Medical Journal of the
Islamic Republic of Iran

Volume 15
Number 4
Winter 1380
February 2002

MONOCLONAL ANTIBODIES TO HUMAN ALPHA-FETOPROTEIN

Z. AMIRGHOFRAN AND A.K. SHEIKHI

From the Department of Immunology,
Shiraz Univeristy of Medical Sciences, Shiraz, I.R. Iran.

ABSTRACT

Alpha-fetoprotein (AFP), a serum glycoprotein belonging to the onco-developmental proteins group, serves as a marker both in cancer research and in studies concerning fetal development and fetal pathophysiology.

Monoclonal anti-AFP antibodies are essential reagents in developing appropriate techniques for measurement of this protein. In this study, in order to produce anti-AFP monoclonal antibody (mAb), AFP was partially purified from cord sera using two-step ion-exchange chromatography on DEAE-cellulose with a final recovery of about 570 μg. MAbs against this preparation was raised by hybridoma technology using Ag8.653 mouse myeloma cells as the fusion partner. Hybridomas appeared in 10% (30/300) of culture wells and of these 2 clones were found to be positive for anti-AFP production. In western blot analysis a 70 kD band from dead fetus serum-but not adult serum-was stained by both mAbs. A sandwich ELISA technique using polyclonal antisera on one side and mAbs on the other side was employed to plot dose response curves. The positive dose dependent reactivity of the mAbs with standard AFP and other AFP containing samples and the negative reaction with normal adult sera lacking AFP showed the specificity of the mAbs for AFP.

MJIRI, Vol. 15, No. 4, 237-242, 2002.
Keywords: Monoclonal antibody, Alpha-fetoprotein

INTRODUCTION

AFP is a fetal plasma protein with a molecular weight of 70,000 Daltons that is produced in large quantities by the fetal liver and yolk sac.[1-3]

Human fetal liver releases AFP at a rate of 19-26 μg/min between 14 and 20 weeks of gestation.[4] The AFP concentration in fetal serum is highest, as high as 3 mg/mL, during the 14th week of gestation, whereafter it decreases as pregnancy progresses.[4] In normal adults the concentration of AFP is extremely low.[1]

This protein has attracted considerable attention since its measurement in amniotic fluid and maternal serum is of clinical value in assessing various pathological conditions of fetal development and monitoring fetal maternal hemorrhage.[5-6] In adults, elevated levels of AFP are found in the plasma of patients with primary hepatoma and non-seminomatous germ cell tumors and have been shown to be helpful in the diagnosis and monitoring of these tumors.[7-9]

Although the functional properties of AFP are not clearly known, it has been proposed that AFP functions as a carrier of essential fatty acids to certain developing cells and as a possible immunosuppressor.[10-11]

A number of immunochemical approaches to AFP have been made, mostly by preparing polyclonal antisera.[12-13] Antisera can be produced by immunizing experimental animals with AFP-containing samples. The resulting sera are useful in different types of experiments. Although, in most situations, AFP levels are determined

Correspondence: Z. Amirghofran, Ph.D., Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. P.O.Box: 71345-1798, Fax: 98-71-334589, 59847 E-mail: amirghz@sums.ac.ir

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by RIA and ELISA techniques, accurate quantitation of an antigen by these methods is dependent on the availability of specific high-titer antibodies directed against the antigen. Monoclonal antibody (mAb) production provides adequate quantities of homogenous and specific antibodies for measurement of AFP.[8,14-15] For the first time Tsung et al. reported the production and characterization of a monoclonal hybridoma antibody specific for human AFP.[7] Subsequently a two-side sandwich enzyme immunoassay was developed by Utila et al.[16] So far, many mAbs have been reported to be produced as immunodiagnostic reagents, in the immunochemical analysis of antigenic determinants of AFP, in the elucidation of the genetic mechanism regulating AFP expression, and in studying the biological activity of AFP or AFP receptors presented on normal and malignant cells.[17-23]

The present study is an attempt to employ hybridoma technology for domestic production of mAbs directed against AFP in culture.

MATERIAL AND METHODS

Specimens

Cord sera and sera from aborted fetuses at 20-24 weeks of gestation were obtained from the delivery room. Samples were pooled and kept frozen at -20°C until use.

Purification of human AFP

A batch and column DEAE-cellulose chromatography was performed for partial purification of AFP from cord sera as described by Wu et al.[24] In this procedure, sodium phosphate buffer (SPB), pH 6.8 with different molarity was used for washing and elution steps. Briefly, in batch DEAE-celluose method, 50 mL of pooled cord sera was dialyzed overnight against 0.005 M SPB as the initial buffer at 4°C. The slurry of 40 g DEAE-cellulose was equilibrated with the initial buffer and then filtered off on a sintered glass funnel. After that, cord sera was added and a slight vacuum was employed. Then the cellulose bed was washed with 0.01 M SPB containing 0.085 M NaCl. In this washing step hemoglobin and the majority of the albumin of the cord sera were passed through. Then the AFP was eluted by 0.01 M SPB containing 0.5 M NaCl. The elution was monitored by measuring the absorbance of the eluate at 280 nm and stopped at an optical density (OD) of less than 0.02. This AFP-containing fraction was concentrated by polyethylene glycol (PEG) and then dialyzed against 0.1 M SPB as the starting buffer for DEAE-cellulose column chromatography

A column was packed with a slurry of 25 g of DEAE-cellulose in 0.01 M SPB and washed with the same buffer until equilibrated. Elution of AFP was performed by a linear gradient of NaCl from 0.05 to 0.4 M in 0.01 M SPB. The conductivity of the eluate fractions was determined by conductometery. The level of AFP was determined by ELISA assay and recorded for each fraction. Samples containing the peak of AFP were pooled and then concentrated.

Monoclonal antibody production and the fusion protocol

BALB/c mice were immunized intraperitoneally with 15-20 μg of partially purified AFP in complete Freund's adjuvant (Sigma, USA). Repeated immunization with three similar doses of antigen in incomplete Freund's adjuvant was performed on days 21, 42 and 56. Mice with higher antiserum levels received the last dose of AFP three days prior to the fusion. Spleen cells from immunized mice were fused with Ag8.653 mouse myeloma cell line using polyethylene glycol 1500 (Sigma, USA). Growing hybridomas were detected 7-10 days after fusion and screened by a modified ELISA asssay as described below to obtain the desired monoclonal antibody producing clones. Positive hybridomas were selected and cloned by the limiting dilution technique.

Screening and ELISA assays

A sandwich ELISA assay using rabbit anti-human AFP, standard human AFP (100 mg/mL) and peroxidase-conjugated rabbit anti-human AFP (all from DAKO, Denmark) was used for measurement of AFP in the samples. Briefly microtitre plates (NUNC, Denmark) were coated with 100 μL of diluted rabbit anti-human AFP (10 mg/L) in 0.05 M carbonate buffer, pH 9.6 for an overnight at 4°C. After washing, different dilutions of the standard AFP and then appropriate dilutions of the peroxidase-conjugated rabbit anti-AFP were added. After washing and addition of the substrate, OD was read at 492 nm. A standard curve with OD at 492 nm as ordinate and the log 10 concentration of standard as abscissa was plotted. This sandwich ELISA method was modified for screening of anti-AFP mAbs by replacing the second antibody (conjugated one) with the supernatant of hybridomas followed by addition of a peroxidase-conjugated goat anti-mouse Ig (DAKO, Denmark).

Western blotting analysis

Dead fetus and normal adult sera were electrophoresed on 10% polyacrylamide gel. After electrophoresis the proteins were transferred on nitrocellulose paper using Novablot 2 (Pharmacia, LKB) for 60 minutes with 100 mA. Following the protein transfer, the paper was blocked overnight in phosphate buffer saline containing 5% bovine serum albumin. After washing, the paper was incubated with an appropriate amount of mAbs for two hours fol-

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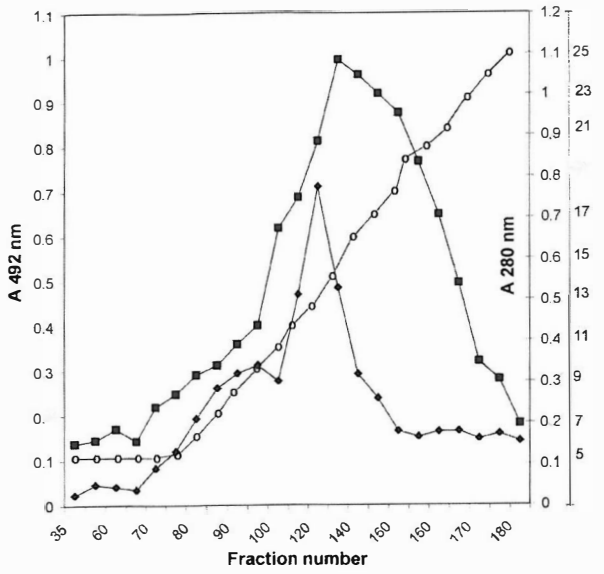


Fig. 1. Purification of AFP by DEAE-cellulose column chromatography. Elution of AFP was carried out by a linear gradient of NaCl (O–O) from 0.05 to 0.4 M in 0.01 M sodium phosphate buffer, pH 6.8. Values in tertiary axis are based on conductivity unit (mS). ●–● represents absorbance (A) at 280 nm and ■–■ represents AFP determined by ELISA assay (at 492 nm). Chromatography was performed at room temperature and 200 drops of eluate per tube was collected.

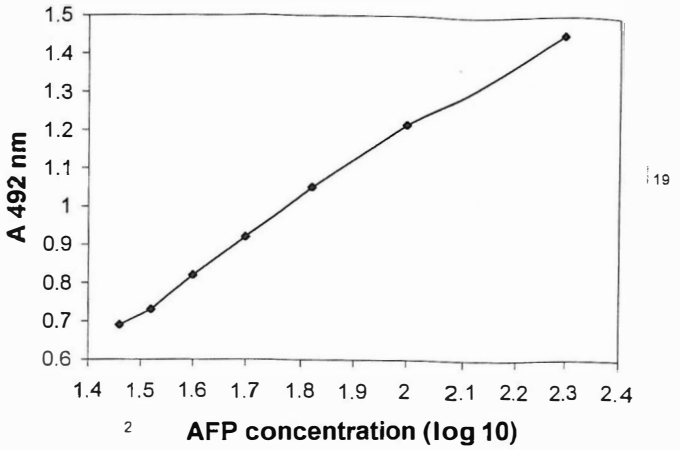


Fig. 2. The standard curve for AFP measurement. AFP concentration (ng/mL) presented as log 10 was determined by sandwich ELISA assay using polyclonal anti-AFP sera in both sides.

lowing addition of a peroxidase-conjugated goat anti-mouse Ig. The membrane was immersed in Supersignal west pico chemiluminescent substrate (Pierce, UK) containing luminol and peroxidase buffer as the detection reagents. The blot was inserted into a cassette with autoradiographic film. The autoradiograph was exposed for 15 minutes and then developed to observe the bands.

RESULTS

In this study cord serum was used for purification of AFP. At the first step a batch DEAE-cellulose chromatography was performed to remove the large quantities of albumin and hemoglobin. The eluted AFP was concentrated by dialyzing against PEG, and then its concentration was determined using the sandwich ELISA technique. From 100 mL of cord serum purified by this method, 570 μg/mL of AFP was totally obtained. In the next step, the concentrated AFP was applied to the DEAE-cellulose column chromatography. This method was performed using a linear gradient of NaCl from 0.05 to 0.4 M in 0.01 M SPB. The linearity of this gradient was shown by determining the conductivity of each fraction and is presented in Figure 1. The OD of each fraction at 280 nm, representing total protein concentration (mainly albumin), was determined. AFP measurement using ELISA method was also carried out for each sample and the results were recorded as OD. As shown in Figure 1, the highest level of AFP was eluted in the tubes numbered 100 to 165 (OD range, 0.62 to 0.5, maximum 1; conductivity range, 10.5 to 22). The tubes relevant to samples measured at 280 nm were omitted and other tubes (130-165) were pooled. After dialyzing against PEG, the sandwich ELISA procedure with serial dilutions of standard AFP and a 1/100 dilution of the semipurified AFP was performed to evaluate the final AFP concentration (Figure 2). 4.7 μg/mL AFP was obtained at this stage (total volume 50 mL). These purification steps were carried out twice and a total of 570 μg AFP was obtained. After immunization of the mice with AFP, somatic hybridization protocol was carried out. Hybridomas grew in 30 wells out of 300. After screening, two clones were found to be positive for anti-AFP production. These hybridomas cells were selected and cloned by limiting dilution technique. An indirect sandwich ELISA using rabbit anti-human AFP-coated plates was used for screening of the hybridomas. As shown in Figure 3 the supernatant of the cloned cells were able to recognize the standard AFP (OD range, 0.61 to 0.91 for C1 and 0.59 to 0.82 for C2). In a similar experiment, different dilutions of adult normal serum (instead of standard AFP) containing a very low amount of AFP (<10 ng/mL) were added to the rabbit anti AFP-coated plates and the ELISA procedure was performed using the supernatants of the two positive clones. The results presented in Figure 3 indicate that the absorbance obtained in this experiment was much lower than the previous experiment using standard AFP (OD range, 0.068-0.09). These results showed

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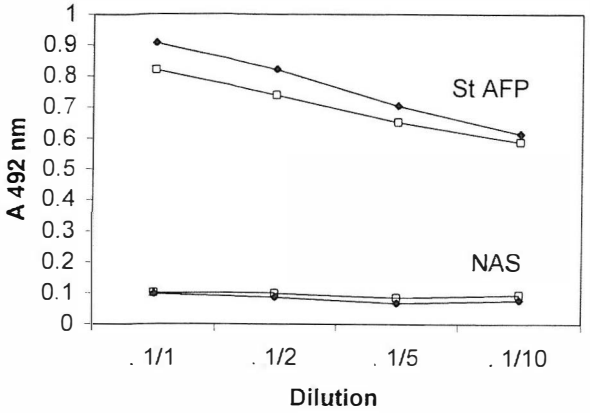


Fig. 3. Sandwich ELISA assay using mAbs C1 (●-●) & C2 (□-□) for various dilutions of standard (St) AFP (100 ng/mL) and normal adult serum (NAS).

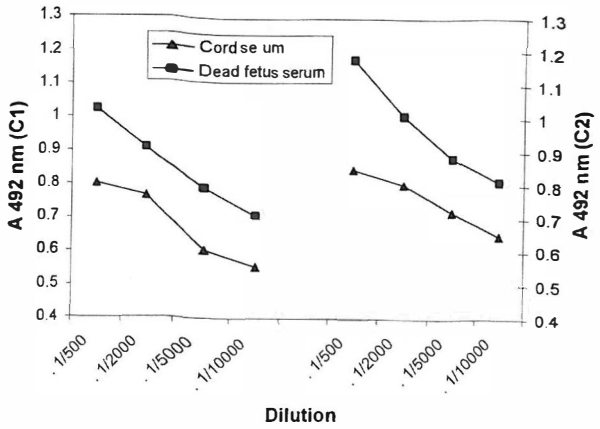


Fig. 4. Sandwich ELISA assay using mAbs (C1 & C2) for various dilutions of cord and dead fetus sera.

the specificity of the produced monoclonal antibodies for AFP and confirmed that both of these antibodies were not able to react with proteins presented in normal adult serum. For further confirmation of the results, samples with higher and lower amounts of AFP were used to show the ability of these mAbs for specifically detecting different concentrations of AFP. In this respect different dilutions of cord, dead fetus and normal adult sera were added to the rabbit anti-human AFP-coated plates and then the hybridoma supernatant was added. As shown in Figure 4, a relatively linear correlation between increase in the dilution and decreasing of OD was obtained. As expected, the concentration of AFP in dead fetus serum was higher than other samples (OD range 0.7-1.2 for C1 mAb and 0.8 to 1.3 for C2 mAb). By this method AFP levels were very low in adult serum (OD < 0.1). Determination of the molecular weight of proteins recognized by C1 and C2 mAbs was performed by immunoblotting analysis. As shown in Figure 5 a band with a molecular weight of 70 kD was stained by both mAbs.

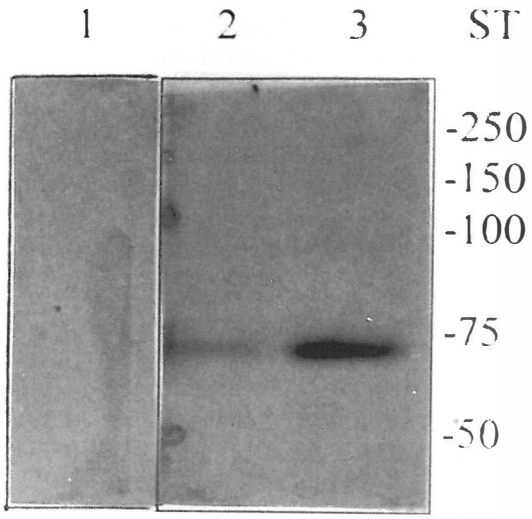


Fig. 5. Western blot analysis of dead fetus (lanes 2 and 3) and adult sera (lane 1) on 10% polyacrylamide SDS gel using C1 (3) and C2 (2) mAbs. (ST, standard molecular weight markers). For adult serum only the reaction with C1 is shown.

DISCUSSION

AFP was first described by Bergstrand and Czar in 1956, and has attracted increasing attention owing to its high clinical usefulness and possible immunoregulatory implications.[25] In order to establish either a sensitive method of AFP detection for the purpose of clinical diagnosis or for continuing the study of its physiological role, development of a hybridoma producing anti-AFP is essential. In this regard, appropriate amounts of purified antigen should be available to immunize mice and provide specific spleen plasma cell partners in the fusion protocol. The major difficulties in purification of AFP is due to the similarity in physiochemical properties between AFP and albumin and the presence of a large amount of albumin in specimens where a small amount of AFP is to be isolated.[26] Moreover, most specimens used for AFP isolation are difficult to obtain. One of the best sources of AFP is the serum obtained from dead fetuses aborted at 12-16 weeks of gestation (AFP concentration, 2 to 3.5 mg/mL).[9] However, despite the large amount of AFP in dead fetus, the low volume of blood samples as well as difficulties in collecting blood from this source has persuaded most investigators to use cord serum for purification of AFP. Wu et al. have presented a complete high yield purification procedure for AFP.[24] This procedure contains several steps of purification including batch DEAE-cellulose and column chromatog-

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raphy, Sephadex G-200, blue sepharose and affinity chromatography. In the present study, since a complete purification of the antigen was not necessary for production of mAbs against AFP, the semi-purification of AFP with a batch wise and column DEAE-cellulose chromatography was performed. As Wu et al. have shown, from 500 mL cord serum, as much as 20 g of albumin has to be removed. To separate this large amount of albumin and also other proteins including hemoglobin, the batch DEAE-cellulose method was developed and used before purification through the column chromatography. In this batch method, albumin and AFP were absorbed by the DEAE-cellulose and the majority of hemoglobin and gamma globulin passed through. Additional washing with 0.1 M SPB containing 0.08 M NaCl removed a large amount of serum albumin. Absorbed AFP was recovered then by eluting with 0.01 M SPB containing 0.5 M NaCl.

In the column chromatography, according to OD of fractions at 280 nm, one peak (tubes No, 100-140) was obtained but measurement of AFP in each sample showed another peak close to the first one (tubes No. 100 to 165). The latter peak occurred at an interval of 10.5 and 22 mS and this range of conductivity was in accordance with that obtained by Wu et al.[24]

Although the amount of total protein in this peak was still high, this was much lower than the primary source and the ratio of albumin to AFP concentration was markedly decreased. The pooled fractions contained enough AFP to immunize mice for the preparation of monoclonal anti-AFP. In this study, since a highly purified AFP is not easily available and is very expensive, development of an indirect ELISA technique using AFP-coated plates for screening of mAbs was not profitable. Therefore, a sandwich ELISA using rabbit anti-AFP in one side of the sandwich was developed.

Bound AFP was then quantitated by completing the sandwich with anti-AFP mAbs. As shown in Figure 3, a reasonable absorbance change over AFP was found with the two mAbs, whereas replacement of standard AFP with normal adult serum (containing all proteins except fetal ones) caused a poor-reactivity and -dose response curve. This observation was supported by results obtained by western bloting analysis in which the two mAbs reacted with a protein presented in the fetus serum but not in normal adult serum. The reactivity of mAbs with a 70 kD molecule was in agreement with the molecular size that has been reported for the AFP molecule.[1-3] These two mAbs also gave parallel dose response curves with AFP-containing samples including dead fetus and cord sera. The linear absorbance with response over these samples showed the proper relation between changes in OD and the concentration of AFP.

In conclusion, our results indicated that the produced mAbs have the capacity to be used in a sandwich-type ELISA assay for detection of human AFP. In this assay, a polyclonal anti-AFP was immobilized by absorption to the walls of microtiter plates and used to trap AFP in the samples. It is possible that by combining the mAbs, a more sensitive and specific ELISA employing mAbs on both sides can be worked out. It is obvious that anti-AFP mAbs of only one specificity may not be used in such a sandwich-type ELISA and the reactivity of the mAbs against two different epitopes should be confirmed.

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