

Relevance of IgE binding to short peptides for the allergenic activity of food allergens

Melanie Albrecht, MSc,^a Yvonne Kühne, MSc,^a Barbara K. Ballmer-Weber, MD,^b Wolf-Meinhard Becker, PhD,^c Thomas Holzhauser, PhD,^a Iris Lauer, PhD,^a Andreas Reuter, PhD,^a Stefanie Randow,^a Sabine Falk,^a Andrea Wangorsch, BSc,^a Jonas Lidholm, PhD,^d Gerald Reese, PhD,^a and Stefan Vieths, PhD^a *Langen and Borstel, Germany, Zürich, Switzerland, and Uppsala, Sweden*

Background: Analysis of IgE antibody binding to epitopes provides information for food allergy diagnosis and management and construction of hypoallergenic candidate vaccines, but the contribution of sequential epitopes to functionally relevant IgE binding is not fully understood. **Objectives:** We sought to study the impact of IgE-binding peptides described as major sequence epitopes in the literature on IgE-binding capacity of 2 selected food allergens.

Methods: IgE-binding peptides of the food allergens Ara h 2 (peanut) and Pen a 1 (shrimp) were identified. Synthetic soluble peptides representing the identified sequences were assessed for their capacity to inhibit IgE binding to the parent allergens by means of ELISA and in mediator release assay. The IgE-binding capacity of unfolded recombinant (r) Ara h 2 was analyzed. A hybrid tropomyosin carrying the IgE-binding regions of Pen a 1 grafted into the structural context of the nonallergenic mouse tropomyosin was applied in ELISA inhibition experiments and ImmunoCAP analysis.

Results: Although IgE-binding peptides representing sections of the allergen sequences were detected, no relevant capacity to inhibit the IgE binding to the parent allergen in ELISA or basophil activation test was observed. Unfolded rAra h 2 showed reduced IgE-binding capacity compared with folded

rAra h 2 and failed to elicit mediator release. Hybrid tropomyosin bound less IgE than rPen a 1 in ImmunoCAP analysis and revealed marginal inhibitory capacity.

Conclusion: Peptides identified as major sequence epitopes on Pen a 1 and Ara h 2 show little contribution to the IgE binding of the allergens studied. (J Allergy Clin Immunol 2009;123:123-133.)

Key words: SPOT technique, B-cell epitopes, allergy, Pen a 1, Ara h 2, IgE binding

Binding of IgE antibodies to specific regions of allergens is a prerequisite for triggering of type I allergic reactions. Such binding sites recognized by antibodies are called *B-cell epitopes* and are frequently categorized as conformational or discontinuous epitopes or as linear or sequential epitopes. In conformational epitopes, the amino acid residues recognized by the binding site of antibodies are distributed discontinuously over the protein sequence and occur in sterical proximity only when the protein is correctly folded. In linear epitopes, the amino acid residues recognized by the antibody make up a continuous section of the protein's amino acid sequence. However, in a folded protein, even a sequential epitope is forced into a certain conformation by the spatial relationship to the rest of the molecule.

In contrast with aeroallergens, many foods and their allergens undergo heat processing before consumption and enzymatic digestion in the stomach and the gut. The food proteins and their fragments encounter the immune system during the passage through the gastrointestinal tract. Because these modified allergens are not in their native conformation, they are more likely to induce immune responses to linearized stretches of the amino acid sequence of the allergens and/or food proteins. As a consequence, the humoral immune response to linear epitopes may be favored. Indeed, linear IgE-binding sequences have been identified on several food allergens, including major allergens of peanut, soybean, wheat, milk, egg, and shrimp.^{1,2}

For identification of linear B-cell epitopes with the SPOT technique, overlapping short synthetic peptides covering the whole amino acid sequence of the protein are coupled to a membrane. From the pattern of the IgE antibody binding to those peptides, the location of regions in the protein containing linear epitopes can be deduced.³⁻⁵

Knowledge of IgE-binding epitopes on allergens is thought to be important for diagnosis and treatment of allergic diseases. Several studies evaluated genetically engineered allergens with reduced IgE-binding capacity but retained T-cell-activating properties, as novel immunotherapeutics with improved safety

From ^athe Division of Allergology, Paul-Ehrlich-Institut, Langen; ^bthe Allergy Unit, Department of Dermatology, University Hospital Zürich; ^cthe Leibniz-Center for Medicine and Biosciences, Research Center Borstel; and ^dPhadia AB, Uppsala. Supported by Paul-Ehrlich-Institut, Langen, Germany.

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Reprint requests: Stefan Vieths, PhD, Paul-Ehrlich-Institut, Division of Allergology, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany. E-mail: viest@pei.de.

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Abbreviations used

CD: Circular dichroism
 MRW: Mean residue weight
 r: Recombinant
 RBL: Rat basophilic leukemia
 red/alk: Reduced and alkylated

and efficacy profiles.⁶⁻⁸ In particular, several candidate vaccines for the treatment of food allergy have been suggested on the basis of IgE epitope mapping data.⁹⁻¹²

In food allergy diagnosis, IgE binding to allergen peptides has been suggested as a clinically relevant biomarker predicting, for example, severity or persistence of the disease. For example, indications for such correlations were found for peanut, milk, and egg allergens by using peptides representing sequential epitopes.¹³⁻¹⁸ Battais et al¹⁹ found a correlation between specific epitope recognition patterns and different clinical manifestations of wheat allergy and celiac disease. Gliadins are rheomorphic proteins lacking a defined conformational structure and are therefore likely to induce antibody responses against sequential structures.

However, in particular when hypoallergenic allergen variants were designed on the basis of results of epitope disruption experiments using alanine scanning or other strategies of systematic mutation, only partial reduction of allergenicity was obtained in several cases.^{9,10,12,20} These observations lead to the suspicion that identified linear IgE-binding sequences may represent partial structures involved in the formation of a full epitope, but not the epitope itself. To generate initial evidence to test this hypothesis, we selected the peanut allergen Ara h 2 and the shrimp tropomyosin Pen a 1, which have been described as containing sequential epitopes, as model allergens. Ara h 2 was chosen as a model for an allergen with a complex 3-dimensional structure stabilized by disulfide bonds.²¹⁻²⁴ Pen a 1, a major shrimp allergen, served as the model for a protein containing predominantly α -helical structures and no disulfide bonds.^{25,26} Short peptides representing the main IgE-binding regions of the allergens were applied in competition experiments. If the IgE-binding sequences recognized by individual patients represented true epitopes, these structures would be able to inhibit IgE binding to the parent allergen significantly or, when applied as monovalent epitope, would be able to block mediator release from basophils stimulated with the full-length allergens. In addition, an attempt was made to convert a nonallergenic mammalian tropomyosin into a highly allergenic molecule by inserting the main linear epitopes of Pen a 1. The data consistently indicated a limited impact of short IgE-binding sequences for the allergenicity of the selected allergen molecules.

METHODS**Subjects and serum samples**

Sera of 2 subjects with peanut allergy and 2 subjects with shrimp allergy were used in this study. As negative control, a human serum pool of 6 subjects without allergy (19, 208, 230, 231, 233, 245) was used. The serum donors with peanut allergy had a case history of anaphylactic reactions to peanut. To ensure the selection of representative sera for this study, inclusion criteria were defined as follows: high specific IgE levels to peanut or shrimp extract and IgE to rAra h 2 or rPen a 1, respectively; severe symptoms described in clinical history; a sufficient amount of serum available; and linear IgE epitopes described for the according food allergen—leading to 2 characteristic sera from individuals with peanut allergy and shrimp allergy.

The 2 sera, designated 163 and JG3, were deposited in the serum collection of Paul-Ehrlich-Institut and Research Center Borstel, respectively. Both had specific IgE to peanut of >100 kU_A/L as determined by ImmunoCAP.

The serum donors with shrimp allergy had a case history of anaphylactic reaction to shrimp (PII-3) or shrimp allergy confirmed by a positive double-blind placebo-controlled food challenge result (PI-1). Both donors were highly sensitized to shrimp, as evidenced by skin and/or specific IgE test results (see this article's Table E1 in the Online Repository at www.jacionline.org). IgG was removed from all sera used in the experiments by 2 passages through Protein G Sepharose (GE Healthcare, Chalfont St Giles, United Kingdom) according to manufacturer's instructions, and the absence of allergen-specific IgG was confirmed by ELISA.

Allergen preparations

Recombinant shrimp tropomyosin (rPen a 1, accession no. DQ151457) was cloned and expressed as described elsewhere.^{27,28} Recombinant Ara h 2 (accession no. L77197 without leader sequence) was cloned and expressed in a folded form as described elsewhere.²⁹ Murine tropomyosin (AAA03725) and a tropomyosin hybrid consisting of the murine tropomyosin backbone grafted with sequences of Pen a 1, previously identified as major IgE antibody binding regions,¹⁰ were obtained by expression of synthetic genes (GENEART, Regensburg, Germany) in a heat-inducible bacterial expression system and protein purification as described (for amino acid sequence, see this article's Fig E1 in the Online Repository at www.jacionline.org).³⁰

To obtain unfolded rAra h 2, the allergen was denatured in 5.3 mol/L urea and 0.13 mol/L TRIS-HCl pH 9.0, reduced with 28 mmol/L dithiothreitol at 55°C for 45 minutes and alkylated at room temperature for 20 minutes in the dark by adding iodacetamide to a final concentration of 55.5 mmol/L. Because of a lack of disulfide bonds, reduction and alkylation experiments were not performed with Pen a 1.

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed on a J-180 S spectropolarimeter (Jasco, Groß-Umstadt, Germany) with constant nitrogen flushing at 20°C. Before analysis, recombinant allergens were dialyzed against 10 mmol/L KH₂PO₄/K₂HPO₄ buffer (pH 7.4), and the protein concentration was adjusted to 5.2 μ mol/L. 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 to 255 nm at 50 nm/min. Ten scans were accumulated, and the mean residue weight ellipticity (Θ_{MRW}) was calculated.

Immunoblot analysis

Recombinant Ara h 2 (1 μ g/cm) or reduced and alkylated rAra h 2 (1 μ g/cm) was separated by SDS-PAGE on 13% total acrylamide (T), 2.67% cross-linker (C) gels under nonreducing conditions using the Laemmli buffer system and transferred onto nitrocellulose membranes (pore size, 0.2 μ m; Schleicher & Schuell, Dassel, Germany) by semidry blotting.³¹ After blocking of free binding sites on the membrane (TBS buffer containing 0.3% Tween 20), the membranes were incubated with sera from patients with peanut allergy (1:6.7 in TBS buffer containing 0.05% Tween 20, 0.1% BSA) overnight. The bound antibodies were detected with alkaline phosphatase-labeled anti-human-IgE (EAST test kit; Allergopharma, Reinbek, Germany; 1:2 in TBS buffer containing 0.05% Tween 20, 0.1% BSA) and visualized by adding the combined substrate 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.³²

Synthesis and characterization of peptides

Overlapping peptides (15 amino acid residues, offset of 3 amino acid residues) of Ara h 2 were synthesized with the Merrifield synthesis (N-alpha-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids) on derivatized cellulose membrane according to the manufacturer's instructions (Intavis Bio-analytical Instruments, Cologne, Germany). At the end of the synthesis, the side chain protection groups of the amino acids were deprotected with a

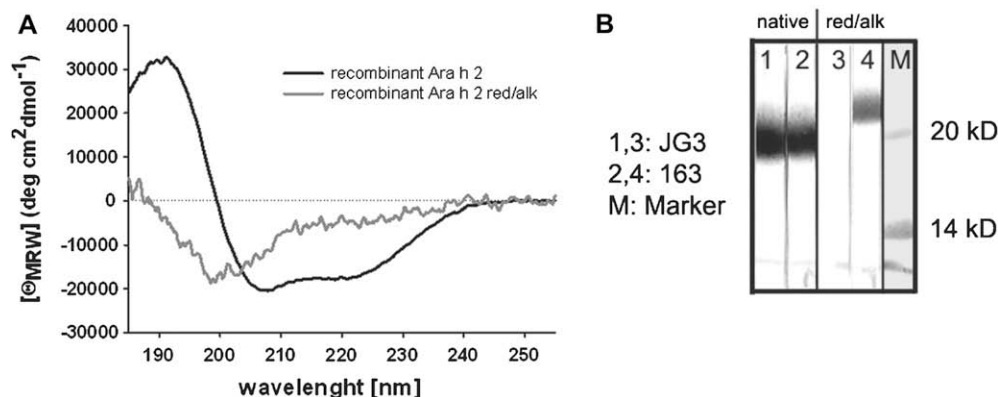


FIG 1. IgE reactivity of different rAra h 2 preparations. **A**, CD spectra of native and denatured (red/alk) recombinant Ara h 2. **B**, IgE reactivity of sera from 2 individuals with peanut allergy with native rAra h 2 compared with denatured (red/alk) Ara h 2 was detected in Western blot analysis.

mixture of dichloromethane/trifluoroacetic acid/triisopropylsilane/water in volume proportions 1:1:0.06:0.04.

Soluble peptides were synthesized as amides on the ends of polyoxyethylene linkers after activation of the amino acids with O-benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluoro-phosphate according to the manufacturer's instructions (Intavis Bioanalytical Instruments, Cologne, Germany). The linkers were cleaved off after synthesis with trifluoroacetic acid/triisopropylsilane/water (92.5:5:2.5). The peptides were precipitated with tert-butyl-methyl-ether, dissolved in water/acetonitrile (80/20), and lyophilized before storage.

The identity of all produced soluble synthetic peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The peptides were diluted 1:2000 in 25 mmol/L NH_4HCO_3 and analyzed as described elsewhere.²⁸ A maximum difference of 30 ppm between experimental and theoretical peptide masses was accepted as proof of identity. Because impurity as well as solubility problems might be an issue influencing the results obtained with these peptides, solubility was checked by means of dot immunoblot analysis and inhibition experiments using peptide solutions (see this article's Figs E2 and E3 in Online Repository at www.jacionline.org).

Soluble HPLC-purified and mass spectrometry-confirmed Pen a 1 peptides were purchased from Intavis Bioanalytical Instruments.

Immunodetection of IgE binding to immobilized sequential peptides

The cellulose membrane was blocked overnight with TRIS-buffered saline containing 0.05% Tween 20, 5% sucrose, and SPOTs Blocking Buffer (Sigma-Genosys, The Woodlands, Tex). The patient sera were incubated overnight at a dilution of 1:80 in blocking buffer. For a specific detection of IgE, the membrane was washed and incubated 1 hour with goat antihuman-IgE antiserum (KPL, Gaithersburg, Md) and, after washing with TRIS-buffered saline + 0.05% Tween, 1 hour with a horseradish peroxidase conjugate rabbit antigoat antibody (Dianova, Hamburg, Germany). After washing, the membrane was incubated with LumiGlo Reserve chemiluminescence substrate (Medac, Hamburg, Germany), and the chemiluminescence was detected with LumiImager (Lumi F1; Roche Diagnostics, Mannheim, Germany).

Inhibition IgE-ELISA

For IgE-ELISA inhibition experiments, Maxisorp plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 125 ng/mL rAra h 2 or 50 ng/mL rPen a 1 dissolved in 50 mmol/L sodium carbonate buffer, pH 9.6. IgG-depleted patients' sera in adequate dilutions were incubated with dilution series of inhibitors overnight at room temperature. Allergen-specific IgE was detected with biotinylated antihuman-IgE antibody (Pharmingen, Heidelberg, Germany) and horseradish peroxidase-labeled streptavidin (Calbiochem, Darmstadt,

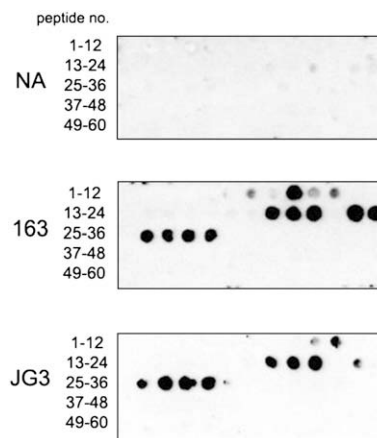


FIG 2. Identification of IgE-reactive peptides of Ara h 2 with epitope mapping of synthetic peptides on SPOT membrane. Chemiluminescence detection of pool serum of patient without allergy (NA) and sera of patient with peanut allergy, 163 and JG3.

Germany). The substrate was 3,3',5,5'-tetramethylbenzidine (Roth, Karlsruhe, Germany). The percentage of inhibition was calculated as follows:

$$\text{inhibition} = \frac{OD_{\text{uninhibited}} - OD_{\text{inhibited}}}{OD_{\text{uninhibited}} - OD_{\text{buffer}}} \times 100 [\%]$$

β-Hexosaminidase release from humanized RBL cells

The mediator release assay followed the protocol established by Vogel et al.³³ Briefly, RBL cells expressing the α-chain of human FcεRI were sensitized with human sera (1:40, 1:70) overnight. After washing, cells were stimulated with antigen (rPen a 1, rAra h 2, reduced and alkylated [red/alk] rAra h 2) in dilution series. Specific mediator release was quantified by measuring β-hexosaminidase activity and expressed as percent of the total β-hexosaminidase content, obtained by lysing cells with Triton X-100 (Sigma-Aldrich, Steinheim, Germany). For inhibition, sera were incubated overnight at room temperature with inhibitors in 20-fold molar excess of the maximal antigen stimulation amount before sensitization of RBL cells.

Determination of specific IgE by ImmunoCAP

Specific IgE levels were determined by using an ImmunoCAP 250 instrument (Phadia AB, Uppsala, Sweden) according to the manufacturer's instructions. Experimental ImmunoCAP tests carrying rPen a 1, recombinant mouse

TABLE I. IgE-reactive peptides in epitope mapping array of Ara h 2 on spots membrane

Peptide number	Peptide sequence	IgE-reactive region	Amino acid position	IgE reactivities to synthetic peptide		Gruber et al, 2004 ⁴⁰ Epitope number	Stanley et al, 1997 ²¹ Peptide number
				163	JG3		
6	A-A-H-A-S-A-R-Q-Q-W-E-L-Q-G-D	I	16–30	+	–	—	1
8	R-Q-Q-W-E-L-Q-G-D-R-R-C-Q-S-Q			+	–	—	2
9	W-E-L-Q-G-D-R-R-C-Q-S-Q-L-E-R			+	+	3	3
10	Q-G-D-R-R-C-Q-S-Q -L-E-R-A-N-L	II	22–42	+	+	6	6
19	R-D-E-D-S-Y-G-R-D-P-Y-S-P-S-Q			+	+		
20	D-S-Y-G-R-D-P-Y-S-P-S-Q-D-P-Y			+	+		
21	G-R-D-P-Y-S-P-S-Q -D-P-Y-S-P-S	III	55–75	+	+	6,7	7
23	P-S-Q-D-P-Y-S-P-S-Q-D-P-D-R-R			+	+	7	
24	D-P-Y-S-P-S-Q-D-P-D-R-R -D-P-Y			+	–	—	
25	S-P-S-Q-D-P-D-R-R-D-P-Y-S-P-S	IV	67–84	+	+	—	—
26	Q-D-P-D-R-R-D-P-Y-S-P-S-P-Y-D			+	+	—	—
27	D-R-R-D-P-Y-S-P-S-P-Y-D-R-R-G			+	+	—	—
28	D-P-Y-S-P-S-P-Y -D-R-R-G-A-G-S	V	73–96	+	+	—	—
				+	+	—	—
				+	+	—	—

TABLE II. IgE-reactive peptides in epitope mapping array of Pen a 1 on spots membrane

Peptide number	Peptide sequence	IgE-reactive region	Amino acid position	IgE reactivities to synthetic peptide		Reese et al, 2005 ¹⁰ Epitope region
				PII-3	PI-1	
1	M-D-A-I-K-K-K-M-Q-A-M-K-L-E-K	I	1–21	+	+	—
2	I-K-K-K-M-Q-A-M-K-L-E-K-D-N-A			+	+	—
3	K-M-Q-A-M-K-L-E-K -D-N-A-M-D-R			+	+	—
4	A-M-K-L-E-K-D-N-A-M-D-R-A-D-T	II	10–30	+	–	—
5	L-E-K-D-N-A-M-D-R-A-D-T-L-E-Q			+	+	—
6	D-N-A-M-D-R-A-D-T -L-E-Q-Q-N-K			+	–	—
9	L-E-Q-Q-N-K-E-A-N-N-R-A-E-K-S	III	25–39	+	–	—
13	E-K-S-E-E-E-V-H-N-L-Q-K-R-M-Q			+	+	A
14	E-E-E-V-H-N-L-Q-K-R-M-Q -L-E			+	+	—
74	A-Y-K-E-Q-I-K-T-L-T-N-K-L-K-A	V	220–240	+	–	—
75	E-Q-I-K-T-L-T-N-K-L-K-A-A-E-A			+	–	—
76	K-T-L-T-N-K-L-K-A-A-E-A -R-A-E			+	–	—
87	E-L-V-N-E-K-E-K-Y-K-S-I-T-D-E	VI	259–279	+	–	—
88	N-E-K-E-K-Y-K-S-I-T-D-E-L-Q-T			+	–	E
89	E-K-Y-K-S-I-T-D-E -L-Q-T-F-S-E			+	–	—

tropomyosin, or hybrid tropomyosin were prepared as described elsewhere.^{34,35} See this article's additional [Methods](#) in the Online Repository at www.jacionline.org.

RESULTS

Destruction of antigen conformation reduces IgE reactivity of patients' sera to Ara h 2

To assess whether the folding of Ara h 2 is important for IgE binding, denatured rAra h 2 was prepared by reduction and alkylation. Comparison with folded rAra h 2 by means of CD analysis revealed that reduction and alkylation led to destruction of secondary structure (Fig 1, A). Western blot analyses of both preparations with sera of 2 individuals with peanut allergy showed that the strong IgE reactivity of the sera to the native allergen was completely abrogated (Fig 1, B, lane 3) or reduced (Fig 1, B, lane 4) by the denaturing treatment of the protein.

Identification of IgE reactive peptides of Ara h 2 and Pen a 1

IgE-binding epitopes of peanut allergen Ara h 2 and shrimp allergen Pen a 1 were mapped by using sets of overlapping synthetic peptides. Sera of subjects with peanut allergy JG3 and 163 (Fig 2) and subjects with shrimp allergy PI-1 and PII-3 displayed strong IgE binding to the synthetic peptides of Ara h 2 and Pen a 1, respectively. The serum pool of the patient without allergy showed no reactivity on spot membrane (Fig 2).

The amino acid sequences of the peptides recognized by patients' IgE antibodies are summarized in Tables I and II. The recognized peptides were assigned to IgE reactive regions as described¹⁰ and, within each region, amino acid sequences shared between the peptides are illustrated in *bold*. Tables I and II also indicate previously described IgE-binding epitopes of Ara h 2 and Pen a 1, among those identified here.

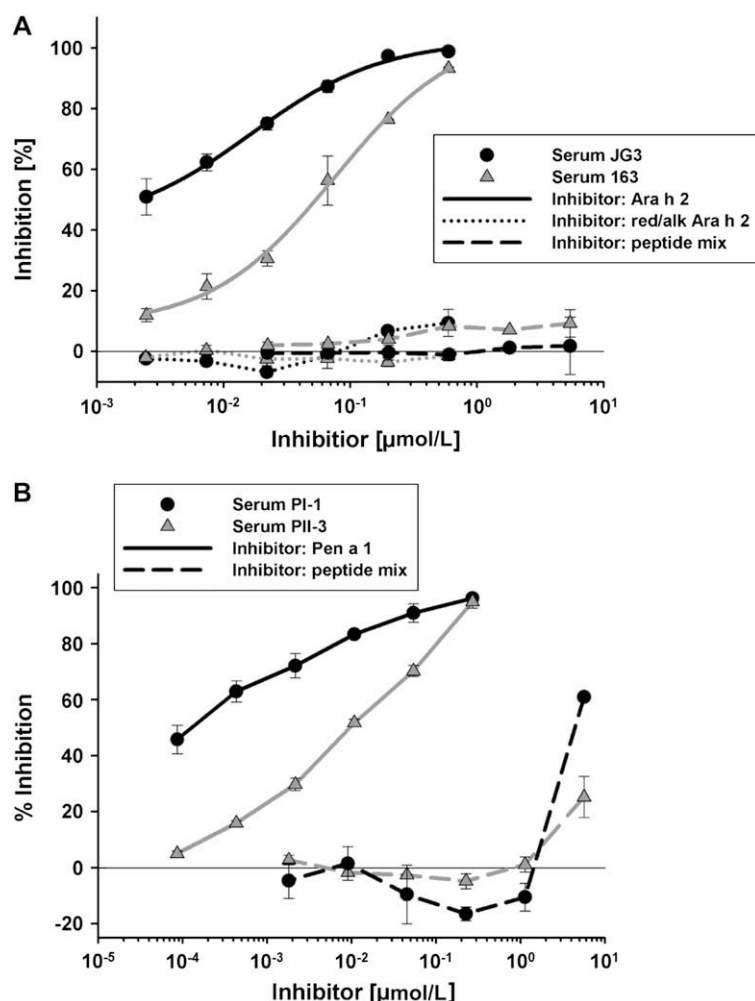


FIG 3. Inhibition of IgE-binding with short peptides in ELISA with rAra h 2 and rPen a 1. Binding of IgE from patients' sera (A, JG3, 163; B, PI-1, PII-3) to allergens coated on microtiter plates (A, rAra h 2; B, rPen a 1) was inhibited by coincubation with the parent allergen (A, rAra h 2; B, rPen a 1), denatured allergen (A, red/alk rAra h 2) or synthetic short peptides representing the main sequential IgE-binding regions identified in this study.

IgE reactivity of sera in ELISA is not inhibited by short peptides or denatured antigen

To analyze the relevance of IgE-binding sequences identified with the SPOT technique, IgE ELISA inhibition experiments were performed. Incubation of IgG-depleted sera from 2 individuals with peanut allergy with a mixture of soluble short peptides representing the identified sequences (Table I) did not interfere detectably with IgE binding to rAra h 2 coated on the solid phase (Fig 3, A). Moreover, no inhibition of IgE binding to rAra h 2 was detectable after incubation with the denatured protein, whereas incubation with folded rAra h 2 caused strong inhibition in a dose-dependent manner leading to 100% reduction of the optical density obtained with sera that were not inhibited (Fig 3, A).

IgE binding to shrimp tropomyosin rPen a 1 on the solid phase was inhibited in similar experiments by incubation of IgG-depleted sera from individuals with shrimp allergy with recombinant Pen a 1 (Fig 3, B). A mixture of peptides, representing the Pen a 1 sequences identified in Table II, was able to cause a certain interference with IgE binding to rPen a 1, but only when applying a 20-fold molar excess of peptides compared

with the amount of Pen a 1 required to achieve a 100% inhibition in this test (Fig 3, B).

IgE specific mediator release is not inhibited by short peptides or denatured antigen

IgE binding to allergens in ELISA reveals little information about their capacity to cross-link receptor-bound IgE on effector cells. Hence, the functional impact of short peptides in inhibiting IgE binding was analyzed in humanized RBL cells, an *in vitro* model of the type I allergic reaction, in which mediator release after receptor cross-linking is quantified by measuring the amount of the surrogate marker β -hexosaminidase.³³

Preincubation of IgG-depleted patients' sera with a mixture of peptides representing the sequences identified in Table I before sensitization of RBL cells did not affect the mediator release after stimulation with recombinant Ara h 2, and stimulation with denatured rAra h 2 did not elicit any mediator release (Fig 4, A).

Preincubation of sera with peptides representing the IgE-binding Pen a 1 sequences identified in Table II did not reduce the capacity of the parent allergen to cause mediator release in the RBL assay (Fig 4, B).

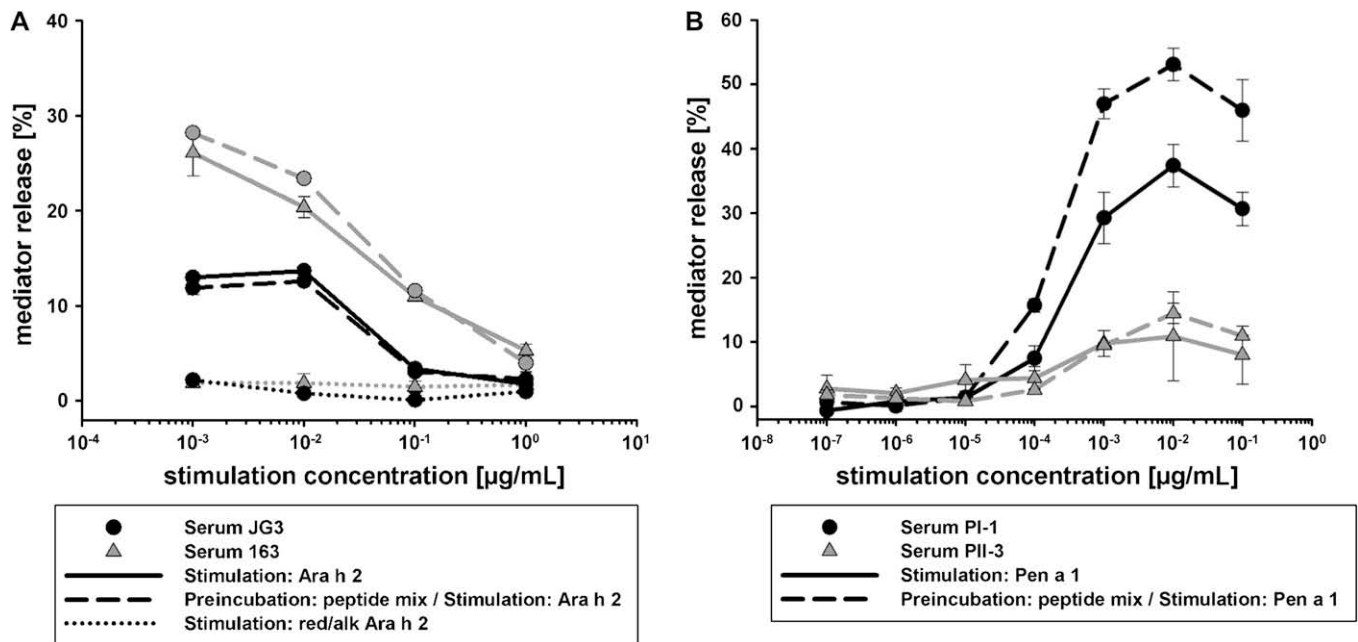


FIG 4. Inhibition of mediator release from humanized RBL cells with short peptides. Mediator release from humanized RBL cells sensitized with human sera (**A**, JG3, 163; **B**, PI-1, PII-3) with (dashed line) or without (solid line) preincubation with short peptides representing the main sequential IgE-binding regions of the parent allergen (**A**, rAra h 2; **B**, rPen a 1) in response to the recombinant allergen (**A**, rAra h 2; **B**, rPen a 1). The dotted line in **A** reflects the mediator release in response to denatured rAra h 2.

IgE binding to rPen a 1 is only partially inhibited by hybrid mouse tropomyosin

To assess the impact of a defined structural context on the IgE-binding potential of short epitope peptides, a hybrid tropomyosin was generated in which the 5 main IgE-binding regions of allergenic Pen a 1^{10,36} were grafted into the nonallergenic mouse tropomyosin. α -Helical folding, typical for tropomyosins, of the hybrid molecule, as well as of Pen a 1 and the mouse tropomyosin, was confirmed by means of CD spectroscopy (Fig 5).

The capacity of the hybrid tropomyosin and the nonallergenic mouse tropomyosin to inhibit IgE binding to adsorbed rPen a 1 was examined in inhibition ELISA experiments. Coincubation of patients' sera with mouse tropomyosin caused inhibition only at the highest inhibitor concentration (Fig 6). Similar results were obtained with the hybrid tropomyosin serving as inhibitor, although some inhibitory effect (about 20%) on binding of IgE from serum PI-1 was also observed at lower inhibitor concentrations.

Because some IgE reactivity to mouse tropomyosin seemed to occur among the subjects with shrimp allergy tested, direct binding of IgE antibodies to rPen a 1, mouse tropomyosin, and the hybrid was studied by ImmunoCAP measurements (sera diluted 1/10). Recombinant Pen a 1 showed about 3 times higher IgE binding (PI-1, 6.8 kU_A/L; PII-3, 15.9 kU_A/L) than the mouse tropomyosin (PI-1, 1.82 kU_A/L; PII-3, 5.66 kU_A/L), whereas the hybrid protein showed intermediate IgE-binding activity (PI-1, 4.32 kU_A/L; PII-3, 12.2 kU_A/L).

DISCUSSION

Identification of IgE antibody-binding B-cell epitopes of allergens has gained increasing interest in recent years in view of novel options for therapy and diagnosis of allergic diseases.^{13,16,37} For experimental reasons, the number of published

studies on linear B-cell epitopes by far exceeds those on identification of conformational epitopes. However, the contribution of the identified sequential epitopes for IgE binding to folded allergens and their impact on Fc ϵ R cross-linking events is not fully understood and has to be assessed for each allergen or allergen group of interest.

In this study, we investigated the contribution of IgE-binding short peptides, commonly described as sequential epitopes, to the IgE-binding capacity of 2 selected food allergens, Ara h 2 from peanut and Pen a 1 from shrimp. In addition, the potential of these peptides to act as monovalent IgE epitopes was investigated by an attempt to block mediator release from basophils passively sensitized with IgE from patients presenting serum IgE against these structures. Ara h 2 is a monomeric protein containing 8 cysteines that contribute to the 3-dimensional folding by forming 4 intramolecular disulfide bonds.^{38,39} Pen a 1 forms a homodimer of 2 parallel α -helices in a coiled-coil manner. It lacks cysteine residues and thus does not possess disulfide bonds.²⁵ Because both of these allergens are well characterized and described to contain linear IgE-binding epitopes,^{10,21,40} they were chosen as model allergens for this study.

Soluble short peptides composing IgE-binding regions of Ara h 2 and Pen a 1, identified by SPOT analysis, were assessed for their ability to inhibit IgE binding to their parent allergen in ELISA and allergen-stimulated mediator release from a basophil cell line. In addition, an unfolded form of rAra h 2, representing all possible sequential epitopes of the allergen, was used in the experiments.

Analysis of IgE binding to Pen a 1 and Ara h 2 peptides on SPOT membranes led to identification of a similar set of peptides as reported in other publications.^{10,21,40} The fact that some of the reported sequences were not reactive with IgE in this study and vice versa may be a result of both to the particular patient sera used and the level of sensitivity of the detection system used.

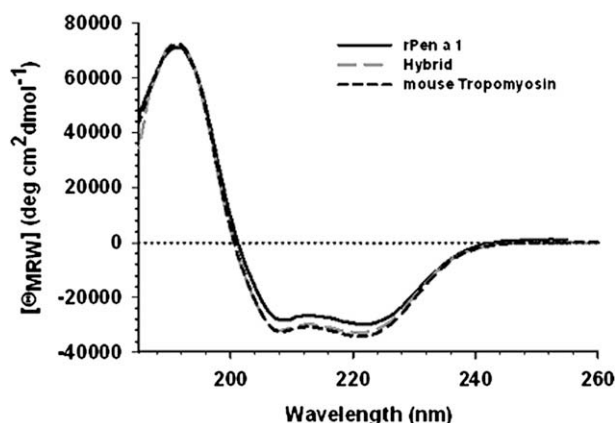


FIG 5. Secondary structure of tropomyosin. Recombinant Pen a 1 (black, solid line), Pen a 1-hybrid (gray, long dashes) and mouse tropomyosin (black, short dashes) were applied to CD spectroscopy to confirm their secondary structure.

The clinical relevance of peptides that can only be identified with highly sensitive systems, indicating low affinity binding, may be questioned.

A mixture containing exactly the soluble short peptides that were identified by SPOT analysis was applied to ELISA inhibition experiments to examine their ability to compete in solution with the native allergen for IgE binding to immobilized rAra h 2 or rPen a 1, respectively. All peptides used as inhibitors were selected on the basis of proven IgE antibody reactivities by means of dot blot immune detection and in part by inhibition experiments (Fig E2 and E3).

In the case of rAra h 2, even though each peptide in the mixture was present at molar concentrations as much as 20-fold higher than that of native rAra h 2, no inhibitory effect was observed. In the RBL cell assay, no inhibitory effect on mediator release was caused by rAra h 2 peptides.

Similar results were obtained with unfolded rAra h 2, which, in comparison to native rAra h 2, displayed reduced IgE-binding capacity in Western blot analysis and drastically reduced capacity to induce mediator release from humanized RBL cells. These results indicate a predominance of IgE recognition of conformational epitopes on Ara h 2 and minor involvement of sequential epitopes. Moreover, the partial reduction of IgE binding in Western blot analysis, in contrast with the nearly complete abrogation of mediator release in RBL assay with unfolded rAra h 2, demonstrates how careful conclusions regarding allergenic activity must be drawn when the data were obtained with techniques that exclusively address antibody-antigen interaction. Studies with the major carrot allergen Dau c 1 and the major birch pollen allergen Bet v 1 also report that destruction of conformation leads to reduced IgE binding.^{41,42} It is possible that the conformation of Ara h 2, stabilized by disulfide bonds, acts to suppress the proteolytic fragmentation of the protein during digestion and thereby extensive sensitization to such submolecular determinants.^{38,43} This is corroborated by the finding that all IgE-binding sequences identified here are located on the surface of Ara h 2 (modeling of Ara h 2 *in silico*; see this article's Fig E4 in the Online Repository at www.jacionline.org). Therefore, it seems highly unlikely that the identified IgE-binding peptides might represent hidden epitopes that are not accessible in the native folded allergen, preventing the inhibition of IgE binding

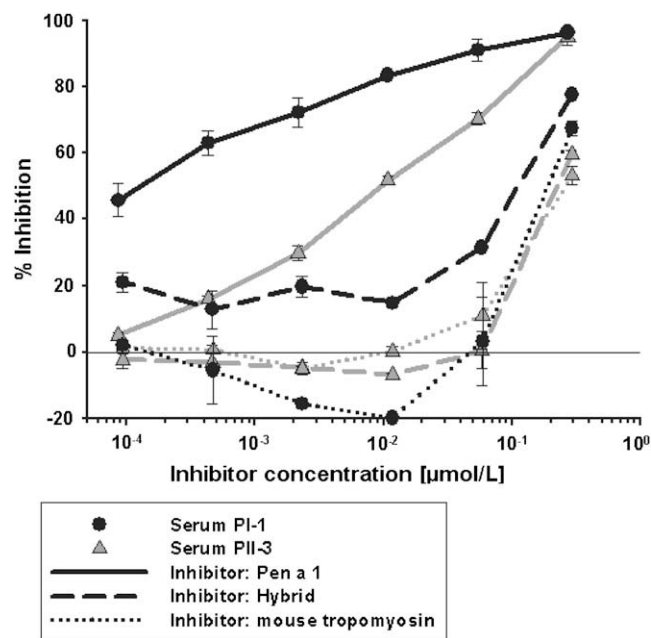


FIG 6. Inhibition of IgE binding to Pen a 1 in ELISA. Binding of IgE to rPen a 1 from patients' sera PI-1 (black circle) or PII-3 (gray triangle) was inhibited by coinubation with recombinant Pen a 1 (solid line), tropomyosin hybrid (dashed line), or recombinant murine tropomyosin (dotted line).

to the native allergen by short peptides. Moreover, if the hidden sequential IgE epitopes were important for the reactivity of Ara h 2, one would expect that the reduced and alkylated allergen in its unfolded form would be able to activate basophils in an IgE-dependent manner, which was clearly not the case. However, there is a residual binding of serum 163 to reduced/alkylated rAra h 2 in Western blot analysis, which is probably a result of low-avidity IgE binding to sequential structures that do not contribute significantly to the allergenic reactivity of the molecule, as proven by the mediator release experiments with unfolded rAra h 2.

Experiments with rPen a 1 and the corresponding short peptides also led to low inhibitory capacity of peptides, although the highest peptide concentration did show some inhibitory effect. This finding is interesting because it can be assumed that tropomyosin, as a result of its coiled-coil structure, would tend to expose many linear sequences to antibodies, potentially favoring the contribution of linear regions to IgE binding. One might argue that the amount of peptide applied in RBL assay might not be enough to inhibit reactions of a serum with high allergen-specific IgE content (such as PII-3). However, when the antibody concentration compared with the amount of peptide is calculated on a molar basis, it becomes clear that there was a ratio of 1 mol specific IgE to 33 mol peptide present during the overnight incubation, which should be sufficient for saturation of binding of high-affinity IgE antibodies.

Overall, when amino acid positions and/or substitutions critical for IgE antibody binding that were identified exclusively with linear overlapping peptides and mutational analysis of epitopes of Pen a 1 and Ara h 2^{9,10} were introduced to the complete protein, T-cell activity (Ara h 2) remained unaltered, and IgE antibody binding capacity and capability to induce mediator release from RBL cells was reduced. However, the relevance and contribution of the results obtained with linear epitopes may vary from

allergen to allergen and experimental design. Alcocer et al⁴⁴ showed that there was very little agreement between the linear IgE epitopes and the actual IgE epitopes located on Ber e 1, the major allergen of Brazil nut, *Bertholletia excelsa*.

To examine whether the structural context in which peptide structures are presented plays a role in IgE binding, a hybrid tropomyosin molecule was generated. The hybrid protein displayed the α -helical folding typical of tropomyosins, but it showed only limited inhibition of IgE binding in ELISA experiments, although the increase in inhibitory capacity of the linear peptides in a structural context indicates that for Pen a 1, the structure is important for its IgE reactivity too. The inhibitory effect observed with the highest inhibitor concentration was also seen with the nonallergenic murine tropomyosin, indicating low-affinity binding. The results suggest at most a minor importance of linear epitopes for the IgE-dependent allergen activity of rPen a 1. In contrast, quantitative measurements of direct IgE binding revealed an increased activity of the hybrid protein compared with murine tropomyosin, suggesting an involvement of the engrafted regions in IgE binding to rPen a 1. However, as rPen a 1 showed stronger IgE binding than the hybrid, determinants of IgE binding in addition to those grafted into the hybrid must be present in rPen a 1.

However, our study has to be considered a proof-of-concept study, and larger study populations need to be investigated before general conclusions on the relevance of sequential epitopes for the activity of food allergens can be drawn. In addition, the low biological activity found for IgE-binding peptides in our study may not affect the diagnostic significance resulting from testing of peptide arrays reported in several studies.¹³⁻¹⁹ Moreover, there may be allergies to different foods for which the effects found in this study might not apply, because recent studies with patients with milk/egg allergy found a proportion of patients for whom IgE to sequential epitopes seems to matter.^{45,46}

In conclusion, our results provided for the first time evidence that sequential epitopes, even though they can readily be identified by direct IgE binding to short peptides, contribute little to the IgE binding of the 2 food allergens studied, particularly in fluid phase antibody/target interaction. Although Pen a 1 peptides displayed low but detectable IgE binding in fluid phase, significant binding occurred when the epitope regions were grafted into the molecular framework of a nonallergenic mammalian tropomyosin. This finding suggests that not only conformational epitopes but also continuous determinants may require a structural context for proper epitope presentation, corroborating the view that stability of a proteins' 3-dimensional structure—especially the in case of food allergens—seems to play an important role in terms of immunogenicity and allergenicity.^{41,47,48}

The findings further serve to illustrate how dramatically different results may be obtained, depending on the experimental approach used for epitope identification and the method used for detection of antibody binding. Assuming that fluid phase binding of IgE antibodies is more relevant in relation to *in vivo* allergenicity, it is possible that solid phase approaches for epitope identification may generate partially misleading results.

Clinical implications: Our data provide evidence that IgE-binding peptides may serve as useful tools in the diagnosis of food allergy but are unlikely to act as full epitopes and cause clinical reactions.

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METHODS

Dot blot of soluble peptides

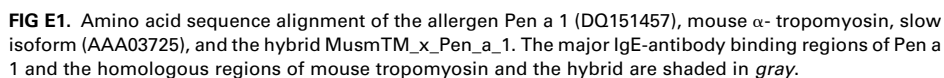
Nitrocellulose membrane (0.2 μ m pore size) was equilibrated with 50 mmol/L carbonate buffer (pH 9.6) for 20 minutes and dried. A volume of 20 μ L of each synthetic peptide was dotted in 1- μ L steps on the membrane. Afterwards, dot-blot membranes were blocked with 0.3% Tween 20 in TRIS-buffered saline for 2 hours and incubated with sera of the nonallergic serum pool and patient with peanut allergy 163 (1:12 in 0.1% BSA in TRIS-buffered saline containing 0.05% Tween20 (TBST)) overnight. The membranes were incubated with monoclonal antihuman-IgE-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, Ala) antibody 1:100,000 in 0.1% BSA in TBST for 1 hour. The substrate LumiGlo Reserve (KPL) was added for 1 minute, and the chemiluminescence signal was exposed on x-ray film for 10 minutes. Between the incubation steps, the membranes were washed 4 times with TBST (0.05% Tween in TRIS-buffered saline) for 5 minutes.

Three-dimensional model of Ara h 2

The amino acid sequence of Ara h 2 (accession no. L77197 without leader sequence) was sent to Swissmodel (swissmodel.expasy.org). The structure was modeled starting with amino acid 22.^{E1} The protein data bank (pdb)-file was illustrated with the DeepView/Swiss-ProtViewer software 3.7 (Swiss Institute of Bioinformatics & GlaxoSmithKline; <http://www.expasy.org/spdbv/>).^{E2} IgE-binding areas were colored green, red, and pink.

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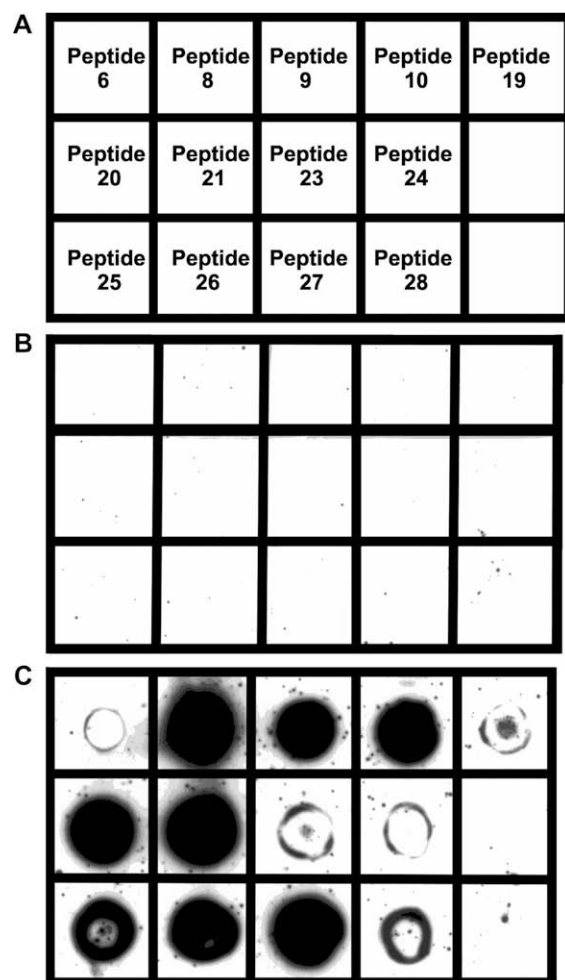


FIG E2. Dot blot of soluble Ara h 2 peptides. **A**, Distribution of soluble peptides on nitrocellulose membrane (50 μ g/peptide). IgE immune detection of soluble Ara h 2 peptides with nonallergic serum pool (**B**) or serum from subject with peanut allergy 163 (**C**).

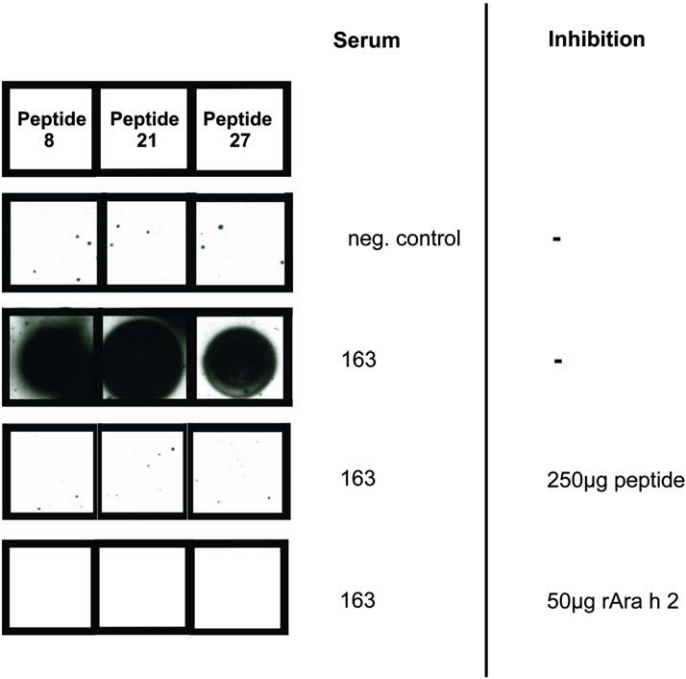


FIG E3. Dot-blot inhibition assay of IgE binding to soluble Ara h 2 peptides. Highly reactive soluble peptides of Ara h 2 were dotted on nitrocellulose membrane, and IgE binding of serum from subject with peanut allergy 163 was either not inhibited or inhibited with 250 µg of the respective peptide or 50 µg rAra h 2. Immune detection with a serum pool from patients without allergy served as control. *neg.*, Negative.

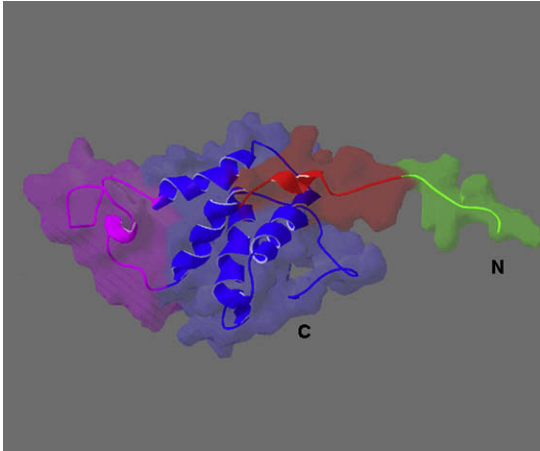


FIG E4. Three -dimensional model of Ara h 2. The model was created with Swissmodel (swissmodel.expasy.org) on the basis of the known solution structure of Ara h 6 (1w2qA).^{E1} The location of IgE-binding areas identified in this study are shown in *green* (peptide 6), *red* (peptides 8-10), and *pink* (peptides 21-28). The figure was illustrated with the pdb-viewer program.

TABLE E1. Characterization of sera from individuals with shrimp allergy

ID	History of anaphylaxis to shrimp	DBPCFC shrimp	SPT shrimp	Prick to prick shrimp	CAP shrimp (kU _A /L)	CAP rPen a 1 (kU _A /L)	CAP nPen a 1 (kU _A /L)
PI-1	No	Pos	Pos	Pos	81.5	76	ND
PII-3	Yes	ND	ND	ND	>100	>100	>100

DBPCFC, Double-blind, placebo-controlled food challenge; *ND*, not done; *Pos*, positive; *SPT*, skin prick test.