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A Simple Direct Enzyme Immunoassay of Antibodies Based on the Differences in Diffusion Rates in a Gel of a Synthetic Antigen and of Its Complex with Antibodies

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ABSTRACT

A simple direct enzyme immunoassay for semiquantitative detection of antibodies is suggested. It is based on the difference in diffusion rates in a gel for a synthetic low-mol-wt antigen and of its complexes with antibodies to be detected. Sensitivity and specificity of the developed assay are equal to an ELISA method. The assay has been tested with antibodies against HIV protein gp41 in rabbit serum. Possible applications and limitations of the method are discussed.

Index Entries: Enzyme immunoassay; homogeneous immunoassay; immunoassay in gel; AIDS; gp41 antibodies.

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) was put forward in 1971. Several dozen variants of ELISA have been worked out since then that differ in the nature of antigen (Ag) or antibody (Ab), in enzyme marker, existence of interphase, configuration of the final complex, equipment, and so forth. There are many reasons for different ELISA procedures, since a variety of problems must be solved. New areas of application for ELISA has been often stimulated by appearance of the new ELISA variants. That is why the development of new procedures is still actual.

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In the present work, we suggest a new variant of ELISA based on a difference in rate diffusion in a gel of synthetic low-mol-wt peptide Ag and of its complex with Ab.

Rationale of the Method

The method is aimed at semiquantitative detection of Abs in biological samples. Ab interacts with synthetic low-mol wt Ag, a peptide in this work. The latter is covalently conjugated with an enzyme marker, horseradish peroxidase.

The rate of protein diffusion in a gel strongly depends on its molecular mass (2); the latter for peroxidase is 44,000 Dalton (3). For a peroxidase conjugate with a low-mol-wt Ag, it does not exceed 50,000 Dalton. In the absence of Ab, the conjugate freely diffuses in agar gel. In the presence of Ab, the complex between the conjugate and Ab with the molecular mass more than 200,000 Dalton is formed (3). Consequently, the rate of diffusion of the conjugate decreases abruptly on complex formation. Hence, the presence of Ab specific to a particular Ab can be detected by comparison of a pathlengths covered by the conjugate in the absence and presence of the sample.

The assay is performed as follows. A layer of agar gel is formed in a Petri dish with round holes made in the gel. A solution containing the conjugate of synthetic Ag with peroxidase and a sample for analysis are introduced in the hole. The gel is incubated for 2–3 h under the conditions ruling out drying. A free conjugate diffuses into the gel covering a distance of 2–3 mm. The complex with Ab does not diffuse remaining in solution inside the hole or sorbed on its walls. After incubation, the gel is washed by distilled water, and a solution of hydrogen peroxide and diaminobenzidine as a chromogeneous substrate is introduced in the hole. The product of the enzymatic reaction precipitates forming circular colored zones in the area of location of the conjugates. The width of the zone and the intensity of a color are the qualitative characteristics of Ab in a sample specific to Ag.

If an area around the hole is colored, the width of the colored zone is 2–3 mm, and the intensity is practically the same as in a control experiment, Ab is absent in a sample ("−"). If an area around the hole is not colored, only the walls of the hole are colored, or a colored zone is of 1-mm width and the intensity is much lower than in a control experiment, Ab is present in a sample ("+"). If a width of the colored zone is 1–3 mm and the intensity is lower than in a control experiment, the final decision about the presence of Ab in a sample is arguable ("±"). Discrimination between positive or negative results can usually be based solely on visual perception and does not need any equipment. For semiquantitative evaluation of Ab, an assay is performed with different dilutions of a sample.

Table 1
Comparison of Assays of Sera from Rabbits Immunized by Synthetic or Recombinant Fragments of Envelope Protein gp41 Using the Developed Method and Solid-Phase ELISA

No	Sequence of the fragment of protein gp41 used for immunization of rabbits	Results of analysis by	
		ELISA	The developed method
1.	598–609	±	±
2.	598–609	–	–
3.	598–609	+	+
4.	598–609	+	+
5.	598–609	+	+
6.	598–609	+	+
7.	598–609	–	+
8.	598–609	±	+
9.	584–604	+	+
10.	584–604	+	±
11.	584–604	–	–
12.	A mixture[a]	+	+
13.	R34	+	–
14.	R35	+	–
15.	R36	+	–
16.	R37	±	[1]

[a]A mixture of sera of rabbits immunized by peptides with sequences of 598–609 and 584–604 was used.

An Example

The assay was tested by the example of Abs in serum of immumized rabbits capable of recognition of the sequence 584–604 of HIV envelope protein gp41. As Ag, we used synthetic peptide corresponding to the sequence 584–604 of HIV envelope protein gp41, but its thiol group was protected by acetamidomethyl function (4). Synthetic or recombinant fragments of protein gp41 that entirely or partially include the 584–604 were used for immunization of rabbits.

Serum of immunized rabbits was diluted 100-fold and further analyzed by the assay developed. Solid-phase ELISA was used for analysis of the same sera with the same dilution. The results are shown in Table 1.

EXPERIMENTAL

Materials

Horseradish peroxidase was purchased from NPO Biolar, Latvia, and substrate, diaminobenzidine and *o*-phenylenediamine from Fluka and

Merck, respectively. Agarose and bovine serum albumin were from Serva, Tween-20 from Sigma (St. Louis, MO), and protein A was a product of Paster Institute of Experimental Medicine, St. Petersburg. Hydrogen peroxide, salts, acids, and bases were all from Reakhim (Russia), the highest purity grade available. Peptide corresponding to the sequence 584–604 of envelope protein gp41 was synthesized in Division of Chemistry of Joint Venture "Constanta."

Methods

The conjugate between the synthetic peptide and peroxidase was obtained using the standard periodate method (*6*). In order to obtain maximal modification of peroxidase, we used 17-fold molar excess of Ag. After finishing the reaction, unbound Ag was removed by dialysis.

An assay was carried out as follows. Molten agar gel (3%, w/w) in 0.1*M* phosphate buffer, pH 7.0, was placed in a Petri dish to give a layer of 5-mm width. Circular holes, 6 mm in diameter, were made in the gel, and a sample (0.1 mL) containing either serum diluted 100-fold or physiological solution as a control, and conjugate (20 μL, 2 μg/mL) were introduced. The Petri dish was covered to prevent a gel layer from drying, and gel was incubated at room temperature for 2 h. After the incubation, the gel was washed by distilled water, and a substrate solution containing 9 mL buffer, diaminobenzidine (1 mL, 5 mg/mL methanol), and aqueous solution of hydrogen peroxide (0.1 mL, 0.176*M*) was introduced. A clear picture of a colored circle appeared after 1 h of incubation. Nevertheless, for unambiguous treatment of the assay, 2 h of incubation are recommended.

An ELISA was performed as follows. Synthetic Ag sorbed on "Constar" polystyrene plates for EIA from a solution (1 μg/mL) in 0.05*M* carbonate buffer, pH 9.5, 4°C, during 18–20 h. After washing unbound Ag by sodium phosphate butter (0.01*M*) containing 0.3*M* NaCl and 0.05% (w/w) Tween-20 (PBS-Tween), 0.1 mL diluted (1:100) serum solution in PBS-Tween containing BSA (5 mg/mL) was placed in each hole in the dish and incubated for 30 min at 37°C. After being washed four times, 0.1 mL of solution of the conjugate of protein A with horseradish peroxidase in PBS-Tween containing BSA (5 mg/mL) was placed in a hole and incubated for 30 min at 37°C. After washing unbound conjugate, substrate solution (*o*-phenylenediamine, 0.4 mg/mL, hydrogen peroxide, 30%, in 0.05*M* citrate-phosphate buffer, pH 5.0, 0.4 μL/mL) was introduced, and after 15 min, the reaction was stopped by 10% (w/w) H_2SO_4. Analysis of data was carried out using a Multiscan photometer at 492 nm.

RESULTS AND DISCUSSION

Table 1 points to a major coincidence of results of analysis by solid-phase ELISA and the developed methods (entries 1–6, 8–12). Entries 1–7 also stress high specificity of the new method at the same level as ELISA.

Actually, for the synthetic Ag used for immunization (sequence 598–609) and for Ab detection (sequence 584–604), only the oligopeptide moiety containing seven amino acids residues is close to a minimal length of an epitope (*7,8*). Nevertheless, six of the seven sera analyzed by the method were detected. The sensitivity of the method was also comparable to that of solid-phase ELISA (data not shown).

Interesting results were obtained in the case of serum of rabbits immunized by recombinant protein (entries 13–16). The latter has a sequence 580–613, thus including the whole sequence of synthetic Ag used for detection of Ab. Nevertheless, negative results were obtained. Meanwhile, ELISA with the same Ag detected the presence of Ab. The inconsistency between the methods can be explained by the fact that in our case, the interaction proceeds in a homogeneous phase, whereas in ELISA, it proceeds in the interphase. It is known (*5*) that a transition from a homogeneous system to a heterogeneous one leads ofter to a substantial change in both thermodynamic and kinetic characteristics of a reaction between Ag and Ab. The same change of specificity is probably observed in entries 13–16 in Table 1. Ab against recombinant protein, a fragment of envelope glycoprotein gp41 (sequence 580–613), is "recognized" by synthetic Ag 584–604 in a heterogeneous procedure, but not in a homogeneous one.

To a definite extent, this is a drawback of the method proposed. Actually, small changes in the conformation of Ab against the recombinant protein in comparison with Ab against synthetic Ag 584–604 result in a dramatic decrease in the binding constant between synthetic Ag and the latter Ab. This substantially limits the application of synthetic low-mol wt Ag, for example, in diagnostic kits for detection of Ab against proteins.

At the same time, high specificity of the method in comparison with ELISA may be very valuable, especially for selective detection of Ab of a definite type in complex mixtures. It is now hard to imagine an area of application of the assay, if any, but its high specificity is more an advantage than a limitation.

CONCLUSION

A new variant of EIA has been developed that resembles the known method of "diffusion in gel" (*9*) by reacting Ab and Ag in a cell of agar gel to produce a circular colored zone. The resemblance is, however, purely superficial. In particular, the stage of sensibilization of a matrix is absent, and the reaction proceeds in a homogeneous phase. The way of separation of reacting and inert complexes in the reaction should also be mentioned as a serious distinction between the methods. In the previously known method (*9*), the separation was achieved at the expense of binding with a solid matrix, whereas in our method, it is achieved by differences in the rates of diffusion in a gel.

In terms of sensitivity and specificity, the assay is equal to ELISA. Among other advantages is the lack of complex equipment. Hence, the method is appropriate for operation in nonspecialized laboratories and field effect conditions. Among the limitations are long-running incubations and applicability for analysis of only Ab (not Ag) only if low-mol wt Ag is available. It is also probable that for a successful realization of the method, enzyme markers of relatively low molecular weight will be preferentially used, for example, peroxidase and laccase.

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