대한의생명과학회지: 제3권 제2호 1997
Korean J. Biomed. Lab. Sci.
Vol. 3, No. 2, p.115~123, December, 1997

Production of Polyclonal Antibody against α-Fetoprotein and Polyclonal Antibody-Based Competitive Enzyme-Linked Immunosorbent Assay for α-Fetoprotein

Michung Yoon†

Department of Biology, Mokwon University, Taejon, 301-729, Korea

Abstract: α-Fetoprotein (AFP) has been a useful marker in screening and/or monitoring patients with hepatocellular carcinoma, gonadal germ cell tumor, gastric carcinoma and neural tube defects. In the present study, it was attempted to produce anti-human AFP polyclonal antibodies and to develop a competitive enzyme-linked immunosorbent assay (ELISA) for the measurement of AFP in human plasma and amniotic fluid. AFP was isolated from amniotic fluid using an isolation procedure consisting of affinity chromatography and preparative polyacrylamide gel electrophoresis. The antibody directed against AFP was raised in rabbits. Double immunodiffusion and Western blotting methods showed that the antiserum was highly specific, reacting with only AFP-containing samples. Standard curve was obtained by using purified AFP and specific antiserum. The assay sensitivity was 5ng/ml and the working range was 5~1,000ng/ml. The within-assay and between-assay coefficient of variance (CV) was 4.5% and 8.5%, respectively. These results indicate that the assay is valuable for the measurement of AFP and found to be simple, reproducible, and accurate.

Key Words: α-fetoprotein, Polyclonal, Antibody, ELISA

INTRODUCTION

AFP is a well-known embryonal serum protein produced by fetal liver cells, yolk sac cells, and some fetal gastrointestinal cells[15]. In yolk sac tumors, teratomas, and hepatomas, production of AFP by tumor cells has been proved, and detection of AFP in patient sera or in tumor tissues has become important in the diagnosis of these tumors[6,11,19,24,27,29]. Assay for AFP is widely used as a diagnostic aid in these malignancies and the oncofetal expression of AFP has made it an important model system for studies concerning the expression of fetal antigens in cancer[3,36]. These studies show that it is significant to develop an assay system for the measurement of AFP. Earlier, I reported many results related with antigen and antibody reaction[1,37,38,39,40]. This paper describes the development of an assay for measurement of AFP, using AFP and anti-AFP polyclonal antibody prepared in my laboratory.

*Received October 31 1997, Accepted after revision December 22, 1997.
†Corresponding author. Reprint requests: Michung Yoon, Ph.D. e-mail) yoon60@mwus.mokwon.ac.kr

MATERIALS AND METHODS

Materials

Amniotic fluid was obtained from Eulji General Hospital (Taejon). CNBr-activated Sepharose

4B was purchased from Pharmacia (Sweden) and the other chemicals were purchased from Sigma (USA).

Preparation of AFP antibody-coupled Sepharose 4B

An AFP antibody-affinity column was prepared by covalently linking 87.5mg of AFP antibody to 5g of CNBr-activated Sepharose 4B. Excess ligand was washed away with coupling buffer and any remaining active groups were blocked with 0.1M Tris-HCl (pH 8.0) for 2 hrs at room temperature. The product was washed with three cycles of alternating pH. Each cycle consists of a wash with 0.1M acetate buffer (pH 4.0) containing 0.5M NaCl followed by a wash with 0.1M Tris buffer (pH 8.0) containing 0.5M NaCl. The product was stored at 4-8℃.

Affinity chromatography on AFP antibody coupled-Sepharose 4B

The dialyzed amniotic fluid with pre-elution buffer, 0.01M phosphate buffer (pH 6.8), was affinity-chromatographed on AFP antibody-Sepharose 4B. Binding molecules were eluted with elution buffer, 0.1M glycine buffer (pH 2.5). Peaked fractions of eluates were dialyzed, lyophilized, and loaded onto 10% SDS-polyacrylamide gel for preparative electrophoresis and the contaminant from AFP preparation was removed by electroelution. Purified AFP was dialyzed against 0.9% NaCl and 0.01% EDTA solution (pH 7.4). The purity was confirmed by 10% SDS-polyacrylamide gel electrophoresis (PAGE).

Radioimmunoassay of AFP

AFP was measured by using radioimmunoassay kit (RADIM, Italia). Reagents and samples were allowed to warm to room temperature and the samples were mixed by inversion before use. 25μl of each standard, control serum and samples were pipetted into the antibody-coated tube and 300μl of assay buffer was added into the all tubes except those of total activity. All tubes were incubated for 30 min at room temperature with orbital shaker. At the end of the incubation, incubation mixture was carefully aspirated from all tubes. All tubes were washed once with 1ml of diluted washing solution and the rims of the tubes were blotted with a towel. 300μl of ^{125}I-monoclonal anti-AFP was pipetted into all tubes and all tubes were incubated for 30 min at room temperature. Tubes were washed and aspirated with aspiration pump. Radioactivity present in the tubes was counted using a gamma counter for 1 min.

Preparation of antiserum against AFP

Purified AFP (50μg) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits. Four weeks later, booster injections were done with freshly prepared emulsion of the antigen and Freund's incomplete adjuvant. Blood was drawn from the rabbits, allowed to clot at 4℃, and the antiserum was recovered by low speed centrifugation.

Immunoblotting

Immunoblotting was performed according to the method of Towbin *et al*[33]. AFP from SDS-PAGE was transferred to nitrocellulose membrane at 40V-constant condition for 4 hrs. After transfer, membrane was soaked in a blocking buffer (10mM sodium phosphate, 150mM NaCl, 0.05% Tween 20, 1% bovine serum albumin, pH 7.5) for 1 hr at room temperature and washed 3 times with a washing buffer (10mM sodium phosphate, 150mM NaCl, 0.05% Tween 20, pH 7.5). Next, the membrane was incubated for 1 hr at room temperature in a washing buffer containing antiserum (10^{-4}-dilution) against AFP. After washing once, the membrane was incubated in the washing buffer containing goat anti-rabbit IgG coupled with horseradish peroxidase (HRP) for 1 hr at room temperature and then washed again. Next, a

freshly prepared solution of peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, was added for the visualization of positive bands.

Double Immunodiffusion

1% (w/v) agar solution was prepared by heating 1g of agar in 100ml of phosphate buffered-saline (PBS) containing 0.02% sodium azide. 25ml of the agar solution was pipetted onto a petri dish (9cm x 9cm) and holes were punched on the solidified agar plate. 60μl of antiserum was loaded into center well, and the same volume of antigen or several other proteins were loaded into the surrounding wells. The plate was incubated overnight at room temperature in a humid chamber.

ELISA

96 well microplates were coated with 0.1ml of PBS containing 5μg/ml of AFP overnight at 4℃. The wells were washed 3 times with 0.2ml of an washing buffer (PBS containing 0.5mg/ml of bovine serum albumin and 0.05% Tween 20). A blocking buffer (1% bovine serum albumin in PBS) was added, the plates were incubated for 1 hr at room temperature with gentle agitation, and then the wells were washed 3 times with the washing buffer. 50μl of standard or samples were added, followed immediately by addition of 50μl of diluted anti-AFP antiserum. The plates were incubated overnight at 4℃. After washing, 0.1ml of horseradish peroxidase-conjugated goat anti-rabbit IgG was added to the wells, the plates were incubated for 1 hr at room temperature and then washed again. 0.1ml of freshly prepared solution of o-phenylenediamine and H_2O_2 was added to all the wells, and the absorbances at 450nm were determined by ELISA reader (BIO-RAD, USA).

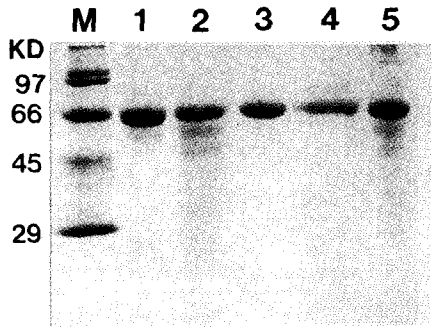

Figure 1. 12% SDS-PAGE analysis of AFP. M, protein standard marker; 1, amniotic fluid; 2, chromatographed AFP fraction; 3, electroeluted AFP; 4, AFP from Sigma; 5, human serum albumin.

RESULTS

Isolation of AFP

AFP-containing fractions were collected from anti-AFP antibody-coupled Sepharose 4B affinity chromatography and the second peak eluted with elution buffer (pH 2.5) contained AFP fractions. The final preparation of immunoreactive AFP was obtained through electroelution from SDS-PAGE. Figure 1 represents the AFP preparation compared with a commercialized AFP (Sigma) on a 12% SDS-PAGE and the lane 3 of AFP fraction exhibited single band with no detectable contaminant protein. AFP content of each preparation such as amniotic fluid, chromatographed AFP fraction and electroeluted AFP fraction was measured by radioimmunoassay and the final yield of AFP was 51% of the starting material (Table 1).

Preparation of antibody against AFP

The antibody directed against AFP antigen was raised in rabbits. The purity and specificity of the antibody were estimated by dou-

Table 1. Summary of percent recovery of human AFP for the various purification steps

Steps	% Recovery
Amniotic fluid	100
1-1) Elution with glycine buffer on affinity chromatography	85
1-2) Pool, concentration	72
2-1) SDS-PAGE and electroelution	60
2-2) Pool, concentration	51

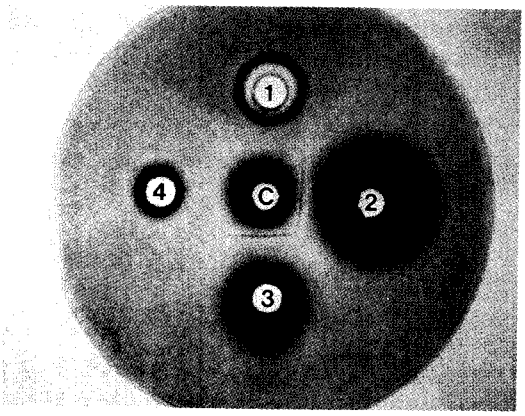

Figure 2. Double immunodiffusion of AFP. C is antiserum against AFP. 1, normal serum; 2, amniotic fluid; 3, AFP; 4, human serum albumin.

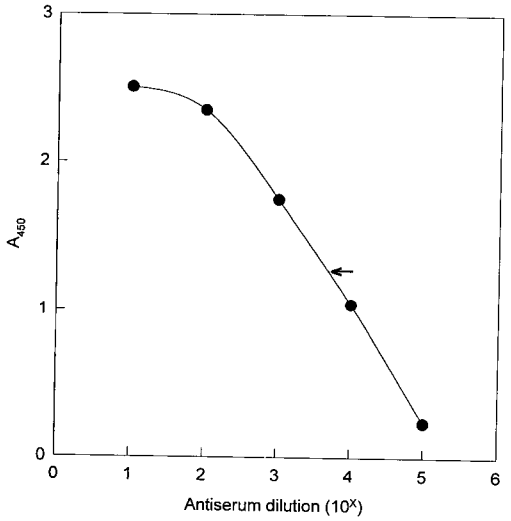

Figure 4. Titration curve of anti-AFP antiserum. The antiserum was diluted as indicated on the abscissa and the arrow indicates antibody titer.

ble immunodiffusion and Western blotting methods. In the double immunodiffusion assay, the antiserum formed single precipitation line with amniotic fluid or AFP fraction but no line was formed with human serum albumin or normal human serum (Fig. 2). In Western blotting analysis, positive band was obtained at a molecular weight-position of 70Kd when amniotic fluid, AFP from our laboratory or AFP from Sigma company was loaded on the gel and analyzed, but no band was obtained

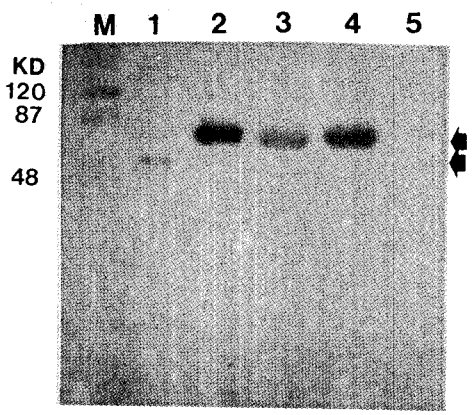

Figure 3. Western blot analysis of AFP. The two arrows indicate AFP bands. M, prestained protein standard marker; 1, amniotic fluid; 2, chromatographed AFP fraction; 3, electroeluted AFP; 4, AFP from Sigma; 5, human serum albumin.

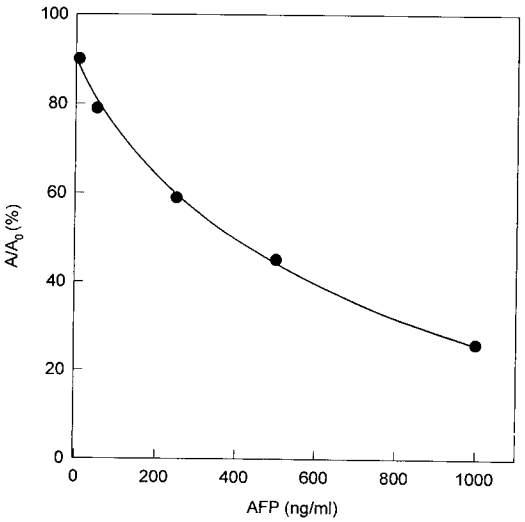

Figure 5. Standard curve of the ELISA for human AFP. A_0 is the absorbance measured in the absence of AFP.

when human serum albumin was used for the analysis (Fig. 3). Therefore, these results clearly indicate that the prepared antiserum reacts with AFP with monospecificity.

Development of ELISA

An optimal dilution of the anti-AFP antiserum used in the competitive ELISA was determined by a titration curve and $5x10^{-3}$ was

Table 2. The specificity of ELISA for AFP

	Cross-reactivity (%) with	
	AFP (5μg/ml)	Human serum albumin (5μg/ml)
Anti-AFP antiserum	100	0.17

the optimal dilution of antiserum used in this assay (Fig. 4). A standard curve was drawn from ELISA results obtained by using serially diluted solutions of the purified AFP (Fig. 5). The working range calibrated from the standard curve was 5~1,000ng/ml of AFP concentrations in this ELISA. The assay sensitivity, minimum detectable concentration of AFP was 5ng of AFP per ml (Fig. 5). About specificity, the antiserum didn't show a detectable cross-reaction with human serum albumin (Table 2). About precision, the within-assay and between-assay CV were 4.5% and 8.5%, respectively (Table 3).

DISCUSSION

The clinical significance of human α-fetoprotein (AFP) as a marker in the diagnosis and monitoring of certain types of cancer as well as screening for neural tube defects has been extensively reviewed[12]. AFP has been one of the first tumor-associated antigens proven useful in the diagnosis and evaluation of therapy of hepatocellular carcinoma and testicular germ cell tumors[2,10,14,27]. Although it is clinically significant to develop an assay system for AFP measurement, its development has not been domestically achieved. In this study, it was attempted to develop an competitive ELISA measuring the AFP concentration, by using purified AFP antigen and polyclonal antibody.

Like other immunoassays, choosing an appropriate standard and documenting its purity and physiological properties are matters of primary concern for the development of immunoassay for AFP. A purification procedure for AFP was employed in isolating mg level of AFP from amniotic fluid. Large amounts of AFP were purified on anti-AFP antibody-coupled Sepharose 4B affinity chromatography, followed by preparative SDS-PAGE. This method isolated AFP in 51% yield of the starting material and resulted in good purity, indicating that the two-step method used in this study is very efficient. In the earlier studies, we had to use several chromatographic procedures, i.e. Affi-gel blue, ion-exchange and concanavalin A-Sepharose affinity[16,18,25,30], for preventing the contamination of human serum albumin because of the similarity of the overall structures of AFP and human serum albumin[17,23,35]. But, the simple approach used in this study - high capacity affinity chromatography and high resolution preparative polyacrylamide gel electrophoresis - overcame the problems of the earlier preparative methods for the isolation of AFP such as low capacity with high resolution or high capacity with low resolution.

Table 3. The precision of ELISA for AFP

AFP (ng/ml)	CV (%)	
	Within-assay	Between-assay
5	4.7	8.8
50	3.9	7.4
250	4.5	9.3
500	5.2	9.2
1000	4.3	8.2
5000	4.6	7.8
Mean	4.5	8.5

Polyclonal anti-AFP antibodies were obtained from rabbits. Antibodies were specific to AFP and did not show any detectable cross-reaction with serum albumin or with any other proteins in normal human serum by double immunodiffusion and Western blotting methods. The polyclonal antibody in antiserum used in this assay was not affinity-purified, but qualities including the specificity were comparable to those of affinity-purified antibodies[8,13], al-

though using an antigen-coupled affinity column to isolate the antibodies might help to recover higher-reactive antibodies in the immunoassays[8,22]. These results suggest that antibodies were well-prepared for the development of the immunoassay system.

In Western blotting analysis, two positive bands were obtained at molecular weight-positions of about 70Kd and 65Kd when amniotic fluid was loaded on the gel and analyzed. It was reported that carbohydrate portions of AFP from several sources should be heterogenous since such neoplastic AFP has differently glycosylated moieties and the carbohydrate extensions differ according to the stages of pregency[9,21,24]. Therefore this result shows the presence of molecular variants of AFP in amniotic fluid.

Assay of AFP in biological fluids and especially in serum has traditionally involved radioimmunoassay or immunoradiometric assay[26]. Among alternative methodologies, luminiscence[20], time-resolved fluorescence[10] and ELISA[34] are commercially available. Among the 5 methodological approaches, radioimmunoassay and ELISA require the least amount of antibody and are the most sensitive, but radioimmunoassay is not convenient for clinical laboratories to use due to safety requirements inherent to the handling of radioactive materials. Thus we used ELISA method to develop an immunoassay for the measurement of AFP concentration in human. In addition, powerful combinations arise when enzymes are used with substrates that release fluorescent[28] or chemiluminescent[4,5] products. A recent review reports the most promising techniques that have been used for both immunoassays and DNA probes[32].

In this experiments, the optimal dilution of primary antibody determined for AFP assay was $5x10^{-3}$ and the working range of AFP concentrations calibrated from standard curve was 5～1,000ng/ml in the competitive ELISA, which is pretty similar to those of previously reported assay[8,20,31]. This assay was thus highly sensitive and suitable to measure the serum or amniotic samples having low concentrations of AFP. The precision in the present result was comparable to that of previously reported AFP ELISA[34,31]. These results indicate that these methods have high sensitivity, high precision and high antigen-binding capacity. In addition, it is urgently required to develop the new method measuring the high level of AFP because one of the great sources of error in highly sensitive immunoassay like ELISA is in dilution of serum or amniotic fluid samples.

In conclusion, the ELISA developed in this report for human AFP offers several advantages: sensitivity, specificity, no need of hazardous radiochemicals, and potential for use with monoclonal antibodies. Its precision and accuracy meet the requirements of a good immunological techniques. The further study is planned to develop an extra ELISA for human AFP employing monoclonal antibodies.

Acknowledgement

I am grateful to Ji-Won Ahn and Wha Yeon Chung for the technical help. This work was supported by a grant from Korea Sanhak Foundation.

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=국문초록=

인간 α-fetoprotein (AFP)에 대한 폴리클로날 항체의 생산 및 α-fetoprotein 측정용 효소면역분석법 (competitive ELISA)의 개발

목원대학교 이공대학 생물학과, 대전, 301-729

윤 미 정†

인간 α-fetoprotein (AFP)은 간암, 위암, 생식기종양 및 신경관이상인 환자를 검사하고 진단하는데 유용한 지표로 알려져 있다. 본 연구에서는 사람의 AFP를 분리정제하여 폴리클로날 항체를 생산하고 인간 혈장과 양수내의 AFP를 측정하기 위한 경쟁적 효소면역분석법을 개발하고자 하였다. 친화크로마토그래피법과 SDS-polyacrylamide 전기영동법을 이용하여 양수로부터 AFP를 분리하였다. 정제된 AFP를 토끼에 주사하여 폴리클로날 항체를 생산하였으며, 이중면역확산법과 Western blot 분석법을 사용하여 본 연구실에서 제조된 항체의 항원 특이성이 대단히 높음을 확인하였다. AFP와 항혈청을 이용하여 표준곡선을 얻었으며, 민감도는 5ng/ml이었고, 작용범위는 5～1,000ng/ml이었다. 분석내 CV는 4.5%이었고, 분석간 CV는 8.5%이었다. 따라서 이러한 결과로 보아 본 연구에서 개발된 경쟁적 효소면역분석법이 AFP를 측정하기에 적절하며, 간암 등의 기초연구에도 많은 기여를 할 것으로 생각된다.

[대한의생명과학회지 3(2): 115-123, 1997년 12월]

†별책요청 저자