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Title: Epitope Resolved Detection of Peanut Specific IgE Antibodies by SPR Imaging

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Epitope Resolved Detection of Peanut Specific IgE Antibodies by SPR Imaging

Min Shen, Amit A. Joshi, Raghu Vannam, Chandra K. Dixit, Robert G. Hamilton, Challa V. Kumar, James F. Rusling, and Mark W. Peczu*^{*}

Abstract: Peanut allergy can be life-threatening and is mediated by allergen-specific immunoglobulin E (IgE) antibodies. Investigation of IgE antibody binding to allergenic epitopes identifies specific interactions underlying the allergic response. Here we report a surface plasmon resonance imaging (SPRi) immunoassay for differentiating IgE antibodies by epitope resolved detection. IgE antibodies are first captured by magnetic beads bearing IgE ϵ -chain specific antibodies and then introduced to an SPRi array immobilized with epitopes from the major peanut allergen glycoprotein *Arachis hypogaea* h2 (Ara h2). Differential epitope responses are achieved by establishing a binding environment that minimizes cross-reactivity while maximizes analytical sensitivity. IgE antibody binding to each Ara h2 epitope was distinguished and quantified from 10 μ L patient serum samples in a 45 min assay. Excellent correlation of Ara h2-specific IgE values was found between ImmunoCAP assays and the new SPRi method.

The prevalence of food allergies, particularly among children, is on the rise and it constitutes as a growing health concern worldwide.^[1-3] Peanut allergy is among the most common food allergies, and it is the leading cause of food allergy deaths.^[4] Exposure to even trace amounts of peanut-derived materials can induce a serious reaction - including anaphylactic shock - to a sensitized individual who is allergic to peanut storage proteins. Treatment requires an immediate administration of epinephrine followed by an emergency room visit. There is a persistent and critical need for improved diagnostic methods that can accurately predict a patient's severity of allergic reactions to peanuts. While the gold standard method in peanut allergy diagnosis is the double-blind, placebo-controlled food challenge, it is expensive, time consuming, and potentially dangerous. The current state-of-the-art procedure is to perform a clinical history and then confirm sensitization with an IgE anti-peanut serology. The immunoCAP, which is a widely employed diagnostic IgE antibody assay, uses

allergosorbents to which both peanut extract and a number of individual allergenic peanut (*Arachis hypogaea*) proteins (Ara h1, 2, 3, 8, and 9) are immobilized.^[5,6] Several studies have shown that the presence and quantity of IgE antibodies specific for the major peanut storage proteins (e.g. Ara h1, 2, and 3) are particularly useful in confirming a clinically relevant sensitization and predicting the relative severity of a reaction following a peanut exposure.^[7,8]

Higher resolution information about specific sequences of Ara h2 recognized by IgE antibodies has been obtained by peptide array methods.^[9-11] In these studies, IgE antibodies selectively and differentially bind to the peptides with overlapping sequences based on the epitopes recognized. Certain regions of a protein, therefore, are inherently more immunogenic than others. The properties of the IgE repertoire logically have a direct impact on the degree of allergic symptoms that an individual will experience. Parameters, such as the total IgE concentration, the diversity of epitopes recognized, the IgE antibody affinity for those epitopes, and the ratio of allergen specific IgE to total IgE, are the most important in this regard.^[12] Our long term goal is to collect concentration and affinity data on circulating IgE antibodies in an epitope resolved manner. This will allow for fundamental investigation of the link between IgE antibody repertoire as measured in serum and objective clinical manifestation of an allergic response. It may also provide information about the severity of allergic response. Here we focus on the differential recognition of Ara h2 epitopes by IgE antibodies using surface plasmon resonance imaging (SPRi).

We previously reported the use of an Ara h2 peptide as a defined epitope to detect a peanut-specific antibody by electrochemical impedance spectroscopy^[13] and resistive-pulse sensing^[14]. More recently, we reported the first immunoarray for IgE antibodies utilizing both peptide and carbohydrate epitopes. However, the measured concentrations of IgE antibodies that bound to each epitope were statistically indistinguishable.^[15] In this study, we have modified the experimental design to improve the selectivity of IgE antibody binding to different epitopes. We present a simple method that is able to differentiate IgE antibody populations in an epitope resolved manner using superparamagnetic beads (MB), a commercial SPRi array system, and an off-line IgE capture strategy. Secondary antibodies (Ab₂) specific for IgE ϵ -chain were immobilized onto the MB to bind IgE antibodies from serum. This tended to lower the non-specific binding of other serum proteins to array surface.^[16-18] Differential epitope responses to allergen specific IgE antibodies were achieved by providing a binding environment designed to detect low IgE concentrations with minimal cross-reactivity. Using this approach, IgE antibodies from peanut sensitized patient sera were distinguishable in their binding to each of the Ara h2 epitopes and they correlated well with results from ImmunoCAP assays using Ara h2 allergosorbent. Statistical analysis of limited patient sample data shows good peanut allergy diagnostic

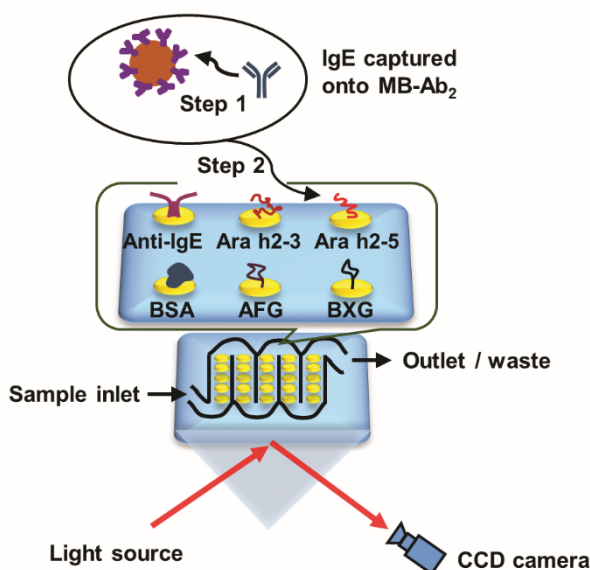
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potential and provides epitope specific immunogenic information that can be correlated with observed IgE-mediated allergy reactions.

The mixture of IgE antibodies were captured off-line from serum using 1 μm Ab_2 coated magnetic beads (MB-Ab_2). The resulting MB-Ab_2 -IgE complexes were washed, and injected into an SPRi array (SpotReady™ 25, GWC Technologies) where they bound to Ara h2 epitopes on individual gold SPRi spots (Scheme 1). The Ara h2 epitopes included two immunogenic peptides, namely Ara h2-3 and Ara h2-5, and two carbohydrate epitopes, i.e., $\alpha(1,3)$ -fucosyl glycoside (AFG) and $\beta(1,2)$ -xylosyl glycoside (BXG). A monoclonal anti-IgE positive control and bovine serum albumin (BSA) negative control were also included. Epitope motifs and control proteins were covalently immobilized onto 25 carboxylate-functionalized gold spots of the SPRi array using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) bioconjugation.^[19] The SPRi array was equipped with a 3D-printed serpentine flow channel to address the epitope coated gold spots (Figure S1), and interfaced with a syringe pump and a sample injector. Before analysis, the gold array was treated by ethanolamine and equilibrated with phosphate buffered saline containing 0.05% Tween 20. The array could be regenerated using a 60 second pulse of 10 mM NaOH for 10 ~ 16 times.^[20]



Scheme 1. Schematic for detection of epitope specific IgE antibodies using SPRi. IgE antibodies were first captured by MB-Ab_2 , washed, and injected into an SPRi array; SPRi responses were then measured on individual array spot that has been immobilized with specific allergenic epitopes.

SPR images were collected in real time for the whole array. Bright spots in the SPR difference image indicated the specific binding of MB-Ab_2 -IgE complexes onto those spots coated with epitopes and anti-IgE, while negligible responses were observed on BSA spots (Figure S3A). Meanwhile, the image pixel intensities were plotted as SPRi responses against time, generating sensorgrams that were used for quantitative analysis

(Figure S3B). The conditions for IgE antibody binding to MB-Ab_2 and SPRi spots were optimized to achieve the best differential epitope responses (Figure S2, S4, and S5).

Different concentrations of a human IgE antibody standard (Thermo Fisher) diluted in calf serum were analyzed to create dose response curves (Figure 1). Limits of detection (LOD) for IgE antibody were 0.5 pg mL^{-1} on anti-IgE spots, 5.0 pg mL^{-1} on peptide spots, and 2.0 pg mL^{-1} on carbohydrate spots, all of which were over 50-fold better than LOD of ImmunoCAP assay. The analytical sensitivity as defined by the slope of calibration curve on anti-IgE spots differed significantly from those on peptide and carbohydrate spots according to t-test ($P < 0.01$). The dynamic ranges of calibration plots were over two orders of magnitude for all the epitope spots. These results illustrate the potential of the SPRi to differentiate epitope specific IgE antibodies in patient samples.

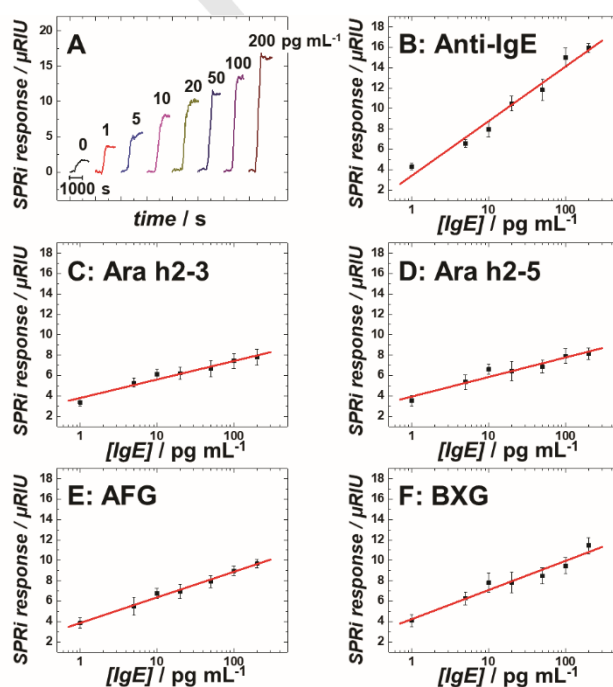


Figure 1. Calibration assays for human IgE antibodies spiked in calf serum: (A) background subtracted SPRi responses for IgEs captured on MB-Ab_2 and injected to anti-IgE immobilized SPRi spots; (B–F) calibration plots of human IgE antibodies measured on SPRi spots coated with (B) anti-IgE, (C) Ara h2-3, (D) Ara h2-5, (E) AFG, and (F) BXG. Error bars represent standard deviations for $n = 4$. μRIU : micro-Refractive Index Unit.

Thirty two coded sera from peanut sensitized patients were provided from the serum bank at the Johns Hopkins University DACI Reference Laboratory. The sera were analyzed in the SPRi array after a 100–10,000 fold dilution to bring their SPRi responses into the linear range of the assay. The measured concentrations in ng mL^{-1} were converted to kU L^{-1} ($1 \text{ kU L}^{-1} = 2.42 \text{ ng mL}^{-1}$)^[21] and further compared with the ImmunoCAP assays (peanut extract and Ara h1, 2, 3, 8, and 9 specificities). The historical threshold 0.35 kU L^{-1} was used as a cutoff for positive and negative results.^[22] As IgE antibodies specific for Ara h2 is considered by many to be the most accurate predictor of a

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risk for a severe reaction in peanut sensitized individuals, two groups were categorized based on having negative ($n = 13$) and positive ($n = 19$) results in levels of IgE anti-Ara h2 (Figure 2A). Significant differences in level of IgE anti-Ara h2-3 peptide epitope were observed between those two groups (Figure 2B, $P < 0.01$, Table S1). On the other hand, measured IgE antibody levels between negative and positive groups were found statistically indistinguishable ($P > 0.01$, Table S1) when using array spots containing peptide epitope Ara h2-5, two carbohydrate epitopes, or anti-IgE antibody (Figure 2C and S7). These results confirm that our new immunoassay strategy resulted in differentiation of IgE antibody binding to each epitope.

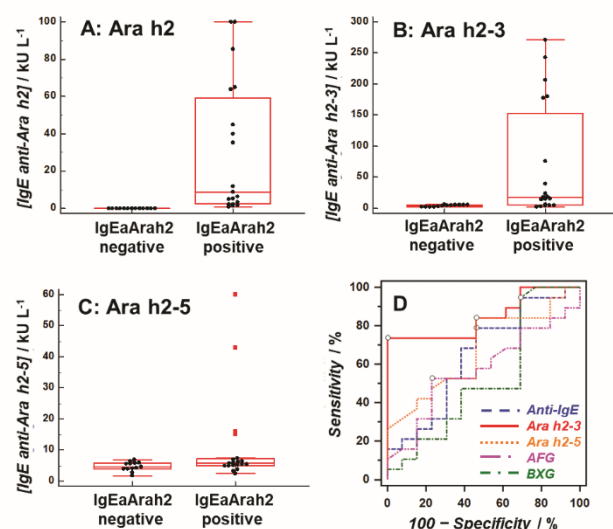


Figure 2. Distributions of IgE antibody levels in individuals having negative ($n = 13$) and positive ($n = 19$) levels of IgE anti-Ara h2 (IgEaArah2): IgE antibodies against (A) Ara h2; (B) Ara h2-3; (C) Ara h2-5; and (D) ROC curves for peanut sensitization confirmation using levels of IgE antibodies against four peanut epitopes and anti-IgE.

Receiver operating characteristic (ROC) analyses^[23] examined the ability of each epitope to confirm the sensitization to peanuts. ROC curves for levels of IgE antibodies against four Ara h2 epitopes and anti-IgE are shown in Figure 2D. The IgE anti-Ara h2-3 exhibited the best prediction for peanut sensitization with the area under the curve (AUC) of 0.85, 73.7% sensitivity and 100.0% specificity (Table 1).

Table 1. AUC, sensitivity, and specificity for peanut sensitization confirmation by ROC analysis.

Level of IgE antibodies specific for	AUC $\pm$ SE ^[a]	Sensitivity, %	Specificity, %
Anti-IgE	0.64 $\pm$ 0.11	79.0	53.9
Ara h2-3	0.85 $\pm$ 0.07	73.7	100.0
Ara h2-5	0.68 $\pm$ 0.10	84.2	53.9
AFG	0.51 $\pm$ 0.12	100.0	53.9

BXG 0.57 $\pm$ 0.11 52.6 69.2

[a] standard error (SE)

Our epitope resolved analytical system depends on minimizing cross-reactivity while maintaining high analytical sensitivity. Its ability to measure IgE levels in the pg mL⁻¹ range with relatively low epitope surface coverage minimizes the cross-reactivity of other IgE antibody specificities. Using MB enables the SPRi to detect pg mL⁻¹ levels of IgE antibodies by increasing both the refractive index of the binding moiety and the apparent binding constant of IgE antibody.^[24] Around 60,000 Ab₂ molecules were estimated on one MB-Ab₂ bioconjugate (Figure S2), which facilitates efficient IgE capture by driving the Ab₂ + IgE $\leftrightarrow$ [Ab₂-IgE] equilibrium toward association. Using the off-line capture approach, IgE antibodies were separated from non-specific serum proteins, resulting in a higher analytical sensitivity and a clearer signal differentiation with each epitope. Importantly, this capture strategy rules out the binding of other anti-peanut antibodies (e.g. IgG₄) to Ara h2 epitopes.^[9]

Since Ara h2-specific IgE antibody levels are important in the evaluation of a patient for peanut allergy, two peptides bearing Ara h2 anti-peanut IgE binding epitopes were evaluated in this study (Ara h2-3 and Ara h2-5). The IgE antibodies specific for Ara h2-3 provide an enhanced correlation for sensitization to peanuts than those for Ara h2-5 epitope (Figure 2 and Table 1). It indicates that Ara h2-3 is a more immunogenic epitope of the Ara h2 protein. These data agree with other epitope mapping studies.^[9-11] Such differences in IgE antibody levels might result from their different positions and secondary structures in the Ara h2 protein (Figure S8)^[25]: Ara h2-3 presents a protruding, flexible surface loop which is easily accessible for IgE antibody binding, while Ara h2-5 maintains a rigid α -helix structure that is buried behind Ara h2-3 in Ara h2 protein. Thus most Ara h2-specific IgE antibodies may bind to the immunodominant epitope of Ara h2-3 rather than Ara h2-5.

In addition, two carbohydrate epitopes (AFG and BXG) were investigated to examine the effect of cross-reactive carbohydrate determinants (CCDs), as xylose and core-3-linked fucose residues from CCDs can also contribute to peanut specific IgE antibody binding.^[26,27] The presence of these sugars on glycoproteins from numerous plant species is thought to be responsible for sensitization profiles due to cross-reactivity. Our results indicate that the level of IgE antibodies specific for AFG or BXG gave a poor correlation with sensitization profiles from the ImmunoCAP using either t-test or ROC analysis (Figure 2D and S7, Table 1 and S1). Our findings, together with others^[28], question the relevance of CCDs in the allergic response.

A model initially proposed by Aalberse et al.^[29] and later demonstrated experimentally by Christensen et al.^[30] is likely operative in our system. In this model, an allergen is initially captured by one IgE antibody anchored on the mast cell surface followed by binding to another IgE in close-proximity. The co-binding of IgE antibodies to the allergen is sufficient to induce degranulation, a key process that can lead to anaphylaxis if it were sufficiently massive. Similarly, in the SPRi assay, IgE

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antibodies are first anchored onto MB-Ab₂, whose size is comparable to a cell. The anchored IgE antibody is then allowed to interact with epitopes on the array spots. The interaction between epitopes of low surface coverage and IgE antibodies of pg mL⁻¹ level reflects the fact that only a subset of circulating allergen proteins can interact with a subset of IgE antibodies and trigger the immune responses.^[31] Throughout the assay, the flow rate creates a dynamic, non-static condition, similar to the circulatory system.

In summary, we describe an epitope resolved assay system for the detection of peanut allergen specific IgE antibodies using SPRI technology. The assay achieves LODs of 0.5–5.0 pg mL⁻¹ using MB and produces differential IgE responses to different Ara h2 epitopes. The SPRI array results correlate well with ImmunoCAP results from repository based sera and provide insights into the molecular epitopes involved in sensitization to peanuts. This analytical approach is applicable to future microarrays that employ an expanded cohort of allergenic epitopes to achieve a clear fingerprint of a patient's sensitization profile and risk for a severe reaction to peanuts.

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Keywords: immunoassay • immunoglobulin E • magnetic bead • peanut allergy • surface plasmon resonance (SPR) imaging

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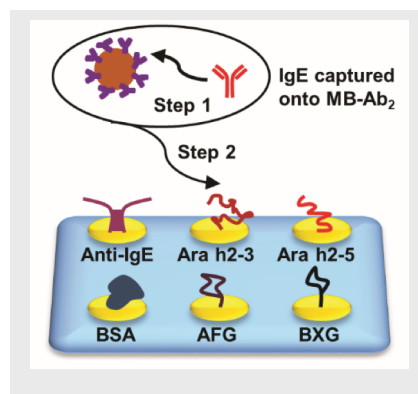
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IgE antibody differentiation: For the first time, epitope resolved detection of peanut specific IgE antibodies was achieved on a surface plasmon resonance imaging (SPRi) immunoarray using superparamagnetic bead-antibody conjugates (MB-Ab₂). The level of IgE antibodies specific for a prominent peptide epitope Ara h2-3 was found promising for predicting peanut sensitization.



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