# IMMUNOAUTORADIOGRAPHIC DETERMINATION OF FETAL AND HUMAN α-FETOPROTEIN

D. A. Él'gort and G. I. Abelev

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A modified immunoautoradiographic method is proposed which increases by 16-32 times the sensitivity of the gel-diffusion reaction. The method combines high sensitivity with absolute specificity. By the use of this method, human α-fetoprotein was detected in a concentration of 0.1-0.05 μg/ml. It was thus possible to discover this protein in the sera of pregnant women, in a high proportion of sera from patients with hepatomas giving a negative result in the ordinary gel-diffusion test, and in certain other cases. The method is universally applicable to all proteins giving precipitation in agar.

α-Fetoprotein (α-fp) is a specific protein found in the serum of human and animal embryos. The specific immunochemical test for primary carcinoma of the liver [8, 4] and for certain teratoblastomas [4, 12] is also a test for α-fp in patients' sera.

The agar diffusion test is usually used to determined α-fp, for in this way it can be identified in complex systems, e.g., in blood serum or tissue extracts. However, the sensitivity of this method is not very high, and α-fp cannot be detected in concentrations below 1-2 μg/ml. For experimental research and the early diagnosis of carcinoma of the liver, it is important to increase the sensitivity of the tests. Recently, Olovnikov and Tsvetkov [7] have proposed an aggregate-hemagglutination method for this purpose. This greatly increases the sensitivity of the test, but deprives it of the resolving power possessed by the agar precipitation test. An immunoautoradiographic method of determining α-fp combining high sensitivity with absolute specificity is now suggested.

## EXPERIMENTAL METHOD

The essence of the method is detection of an invisible precipitate by means of radioactive labeled antibodies against the antibodies forming this precipitate. The invisible precipitate, once it has become radioactive, gave an image on x-ray film. This method, previously suggested by Rowe [13] for simple agar diffusion, was applied to double gel diffusion and used to determine α-fp.

The test system, consisting of monospecific rabbit antiserum α-fp and of α-fp itself, was diluted 8, 16, and 32 times. However, the precipitation band formed in agar is invisible. The agar plates were washed to remove nonreacting substances and treated with antibodies against rabbit γ-globulin, labeled with $I^{131}$ or $I^{125}$. After all unbound radioactivity had been removed by washing, the plates were dried and kept for 1-7 days in contact with photographic film. The invisible radioactive precipitate present in the agar gave an image on the film.

A test system of human α-fp, generously provided by S. D. Perova, was used. This test system has been described by Abelev et al. [4]. Ascites fluid of a patient with primary carcinoma of the liver is its antigen, and the serum of a rabbit immunized with human fetal serum and exhausted by donor's serum is its antiserum. This test system was diluted 8, 16, and 32 times by comparison with the optimal dilution

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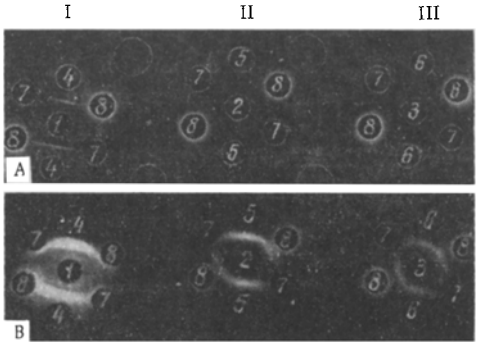


Fig. 1. Determination of $\alpha$-fp in serum by ordinary precipitation and by immunoautoradiography: A) stained preparation; B) autoradiograph of same preparation; I) test system for ordinary precipitation; II) same test system diluted 1:8; III) same test system diluted 1:32; 1) antiserum of test system (AS); 2) AS in dilution 1:8; 3) AS in dilution 1:32; 4) antigen of test system (AG); 5) AG in dilution 1:8; 6) AG in dilution 1:32; 7) serum for testing; 8) physiological saline.

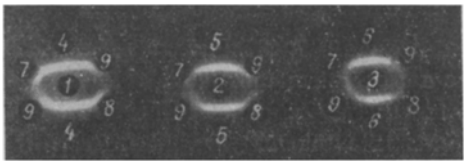

Fig. 2. Increase in the sensitivity of $\alpha$-fp detection by concentrating the test serum. Autoradiograph: 1) AS diluted 1:8; 2) AS diluted 1:16; 3) AS diluted 1:32; 4) AG diluted 1:8; 5) AG diluted 1:16; 6) AG diluted 1:32; 7) test serum; 8) test serum concentrated by means of Lifogel (30 mg Lifogel added to 0.2 ml serum); 9) physiological saline.

for ordinary precipitation. The sample to be investigated was tested with each of the three test systems. During the work, strict watch had to be kept to ensure optimal ratios between antigen and antiserum in the test system. These ratios may alter slightly as the invisible zone is entered, so that before the test material is examined, optimal dilutions of the components of the test system must be selected and the dilution at which the invisible precipitate still remains in the agar and gives an image on the film must be determined. To obtain more reliable results, not only the last dilution of the test system, but the two preceding dilutions, were used.

Antibodies against rabbit $\gamma$-globulin were obtained from ass antiserum by the use of an immunosorbent. This was $\gamma$-globulin, polycondensed with glutaraldehyde [10]. Either $I^{125}$ (Amersham, England) or the Soviet $I^{131}$ were used for labeling. The method of iodination used was based on the use of ICl as iodine carrier [11]. Antibodies (4 mg) were labeled with 5-10 mCi iodine. Unbound iodine was removed by dialysis. The resulting preparation was made up to 100-200 ml with borate buffer, pH 8.0. Normal horse serum was added to the buffer (2 ml to 100 ml buffer) as carrier protein to prevent self-irradiation of the preparation, and merthiolate was added as antiseptic.

The test was carried out as follows. A layer consisting of 3 ml 2% Difco agar in physiological saline was applied to a slide. The precipitation test was carried out by the standard method [6], but the diluted test system was used. Wells for reagents were 3 mm in diameter and the distance between the wells 3 mm. The slides were kept in a humid chamber for 2-3 days and then washed. For rapid washing the agar plate was transferred to a Kapron sieve, stretched over a special frame, and fixed with soft rubber rings [1]. The physiological saline was rinsed off with phosphate buffer, pH 7.0, with stirring overnight. After washing, the sieve with the agar plate was immersed for 1 h in the radioactive antibodies and left overnight in the humid chamber. It was then washed again for 24 h to remove unbound radioactivity, just as in the preceding stage. The agar plate was again transferred to a slide, fixed in 80% ethanol for 2 h to remove salts, and then covered with filter paper and dried in an incubator at 50°C. Fluorographic film was pressed against the dried film by means of a clean slide and rubber rings, with the emulsion toward the agar, the whole specimen was wrapped in black paper, and then kept in a dark room for 1-7 days. The film was developed with high-contrast D-11 developer. The technique of immunoautoradiography has been fully described previously [2].

A further increase in sensitivity was obtained by preliminary concentration of the test specimens with Lifogel. To 0.2 ml of the specimen, 30 mg of Lifogel (Gel Manuf. Co., England) was added and the specimens were kept in a refrigerator overnight. In this way the protein concentration was increased by 3-4 times.

## EXPERIMENTAL RESULTS

The method described above increased the sensitivity of double diffusion in gel by 16-32 times. Whereas $\alpha$-fp could be detected by ordinary precipitation in agar in a concentration of 2-4 $\mu$g/ml, the same

protein could be detected by autoradiography in concentrations of 0.06-0.126 μg/ml. The results of a typical experiment are shown in Fig. 1.

As a result of the preliminary concentration of the test sample by 4 times, α-fp could be detected in a concentration of 0.0125-0.032 μg/ml (Fig. 2).

This sensitivity was sufficient to enable solutions to be found for a number of problems which had proved insoluble before this experimental approach was available.

One such problem is that of the "$\alpha_f$-negative" hepatomas, i.e., hepatomas not producing α-fp [4]. It was not known whether the difference between $\alpha_f$-negative and $\alpha_f$-positive hepatomas is qualitative or due to quantitative differences in α-fp production. In tests of $\alpha_f$-negative sera from patients with hepatomas, more than 40% of them were found to be $\alpha_f$-positive. Another example of the successful use of this method was the detection of α-fp in the blood of pregnant women. This protein can, of course, be detected in the blood of pregnant mice by precipitation in agar [3], but it cannot be found in human blood under these conditions. By the use of the autoradiographic method, α-fp was found in the sera of all pregnant women after the 15th week.

This method can evidently be applied to all proteins giving precipitation in agar. For instance, the writers have used it successfully to study group-specific leukemic antigen in mice and were able to detect this antigen in healthy mice of various lines [5].

The method can also be used to determine whether a precipitating factor which has been found belongs to the γ-globulin class, i.e., whether an observed reaction in fact is an antigen–antibody reaction. It can be used, evidently, for immunoelectrophoretic analysis. The writers are grateful to Dr. Rowe (WHO Immunoglobulin Reference Laboratory) for information about his method before publication.

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