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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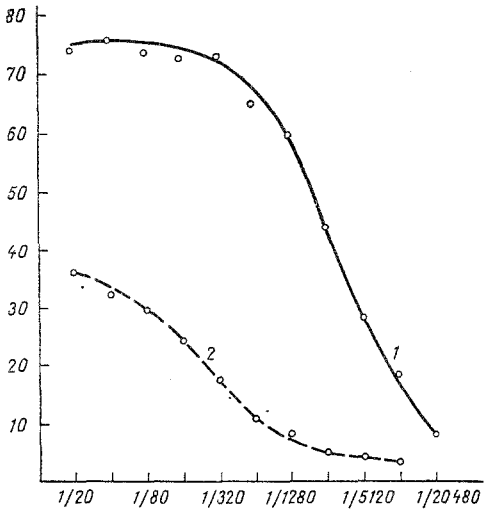

Fig. 1. Titration curves. 1) Titration curve for mouse AFP. To a series of successive dilutions of antiserum about 25 ng mouse ^{125}I-labeled AFP was added; a dilution of 1/300 was chosen for plotting the standard inhibition curve. 2) Titration curve for human AFP. To a series of successive dilutions of antiserum about 40 ng human ^{125}I-labeled AFP was added; standard inhibition curves were plotted for dilution of 1/100 and 1/60. Ordinate: here and in Figs. 2 and 3, percentage of binding; abscissa, dilution of antiserum.

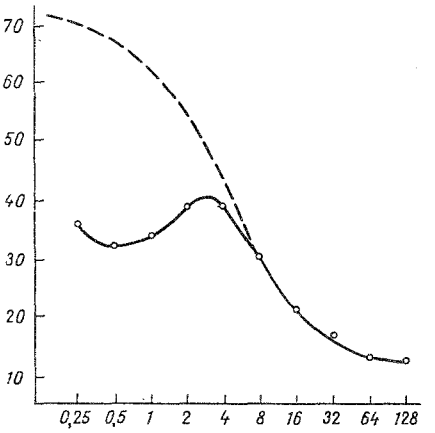

Fig. 2. Standard inhibition curve for mouse AFP. Broken line shows usual shape of calibration curves. Abscissa, quantity of unlabeled mouse AFP (in mg/20 µl).

proteins can be determined quantitatively within a range of concentrations down to 10^{-9} g/ml. The method is being increasingly applied in oncologic practice for the diagnosis and assessment of the clinical dynamics of various tumors by tests using immunological markers of tumors. Many variants of the method differing in ways of separating the antigen–antibody (AG–AB) complexes from free AG are known: salting out with ammonium sulfate, the double antibody method, the use of immunosorbents, and so on [3-5].

This paper describes an express method of radioimmunological determination of α-fetoprotein (AFP), a marker of hepatocellular carcinoma and teratoblastomas, based on electrophoretic fractionation of free and bound AG in agarose gel. The method was developed for clinical use for the following reasons: its comparative simplicity in use, and the possibility of rapid and simultaneous analysis of a large series of samples.

Most investigators have given no details of the behavior of the AG–AB system in the radioimmunological method outside the zone of the linear region of the standard inhibition curve, although in some radioimmunological systems this curve rises to a clearly defined peak [3].

Results obtained for mouse and human AFP are described below.

EXPERIMENTAL METHOD

Antigens and Antisera

Purified preparations of mouse and human AFP and antisera against them were generously provided by A. I. Gusev and A. K. Yazova (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR). Antigens isolated by disk electrophoresis in polyacryalmide gel [1] contained only traces of antigenic impurities. Antiserum against

mouse AFP contained traces of AB against a component of adult mouse serum. A monospecific rabbit antiserum from the immunodiagnostic kit for primary carcinoma of the liver and teratoblastoma (series 4), produced by the N. F. Gamaleya Institute, was used as the antiserum against human AFP.

Iodination of the Antigen

Mouse and human AFP were labeled with ^{125}I with the aid of chloramine T [2]. After iodination, the labeled AG was separated from free ^{125}I by gel filtration on Sephadex G-25. Fractions containing labeled AG were pooled and dialyzed against 0.05 M phosphate buffer, pH 7.3. To dilute the AG an 0.5% solution of bovine serum albumin (BSA) in the same buffer was used. The specific radioactivity of the preparation was $4 \cdot 10^6$ cpm/μg for mouse AFP and $8 \cdot 10^5$ cpm/μg for human AFP.

Separation of Immunologically Bound and Free Antigen

Electrophoretic fractionation was carried out in 1.1% agarose gel in an apparatus made from transparent plastic specially for this purpose. The apparatus consists of an electrophoresis slab with parallel grooves isolated from each other and a die for making wells. Each compartment of the apparatus is filled with liquid agarose. After polymerization of the gel, the die was carefully removed and a series of standard wells, the bottoms of which were filled with gel, was formed in the agarose. By using a die, standard conditions for starting and for electrophoresis of a series of samples were ensured. After incubation of AG with the corresponding serum, part of each sample was introduced into a well and electrophoresis carried out in veronal–medinal buffer, pH 8.6 (ionic strength 0.05) at 80 V (the strength of the current under these circumstances was 50 mA) for 1 h. To determine the mobility of the immunoglobulins pyronine was used. The position of free ^{125}I-labeled AFP on the electrochromatogram was determined from the position of BSA, stained with bromphenol blue. Each electrochromatogram was cut into two fractions: the cathode end, corresponding to the position of the immunoglobulins, and the anode end, corresponding to the position of free ^{125}I-labeled AFP. The radioactivity of each fraction was measured in a Nuclear Chicago gamma counter and the percentage of immunologically bound ^{125}I-labeled AFP calculated.

Titration of Antiserum

To each of a series of samples containing 20 μl antiserum against AFP in successive dilutions from 1:20 to 1:20,480, 20 μl of the ^{125}I-labeled AFP solution was added. The mixture was incubated at 37°C for 1 h, after which the samples were subjected to electrophoresis and the percentage of immunologically bound AG determined. The titration curves are shown in Fig. 1.

Plotting Standard Inhibition Curves

When plotting the inhibition curves for mouse AFP, serial dilutions of unlabeled mouse AFP in doses of 0.25–128 ng in 20 μl were added to a series of samples each containing 20 μl

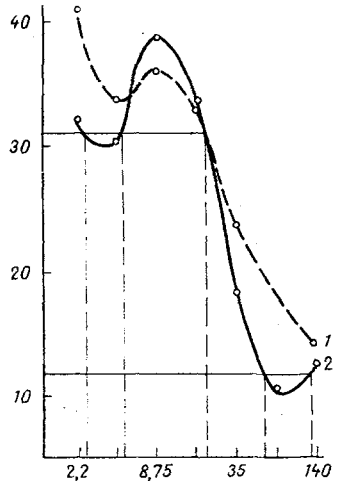

Fig. 3. Standard inhibition curves for human AFP. 1) Standard inhibition curve for antiserum diluted 1/100; 2) diluted 1:60. Several values of concentration equivalent to the same experimental value for the percentage of bound labeled AG are indicated in this figure. Abscissa, quantity of unlabeled human AFP (in mg/20 μl).

antiserum in a standard dilution of 1/300; the samples were incubated at 37°C for 1 h, after which 20 µl of mouse ^{125}I-labeled AFP (about 25 ng) was added to each sample and incubation was continued for a further hour, after which the percentage of immunologically bound mouse ^{125}I-labeled AFP was determined. It will be clear from Fig. 2 that the linear region of the inhibition curve corresponds to a narrow range of concentrations of unlabeled AG. In the region of low concentrations of AG, a zone of inhibition of the AB–AG reaction was observed, i.e., a decrease in the percentage of bound labeled AG.

Standard inhibition curves for human AFP (Fig. 3) were plotted for dilutions of antiserum of 1/100 and 1/60 and a range of concentrations of unlabeled AG from 2 to 140 ng in 20 µl. These curves also had a linear region, but in the region of low concentrations the zone of inhibition of the AB–AG reaction was narrow.

Standard inhibition curves for the two related antigens mouse and human AFP, plotted for a wide range of concentrations, revealed the same distinguishing feature: a zone of inhibition of the AB–AG reaction in the region of low AG concentrations. The wave-like shape of the curves differs significantly from the shape of the ordinary calibration curves. A similar disturbance of the uniform course of the curves also was observed in the zone of high AG concentrations (Fig. 3). On electrophoretic separation of AG bound with AB from free AG, one explanation of the results could be the formation of AB–AG complexes of different composition, with different solubility (electrophoretic mobility) depending on the AB/AG ratio in the original mixture. Another explanation may be that during the binding of one combining site of AB by AG, in the zone of a large excess of AB the affinity of a second combining site for AG rises sharply [3]. This could be the cause of formation of the peak of binding of labeled AG when the inhibition curve comes out on a plateau. A similar pattern also was observed in the standard method of performing the radioimmunological test [3].

When the radioimmunological method is used under experimental and clinical conditions, allowance must evidently be made for the behavior of the AB–AG system, for several values of the concentration of AG determined in the sample, differing by almost an order of magnitude, may correspond to the same experimentally determined value of the percentage of bound labeled AG (Fig. 3).

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