An improved method for the detection of alpha-fetoprotein in serum

—Enhancement of double diffusion precipitin reaction by cadmium ion, and a combined duo antibody method for single radial immunodiffusion—

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INTRODUCTION

The detection of alpha-fetoprotein (AFP) in the serum of hepatoma patients has been proved to be of diagnostic value. A double diffusion precipitin reaction in agar gel, the Ouchterlony method, is found to be specific for hepatoma, although a few exceptions are present for teratoblastoma and certain gastric cancer. The rate of positive reaction on a considerable number of hepatoma patients in Japan was reported to be 78%[1], but the rate was increased to about 90% by a radioimmunoassay (RIA) method. However, at the same time it resulted conversely in a decrease in the specificity for the diagnosis of hepatoma. The present investigation is designed to increase the photographic contrast of the precipitin line in gels with the aid of a heavy metal ion, namely cadmium nitrate as originally reported by A. J. Crowle[2].

A combined duo antibody method for single radial immunodiffusion to improve the sensitivity in detecting AFP in the serum was also described.

MATERIALS AND METHODS

The sera of 95 patients with various liver diseases admitted to our clinic were tested for AFP.

Counter-current (cross-over) electrophoresis was conducted in a 1.2% agar-agarose (1:1) gel buffered with a veronal buffer at pH 8.6, $\mu=0.025$. Circular wells were cut in the agar-agarose layer along the electrophoretic axis approximately 5–6 mm apart midway between the cathode and the anode according to Kohn's method[3]. A constant current of approximately 3 milliamps/cm width of agar-agarose plate was introduced for 25 minutes. Then the gel plates were immersed in 1/1,000 M cadmium nitrate saline

solution for 10 minutes, and followed with a treatment with 0.5% Tween 80 saline solution for 24 hours. The precipitin line became distinct and the results of photography improved. Thus the discrimination of a positive reaction against negative results became easy, which resulted in the increase in the sensitivity of the test. A purified AFP donated by Professor Hirai of the Hokkaido University Medical School and also the reference AFP solution from the Behring-Werke Co. were used to prepare a standard AFP solution. The reference solutions of AFP were diluted with saline including gelatin at a rate of 1%, and made a serial known concentration of AFP ranging from 22 mg/dl down to 0.042 mg/dl. The set of AFP solution served to test the sensitivity of the method. Next, the procedure was applied to test the sera of the patients with various liver diseases for AFP. Simultaneously, the amount of AFP was measured by either Mancini's method or radioimmunoassay utilizing an α Feto-125 kit of the Dainabot RI Laboratories. Thus the sensitivity of the method was evaluated and a comparison was run between the data obtained by the basic experiments and the data by clinical observations.

The determination of serum AFP by combined duo antibodies were carried out as follows. Anti-human AFP-rabbit serum was mixed in the agar-agarose plate at a rate of 5%. The wells were charged with 5 μl of the different concentrations of AFP solution ranging from 22 mg/dl down to 0.011 mg/dl. The plates were placed in a cold room for 72 hours after the plates were left standing at room temperature for several hours. Next, the plates were washed using saline until the non-reacted antiserum in the plates were no longer remained. This required about 24 hours. Then the wells were charged with the second antibody, anti-rabbit γG-goat serum and the diameter of disclosed precipitin ring was measured.

RESULTS

Fig. 1 illustrates the results of counter-current electrophoresis. The upper wells with a diameter of 3 mm were charged with 5 μl of anti-human AFP rabbit serum, and the lower wells with a diameter of either 3 mm or 6 mm were charged with AFP solutions of different concentrations ranging from 22 mg/dl down to 0.042 mg/dl. The photograph in the upper part gives the simple cross-over electrophoresis before the treatment. The photograph in the middle illustrates the enhancing effect of cadmium ion on the precipitin line. The photograph at the base illustrates the clarification of the gel by treatment with Tween 80. The cloudy line blurred by lipoproteins was removed by the procedure without obscuring the precipitin line formed by antigen-antibody aggregate. The lower limit of AFP de-

Sensitivity test of IEP
— Enhancing effect of cadmium ion on specific precipitation —

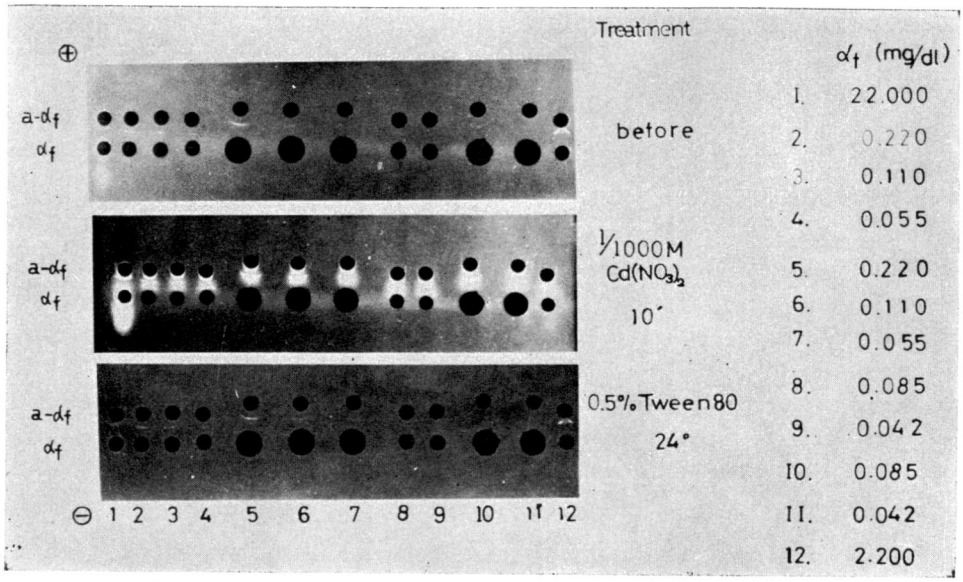

Fig. 1.

tected by this method is as low as 0.042 mg/dl.

Sera of 6 male patients with fulminant hepatitis were tested for AFP by this method. The age of the patients ranged from 46 to 66 years, and the day of examination from the onset of the disease ranged from 7th to 79th day. One of the 6 patients showed positive for AFP for a limited time. In another patient, the concentration of the serum AFP was measured as 175 ng/ml, but showed negative by the qualitative method.

Three patients with acute viral hepatitis were all negative. Likewise, 20 patients with chronic aggressive hepatitis of a moderate activity were negative, whereas 1 out of 7 patients with chronic aggressive hepatitis of a severe activity was temporarily positive. The amount of serum AFP determined by RIA method ranged from 11 to 450 ng/ml. The biopsy findings of the liver revealed an extensive collapse of the parenchymal cells with active regeneration, however, the correlation of serum AFP with the liver cell regeneration in histology was not convincing.

One of 21 patients with liver cirrhosis was positive for AFP for as long as 7 weeks. The amount of serum AFP of the patient measured by the RIA method showed from 218 to 425 ng/ml. The activity of serum transaminases varied from 90 to 170 units. Peritoneoscopy revealed a typical nodular cirrhosis, and the histological diagnosis was postnecrotic cirrhosis.

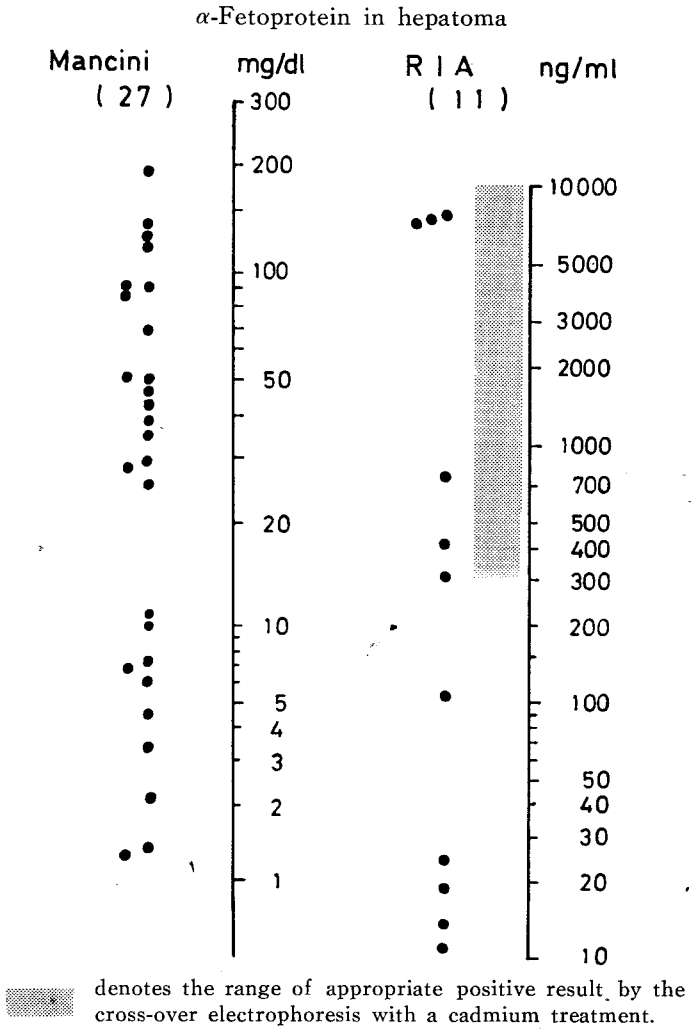

Fig. 2.

A marked nodularity suggesting an active regeneration of the liver cells was observed, but there was no malignancy.

Sera from the histologically verified hepatoma patients were tested for AFP. Out of 38 examined patient sera, 33 (87%) showed positive, whereas the rate of positivity was 71.1% by the micro-Ouchterlony method. The result was ascertained by measuring the same samples by either the Mancini method or the RIA method. The qualitative method described here showed diagnostic values for the detection of serum AFP at concentrations ranging from 10,000 ng/ml down to 300 ng/ml (Fig. 2).

For the quantitative determination of AFP, Mancini's single radial immunodiffusion method was used as a routine laboratory test. Again, the minimum amount of AFP determined was as low as 0.6 mg/dl.

The combined duo antibody methods, using anti-human AFP-rabbit serum as the first antibody, and anti-rabbit γG-goat serum as the second antibody increased the sensitivity by about 10 times as compared with the control. Fig. 3 illustrates such an example. The immunoplate on the left shows the precipitin ring around the wells charged with tested sample as low as 0.688 mg/dl in concentration, whereas the plate on the right shows distinct rings around the wells charged with the samples as low as 0.086 mg/dl.

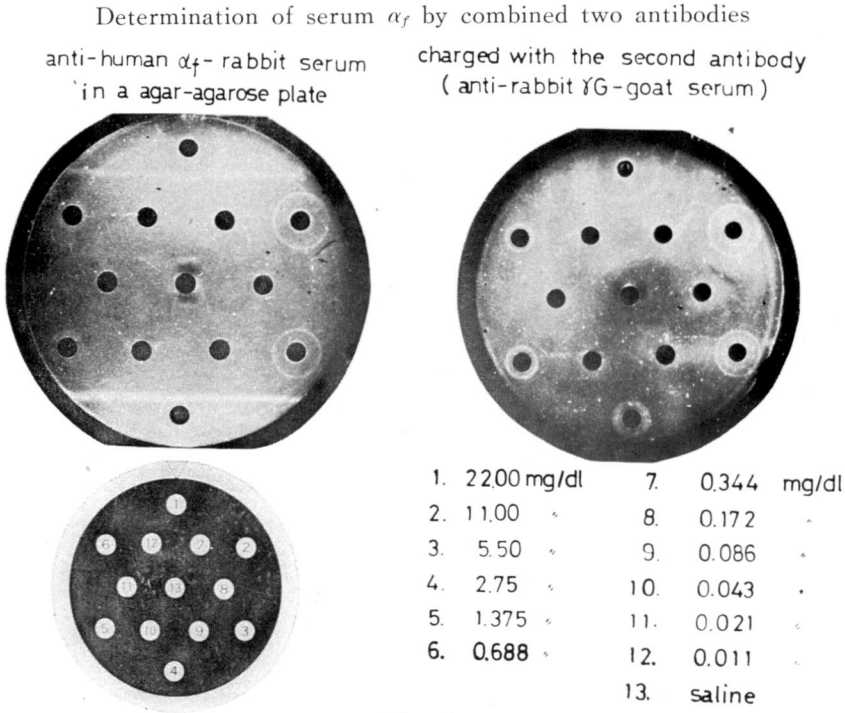

Fig. 3.

DISCUSSION

The two methods described here were intended to increase the sensitivity of precipjtin reaction in the gel, so that the diagnostic value of AFP in the sera of hepatoma patients and the patients with other various liver diseases would be increased. The enhancing effect of cadmium ion on the invisible antigen-antibody aggregates formed in plates was first described

by Crowle[2]. At concentrations ranging from 0.05% down to 0.0001% in barbital buffered agar, the effect of this salt varied from enhancement to inhibition. In the present study, we observed the concentration of cadmium nitrate, 1/1,000 M, was satisfactory to visualize invisible antigen-antibody aggregates in plates.

Since precipitin reactions may vary considerably as different agar solvents are employed, the enhancing activity of cadmium nitrate in agar-agarose (1 : 1) dissolved in barbital buffer was superior to those dissolved in either physiologic saline or distilled water. Crowle also noted the differences in the enhancing effect of cadmium on rabbit antisera and guinea pig antisera. Cadmium enhanced rabbit antiserum activity in barbital buffer but not in physiologic saline or distilled water. Guinea pig antisera, however, were favorably affected in both saline and the buffer, but the specific enhancing effect of cadmium was not evident with these sera in distilled water.

The enhancing effect appeared at concentrations far below those at which it denatures proteins. Moreover, several other heavy metal cations such as mercury well recognized for their denaturing activity, so far have not been observed to enhance specific precipitation.

Unspecific proteins visualized by cadmium simultaneously were successfully removed by Tween 80. The majority of the removed proteins are thought to be lipoproteins.

A clinical application of this method was presented. The diagnostic value for hepatoma patients was ascertained. The test for AFP at concentrations of 10,000 ng/ml down to 300 ng/ml is suitably carried out by this method. Thus about 87% of histologically verified hepatoma showed positive for AFP by this method.

The quantitative determination of serum AFP by the conventional single radial immunodiffusion method was not as sensitive as RIA, but the method described here increased the sensitivity by 10 times. The minimum amount determined was 0.086 mg/dl.

CONCLUSION

By applying cadmium ions to antigen-antibody aggregates in gels, the diagnostic value of cross-over electrophoresis in detecting serum AFP was increased. Positive results were obtained at a rate of 87% in histologically verified hepatoma patients. Increased AFP in the sera of a few patients with various liver diseases was also observed by this method. Such patients had fulminant hepatitis, chronic aggressive hepatitis of a severe activity and liver cirrhosis.

A combined duo antibody method for quantitation of serum AFP by single radial immunodiffusion precipitin reaction was also described.

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