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Two-step Enzyme Immunoassay for the Determination of Serum α-Fetoprotein[1)]

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We have developed a competitive enzyme immunoassay to determine serum α-fetoprotein for use in clinical diagnosis. β-Galactosidase is employed as a label enzyme with anti-human α-fetoprotein antibody-coated polystyrene beads. By the proposed method, human α-fetoprotein could be determined in the range of 0.1 to 100 μg/ml within 2 h. Recovery of α-fetoprotein from various serum samples was good. The correlation coefficient between the proposed method and the sandwich enzyme immunoassay, which is commercially available, was 0.969. Coefficients of variation in the within- and between-assays were 7.7 to 9.3% and 6.6 to 20.7%, respectively. This method is rapid, simple and accurate, and is eminently suitable for use as a screening procedure for hepatoma and neural tube defects.

Keywords——enzyme immunoassay of human α-fetoprotein; competitive enzyme immunoassay; α-fetoprotein; oncodevelopmental protein; hepatoma; neural tube defect

Since α-fetoprotein (AFP) is increased in sera of patients with hepatocellular carcinoma or embryonal carcinoma,[2)] it was designated as a carcinofetal or oncodevelopmental protein. AFP also frequently appears in sera of patients with hepatoma and it is considered that an increased AFP level in serum is significant for the diagnosis of this condition. Endo[3)] reported that more than 200 ng/ml of serum AFP was detected in about 80% of patients with hepatoma. Recently, it has been reported that the AFP level in the mother's serum increases to between 0.1 to 5 μg/ml[4)] if the fetus has a neural tube defect.

Immunological methods for the determination of AFP have already been reported, for example, single radial immunodiffusion[5)] or rocket immunoelectrophoresis,[6)] but they were not sensitive enough to detect the AFP levels in sera of patients with hepatoma. The development of highly sensitive radioimmunoassay (RIA)[3,7-9)] enabled us to detect even small increases of AFP levels. However, this has disadvantages regarding the stability, storage and disposition of radioactive materials. Enzyme immunoassay (EIA) has replaced RIA in many clinical laboratories. The sandwich EIA[10,11)] was widely employed; it is sensitive, but the procedure is time-consuming and troublesome in that it requires first and second immunoreactions. Recently, sandwich EIA employing monoclonal antibody was reported.[12)] The latter employed monoclonal antibodies reacting with different antigenic determinants on the AFP molecule, and had the advantages of rapidity, simplicity and sensitivity. On the other hand, homogeneous EIA methods were devised, for example, particle counting immunoassay,[4)] but severe interference by serum components was encountered.

In the present paper, we have developed a competitive EIA procedure as a rapid and simple assay with sufficient sensitivity to assay AFP in sera of patients with hepatoma and neural tube defects.

Materials and Methods

Materials——β-Galactosidase was obtained from Boehringer Mannheim, 4-methylumbellirefyl-β-D-galactopyranoside (4MUG) was from Koch-Light Laboratories Ltd., *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce Chemicals Co., Sepharose 6B, Sephadex G-150, Sephadex G-25, Con

A-Sepharose 4B and Protein A-Sepharose CL-4B were from Pharmacia Fine Chemicals, polystyrene beads were from Ichiko Co., dioxane, bovine serum albumin (BSA), 2-mercaptoethanol and α-Fetoprotein B-Test Wako (Code 276-80801; label enzyme, horse radish peroxidase; solid phase, glass beads) were from Wako Pure Chemical Industries, and Scat 20-X was from Nakarai Chemical. Other reagents were of analytical grade.

Purification of AFP——AFP was purified from human cord serum according to the previous paper[13] by employing an immunoadsorbent column and Sephadex G-150 gel filtration. Purified AFP showed a single protein band on disc and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and a single precipitin line on two-dimensional immunoelectrophoresis. One mg of purified AFP was found to be equivalent to 1.02 I.U.

Preparation of AFP Antibody——AFP antibody was obtained by immunization of a rabbit with 100 μg of purified AFP emulsified in Freund's complete adjuvant. The crude IgG was prepared by fractionation with 33% saturation of $(NH_4)_2SO_4$, followed by passage through a DEAE-cellulose column (3.5 × 28 cm) equilibrated with 20 mM potassium phosphate buffer (pH 8.0). It was further purified by adsorption on a Protein A-Sepharose CL-4B column (1.5 × 2 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2) and by elution with 1 M acetic acid. The specificity of the antibody was confirmed by two-dimensional immunoelectrophoresis, which showed a single precipitin line.

Preparation of AFP Antibody-coated Polystyrene Beads——AFP antibody-coated polystyrene beads were prepared by the method of Kato *et al.*[14] Polystyrene beads were washed thoroughly with 10% Scat 20-X and soaked with AFP antibody solution diluted to 10 μg/ml with 0.25 M sodium phosphate buffer (pH 7.2) at 30°C for 2 h, and then allowed to stand at 4°C overnight. Next, they were thoroughly washed with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3 and 0.1% BSA (buffer A) and stored at 4°C in buffer A until use.

Preparation of AFP-β-galactosidase Conjugate——AFP-β-galactosidase conjugate (AFP-β-gal) was prepared with MBS as a crosslinking reagent by the method of O'Sullivan *et al.*[15] A solution of 800 μg of purified human AFP in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) and 15 μl of MBS (20 mg/ml) dissolved in dioxane were mixed and kept at 30°C for 1 h. The mixture was applied to a Sephadex G-25 column (1.5 × 35 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$ and 50 mM NaCl. Immediately, 1.5 mg of β-galactosidase was mixed with the AFP fraction eluted from the column and incubated at 30°C for 1 h. The reaction was terminated by the addition of 40 μl of 1 M 2-mercaptoethanol. An aliquot of the conjugate was applied to a Con A-Sepharose 4B column (1.5 × 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 10 mM $CaCl_2$, and eluted with the same buffer containing 1 M methyl-α-D-glucopyranoside. Then the AFP-β-gal fraction was applied to a Sepharose 6B column (1.5 × 48 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and 1 mM $MgCl_2$. The AFP-β-gal fraction (AFP; 24 μg/ml and β-galactosidase; 1.15 unit/ml) was stored at 4°C and diluted 40-fold with buffer A just before use.

Standard Assay Procedure——A mixture of 0.1 ml of AFP-β-gal, 20 μl of sample or AFP standard and 0.2 ml of buffer A was incubated with one AFP antibody-coated polystyrene bead at 37°C for 60 min in a test tube (1.5 × 10.5 cm). Then the bead was washed 3 times with 1.5 ml of buffer A and transferred to another test tube containing 0.2 ml of buffer A. The enzyme activity was assayed using 4MUG as a substrate. The reaction was begun by adding 0.1 ml of 0.3 mM 4MUG and the mixture was incubated with shaking at 37°C for 15 min. The reaction was terminated by the addition of 2.5 ml of 0.1 M glycine-NaOH buffer (pH 10.3). The amount of liberated 4-methylumbelliferone was measured with a Shimadzu spectrofluorometer model RF 510 at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. One unit of β-galactosidase activity is defined as that amount which hydrolyzes one μmol of 4MUG/min under the conditions described above.

Results and Discussion

The Effect of AFP Antibody Concentration on the Dose-Response Curve

Polystyrene beads were coated with various concentration of AFP antibody (0.03 to 100 μg/ml). Ten μg/ml of AFP antibody gave an adequate dose-response curve (0.1 to 100 μg/ml) for the clinical diagnosis of hepatoma, but concentrations of less than 10 μg/ml of AFP antibody resulted in a relatively high blank value and a reduction of the working range of the assay. With 100 μg/ml of AFP antibody, the calibration curve covered a higher range of AFP than with 10 μg/ml.

The Effect of AFP-β-gal Concentration on the Dose-Response Curve

The concentration of AFP-β-gal was examined using polystyrene beads with AFP antibody (10 μg/ml). The AFP-β-gal fraction described in "Materials and Methods" was diluted up

to 400-fold. A 40-fold dilution of AFP-β-gal gave an adequate dose-response curve. AFP-β-gal dilutions of less than 40-fold reduced the working range of the assay, and greater dilutions resulted in reduced sensitivity at low concentrations of AFP.

Analysis Time

The optimum time for competitive reaction between antigen and enzyme-labelled antigen against the AFP antibody-coated polystyrene beads was also investigated. A reaction time of 60 min was selected as giving a precise result without loss of activity, but times of less than 60 min resulted in marked variation of the calibration curve. β-Galactosidase bound to the bead exhibited a linear increase of fluorescence proportional to the enzyme reaction time, and enzyme reaction for 15 min was found to be adequate in terms of the detection range of the assay. Thus, the total time required to measure even 100 serum samples was within approximately 2 h.

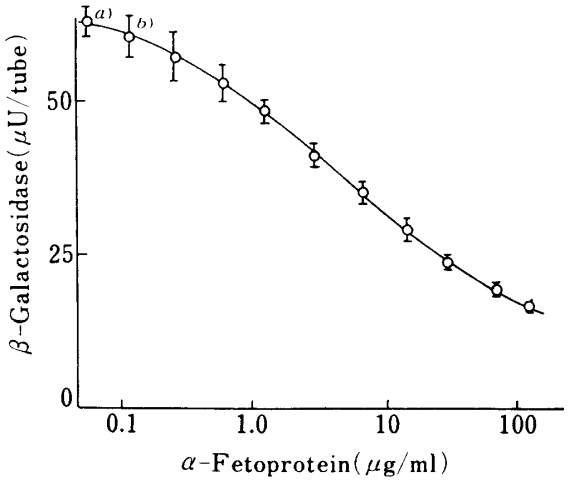

Fig. 1. Calibration Curve for the Determination of α-Fetoprotein

Twenty μl of sample, 0.1 ml of AFP-β-gal, 0.2 ml of buffer A and one AFP antibody-coated polystyrene bead were added to a test tube, and incubated at 37°C for 60 min. The enzyme activity was determined at 37°C for 15 min. *a*) and *b*) indicate values which are different from the control at the $p<0.05$ and $p<0.01$, respectively. The other values are different from the control at the $p<0.005$ level.

Calibration Curve

A calibration curve for human AFP is shown in Fig. 1. Human AFP could be determined in the range of 0.1 to 100 μg/ml by the proposed method. The detection range was sufficient for use in clinical diagnostic tests. From the viewpoint of sensitivity, the proposed method is inferior to sandwich EIA.[11,12] However, the ability to detect 100 ng/ml of AFP in sera of patients with hepatoma or neural tube defects, provides significant information for clinical diagnosis.

Precision of the Assay

The within-assay and between-assay coefficients of variation (C.V.) of the proposed method were clarified by performing each assay in duplicate with various concentrations of AFP. The results are shown in Table I. The within-assay ($n=10$) C.V. was 7.7 to 9.3% and the between-assay ($n=7$) C.V. was 6.6 to 20.7%. These variations are acceptable for clinical tests using immunological techniques.

Table I. Precision of the Proposed Assay as applied to the Determination of AFP

Concentration (μg/ml)[a]	C.V. (%)
Within-run[b]	
0.96	7.7
4.42	9.3
36.3	7.8
Between-run[c]	
3.86	20.7
9.63	6.6
41.4	14.8

a) These assays were done on pooled serum supplemented with standard AFP.
b) Ten assays repeated within a day with one standard curve.
c) Assay repeated during 7 d, employing AFP-β-gal diluted 40-fold with buffer A just before use.

Recovery of AFP

A series of 10 serum samples from patients with various diseases was collected. No trace of AFP could be detected in the above samples by the proposed method. Aliquots of purified AFP at the indicated concentrations were added to the serum samples. As shown in Table II, the average recovery was from 99.2 to 101%. These results show that the serum components scarcely influenced the proposed method.

TABLE II. Analytical Recovery of AFP in the Proposed Method

No. of samples	AFP		Recovery (%) (Mean ± SD)
	Added (μg/ml)	Recovered (μg/ml)	
10	1.2	1.21	101 ±5.9
10	12	12.0	100 ±4.7
10	30	29.7	99.2±4.9

A series of 10 serum samples of patients with various diseases was collected. No trace of AFP could be detected in the above samples by the proposed method. An aliquot of purified AFP at the indicated concentration was added to each serum sample.

Correlation between the Proposed Method and the Conventional Method

The correlation of human AFP concentration determined by the proposed method and by the conventional sandwich EIA method was investigated using 30 serum samples. The results are shown in Fig. 2. There was a good correlation and the correlation coefficient for these assays was 0.969.

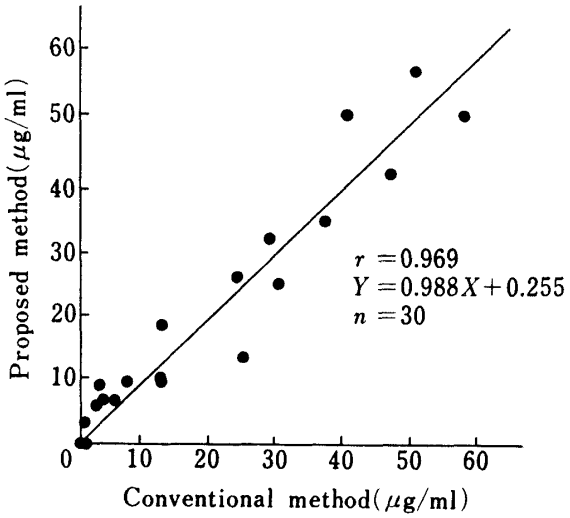

Fig. 2. Correlation of α-Fetoprotein Levels determined by the Proposed Method and the Conventional Method

AFP is a fetal glycoprotein. It is well known that AFP is synthesized in the yolk sac, fetal liver and gastrointestinal tract.[16,17] It is present in low concentration in normal adult serum but in large amounts in maternal and fetal sera and in amniotic fluid. Its reappearance in the sera of patients with hepatoma or neural tube defects, indicates that AFP plays an important role *in vivo*. Therefore, the determination of AFP level in serum may give significant information for reaching a clinical diagnosis.

Various methods for the measurement of AFP have been reported, and most of them rely on the antigenicity of AFP. Typical methods used in clinical diagnostic tests, are single radial immunodiffusion,[5] rocket immunoelectrophoresis,[6] RIA[3,7-9] and EIA.[4,10-12] Single radial immunodiffusion and rocket immunoelectrophoresis are insensitive and not very suitable for use in clinical tests. However, RIA and EIA are highly sensitive and can detect even a small increase of AFP in normal adult serum. The sensitivity and specificity of RIA and EIA are of the same order, and the results of EIA are compatible with those obtained by RIA. The advantages of EIA over RIA include the greater stability of reagents and avoidance of the use of radioactive materials.

In recent years, sandwich EIA[10-12] has been widely employed. Homogeneous EIA is a much better method on the basis of its simplicity and rapidity, but it has been reported that

particle counting immunoassay,[4] one of the homogeneous EIA methods, is strongly interfered with by serum components.

We have developed a competitive EIA for application to clinical diagnosis. As mentioned previously, 0.2 μg/ml of AFP is detected in about 80% of patients with hepatoma,[3] and the appearance of 0.1 μg/ml of AFP is now regarded as a diagnostic threshold. The detection range of the proposed method is 0.1 to 100 μg/ml and is adequate for clinical diagnosis. However, the sensitivities of conventional RIA and EIA are at the ng/ml level in most cases when applied to the detection of AFP level of patients with hepatoma. In practice, by the proposed method, 0.5, 0.7 and 0.8 μg/ml of AFP were detected in 3 of 6 serum samples of patients with liver cancer. Moreover, the proposed method is scarcely affected by serum components, as shown in Table II. In conclusion, the proposed method is rapid, simple and direct, and is eminently suitable for application as a clinical diagnostic test. AFP-β-gal conjugate and AFP antibody-coated polystyrene beads prepared according to the proposed method are both stable for at least 6 months.

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