

Reduced Allergenic Potency of VR9-1, a Mutant of the Major Shrimp Allergen Pen a 1 (Tropomyosin)¹

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The major shrimp allergen, tropomyosin, is an excellent model allergen for studying the influence of mutations within the primary structure on the allergenic potency of an allergen; Pen a 1 allows systematic evaluation and comparison of Ab-binding epitopes, because amino acid sequences of both allergenic and nonallergenic tropomyosins are known. Individually recognized IgE Ab-binding epitopes, amino acid positions, and substitutions critical for IgE Ab binding were identified by combinatorial substitution analysis, and 12 positions deemed critical were mutated in the eight major epitopes. The mutant VR9-1 was characterized with regard to allergenic potency by mediator release assays using sera from shrimp-allergic subjects and sera from BALB/c, C57BL/6J, C3H/HeJ, and CBA/J mice sensitized with shrimp extract using alum, cholera toxin, and *Bordetella pertussis*, as adjuvants. The secondary structure of VR9-1 was not altered; however, the allergenic potency was reduced by 90–98% measuring allergen-specific mediator release from humanized rat basophilic leukemia (RBL) cells, RBL 30/25. Reduced mediator release of RBL-2H3 cells sensitized with sera from mice that were immunized with shrimp extract indicated that mice produced IgE Abs to Pen a 1 and to the same epitopes as humans did. In conclusion, data obtained by mapping sequential epitopes were used to generate a Pen a 1 mutant with significantly reduced allergenic potency. Epitopes that are relevant for human IgE Ab binding are also major binding sites for murine IgE Abs. These results indicate that the murine model might be used to optimize the Pen a 1 mutant for future therapeutic use. *The Journal of Immunology*, 2005, 175: 8354–8364.

Shrimp allergy is a potentially life-threatening disease. Pen a 1, the tropomyosin of brown shrimp, *Penaeus aztecus*, is the only major allergen of this particular shrimp species and is recognized in >80% of shrimp-allergic subjects (1). Pen a 1 inhibited at least 75% of patients' IgE radioallergosorbent test reactivity to whole body shrimp extract, indicating that Pen a 1 is also responsible for most of the allergenic activity of shrimp (2). Tropomyosin is a major allergen in other Crustacea species (3–6) and in dust mite (7–11), cockroach (12, 13), lobster (4), squid (14), and other mollusks (15–19), suggesting that tropomyosin may be regarded as an invertebrate pan-allergen (20). Indeed, cross-reactivity between cockroaches and mites with Crustacea was attributed to tropomyosin (4, 21, 22).

In the first systematic analysis of IgE-binding regions of Pen a 1, Ayuso et al. (2) synthesized a set of 46 overlapping peptides, 15 aa long, spanning the 284-aa-long Pen a 1 molecule. Peptides were tested for IgE binding with sera from 18 shrimp-allergic subjects. Based on frequency and intensity of IgE binding, five major allergenic regions were identified: region 1 (Pen a 1^{43–57}), region 2 (Pen a 1^{85–105}), region 3 (Pen a 1^{133–153}), region 4 (Pen a 1^{187–201}),

and region 5 (Pen a 1^{247–284}). The IgE binding regions seemed to occur at regular intervals in the molecule spaced approximately every sixth heptad (approximately every 42 aa), suggesting some relation with the coiled-coil structure of tropomyosin.

Although the amino acid sequence identity between invertebrate and vertebrate tropomyosins is relatively high, ranging from 51–58% (20), vertebrate tropomyosins have not been described as allergenic (23) and do not bind IgE from shrimp-allergic subjects, most likely due to phenomena of immunological tolerance. This observation offered the opportunity to stepwise convert an allergenic tropomyosin, Pen a 1, into a potentially nonallergenic form while preserving structure and function. Such a genetically engineered protein could represent a candidate vaccine for treatment of this potentially severe food allergy for which, until now, no curative treatment was available.

Thus, the overall aim of the present study was to generate and characterize a mutant of the allergen Pen a 1 carrying amino acid substitutions in major IgE-reactive epitopes. First, individually recognized epitopes within the five regions were identified by testing short (5–15 aa long), overlapping (offset of one or two amino acid positions) peptides for IgE Ab binding of sera that had been used for the identification of IgE-reactive regions (2). Second, amino acid positions and substitution critical for IgE Ab binding were identified by combinatorial substitution analysis, and IgE-reactive peptides were converted into the homologous sequence of nonallergenic vertebrate tropomyosins and tested for alterations of their IgE Ab reactivities. Third, based on these results, substitutions were selected that abolished the IgE Ab reactivities of the Pen a 1 epitopes, and a Pen a 1 mutant was generated by site-directed mutagenesis and characterized with regard to allergenic potency by mediator release assays using sera of shrimp-allergic subjects and mice sensitized with shrimp extract.

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Materials and Methods

Subjects' sera

Sera ($n = 10$) from atopic shrimp-allergic subjects with significant reactivity to the main IgE-binding regions (2) were used to identify the IgE-binding epitopes within these regions. All subjects had a history of respiratory (wheezing and shortness of breath), dermatologic (urticaria and angioedema), and/or gastrointestinal (nausea, vomiting, and diarrhea) symptoms occurring within 1 h after ingestion of shrimp, positive immediate skin prick test (wheal >3 mm) to brown shrimp extract, elevated shrimp-specific IgE by radioallergosorbent test (binding, $>3\%$) (24, 25), and strong IgE reactivity to purified shrimp tropomyosin by immunoblot analysis (1). For the mediator release experiments, additional sera ($n = 4$) from shrimp-allergic subjects that had not been used for epitope characterization were included.

Identification of Pen a 1 epitopes by SPOTS analysis

Overlapping peptides were synthesized using F-moc amino acids on derivatized cellulose membranes according to the manufacturer's instructions (SPOTS Epitope Mapping System; Genosys Biotechnologies). To identify the IgE-binding epitopes (shortest peptide with maximal IgE Ab reactivity) 5- to 15-aa-long peptides with offsets of one or two residues were synthesized spanning the previously identified main IgE-binding regions of Pen a 1 (region 1, Pen a 1^{43–57}; region 2, Pen a 1^{85–105}; region 3, Pen a 1^{133–153}; region 4, Pen a 1^{187–201}; region 5, Pen a 1^{247–284}) (2). These peptide libraries were probed with patients' sera for specific IgE Abs as described previously (2). Briefly, the membranes were rinsed in methanol, washed with TBS (pH 7.5), and incubated in blocking solution (Genosys Biotechnologies) for 2 h and overnight with the patient's serum diluted 1/5 with blocking solution. After washing with TBS-0.5% Tween 20 (pH 7.5), IgE reactivities were detected using 0.8 μ Ci/membrane of ¹²⁵I-labeled horse anti-human IgE (Sanofi Diagnostics Pasteur) diluted in blocking solution. The next day, the membranes were washed, placed between plastic sheets, and exposed to x-ray film for 72 h. The IgE Ab reactivity was graded by agreement by four investigators, grading the intensities independently for each other.

Combinatorial substitution analysis for the identification of amino acids critical for IgE Ab binding

Amino acid sequences of vertebrate tropomyosins were used as templates to select the mutations: individual IgE-binding epitopes were gradually converted into the homologous sequences of vertebrate (nonallergenic) tropomyosins. For each identified major epitope, a set of modified peptides was synthesized that contained all possible combinations of substitutions. For example, if the allergenic epitope differed in five positions compared with the homologous sequence of a nonallergenic tropomyosin, 32 peptides were tested. Vertebrate nonallergenic muscle tropomyosins of chicken (*Gallus gallus*, α -tropomyosin, Galg α TM, GenBank accession no. CAA41056; β -tropomyosin, Galg β TM, GenBank accession no. P19352), swine (*Sus scrofa*, α -tropomyosin, Sussa α TM, GenBank accession no. CAA46986, 1C1G_D), rabbit (*Oryctolagus cuniculus*, α -tropomyosin, Oryc α TM, GenBank accession no. TMRBA; β -tropomyosin, Oryc β TM, GenBank accession no. AAK77199), and trout (*Salmo trutta*, SaltTM, GenBank accession no. CAA91251, CAA91434) were used as templates.

Preparation of shrimp extract and purification of natural and recombinant Pen a 1

Extract from locally (New Orleans, LA) purchased brown shrimp, *P. aztecus*, was prepared as described previously (24). Natural Pen a 1 was purified from shrimp extract by preparative SDS-PAGE (model 491 Prep-Cell; Bio-Rad). Briefly, shrimp extract was separated on the 28-mm internal diameter column using Laemmli discontinuous SDS-PAGE buffer system (26). A 15-mm-high stacking gel (5% T; 1.5% C) poured on top of the 65-mm-high separation gel (11% T, 1.5% C) was used to separate shrimp proteins, and the fractions containing Pen a 1 were identified by SDS-PAGE, silver staining, and immunoblotting; pooled; and dialyzed against water.

For the production of recombinant Pen a 1, total RNA was isolated from snap-frozen shrimp (RNeasy-Total RNA-Isolation kit; Qiagen), and 5' RACE was performed using a gene-specific internal primer (5'-CTGCTCTTAACCGCCGATCCAGC-3') deduced from published partial Pen a 1 cDNA sequence (20). Full-length cDNA (GenBank accession no. DQ151457) was obtained by PCR, purified, cloned into the pCR4-TOPO vector, expanded in TOP10 cells, and sequenced. For the preparation of rPen a 1 BL 21 (DE3) competent cells (Novagen) were transformed by heat shock with a pET 101/D-TOPO vector coding for Pen a 1 and a C-terminal hexahistidine tag (49), incubated first in SOC medium for 1 h at 37°C, followed by culture on ampicillin-containing Luria-Bertoni agar plates

overnight at 37°C. A colony was picked and expanded, and Pen a 1 expression was induced with isopropyl- β -D-thiogalactopyranoside. Frozen pellets were thawed at 37°C and incubated with lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, and 2 mM imidazole (pH 8.0)). After three cycles of freezing and thawing, rPen a 1 was purified by immobilized metal affinity chromatography using (Ni-NTA agarose; Qiagen) according to the manufacturer's instructions. Because the last mutation introduced into Pen a 1 at position 46 (L46M, epitope 1) created a new bacterial ribosome-binding site (Shine-Dalgarno sequence) (27), the expressed truncated polypeptide had to be separated from full-length Pen a 1 by preparative SDS-PAGE using a 4.5-cm-high separating gel.

Generation of Pen a 1 mutant VR-9/1 by site-directed mutagenesis

Pen a 1 cloned into the pET 101/D-TOPO vector (49) was used as the basis for the generation of the Pen a 1 mutant VR-9/1; the Quick Change Site-Directed Mutagenesis Kit and the Quick Change Multi Site-Directed Mutagenesis kit (Stratagene) were used. Primers (MWG Biotech) were designed according to the manufacturer's instructions. The primers were named according to the target epitopes that were mutated (e.g., 5b) and the resulting amino acid substitutions and positions (e.g., S269F) and their direction, i.e., forward (fwd) or reverse (rev) primer. For introduction of single mutations into epitopes 1, 4, 5a, and 5b, the primers PA1-L46M-L53T-fwd (5'-GGTTCACAACCTTCAGAAGAGGATGCAGCAACTTGAAGAACCACC-3'), PA1-L46M-L53T-rev (5'-CCAAGTGTGTGG AAGTCTTCTCCTACGTCGTTGAACCTCTTGCTGG-3'), PA4-V191S-V199N-fwd (5'-GGTGAATCAAAGATCGTCGAGCTTGAGGAAGAG CTGCGTGTCTGTTGGC-3'), PA4-V191S-V199N-rev (5'-CCACTTAG TTTCTAGCAGCTCGAACTCCTTCTCGACGCACAGCA ACCG-3'), PA5aII-L260V-fwd (5'-GGTCGACAGGCTTGAAGACGAAGTGGT AACGAAAAGGAGAAGTACAAGTCC-3'), PA5aII-L260V-rev (5'-CCAGCTGTCCGAACCTCTGCTTGACCAATTGCTTTTCTCTT CATGTTACAGG-3'), PA5b-S269F-fwd (5'-CGAAAAGGAGAAGTAC AAGTCCATTACCGACGAGCTGGACC-3'), and PA5b-S269F-rev (5'-GCTTTTCTCTTCATGTTTCAAGTAAAGGCTGTCTCGACCTGG-3') were used. For mutating epitopes 2, 3a, 3b, 5a, 5b, and 5c, the Multi Site-Directed Mutagenesis Kit was applied, requiring only the forward primers PA2-L95V-fwd (5'-CCGCCGATCCAGCTGCTCGAGGAG GACCTGG-3'), PA3a-S136K-fwd (5'-CGAGAACCGCTCCCTGTCCG ACGAGGAGCGCATGGACGCCC-3'), PA3b-E145D-fwd (5'-GCGC ATGGACGCCCTGGAGAACCAGCTCAAGGAGGC-3'), PA5aI-R255D-fwd (5'-GCTCCAGAAGGAGGTCGACAGGCTTGAAGA CGAAGTGG-3'), and PA5c-T277A-F278L-fwd (5'-CCGACGAGCTG GACCAGACTTTCAGCGAACTGTCTGGC-3'). All mutagenesis reactions were run in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems). XL1-Blue supercompetent cells (Stratagene) were transformed with the product of the single mutagenesis steps, and XL10-Gold ultracompetent cells (Stratagene) were transformed with the ssDNA from multisite-directed mutagenesis steps. After each mutagenesis, plasmid DNA was purified (Plasmid Mini Kit; Qiagen) and sequenced (MWG Biotech).

Circular dichroism (CD)⁴ spectroscopy

Natural and recombinant Pen a 1 were dialyzed against 10 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.4), and protein concentrations were adjusted to 5.2 μ M. Circular dichroism spectroscopy was performed on a J-810 S spectropolarimeter (Jasco) with constant nitrogen flushing at 20°C. For wavelength analysis, Pen a 1 was scanned with a step width of 0.2 nm and a band width of 1 nm. The spectral range was 185–255 nm at 50 nm/min. Ten scans were accumulated, and the mean residue molar ellipticity (2_{MRV}) was calculated.

Animal models of shrimp allergy

For development of a suitable mouse model of shrimp allergy, 5- to 6-wk-old female mice (BALB/cJ, C57BL/6J, C3H/HeJ, and CBA/J; The Jackson Laboratory) were sensitized with shrimp extract using *B. pertussis*, cholera toxin, and alum as adjuvants, respectively. Using cholera toxin, mice received 10, 1, or 0.1 mg of shrimp extract by gavage on days 1, 7, 21, and 35, respectively (28). Each dose contained 10 μ g of cholera toxin (List Biological Laboratories). The mice were killed and bled on day 42. On day 1, mice were injected i.p. with 1 mg of shrimp plus 100 μ g of *B. pertussis* organism. The mice received on days 28 and 35, 50 μ g of shrimp extract without adjuvant and were killed and bled on day 42. Using aluminum hydroxide (Pierce) as adjuvant, mice received 10 μ g of shrimp extract plus

⁴ Abbreviations used in this paper: CD, circular dichroism; RBL, rat basophilic leukemia; %T, percent total acrylamide; %C, percent cross-linker.

1 mg of $\text{Al}(\text{OH})_3$ in 0.2 ml of PBS i.p. on days 1, 7, 14, 21, 28, and 35. The mice were killed and bled on day 42. The experiments were approved by the appropriate Tulane University institutional review committee.

Mediator release from wild-type and transfected RBL cells

The mediator release assays followed a protocol established by Hoffmann et al. (29, 30). Briefly, RBL-2H3 (DSMZ) or RBL 30/25 cells expressing the α -chain of the human Fc ϵ RI (31) were plated in 96-well flat-bottom cell culture plates (Nunc) at 1.5×10^5 cells/well. The cells were passively sensitized with IgE-containing mouse (1/50) and human sera, respectively. The sensitization of RBL 30/25 cells was optimized by titrating serum against challenge Ag concentration in a checkerboard format for each individual human serum. After washing, the cells were stimulated with Ag (*P. aztecus* extract, Pen a 1). The Ag-specific release was quantified by measuring β -hexosaminidase activity and expressed as a percentage of the total β -hexosaminidase content that was obtained by lysing the cells with Triton X-100. For measurements of spontaneous release and possible unspecific effects, naive RBL cells were incubated with Tyrode's buffer, cell culture medium, and Ag without specific Abs, respectively.

Results

Individual IgE-binding epitopes of Pen a 1 are centered around common cores and vary in size

The individually recognized epitopes of region 1 (Fig. 1A) were identical for all four subjects tested (Pen a 1^{43–55}), VHNLQKRMQLEN) and consisted of 13 aa residues. Pen a 1^{43–55} differed at nine or 10 aa positions from homologous vertebrate tropomyosin sequences. Thus, the sequence identity was very low (23–31%), and the sequence similarity ranged from 39–54%.

The IgE-binding epitopes of region 2 were 15 (Pen a 1^{87–101}) and 11 (Pen a^{91–101}) aa long (Fig. 1B). The longer peptide bound IgE Abs from three of the four subjects tested; only one subject showed specific IgE Ab reactivity to the shorter, 11-aa-long peptide (Pen a 1^{91–101}). This shorter peptide defined the sequence that was commonly recognized by all four individuals (epitope core). The individual Pen a 1 epitopes differed in two or three amino acid positions from those of the homologous vertebrate sequences. Thus, sequence identity (73%) and similarity (87–100%) of this epitope to homologous vertebrate sequences were much higher than those of Pen a 1^{43–55}.

Region 3 (Pen a 1^{133–153}) split into two overlapping epitopes that were centered around epitope cores 3a (Pen a 1^{137–141}) and 3b (Pen a 1^{144–151}; Fig. 1C). The lengths of the identified 3a epitopes varied greatly, ranging from eight to 12 aa. The average length was approximately eight to nine residues; however, one subject (no. 1) bound to a 12-aa-long peptide. Only two subjects (no. 10 and 18) recognized the same epitope (Pen a 1^{137–145}). For most (five of six) subjects, the Pen a 1 epitopes differed in four amino acid positions from those of the homologous vertebrate sequences, resulting in sequence identities of 42–56%, and the sequence similarities ranging from 63–89% (Table I). The epitope core showed a sequence identity of 80% and a sequence similarity of 100%, respectively. Epitope 3b was recognized by subjects 4 and 6. The minimal IgE-binding sequence bound by IgE Abs of subject 6 is Pen a 1^{144–151} (LENQLKEA). This sequence represents the core of this epitope, because this is contained in the sequence recognized by subject 4 (Pen a 1^{139–153}, ERMDALENQLKEARF). Up to seven amino acid differences exist between the individually recognized Pen a 1 epitopes and the homologous vertebrate sequences; the sequence identities ranged from 53–88%, and the sequence similarities were between 74 and 88%.

All individual epitopes of region 4 recognized by the three subjects tested began with position 187 and varied in length from 11–15 aa residues (Fig. 1D). The shortest of the three epitopes (ESKIVELEEEL) was considered to be the epitope core. Vertebrate tropomyosins differ from this core in three or four positions; up to seven differences between the individual epitopes with their

homologous Pen a 1 sequences resulted in identities of 53–82% and similarities of 67–91%, respectively.

The C terminus of Pen a 1 (region 5) was identified as the largest of the five IgE-binding regions; it stretches from aa positions 247–284 (Fig. 1E). Within this region, three epitopes were identified. The first epitope of region 5 was centered around the core Pen a 1^{251–259} and was recognized by all four subjects; the core differed in three positions from nonallergenic, vertebrate tropomyosins. Two of three individually recognized epitopes were larger (12 residues) than the average epitope size (nine residues). Three of the four subjects tested reacted with the second epitope in region 5, Pen a 1^{266–273} (KYKSITDE). The sequence of epitope 5b was the minimal IgE-binding site for all four subjects; no other epitope showed stronger IgE Ab-binding activity. The third epitope in region 5 (Pen a 1^{273–281}) was only recognized by subject 4. Amino acid sequence identities with vertebrate tropomyosins were 54–57% (epitope 5a), 63% (epitope 5b), and 33% (epitope 5c); the corresponding similarities were 69–78% (epitope 5a), 88% (epitope 5b), and 56–78% (epitope 5c), respectively.

Degree of sequential difference between IgE-binding Pen a 1 amino acid sequences and nonallergenic vertebrate tropomyosins is not predictive for allergenicity of given Pen a 1 peptide

Sequence identities and similarities of individual Pen a 1 epitopes and epitope cores with homologous vertebrate tropomyosins were calculated. Overall identities and similarities ranged from 23–82 and 39–100%, respectively. A statistical comparison of sequence identities and similarities of individual epitopes with homologous vertebrate tropomyosin sequences was performed, and the calculated values were statistically compared by one-way ANOVA to identify vertebrate tropomyosins whose homologous sequences resemble the Pen a 1 epitopes. The differences in mean identities and similarities among the sequences were not great enough to exclude the possibility that the difference was due to random sampling variability; there was no statistically significant difference (sequence identities, $p = 0.997$; sequence similarities, $p = 0.807$). Thus, it was not possible to predict Pen a 1 epitopes based on the degree of differences between the amino acid sequence of Pen a 1 and those of nonallergenic tropomyosins.

Three substitutions may be necessary to abolish IgE binding of the major Pen a 1 epitopes

The results of the combinatorial substitution analyses demonstrated that none of the vertebrate sequences bound IgE Abs from shrimp-allergic subjects, confirming that vertebrate tropomyosins had limited or no cross-reactivity with the major Pen a 1 epitopes. Fig. 2 shows an example for combinatorial substitution analysis; the Pen a 1 epitope 5a, recognized by subject 10, was mutated into the homologous sequences of chicken α -tropomyosin (GalgaTM) and chicken β -tropomyosin (GalgbTM). All binding was compared with spot 1, the epitope sequence in Pen a 1. Most amino acid substitutions reduced or abolished IgE binding of subject 10 to epitope 5c.

Amino acid substitutions and their effect on the IgE-binding capability of modified Pen a 1 epitopes were categorized according to different criteria: 1) the minimal number of amino acid substitutions that render an epitope nonreactive, 2) the maximal number of amino acid substitutions that do not affect IgE Ab binding, and 3) the importance of amino acid position and nature of the substitution.

Twenty epitope-subject combinations were studied by combinatorial substitution analysis. When IgE Ab reactivity of all peptides carrying exactly one substitution was compared with that of wild-type Pen a 1 epitopes, only 57.6% of the peptides tested did

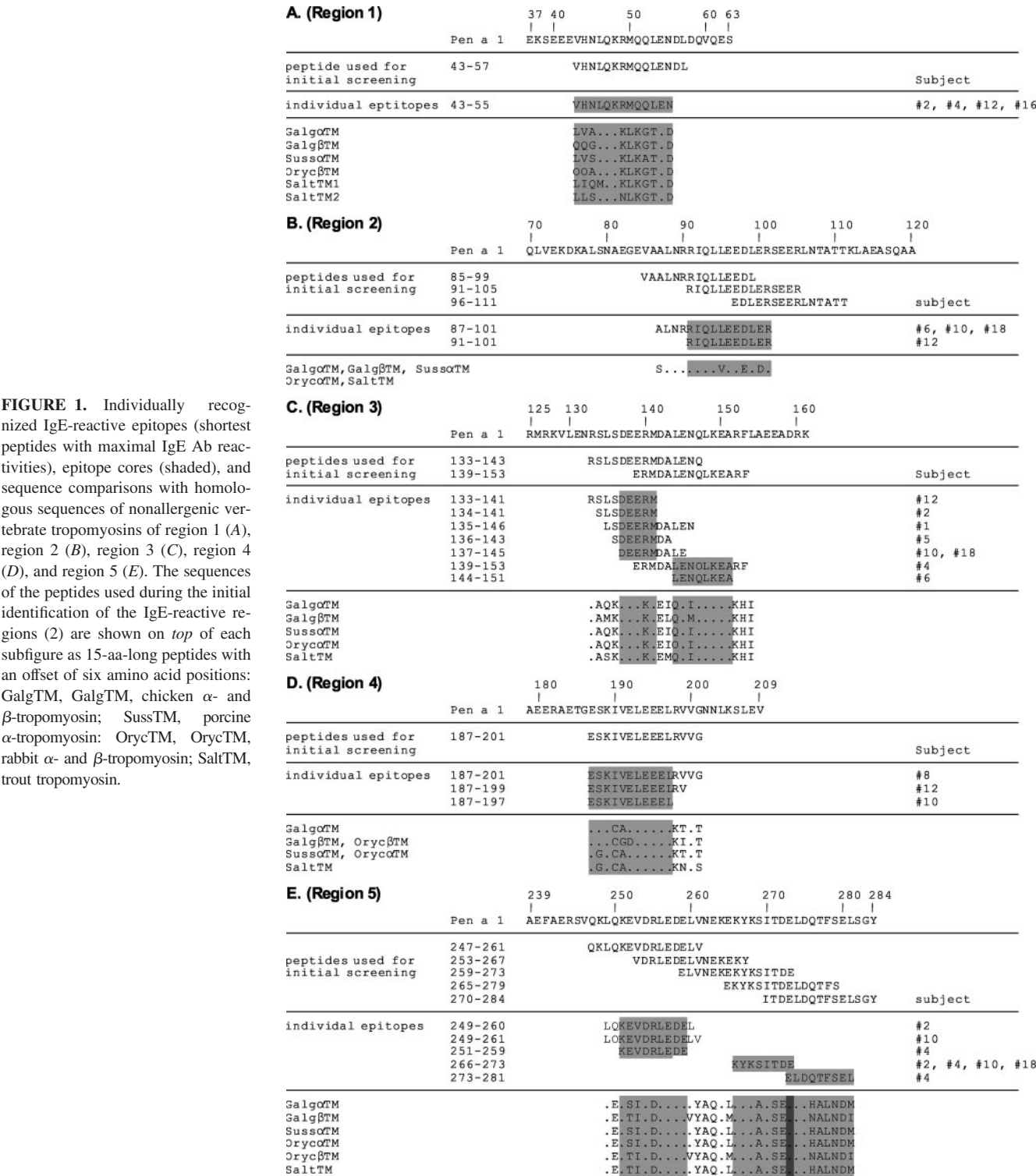


FIGURE 1. Individually recognized IgE-reactive epitopes (shortest peptides with maximal IgE Ab reactivities), epitope cores (shaded), and sequence comparisons with homologous sequences of nonallergenic vertebrate tropomyosins of region 1 (A), region 2 (B), region 3 (C), region 4 (D), and region 5 (E). The sequences of the peptides used during the initial identification of the IgE-reactive regions (2) are shown on top of each subfigure as 15-aa-long peptides with an offset of six amino acid positions: GalgTM, GalgTM, chicken α - and β -tropomyosin; SussoTM, porcine α -tropomyosin; OrycTM, OrycTM, rabbit α - and β -tropomyosin; SaltTM, trout tropomyosin.

but did not abolish, IgE binding, this number rose to 89.2%. The influence of the nature of the substitutions was considered by assessing the changes in IgE Ab reactivity associated with conservative and nonconservative substitutions. Nonconservative substitutions abolished IgE Ab reactivity at a rate of 60.9%; including peptides with reduced IgE binding increased this rate to 79.7%. An interesting and surprising finding was the occasionally increased IgE binding capacity of some mutated epitopes carrying one or two substitutions that originated from a vertebrate tropomyosin sequence even though the unmodified vertebrate tropomyosin

Table I. Critical amino acid positions and substitutions^a

Epitope	TMC Sera				Substitution		
					position:	from:	to:
1	2	4		16	<u>46</u>	<u>Leu</u>	<u>Met^c</u>
	2	4		16	<u>53</u>	<u>Leu</u>	<u>Thr^{nc}</u>
2			6	10	<u>95</u>	<u>Leu</u>	<u>Val^{nc}</u>
			6		<u>95</u>	<u>Leu</u>	<u>Phe^{nc}</u>
			6		<u>98</u>	<u>Gln</u>	<u>Glu^{nc}</u>
				10	<u>100</u>	<u>Glu</u>	<u>Asp^c</u>
3a	2			12	<u>135</u>	<u>Leu</u>	<u>Gln^{nc}, Met^{nc}, Ser^{nc}</u>
	1	2	5	10	<u>136</u>	<u>Ser</u>	<u>Lys^{nc}</u>
			5	10	<u>140</u>	<u>Arg</u>	<u>Lys^c</u>
	1		5	10	<u>142</u>	<u>Asp</u>	<u>Glu^c</u>
3b		4	6		<u>144</u>	<u>Leu</u>	<u>Gln^{nc}</u>
		4	6		<u>145</u>	<u>Glu</u>	<u>Asp^c</u>
4			8	12	<u>190</u>	<u>Val</u>	<u>Ser^{nc}</u>
			8	12	<u>199</u>	<u>Val</u>	<u>Asn^{nc}</u>
5a				10	<u>250</u>	<u>Gln</u>	<u>Glu^{nc}</u>
				10	<u>252</u>	<u>Glu</u>	<u>Ser^{nc}</u>
	2	4		10	<u>255</u>	<u>Arg</u>	<u>Asp^{nc}</u>
				10	<u>260</u>	<u>Leu</u>	<u>Val^c</u>
5b	2	4		10	<u>269</u>	<u>Ser</u>	<u>Phe^{nc}</u>
5c		4			<u>277</u>	<u>Thr</u>	<u>Ala^{nc}</u>
		4			<u>278</u>	<u>Phe</u>	<u>Leu^{nc}</u>
		4			<u>280</u>	<u>Glu</u>	<u>Gln^{nc}</u>
		4			<u>281</u>	<u>Leu</u>	<u>Met^{nc}</u>
		4			<u>281</u>	<u>Leu</u>	<u>Ile^c</u>

^a for every epitope the positions and substitutions are shown. The substitution at position 269, for example, substituting a phenylalanine (Phe) for a serine (Ser) abolished the IgE Ab reactivity to epitope 5b for sera of subject nos. 2, 4, 10, and 18. The substitutions introduced into wild-type Pen a 1 to generate the Pen a 1 mutant VR9-1 are underlined and the substitution type (conservative: e.g., Val^c nonconservative: e.g., Val^{nc}) is labeled.

sequences did not bind any IgE Abs. An example of this is shown in Fig. 2.

Critical amino acid positions identified for all eight major Pen a 1 epitopes

The mutated peptides were analyzed with regard to positions within individual epitopes that were critical for IgE Ab binding. A critical position was defined as a position that, when substituted, abolished the IgE Ab reactivity of any peptide containing this mutation. The results of this analysis, including the amino acids that have to be substituted, are summarized in Table I.

A single substitution was sufficient to abolish the IgE Ab reactivity of epitopes 2, 3a, 3b, and 5b, respectively, because all subjects showed strong IgE Ab reactivities to these epitopes. A single substitution at position 255 was sufficient to abolish IgE Ab reactivity in three of four subjects who reacted with epitope 5a. Because the IgE Ab reactivity of subject 12 was not abolished by this substitution, a substitution at position 260 was necessary to render this epitope nonreactive for all four subjects. To abolish the reactivity of the remaining three epitopes, epitopes 1, 4, and 5c, a combination of two substitutions was required to render these epitopes non-IgE binding.

Mutant of Pen a 1, VR9-1, showed a CD spectrum consistent with predominantly α -helical secondary structure

Based on the results of the combinatorial substitution analysis, the Pen a 1 mutant VR9-1 was generated, carrying 12 mutations at positions 46, 53, 95, 136, 145, 190, 199, 255, 260, 269, 277, and 278 (Table I). The majority (nine of 12) were nonconservative substitutions. Because only homologous amino acids from the non-allergenic tropomyosins were introduced into Pen a 1, the secondary structure of the mutated Pen a 1 variant VR9-1 was expected to be intact. This was confirmed by CD analysis; the CD spectrum of the Pen a 1 mutant VR9-1 was, even though not fully identical

with those of natural and recombinant wild-type Pen a 1, typical for α -helical proteins and indicated that the mutant probably adopted a folded state (Fig. 3A).

Markedly reduced allergenic potency of the Pen a 1 mutant

When humanized RBL-30/25 were sensitized with human IgE Abs, the mediator release induced by natural and recombinant Pen a 1 was always higher than that induced by the Pen a 1 mutant, VR9-1 (Fig. 4). The allergenic potency, calculated as the increase in VR9-1 concentration required to induce 50% of the maximal release in relation to wild-type Pen a 1, was reduced by 10- to 40-fold. The maximal release that was reached after challenging sensitized RBL-30/25 cells was often similar to that of wild-type Pen a 1. In only one case did the shape of the curve suggest that VR9-1 might not reach the level of mediator release of the wild-type Pen a 1 (Fig. 4D).

IgE Ab response of C57BL/6J, BALB/cJ, C3H/HeJ, and CBA/J mice to shrimp using B. pertussis, alum, and cholera toxin as adjuvants

To develop a mouse model of shrimp allergy that mimics the immune response of human shrimp-allergic individuals, mice of the strains BALB/cJ, C3H/HeJ, C57BL/6J, and CBA/J were sensitized with shrimp extract using cholera toxin (oral), aluminum hydroxide (i.p.), and B. pertussis (i.p.) as adjuvants. The β -hexosaminidase release upon allergen challenge with shrimp extract was measured. Of the four mouse strains tested, the strongest IgE response was observed in C3H/HeJ and CBA/J mice (Fig. 5, C and D), with some activity in sera from sensitized BALB/c mice (Fig. 5B). C57BL/6J mice did not produce appreciable amounts of IgE Abs with any of the adjuvants used, even though immunoblot analysis demonstrated that C57BL/6J mice produced shrimp-specific IgG Abs (data not shown). Alum and cholera toxin were the most effective adjuvants to induce IgE Abs to shrimp extract. However,

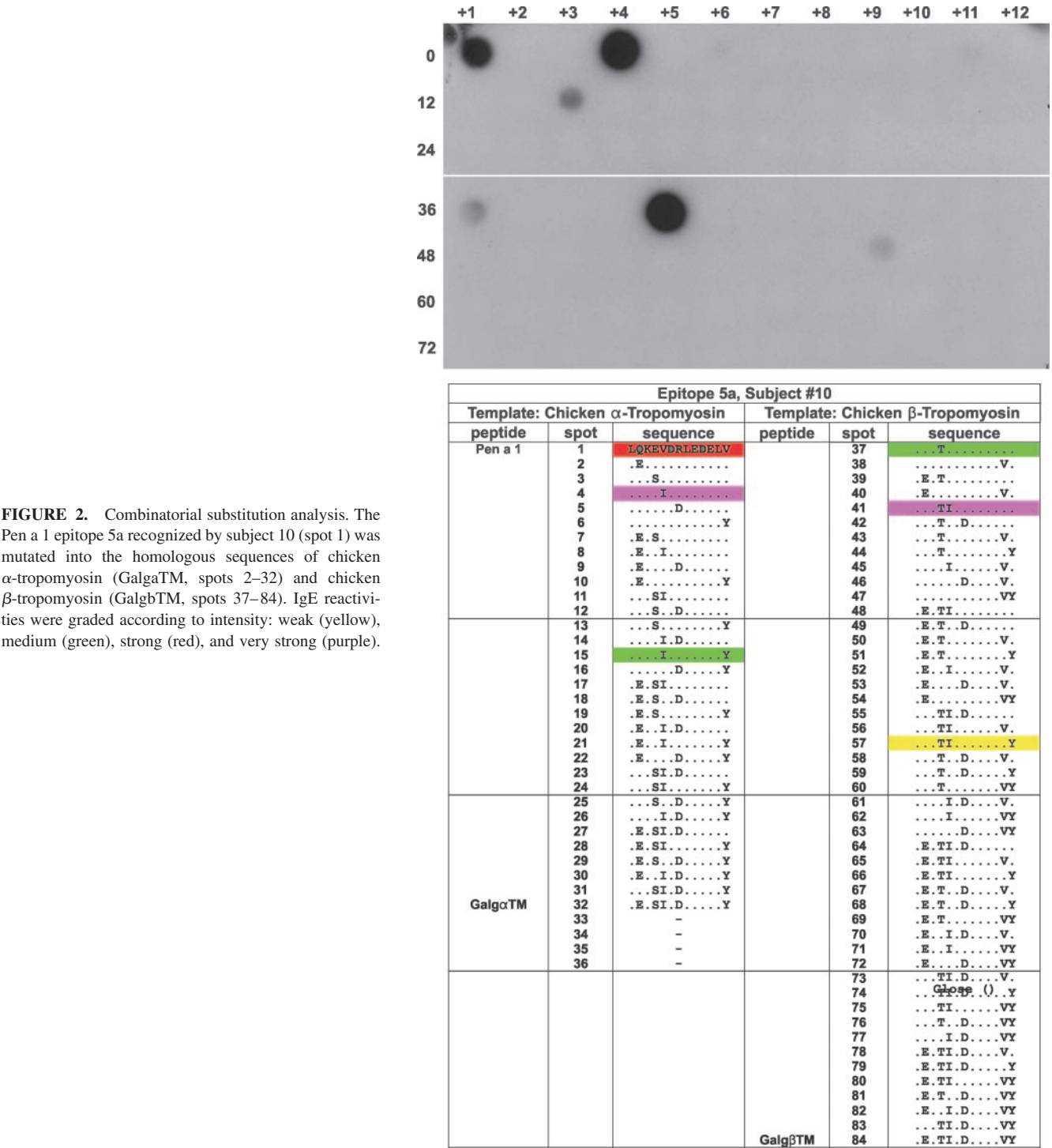


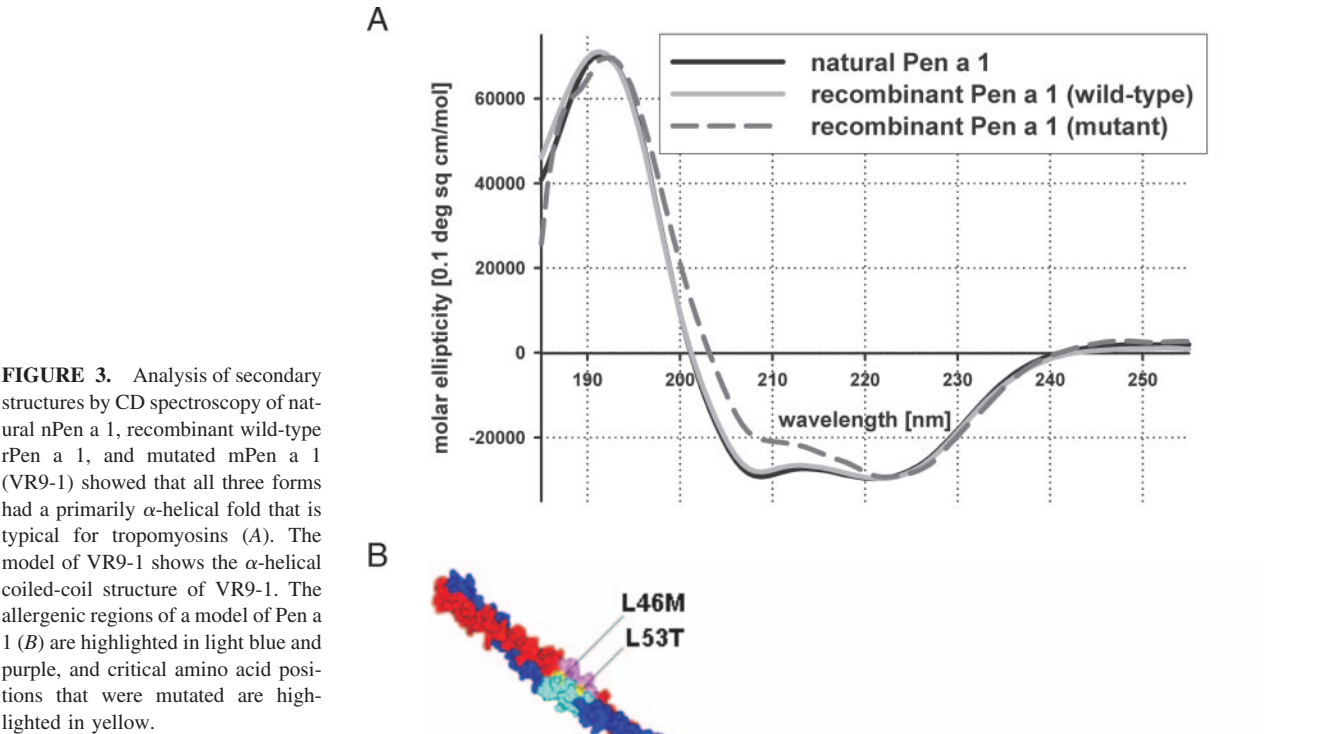
FIGURE 2. Combinatorial substitution analysis. The Pen a 1 epitope 5a recognized by subject 10 (spot 1) was mutated into the homologous sequences of chicken α -tropomyosin (GalgaTM, spots 2–32) and chicken β -tropomyosin (Galg β TM, spots 37–84). IgE reactivities were graded according to intensity: weak (yellow), medium (green), strong (red), and very strong (purple).

only C3H/HeJ and CBA/J mice that received cholera toxin as adjuvant produced IgE Abs to Pen a 1, and only C3H/HeJ produced Pen a 1-specific IgE using cholera toxin and aluminum hydroxide as adjuvants, respectively.

Mice and human allergic subjects produce IgE Abs to the same epitopes of Pen a 1

The sera of BALB/cJ, C3H/HeJ, and CBA/J were tested using natural Pen a 1, recombinant Pen a 1, and mutated Pen a 1 to challenge sensitized RBL-2H3 cells. In general, the sera from C3H/HeJ and CBA/J mice mediated the highest mediator release. However, sera from CBA/J mice that had been sensitized with

shrimp extract plus alum as adjuvant did not trigger any release of β -hexosaminidase. The responses induced by sera from BALB/c mice were weak; only the sera from animals sensitized with 1 mg of shrimp extract plus cholera toxin showed a weak response to Pen a 1 (release, <8.0%). In C3H/HeJ and CBA/J mice, the highest response to wild-type Pen a 1 was observed after sensitization with 1 mg of shrimp extract plus cholera toxin (Fig. 6, B and D), even though the responses of mice sensitized with 0.1 mg of shrimp extract plus cholera toxin were almost identical with those of mice sensitized with 1 mg of extract plus cholera toxin (Fig. 6, A and B). Oral sensitization of C3H/HeJ and CBA/J mice with the highest dose of 10 mg resulted in a reduced IgE Ab response; the



maximal releases of ~ 15 and 28% were reduced to 10 and 20% , respectively.

The mediator release that was induced by the mutant Pen a 1, VR9-1, was much lower than that mediated by wild-type Pen a 1 (Fig. 6). It never reached the 50% of maximal release caused by

wild-type Pen a 1. The reduction of allergenic potency of VR9-1 ranged from 30 -fold (Fig. 6B) to 1000 -fold (Fig. 6C). However, on the average, the allergenic potency of VR9-1 was reduced by ~ 300 -fold. For some combinations (e.g., C3H/HeJ orally sensitized with 10 mg of shrimp extract plus cholera toxin), the

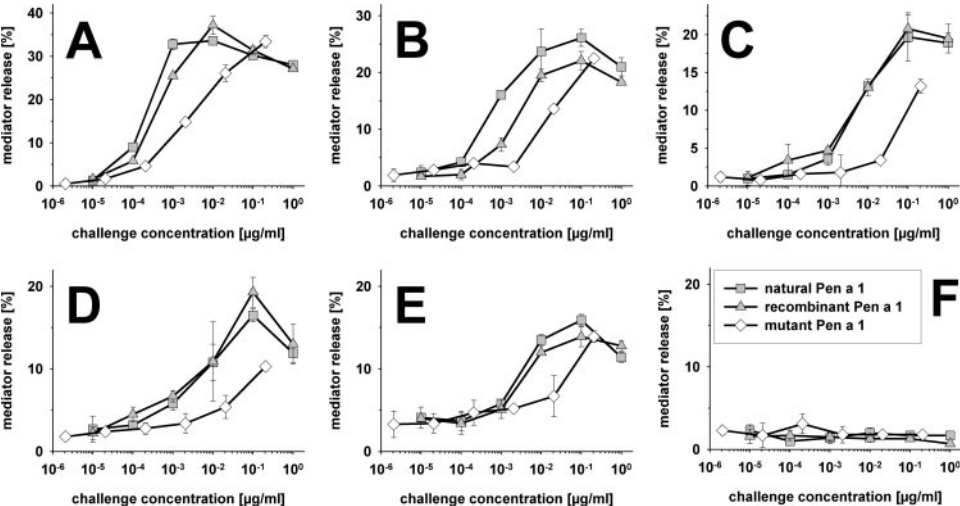
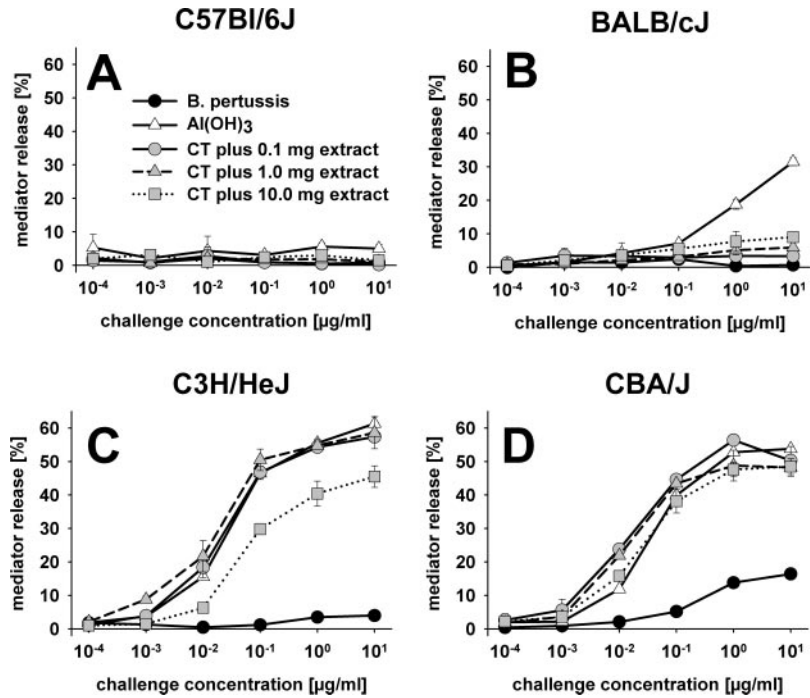


FIGURE 4. Allergenic potency of the Pen a 1 variant VR9-1 in the human system: mediator release from RBL-30/25 cells sensitized with sera from shrimp-allergic subjects (A, TMC-10; B, TMC-4; C, BC-1; D, BC-2, TMC-12) or serum of a nonallergic subject (F, PEI-) induced by wild-type (natural and recombinant Pen a 1, 10.0 pg/ml to 1.0 μ g/ml) and mutated Pen a 1 (2 pg/ml to 0.2 μ g/ml).

FIGURE 5. Mediator release from RBL-2H3 cells sensitized with sera from C57BL/6J, BALB/cJ, C3H/HeJ, and CBA/J using shrimp extract (100.0 $\mu\text{g/ml}$ to 10.0 $\mu\text{g/ml}$) as the challenge allergen. Shrimp extract was used as immunogen, and *B. pertussis*, alum, and cholera toxin were used as adjuvants.



reduction could not be calculated due to the low activity of all Pen a 1 forms or the abolished activity of the Pen a 1 mutant VR9-1.

Discussion

Development of safe and effective vaccines for allergen-specific immunotherapy aims to preserve the therapeutic properties of the allergen and to reduce the IgE Ab-binding capacity. Strategies for generation of hypoallergenic variants of allergens include denaturation (e.g., treatment with glutaraldehyde and formaldehyde) and alteration of conformation (e.g., by introduction of proline residues into α -helices or elimination of disulfide bonds). Furthermore, mutations at amino acid positions that are critical for IgE Ab binding

may result in allergen variants with reduced IgE Ab binding capacity.

Pen a 1 is an excellent model allergen and presents a rare opportunity for systematically studying the impact of mutations on the allergenic potency, because sequences of both allergenic and nonallergenic tropomyosins are known. A detailed analysis, as presented in this study, has not been performed with any other allergen. Based on the identified five major IgE Ab-binding regions of Pen a 1 (2), the aims were identification of individual epitopes, identification of amino acid positions critical for IgE, and generation and characterization of a Pen a 1 mutant with regard to its allergenicity.

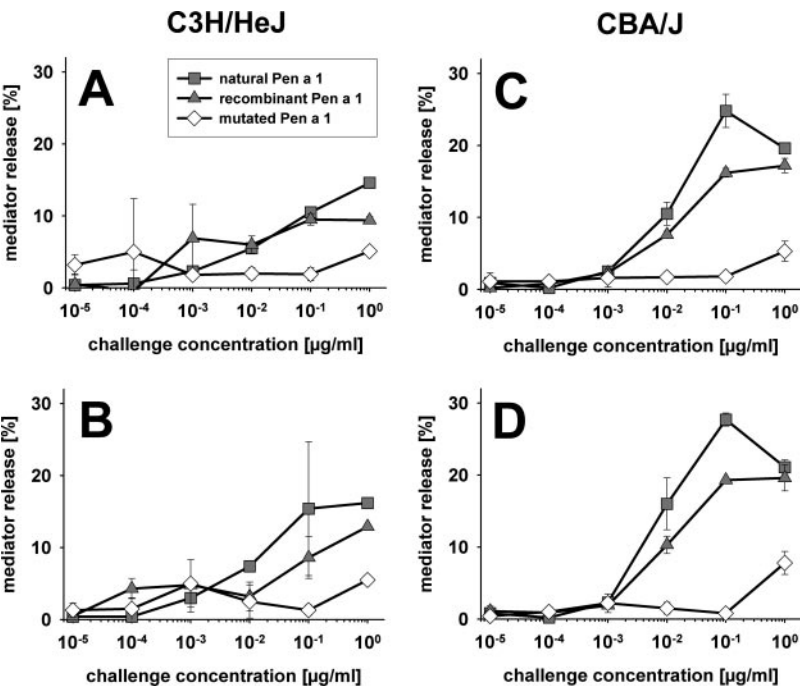


FIGURE 6. Allergenic potency of the Pen a 1 variant VR9-1 in the murine system: mediator release from RBL-2H3 cells sensitized with sera from C3H/HeJ, and CBA/J using natural wild-type, recombinant wild-type, and mutant Pen a 1 as the challenge allergen (10.0 pg/ml to 1.0 $\mu\text{g/ml}$). Shrimp extract (A and C, 0.1 mg/dose ; B and D, 1.0 mg/dose) was used as immunogen, and cholera toxin was used as adjuvant.

Epitope identification of food allergens provides information on the structural basis of protein allergenicity and offers the rationale of how to modify the allergen and render it non-IgE binding. To date, immunotherapy of food allergies is not the course of treatment, because potent allergenic foods, such as peanut, tend to induce severe adverse reactions (32, 33). However, reducing or abolishing the IgE Ab binding capacity and preserving T cell reactivity are currently discussed as a promising new strategy for the treatment of allergies (34, 35). Because Pen a 1 is responsible for at least 75% of the shrimp-specific IgE Abs (1), the IgE Ab-binding capacity of Pen a 1 has to be reduced substantially if it is to be used as a therapeutic reagent. Strategies, such as destroying the conformation by eliminating disulfide bonds are not feasible, because invertebrate tropomyosins do not have disulfide bonds, and processing, such as boiling of the shrimp, does not alter the structure of Pen a 1, as was shown by CD spectroscopy with immunologically active Pen a 1 purified from cooked shrimp (36).

The epitopes and amino acid substitutions rendering these epitopes nonbinding were identified by determining the IgE Ab reactivities of individual sera to overlapping peptides of various lengths. Ab binding sites are thought to be dependent on the native conformation of the allergen, and epitope identification using short overlapping peptides has been criticized in that this strategy does not produce meaningful results. However, this strategy has been used to identify major epitopes of peanut allergens (37–39), and studies of milk and peanut allergens suggested (40, 41) associations between IgE Ab reactivities to linear peptides and severity of disease.

Eight IgE-binding epitopes within the five major IgE Ab-binding regions were identified. As expected, the larger regions 3 and 5 contained two and three epitopes, respectively. Epitopes were centered on epitope cores; the lengths of the peptides recognized by IgE Abs of individual shrimp-allergic subjects varied considerably. Some individual epitopes were as short as eight amino acid residues, whereas others only showed their maximal IgE Ab-binding capacity at a length of 13 aa residues. The locations of individually recognized epitopes were identical for all subjects tested only for epitopes 1 and 5b; the other six epitopes varied considerably around the epitope cores.

The most frequently used strategy to identify amino acid positions critical for IgE Ab binding is to replace each amino acid with alanine (alanine scan) (38, 39, 42–44). Instead of an alanine scan, amino acids located at homologous positions of nonallergenic tropomyosins were replaced in the allergenic peptides. It was expected that such a variant would retain its natural conformation. The predominant effect of amino acid substitutions on Pen a 1 epitopes was the reduction of their IgE Ab binding capacity. Non-conservative substitutions in the center of the epitope sequence seemed to be the most effective way to reduce or abolish the IgE Ab capacity of Pen a 1 epitopes. This held true when the substitutions at the critical amino acid positions were analyzed. Non-conservative substitutions in the center of an epitope abolished IgE Ab binding of 44.4% of mutated Pen a 1 epitopes, whereas conservative substitutions in the peripheral parts abolished IgE Ab binding of only 13.9% of the modified epitopes. In addition to the position and nature of substitutions, their number was important. The mutational analysis showed that one substitution might be sufficient to abolish IgE Ab reactivity of a majority of modified peptides. However, ~40% of peptides carrying one substitution still bound some IgE Abs. At least three substitutions per epitope were necessary to abolish the IgE Ab reactivity in >99% of the mutated epitopes.

As expected, the secondary structure of the Pen a 1 mutant VR9-1 was not much affected by the 12 mutations, as was shown

by CD spectroscopy; thus, any changes in the allergenic potency of Pen a 1 mutant had to be attributed to mutations at the critical amino acid positions. To what degree would the IgE Ab-binding capacity be reduced if a mutated Pen a 1 molecule carried substitutions at all critical amino acid positions? In the case of shrimp tropomyosin, experiments by Shanti et al. (5) suggested that small peptides could be used to characterize epitopes of shrimp tropomyosin, because inhibition of IgE Abs specific for the tropomyosin from *Penaeus indicus*, Pen i 1, with peptides consisting of nine or 17 aa residues reached up to 50%. Our data obtained with the VR9-1 mutant provide the experimental proof for this concept.

Because several critical positions and substitutions were identified for a particular epitope, the smallest number of substitutions that abolished IgE Ab binding for the largest possible number of subjects who reacted to a particular epitope were mutated. The allergenic potency of the mutant Pen a 1 was evaluated by measuring mediator release from both wild-type and humanized RBL cells.

In our view, functional assays such as the RBL assays, are more meaningful tests to evaluate the changes in allergenic potency, and studies of the major allergen Bet v 1 have shown (45) that a significant reduction of allergenic potency measured by inhibition tests does not necessarily translate into reduced basophil activation capacity. Because most shrimp-allergic subjects reacted to major as well as minor IgE-binding sites (2), these substitutions may not abolish IgE Ab binding to Pen a 1 completely. Based on the reactivity scores to the 46 overlapping 15-aa-long, spanning the entire Pen a 1 sequence, Ayuso et al (2) estimated that mutations at the critical amino acid positions might reduce the average IgE binding capacity by at least 66%. The results of this study supported this estimation. The allergenic potency determined by mediator release assay showed a reduction of 10- to 40-fold, which translates into a reduction of allergenic potency of 90–98%. However, the maximal releases reached in the RBL assay were frequently similar to that of wild-type Pen a 1. These results suggested that IgE Abs bound to other epitopes, and/or that the substitutions only weakened the binding between IgE Ab and epitope. This idea is supported by previous results (2) showing that, in addition to the eight major epitopes that were mutated, additional individual epitopes exist.

Animal models of allergy and food allergy, in particular, are needed to test new vaccines and to better understand the mechanisms of sensitization, clinical manifestation, and underlying immunological mechanisms. The sensitization experiments with the different mouse strains using shrimp extract and different adjuvants (alum, *B. pertussis*, and cholera toxin) showed impressive strain differences with regard to the IgE Ab response. C3H/HeJ mice in combination with cholera toxin as adjuvant currently seem to be the best mouse model for food allergy. As shown in other studies comparing the allergen-specific induction of IgE Abs, C3H/HeJ mice showed an increased allergic response to peanut compared with BALB/c mice (46, 47). Seitzer et al. (48) demonstrated that C57BL/6 mice did not respond well to timothy grass pollen allergen. These published results and the data presented in this study seem to suggest that mouse strain selection is critical, and that relying on BALB/c mice as an experimental system in allergy research must be carefully evaluated in each case.

If an animal model were to be used for the development and/or optimization of an allergen vaccine for treating humans, the IgE Ab response of the animals should at least be directed toward epitopes that are relevant for human IgE Abs as well. The relevance of the established mouse model was demonstrated using wild-type Pen a 1 and the Pen a 1 mutant VR9-1 in the RBL

mediator release assay. Because the Pen a 1 mutant VR9-1 contained only substitutions at amino acid positions that were identified with human sera and had a drastically reduced allergenic potency in a mediator release assay with sera from mice that were sensitized with wild-type Pen a 1, it must be concluded that mice produce IgE Abs to the same epitopes as humans did. Because the reduction of allergenic potency was significantly higher in the murine system than in the human system, it may be tempting to speculate that the apparently restricted number of epitopes recognized by murine IgE Abs resulted from differences between the natural exposure of allergic subjects to shrimp and the artificial and relatively short immunization scheme used to sensitize animals. In contrast to mice, sensitization of shrimp-allergic subjects probably developed over a long period of time and exposure to additional tropomyosins other than shrimp tropomyosin. We have previously shown that IgE Ab reactivity to shrimp occurred in unexposed individuals (22); house dust mite or cockroach-allergic subjects showed significant IgE Ab reactivity to Pen a 1, even though all subjects were strictly observing Jewish tradition, which regards seafood as a non-Kosher food. Based on inhibition experiments, the IgE Ab reactivity to shrimp tropomyosin appeared to be due to cross-reacting tropomyosins of house dust mite and cockroach.

In conclusion, the Pen a 1 mutant VR9-1, carrying 12 aa substitutions in the eight major IgE-binding regions and/or epitopes, showed the characteristic α -helical structure of tropomyosins. The mediator release experiments demonstrated a significant reduction in the allergenic potency of VR9-1. This reduction was more pronounced in the murine model than in the human model. Thus, the epitopes relevant for human IgE Ab binding are also major binding sites for murine IgE Abs. The results of this study indicated that the murine model might be used to further optimize the Pen a 1 mutant for therapeutic use.

Disclosures

The authors have no financial conflict of interest.

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