Acta Medica Okayama

Volume 38, *Issue* 4 1984 *Article* 11

AUGUST 1984

Electrophoresis and electro-affinity transfer with specific antibodies to alpha-fetoprotein for detection of circulating immune complexes of alpha-fetoprotein.

Kazuhisa Taketa* Eriko Ichikawa†

Hiroko Taga‡ Hidematsu Hirai**

*Kagawa University,

†Kagawa University,

‡Tumour Laboratory,

**Tumour Laboratory,

Electrophoresis and electro-affinity transfer with specific antibodies to alpha-fetoprotein for detection of circulating immune complexes of alpha-fetoprotein.*

Kazuhisa Taketa, Eriko Ichikawa, Hiroko Taga, and Hidematsu Hirai

Abstract

A combination of agarose gel electrophoresis and a newly developed technique of electro-affinity transfer was applied to the detection of circulating immune complexes of human alpha-fetoprotein (AFP) and anti-AFP. After electrophoretic transfer to nitrocellulose membrane, to which affinity-purified polyclonal horse antibodies to human AFP were bound, the membranes were treated with or without rabbit immunoglobulins to human AFP, followed by overlaying with horseradish peroxidase-labeled goat anti-rabbit IgG for color development. Artificial complexes formed in vitro from human AFP and rabbit anti-AFP were clearly separated from free AFP by the agarose electrophoresis. The complexes were stained 20-40% as dark as the equivalent amount of free AFP by treatment with rabbit anti-AFP, and 10-20% as dark without the antibody treatment over a wide range of antigen-antibody ratios.

KEYWORDS: circulating immune complexes, ?-fetoprotein, electrophoresis, electroaffinity transfer, enzyme immuno-detection

---

*PMID: 6208755 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **38,** (4), 409-413 (1984)

—— BRIEF NOTE ——

ELECTROPHORESIS AND ELECTRO-AFFINITY TRANSFER WITH SPECIFIC ANTIBODIES TO α-FETOPROTEIN FOR DETECTION OF CIRCULATING IMMUNE COMPLEXES OF α-FETOPROTEIN

Kazuhisa TAKETA, Eriko ICHIKAWA, Hiroko TAGA* and Hidematsu HIRAI*

*Health Research Center, Kagawa University, Takamatsu 760, Japan and *Tumour Laboratory, Kokubunji 185, Japan*

Received May 7, 1984

Abstract. A combination of agarose gel electrophoresis and a newly developed technique of electro-affinity transfer was applied to the detection of circulating immune complexes of human α-fetoprotein (AFP) and anti-AFP. After electrophoretic transfer to nitrocellulose membrane, to which affinity-purified polyclonal horse antibodies to human AFP were bound, the membranes were treated with or without rabbit immunoglobulins to human AFP, followed by overlaying with horseradish peroxidase-labeled goat anti-rabbit IgG for color development. Artificial complexes formed *in vitro* from human AFP and rabbit anti-AFP were clearly separated from free AFP by the agarose electrophoresis. The complexes were stained 20-40 % as dark as the equivalent amount of free AFP by treatment with rabbit anti-AFP, and 10-20 % as dark without the antibody treatment over a wide range of antigen-antibody ratios.

Key words : circulating immune complexes, α-fetoprotein, electrophoresis, electro-affinity transfer, enzyme immuno-detection.

A number of tests have been developed for the detection of circulating immune complexes, and some of them are directed to the identification of specific antigens (1-3). The methods employing the separation of complexes and solubilization of antigens to be detected must be used with caution to avoid errors resulting from contamination or coprecipitation of the free antigen at high degrees of antigen excess, which is presumed to be the situation for circulating immune complexes of AFP.

Asano *et al.* (4) suggested the possibility of the presence of autoantibodies to AFP, although no direct evidence for AFP-anti-AFP complexes has been obtained. On the other hand, enzyme-linked immunoglobulins have been reported in one to five out of thousand subjects without specified diseases (Progress note (1981-82) on Enzyme-Linked Immunoglobulins, Incidence and Distribution in Japan reported by a working group of Tsuyoshi Kanno and others). The high inci-

dence rates are partly due to the altered isozyme pattern, which gives a greater sensitivity than others to the detection of circulating immune complexes. For example, a case of lactate dehydrogenase-linked IgG (κ and λ) had no symptoms of autoimmune diseases and a test for circulating immune complexes was negative when serum was analyzed by a Clq solid-phase enzyme immunoassay (5) (unpublished observation; K.Taketa and M.Sato, Yura Hospital, Tamano, Japan). The electrophoretic technique will be better suited for the detection of presumed AFP-anti-AFP complexes if the antibody-bound AFP is visualized by application of a sensitive technique of enzyme amplification. A specific antibody solid-phase assay is also feasible provided that more than one extra antibody-binding site is left on the immune complex antigen.

In the present communication, a method for the detection of immune complexes of AFP is described based on the assumption that the autoantibodies to AFP in patients with hepatocellular carcinomas exist at various degrees of antigen excess. Since no human antibody to AFP is available at present, rabbit antibodies to AFP were used to preform artificial complexes of human AFP and anti-AFP *in vitro.*

Materials and Methods. As a source of AFP, ascites fluid obtained from a patient with hepatocellular carcinoma was used. Agarose gel electrophoresis was performed as described previously (6) by applying samples (4 μl/5 mm slit) containing 16 ng of AFP and varied amounts of rabbit immunoglobulins to human AFP (DAKO-immunoglobulins, Ltd., Copenhagen). After electrophoresis, AFP and AFP-containing complexes were specifically transferred to horse anti-AFP-treated nitocellulose membranes (Bio-Red Lab., Richmond) by electrophoresis (40 V-200 mA, 1 h at room temperature) with a Marysol Gel-Membrane-Transfer apparatus (Model KS-8450GMT, Marysol Industry Co., Ltd.) and a buffer system of Tris (41 mM)-boric acid (40 mM), pH 8.3 (7). This new technique is a variation of electroblotting (7) and filter affinity transfer (8), and referred to as electro-affinity transfer. The transferred membranes were soaked in 1 % gelatin/Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 2 min, transferred to 1 % gelatin/TBS with or without 500-fold diluted rabbit anti-AFP and incubated for 30 min (or overnight at 4 ℃ with identical results). After washing twice with TBS for 10 min each time, the membranes were incubated for 30 min with 1,000-fold diluted affinity-purified goat anti-rabbit lgG-horseradish peroxidase conjugate in 1 % gelatin/TBS and washed twice. Color was developed with diaminobenzidine (9). Densitometric scanning was performed with an Atago Quick Densitomaster (Atago Optical Works, Co., Ltd.) after treating the dried membranes with decalin.

Horse anti-sera raised against human AFP, which was purified from ascites fluid of hepatocellular carcinoma patients by the method of Nishi (10), were purified by ammonium sulfate fractionation and affinity chromatography with an immunoadsorbent colum of Sepharose 4B coupled with similarly purified human

AFP according to a method described previously (11). One mg of the affinity-purified antibodies reacted with 255 μg of AFP in a quantitative precipitation reaction. The antibodies were diluted with TBS to give a concentration of 50 μg/ml and reacted with nitrocellulose membranes (10 ml/sheet of 5 × 10 cm^2) under a gentle shaking for 30 min. The membranes were blotted with filter paper and fixed with glutaraldehyde vapor (12) for 30 min, blocked with 3 % gelatin/TBS for 30 min, blotted, fixed again with glutaraldehyde vapor, washed with TBS and dried *in vacuo* for storage at 4 ℃. The anti-AFP-coated nitrocellulose membranes were soaked in the Tris-boric acid buffer and used for electro-affinity transfer.

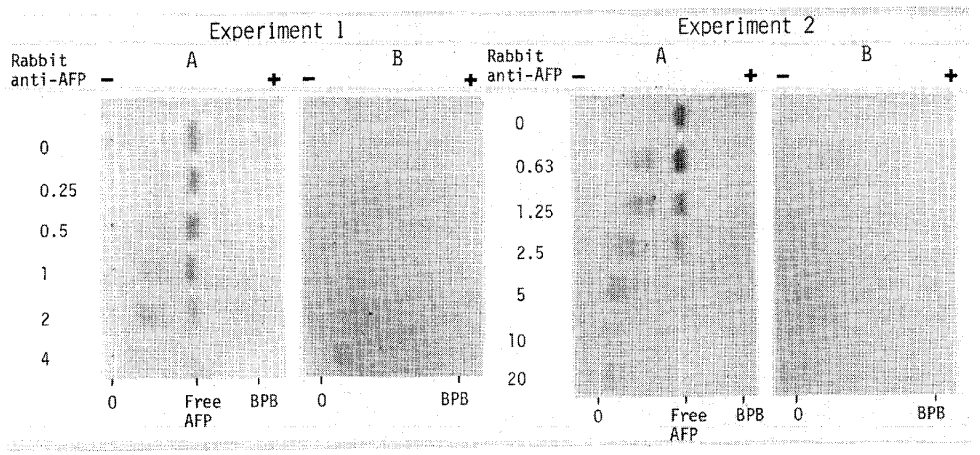

Fig. 1. Electrophoretic bands of free and anti-AFP-bound AFP. Results of two experiments with different ratios of anti-AFP to AFP are given. AFP and anti-AFP were mixed and kept at room temperature for 30 min before application. Electrophoreses in agarose gele were run until bromophenol blue (BPB) moved 3.5 cm (or free AFP moved 2.0 cm). Amounts of rabbit anti-AFP used are given in ml/mg AFP. A, transferred membranes treated with rabbit anti-AFP; B, untreated. 'O' denotes the origin.

Results and Discussion. AFP bands separated by electrophoresis and transferred to nitrocellulose membranes are shown in Fig. 1. When the transferred nitrocellulose membranes were further overlaid with rabbit immunoglobulins to human AFP (Fig. 1A), sharp anodic bands were demonstrated together with broad bands of intermediate mobilities. The intensity of anodic bands decreased as the amount of anti-AFP in the samples was increased, indicating that the fast moving bands represent free AFP. On the other hand, the intermediate bands appeared only in samples with anti-AFP, and their mobility was reduced as the amount of anti-AFP added to the sample was increased. In other experiments, free antibodies were shown to stay at the origin under the present electrophoretic conditions. Thus, the intermediate bands represent AFP-anti-AFP complexes. The reduced mobility of the complex bands with increased anti-AFP may be re-

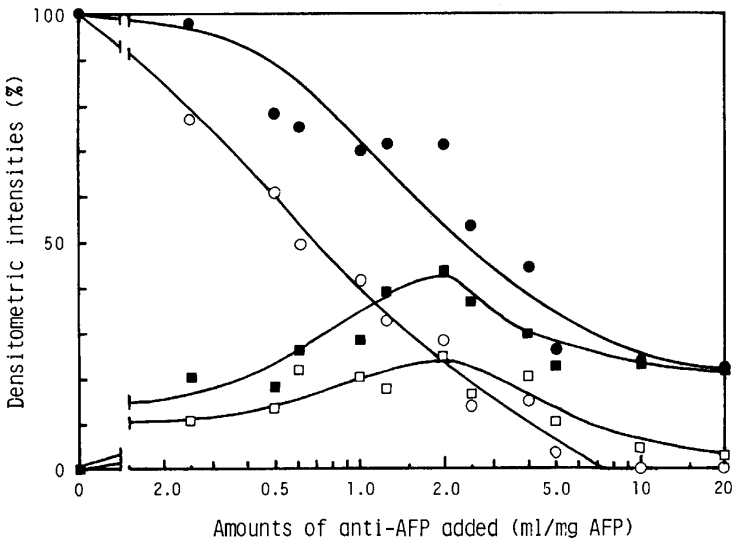

Fig. 2. Densitometric profiles of free and anti-AFP-bound AFP with varied ratios of anti-AFP to AFP. Densitometric scanning was carried out at 500 nm to obtain peak areas of AFP bands presented in Fig. 1. Intensities of AFP bands not mixed with anti-AFP were taken as 100 % (16 ng). ○, free AFP; ■, AFP-anti-AFP complexes treated further with anti-AFP; ●, ○ + ■; and □, AFP-anti-AFP complexes without further treatment with anti-AFP.

lated to the increased number of antibodies bound per molecule of AFP.

The results of densitometric scanning of the bands are graphed in Fig. 2. The intensity of immune-complex bands increased to some extent as the amount of anti-AFP added to the sample was increased up to 2 ml/mg AFP and then decreased with larger amounts of anti-AFP. One ml of anti-AFP was equivalent to 1 mg of AFP in a quantitative precipitation reaction. The sum of band intensities of free AFP and antibody-bound AFP tended to decrease as the amount of anti-AFP added became larger, probably due to saturation of antibody-binding sites of AFP and inhibition of AFP binding to the membrane antibodies, although more than 20 % of the initial intensity of free AFP was obtained even with antibodies added in excess.

When the membrane, to which AFP-anti-AFP complexes were transferred, was not overlaid with rabbit anti-AFP, free AFP was not visualized and the complexes were stained much more weakly (Figs. 1B and 2). This result indicates that a considerable number of extra antibody-reactive sites are available on the AFP molecules that have already bound at least two antibodies, the rabbit anti-AFP added to samples and the horse anti-AFP coupled to the nitrocellulose membrane. Since this result was found for the AFP reacted with excess antibodies, the first antibody (rabbit anti-AFP), which had already bound to AFP, might have been repelled or replaced by the second antibody (horse anti-AFP) on the

membrane. Whatever the mechanism involved may be, it is more sensitive to stain the immune complex-bound AFP by reacting with additional anti-AFP than to stain the immunoglobulins originally bound to AFP even in the presence of excess antibody. Thus, the present method provides keys to finding human auto-antibodies to AFP and can be used in conjunction with the ordinary method by overlaying with labeled antibodies produced against human immunoglobulins in order to identify AFP-anti-AFP (human) immune complexes.

REFERENCES

1. Anh-Tuan, N. and Novak, E.: Detection and quantitation of hepatitis-B surface antigen immune complexes (HBsAg-ICs) by an antigen-specific method. I. Detection and quantitation of *in vitro* prepared HBsAg-ICs. *J. Immunol. Methods* **33**, 293-300, 1980.
2. Brown, S.E., Steward, M.W., Viola, L., Howard, C.R. and Murray-Lyon, I.M.: Chronic liver disease : the detection and characterization of circulating immune complexes. *Immunology* **49**, 673-683, 1983.
3. Schüpbach, J., Kalyanaraman, V.S., Sarngadharan, M.G., Bunn, P.A., Blayney, D.W. and Gallo, R.C.: Demonstration of viral antigen p24 in circulating immune complexes of two patients with human T-cell leukaemia/lymphoma virus (HTLV) positive lymphoma. *Lancet* **1**, 302-304, 1984.
4. Asano, T., Yamada, N., Ochiai, T., Sato, H. and Fukao, T.: Presence of anti-AFP-antibody producing B cells in peripheral blood lymphocyte of hepatocellular carcinoma patient. *Jpn. J. Gastroenterol.* **81**, 278, 1984 (in Japanese).
5. Nakamura, T. and Yokota, H.: 'Determination of immune complexes by Clq solid-phase enzyme immunoassay'. *Jpn. J. Clin. Pathol.* **27** Suppl., 336, 1979 (in Japanese).
6. Taketa, K., Toguchi, E., Izumi, M. and Takeo, K.: Amplification of immunoprecipitates with horseradish peroxidase-labeled protein A : Application to studies of interaction between α-fetoprotein and lectins by affinity electrophoresis. In *Electrophoresis '82*, ed. D. Stathakos, Walter de Gruyter, Berlin, pp. 577-585, 1983.
7. Gershoni, J.M. and Palade, G.: Electrophoretic transfer of proteins from sodium dodecyl suifate-polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* **124**, 396-405, 1982.
8. Erlich, H.A., Levinson, J.R., Cohen, S.N. and McDevitt, H.O.: Filter affinity transfer. A new technique for the *in situ* identification of proteins in gels. *J. Biol. Chem.* **254**, 12240-12247, 1979.
9. Taketa, K.: Enzymatic amplification of electroimmunoprecipitates in agarose gels with horseradish peroxidase-labeled protein A. *Electrophoresis* **4**, 371-373, 1983.
10. Nishi, S.: Isolation and characterzation of a human fetal α-globulin from sera of fetus and a hepatoma patient. *Cancer Res.* **30**, 2507-2513, 1973.
11. Nishi, S. and Hirai, H.: A new radioimmunoassay of α-fetoprotein and carcinoembryonic antigen. In *Protides of the Biological Fluids, 23rd Colloquium*, ed. H. Peeters, Pergamon Press, Oxford, pp. 303-307, 1976.
12. Polvino, W.J., Saravis, C.A., Sampson, C.E. and Cook, R.B.: Improved protein analysis on nitrocellulose membranes. *Electrophoresis* **4**, 368-369, 1983.