ELSEVIER

Journal of Immunological Methods 300 (2005) 24–31

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Development of a sensitive, colorometric microarray assay for allergen-responsive human IgE

Stewart J. Lebrun[a,b,*], Wasinee N. Petchpud[a], Agnas Hui[a], Calvin S. McLaughlin[a,c]

[a]*Miragene, Inc., 5475 E. La Palma, Suite 206, Anaheim, CA 92807, United States*[1]
[b]*University of California-Irvine, Department of Community and Environmental Health, Irvine, CA 92697, United States*
[c]*University of California-Irvine, Department of Biological Chemistry, Irvine, CA 92697, United States*

Received 7 July 2004; received in revised form 13 October 2004; accepted 30 January 2005
Available online 13 April 2005

Abstract

With the existence of several thousand unique human allergens, a multiplex format, such as protein microarrays, is an attractive option for allergy screening. To determine the feasibility and sensitivity of using an enzyme-based, colorimetric protein microarray assay, three common allergens (mold, dustmite, grass) were arrayed and added sera assayed for responsive human IgE. Normal, low positive, and negative control samples were assayed to determine optimal reaction parameters. Sensitivity of the assay (in international units, IU) was determined by constructing a standard curve using World Health Organization (WHO) standards. The system described here can reliably detect allergen-specific IgE below 0.35 IU, the current WHO standard cutoff. By taking advantage of the sensitivity of enzyme-linked immunosorbent assays (ELISAs) and the multiplex format of microarrays, we have achieved a high throughput system, capable of screening patients for allergen-susceptibility with optimal sensitivity.

Keywords: Protein microarray; Allergy testing; IgE in vitro test

1. Introduction

Type I allergy is an immunoglobulin E (IgE)-mediated hypersensitivity disease affecting greater than 25% of the world's population (Kay, 1997; Wills-Karp et al., 2001). The pivotal event leading to appearance of classic symptoms (e.g. allergic rhinitis, urticaria, angioedema, anaphylaxis) is the recognition of an allergen by allergen-specific IgE antibodies

Abbreviations: IgE, immunoglobulin type E; IU, International Unit; WHO, World Health Organization; ELISA, enzyme-linked immunosorbent assay; AP, alkaline phosphatase; IgG-AP, immunoglobulin type G coupled AP; BCIP/NPT, 5-Br-4-Cl-3-indoyl phosphate/neomycin phosphotransferase.

* Corresponding author. Miragene, Inc., 5475 E. La Palma, Suite 206, Anaheim, CA 92807, United States. Tel.: +1 714 701 9573; fax: +1 714 701 9583.
E-mail addresses: stewartlebrun@hotmail.com, lebruns@uci.edu (S.J. Lebrun).
[1] mirageneinc@yahoo.com.

0022-1759/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2005.01.019

circulating in the bloodstream as well as those present on the patient's mast cells and basophils (Ishizaka et al., 1966; Deinhofer et al., 2004).

Diagnosing type I allergies have traditionally involved in vivo provocation tests (Bousquet et al., 1998) which can only determine if a patient is sensitized to an allergen source but cannot clearly identify the disease-eliciting component of the allergen extract (Valenta et al., 1999). In order to identify and characterize the allergenic components of the extract, panels of recombinant allergens have been produced, assembled into protein chips, and used diagnostically for IgE profiling (Deinhofer et al., 2004; Harwanegg et al., 2003; Hiller et al., 2002; Kim et al., 2002; Jahn-Schmid et al., 2003). To date, these allergen microarrays have utilized fluorescence detection of the IgE, either using fluorescently labeled antihuman IgE (Deinhofer et al., 2004; Harwanegg et al., 2003; Hiller et al., 2002; Jahn-Schmid et al., 2003) or by first conjugating the IgE pool with biotin and using fluorescently labeled streptavidin (Kim et al., 2002).

Existing IgE assays are conducted using enzymatic amplification, presumably to compensate for the low abundance of circulating IgE. These assay systems require large amounts of allergen to be attached to a porous test disk or other substrate, typically by overnight incubation of the 3-dimensional disk with the allergen, and then subsequent application of a relatively large amount (50 μl) of patient serum to the disk. The non-binding IgE is then washed away and a secondary antibody with an enzyme linked to it (typically alkaline phosphatase, AP) is added. In such a colorimetric assay, an added substrate is enzymatically converted to a product that absorbs light at a particular wavelength. The color density of the resulting solution is measured spectrophotometrically, compared to World Health Organization (WHO) standards, and a quantitative estimate of allergen-specific IgE can be determined.

The primary difference between the typical fluorescent microarray and the IgE assay is that the detection scheme for the fluorescent microarray is not enzyme-linked and does not provide equivalent sensitivity to the original allergy ELISAs. Microarrays do, however, allow the simultaneous, multiparametric determination of specific subclasses of antibodies directed against many pathogenic antigens (Bacarese-Hamilton et al., 2004). In addition, binding capacities of current microarray substrates (e.g. two-dimensional glass) may not bind as well as three-dimensional porous substrates based on predicted steric hindrance.

We decided to combine the sensitivity of the allergy ELISA assays with the multiplex advantages of microarray technology by developing a novel assay for allergen-responsive human IgE. The method we developed uses a three-dimensional, highly porous substrate (thickness 0.1 μm) that binds a substantial amount of protein and uses the same basic ELISA sandwich and detection scheme as that in current in vitro IgE testing (Emanuel, 2003). Our preliminary studies demonstrate that we can detect as little as 0.1 IU IgE using this enzyme-linked, colorimetric-based system. The Zeta-Grip™ protein microarrays have previously been shown to be a low cost, highly sensitive, miniaturized way to conduct ELISA-like assays. This method is well suited to the application of allergens where patients are typically screened for susceptibility to several hundred suspected allergens at one time.

2. Methods

2.1. Materials

All of the patients and control serum as well as allergens (mold, Timothy grass or dustmite) were obtained confidentially and within federal guidelines for human tissues.

2.2. Non-contact spotting

The Biodot Biojet dispensing system was used according to the manufacturer's instructions (www.biodot.com). Allergen extract solutions were filtered using a 0.2 μM filter, dialyzed twice against 2 l PBS over 6 h at 8 °C, and spotted at a concentration of 0.1 mg/ml.

2.3. Generation of standard curve

Using non-contact spotting, we printed chips with non-specific anti-human IgE in amounts ranging from 0.25 to 8 pg. We then incubated different WHO standard samples (known amounts of human IgE in equine serum) with these chips and developed them

using 250 μl mouse anti-human IgG-AP/10 ml buffer (PBS or TBS, see below). The chips were then scanned and normalized (values derived by scanning were divided by calibration standard values). These normalized intensities were used to relate sample signal intensity (on *y*-axis) with IU (on *x*-axis).

2.4. Microarray assay and development

The allergen extracts were aliquoted into a 384 well plate (0.5 μg protein/well) and robotically transferred to the chip substrate (Zeta-Grip™; Lebrun, 2004) using an array printer (www.biodot.com). The antigens were spotted based on equivalent activity values (determined using ELISA and WHO standards), ranging between 0.01 and 1 pg/nl. A set of spots is included on each array for normalization and is predetermined to be just below saturation. The normalization of spots corrects for chip-to-chip differences and maintains signal in a quantifiable region (if unknown signal is equal to or greater than the normalization signal, it is considered oversaturated

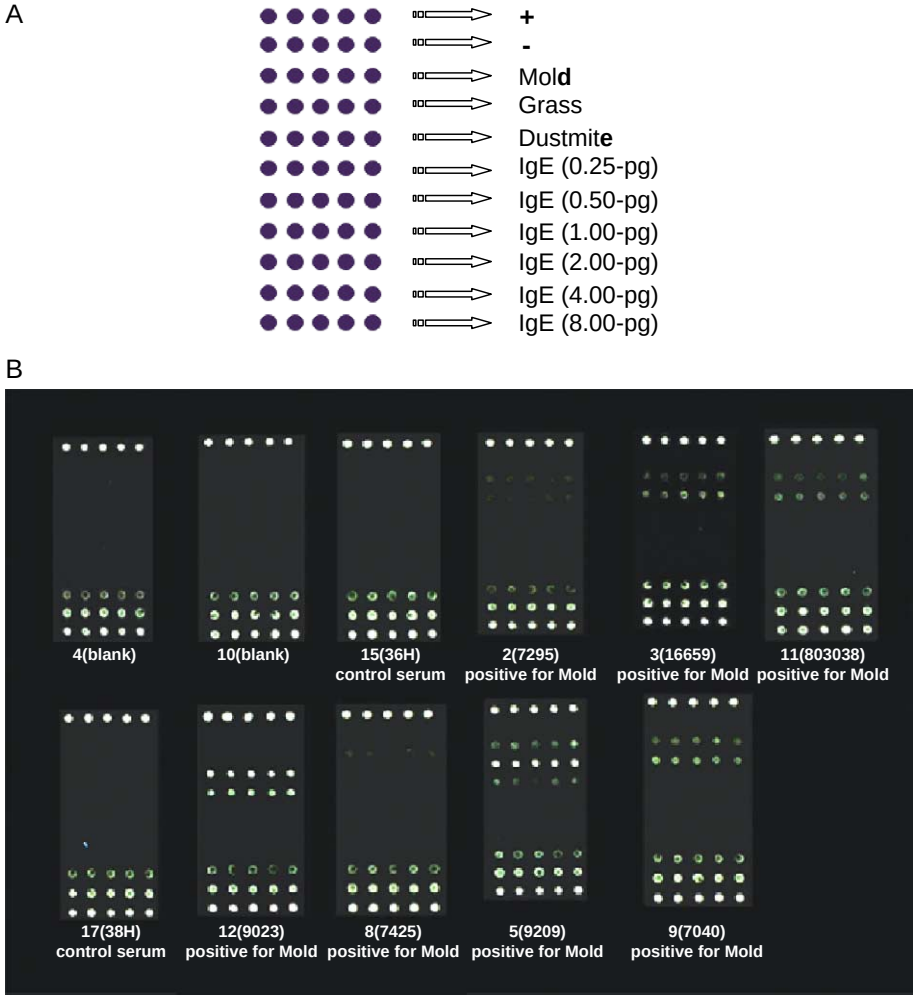

Fig. 1. Non-contact printing of microarray chip. (A) Typical spotting pattern template. Standard and experimental values used for quantitative data analysis are the average of 15 spots (3 chips) normalized to the positive control ("+"), negative control ("−"). Mold, grass, and dustmite allergens were spotted at 0.01–1 pg/nl in 5 μl buffer. Non-specific IgE was spotted at the indicated amounts. (B) Reaction of allergens by patients [2(7295), 3(16659), 11(803038), 12(9023), 8(7425), 5(9209), and 9(7040)], equine control [15(36H) and 17(38H)], or with no serum present [4(blank) and 10(blank)].

and not quantifiable). The dynamic range of the assay is 3 orders of magnitude. All printed chips were labeled and individually placed into sterile plastic dishes, pre-filled with proprietary blocker. Loaded dishes were then placed on an ELISA shaker (Titerplate Shaker, Labline Instruments, IL) and rotated (to allow for continuous mixing) at room temperature (25 °C) for 1 h. Next, diluted patient serum at desired titer (known generally as 'primary antibody') was added to each dish (with the slide and blocker) and continuously mixed for 1 h. Solutions were appropriately discarded, and slides were washed 3 times by adding 10 ml of TBS wash buffer. Anti-IgE-AP (20 μl) was added for detection of patient IgE antibodies. Chips were washed as described above. After the last wash, 10 ml proprietary detector was added. This reagent is catalyzed by alkaline phosphatase and further developed as in a standard ELISA (Van Weemen, 1985). The dishes were shaken for 1 h at room temperature. Solutions were discarded from the dishes, and slides were again washed three times. After discarding the last wash, 10 ml developer was added to the dish and shaken at room temperature for 15 min. The developer was discarded and distilled water was added to the dishes to stop further development for 2 min after which, chips were left to air-dry overnight. Developed chips were then scanned using the Miragene scanning system, where the resulting image is then quantified using commercial microarray software, such as ArrayVision™ (www.imagingresearch.com/products/ARV.asp), Molecularware™ (www.molecularware.com), or TIGR™ (www.tigr.org).

CAP values were provided pre-measured by Pharmacia, performed by standard methods (www.diagnostics.com).

2.5. Optimization studies

Since initial studies yielded sensitivity to the WHO standard 0.35 IU cutoff, we attempted to increase assay sensitivity. Patient serum of known titer was diluted with equine horse serum to produce samples with detectable human allergen-specific IgE as low as 0.05 IU. In order to increase sensitivity, the following parameters were titrated and optimized for maximal results: temperature: 8 °C (overnight), 25 °C for 1 h, 37 °C for 1 h, and 42 °C for 1 h; secondary antibody: 2 different mouse anti-human IgE-AP (Southern Biotech and BD Pharmingen), 2 different goat anti-human IgE-AP (Antibodies Inc. and Pierce Biotechnology); titration of secondary: 20–500 μl/10 ml buffer; buffer type: PBS vs. TBS.

3. Results/discussion

As shown in Fig. 1, we successfully demonstrate the spotting of crude allergen extract by the non-contact spotting method described. Panel A shows the printing template while panel B shows that spotting consistency revealed little carryover between adjacent spots. While spot morphology was slightly inconsistent, this phenomenon is easily corrected by drying chips at 8 °C with 80% humidity. One can see that patients originally diagnosed positive to a single allergen (mold) are often responsive to other allergens as well (grass, dustmite). This observation was later confirmed using a typical CAP assay (Table 1), illustrating that allergic phenomena are very complex and likely involve multiple allergens. The movement of allergy testing to multiplex assays is, therefore, appropriate for optimizing allergen-screening methods. Previously studies using allergen microarrays have been performed utilizing purified recombinant allergen proteins using contact or hand-spotting (Valenta et al., 1999; Harwanegg et al., 2003; Hiller et al., 2002; Kim et al., 2002; Jahn-Schmid et al., 2003) and are limited in the amount of protein that can be printed, hence limiting the optimal signal generation. These systems have not found widespread clinical utility and have not been validated on arrays as being equivalent to existing IgE allergy assays that have used crude extracts.

Table 1
Comparison of CAP and microarray IU values

Allergen	CAP	Microarray
Dustmite	3.3	4
	6.6	8.7
	0.9	1.1
Timothy grass	2.3	4.2
	2.1	1.8
	7.0	9.8
Mold	5.6	3.2
	17.2	18.4
	4.4	7.0

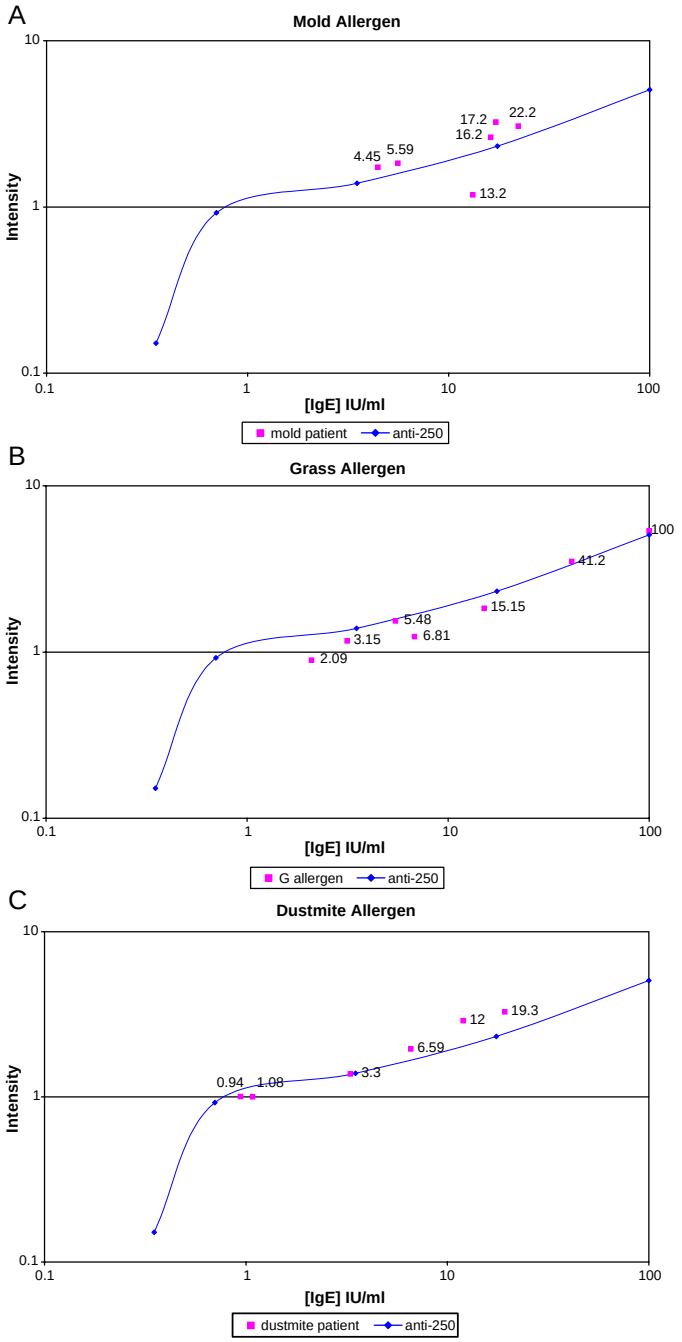

Fig. 2. Assay sensitivity for patient samples. Indicated allergens were spotted as described in the methods and assayed against patient sera to (A) mold, (B) grass, and (C) dustmite. Results are representative of 1600 assays.

In order to validate the detection of IgE reactivity to spotted allergens by colorimetric microarray assay, we assayed patient sera to mold (Fig. 2A), Timothy grass (Fig. 2B), and dustmite (Fig. 2C) and compared our results to our calibration curve generated with WHO standards. These patient samples had known CAP values and hence a direct comparison of assays can be made. Our assay allowed detection of IgE below the current WHO cutoff of 0.35 IU, making this assay as sensitive as non-microarray IgE tests. Coupled with the multiplexing advantages of microarray technology, our assay is a substantial improvement over these traditional in vitro tests. Additionally, the use of enzyme-linked detection adds a further improvement over other allergen arrays that rely on a lesser sensitive fluorescent detection scheme (Deinhofer et al., 2004; Harwanegg et al., 2003; Hiller et al., 2002; Kim et al., 2002; Jahn-Schmid et al., 2003).

To further optimize the sensitivity of our method, we tested a variety of assay parameters. Fig. 3 shows incubation of chips with patient sera in PBS or TBS buffers. While we found that 37 °C incubations with 1:250 dilution of Southern Biotech's mouse anti-human IgE-AP secondary antibody gave greatly

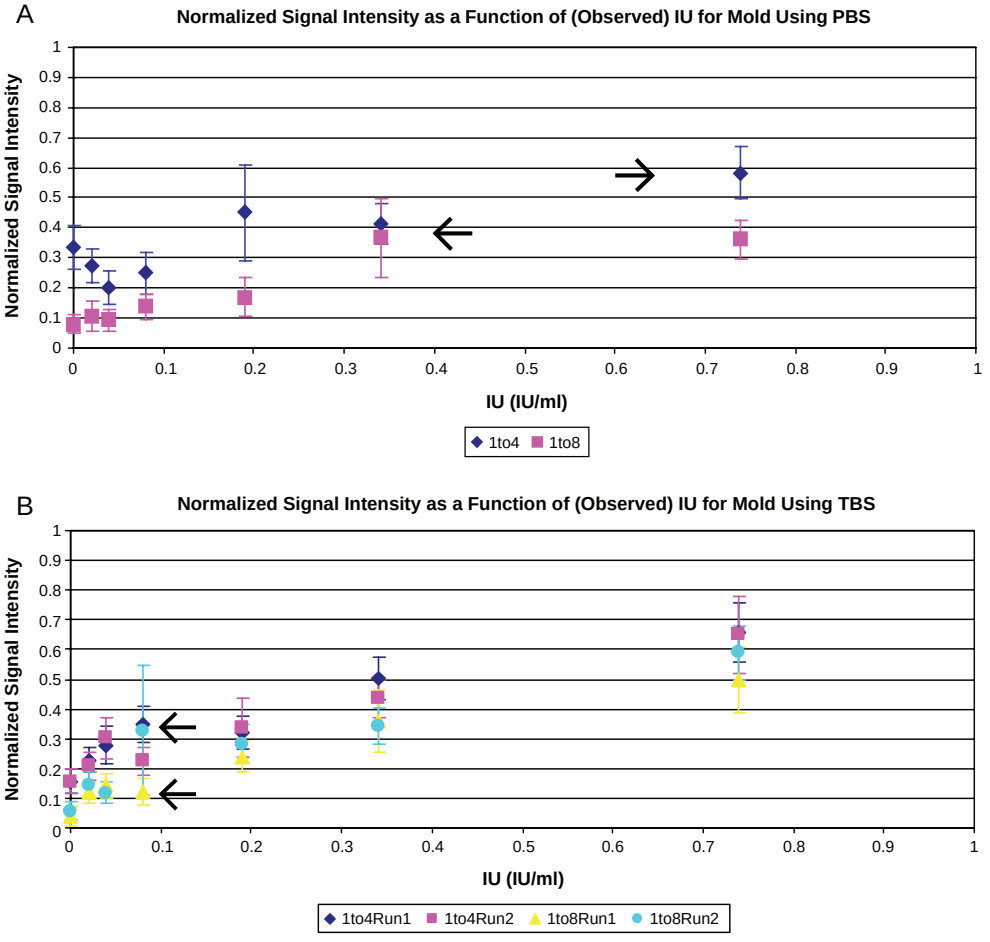

Fig. 3. Assay buffer optimization. (A) Plotted data illustrating the normalized signal intensity of samples assay washed in PBS as a function of IU for patient 8591. Data focuses on the mold allergen, and plots the observed IU. Data represent the average signal intensity per IU along with their corresponding standard error 250 assays. "1 to 4" and "1 to 8" indicate the dilution of the mold allergen. (B) Plotted data illustrating the normalized signal intensity of samples assay washed in TBS as a function of IU for patient 8591 as in (A). "1 to 4" and "1 to 8" indicate the dilution of the mold allergen and "Run 1" and "Run 2" denote different sets of data.

improved results than other temperature and antibody manipulations (data not shown), an additional increase in assay sensitivity was achieved by using a TBS buffer over PBS. As our colorimetric assay, utilizing alkaline phosphatase, converts a soluble BCIP/NPT reagent to the colored diformazan product, we wanted to test whether substituting a non-phosphate-containing buffer would improve assay sensitivity. It is believed that excess phosphate competes for binding to the phosphatase and may inhibit this conversion reaction. As shown in Fig. 3, assays performed in TBS provided increased sensitivity to those performed in PBS (compare panel B sensitivity <0.1 IU to panel A sensitivity >0.7 IU).

Our optimized multiplexed assay has many advantages over conventional in vitro IgE tests. Because of the advantages of microarray technology, we are able to reduce the amount of patient sera required and test multiple allergens at once, compared to assays which only screen a single allergen at a time and hence require much more sera and take substantially more time.

Our success in assaying crude and semi-purified allergen extracts allows us to collate results with those using recombinant allergens and establish biochemical characterizations of the protein components which cause allergic reaction. The use of crude extracts allows the allergen epitopes recognized by the IgE to be more representative of the complex allergen epitopes in vivo. We will be able to test a large population against thousands of allergens and potentially determine which reactive epitopes are critical for mediating the antibody response. Such insight will allow future advances in understanding the nature of allergic antibody reactions and will aid the generation of specific therapeutics to treat allergy.

We show that the combination of an optimized enzyme-linked assay, three-dimensional chip substrate, and protein microarray results in a very sensitive allergen assay. Non-contact printing allows use of crude, semi-purified, or recombinant allergens, expanding the menu of allergens and sources that can be used for future allergen microarray assays.

We predict that for a single analyte, the equivalency of our assay to existing validated tests will be sufficient to allow to proceed to FDA approval based on equivalency to existing in vitro allergen assays. When several allergens are shown to be equivalent, additional FDA applications will be required to show that multiplexing produces results that are equivalent to individual testing. Fluorescent microarrays will have a more difficult path to demonstrate equivalency since traditional, validated tests use an enzyme-linked method of detection and fluorescent systems have not been in use for this type of application. We envision the application of our method for high throughput, multiplexed clinical allergy testing.

Acknowledgements

Thanks to Michael O'Neill for providing support for this project.

References

Bacarese-Hamilton, T., Ardizzoni, A., Gray, J., Crisanti, A., 2004. Protein arrays for serodiagnosis of disease. Methods Mol. Biol. 278, 271.

Bousquet, J., Lockey, H.J., Malling, J., 1998. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J. Allergy Clin. Immunol. 102, 558.

Deinhofer, K., Sevcik, H., Balic, N., Harwanegg, C., Hiller, R., Rumpold, H., Mueller, M.W., Spitzauer, S., 2004. Microarrayed allergens for IgE profiling. Methods 32, 249.

Emanuel, I.A., 2003. In vitro testing for allergy diagnosis. Otolaryngol. Clin. North Am. 36, 879.

Harwanegg, C., Laffer, S., Hiller, R., Mueller, M.W., Kraft, D., Spitzauer, S., Valenta, R., 2003. Microarrayed recombinant allergens for diagnosis of allergy. Clin. Exp. Allergy 33, 7.

Hiller, R., Laffer, S., Harwanegg, C., Huber, M., Schmidt, W.M., Twardosz, A., Barletta, B., Becker, W.M., et al., 2002. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. FASEB J. 16, 414.

Ishizaka, K., Ishizaka, T., Hornbrook, M.M., 1966. Physicochemical properties of human reagenic antibody: IV. Presence of a unique immunoglobulin as a carrier of reagenic activity. J. Immunol. 97, 75.

Jahn-Schmid, B., Harwanegg, C., Hiller, R., Bohle, B., Ebner, C., Scheiner, O., Mueller, W.M., 2003. Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. Clin. Exp. Allergy 33, 1443.

Kay, A.B., 1997. Allergy and Allergic Disease. Blackwell Science, Oxford.

Kim, T.-E., Park, S.-W., Cho, N.-Y., Choi, S.-Y., Yong, T.-S., Nahm, B.-H., Lee, S., Noh, G., 2002. Quantitative measurement of serum allergen-specific IgE on protein chip. Exp. Mol Med. 34, 152.

Lebrun, S.J., 2004. Development of a low-cost, colorimetric protein array system to measure human autoimmune disease markers. Preclinica 2 (4), 267–274.

Valenta, R., Lidholm, J., Niederberger, V., Hayek, B., Kraft, D., Gronlund, H., 1999. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clin. Exp. Allergy 29, 896.

Van Weemen, B.K., 1985. ELISA: highlights of the present state of the art. J. Virol. Methods 10, 371.

Wills-Karp, M., Santeliz, J., Karp, C.L., 2001. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat. Rev., Immunol. 1, 69.