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Clinical Observations

ENZYMO - ROCKET ELECTROPHORETIC ASSAY AND CROSSED AFFINITY ENZYMOIMMUNOELECTROPHORESIS AND ITS APLICATION IN DIAGNOSIS OF PLIMARY LIVER CANCER

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A sensitive and accurate quantitative method of alpha-fetoprotein (AFP), enzymo-rocket electrophoretic assay (EREA), was developed by introducing horseradish peroxidase-labeled anti-human AFP antibody into rocket electrophoresis. The lower limit of quantitation by this method is about 10 ng/ml of AFP. The dose-response curve covers a broader range of concentrations of AFP (10—4000 ng/ml) than RIA (0—400 ng/ml). The accuracy and precision is comparable to that of RIA (r = 0.986). Serum AFP measured in 100 patients with primary liver cancer by this method, 88% had levels over 25 ng/ml.

The crossed affinity enzymoimmunoelectrophoresis is a combination of lentil lectin (LCA) affinity electrophoresis and enzymo-rocket electrophoresis, it has been possible to separate the AFP into two variants, LCA-reactive (LCA-R) and LCA-nonreactive (LCA-N) fractions. The advantages of this method are high sensitivity, rapid (6—7 h), and can be effectively used to differentiate the primary liver cancer and benign liver disease.

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Measurement of AFP in serum is well established to be useful for diagnosis, not only of primary liver cancer but also of some congenital anomalies. At present two radioimmunological techniques have been applied for the determination of AFP. Radioimmunoassay[1] (RIA) and radio-rocket electrophoretic assay (RREA)[2] are sensitive and permit precise quantification. However, the isotope labels may decay rapidly, so that the conjugates have a short shelf-life; expensive equipment is necessary for RIA; the assay time is too long for the RREA; and, because of the medical hazards, they must be handled by highly trained personnel.

We have developed a new method of enzymo-rocket electrophoretic assay which is rapid, sensitive, accurate and can be undertaken with ordinary laboratory equipment. The enzymatic label

in this method is far more stable than the radioactive iodine label of radioimmunological techniques.

On the basis of above method, we have developed another method of crossed affinity enzymoimmunoelectrophoresis which is a combination of LCA affinity electrophoresis and enzymo-rocket electrophoresis suitable for rapidly direct analysis of AFP variants at total AFP levels as low as 150 ng/ml in routine laboratory. It is a useful test for differentiating between primary liver cancer and benign liver disease.

MATERIALS AND METHODS

Isolation of Horse Anti-human AFP Antibodies

The IgG fraction of horse anti-human AFP serum was isolated by a combination of 33% ammonium sulfate precipitation and DEAE cellulose chromatography. After concentration, the effluent was shown to be pure IgG in the Ouchterlony test and by immunoelectrophoresis. The protein concentration was determined from absorbence measurements at 280 nm.

Preparation of Horseradish Peroxidase-antibody Conjugate

Commercial horseradish peroxidase (HRPO) (Rz = 3.0) was coupled to the antibodies according to the procedure of Tijssen and Kurstak.[3] The optimal concentration of the conjugate was determined by enzyme linked counterelectrophoretic assay.[4] The conjugate (lyophylized product) was stable at −20℃ for at least one year.

Preparation of Lentil Lectin

Isolation of LCA was performed according to the procedure of Howard et al..[5]

AFP Standard

National standard of human AFP was donated by the National Institute for the Control of Pharmaceutical and Biological Products, which is designated by WHO as its "Collaborative Center for Drug Quality Assurance".

1% Solution of Agarose

One gram of agarose was added to 100 ml of 0.05 M pH 8.6 Tris-barbital buffer. The solution should be boiled for 10 min to ensure that the agarose is completely dissolved.

Substrate Solution

20 mg of 3-amino-9-ethylcarbazole was dissolved in 2.5 ml N, N-dimethyl formamide and mixed with 50 ml 0.05 M pH 5.0 acetate buffer, 25 μl 30% H_2O_2 was added immediately before use.

Enzymo-rocket Electrophoretic Assay

8.8 ml of melted 1% agarose solution and 1 ml of 30% polyethylene glycol Mw 6000 was pipetted into a test tube. The solution was allowed to cool to 55℃, and 0.2 ml enzyme labelled antibody was added during careful mixing. The mixture was poured onto a 8 × 10 cm glass plate. After congelation, at the border of the cathode a row of wells (3 mm in diameter or 2 × 5 mm oval wells) was cut. Ten μl of standard sera or test samples were applied to sample wells and electrophoresis was carried out at 6—7 V/cm at 15—25℃ for 2—3 h. After the electrophoresis the gel plate was covered with a piece of filter paper and several layers of tissue paper and pressed to obtain a uniform blotting of the gel. The blotting pad was removed, and the filter paper in contact with the gel was moistened with saline solution and removed. The gel plate was placed in saline solution for 5 min and then pressed and washed again. The gel plate was stained for HRPO activities with substrate solution.

After staining measurements were made of the height of the peaks (from the uper edge of the well to the top of the rocket) and standard curve was plotted, from which the values of AFP concen-

trations in the test samples were determined.

Procedure of Crossed Affinity Enzymoimmunoelectrophoresis

First Dimension Electrophoresis

2.5 ml of melted 1% agarose solution was pipetted into a test tube. The solution was allowed to cool to 55℃, and 0.2 ml of 0.3% LCA was added (the final concentration of LCA was 0.22 mg/ml). The mixture was poured onto the lower 10 × 2 cm of the 10 × 8 cm glass plate. After congelation, at 1 cm and 5 cm from the border of the cathod two sample wells (2 × 5 or 3 × 8 mm) was cut. 10—20 μl of sera were applied and electrophoresis was carried out at 15—25℃ at 7 v/cm for 90—120 min.

Second Dimension Electrophoresis

6 ml of melted 1% agarose solution was piptted into a test tube. 0.7 ml of 30% polyethylene glycol Mw 6000 was mixed with agarose. The solution was allowed to cool to 55℃, and 0.2 ml enzyme labelled antibody was added. The mixture was poured onto the upper part of the glass plate. After congelation, electrophoresis was carried out at 7 v/cm for 150 min.

Pressing and washing of the gel and enzymatic reaction was performed as described above.

Calculation of Percentages of LCA-reactive AFP

The peak nearer the origin in the first dimension gel was designated as LCA-reactive (LCA-R), and the peak moving farther from the origin as LCA-nonreactive (LCA-N). The area of the peaks were measured and the percentages of LCA-R were calculated as follow:

$$\text{LCA-R\%} = \text{LCA-R}/(\text{LCA-R} + \text{LCA-N}) \times 100\%$$

Radioimmunoassay of AFP

Radioimmunoassay of AFP was performed with AFP-RIA kit (supplied by the Shanghai Institute of Biological Products, Ministry of Public Health, People's Republic of China), which is double antibody method of producing competitive sensitive reactions making quantitation of AFP possible.

RESULTS

Dose-response Curve and Working Range of EREA

The photograph of the pattern of enzymo-rocket electrophoresis for standart AFP is shown in Figure 1, where the peak heights are proportional to the concentration of the AFP. The standard dose-response curve constructed from the pattern above is shown in Figure 2. The limit of the detection of AFP by this method was found to be about 10 ng/ml and the working range of the assay was 10—4000 ng/ml.

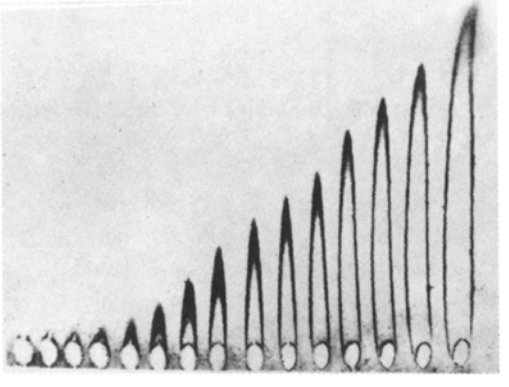

Fig. 1. A photo of pattern of enzymo-rocket electrophoresis of AFP standards. In the application wells (left to right) 5, 5, 10, 20, 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 3000, 4000 ng/ml. The agarose gel contains HRPO labelled antibody and 3% polyethylene glycol Mw 6000. The electrophoresis was at 7 v/cm for 2.5 h at 25℃. Chromogen was 3-amino-9-ethylcarbazole.

Precision of the Assay

The within-assay repeatability of this method was estimated by quantitating 25 sera. Each pair of duplicates being assayed as nonadjacent samples within a single batch. The coefficient of variation was 1.2% for the range 20—4000 ng/ml.

The between-assay repeatability was estimated by running 15 samples on five different days and recording the results each time from a different dose-respone curve. The coefficient of variation was 6.3% for the range 20—4000 ng/ml.

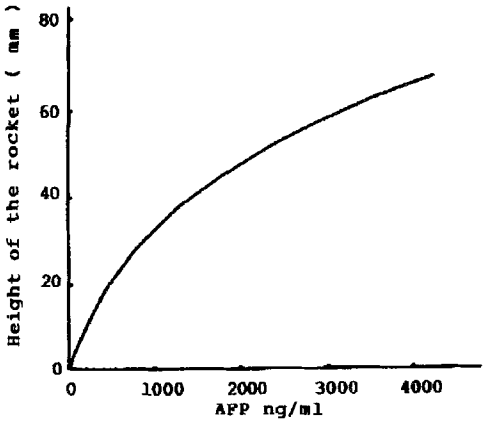

Fig.2. Dose-response curve obtained by enzymo-rocket electrophoresis of AFP standards. Technical details see Figure 1.

Comparison between EREA and RIA

The specificity and accuracy of this method were examined by determining the same samples both by EREA and RIA. Fifty sample sera (37 cases of hepatocellular carcinoma and 13 cases of hepatitis) were assayed as shown in Figure 3. The values by the two methods were in good agreement. The values obtained by EREA and RIA correlate with a correlation coefficient of 0.986 ($P < 0:001$). The equation of regression line was: $Y = 1.006X + 1.821$.

Determination of Serum AFP of Patients with Primary Liver Cancer

AFP was measured in 100 patients with histologically proved primary liver cancer by this method. Serum AFP levels ranged from $25 - 3 \times 10^5$ ng/ml. 12% had normal levels (less than 25 ng/ml) and 88% had levels above normal.

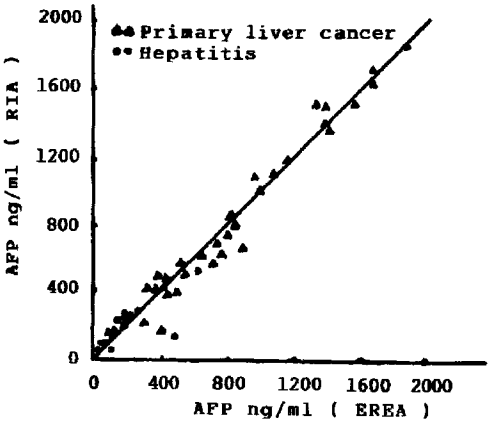

Fig.3. Correlation between AFP concentration of 50 samples measured by EREA and RIA. The correlation coefficient was 0.986 ($P < 0.001$) and the linear regression equation was $Y = 1.006X + 1.821$.

The LCA Affinity Pattern of AFP

Using crossed affinity enzymoimmunoelectrophoresis, it has been possible to obtain directly the LCA affinity patterns of AFP present in serum. The photographs of the LCA affinity pattern of serum AFP from patients with primary liver cancer are shown in Figure 4. where the AFP was separated into two molecular variants, LCA-R and LCA-N fractions. It has resulted in clear and sharp electrophoretic patterns.

LCA Reactive AFP in Serum of Patients with Primary Liver Cancer

Table 1 summarizes the AFP concentration in serum and the percentage in the lentil lectin reactive form for 47 cases of patients with primary liver cancer which was comfirmed by histological exa-

Table 1. LCA reactive AFP in serum of 47 patients with primary liver cancer

Case number	Total AFP ng/ml	LCA-R %	Case number	Total AFP ng/ml	LCA-R %
1	40000	60	25	17058	69
2	340	38	26	1500	62
3	1890	29	27	9530	45
4	317	67	28	1030	40
5	592	44	29	1020	61
6	680	45	30	26000	25
7	1500	88	31	20000	21
8	101924	0	32	700	71
9	102704	55	33	1488	80
10	4000	59	34	1590	52
11	5016	67	35	1410	67
12	230	67	36	1970	71
13	1010	61	37	1788	57
14	22900	0	38	400	67
15	1720	68	39	12800	77
16	386	62	40	8000	72
17	27600	56	41	400	60
18	800	67	42	400	81
19	500	60	43	800	80
20	4000	72	44	790	90
21	8964	20	45	3000	67
22	20000	75	46	5415	48
23	5933	67	47	16000	46
24	800	42			

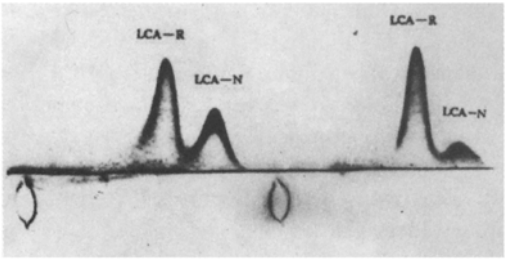

Fig. 4. A photo of the LCA affinity pattern of serum AFP from patients with primary liver cancer. The concentration of AFP was 614 ng/ml (left) and 500 ng/ml (right). The sample size of 10 μl was used.

mination. The mean value of the LCA-R% was $61.3 \pm 14.6\%$ ($\bar{x} \pm s$). LCA-R AFP equal to or higher than 25% was considered to diagnose for primary liver cancer. The positive rate in patients with primary liver cancer was 91.5% (43/47). The false positive rate in 46 patients with benign liver disease (18 cases of cirrhosis, 26 cases of acute and chronic hepatitis, 2 cases of diseases of the biliary tract) was 2.2% (1/46).

DISCUSSION

As indicated above, the sensitivity threshold of

the enzymo-rocket electrophoresis is between that of RIA and RREA. Some RIA procedure achieve a lower detection limit, but they gain nothing in clinical usefulness. The accurate assay range of this method (10—4000 ng/ml) is over 40 fold for RIA (0—100 ng/ml) and 10 fold for RREA (25—400 ng/ml). This means that samples containing a wide variety of AFP concentrations may be directly tested, without dilution.

The accuracy and precision of this method is comparable to that of RIA. The correlation coefficient was 0.986 ($P < 0.001$) and the equaiton of variation was $Y = 1.006X + 1.821$. The within-assay coefficient of variation was 1.2% and the between-assay coefficient of variation was 6.3%. Precision of the present assay was thus fully comparable to that achieved by RIA procedures (C. V. = 3.2% and 7.2%) by Forrester et al..[1]

In conclusion, this new method provides a relatively simple, rapid, sensitive and reproducible means of measurement of AFP.

Alpha-fetoprotein is a glycoprotein which has been used to diagnosis of primary liver cancer, but increased levels of AFP in serum are also observed in benign liver disease such as cirrhosis or acute and chronic active hepatitis. There is no clear-cut serum AFP concentration in benign and malignant liver disease. Breborowicz and Kuromatsu, et al.,[6] have shown that estimation of lentil lectin reactive AFP in serum provides a useful means for the differential diagnosis of hepatic disease. Many investigators have separated AFP into lectin-reactive and lectin-nonreactive fractions with crossed affinoimmuno-electrophoretic technique. While producing excellent results at higher levels of AFP, this method has lacked the sensitivity needed for analysis of serum specimens with low AFP levels. The crossed affinity radioimmunoelectrophoresis developed by Ker - ckaert, et al.[7] or an improved crossed immuno - affinoelectrophoretic technique by Albanes et al.[8] permits analysis of AFP variants when total AFP is below 1000 ng/ml. But the procedures of these methods are tedious and time-consuming.

Using our new method crossed affinity enzymoimmunoelectrophoresis, it has been possible to obtain directly the LCA affinity patterns of AFP, and has resulted in clear and sharp electrophoretic patterns. With this method, specimens having larger amounts of total AFP (over 800 ng/ml) adjusted by serum dilution to a range of 400—800 ng/ml were examined. In this range of AFP concentration, a sample size of 10 μl was used. With specimens having lower concentration of AFP (150—400 ng/ml), a sample size of 20—30 μl was used. In these cases, the final concentration LCA in the first dimension gel was 0.5 mg/ml.

The advantages of this method are high sensitivity (the lower limit of analysis is 1—2 ng of AFP), rapid (results are obtained within 6—7 h), more distinct pattern, and high stability of the labeled antibody.

The positive rat of LCA-R $\geqslant$ 25% in 47 patients with primary liver cancer was 91.5%, and in 46 benign liver disease was 2.2%. Evidently, this new technique can be used effectively to discriminate these two conditions.

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