

Microarrays for the Screening of Allergen-Specific IgE in Human Serum

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The described in vitro test system for allergy diagnosis is based on microscope glass slides activated with (3-glycidyloxypropyl)trimethoxysilane. Allergen solutions are immobilized as small droplets (~10 nL) on the activated glass slides with a piezoelectric arrayer. In contrast to other tests for specific IgE, such as Pharmacia CAP FEIA, AlaSTAT, or FAST, only a 25- μ L serum sample is needed for the screening of allergen-specific IgE against a multitude of allergens and the test can be performed in less than 1 h. Compared with multiallergen dipstick screening tests (e.g., IgEquick, CMG Immunodot) based on multi-allergen-coated nitrocellulose strips, the measurement of the microarray-based system can be performed automatically. The chemiluminescence intensities are detected with a sensitive CCD camera. Allergen extracts and recombinant/purified allergens (24 preparations) have been used on the same modified surface for the screening of allergen-specific IgE. With these disposable microarray slides, it is possible to distinguish between patients with and without elevated levels of allergen-specific IgE. Repeated measurements of serum samples demonstrated a sufficient reproducibility. Detection limits (μ g/L) of 0.35 (r Bet v1), 0.16 (PLA₂), and 1.9 (Der p1) were achieved.

Since the discovery of immunoglobulin E (IgE)^{1,2} by Ishizaka et al. and Johansson et al. and its key role in allergic reactions, the importance of the development of test systems measuring IgE levels in serum samples has widely been recognized. In 1967, the first radioallergosorbent test (RAST) for allergen-specific IgE in serum was described.³ Later, radioactive labels used in the RAST were replaced by many different procedures based on chromogenic enzyme immunoassay (EIA) or fluorescence enzyme immunoassay (FEIA) techniques, but only a few have become routine methods for allergy diagnosis in both research and practice.^{4–8}

In clinical routine, the most common in vitro technique is the Pharmacia CAP System (PCS) for total and allergen-specific IgE (specific IgE FEIA, Pharmacia, Uppsala, Sweden). There are also other methods (e.g., FAST FEIA, MAST Diagnostica, Reinfeld, Germany; HYTEC EIA, Hycor Biomedicals, Kassel, Germany) commercially available.⁹ Some methods are based on liquid-phase inhibitor assays (e.g., AlaSTAT, DPC Biermann, Los Angeles, CA) or multiallergen-coated nitrocellulose strips (e.g., IgEquick, Teomed AG, Greifensee, Switzerland; CMG Immunodot, Trimedial AG, Brüttisellen, Switzerland).^{10,11} The measurements obtained with the CAP System are expressed in units (kU/L) or in specific IgE classes (0–6).⁸ The assignment of units to IgE classes¹² is shown in Table 1. Any analytical technique for the examination of specific IgE levels should be able to distinguish the classes 0 and 1. Class 0 comprises IgE concentrations of up to 0.84 μ g/L, which is equivalent to 0.35 kU/L. The CAP System contains a cellulose polymer densely conjugated with allergen extracts or recombinant allergens. In general, allergen extracts are mixtures of different allergenic and nonallergenic proteins.¹³ Carbohydrate components, e.g., from glycoproteins, are also known to be of relevance in this context. Gel electrophoresis in combination with immunoblotting techniques allowed the determination and purification of the major allergenic compounds of several allergen mixtures.¹³ Recombinant DNA technology offers the possibility to produce recombinant allergenic proteins.¹⁴ Over the past 10 years, the most important allergens from tree and grass pollens, mites, animal epithelia, insect venoms, and foods have been cloned, sequenced, and expressed.^{15,16} Further studies examined the usefulness of recom-

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Table 1. Classification of Specific IgE Levels (Pharmacia CAP System)

class	specific IgE (kU/L)	specific IgE (μ g/L) ^a	level of specific IgE ^b
0	<0.35	<0.84	absent or undetectable
1	0.35–0.70	0.84–1.68	low
2	0.70–3.5	1.68–8.4	moderate
3	3.5–17.5	8.4–42	high
4	17.5–50	42–120	very high
5	50–100	120–240	very high
6	>100	>240	very high

^a 1 kU equals 2.4 μ g of IgE. ^b Tentative assignment of clinical relevance.

binant allergens for diagnosis and therapy of allergic diseases.¹⁷ An increasing number of patients are suffering from immediate-type allergic diseases such as allergic rhinoconjunctivitis, atopic eczema, and food and drug allergies.¹⁸ Although it is possible to measure a multitude of allergen-specific IgEs by immunoassays in the patient's blood, these tests are expensive, time-consuming (for the mentioned commercial systems the duration of one test is about 3–4 h), and need a rather high volume of reagents and serum sample (50 μ L/well) in some varieties. Taking into account that allergies are on the increase,¹⁸ it is highly desirable to develop a fast and economic screening technology for the detection of allergen-specific IgE in serum samples, which allows the simultaneous analysis of hundreds of allergens in a single run. Multiallergen dipstick tests were a first step for miniaturization and cost savings,^{11,19} but most of them lack automatization. Another strategy for miniaturization are microarrays produced with spotting devices enabling the immobilization of proteins in the lower nanoliter range on defined positions on a surface.²⁰ Already in one of the first publications regarding protein microarrays in 1989, the application in allergy diagnosis was proposed.²¹ Surprisingly, the first experimental microarray system for allergy diagnosis²² was not reported until the year 2000. In 2001, a microarray biosensor based on chemiluminescence was presented.²³ This system had been previously developed for the highly sensitive detection of pesticides and other environmental contaminants in drinking water,²⁴ and an improved system is now to be commercialised by ATTO-TEC (Siegen, Germany). Recently, an allergen microarray based on fluorescence detection was published.²⁵ The presented study is an application of the parallel affinity sensor array (PASA) technology to automatically performed allergy diagnosis.²³ Purified recombinant and natural

allergens as well as allergen extracts were immobilized on glass slides for the detection of allergen-specific IgE. The label peroxidase was detected by enhanced luminol chemiluminescence.

EXPERIMENTAL SECTION

Reagents and Chemicals. The support material (microscope glass slides, Merck, Darmstadt, Germany) was cleaned using a 1:1 mixture of methanol and HCl (36%) for 30 min at room temperature, washed with water (HPLC grade, Millipore, Milli-Q Plus 185), and immersed in H₂SO₄ (98%) for 30 min. The glass slides were rinsed with water and dry methanol and dried under a nitrogen stream. Activation of the glass slides was achieved with a solution of 1% (3-glycidyloxypropyl)trimethoxysilane (GOPS; Sigma-Aldrich, Taufkirchen, Germany) in dry toluene (Sigma-Aldrich) for 18 h at room temperature. After washing steps with toluene and methanol, the glass slides were dried under nitrogen and stored until use in a desiccator. Other silanes were obtained from ABCR (Karlsruhe, Germany). The raw pollen material was purchased from Allergon (Ängelholm, Sweden); bee venom, phospholipase A₂ (PLA₂), casein, gliadin, streptavidin–HRP, and BSA were from Sigma-Aldrich. Apple, celery, cat epithelia, and mold allergen (*Aspergillus fumigatus*, *Penicillium notatum*) extract solutions for prick tests from Allergopharma (Reinbek, Germany) were used. Unroasted peanuts were obtained from a local Chinese food store. The recombinant allergens rCor a1, rAln g1, rPhl p1, rApi g1, rMal d1, rBet v2, and rAlt a1 were obtained from Biomay (Linz, Austria); rFel d1, affinity purified Der p1, and Der f1 were from Indoor Biotechnologies (Cardiff, U.K.). Myeloma IgE and purified IgG were obtained from Calbiochem-Novabiochem (Bad Soden, Germany), monoclonal rat anti-human IgE antibody (LO-HE-10) was from Technopharm (Paris, France), and the polyclonal affinity-purified horseradish peroxidase-labeled goat anti-human IgE antibody was from Dunn (KPL, Reinbek, Germany). The protein tests were performed with the BCA microassay from Pierce (Rockford, IL). Microdon MTPs were from Greiner (Nürtingen, Germany), and NHS-activated amine-binding plates (NOS) were from Corning (Cardiff, U.K.). The reference measurements were performed with the Pharmacia-CAP System (CAP specific IgE-FEIA, Pharmacia).

General Setup. The pipetting system consisted of a three-dimensional stage, a controller (multi-dos), a personal computer, piezoelectric nanoliter pumps (standard micropipet manufactured by silicon etching), and a liquid handling system (syringe pumps) for priming and washing of the silicon pumps (GeSiM, Grosserkmannsdorf, Germany). A standard 96-well MTP was used as reagent reservoir. The evaporation of the small drops was delayed by the use of 0.5% trehalose or 1% glycerol. The chosen conditions were five droplets, spot diameter $\sim$ 200 μ m, $U = 60$ V, and $f = 300$ Hz. The coating was performed for 18 h at 4 °C. After the blocking step, the slides were washed with WB (60 mM KH₂PO₄, 420 mM K₂HPO₄, 870 mM NaCl, 0.3% Tween-20), and HPLC-water (0.5% trehalose) and dried under a nitrogen stream.

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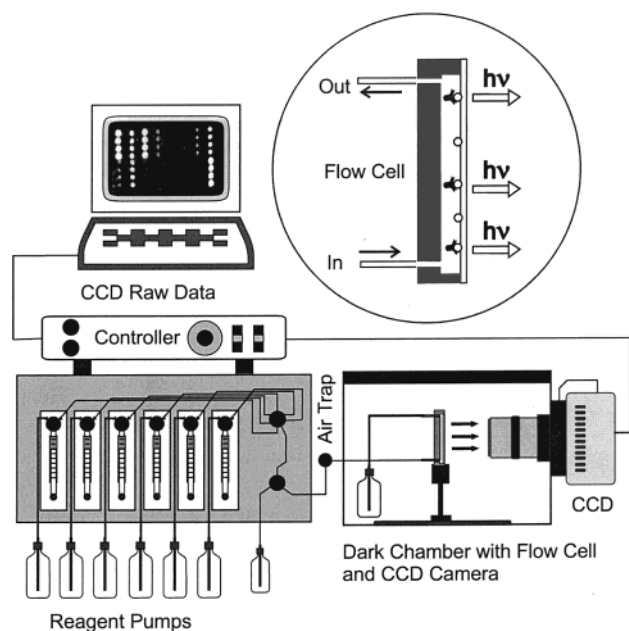


Figure 1. General setup of the parallel affinity sensor array (PASA) system for chemiluminescence detection and a closeup of the flow cell.

Incubation with serum samples was performed with chamber slides (200 μ L, Sigma-Aldrich). The reaction with the HRP-labeled antibody was done using a cover slip (Hybri-slip, 22 $\times$ 40 mm, Sigma-Aldrich). After a washing step, the slide was placed in the flow cell (approximately cell volume 150 μ L) of the PASA system (Figure 1) and 500 μ L of CL substrate (SuperSignal ELISA Femto or West Pico, Pierce) was pumped into the flow cell. Light emission was accumulated for a specified time using a 2 $\times$ 2 binning. Data evaluation was performed with the program Image-tool (Version 2.0, Wilcox, Dave Mc David, UTHSCA, University of Texas Health Science Center, San Antonio, TX). A trimmed mean (25%) in combination with a Winsorized standard deviation was used to calculate robust statistical parameters.²⁶

Extraction of Allergens. For preparation of allergen extracts, 10% raw pollen material (w/v) was extracted with 0.125 M ammonium hydrogencarbonate (0.015 M sodium azide, 20 mM EDTA, 5 mM EACA, pH 7.5). After the material was suspended for 1 h in this solution, the medium was centrifuged (14,000g, 10 min, 4 $^{\circ}$ C). The supernatant was collected, and the insoluble residue was extracted again for 1 h. After a filtration step (cellulose acetate, 0.45 μ m, Sartorius, Göttingen, Germany), the supernatant was dialyzed (Slide-A-Lyzer dialysis cassettes, cutoff 10 kDa, Pierce) against water/PBS (10:1) for 24 h and then lyophilized. Prick test solutions were also dialyzed and lyophilized. A 10-mg sample of dry substance was subsequently diluted with 1 mL of water/PBS buffer. To characterize the obtained allergen extracts, the protein content was determined using the BCA microassay. For this purpose, the allergen extracts were diluted 1:100–1:1000 with PBS. A 150- μ L aliquot of this solution was added to a MTP. A 150- μ L aliquot of a BSA solution (0.125–200 mg/L) was used for calibration. By addition of 150 μ L of the BCA microassay solution, a color change to green-purple occurs. After a 30-min incubation at 36 $^{\circ}$ C, the purple color was measured at 550 nm.

Immobilization of the Reagents on Glass Slides. Crude allergen extracts (total protein content 5–10 g/L) and recombinant allergens (protein concentration 1–2 g/L) were diluted (1:40 or 1:100, respectively) with CB 1 (300 mM Na₂HPO₄, pH 8.5, 0.5% trehalose, 1 mM EDTA). Myeloma IgE (1 g/L, 1:100) and human IgG (10 g/L, 1:1000) were diluted with CB 2 (15 mM Na₂CO₃, 25 mM NaHCO₃, pH 9.8, 1% glycerol). The monoclonal anti-human-IgE–Ab LO-HE-10 (1 g/L) was diluted 1:100 with CB 2 containing 1% glycerol and 0.01% Tween-20 (protein grade).

Determination of Total and Allergen-Specific IgE on GOPS Slides. Unreacted surface groups were blocked with BB (1% BSA, 0.5% glycine in Tris-HCl, pH 8.3) for 2 h at room temperature. Serum samples were diluted 1:8 with IB (PBS/0.1% BSA/0.01% Tween, 200 μ L, 30 min, 36 $^{\circ}$ C). The anti-human-IgE Ab was diluted 1:500 with IB and incubated for further 30 min at room temperature. After a washing step, the slide was placed in the flow cell of the PASA system and Super Femto CL substrate was pumped into the flow cell. Light emission was accumulated for 60 s.

Determination of Allergen-Specific IgE on Microtiter Plates. Recombinant allergens were diluted (1:400 with PBS, pH 7.8 for Microton MTPs and 1:400 with Na₂HPO₄, pH 8.5 for NHS MTPs) and 100 μ L of each allergen solution was filled into the wells of the corresponding MTP. Coating was performed for 18 h at room temperature. After a washing step with WB, the blocking of unreacted surface places was done with 1% BSA in PBS (Microton) or 1% BSA/0.5% glycine in tris-HCl (NHS) for 2 h at room temperature. Incubation with human serum samples was performed with 50 μ L of the diluted serum samples (1:8 in IB) for 1 h at room temperature. After a washing step, 100 μ L of 1:5000 diluted HRP-labeled anti-human-IgE Ab was filled in the wells for 45 min at room temperature. The color development (25 mL of citrate buffer, 300 μ L of 1% TMB solution in methanol, and 100 μ L of 1% H₂O₂ solution) was stopped after 10 min with 5% H₂SO₄ solution. The absorbance was read at 450 nm with an eight-channel photometer (TR 340, SLT, Overath, Germany).

Estimation of Detection Limits. For the estimation of the detection limits for allergen-specific IgE, the corresponding proteins (e.g., rBet v1, PLA₂, Der p1, Der f1, and LO-HE-10) were immobilized on different GOPS glass slides. A serum sample with known specific IgE class against the allergen extract, e.g., a birch pollen-positive serum sample in case of rBet v1, was diluted 1:1 with a heat-denatured (2 h, 56 $^{\circ}$ C) blank serum sample. The obtained serum samples of different RAST classes were diluted 1:4 with IB. Incubation with serum sample and HRP-labeled anti-human-IgE Ab was performed as already described. The West Pico CL substrate was pumped into the flow cell, and light emission was accumulated for 90 s.

Patients and Sera. According to the test results obtained by the reference measurements, patients were selected as listed in Table 2. Samples with and without elevated levels of specific IgE have been used for determination.

RESULTS AND DISCUSSION

Test Performance. For the evaluation of a suitable surface chemistry, different surface modifications of microscope glass slides were tested, such as trimethylchlorosilane, phenyltriethoxysilane, (3-aminopropyl)trimethoxysilane, (4-aminobutyl)-triethoxysilane, *N*-(2-aminoethyl)-3-aminopropyl-trimethoxysilane, (3-glycidyloxypropyl)trimethoxysilane, 2-(3,4-epoxycyclohexyl)ethyl-

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Table 2. Summary and Description of Used Serum Samples^a

serum no.	symptoms	specific IgE	microarray reactivities
3	ae	bi, al, ti, ry	pc, ry, wh, ti, rPhl p1
4	ae, a	dpt, ce, ti	pc, Der p1, Der f1
6	i	bv, wv	pc, PLA ₂ , bv, ti, ry, lys
16	ra	bi, al, ti, ry, wh, ao (f, a)	pc, bi, rBet v1, ti, rPhl p1, al, rAln g1, ry, wh
24	ae	negative	pc
26	pso	negative	pc
32	cu	negative	pc
59	ae	bi, al, ti, ry, wh, ao (f, ce)	pc, bi, rBet v1, ti, al, rAln g1, ry, wh
74	i	negative	pc
82	i	bv	pc, PLA ₂ , bv, Der p1, Der f1, al, ti
88	ae	ti, bi, ry, ce, dpt, cel, ao (f)	pc, rFel d1, rBet v2, rAln g1, ha, ry, wh, ti, ce, cel
89	ra	ti, ry, wh	pc, rBet v1, rBet v2, ti, rPhl p1, al, rAln g1, ry, wh
94	i	bv	pc, rBet v1
96	ra, ae	bi, al, ha, ti, ry, wh, ao (g, t)	pc, rBet v1, ti, rPhl p1, ry, wh
109	ra	bi, al, ha, ti, ry, wh, ao (f)	pc, rBet v1, bi, ti, rPhl p1, al, rAln g1, ry, wh
123	ra	ti, dpt, df, ao (mo)	pc, Der f1, ry
129	ae	negative	pc
131	ae	bi, al, ha, ti, ry, wh, ao (f)	pc, rBet v1, ti, rPhl p1, al, rAln g1, ry, wh
136	pso	negative	pc
145	ra, ae	bi, al, ti, ao (f)	pc, rBet v1, rAln g1, rCor a1
146	ra	ti, wh, ry, bi, ha, ao (f, mi)	pc, rBet v1, bi, rPhl p1, al, rAln g1
147	ra	bi, al, ha, ry, wh, ao (mo)	pc, rBet v1, rBet v2, ti, rPhl p1, al, rAln g1, ry, wh
163	ae	bi, al, ti, dpt, df	pc, rBet v1
165	ra	bi, al, ha, cel, ao (f, a, mi)	pc, bi, rBet v1, al, rAln g1, cel, rApi g1, rCor a1
170	ra	bi, ti, ry, wh, ao (f, g, t)	pc, ti, rPhl p1, ry, wh
186	ra	bi, al, ti, ry, wh, ce, ao (a, f)	pc, rFel d1, rBet v1
187	ra	ti, ry, wh	pc, rAlt a1
188	ra, a	bi, al, ha, ti, ry, wh, ce, ao (f, g, t)	pc, rBet v1, rFel d1
399	ae	ap, ao	pc, rAlt a1
1759	ae, a	alt, ao	pc, rBet v1, rAln g1, rPhl p1, rCor a1
2501	ae, ra	alt, ao	pc, rAlt a1, Der p1, Der f1, PLA ₂ , bi, rBet v1,
2537	ae, ra	ap, ao	pc, rBet v1, rCor a1, Der p1, Der f1

^a ae, atopic eczema; a, asthma; pso, psoriasis; cu, chronic urticaria; i, insect venom allergy; ra, allergic rhinitis; dpt, extract of mite (*Dermatophagoides pteronyssinus*); df, extract of mite (*Dermatophagoides farinae*); ao, and others (allergens were not tested with our system); a, animals; f, food; g, grass; mi, mites; mo, moulds; pc, positive controls (anti-human IgE, myeloma IgE; streptavidin-HRP); bi, birch; al, alder; cel, cellery; ce, cat epithelia; ha, hazelnut; ti, timothy; ry, rye; wh, wheat; lys, lysozyme; bv, bee venom; wv, wasp venom; PLA₂, phospholipase A2; alt, *Alternaria alternata*; ap, apple.

trimethoxysilane, 3-(triethoxysilyl)propylsuccinic anhydride, and others (data not shown). For the enhancement of hydrophilicity, mixtures with *N*-(3-triethoxysilylpropyl)gluconamide and ureido-propyltrimethoxysilane were used. However, none of them were fully satisfactory as a general immobilization surface. Nevertheless, the best results could be obtained with (3-glycidyloxypropyl)-trimethoxysilane-modified glass surfaces. The mixed binding mode of GOPS slides seems to be a good compromise to achieve sufficient binding of most allergens with very low background signals. A higher spot volume (five droplets on the same point, ~10 nL) was used to improve reproducibility. Comparing data from microtitration plates (MTP) and microarrays, it should be considered that MTP surfaces are less likely to dry up, which might lead to a better preservation of the activity of sensitive proteins. The small reagent drops of microarrays dry very fast without particular countermeasures (during some seconds). Although the addition of stabilizers (e.g., glycerol, poly(ethylene glycol), trehalose, and saccharose) suppresses the denaturation of proteins during the spotting procedure, the high viscosity slows down the coating process considerably. In our case, the best results were obtained with 0.5% trehalose and 1 mM EDTA for most of the proteins. Nevertheless, high humidity during the microarray production seems to be preferable to prevent denaturation and to achieve high coating efficiencies. After the immobilization of the allergenic proteins, the surface was blocked

to reduce nonspecific binding. It has to be considered that the patient could also be allergic to the blocking reagent (e.g., milk casein). We used a solution of BSA/glycine in Tris-HCl as a blocking reagent. The flow chamber of the PASA system consists only of one reaction chamber. To allow the measurement of several allergen chips in parallel, the mentioned chamber slides in combination with cover slips for incubation were used. In the case of allergen extracts, immobilization of the main allergenic proteins was sometimes difficult because of an unfavorable ratio of allergenic to nonallergenic proteins. Therefore, we also used recombinant and purified proteins. Before the screening tests on the chip format were started, positive serum samples for the individual recombinant/purified allergens were identified with MTP assays (results not shown).

Comparison between MTP and GOPS Chip Surface. It can be seen in Figure 2 that in most cases a correlation of the recombinant/purified allergen-specific IgE signals between MTPs and GOPS-modified glass slides was observed. The test results of GOPS slides seemed to be closer to test results obtained with the hydrophobic Microlon MTP than to the hydrophilic NHS-activated MTP, indicating that an adsorptive binding mechanism on the hydrophobic GOPS slide is predominant in relation to the covalent attachment of the protein's amine groups to the epoxy groups of the surface. The recombinant protein rMal d1 (17 kDa, Bet v1 homologue) was not recognized either on MTPs or on

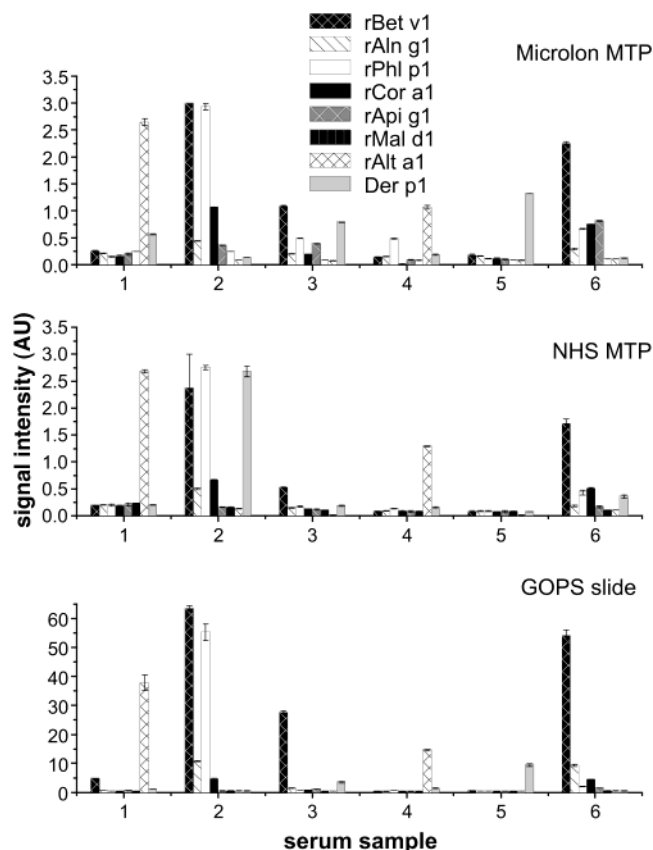


Figure 2. Comparison between test results of allergen-specific IgE obtained with microtiter plates (hydrophobic Microton and hydrophilic NHS) and GOPS-activated glass slides: 1, serum 399; 2, serum 1759; 3, serum 146; 4, serum 187; 5, serum 4; 6, serum 145.

GOPS-modified slides. Commercially available rMal d1 seems to be less reactive than its natural counterpart.²⁷

Screening of Serum Samples with Recombinant/Purified Allergens and Allergen Extracts. Sixteen different substances (four purified and nine recombinant allergens) including two positive controls (monoclonal anti-human-IgE–Ab, streptavidin–HRP) and one negative control (purified human IgG) were immobilized on different GOPS slides. As serum samples, 5 negative controls (patients without elevated levels of specific IgE) and 12 positive serum samples with a different pattern of specific IgE—see Table 2—were used. The positive controls were recognized on every slide (Figure 3), indicating that the spotting procedure, the surface chemistry, and the detection antibody seem to work properly. The standard error of the mean for the streptavidin–HRP signals on a single chip surface was between 2.6 and 7.6%. Between 17 GOPS slides, the standard error was 19.7 (mean) or 7.9% (25% trimmed mean, Winsorized sample mean). Cross-reactivity of 4% of the used anti-human-IgE–Ab with IgG could be determined with the immobilized human IgG. Regarding the negative controls, no false positive signal for a single allergen has been obtained. Therefore, it was possible to distinguish between “negative” and “positive” patients using the microarray system. As can be seen in Figure 3, every serum sample has its own signal pattern. In the case of rApi g1 and rCor

a1, only weak signals were obtained. Working with recombinant proteins, a lacking spot on a multiallergen chip in the test could have different reasons. An insufficient immobilization of the corresponding protein on the surface, a partial or complete denaturation of the immobilized protein, or the lack of allergen-specific IgE in the patient's serum sample could be reasons for missing signals. The fact that we have obtained positive signals with serum samples 188, 145, and 146 in MTPs (results not shown) indicates that the immobilization procedure may not be suitable for rApi g1 and rCor a1. Api g1 and Cor a1 belong to the family of food allergens. Direct ingestion of food allergens also stimulates the production of allergen-specific IgG antibodies. The concentration of allergen-specific IgG can be 10 000 times higher than allergen-specific IgE. Therefore, immobilized allergens may also be blocked by specific IgG,²⁸ leading to an underestimation of IgE. Positive signals could be obtained for rAlt a1, rBet v1, rBet v2, rPhl p1, rAln g1, Der p1, Der f1, and PLA₂. In addition, the measurements showed a sufficient reproducibility for the repeated measurements of serum samples (Figure 3). A combination of allergen extracts (birch, alder, timothy, rye, wheat) and recombinant allergens (rBet v1, rBet v2, rPhl p1, rAln g1) demonstrated (Table 2) that patients with specific IgE against birch pollen extracts showed either rBet v1 (serum samples 59, 89, and 145) or rBet v2 (serum samples 88 and 89) signals. Serum sample 158 was tested negative against birch pollen and alder allergens with the reference measurements and also with the microarray-based system. False negative signals in comparison with the reference measurements were obtained for both tree pollen allergens in the case of serum sample 3. Positive signals for the alder extract correlated in most cases with a positive signal for the recombinant allergen rAln g1. Serum sample 88 showed only signals for the alder extract. Besides one exception (serum sample 59), a positive signal for the timothy extract correlated also with a positive signal for the recombinant protein rPhl p1. Allergen extracts are mixtures of different allergic and nonallergic proteins. At least seven different allergen-relevant proteins (rPhl p1, rPhl p2, rPhl p4, rPhl p5, rPhl p6, rPhl p12, rPhl p13) of timothy extracts are known.²⁹ Therefore, it is possible that the patient of serum 59 is allergic to another allergen of the timothy group, similar to serum 88 in the case of birch pollen allergy. In the next step, the array for the screening test was extended to 24 allergenic substances. No false positive signals for the negative control (serum sample 32) were detected (Figure 4). The positive control (myeloma IgE) could be observed on every slide, but there was a variation in signal intensity for the different chips. Signals for the total IgE levels were lower in the case of patients without elevated specific IgE and serum sample 123 in comparison to serum sample 88 and 82. Therefore, the signal for the total IgE level could also be used as an internal quality control for interpretation of the test results. Serum sample 82 was taken from a patient allergic to bee and wasp venom. Positive signals for Der p1, Der f1, PLA₂, bee venom, alder, and timothy were obtained. Signal intensities for bee-related allergens were much higher than the intensities for the other allergens. Positive signals for rBet v2, rye, wheat,

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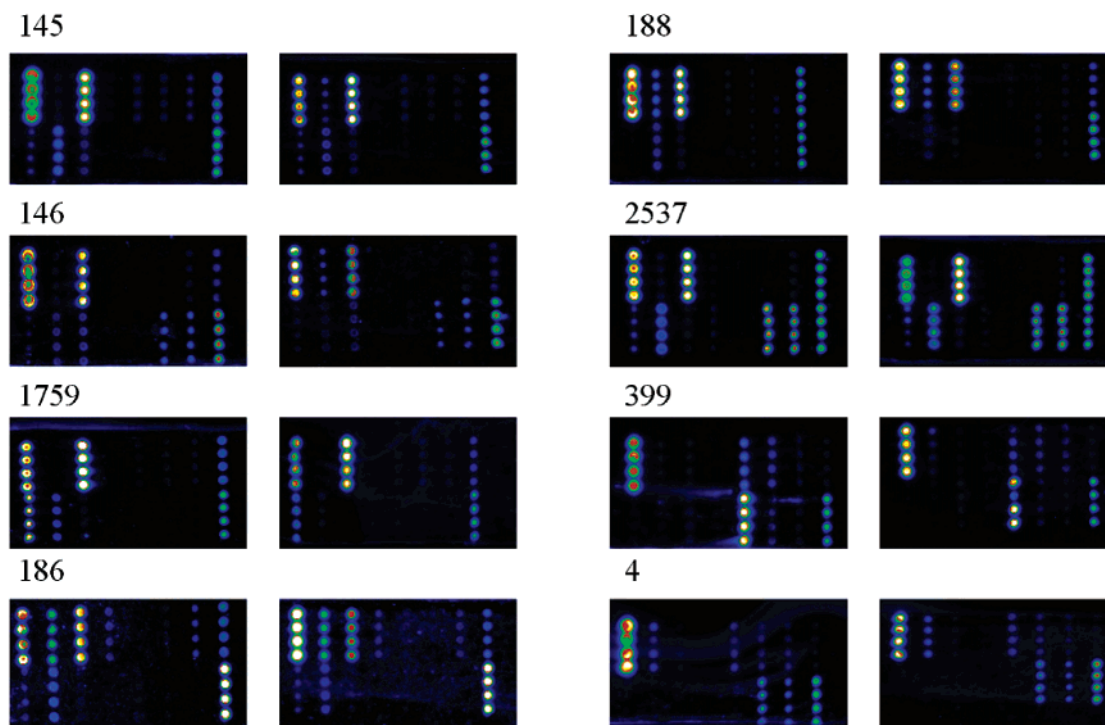


Figure 3. Reproducibility (duplicates) of the screening of allergen-specific IgE against recombinant and purified allergens immobilized on GOPS-modified glass slides represented by CCD raw data; vertical spot-to-spot distance, 750 μm (center): 1, anti-human-IgE LO-HE-10; 2, rFel d1; 3, rBet v1; 4, rBet v2; 5, PLA₂; 6, bee venom; 7, IgG; 8, rAln g1; 9, rPhl p1; 10, rCor a1; 11, rApi g1; 12, rMal d1; 13, rAlt a1; 14, Der p1; 15, Der f1; 16, streptavidin-HRP.

Table 3. Estimation of Detection Limits^a for Specific IgE on GOPS-Modified Glass Slides

allergen	serum sample	IgE reference values ^b ($\mu\text{g/L}$) (extract)	LOD ($\mu\text{g/L}$) (3s method)
r Bet v1	96	38.0 (birch pollen)	0.35
PLA ₂	82	54.6 (bee venom)	0.16
bee venom	82	54.6 (bee venom)	1.03
mellitin	82	54.6 (bee venom)	4.80
Der p1	4	240 (<i>D. pteronyssinus</i>)	1.85
Der f1	4	240 (<i>D. pteronyssinus</i>)	4.62

^a The detection limit was determined by dilution of serum samples of known specific IgE class (1 IU = 2.4 ng IgE) against the specified allergen extract (e.g., a birch pollen positive serum sample in case of rBet v1) with an IgE heat-denatured (2h, 56 °C) serum sample of a patient without elevated specific IgE. ^b The IgE levels [$\mu\text{g/L}$] have been determined against the corresponding allergen extract solution.

timothy, rPhl p1, cat, and rFel d1 could be obtained for serum sample 88. Only weak signals could be obtained for hazel, ragweed, and celery.

Estimation of Detection Limits. The detection limits of IgE against rBet v1, Der p1, Der f1, and PLA₂ have been estimated. One has to consider that the “reference” values have been determined against the corresponding allergen extract solution and not against the pure allergen. Therefore, the mentioned IgE value of our reference measurement represents a sum parameter of all birch pollen allergen-related IgE. Positive signals in the case of allergen extracts could be caused either by specific reaction between allergen and allergen-specific IgE or by a “cross-reaction” of human IgE with other components (e.g., lectins)^{30,31} of the allergen extracts. Hence, the determined detection limits are

worst-case estimations. Table 3 shows that it is possible to distinguish between specific IgE class 0 (0.35 $\mu\text{g/L}$) and specific IgE class 1 (0.69 $\mu\text{g/L}$) in the case of rBet v1, PLA₂, and Der p1.^{9,15} With the detection limit of Der f1-specific IgE, classes 1 and 2 could be discriminated. The higher detection limit for Der f1 may also be an effect of the use of purified allergens instead of allergen extracts. The patient of this serum sample seems to have more allergen-specific IgE against Der p1 than against Der f1. Inhibition experiments (results not shown) demonstrated that the positive signal for mellitin was caused by a reaction of PLA₂-specific IgE. The higher detection limit of immobilized bee venom instead of PLA₂ could be explained by the fact that bee venom represents a mixture of at least three different proteins (PLA₂, hyaluronidase, mellitin). Therefore, a competition for binding places took place leading to a lower amount of PLA₂ immobilized on the surface, resulting in an apparent elevation of the detection limit. Serum samples with higher IgE concentrations (greater than specific IgE class 3, results not shown) could not be classified directly, because the microarray surface would be saturated. Appropriate dilution of serum samples solved this problem.

CONCLUSIONS

We have demonstrated that a series of allergens both recombinant and purified (rBet v1, rBet v2, PLA₂, Der p1, Der f1, rPhl p1, rAln g1, rFel d1, rAlt a1) could be successfully immobilized from small droplets on GOPS-modified glass slides. Some difficulties occurred with the recombinant food proteins rMal d1, rApi

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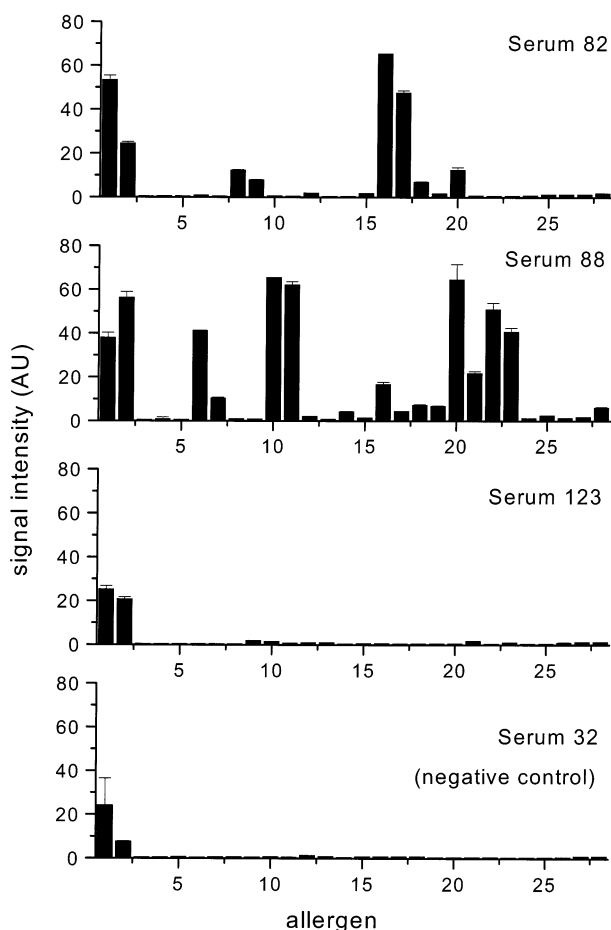


Figure 4. Screening of four serum samples with 24 individual allergenic proteins (allergen extracts and recombinant allergens) Immobilized recombinant/purified allergens and allergen extracts: 1, myeloma IgE; 2, anti-human IgE LO-HE-10; 3, BSA; 4, birch; 5, rBet v1; 6, rBet v2; 7, hazel; 8, Der p1; 9, Der f1; 10, rye; 11, wheat; 12, casein; 13, gliadin; 14, ragweed; 15, IgG; 16, PLA₂; 17, bee venom; 18, alder; 19, rAln g1; 20, timothy; 21, rPhl p1; 22, cat epithelia; 23, rFel d1; 24, apple; 25, peanut; 26, mold (*A. fumigatus*); 27, mold (*C. herbarum*); 28, celery.

g1, and rCor a1. Misfolding, surface denaturation, lack of suitable sera, or a competitive effect with allergen-specific IgG could be the causes for this problem. Using recombinant allergens as well as allergen extracts, it was possible to distinguish between patients with and without elevated levels of specific IgE. The use of both recombinant allergens and allergen extracts has its merits. Using recombinant allergens, the interpretation is greatly facilitated, since only one reagent species per spot has to be considered. On the other hand, allergen extracts are composed of many different

allergenic compounds, which might be unknown in detail. Therefore, the use of allergen extracts makes false negatives less likely. The combination of both reagent types on a microarray synergistically combines their strengths. Repeated measurements of some serum samples demonstrated a sufficient reproducibility. Detection limits of IgE against rBet v1 (0.35 $\mu\text{g/L}$) and PLA₂ (0.16 $\mu\text{g/L}$) show that it is possible to distinguish between specific IgE classes 0 and 1. Furthermore, only 25 μL of serum was necessary for the screening against 24 allergenic proteins and the test was performed in less than 1 h. In comparison to MTPs, the capacity of such a microarray surface is much higher considering the number of possible immobilized allergens. Now even the differentiation of IgE against allergen isoforms and the inclusion of carbohydrate microarrays ("glycoarrays")³² comes into reach. This seems to be impossible by any other conventional method for the analysis of specific IgE in human serum. Microarray-based technology can be considered to be a successful strategy to enable a highly multidimensional allergy diagnosis using all available relevant allergen compounds in combination with allergen extracts. Finally, a preselection of suspicious allergens for in vitro diagnosis would not be necessary anymore, since an allergen microarray including all known allergens and allergen extracts would not cost much more than a single specific IgE determination today and avoids the risk of anaphylactic shock caused by the use of in vivo tests.

Abbreviations: Ab, antibody; BB, blocking buffer; BCA, bicinchoninic acid; BSA, bovine serum albumin; CB, coupling buffer; CL, chemiluminescence; EACA, ϵ -aminocaproic acid; EDTA, ethylenediaminetetraacetate; GOPS, (3-glycidyloxypropyl)trimethoxysilane; HRP, horseradish peroxidase; IB, incubation buffer; MTP, microtiter plate; NHS, *N*-hydroxysuccinimide; PASA, parallel affinity sensor array; PBS, phosphate-buffered saline; PLA₂, phospholipase A₂; TMB, 3,3',5,5'-tetramethylbenzidine; Tris, tris-(4-hydroxymethyl)methylamine; WB, washing buffer.

ACKNOWLEDGMENT

The authors thank ALK-Abelló, Hørsholm, Denmark, for the supply of rBet v1 and the polyclonal and monoclonal antibodies against rBet v1, r-biopharm, Darmstadt, Germany, for financial support and the supply of allergen extracts, and the Technische Universität München, Germany, for financial support in the program "Medizintechnik".

Received for review August 1, 2002. Accepted November 12, 2002.

AC026016K

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