

Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E

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Summary

Background The availability of recombinant allergens and recent advances in biochip technology led to the development of a novel test system for the detection of allergen-specific IgE.

Objective To test the performance of this allergen microarray in a serological analytical study.

Methods Standard allergens contained in grass pollen (Phl p 1, Phl p 2, Phl p 5 and Phl p 6) and tree pollen (Bet v 1 and Bet v 2) were used as a model system. The detection of allergen-specific serum IgE using microarrays was compared with standard test systems: CAP/RAST and an in-house ELISA. In order to test the analytical sensitivity of the assays, geometric dilutions of a serum pool containing high levels of pollen-specific IgE from allergic individuals were tested in each system. To assess the analytical specificity, the sera of 51 patients with presumptive allergic symptoms were collected before diagnosis. Thereafter, the results for grass/tree-pollen-specific IgE were compared.

Results The microarray has a good dynamic range similar to the CAP/RAST system. Microarray and ELISA showed comparable analytical sensitivity exceeding the CAP/RAST system. With respect to the analytical specificity, no significant cross-reactivity of the allergens was observed. For two of the allergens tested, weak positive signals were detected in the microarray test system, whereas they were not detectable by CAP/RAST.

Conclusion A good correlation of presently used methods to detect serum IgE and the novel microarray test system was observed. As a next step, a careful validation of this method for a multitude of allergens and a thorough clinical evaluation has to be provided.

Microarray testing of allergen-specific IgE can be presumed to be the method of choice for a prospective component-resolved diagnosis of Type I allergy, and the basis for the design and monitoring of a patient-tailored specific immunotherapy in the future.

Keywords IgE, microarray technology, recombinant allergen, Type I allergy

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Introduction

The diagnosis of Type I allergy is currently performed using allergen extracts containing a mixture of allergenic and non-allergenic molecules that are difficult to standardize. Using these extracts for *in vivo* testing (skin prick test; intradermal test; nasal, oral or bronchial provocation), measurement of allergen-specific serum IgE (RAST) to a restricted number of allergen extracts, according to the patient's case history, is performed. Thus, diagnosis defines the source, but not the allergenic molecule(s) eliciting the disease. In the last decade, advances in molecular allergology made it possible to clone and produce the allergens of major allergenic sources (pollens, mites, foods, moulds, venoms) in recombinant form. These proteins are easily available in well-defined large-scale

quantities and have been shown to retain the immunological properties of the natural protein [1–3]. Using these recombinant molecules, improvement of diagnosis is possible, e.g. by spiking allergen extracts with low abundant allergens [4]. In a further development, a 'component-resolved diagnosis' has been suggested for a more specified diagnosis, revealing the antibody reactivity profile of an allergic patient and identifying the disease-eliciting molecules. The results of these analyses could be the basis for patient-tailored allergen preparations to improve the specificity and efficacy of specific immunotherapy and reduce the risk of sensitizing patients to other allergens present in an allergen extract to which they are not yet sensitized [2, 5, 6].

Biochip technology has advanced in recent years, leading to the development of DNA microarrays, a powerful technology that makes it possible to monitor the expression levels of a multitude of genes on miniaturized formats. Subsequently, protein microarray technology – technically more challenging due to the heterogeneity of the molecules and the need to preserve their tertiary structure after binding to the chip – has

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developed protein microarrays designed for the detection and quantification of proteins, respectively their function [7–9].

Together, the progress in molecular allergology and biochip technology led to the development of a miniaturized allergy test [6, 10]. The principle of this allergen chip is a reverse immunoassay consisting of recombinant allergens in nanolitre quantities as capture molecules immobilized on glass slides. The analyte, i.e. low volumes of serum, is screened for allergen-specific IgE by fluorescence-labelled anti-IgE. Thus, a simultaneous analysis of a multitude of single allergens can be probed for IgE binding, allowing the identification of the disease-causing molecules, i.e. also the target molecules for specific immunotherapy in a single test. In addition, such an allergen microarray would be useful in monitoring reactivity profiles following natural allergen exposure, pharmacotherapy or following immunotherapy.

The objective of this study was to test the analytical performance, i.e. reproducibility, sensitivity and specificity, of this novel allergen microarray for the detection of allergen-specific serum IgE. Using four grass and two tree pollen allergens as paradigms, microarray testing was compared with conventional diagnostic methods: the CAP/RAST system and an in-house ELISA assay.

Materials and methods

Purified recombinant allergens

In this study, we concentrated on standard allergens in grass and tree pollen: the recombinant proteins Phl p 1 [11], Phl p 2 [12], Phl p 5 [13], Phl p 6 [14] Bet v 1 [15] and Bet v 2 [16] used in microarrays and ELISAs were obtained from Biomay (Vienna, Austria).

Patients

Serum samples from allergic patients were obtained from the Allergy Clinic Reumannplatz, Vienna, Austria. The diagnosis of Type I allergy was based on case history, skin prick testing and IgE serology performed with the CAP/RAST system. Total IgE ranged from 4.2 to >2000 kIU/L (median: 81.9 kIU/L). For control purposes, sera from 11 atopic individuals allergic to house dust mite (total IgE: >200 IU/mL; CAP/RAST class >4), but not to grass or birch pollen (CAP/RAST negative), were used.

Serological parameters

CAP/RAST Specific IgEs to various allergens (timothy pollen extract, Bet v 1, Bet v 2) were determined by the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden; CAP/RAST) according to the standard procedure. Values indicate protein units (PRU) with a cut-off of 0.35 PRU.

ELISA Binding of patients' IgE to recombinant allergens was determined by ELISA. Phl p 1, Phl p 2, Phl p 5, Phl p 6, Bet v 1 or Bet v 2 were coated onto ELISA plates (Maxisorb, NUNC, Roskilde, Denmark) in carbonate buffer (pH 9.6) at a concentration of 2 µg/mL (50 µL/well), overnight at 4 °C. The plates were washed twice with phosphate-buffered saline

(PBS) –0.05% Tween 20, and blocked for 5 h with 100 µL of 1% human serum albumin in PBS–0.05% Tween 20, at room temperature. Fifty microlitres of the patients' – or control sera were added at a dilution of 1:2 and incubated overnight at 4 °C. Plates were washed five times with PBS–0.05% Tween 20. As the detection antibody, 50 µL of horse anti-human IgE (Allercoat, Bio-Rad, Munich, Germany) conjugated with alkaline phosphatase diluted 1:2 was added in PBS–1% human serum albumin and incubated overnight at 4 °C. After washing five times with PBS–0.05% Tween 20, 100 µL of the substrate *p*-nitrophenyl-phosphate was applied at a concentration of 1 mg/mL in diethanolamine buffer (pH 9.8). Plates were incubated in the dark for 2 or 4 h, respectively, at 37 °C and the colour reaction was stopped by the addition of 100 µL of 0.1 N NaOH. Subsequently, optical density (OD) was measured at 405 nm, using 550 nm as reference. As internal controls, the experiment was also performed using uncoated plates, and coated plates without serum. Sera from 11 atopic individuals with other allergies were used as control. The detection limit for specific IgE binding was defined to be greater than 3.5 SD from the mean values obtained with these control sera.

Preparation of allergen chips

Allergen microarray Conventional microscopy glass slides coated with a customized Teflon mask were purchased from Structure Probe Inc. (SPI, Westchester, PA, USA). The Teflon mask was designed to create 7 × 7 mm glass areas surrounded by 2 mm Teflon on all sides. Inside every one of these arrays, up to 800 individual proteins can be immobilized (Fig. 1). Because of the hydrophobic nature of the Teflon mask, spatially separated reaction wells containing identical arrays of proteins can be incubated with different samples on one and the same slide. Before surface activation, slides were cleaned thoroughly and rendered amine-reactive by using conventional silane and cross-linking chemistries. Allergens were dissolved to a final concentration of 2 mg/mL in 150 mM sodium phosphate buffer (pH 8.4). Before spotting, all allergens were checked by gel electrophoresis and Western blotting. The spotting process was performed with a GMS 417 Microarrayer (Affymetrix, Santa Clara, CA, USA). This spotter is a contact spotter and works by the principle of the so-called pin-and-ring technology. Upon contact with the slide surface, approximately 1 nL of the allergen solution is deposited by the pin. All allergens were spotted as vertical triplicates (Fig. 1).

Allergen chip assay

After protein spotting, arrays were washed two times for 5 min in TBS-T buffer (150 mM sodium chloride, 10 mM Tris base, 0.5% Tween 20, pH 8.0), rinsed with deionized water and dried under nitrogen flow. The individual arrays were then incubated with 20 µL 1:5 diluted human serum for 60 min. Arrays were washed again two times in TBS-T (5 min), rinsed with deionized water and dried under nitrogen flow. A fluorescence-labelled antibody (Alexa Fluor 546 protein labelling kit, Molecular Probes, Leiden, the Netherlands) against human IgE was diluted 1:1000 in TBS-T containing 1 mg/mL bovine serum albumin as a blocking

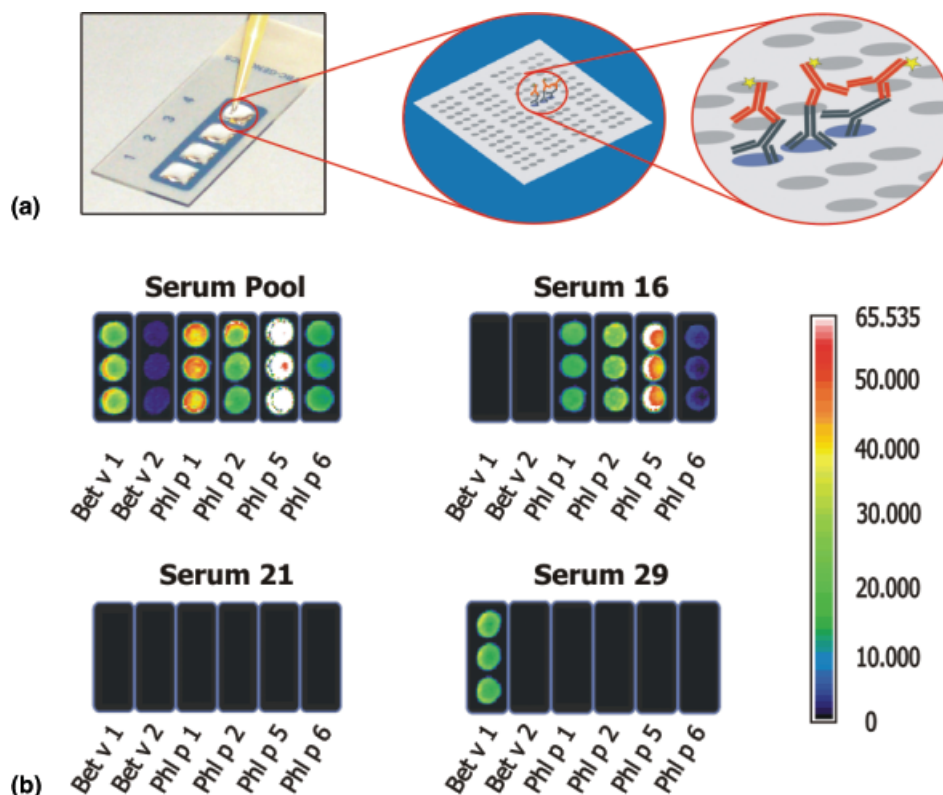


Fig. 1. Schematic description of microarray testing of allergen-specific IgE. (a) Allergens are spotted onto slides in triplicate. Serum incubation is followed by a secondary anti-human IgE Ab for detection. The mean fluorescence intensity is determined at two different laser settings. (b) Four representative serum samples are shown displaying different IgE reactivity profiles.

agent. Arrays were incubated for 30 min with the detection antibody. After three final washing steps in TBS-T and a short rinse with water, arrays were dried under nitrogen flow and scanned with a GMS 428 scanner (Affymetrix, Santa Clara, CA, USA).

Image and data acquisition

Images were acquired with two different laser power settings. For the first scan, 70% of the maximum laser power (50 db) was chosen to obtain the highest possible signal intensities with acceptable background noise. In the second scan, the laser power was reduced to 35 db to prevent saturated signals (65.535). Both images were then analysed with the GenePix image analysis software (version 3.0.6.89, Axon Instruments, Union City, CA, USA). From the three intensity values obtained for each allergen, the mean value was calculated and used for further analysis.

Data extrapolation

The theoretical linear range of a microarray scanner is between 0 and 65.535. Practically, linearity begins at signal intensities of approximately 3.000 and ends at approximately 55.000. To broaden this limited range of linearity, we used the following extrapolation procedure. Signals from both images, which were either between 3.000 and 55.000, were employed to calculate an extrapolation factor, by dividing the signals obtained from the high laser power image by the signals of the low laser power image. The resulting factor is almost

constant for signals in this range. All signals above 55.000 in the high laser power image were then replaced by the according values of the image scanned with the weaker laser power and multiplied by the extrapolation factor. Using this procedure, the linear range can be extended almost 10-fold if the laser power is chosen correctly.

The detection limit for specific IgE binding was defined to be greater than 3.5 SD from the mean fluorescence values obtained with control sera for each allergen. All values below that cut-off were considered to be negative.

Statistics

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Paired correlations were calculated by the Spearman rank test.

Results

Reproducibility of microarray-based IgE determination

Allergen microarrays provide a novel technique to detect allergen-specific IgE. We sought to test the performance of such microarrays using standard allergens of grass and tree pollen as well-characterized model allergens. First, the inter-assay precision of microarray array testing for allergen-specific IgE was evaluated by serial testing. A serum pool prepared from the sera of six patients containing high levels

of grass pollen-specific IgE (CAP/RAST class 6; >100 PRU) and also elevated levels of birch pollen-specific IgE (CAP/RAST class 4; 26.5 PRU) was tested at a concentration of 1:5 on 20 replicate microarrays. IgE specific for the major grass pollen allergens Phl p 1, Phl p 2, Phl p 5, Phl p 6 as well as the major birch pollen allergen Bet v 1 and Bet v 2 was determined (Table 1). The coefficient of variation (CV; standard deviation in % of mean value) varied from 17% to 44% for Phl p 6. The slide-to-slide variation for each allergen was measured by calculating the variation of four arrays on one and the same slide using the serum mentioned above. The within-slide variation for the individual allergens varied from 8% to 40% (Table 1). The batch-to-batch imprecision was determined by testing the same serum pool with seven slides on 3 consecutive days, using a different batch of slides on each of the three days. The overall CV values calculated for the individual allergens ranged from 25% to 47% (Table 1). In all three test series, the highest imprecision was observed for Phl p 6.

Analytical sensitivity

To assess the analytical sensitivity of microarray tests for allergen-specific IgE, the above-mentioned serum pool was tested in eightfold geometric dilutions starting at 1:3. The values obtained with the microarray system were compared with the measurements obtained by CAP/RAST and ELISA at the same dilutions (Tables 2 and 3). For grass pollen allergens, CAP/RAST was performed with timothy pollen extract, but the tree pollen allergens Bet v 1 and Bet v 2 were tested with the respective recombinant proteins. The sensitivity of the microarray system was similar to the sensitivity

obtained with ELISA. The CAP/RAST assay turned out to be less sensitive, most strikingly for Bet v 1. Bet v 1-specific IgE was detectable from a dilution of 1:81 in CAP/RAST, but still gave a signal at 1:2187 in both of the other systems (Table 3). The linearity of the microarray and the CAP/RAST both extended up to high serum concentrations, whereas the dynamic range of the ELISA system was typically much more limited. The correlation was calculated using the CAP/RAST values for timothy pollen extract and the sum of the values obtained with Phl p 1, 2, 5 and 6 using the microarray or ELISA. A coefficient of correlation (ρ) of 0.994 (significance: $P < 0.01$) was obtained for the comparison of microarray and CAP/RAST and $\rho = 0.917$ ($P < 0.01$) for ELISA. The correlation between CAP/RAST and ELISA was 0.988 ($P < 0.01$). The correlation of microarray results and ELISA values for single protein determinations ranged from $\rho = 0.975$ to 0.988 ($P > 0.01$) for the respective recombinant timothy allergens. With respect to the birch pollen allergens, the correlations of the microarray values compared with CAP/RAST were $\rho = 0.939$ ($P < 0.01$) for Bet v 1 and $\rho = 0.713$ ($P < 0.05$) for Bet v 2. In comparison with ELISA data, correlations of $\rho = 0.994$ ($P < 0.01$) and $\rho = 0.829$ ($P < 0.05$) were obtained, respectively. CAP/RAST and ELISA correlated with $\rho = 0.931$ ($P < 0.01$) for Bet v 1 and $\rho = 0.930$ ($P < 0.01$) for Bet v 2.

Analytical specificity

To assess the specificity of IgE testing by microarray, sera of 51 patients visiting an allergy clinic were collected randomly. The only selection criterion was that, according to their case history, these patients were likely to suffer from Type I

Table 1. Reproducibility of microarray testing of grass- and birch pollen-specific IgE

Reproducibility	Phl p 1	Phl p 2	Phl p 5	Phl p 6	Bet v 1	Bet v 2
Inter-assay	17*	26	17	44	26	18
Within-slide	8	19	15	40	23	12
Batch-to-batch	25	27	36	47	33	31

*Coefficient of variation (CV; standard deviation in % of mean value) pooled serum derived from six patients allergic to timothy pollen (CAP/RAST > 100 PRU) and birch pollen (CAP/RAST 26.5 PRU) was tested at a concentration of 1:5. Microarray conditions are described in Results.

Table 2. Comparison of assay sensitivity to detect grass pollen-specific IgE using microchip array, CAP/RAST and ELISA

Dilution of serum pool*	Microchip array (fluorescence units)				CAP/RAST (PRU)	ELISA (OD)			
	Phl p 1	Phl p 2	Phl p 5	Phl p 6	Phleum extract	Phl p 1	Phl p 2	Phl p 5	Phl p 6
1:3	230 334	119 692	377 019	82 589	81.1	>2.5	>2.5	>2.5	>2.5
1:9	97 997	44 607	134 434	24 303	28.4	>2.5	>2.5	>2.5	>2.5
1:27	11 953	8164	55 659	5660	10.1	>2.5	1.811	>2.5	1.363
1:81	5040	5651	20 063	2510	4.1	2.209	0.947	1.527	0.543
1:243	1811	1843	6575	1128	1.6	0.901	0.355	0.648	0.227
1:729	895	979	2113	Negative†	0.5	0.401	0.160	0.242	0.108
1:2187	353	Negative	627	Negative	Negative	0.166	0.085	0.119	Negative
1:6561	Negative	Negative	440	Negative	Negative	Negative	Negative	Negative	Negative

*Serum was pooled from six patients allergic to timothy pollen (CAP/RAST > 100 PRU) individual allergens were tested with microchip and ELISA, CAP/RAST determination was done with allergen extract. †For CAP/RAST: <0.35; for microchip and ELISA: <mean+3.5 SD of control sera.

Table 3. Comparison of assay sensitivity to detect birch pollen-specific IgE using microarray chip, CAP/RAST and ELISA

Dilution of serum pool*	Microchip array (fluorescence units)		CAP/RAST (PRU)		ELISA (OD)	
	Bet v 1	Bet v 2	Bet v 1	Bet v 2	Bet v 1	Bet v 2
1:3	134 650	6295	12.9	1.5	> 2.5	1.022
1:9	47 470	2061	5.1	0.6	> 2.5	0.378
1:27	10 319	1284	1.7	0.4	1446	0.176
1:81	7493	1643	0.5	Negative†	0.733	0.098
1:243	2753	1327	Negative	Negative	0.305	Negative
1:729	998	802	Negative	Negative	0.138	Negative
1:2187	Negative	Negative	Negative	Negative	0.074	Negative
1:6561	Negative	Negative	Negative	Negative	Negative	Negative

*Serum was pooled from six patients allergic to birch pollen (CAP/RAST: 26.5 PRU). †For CAP/RAST: <0.35; for microchip and ELISA: <mean+3.5 SD of control sera.

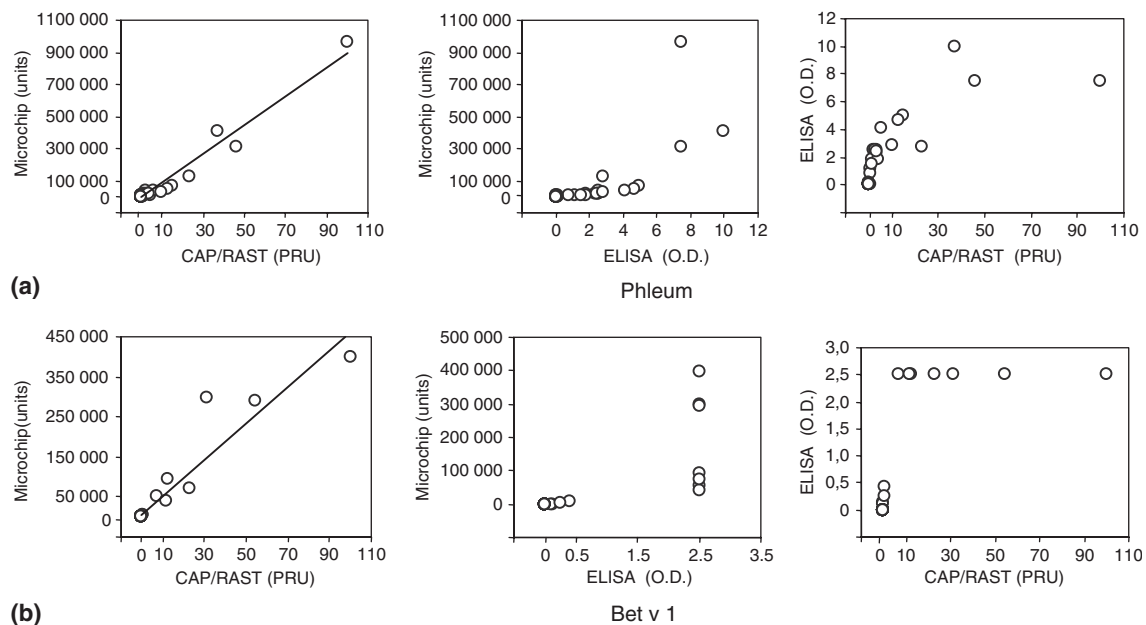


Fig. 2. Comparison of allergen-specific IgE detection using allergen microarray, CAP/RAST and ELISA. The sera of 51 presumptive allergic patients were screened for IgE reactivity to grass and tree pollen allergens. (a) CAP/RAST was performed with grass pollen extract and compared with the summed-up results for Phl p 1, Phl p 2, Phl p 5 and Phl p 6 obtained with microarray and ELISA. (b) IgE reactive with Bet v 1 was compared in all systems.

allergy. IgE specific for timothy extract, Bet v 1 and Bet v 2 was determined using the CAP/RAST system. In parallel, the sera were analysed by microarray and ELISA for IgE against the respective recombinant allergens. Within these 51 patients, the CAP/RAST system detected 20 patients with timothy pollen-specific IgE of different RAST classes with PRUs ranging from 0.4 to >100 (median: 3, 6). Using microarray or ELISA, IgE specific for one or more grass pollen allergens was also detected in 18 of these 20 sera. The two 'negative' sera were identical for both test systems and represented the weakest positive data in the CAP/RAST system (PRU: 0.4 and 0.69). In general, no significant cross-reactivity was observed in microarrays and ELISA. For example, several sera highly positive for one allergen were totally negative for any of the other allergens tested. Some other sera were selectively positive for more than one, but not all allergens (data not shown). Both the microarray and the

ELISA resulted in three positive values for Phl p 1 that were negative with CAP/RAST, one of them derived from the identical serum. Within the 18 sera that were recognized by all three assays, three discordant values were obtained for Phl p 6, i.e. positive in ELISA with OD values in a lower range (0.109, 0.120, 0.375) and negative in microarray testing. With respect to Phl p 2, three sera were positive in ELISA in a very low range (0.090, 0.079, 0.088), but negative with microarray. The correlation of the summed-up microarray values compared with CAP/RAST was $\rho = 0.914$ ($P < 0.01$). The correlation of the summed-up microarray values compared with the combined ELISA data was $\rho = 0.934$ ($P < 0.01$). The correlation between the combined ELISA data and CAP/RAST was $\rho = 0.917$ ($P < 0.01$). These correlations are illustrated in Fig. 2a. ELISA- and microarray-derived values for single allergens correlated from $\rho = 0.681$ ($P < 0.01$) for Phl p 6 to $\rho = 0.998$ ($P < 0.01$) for Phl p 5.

Within the 51 sera tested, the CAP/RAST system detected 10 sera positive for birch pollen-specific IgE, nine for Bet v 1 (median: 12.0; range: 0.82 to > 100 PRU) and one for Bet v 2 at a low titre (0.56 PRU). The ELISA test system yielded congruent results. Microarray testing recognized all nine sera IgE-positive for Bet v 1. The correlations of the values obtained with microarray and CAP/RAST were $\rho = 0.999$ ($P < 0.01$) and, compared with ELISA, $\rho = 0.921$ ($P < 0.01$). The correlation between CAP/RAST and ELISA was $\rho = 0.920$ ($P < 0.01$) (Fig. 2b). The Bet v 2-positive serum was not detected by the microarray and two additional positive reactions were obtained for Bet v 2.

Discussion

DNA microarrays have emerged as a powerful technology to analyse gene expression and variations in gene sequences. Multiple probes can be tested in a single experimental set-up. Subsequently, the concept of using microarrays as a diagnostic test system has also been applied for testing serum samples to multiple antigen probes in order to reveal Ab binding, e.g. to autoantigens [17–20]. Recently, the detection of allergen-specific IgE, which is several magnitudes lower abundant in serum, has been reported to be feasible by microarrays [10, 21–24]. In this serologic study, the technical performance of a novel allergen microarray [10] was assessed. Analytical sensitivity and specificity of microarray testing was compared with CAP/RAST and ELISA as standard methods of IgE analysis.

With respect to the model allergens used, microarray and ELISA appeared to be more sensitive than the CAP/RAST system. In the case of grass pollen allergens, the lower sensitivity of CAP/RAST could be explained by the use of pollen extract, which may contain suboptimal concentrations of particular allergens. On the other hand, the sensitivity of CAP/RAST for recombinant Bet v 1 was even lower. The dynamic range of the microarray was comparable to the CAP/RAST system and much higher than the range provided by ELISA, although the microarray carried a much lower amount of immobilized protein to be detected as compared with the conventional test systems. In general, the specificity of the assays was comparable, with the exception of two allergens, Phl p 1 and Bet v 2, where a few weak positive results were detected in microarray and ELISA, whereas CAP/RAST was negative. However, the clinical relevance of IgE in those low serum concentrations may be questionable.

The CV values of the microarray-derived data were in the range of 17–26%, except for Phl p 6 with 44% (Table 1) for assays performed on one day and with one batch of allergen chips. CV values were slightly higher when different batches were used on consecutive days, but did not exceed 33%, with the exception of Phl p 6 (47%). Notably, typical CV values for the immobilization of biomolecules when using commercially available spotters are specified to lie between 20% and 25%. Thus, the variation may result mainly from chip production. As proteins display diverse physicochemical properties, the application of certain allergens in microarrays may cause problems. In this study, Phl p 6 displayed a great variation in the reproducibility test, and discordant results for

some test sera, when compared with ELISA, were obtained for Phl p 6. Consequently, for the set-up of a diagnostic microarray, each protein should therefore be carefully validated.

Generally, the microarray-based IgE test accelerates immune diagnosis significantly by analysing in parallel all relevant diagnostic parameters of interest. The amount of information that can be obtained from these protein arrays is significantly increased, while the sample quantity is relatively low. A reduced serum volume is of relevance, e.g. in allergy diagnosis of infants and young children. A component-resolved diagnosis generates valuable information with respect to the individual sensitization pattern of a given patient. This detailed diagnosis can provide valuable information for the clinician and the patient. For example, if IgE binding to a cross-reactive compound occurs, prophylactic measures (like the avoidance of special food stuff) could be induced. Panels of recombinant allergens for special diagnostic purposes could replace natural allergen extracts that cause special diagnostic problems such as latex or food allergens. It has been shown for tree and grass pollen allergens that a panel of a few recombinant allergens is sufficient for diagnosis [5, 25]. At present, the use of recombinant proteins is limited to allergenic glycoproteins without relevant carbohydrate epitopes. However, novel expression systems that perform post-translational modifications such as glycosylation or carbohydrate mimotopes are being developed [26, 27]. Importantly, a component-resolved diagnosis would be the basis for immunotherapy with patient-tailored allergens or hypoallergenic forms for specific immunotherapy.

In conclusion, complementary to a sound clinical examination of the patient, microarrays are a valuable option for the testing of allergen-specific IgE. However, still a wider range of different allergens have to be tested for use in microarrays. After a careful validation and, most importantly, clinical testing, allergen microarrays will allow a component-resolved diagnosis, which is indispensable for future component-resolved immunotherapy of Type I allergy.

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