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Detection of Allergen-specific IgE on Microarrays by Use of Signal Amplification Techniques, Tito Bacarese-Hamilton,¹ Letizia Mezzasoma,² Colin Ingham,¹ Andrea Ardizzone,¹ Ruggero Rossi,² Francesco Bistoni,³ and Andrea Crisanti^{1*} (¹Department of Biology, Imperial College of Science, Technology and Medicine, London SW7 2AZ, United Kingdom; ²Dipartimento di Medicina Clinica e Sperimentale, Università degli Studi di Perugia, Policlinico Monteluce, 06100 Perugia, Italy; ³Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università degli Studi di Perugia, Via del Giochetto, 06100 Perugia, Italy; *author for correspondence: fax 44-207-5945439, e-mail acrs@ic.ac.uk)

Allergy is estimated to be the sixth leading cause of chronic disease in the US, and the number of people exhibiting symptoms of an allergic response to various natural and synthetic compounds has increased dramatically in the last decade (1). The prevalence of food allergies is particularly evident in children; some reports estimate that the frequency of food allergies in infants is 2-5% (2). The identification of the specific IgE responsible for the clinical symptoms can be a costly and lengthy procedure. Various immunoassays, such as ELISA (3), the Radio Allergo Sorbent Test (4), and high-capacity solid-phase tests, e.g., the CAP system (Pharmacia) (5), are currently used in the diagnosis of allergies and have the inherent sensitivity and specificity to detect IgE in human serum. However, all of these assays are time-consuming, require large quantities of serum samples, and use poorly characterized antigen preparations. Moreover, none of the assays currently used in the diagnosis of allergies has the throughput to screen for the most common allergens, which are estimated to exceed 300.

High-density ordered arrays of molecules (microarrays) (6,7) may circumvent most of the current limitations in the diagnosis of allergies by allowing simultaneous and multiparametric analysis of serum reactivity against a variety of antigens. Protein microarrays in combination with fluorochrome-labeled secondary antibodies have been used to reveal the presence in human serum of IgG and IgM directed against microbial antigens (8). However, this assay format is not suitable for the serodiagnosis of allergies. A signal enhancement procedure is required to reveal the presence of subnanomolar concentrations of analytes, such as serum-specific IgE. One such method has recently been described (9); here we report on an alternative methodology.

We developed a high-sensitivity assay to reveal the presence of specific IgE in human sera that combines allergen microarrays with tyramide signal amplification. Using contact printing with high-speed robotics (Total Array System; BioRobotics), we have arrayed on silanized

glass microscope slides (CEL Associates) a variety of allergens together with human IgE at different concentrations. The allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria alternata*, *Olea europaea*, *Artemisia vulgaris*, *Dactylis glomerata*, and house dust) were supplied by Radim S.p.A. Human IgE (purified from myeloma plasma) was purchased from Calbiochem Corporation. Allergens were extracted with 1× phosphate-buffered saline (PBS; 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, 8 g/L NaCl, pH 7.4) containing Tween 20 (0.1 mL/L) and printed using the same solution. Human IgE was printed using 1× PBS containing 0.1 mL/L Tween 20 and 1 g/L sodium dodecyl sulfate. Allergen preparations were spotted at 10 g/L, each spot being ~1 nL (10 ng). Arrays consisted of a 7 × 7 matrix that included the IgE internal calibration curves in duplicate and the allergens in quadruplicate (see Fig. 1B). Slides were handled and stored as described previously (8). Printed slides were incubated overnight at room temperature with a solution containing 20 g/L bovine albumin in PBS to block non-specific antibody binding. An adhesive tape (Abgene Limited) was used to contain samples/reagents within the array area. Sera (100 μL) were incubated at room temperature for 60 min. After washing (five times; each time with 1 mL of PBS containing 0.1 mL/L Tween 20), the following secondary antibodies were assessed for their ability to reveal serum IgE bound to the printed allergens:

1. Alexa 532-labeled anti-human IgE (OEM Concepts Inc.) at a final concentration of 27 mg/L in a solution of 2× PBS containing 10 g/L bovine serum albumin (BSA) and 0.1 mL/L Tween 20
2. A biotinylated anti-human IgE (KPL) at a final concentration of 10 mg/L in a solution of 2× PBS containing 10 g/L BSA and 0.1 mL/L Tween 20, followed by Alexa 546-streptavidin (Molecular Probes Inc.) at dilution of 1:50 as detailed in the product data sheet
3. A horseradish peroxidase (HRP)-labeled anti-human IgE (KPL) at a final concentration of 10 mg/L in a solution of 2× PBS containing 10 g/L BSA and 0.1 mL/L Tween 20; bound secondary antibody was detected by incubating the slides with Alexa 546 tyramide conjugate (Molecular Probes) diluted 1:100 with Molecular Probes diluent, as detailed in the product information (10)
4. A biotinylated anti-human IgE at a final concentration of 1 mg/L, followed by incubation with HRP-streptavidin at a dilution of 1:100 as detailed in the product information (10); bound streptavidin-antibody complexes were detected by incubating the slides with Alexa 546 tyramide conjugate (Molecular Probes) diluted 1:100 with Molecular Probes diluent (10)

All incubations were conducted at room temperature: 60 min for the secondary antibodies and 15 min for the tyramide reagents. Before the fluorescence was read in the scanner (S5000; Packard Biosciences), the slides were washed and dried at 37 °C. Images were generated with the ScanArrayTM software provided by Packard Bio-

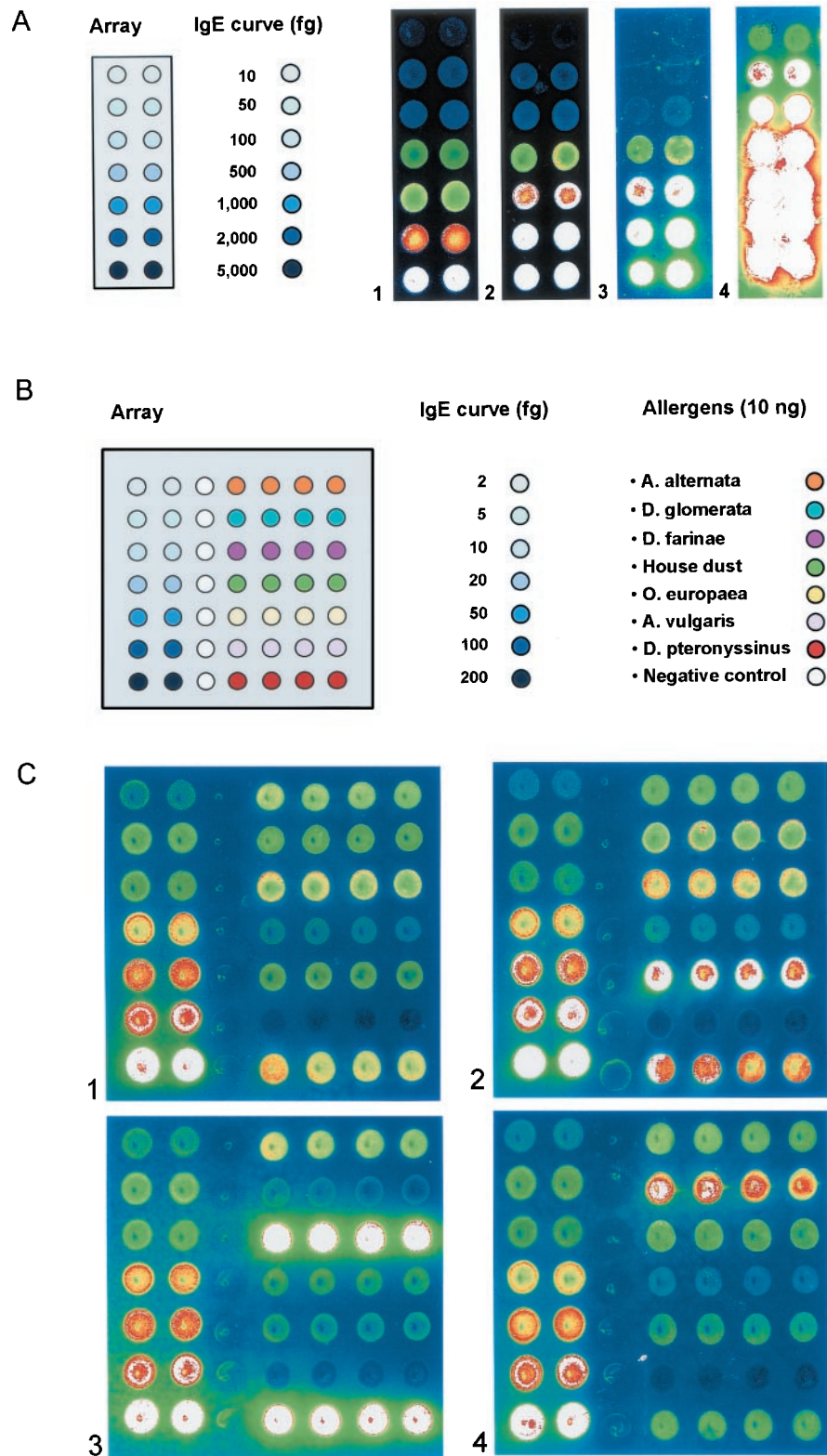


Fig. 1. Schematic of arrays used in the study and fluorescent scans of IgE curve and allergens incubated with serum samples.

(A), detection of IgE by the four different protocols. 1, Alexa 532-labeled anti-human IgE; 2, biotinylated anti-human IgE followed by Alexa 546-streptavidin; 3, HRP-labeled anti-human IgE followed by Alexa-546 tyramide conjugate; 4, biotinylated anti-human IgE followed by HRP-streptavidin and Alexa 546 tyramide conjugate. (B), schematic representation of the array. Colored circles indicate the positions where the allergens were printed in quadruplicate and the internal IgE calibration curve was printed in duplicate; the blank (dilution buffer) is represented as white circles. (C), fluorescent scan of allergen arrays incubated with four different sera (1–4) and developed using the tyramide amplification protocol.

science and quantified using the QuantArray™ software provided by the same company.

A dose-response curve generated by printing increasing amounts of IgE in duplicate was processed with the

four reaction protocols detailed above. Quantification of the resulting curves (Fig. 1A) against a non-IgE control gave signal-to-background ratios for the first non-zero calibrator (10 fg) of ~1.3 for antibody protocols 1, 2, and

Table 1. Comparison of microarray and ELISA for serum reactivity.

Allergens	ELISA		Microarray			
	Positive	Negative	Positive	Negative	Sensitivity, %	Specificity, %
<i>D. pteronyssinus</i>	11/22	11/22	13/22	9/22	90.9	72.7
<i>D. farinae</i>	8/16	8/16	9/16	7/16	87.5	75
<i>O. europaea</i>	7/22	15/22	8/22	14/22	71.4	80
<i>A. alternata</i>	3/20	17/20	4/20	16/20	100	94.1
<i>A. vulgaris</i>	2/22	20/22	2/22	20/22	50	95
<i>D. glomerata</i>	6/22	16/22	7/22	15/22	83.3	87.5

3 and 2.2 for protocol 4. Corresponding values for the second non-zero calibrator (50 fg) were 2.5, 4.3, 1.0, and 9.1 for the four protocols, respectively. The dose-response curve in protocol 4 was optimized to increase linearity (Fig. 1C). This produced signal-to-background ratios of 6.7 for the 10-fg calibrator and 14.9 for the 50-fg calibrator. The slope of the linear portion of the dose-response curve generated with this amplification protocol was 5.6, whereas the slope for the curve generated with the protocol that did not include amplification (protocol 1) was only 0.1.

The assay incorporating the tyramide amplification system was sensitive enough to detect <1 fg of allergen-bound IgE from human serum (Fig. 1C). The corresponding value for the assay without tyramide amplification was 24 fg. The detection limit was determined by measuring the reactivity of a serum sample against replicates of a nonspecific protein printed on slides as described previously (8). The detection limit, defined as the mean photomultiplier counts plus 2 SD of the resulting signal, was interpolated from the IgE calibration curve. Two sera classified as positive (class score 3) in an ELISA and two negative sera were assessed using the protocols with and without amplification. Neither of the positive sera were detectable in the assay without amplification, i.e., the sensitivity was unacceptable. No further sera were analyzed with the protocol that did not include amplification.

The assay using amplification protocol 4 was selected to analyze 22 serum samples, collected from patients attending an allergy clinic, against the arrayed allergens. A schematic representation of the array used throughout the study is shown in Fig. 1B. Each serum sample investigated for its reactivity against the panel of arrayed allergens was also analyzed with a commercial ELISA assay (Radim S.p.A.). In the ELISA protocol, the negative serum was defined as having an allergen-specific IgE concentration <1 kIU/L or a class score of 0 or 1. Sera having an IgE concentration >1.0 kIU/L or class scores ≥ 2 were regarded as positive. In the microarray immunoassay, a cutoff value for each allergen was calculated using reference sera that were negative for serum IgE as determined by the ELISA. These cutoff values incorporated the 95th percentile of these negative reference sera. We compared the ELISA and the microarray assay for their ability to reveal specific IgE in the sera of the patients (Table 1).

The commercial ELISA used in this study did not

include reagents for house dust; therefore, no comparison was performed between the ELISA and the microarray assay for this allergen. In addition, insufficient serum was available to analyze all samples for *D. farinae* and *A. alternata*. This analysis revealed that the overall diagnostic performance, as defined by clinical sensitivity and specificity, of the microarray immunoassay was very good for the panel of allergens evaluated in this study. Notably, an allergen microarray assay developed with an indirect fluorescence (nonamplified) protocol was unable to detect serum reactivities less than score class 4 (as determined by ELISA) and hence had limited clinical applicability.

Tyramide signal amplification uses the catalytic activity of HRP to generate high-density labeling of a target protein or nucleic acid sequence in situ (10). This procedure has previously been used for nucleic acid detection (11) and immunohistochemistry (12). We show here that the tyramide amplification system dramatically enhances the performance of protein microarray assays. A previous report (9) described the use of a signal amplification protocol for the detection of allergen-specific IgE on microarrays with an alternative method (rolling circle amplification). Both methods gave comparable diagnostic performance when compared with an ELISA method.

In conclusion, signal amplification using HRP conjugates and a fluorescent HRP substrate is more sensitive than detection with fluorescently labeled anti-IgE or the combination of biotin-labeled anti-IgE and fluorescently labeled streptavidin. Although we studied a small number of patient sera against a limited number of allergens, the data show good concordance between the microarray immunoassay and commercial ELISAs. Automation of the assay format and calibration of the results in kIU/L will provide allergy specialists with fully quantitative, multi-analyte determinations that will increase cost-effectiveness and limit the need for skin-prick testing for the diagnosis of allergies.

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