

Microarrayed allergens for IgE profiling

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Abstract

Diagnosis of type I allergy is based on anamnesis, provocation testing, and serological determination of total and specific IgE. Currently, in vivo and in vitro diagnostic tests employ allergen extracts prepared from various allergen sources (e.g., pollen, mites, animal dander, moulds, foods, venoms, etc.). The application of recombinant DNA technology to the field of allergen characterization has allowed to reveal the molecular nature of the most common allergens. To date a continuously increasing number of allergen sequences has become available and panels of recombinant allergens—assembling the epitope complexity of natural allergen sources—can be produced. The use of recombinant allergens instead of crude, natural extracts for allergy diagnosis allows us to determine the individual IgE reactivity profile of each patient. To enable a comprehensive analysis of the patient's IgE binding pattern to a large number of individual allergens, a new type of serological test is required. In this paper, we applied microarray technology to create a multi-allergen test system, based on microarrayed recombinant allergens.

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1. Introduction

Type I allergy represents an IgE-antibody-mediated hypersensitivity disease, which affects more than 25% of the population [1,2]. The central event which leads to the appearance of type I allergic clinical symptoms (e.g., allergic conjunctivitis, allergic rhinitis, allergic asthma bronchiale, urticaria, angioedema, and anaphylaxis) is the recognition of an allergen by allergen-specific IgE-antibodies present on patient's mast cells and basophils as well as in patient's serum. The allergen-mediated crosslinking of effector cell-bound IgE antibodies leads to the rapid release of biologically active mediators (e.g., histamine and leukotrienes) and to immediate clinical symptoms [3,4].

Diagnosing type I allergies involves accurate anamnesis and performance of in vivo provocation tests (e.g., skin prick tests, oral food challenge tests) [5]. If clinical

symptoms, case history, and results of provocation tests are indicative of type I allergy, the determination of allergen-specific IgE-antibodies is necessary to confirm the diagnosis of IgE-mediated allergy [6]. Currently, provocation tests as well as serological test systems employ allergen extracts prepared from various allergen sources (e.g., pollens, mites, moulds, foods, animal dander, and insect venoms). It is thus only possible to determine if a patient is sensitized to undefined allergens in a given allergen source, but the disease-eliciting components cannot be identified by these tests [7].

The application of recombinant DNA technology to the field of allergen characterization has allowed one to reveal the molecular nature of allergenic components. A continuously increasing number of allergen sequences has become available and panels of recombinant allergens—assembling the epitope-complexity of natural allergen sources—can be produced, offering the possibility to determine the individual IgE reactivity pattern of each patient and to identify the disease-eliciting allergen molecules [8–10]. To enable comprehensive analyses of patients' individual IgE-reactivity profiles to a great

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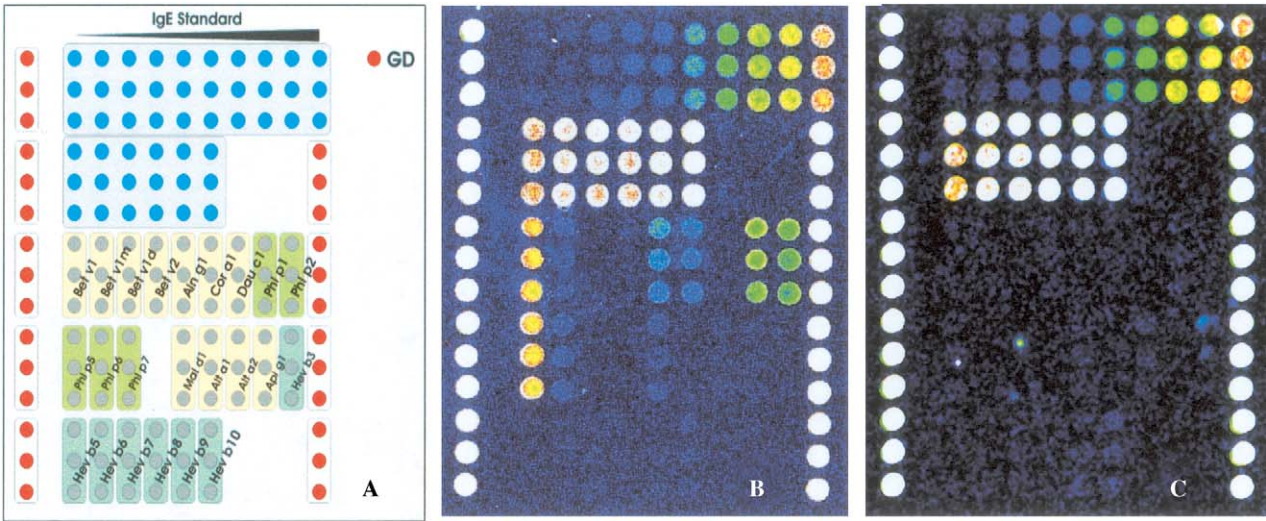
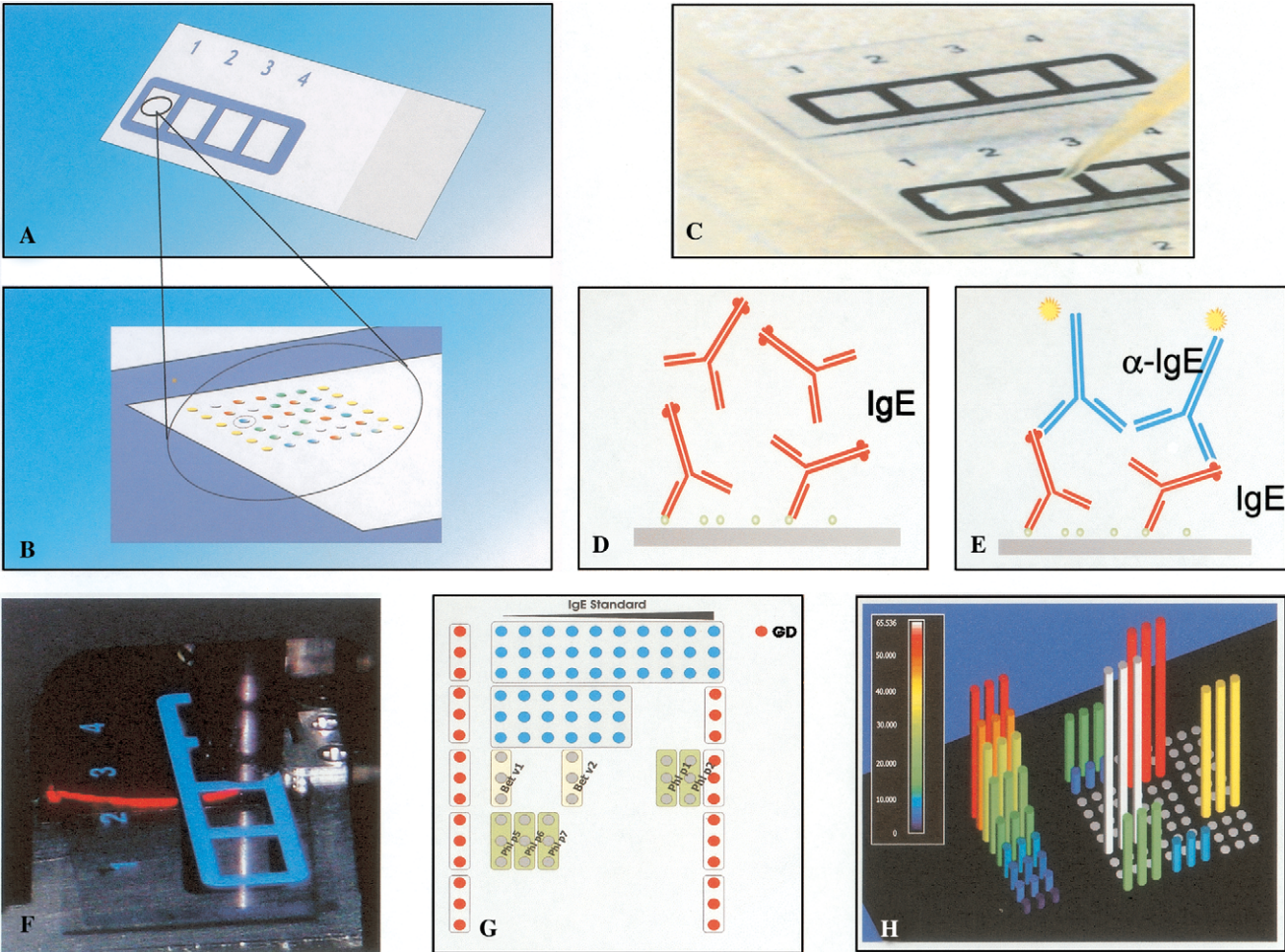


Fig. 1. A multi-allergen test system based on microarrayed recombinant allergens for component-resolved allergy diagnosis. (A) A standard calibration curve of purified human IgE and recombinant tree pollen allergens (Bet v 1, Bet v 1m, Bet v 1a, Bet v 2, Aln g 1, and Cor a 1), recombinant food allergens (Dau c 1, Mal d 1, and Api g 1), recombinant grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, and Phl p 7), recombinant mould allergens (Alt a 1 and Alt a 2), and recombinant latex allergens (Hew b 3, Hev b 5, Hev b 6, Hev b 7, Hev b 8, Hev b 9, and Hev b 10) were spotted in triplicates on the allergen chip. GD: guideline dots for scanning. (B) Image of a pollen-allergic patient with IgE reactivity to rBet v 1, rAln g 1, rCor a 1, rPhl p 2, and rPhl p 5. (C) Image of a non-allergic individual (control).



number of individual, recombinant allergens, a new type of serological test is required. Taking advantage of microarray technology we have developed a multi-allergen assay, which contains recombinant allergens that are printed onto a glass slide and can be tested with minute amounts of serum (20 μ l) in a single step (Figs. 1 and 2). The chip technology can be used to test simultaneously IgE reactivity to up to 5000 components in a single step. The multi-allergen test system, based on microarrayed recombinant allergens, was evaluated by determination of complex IgE reactivity profiles of sera obtained from tree and grass pollen-allergic patients. Results were compared with results obtained by standard routine serological measurements employing recombinant pollen allergens (see Fig. 3).

2. Description of methods

2.1. Production of chips containing microarrayed recombinant allergens

Conventional microscopy glass slides with a customized teflon mask (SPI Technologies, Westchester, USA), designed to create 7×7 mm rectangular glass areas representing four individual reaction wells surrounded by 2 mm teflon, were incubated in a 1:1 mixture of methanol and concentrated hydrochloric acid overnight and sonicated in acetone to remove surface contaminations. The surface was rendered amine-reactive by using conventional silane and coated with an amine-reactive polymer to allow efficient covalent attachment of recombinant proteins and optimum accessibility to analyte antibodies. Recombinant allergens were obtained from Biomay (Biomay, Vienna, Austria; www.biomay.at) and dissolved to a final concentration of 2 mg/ml in 150 mM sodium phosphate buffer (pH 8.4). Spotting of recombinant allergens onto glass slides was performed in an ordered array using the GMS 417 Microarrayer (Affymetrix, Santa Clara, CA), which represents a contact spotter employing pin-and-ring technology: the spotter's metal ring, with a diameter of 1 mm, was dipped into the protein solution and 1 μ l of the sample fluid was taken up by the pin generating a thin film of fluid inside the ring. A metal pin, with a diameter of 125 μ m at the tip, penetrated the ring to allow contact between the surface and the protein solution. Depending on the surface tension of each protein solution the diameters of arrayed spots were between

180 and 250 μ m corresponding to approximately 1 ng of protein per spot. Each recombinant allergen was spotted in triplicates onto the surface in each reaction well of the glass slide. After spotting, allergen chips can be stored at room temperature until use.

2.2. Assay performance

Microscopy slides containing allergen chips were washed for 120 min in TBS-T buffer (150 mM sodium chloride, 10 mM Tris base, and 0.5% Tween 20, pH 8.0), rinsed with deionized water, and dried. Slides were placed into a humid chamber and 20 μ l of undiluted serum from pollen-allergic patients was applied to the individual reaction wells. After incubation with patients' sera for 180 min at room temperature in the humid chamber, slides were rinsed and washed for 15 min in TBS-T, for 5 min in deionized water and dried. To detect bound IgE-antibodies, allergen chips were incubated for 60 min at room temperature with 20 μ l of a fluorescence-labelled anti-human IgE antibody (Pharmingen, San Diego, CA, Alexa Fluor 546 protein labelling kit, Molecular Probes, Leiden, Netherlands) diluted 1:1000 in TBS-T containing 5% milk powder. Subsequently, slides were washed twice for 10 min with TBS-T, rinsed with deionized water, dried, and stored in the dark until scanning.

2.3. Image and data acquisition, quantification of results

Images were acquired by scanning of allergen chips with a GMS 428 laser scanner (Affymetrix, Santa Clara, CA) with two different laser power settings. In the first scan, 70% of the laser power (50 db) was employed to obtain the highest possible signal intensities with acceptable background noise. In a second scanning step, laser power was reduced to 35 db in order to prevent saturated signals. Both images of each spot were analysed using the GenePix image analysis software (Version 3.0.6.89, Axon Instruments, Union City, CA).

Signals from images with fluorescence intensities between 3000 and 30,000 were used to calculate an extrapolation factor. The factor was calculated by dividing the signal intensities from the image scanned with high laser power (50 db) by the signal intensities from the image scanned with less laser power (35 db). Signals from images with fluorescence intensities above 30,000 were replaced by the corresponding signal of the image scanned with 35 db and multiplied with the extrapolation factor.

Fig. 2. Overview: chip-based allergy diagnosis. (A) Each allergen chip consists of four individual reaction wells containing microarrayed recombinant allergens. (B) Schematic representation of microarrayed recombinant allergens. (C) Incubation of allergen chips with only 20 μ l serum allows allergen-specific IgE antibodies to bind to spotted allergens (D). (E) Detection with fluorescence-labelled anti-IgE antibodies. (F) Scanning of allergen chips with a laser scanner. Each allergen array contains an IgE standard calibration (G) curve for quantitative analyses of results to allow the determination of the IgE reactivity profile (H).

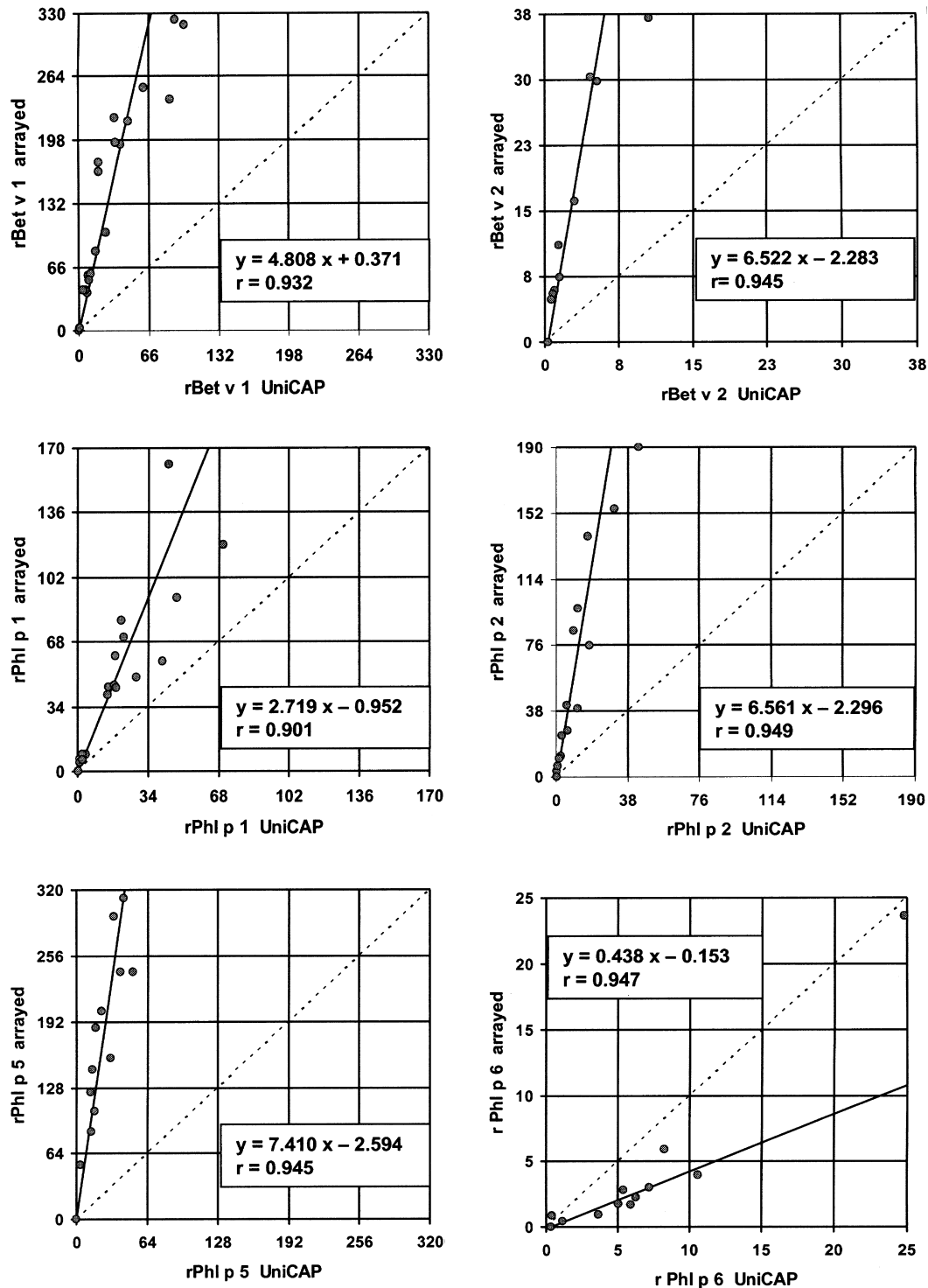


Fig. 3. Correlation of IgE reactivities to microarrayed allergens with results obtained by CAP measurements. The individual IgE reactivity profiles of sera from 26 pollen allergic patients were determined by the multi-allergen test system based on microarrayed recombinant allergens. Sera were further tested for IgE binding to rBet v 1, rBet v 2, rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, and rPhl p 7. Results were correlated by linear regression analyses. Correlations were calculated for rBet v 1, rBet v 2, rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6.

To score IgE binding to a recombinant allergen as positive, the following criteria had to be fulfilled: (i) The fluorescence intensities of each of the triplicates spotted of one allergen had to be above the overall signal cut-

off, which was calculated by the mean fluorescence signal determined for non-atopic human sera plus three standard deviations. (ii) The difference between the mean and median fluorescence intensity of each spot

had to be less than 30%. (iii) The coefficient of variation between the fluorescence intensities of all triplicates had to be less than 30%. (iv) An expected position of each spot was calculated in relation to the guiding dots of the protein array. The deviation of the determined position from the expected position of each spot had to be less than 10%. Java script based software was used to establish automatic filtering and quality control of data. If one of these criteria was not fulfilled, visual inspection of the image was requested. If all criteria were met, the mean value of the triplicates was used for further calculations.

On each allergen chip, a standard calibration curve of purified human IgE (concentrations: 1, 5, 15, 30, 60, 100, 150, 225, and 300 µg/ml, corresponding approximately to 1–300 pg IgE per spot) was spotted in triplicates and detected by the fluorescence-labelled anti-human IgE antibody. After a log/log transformation of IgE-concentrations and resulting fluorescence signals, the linear regression parameters were calculated and applied to the fluorescence data of allergen-specific signals.

2.4. Evaluation of the multi-allergen array and comparison of results

The multi-allergen test containing microarrayed recombinant birch and grass pollen allergens (rBet v 1, rBet v 2, rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, and rPhl p 7) was evaluated by determination of complex IgE reactivity patterns of sera obtained from 26 pollen-allergic patients. Sera were further tested for IgE binding to rBet v 1, rBet v 2, rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, and rPhl p 7 using the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Results were correlated by linear regression analysis according to Passing and Bablok [11].

Although the allergen chip does not immobilize allergen in excess of IgE antibodies, excellent correlations were found for recombinant birch and grass pollen allergens (rBet v 1, $r = 0.932$; rBet v 2, $r = 0.945$; rPhl p 1, $r = 0.901$; rPhl p 2, $r = 0.949$; rPhl p 5, $r = 0.945$; and rPhl p 6, $r = 0.947$; and Fig. 3). rPhl p 7, a minor grass pollen allergen, was detected only by 2 out of 26 sera therefore, no correlation was calculated in case of rPhl p 7. Nevertheless, both sera were scored positive in both test systems and all other sera were diagnosed negative, demonstrating that both test systems yielded corresponding results.

3. Concluding remarks

During the last decade, a continuously increasing number of recombinant allergens has been produced by recombinant DNA technology and panels of recombinant allergens—assembling the epitope-complexity

of natural allergen sources—can now be used to determine the individual patient's sensitization profile. Component-resolved diagnosis based on recombinant allergens is superior to extract-based testing for several reasons: Allergen extracts prepared from allergen-containing biological materials are difficult to standardize because allergens may become degraded due to instability or may be under-represented [12], leading to false-negative results. Furthermore, natural allergen extracts may be contaminated with allergens from other allergen sources (e.g., mite allergens in animal dander extracts), leading to false-positive results [13]. Diagnostic tests based on natural allergen extracts cannot identify the disease-eliciting components and cannot be used to discriminate between patients who are sensitized against few allergen molecules and patients who are polysensitized against a great variety of immunologically unrelated allergens. The latter information is necessary to assist the clinician in choosing optimal therapy strategies among available treatment forms (symptomatic treatment versus immunotherapy).

Type I allergy can be elicited by allergens derived from a great number of different allergen sources (e.g., pollens, mites, foods, animal danders, moulds, insect venoms, etc.). Today the most common allergen components have been produced by recombinant DNA technology providing the basis for multiallergen testing. However, the currently available allergy test systems are either single allergen tests or allow simultaneous testing of only a limited number of allergens. Furthermore, these tests consume rather large volumes of serum and thus make multiallergen testing costly, time-consuming, and laborious. Microarray technology allows one to immobilize a large number of proteins onto small surfaces and thus represents one possibility to test IgE reactivities against a great number of allergen components in a single step requiring low volumes of serum. The feasibility of using allergen-chips or arrayed allergens for multiallergen testing has been recently reported [14–16].

Here, we demonstrate for a selected panel of well-characterized recombinant pollen allergens that chip-based allergy diagnosis may equal well-established allergy test systems in terms of sensitivity and specificity. The multi-allergen test system, based on microarray technology, presented in this study enables one to determine a patient's individual IgE reactivity profile against up to 400 individual allergens using only 20 µl of serum whereas conventional allergen tests consume up to 50 µl serum per tested allergen. One big advantage of chip-based allergy diagnosis is therefore that the IgE-reactivity profile to a large number of allergen components can be determined in a single test (Figs. 1 and 2).

However, it must also be admitted that several problems must be properly addressed before allergen

chips can be used for routine testing. Microarray assays are generally prone to produce artificial signals, even if experiments are conducted with utmost care, because defects of the glass substrate, accumulation of dust particles on the surface, and partial or complete dehumidification may give rise to artificially increased signals and such defects cannot be simply visualized by the operator. Therefore, as described in detail above, strict rules were developed to score a result as positive.

Another problem of the multi-allergen test is that CV values (batch to batch variation) are around 30%, due to the inaccuracy of microarray spotters, a problem that might be overcome by improvement of spotting technology.

The fact that the allergen chip does not contain allergen in excess to IgE antibodies seems to turn out rather as an advantage because it mimics more closely the situation in the patient where allergens are presented in minute amounts. Although working in allergen excess has been postulated for many years as a rule to increase the sensitivity of in vitro tests, it has been reported that allergen-specific IgE levels do not always correlate with biological and clinical sensitivity [17]. Our assumption that testing with low amounts of allergens does not necessarily represent a disadvantage was indeed corroborated by the finding that excellent correlations with a standard routine test working in allergen excess were observed.

We are well aware that substantial additional work will be required to manufacture chips containing complete repertoires of allergens for reliable in vitro testing and that new forms of analysis for the complex test results need to be developed. However, we believe that chip-based multiallergen testing with recombinant allergens will greatly improve future allergy diagnosis.

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