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Lectin affinity electrophoresis of alpha-fetoprotein detected by immunoenzymatic and chemiluminescent amplification followed by direct scanning.

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Abstract

Microdetermination of alpha-fetoprotein (AFP) glycoforms by lectin affinity electrophoresis followed by chemiluminescence reaction using horseradish peroxidase (POD) or alkaline phosphatase (ALP) in antibody-affinity blotting was developed. The intensity of chemiluminescence obtained by ALP was greater than that by POD; however, the coefficient of variation with POD was less than that with ALP. The optimized sensitivity of the chemiluminescence method with POD was two times that of the most sensitive colorimetric method currently available in terms of the chemiluminescence intensity per unit AFP concentration. The lower detection limit by the chemiluminescence method with POD (0.5 ng/ml) was much lower than that by the colorimetric method (3 ng/ml). Both methods gave identical percentages of lentil lectin- and erythroagglutinating phytohemagglutinin-reactive minor bands using a serum with 52 ng/ml AFP. This result indicates that microdetermination of AFP glycoforms by chemiluminescence after lectin-affinity electrophoresis was more sensitive than currently available methods and that it is potentially useful for clinical application

KEYWORDS: alpha-fetoprotein, lectin affinity electrophoresis, chemiluminescence, antibody-affinity blotting, avidin-biotin complex

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Lectin Affinity Electrophoresis of α -Fetoprotein Detected by Immunoenzymatic and Chemiluminescent Amplification Followed by Direct Scanning

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Microdetermination of α -fetoprotein (AFP) glycoforms by lectin affinity electrophoresis followed by chemiluminescence reaction using horseradish peroxidase (POD) or alkaline phosphatase (ALP) in antibody-affinity blotting was developed. The intensity of chemiluminescence obtained by ALP was greater than that by POD; however, the coefficient of variation with POD was less than that with ALP. The optimized sensitivity of the chemiluminescence method with POD was two times that of the most sensitive colorimetric method currently available in terms of the chemiluminescence intensity per unit AFP concentration. The lower detection limit by the chemiluminescence method with POD (0.5 ng/ml) was much lower than that by the colorimetric method (3 ng/ml). Both methods gave identical percentages of lentil lectin- and erythroagglutinating phytohemagglutinin-reactive minor bands using a serum with 52 ng/ml AFP. This result indicates that microdetermination of AFP glycoforms by chemiluminescence after lectin-affinity electrophoresis was more sensitive than currently available methods and that it is potentially useful for clinical application.

Key words: alpha-fetoprotein, lectin affinity electrophoresis, chemiluminescence, antibody-affinity blotting, avidin-biotin complex

Lectin affinity electrophoresis of α -fetoprotein (AFP) coupled with antibody-affinity blotting developed by Taketa *et al.* (1) has been widely used for the early detection of hepatocellular carcinoma (HCC) (2,

3) because of the high specificities and sensitivities of the separated AFP glycoforms, AFP-L3 and AFP-P4. The HCCs detected currently by imaging modalities, such as ultrasonography, computed tomography or angiography, are mostly small in size and are associated with low serum levels of AFP, frequently below 20 ng/ml (4). As a result, there is a wide overlap in the range of AFP levels between HCC and precancerous cirrhosis, which also shows increased serum AFP levels frequently. Therefore, the development of highly sensitive assay methods of AFP glycoforms is urgently needed. Chemiluminescence is one of the candidate techniques to be applied to improve the sensitivity for detection of separated AFP glycoform bands.

Taketa (5) developed a chemiluminescence method which uses horseradish peroxidase (POD) and luminol as its substrate for the detection of separated AFP bands on nitrocellulose (NC) membranes after lectin affinity electrophoresis. By this method, the chemiluminescence was detected on X-ray films by direct contact printing. Although the sensitivity of detecting separated AFP bands was higher by this method; the reproducibility of the quantitation of relative chemiluminescence intensities of separated AFP bands was not always good, because manual control of the exposure time for printing a short-lived chemiluminescence on X-ray films introduced variation.

An apparatus for the direct scanning of chemiluminescence became available recently and it was used in the present study for the detection of AFP bands in lectin affinity electrophoresis. Vesterberg *et al.* recently developed a sensitive method for the quantification of proteins

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separated by electrophoresis. They used their own newly developed luminometer for the detection of chemiluminescence produced by 3-(4-methoxyspiro [1, 2-dioxetane-3, 2'-(5'-chloro) tricyclo [3.3.1.1.] decan]-4-yl) phenylphosphate (CSPD) or Lumi-Phos 530 (Lumigen Inc., Southfield, MI, USA) (6). However, the direct scanning of separated proteins on membranes has not been published yet. The present report describes the sensitivity and reproducibility of newly-developed methods that use a chemiluminescence-scanning apparatus for quantitation of AFP glycoforms separated by lectin affinity electrophoresis.

Materials and Methods

Electrophoresis for preparation of standard curves. Standard curves of AFP were prepared by impregnating known amounts of AFP into agarose gels and transferring them to NC membranes which were precoated with purified horse anti-human AFP antibodies or with mouse anti-human AFP monoclonal antibody [NB-11 (10 mg/ml), Nippon Bio-Test Laboratories Inc., Tokyo, Japan] as described previously (1). The anti-human AFP antibodies were purified by immunoadsorbent column chromatography with human AFP and elution with 8M urea. For electrophoresis, 1% agarose gels (Agarose M with an electroendosmosis of -0.13, Pharmacia LKB, Biotechnology AB, Uppsala, Sweden), 1 mm thick, were prepared on GelBond films (FMC Corporation, Rockland, MN, USA) with a mold having projections of $0.8 \times 5.0 \times 1.0$ mm in parallel with each other at a distance of 10 mm and at intervals of 5 mm to make troughs. The agarose gel was prepared in a gel buffer consisting of barbital/barbital-Na, (ionic strength 0.025, pH 8.6). Japanese AFP standard (Nippon Bio-Test Laboratories) was serially diluted with the gel buffer and bromophenol blue (BPB) at a final concentration of 1 mg/ml, and 4 μ l of the diluted solutions were applied to the troughs. Electrophoresis was run by applying a voltage gradient of 20 volts/cm using a model TC-3 electrophoretic chamber (Kayagaki-Irika-Kogyo, Tokyo, Japan) until free BPB migrated 10 mm from the origin.

Lectin affinity electrophoresis. Lectin affinity electrophoresis was carried out in agarose gels containing 0.2 mg/ml lentil lectin (LCA, Seikagaku Co., Tokyo, Japan) or 0.5 mg/ml erythroagglutinating phytohemagglutinin (E-PHA) (E₄-PHA, Seikagaku Co.) by applying 4 μ l of samples containing AFP at indicated

concentrations. Electrophoresis was run in the same manner as described above until free BPB migrated 5.5 cm from the origin.

Antibody-affinity blotting. After electrophoresis, AFP bands in agarose gels were transferred by 20 min capillary blotting to NC membranes (Bio-Rad Laboratories, Richmond, CA, USA) which were precoated with anti-human AFP antibodies.

Immuno-chemiluminescent amplification. The AFP transfers were treated with rabbit anti-human AFP antibodies (Dakopatts, Copenhagen, Denmark) diluted 1,000-folds, unless otherwise indicated, in 1% gelatin-containing Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 30 min, followed by biotinylated goat anti-rabbit IgG diluted 2,000-fold, unless otherwise indicated, in the gelatin-TBS for 20 min and then by 1,000-fold-diluted avidin-biotin POD complex (ABC-POD) for 20 min. The enzyme reaction was initiated by the addition of a luminol and paraiodophenol solutions with hydrogen peroxide immediately after mixing them. A high-sensitivity immunoblotting (R) kit ABC-POD (WAKO Pure Chemical Industries, Osaka, Japan) was used for this purpose.

For alkaline phosphatase (ALP) reaction, the anti-AFP (1,000-fold-diluted)-treated AFP transfers were allowed to react with the 2,000-fold-diluted biotinylated goat anti-rabbit IgG, followed by 2,000-fold-diluted avidin for 30 min and then by 2,000-fold-diluted biotinylated ALP for 30 min using a Vectastain ABC-AP kit, consisting of affinity-purified biotinylated goat anti-rabbit IgG, avidin and biotinylated alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA). The chemiluminescence reaction was allowed to proceed with Lumipuls, (Fujirebio, Inc., Tokyo, Japan) containing a solution of AMPPD [3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt]. AFP bands transferred to NC membranes were also visualized as formazan stain by the tetrazolium method of Taketa (7).

All the reactions were allowed to proceed at room temperature. Washing was done with 0.05% Tween 20-TBS for 5 min twice after each step. The intensity of chemiluminescence was determined by scanning the reacted NC membranes with a Lumi-Scan (Microtec Co., Funabashi, Japan) at a maximum speed of 0.25 cm/sec. The intensity is expressed as the peak area (mV · sec) of the Lumi-Scan chart.

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Results

Optimization of chemiluminescence assay.

The assay conditions which maximize the chemiluminescence intensity of the AFP bands were studied by following the time course of chemiluminescence produced by POD with luminol as a substrate and paraiodophenol as an enhancer. The results are given in Fig. 1. The maximum intensity was obtained at 30 sec of incubation. The chemiluminescence intensity decreased rapidly thereafter with prolonged soaking in the substrate solution. The intensity became nearly half the maximum in 2 min and dropped below one-tenth after 5 min of incubation. This indicated a rapid completion of the reaction as well

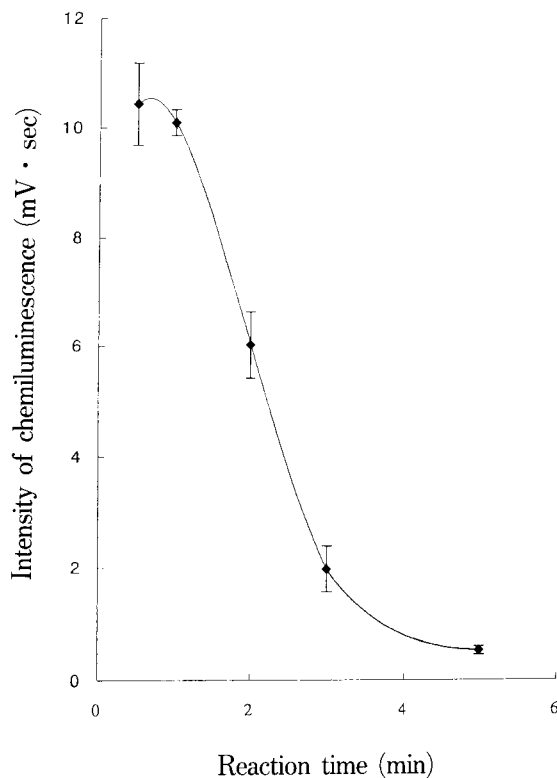


Fig. 1 Intensities of chemiluminescence of α -fetoprotein (AFP) bands in the peroxidase (POD) and luminol reaction. AFP transfers treated with 2,000-fold-diluted biotinylated antibody and ABC-POD were incubated with a mixture of luminol paraiodophenol and hydrogen oxide for the indicated periods of time and immediately scanned. The concentration of AFP applied was 20 ng/ml. Mean values and standard deviations (vertical lines) in the 20 determinations are given. Intensity of chemiluminescence is expressed the peak area in scanning.

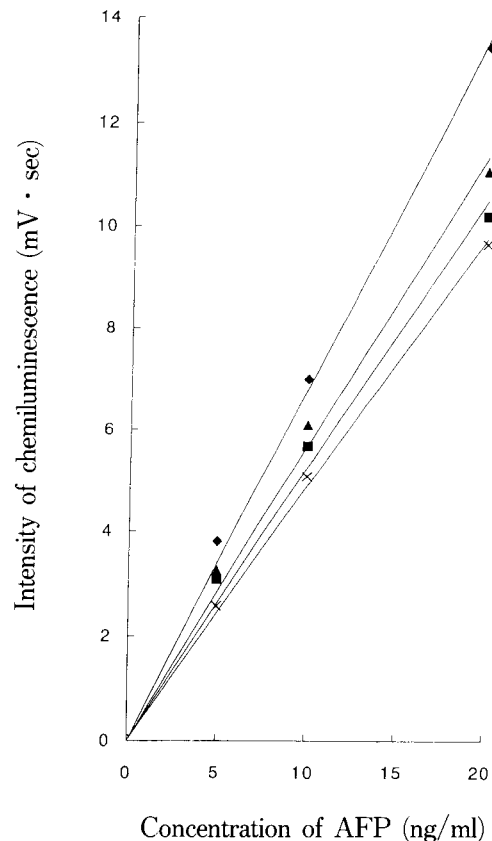


Fig. 2 AFP concentration and chemiluminescence intensities, with different dilutions of antibodies. \blacklozenge , 1,000-fold-dilution of rabbit antihuman AFP antibodies and 1,000-fold-dilution of biotinylated antibodies; \blacksquare , 2,000- and 1,000-; \blacktriangle , 1,000- and 2,000- and; \times , 2,000- and 2,000-fold, respectively.

as a rapid decay of the luminol chemiluminescence.

The effect of different concentrations of rabbit anti-AFP and biotinylated goat anti-rabbit IgG antibodies on the intensity was studied and the results are given in Fig. 2. There were no marked differences in the chemiluminescence intensity among the different combinations of antibodies. The maximum intensity was obtained with 1,000-fold-diluted rabbit anti-AFP and 1,000-fold-diluted biotinylated goat anti-rabbit IgG antibodies. However, the background intensity became significantly high under this condition, and 2,000-fold-diluted biotinylated goat anti-rabbit IgG antibodies and 1,000-fold-diluted rabbit anti-AFP antibodies were used in the following studies. Figure 2 also shows that the reaction was nearly linear

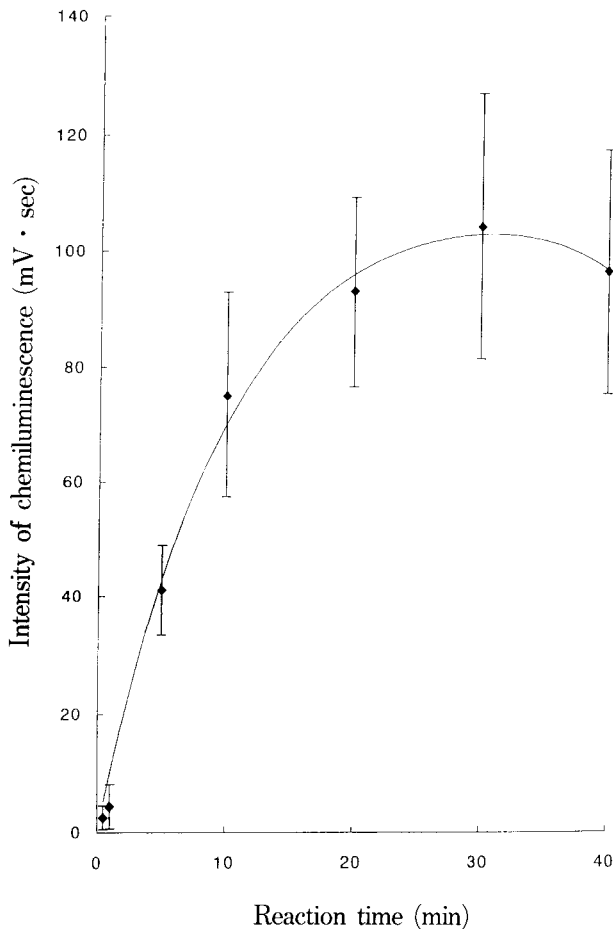


Fig. 3 Intensities of chemiluminescence of AFP bands in alkaline phosphatase (ALP) and AMPPD reaction. AFP transfers treated with avidin and biotin-labeled ALP were incubated with AMPPD for the indicated periods of time and immediately scanned. The concentration of AFP applied was 20 ng/ml. Other symbols and abbreviation, see the legend to Fig. 1.

with respect to the AFP concentration. It was also noted that the light emitted by the peroxidase reaction decayed rapidly. Accordingly, when POD was used, scanning of AFP bands was made at the maximum scanning speed of 0.25 cm/sec starting 30 sec after the substrate was added.

On the other hand, the chemiluminescence evoked by the ALP reaction increased gradually in intensity until approximately 30 min and then declined as shown in Fig. 3. Therefore, the membranes for ALP reaction were incubated for 20 min and scanned at a speed of 0.25 cm/sec.

Although the chemiluminescence produced by ALP

and AMPPD remained longer, the chemiluminescence of the background also increased with time. The mean background intensity of the ALP reaction after 1 min was 1.1 ± 0.5 mV · sec and that of the POD reaction after 1 min was 0.3 ± 0.05 mV · sec.

The chemiluminescent intensities of the AFP bands obtained with POD and ALP are compared in Fig. 4. The relationship between the chemiluminescence intensity and the concentration of AFP was linear in both assays at least up to 20 ng/ml AFP. The intensity per unit AFP concentration was greater with ALP than with POD, although the variability was much greater with ALP than with POD.

The chemiluminescence intensities obtained with POD were compared with the intensities of the AFP bands

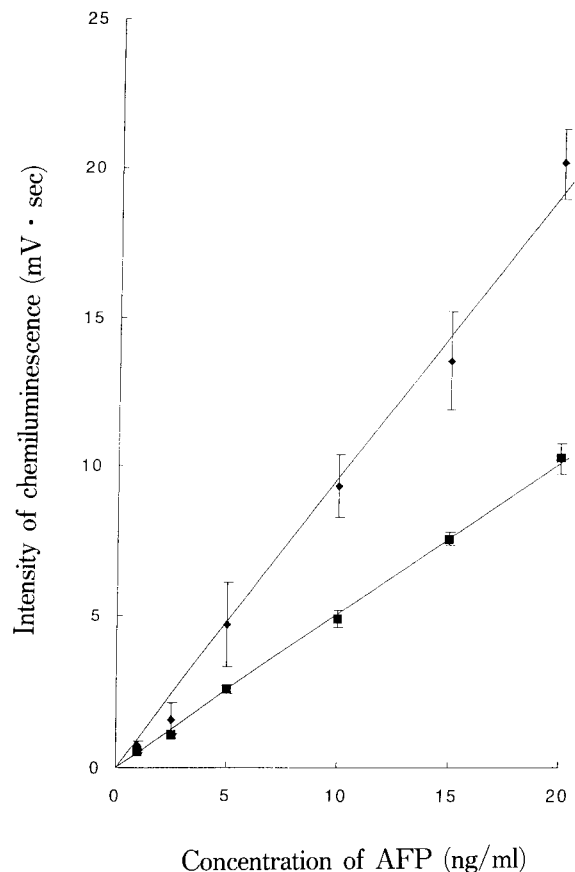


Fig. 4 The chemiluminescence intensities plotted against AFP concentration for POD and ALP reactions. ◆, ALP reaction; and ■, POD reaction. Other symbols and abbreviations, see the legend to Figs 1 and 3.

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obtained by the colorimetric method with POD in terms of the peak area in scanning. The intensities of chemiluminescence were significantly greater than those of formazan stain at AFP concentrations below 5 ng/ml as shown in Fig. 5. The peak area per unit AFP concentration by the POD chemiluminescence method was two times that by the colorimetric method. The detection limit of AFP chemiluminescence (0.5 ng/ml) was lower than that of formazan stain (3 ng/ml) as judged by the significant difference between the two adjacent concentrations of AFP.

Lectin affinity electrophoresis of AFP.

AFP bands separated by affinity electrophoresis with LCA and E-PHA and visualized by chemiluminescence

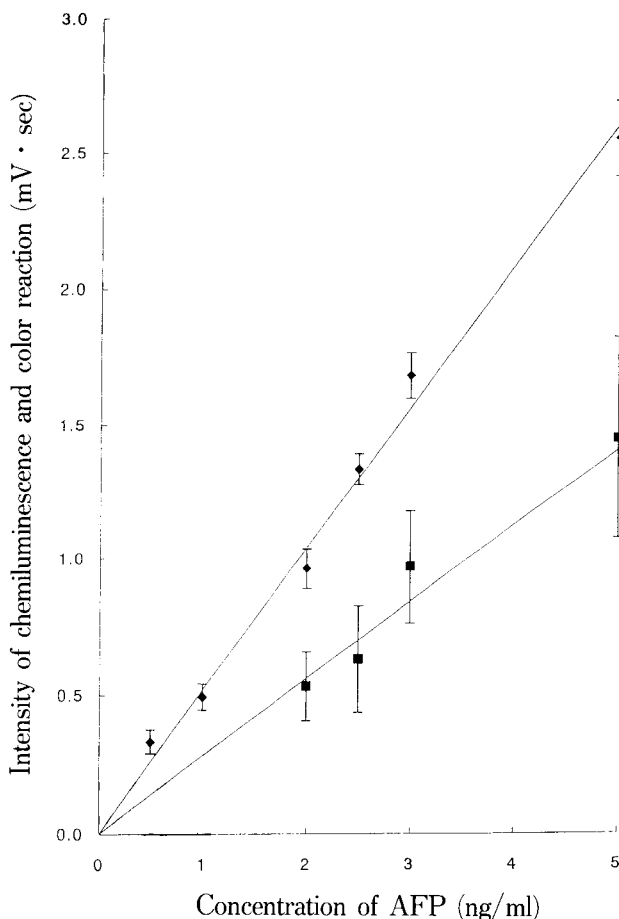


Fig. 5 Peak area of chemiluminescence intensity with POD and that of formazan stain plotted against AFP concentration. ◆, POD reaction; and ■, formazan stain. Other symbols and other abbreviation, see the legend to Fig. 1. Intensity of color production is expressed as peak area.

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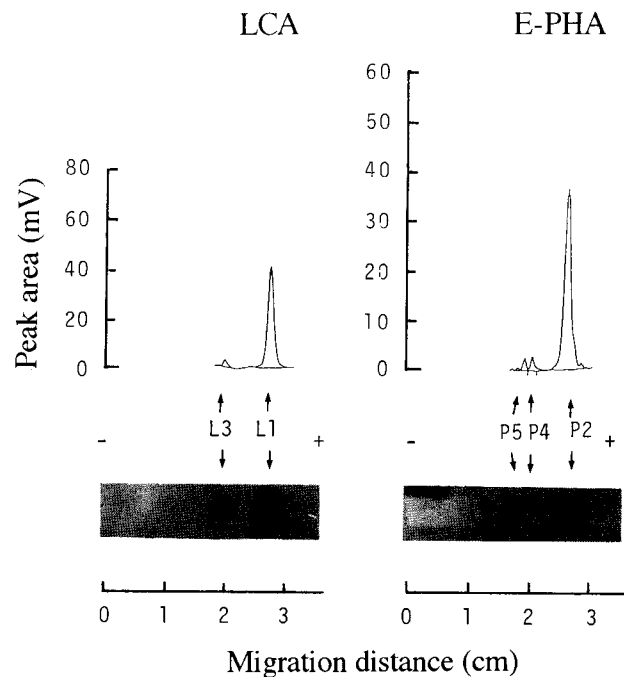


Fig. 6 Scanning profiles of AFP bands separated by lectin affinity electrophoresis and visualized by chemiluminescence reaction with POD and luminol and by POD and tetrazolium. Percentages of minor AFP bands, AFP-L3, AFP-P4 and AFP-P5, in chemiluminescence assay were 3.1, 6.6 and 7.8%, respectively, and those in color reaction were 2.2, 6.0 and 7.1%, respectively.

with POD are compared with those obtained by the color reaction of POD (Fig. 6). Patients having a serum level AFP of 52 ng/ml was diagnosed as liver cirrhosis, and 52 ng/ml of AFP could be separated and detected as minor bands. The relative intensities of the AFP bands, AFP-L1 and AFP-L3 (which were obtained with LCA) and of AFP-P2, AFP-P4, and AFP-P5 (which were obtained with E-PHA in the two different POD reactions for visualization of bands) were practically identical, as seen from the recording chart and the stained formazan bands shown in the same figure.

Discussion

The minimum concentration of AFP detected by the present chemiluminescent method with POD and luminol was lower than that of formazan bands. The detection limit by the chemiluminescence method was 0.5 ng/ml

while that in the color reaction was 3 ng/ml. When the peak areas of the AFP band per unit AFP concentration were compared, the extent of the increase in sensitivity was two-fold with POD and luminol over that with POD and tetrazolium. The extent of the amplification by chemiluminescence varied largely depending on the time of scanning after starting the reaction and also on the speed of scanning as the extent is readily from the time course of chemiluminescence of luminol. The chemiluminescence of ALP was relatively stable, although the background staining became significant and the actual signal-to-noise ratio was not as large as with luminol. The results obtained with POD and luminol on AFP separated by affinity electrophoresis agreed well with those obtained by the tetrazolium method, suggesting that the present method can be applied to clinically. Vesterberg *et al.* (6) showed a linear relationship between the protein concentration and the chemiluminescence intensity, although no attempt was made to apply their method to lectin affinity electrophoresis.

The sensitivities of lectin affinity electrophoresis of AFP attained by other methods are in similar ranges. Higashino *et al.* (8) detected 10 ng/ml of AFP after semi-purification and concentration by evaporation. Ajdukiewicz *et al.* (9) employed affinity column fractionation and concentration before analyzing AFP. Although these methods yielded desirable results, they are too time consuming and expensive to be applied to routine clinical materials. Thus, the present chemiluminescence method is useful for routine clinical application for patients with a high risk for HCC and with low serum levels of AFP. Theoretically, the analysis of separated AFP bands with an image analyzer for chemiluminescence would be better suited for avoiding the time difference in scanning the

separated AFP bands. The principle usefulness of the present method bears a fundamental importance in a sense that it has a potential to be applied to such image analysis and in turn to the secondary prevention of HCC.

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