

Allergenicity of peanut component Ara h 2: Contribution of conformational versus linear hydroxyproline-containing epitopes

Hervé Bernard, PhD,^{a,b} Blanche Guillon, MSc,^{a,b} Marie-Françoise Drumare, MSc,^{a,b} Evelyne Paty, MD,^c Stephen C. Dreskin, MD, PhD,^d Jean-Michel Wal, PhD,^{a,b,*} Karine Adel-Patient, PhD,^{a,b} and Stéphane Hazebrouck, PhD^{a,b}
 Jouy-en-Josas, Gif-sur-Yvette, and Paris, France, and Aurora, Colo

Background: The 2S-albumin Ara h 2 is the most potent peanut allergen and a good predictor of clinical reactivity in allergic children. Posttranslational hydroxylation of proline residues occurs in DPYSP^{OH}S motifs, which are repeated 2 or 3 times in different isoforms.

Objectives: We investigated the effect of proline hydroxylation on IgE binding and the relative contributions of linear and conformational epitopes to Ara h 2 allergenicity.

Methods: Peptides containing DPYSP^{OH}S motifs were synthesized. A recombinant variant of Ara h 2 without DPYSP^{OH}S motifs was generated by means of deletion mutagenesis. IgE reactivity of 18 French and 5 American patients with peanut allergy toward synthetic peptides and recombinant allergens was assessed by using IgE-binding inhibition assays and degranulation tests of humanized rat basophilic leukemia cells.

Results: Hydroxyproline-containing peptides exhibited an IgE-binding activity equivalent to that of the unfolded Ara h 2. In contrast, corresponding peptides without hydroxyprolines displayed a very weak IgE-binding capacity. Despite removal of the DPYSP^{OH}S motifs, the deletion variant still displayed Ara h 2 conformational epitopes. The IgE-binding capacity of Ara h 2 was then recapitulated with an equimolar mixture of a hydroxylated peptide and the deletion variant. Hydroxylated peptides of 15 and 27 amino acid residues were also able to trigger cell degranulation.

Conclusions: Sensitization toward linear and conformational epitopes of Ara h 2 is variable among patients with peanut allergy. Optimal IgE binding to linear epitopes of Ara h 2 requires posttranslational hydroxylation of proline residues. The absence of hydroxyprolines could then affect the accuracy of component-resolved diagnostics by using rAra h 2. (*J Allergy Clin Immunol* 2014;■■■■:■■■■-■■■■.)

Key words: Food allergy, peanut allergen, posttranslational modifications, hydroxyproline, IgE-binding, conformational and linear epitopes

Peanut is one of the most common causes of severe allergic reactions to food.^{1,2} IgE-mediated peanut allergy affects more than 1% of children and is outgrown by only 20% of patients.³

Until now, 12 allergens (Ara h 1 to Ara h 13, with Ara h 3/4 describing the same protein) have been identified in peanut (*Arachis hypogaea*).⁴ Ara h 1, Ara h 2, and Ara h 3 were initially recognized as the major peanut allergens.⁵⁻⁷ Recently, Ara h 2 and Ara h 6 were described as being the most clinically relevant peanut allergens, as observed *in vitro* with effector cell-based assays and *in vivo* with skin prick tests.⁸⁻¹⁷ Accordingly, the IgE response to Ara h 2 has been reported to be a good predictor of clinical allergy in children,^{18,19} and the IgE response to Ara h 6 could also provide good diagnostic performance.^{20,21}

Ara h 2 and Ara h 6 belong to the 2S-albumin family. They share a compact conformation characterized by 5 α -helical structures and stabilized by a network of 4 conserved disulfide bridges.²² This arrangement provides a core structure highly resistant to proteolysis so that treatment of 2S-albumins with digestive enzymes does not have a significant effect on their allergenicity.²³⁻²⁷ Ara h 2 and Ara h 6 are 59% homologous, but compared with Ara h 6, 2 insertions of 14 and 26 amino acid residues occur in the Ara h 2 major isoforms Ara h 2.01 and Ara h 2.02.^{6,28} These insertions are exposed on a flexible surface loop and comprise the repeated DPYSP^{OH}S motif, with the second proline being hydroxylated.^{23,29} The DPYSP^{OH}S-containing domain has been reported to be a major linear IgE-binding epitope.^{30,31} The effect of proline hydroxylation on Ara h 2 IgE reactivity has not yet been investigated, although this posttranslational modification has been shown to influence IgE binding to Phl p 1, a major allergen from timothy grass pollen.³² On the other hand, conformation of 2S-albumins is also essential for the allergenic potency because suppression of disulfide bridge formation in Ara h 2 and Ara h 6 by means of chemical reduction or site-directed mutagenesis reduced their IgE reactivity significantly.^{26,30,33-35} In this regard some studies reported a predominance of IgE recognition of conformational epitopes on Ara h 2, whereas others suggested a higher proportion of IgE binding to linear epitopes.^{30,33,36}

From ^aINRA, UR 496, Unité d'Immuno-Allergie Alimentaire, Jouy-en-Josas; ^bCEA, iBiTecS/Service de Pharmacologie et d'Immunoanalyse, Gif-sur-Yvette; ^cUniversité Paris Descartes-Assistance Publique des Hôpitaux de Paris, Hôpital Necker Enfants Malades, Paris; and ^dthe Division of Allergy and Clinical Immunology, Department of Medicine, University of Colorado Denver, Aurora.

*Jean-Michel Wal, PhD, is currently affiliated with AgroParisTech-SVS Department, Paris, France.

Supported by AlimH department of INRA and grant R01-AI099029 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (to S.C.D.).

Disclosure of potential conflict of interest: S. C. Dreskin has received research support from the National Institutes of Health; is a member of the American Board of Allergy and Immunology; has received consultancy fees from the Clinical Immunization and Safety Assessment (CISA) Network (administered by Vanderbilt University) and Pfizer; has provided expert testimony for Pinnacol Assurance, for Frasca, Joiner, Goodman and Greenstein P.C., and for the Medical Expert Panel of the Vaccine Injury Compensation Program, Health Resources and Services Administration (HRSA); has received research support from Genentech; and has received lecture fees from Pri-Med Institute (pmiCME). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 13, 2014; revised October 3, 2014; accepted for publication October 6, 2014.

Corresponding author: Stéphane Hazebrouck, PhD, Unité d'Immuno-Allergie Alimentaire, INRA, iBiTec-S, SPI-Bât 136, CEA de Saclay, 91191 Gif-sur-Yvette cedex, France. E-mail: stephane.hazebrouck@cea.fr.

0091-6749/\$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2014.10.025>

Abbreviations used

CD:	Circular dichroism
MALDI-TOF:	Matrix-assisted laser desorption ionization time-of-flight instrument
p ^{OH} :	Hydroxyproline
r/a:	Reduced and S-alkylated
RBL:	Rat basophilic leukemia
RP-HPLC:	Reversed-phase high-performance liquid chromatography

In the present work we aimed to determine the relative contributions of linear and conformational epitopes to the allergenic potency of Ara h 2. For this purpose, we first characterized the IgE reactivity of a stably unfolded nAra h 2 and of a properly refolded rAra h 2. Discrepancies of IgE-binding capacity between the recombinant and native allergens led us to investigate the influence of proline hydroxylation. Therefore we compared the IgE reactivity of 18 French and 5 American patients with peanut allergy to nAra h 2 and rAra h 2 and toward synthetic peptides containing DPYSP^{OH}S motifs with or without hydroxyprolines. Moreover, considering that the DPYSP^{OH}S-containing domain is located on a flexible surface loop in Ara h 2 and is absent in Ara h 6, this domain was not expected to contribute significantly to the stability of the global fold of 2S-albumins.^{23,37} Thus we generated a recombinant variant of Ara h 2 lacking the DPYSP^{OH}S motifs to investigate the contribution of conformational epitopes to the IgE reactivity of Ara h 2 without any IgE binding to the linear hydroxyproline-containing epitopes.

METHODS**Human sera**

French sera for this retrospective study were collected from 18 children with peanut allergy recruited at the Paediatric Allergy Clinic of Hôpital Necker-Enfants Malades after informed consent from the patients' parents (see Table E1 in this article's Online Repository at www.jacionline.org and Bernard et al³⁸). All serum samples were collected during routine clinical practice and studied in accordance with the purpose of the initial study. Based on their medical history, symptoms of the IgE-mediated peanut allergy involved the skin, respiratory tract, gastrointestinal tract, and cardiovascular system. Five sera from American patients with peanut allergy with a strong history of peanut-induced immediate hypersensitivity and peanut-specific IgE levels of 13 KAU/L or greater (ImmunoCAP; Phadia, Uppsala, Sweden) in serum were collected within 6 months of this study (see Table E2 in this article's Online Repository at www.jacionline.org). All adult patients and the parents or guardians of minors signed informed consent forms. Minors who were greater than 6 years of age signed an assent form. The University of Colorado Denver Institutional Review Board approved this study.

Allergen preparations

The 2S-albumins Ara h 2 and Ara h 6 were purified from whole peanut protein extract prepared with commercially roasted peanuts (Virginian variety), as previously described.³⁵ Separation of the different isoforms of Ara h 2 is described in the Methods section in this article's Online Repository at www.jacionline.org.

The gene encoding Ara h 2.01 (Swiss-Prot accession no. Q6PSU2-2, Fig 1) was synthesized by using codons optimized for bacterial expression (Genscript USA, Piscataway, NJ) and inserted into the *Escherichia coli* expression plasmid pET9c (Novagen-Merck, Darmstadt, Germany). The variant rAra h 2.Δ was obtained by replacing the domain GRDPYSPSQD PYSPSP of rAra h 2.01 by the dipeptide DS naturally occurring in Ara h 6

(Fig 1). Expression, purification, and refolding of recombinant proteins are described in the Methods section in this article's Online Repository. Refolding of the recombinant proteins was verified by means of circular dichroism (CD) spectroscopy, as previously described.²⁶

Reduction and S-alkylation of Ara h 2 (all isoforms) and Ara h 6 were performed, as previously described, to prepare stably unfolded 2S-albumins.³⁵

Peptides

Several peptides comprising major linear IgE-binding epitopes, as initially reported by Stanley et al,³¹ were synthesized by taking into account the post-translational hydroxylation of proline residues. The peptide 1-21 (RQQWELQGDRRCQSQLERANL) covered the N-terminal part of Ara h 2 (Fig 1). The peptide containing 2 hydroxyprolines (pep 2P^{OH}) corresponded to the domain found in the Ara h 2.01 isoform (DPYSP^{OH}SQDPYSP^{OH}SPY). The peptide containing 3 hydroxyprolines (pep 3P^{OH}) corresponded to the domain found in the Ara h 2.02 isoform (DPYSP^{OH}SQDPYSP^{OH}SQDPDRRD-PYSP^{OH}SPY; Fig 1). Corresponding control peptides without hydroxyprolines (pep 2P and pep 3P) were also synthesized. The different peptides were purified by means of reversed-phase high-performance liquid chromatography (RP-HPLC) and analyzed by using matrix-assisted laser desorption ionization time-of-flight instrument (MALDI-TOF; see the Methods section in this article's Online Repository). Characterization of the synthetic peptides by means of CD spectroscopy did not reveal a significant presence of α-helix or β-sheet secondary structures (see Fig E1 in this article's Online Repository at www.jacionline.org). Gel permeation chromatography did not evidence the formation of peptide aggregates under physiologic conditions (see Fig E2 in this article's Online Repository at www.jacionline.org).

IgE immunoreactivity analysis

In agreement with the assumption from Albrecht et al³⁰ that "fluid-phase binding of IgE antibodies is more relevant in relation to *in vivo* allergenicity," we developed a reverse enzyme allergosorbent test that is not based on the binding capacity of allergens immobilized on the solid phase but measures the binding of labeled allergens by patients' IgE antibodies captured by an anti-human IgE mAb immobilized on the solid phase.³⁹ In this test plates were first coated with the anti-human IgE mAb LE27. Fifty microliters per well of serum from each patient at adequate dilutions was incubated overnight at 4°C. After washing, 25 μL of inhibitors (ie, increasing concentrations of the tested molecule) and 25 μL of labeled nAra h 2 were mixed and incubated for 4 hours at room temperature. Labeled nAra h 2 (all isoforms) used for this IgE-binding assay were prepared by means of covalent linkage of the native protein to the tetrameric form of acetylcholinesterase.⁴⁰ Ellman's reagent was then used as the acetylcholinesterase chromogenic substrate, and absorbance at 414 nm was measured.⁴¹ Results were expressed as B/B₀. B₀ and B represent the amount of labeled nAra h 2 bound to immobilized IgE antibodies in the absence or presence of a known concentration of inhibitor, respectively. The concentration inhibiting 50% of the IgE binding to labeled allergen was evaluated by using GraphPad Prism 5.01 software (GraphPad Software, La Jolla, Calif).

Mediator release assay

The degranulation assay was performed with rat basophilic leukemia (RBL) SX-38 cells, as previously described.⁹ Cells were passively sensitized with IgE antibodies immunopurified from individual sera, as previously described.⁹ Mediator release was induced by incubation with different concentrations of synthetic peptides and native or recombinant allergens and was determined by measuring β-hexosaminidase activity. Results were expressed as a percentage of the reference release induced with anti-human IgE (LE27 clone, 100 ng/mL).

Statistical analysis

Data were analyzed by using the nonparametric Wilcoxon matched-pairs signed-rank test. Statistical analyses were performed with GraphPad Prism 5.01 software.

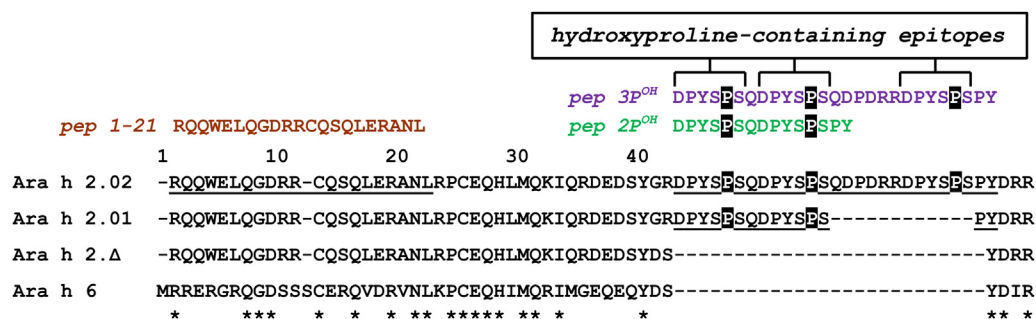


FIG 1. Sequence comparison of the N-terminal part of Ara h 2.02 (Ara h 2.0201, UniProt accession no. Q6PSU2), Ara h 2.01 (Ara h 2.0101, Q6PSU2-2), the deletion variant Ara h 2.Δ, and Ara h 6 (Q647G9). Numbering of Ara h 6 is shown, and hydroxyprolines in the DPYSP^{OH}S motifs are shown with shaded letters. Identical residues between Ara h 2 and Ara h 6 sequences are indicated with asterisks. The peptide (pep) 1-21 covered the N-terminal part of Ara h 2. The peptide containing 2 DPYSP^{OH}S motifs and thereby 2 hydroxyprolines (pep 2P^{OH}) corresponded to the domain found in the Ara h 2.01 isoform. The peptide containing 3 DPYSP^{OH}S motifs and thereby 3 hydroxyprolines (pep 3P^{OH}) corresponded to the domain found in the Ara h 2.02 isoform.

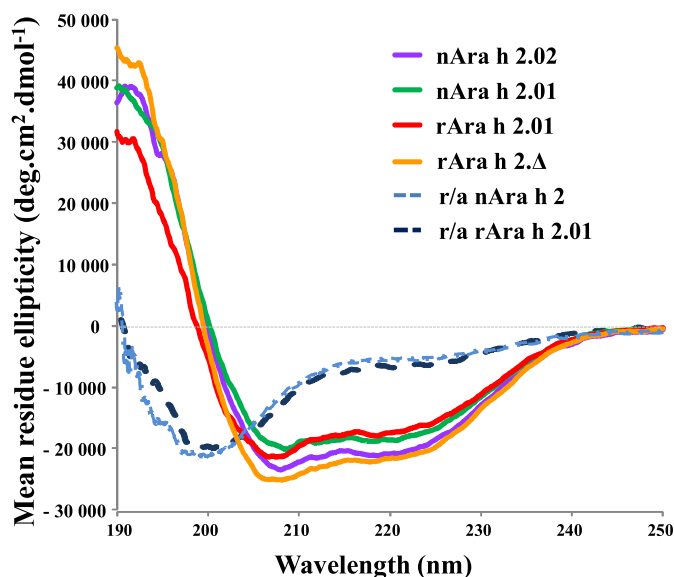


FIG 2. CD analysis shows a comparison of different isoforms of nAra h 2 and rAra h 2, the recombinant variant rAra h 2.Δ without the DPYSP^{OH}S motif, and r/a nAra h 2 and rAra h 2. The x-axis shows the wavelength, and the y-axis shows the molecular ellipticity.

RESULTS

Effect of reduction and S-alkylation on Ara h 2 IgE reactivity

We first wanted to evaluate the loss of IgE reactivity induced by chemical reduction of the disulfide bridges and the resulting suppression of the conformational epitopes. The unfolded state of reduced and S-alkylated (r/a) Ara h 2 was confirmed by CD spectra with a single spectrum minimum close to 200 nm instead of the 2 broad minima at 208 and 222 nm, which is typical for the α -helical secondary structures largely present in nAra h 2 (Fig 2). This denaturing treatment reduced considerably the IgE-binding capacity of nAra h 2 (Fig 3). However, for 9 of 18 patients (sera 313, 576, 101, 102, 486, 572, 841, 109, and 907), r/a nAra h 2 retained a significant IgE reactivity, with the concentration inhibiting 50% of IgE binding to labeled allergen ranging from 0.5 to 750 nmol/L. Two patients (109 and 907) even displayed

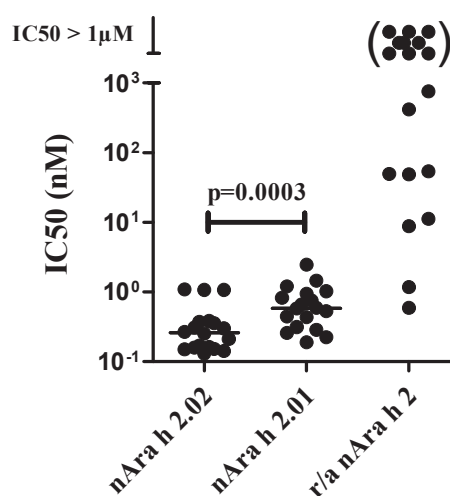


FIG 3. Effect of reduction and S-alkylation on the IgE-binding capacity of nAra h 2. Competitive inhibition binding of IgE antibodies from 18 patients with peanut allergy to nAra h 2 was performed individually, and the 50% inhibitory concentration (IC_{50}) was determined. For 9 of 18 tested sera, 50% inhibition of IgE binding to nAra h 2 by r/a nAra h 2 was not reached at a concentration of 1 μ mol/L.

an IgE response to r/a nAra h 2 comparable with that against the native allergen. Of note, the isoform nAra h 2.02 displayed a slightly but significantly higher IgE-binding capacity than nAra h 2.01 ($P = .0003$). As shown in Fig 4, A, with 4 representative sera, and in Fig E3 in this article's Online Repository at www.jacionline.org, the difference in IgE reactivity between nAra h 2.01 and nAra h 2.02 increased concomitantly with the IgE-binding capacity of r/a nAra h 2.

IgE-binding capacity of rAra h 2 versus nAra h 2

We then wanted to assess whether a recombinant form of Ara h 2.01 shared similar allergenic properties with its native counterpart. It thus appeared that rAra h 2 and nAra h 2 displayed different patterns of IgE reactivity among the patients (Fig 4, A, and see Fig E3). Interestingly, the IgE reactivity of rAra h 2.01 was inversely proportional to that of r/a nAra h 2. Indeed nAra

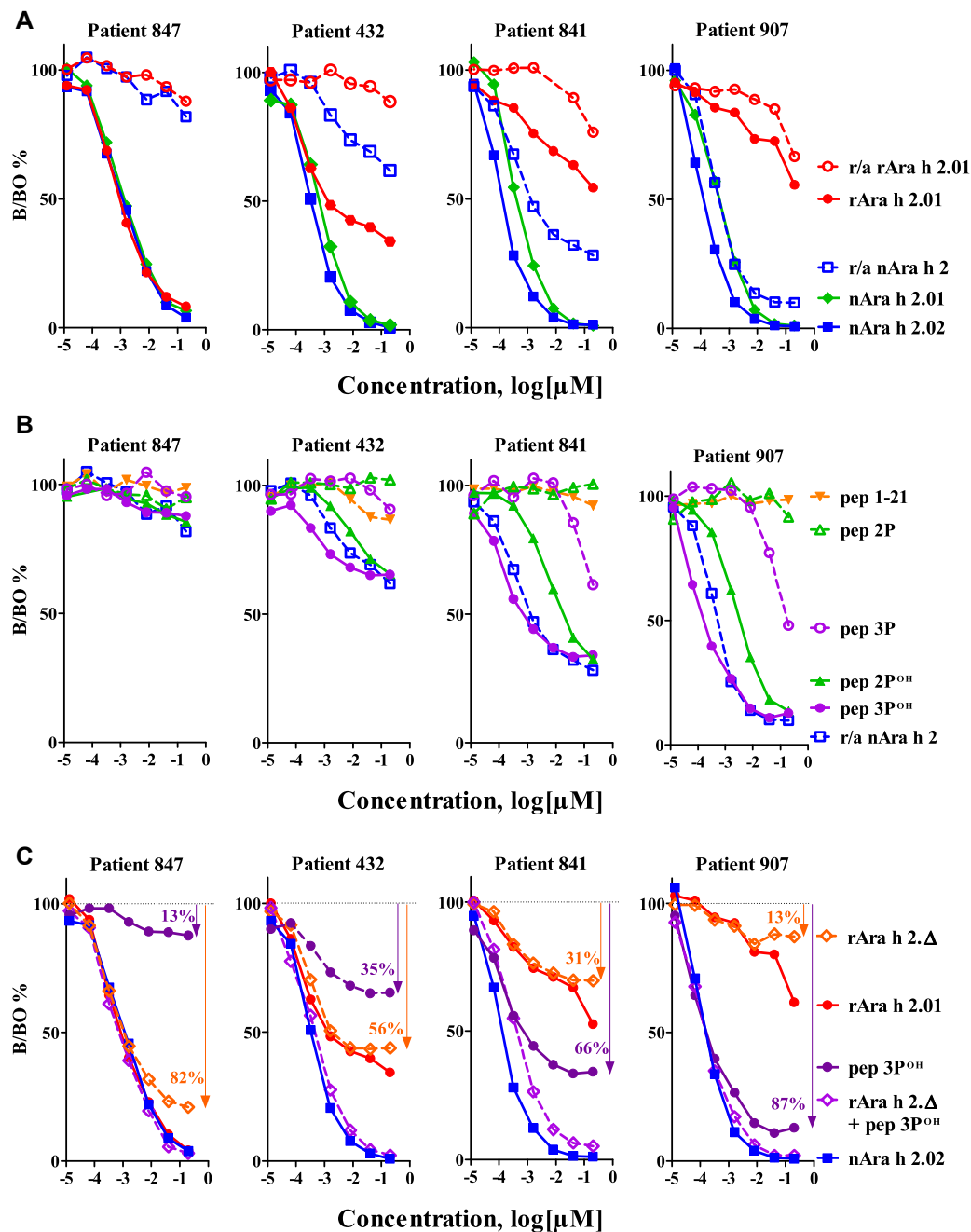


FIG 4. Competitive inhibition of IgE binding to nAra h 2 for 4 representative sera. **A**, Comparison of the IgE-binding capacity of nAra h 2 and rAra h 2 and effect of reduction and S-alkylation. **B**, Influence of proline hydroxylation on the IgE-binding capacity of synthetic peptides overlapping the DPYSP^{OH}S-containing domain of Ara h 2. **C**, Recapitulation of the IgE-binding capacity of nAra h 2.02 with an equimolar mixture of synthetic peptide pep 3P^{OH} and the deletion variant rAra h 2.Δ. The relative contribution of linear (purple) and conformational (orange) epitopes to the IgE reactivity of nAra h 2 are estimated with the inhibitory capacity of pep 3P^{OH} and rAra h 2.Δ, respectively, and are shown for each patient. Sera 432, 841, 847, and 907 were diluted 1:200, 1:500, 1:300, and 1:500, respectively. Complementary data for 14 French patients and 5 American patients are shown in Figs E3 and E4 in this article's Online Repository.

h 2 and rAra h 2 were similarly bound by IgE antibodies from patient 847 but r/a nAra h 2 was not recognized, thus indicating that most Ara h 2-specific IgE antibodies from patient 847 recognized conformational epitopes. In this regard rAra h 2.01 also appeared to be properly refolded, as confirmed by using CD analysis with the predominance of α -helical structures

(Fig 2). Conversely, rAra h 2.01 was poorly recognized by IgE antibodies from patient 907, whereas r/a nAra h 2 displayed IgE reactivity almost as high as that of the native allergen. In this case most of the Ara h 2-specific IgE antibodies from patient 907 recognized linear epitopes. Patients 432 and 841 displayed intermediate IgE reactivity toward rAra h 2.01 and r/a nAra h 2

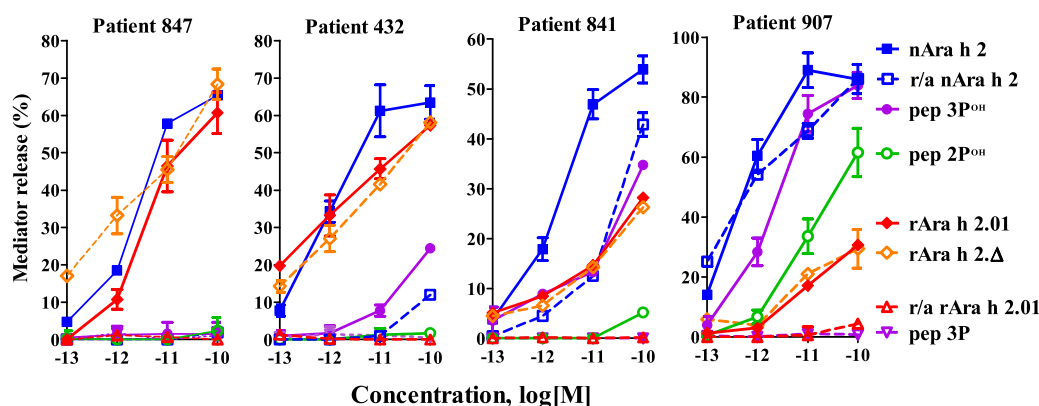


FIG 5. Mediator release assay with RBL SX-38 cells sensitized with immunopurified IgE antibodies from 4 patients with peanut allergy in response to increasing concentrations of different Ara h 2 variants and synthetic peptides. The x-axis shows the concentration of the tested molecule, and the y-axis shows the percentage of the reference release induced with the anti-human IgE mAb LE27.

(see also Fig E3). It was then noteworthy that even after reduction and S-alkylation, the IgE-binding capacity of the recombinant allergen remained lower than that of the native form. The influence of posttranslational modifications that naturally occur in peanut seeds but not in prokaryotes was then further investigated.

Effect of proline hydroxylation on Ara h 2 IgE-binding capacity

Four peptides containing 2 or 3 DPYSP^{OH}S motifs, as found in nAra h 2.01 and nAra h 2.02, respectively, were synthesized with or without hydroxyprolines (Fig 1). As shown by patients 841 and 907, peptides with hydroxyprolines displayed an IgE-binding capacity at least 1000-fold higher than that of peptides without hydroxyprolines (Fig 4, B). Inhibition of IgE binding to nAra h 2 was always more efficient with the 27-residue-long peptide 3P^{OH}, with 3 DPYSP^{OH}S motifs, than with the 15-residue-long peptide 2P^{OH}, with only 2 DPYSP^{OH}S motifs. Surprisingly, the IgE-binding capacity of the 27-residue-long peptide was as high as that of the full-length r/a nAra h 2 for all the tested sera (Fig 4, B, and see Fig E3), thus suggesting that pep 3P^{OH} was bound by nearly all of the IgE antibodies recognizing linear epitopes. Peptide 1-21 did not exhibit any significant IgE-binding capacity for any of the French sera.

Evaluation of the relative contribution of linear versus conformational epitopes to Ara h 2 IgE-binding capacity

A recombinant variant of Ara h 2 lacking the DPYSP^{OH}S-containing domain, rAra h 2.Δ, was generated by means of deletion mutagenesis (Fig 1). Suppression of this disordered domain did not prevent rAra h 2.Δ to refold properly (Fig 2). Furthermore, rAra h 2.Δ and rAra h 2.01 were bound by IgE antibodies with an apparent similar affinity, thus confirming that most of the conformational epitopes were preserved on rAra h 2.Δ (Fig 4, C).

IgE binding to conformational epitopes could then be specifically inhibited with the deletion variant rAra h 2.Δ, and IgE binding to linear epitopes could be inhibited with the synthetic peptide pep 3P^{OH}. Accordingly, an equimolar mixture of pep 3P^{OH} and rAra h 2.Δ exhibited an inhibitory capacity

similar to that of nAra h 2 (Fig 4, C). The relative contribution of linear and conformational epitopes to the IgE reactivity of Ara h 2 was then evaluated for each patient. For patient 847, around 82% of the IgE binding to Ara h 2 was due to the recognition of conformational epitopes, whereas around 87% of the IgE binding to Ara h 2 was due to the recognition of linear epitopes for patient 907 (Fig 4, C). Five sera from American patients with peanut allergy were similarly tested (see Fig E4 in this article's Online Repository at www.jacionline.org). As observed with French patients, hydroxylation of the synthetic peptides was required to obtain significant IgE binding to the synthetic peptides. IgE binding to linear epitopes also appeared to be restricted to the DPYSP^{OH}S-containing domain. Of note, peptide 1-21 displayed a rather significant IgE-binding capacity for 1 serum, although still with a much lower affinity than peptide 3P^{OH} (serum D119; see Fig E4, A). American patients also displayed variable levels of sensitization toward linear and conformational epitopes of Ara h 2 (see Fig E4, B).

Allergenic activity of the DPYSP^{OH}S-containing peptides

The capacity of the different variants and synthetic peptides from Ara h 2 to cross-link IgE/FcεRI complexes was evaluated with a degranulation assay of RBL SX-38 cells. As expected, when cells were passively sensitized with immunopurified IgE antibodies from patient 847, the synthetic peptides did not display any allergenic activity, and only properly folded allergens (ie, nAra h 2 and rAra h 2) were able to induce cell degranulation (Fig 5). In this case we also confirmed that the synthetic peptides did not possess any intrinsic ability to induce mediator release and that basophil degranulation was actually dependent on the presence of specific IgE antibodies. Indeed, when cells were loaded with IgE antibodies from patient 907, the pep 3P^{OH} and, to a lesser extent, pep 2P^{OH} could trigger cell degranulation almost as efficiently as the full-length allergen. Unfolded r/a nAra h 2 also retained a strong allergenic potency. In contrast, r/a rAra h 2 and the synthetic peptides without hydroxyprolines, pep 3P and 2P (data not shown), did not display any allergenic potency. The pattern of cell degranulation induced with IgE antibodies from patients 432 and 841 were in line with IgE-binding assays because the potency

of the hydroxylated peptides correlated with that of r/a nAra h 2 and with a decreasing potency of the recombinant allergens (Fig 5).

DISCUSSION

Being the most potent allergens from peanut, the 2S-albumins Ara h 2 and Ara h 6 are attractive target molecules for therapeutic applications. Treatment of allergic mice with purified Ara h 2 and Ara h 6 has been shown to provide an equivalent level of desensitization as crude peanut extract.¹⁴ The use of stably unfolded 2S-albumins has also been proposed for the development of safer immunotherapeutic treatments.^{26,34} However, the presence of major linear IgE-binding epitopes persisting in unfolded allergens could limit the advantages of such allergoids. In this regard determination of the relative importance of linear and conformational IgE-binding epitopes in Ara h 2 allergenicity could be of interest to optimize specific immunotherapy.

As previously observed with Ara h 6, reduction and S-alkylation of nAra h 2 led to a substantial decrease in IgE-binding capacity.^{26,33} Nevertheless, approximately 50% of the tested sera exhibited significant IgE reactivity to r/a nAra h 2. Similarly, Starkl et al³⁴ observed that the residual IgE-binding capacity of r/a nAra h 2 compared with untreated nAra h 2 was highly variable among patients with peanut allergy. In contrast, we never evidenced such residual IgE reactivity for r/a Ara h 6 when using the same competitive fluid-phase assay.²⁶ Even the patients displaying the highest IgE reactivity to r/a nAra h 2 did not recognize r/a Ara h 6, thus suggesting that only nAra h 2 contains immunodominant linear IgE-binding epitopes (see Fig E5 in this article's Online Repository at www.jacionline.org).

For the first time, posttranslational modifications were shown to be critical for IgE binding to Ara h 2 linear epitopes, and this explained the differences in IgE reactivity between nAra h 2 and rAra h 2.²³ Thus we demonstrated the importance of proline hydroxylation in the motif DPYSP^{OH}S, which was the sole major linear IgE-binding epitope of nAra h 2 in our experimental setup. Accordingly, the inhibitory capacity of r/a nAra h 2 was completely recapitulated with a single peptide containing 3 DPYSP^{OH}S motifs. Although domain 1-21 was reported to contain immunodominant IgE-binding epitopes,³⁰ the corresponding synthetic peptide rarely displayed a significant inhibitory capacity. The weak IgE-binding capacity of r/a rAra h 2 also confirmed the absence of a major linear epitope when prolines are not posttranslationally modified. This result is in agreement with those of Albrecht et al,³⁰ who reported that a mix of Ara h 2–derived peptides did not interfere detectably with the IgE binding to a recombinant form of Ara h 2. In contrast, Bublin et al³⁶ observed that a considerable proportion of IgE binding to Ara h 2, Ara h 1, and Ara h 3 could be inhibited by a mix of 3 synthetic peptides containing the N-terminal region of Ara h 2 and the repeated motif DPYSPS without hydroxyproline. A weak IgE reactivity of the nonhydroxylated peptide pep 3P was also detected with patients 841 and 907. However, the clinical relevance of this low-affinity binding is questionable because pep 3P displayed no allergenic potency when pep 3P^{OH} induced cell degranulation.

Another important finding was therefore the ability of the 15- and 27-residue-long peptides pep 2P^{OH} and pep 3P^{OH} to induce basophil degranulation. This result was rather unexpected for

such short peptides, especially when considering that these peptides did not form aggregates under physiologic conditions (see Fig E2), as previously reported for other allergenic peptides of less than 3 kDa.^{42,43} The allergenic activity of these peptides thus suggests that their repeated DPYSP^{OH}S motifs can be bound simultaneously by at least 2 IgE antibodies. Considering the minimum peptide size that could optimally cross-link IgE/FcεRI complexes, as calculated by Bannon et al⁴⁵ with data from Kane et al,^{44,45} the trivalent peptide pep 3P^{OH} was just long enough to induce efficient cell degranulation. Surprisingly, with only 15 amino acid residues, the bivalent peptide pep 2P^{OH} still displayed allergenic activity, although much lower than that of pep 3P^{OH}. Thus, according to its higher valency, pep 3P^{OH} displayed a higher IgE-binding capacity and a higher allergenicity than pep 2P^{OH}, which is in agreement with previous studies, suggesting a higher allergenicity of nAra h 2.02 compared with nAra h 2.01.^{31,46}

In addition, the allergenic potency of the DPYSP^{OH}S-containing peptides provides new insight into the residual allergenicity of hydrolyzed peanut proteins. It has been shown that Ara h 2 digested with trypsin/chymotrypsin displayed a minimal reduction in IgE-binding capacity and allergenicity.²³ Recently, Shi et al²⁷ reported that even with an extensive reduction in the size of the IgE-binding peptides and a substantial decrease of IgE-binding capacity, peanut flour hydrolysates still displayed high allergenic potency because of Ara h 2 fragments. Accordingly, our data showed that any peptide containing more than 1 DPYSP^{OH}S motif, even those of less than 3 kDa, could induce mast cell degranulation and thereby present an allergenic risk. The preparation of hydrolysate as an alternative to native peanut flour proteins in immunotherapy could then take into account the specific detection of such short peptides. Moreover, as shown with patient 432, even when the contribution of linear epitopes to nAra h 2 allergenicity is not predominant, r/a nAra h 2 and pep 3P^{OH} could still present an anaphylactic risk (Fig 5). In this regard the use of hypoallergens without hydroxyprolines, such as r/a rAra h 2 or r/a rAra h 2.Δ, might be preferred to unfolded native allergens for the development of future specific immunotherapy.

Finally, the fact that the immunodominant linear IgE-binding epitopes of Ara h 2 are almost exclusively located in the DPYSP^{OH}S-containing domain permitted us to use the peptide pep 3P^{OH} and the variant rAra h 2.Δ to quantify the relative contribution of linear and conformational epitopes to the IgE binding. The level of sensitization to linear and conformational epitopes appeared then to be quite variable among patients, and we did not evidence a predominant IgE recognition of a certain type of epitopes over the other one. In fact, when testing 2 different pools of sera from French or American patients with peanut allergy, the contributions of linear and conformational epitopes to the IgE-binding capacity of Ara h 2 were globally equivalent in both cases (data not shown). In this regard comparison of Ara h 2 IgE reactivity between sera from French and American patients did not reveal any significant differences. The absence of proline hydroxylation in rAra h 2 could then significantly affect the accuracy of component-resolved diagnostics for most patients with peanut allergy by underestimating the IgE response to Ara h 2. Recently, Lin et al⁴⁷ used a bioinformatics approach to identify patients with symptomatic peanut allergy using a peptide microarray immunoassay. The use of hydroxylated peptides as biomarkers could certainly increase

the prediction performance. Moreover, sensitization to linear epitopes has been associated with persistent allergy to milk and egg.^{48,49} The level of specific IgE responses to the DPYSP^{OH}S-containing domain could then provide additional information for the diagnosis and management of patients with peanut allergy.

In conclusion, our study demonstrated the critical influence of posttranslational modifications on the allergenic potency of Ara h 2. It also evidenced that short peptides encompassing the DPYSP^{OH}S-containing domain still constitute a potential risk for patients with peanut allergy. These results provide new insight into the allergenic activity of the most potent peanut allergen. Considering the diversity in the serology of patients with peanut allergy in various parts of the world,⁵⁰ it would be also interesting to determine whether the pattern of sensitization toward the DPYSP^{OH}S-containing domain and the conformational epitopes of Ara h 2 could be correlated to different methods of peanut processing and consumption.

We thank Sandrine Ah-Leung for skilled technical assistance for the degranulation assay.

Key messages

- Absence of hydroxyprolines in recombinant Ara h 2 might affect the accuracy of component-resolved diagnostics.
- Short peptides of Ara h 2 encompassing linear hydroxyproline-containing epitopes can trigger RBL mast cell degranulation.
- Relative contributions of linear and conformational epitopes to Ara h 2 allergenicity are variable among patients with peanut allergy.

REFERENCES

- Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, et al. The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 2007;120:638-46.
- Sicherer SH, Munoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol* 2010;125:1322-6.
- Skolnick HS, Conover-Walker MK, Koerner CB, Sampson HA, Burks W, Wood RA. The natural history of peanut allergy. *J Allergy Clin Immunol* 2001;107:367-74.
- Radauer C, Nandy A, Ferreira F, Goodman RE, Larsen JN, Lidholm J, et al. Update of the WHO/IUIS Allergen Nomenclature Database based on analysis of allergen sequences. *Allergy* 2014;69:413-9.
- Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* 1991;88:172-9.
- Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992;90:962-9.
- Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 1999;103:535-42.
- Palmer GW, Dibbern DA Jr, Burks AW, Bannon GA, Bock SA, Porterfield HS, et al. Comparative potency of Ara h 1 and Ara h 2 in immunochemical and functional assays of allergenicity. *Clin Immunol* 2005;115:302-12.
- Blanc F, Adel-Patient K, Drumare MF, Paty E, Wal JM, Bernard H. Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors. *Clin Exp Allergy* 2009;39:1277-85.
- Chen X, Zhuang Y, Wang Q, Moutsoglou D, Ruiz G, Yen SE, et al. Analysis of the effector activity of Ara h 2 and Ara h 6 by selective depletion from a crude peanut extract. *J Immunol Methods* 2011;372:65-70.
- Flinterman AE, van Hoffen E, den Hartog Jager CF, Koppelman S, Pasmans SG, Hoekstra MO, et al. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy* 2007;37:1221-8.
- Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 2004;34:583-90.
- Koppelman SJ, de Jong GA, Laaper-Ertmann M, Peeters KA, Knulst AC, Hefle SL, et al. Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. *Clin Exp Allergy* 2005;35:490-7.
- Kulis M, Chen X, Lew J, Wang Q, Patel OP, Zhuang Y, et al. The 2S albumin allergens of *Arachis hypogaea*, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice. *Clin Exp Allergy* 2012;42:326-36.
- Peeters KA, Koppelman SJ, van Hoffen E, van der Tas CW, den Hartog Jager CF, Penninks AH, et al. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clin Exp Allergy* 2007;37:108-15.
- Porterfield HS, Murray KS, Schlichting DG, Chen X, Hansen KC, Duncan MW, et al. Effector activity of peanut allergens: a critical role for Ara h 2, Ara h 6, and their variants. *Clin Exp Allergy* 2009;39:1099-108.
- Zhuang Y, Dreskin SC. Redefining the major peanut allergens. *Immunol Res* 2013;55:125-34.
- Dang TD, Tang M, Choo S, Licciardi PV, Koplin JJ, Martin PE, et al. Increasing the accuracy of peanut allergy diagnosis by using Ara h 2. *J Allergy Clin Immunol* 2012;129:1056-63.
- Klemans RJ, Otte D, Knol M, Knol EF, Meijer Y, Gmelig-Meyling FH, et al. The diagnostic value of specific IgE to Ara h 2 to predict peanut allergy in children is comparable to a validated and updated diagnostic prediction model. *J Allergy Clin Immunol* 2013;131:157-63.
- Koid AE, Chapman MD, Hamilton RG, Van Ree R, Versteeg SA, Dreskin SC, et al. Ara h 6 complements Ara h 2 as an important marker for IgE reactivity to peanut. *J Agric Food Chem* 2014;62:206-13.
- Klemans RJ, Knol EF, Bruijnzeel-Koomen CA, Knulst AC. The diagnostic accuracy of specific IgE to Ara h 6 in adults is as good as Ara h 2. *Allergy* 2014;69:1112-4.
- Moreno FJ, Clemente A. 2S Albumin storage proteins: what makes them food allergens? *Open Biochem J* 2008;2:16-28.
- Lehmann K, Schweimer K, Reese G, Randow S, Suhr M, Becker WM, et al. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. *Biochem J* 2006;395:463-72.
- Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol* 2002;169:882-7.
- Suhr M, Wicklein D, Lepp U, Becker WM. Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Mol Nutr Food Res* 2004;48:390-9.
- Hazebrouck S, Guillon B, Drumare MF, Paty E, Wal JM, Bernard H. Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds. *Mol Nutr Food Res* 2012;56:548-57.
- Shi X, Guo R, White BL, Yancey A, Sanders TH, Davis JP, et al. Allergenic properties of enzymatically hydrolyzed peanut flour extracts. *Int Arch Allergy Immunol* 2013;162:123-30.
- Chatel JM, Bernard H, Orson FM. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *Int Arch Allergy Immunol* 2003;131:14-8.
- Li J, Shefcheck K, Callahan J, Fenselau C. Primary sequence and site-selective hydroxylation of prolines in isoforms of a major peanut allergen protein Ara h 2. *Protein Sci* 2010;19:174-82.
- Albrecht M, Kuhne Y, Ballmer-Weber BK, Becker WM, Holzhauser T, Lauer I, et al. Relevance of IgE binding to short peptides for the allergenic activity of food allergens. *J Allergy Clin Immunol* 2009;124:328-36.
- Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, et al. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophys* 1997;342:244-53.
- Petersen A, Schramm G, Schlaak M, Becker WM. Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. *Clin Exp Allergy* 1998;28:315-21.

33. Apostolovic D, Luykx D, Warmenhoven H, Verbart D, Stanic-Vucinic D, de Jong GA, et al. Reduction and alkylation of peanut allergen isoforms Ara h 2 and Ara h 6; characterization of intermediate- and end products. *Biochim Biophys Acta* 2013;1834:2832-42.
34. Starkl P, Felix F, Krishnamurthy D, Stremnitzer C, Roth-Walter F, Prickett SR, et al. An unfolded variant of the major peanut allergen Ara h 2 with decreased anaphylactic potential. *Clin Exp Allergy* 2012;42:1801-12.
35. Bernard H, Mondoulet L, Drumare MF, Paty E, Scheinmann P, Thai R, et al. Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. *J Agric Food Chem* 2007;55:9663-9.
36. Bublin M, Kostadinova M, Radauer C, Hafner C, Szepefalusi Z, Varga EM, et al. IgE cross-reactivity between the major peanut allergen Ara h 2 and the nonhomologous allergens Ara h 1 and Ara h 3. *J Allergy Clin Immunol* 2013;132:118-24.
37. Mueller GA, Gosavi RA, Pomes A, Wunschmann S, Moon AF, London RE, et al. Ara h 2: crystal structure and IgE binding distinguish two subpopulations of peanut allergic patients by epitope diversity. *Allergy* 2011;66:878-85.
38. Bernard H, Paty E, Mondoulet L, Burks AW, Bannon GA, Wal JM, et al. Serological characteristics of peanut allergy in children. *Allergy* 2003;58:1285-92.
39. Bernard H, Drumare MF, Guillon B, Paty E, Scheinmann P, Wal JM. Immunochemical characterisation of structure and allergenicity of peanut 2S albumins using different formats of immunoassays. *Anal Bioanal Chem* 2009;395:139-46.
40. Bernard H, Creminon C, Yvon M, Wal JM. Specificity of the human IgE response to the different purified caseins in allergy to cow's milk proteins. *Int Arch Allergy Immunol* 1998;115:235-44.
41. Ellman GL, Courtney KD, Andres V Jr, Feather-stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88-95.
42. Bogh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills EN, et al. Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clin Exp Allergy* 2009;39:1611-21.
43. King TP, Wade D, Coscia MR, Mitchell S, Kochoumian L, Merrifield B. Structure-immunogenicity relationship of melittin, its transposed analogues, and D-melittin. *J Immunol* 1994;153:1124-31.
44. Bannon GA, Ward JM, Dobert RC, Fuchs RL. *Biotechnology and genetic engineering*. Food allergy. Hoboken (NJ): John Wiley & Sons Ltd; 2013; 68-89.
45. Kane P, Erickson J, Fewtrell C, Baird B, Holowka D. Cross-linking of IgE-receptor complexes at the cell surface: synthesis and characterization of a long bivalent hapten that is capable of triggering mast cells and rat basophilic leukemia cells. *Mol Immunol* 1986;23:783-90.
46. Hales BJ, Bosco A, Mills KL, Hazell LA, Loh R, Holt PG, et al. Isoforms of the major peanut allergen Ara h 2: IgE binding in children with peanut allergy. *Int Arch Allergy Immunol* 2004;135:101-7.
47. Lin J, Bruni FM, Fu Z, Maloney J, Bardina L, Boner AL, et al. A bioinformatics approach to identify patients with symptomatic peanut allergy using peptide microarray immunoassay. *J Allergy Clin Immunology* 2012;129:1321-8.
48. Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2002;110:293-7.
49. Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA. Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy* 2007;62:758-65.
50. Vereda A, van Hage M, Ahlstedt S, Ibanez MD, Cuesta-Herranz J, van Odijk J, et al. Peanut allergy: clinical and immunologic differences among patients from 3 different geographic regions. *J Allergy Clin Immunol* 2011;127: 603-7.

METHODS

Recombinant allergens: Design, expression, refolding, and purification

The gene encoding Ara h 2.01 (Swiss-Prot accession no. Q6PSU2-2, Fig 1) was synthesized by using codons optimized for bacterial expression (Genscript USA) and inserted into the *E coli* expression plasmid pET9c (Novagen-Merck). A 6xHis Tag and the HRV 3C protease cleavage site were added to the N-terminus of the recombinant allergen. The variant rAra h 2.Δ was generated by means of PCR amplification of the expression plasmid without the sequence corresponding to the hydroxyproline-containing domain with the primers ATGAGTCGATCGTATGAGTCATAAGAGTCTTCAT CACG and CGTATTCACCGTCGC and self-ligation of the PCR product restricted with *PvuI* enzyme.

Overnight cultures of transformed *E coli* BL21 (DE3) were used to inoculate fresh medium at a dilution of 1:40. Cultures were grown at 37°C until the OD at 600 nm reached 0.5. Protein expression was induced by adding isopropylthio-β-D-galactoside (0.5 mmol/L) for 5 hours. After centrifugation, bacterial pellets were stored at −20°C until extraction. A frozen pellet corresponding to 1 L of culture was resuspended in 100 mL of NaH₂PO₄/Na₂HPO₄ (50 mmol/L, pH 8)/NaCl (0.5 mol/L) buffer with protease inhibitors. After sonication and centrifugation (10 minutes at 5000g and 4°C), the pellet was resuspended in 100 mL of extraction buffer (50 mmol/L NaH₂PO₄/Na₂HPO₄ [pH 8], 0.5 mol/L NaCl, 8 mol/L urea, 5 mmol/L dithiothreitol, and protease inhibitors) for 2 hours on a rotary mixer at room temperature to solubilize inclusion bodies. His-tagged allergen was then purified with a HisTrap FF Crude column (GE Healthcare, Fairfield, Conn).

Refolding of recombinant protein was performed by using direct dilution of the His-tagged purified fraction diluted at 0.2 mg/mL in refolding buffer (0.5 mol/L Tris [pH 8], glycerol 20%, 0.4 mol/L L-arginine, and 2 mmol/L glutathione disulfide) and incubation overnight at 4°C with energetic shaking. After dialysis against 20 mmol/L Tris, the fraction of refolded allergen was purified by means of RP-HPLC, as described previously.^{E1} Recombinant allergens were then characterized by using gel electrophoresis, MALDI-TOF analysis, and CD spectroscopy.

Purification of Ara h 2 isoforms

Whole peanut protein extract was prepared, as previously described.^{E2} Dialyzed extract was fractionated by means of precipitation with ammonium sulfate, which was added to 40% saturation. After centrifugation, the pellet was discarded, and the supernatant was dialyzed against 20 mmol/L phosphate buffer (pH 7.4). After addition of 0.5 mol/L NaCl, the dialysate was submitted to affinity chromatography by using Concanavalin A-Sepharose 4B (Sigma, St Louis, Mo). The flow-through fraction was dialyzed against 20 mmol/L Tris buffer (pH 7.4). 2S-albumins were separated by using a combination of preparative ion exchange and reversed-phase chromatography. Fractions containing isoforms of Ara h 2 were resuspended in buffer A (4 mol/L urea and 5 mmol/L Tris [pH 8.0]) and further purified by means of anion exchange chromatography with a Source 30Q column (1.6 × 10 cm) and an AKTA purifier system (GE Healthcare). Isoforms were separately eluted with a 100-minute linear gradient from 0% to 25% of buffer B (4 mol/L urea, 5 mmol/L Tris, and 1 mol/L NaCl [pH 8.0]).

Reduction and S-alkylation

Reduction of 2S-albumins was performed in 4 mol/L urea, 200 μmol/L EDTA, and 20 mmol/L dithiothreitol for 2 hours at 56°C. After cooling at

room temperature, alkylation was performed by adding iodoacetamide (200 mmol/L) in the dark over 4 hours. After dialysis against potassium buffer (0.05 mol/L, pH 7.4), r/a 2S-albumins were characterized by using gel electrophoresis, MALDI-TOF analysis, and CD spectroscopy.

Peptide synthesis

Peptides were synthesized by using a standard solid-phase synthesis with the 9-fluorenyl-methoxycarbonyl continuous-flow method (peptide synthesizer 433A; Applied Biosystems, Foster City, Calif). Following standard procedures, including TFA cleavage and ether precipitation, crude peptides were purified by means of RP-HPLC. The purified fraction was resuspended in potassium buffer (0.1 mol/L, pH 7.4), and peptides were characterized by using MALDI-TOF analysis.

Mass spectrometric characterization

Mass determination was carried out with MALDI-TOF (Voyager-DE STR; Applied Biosystems, Foster City, Calif) operating at 20-kV acceleration voltage and equipped with a nitrogen UV laser (337 nm). Mass spectrometric analysis was performed on peptides or purified proteins mixed in a 1:1 ratio with a matrix solution of α-cyano-4-hydroxycinnamic acid or sinapinic acid. Analysis was performed in a reflector or in a linear mode.

CD analysis

CD measurements were performed at 20°C on a J-810 Spectropolarimeter (Jasco Analytic Instruments, Easton, Md) by using 0.1-cm path length cells. A concentration of 0.1 mg/mL in 20 mmol/L phosphate buffer (pH 7.4) was prepared for the natural and recombinant isoforms of Ara h 2 samples. The spectra were recorded from 190 to 250 nm at a scanning speed of 100 nm/min with a 1-second time constant, a 0.1-nm resolution, and a 2-nm constant band pass. Three spectra were accumulated in each case. The averaged spectra were corrected by subtracting the baseline spectra obtained with the buffer alone under identical conditions. Mean residue weight ellipticities were calculated and expressed as follows:

$$\text{in units of degrees} \times \text{cm}^2 \times \text{dmol}^{-1}.$$

Gel permeation chromatography

Gel permeation chromatography was performed to characterize the formation of peptide aggregates. Synthetic peptides were analyzed under physiologic conditions at room temperature on a Stability GFC 50, 300 × 8-mm column (CIL, Cluzeau, France) coupled to an AKTA purifier system (GE Healthcare Life Sciences). A sample of peptide (100 μL of 1–2 mg/mL) was applied to the column and eluted at 0.5 mL/min with phosphate buffer (150 mmol/L KCl and 50 mmol/L K₂HPO₄/KH₂PO₄ [pH 7.4]). The eluate was monitored by using UV absorbance at 220 nm. The column was calibrated by using synthetic peptides with molecular weights of 1 and 6 kDa.

REFERENCES

- E1. Clement G, Bosquet D, Mondoulet L, Lamourette P, Bernard H, Wal JM. Expression in *Escherichia coli* and disulfide bridge mapping of PSC33, an allergenic 2S albumin from peanut. *Protein Expr Purif* 2005;44:110-20.
- E2. Bernard H, Mondoulet L, Drumare MF, Paty E, Scheinmann P, Thai R, et al. Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. *J Agric Food Chem* 2007;55:9663-9.

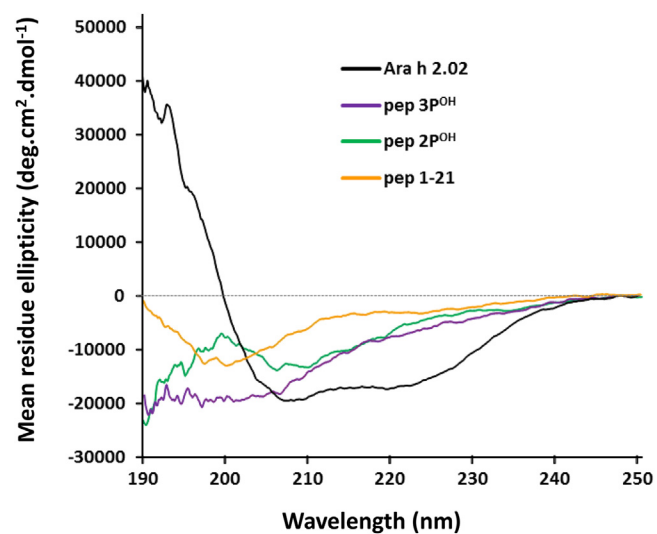


FIG E1. CD analysis shows a comparison of different synthetic peptides and nAra h 2.02. The *x-axis* shows the wavelength, and the *y-axis* shows the molecular ellipticity.

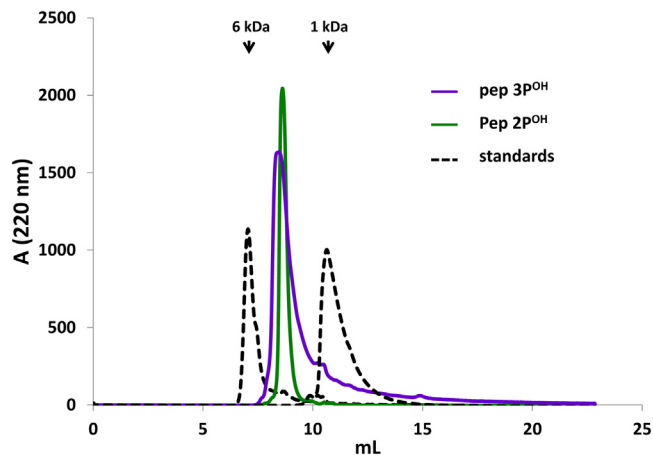


FIG E2. Analytic gel permeation chromatography of hydroxyproline-containing peptides under physiologic conditions. Standard molecular weight markers are shown across the top of the graph. The *x-axis* shows the elution volume, and the *y-axis* shows absorbance at 220 nm.

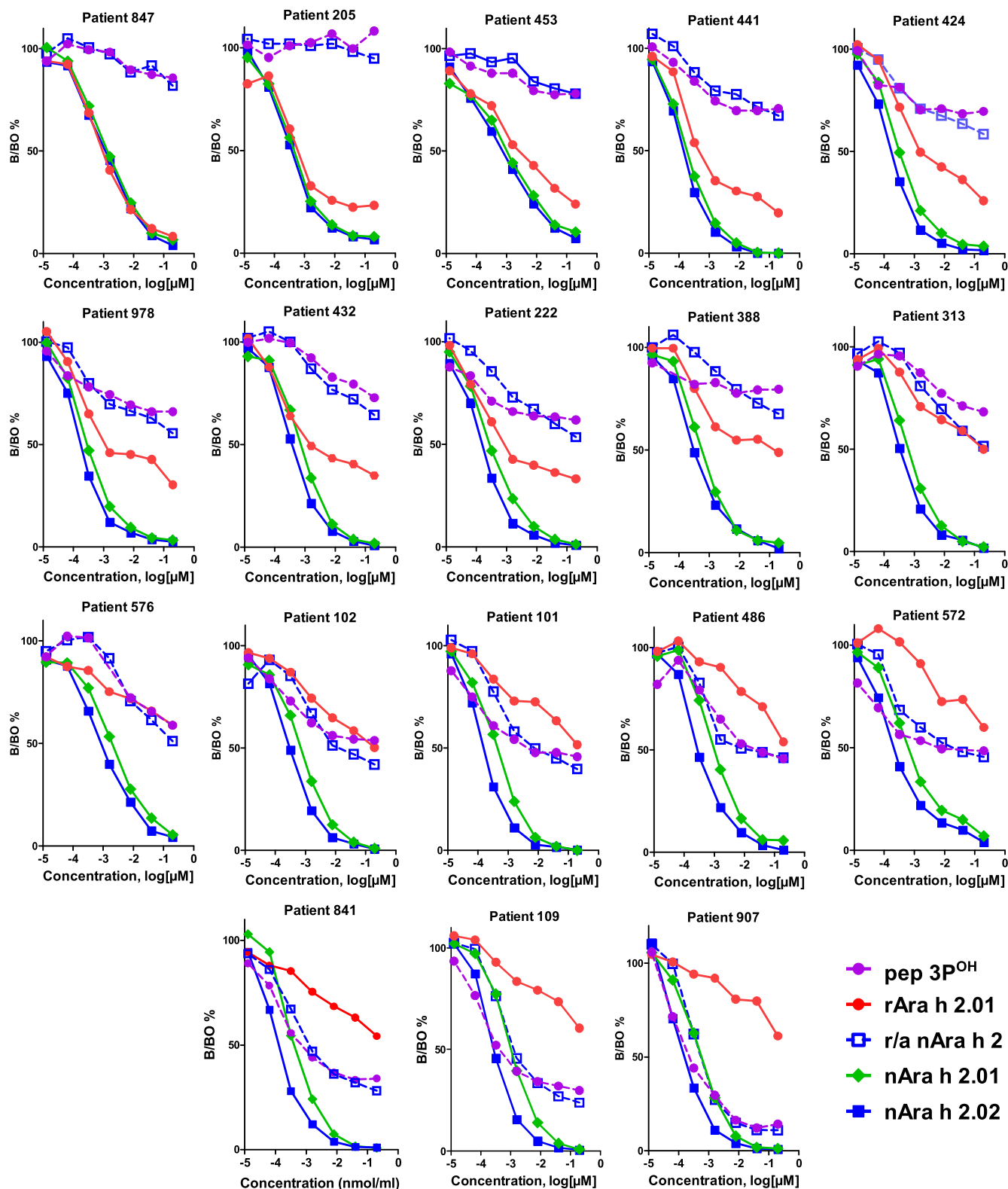


FIG E3. Comparison of the IgE-binding capacity of nAra h 2 and rAra h 2 and effect of reduction and S-alkylation. The IgE reactivity of r/a nAra h 2 was recapitulated with the synthetic peptide pep 3P^{OH} and was inversely proportional to that of rAra h 2.01. Competitive inhibition of IgE binding to nAra h 2 is shown for 18 sera from patients with peanut allergy.

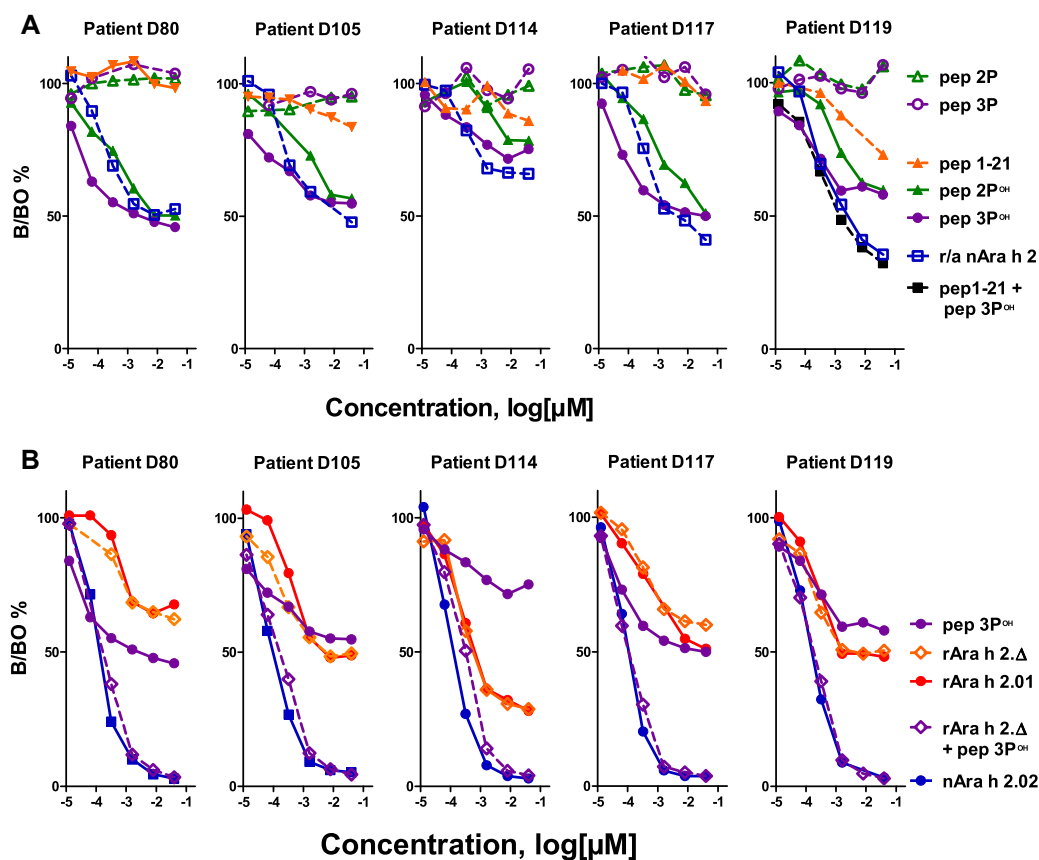


FIG E4. A, Influence of proline hydroxylation on the IgE-binding capacity of synthetic peptides containing 2 or 3 DPYSP^{OH}S motifs. Competitive inhibition of IgE binding to nAra h 2 for 5 sera from American patients with peanut allergy is shown. Of note, the IgE-binding capacity of r/a nAra h 2 was recapitulated with an equimolar mixture of pep 1-21 and pep 3P^{OH} for patient D119. **B**, The IgE-binding capacity of nAra h 2.02 is recapitulated with an equimolar mixture of the synthetic peptide pep 3P^{OH} and the deletion variant rAra h 2.Δ.

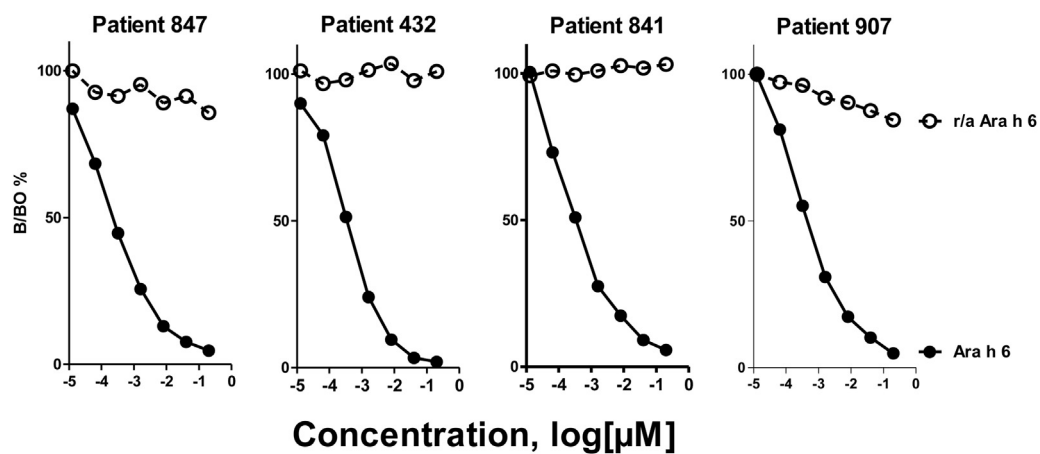


FIG E5. IgE-binding capacity of Ara h 6 versus r/a Ara h 6. Competitive inhibition of IgE binding to Ara h 6 for 4 representative sera is shown.

TABLE E1. Clinical features and IgE responses of French patients with peanut allergy

Patient no.	Age/sex	Symptoms	Specific IgE levels to peanut proteins (IU/mL)				Total IgE (IU/mL)
			Ara h 1	Ara h 2	Ara h 3	Ara h 6	
101	9/M	U	168	237	321	239	532
102	11/M	QO, GU, V	164	158	279	197	796
109	8/F	A	325	446	538	529	2100
205	9/F	V, CP	5	6	8	12	806
222	6/M	GU, V	93	130	130	162	443
313	4/M	GU	111	133	163	138	5301
388	6/F	LO	204	174	341	235	1352
424	11/F	GU	81	64	95	90	325
432	4/F	LO, GU, V	74	87	108	106	361
441	9/M	LO, U, V	42	73	69	75	479
453	10/F	U	4	13	10	10	4523
486	7/M	A, R	41	65	96	78	412
572	7/F	GU	37	34	48	50	137
576	6/M	GU	13	30	33	28	114
841	6/M	LO, U, V	192	246	273	312	1283
847	5/M	LO, AS	176	221	220	159	697
907	9/F	LO, GU, V	555	667	916	935	1822
978	8/F	U, V	58	45	80	53	254

A, Asthma; AO, angioedema; AS, anaphylactic shock; CP, cutaneous pruritus; F, female; GU, generalized urticaria; LO, laryngeal edema; M, male; QO, Quincke edema; R, rhinitis; U, urticaria; V, vomiting.

TABLE E2. Clinical features and IgE responses of American patients with peanut allergy

Patient no.	Age/sex	Symptoms	Specific IgE levels to peanut proteins (IU/mL)				
			WPPE	Ara h 1	Ara h 2	Ara h 3	Ara h 6
D80	13/M	V	156	107	95	122	112
D105	11/M	GU, AO, V	13.5	8.6	8.6	8.5	7.5
D114	28/F	GU, AO	24	15.9	10.6	20.2	9.5
D117	9/M	GU, AO, V	14.5	11.7	9.3	12	8.3
D119	15/M	GU, LO, A	64	45	40	51	55

A, Asthma; AO, angioedema; F, female; GU, generalized urticaria; LO, laryngeal edema; M, male; V, vomiting; WPPE, whole peanut protein extract.