A HIGHLY SENSITIVE ELECTROIMMUNODIFFUSION METHOD OF ANTIGEN DETECTION ON CELLULOSE ACETATE FILMS

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A highly sensitive electroimmunodiffusion method of detecting antigens on cellulose acetate films is suggested. There are three stages: concentration of the antigen in a discontinuous buffer system on cellulose acetate films; detection of the antigen on the same films by immunodiffusion using a standard test system; staining the washed films with a protein stain to detect precipitation bands if the reaction takes place in the visible zone, or further treatment by "growing" precipitates with antiglobulin antibodies or by autoradiography. The method can detect nanogram amounts of α-fetoprotein and can be used to discover antigens of different molecular weight and electrophoretic mobility.

KEY WORDS: *α-fetoprotein; antigens; cellulose acetate films; electroimmunodiffusion.*

Immunodiffusion methods with high specificity have comparatively low sensitivity and cannot detect an individual antigen in a concentration under 1-3 μg/ml.

The writers previously suggested a method of electrophoresis and precipitation (EPAG) increasing the sensitivity of the double diffusion in gel method by 1000 times while preserving its specificity and resolving power [1]. The EPAG consists of three stages: concentration of the antigen in a discontinuous buffer system in polyacrylamide gel, determination of the antigen by immunodiffusion with maximally diluted test system in the same gel, and detection of invisible precipitated by ^{125}I-labeled antibodies against γ-globulin of the immune serum, followed by autoradiography.

In the method now described, instead of using polyacrylamide gel, it is suggested that the reaction be carried out on cellulose acetate film, therefore considerably simplifying the technique of the reaction and reducing the time taken for it by two thirds.

Films of gelatinized cellulose acetate (Cellogel, from Serva, West Germany) measuring 2.5 × 12 and 4 × 17 cm were used, and strips of the required size were cut from them. The work was done with human α-fetoprotein (AFP), using the "immunodiagnostic serum for primary liver cancer and teratoblastoma" produced by the N. F. Gamaleya Institute of Epidemiology and Microbiology. The AFP content in the antigen of the immunodiagnostic serum was 50-55 μg/ml. The antiserum was monospecific during immunodiffusion.

The ^{125}I-labeled antibodies against rabbit immunoglobulin were obtained from "Izotop" (Moscow). One working dose was diluted in 300 ml (0.013 mg antibodies in 1 ml, 0.4 mCi ^{125}I to 1 mg antibodies).

Antiserum against rabbit immunoglobulin was obtained by hyperimmunization of a donkey with rabbit IGG and was generously provided by P. Z. Budnitskaya. Rabbit antiserum against horse globulin was produced at the N. F. Gamaleya Institute of Epidemiology and Microbiology.

Buffer 1 was 0.06 M Tris with HCl to pH 6.7; buffer 2 was 0.012 M Tris with 0.19 M glycine, pH 8.3. The agar drops were applied by a 5-μl capillary microsyringe (Partigen Dispenser, from Behringwerke, West Germany).

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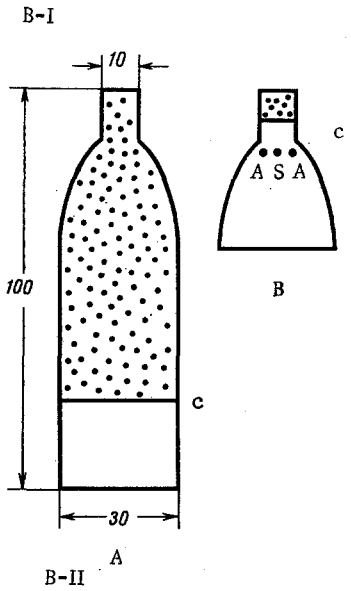

Fig. 1. Principle of electroimmunodiffusion. A) Concentration of antigen: B-I) buffer 1 (Tris-HCl, pH 6.7); B-II) buffer 2 (Tris-glycine, pH 8.3); c) zone of concentrate; B) detection of antigen by immunodiffusion: A) antigen of test system; S) antiserum of test system.

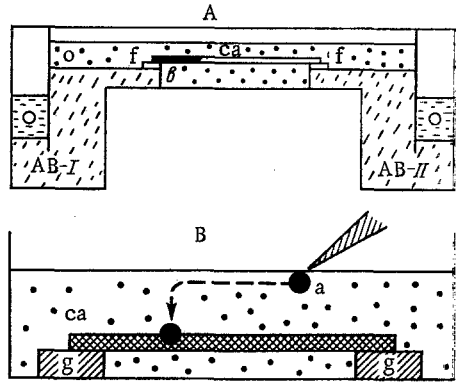

Fig. 2. Electrophoresis and immunodiffusion. A) Apparatus for electrophoresis: AB-I, AB-II) 1% agarose in buffer 1 and 2, respectively; ca) cellulose acetate film; f) filter paper soaked with buffer 1 and 2, respectively; o) mineral oil; B) immunodiffusion; a) agar drop; ca) film; g) glass supports.

Concentration of Antigen

The principle of the method is illustrated in Fig. 1. The first stage is concentration of the antigen. For this purpose strips of Cellogel measuring 10 × 3 cm are cut and immersed in a solution of the antigen made up in buffer 1, for not less than 30 min. Bromphenol blue (BPB) is added to the solution until the color is just detectable. The excess of fluid is removed from the strips, which are arranged around the edge of a petri dish. The strip is placed in an electrophoresis apparatus (Fig. 2A) under mineral oil. The edges of the films lie between strips of filter paper saturated with the corresponding buffer. Electrophoresis is carried out with a constant current of 0.2 mA per cm width of film. The voltage rises in the course of the experiment from 50 to 250-300 V. Concentration can be carried out overnight (15-16 h) at 30-35 V. Electrophoresis under a layer of mineral oil, which does not mix with the aqueous phase of the film, prevents it from drying and ensures uniform electrophoresis at a high potential gradient.

Immediately after the current is switched on a Kohlrausch boundary is formed and is clearly visible because of the concentration of the BPB in it. In this zone all proteins contained in the preparation are concentrated. Serum albumin migrates as a blue zone before the BPB, whereas hemoglobin forms a stained zone along the cathodal border of the BPB. Electrophoresis is stopped when the Kohlrausch boundary is 2-3 mm inside the narrow part of the film (Fig. 1B). After the end of electrophoresis the strip of Cellogel is cut out as shown in Fig. 1B.

Immunodiffusion

The film in oil is placed on moist filter paper and three hollows are made in it for the components of the test system (Fig. 1B). A plastic needle with curved end of a Plexiglas dye with ball bearings 2 mm in diameter mounted in it is used for this purpose. The film is then transferred to a petri dish filled with mineral oil and placed on glass supports so that the central part, where the reaction takes place, is not in contact with the bottom of the dish (Fig. 2B).

The antigen and serum of the test system are applied to the film as agar drops. To do this, the required dilution of antigen is mixed at 40-45°C with an equal volume of melted 1%

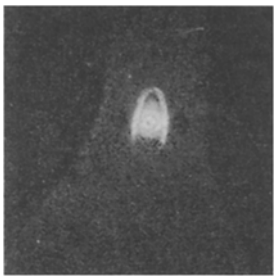

Fig. 3. Autoradiograph for detection of AFP. Test system 1:64, AFP 1 ng/ml. Exposure 1 night.

agar and 5 μl of the warm solution is transferred by a capillary syringe to the surface of the mineral oil. The drop remains in the suspended state, apparently fixed below to the surface of the mineral oil layer (Fig. 2B). From 3 to 5 min after the agar drop has solidified it is moved by a dissection needle to the required position and placed in the hollow of the film (Fig. 2B). The test system is spread over the film as shown in Fig. 1B. Introduction of the components of the test system as drops of gel prevents their rapid absorption into the film and ensures a gradual and uniform diffusion of antigen and antibodies from the "reservoirs" in the film. The dishes are allowed to stand overnight at room temperature. After the end of immunodiffusion the films are taken from the dishes, the oil is removed, and they are transferred to a jar with physiological saline. They are washed with stirring for 30-60 min depending on the quantity of protein in the concentration zone. The washed films are stained with Amido Black (according to the instructions provided by the firm for Cellogel), if the reaction takes place in the visible zone, or they undergo further treatment to reveal "invisible" precipitation bands.

Intensification of the precipitation bands was carried out by "growing" the precipitate with antiglobulin antibodies [2]. The washed strips of film were placed for 15 min in donkey antiserum against rabbit γ-globulin (1/10) and washed with buffered physiological saline (30 min). They were then again treated with antiserum (1/10) against horse γ-globulin (15 min), washed, and stained with Amido Black.

Detection of the precipitation bands by autoradiography was carried out by the method described previously [3], but with a reduction in the film processing time. Exposure in ^{125}I-labeled antibodies lasted 1 h at room temperature and the duration of washing with 5 liters physiological saline was about 2 h.

The washed films were cleared, dried by the method recommended by the firm for Cellogel, and exposed overnight on photographic film.

Determination of antigens in blood serum requires preliminary dialysis of the serum against buffer 1 and dilution 4-8 times with the same buffer before the experiment. An excess of BPB must be added to the serum to ensure complete staining of the albumin zone.

TABLE 1. Sensitivity of Different Forms of Electroimmunodiffusion

Method of revealing precipitate	Treatment of precipitate with antiglobulin	Maximal sensitivity of AFP, ng/ml				
		dilutions of test system				
		1:8	1:16	1:32	1:64	1:128
Staining	+	10	0	0	0	0
Autoradiography	+	10	5—2,5	0	0	0
	+	—	—	—	1	1

Legend. +) Successive treatment with donkey antirabbit antibodies and rabbit antibodies against horse globulin; 0) bands of test system not revealed; —) no determination carried out.

The sensitivity of the method, depending on the dilution of the test system, and the method of revealing the precipitate are given in Table 1. Determinations were carried out with a highly diluted preparation of AFP with negligible protein loading. Staining the precipitates reveals AFP with a sensitivity of 5-10 ng/ml, whereas autoradiography increases the sensitivity of the determination to 1 ng/ml or even less (Fig. 3).

This investigation thus showed that cellulose acetate films, especially the Cellogel type, are an ideal supporting medium for work with interrupted buffer systems and are not inferior to polyacrylamide gel. They enable antigens to be easily concentrated from very dilute solutions and antigens to be obtained in a compressed, highly localized zone. It is very important that antigens with different molecular weights should migrate easily in the films used. By carrying out electrophoresis under a layer of mineral oil the techniques of the work and design of the apparatus are greatly simplified, for the main difficulty of work with films — their irregular drying during the experiment — is prevented. Cellulose acetate films have long been used in immunodiffusion and counterimmunoelectrophoresis experiments [4].

The main difficulty in this case is irregular absorption of the reacting components into the film. The method of introducing reagents as agar drops suggested in this paper completely abolishes this difficulty also. The enormous advantage of films is that they are quickly and easily washed to remove unreacted proteins. This means that all the stages of processing of the precipitates can be completed in a few hours and the total duration of the analysis reduced from 7 to 2 days.

Performance of the EPAG method on cellulose acetate films thus produces a simple, rapid, and highly reproducible immunodiffusion test with a sensitivity several thousands times greater than that of the standard immunodiffusion reaction yet completely preserving all its advantages.

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RADIOIMMUNOELECTROPHORETIC DETERMINATION OF α-FETOPROTEIN. CHARACTERISTICS OF STANDARD INHIBITION CURVES

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An express method of radioimmunological determination of mouse and human α-fetoprotein using electrophoretic fractionation of immunologically bound and free antigen is suggested. The method is comparatively simple in use and enables a large series of samples (up to 40) to be analyzed simultaneously within a short time (5 h). The behavior of the antibody–antigen system during plotting of standard inhibition curves was analyzed. Inhibition of the antibody–antigen reaction was found in the zone of low and high concentrations. The effect described calls for further study.

KEY WORDS: *α-fetoprotein; radioimmunoelectrophoresis; express method.*

In recent years the radioimmunological method has been extensively used in biology and medicine. Among its more important advantages are specificity and high sensitivity, so that

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