



Research paper

Development of hydrogel biochip for in vitro allergy diagnostics



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ARTICLE INFO

Article history:

Received 15 October 2013

Received in revised form 27 February 2014

Accepted 4 March 2014

Available online 12 March 2014

Keywords:

Protein microarray

Biochip

IgE in vitro test

Allergy diagnostics

ABSTRACT

A hydrogel biochip was developed for the simultaneous quantitative determination of sIgE for 21 allergens and total IgE in human serum. The biochips are manufactured by photoinduced copolymerization of different molecules (allergens and antibodies) with gel-forming monomers resulting in the formation of three-dimensional hydrogel elements (1 nl gel drops). After incubation of the biochip with the serum, the results are visualized using fluorescently labeled anti-IgE antibodies. Using biochips, serum samples from allergic patients and healthy donors were analyzed and good correlation with the results obtained using commercial EIA test systems of generally recognized quality (Dr. Fooke Laboratorien GmbH, Germany) was observed.

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1. Introduction

Classical diagnostics of type I allergies involve skin-prick test (SPT). However, this in vivo approach may cause complications and suffers a number of limitations: patient's age, possibility of disease aggravation, dermatographism, etc. (Bernstein et al., 2004; dos Santos et al., 2009). Therefore, for a screening, in vitro methods are preferable.

Historically, the first in vitro laboratory method for sIgE assays was the Radio-Allergo-Sorbent Test (RAST) (Ceska and Lundkvist, 1972), which was later modified by using a chromogenic or fluorescence system of signal detection. Currently, the enzyme immunoassay in various versions together with chemiluminescence or fluorescence is the method of choice for most clinical laboratories (Hamilton and Adkinson, 2004). Despite its advantages such as equipment versatility and good analytical characteristics, one EIA analysis of IgE specific for one

allergen requires about 50 µl of blood serum, which makes it difficult to perform screening for a wide panel of allergens due to the necessity of using large amounts of the sample.

Biological microarrays or biological microchips (biochips) provide an alternative to EIA. Biochips are arrays of elements, each of which contains an individual molecular probe. Protein microchips, a logical extension of DNA microchips allow simultaneous multiplex assays to be carried out using a minimal amount of sample (Sutandy et al., 2013; Hall et al., 2007).

Analysis of allergen-specific IgE on microchips is performed using a multiplex solid-phase immunoassay, where the allergens are immobilized on a support. Specific IgE from a patient's serum are trapped by immobilized allergens and then developed with labeled isotype-specific antibodies (Harwanegg and Hiller, 2005; Kim et al., 2002).

Several types of microarrays for sIgE assays are described in the literature (Bacarese-Hamilton et al., 2002; Lebrun et al., 2005; Cretich et al., 2010). The main challenge for protein immobilization on biochips is the retention of protein stability and function after immobilization. The majority of described

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biochips are planar with allergens immobilized directly onto the support surface (Hartmann et al., 2009). The drawbacks of planar biochips are uncontrolled immobilization efficiency and denaturation of proteins on the carrier surface resulting in a loss of protein functional activity and insufficient concentration of active proteins in biochip elements resulting in low analytic sensitivity.

Currently only one test system in microchip format is commercially available, namely, ImmunoCAP ISAC® (Thermo Fischer Scientific Inc., Sweden) (Gadisseur et al., 2011). This microchip contains recombinant and highly purified allergen components immobilized as spots on a support (two-dimensional microchips). The test-system allows for semi-quantitative determination of sIgE and interpretation of the sIgE levels according to the four classes of allergic reactions: negative, low, medium, and high.

The aim of this work was to develop a biochip for the quantitative assay of allergen-specific and total IgE using the technology of three-dimensional gel-based microchips developed by EIMB RAS, Moscow. In this work we have used allergens mostly as protein extracts from natural sources. The results obtained from the assay of sera from different patients using biochips were compared to the commercial EIA test systems of generally recognized quality (Specific IgE REAST, Total IgE-HRP EIA; Dr. Fooke Laboratorien GmbH, Germany). We chose these assay systems as a reference because they allow using allergen extracts, highly purified allergens and recombinant allergens for the detection and quantification of sIgE whereas the ImmunoCAP ISAC system does not support using allergen extracts and, hence, cannot provide complete reference data for the new hydrogel biochip. In addition, we have used the same allergen preparations in both the hydrogel biochips and the REAST system.

Microchips are manufactured by the photo-induced copolymerization technology (Rubina et al., 2003; WO, 2003/033539) where molecular probes are immobilized in situ in the bulk of hemispherical gel elements. This immobilization method is preferable if molecular probes of protein nature are used, since this prevents the denaturation of protein molecules and provides a native hydrophilic environment. Owing to the high immobilization efficiency, the hydrogel biochip technology allows one to work not only with individual components but also with heterogeneous samples, e.g., full-protein allergen extracts from natural sources. Several test-systems based on hydrogel protein biochips have been successfully applied for laboratory diagnostics, e.g., biochips with immobilized antibodies for the quantitative assay of tumor markers (Rubina et al., 2008; Gryadunov et al., 2011; Zubitsova et al., 2013).

The biochips for allergy diagnostics described in this paper contained allergens which are the most frequent triggers of allergic reactions in Europe and near-Moscow region in Russia; several recombinant allergens were also included (total—21 allergens).

2. Materials and methods

The following reagents were used in this study: polyvinyl alcohol 50,000 Da, polyvinylpyrrolidone 360,000 Da, Tween-20, Sephadex G-25 coarse (Sigma, USA), Bind Silane (Amersham Pharmacia Biosciences, USA), and Amersham Cy5 Mono NHS Ester (GE Healthcare, UK). Antibody conjugates were purified on

Micro Bio-Spin Chromatographic Columns (Bio-Rad, USA). Allergen extracts, recombinant allergens, IgE and anti-IgE were provided by Dr. Fooke Laboratorien GmbH, Germany. Chemical reagents were obtained from commercial sources and used without further purification. For the biochip immobilization, the allergen extracts and recombinant allergens were adjusted in protein concentration to achieve maximum sensitivity and specificity of the total and sIgE assay. Glass slides for biochip manufacturing were from Corning, USA (Corning 2947 microscope slides).

Serum samples were collected from the Federal State Budgetary Institution “Polyclinic No. 1” of the Business Administration for the President of the Russian Federation and from the serum bank at Dr. Fooke Laboratorien, Germany. As the aim of our study was the technical evaluation of the hydrogel biochip in comparison to the established reference methods, it was not required to include the clinical diagnostic information. The clinical evaluation of the biochip is planned for future studies.

The kits for sIgE determination “Specific IgE REAST” (ALLERG-O-LIQ) and total IgE determination “Total IgE HRP EIA” were provided by Dr. Fooke Laboratorien GmbH, Germany.

The following buffer solutions were used: PBS (0.01 M phosphate buffer, pH 7.2, 0.15 M NaCl), PBST (PBS, 0.1% Tween-20). All solutions were prepared using Milli-Q deionized water.

2.1. Biochip manufacturing

Biochips were manufactured by the technique developed at EIMB RAS as reported previously (Rubina et al., 2003). Polymerization mixtures containing gel-forming monomers together with the proteins to be immobilized were spotted as 1 nl drops onto the activated surface of glass slides using a QArray pin robot (Genetix, UK). The protein concentration of allergens in polymerization mixtures was between 1.0 and 6.0 mg/ml. Polymerization of gel elements was carried out under UV light with a wavelength of 350 nm (GTE Sylvania lamp F15T8/350BI, UK) at a distance of 8 cm from the lamp for 50 min at 20 °C in nitrogen flow. The 100 µm robot pin produced semispherical drops of 80 µm in diameter. After polymerization, the biochips were washed for 40 min in PBST and then rinsed with water. To suppress non-specific binding, the biochips were additionally treated with blocking solution: PBS buffer containing 1% polyvinyl alcohol.

2.2. Biochip structure and assay procedure

The biochip for the simultaneous quantitative assay of sIgE for 21 allergens and total IgE in serum samples (Fig. 1) contained gel elements with immobilized allergens (extracts, highly purified and recombinant allergens) belonging to different groups:

tree pollen allergens: t2 (alder), t3 (birch), Bet v1* (birch), Bet v2* (birch), t4 (hazelnut);
grasses and corns: g6 (timothy grass), g12 (cultivated rye);
animal allergens: e1 (cat, dander), Fel d1* (cat, dander), e5 (dog, dander);
house dust mites: d1 (*Dermatophagoides pteronyssinus*), d2 (*Dermatophagoides farinae*), Der p1** (*Dermatophagoides*

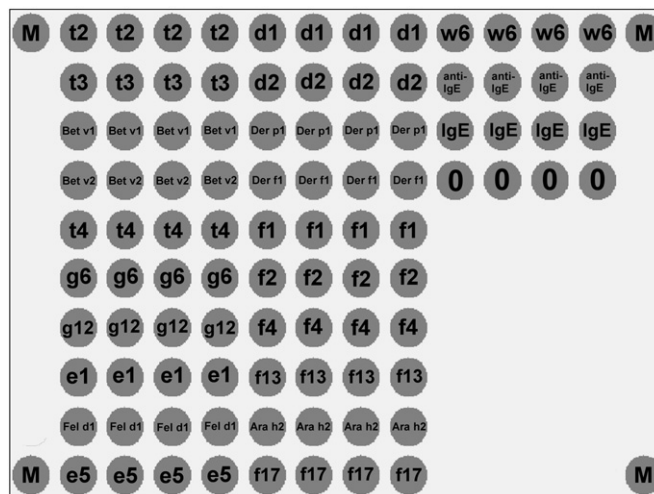


Fig. 1. Layout of the biochip for the assay of 21 allergen-specific and total IgE. Gel elements contain immobilized allergens t2, t3, Bet v1, Bet v2, t4, g6, g12, e1, Fel d1, e5, d1, d2, Der p1, Der f1, f1, f2, f4, f13, Ara h2, f17, w6 (designation of allergens is given in the text), anti-IgE antibodies, and IgE antibodies. 0—empty gel elements without immobilized substances, M—marker elements in the corners of biochip containing immobilized Cy5 fluorescent dye.

pteronysinus, highly purified), Der f1** (*Dermatophagoides farina*, highly purified);

food allergens: f1 (egg white), f2 (cow's milk), f4 (wheat flour), f13 (peanut), Ara h2* (peanut), f17 (hazelnut);

weeds: w6 (mugwort);

(*—recombinant allergens, **—highly purified native allergens).

In addition to full protein extracts, several highly purified and recombinant allergens were added into the microchip structure. Bet v1 is the major allergen in birch pollen. Bet v2—minor allergen in birch pollen—may serve as a marker of cross-reactivity indicating sensitization to other pollen or plant food. Fel d1 is the major allergen in cat, Der p1 and Der f1 are specific markers for sensitization to dust mites. Ara h2 is considered to be a marker for genuine sensitization to peanut (Sastre, 2010).

The biochip also contained gel elements with immobilized antibodies: anti-IgE, human IgE as a functional control, gel elements containing no immobilized proteins to control for nonspecific sorption, marker elements in the corners containing Cy5-dye for providing correct biochip orientation during the assay of fluorescence image using ImageAssay software. Each protein was immobilized in four similar gel elements to improve analysis reproducibility.

2.3. Preparation of fluorescently labeled developing antibodies

Monoclonal anti-IgE antibodies were labeled with Cy5 dye according to the manufacturer's instructions. Purification was performed on a Micro-Bio-Spin column with Sephadex G-25 medium equilibrated with PBS buffer. The number of dye molecules incorporated into protein molecule was determined spectrophotometrically by the absorbance at 647 nm for Cy5 dye and 280 nm for the protein. The conditions for labeling

were chosen in such a way that the bonding dye/protein molar ratio was 3: 1.

2.4. Carrying out analysis on biochips

Serum samples or calibrators (60 µl) were applied to biochips and the biochips were incubated for 20 h at 37 °C. The biochips were then washed with PBST for 20 min and a solution of Cy5-labeled anti-IgE antibodies (50 µl) was applied to each biochip. The biochips were incubated for 1 h at 37 °C. After the final washing (30 min, PBST), fluorescence signals were recorded.

2.5. Fluorescence measurements and processing of results

Fluorescence measurements were carried out on a portable fluorescence biochip analyzer (EIMB RAS, Moscow) with laser excitation using 650/670 nm filters (excitation/emission). Calculation of fluorescence signal intensity from each biochip element and determination of the concentrations of all sIgE were carried out using ImageAssay software developed in EIMB RAS. The intensity of fluorescence from gel elements with immobilized allergens and antibodies was calculated as the mean signal from four similar gel elements.

2.6. Statistics

ROC analysis and Pearson correlation were performed using MedCalc software (MedCalc Software, Belgium), version 12.

3. Results and discussion

The technology of three-dimensional hydrogel microchips created at EIMB RAS was used for the development of a test-system for the simultaneous quantitative assay of sIgE for 21 allergens and total IgE. The technology allows for

immobilizing both allergen extracts and recombinant or highly purified components. The three dimensional hydrogel provides preservation of the native conformation of immobilized proteins and full accessibility of immobilized proteins for binding to a specific antibody. The immobilized substances are uniformly distributed inside the hydrogel elements resulting in higher sensitivity and reproducibility of the assay when compared to planar (surface) microarrays (Rubina et al., 2008).

Incubation of a serum sample on a biochip results in the formation of specific complexes: immobilized allergen–sIgE and immobilized anti-IgE–IgE. After serum incubation, the biochip is developed using Cy5-labeled anti-IgE antibodies. The typical fluorescence images obtained after carrying out the assay are shown in Fig. 2. Fluorescence signals from biochip gel elements containing immobilized anti-IgE are proportional to the concentration of total IgE in the sample; signals from biochip gel elements with immobilized allergens are proportional to the concentration of corresponding sIgE.

3.1. Calibration curve

Our goal was to create a biochip for the simultaneous quantitative determination of total IgE and sIgE against 21 allergens. For this purpose, the same calibration curve has to be used for the assay of total IgE and all 21 sIgE. For the construction of the calibration curve we have used the biochip gel elements with immobilized anti-IgE. Fluorescence signals were determined after incubating the biochip with 0.35; 0.7; 3.5; 5; 17.5; 20; 50; 100; 200; 1000 IU/ml calibrators, calibrated to WHO 75/

502 reference standard. The concentrations of the calibrators correspond to the internationally accepted calibration system. The correlation of the fluorescence units with IU/ml values is shown in Fig. 3. The variation coefficient of fluorescence signals for each calibrator did not exceed 15%.

To use the same calibration curve for all assayed sIgE, the signals from biochip elements with immobilized allergens must correlate with the calibration curve. This was achieved by adjusting the concentrations of biochip-immobilized allergen extracts and recombinant allergens. For this purpose, special biochips were manufactured containing gel elements with allergens immobilized in different concentrations. These biochips were tested with sera with known sIgE concentration (determined by the REAST method, Dr. Fooke Laboratorien GmbH, Germany). The resulting fluorescence signals were compared to the signals on the calibration curve shown in Fig. 3. For each allergen, the concentration which shows the best agreement with the sIgE concentration of the reference serum samples was chosen. This way the immobilization concentrations for all 21 allergens were selected and these concentration were further used for biochip manufacturing.

3.2. Analysis of serum samples

For the positive versus negative classification of the samples we used the cut-off 0.35 IU/ml in accordance with the international calibration system. This cut-off is also used for the REAST reference method.

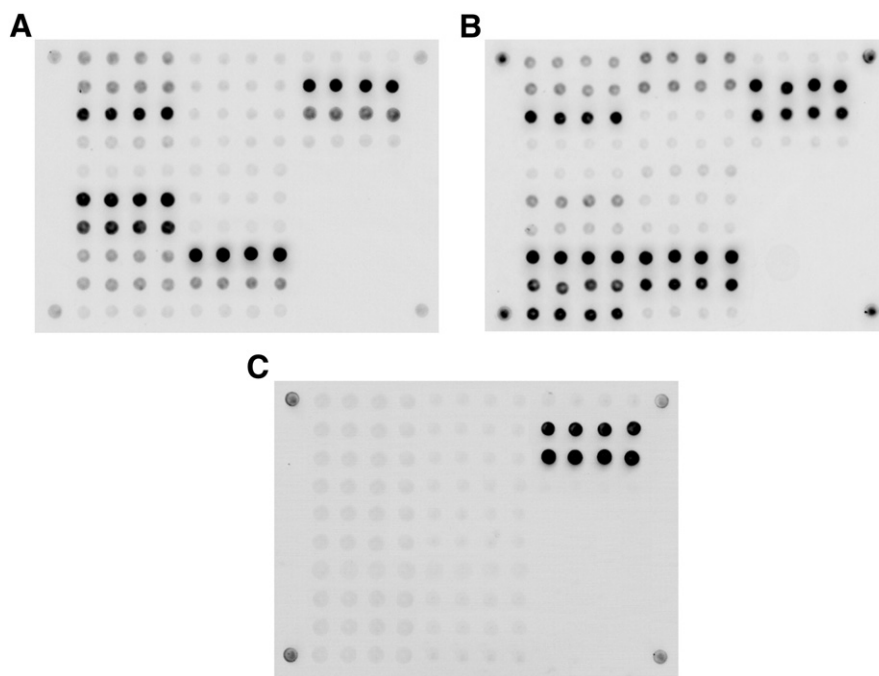


Fig. 2. Fluorescence images of biochips obtained from a fluorescence biochip analyzer after carrying out the assay. (A)—analysis of serum with high levels of sIgE to t2, t3, Bet v1, g6, g12, e1, Fel d1, f13, Ara h2; (B)—analysis of serum with high levels of sIgE to t2, t3, Bet v1, g6, g12, e1, Fel d1, e5, d1, d2, f13, Ara h2; (C)—analysis of serum from a non-allergic patient without increased levels of sIgE. The layout of the biochip is presented in Fig. 1.

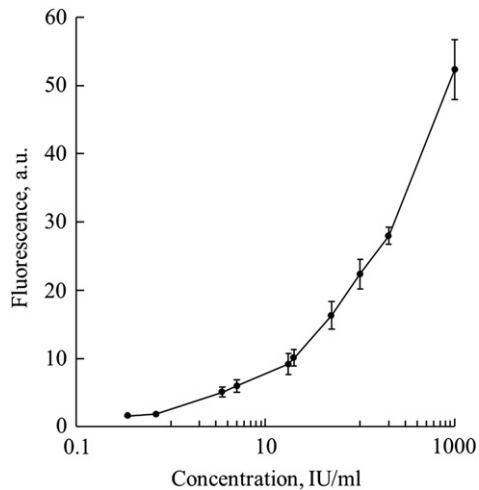


Fig. 3. Example of the calibration curve for the assay of total and sIgE obtained using calibrators adjusted to the WHO IgE 75/502 reference standards.

The biochip-based assay provided specific IgE concentrations against 21 allergens and total IgE concentration for each serum sample. 221 serum samples including 92 samples

from healthy donors were analyzed. The results of the measurements were compared to those obtained by the “ALLERG-O-LIQ” and “Total IgE HRP EIA” systems (Dr. Fooke Laboratorien GmbH, Germany).

The analysis of the data revealed the following Area under the Curve (AUC) values for the Receiver Operating Curve (ROC) Analysis:

0.99 for t3, f13, Fel d1 allergens;
 0.97–0.98 for t2, w6, g12 allergens;
 0.94–0.95 for g6, t4, d2 allergens;
 0.92 for e5, f1 allergens;
 0.89 for d1 allergen;
 0.84–0.85 for e1, f2 allergens;
 not enough data for f4, f17, Bet v1, Bet v2, Der p1, Der f1, Ara h2 allergens.

The examples of the ROC plots for t3, w6, g6 and f13 allergens are shown in Fig. 4. For most allergens the obtained AUC value was in the range of 0.92–0.99. The interpretation of the AUC values is as follows: >0.9 indicates high accuracy, 0.7–0.9—moderate accuracy, 0.5–0.7—low accuracy, and 0.5—a chance result (Akobeng, 2007).

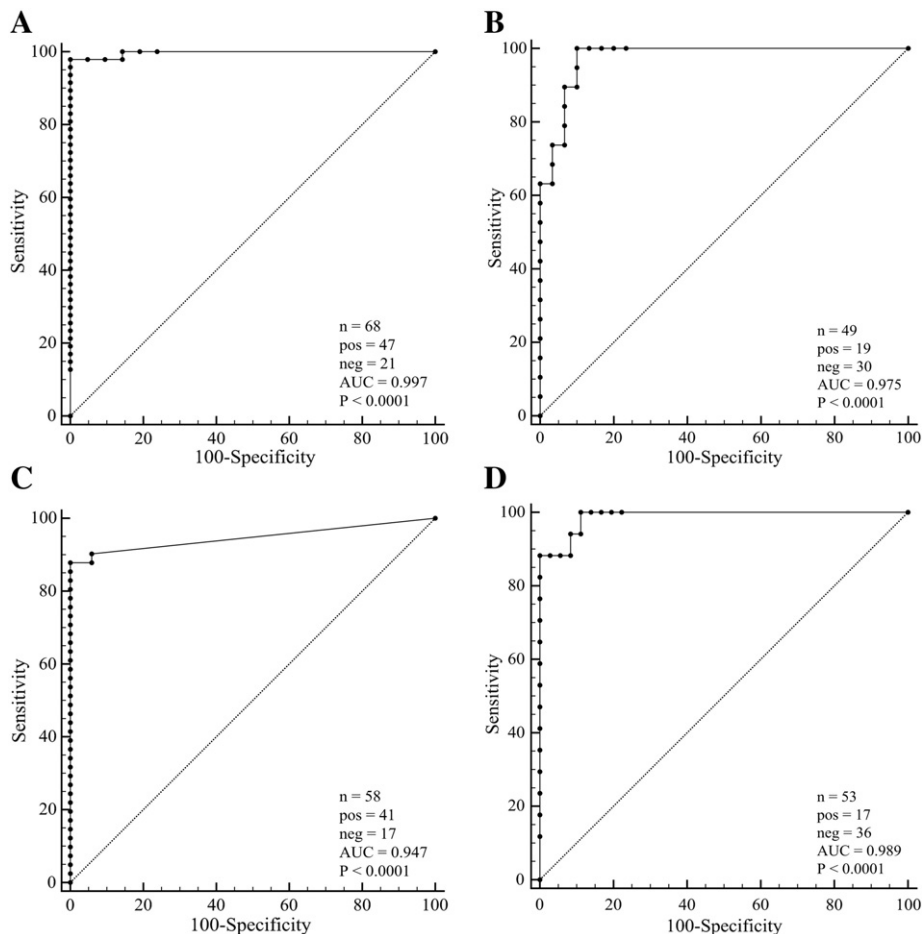


Fig. 4. ROC plots for t3 (A), w6 (B), g6 (C), and f13 (D) allergens: comparison of the data obtained on biochips with the data for the same allergens measured in the ALLERG-O-LIQ system.

Fig. 5 presents the results of Pearson's correlation of the data obtained with the biochip (for positive samples) vs those obtained with ALLERG-O-LIQ for different allergens and biochip vs EIA for total IgE. Pearson's correlation coefficient was near 0.8 in all cases. However it should be mentioned that for some

serum samples biochips showed higher results as compared to the standard systems, especially for the determination of total IgE concentration. This can be explained by the fact that the allergen preparations used in both systems were similar but not identical; for total IgE determination, different antibodies

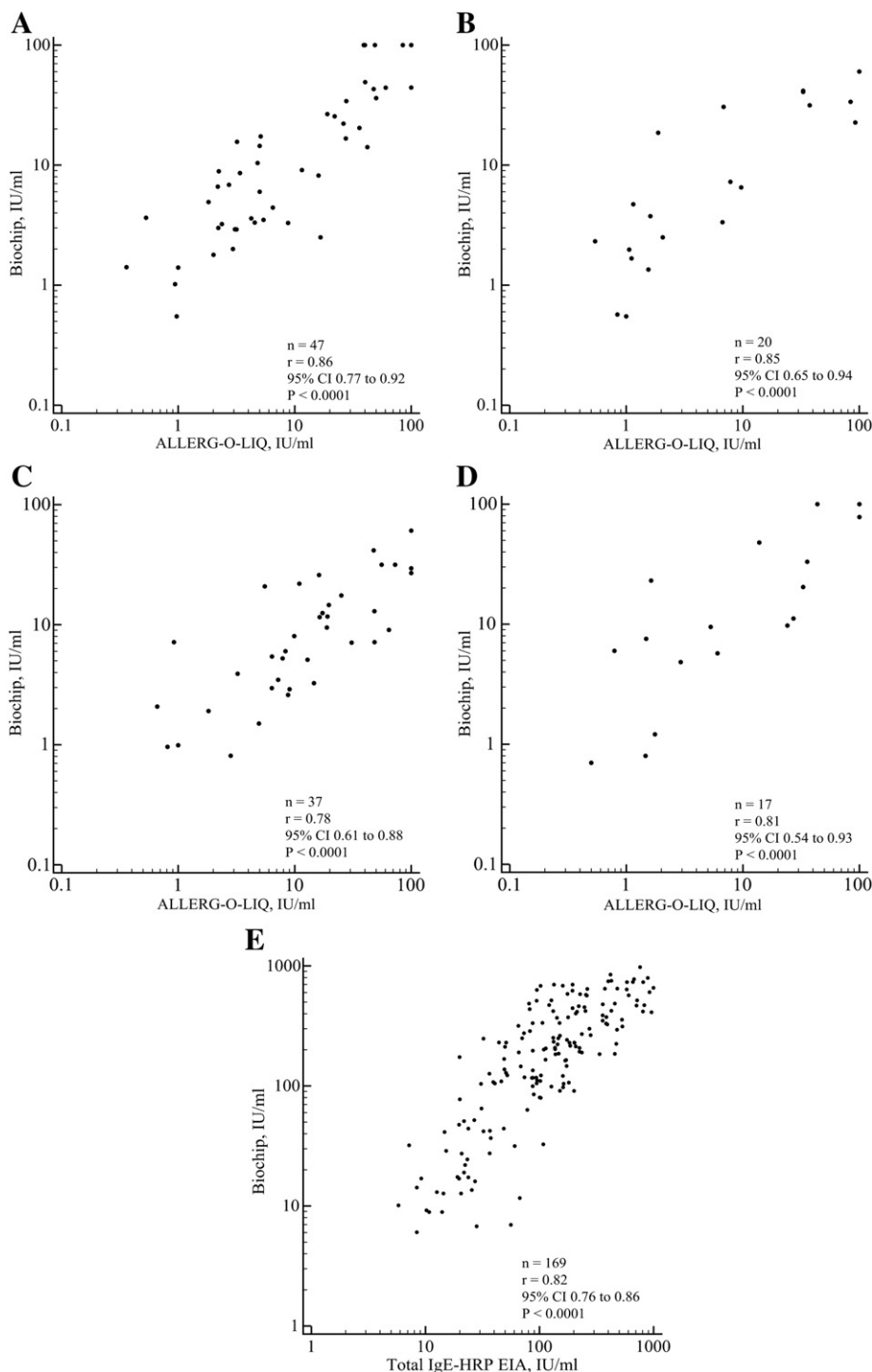


Fig. 5. Pearson correlations for the results obtained with biochips and "ALLERG-O-LIQ" or "Total IgE HRP EIA" systems: for t3 (A), w6 (B), g6 (C), f13 (D) allergens, and total IgE (E).

(anti-IgE) were used as capture antibodies (for immobilization in biochip elements and in plate wells for EIA system) and as developing antibodies.

In general, analysis of the results in Fig. 4 and 5 allowed us to conclude that the results of simultaneous determination of sIgE and total IgE in serum on the biochip show good correlation with the results obtained by separate determinations of sIgE and total IgE concentrations using commercial EIA test systems of generally recognized quality (Dr. Fooke Laboratorien GmbH, Germany).

3.3. Biochip stability

As has been shown previously for hydrogel biochips with immobilized antibodies, the storage period for protein biochips was not less than 2 years (Rubina et al., 2008). Biochips with immobilized allergens are stable for at least half a year under storage at 2–8 °C based on ongoing stability studies (data not shown).

4. Conclusions

The developed biochip-based system allows carrying out simultaneous quantitative assays of 21 sIgE belonging to different allergen groups and total IgE. Good correlation with the results obtained using commercial EIA test systems of generally recognized quality (Dr. Fooke Laboratorien GmbH, Germany) was shown. The number of immobilized allergens, including recombinant and highly purified allergens, is nearly unlimited. Simultaneous use of both allergen extracts and the corresponding relevant recombinant allergens would enable not only a reliable determination of sIgE concentrations to specific allergens but also a component-resolved sIgE diagnostics, the results of which could greatly improve the decision-making with respect to the selection of allergen-specific immunotherapies.

Further studies should be performed to demonstrate the clinical value of microarray-based, multiplexed specific IgE testing in allergy diagnostics.

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