

Identification of IgE sequential epitopes of lentil (Len c 1) by means of peptide microarray immunoassay

Andrea Vereda, MD, PhD,^{a,b,c,*} Doerthe A. Andreae, MD, PhD,^{a,*} Jing Lin, PhD,^a Wayne G. Shreffler, MD, PhD,^a Maria Dolores Ibañez, MD, PhD,^b Javier Cuesta-Herranz, MD, PhD,^c Ludmilla Bardina, MSc,^a and Hugh A. Sampson, MD^a New York, NY, and Madrid, Spain

Background: Lentils are often responsible for allergic reactions to legumes in Mediterranean children. Although the primary sequence of the major allergen Len c 1 is known, the location of the IgE-binding epitopes remains undefined.

Objective: We sought to identify IgE-binding epitopes of Len c 1 and relate epitope binding to clinical characteristics.

Methods: One hundred thirty-five peptides corresponding to the primary sequence of Len c 1 were probed with sera from 33 patients with lentil allergy and 15 nonatopic control subjects by means of microarray immunoassay. Lentil-specific IgE levels, skin prick test responses, and clinical reactions to lentil were determined. Epitopes were defined as overlapping signal above interslide and intraslide cutoffs and confirmed by using inhibition assays with a peptide from the respective region. Hierarchic clustering of microarray data was used to correlate binding patterns with clinical findings.

Results: The patients with lentil allergy specifically recognized IgE-binding epitopes located in the C-terminal region between peptides 107 and 135. Inhibition experiments confirmed the specificity of IgE binding in this region, identifying different epitopes. Linkage of cluster results with clinical data and lentil-specific IgE levels displayed a positive correlation between lentil-specific IgE levels, epitope recognition, and respiratory symptoms. Modeling based on the 3-dimensional structure of a homologous soy vicilin suggests that the Len c 1 epitopes identified are exposed on the surface of the molecule.

Conclusion: Several IgE-binding sequential epitopes of Len c 1 have been identified. Epitopes are located in the C-terminal

region and are predicted to be exposed on the surface of the protein. Epitope diversity is positively correlated with IgE levels, pointing to a more polyclonal IgE response. (*J Allergy Clin Immunol* 2010;126:596-601.)

Key words: Lentil allergy, peptide microarray, epitope recognition, epitope diversity, Len c 1

Lentils, together with chickpea, are important causes for IgE-mediated food hypersensitivity in the Mediterranean.¹⁻⁴ Other legumes, such as peanut and soybean, are the legumes most frequently involved in allergic reactions in the United States, the United Kingdom, and Japan. The major allergens from soybean and especially peanut have been extensively studied⁵⁻¹⁰; however, more interest has been shown recently in chickpea and lentil allergens.¹

In Spain allergy to lentils is the fifth most common cause of food allergy in the pediatric population.¹¹ Ten percent of children with food allergy have a convincing clinical history of allergy to lentils. Lentils frequently induce systemic symptoms, such as wheezing, rhinorrhea, and generalized urticaria.² Lentils have also been implicated in food-dependent exercise-induced anaphylaxis.⁴

More than 50% of subjects with lentil allergy are also allergic to chickpea and green pea.² Inhibition experiments and oral challenge tests suggest a high degree of *in vitro* and *in vivo* cross-reactivity.²

Several allergens from lentil have been characterized to date, including Len c 1.01, Len c 1.02, and Len c 2.

Len c 1.01 (Len c 1) is a protein of approximately 50 kd that has been identified as a mature vicilin chain.¹² Three genetic isoforms of this allergen have been described: Len c 1.0101, Len c 1.0102, and Len c 1.0103. Len c 1.02 is a 12- to 16-kd protein and the β -subunit of lentil vicilin that is probably produced by means of posttranslational proteolytic processing of the precursor Len c 1.01. Len c 2 is a distinct 66-kd protein corresponding to a seed-specific biotinylated protein.^{12,13} A clear structural relationship between Len c 1 and several allergens of the vicilin family, including Ara h 1, Jug r 2, Ana o 1, Ses i 3, and subunits of soybean conglycinin, has been described.¹⁴ Studies have shown IgE binding to a 50-kd band in lentil extract in more than 65% of patients,¹⁵ which was later described as Len c 1. Seventy-seven percent of the patients with lentil allergy recognized the purified Len c 1.¹² However, the location of the IgE-binding sequential epitopes of the major lentil protein Len c 1 remains unknown.

Here we report the mapping of IgE-binding epitopes of Len c 1 using a peptide microarray-based immunoassay. This sensitive

From ^athe Elliot and Roslyn Jaffe Food Allergy Institute, Division of Allergy and Immunology, Department of Pediatrics, Mount Sinai School of Medicine, New York; ^bthe Allergy Service, Hospital Infantil Universitario Niño Jesús, Madrid; and ^cServicio de Alergia, Fundación Jiménez Díaz, Madrid.

*These authors contributed equally to this work.

A. V. was funded by the World Allergy Organization; H. A. S. and W. G. S. have received research support from the National Institutes of Health/National Institute of Allergy and Infectious Diseases and the Food Allergy and Anaphylaxis Network.

Disclosure of potential conflict of interest: A. Vereda has a Fellowship with the World Allergy Organization. H. A. Sampson is a consultant for Allertein, LLC; has received research support from the Food Allergy Initiative and the National Institute of Allergy and Infectious Diseases/National Institutes of Health; is a consultant and scientific advisor for the Food Allergy Initiative; and is a 45% owner of Herbal Springs, LLC. The rest of the authors have declared that they have no conflict of interest.

Received for publication January 11, 2010; revised June 1, 2010; accepted for publication June 10, 2010.

Reprint requests: Hugh A. Sampson, MD, Department of Pediatrics, Box 1198, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574. E-mail: hugh.sampson@mssm.edu.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2010.06.023

Abbreviations used

HSA: Human serum albumin
PBS-T: PBS containing 0.05% Tween 20
SPT: Skin prick test

technique allowed us to rapidly study several sets of the protein simultaneously using only a minute quantity of sera.

METHODS

Patients

Thirty-three patients with lentil allergy were recruited from the Hospital Fundación Jiménez Díaz (Madrid, Spain; $n = 5$), from the Hospital Niño Jesús (Madrid, Spain; $n = 24$), and from Mount Sinai Medical Center (New York, NY; $n = 4$) from 2004 to 2006. Written informed consent was obtained from all subjects (or the legal guardians for children) before their inclusion in the study. The diagnosis of IgE-mediated lentil allergy was made by an allergist on the basis of a convincing history (objective symptoms) of an acute reaction (<30 minutes) after lentil ingestion together with evidence of specific IgE antibodies (positive skin prick test response, lentil-specific IgE >0.35 kU_A/L, or both).

Lentil (*Lens esculenta*)-specific IgE levels were measured with the ImmunoCAP System FEIA (Phadia, Uppsala, Sweden). The assay had a lower detection limit of 0.35 kU_A/L and an upper limit of 100 kU_A/L, with higher values reported as greater than 100 kU_A/L.

Skin prick tests (SPTs) with lentil (*Lens culinaris*) extract (Leti, Madrid, Spain) were performed by using a standard technique with a needle (ALK-Abelló, Madrid, Spain). The mean of the wheal's largest diameter and its perpendicular in millimeters was measured after 15 minutes. A result was considered positive when the mean diameter of the wheal was at least 3 mm larger than the wheal elicited by the negative control. Histamine (10 mg/mL) and saline solutions were used as positive and negative controls, respectively. When this commercial extract was not available, results of prick-to-prick skin tests performed with cooked lentils were reported as positive or negative. Clinical information was gathered by using a specific questionnaire. This information included a detailed clinical history, including the characteristics of reactions to lentil exposure, other plant-related food allergies, pollen allergy, and personal and family histories of atopic diseases.

Preparation of microarray: Peptides, slides, and printing

Peptide libraries, slides, and printing were prepared and performed as described elsewhere.^{16,17}

Peptide microarray immunoassay

Immunolabeling was performed as previously described, with some modifications.^{8,10,16,18} In brief, an area around the arrays was demarcated with a hydrophobic pen (DakoCytomation Pen; DAKO, Glostrup, Denmark). The slides were first rinsed with PBS containing 0.05% Tween 20 (PBS-T), and nonspecific binding sites were blocked with 400 μ L of 1% human serum albumin (HSA) in PBS-T (PBS-T/HSA) for 60 minutes at room temperature. After removing the PBS-T/HSA from the slide surface by means of aspiration, 50 μ L of patient serum diluted 1:5 in PBS-T/HSA was applied and allowed to incubate for 24 hours at 4°C. Slides were washed with PBS-T and incubated for 24 hours at 4°C with a cocktail of 2 monoclonal biotinylated anti-human IgE antibodies (Invitrogen, Carlsbad, Calif, and BD Biosciences PharMingen, San Jose, Calif) diluted 1:250 each.

Slides were washed with PBS-T, incubated for 4 minutes with 1 mmol/L EDTA in PBS-T, washed again with PBS-T, and equilibrated for 1 minute with Dendrimer Buffer (Genisphere, Philadelphia, Pa), followed by incubation for 3 hours at room temperature with Anti-Biotin-Dendrimer-Oyster 550 (Genisphere) in Dendrimer Buffer at 0.6 μ g/mL with addition of 0.02 μ g/mL salmon sperm DNA.

All incubations were performed in the dark in a humidity chamber (Binding Site, Birmingham, United Kingdom) on an orbital rotating platform with gentle agitation. Two immunolabelings were performed without a patient's serum to test for nonspecific binding of secondary antibody or dendrimers to peptides. The immunoassay was performed as explained above, except for substitution of serum by blocking solution.

The slides were then washed with PBS-T and 15 mmol/L Tris buffer, centrifuge dried, washed with 0.1 $\times$ PBS, centrifuge dried, washed again with 0.05 $\times$ PBS, centrifuge dried, and scanned with a ScanArray Gx Scanner (PerkinElmer, Waltham, Mass). Images were saved as TIFF files.

Peptide microarray inhibition experiment

A peptide inhibition assay was carried out in which the array was immunolabeled as described above except that the serum pool (diluted 1:50 in blocking buffer) was preincubated with 1 μ L of peptide at a concentration of 1 mmol/L (1 peptide per array) for 2 hours at gentle agitation. The same serum pool without added peptides was processed in parallel and incubated under the same conditions as the control.

Data analysis

Lentil-specific IgE level, age, age of onset of lentil allergy, and lentil SPT size were summarized as medians with interquartile ranges. χ^2 (or Fisher tests, if necessary) and Wilcoxon tests were used for comparisons between groups. Correlation between variables was measured with the Pearson correlation coefficient.

Regarding peptide microarray data, fluorescence signal from one channel (for IgE binding) was digitized with the ScanArray Express Microarray Analysis System (PerkinElmer), exported as comma-delimited (CSV) files, and analyzed with R analysis (R: A language and environment for statistical computing, version 2.5.0; R foundation for Statistical Computing, Vienna, Austria; <http://r-project.org>).

For hierarchical cluster analysis, the absolute signal intensity data (in digital fluorescence units) of all patients on all array elements was used. Clusters were determined by using cluster software (<http://rana.lbl.gov/EisenSoftware.htm>) with the average linkage algorithm and uncentered measurements for features, array elements remained unchanged. Results were displayed with Java TreeView 1.60 (<http://rana.lbl.gov/EisenSoftware.htm>).

RESULTS

Description of patients

Thirty-three patients with lentil allergy were included in the study. Median age at inclusion was 8.0 years (interquartile range, 5.0-13.0 years). The majority of patients had family and personal histories of atopy: 24 patients had allergic rhinitis, 21 had asthma, and 14 had atopic dermatitis. Sixteen (48.5%) patients were allergic to pollen. General characteristics of the patients are included in Table I.

The median age of onset for lentil allergy was 2 years (interquartile range, 1.0-3.0 years). The lentil-specific IgE level at the time of enrollment in the study was 19.0 kU_A/L (interquartile range, 9.9-58.0 kU_A/L), and the lentil SPT response was 5.0 mm (interquartile range, 3.4-6.9 mm). Most subjects reported systemic symptoms after lentil ingestion (87.9%), mostly generalized urticaria, and upper, lower, or both respiratory tract symptoms (wheeze, cough, shortness of breath, and rhinoconjunctivitis).

A significant number of patients reported symptoms after ingestion of other legumes: 29 of 33 patients reacted to green pea, 26 of 28 reacted to chickpea (28 patients had eaten chickpea previously and therefore were able to answer), 25 of 32 reacted to bean, 12 of 27 reacted to peanut, and 5 of 19 reacted to soy. With respect to reactions to tree nuts, 6 of 22 reacted to almond, 4 of 19 reacted to pistachio, 3 of 20 reacted to walnut, 4 of 19 reacted to hazelnut, and 2 of 16 reacted to cashew.

TABLE I. Patients' characteristics

ID	Age (y)/sex	Pollen allergy	CAP to lentil (kU _A /L)	Age of lentil allergy onset (y)	Symptoms with lentil	SPT	Provocation with lentil	Allergy to tree nuts and sesame	Allergy to legumes (including peanut)
1	14/M	No	≥100	4	U, OAS, DS, A, ANA	Positive	ND	No	Pea, bean, chickpea
2	13/M	Yes	24.20	3	OAS	ND	Positive	No	Pea, bean, chickpea, peanut
3	13/M	Yes	26.20	2	OAS, U, DS, ANA	Positive	Positive	No	Pea, bean, chickpea
4	12/M	Yes	17.40	2	OAS, R, A, U, ANA	Positive	Positive	No	Pea, bean, chickpea
5	16/M	Yes	22.00	1	OAS	Positive	Positive	No	Pea
6	11/M	Yes	9.67	2	OAS, R, A, U, ANA	Positive	Positive	No	Bean, pea
7	12/M	Yes	8.02	7	R, A, U, ANA	Positive	Positive	Almond	Chickpea, pea, peanut
8	13/F	Yes	≥100	2	OAS, R, A, U, DS, ANA	Positive	ND	No	Chickpea, pea, bean
9	10/F	No	59.20	2	R, A, U	Positive	Positive	No	Pea, bean, chickpea, peanut
10	8/M	Yes	21.00	1	U, DS, ANA	Positive	ND	Almond	Chickpea, pea, bean
11	6/M	Yes	65.40	1	R, A, U, ANA	Positive	Positive	No	Chickpea, pea, bean
12	5/F	ND	30.10	3	OAS, R, A, U	ND	Positive	Almond	Bean, pea, peanut
13	7/M	No	≥100	1.2	OAS, R, A, U, DS, ANA	Positive	ND	No	Chickpea, pea, bean
14	7/M	No	11.30	3	OAS, U	Positive	Positive	No	Pea, bean, chickpea, peanut
15	5/M	Yes	16.30	4	OAS, R, A, U, ANA	Positive	Positive	No	Chickpea
16	3/M	No	14.50	2	OAS, R, A, U, DS, ANA	Positive	ND	No	Pea, bean, chickpea, peanut
17	6/M	No	49.90	1.4	Systemic	Positive	Positive	No	Chickpea, pea, bean
18	4/M	No	3.80	2	OAS, R, A, U, ANA	Positive	Positive	No	Pea, bean, chickpea, peanut
19	32/M	Yes	0.77	1	OAS, R, A, DS, U, ANA	Positive	ND	Pistachio, cashew, walnut, hazelnut, almond	Pea, bean, chickpea, peanut
20	5/F	No	9.82	1	OAS, R, A, U, ANA	Positive	Positive	Pistachio, hazelnut, walnut, almond	Chickpea, pea, bean
21	4/M	Yes	56.70	2	OAS, R, A, U, ANA	Positive	Positive	No	Chickpea, pea, bean
22	3/M	Yes	≥100	2	Systemic	Positive	Positive	No	Chickpea, pea, bean, soy
23	5/F	No	≥100	1	OAS, U	Positive	ND	No	Chickpea, pea, bean
24	5/F	No	19.00	2	DS	Positive	Positive	No	Chickpea, pea
25	2/M	ND	16.00	1	OAS	Positive	Positive	No	Chickpea, pea, bean
26	49/F	No	1.61	2	OAS, U, R, A	Positive	ND	No	Chickpea, pea, bean, soy
27	5/M	No	3.60	1	OAS, U, ANA	Positive	ND	No	No
28	5/F	No	9.92	1	U, DS, ANA	Positive	ND	No	Chickpea, pea, bean, soy
29	19/F	No	1.78		R, U, A, DS, angioedema	Positive	ND	Pistachio, hazelnut, walnut, almond	Chickpea, peanut
30	10/M	Yes	14.50	6	R, A, U, DS, ANA	Positive (PP)	ND	No	Bean, pea, peanut
31	37/F	No	10.40	5	OAS, R, A, U, DS, ANA	ND	ND	Pistachio, cashew, hazelnut, sesame	Chickpea, pea, peanut, soy
32	9/F	Yes	35.50	4	OAS, R, A, U, DS, ANA	Positive (PP)	ND	Sesame	Bean, pea, peanut, soy
33	12/F	Yes	73.70	1	OAS, R, A, U, ANA	Positive (PP)	ND	No	No

A, Asthma; ANA, anaphylaxis; DS, digestive symptoms; F, female; M, male; ND, not done or not determined; OAS, oral allergy syndrome; PP, prick-prick test with cooked lentils; R, rhinoconjunctivitis; SPT, skin prick test; U, urticaria.

Peptide microarray immunoassay: Epitope recognition

Commercially synthesized peptides of 15 amino acids in length with an overlap of 12 amino acids corresponding to the primary sequence of Len c 1 were robotically arrayed in 2 sets of triplicates and attached to derivatized glass slides. The peptide arrays were assayed in parallel with sera from the 33 patients with lentil allergy and 15 healthy volunteers.

Based on previous experiments,^{8-10,18} we expected to see a non-random distribution of signal intensity corresponding to antibody recognition of overlapping peptides, even though the peptides were printed on different locations on the array. Fig 1 displays the median signal (in digital fluorescence units) by peptide ordered

according to the primary sequence of Len c 1 from the N-terminus to the C-terminus. As expected, signal intensity is present in peaks in distinct regions of the protein, represented by sets of overlapping peptides, indicating recognition of specific sequences.

The region at the C-terminal side (peptides 106-135) of the molecule appears to be specifically recognized by 84.8% of the patients with lentil allergy above interslide and intraslide cutoff values. The intensity of the IgE signal was significantly higher in the allergic group ($P < .001$).

Some signal associated with peptides in the N-terminal side of the molecule and a region in the center of the molecule (peptides 70-89) can be seen in both allergic patients and negative control subjects. Nonspecific artifact was suspected. The nonspecificity of these

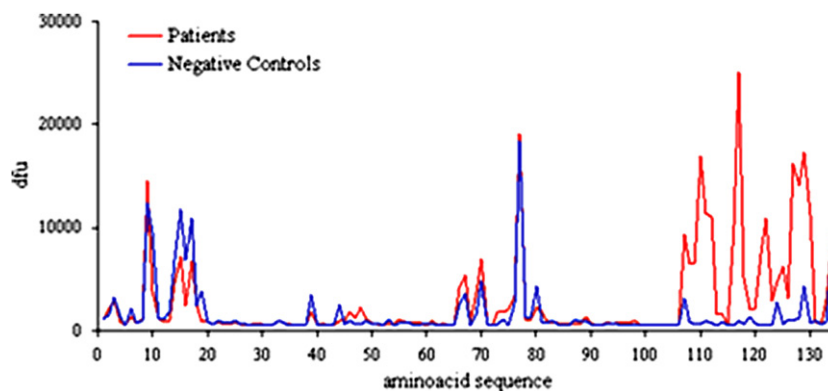


FIG 1. Average signal intensity (in digital fluorescence units [dfu]) plotted from the N-terminal to the C-terminal position of the Len c 1 sequence for patients (red) and negative control subjects (blue).

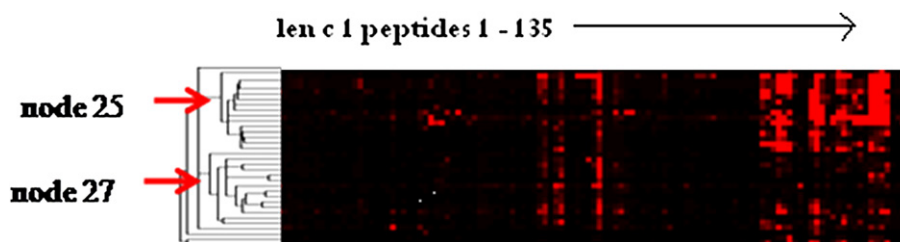


FIG 2. Treeview display of hierarchic cluster analysis of IgE signal (positive in increasing brightness of red and negative in black). Peptides are in columns, and individual patients (arrays) are in rows. The arrows indicate peptide clusters (nodes) that were chosen for additional analysis of patients' characteristics. See the Methods and Results sections for details.

signals was confirmed by 2 immunolabelings substituting a patient's sera with blocking solution. Without serum incubation, fluorescence was absent in the C-terminal region of the molecule but was still present in the N-terminus and the central part (peptides 70-89). Inhibition experiments were not performed for these regions. We focused our attention on the C-terminal portion of the molecule.

As previously described, dominant antigenic regions can be identified by using this approach, although there is a high interpatient heterogeneity. Some subjects' IgE antibodies appear to recognize slightly different peptides; others recognize more or less regions than the majority. Fig 2 displays individual heterogeneity in recognition patterns for the 33 patients. Red pixels represent the intensity of individual IgE binding by pixel intensity. Patients are displayed in rows, and subsequent peptides are displayed in columns. Negative control subjects are not included in this analysis because no above-cutoff binding to the described region was detected. The region from peptides 106 to 135 is recognized by the majority of patients. Differences, however, exist in the number of peptides recognized and intensity of binding. Patients cluster in 2 main groups: node 25 ($r = 0.729$) and node 27 ($r = 0.6768$; Fig 2). The lentil-specific IgE level is different between those 2 groups. Patients clustering in node 25 have a significantly higher lentil-specific IgE level than the rest of the patients ($P < .001$; median of 62.3 kU_A/L [interquartile range, 17.1-100.0 kU_A/L] vs median of 11.3 kU_A/L [interquartile range, 8.0-22.0 kU_A/L]). The patients clustered in node 25 also have more respiratory symptoms, including rhinoconjunctivitis and wheezing after exposure to lentil ($P = .024$). Patients clustering in node 27 have a significantly lower median lentil-specific IgE level than the rest of the patients ($P < .005$; median of 12.9

kU_A/L [interquartile range, 6.5-21.3 kU_A/L] vs median of 49.9 kU_A/L [interquartile range, 14.5-100.0 kU_A/L]).

Lentil-specific IgE levels positively correlated with both the number of positive peptides ($r = 0.514$, $P < .005$) and the signal intensity ($r = 0.799$, $P < .0001$) for the region of interest (peptides 106-135).

Inhibition experiments

Pooled sera were incubated for 2 hours with individual peptides before immunolabeling, as described above, to confirm the sequence specificity of IgE signal. Four peptides from the region of interest were chosen for inhibition studies (peptides 110, 117, 123, and 128).

Signal from overlapping peptides was attenuated by using single peptides as inhibitors. For example, peptide 110 blocked epitope 106 to 113. Peptide 117 blocked epitope 115 to 119. Peptide 123 blocked epitope 120 to 123, and peptide 128 blocked epitope 126 to 129 (Fig 3). Four distinct epitopes could be identified in the C-terminal region of the Len c 1 protein. The location of these epitopes on the Len c 1 sequence is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

Modeling using the known 3-dimensional structure of another vicilin (soy β -conglycin, PDB 1UIK) predicts that these epitopes of Len c 1 are exposed on the surface of the molecule (Fig 4).

DISCUSSION

This study focused on the specificity and diversity of the IgE response to the major lentil allergen Len c 1. We have identified 4 putative epitopes in the C-terminus. No epitopes in Len c 1 have been previously identified. We could also demonstrate that IgE

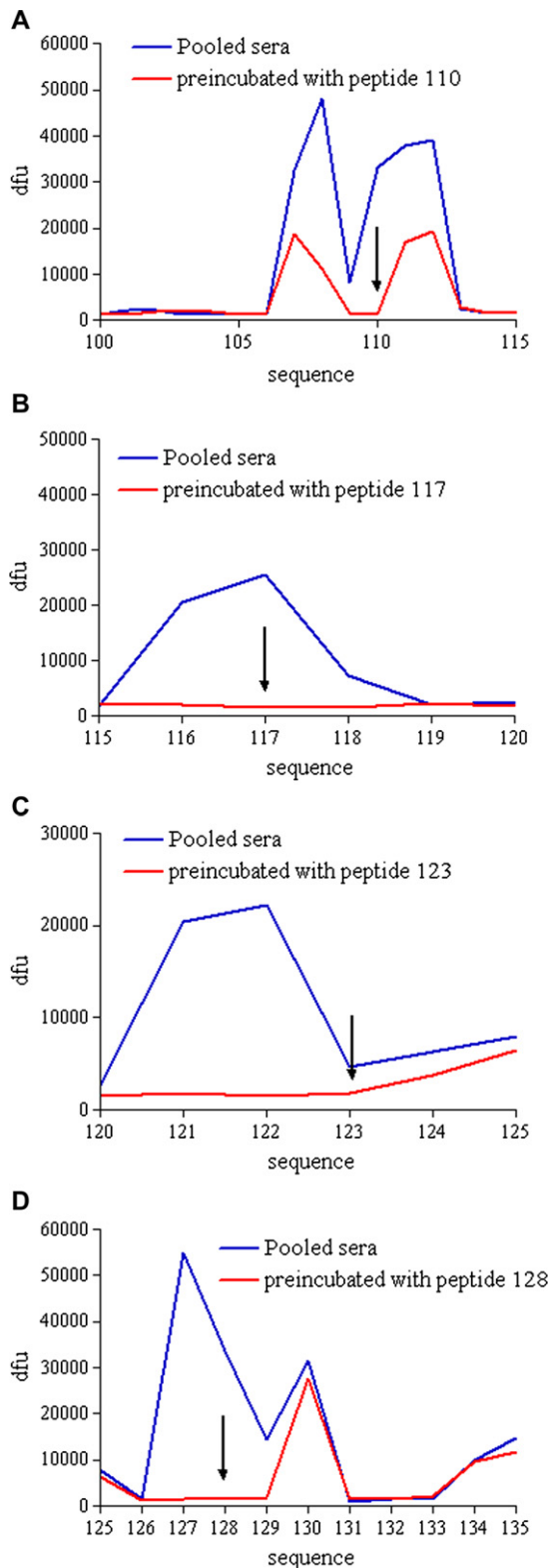


FIG 3. Average signal intensity (in digital fluorescence units [dfu]) plotted from the N-terminal to the C-terminal position of the Len c 1 peptide for pooled sera preincubated with a single peptide for 2 hours (red) and pooled sera without preincubation (blue). Four separate experiments with different peptides were conducted (A-D). The arrows indicate the localization of the peptide used for the inhibition.

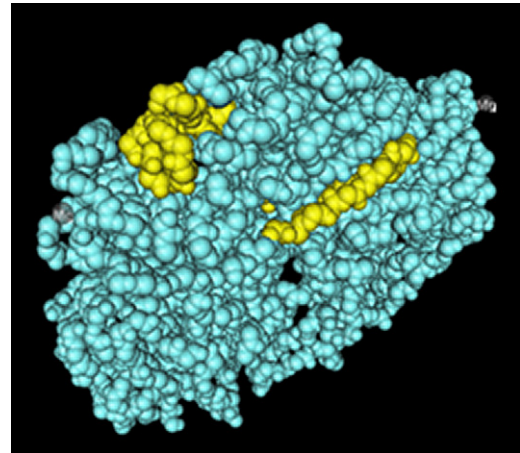


FIG 4. Epitopes 1 and 4 as defined in this study superimposed on the 3-dimensional structure of the vicilin soy β -conglycin. Epitopes are located on the outside of the molecule. On top is epitope 1, and below is epitope 2.

levels and clinical characteristics correlated with features of epitope recognition. This is consistent with previous studies of IgE recognition patterns to sequential (linear) epitopes, which have been associated with the natural history of food allergy in individual patients¹⁸⁻²⁰ and the severity of allergic reactions.⁸⁻¹⁰

Epitope detection and definition have been a main focus of several studies in allergy, all of which are confronted with the problem of definition criteria.^{8-10,18,21} Epitopes might be defined by a set of overlapping peptides that are identified by a certain percentage of patients. An important uncertainty, however, lies in the definition of the start and end points of the epitope. For example, in the case of Len c 1, there are 4 major signal peaks in the region from peptides 106 to 135 (Fig 1). However, no clear distinction can be made between single epitopes in the region by taking into account only the percentage of patients with positive binding. Cluster analysis of binding patterns of multiple peptides comprised of different amino acid numbers covering this sequence of the protein,⁹ offers one possible approach to address this vagueness. The specific design of our experiment did not include peptide sequences of different lengths covering this sequence of the protein. Therefore the cluster analysis performed here was solely used to link binding patterns to patients' characteristics.

Another approach is the implementation of inhibition experiments. In our study preincubation of patients' sera with a peptide selected from the area of interest showed a clear inhibition of IgE binding to that peptide, and because of sequence overlap, inhibition of the adjacent peptides could also be seen.

All 4 of the epitopes that we have defined in this assay are predicted to be located on the surface of the molecule by means of 3-dimensional modeling based on the sequence of the homologous β -conglycin of soy. Highlighting the Len c 1 epitopes on a related food protein with a known 3-dimensional structure (β -conglycin α subunit of soybean)¹⁴ indicates that all 4 epitopes are exposed on the surface of the protein. Vicilins, such as those of soybean and lentil, are very stable proteins²² that are largely resistant to heat and digestion. A recent publication emphasizes the extreme heat resistance of some lentil allergens.¹ Therefore we can assume that Len c 1-reactive patients probably react to the whole protein as a trimer. The epitope location on the outer surface of the molecule makes them readily accessible for IgE antibodies.

As mentioned above, a positive correlation between the number of peptides recognized by a subject and the level of peanut-specific IgE (correlation factor, 0.579) has been studied and published previously.⁸ Our study shows that peptide recognition in numbers of positive peptides and binding intensity to epitopes is positively correlated to lentil-specific IgE levels (number: $r = 0.514$, $P < .005$; intensity: $r = 0.799$, $P < .0001$).

However, not all patients with lentil allergy had detectable Len c 1 epitope binding, as determined by using the microarray immunoassay. In our study 84.8% of patients with lentil allergy recognized peptide epitopes of Len c 1. The remaining patients might have antibody to conformational epitopes only, which are not detected with this experimental setup, or to other lentil proteins. Other studies have confirmed that Len c 1-specific antibodies are not present in all patients with lentil allergy; approximately 65% of patients with lentil allergy recognize a 50-kd band in lentil extract,⁵ later described as Len c 1, and López-Torrejón et al¹² reported a positive recognition by 77% of patients with lentil allergy studied. These results indicate that Len c 1 is a major lentil allergen but not the only one responsible for symptoms.

Panallergens, or common protein families (eg, vicilins), might result in cross-reactivity among foods. The two clones of the lentil allergen Len c 1.02 were shown to have greater than 50% identity with other vicilins, including the major peanut allergen Ara h 1, and with soybean conglutinin subunits.¹² In a study of 18 Spanish patients with pea allergy, many also reacted to lentil. Vicilin and con-vicilin were shown to be potential major allergens from pea, and these cross-reacted with the major lentil allergen Len c 1.¹³ The 3-dimensional models of vicilin allergens (Ara h 1 from peanut, Len c 1 from lentil, and Pis s 1 from pea) built by homology-based modeling from the soybean β -conglycinin were shown to be very similar, helping to account for their IgE-binding cross-reactivity.¹⁴

Many of the patients included in this study had symptoms when exposed to other legumes, mainly chickpea (92.9%) and pea (87.9%). Many of the patients (12/27) reacted to peanut. In the group of patients studied here, the lentil-specific IgE levels appeared to correlate with the number of epitopes bound and the intensity of binding rather than the presence of tolerance/reaction to other legumes. Further analysis of IgE binding to vicilins with a larger number of patients is needed to fully understand the above-described legume *in vivo* and *in vitro* cross reactivity² at an epitope level.

Performing the same experiments in Len c 1 using sera from patients allergic to peanut or chickpea but tolerant to lentil and analysis of Ara h 1 epitope binding in a larger cohort of patients with lentil allergy, including a comparison of peanut-tolerant subjects and patients with peanut allergy (with similar specific IgE levels), might further clarify the mechanism of legume cross-reactivity. We believe that understanding the molecular basis of clinical reactivity will help us in counseling our patients more accurately. Peptide microarray immunoassays offer a versatile platform to address this issue. Our results provide a first step in helping to resolve the above-described questions.

In conclusion, we have identified the IgE binding epitopes of a major lentil allergen, Len c 1, using a peptide microarray. Len c 1 epitopes detected by this assay appear to be located on the surface of the molecule. The number of peptides recognized and the intensity of the signal are correlated with lentil specific IgE.

We thank Michelle Mishoe for the lentil-specific IgE determination and Galina Grishina and Rosalia Ayuso for their helpful discussion of the data. Andrea Vereda thanks the World Allergy Organization for their support.

Key messages

- This study is the first to explore and locate IgE-binding B-cell epitopes of lentil allergens by using a peptide microarray immunoassay.
- Combining the findings of the microarray immunoassay with clinical characteristics and IgE levels shows a positive correlation.

REFERENCES

- Cuadrado C, Cabanillas B, Pedrosa MM, Varela A, Guillaumon E, Muzquiz M, et al. Influence of thermal processing on IgE reactivity to lentil and chickpea proteins. *Mol Nutr Food Res* 2009;53:1462-8.
- Martínez San Ireneo M, Ibáñez MD, Sánchez JJ, Carnés J, Fernández-Caldas E. Clinical features of legume allergy in children from a Mediterranean area. *Ann Allergy Asthma Immunol* 2008;101:179-84.
- Kalogeromitos D, Armenaka M, Galatas I, Capellou O, Katsarou A. Anaphylaxis induced by lentils. *Ann Allergy Asthma Immunol* 1996;77:480-2.
- Orhan F, Karakas T. Food-dependent exercise-induced anaphylaxis to lentil and anaphylaxis to chickpea in a 17-year-old boy. *J Investig Allergol Clin Immunol* 2008;18:465-8.
- Krishnan HB, Kim WS, Jang S, Kerley MS. All three subunits of soybean beta-conglycinin are potential food allergens. *J Agric Food Chem* 2009;57:938-43.
- Holzhauser T, Wackermann O, Ballmer-Weber BK, Bindslev-Jensen C, Scibilia J, Perono-Garoffo L, et al. Soybean (Glycine max) allergy in Europe: Gly m 5 (beta-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *J Allergy Clin Immunol* 2009;123:452-8.
- Jin T, Guo F, Chen YW, Howard A, Zhang YZ. Crystal structure of Ara h 3, a major allergen in peanut. *Mol Immunol* 2009;46:1796-804.
- Flinterman AE, Knol EF, Lencer DA, Bardina L, den Hartog Jager CF, Lin J, et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. *J Allergy Clin Immunol* 2008;121:737-43.
- Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA. Microarray immunoassay: association of clinical history, *in vitro* IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 2004;113:776-82.
- Shreffler WG, Lencer DA, Bardina L, Sampson HA. IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. *J Allergy Clin Immunol* 2005;116:893-9.
- Ibáñez MD, Martínez M, Sánchez JJ, Fernández-Caldas E. [Legume cross-reactivity]. *Allergol Immunopathol (Madr)* 2003;31:151-61.
- López-Torrejón G, Salcedo G, Martín-Esteban M, Díaz-Perales A, Pascual CY, Sanchez-Monge R. Len c 1, a major allergen and vicilin from lentil seeds: protein isolation and cDNA cloning. *J Allergy Clin Immunol* 2003;112:1208-15.
- Sanchez-Monge R, Pascual CY, Díaz-Perales A, Fernandez-Crespo J, Martín-Esteban M, Salcedo G. Isolation and characterization of relevant allergens from boiled lentils. *J Allergy Clin Immunol* 2000;106:955-61.
- Barre A, Borges JP, Rouge P. Molecular modelling of the major peanut allergen Ara h 1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. *Biochimie* 2005;87:499-506.
- Ibáñez SD, Martínez SI, Marañón LF, Fernández-Caldas E, Alonso LE, Laso BT. Specific IgE determinations to crude and boiled lentil (*Lens culinaris*) extracts in lentil-sensitive children and controls. *Allergy* 1999;54:1209-14.
- Lin J, Bardina L, Shreffler WG, Andrae DA, Ge Y, Wang J, et al. Development of a novel peptide microarray for large-scale epitope mapping of food allergens. *J Allergy Clin Immunol* 2009;124:315-22.
- Lin J, Bardina L, Shreffler WG. Microarrayed allergen molecules for diagnostics of allergy. *Methods Mol Biol* 2009;524:259-72.
- Cerecedo I, Zamora J, Shreffler WG, Lin J, Bardina L, Dieguez MC, et al. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J Allergy Clin Immunol* 2008;122:589-94.
- 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.
- Wang J, Lin J, Bardina L, Goldis M, Nowak-Węgrzyn A, Shreffler WG, et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J Allergy Clin Immunol* 2010;125:695-702.
- Matsuo H, Kohno K, Niihara H, Morita E. Specific IgE determination to epitope peptides of omega-5 gliadin and high molecular weight glutenin subunit is a useful tool for diagnosis of wheat-dependent exercise-induced anaphylaxis. *J Immunol* 2005;175:8116-22.
- Breiteneder H, Radauer C. A classification of plant food allergens. *J Allergy Clin Immunol* 2004;113:821-30.

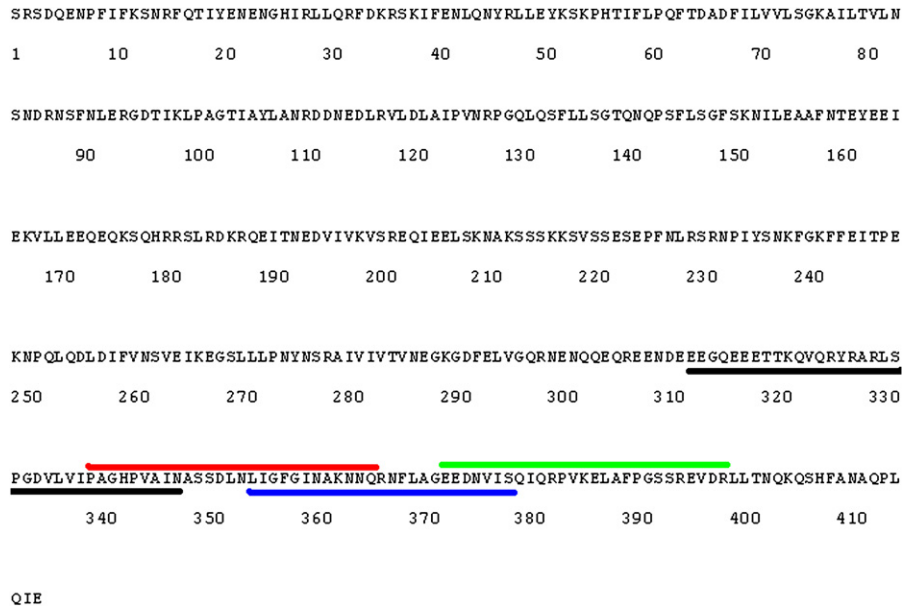


FIG E1.: Location of the four defined epitopes on the len c 1 sequence. The defined epitopes have partly overlapping sequences.

- Epitope 1 (EEGQEEETTKQVQRYRARLSPGDVLVIPAGHPVA IN) —
- Epitope 2 (PAGHPVA INASSDLNLIGFGINAKNNQ) —
- Epitope 3 (LIGFGINAKNNQRNFLAG EEDNVIS) —
- Epitope 4 (EEDNVISQIQRPVKELAFPGSSREVDR) —

FIG E1. Sequence with highlighted epitopes.