Allergen Valency, Dose, and FcεRI Occupancy Set Thresholds for Secretory Responses to Pen a 1 and Motivate Design of Hypoallergens

Avanika Mahajan,* Lama A. Youssef,[†,‡] Cédric Cleyrat,* Rachel Grattan,* Shayna R. Lucero,* Christopher P. Mattison,[§] M. Frank Erasmus,* Bruna Jacobson,[¶] Lydia Tapia,[¶] William S. Hlavacek,[‖,#] Mark Schuyler, and Bridget S. Wilson***

Ag-mediated crosslinking of IgE–FcεRI complexes activates mast cells and basophils, initiating the allergic response. Of 34 donors recruited having self-reported shrimp allergy, only 35% had significant levels of shrimp-specific IgE in serum and measurable basophil secretory responses to rPen a 1 (shrimp tropomyosin). We report that degranulation is linked to the number of FcεRI occupied with allergen-specific IgE, as well as the dose and valency of Pen a 1. Using clustered regularly interspaced palindromic repeat–based gene editing, human RBL$^{r\alpha KO}$ cells were created that exclusively express the human FcεRIα subunit. Pen a 1–specific IgE was affinity purified from shrimp-positive plasma. Cells primed with a range of Pen a 1–specific IgE and challenged with Pen a 1 showed a bell-shaped dose response for secretion, with optimal Pen a 1 doses of 0.1–10 ng/ml. Mathematical modeling provided estimates of receptor aggregation kinetics based on FcεRI occupancy with IgE and allergen dose. Maximal degranulation was elicited when ∼2700 IgE–FcεRI complexes were occupied with specific IgE and challenged with Pen a 1 (IgE epitope valency of ≥8), although measurable responses were achieved when only a few hundred FcεRI were occupied. Prolonged periods of pepsin-mediated Pen a 1 proteolysis, which simulates gastric digestion, were required to diminish secretory responses. Recombinant fragments (60–79 aa), which together span the entire length of tropomyosin, were weak secretagogues. These fragments have reduced dimerization capacity, compete with intact Pen a 1 for binding to IgE–FcεRI complexes, and represent a starting point for the design of promising hypoallergens for immunotherapy. ***The Journal of Immunology*****, 2017, 198: 1034–1046.**

Food allergies are IgE-mediated immunological reactions that affect up to 10% of the human population (1). Increased consumption of seafood, particularly shrimp, during the past few decades has led to increased incidences of allergic reactions. More than 6 million Americans suffer from seafood allergies (2). Ingestion of shellfish can cause a potentially life-threatening anaphylactic reaction and poses a growing worldwide health problem (3). The major allergen in shrimp has been identified as the muscle protein tropomyosin, a coiled-coiled dimer composed of two identical α helices of 33 kDa each (4). Pen a 1, the tropomyosin of brown shrimp (*Penaeus aztecus*), is one of the most common shrimp allergens and is reactive with circulating IgE from >80% of shrimp-allergic patients (5). Tropomyosin is not only a major allergen in crustacean species (e.g., crabs and lobsters) (6, 7), but also in mollusks (e.g., squid and snails) (8), house dust mites (9), and cockroaches (10). The concept of tropomyosin as a pan-reactive invertebrate allergen is strongly supported by studies describing shrimp crossreactive IgE in the serum of individuals strictly following a kosher diet that restricts shrimp (11).

Previous studies have identified five major IgE binding regions within Pen a 1, which bear approximately eight epitopes based on analysis of synthetic overlapping peptides using the SPOTS technique (12). These studies provided critical insight into shrimp allergy, through characterization of IgE binding to Pen a 1–derived peptides as well as early evaluation on their ability to modulate the activation of mast cells (13, 14). As with other allergic reactions, activation of mast cells and basophils occurs when a multivalent allergen crosslinks specific IgE that is tightly bound to FcεRI on the cell surface (15, 16).

Allergen-specific immunotherapies (allergen immunotherapy, specific immunotherapy) have been used for more than a century (17) to alter adaptive immune responses, with the best successes in aeroallergens and insect hypersensitivity (18). Present guidelines reflect the standards of practice in allergy clinics, including the common use of stock extracts that contain whole allergens

*Department of Pathology, University of New Mexico, Albuquerque, NM 87131; [†]Department of Pharmaceutics and Pharmaceutical Technology, School of Pharmacy, Damascus University, Damascus, Syria; [‡]National Commission for Biotechnology, Damascus, Syria; [§]Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, LA 70124; [¶]Department of Computer Sciences, University of New Mexico, Albuquerque, NM 87131; [‖]Theoretical Biology and Biophysics Group, Theoretical Division, Los Alamos National Laboratory, Los Alamos, NM 87545; [#]Theoretical Biology and Biophysics Group, Center for Nonlinear Studies, Los Alamos National Laboratory, Los Alamos, NM 87545; and **Department of Medicine, University of New Mexico, Albuquerque, NM 87131

ORCIDs: 0000-0001-8013-7856 (L.A.Y.); 0000-0002-1928-6497 (C.C.); 0000-0003-1204-6615 (R.G.); 0000-0002-8046-5048 (M.F.E.); 0000-0002-7822-7091 (L.T.); 0000-0003-4383-8711 (W.S.H.); 0000-0002-3775-4450 (B.S.W.).

Received for publication August 2, 2016. Accepted for publication November 30, 2016.

This work was supported by National Institutes of Health Grant P50GM085273 (to B.S.W.) and National Science Foundation Career Award III-1553266 (to L.T.). This work was also supported in part by funds from the U.S. Department of Agriculture, Agricultural Research Service (to C.P.M.).

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Address correspondence and reprint requests to Dr. Bridget S. Wilson, University of New Mexico, Cancer Research Facility, 205A, 2325 Camino de Salud NE, Albuquerque, NM 87131-5301. E-mail address: bwilson@salud.unm.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: CD, circular dichroism; CRISPR, clustered regularly interspaced palindromic repeat; gRNA, guide RNA; h, human; hIgE$^{Alexa\text{-}488}$, Alexa Fluor 488–conjugated hIgE; IgE$^{Pen a1}$, Pen a 1–specific IgE; m, mouse; SGF, simulated gastric fluid.

(19, 20). In addition to commercial extracts, there is an increased movement toward development of recombinant proteins that will offer better standardization (21). However, the potential risk of adverse events, for both s.c. administration (s.c. immunotherapy) and newer sublingual (sublingual immunotherapy) delivery, has driven research toward alternative approaches to traditional immunotherapy (17, 22). Promising new results have suggested that early exposure of infants to peanuts and peanut products can lead to long-term tolerance (23, 24). Oral food challenges are commonly practiced in allergy clinics (25), which are time-intensive and clinically supervised procedures. There is a critical need for additional knowledge about the properties of food allergens (26, 27) leading to the design of safe and effective diagnostic tools and immunotherapies.

The present strategies for the development of hypoallergens for immunotherapy include genetically engineered allergens or chemically fixed allergoids that reduce or abolish IgE reactivity while preserving the T cell reactivity for the modulation of an immune response (22). One limitation of these approaches is that hypoallergens that lack IgE-binding epitopes altogether may not elicit desirable IgG production capable of blocking IgE binding to allergens. We propose that rationally designed hypoallergens can be created by carefully integrating the properties of allergen valency and structure, as they relate specifically to the crosslinking of IgE–FcεRI complexes on the surface of mast cells and basophils. Our goal is to create a formulation that retains the full spectrum of IgE-binding and T cell epitopes while avoiding FcεRI crosslinking conditions that trigger the release of inflammatory mediators. The design of such hypoallergens requires detailed insight regarding the allergen-mediated aggregation of the FcεRI, including the number and orientation of the ITAMs (28) in the FcεRI γ subunits within allergen–IgE–FcεRI complexes and the formation of activation-competent signaling clusters in the plasma membrane (29). To retain the potential for the immunotherapy formulation to shift the Th1/Th2 paradigm to the recruitment of regulatory T cells, researchers should also consider the available information about T cell epitopes for specific allergens (30).

As proof of principle for the improved design of hypoallergens based on structural limitations for activating the FcεRI, we began with characterization of Pen a 1–mediated responses in human basophils from allergic subjects. We recruited shrimp-allergic subjects to evaluate the range of responses based on IgE reactivity against shrimp extract, as well as basophil histamine release. Using a clustered regularly interspaced palindromic repeat (CRISPR)–engineered RBL cell line (human [h]RBL$^{r\alpha KO}$) and a rule-based theoretical model, we characterized the relationship between Pen a 1–specific IgE dose, the number of FcεRI occupied, and Pen a 1 dose-dependent mast cell degranulation. Five overlapping recombinant fragments spanning the sequence of Pen a 1 were generated to characterize their allergenic/degranulation activity. We show that although recombinant Pen a 1 fragments are IgE reactive and are likely a mix of monomers and dimers, the resulting FcεRI aggregates have reduced potency for cell activation. We discuss the potential for these hypoallergenic fragments to form the basis of safe and effective immunotherapy.

Materials and Methods

Cell isolation and histamine release assays

Histamine release was measured as described previously (31, 32). Briefly, Percoll gradient centrifugation was used to prepare basophil-enriched cell populations (1–55% basophils) from 54 ml of anticoagulated blood. Enriched cell fractions were activated by incubating with anti-IgE Ab, Pen a 1 full-length peptides, or digested fragments at 37˚C. Spontaneous degranulation was measured by adding HBSS to the cells. Calcium ionophore A23187 was used as an internal positive control. Cells were activated for 30 min and reactions were terminated by addition of excess ice-cold buffer. After centrifugation, histamine in cell supernatants was measured by ELISA (Genway Biotech) according to the manufacturer's instructions. Total histamine was measured in supernatants generated by lysis of identical cell aliquots using three freeze-thaw cycles. The net FcεRI-mediated histamine released in response to ligand was expressed as a percentage of total histamine release after subtraction of spontaneous release.

Study subjects

Individuals with shrimp allergy were recruited based on self-reporting. Shrimp allergy was confirmed by an ImmunoCAP assay performed at TriCore Reference Laboratories (Albuquerque, NM), which determines IgE reactivity with crude shrimp extract. Values of >0.35 kU/l were considered positive. Subjects that were positive for shrimp allergy, as well as control subjects, were clinically assessed. Total IgE levels were measured by the ImmuneTech Reference Laboratory (Foster City, CA). As a source of standardized Pen a 1–specific IgE, serum of shrimp-allergic individuals was purchased from PlasmaLab International (Everett, WA). Serum samples were stored at −20˚C.

Purification of Pen a 1–specific IgE

Pen a 1–specific IgE (IgE^{Pena1}) was purified on an affinity column, prepared using full-length rPen a 1 according to the manufacturer's instructions (Abcam, Cambridge, MA). IgG was removed by incubating elution fractions with protein A/G beads (Invitrogen, Grand Island, NY) for 2 h. Absence of IgG in fractions was confirmed by immunoblotting with anti-IgG HRP (Invitrogen).

CRISPR-edited human RBL$^{r\alpha KO}$ cells

Cas9-mediated DNA cleavage was used to knock out both alleles encoding endogenous rat α subunit in RBL-2H3 cells. A highly specific guide RNA (gRNA), directed against the first exon of the rat FcεRIα genomic sequence, was designed using the CRISPR Design portal (http://crispr.mit.edu/) and then subcloned into the PX458 vector (Addgene, plasmid no. 48138) for simultaneous expression of the gRNA, wild-type Cas9, and a GFP reporter. For subcloning, two partially complementary oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA) and assembled by PCR. The sequences were as follows: rat FcεRIα knockout gRNA, forward, 5′-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGAGTGTCCTTGGACCCACCG-3′, reverse, 5′-GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGGTGGGTCCAAGGACACTC-3′.

Gel-purified PCR products were cloned into BbsI-digested PX458 using Gibson Assembly (New England BioLabs, Ipswich, MA) following the manufacturer's specifications. The engineered plasmid was used to transfect RBL-2H3 cells using the Amaxa system (Lonza, Basel, Switzerland). Positive, GFP-expressing cells were selected by flow cytometry using an iCyt cell sorter. After transient GFP expression was cleared, transfected cells were primed overnight with mouse (m)IgE labeled with Pacific Blue 410 at 1 μg/ml. GFP-negative and Pacific Blue 410–labeled mIgE-negative cells were selected by flow cytometry and named hRBL$^{r\alpha KO}$. These cells were subsequently transfected with a vector containing the cDNA coding for hFcεRIα and geneticin resistance. Stably transfected cells flow sorted after overnight labeling with Alexa Fluor 488–conjugated hIgE (hIgE$^{Alexa\text{-}488}$) at 1 μg/ml and positive cells were named hRBL$^{r\alpha KO}$.

Evaluation of IgE binding

hRBL$^{r\alpha KO}$ cells were primed with hIgE$^{Alexa\text{-}488}$ as indicated in the figure legends. Cells were then harvested, washed twice to remove unbound IgE, and cell-associated fluorescence was measured in a BD LSRFortessa flow cytometer (BD Biosciences). To estimate the association rate constants, suspension cultures of hRBL$^{r\alpha KO}$ or RBL-2H3 cells (5×10^5 cells in 400 μl of medium) were incubated with hIgE$^{Alexa\text{-}488}$ or mIgE at doses ranging from 60 to 20,000 μg/ml for 30 and 60 min, followed by quantification of binding by flow cytometry. To measure the rate of hIgE dissociation from chimeric FcεRI, suspension cells (5×10^5 cells in 400 μl of medium per well) were primed with 4 μg/ml hIgE$^{Alexa\text{-}488}$ for 2 d. After washing, resuspended cells were incubated with 10 μg/ml unlabeled IgE for defined intervals and loss of labeled IgE was monitored by flow cytometry. For quantification of bound hIgE under priming conditions used for degranulation assays, hRBL$^{r\alpha KO}$ cells were primed for 2 h with hIgE$^{Alexa\text{-}488}$ as described in the figure legends. The number of FcεRI bound to IgE on the cell surface was determined based on calibration using Quantum MESF

standard beads (Bangs Laboratories, Fishers, IN) and measured fluorescein/protein ratios for each batch of $IgE^{Alexa-488}$.

Model for IgE–FcεRI binding

Interaction of IgE with FcεRI is consistent with a simple reaction scheme (33, 34): L + R = B, where L represents free IgE, R represents free FcεRI, and B represents the IgE–FcεRI complex. Binding is not diffusion controlled (35), and internalization/turnover of the IgE–FcεRI complex is minimal (36). Under assumptions of mass-action kinetics and conservation of IgE and FcεRI (over time scales of interest), the time-dependence of IgE–FcεRI abundance is governed by the following equation:

where $\alpha = 2(k_f L_0 R_0 - k_r B_0)$, $\beta = k_f(L_0 + R_0) + k_r$, and $\gamma = [k_f^2(L_0 - R_0)^2 + 2k_f k_r(L_0 + R_0 + 2B_0) + k_r^2]^{1/2}$. Where, B_0, L_0, and R_0 represent the abundances of IgE–FcεRI complex, free IgE, and free FcεRI at time $t = 0$; $B(t)$ is the time-dependent abundance of IgE–FcεRI; k_f is the forward rate constant for IgE binding to FcεRI; and k_r is the reverse rate constant. The equilibrium abundance of IgE–FcεRI complex,

In experiments where the amount of unlabeled IgE in solution is in large excess of labeled IgE on cells, such that rebinding of dissociated labeled IgE is negligible, decay of labeled receptor-bound IgE is governed by the following equation, which is a special case of the equation given above: $B(t) = B_0 \exp(-k_r t)$. Parameters were estimated using nonlinear least squares fitting (37). Confidence limits on estimates were determined using bootstrapping (38).

From flow cytometric measurements of decay of labeled cell-surface hIgE in the presence of excess unlabeled hIgE in solution, we estimated that $B_0 = 1.27 \times 10^5$ copies per cell and $k_r = 1.53 \times 10^{-5}$ s^{-1}. The 68% confidence limits on these estimates are (1.26, 1.28) $\times 10^5$ copies per cell and (1.43, 1.68) $\times 10^{-5}$ s^{-1}.

To characterize the kinetics of hIgE association with hFcεRI, we set k_r at our estimated value of 1.53×10^{-5} s^{-1}. For mIgE interaction with hFcεRI, we set k_r at ρ_{MH} (1.53×10^{-5} s^{-1}) = 2.20×10^{-5} s^{-1}, where ρ_{MH} = 9.89/6.87 is a ratio of dissociation rate constants measured by Ishizaka et al. (39) for mIgE and hIgE binding to hFcεRI. For mIgE interaction with rat FcεRI, we set k_r at ρ_{RH} (1.53×10^{-5} s^{-1}) = 2.20×10^{-5} s^{-1}, where ρ_{RH} = 2.73/6.87 is the ratio of dissociation rate constants measured by Sterk and Ishizaka (40) and Ishizaka et al. (39) for mIgE binding to rat FcεRI and hFcεRI. Our parameter estimates are insensitive to the particular fixed values chosen for dissociation rate constants, provided that these values are sufficiently small (i.e., such that the bond lifetime $1/k_r$ is much longer than the duration of the experiment).

Theoretical modeling of allergen-mediated FcεRI aggregation

In our rule-based model, IgE-bound receptors have a valency of 2 and dimerized Pen a 1 molecules have 10 binding sites. We simplify the model by assuming that all binding sites have the same affinity. Receptor–Ag complexes are limited to 20 receptors per aggregate, as slow diffusion of large complexes and constraints on accessibility are expected to constrain aggregate size. All dissociation events have the same off-rate ($k_{off} = 0.01$ s^{-1}). Unbound Ag is assumed to diffuse over the entire three-dimensional volume of the well; the on-rate for receptor binding (k_{on}) is given by the off-rate (k_{off}) divided by the dissociation constant (K_D). However, if the Ag is already bound to one or more receptors on the cell surface, its on-rate is effectively higher, as there is a reduction of the number of df of the search space for binding events. In our model, the crosslinking on-rate is given by k_{on} times the crosslinking factor α, a dimensionless constant. Rules also reflect overall concentration of Ag (i.e., limiting or in excess as compared with the number of receptors). We simulated the entire cell in BioNetGen, with the number of receptors and Pen a 1 molecules derived experimentally; the volume of extracellular fluid is set at 2.3×10^{-6} ml. The molecular mass of Pen a 1 is 72,000 g/mol. For each IgE priming condition, we have 2692 molecules of IgE (120 ng/ml), 1211 molecules of IgE (60 ng/ml), 716 molecules of IgE (30 ng/ml), and 324 molecules of IgE (15 ng/ml). Pen a 1 concentration is varied logarithmically, from 10^{-4} to 10^4 ng/ml, for a total of nine data points. We run our model with BioNetFit (41), an optimization algorithm that couples BioNetGen rule–based model computations and a genetic algorithm that finds the values of free parameters that give a best fit for the output of the simulation as compared with experimental results. The free parameters that are optimized during fitting are the dissociation constant (K_D = off-rate/on-rate) and a crosslinking factor α. These parameters are varied randomly within constraints. The K_D is varied between 10^{-10} and 10^{-8} M whereas α is varied between 1 and 5000. For fitting, we make the simple assumption (42) that secretion percentage is directly proportional to the number of receptors in aggregates, thus directly comparing simulation output (number of receptors in aggregates) to experimental degranulation data. This is a first approximation of the relationship between aggregation and secretion because the two events are separated by a series of subsequent intracellular events, implicating a more complex relationship. Thus, to test the hypothesis that the two distinct observables can indeed be compared in this manner, we perform training and testing on the data. First, we determine the free parameters of the model for the IgE concentration of 120 ng/ml using BioNetFit. Then, we use these same parameters for simulations of the same model under different IgE concentrations (60, 30, and 15 ng/ml) and compare the number of receptors in aggregates to the percentage secretion in each case. The secretion percentage data for [IgE] = 120 ng/ml is normalized to the highest secretion value, resulting in a set of values between 0 and 1. This dataset is the input file used in BioNetFit. The simulation output that is compared with the normalized secretion is the number of receptors in aggregates divided by total number of receptors. BioNetGen runs are stochastic simulations performed with NFsim (42). During the fit, data were collected in 18-s intervals and reported as the overall fraction of receptors in aggregates at 3 min. After the fit, constants were used in BioNetGen with NFsim to compare the total number of receptors in aggregates to the percentage secretion for each IgE concentration. The number of receptors in aggregates was estimated from kinetics by calculating the area under the curve from 0 to 3 min.

Electron microscopy

Methods for preparation of mast cell membrane sheets and immunogold labeling FcεRI have been described (29). Digital images were acquired using a Hitachi H600 transmission electron microscope, followed by image processing to capture FcεRI gold particle distribution and use of published methods for statistical analysis of clustering. Statistical differences were also computed using one-way ANOVA test in MATLAB.

Cloning

Pen a 1 cDNA (GenBank no. DQ151457.1, *Farfantepenaeus aztecus*) was synthesized by Genewiz (South Plainfield, NJ). Five fragments of Pen a 1 were amplified using PCR and cloned into pET101 vector (Invitrogen). Primers were synthesized by Integrated DNA Technologies. All constructs were expanded in One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen), and plasmid DNA was isolated using NucleoSpin plasmid kits (Macherey-Nagel, Duren, Germany). Sequencing was performed using Genewiz. Primers used for amplification are as follows: forward 1, 5′-CACCATGGAC GCCATCAAGAAGAAGATGC-3′; reverse 1, 5′-AGAGAGGGCCTTGTCCTTGTC-3′; forward 2, 5′-CACCATGAACATCCAGCTTGTGGAGAA-3′; reverse 2, 5′-GTTCTCGAGCACCTTGCGCAT-3′; forward 3, 5′-CACCATGAAGGTGCTCGAGAACC-3; reverse 3, 5′-CTCCTCAGCACGCTCAAGGT-3′; forward 4 5′-CACCATGGACCTTGAGCGTG-3′; reverse 4, 5′-CTCAGCCGCCTTCAGCTTGTT-3′; forward 5, 5′-CACCATGCAGATTAAGACACTTACCAACAAG-3′; reverse 5, 5′-GTAGCCAGACAGTTCGCTGAAAGTCT-3′.

Expression and purification of recombinant allergens

All recombinant proteins were expressed in BL21 star(DE3) cells (Invitrogen) induced with 1 mM isopropylthio-β-galactoside for 4 h. Cells were harvested by centrifugation and stored at −80°C overnight. On the next day, pellets were dissolved in native buffer (50 mM sodium phosphate, 0.5 M NaCl, 1 mg/ml lysozyme, protease inhibitors [pH 8]). Cells were sonicated using the Misonix system, and cell debris was removed by centrifugation at 5000 rpm for 15 min. Proteins were extracted and purified using an Ni-NTA purification system (Invitrogen) according to the manufacturer's instructions using native or denaturing buffer conditions. Proteins were dialyzed against PBS (pH 7.4) followed by final purification using size-exclusion chromatography using the SEC-s3000 column (Phenomenex, Torrance, CA) and the Agilent 1100 system. Concentrations were determined by densitometric analysis of SDS-PAGE gels stained with Coomassie brilliant blue, using known amounts of natural shrimp tropomyosin (Indoor Biotechnologies, Charlottesville, VA) as standard.

Circular dichroism spectroscopy

Proteins dialyzed against PBS were adjusted to a concentration of 200 μg/ml. Circular dichroism (CD) spectroscopy was performed with a model 420 AVIV spectropolarimeter (AVIV Biomedical) with a constant nitrogen flushing at 20°C. Proteins were scanned with a spectral range of 185–255 nm, with a step width of 0.2 nm and bandwidth of 1 nm.

Immunoblotting analysis

Proteins were fractionated by SDS-PAGE under reducing conditions using 4–12% gradient precast gels (Invitrogen), and bands were detected by Coomassie staining. For immunoblotting, Pen a 1 and fragments separated

by SDS-PAGE were transferred onto nitrocellulose membranes using the iBlot (Thermo Fischer), blocked with 3% BSA in TBST (0.1% Tween 20 in TBS), and incubated overnight with the pool of patient sera diluted in blocking buffer. Bound IgE was detected using 1:1000 dilution of anti-IgE HRP (Invitrogen). For detection and quantification of purified IgE, elution fractions mixed with sample buffer without DTT were run on SDS-PAGE, transferred onto nitrocellulose membranes, and blocked and detected with anti-IgE HRP as described above. In between incubations, membranes were washed three times with TBST for 5 min each with gentle shaking at room temperature. For dot blots, 0.5 μg of each protein was spotted onto a nitrocellulose membrane, followed by blocking with 5% milk in TBST for 1 h at room temperature. Membranes were incubated overnight with either normal or IgG-depleted patient serum diluted in blocking buffer, then washed with TBST. Bound Ab was detected using a 1:1,000 dilution of anti-IgE HRP or 1:10,000 dilution of anti-IgG HRP. For depletion of IgG, 800 μl of serum was incubated with 2 ml of protein G beads (Thermo Fisher Scientific, Waltham, MA) for 2 h at room temperature. After incubation, beads were spun down and serum was recovered.

ELISA assay for reactivity of IgE in human plasma to Pen a 1

In brief, recombinant Pen a 1 or fragments were coated on the bottom of a high-binding, flat-bottom 96-well plates (Corning, Corning, NY) at 1.25 μg/ml in carbonate-bicarbonate buffer (pH 9.6). Plates were blocked with 5% nonfat dry milk in PBST overnight, followed by incubation with atopic sera at 1:20 dilution for 1 h at room temperature. For IgE detection, wells were thoroughly washed and incubated with polyclonal goat anti-human IgE-HRP (Invitrogen). OD was read at 450 nm following detection with a 1-Step ultra tetramethylbenzidine ELISA substrate (Thermo Fisher Scientific) and 2 M sulfuric acid as stop solution.

Digestion of Pen a 1

Digestion of recombinant tropomyosin was performed using simulated gastric fluid (SGF; 35 mM NaCl, 84 mM HCl [pH 1.2]) conditions as described in Mattison et al. (43). Recombinant tropomyosin (1 μg) was added to SGF containing half-log (3.16-fold) dilutions of porcine pepsin (Sigma-Aldrich, St. Louis, MO) beginning with 40 U of enzyme. Pepsin was diluted in SGF prior to use and was omitted from the 0 time point sample. Reactions were incubated at 37°C for 10 min and then halted by the addition of Tris buffer (pH 8.5) to 100 mM on ice. Samples were frozen and stored at −80°C prior to analysis.

Basophil purification and acid stripping of IgE

Venous blood was collected from normal donors at United Blood Services (Albuquerque, NM). Basophils were isolated and enriched from blood by using HetaSep for RBC depletion and EasySep human basophil enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) for negative selection according to the manufacturer's instructions. Enriched basophils were stripped of bound IgE by incubating with 0.01 M lactate-buffered NaCl, KCl solution (pH 3.9) for 20 s followed by neutralization with Tris buffer. Stripped basophils were primed with media containing shrimp-reactive serum (20%), followed by stimulation and collection of supernatant for histamine release as described above.

Results

Variability in Pen a 1–specific IgE levels and basophil responsiveness in shrimp-allergic subjects

Shrimp-allergic patients were recruited based on self-reporting and clinical history, followed by testing for positive reaction to shrimp extract (Supplemental Fig. 1A). As shown in Fig. 1A, only 12 of the 34 subjects (35%) had clinically detectable levels of shrimp-reactive IgE. Data in Fig. 1B report shrimp-specific IgE levels for the 10 positive subjects, based on the Mid America Clinical Laboratories management options for ImmunoCAP-specific IgE blood tests. Also reported in Fig. 1B are histamine release data from donor basophils after stimulation with 0.1 μg/ml rPen a 1, a principal allergen in shrimp extraction. As controls, we also report histamine release in response to 5 μg/ml anti-IgE. Statistical analysis indicates that there is poor correlation between the specific/total IgE ratio and Pen a 1–mediated histamine release (Fig. 1B, Supplemental Fig. 1B). There is also no correlation between anti-IgE–mediated secretion and specific/total IgE ratio.

Dose response studies over a range of rPen a 1 (0.001–5 μg/ml) were also performed using basophils from these donors. Data are shown for donors B, D, and F, which represent a wide range of shrimp-reactive IgE concentrations (expressed in kU/l). Because the circulating levels of specific IgE do not reliably predict degranulation responses, we speculate that other factors contribute to overall responses. These factors could be circulating cytokines, IgE epitope specificity, aggregation potential, and the percentage of the individual's total IgE repertoire that is Ag specific. Although there was considerable donor-to-donor variation in the total histamine release, the optimal dose of Pen a 1 typically fell between 0.1 and 1 μg/ml.

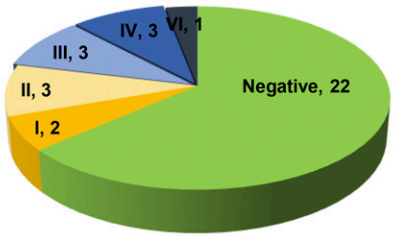
Quantification of dose-dependent Pen a 1–mediated degranulation responses and corresponding FcεRI occupancy

For further studies, we employed RBL cells modified for exclusive expression of the human α subunit of FcεRI (hRBL$^{r\alpha KO}$). To accomplish this, the rat FcεRI α subunit was disrupted in RBL-2H3 cells using CRISPR-Cas 9 gene editing methods, followed by transfection with an expression vector coding for the hFcεRI α subunit and flow sorting for hIgE binding. The stably transfected cells are thus unique, in that there are no rodent FcεRI α tetrameric receptors to compete with the human α-bearing FcεRI tetramers for coupling with signaling partners. The specificity of the chimeric FcεRI (human α, rat β/γ) in these cells for hIgE was verified by flow cytometry analysis (Supplemental Fig. 2). Additional characteristics of the hRBL$^{r\alpha KO}$ model system include: 1) low surface accumulation of IgE–FcεRI complexes after prolonged incubation of hRBL$^{r\alpha KO}$ cells with hIgE, which peaks within 3–4 d (Supplemental Fig. 2B); and 2) hIgE binds to the chimeric FcεRI with a k_f of $6.23 \times 10^3\ M^{-1}\ s^{-1}$ and dissociates at a k_r of $1.53 \times 10^{-5}\ s^{-1}$, with a $K_D = k_r/k_f = 2.4$ nM (Supplemental Fig. 3, Supplemental Table I). The marked rise in hFcεRI levels over days is consistent with prior reports of IgE-mediated stabilization of hFcεRI (44). For most experiments in hRBL$^{r\alpha KO}$ cells, IgE priming was consistently performed for 2 h (maximal 62,000 IgE-bound FcεRI, see Table I). This is important to compare results with stripped human primary basophils (see Fig. 6), because basophils have a limited lifetime after isolation from peripheral blood. Additionally, results in Supplemental Fig. 2C show that 2 h priming conditions avoid the need to account for receptor internalization and recycling.

Fig. 2 reports secretion data from hRBL$^{r\alpha KO}$ cells after 2 h priming with a range of concentrations of IgE^{Pena1} that was affinity purified from shrimp-reactive plasma. Cells were activated with increasing doses of rPen a 1 or anti-IgE (as a positive control). The data are plotted two ways: as a function of IgE priming conditions (Fig. 2A, 2B) or as a function of crosslinker concentration (Fig. 2C, 2D). Saturating concentrations of IgE occurred at ~500 ng/ml (Fig. 2A, 2B). The typical bell-shaped secretory response, seen with structurally defined ligands (45) as well as natural allergens (46), is reproduced in the hRBL$^{r\alpha KO}$ cells challenged with Pen a 1 (Fig. 2C). The optimal dose of rPen a 1 was 10 ng/ml under all priming conditions, underscoring that the number of receptors occupied with IgE^{Pena1} is a key factor in setting the threshold for response.

We used a flow-based assay to quantitate occupancy of FcεRI with allergen-specific IgE (Table I). The minimal IgE^{Pena1} priming conditions for stimulating secretion from hRBL$^{r\alpha KO}$ cells occurred after 2 h exposure to IgE^{Pena1} at 15 ng/ml (6.25 kU/l) (47). This translates to a minimum of 300 FcεRI required for measurable responses to Pen a 1 (Fig. 2C, yellow line). Maximal secretory responses to rPen a 1 were achieved when <3000 FcεRI were primed with IgE^{Pena1} (Fig. 2C, red line, Table I).

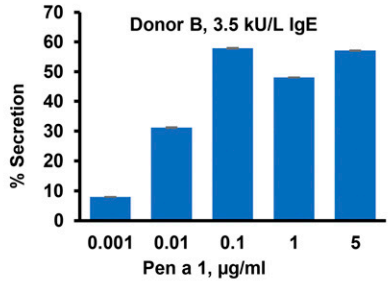
A

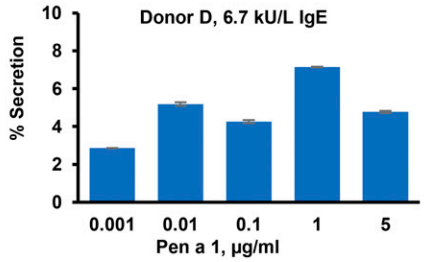


class	KU/L	Interpretation
0	<0.35	Negative for Allergen
I	0.35-0.69	Low Positive
II	0.70-3.49	Moderate Positive
III	3.5-17.49	High Positive
IV	17.5.52.49	High Positive
V	52.5-99.99	Very High Positive
VI	>100	Very High Positive

B

Donor	Shrimp-specific IgE KU/L	Total IgE kU/L	Ratio of shrimp-specific IgE to Total IgE	Basophil Releasability	
				% Secretion with 0.1 μg/ml Pen a1	% Secretion with 5 μg/ml Anti-IgE
A	4.6	N/A	N/A	6.6	92.3
B	3.5	43.2	0.08	57.9	76.3
C	0.7	53.7	0.01	8.4	54.9
D	6.7	30.4	0.22	4.9	90.4
E	49.8	92	0.54	72	58
F	17.7	165.1	0.11	30.6	72.9
G	0.5	128.8	0.00	2.4	7.1
I	0.6	49.4	0.01	30	75
J	34.3	531	0.06	21	55
L	1.8	39.7	0.05	2.28	27.6





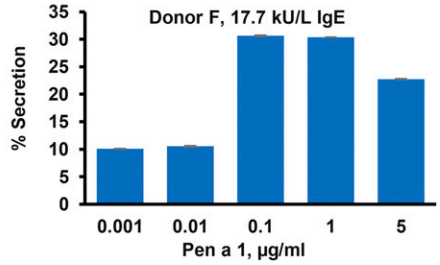


FIGURE 1. Pen a 1–specific IgE levels and basophil releasability in shrimp-allergic blood donors. (**A**) Scoring system for classification of allergic patients based on circulating levels of specific IgE. Pie chart shows the distribution of self-reported shrimp-allergic donors according to the classification. (**B**) Inset table indicates the circulating levels of specific IgE, total IgE, ratio of specific to total IgE, and corresponding basophil secretory response upon stimulation with either 0.1 μg/ml rPen a 1 or 5 μg/ml anti-IgE in allergic donors. Plots show the dose-dependent basophil releasability profile upon challenge with rPen a 1 for three allergic donors.

Rule-based model predicts FcεRI aggregation kinetics over a range of Pen a 1 doses

We next applied a rule-based mathematical model to estimate the number of IgE–FcεRI in aggregates as a function of IgE occupancy and allergen dose. Results of the optimized rule-based model (see *Materials and Methods*) are shown in Fig. 2E, comparing the computed aggregation kinetics for 2692 IgE–FcεRI complexes as a function of Pen a 1 dose. As expected, high doses of Ag bind and initiate receptor aggregation within seconds. Dotted lines in Fig. 2F report the total number of receptors bound in aggregates of any size over the simulation time course, based on the total area under the curve for each condition of IgE priming over the entire range of Ag doses used. For comparison, dashed lines in Fig. 2F provide the experimentally measured values for secretion used for fitting in the mathematical model.

The general shape of the dose-response curve, which increases as the allergen concentration increases until it peaks and then monotonically decreases for higher allergen concentrations, meets theoretical expectations (48). We expect that at low allergen concentrations, there will be small aggregates due to the low number of allergens per cell. As the allergen concentration rises, the average aggregate size will correspondingly increase until it reaches a size that causes maximum response. Further increases in allergen dose begin to favor monovalent allergen–receptor binding, which limits crosslinking. These results are consistent with prior explanations for "high-dose inhibition" of human basophils (49).

Table I. Quantification of IgE-bound FcεR

IgE (ng/ml)	IgE (*n*)	% Receptor Occupied
20,000	62,632	100
15,000	58,432	93
10,000	52,500	83
8000	49,450	78
4000	37,909	60
1000	17,641	28
120	2,692	4.00
60	1,211	1.89
30	716	1.10
15	324	0.44
7.5	162	0.26
3.75	129	0.16

hRBL$^{r\alpha KO}$ cells were primed for 2 h with hIgE$^{Alexa\text{-}488}$ over a specified range of IgE concentration, and fluorescence was quantified using flow cytometry. Numbers of FcεRI bound to IgE on the cell surface were measured using Quantum MESF standard beads and a fluorescein/protein ratio of IgE$^{Alexa\text{-}488}$. Results are expressed as the mean of two independent experiments.

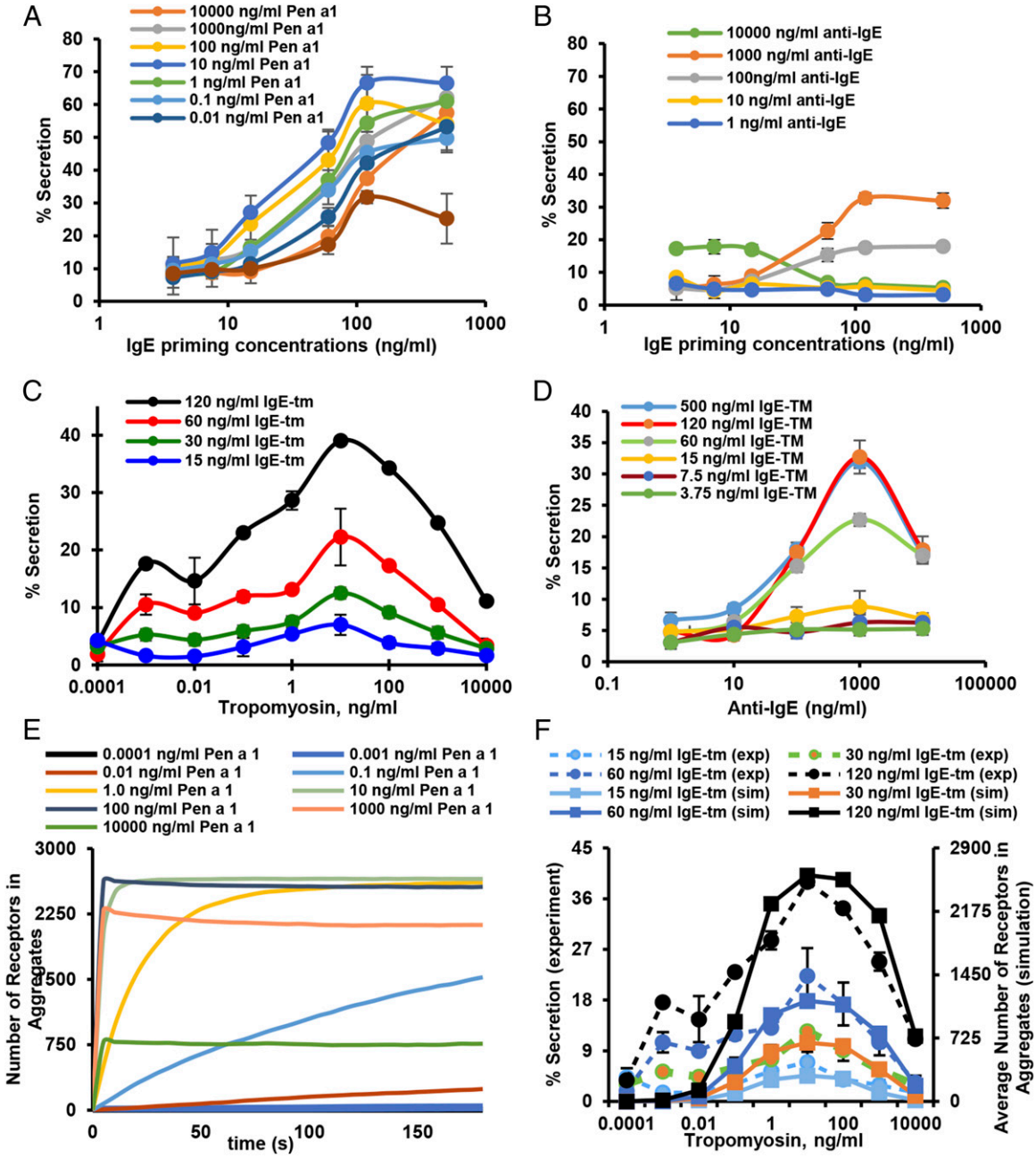


FIGURE 2. Threshold and dose-dependency of Pen a 1–mediated response. (**A**–**D**) hRBL$^{r\alpha KO}$ cells were primed with IgE^{Pena1} as indicated, incubated with a range of anti-IgE or Pen a 1 concentrations to crosslink IgE–FcεRI complexes, and degranulation responses were measured. Error bars represent SD. Results are representative of three independent experiments. (**E**) Simulated number of IgE–FcεRI complexes in aggregates as a function of time for each Pen a 1 concentration. (**F**) Comparison of percentage secretion (dashed lines) with average number of receptors in aggregates of any size, computed via integration of kinetic data as shown in (E). Curves in (E) and (F) are averages of >20 stochastic runs of the rule-based model with BioNetGen. Error bars represent SD. Values for the dissociation constant and crosslinking factor were $K_D = 4.21 \times 10^{-1}$ nM and $\alpha = 3.98 \times 10^3$.

Pen a 1 induces FcεRI clustering

We next evaluated the spatial redistribution of FcεRI on the surface of Pen a 1–treated cells, using established electron microscopy methods. hRBL$^{r\alpha KO}$ cells were primed with a saturating conditions of IgE^{Pena1}, then incubated with and without Pen a 1 prior to preparing membrane sheets ("rip-flips") on electron microscopy grids. Samples were labeled with anti-FcεRIβ immunogold and imaged by transmission electron microscopy. Images in Fig. 3A show that resting receptors are distributed across the cell membrane, alone and in small clusters that likely represent transient coconfinement in membrane domains (50). Fig. 3B and 3C show that activation with Pen a 1 (10 or 100 ng/ml) leads to an increase in the size of FcεRI clusters on the plasma membrane (arrows). These clusters appear to represent linear and branching chains (white arrowheads), as well as globular patches (black arrows) that likely represent heterogeneous mixtures of Pen a 1–IgE–FcεRI aggregates of various sizes.

Simulated gastric digestion produces rPen a 1 IgE-binding peptides with modestly attenuated mediator release

As a food allergen, Pen a 1 undergoes proteolytic cleavage during digestion. To evaluate the changing profile of Pen a 1 as a secretagogue during this process, rPen a 1 was incubated with pepsin

under conditions designed to mimic gastric digestion. Immunoblot analysis of digested rPen a 1 (Fig. 4A, 4B) revealed that IgE from the serum of an atopic donor strongly recognized major fragments migrating at 17 and 33 kDa that persist even after 10 min digestion with 0.4–12.7 U pepsin. These fragments retain their ability to crosslink IgE^{Pena1}–FcεRI complexes and robustly stimulate secretion, as shown in Fig. 4C. Extensive digestion (40 U pepsin) was required to reduce the secretory response of $hRBL^{r\alpha KO}$ cells primed with IgE^{Pena1} (Fig. 4D), consistent with the life-threatening clinical sensitivity of allergic subjects during exposure to dietary shrimp tropomyosin.

Design and characterization of Pen a 1–derived polypeptides

While pepsin-cleaved fragments of natural Pen a 1 are potent initiators of degranulation, it has been reported that short, rPen a 1–derived polypeptides encompassing a single predominant IgE binding epitope have diminished capacity (51). As shown in Fig. 5A, we designed and expressed five sequential Pen a 1 recombinant polypeptides ($FR1^{1–79}$, $FR2^{68–127}$, $FR3^{121–181}$, $FR4^{172–236}$, and $FR5^{224–284}$), each 60–79 aa in length. These products covered the entire sequence of Pen a 1, retained known IgE and T cell epitopes (13, 52), and had overlaps of 7–12 aa. Each polypeptide covered one major IgE binding region based on prior studies (12). Recombinant His-tagged proteins were expressed in *E. coli*, purified using Ni-NTA columns and separated using size-exclusion chromatography to yield a single major band on SDS-PAGE (Fig. 5B) and identified with an anti-His Ab on a Western blot (Fig. 5C). The minor, slower migrating bands in Fig. 5C suggest that minute fractions of PP1, PP2, and PP5 migrate as dimers.

Secondary structures of recombinant intact Pen a 1 and the truncated polypeptides were evaluated using CD (Fig. 5D). rPen a 1 exhibited a typical α helical structure, with characteristic minima at 208 and 222 nm and maxima at 193 nm (53). The Pen a 1–derived polypeptides exhibited varying amounts of α helical content that were significantly lower than those of the parent molecule. Because tropomyosin coiled-coil dimerization is dependent on α helical structure (54), this is consistent with the blotting results that estimate that only a small fraction of the recombinant polypeptides are in the dimer state in these solutions.

The binding of IgE from serum from a representative shrimp-allergic subject was confirmed by Western blotting (Fig. 5E, left), as well as ELISA-based measurements (Fig. 5F). As expected, intact rPen a 1 showed strong binding to IgE in shrimp-positive serum. Of the truncated products produced as recombinant polypeptides, PP1, PP2, and PP3 were highly reactive in immunoblots probed with IgE from the same serum, with modest reactivity with PP5 and weak reactivity with PP4 (Fig. 5E, left panel). Results were confirmed by ELISA (Fig. 5F). Note that IgE from control plasma (i.e., negative by the ImmunoCAP assay) failed to bind either whole Pen a 1 or the truncated polypeptides (data not shown).

The binding of peptides to IgG present in the same shrimp-positive serum was evaluated using dot blot analysis. Proteins were spotted on nitrocellulose membrane and incubated with serum, followed by detection with anti-IgG HRP or anti-IgE HRP (Fig. 5G). Similar to IgE, IgG against all peptides and intact Pen a 1 was detected. Specificity was confirmed by the absence of binding detection when IgG-depleted serum was used.

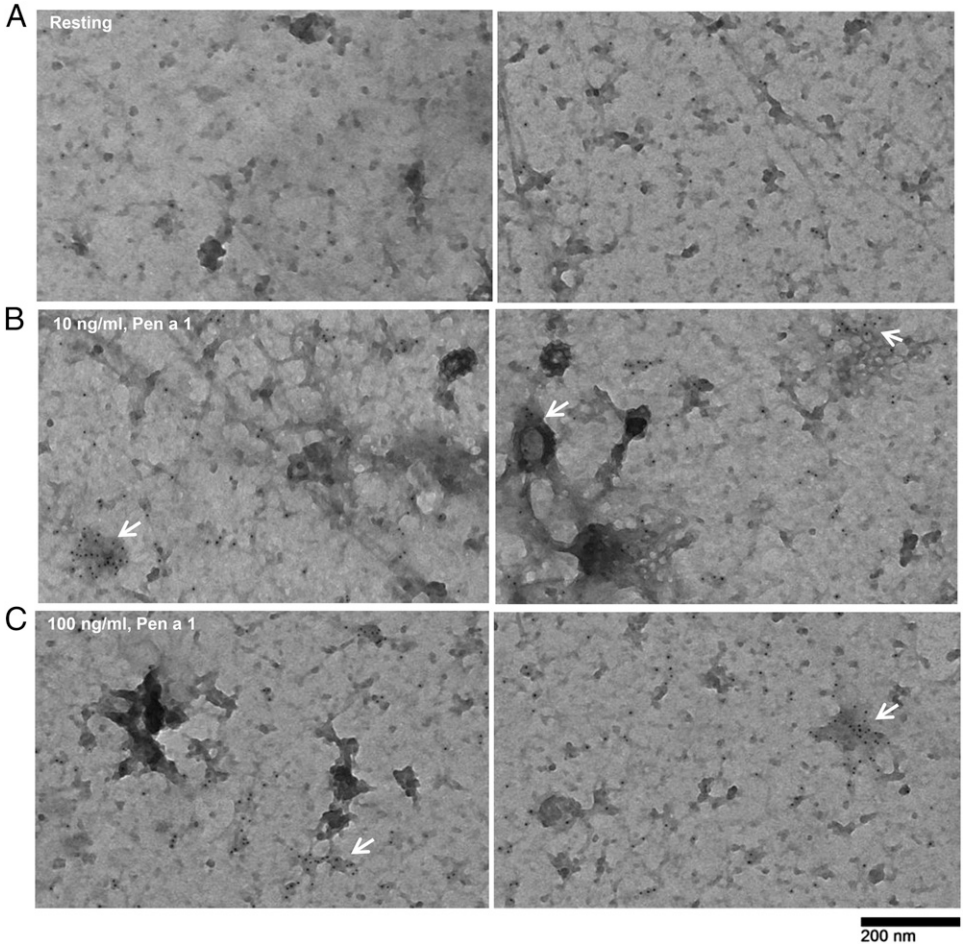


FIGURE 3. Pen a 1 crosslinking induces FcεRI clustering on the cell surface. (**A**–**C**) Transmission electron microscopy images of membrane sheets prepared from cells primed with 500 ng/ml IgE^{Pena1} overnight, followed by stimulation with 0, 10, or 100 ng/ml Pen a 1 and immunogold labeling (6 nm gold) for FcεRIβ. Arrows point to signaling patches, typical after addition of Ag. Scale bar, 0.2 μm.

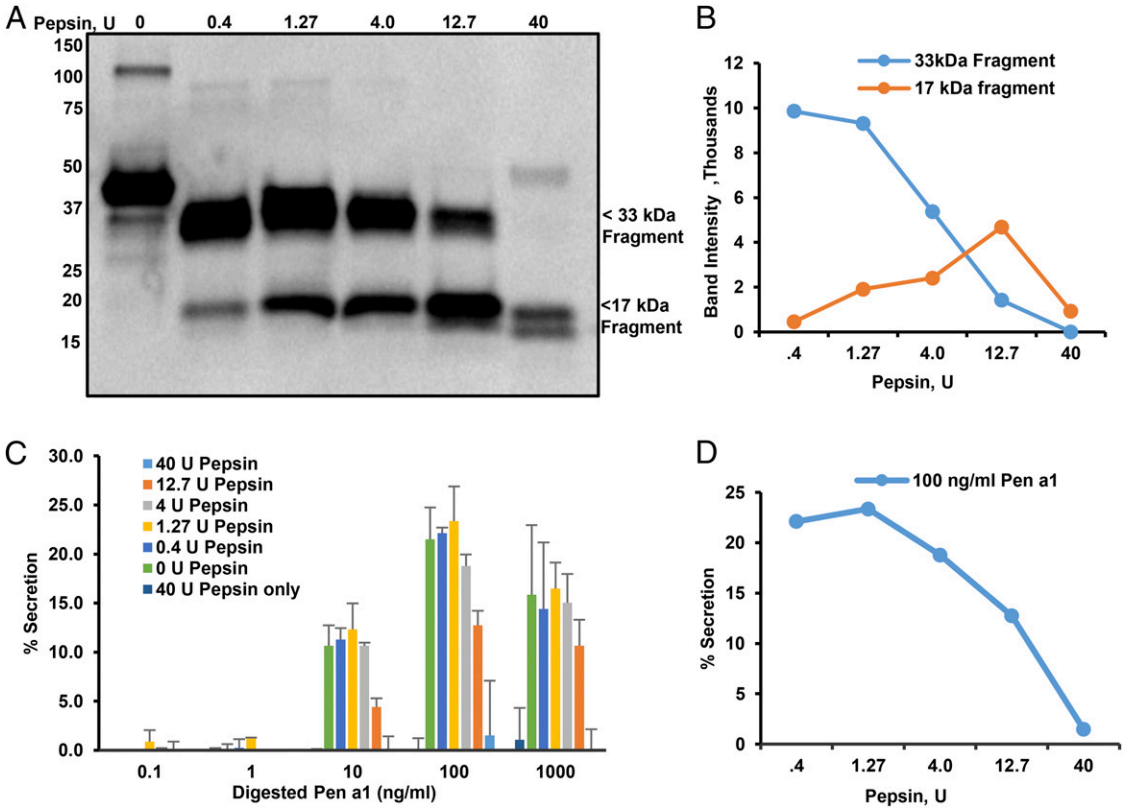


FIGURE 4. Effect of pepsin digestion on IgE binding to Pen a 1 and basophil degranulation. (**A**) Immunoblot analysis of IgE from serum of atopic individual binding to rPen a 1 digested with increasing concentrations of pepsin for 10 min at 37˚C. Bound IgE was detected using HRP-conjugated anti-IgE. (**B**) Quantification of two major fragments obtained with rPen a 1 digestion in SDS-PAGE (B). (**C**) hRBL-2H3 cells were primed with serum of an atopic individual and challenged with rPen a 1 digested with increasing concentrations of pepsin (concentrations shown in legend). (**D**) Quantification of secretory responses induced by 100 ng/ml rPen a 1 digested with increasing concentrations of pepsin from (B).

rPen a 1–truncated polypeptides are poor secretagogues and act as competitive inhibitors for intact Pen a 1

Based on data in Fig. 5, our preparations of truncated Pen a 1 polypeptides are predominantly monomers, with a small fraction of dimers. These peptides have been reported in the literature to each have a single dominant linear epitope or an overlapping string of epitopes (12). We expected that preparations with a large fraction of monomers (valency of 1) and small fraction of dimers (valency of 2) would be a poor aggregating stimulus for IgE–FcεRI complexes. To test this, hRBL$^{r\alpha KO}$ cells were sensitized with IgE by incubation with serum from a shrimp-allergic subject, followed by a challenge with whole rPen a 1 or each of the five polypeptides alone (Fig. 6A). We observed reduced secretory response to each of five fragments when administered alone, even when challenging cells with the highest dose (1 μg/ml). Even when all five truncated polypeptides were pooled, where cross-reactivity between epitopes might enhance crosslinking of IgE–FcεRI complexes, the secretory response was approximately half that of intact Ag.

Results in Fig. 6B show that Pen a 1–truncated polypeptides also compete with intact Pen a 1 to competitively inhibit mediator release from hRBL$^{r\alpha KO}$ cells. IgE-sensitized cells were incubated first for 5 min with 1 μg/ml Pen a 1, followed by addition of pooled fragments at defined concentrations for 25 min. Competitive inhibition by the truncated polypeptides was dose-dependent.

Finally, we confirmed these results using primary cells (Fig. 6C). Human basophils were freshly isolated from two donors by negative selection procedures from the blood of a normal donor, then stripped of native IgE and primed for 1 h with 20% serum from the same shrimp-allergic subject as described above. The basophils were then challenged with either intact rPen a 1 or the truncated Pen a 1 polypeptides. Results for donor 1 show that basophil histamine release was markedly lower following challenge with PP1, PP3, and PP5 and not measurable to PP4. High concentrations of PP2 did stimulate histamine release that was nearly as robust as intact allergen, suggesting that the donor's IgE repertoire is enriched in IgE recognizing epitopes in the PP2 peptide and/or that primary cells may have an even lower threshold for activation than the hRBL$^{r\alpha KO}$ cell line. For donor 2, Pen a 1 elicited a higher response at 100 and 1000 ng/ml concentrations when compared with all polypeptides added individually or pooled together. However, the difference was less pronounced with 100 ng/ml PP1 and PP2 that stimulated relatively high levels of histamine. The low concentration of 10 ng/ml caused a barely detectable histamine release regardless of stimulant. These results demonstrate the donor-to-donor variability resulting from differences in activation thresholds and underscore the need to validate results obtained with cultured cells with primary basophils or mast cells.

Discussion

The sequence, structure, and stability of the major shellfish allergen Pen a 1 has been well characterized (55). B and T cell epitopes of Pen a 1 have also been identified using extensive binding studies (56, 52). However, little is known about the relationship between threshold or activating doses of Pen a 1 and their relationship to the IgE receptor (FcεRI) aggregate properties and effector cell responses that constitute allergy. Because the density of cell surface

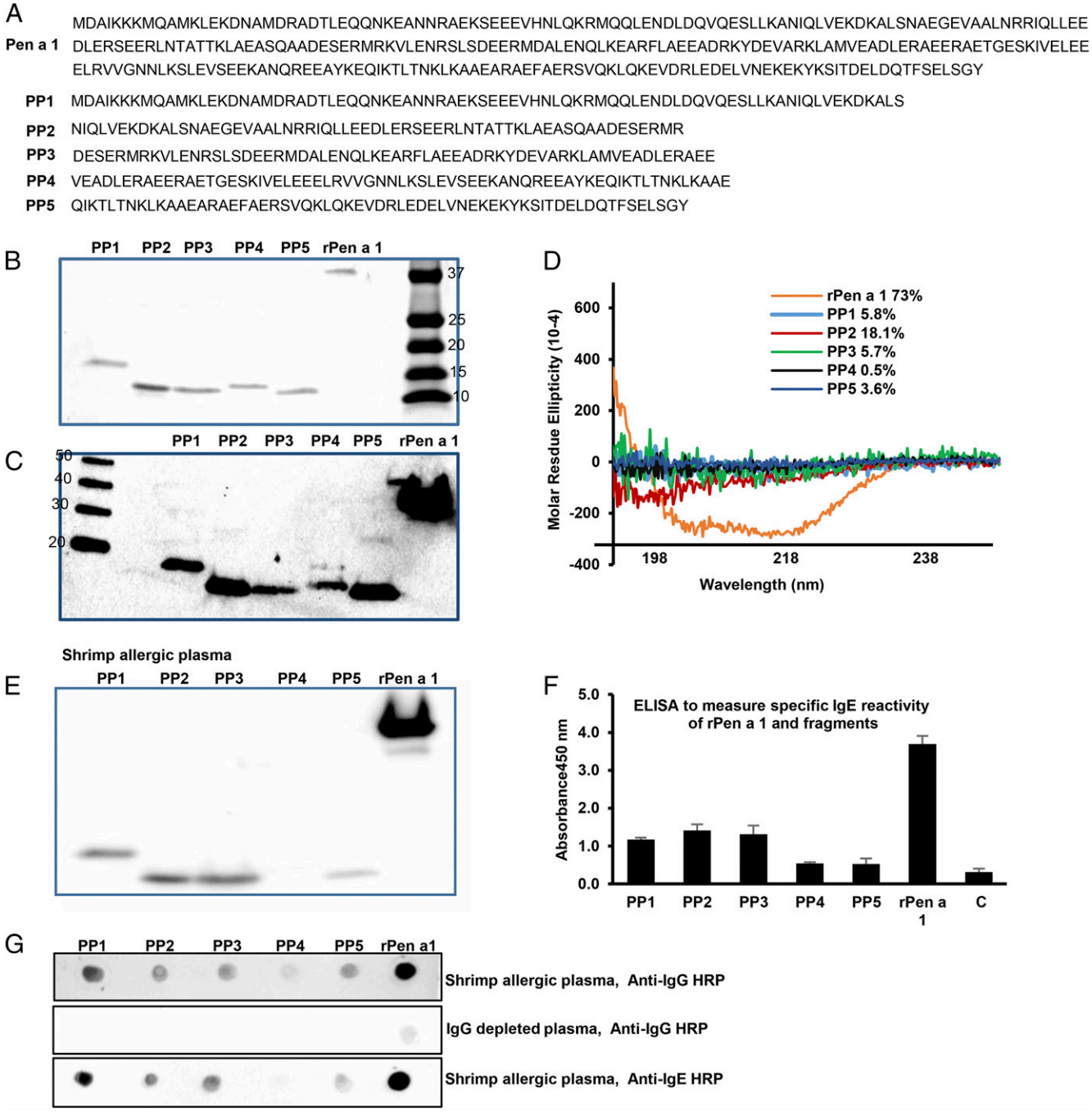


FIGURE 5. Design and recombinant expression of Pen a 1 and fragments. (**A**) Amino acid sequence of Pen a 1 and the five fragments. Sequence does not include N-terminal His tag. (**B**) SDS-PAGE. Size-exclusion chromatography–purified proteins were separated and detected by Coomassie staining. (**C**) Anti-His Western blotting. Size-exclusion chromatography–purified proteins were separated and detected by probing with anti-His Abs. (**D**) CD spectroscopy in PBS. Intact Pen a 1 but not the fragments showed a typical spectrum for α helix with minima at 208 and 222 nm. The percentage of α helical content was calculated for each molecule by using mean residue ellipticity at 222 nm as described previously, (**E**) Immunoblot analysis of IgE binding to rPen a 1 and its fragments with serum of atopic (shrimp-allergic) individual. Bound IgE was detected using HRP conjugated anti-IgE. (**F**) ELISA to measure reactivity of IgE from atopic sera to rPen a 1 or fragments. Error bars represent SD. C, control serum. (**G**) Dot blot analysis of IgE or IgG binding to rPen a 1 and its fragments with normal or IgG-depleted serum from atopic (shrimp-allergic) individual. Bound Ab was detected using HRP-conjugated anti-IgE or anti-IgG.

FcεRI expression in basophils and mast cells is generally expected to correlate with circulating/free levels of IgE (57, 58), the detection of IgE Abs to shrimp or specific epitopes of tropomyosin in serum is assumed to be clinically relevant (56, 59). The magnitude of effector cell responses have been shown to depend on the ratio of allergen-specific IgE to total IgE, which varies with age, total serum IgE, and heterogeneity in IgE (60). In our study, we did not observe correlations between specific IgE levels in individual blood samples and the magnitude of responses of basophils isolated from the same donor source upon challenge with allergen. Therefore, a number of factors contribute to variability among individuals, including the basal levels of inflammatory cytokines such as IL-3 that are well known to potentiate FcεRI-stimulated responses (61). Additionally, there is a persistent and wide gap between the number of individuals who think they have food allergies and the true prevalence (1).These results support the use of clinically approved in vitro basophil activation tests (62) to evaluate allergen reactivity for patients in the allergy clinic.

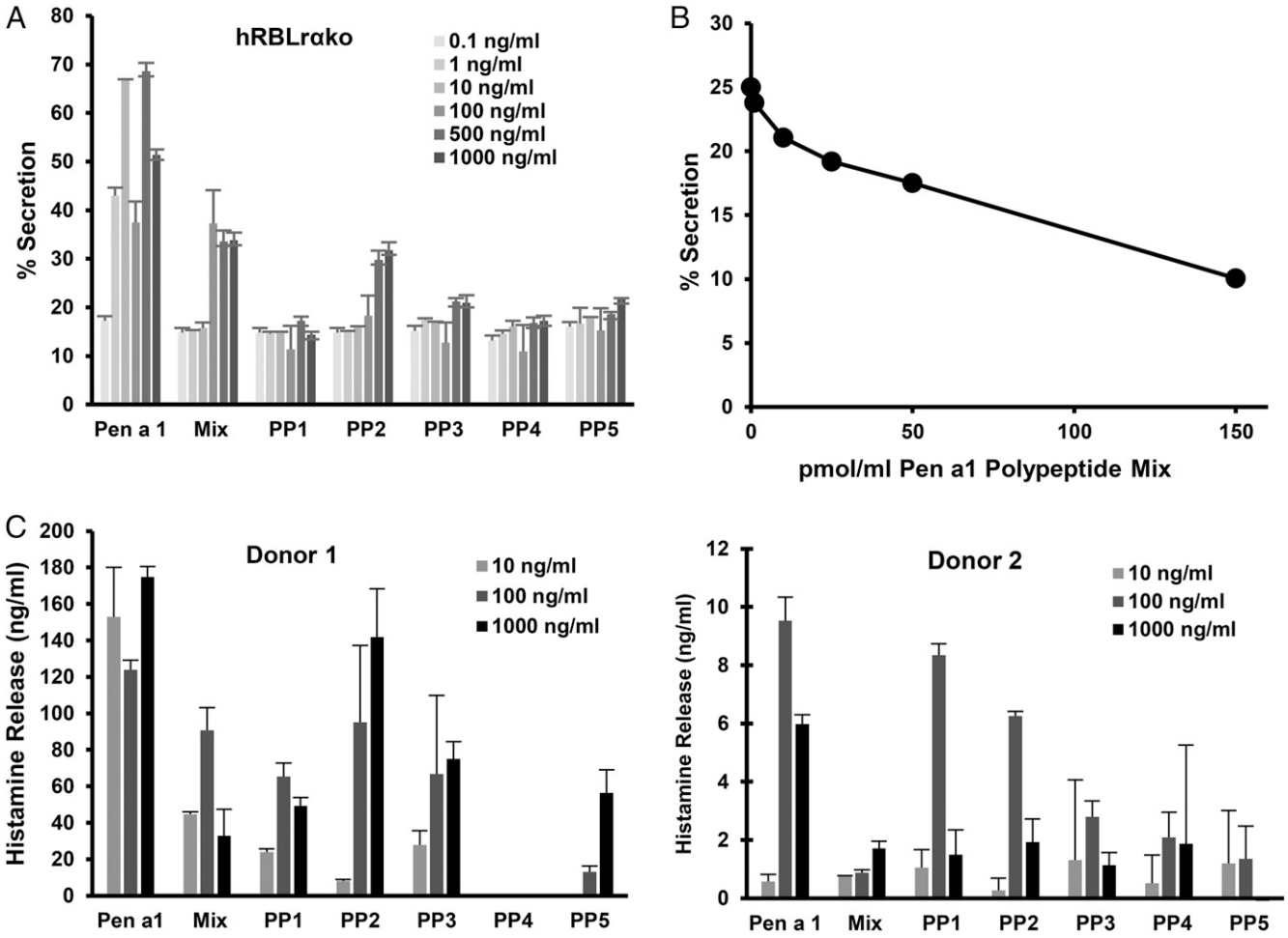


FIGURE 6. Pen a 1 fragments cause reduced cell activation and inhibit responses to intact Pen a 1. (**A**) hRBL$^{r\alpha KO}$ cells were primed with atopic sera and stimulated with rPen a 1 and fragments (concentrations shown in legend). Cells were incubated with serum from a shrimp-allergic individual for 2 h before addition of rPen a 1, isolated fragments, or fragments pooled together (concentrations shown in legend). (**B**) Addition of fragment mix in specified molar concentrations to cells preincubated with 1 μg/ml Pen a 1 for 5 min. Degranulation response is based on the percentage of total β-hexosaminidase released from cells after stimulation for 30 min. (**C**) Histamine release in basophils is expressed in nanograms per milliliter, after subtraction of spontaneous release. Basophils isolated from donors were stripped of bound IgE, primed with atopic serum, and stimulated with indicated allergen. Error bars represent SD. FR1–FR5, Pen a 1 fragments 1–5; Mix, five Pen a 1 fragments pooled together in equivalent amounts at indicated concentrations.

In this study, we report, to our knowledge, the first use of hRBL$^{r\alpha KO}$ cells that exclusively express the hFcεRI α subunit. Our characterization of this cell line (Supplemental Figs. 2, 3) reveals a slower on-rate for IgE binding compared with previous estimates for hFcεRI on primary cells (39), but with a somewhat higher affinity than transgenic mice expressing chimeric FcεRI comprised of human α subunit with mouse β and γ2 (2.4 versus 6.4 nM) (63).

In this study, we initially characterized the relationship between Pen a 1 and IgE^{Pena1} priming using both experimental and rule-based modeling approaches, enabling us to determine the relationship between the number of FcεRI occupied and cellular responses. Because the occupancy of FcεRI with IgE is saturable, we expected that a key variable is the fraction of the total IgE repertoire that is allergen specific. Our data using "humanized" RBL cells indicate that measurable degranulation responses to Pen a 1 can be achieved with only a few hundred receptors engaged on the cell surface, whereas maximal secretion can occur when <2700 FcεRI are primed with allergen-specific IgE. Human basophils express from 70,000 to 230,000 FcεRI on the cell surface (64, 65). Thus, if a similar threshold applies to human primary cells expressing 230,000 FcεRI, occupancy of between 0.0017 and 0.01% of FcεRI with Pen a 1–specific IgE might be sufficient to trigger histamine release. We note that although hRBL$^{r\alpha KO}$ cells do serve as a useful surrogate for primary basophils, ~10-fold higher Pen a 1 doses were required for optimal degranulation from basophils from individual, shrimp-allergic donors (see sonors B and F in Fig. 1).

Other key variables for FcεRI activation are the valency and dose of allergen, which together exert strong influence over the aggregation potential of a given allergen. We developed a rule-based model to generate predictions of receptor aggregation achieved over a range of IgE and Pen a 1 doses. The mathematical model matches aggregating conditions that are optimal for Pen a 1–mediated degranulation, which crosslink ~2000 receptors for 120 ng/ml IgE, and predicts a higher number of receptors in aggregates at maximum secretion for 15, 30, and 60 ng/ml IgE. Using immunoelectron microscopy of membrane sheets (Fig. 3), we observed that receptor clusters grow in size under these crosslinking conditions. Some of the larger clusters seen by electron microscopy are likely to be composites of smaller aggregates in the same signaling patch (29).

Specific immunotherapy using natural allergen extracts is the most common treatment option employed today for allergen-specific disease modification (66). The risk of adverse reactions during immunotherapy for food allergens is considerable (67).

Avoidance remains the primary recommendation for patients diagnosed with shrimp allergy, with one recent study evaluating sublingual immunotherapy with shrimp extract (68). It is particularly remarkable that defined formulations of recombinant tropomyosin have not been evaluated for shrimp-specific immunotherapy, despite the successful use of recombinant allergens in clinical trials with grass and birch pollen allergens (69, 70). Caution is understandably merited for the full-length Pen a 1 protein, owing to its unique filamentous, dimer structure and well-spaced epitopes. With a valency of ≥10, tropomyosin is an exceptionally efficient crosslinking stimulus for the IgE receptor. It makes sense to consider these structural features for the design of hypoallergenic allergy vaccines (71). Improved safety profiles might be expected after reengineering Pen a 1 to have reduced or abolished IgE binding (13) or by lowering the crosslinking capacity (14, 51). Mutagenesis and epitope deletion strategies have been shown to reduce allergenicity in the shrimp allergen Met e 1 (72). Because tropomyosin is a pan-allergen, causing adverse reactions across shellfish and other invertebrate species (e.g., dust mite) (73), it is particularly important to reevaluate these strategies with the goal of developing Pen a 1 hypoallergen formulations that retain T cell epitopes and lower the potential for adverse events during therapy.

One of the methodologies for reducing IgE binding includes destroying the structural conformation of the allergen with heat-induced denaturation or in vivo digestion. However, the structure of Pen a 1 is extremely stable even after boiling (74), retaining full allergenicity after heat treatment (75). We characterized the IgE binding and effector cell activation of Pen a 1 under conditions that mimic gastric digestion. The profile of digested fragments obtained in our study differed from previously reported studies (76, 77), possibly because of differences in IgE epitopes recognized by patients in the different studies as well as experimental variables (pepsin concentrations, incubation times, and protein production). Importantly, we show that extensive digestion is needed to lower the allergenic potential of Pen a 1. Apart from the already established route of gut absorption, allergic symptoms have been shown to emerge with mere inhalation of cooking vapors or handling of seafood (78), thus serving as an alternative route of triggering an allergic response.

Protein engineering represents an important strategy to reduce allergen valency and allergenicity (71). We designed overlapping recombinant fragments that span the entire length of Pen a 1 and evaluated IgE binding and effector cell responses. Similar to digested Pen a 1, these polypeptides retained the ability to bind IgE in both denatured and native forms. These polypeptide formulations each bear a single major linear IgE epitope or have a group of adjacent epitopes that would likely be effectively monovalent due to steric constraints once IgE was bound to one epitope. The Pen a 1–derived polypeptides were relatively weak stimulators of hRBL$^{r\alpha KO}$ cells primed with allergen-specific IgE, even at high concentrations and when pooled together. Their low α helical content suggests that most polypeptides remain monomeric in solution and are thus monovalent. For the small fraction that do form dimers based on SDS-PAGE analysis, it is intriguing that the coiled-coil orientation of two identical epitopes in any pair of dimers renders their distance very close (<2 nm). Our preparations showed reduced allergenicity by comparison with an earlier study (51), presumably due to differences in design, affinity purification, and/or concentration steps that will be critical to carry forward for potential clinical development.

The Pen a 1–derived polypeptides also had a markedly reduced capacity to stimulate normal human basophils from two individual donors. Both of these basophil preparations were subjected to rapid acid stripping of bound IgE, followed by priming with the same source of allergic serum containing Pen a 1–specific IgE. The differences in histamine releasability for these basophils after challenge with intact or fragmented Pen a 1 likely reflect differences that are unique to the donors, including basal levels of surface FcεRI and exposure of the basophils in vivo to circulating cytokines.

We expect that differences in primary cells versus hRBL$^{r\alpha KO}$ cells reflect variable expression levels of signaling molecules such as Syk, Lyn, and SHIP, which serve as positive and negative regulators of mediator release (31, 79). Studies with birch pollen allergen Bet v 1 additionally suggest that the basophil activation assay may not always correlate with the potency of an allergen in triggering an in vivo response (80).

In conclusion, we have demonstrated the concept of dividing a linear complex allergen such as tropomyosin into overlapping recombinant polypeptides. In total, they retain all known IgE and T cell epitopes while simultaneously reducing allergenicity. These formulations represent a starting point for the design and development of a therapeutic vaccine for shrimp allergy that is efficacious and safe with minimal side effects in patients. It is notable that IgG against all polypeptides was also detected in allergic serum, thus confirming that these polypeptides bear some IgG-binding epitopes. Further studies are now warranted to examine the potential of these polypeptides to induce a protective blocking Ab response in vivo.

Acknowledgments

We acknowledge the University of New Mexico Clinical and Translational Science Center for recruitment of human subjects, as well as the University of New Mexico Electron Microscopy Facility, Center for Advanced Research Computing, and Cancer Center Core Facilities for Flow Cytometry and Fluorescence Microscopy. We thank Dr. Eva Chi and Adeline Fanni for help with HPLC experiments.

Disclosures

The authors disclose a patent pending related to hypoallergen design based upon this work.

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