

Multiplex Assay of Allergen-Specific and Total Immunoglobulins of E and G Classes in the Biochip Format

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Allergy is an acute immune response of the body to a normally harmless substance, allergen, which develops under a repeated exposure. Allergic reactions are mediated primarily by immunoglobulins of classes E (IgE) and G (IgG). IgE is responsible for the manifestation of the immediate-type allergy (type I, the so-called IgE-mediated hypersensitivity of the body); as much as 20–50% of the population of developed countries is prone to it. In addition to determining the levels of specific IgE (sIgE), for differential diagnosis of food allergy and food intolerance, which can be the cause of many chronic diseases, it is also necessary to determine the levels of specific IgG4 (sIgG4) to numerous food allergens. In addition, the determination of the level of sIgG4 and the IgG4/IgE ratio is important to monitor immunotherapy.

The existing in vitro allergy diagnostic methods are aimed at semiquantitative and quantitative determination of sIgE and sIgG4 in patient's blood serum. The majority of methods make it possible to determine the level of only sIgE or sIgG4 in a single assay.

Modern diagnosis of allergic diseases requires parallel (multiplex), highly sensitive analysis that allows simultaneous determination of different sIgE and sIgG4 in a single assay. A promising tool for developing such methods are biological microchips (biochips)—arrays of cells containing a large set of immobilized allergens and allowing determination of a variety of different immunoglobulins in a single experiment using a minimal number of samples.

The ImmunoCAP ISAC microchip (Thermo Fischer) includes 103 natural and recombinant allergens, which are immobilized on a substrate in the form of spots (two-dimensional microchip). This microchip makes it possible to determine the levels of sIgE

in a sample and classify them in accordance with the four classes of allergic reactions (negative, low, medium, and high). This chip, however, cannot be used to analyze sIgG4.

In this paper, we propose a procedure for a simultaneous determination of 32 different sIgE and sIgG4 in a single assay on a hydrogel-based biochip (three-dimensional microchip). Biochips are produced by the polymerization immobilization technology developed at the Institute of Molecular Biology, Russian Academy of Sciences [1, 2]. This technology is based on the photoinduced copolymerization of molecular probes (proteins, oligonucleotides, or glycans) with gel-forming monomers during the formation of hydrogel cells of the biochip. The molecular probes immobilized in hemispherical gel cells (microdroplets) are uniformly distributed throughout the volume of the drop; they are sufficiently distant from one another (1000 Å) and do not come into contact with the hydrophobic substrate. This is especially important for immobilization of protein probes, which can undergo partial or complete denaturation as a result of such contacts. In addition, the use of three-dimensional gels raises their capacity, increasing the possible amount of the immobilized protein, which leads to an increase in the sensitivity of the assay [1–5].

Biochip structure. A biochip for a simultaneous determination of 32 sIgE and sIgG4 in blood serum contains different classes of allergens (including recombinant) immobilized in hydrogel elements:

- (1) the class of tree pollen allergens (t2, t3, bet v1¹, and bet v2¹);
- (2) the class of weeds and flowers (w1, w6, and w5);
- (3) the class of meadow grass allergens (g3, g4, g5, g6, g12, phl p1¹, phl p5¹, phl p7¹, and phl p12¹);
- (4) the class of fungal allergens, molds and yeasts (m5 and m6);
- (5) the class of epidermal allergens (e1 and e5);
- (6) the class of household allergens, mites (d1 and d2);

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¹ Recombinant allergens.

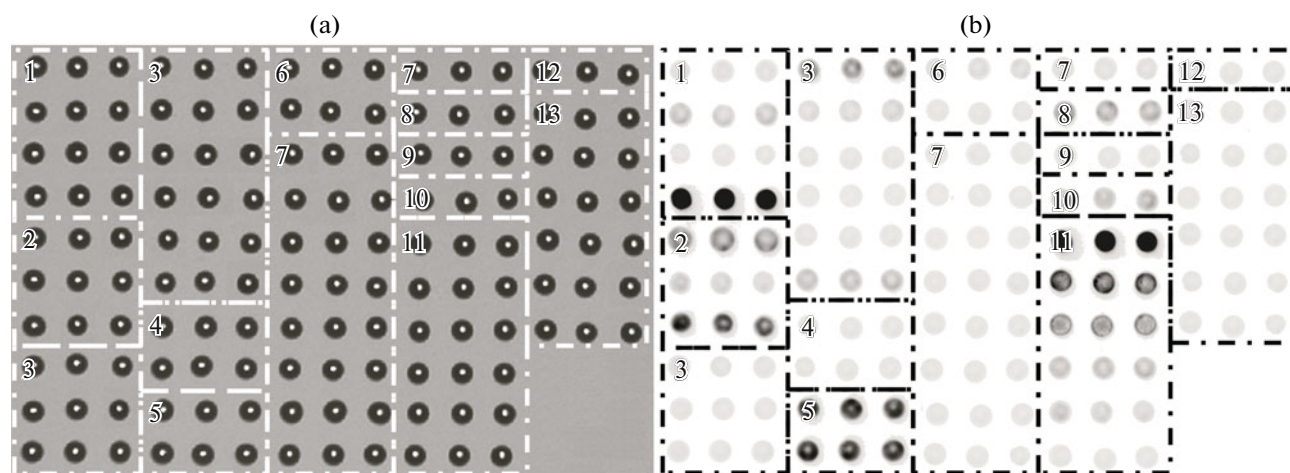


Fig. 1. Image of the biochip for a simultaneous determination of sIgE and sIgG4: (a) an enlarged photo image; (b) an image obtained using a fluorescence biochip analyzer recording the signals from developing antibodies labeled with the fluorescent dye Cy5 under excitation with a laser with $\lambda = 650$ nm. The biochip design: 1–8, immobilized allergens; 9, cells without immobilized proteins; 10, immobilized anti-IgE; 11, immobilized IgE at different concentrations; 12, immobilized anti-IgG4; 13, IgG4 immobilized at different concentrations.

(7) the class of food allergens (f1, f2, f4, f7, f13, f14, f17, f39, and f49); and

(8) the carbohydrate determinant CCD¹.

The allergens for immobilization on the biochip were kindly provided by Dr. Fooke Laboratorien GmbH, Germany.

In addition to the allergens, biochips contain the cells with immobilized antibodies: human anti-IgE and anti-IgG4, IgE, and IgG4 at different concentrations as well as the cells that do not contain immobilized proteins to control nonspecific interactions. Each protein was immobilized in three identical gel cells (Fig. 1).

The biochip developed by us is intended for determination of both specific and total IgE and IgG4 in blood serum samples in a single assay. Figure 2 shows the scheme of processes occurring in the gel cells of the biochip.

During the interaction of serum with immobilized allergens, the complexes of allergens with sIgE and sIgG4 are formed. After the treatment of the biochip with the developing solution containing a mixture of antibodies against sIgE and sIgG4, labeled with different detectable labels (e.g., anti-IgE-Cy5 and anti-IgG4-Cy3), the content of sIgE and sIgG4 for a given allergen was determined simultaneously on the basis of recorded fluorescence signals from each cell with immobilized allergen (Fig. 2, 1).

The design of the biochip makes it possible to determine the concentration of total IgE and IgG4 in the same assay. In cells with immobilized anti-IgE and anti-IgG4, the complexes with IgE and IgG4 from the sample are formed. After the treatment of the biochip with the developing solution containing a mixture of anti-IgE-Cy5 and anti-IgG4-Cy3 conjugates, the sig-

nals from the corresponding cells allowed IgE and IgG4 in the sample to be determined (Fig. 2, 2).

To simplify the procedure of analysis and data processing, the biochip contained arrays of cells with immobilized IgE and IgG4 at increasing concentrations, which served as internal standards [4, 6]. The treatment of the biochip with a mixture of fluorescently-labeled anti-IgE and anti-IgG4 led to the formation of complexes of immobilized IgE and IgG4 with appropriate conjugates. The fluorescent signals from these cells were used to construct the standard curves for the determination of concentrations of both total and specific IgE and IgG4 (Fig. 2, 3).

The registration of signals, data processing, and determination of concentrations of IgE and IgG4 in the sample were performed with the fluorescence biochip analyzer developed at the Institute of Molecular Biology, Russian Academy of Sciences, which allows simultaneous detection of fluorescence images obtained at two different wavelengths [7] using the ImaGelResearch software (Institute of Molecular Biology). The fluorescence intensity of the cells with the immobilized compound was calculated as the average signal from three identical gel elements. The conditions of measurement of fluorescent signals at two wavelengths were adjusted so that the signals obtained for equal concentrations of detected IgE and IgG4 coincided.

The analysis procedure. To perform analysis, 60 μ L of a blood serum sample was incubated on the biochip at 37°C for 20 h, after which the biochip was washed by incubation for 20 min in PBST. Then, 60 μ L of a solution of developing antibodies (anti-IgE-Cy5 and anti-IgG4-Cy3) was applied, and the biochip was incubated once again at 37°C for 1 h. After the final wash (30 min, PBST), the fluorescent signals was

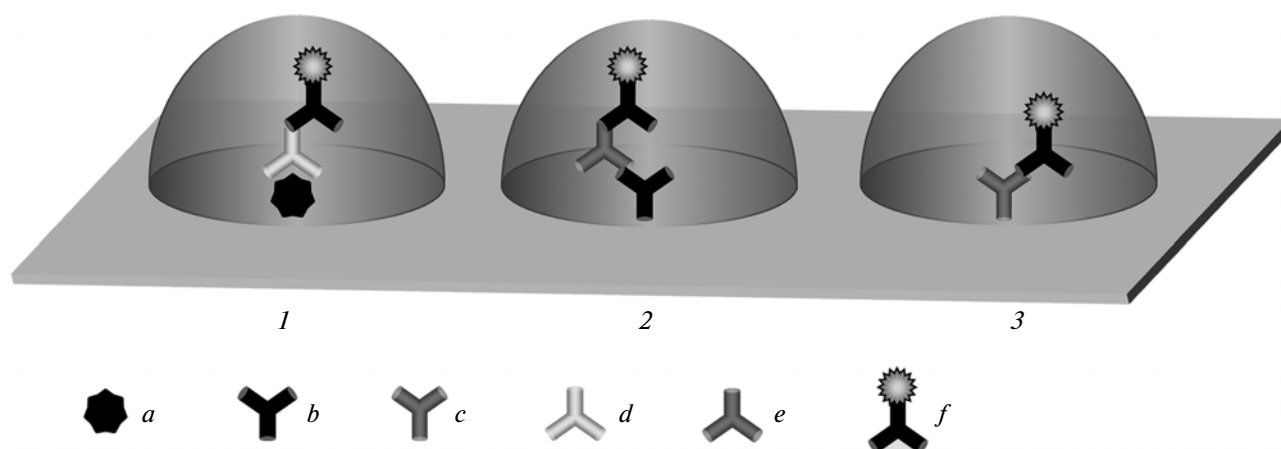


Fig. 2. Schematic representation of the processes occurring in the cells of the biochip: (1) cells with immobilized allergens; (2) cells with immobilized anti-IgE or anti-IgG4; (3) cells with immobilized IgE and IgG4. Designations: *a*, immobilized allergen; *b*, immobilized anti-IgE or anti-IgG4 antibodies; *c*, immobilized IgE or IgG4; *d*, sIgE or sIgG4 in the sample; *e*, total IgE and IgG4 in the sample; *f*, anti-IgE or anti-IgG4 antibodies conjugated with a fluorescent dye.

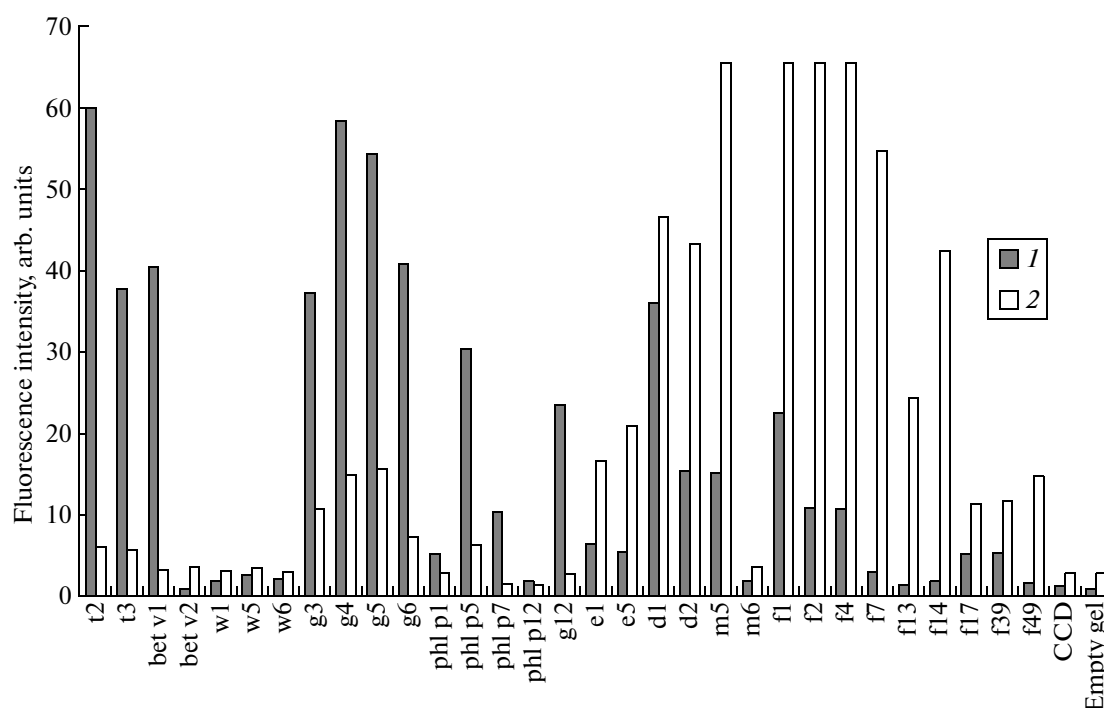


Fig. 3. Fluorescent signals obtained during simultaneous detection of sIgE and sIgG4 on the biochip: (1) fluorescent signals recorded at $\lambda_{\text{ex}} = 650$ nm for anti-IgE-Cy5 and (2) fluorescent signals obtained at $\lambda_{\text{ex}} = 540$ nm for anti-IgG4-Cy3.

recorded at two wavelengths (absorption/emission wavelengths for Cy5 and Cy3 are 650/670 and 540/560 nm, respectively).

As a result, an individual profile of fluorescent signals for all sIgE and sIgG4 was obtained for each blood serum sample (Fig. 3). On the basis of signal values and the internal calibration curves, the content of each sIgE and sIgG4 was determined.

Determination of sIgE in blood serum with a high sIgG4 content. It is known that IgG account for 75–

80% of immunoglobulins in blood serum, whereas the proportion of IgE accounts for only about 0.002%. Thus, the concentration of IgG in the blood is several orders of magnitude greater than the concentration of IgE (10–20 mg/mL for IgG and ~50 ng/mL for IgE) [8].

It is also known that, in a number of allergic diseases, including food allergies, blood serum may contain high quantities of sIgG4. They may compete with sIgE for the binding to allergens immobilized on the support, which gives an inaccurate, underestimated

Concentrations of allergen-specific and total IgE in blood sample before and after sample preparation to remove IgG, obtained using the Specific IgE REAST kits (Dr. Fooke)

Allergen	Concentration, IU/mL	
	before treatment	after treatment
Total IgE	711	695
t7 White oak	6.3	5.2
g3 Cocksfoot	0.6	0.6
g4 Meadow fescue	1.6	1.5
g5 Perennial ryegrass	2.6	2.6
g6 Timothy grass	3.2	3.1
g12 Rye	5.1	4.6
d2 Dermatophagoides farinae (mite)	3.7	3.6
m5 Candida albicans (mold fungus)	1.9	1.8
f1 Egg-white	47.4	46.5
f2 Cow milk	100	99
f4 Wheat flour	16.6	15.4

sIgE concentration [9]. In addition, blood serum may contain IgG autoantibodies against IgE, which form the IgG–IgE complex. The presence of such complexes during analysis hampers the determination of IgE, because they prevent further binding of IgE or sIgE to the species-specific developing antibodies [10].

In some variants of test systems for the analysis of sIgE performed in the MTP-format, this problem is

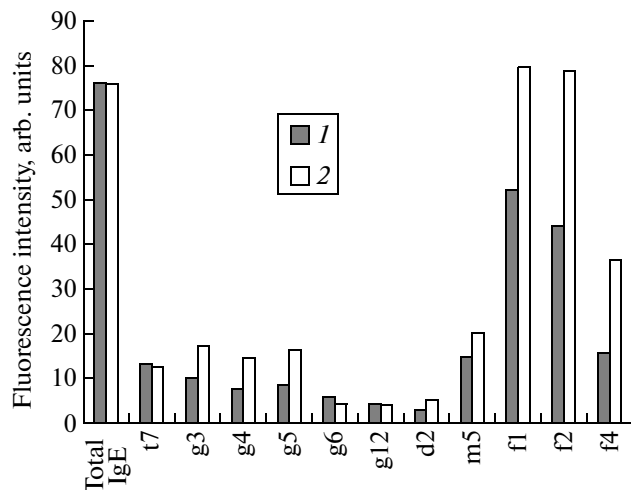


Fig. 4. Fluorescent signals from the biochip elements during simultaneous analysis of specific and total IgE in a human blood serum sample (1) before and (2) after pretreatment of the sample to eliminate IgG. The biochips were incubated with the sample and developed with the antibodies against IgE labeled with the fluorescent dye Cy5.

solved by using the reverse immunoassay technique (the so-called reverse allergen sorbent test (REAST)), which eliminates the competition of allergen-specific antibodies of other classes. This approach is used, for example, in the Specific IgE REAST test (Dr. Fooke Laboratorien, GmbH, Germany). In this case, human anti-IgE are immobilized on a microtitre plate. After incubation with blood serum samples, anti-IgE–sIgE complexes are formed, whereas other components of the serum, including sIgG4, which are incapable of specific binding, are eliminated by washing. Then, the formed complex interacts with a biotinylated allergen and is treated with streptavidin conjugated to an enzyme. This eliminates the possibility of interaction of an allergen with sIgG.

To solve this problem with respect to the assay on the biochip, we have developed a sample preparation procedure that is based on the elimination of IgG by affinity chromatography of blood serum sample prior to analysis. This procedure makes it possible to eliminate the competition between sIgE and sIgG4 for the binding to the allergens immobilized in the biochip cells.

Chromatography was performed on a Sepharose column containing immobilized protein G, which specifically interacts with IgG (Protein G Sepharose 4 Fast Flow, GE Healthcare, United States; the sorbent capacity was > 10 mg/mL). The concentration of specific and total IgE before and after the washing procedure was monitored by enzyme immunoassay using the Specific IgE REAST kits for quantitation of total and allergen-specific IgE (Dr. Fooke Laboratorien, GmbH, Germany). As can be seen from the data summarized in the table, after the removal of IgG from a blood serum sample, the concentration of IgE (both total and allergen-specific) almost did not change.

Figure 4 shows the results of analysis of a blood serum sample performed on a biochip both without elimination of IgG and with the use of the proposed sample preparation procedure. As can be seen from the diagram, for sIgE, for example, for f1, f2, f4, g3, g4, and g5 allergens, the sample purification procedure makes it possible to adjust the specific signal value. Thus, the use of the sample preparation procedure allowed us to prevent obtaining the false-underestimated concentrations of IgE and improve the accuracy of analysis.

Thus, we propose the following assay procedure with the use of the allergochip: first, a simultaneous determination of the allergen-specific IgE and IgG4 is performed in a sample using two fluorescent labels (e.g., Cy3 and Cy5). If high concentrations of sIgG4 antibodies are detected (i.e., in the case of food allergies), it is recommended to eliminate IgG antibodies by affinity chromatography as described above and then repeat the assay of the sample on the biochip to obtain more reliable results of sIgE determination.

The biochip for a simultaneous assay of allergen-specific and total IgE and IgG4 is currently being

developed and will include a multiply increased number of clinically relevant allergens.

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