IgE and IgG_4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2

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Background: Detailed assessment of antibody responses to allergens reveals clinically relevant information about both host response and antigen structure. Microarray technology offers advantages of scale and parallel design over previous methods of epitope mapping.

Objective: We designed a redundant peptide microarray for IgE and IgG_4 epitope mapping of the previously characterized peanut allergen, Ara h 2.

Methods: Six complete sets of overlapping peptides were commercially synthesized and site-specifically bound to epoxy-derivatized glass slides in triplicate. Peptides were 10, 15, or 20 amino acids in length with an offset of either 2 or 3 amino acids. A total of 10 control and 45 peanut-allergic sera were assayed. Specific IgE and IgG4 were detected by using fluorochrome-labeled monoclonal secondary antibodies.

Results: By using 15-mer and 20-mer peptides, we could define 11 antigenic regions, whereas only 5 were identifiable using 10-mers. Controls and patients produced IgG_4 recognizing a comparable number of Ara h 2 peptides, although the dominant epitopes were distinct. As expected, patient IgE bound a larger number of Ara h 2 peptides (9.4% vs 0.9%). IgE and IgG_4 epitopes recognized by patients were largely the same, and there was a positive association between IgE and IgG_4 signal, suggesting coordinate regulation. Cluster analysis of peptide binding patterns confirmed the specificity of antibody-peptide interactions and was used to define 9 core epitopes ranging from 6 to 16 residues in length—7 of which (78%) agreed with previous mapping.

Conclusion: Epitope mapping by microarray peptide immunoassay and cluster analysis reveals interpatient heterogeneity and a more detailed map. (J Allergy Clin Immunol 2005;116:893-9.)

Key words: *Peptide/protein microarray, Ara h 2, epitope mapping, antibody diversity, IgE, IgG_4, peanut allergy*

Antigen-specific B-cell responses are accessible to study with minimal invasiveness because of the presence of their specific antigen receptors in relatively abundant quantities in serum and their capacity to recognize cognate antigen directly.[1] The investigation of antibody responses may reveal information about both host response and antigen structure.

The measurement of a polyclonal B-cell response to a complex antigen is the sum of several variables, including the concentration of each specific antibody clone, the affinity of each clone, and the clonal diversity that together determine the fraction of target (antigen) that is bound.[2-5]

Diversification, along with affinity maturation and class switch recombination, is the result of immune progression—a process that is specifically regulated[6-8] and may provide insight into the host immune response. For example, clinical correlations have been made among antibody diversity and clinical autoimmunity,[9] anti-infective immunity,[10] and clinical allergy.[11-13] We have previously shown a correlation between specific IgE diversity and clinical allergy severity as well as *in vitro* effector function.[14]

Antigen arrays can be used to measure clonal diversity.[15] As the antigenic complexity of each arrayed element decreases, fewer epitopes are represented, and therefore, fewer distinct clonal responses are measured—approaching a theoretical limit of 1 array element to 1 antibody clone. This can be accomplished by using short synthetic peptides derived from the primary sequence of the corresponding antigen of interest.

Synthetic peptide arrays may also be used to elucidate antigenic structure. Investigators[16] including Geysen et al[17,18] pioneered the use of peptide arrays to measure the polyclonal response in outbred animals to study epitope structure. Many investigators have used small overlapping arrays of peptides to define continuous B-cell epitopes of numerous antigens, including many allergens.[13,19-27]

Because of its ability to assay thousands of targets in parallel by using small volumes of sample, the microarray immunoassay is ideally suited for the determination of individual polyclonal epitope recognition patterns.[28] Furthermore, signal intensity for microarray-based immunoassays is more dependent on affinity and tolerant of low concentration,[3-5] making it an ideal platform for the

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Abbreviations used
dfu: Digital fluorescence units
HAS: Human serum albumin
PBS-T: PBS with 0.5% Tween 20

detection of epitope-specific IgE and IgG_4—immunoglobulins present at low concentrations in serum.

We sought to adapt protein microarray technology for the measurement of antibody clonal diversity and characterization of antigenic structure in the context of a T_H2-skewed immune response to a major dietary antigen, Ara h 2, from peanut (*Arachis hypogaea*). Ara h 2 is an abundant 17/19-kd glycoprotein member of the lipid transfer protein family of seed storage proteins that is widely recognized by specific IgE from individuals allergic to peanut.[14,29-32]

We describe here the novel use of the microarray immunoassay with hierarchical cluster analysis to define IgE and IgG_4 epitopes of a major dietary allergen. We also compare the sensitivity of peptides of varying lengths for the detection of specific antibody on this substrate.

METHODS

Peptides, reagents, and array production

Six sets of peptides 10, 15, and 20 amino acids in length with an offset of 2 or 3, covering the sequence of the major peanut allergen, Ara h 2 (GeneBank accession, AAK96887), were commercially synthesized (Mimotopes, Pty Ltd, Raleigh, NC).

Stock solutions of peptides were dissolved in 40% acetonitrile, 20% dimethyl sulfoxide, to 1.6 mol/L. Working dilutions were then made in Protein Printing Buffer (Telechem, Sunnyvale, Calif) to a final concentration of 100 μmol/L and stored in 384-well polypropylene plates (Matrix Technologies, Hudson, NH). Triplicate spots were printed on epoxy-derivatized slides (SuperEpoxy Substrate, Telechem, Sunnyvale, Calif) 350 nm apart by using a GMS Arrayer (Affymetrix, Inc, Santa Clara, Calif). AlexaFluor-labeled protein was used to print positional reference controls. Negative control features (buffer alone and nonbinding peptides) and positive control (recombinant Ara h 2[33]) were also printed. Arrays were stored in a desiccator until use. Identical 2304-feature arrays were used for all experiments.

Anti-IgE and Anti-IgG_4 mAbs (both Pharmingen, San Jose, Calif) covalently conjugated to fluorescent markers (AlexaFluor 647 and 546, respectively) were prepared by reaction with activated *N*-hydroxysuccinimidyl-ester fluorochromes according to the manufacturer's protocol (Molecular Probes, Eugene, Ore). Final molar ratios of fluorochrome to immunoglobulin were between 5 and 9.

Forty-five patient sera with documented clinical reactivity and high peanut specific IgE values (median > 100 kU/L; Immuno CAP System FEIA; Pharmacia, Uppsala, Sweden) were recruited from the Mount Sinai Allergy Clinic. Ten nonatopic control sera were taken from healthy volunteers with no history of food adverse reactions.

Immunolabeling

An area around the printed grids was encircled with a hydrophobic PAP Pen (DAKO, Glostrup, Denmark) and blocked for 1 hour with 200 μL PBS with 0.5% Tween 20 (PBS-T) with 1% human serum albumin (HSA) at room temperature. Diluted patient serum (1:5 in PBS-T/HSA) was applied to the array and was incubated overnight on a rotator at ambient temperature. Titration (see Fig E1 in the Online Repository in the online version of this article at www.jacionline.org) and kinetics experiments (not shown) were previously performed to determine optimum conditions. Slides were then washed with PBS-T and incubated for 150 minutes with 200 μL anti-IgE and anti-IgG_4 cocktail (1:16,000 PBS-T/HSA) at room temperature in the dark. All incubations were performed in a humidity chamber (Binding Site, San Diego, Calif). Subsequently, the slides were washed 3 times for 3 minutes in PBS-T in a washing station (Telechem, Sunnyvale, Calif), followed by 3 times rinsing in deuterium, and spun dry.

Analysis

The feature and background pixel intensities were determined with ScanAlyze software (Michael Eisen; http://rana.lbl.gov/EisenSoftware.htm), and the net signal was exported and expressed as digital fluorescence units (dfu). A Microsoft Excel Visual Basic Application (Microsoft, Renton, Wash) created by our laboratory was used to relate the grid data to the corresponding peptide identity and to calculate the means and SDs of the triplicates. Array elements with coefficient of variance > 30% were excluded from analysis. The mean plus 2 SD log-transformed of the buffer alone negative controls was used as an intraslide cutoff to establish background. An interslide cutoff was calculated as the mean plus 2 SD intensity of the corresponding signal from the nonatopic control sera. For calculation of percent of patients with positive IgE binding, both intraslide and interslide cutoffs had to be exceeded. For hierarchical cluster analysis, the IgE/IgG_4 ratio of log-transformed data from all 55 patients and controls on all peptide elements was used. Clusters were determined by using Cluster software (http://rana.lbl.gov/EisenSoftware.htm) using the complete linkage algorithm and centered measurements for both arrays and features. Results are displayed by using Java TreeView 1.0.8 (http://rana.lbl.gov/EisenSoftware.htm). Additional analysis and graphical display were performed by using Prism (GraphPad Software, San Diego, Calif).

RESULTS

Commercially synthesized peptides of different lengths (10, 15, or 20 amino acids) and different degrees of overlap (2 or 3 offset), corresponding to the sequence of Ara h 2, were robotically arrayed in triplicate and site-specifically attached to derivatized glass slides; we assayed these peptides in parallel with sera from 45 individual patients allergic to peanut and 10 nonallergic controls.

Fig 1 displays scatter plots of IgE versus IgG_4 signal for negative control array elements probed with patient sera *(A)*, Ara h 2 peptide array elements probed with nonallergic control sera *(B)*, or Ara h 2 peptide array elements probed with patient sera *(C)*. As can be seen from Fig 1, *A*, a low level of background signal because of autofluorescence and nonspecific primary or secondary antibody binding exists (<0.5% for each channel). The central tendency of signal for negative control elements (for both controls and patients), normal control sera on peptide elements, or patients on peptide elements is the same—that is, a minority of antibody peptide interactions results in a positive signal. From controls not allergic to peanut,