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Radioimmunoassay for Human Alpha$_1$-Fetoprotein

(cancer/diagnosis/hepatoma/hepatitis/embryonic)

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ABSTRACT **A method of radioimmunoassay has been developed for the quantitation of alpha$_1$-fetoprotein in human serum. The assay requires 20 μl of serum, can be completed in 8 hr, and can reproducibly detect concentrations of 20 ng of alpha$_1$-fetoprotein per ml of serum. Hence, the method is about 500-fold more sensitive for the detection of alpha$_1$-fetoprotein than the Ouchterlony technique currently in general use. The procedure is of potential clinical value as an aid in the diagnosis of cancer and a number of noncancerous hepatic diseases.**

In 1963, Abelev reported that some chemically-induced mouse hepatomas synthesized an alpha$_1$-globulin that was absent from normal adult mouse tissues. This constituent was, however, present in the tissues and sera of embryonic and neonatal mice (1). Subsequently, the alpha$_1$-globulin associated with mouse hepatomas was also detected in the sera of mice without liver tumors during the process of liver regeneration after partial hepatectomy (2). A similar alpha$_1$-globulin component was found by Tatarinov in the sera of patients with primary hepatomas, and in the sera of human fetuses and newborn infants (3). This material has been named human alpha$_1$-fetoprotein (AFP).

With use of the Ouchterlony technique for the detection of AFP as an aid in the diagnosis of primary liver tumors, the frequency with which positive results have been obtained has varied from less than 50% to over 80% in different areas of the world (4). The demonstration of such variability has led to the suggestion that there may be etiologic and/or pathogenetic differences in the development of hepatomas in populations in different geographic locales (5). However, the failure to detect AFP by immunoprecipitation in gel media in certain patients with hepatoma may simply reflect the limited sensitivity of the procedure (6). This possibility is, in fact, supported by data from recent studies that used different technology for the detection of human and rat AFP (7–9).

The objectives of the present investigation were, therefore, to develop a sensitive and quantitative radioimmunoassay for the detection of AFP in human serum, and to perform a preliminary assessment of this procedure as an aid in the diagnosis of human disease.

MATERIALS AND METHODS

Preparation of Monospecific Anti-AFP Antiserum. 10 to 20-Week-old fetuses, obtained at therapeutic abortion, were the source of human fetal serum. Antiserum prepared against fetal serum was produced by repeated intramuscular injection of goats with human fetal serum emulsified in Freund's complete adjuvant. In order to render the antiserum monospecific for AFP, it was absorbed with serum, from a normal adult male, that had been lyophilized and dialyzed against water, at a concentration of 200 mg of lyophilized serum per ml of antiserum. After absorption, the antiserum was centrifuged at 40,000 × *g* for 20 min. This preparation was denoted as anti-AFP antiserum.

Purification of Human AFP was accomplished by the technique of Nishi (10), as modified by Silver *et al.* (11). The method involves the immunoprecipitation of AFP from fetal serum with goat anti-AFP antiserum, and the subsequent acid dissociation and separation of α_1-fetoprotein–antibody complexes by gel chromatography. The details of the procedural modifications have been extensively described by Oakes *et al.* for the purification of rat AFP (9).

Specificity and Purity of the Anti-AFP Antiserum and AFP. The specificity of the anti-AFP antiserum was determined by the Ouchterlony technique, immunoelectrophoresis, and radioimmunoelectrophoresis. The purity of the final AFP preparation was assessed by the Ouchterlony technique and by radioimmunoelectrophoresis; high-resolution Singul-X x-ray film was used for the latter procedure (A. B. Ceaverken, Strangas, Sweden). In addition, the capacity of anti-AFP antiserum to precipitate [^{125}I]AFP in 50%-saturated ammonium sulfate was used as another indication of AFP purity.

Radioimmunoassay for AFP. Purified AFP was radiolabeled with ^{125}I by the chloramine-T method (12). [^{125}I]AFP used in the radioimmunoassay was diluted in normal adult male human serum, or 3% bovine gammaglobulin, both of which were diluted 1:100 in borate buffer (0.05 M, pH 8.6). The solutions of [^{125}I]AFP used in the assay were prepared so that a 500-μl aliquot contained 10,000–20,000 cpm. Since the specific activity of [^{125}I]AFP was 20–40 μCi/μg, each 500-μl aliquot of [^{125}I]AFP contained 1–2 ng of AFP. Solutions containing known amounts of purified AFP, used in obtaining standard inhibition curves, were prepared in either 3% bovine gammaglobulin or undiluted normal human serum. Anti-AFP antiserum used in the assay was diluted in either normal human serum or 3% bovine gammaglobulin diluted 1:20 in borate buffer. AFP was then radioimmunoassayed by the technique of coprecipitation-inhibition in 50%-saturated ammonium sulfate previously described by this laboratory for the radioimmunoassay of carcinoembryonic antigen (13).

AFP Determination in Human Sera. Sera for testing were collected and stored at 4° until assayed. Concentrations of

Abbreviation: AFP, α_1-fetoprotein.

circulating AFP were determined, on the basis of a double-blind design, in the sera obtained from 110 patients having one of the following clinical and/or histopathologic diagnoses: hepatoma, embryonal carcinoma of the testis, nonmalignant hepatobiliary disorders, or other neoplasms. Control sera were obtained from 17 healthy adult laboratory personnel of both sexes, and from 33 patients admitted to the orthopedic ward of the Montreal General Hospital for traumatic fractures without clinical or laboratory evidence of major organ dysfunction.

RESULTS

Specificity of Anti-AFP Antiserum. The anti-AFP antiserum used was monospecific by the Ouchterlony technique and by immunoelectrophoresis. The antiserum failed to react in the Ouchterlony plate with normal human serum, but produced a single precipitin line with human fetal serum (Fig. 1). Furthermore, the precipitin line that developed against fetal serum gave a reaction of identity with serum obtained from a patient with hepatoma. On immunoelectrophoresis, the anti-AFP antiserum reacted with a single constituent of fetal serum that had the electrophoretic mobility of an alpha-globulin (Fig. 2).

Purity of AFP. The purified AFP preparation, which gave a single intense line with anti-AFP antiserum by the Ouchterlony technique, did not react with anti-normal human serum or anti-goat gammaglobulin antisera. The purified preparation was examined by the more sensitive technique of radioimmunoelectrophoresis (Fig. 2). Fetal serum was used as a carrier for the [^{125}I]AFP in this procedure. With an antiserum directed against human fetal serum, the immunoelectrophoresis pattern revealed several precipitin lines. However, the radioimmunoelectrophoresis autoradiograph, after exposure for 1 hr, demonstrated a single intense radioactive line corresponding in position to the AFP line on immunoelectrophoresis. If the film was exposed for 24 hr, a faint radioactive arc appeared anodal to the AFP line. This arc corresponded exactly to the albumin portion of the carrier fetal serum seen on immunoelectrophoresis. With much more prolonged exposure of the x-ray film, trace amounts of goat gammaglobulin could be detected by radioimmunoelectrophoresis in the [^{125}I]AFP preparation.

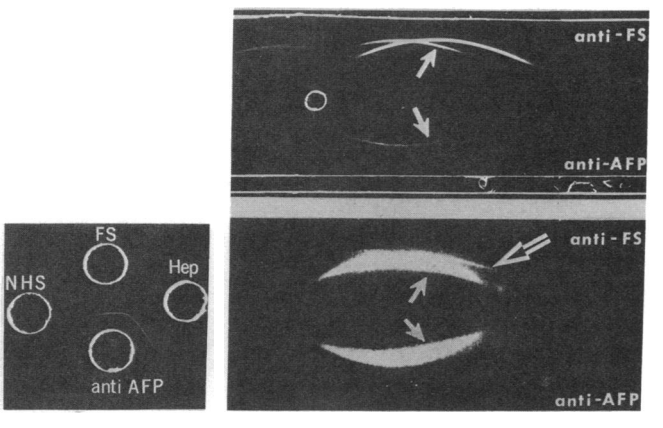

FIG. 1 (*left*). Ouchterlony plate. Anti-AFP antiserum fails to react with normal human serum (*NHS*), but there is a single precipitin line and reaction of identity with human cord serum (*FS*) and hepatoma (*Hep*) serum.

FIG. 2 (*right*). *Top:* Immunoelectrophoresis. The fetal serum (*FS*) in the center well reacts with anti-FS antiserum and with the anti-AFP antiserum. A single line appears with the anti-AFP antiserum. *Bottom:* Radioimmunoelectrophoresis. Radioautograph of the above immunoelectrophoresis. Purified [^{125}I]AFP was added to the carrier *FS*. The *white arrows* indicate the AFP line, and the *black arrow* indicates a faint radioactive albumin line.

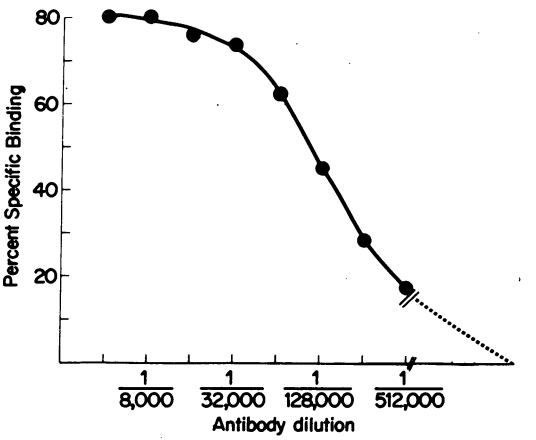

FIG. 3. Antibody titration curve of [^{125}I]AFP incubated with serial 2-fold dilutions of anti-AFP antiserum. % Specific binding is determined as follows: (^{125}I precipitated in antibody excess)–(^{125}I nonspecifically precipitated in the absence of antibody) divided by (total ^{125}I added–^{125}I nonspecifically precipitated in the absence of antibody).

As an additional measure of purity of the AFP preparation, the capacity of anti-AFP antiserum to bind to [^{125}I]AFP was measured by precipitation in 50% saturated $(NH_4)_2SO_4$. 80% of a freshly labeled preparation of [^{125}I]AFP was *specifically* precipitated by this procedure (Fig. 3).

Specificity of the Radioimmunoassay. The specificity of the immunologic reaction between [^{125}I]AFP and the anti-AFP antiserum was revealed by radioimmunoelectrophoresis. Despite the presence of trace contaminants in the purified AFP preparation, only a single radioactive precipitin arc developed between the [^{125}I]AFP and the anti-AFP antiserum (Fig. 2).

Radioimmunoassay for AFP. An antibody titration curve was prepared by reaction of [^{125}I]AFP with serial 2-fold dilutions of the anti-AFP antiserum (Fig. 3). 12% of the radiolabeled AFP was nonspecifically coprecipitated by ammonium sulfate in the absence of anti-AFP antibody. A maximum specific binding of 80% of the [^{125}I]AFP was obtained in the presence of anti-AFP antibody excess. (See caption of Fig. 3 for the definition of "specific binding".) A dilution of antiserum of 1:64,000, which provided conditions of antigen excess and which specifically bound 65% of the added radiolabel, was used to obtain the standard coprecipitation-inhibition curve (Fig. 4).

The standard inhibition curve demonstrated a linearity between 0.4 and 5 ng (in 20 μl) of purified AFP solution. This corresponds to serum concentrations between 20 and 250 ng of AFP per ml of serum. At concentrations of AFP between 0.125 and 0.4 ng/20 μl of serum, the shallow slope of the curve does not allow accurate quantitation and reproducibility of data. Essentially similar curves were obtained with normal human serum or bovine gammaglobulin as diluents of both radiolabeled antigen and antiserum. The radiolabeled pro-

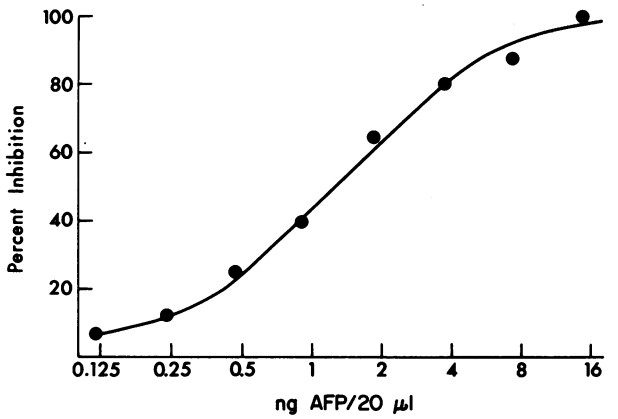

FIG. 4. Standard coprecipitation-inhibition curve for quantitation of serum AFP concentrations. The curve indicates the ng of AFP in a 20-μl sample volume that will produce a corresponding inhibition of coprecipitation of [^{125}I]AFP.

tein remained stable at 4° for at least 45 days after the time of the initial labeling procedure.

Concentrations of AFP in Human Sera. The results of AFP determinations in normal individuals and in patients with various diseases are listed in Table 1, and are represented graphically in Fig. 5. None of the normal sera demonstrated AFP values above 20 ng/ml of serum. Since this value represents the lowest concentration at which AFP could be measured with confidence, only concentrations above 20 ng/ml were considered abnormal for adult serum.

18 of the 19 sera from patients with hepatomas demonstrated clearly elevated AFP values, ranging from 160 ng/ml to 5.5 mg/ml. The single hepatoma patient with an AFP concentration of less than 20 ng/ml had a clinical and histopathologic diagnosis of cirrhosis, hemachromatosis, and hepatoma. Of the 6 patients with embryonal cell carcinoma of the testis, 5 had distinctly elevated levels, ranging from 1.1 to 130 μg/ml. In the viral hepatitis group, 6 of 11 patients had elevated AFP values, ranging from 24 to 87 ng/ml. In this category, 2 of 6 sera positive for hepatitis-associated antigen and 4 of 5 sera negative for this antigen were positive for AFP.

4 of 37 Patients with various nonmalignant hepatic disorders other than viral hepatitis had increased levels of AFP. The clinical diagnoses of these 4 patients were Laennec's cirrhosis, alcoholic hepatitis, obstructive jaundice of unknown cause, and hepatitis in a patient who had had halothane anesthesia. The sera of another group of 37 patients with various neoplasms arising in sites other than the liver or testis were also examined for elevated levels of AFP. Increased amounts of circulating AFP were found in 3 of 8 patients with bronchogenic carcinoma, 1 patient with gastric carcinoma, 1 patient with Hodgkin's disease, and 1 patient with acute myelogenous leukemia. The other gastrointestinal neoplasms studied were not associated with elevated levels of AFP.

DISCUSSION

A combination of immunochemical and physicochemical methods were used in the development of a sensitive radioimmunoassay method for the measurement of AFP in human sera. The goat anti-AFP antiserum, used subsequently to purify the AFP, was monospecific, as demonstrated by the techniques of double diffusion in agar gel and radioimmunoelectrophoresis. Although the preparation of AFP used in the radioimmunoassay contained trace amounts of human albumin and goat gammaglobulin, the immunologic reactivity between the AFP preparation and the anti-AFP antiserum used in the assay was monospecific by radioimmunoelectrophoresis.

With these reagents, a radioimmunoassay based on the technique of coprecipitation-inhibition in 50%-saturated ammonium sulfate was established. The assay, which can be completed in 8 hr, requires 20 μl of serum, and can reproducibly detect serum AFP levels of 20 ng/ml. In contrast to this degree of sensitivity, our results indicate that the Ouchterlony technique, commonly used for clinical purposes, cannot detect AFP levels below 10,000 ng/ml.

The overall biologic and clinical significance of AFP measurement will require extensive prospective studies in a large number of patients with a wide spectrum of clinical disorders. Among the unresolved problems is the question of whether

TABLE 1. *Concentrations of alpha$_1$-fetoprotein in human sera*

Clinical category	Number of patients	Number AFP-positive	Range of AFP (ng/ml)
Normal adults	50	0	<20
Hepatomas	19	18	160–5,500,000
Embryonal carcinomas of testes	6	5	1100–130,000
Other neoplasms			
Bronchogenic carcinoma	8	3	20–418
Gastrointestinal tract cancer			
Stomach	4	1	27
Pancreas	2	0	
Biliary tree	2	0	
Colon	7	0	
Rectum	3	0	
Lymphoreticular malignancy			
Hodgkin's disease	5	1	22
Leukemia	3	1	33
Reticulum cell sarcoma	1	0	
Multiple myeloma	1	0	
Urinary bladder carcinoma	1	0	
Viral hepatitis	11	6	24–87
Nonmalignant hepatobiliary disorders			
Laennec's cirrhosis	7	1	24
Alcoholic hepatitis	3	1	56
Chronic active hepatitis	5	0	
Cryptogenic cirrhosis	2	0	
Primary biliary cirrhosis	3	0	
"Halothane hepatitis"	2	1	87
Obstructive jaundice	8	1	34
Ascending cholangitis	1	0	
Hemochromatosis	2	0	
Gilbert's syndrome	1	0	
Hepatomegaly, etiology unknown	3	0	

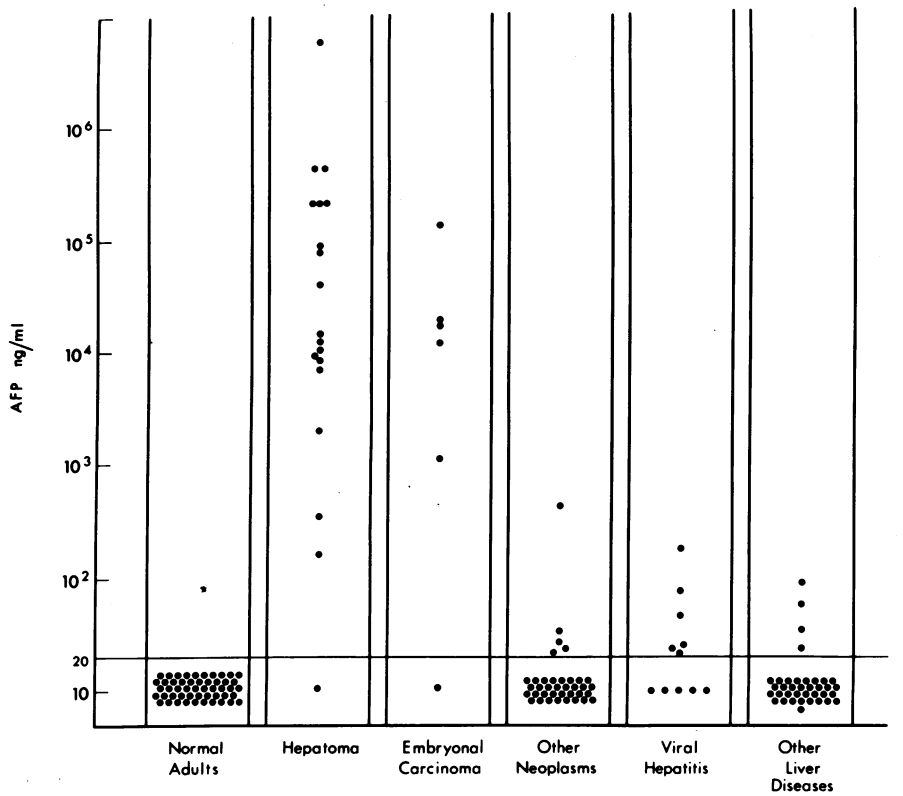

FIG. 5. AFP levels measured in normal individuals and in patients with various clinical disorders. AFP values plotted on a log scale were expressed in ng/ml of serum. Data from Table 1.

trace quantities of AFP ever occur in normal adult sera. Based on studies by the technique of solid-phase immunoadsorption, it has recently been suggested that AFP concentrations below 20 ng/ml may normally be present in adult human sera (8). In the present study, 20 ng/ml was the lower limit of serum AFP concentration that could be reliably detected; all normal sera examined gave values below this level. Hence, we can not comment concerning the possibility of trace quantities of AFP in normal adult human serum.

In the present series of patients studied, 18 of 19 with hepatoma had elevated AFP levels by the radioimmunoassay method, although only 12 of the same patients had positive Ouchterlony tests for AFP. Thus, the previously reported variations in incidence of AFP-producing hepatomas in different populations and in different geographic areas may, in part, be attributed to the lack of sensitivity of the diagnostic methods used. Nevertheless, one patient in this series who had a hepatoma did not have an AFP level above 20 ng/ml. This observation suggests either that AFP was present below the limits of sensitivity of the technique, or that the production of AFP by hepatomas can be intermittent. A further possibility is that hepatomas can be divided into two categories on the basis of their ability to synthesize and release AFP. It is conceivable that hepatomas that do not secrete AFP may produce another fetal antigenic marker, such as carcinoembryonic antigen. In tentative support of this hypothesis is a recent observation made in our laboratory. A patient who developed a thorotrast-induced hepatoma had carcinoembryonic antigen present in both her serum and in an extract of the tumor. AFP was not found either in the serum of this patient by the Ouchterlony technique or in the tumor extract by the radioimmunoassay method. Unfortunately, no serum from this patient is currently available for AFP measurement by radioimmunoassay.

The elevated levels of AFP found in patients with embryonal cell carcinomas of the testis are presumably a reflection of the pluripotential differentiation of this form of tumor. The positive sera in the groups with viral hepatitis and other nonmalignant hepatic disorders had much lower AFP levels than the hepatoma and embryonal cell carcinoma groups. The low positive levels in viral hepatitis and other nonmalignant hepatic disorders may reflect hepatic tissue damage and/or regeneration of liver tissue in the absence of malignant transformation, Thus, one might speculate that hepatic cells can secrete AFP in the process of gestation, neoplasia, or regeneration. The biologic significance of AFP associated with nonhepatic neoplasms remains unclear, and will require further studies in which both AFP and carcinoembryonic antigen are measured in the same patients. Preliminary data from this laboratory on a small number of patients suggest that it is unusual for both antigens to be present, simultaneously, in the circulations of individuals with neoplasms. Thus, AFP may serve as a complementary adjunct to carcinoembryonic antigen in the diagnosis of human cancer.

In view of the current interest in immunologic tests as clinical aids in the diagnosis and management of human cancer, the radioimmunoassay for AFP would appear to have several potential applications in clinical medicine. From the data presented, it is clear that patients with hepatomas and embryonal cell carcinomas of the testis can be readily distinguished from healthy individuals. Furthermore, the majority

of these patients can be distinguished from patients with nonmalignant hepatobiliary disease by their much higher levels of AFP. Studies reported by other investigators have suggested that there is a correlation between changes in serum AFP levels and the clinical course of liver disease, but these observations await confirmation by sensitive assay techniques in larger numbers of patients (14). Therefore, it is more than likely that a radioimmunoassay for the measurement of AFP will eventually become part of a battery of immunodiagnostic tests for the assessment of human malignant disease.

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