ULTRAMICROMETHOD FOR DETECTING ANTIGENS

G. I. Abelev and S. D. Perova

UDC 576.8.097.2.07

An ultramicromethod for determining α-fetoprotein (AFP), capable of detecting as little as 10^{-8} mg AFP in a concentration of (1-2) · 10^{-6} mg/ml, is suggested. The antigen is concentrated in a capillary tube by electrophoresis in polyacrylamide gel. The concentrated zone migrates into the slab of gel containing antiserum. After free diffusion of the antigen, precipitation rings are formed and their area is proportional to the quantity of antigen. The precipitates are revealed by treatment with I^{125}-antibodies against immune serum γ-globulin followed by autoradiography.

KEY WORDS: α-fetoprotein; ultramicromethod of detecting antigens.

The most sensitive immunodiffusion method for determining antigens is electrophoresis–precipitation in polyacrylamide gel (EPPG) [1]. To the sensitivity of the radioimmunological test, EPPG adds the specificity of immunodiffusion tests. In this paper the writers suggest a microvariant of the EPPG capable of detecting minimal absolute quantities of antigen (of the order of 10^{-8} mg) in a concentration of 10^{-5}-10^{-6} mg/ml and in a volume of 1-20 μl. The method is suitable for the determination of human and animal α-fetoprotein (AFP).

The principle of the method is illustrated in Fig. 1. The antigen, polymerized in concentrated polyacrylamide gel (PAG), is introdued into a capillary tube (Fig. 1, I). On electrophoresis in a stepwise system of buffers the antigen is concentrated and, condensed into a "microdrop," it migrates from the capillary tube into a flat PAG slab mixed with monospecific antiserum (Fig. 1, IV). Electrophoresis is stopped immediately after migration of the antigen, which diffuses freely into the gel to form precipitation rings just as in Mancini's method of linear diffusion in gel [4]. The area of the ring is proportional to the quantitiy of antigen in the "microdrop." To detect minimal quantities of antigen, very high dilutions of serum which form invisible precipitation rings are used. These precipitates are detected by treating the gel with I^{125}-antibodies against immune serum γ-globulins followed by autoradiography [3, 5].

The capillary tubes used in the work had an internal diameter of 0.5 mm, external 1 mm, length 35 mm, and volume 7 μl (Partigen Dispenser, Behringwerke, West Germany). In some experiments capillary tubes with volumes of 1, 2, 3, and 20 μl (Labora, Mannheim, West Germany) were used. The chamber for electrophoresis consisted of a glass slab (9 × 12 cm) with side pieces 1.5-2 mm high glued around it. A plexiglas lid with slots for the side pieces and with gutters for the capillary tubes is placed on the glass slab. The depth of the slots determines the width of the space thus formed, namely 0.5-0.6 mm. The 20 gutters for capillary tubes consist of semicylindrical hollows in the plexiglas lid 0.5 mm deep, 1 mm wide, and 30 mm high. The lid is smeared with sealing compound to make an airtight joint with the sides and secured with plexiglas screw clamps. The lower part of the chamber is covered with adhesive tape. The chamber filled with the gel is placed in a vertical electrophoresis apparatus of any design.

The concentrating gel consisted of 4% Cyanogum 41 (Serva) in tris–HCl buffer [0.06 M Tris, HCl to pH 6.7 (buffer I)] with the addition of TEMED, and also bromphenol blue (BPB) and mouse hemoglobin (Hb)

Laboratory of Immunochemistry of Tumors, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR O. V. Baroyan.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 79, No. 6, pp. 120-123, June, 1975. Original article submitted November 15, 1974.

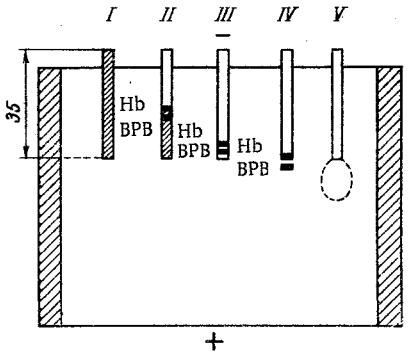

Fig. 1

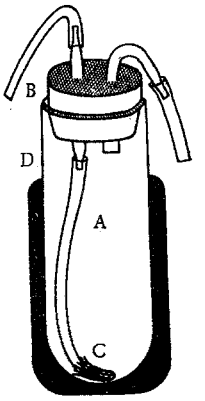

Fig. 2

Fig. 1. Scheme showing principles of method: I) capillary tube filled with antigen in concentrating gel; II) concentration of antigen during electrophoresis; upper limit of reference standards bromphenol blue (BPB) and mouse hemoglobin (Hb) can be seen; III) moment of stopping electrophoresis on transferring capillary tubes to a new chamber; IV) position of reference standards when electrophoresis stopped in second chamber; V) diffusion of antigen into gel containing antiserum and formation of precipitation ring.

Fig. 2. Vessel for labeling and washing gel slab: A) centrifuge jar with capacity 25-30 ml; B) teflon tube 1.2 mm in diameter through which labeled antibodies and washing solution are supplied and withdrawn; C) teflon tube with end covered with capron gauze to prevent damage to gel; D) lead cylinder.

as reference standards. The concentrations of BPB and Hb were chosen empirically in order to give clearly visible zones during migration in the capillary tube. Immunodiffusion was carried out in large-pore gel made up in buffer I in the following ratios: 30.5% acrylamide (10 ml)* + 1% N,N'-methylene-bisacrylamide (10 ml)* + 38 ml buffer I + 0.1 ml TEMED + 75 g sucrose + the necessary quantity of antiserum and ammonium persulfate in a concentration of 1 mg/ml [6].

Monospecific sera against human and mouse AFP [2] and AFP-containing material with a known content of AFP were used. To determine the sensitivity of the method a series of double dilutions were prepared in a solution of concentrating gel containing bovine serum in a dilution of 1:400, with an antigen concentration of between 10 μg/ml and 0.1 ng/ml. Washing the slabs of gel to remove unbound protein was carried out either mechanically [1] or electrophoretically. Electrophoretic washing was carried out in a block of 5.6% PAG consisting of two parts: PAG in buffer I (anodic part of the block) and PAG in tris–glycine buffer II (Tris 0.012 M, glycine 0.19 M, pH 8.3; cathodic part). The height of the block was 12-14 mm. Slabs of gel were placed on a transverse section through the block, 2-3 mm on the anodic side of the boundary between the gels. For this purpose, a strip 2-3 cm wide was cut out of the block. A cut was made at the required distance from the boundary of separation, the block was divided, and slabs of PAG to be washed were placed on the free section of the gel. The parts of the block were then moved together, the excised strip of gel was replaced and, in this way, the continuity of the block was restored. During electrophoresis (about 2 V/cm) a Kohlrausch boundary was formed at the boundary between the buffers, and as it passed across the slabs of gel toward the anode it "extracted" all the proteins from them irrespective of their electrophoretic mobility. For convenience of observation of the moving boundary, BPB was added to the gel of the anodic part of the block before it was polymerized. Electrophoresis was stopped when the moving boundary of BPB was 15-20 mm from the slabs of gel to be washed, which usually happened after about 2 h.

The precipitates were labeled in a glass vessel (Fig. 2) with purified monospecific sheep's antibodies against rabbit γ-globulin, labeled with I^{125} (13 μg antibodies in 1-ml 0.6-mCi I^{125} in 1 mg [1]). Antibodies were added to the vessel under slight pressure and were withdrawn after incubation by means of a low vacuum.

*In buffer I.

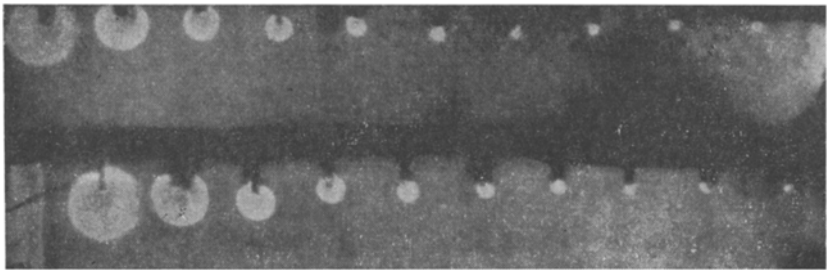

Fig. 3. Autoradiographs of precipitation rings of mouse and human α-fetoprotein (AFP). Mouse AFP in dilutions of 500 to 1 ng/ml. Antiserum in dilution of 1:1200. Capacity of capillary tube 7 μl. Electrophoretic washing at all stages. Exposure 15 h.

Conduct of the Experiment. Ammonium persulfate was added to the concentrating gel solution containing the antigen immediately after the capillary tube was immersed in it so that the solution was drawn up inside the tube. After polymerization the upper end of the capillary tube was cut off so as to remove the top meniscus of solidified gel. The capillary tubes were transferred to a chamber where they were held in the corresponding gutters. The chamber was first filled with 5.6% gel in buffer I, which was polymerized after immersion of the capillary tubes in it. The electrode vessels were filled with buffer II. Bubbles were carefully removed from the top parts of the capillary tubes. Electrophoresis was carried out at 6-8 V/cm and with a current of about 10 mA; under these conditions BPB and Hb were concentrated in the capillary tube into clearly visible and rapidly migrating zones. The mobility of these zones varied a little in different capillary tubes and for that reason electrophoresis was stopped whenever the BPB approached the open end of one of the capillary tubes, and that tube was transferred to the moist chamber.

After the end of the first stage of electrophoresis, the second chamber, identical in construction and dimensions with the first, was filled with a solution of large-pore gel containing antiserum. All the capillary tubes were placed in the as yet unsolidified solution, after which bubbles were carefully removed from the gel and, in particular, from the lower ends of the capillary tubes, for the gel does not polymerize at the boundary with the air. After polymerization of the gel, electrophoresis was continued, during which the reference standards migrated simultaneously from the capillary tubes. Electrophoresis was stopped when Hb migrated from the capillary tube. With the recommended ratio between buffers in the capillary tube and the slab of gel, the rate of migration of proteins from the capillary tube was retarded and the zone of antigen was slightly compressed, forming a compact ring.

The chamber in the assembled form was placed in an exsiccator for 2 days at room temperature or for 20 h at 37°C. A thin slab of gel does not dry under the lid and free diffusion of antigen is undisturbed. After incubation the lid of the chamber was removed and a strip of gel about 10 mm wide, containing rings of precipitates, was carefully cut out. The strip was transferred to gauze for mechanical washing or into a block of gel for electrophoretic washing. The washed slabs were placed in a vessel for treatment with labeled antibodies for 1 h at room temperature and were then left overnight at 4°C. After labeling the vessel was connected to a 10-liter bottle for continuous washing for 15-18 h. To speed up the process of washing out the labeled antibodies, the slabs were washed in the vessel for 1 h with a fast current of physiological saline, after which electrophoresis was carried out as described above. Slabs of gel, flooded with 1% agar and covered with moist filter paper, were dried under a fan and exposed overnight on RF-3 film [1]. By incubation at 37°C and with the use of electrophoretic washing, the results could be obtained after 3 days.

Determination of AFP. Human and mouse AFP were detected in concentrations down to 1-2 ng/ml by the use of capillary tubes with capacities of 7 and 2 μl. The absolute quantities of AFP were down to 5-15 pg respectively per sample. On detection of the precipitation rings by staining the gel with Amido Black, visible precipitation rings were found only with antiserum in dilutions of 1:25 and 1:50, but when autoradiography was used, they were found in dilutions down to 1:4000 and 1:8000, with a proportional increase in the sensitivity of the determination. Dilutions of 1:1000 and 1:2000 were optimal for the work (Fig. 3). The areas of the precipitation rings, calculated from their diameters, were expressed as ratios of each other in consecutive double dilutions of antigen, with approximation toward 2. For instance, calculations for 11 independent experiments gave a ratio of 1.88, with variation in individual experiments from 1.61 to 2.25. Deviations from two increased with a decrease in the diameters of the rings.

The authors are grateful to A. K. Yazova for providing the preparations of AFP and anti-AFP for the experiments, to D. A. Él'gort for labeling the antibodies with I^{125}, and to O. A. Muratova for technical assistance. The work was partly subsidized by the immunologic division of WHO.

LITERATURE CITED

1. G. I. Abelev, Byull. Éksperim. Biol. i Med., No. 11, 122 (1973).
2. A. I. Gusev and A. K. Yazova, Byull. Éksperim. Biol. i Med., No. 4, 120 (1970).
3. D. A. Él'gort and G. I. Abelev, Byull. Éksperim. Biol. i Med., No. 2, 118 (1971).
4. G. Mancini, Q. Carbonara, and J. Heremans, Immunochemistry, 2, 235 (1965).
5. D. Rowe, Lancet, 1, 1340 (1970).
6. O. Vesterberg, Sci. Tools, 20, 22 (1973).