ONCOLOGY

Biological Microchip for Simultaneous Quantitative Immunoassay of Tumor Markers in Human Serum

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The biochip was constructed for simultaneous assay of total and free prostate-specific antigen, α-fetoprotein, cancer embryonic antigen, human chorionic gonadotropin, and neuron-specific enolase. These biochips represent an array of gel elements with covalently immobilized proteins. The major analytic characteristics of the developed method were obtained. It was shown that the results of simultaneous assay of six tumor markers in blood serum well correlated with routine measurements of each marker using enzyme immunoassay kits. This approach allowed us to reveal the hook effect of high concentrations during biochip assay, which prevents distortion of the diagnostic picture at high concentration of the analyte in the sample.

Key Words: *hydrogel microchips; protein microchips; tumor markers; simultaneous immunoassay; hook effect*

Early diagnostics is an urgent problem of oncology. Measurement of tumor markers in blood serum is widely used for early diagnostics of primary tumors and metastatic dissemination, as well as for monitoring of therapeutic treatment. Ideal tumor marker should be characterized by high specificity and sensitivity to a certain type of tumor. However, the majority of tumor markers do not satisfy these requirements. Simultaneous evaluation of several tumor markers increases the efficacy of screening analysis and provides the possibility for differential diagnostics of the diseases. The majority of immunohistochemical methods allow us to study only one marker in the clinical sample. Therefore, multiplex systems (*e.g.*, biological microchips) hold much promise for cancer diagnostics.

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We developed the method for simultaneous quantitative evaluation of six tumor markers in blood serum on a biological microchip. Experiments were performed with the following serum markers: total prostate-specific antigen (PSA_{tot}), free prostate-specific antigen (PSA_{fr}), α-fetoprotein, cacinoembryonic antigen (CEA), human chorionic gonadotropin (HCG), and neuron-specific enolase (NSE).

MATERIALS AND METHODS

Blood serum samples were obtained from the Laboratory of Clinical Immunology and Urology Department (Russian Cancer Research Center), B. V. Petrovsky Russian Research Center of Surgery (Russian Academy of Medical Sciences), and Central Clinical Hospital and Outpatient Department (Department for Presidential Affairs of the Russian Federation). The study was performed with purified PSA, α-fetoprotein,

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CEA, HCG, and monoclonal antibodies to α-fetoprotein (XFP2 and XFP4) and HCG (XK77 and XK27; Hema Medica). The antigen NSE was gifted by Prof. P. G. Sveshnikov (All-Russian Research Center of Molecular Diagnostics and Therapy). We used antibodies to PSA (PSA30, PSA36, and PSA66), CEA (CEA12-140-1 and CEA12-140-10), and NSE (NSE17 and NSE21; Fujirebio Diagnostics, CanAg).

The concentration of antigens in the solution and blood serum was measured by enzyme immunoassay (EIA) with Fujirebio Diagnostics kits (CanAg) for PSA_{tot}, PSA_{fr}, α-fetoprotein, CEA, and NSE. HCG was assayed with Immunotech kits. The measurements were performed according to manufacturer's recommendations.

Hydrogel biochips were constructed by the patented polymerization-mediated immobilization method [1]. This method was developed under supervision of Acad. A. D. Mirzabekov (V. A. Engel'gardt Institute of Molecular Biology) [5,6]. The biochips were treated with a blocking buffer (1% solution of polyvinyl alcohol in phosphate buffered saline) for 1 h.

Antibodies were stained with a fluorescent dye Cy5 according to manufacturer's recommendations (Amersham Pharmacia Bioscience). A developing mixture for immunoassay on the microchip was composed of antibodies to PSA (PSA66-Cy5, 21 μg/ml), α-fetoprotein (XFP2-Cy5, 72 μg/ml), CEA (CEA12-140-10-Cy5, 18 μg/ml), HCG (XK77-Cy5, 12 μg/ml), and NSE (NSE21-Cy5, 30 μg/ml) in phosphate buffered saline (0.15% polyvinyl alcohol, 0.15% polyvinylpyrrolidone, 1% bovine serum albumin, 0.6% Tween 20, and 0.01% methylisothiazolone hydrochloride).

Calibration curves were constructed with blood serum from male donors. Each calibration sample contained six tumor markers.

Sandwich immunoassay on biochips was performed as follows. A mixture of fluorescently-labeled developing antibodies (10 μl) was added to the analyzed sample or calibration sample (50 μl). Incubation on the biochip was performed at 37°C for 20 h. Washout was performed with phosphate buffered saline and 0.1% Tween 20 for 30 min. Fluorescence signals were recorded.

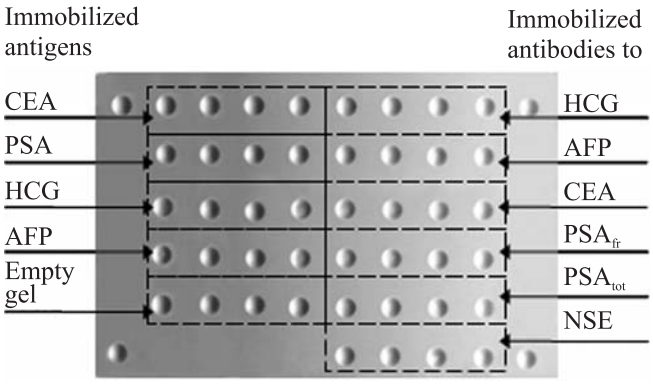

Fig. 1. Biochip for a study of tumor markers (in transmitted light). The biochip contains gel elements (diameter 100 μ, volume 0.1 nl; 4 identical elements in the row) with immobilized monoclonal antibodies to six tumor markers, immobilized antigens, and blank ("empty") gels. Here and in Figs. 2 and 3: AFP, α-fetoprotein.

Fluorescence was measured on a biochip portable fluorescence analyzer with laser excitation (Institute of Molecular Biology) using 650/750-nm filters (excitation/recording) [2]. Fluorescence signals from each well of the biochip and concentrations of all tumor markers were studied by means of ImagelAssay software (Institute of Molecular Biology). The intensity of fluorescence was calculated as the median signal from four gel wells.

RESULTS

Figure 1 shows the biochip structure for quantitative immunoassay of six markers of tumor diseases. The biochip consisted of gel elements with immobilized monoclonal antibodies to tumor markers and gel elements with immobilized antigens CEA, PSA, HCG, and α-fetoprotein. This biochip did not contain NSE antigen: due to high lability of the enzyme, fluorescence signals from immobilized NSE cannot be detected. Marker points with a florescent dye Cy5 were

TABLE 1. Immunoassay of Tumor Markers on Biochips

Tumor marker			Normal serum concentration	Analytical sensitivity	Measurement range	
					C1	C2
PSA, ng/ml	total	below 4 (men under 50 years of age)	0.3	0.3-70.0	1500	20 000
	free	PSA_{fr}/ PSA_{tot}>0.15	0.2	0.2-60.0	1000	20 000
α-Fetoprotein, ng/ml		below 10 (men and nonpregnant women)	1.0	1.0-600.0	4000	7000
CEA, ng/ml		below 5; for smokers, below 10	0.5	0.5-100.0	2000	15 000
HCG, U/ml		below 10 (men and nonpregnant women)	3.0	3.0-400.0	7000	300 000
NSE, ng/ml		below 12.5	2.0	2.0-150	3000	125 000

put at the biochip edge (orientation of the biochip during measurements).

During biochip analysis, triple complexes consisting of the immobilized antibody, antigen, and developing antibody (sandwich analysis) are formed. The interaction of immobilized antigens with fluorolabeled developing antibodies results in the formation of binary complexes.

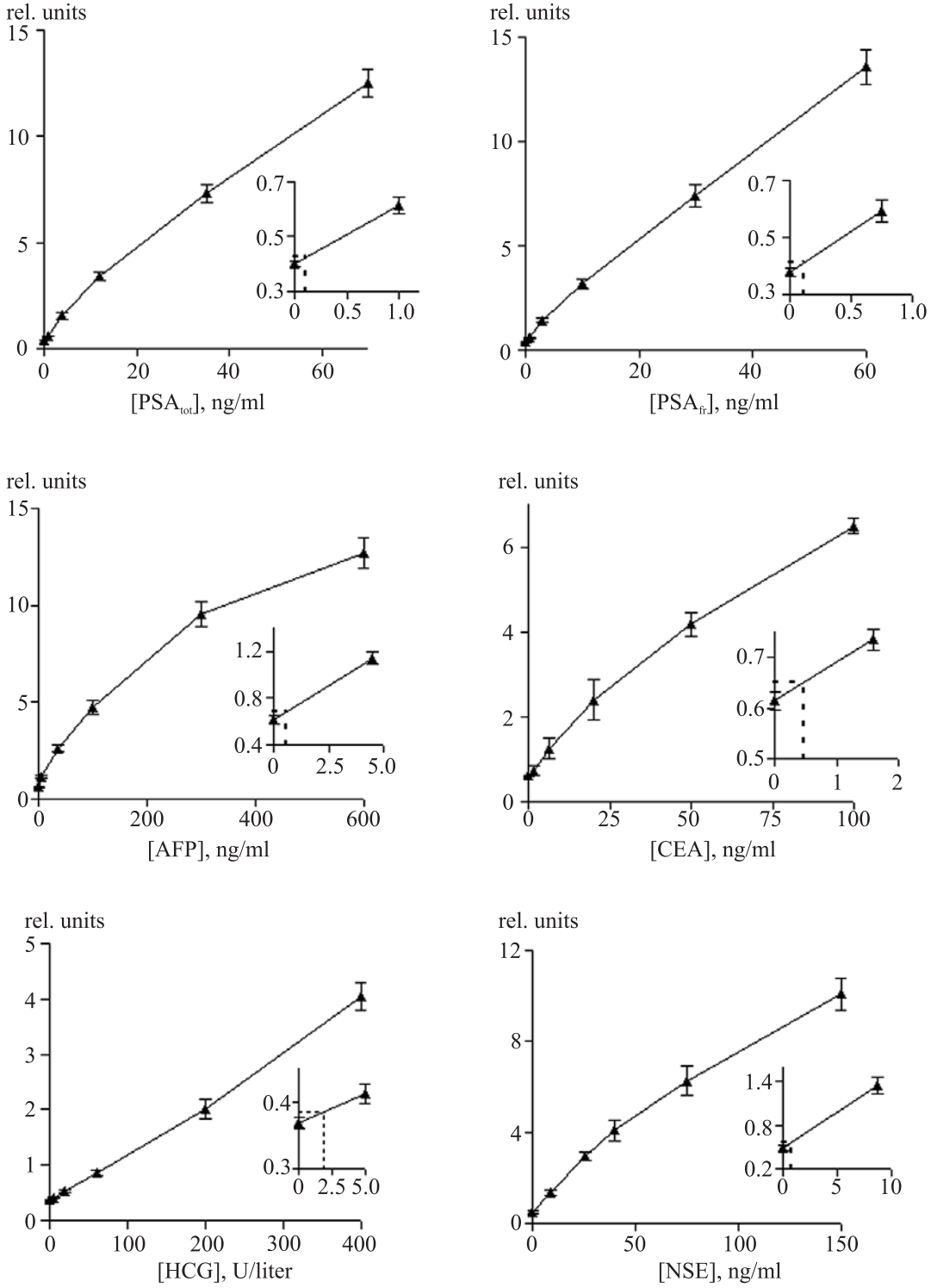

Fig. 2. Sandwich immunoassay. Dependence curves for the fluorescence signal from gel elements with immobilized antibodies to PSA$_{tot}$, PSA$_{fr}$, α-fetoprotein, CEA, HCG, and NSE on the concentration of test antigen in the calibration sample (calibration curves). Each point on the calibration curve corresponds to the mean value of measurements with 10 biochips. Insertions: initial segments of the corresponding curves for a study of the analytical sensitivity for each tumor marker.

For quantitative evaluation of antigen concentration by immunofluorescence analysis on the biochip, the dependence curve of the fluorescence signal from gel elements with immobilized antibodies on the concentration of this antigen in the calibration sample (calibration curve). To simplify the analysis, the concentrations of all tumor markers (n=6) in calibration samples were within the range required for clinical analysis of blood serum (Table 1). A mixture of antibodies to six tumor markers was used as developing antibodies. Six calibration curves were constructed simultaneously.

A special series was conducted on 60 biochips (10 calibration curves) to evaluate the reproducibility and analytical sensitivity of the method. Straight-line interpolation was used for all calibration curves with biochips (Fig. 2). The variation coefficient for different concentrations of the test antigens did not exceed 15%. The analytical sensitivity of this assay (lowest estimated concentration of each tumor marker) was determined as a concentration corresponding to a fluorescence signal exceeding the signal of repeatedly analyzed zero calibration sample (10 times) by at least 2 standard deviations (Fig. 2, Table 1).

Biochips were used for the simultaneous evaluation of six tumor markers in blood serum from oncology patients and healthy donors. The results of simultaneous assay of six tumor markers in blood serum well correlated with routine measurement of each marker using EIA kits. The correlation coefficients appeared as follows: PSA_{tot}, 0.993 (n=25, p<0.01); PSA_{fr}, 0.994 (n=22, p<0.01); α-fetoprotein, 0.989 (n=24, p<0.01); CEA, 0.986 (n=18, p<0.01); HCG, 0.981 (n=20, p<0.01); and NSE, 0.989 (n=18, p<0.01).

A special series was performed to study the hook effect of high concentrations. The hook effect of high concentrations occurs in immunodiagnostic systems (sandwich analysis) with high concentration of the analyte. Under these conditions, the solid-phase signal inversely depends on analyte concentration [4]. The hook effect of a one-step sandwich immunoassay is related to blockade of binding sites for immobilized and developing antibodies at high concentration of an analyte in the sample. Uncontrolled hook effect leads to distortion of the diagnostic data: high concentration of the analyte in blood serum is interpreted as low concentration.

In the majority of the test systems with solid-phase EIA, the hook effect can be evaluated and controlled by means of simultaneous measurements with the sample and its consecutive dilutions [3]. However, this approach adds complexity to the analysis. We proposed an original method for detection of the hook effect of high concentrations. This method is based on measurement of signal intensity from biochip wells with immobilized antigens.

At high concentration of the analyte in the sample, the biochip-immobilized antigen competes with the antigen in the solution for binding with developing antibodies. At high concentration of the antigen in the solution, we revealed a decrease in fluorescence signals from the wells with immobilized antibodies and immobilized antigen (Fig. 3). At varying concentrations, the signal from the immobilized antigen remains unchanged and does not depend on antigen concentration in the solution (Fig. 3, *b*, curve *2*). The fluorescence signal from immobilized antibodies (Fig. 3, *a*, curve *1*) and immobilized antigen (Fig. 3, *a*, curve *2*) was reduced at high concentrations. Similar results were obtained for

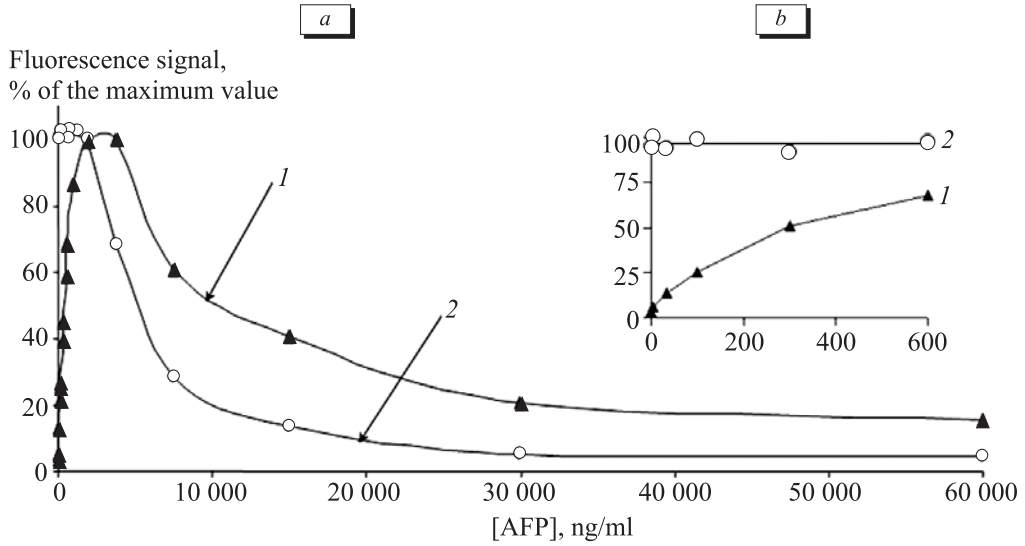

Fig. 3. Hook effect of high concentrations. (*a*) Dependence of the fluorescence signal from gel elements with immobilized antibodies to α-fetoprotein (*1*) and gel elements with immobilized α-fetoprotein (*2*) on the concentration of α-fetoprotein. The hook effect is evaluated from a decrease in the fluorescence signal from gel elements with immobilized α-fetoprotein. (*b*) Initial segments of the corresponding curves (varying concentrations).

all tumor markers. These data were taken into account to study the hook effect during an analysis of serum sample with high concentration of an analyte. The hook effect was characterized by two concentrations of the antigen in test sample. C1 corresponds to the concentration at which the recorded signal is inversely related to antigen concentration. C2 is the concentration when the fluorescence signal becomes lower than that from the maximum calibrator for test antigen. Table 1 shows C1 and C2 for all tumor markers.

ImagelAssay software allows us to perform quantitative study of all tumor markers and to reveal the hook effect in the automatic mode. When this software detects the decrease in fluorescence signals from the wells with immobilized antigens during the analysis of the test sample (as compared to signals from biochips in the construction of calibration curves), the dialog box shows a message to dilute the sample or indicates the presence of a specific antigen at high concentration in the sample.

The proposed method allows us to perform simultaneous quantitative immunoassay of six tumor markers in blood serum on the microchip. The hook effect of high concentrations can be evaluated during this analysis.

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