NEW BIOMEDICAL TECHNOLOGIES

Methodology of Immunoassays with Immunosorbent Suspensions

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A new principle of immunoassays is proposed, based on the sandwich technique, which combines various effects of a standing ultrasound wave on the ingredients and product of the immunoassay. Acoustic currents intensify the mass exchange and prevent nonspecific sorption, while flow ultrasonic selection ensures that the immunosorbent suspension is retained and the unbound substances are separated from the reaction products at each stage of the test. Such a combination greatly speeds up the test and the analysis of its results, and achieves a hitherto unattainable sensitivity and reliability of analysis of biosamples with high levels of admixtures. The new principle opens the door to studies with fluorescent, enzymatic, and radioactive labels.

Key Words: *immunoassay; field of standing ultrasound wave; fluorescent, enzymatic, and radioactive labels*

Heterogeneous methods (with phase separation), making use of enzymatic, fluorescent, or radioactive labels, are the most frequently used techniques of immunoassay. Such immunoassays involve a succession of incubations of the immunoactive layer immobilized on the surface of a solid carrier with the test sample and conjugate together with, and the removal of substances failing to bind after each incubation. The use of an enzymatic label requires an extra incubation with the substrate. Performing these operations using traditional methods is time consuming, sometimes taking several hours. Incubation takes the longest time, due to the slow diffusion of the immunoactive ingredients through the liquid-immobilized immunoactive layer interface, that is, the low rate of mass exchange between the free and immobilized ingredients of the tests. In addition, nonspecific sorption, compromising the sensitivity and reliability of the test, is a serious hindrance of immunoassay.

We propose a new principle of immunoassays for heterogeneous methods of analysis [3] that eliminates many of the effects of artifacts and shortens analysis time.

MATERIALS AND METHODS

A field of a standing ultrasound (US) wave was superimposed onto a suspension of immunosorbents moving in a fluid flow. The parameters of the US field met the requirements for retaining the immunosorbents in the US field (the forces of pressure and friction exerted on the immunosorbents by the fluid flow do not exceed the forces of radiation pressure exerted on the immunosorbents by the standing US wave field). The rest of the ingredients were then passed through the immunosorbent layers retained by the US field, the sequence being the same as in traditional heterogeneous analysis. In the

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TABLE 1. Sensitivity Assessment

Immunoactive object	Sensitivity, bacterial bodies/cm³ (dilution)	
	of device	of reference method
Francisella tularensis	1×10^3	1×10^5
Tetanus anatoxin	(1:1000)	(1:200)
Brucella	5×10^3	1×10^5
Smallpox vaccine virus	(1:20)	(1:10)
D. sibiricus rickettsiae	(1:2000)	(1:250)

case of an enzyme immunoassay the flow was arrested and the immunosorbent layers were allowed to stay in the substrate after the last ingredient (substrate) had been delivered. The US field was then removed and the test result recorded with the appropriate label.

Let us consider the effect of a US field on the processes occurring on the surface of an immunosorbent. Immunosorbent layers formed by the US field are obstacles impeding US propagation. Acoustic currents representing a unidirectional transfer of mass are known to form near obstacles placed in a US field [1]. In our case the pattern of mass transfer is closed or turbulent, with a certain scale (linear size of vortex) $L<\lambda$ and $L\approx\lambda$, where λ is the length of the US wave. Acoustic currents of such a scale are known as Schlichting and Rayleigh currents. The former takes the form of vortex flows in a boundary layer (liquid phase — immunosorbent layer) and accelerates the diffusion of substances across the interface. The latter takes the form of vortex flows forming on the external part of the interface eddies as a result of interaction between the fluid layers, and it intensifies the mass exchange with the boundary layer. Hence, the acoustic currents that emerge create the conditions necessary to speed up the immunoassay and to reduce the nonspecific sorption. It is noteworthy that immunosorbent layers form at an interval equal to half the US wavelength, which is comparable to the scale of the developing acoustic currents, that is, the above conditions are fulfilled for the entire volume of sonicated suspension, which in turn renders the immunoassay as a whole effective.

RESULTS

For experimental validation of the new method we selected a heterogeneous immunoassay with fluorescent antibodies and an immunosorbent consisting of a suspension of agarose or sepharose beads 15 μ in diameter. A device for flow US selection of suspensions with a 25 μl cell has been developed. The device provides for a 3 MHz US field with a 6 J/m³ energy density, thermostatic control of the working volume of the flow cubicle, and passage of the immunoassay ingredients at a rate of up to 1 ml/min after their preliminary introduction with a pipette dispenser into a microfunnel attached to the cell aperture. The device can be hooked up to a fluorescence microscope fitted with a photometric attachment. The sensitive zone of the microscope is spatially combined with the working volume of the cell.

The following reagents were used:

- immunosorbent: agarose antitularemia, equine; agarose to tetanus anatoxin, equine; agarose antirickettsial to *D. sibiricus,* equine; agarose antismallpox, rabbit; sepharose antibrucella, rabbit.
- antigens: tularemia diagnostic agent; tetanus anatoxin; brucellosis diagnostic agent; smallpox vaccine; rickettsiosis diagnostic agent.
- fluorescent immunoglobulins: tularemic; tetanus; brucellosis; smallpox; antimurine; rhodamine-labeled bovine albumin.
- washing liquid — phosphate buffer solution.

After an experimental study of the basic regimes, the following procedure of analysis was selected. Immunosorbent (0.05 ml of a 0.25% suspension) is introduced into the microfunnel and so delivered to the cell that 1-2 μl are left in the microfunnel in order to prevent air bubbles from forming during delivery of the next ingredient. The ultrasound is switched on. The test sample (0.05 ml) is placed in the microfunnel and delivered to the cell, and the sample is exposed to the immunosorbent for 2 min. Phosphate buffer solution (1 ml) is placed in the microfunnel and passed through the cell at a rate of 1 ml/min. Fluorescent immunoglobulins (0.05 ml of a working dilution for the traditional test) are delivered to the cell through the microfunnel. The immunosorbent is exposed to the immunoglobulin solution for 1 min. Phosphate buffer solution (1 ml) is placed in the microfunnel and passed through the cell at a rate of 1 ml/min. The US field is removed and the informative signal from the reaction product is recorded using the photometric attachment.

Immunoassay in a field of standing wave takes just 1-2 min, or only one-tenth of the time required in the traditional test. With heterologous ingredients, the level of residual fluorescence of the immunosorbents falls to the baseline (not caused by fluorescence) value, which cannot be achieved in the traditional test.

Trials of the method were carried out in 2 stages. In the first stage the threshold concentrations of detectable immunoactive objects were determined, which were then used to prepare coded samples. The data of traditional tests were the control. In the

second stage the coded samples were examined. The specificity and reproducibility of the method were assessed from the data of this stage. In addition, interfering admixtures were used in the second stage, namely heterologous microorganisms and toxins in threshold and higher concentrations, as well as dust and some bioactive substances in total concentrations of 0.125 to 0.25 mg/ml.

The results of the sensitivity assessment are presented in Table 1. The sensitivity of the analyzer is shown to be 2 to 100 times higher than that of the traditional method with visual assessment. The interference admixtures lowered the potential sensitivity of the device only in a case where the virus was detected in the vaccine, because it contained tissues and other biological material up to hundreds of microns in size.

The specificity was assessed by the method recommended for evaluating the classification quality [2]. The study showed that some samples did not contain the desired antigen.

Investigations of 65 samples yielded the following results:

- sensitivity of detection 1,
- specificity of detection ≈ 0.95,
- relative risk $R \rightarrow 0$,
- fraction of false-positive results 1/45,
- fraction of false-negative results 0.

For determining the reproducibility, 20 variants of different samples were tested five times each. The results failed to be reproduced in only 2 cases.

Moreover, the US regime used in the experiments greatly speeded up the enzymatic reactions.

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