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Short Communication

Production of Monoclonal and Polyclonal Antibodies against Human Alphafetoprotein, a Hepatocellular Tumor Marker

SHU-FEN CHOU,[1,2] WEN-LIN HSU,[3] JING-MIN HWANG,[3] and CHIEN-YUAN CHEN[2]

ABSTRACT

The objective of this study is to produce and purify monoclonal antibodies and polyclonal antibodies (PAbs) against human alphafetoprotein (AFP). Hyperimmune ICR mice produced PAbs after injection with 0.5 mL pristane, and were injected with NS-1 myeloma cells 2 weeks later. Hyperimmune Balb/c mice were used for the production of MAbs. Mice were immunized four times, given a final boost, and their spleen cells were collected and fused with NS-1 myeloma cells under the presence of PEG 1500. The fused cells were then selected in the hypoxanthine, aminopterine, and thymidine (HAT)-RPMIX medium. Anti-AFP antibody-secreting hybridoma cell lines with high titer were cloned by enzyme-linked immunosorbent assay (ELISA) and then subcloned by limiting dilution in 15% fetal bovine serum (FBS), hypoxanthine, thymidine (HT)-RPMIX medium. Twelve murine hybridoma producing anti-AFP MAbs were obtained and designated as A73F3, A73E8, B73C5, A73G3, A73F8, 67B3, B73C2, B73E1, A73G2, B73G7, B73D7, and B73F4. Isotypes of these MAbs were identified as IgG_1 heavy chain and κ light chain. The MAbs with high purity were obtained by affinity chromatography. The purity analysis of AFP and the MAbs was performed by capillary electrophoresis.

INTRODUCTION

ALPHAFETOPROTEIN (AFP) is a glycoprotein with molecular weight between 65–70 kDa. It was identified by Bergstrand[1] in 1956 as X-component in human cord blood. In 1963, Abelev et al.[2] demonstrated that this embryonal α-globulin was not only detected in normal pregnant mice and the sera of newborn mice, but also in mice bearing hepatocellular carcinomas. In 1964, Tatarinov[3] identified AFP in the sera of patients with primary liver tumors. It appears that AFP is first produced in the yolk sac and later in the liver of the embryo. The synthesis of AFP in the liver subsides and is depressed after birth and can be found in only extremely low amounts in the adult except in the presence of hepatomas or teratomas. Normal value of AFP concentration in a healthy adult serum is <20 ng/mL.

From 1975 Köhler and Milstein[4] successfully fused antibody-producing mouse spleen cells with mouse myeloma cells, the fusion of somatic cells has been carried out for many years with a variety of different aims. Monoclonal antibodies (MAbs) have been widely applied in various fields including immunoassay, immunotherapeutic techniques (radioimmunolocalization and radioimmunotherapy), and affinity chromatography, etc.[5] Antibodies (and antigens) have been used for many years for specific detection of their complementary partners. Immunoassay is the predominant analytical technique for quantitative measurements, being used over a wide concentration range and in many different biological matrices. The unique feature of immunoassay that provides desired specificity is the complementary reaction (both in chemical binding and spatial orientation of reaction groups) between antigen and antibody.[6]

In this paper, monoclonal and polyclonal anti-human AFP antibodies were produced, characterized, and purified. Furthermore, the purities of the commercial human AFP and anti-AFP MAbs produced were analyzed. These antibodies produced will

[1]Department of Food Health, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan, China.
[2]Department of Agriculture Chemistry, National Taiwan University, 1, Sec. 4, Roosevelt Road, Taipei, Taiwan, China.
[3]Department of Radiation Oncology, Tri-Service General Hospital, 8, Sec. 3, Ting-Chow Rd., Taipei, Taiwan, China.

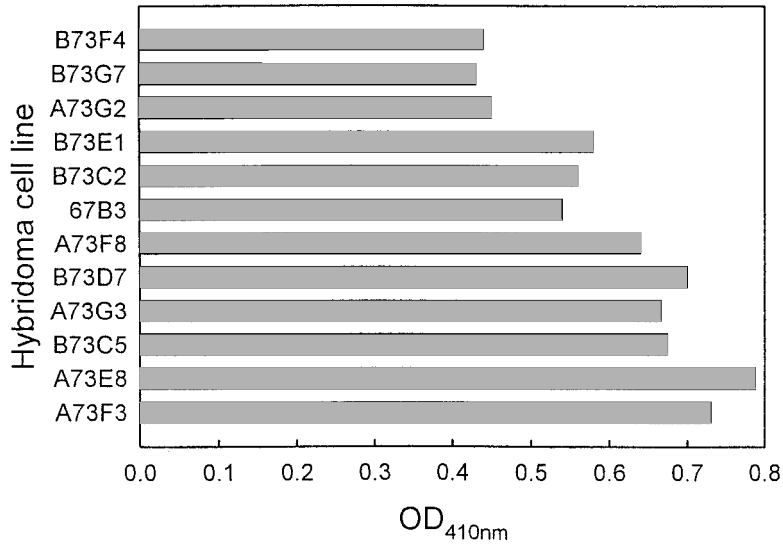


FIG. 1. Selection for anti-human AFP MAbs-secreting hybridoma cell lines with high titer by ELISA.

be used for application in the development of tumor marker immunosensors.

MATERIALS AND METHODS

Reagents

The following reagents were used: (1) AFP from human cell culture-derived (Calbiochem-Novabiochem Intl, La Jolla, CA) M.W. 70,000; sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) $\geq 95\%$; (2) RPMIX: RPMI 1640 (Seromed, Berlin, Germany) was supplemented with fetal bovine serum (FBS)(Hyclone, Logan, Utah) 12%, L-glutamine (200 mM, GibcoBRL, Grand island, NY) 1%, Pen-Strep (10,000 U penicillin G and 10 mg streptomycin/mL solution, 100×, GibcoBRL) 1%, fungizon (250 μg/mL, GibcoBRL) 1% and sodium pyruvate (100 mM, GibcoBRL) 1%; (3) Freund's adjuvant (complete and incomplete, GibcoBRL); (4) Peroxidase conjugated goat anti-mouse IgA, IgG, IgM (Capple, Malvern, PA); (5) ABTS (2,2-azino-di-[3-ethyl-benzthiazoline sulfonate]diammoniumsalt) (Sigma, St. Louis, MO); (6) PEG1500

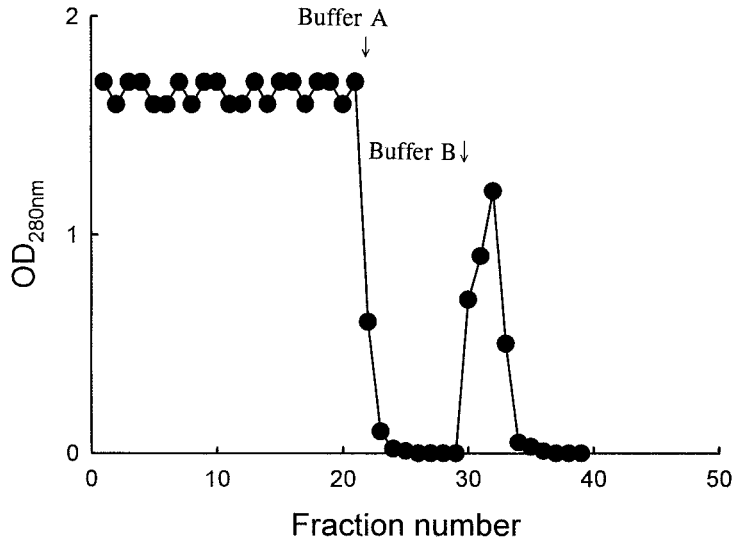


FIG. 2. Affinity chromatogram of anti-human AFP MAbs-secreting hybridoma cell (A73G3) culture supernatant using Hitrap Protein A purification column. Twenty milliliters of the culture supernatant was applied into the column. Buffer A (Starting buffer) is a solution containing 0.05 M Tris-HCl, 3 M NaCl (pH 7.8). Buffer B (Elution buffer) is a 0.1 M citrate buffer (pH 5.0). Flow rates of washing and equilibration were 4 mL/min, and rates of sample application and elution were 1 mL/min (1 mL/fraction).

(polyethylene glycol 1500)(Roche Diagnostics GmbH, Mannheim, Germany); (7) HAT (hypoxanthine 10 mM, thymidine 1.6 mM, aminopterin 1.76 mg/100mL) (GibcoBRL); (8) HT (hypoxanthine 10 mM, thymidine 1.6 mM) (GibcoBRL, Grand Island, NY); and (9) Hitrap Protein A column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Materials

The NS-1 myeloma cell line was a gift from Dr. Rong Huay Juang in the Agriculture Chemistry Department of National Taiwan University, Taiwan, R.O.C. Balb/c mice (6 to 8 weeks old, male) were obtained from the Experimental Animal Center of the Medical College of National Taiwan University, Taiwan, R.O.C.

Immunization

Balb/c and ICR mice were given the initial intraperitoneal (i.p.) immunization with 50 μg antigen (human AFP) in complete Freund's adjuvant (GibcoBRL) and then boosted the antigen in incomplete Freund's adjuvant (Gibco) at 3-week intervals.[7] After 3 months, the ICR mice were used to produce ascites and the spleens from the Balb/c mice were used in the production of hybridoma cells.

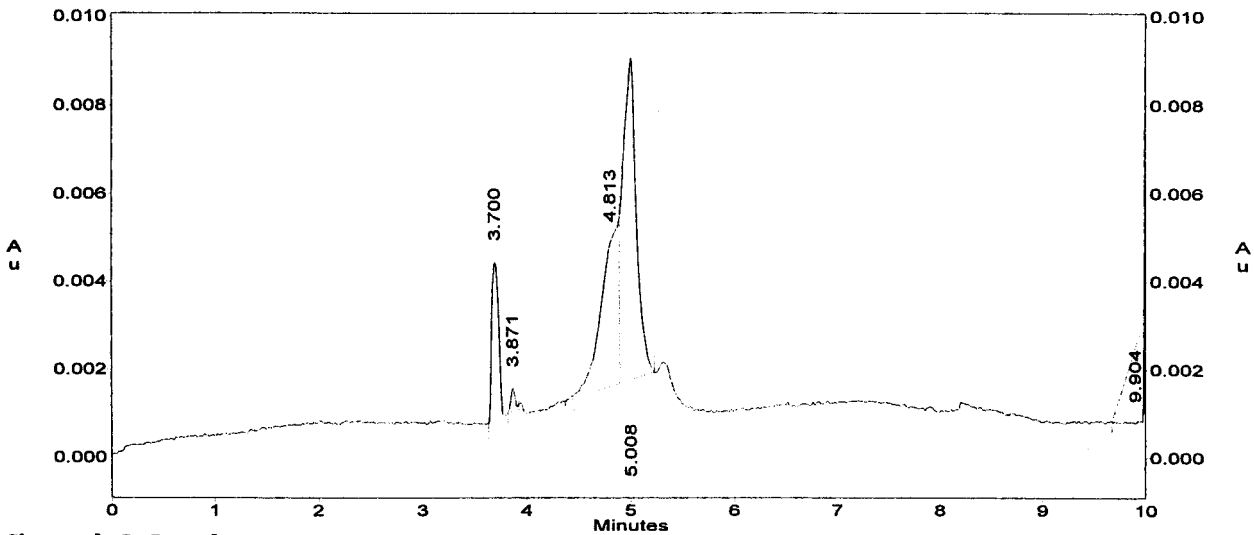
Production of polyclonal antibodies

The hyperimmunized ICR mice were injected with 0.5 mL pristane (2,6,10,14-tetramethyldecanoic acid). Two weeks later, mice were injected with 10^6 NS-1 cells. The fluid was tapped when the mice were noticeably enlarged, but before the mice had difficulty moving. After centrifugation at 3000 × g for 10 min, supernatant was carefully removed and the oil layer was discarded.[7,8]

Production of MAbs

Seven days before fusion and at least 3 weeks after the previous injection, a hyperimmunized mouse was given a final boost of 50 μg antigen in phosphate-buffered saline (PBS) (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). The spleen was then removed and spleen cells (10^8) fused with NS-1 myeloma cells (10^7) using PEG 1500. Fused cells were selected in HAT-RPMIX medium. Anti-AFP antibody-secreting hybridoma cell lines with high titer were cloned by enzyme-linked immunosorbent assay (ELISA) and then subcloned by limiting dilution in 15% fetal bovine serum (FBS), hypoxanthine, thymidine (HT)-RPMIX medium.[7,8]

The production of MAbs was scaled up by tissue culture in flasks. Cultures were allowed to grow until the hybridomas died



Channel A Results

Peak	Time	Name	Height (µAU)	Area	Area %	Corrected Area
1	3.70		3558	16106	10.933	3627
2	3.87		528	1598	1.085	344
3	4.81		3243	41643	28.267	7211
4	5.01		7298	66881	45.399	11128
5	9.90		1707	21091	14.317	1775
Totals :			16334	147319	100.000	24085

FIG. 3. The purity analysis of commercial human AFP using capillary electrophoresis. AFP from human cell culture-derived was purchased from CalBiochem-Novabiochem International, La Jolla, CA.

and tissue culture supernatants were collected. Debris was removed by centrifugation (1000 × g, 10 min) and supernatants were decanted from the cell pellet.[7,8] Supernatant titer was measured by ELISA.

Procedure of determining antibodies by ELISA

Fifty micrograms per milliliter of antigen (human AFP) was adsorbed into a 96-well microtiter plate at 4°C overnight. After coating, the plate was washed twice with PBS (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). 0.2 mL of gelatin-NET solution (gelatin 0.5%, NaCl 0.15 M, ethylenediaminetetraacetic(EDTA) · 2Na 5 mM. Tween 20 0.05%, Tris base 50 mM, pH 8.0) was added for blocking at room temperature. After 1 h, the plate was washed twice with PBST ($NaH_2PO_4 \cdot 2H_2O$ 10 mM, NaCl 0.13 M, Tween 20 0.05%, pH 7.0). 0.1 mL of antibody solution was added to the wells and incubated at 37°C for 30 min, then 4°C for 30 min. After antibody–antigen reaction, the plate was washed three times with phosphate-buffered saline containing 0.2% Tween 20 (PBST) and 0.1 mL of peroxidase conjugated goat anti-mouse antibody was added to the wells and incubated at 37°C for 30 min, then at 4°C for 30 min. After 1 h of incubation, the plate was washed three times with PBST and the enzyme substrate, H_2O_2 and ABTS was added. Absorbance of the colored reaction product at 405 nm was measured by an automated ELISA reader (MR5000, Dynatech, Alexandria, VA).[7,8]

Monoclonal antibodies characterization

Monoclonal cell culture supernatant (0.1 mL) was added to the ELISA plate, which had adsorbed the antigen. After 1 h of incubation at room temperature, the plate was washed three times with PBST. After blocking, eight kinds of isotype goat anti-mouse Ig-peroxidase conjugates were then added to the plate for 1 h of incubation. The plate was washed three times with PBST and the absorbance at 405 nm was measured.

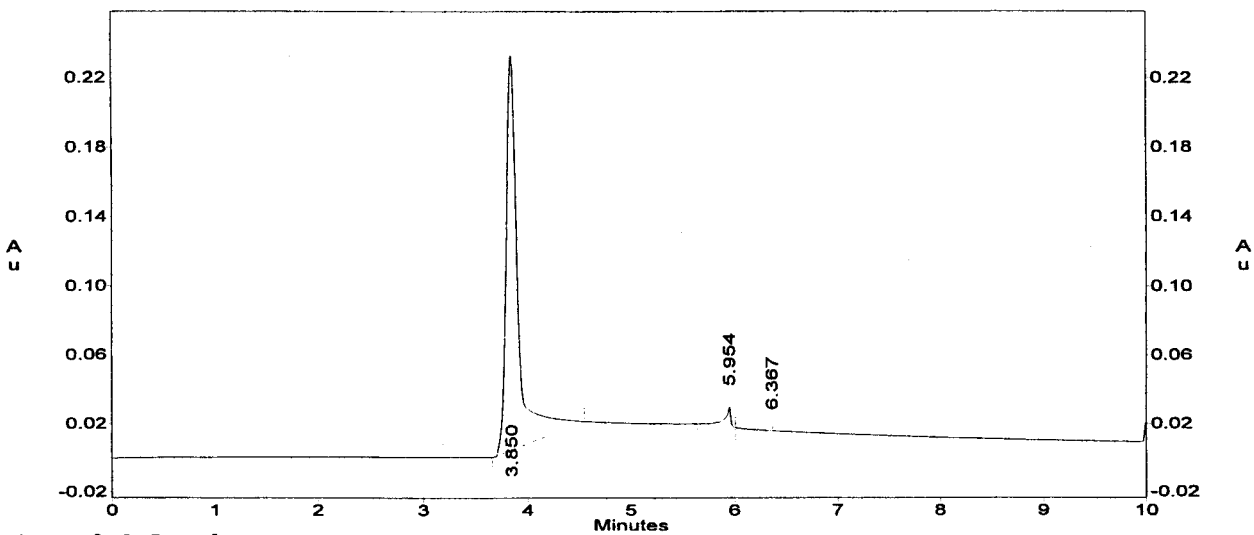
Purification of MAbs and PAbs using Hitrap protein A purification column

The sample was pretreated with ammonium sulfate precipitation. The Hitrap protein A column was equilibrated with at least two column volumes of starting buffer (20 mM sodium phosphate, pH 7.0). The sample was then applied by pumping it into the column, then the column was washed with the starting buffer for 10 column volumes or until no material appeared in the effluent and eluted with elution buffer (0.1 M citric acid buffer, pH 5.0) 1–3 column volumes. The purified IgG fraction was desalted by dialysis. Flow rates of washing and equilibration were 4 mL/min and rates of sample application and elution were 1 mL/min.

RESULTS

Production and purification of PAbs

Ascites formation could be induced in hyperimmune ICR mice (serum titer 1:6000) when injected with pristane and then NS-1 myeloma cells after 2 weeks. The highest dilution fold of the ascites determined by ELISA was 3125-fold. The ascites were purified using Hitrap Protein A column. A single peak of



Channel A Results

Peak	Time	Name	Height (µAU)	Area	Area %	Corrected Area
1	3.85		227680	1842279	96.967	398762
2	5.95		11492	51303	2.700	7180
3	6.37		21	6319	0.333	827
Totals :			239193	1899901	100.000	406769

FIG. 4. The purity analysis of purified anti-human AFP MAbs (A73G3) by capillary electrophoresis. The purified anti-human AFP MAbs (A73G3) were prepared using Hitrap Protein A purification column.

protein fraction ($OD_{280\ nm}$ = 2.85) was obtained when elution buffer (Buffer B) was applied to the column (data not shown).

Production and classification of MAbs

The 12 high-titer MAbs-producing hybridoma cell lines selected and designated as A73F3, A73E8, B73C5, A73G3, B73D7, A73F8, 67B3, B73C2, B73E1, A73G2, B73G7, and B73F4 are shown in Fig. 1. The isotypes of MAbs secreted by hybridoma cell lines A73F3, A73E8, B73C5, A73G3, B73D7, A73F8, 67B3, B73C2, B73E1, A73G2, B73G7, and B73F4 were classified as IgG_1 heavy chain and κ light chain using mouse-hybridoma subtyping kit.

Purification of MAbs

The supernatant containing anti-AFP MAbs A73G3 was purified using Hitrap Protein A column. An affinity chromatogram is shown in Fig. 2. A single peak of protein fraction ($OD_{280\ nm}$ = 1.25) was obtained when elution buffer (Buffer B) was applied to the column.

Purity analysis of antigen and MAbs

The purities of human AFP and MAbs were analyzed by the Capillary Electrophoresis (P/ACE™ System MDQ, Beckman Coulter Taiwan Inc, Taiwan Branch) in Optoelectronic Biomedicine Center of Medical College of National Taiwan University, Taiwan, R.O.C. Figures 3 and 4 show the purity analysis of the antigen (human AFP) and MAbs were analyzed using capillary electrophoresis. The results demonstrated the commercial human AFP was not purified and the MAbs obtained were of high purity.

DISCUSSION

AFP is a kind of complete antigen that when mixed with Freund's adjuvant can stimulate a good response after injection into mice. A successful fusion procedure brings cells together with an optimal frequency of interactions between the two "parent" cell types. Unfused myeloma cells were dying out as a result of the aminopterin block. Spleen cells were dying out, with the exception of macrophages and/or fibroblasts, which might be establishing themselves and beginning to divide.[7] There appeared to be a correlation between the appearance of such cells and subsequent good yield of hybrids. The hybrids were characteristically round with a clear membrane under phase contrast. When the medium in the culture turned yellow, the cultures were screened to determine antibody production and positive colonies by ELISA were selected for expansion and subcloning. Limiting dilution was performed by adding 15% FBS HT-RPMIX medium to replace the conventional method (using feeder cells). The class and subclass should be determined on MAbs prepared in culture, rather than in mice because the latter will contain the other classes and subclasses of antibodies originating from the mouse but not the hybridoma. Tissue culture in flasks was used for scale-up of anti-AFP MAbs. Tissue culture might be maintained for long periods.

The result of the purity analysis for the purified MAbs demonstrated the purification method and the conditions of the purification process using Hitrap Protein A purification column were suitable.

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Address reprint requests to:
Chien-Yuan Chen, Ph.D.
Department of Agriculture Chemistry
National Taiwan University
1, Sec. 4, Roosevelt Rd.
Taipei, Taiwan
China

E-mail: chenyuan@ccms.ntuedu.tw

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