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

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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 studied[5-10]; 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

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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 μL 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 μL 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 μg/mL with addition of 0.02 μg/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× PBS, centrifuge dried, washed again with 0.05× 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 μL 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.