanica*) was measured by ImmunoCAP (ThermoFisher Scientific, Sweden) (cutoff: >0.35 kU_A/L). SPT commercial extracts had to be the ones available in each hospital: shrimp (Spain: Leti, Spain; USA: Alk-Abelló, Spain; Brazil: Hollister-Stier Laboratories, USA), DP, and cockroach (Spain and USA: Alk-Abelló, Spain; Brazil: IPI-ASAC, Spain). Tests were performed according to the European guidelines.[31]

Briefly, DBPCFCs were performed with increasing doses of ground boiled shrimp obtained from a local market (500 mg to a maximum of 8 g) or placebo (lactose) in a chocolate pudding.[21,22,30] Nobody was lactose intolerant or allergic to any pudding components. If the DBPCFC was negative or inconclusive, an open oral challenge receiving a total of 12 cooked shrimps (equivalent to 30 g) was performed. Responses were considered positive if objective signs of allergy were observed.

Purification of recombinant proteins

Ten recombinant (r) proteins were evaluated. TM (Lit v 1), AK (Lit v 2), MLC (Lit v 3), SCP isoform alpha (SCP-alpha, Lit v 4), hemocyanin (Hemo, GenBank Acc. HQ709161), and FABP

(GenBank Acc. KF471026) were from pacific white shrimp (*Litopenaeus vannamei*). SCP isoform beta (SCP-L-beta, GenBank Acc. EH035763) and 2 troponin C proteins (GenBank Acc. FE535729 [TpC-L1] and FD699849 [TpC-L2]) (FE and FD amino acid sequences: 42.2% identity; 55% similarity) were from the American lobster (*Homarus americanus*). The third troponin C (TpC-GC, GenBank Acc. DN634859) (FD and DN: 44% identity/58.4% consensus; FE535729 and DN: 75% identity/84% consensus) was from green crab (*Carcinus maenas*). Shrimp cDNAs were obtained from the Mount Sinai Food Allergen Repository.[13] TpC and SCP-beta clones were kindly provided by Dr. Towle.

Recombinant proteins were expressed on induction with isopropyl-β-D-thio-galactoside of bacterial cultures as previously described.[9,11,13,15,17] All proteins, except rHemo, were purified under native conditions using His 60 Ni Buffer Set (Clontech, Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's protocol. rHemo was purified under denaturing conditions using His-Bind Ni^{2+}-chelating NTA-matrix resin (Novagen, Merck KGaA, Darmstadt, Germany).[17] Purified proteins were dialyzed in phosphate buffer saline (Slide-A-Lyzer Dialysis Cassettes, Pierce, Rockford, IL, USA), analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and stained with Simply Blue SafeStain (Invitrogen, LifeTechnologies, Carlsbad, Calif, USA). Concentrations were determined with Commassie Plus Protein Assay (Pierce).

Dot blotting and immunoblotting studies

IgE reactivity to recombinant proteins and boiled and raw *L. vannamei* was tested with individual sera from all subjects. One microgram of each allergen was applied on the nitrocellulose membrane using the Minifold Dot Blotter (Schleicher and Schuell, Keene, NH), following the manufacturer's protocol. Proteins of whole extracts were separated by SDS-PAGE (Nupage 4% to 12% Zoom Gels; Invitrogen, Calif) and transferred onto an Immobilon-P membrane (Millipore, Mass). After washing and blocking, membranes were incubated with diluted sera (1:5 to 1:20 depending on shrimp-sIgE, f24) for 1 h at room temperature. Iodine 125-labeled goat antihuman IgE (DiaMed, Windham, Me) was used as a secondary antibody, 1 h at room temperature. Membranes were exposed to Kodak Imaging Film (Carestream Health Inc., Rochester, NY) for 2-4 days followed by a longer exposure (16-20 days). Results were evaluated independently by 3 investigators in a qualitative manner (positive and/or negative IgE binding to the spot and/or protein compared to background) and data were combined to analyze frequencies of recognition.

IgE and IgG4 epitope mapping

Overlapping peptides (15-mers, 10-amino-acid overlapping) of the primary sequence of 5 shrimp (*L. vannamei)* allergens—Lit v 1 (TM), Lit v 2 (AK), Lit v 3 (MLC), Lit v 4 (SCP), and TpC (sequence translated from the expressed sequence tag (EST)-nucleotide sequence of eyestalk Acc. FE066626)—were commercially synthesized (JPT Peptide Technologies, Berlin, Germany; sequences in Table E1 in this article's Online Repository at www.jaci-inpractice.org). Peptides were diluted and printed, and immunolabeling was performed as previously described.[30,32]

The fluorescence signal of each spot was digitized and data were analyzed. An individual peptide sample was considered positive if its z score exceeded 3 (ie, signal was significantly above background, $P <$ .003). TileMap, bioinformatics software,[33] which had been used for epitope identification in previous studies,[32,34] was applied to identify IgE and IgG4 binding sites with statistical differences between groups. As hundreds of peptides were analyzed simultaneously, the false discovery rate (FDR) was calculated to adjust for multiple comparisons. A $P < .01$ and FDR < 0.05 were considered significant.

Statistical analysis

Quantitative variables were described by median, range, interquartile range, and qualitative variables with absolute frequencies and percentages. Differences in sIgE levels were assessed using the nonparametric Kruskal-Wallis test. The Fisher exact test was used to compare frequencies of IgE reactivity between groups ($P < .05$ significant). The number of positive peptides bound by IgE, IgG4, or both was analyzed and compared between groups (nonparametric Kruskal Wallis and post-Dunn's test, $P < .05$) (GraphPad Prism 4.0c, GraphPad Software Inc., San Diego, Calif, USA).

Sensitivity, specificity, likelihood ratios (positive [LH+] and negative [LH−]) and area under the curve (AUC) values were calculated using two-by-two contingency tables and receiver-operating-curves analysis.[35]

RESULTS

Study design: subjects characterization

Eighty-six individuals with a positive shrimp SPT were recruited (35% Spain, 34% Brazil, and 31% USA). Of those, 74 (86%) reported a history of allergic reactions after shrimp ingestion and 12 (14%) did not. Of the 74, 58 were allergic (38 positive DBPCFC and 20 recent anaphylaxis; Group 1, G1) and 16 (22%) tolerant (negative DBPCFC; Group 2a, G2a). Subjects sensitized to shrimp but not reporting allergic reactions were also challenged, being all negative (Group 2b, G2b). All negative DBPCFCs were confirmed with an open challenge. As control groups, 12 subjects allergic to HDM and/or cockroach without sensitization to shrimp or the reported history of shrimp-allergic reactions were recruited and challenged, being all negative (Group Control 1, GC1), as well as 5 nonatopic individuals (Group Control 2, GC2). The study is outlined in Figure E1 (in this article's Online Repository at www.jaci-inpractice.org).

Demographics, frequency of sensitization to shrimp by SPT, HDM, and cockroach, sIgE levels, history of shrimp-allergic reactions, and DBPCFC outcomes are summarized in Table I and detailed in Table E2 (in this article's Online Repository at www.jaci-inpractice.org).

G1 had higher shrimp-sIgE levels than all other groups (vs G2a: $P < .001$; vs G2b: $P < .01$). There were no significant differences between G2a and G2b. DP sensitization was very frequent in all groups (94% to 100%), whereas the frequency of cockroach sensitization was more variable among groups (few data not available).

IgE recognition of recombinant allergens differed among groups

The frequency of IgE binding to each allergen per group (Figure 1) and significant differences between groups was analyzed (Table E3 in this article's Online Repository at www.jaci-inpractice.org).

In G1, subjects showed more diversity of allergen recognition (ie, number of different allergens showing IgE binding) (3 [0-9]) than G2a (1.5 [0-5], $P < .05$), G2b (2 [0-3], ns) and GC1 (1.5 [0-2], $P < .01$). Tropomyosin was the most frequently recognized, alone (7/58, 12.1%) and in combination with SCPs (18/58, 31%) or MLC (21/58, 36.2%). This was rare among tolerant subjects.

TABLE I. Characteristics of the subjects recruited for the study

	Group 1	Group 2a	Group 2b	Group Control 1	Group Control 2
N	58	16	12	12	5
Gender: male, % (no.)	50% (29)	50% (8)	50% (6)	17% (2)	40% (2)
Age: median [range]	17 [3-52]	25 [5-45]	29 [13-47]	32.5 [16-60]	36 [24-60]
Sensitization rate by SPT					
Shrimp	100% (58/58)	100% (16/16)	100% (12/12)	0% (0/12)	0% (0/5)
Dust mite (DP)	94.6% (53/56)	93.8% (15/16)	100% (12/12)	100% (12/12)	0% (0/5)
Cockroach	78.8% (41/52)	40% (6/15)	63.6% (7/11)	16.7% (2/12)	0% (0/5)
Specific IgE (kU_A/L), median[range]					
Shrimp	27.10 [<0.35 to >100]	0.52 [<0.35 to 16.5]	1.53 [<0.35 to 5.7]	<0.35	<0.35
Dust mite (DP)	14.0 [<0.35 to >100]	2.24 [<0.35 to 60.2]	54.1 [1.89 to >100]	8.35 [<0.35 to >100]	<0.35
Cockroach	2.7 [<0.35 to >100]	0.79 [<0.35 to 3.6]	<0.35 [<0.35 to 1.29]	<0.35 [<0.35 to 0.53]	<0.35
Shrimp ImmunoCAP negative tests	1/58	4/16	3 (1 NA)/12	12/12	5/5
History shrimp reaction	+	+	-	-	-
DBPCFC	+	-	-	-	-

DP, *Dermatophagoides pteronyssinus*; *DBPCFC*, double-blind placebo-controlled food challenge; "+", positive; "-", negative; *SPT*, skin prick test; *NA*, data not available.
*38 positive DBPCFC and 20 with recent anaphylaxis.

In G2a, TM, MLC, and AK were the allergens more frequently recognized, whereas subjects in G2b recognized mainly AK and Hemo. None from G2b recognized MLC, SCPs, or TpCL1/L2 and only one recognized TM, TpC-GC, and FABP. The frequency of recognition of TpCs and FABP was lower than that for other allergens in all groups. GC1 recognized Hemo and AK (75% both proteins).

Certain subjects did not show IgE binding to any recombinant allergen (Table E3 in this article's Online Repository at www.jaci-inpractice.org). However, all G1 and G2a subjects showed IgE binding around 35-40 kDa and/or high molecular weight proteins on immunoblot. Also 1 of the 2 in G2b and 2 of the 4 in GC1 recognized similar proteins. Nobody in GC2 recognized any protein by dot blot or immunoblotting (not shown).

Sensitization to SCPs and TM was found more frequent in allergic than tolerant subjects who reported a history of shrimp-allergic symptoms

The comparison of subjects with the reported history of shrimp-allergic symptoms (n = 74) showed that the reactive individuals (found by a challenge or with a recent history of anaphylaxis, G1) and those found tolerant by a challenge (G2a) differed with statistical significance in the frequency of recognition of SCPs and TM. Of the 74 allergic individuals, 31% (18) had IgE binding to both SCP and TM, whereas only 6.6% (1/16) of the tolerant ones did so (P = .0549, a trend to significance).

Individuals from G1 and G2b (tolerant but without history of reactions) differed in the frequency of recognition of TM, SCPs, MLC (more frequent in G1), and Hemo (more frequent in G2b).

G2a and G2b subjects only differed in Hemo recognition (more frequent in G2b). If G2a and G2b were considered together (as they all were sensitized to shrimp but passed the shrimp challenge, tolerant, n = 28) and compared with G1 (allergic, n = 58), then also MLC was found more frequent in challenge positive subjects (*P* values in Table E3 in this article's Online Repository at www.jaci-inpractice.org). Moreover, this analysis showed that the sensitization profile $TM^+SCP^+MLC^-$ only occurred in allergic subjects (15.5%). $TM^-SCP^-MLC^-$ was more frequent among tolerant than allergic subjects (75% vs 12.1%; $P < .0001$). Sensitization to $TM^+SCP^+MLC^+$ and $TM^+SCP^-MLC^+$ was more frequent among allergic (15.5% and 20.7%, respectively) than tolerant subjects (3.6% and 10.7%) but without significance. $TM^-SCP^+MLC^+$ was not found in any group.

Individuals with shrimp allergy (G1) compared with control group with HDM allergy (GC1, not sensitized to shrimp) more frequently recognized TM, SCPs, and MLC. G2a more frequently recognized TM (and a trend to significance for Hemo) than GC1, whereas G2b did not show significant differences to this control group (*P* values in Table E3 in this article's Online Repository at www.jaci-inpractice.org).

Within G1, the only significant difference between children (n = 33) and adults (n = 25) was for SCP-L-beta (higher in children, $P = .0128$). Among tolerant subjects who reported a history of symptoms (G2a, 5 children/11 adults), no significant differences were found.

Epitope mapping: IgE and IgG4 binding sites

IgE and IgG4 binding to peptides were tested for a subset of subjects (G1, n = 21; G2a, n = 16; G2b, n = 12; Figures E2 and E3 in this article's Online Repository at www.jaci-inpractice.org). Figure E4 and Table E4 (in this article's Online Repository at www.jaci-inpractice.org) summarize the binding sites and/or epitopes for each allergen showing significant differences between groups based on the TileMap analysis.

IgE and IgG4 binding sites largely overlap. Minor differences in peptide recognition were observed between G2b and G2a.

Regarding IgE binding, tropomyosin had the largest number of differential binding sites. For IgG4, this occurred for AK. For SCP, differential binding sites were mainly observed for IgG4 rather than IgE. Almost no differences were observed for MLC and none for TpC (Table E4 in this article's Online Repository at www.jaci-inpractice.org). Among the subjects with the reported history of shrimp-allergic symptoms tested by a microarray (n = 37), challenge-positive individuals (G1) recognized more frequently the IgE epitopes and/or binding sites than those found tolerant (G2a, $P < .05$), except epitopes AKp28-29,

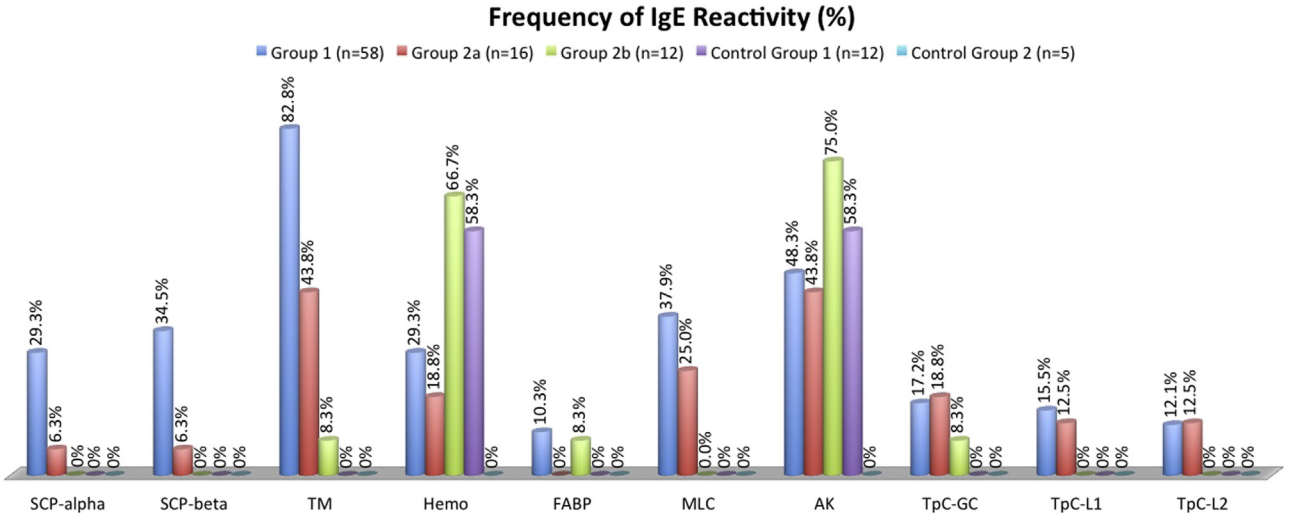

FIGURE 1. Frequency of IgE recognition of recombinant allergens per group. Histogram showing the frequency of IgE recognition per protein and per group, expressed as percentages (%). *SCP*, sarcoplasmic calcium-binding protein, -alpha and L-beta; *TM*: tropomyosin; *Hemo*, hemocyanin; *FABP*, fatty-acid-binding protein; *MLC*, myosin light chain; *AK*, arginine kinase; *TpC*, troponin C (-GC, -L1, -L2).

TMp38-39, and SCPp18-19 (although the last 2 showed a strong trend to significance, $P = .0501$; Table E5 in this article's Online Repository at www.jaci-inpractice.org).

Allergic subjects had more IgE reactive and IgE/IgG4 reactive peptides than tolerant ones

For each allergen, the number of IgE and IgG4 positive peptides per subject was analyzed (Table II). Allergic subjects had more IgE reactive peptides, as well as more peptides recognized by both IgE and IgG4, than tolerant individuals across all allergens. The difference in peptides bound by IgG4 between allergic and tolerant individuals was only significant for TM, MLC, and AK. No correlation was found between IgE and IgG4 binding, either in shrimp-allergic or tolerant individuals (not shown).

Properties of recombinant allergens and epitopes as diagnostic tests

Only the shrimp extract (f24), individual proteins, and their epitopes showing strictly statistically significant differences in the frequency of IgE recognition between allergic and tolerant subjects could be considered as candidates for diagnostic tests (Table E5 in this article's Online Repository at www.jaci-inpractice.org). The combination TM+SCP+ and the epitopes TMp38-39 and SCPp18-19 showed a strong trend to significance (data not included in this analysis).

Shrimp extract had high sensitivity (98.3%), low specificity (25.0%), and very low LH− (0.07). SCP LH+ was found higher (5.5) than TM and shrimp extract (1.9 and 1.3, respectively). TM and SCP allergens had an AUC value similar to shrimp extract.

Epitopes showed better AUC values than whole proteins and shrimp extract (f24). TM epitopes had high specificity (except TMp33-35, 68.8%) and variable sensitivity (33.3% to 85.7%). TMp51-55 had the highest AUC value among all epitopes (0.804). Even though AK and MLC allergens were not more frequently recognized by allergic subjects than tolerant individuals who reported symptoms, several epitopes of these proteins were found clinically relevant and some with the highest AUC values (0.732-0.763) (Table III).

DISCUSSION

Several shellfish allergens have been cloned and characterized during the past decade[1,2] and shrimp IgE epitopes identified.[29] However, more information about the clinical relevance of sensitization to them is still required. This study sought to identify profiles of specific allergen and epitope sensitization associated with clinical reactivity to shrimp. To our knowledge, this is the largest study that provides a molecular diagnosis of shrimp allergy, and the first assessment of IgG4 epitope specificity to shrimp allergens.

Subjects with a positive shrimp SPT were recruited and grouped according to their history of allergic reactions to shrimp and results from the DBPCFC (with the limitation that 20 individuals with a recent history of anaphylaxis were not challenged and considered allergic). Given the extensive cross-reactivity between crustaceans and other arthropods and other invertebrates,[1,2,23,24] a control group allergic to HDM and/or cockroach, but not sensitized to shrimp, was included. The frequency of HDM sensitization was very high in all groups, as previously observed,[13] whereas sensitization to cockroach was less frequent, possibly due to a low exposure in some areas of recruitment and few missing data. These sensitizations in G1, G2a, and G2b could be due to a primary sensitization, or secondary to cross-reactivity among homologous proteins in shrimp, HDM, or cockroach. Neither mites nor cockroaches seem to be very efficient inducers of IgE to tropomyosin and other muscle-associated (nonexcreted) proteins compared with shrimp.[36] Sensitization to other arthropod allergens could occur through different routes, including the skin (dust mites in eczema). Thus, the high homology between proteins in different species, the route, and the frequency of exposure could influence sensitization. Actually, shrimp-sIgE levels might need to be interpreted in

TABLE II. Number of positive peptides bound by IgE, IgG4, and both, per allergen

	AK			MLC			SCP			TM			TpC		
	1	2a	2b	1	2a	2b	1	2a	2b	1	2a	2b	1	2a	2b
No. of peptides bound by IgE															
Median	8	2	4	4	1	2.5	3	2	2.5	6	0	1	2	0.5	1.5
Range	1-30	0-16	1-20	1-14	0-8	1-14	2-11	2-12	2-12	0-20	0-9	0-5	0-7	0-8	0-5
IQ range (25% to 75%)	4-15.5	1-3	2.5-10	1.5-6.5	1-1	1-4.5	2-6	2-2	2-4.5	2.5-9	0-2	0-2.5	1-4	0-1	0-3.5
KW *P* value		.0013			.0016			.0143			<.0001			.0221	
Dunn's posttest *P* value															
1 vs 2a		<.001			<.01			<.05			<.001			<.05	
1 vs 2b		ns			ns			ns			<.01			ns	
No. of peptides bound by IgG4															
Median	9	2.5	1.5	2	1	1	6	3	2	4	0	0	2	0	0.5
Range	2-31	0-26	0-20	0-13	0-6	0-5	2-14	2-12	0-10	0-16	0-9	0-4	0-6	0-7	0-4
IQ range (25% to 75%)	5.5-22	2-9.5	1-6.5	1-7.5	0.5-1.5	0-2	2-7.5	2-7	2-5.5	0-8	0-1.5	0-2	0-4	0-3	0-2.5
KW *P* value		.002			.007			ns			.0112			ns	
Dunn's posttest *P* value															
1 vs 2a		<.05			<.05			-			<.05			-	
1 vs 2b		<.01			<.05			-			<.05			-	
No. of peptides bound by IgE and IgG4															
Median	3	1	1	1	1	1	3	2	2	2	0	0	1	0	0
Range	1-25	0-6	0-6	0-11	0-1	0-2	2-7	2-3	0-7	0-10	0	0-2	0-5	0-1	0-2
IQ range (25% to 75%)	2-8	1-1	0.5-3.5	1-3.5	0-1	0-2	2-4	2-2	1.5-3	0-4.5	0	0-2	0-2	0	0-0.5
KW *P* value		<.0001			.0179			.0155			.0002			.0337	
Dunn's posttest *P* value															
1 vs 2a		<.001			<.05			<.05			<.001			<.05	
1 vs 2b		<.01			ns			ns			<.05			ns	

AK, arginine kinase; *TM*, tropomyosin; *SCP*, sarcoplasmic calcium-binding protein; *MLC*, myosin light chain; *TpC*, troponin C.
For each allergen, the number of positive peptides (ie, *z* score above 3) bound by IgE, IgG4, or both in each subject was analyzed. Medians of the number of positive peptides bound by each immunoglobulin were compared between allergic (G1), tolerant with history of allergic symptoms (G2a), and tolerant without history of allergic symptoms (G2b) individuals (one-way ANOVA nonparametric analysis: Kruskal-Wallis (KW) and Dunn's posttest, $P < .05$ considered significant). *IQ*, interquartile range; *ns*, not significant; "-", no posttest possible because KW *P* value >.05, not significant.

TABLE III. Properties of shrimp extract, recombinant allergens, and IgE epitopes as diagnostic tests

Allergen	Epitope/binding site ID	SE (%)	SP (%)	LH+	LH−	AUC
Shrimp		98.3 (90.8-99.9)	25.0 (7.3-52.4)	1.3	0.07	0.616 (0.445-0.788)
TM		82.8 (70.6-91.4)	56.3 (29.9-80.3)	1.9	0.3	0.633 (0.468-0.797)
SCPs		34.5 (22.5-48.1)	93.8 (69.8-99.8)	5.5	0.7	0.641 (0.503-0.779)
AK	AKp15-17	71.4 (47.8-88.7)	75 (47.6-92.7)	2.86	0.4	0.73 (0.56-0.900)
	AKp34-36	38.1 (18.1-61.6)	100 (79.4-100)	-	0.6	0.691 (0.521-0.860)
	AKp41-42	47.6 (25.7-70.2)	100 (79.4-100)	-	0.5	0.738 (0.578-0.898)
	AKp67-68	81.0 (58.1-94.6)	68.8 (41.3-89.0)	2.59	0.3	0.749 (0.582-0.915)
MLC	MLCp24-26	71.4 (47.8-88.7)	81.3 (54.4-96.0)	3.8	0.4	0.763 (0.603-0.924)
TM	TMp1-3	42.9 (21.8-66.0)	100.0 (79.4-100)	-	0.6	0.714 (0.549-0.88)
	TMp13-14	33.3 (14.6-57.0)	100.0 (79.4-100)	-	0.7	0.667 (0.493-0.840)
	TMp25-26	38.1 (18.1-61.6)	100.0 (79.4-100)	-	0.6	0.690 (0.521-0.860)
	TMp29-30	47.6 (25.7-70.2)	93.8 (69.8-99.8)	7.62	0.6	0.707 (0.539-0.875)
	TMp33-35	71.4 (47.8-88.7)	68.8 (41.3-89.0)	2.29	0.4	0.701 (0.526-0.876)
	TMp51-55	85.7 (63.7-97.0)	75 (47.6-92.7)	3.43	0.2	0.804 (0.650-0.957)

Sensitivity, specificity, likelihood ratios (positive [LH+] and negative [LH−]), and area under the curve (AUC) values of the recombinant allergens and IgE epitopes found with statistically significant differences in terms of the frequency of recognition between subjects with positive challenge (G1) and negative challenge but sensitized to shrimp and reporting symptoms of shrimp allergy (G2a) were calculated using two-by-two contingency tables and receiver-operating-curves analysis.[35] *SE*, sensitivity; *SP*, specificity, both expressed as percentages (%). *LH+*, positive likelihood ratio; *LH−*, negative likelihood ratio; *SCP*, sarcoplasmic calcium-binding protein; *TM*, tropomyosin. Shrimp extract (f24, ImmunoCAP, ThermoFisher Scientific). Epitopes are identified with the abbreviation of the allergen name plus the peptide (p) number/ID.

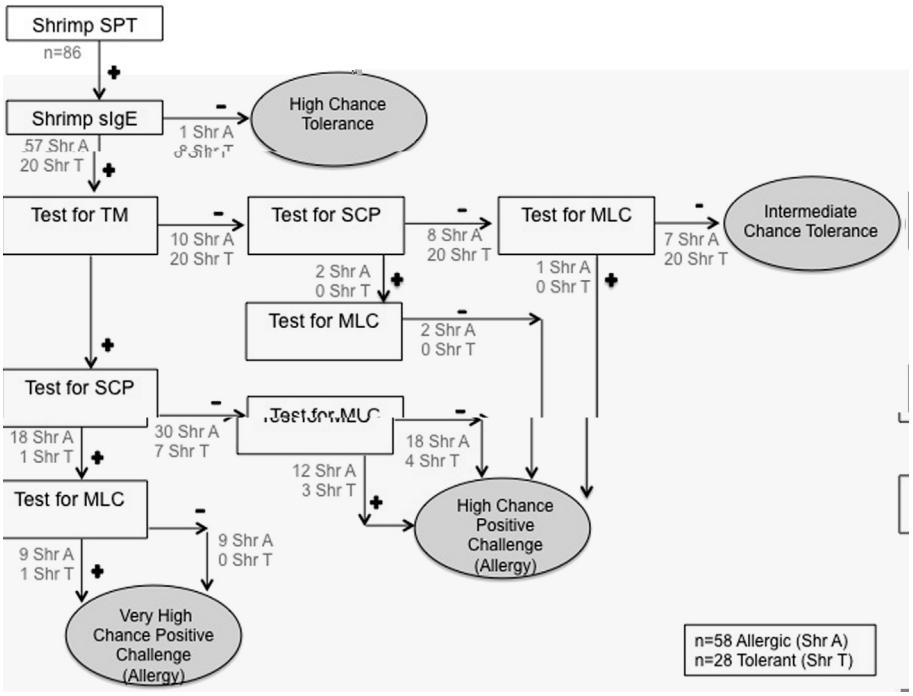

FIGURE 2. Flow diagram for shrimp allergy diagnosis. Suggested protocol using allergenic components for examining patients who are initially found sensitized to shrimp by a skin prick test regardless of whether they report allergic symptoms or not. For each test outcome the absolute number of real shrimp allergic (Shr A, subjects diagnosed by positive challenge or recent anaphylactic reaction; n = 58) and shrimp tolerant (Shr T, subjects with negative challenge; n = 28) subjects is provided.

the context of environmental exposures to cockroach and dust mite.[37]

Individuals with shrimp allergy showed more diversity of IgE recognition at both allergen and epitope level than tolerant subjects. Subjects with HDM and/or cockroach allergy not sensitized to shrimp recognized AK and hemocyanin. Minor differences were found among shrimp-tolerant subjects with and without a clinical history of allergic reactions to shrimp. Among subjects who reported shrimp-allergic symptoms, individuals with positive challenge and/or recent anaphylaxis more frequently recognized TM and SCPs, and having both proteins positive had a strong trend to significance. Sensitization to these components appears to be important in differentiating clinical reactivity to shrimp. An SCP positive test had the best LH+, it is more specific than TM and the shrimp extract, and it is very likely to result in a positive challenge. SCPs from lobster and shrimp show high degree of amino acid identity and homology. Therefore, the clinical relevance of sensitization to different shellfish SCPs in subjects with shrimp allergy may also include reactivity to lobster.

When individuals found tolerant by a challenge were considered as one group (G2a and G2b) and compared with allergic (G1) subjects, then also MLC was found more frequent in allergic ones. This information could help for the diagnosis workup of subjects with an unknown history of shellfish allergy but with a positive serum sIgE or SPT to shrimp extract. Interestingly, allergic individuals compared with those tolerant without reported symptoms more frequently recognized these 3 proteins (ie, TM, SCP, and MLC). It is possible that MLC may play a role in predicting shrimp allergy. Further studies including larger groups of shrimp-tolerant individuals sensitized to shrimp with and without a history of allergic reaction would be of interest to address this point.

IgE reactivity to whole allergen extracts without clinical correlation has been attributed to sensitization to cross-reacting allergens in other foods or environmental allergens.[36,38,39] Most clinically irrelevant shrimp positive tests have been reported to be due to cross-reactive responses to tropomyosin.[23,24] However, Yang et al[21] described a low frequency of sensitization to shrimp tropomyosin in their group of individuals with mite allergy and a high frequency of sensitization to shrimp without clinical relevance. Very recently, Gamez et al[20] found ubiquitin, alpha-actinin, and AK shrimp allergens as responsible for mite-seafood cross-reactivity. In our study, some subjects with this profile (G2a and G2b) recognized tropomyosin, but most recognized AK and hemocyanin (both not tested by Yang et al.,[21] and hemo not evaluated by Gamez et al[20]), like control group C1. Thus, we identified not only clinically relevant components in shrimp allergy, but also AK and hemocyanin as likely markers of cross-reactivity with other arthropods and/or invertebrates (at least, HDM and cockroach). Cross-inhibition studies should be included in future studies to assess their role in cross-reactivity phenomena. Only a few subjects in G1 recognized TpC and FABP, which appeared to be specific for shrimp allergy. However, this should be confirmed in larger studies because green-crab and lobster troponins share about 50% to 60% homology with shrimp-TpC, which may explain the low frequency in the population studied.

Many recombinant allergens, such as tropomyosin, have been shown to behave similarly to their native counterparts.[40,41]

However, this is not universal for all allergens and may explain why some subjects did not recognize any recombinant proteins on dot-blot immunolabeling but showed IgE reactivity to proteins of boiled and raw shrimp extracts. Similarly, the low sensitivity observed of some recombinant allergens, for instance SCP or MLC, may be due to posttranscriptional modifications of the native allergens that differ from the recombinant counterparts; besides that they might be minor allergens in our population of study. The black-tiger-shrimp rSCP has been shown to have the same IgE reactivity as the natural counterpart (unfortunately the pacific-white-shrimp rSCP was not assessed).[42]

Although SCP and MLC have been reported to be particularly important in children,[13,15,29,43] no strong trend was observed. Differences previously observed might be due to the fact that subjects were not challenged and more adults with negative challenges might have been included.

Ayuso et al[29] identified IgE-binding epitopes of 4 shrimp allergens (TM, AK, MLC, and SCP). Hereby, another shrimp allergen, TpC, and IgG4 binding data are included. Subjects recruited come from a population similar to previous studies, but groups are larger and include different geographic areas.

Several informative epitopes and/or binding areas have been identified that may be important in predicting clinical reactivity. Especially, TMp51-55 showed the highest AUC value, thus being of good value as a diagnostic test to confirm allergy.[35] The properties of epitope SCPp16-18 as a diagnostic test in the comparison of G1 versus G2a could not be assessed, but when comparing allergic versus all tolerant individuals (G2a and G2b), it showed a high LH+ (4.7) like the whole allergen SCP. The predictive value of this epitope should be further evaluated.

Although IgE and IgG4 binding areas largely overlap, as has been previously described in microarray studies,[44] more differential IgE recognition sites were observed for TM, a protein associated with positive challenge outcome, whereas more different IgG4 binding sites were found in AK, an allergen that appears more responsible for cross-reactivity (shown in this study). Still some AK epitopes that differ between allergic and tolerant subjects have been found. The IgE antigenic areas identified hereby corresponded well to epitopes previously described.[29] Current results significantly coincide with the results of our pilot study[30] also intended to identify clinically relevant epitopes, and moreover additional binding sites have been identified (Table E6 in this article's Online Repository at www.jaci-inpractice.org).

Testing for serum sIgE to the shrimp extract would be the second step (after the SPT) in the routine diagnosis of shrimp allergy, because it is very sensitive and has the lowest LH− ratio (when compared with all recombinant proteins), which means that a negative result almost rules out shrimp allergy. However, given its poor specificity and sensitivity for some allergens, further testing of components is required. TM and SCP would increase specificity, whereas sensitization to AK and/or hemocyanin without TM, SCP, and/or MLC would point that sensitization to shrimp is more likely due to cross-reactivity with other arthropods and/or other invertebrates, such as HDM, than real shrimp allergy. Although the frequency of recognition in our cohort is low, an SCP positive test is very likely to result in a positive challenge (18 allergic subjects/1 tolerant if SCP+ after TM+; 2 allergic subjects/0 tolerant if SCP+ after TM−; Figure 2). If possible, detection of sIgE to certain epitopes of TM, MLC, AK and possibly SCPp16-18 of SCP, could be also of interest, because it increases specificity and seems to perform better than whole allergens as diagnostic tests.

Similar to peanut and milk allergy,[44,45] clinical reactivity would be associated with IgE broader epitope recognition of shrimp allergens. Interestingly, for TM, MLC, and AK, we observed IgG4 broader epitope recognition in allergic individuals than tolerant ones. The role of IgG4 in these differences is difficult to address, because this antibody has been related to chronic antigen exposure and it has been shown in other context to inhibit the activity of IgE by competing for binding sites becoming somehow protective.[46-48] Individuals with shrimp allergy produce both IgE and IgG4 antibodies, and clinical reactivity might depend on antibody levels, antibody affinity (not analyzed here), or other factors. The relevance of these findings of IgG4 in shrimp allergy needs to be further investigated.

In conclusion, among subjects who report shrimp-allergic symptoms, sensitization to TM and SCPs (whole allergens and specific epitopes) is associated with a positive challenge to shrimp. MLC may also be associated with clinical reactivity to shrimp. Arginine kinase and hemocyanin seem to be the markers of cross-reactivity with other arthropods and/or invertebrates, but still some AK epitopes may be clinically significant. Detection of sIgE to these allergenic components and epitopes could provide more specificity for shrimp allergy diagnosis.

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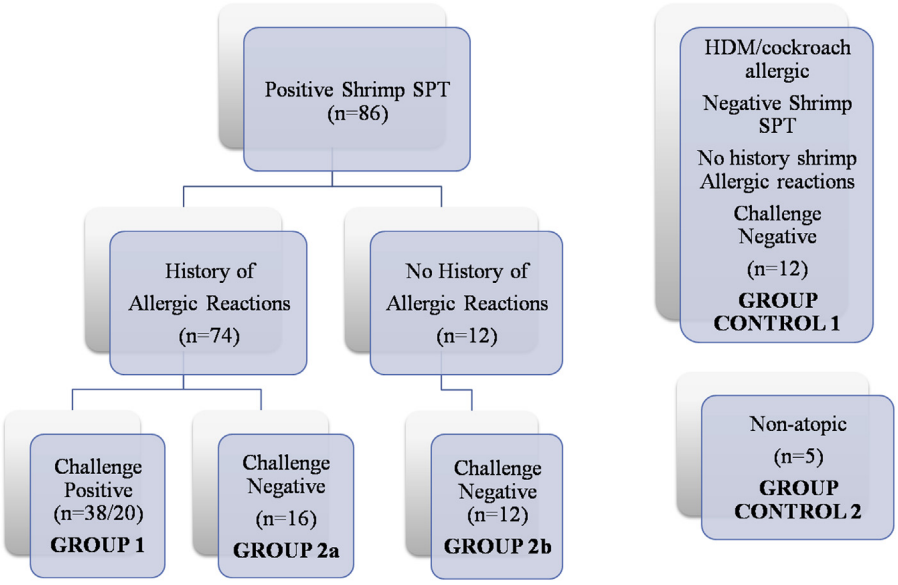

FIGURE E1. Study design: subjects recruited. Subjects with shrimp positive skin prick test (SPT) were recruited and grouped based on history of shrimp allergic reactions and challenge outcome. Two control groups were included, one with subjects allergic to HDM and/or cockroach with no sensitization to shrimp or reported histories of allergic reactions after ingestion, and another one with nonatopic individuals.

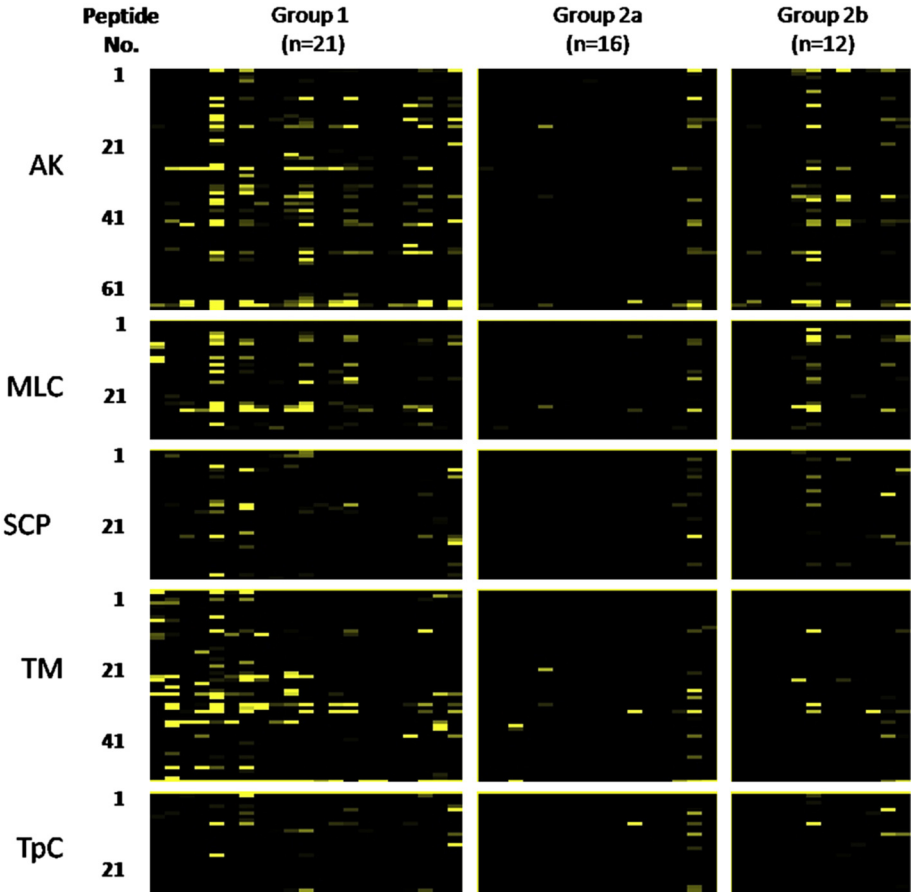

FIGURE E2. Heat map representing IgE binding in the peptide microarray. All peptides from 5 shrimp allergens (*SCP*, sarcoplasmic calcium-binding protein; *TM*, tropomyosin; *MLC*, myosin light chain; *AK*, arginine kinase; *TpC*, troponin C) are represented in rows. The recognition of peptides by each patient is shown in columns. The intensity of binding is presented with average *z* score in a grading scale ranging from less than 3 (black) to 5 or greater (yellow).

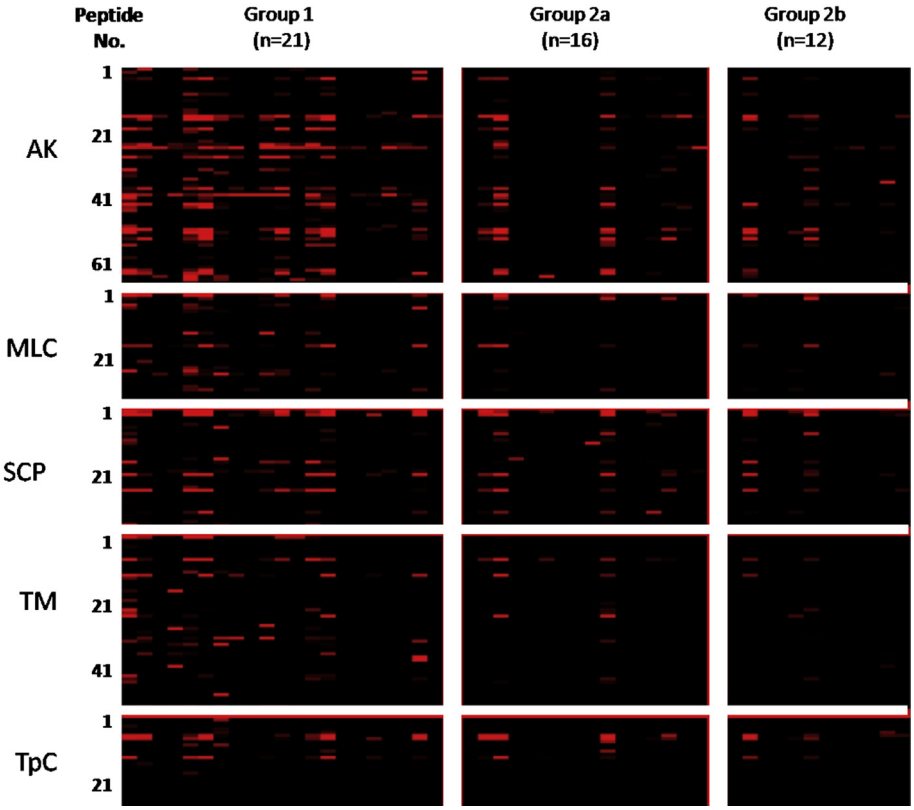

FIGURE E3. Heat map representing IgG4 binding in the peptide microarray. All peptides from 5 shrimp allergens (*SCP*, sarcoplasmic calcium-binding protein; *TM*, tropomyosin; *MLC*, myosin light chain; *AK*, arginine kinase; *TpC*, troponin C) are represented in rows. The recognition of peptides by each patient is shown in columns. The intensity of binding was represented with average *z* scores in a grading scale ranging from less than 3 (black) to 5 or greater (red).

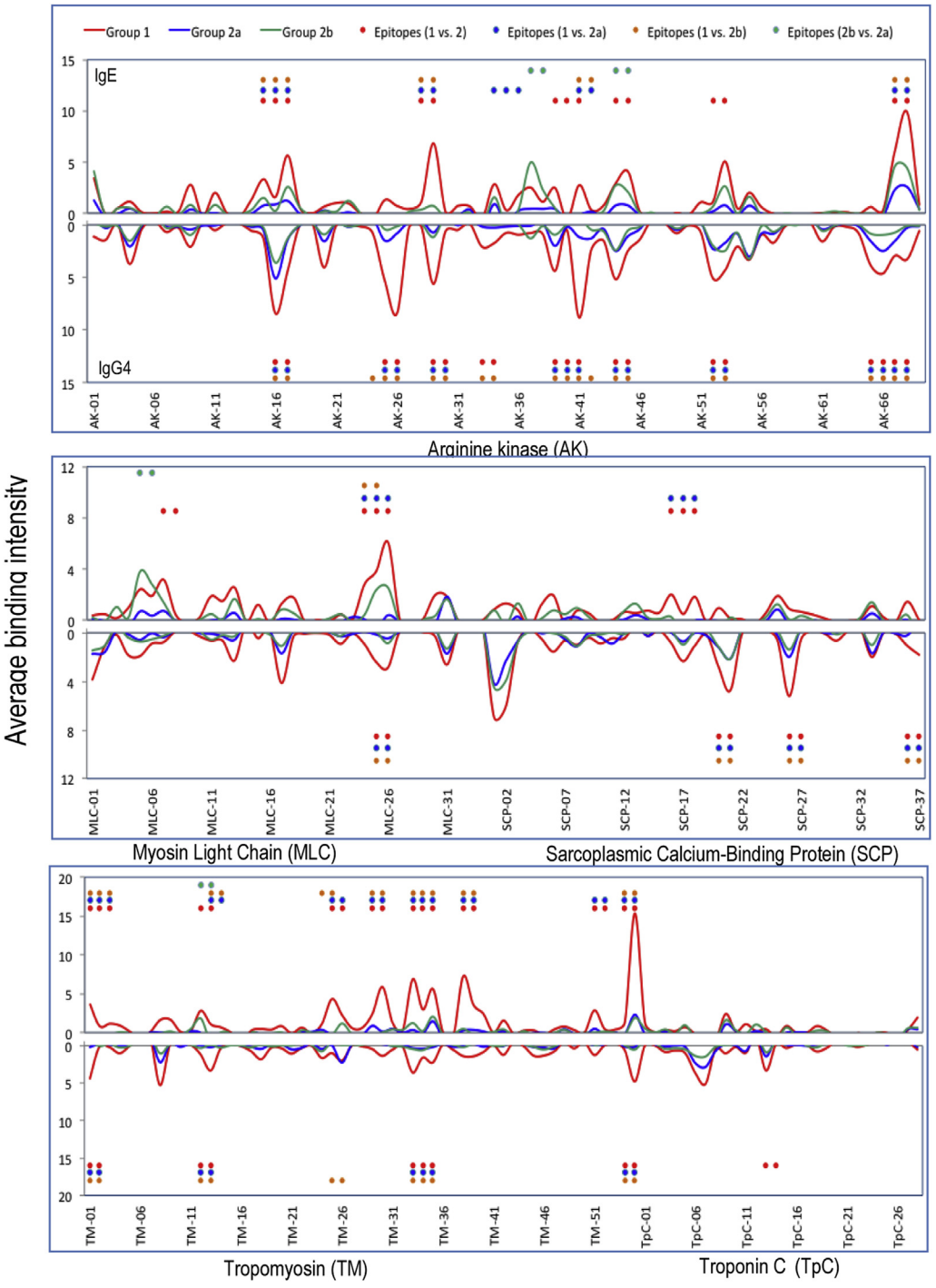

FIGURE E4. TileMap analysis. Lines show average binding intensity per group (G1, red; G2a, blue; G2b, green). Dots represent binding sites with statistically significant differences between the groups compared (G1 vs G2 [G2a+G2b], red; G1 vs G2a, blue; G1 vs G2b, yellow; G2b vs G2a, green). On the x-axis, the protein and the peptide number are shown. Significant binding sites have $P < .01$, FDR < 0.05. The top half of each graph corresponds to IgE binding, whereas the bottom half corresponds to the IgG4 binding.

TABLE E1. Sequences of the synthetic overlapping peptides tested in the microarray

AK		TM		SCP		MLC		TpC	
Pp #	Sequence	Pp #	Sequence	Pp #	Sequence	Pp #	Sequence	Pp #	Sequence
AK-01	MADAAVIEKLEAGFK	TM-01	MDAIKKKMQAMKLEK	SCP-01	MAYSWDNRVKYVVRY	MLC-01	MSRKSGSRSSSKRSK	TpC-01	MDSLDEEQIETLRKA
AK-02	VIEKLEAGFKKLEAA	TM-02	KKMQAMKLEKDNAMD	SCP-02	DNRVKYVVRYMYDID	MLC-02	GSRSSSKRSKKSGGG	TpC-02	EEQIETLRKAFDSFD
AK-03	EAGFKKLEAATDCKS	TM-03	MKLEKDNAMDRADTL	SCP-03	YVVRYMYDIDNNGFL	MLC-03	SKRSKKSGGGSNVFD	TpC-03	TLRKAFDSFDTEKTG
AK-04	KLEAATDCKSLLKKY	TM-04	DNAMDRADTLEQQNK	SCP-04	MYDIDNNGFLDKNDF	MLC-04	KSGGGSNVFDMFTQR	TpC-04	FDSFDTEKTGSITAE
AK-05	TDCKSLLKKYLTKEV	TM-05	RADTLEQQNKEANNR	SCP-05	NNGFLDKNDFECLAV	MLC-05	SNVFDMFTQRQVAEF	TpC-05	TEKTGSITAETIATI
AK-06	LLKKYLTKEVFDKLK	TM-06	EQQNKEANNRAEKSE	SCP-06	DKNDFECLAVRNTLI	MLC-06	MFTQRQVAEFKEGFQ	TpC-06	SITAETIATIMRMMG
AK-07	LTKEVFDKLKDKKTS	TM-07	EANNRAEKSEEEVHN	SCP-07	ECLAVRNTLIEGRGE	MLC-07	QVAEFKEGFQLMDRD	TpC-07	TIATIMRMMGVKISE
AK-08	FDKLKDKKTSLGATL	TM-08	AEKSEEEVHNLQKRM	SCP-08	RNTLIEGRGEFSADA	MLC-08	KEGFQLMDRDKDGVI	TpC-08	MRMMGVKISEKNLQE
AK-09	DKKTSLGATLLDVIQ	TM-09	EEVHNLQKRMQQLEN	SCP-09	EGRGEFSADAYANNQ	MLC-09	LMDRDKDGVIGKTDL	TpC-09	VKISEKNLQEAIAET
AK-10	LGATLLDVIQSGVEN	TM-10	LQKRMQQLENDLDQV	SCP-10	FSADAYANNQKIMRN	MLC-10	KDGVIGKTDLRGTFD	TpC-10	KNLQEAIAETDEDGS
AK-11	LDVIQSGVENLDSGV	TM-11	QQLENDLDQVQESLL	SCP-11	YANNQKIMRNLWNEI	MLC-11	GKTDLRGTFDEIGRI	TpC-11	AIAETDEDGSGLLEF
AK-12	SGVENLDSGVGIYAP	TM-12	DLDQVQESLLKANIQ	SCP-12	KIMRNLWNEIAELAD	MLC-12	RGTFDEIGRIATDQE	TpC-12	DEDGSGLLEFEEFVE
AK-13	LDSGVGIYAPDAEAY	TM-13	QESLLKANIQLVEKD	SCP-13	LWNEIAELADFNKDG	MLC-13	EIGRIATDQELDEML	TpC-13	GLLEFEEFVELSAKF
AK-14	GIYAPDAEAYTLFAP	TM-14	KANIQLVEKDKALSN	SCP-14	AELADFNKDGEVTVD	MLC-14	ATDQELDEMLADAPA	TpC-14	EEFVELSAKFLIEED
AK-15	DAEAYTLFAPLFDPI	TM-15	LVEKDKALSNAEGEV	SCP-15	FNKDGEVTVDEFKQA	MLC-15	LDEMLADAPAPINFT	TpC-15	LSAKFLIEEDEEALK
AK-16	TLFAPLFDPIIEDYH	TM-16	KALSNAEGEVAALNR	SCP-16	EVTVDEFKQAVQKHC	MLC-16	ADAPAPINFTMLLNM	TpC-16	LIEEDEEALKAELRE
AK-17	LFDPIIEDYHVGFKQ	TM-17	AEGEVAALNRRIQLL	SCP-17	EFKQAVQKHCQGKKY	MLC-17	PINFTMLLNMFAERQ	TpC-17	EEALKAELREAFRIY
AK-18	IEDYHVGFKQTDKHP	TM-18	AALNRRIQLLEEDLE	SCP-18	VQKHCQGKKYGDFPG	MLC-18	MLLNMFAERQTGESD	TpC-18	AELREAFRIYDKEGN
AK-19	VGFKQTDKHPNKDFG	TM-19	RIQLLEEDLERSEER	SCP-19	QGKKYGDFPGAFKVF	MLC-19	FAERQTGESDDDDVV	TpC-19	AFRIYDKEGNGFITT
AK-20	TDKHPNKDFGDVNSF	TM-20	EEDLERSEERLNTAT	SCP-20	GDFPGAFKVFIANQF	MLC-20	TGESDDDDVVAKAFL	TpC-20	DKEGNGFITTDVLKE
AK-21	NKDFGDVNSFVNVDP	TM-21	RSEERLNTATTKLAE	SCP-21	AFKVFIANQFKAIDV	MLC-21	DDDVVAKAFLAFADE	TpC-21	GFITTDVLKEILAEL
AK-22	DVNSFVNVDPEGKFV	TM-22	LNTATTKLAEASQAA	SCP-22	IANQFKAIDVNGDGK	MLC-22	AKAFLAFADEEGNID	TpC-22	DVLKEILAELDPRLT
AK-23	VNVDPEGKFVISTRV	TM-23	TKLAEASQAADESER	SCP-23	KAIDVNGDGKVGLDE	MLC-23	AFADEEGNIDCDTFR	TpC-23	ILAELDPRLTPADLE
AK-24	EGKFVISTRVRCGRS	TM-24	ASQAADESERMRKVL	SCP-24	NGDGKVGLDEYRLDC	MLC-24	EGNIDCDTFRHALMT	TpC-24	DPRLTPADLENIIEE
AK-25	ISTRVRCGRSMQGYP	TM-25	DESERMRKVLENRSL	SCP-25	VGLDEYRLDCITRSA	MLC-25	CDTFRHALMTWGDKF	TpC-25	PADLENIIEEVDEDG
AK-26	RCGRSMQGYPFNPCL	TM-26	MRKVLENRSLSDEER	SCP-26	YRLDCITRSAFAEVK	MLC-26	HALMTWGDKFSSQEA	TpC-26	NIIEEVDEDGSGTLD
AK-27	MQGYPFNPCLTESQY	TM-27	ENRSLSDEERMDALE	SCP-27	ITRSAFAEVKEIDDA	MLC-27	WGDKFSSQEADDALD	TpC-27	VDEDGSGTLDFDEFM
AK-28	FNPCLTESQYKEMEA	TM-28	SDEERMDALENQLKE	SCP-28	FAEVKEIDDAYNKLT	MLC-28	SSQEADDALDQMDID	TpC-28	SGTLDFDEFMEMMNG
AK-29	TESQYKEMEAKVSST	TM-29	MDALENQLKEARFLA	SCP-29	EIDDAYNKLTTEDDR	MLC-29	DDALDQMDIDDGGKI		
AK-30	KEMEAKVSSTLSSLE	TM-30	NQLKEARFLAEEADR	SCP-30	YNKLTTEDDRKAGGL	MLC-30	QMDIDDGGKIDVQGV		
AK-31	KVSSTLSSLEGELKG	TM-31	ARFLAEEADRKYDEV	SCP-31	TEDDRKAGGLTLERY	MLC-31	DGGKIDVQGVIQMLT		
AK-32	LSSLEGELKGTYYPL	TM-32	EEADRKYDEVARKLA	SCP-32	KAGGLTLERYQDLYA	MLC-32	DVQGVIQMLTAGGGD		
AK-33	GELKGTYYPLTGMSK	TM-33	KYDEVARKLAMVEAD	SCP-33	TLERYQDLYAQFISN	MLC-33	IQMLTAGGGDDAAAE		
AK-34	TYYPLTGMSKEVQQK	TM-34	ARKLAMVEADLERAE	SCP-34	QDLYAQFISNPDESC	MLC-34	AGGGDDAAAEEA		
AK-35	TGMSKEVQQKLIDDH	TM-35	MVEADLERAEERAET	SCP-35	QFISNPDESCSACYL				
AK-36	EVQQKLIDDHFLFKE	TM-36	LERAEERAETGESKI	SCP-36	PDESCSACYLFGPLK				
AK-37	LIDDHFLFKEGDRFL	TM-37	ERAETGESKIVELEE	SCP-37	SACYLFGPLKVVQ				
AK-38	FLFKEGDRFLQAANA	TM-38	GESKIVELEEELRVV						

AK-39	GDRFLQAANACRYWP	TM-39	VELEEELRVVGNNLK
AK-40	QAANACRYWPAGRGI	TM-40	ELRVVGNNLKSLEVS
AK-41	CRYWPAGRGIYHNDN	TM-41	GNNLKSLEVSEEKAN
AK-42	AGRGIYHNDNKTFLV	TM-42	SLEVSEEKANQREEA
AK-43	YHNDNKTFLVWVNEE	TM-43	EEKANQREEAYKEQI
AK-44	KTFLVWVNEEDHLRI	TM-44	QREEAYKEQIKTLTN
AK-45	WVNEEDHLRIISMQM	TM-45	YKEQIKTLTNKLKAA
AK-46	DHLRIISMQMGGDLG	TM-46	KTLTNKLKAAEARAE
AK-47	ISMQMGGDLGQVFRR	TM-47	KLKAAEARAEFAERS
AK-48	GGDLGQVFRRLTSAV	TM-48	EARAEFAERSVQKLQ
AK-49	QVFRRLTSAVNEIEK	TM-49	FAERSVQKLQKEVDR
AK-50	LTSAVNEIEKRIPFS	TM-50	VQKLQKEVDRLEDEL
AK-51	NEIEKRIPFSHHDRL	TM-51	KEVDRLEDELVNEKE
AK-52	RIPFSHHDRLGFLTF	TM-52	LEDELVNEKEKYKSI
AK-53	HHDRLGFLTFCPTNL	TM-53	VNEKEKYKSITDELD
AK-54	GFLTFCPTNLGTTVR	TM-54	KYKSITDELDQTFSE
AK-55	CPTNLGTTVRASVHI	TM-55	TDELDQTFSELSGY
AK-56	GTTVRASVHIKLPKL		
AK-57	ASVHIKLPKLAANRE		
AK-58	KLPKLAANREKLEEV		
AK-59	AANREKLEEVAGKYN		
AK-60	KLEEVAGKYNLQVRG		
AK-61	AGKYNLQVRGTRGEH		
AK-62	LQVRGTRGEHTEAEG		
AK-63	TRGEHTEAEGGIYDI		
AK-64	TEAEGGIYDISNKRR		
AK-65	GIYDISNKRRMGLTE		
AK-66	SNKRRMGLTEFQAVK		
AK-67	MGLTEFQAVKEMQDG		
AK-68	FQAVKEMQDGILELI		
AK-69	EMQDGILELIKIEKE		

AK, arginine kinase; *TM*, tropomyosin; *SCP*, sarcoplasmic calcium-binding protein; *MLC*, myosin light chain; *TpC*, troponin C; *Pp #*, peptide number.

TABLE E2. Detailed description of the subjects recruited

	Subject	Gender	Age (y)	Country	Symptoms	DBPCFC	Shrimp	DP	Cockroach
Group 1	1	M	10	USA	U, A	ND	49.5	9.5	12
	2	M	17	USA	AE, C, A	ND	52.1	9.7	12
	3	M	13	USA	Asth, AE	+	82.4	22.4	32
	4	F	12	USA	C, Asth	+	74.2	12.1	0.4
	5	F	3	USA	U, A	ND	100	>100	74
	6	M	8	USA	GI, RC, U	+	100	23	30
	7	F	8	USA	AE, U, A	ND	30	7	42
	8	F	38	USA	AE, U	+	>100	>100	17
	9	M	34	USA	AE, U, RC	+	>100	>100	<0.35
	10	M	16	USA	A	ND	>100	>100	8.9
	11	M	33	USA	A	ND	65	40.3	22.6
	12	F	22	USA	AE, RC	+	47.83	59.4	12.1
	13	F	40	USA	AE, RC, A	ND	14	8.96	3.2
	14	F	34	USA	A	ND	1.07	1.15	2.4
	15	M	30	USA	AE, U	+	15	24	35
	16	M	40	USA	AE, U, A	+	30.3	NA	NA
	17	M	6	USA	A	ND	>100	9.3	NA
	18	M	19	USA	U, AE, A, GI	+	8	0.5	0.5
	19	M	5	USA	AE	+	>100	27	33
	20	M	22	USA	OAS, GI	+	78	>100	37
	21	M	12	USA	U	+	>100	>100	NA
	22	F	34	USA	AE, C, A	ND	21	3.8	1.7
	23	F	10	USA	U, C, A	ND	>100	51	NA
	24	F	16	USA	AE, U, A	ND	21	7	3
	25	M	15	USA	A	ND	13	<0.35	<0.35
	26	M	9	USA	A	ND	>100	25	NA
	27	F	9	USA	U, AE, GI	+	96.8	24	NA
	28	F	33	Sp	A, Asth	ND	100	46	46.2
	29	M	8	Sp	A	ND	100	14.1	25.3
	30	F	7	Sp	A	ND	100	11.1	18.1
	31	F	15	Sp	U, RC	+	96.7	18.7	30.3
	32	M	5	Sp	U	+	50.4	10.3	14.8
	33	M	14	Sp	U, A	+	24.2	10.5	10.2
	34	M	3	Sp	A	+	13.7	0.86	1.44
	35	M	9	Sp	U, AE	+	11.7	5.75	5.73
	36	F	29	Sp	A, Asth	ND	7.03	NA	NA
	37	M	33	Sp	A	ND	6.15	2.27	1.88
	38	F	27	Sp	A	ND	5.6	<0.35	<0.35
	39	M	9	Sp	U, A, GI	+	5.5	2.15	1.02
	40	F	16	Sp	GI	+	5.24	26.7	2.27
	41	M	33	Sp	OAS, GI	+	2.72	13.9	1.35
	42	M	48	Sp	OAS, GI	+	2.47	0.82	2.3
	43	F	12	Sp	U, Asth	+	5.9	1.56	0.99
	44	M	8	Sp	AE	+	14.4	1.94	3.04
	45	F	27	Br	U, AE	+	58.6	22.6	0.48
	46	F	37	Br	AE, C, Asth	+	52.4	NA(SPT+)	NA(SPT−)
	47	F	16	Br	U	+	0.6	NA(SPT+)	NA(SPT−)
	48	M	39	Br	U	+	0.81	NA(SPT−)	NA(SPT−)
	49	M	17	Br	A	+	>100	34.5	<0.35
	50	F	25	Br	A	+	2.02	3.79	<0.35
	51	F	17	Br	AE, U	+	0.92	NA(SPT+)	NA(SPT−)
	52	F	12	Br	A	+	4.66	67.8	0.82
	53	F	18	Br	A	+	41.8	24.6	<0.35
	54	F	39	Br	U, GI	+	0.74	73.9	<0.35

(continued)

TABLE E2. *(Continued)*

	Subject	Gender	Age (y)	Country	Symptoms	DBPCFC	Shrimp	DP	Cockroach
	55	M	18	Br	OAS, AE	+	5.53	8.32	<0.35(SPT+)
	56	F	28	Br	AE, U	+	<0.35(SPT+)	27.2	<0.35
	57	F	50	Br	AE, C	+	15.1	8.97	<0.35
	58	F	52	Br	U	+	0.75	44.3	NA(SPT+)
Group 2a	1	M	10	Br	U	-	0.37	14.1	NA(SPT−)
	2	F	20	Br	U	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT−)
	3	F	34	Br	OAS, C	-	< 0.35(SPT+)	2.24	<0.35
	4	F	28	Br	U	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT−)
	5	F	45	Br	OAS	-	0.44	4.05	<0.35
	6	M	15	Br	AE	-	< 0.35(SPT+)	NA(SPT+)	NA(SPT−)
	7	F	29	Sp	U	-	0.44	0.75	<0.35
	8	M	31	Sp	U, AE, A	-	0.62	0.46	2.76
	9	F	18	Sp	OAS	-	0.55	1.26	<0.35
	10	F	41	Sp	RC	-	0.48	9.75	<0.35
	11	M	22	Sp	OAS	-	2.32	4.13	2.37
	12	M	31	Sp	U	-	5.58	12.2	3.19
	13	F	29	Sp	U	-	1.45	60.2	1.24
	14	M	7	Sp	GI	-	0.6	<0.35	NA
	15	M	7	Sp	RC	-	16.5	2	3.56
	16	M	5	Sp	U, GI	-	0.81	0.68	0.79
Group 2b	1	F	32	Br	-	-	<0.35(SPT+)	80.5	<0.35(SPT+)
	2	F	21	Br	-	-	1.54	57.7	<0.35(SPT+)
	3	M	13	Br	-	-	1.19	50.4	<0.35(SPT+)
	4	M	19	Br	-	-	1.53	3.26	0.95
	5	F	47	Br	-	-	2.45	22.9	0.88
	6	M	40	Br	-	-	<0.35(SPT+)	>100	<0.35
	7	F	31	Br	-	-	1.09	>100	<0.35
	8	F	47	Br	-	-	<0.35(SPT+)	1.89	<0.35
	9	M	25	Br	-	-	1.98	>100	<0.35
	10	M	27	Sp	-	-	5.7	NA(SPT+)	0.47
	11	M	15	Sp	-	-	2.7	8.56	1.29
	12	F	45	Sp	-	-	<0.35(SPT+)	NA(SPT+)	NA
Control group 1 (GC1)	1	F	28	Sp	-	-	<0.35(SPT−)	2.36	<0.35
	2	F	52	USA	-	-	<0.35(SPT−)	0.41	<0.35
	3	F	60	USA	-	-	<0.35(SPT−)	4.3	<0.35
	4	M	42	Br	-	-	<0.35(SPT−)	<0.35 (SPT+)	<0.35
	5	F	25	Br	-	-	<0.35(SPT−)	>100	0.53
	6	F	17	Br	-	-	<0.35(SPT−)	2.22	<0.35
	7	F	39	Br	-	-	<0.35(SPT−)	12.4	<0.35
	8	F	37	Br	-	-	<0.35(SPT−)	2.71	<0.35
	9	F	17	Br	-	-	<0.35(SPT−)	13.8	<0.35
	10	M	37	Br	-	-	<0.35(SPT−)	>100	<0.35(SPT+)
	11	F	16	Br	-	-	<0.35(SPT−)	21.9	<0.35
	12	F	25	Br	-	-	<0.35(SPT−)	52.6	<0.35
Control group 2 (GC2)	1	F	36	USA	-	-	<0.35(SPT−)	<0.35(SPT−)	<0.35(SPT−)
	2	M	40	USA	-	-	<0.35(SPT−)	<0.35(SPT−)	<0.35(SPT−)
	3	F	24	USA	-	-	<0.35(SPT−)	<0.35(SPT−)	<0.35(SPT−)
	4	F	60	USA	-	-	<0.35(SPT−)	<0.35(SPT−)	<0.35(SPT−)
	5	M	30	USA	-	-	<0.35(SPT−)	<0.35(SPT−)	<0.35(SPT−)

Gender: *M*, male; *F*, female. Symptoms of allergic reactions to shrimp: *AE*, angioedema; *A*, anaphylaxis; *Asth*, asthma; *C*, cough; *GI*, gastrointestinal disorders; *OAS*, oral allergy syndrome; *RC*, rhinoconjunctivitis; *U*, urticaria. *DBPCFC*, double-blind placebo-controlled food challenge: "+", positive; "-", negative. Shrimp, *Dermatophagoides pteronyssinus* (DP), and cockroach-specific IgE levels expressed as kU_A/L; *NA*, not available; *SPT*, skin prick test; *ND*, not done (previous anaphylaxis).

TABLE E3. IgE reactivity to recombinant allergens per group and statistically significant differences in the frequency of recognition

Allergen recognition	Group 1 n = 58	Group 2a n = 16	Group 2b n = 12	Control G1 n = 12	Control G2 n = 5
Absolute number of allergens recognized*	3 (0-9)	1.5 (0-5) $P < .05$	2 (0-3) ns	1.5 (0-2) $P < .01$	0
Frequency of recognition					
SCP-alpha	17 (29.3%)	1 (6.3%)	-	-	-
SCP-L-beta	20 (34.5%)	1 (6.3%)	-	-	-
TM	48 (82.8%)	7(43.8%)	1 (8.3%)	-	-
Hemo	17 (29.3%)	3 (18.8%)	8 (66.7%)	7 (58.3%)	-
FABP	6 (10.3%)	-	1 (8.3%)	-	-
MLC	22 (37.9%)	4 (25.0%)	-	-	-
AK	28 (48.3%)	7 (43.8%)	9 (75.0%)	7 (58.3%)	-
TpC-GC	10 (17.2%)	3 (18.8%)	1 (8.3%)	-	-
TpC-L1	9 (15.5%)	2 (12.5%)	-	-	-
TpC-L2	7 (12.1%)	2 (12.5%)	-	-	-
None	3 (5.2%)	4 (25%)	2 (16.7%)	4 (33.3%)	5 (100%)

Comparisons	Proteins with statistically significant difference	*P* value
G1 vs G2a	SCP-L-beta	.0298
	SCP-alpha and/or -L-beta (SCPs)	.0298
	TM	.0032
G1 vs G2b	SCP-alpha	.031
	SCP-L-beta	.0145
	TM	<.0001
	Hemo	.0209
	MLC	.0133
G1 vs (G2a+G2b)	SCP-alpha	.0049
	SCP-L-beta	.0012
	TM	<.0001
	MLC	.0271
	SCP-alpha	.031
	SCP-L-beta	.0145
	TM	<.0001
	MLC	.0133
G2a vs GC1	TM	.0103
	Hemo	.0497
G2b vs GC1	-	-
G2a vs G2b	Hemo	.0189

SCP, sarcoplasmic calcium-binding protein, -alpha and -beta; *TM*, tropomyosin; *Hemo*, hemocyanin; *FABP*, fatty-acid-binding protein; *MLC*, myosin light chain; *AK*, arginine kinase; *TpC*, troponin C (-L1, -L2, -GC).

The Fisher exact probability test was applied to assess statistically significant differences between the frequencies of IgE reactivity of each group to individual recombinant proteins.

*The median (range) of allergens recognized by each group and *P* values corresponding to the comparison between the median of Group 1 and the median of the other groups.

TABLE E4. Summary of the differential binding sites identified between groups

	G1 vs G2a		G1 vs G2b		G2b vs G2a	
Allergen	**IgE**	**IgG4**	**IgE**	**IgG4**	**IgE**	**IgG4**
AK	15-17	16-17	15-17	16-17	37-38	44-45
	28-29	25-26	28-29	24-26	44-45	
	34-36	29-30	41-42	29-30		
	41-42	39-41	67-68	33-34		
	67-68	44-45		39-42		
		52-53		44-45		
		65-68		52-53		
				65-68		
MLC	24-26	25-26	24-25	25-26	5-6	
SCP	16-18	20-21		20-21		
		26-27		26-27		
		36-37		36-37		
TM	1-3	1-2	1-3	1-2	12-13	
	13-14	12-13	12-13	12-13		
	25-26	33-35	24-25	25-26		
	29-30	54-55	29-30	33-35		
	33-35		33-35	54-55		
	38-39		38-39			
	51-55		54-55			
TpC	-	-	-	-	-	-

SCP, sarcoplasmic calcium-binding protein; *TM*, tropomyosin; *MLC*, myosin light chain; *AK*, arginine kinase; *TpC*, troponin C.
Binding sites identified as significantly higher bindings when comparing groups using TileMap (FDR < 0.05; $P < .01$). Numbers in the table refer to the number of the peptide for each allergen (peptide ID).

TABLE E5. IgE recognition of shrimp extract, individual allergens, and epitopes showing statistically significant differences between groups

Allergen	**Freq. G1 (%) n = 58**	**Freq. G2a (%) n = 16**	***P* value Fischer's test**
SCPs	34.5	6.3	.0298
TM	82.8	18.8	<.0032
TM+SCPs+	31.0	6.6	.0549*
Shrimp extract (f24)	98.3	75.0	.0068
Epitope/binding site ID	**Freq. G1 (%, n = 21)**	**Freq. G2a (%, n = 16)**	***P* value Fischer's test**
AKp15-17	71.4	25.0	.0081
AKp34-36	38.1	0.0	.0056
AKp41-42	47.6	0.0	.0017
AKp67-68	81.0	31.3	.0059
MLCp24-26	71.4	18.8	.0025
SCPp16-18	38.1	6.3	.0501*
TMp1-3	42.9	0.0	.0046
TMp13-14	33.3	0.0	.0124
TMp25-26	38.1	0.0	.0056
TMp29-30	47.6	6.3	.0100
TMp33-35	71.4	31.3	.0220
TMp38-39	38.1	6.3	.0501*
TMp51-55	85.7	25	.0005

Only the shrimp extract (f24, ImmunoCAP), individual allergens (alone or in combination), and their epitopes showing significant differences in the frequency of IgE recognition between allergic (G1) and tolerant (G2) subjects could be considered as candidates for diagnostic tests (Fisher exact probability test, $P < .05$). Frequencies of IgE recognition are expressed as percentages (%). *SCPs*, sarcoplasmic calcium-binding protein (-alpha and -beta); *TM*, tropomyosin; *MLC*, myosin light chain; *AK*, arginine kinase. Epitopes are identified with the abbreviation of the allergen name plus the peptide (p) number/ID.
*trend to significance.

TABLE E6. Comparison of results with previous studies

Allergen	**Peptide #**	**AA #**	**Previously described epitope* (AA #)**
AK	15-17	71-95	3 (64-96)
	28-29	136-155	4a (121-141) 4b (142-159)
	34-36	166-190	-
	41-42	201-220	-
	67-68	331-350	7 (319-342)†
MLC	24-26	116-140	5 (118-141)†
SCP	16-18	76-100	-
TM	1-3	1-25	1 (1-36)†
	13-14	61-80	3 (61-81)
	25-26	121-140	5a (115-150)†
	29-30	141-160	5b (142-162)†
	33-35	161-185	5c (157-183)†
	38-39	186-205	6 (190-210)
	51-55	251-285	7 (246-284)†

"Peptide #" refers to the peptide number/ID in the current study, "AA #" refers to the corresponding amino acid sequence, and the "Described Epitope (AA #)" shows the ID of the epitope previously identified and in brackets the corresponding amino acid sequence for that epitope.
*Epitopes described by Ayuso et al.[29]
†Epitope also found statistically significant different between allergic and tolerant individuals in Ayuso et al.[30]