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Production and Characterization of anti-AFP Monoclonal Antibodies

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Monoclonal antibodies (MAbs) against human alpha-fetoprotein (AFP) was produced by hybridizing SP 2/0-Ag 14 mouse myeloma cells with spleen cells of Balb/c mice immunized with purified AFP. Two subclones (D-6 and E-6) were expanded as ascite tumors in syngenic mice, and from ascitic fluid immunoglbulins were pruified. Each antibody was identified to be homogeneous by several criteria, and the affinity constant of D-6 and E-6 MAb to AFP was calculated to be 4.2×10^{-8} and 6.4×10^{-8} M^{-1}, respectively. With these MAbs sensitive and accurate enzyme linked immunosorbent assay mothod was established.

KEY WORDS: Alpha-fetoprotein, Monoclonal antibody, Enzyme immunoassay

Alpha-fetoprotein (AFP) is a major plasma α_1-glycoprotein present in the early stage of development in human and animals, which virtually disappears in adult life. Reappearance of AFP in adult serum often signals several malignant disease, particulary hepatocelluar carcinoma, teratoblastoma and gall bladder carcinoma (Rouslaliti and Seppala, 1979; Brown *et al* 1992). Elevated serum AFP levels in amniotic fluid or maternal serum during pregnancy also have been found to be related to neural tube defects, Down's syndrome and other pregnancy disorders (Fuhrmann and Weitzel, 1985; Korner *et al.*, 1986; Macri *et al.*, 1992; Sundaram *et al.*, 1992). The AFP, therefore, has been regarded as one of the remarkable tumor-associated antigens, and the determination of AFP levels has been used in diagnosis of adult cancers associated with AFP as well as in prenatal diagnosis of certain pregnancy disorders (Rouslahti *et al.*, 1982; Chen *et al.*, 1984 ; Phillips *et al*, 1992).

Immunological assay systems are of importance in characterizing the antigenic structure of AFP molecule and can allow the elucidation of molecular structure and properties, which physiological functions of this protein could be deducible from (Mayforth, 1993). A major advance in molecular immunology is the production of monoclonal antibodies (MAbs) by somatic cell fusion technique (Kohler and Milstein, 1975), which leads to an enormous breakthrough in the ability of making large quantities of uniform, homogeneous antibody reagents (Liddell and Cryer, 1991).

To investigate the structure-function relationship and to establish the sensitive assay methods, we have produced and characterized the anti-AFP MAbs by hybridizing SP 2/0-Ag 14 mouse myeloma cells and spleen cells of Balb/c mouse immunized with human AFP. Also the enzyme immunoassay reagents produced with these MAbs were evaluated in terms of their sensitivity and specificity.

Materials and Methods

Inbred Balb/c mice were obtained from Animal Breeding Center, Seoul National University. Human alpha-fetoprotein (AFP) purified from fetal

tissues was used for immunogen. The detailed procedures for the isolation of AFP were described elsewhere (Kang *et al.*, 1986).

Hybridoma

Eight-week old female Balb/c mice were immunized with 100 μg of AFP emulsified 1:1 in complete Freund's adjuvant (Difco) 4 to 5 times at 2 week intervals. Serum was collected before each immunization by bleeding from tail and checked the antibody production by double gel immunodiffusion. Three days prior to fusion, the mice were boosted with an *i.v.* injection of 30 μg of AFP in 0.1 ml of sterile PBS. Hybridization of immune spleen cells with SP 2/0-Ag 14 mouse myeloma cells at a 10:1 ratio was carried out as described previously (Kang *et al.*, 1988, 1993; Kim *et al.*, 1988).

The cells were distributed in 96-well culture plates at 2×10^5 cells/well in HAT media, and the hybrid cells were selected during 2 weeks. Culture of antibody-secreting hybridoma was screened by enzyme linked immunosorbent assay (ELISA), and subcultured in normal DMEM medium. Hybridomas found to secrete anti-AFP antibody were cloned three times by limiting dilutions in 96-well microculture plates in the presence of 1.0×10^5 mouse peritoneal cells or normal mouse spleen cells as feeder cells (Goding, 1980)

Isolation and Characterization of anti-AFP Monoclonal Antibodies

Antibody-producing hybrid cells were injected intraperitoneally into Balb/c recipients primed with pristane (2, 6, 10, 14-tetramethyl pentadecane) and developed ascite tumors. Immunoglobulin from ascitic fluid and from culture media was purified by precipitation with 50% saturated $(NH_4)_2SO_4$, followed by protein A-Sepharose chromatography (Ey *et al.*, 1978).

The affinity of the antibody and its concentrations were determined by equilibrium solution radiobinding assay using ^{125}I-labeled AFP by chloramine T as previously described (Kang *et al.*, 1988). The bound antigen-antibody fraction was precipitated by addition of polyethylene glycol 6,000 in 0.05 M phosphate buffered-saline (PBS) containing horse serum. About 5% of the added radioactivity was precipitated non-specifically in these tubes. The ratio of bound/free AFP was calculated to perform Scatchard analyses. From the slope of Scatchard plot the affinity constant of each monoclonal antibody was calculated.

Immuloglobulin subclasses were checked by murine monoclonal antibody screening/isotyping kit (Boehringer Mannheim). Isoelectric focusing was carried out in 5% polyacryamide gel with a pH gradient of 3-10 (Bio-Rad).

Preparation of Enzyme-Antibody Reagent

Conjugation of anti-AFP monoclonal antibody with alkaline phosphatase (ALP) was carried out by one step glutaraldehyde method (Avrameas, 1969). Briefly, 0.6 ml aliquot of a suspension (5 mg/ml) of ALP (type VII-S, from bovine intestinal mucosa, Sigma) in 2.6 M ammonium sulfate was centrifuged for 10 min at 1,000 rpm. The pellet was mixed with 0.2 ml of anti-AFP MAb (5 mg/ml). After overnight dialysis against PBS, 8% glutaraldehyde was added to a final concentration of 0.2%. After 2 hr at room temperature, the solution was diluted to 1 ml with PBS and dialyzed against PBS extensively. ALP-conjugated antibody was separated from free antibody and enzyme by chromatography on a Sephadex G-200 column.

Enzyme linked Immunosorbent Assay

Non-competitive ELISA was performed according to Engvall's method (1980). Wells in microtiter plate were coated with 100 μl of purified MAb (D-6, 1 μg/ml) in borate buffered-saline and incubated at 37°C for 2 hr, followed by blocking with 200 μl of 1% gelatin in PBS at 37°C for 1 hr. The wells were washed with PBS-Tween, and 100 μl of various concentrations of AFP in PBS-Tween or unknown samples were added to the wells. After incubation at 37°C for 2 hr and subsequent washing, the wells were filled with 100 μl of conjugated antibody (E-6) and the plate was incubated at 37°C for 2 hr. After another extensive washings, 100 μl of *p*-nitrophenyl phosphate (4 mg/ml in carbonate buffer, pH 10.0) was added as enzyme substrate, and the plates were incubated for 30 min at room temperature. The color development was stopped by adding 50

μl of 2 M NaOH, and the absorbance was read at 405 nm with a Titertek Multiskan (Flow Lab.).

Results and Discussion

The huaman alpha-fetoprotein used as an antigen was purified to homogeneity from fetal tissues through the procedures of salt extration, DEAE-cellulose, concanavalin A-Sepharose, Cibacron blue F3GA-agarose, and immuno-adsorbent affinity chromatography as reported previously (Kang *et al.*, 1986). The final AFP preparation showed a single band on polyacrylamide gel electrophoresis (Fig. 1) and a single precipitin arc on immunoelectrophoresis against anti-human cord serum and anti-human AFP but no precipitin arc against anti-normal human serum.

The culture supernatants of HAT-selected hybrid clones were screened for the presence of anti-AFP antibodies by ELISA, where eight out of 180 hybridoma-containing wells were found to secrete antibodies that react to AFP. One of the anti-AFP producing hybridomas was cloned three times by limiting dilutions in 96-well culture plates with feeder cells. As shown in Fig. 2, six stable clones (D-6, D-7, E-6, F-6, H-5 and H-6) were appeared to produce monoclonal anti-AFP antibodies. Moreover, each MAb showed different reactivity, suggesting the differences in the affinity of MAb to AFP and in the productivity of hybridoma clone. These six stable clones were subjected to multiplication in cultures for storage in liquid N_2.

The Scatchard plots of D-6 and E-6 MAbs from the date obtained by equilibrium solution radiobinding assay of two antibody preparations were presented, along with that of immune serum from one of the mice whose spleen cells were used for fusion (Fig. 3). Our antibodies produced linear plots, while the immune antibody produced a curved concaving-up plot. The calculated affinity constant of D-6 and E-6 MAb from the slope of each scatchard plot was 4.2×10^{-8} and $6.4 \times$

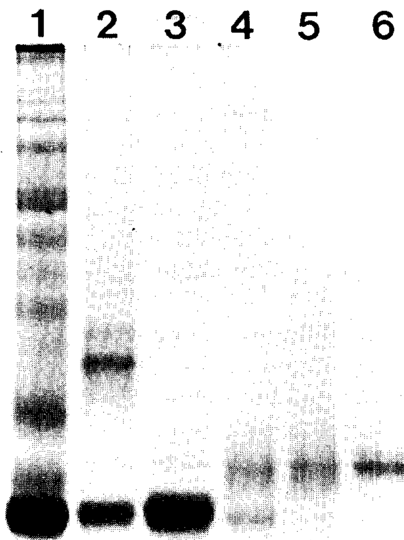

Fig. 1. Alkaline polyacrylamide (7.5%) gel electrophoresis of AFP preparation after each purification step. Migration downward. Coomassie blue G in 2% perchloric acid stained. Slot 1: human cord serum; 2: human fetal extract; 3: AFP preparation after DEAE-cellulose; 4: AFP preparation after Con A-Sepharose; 5: AFP-preparation after Blue-Sepharose; 6: finally purified AFP after immunoadsorbent chromatography.

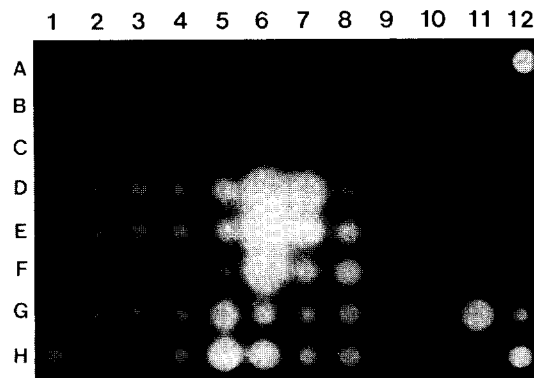

Fig. 2. Autoradiogram of hybridoma culture supernatants for anti-AFP antivody activity. The 6 wells of microtiter plate were coated with 10 μg/ml of AFP and blocked wiht 5% BSA, followed by addition of 50 μl of culture supernatants except four corner wells. ^{125}I-Anti-mouse IgG (20,000 cpm/50 μl) prepared by chloramine T method was used as tracer. Final column (12) was used as control, and the four corners (A1, A12, H1, H12) as plate markers and G11 as tracer marker.

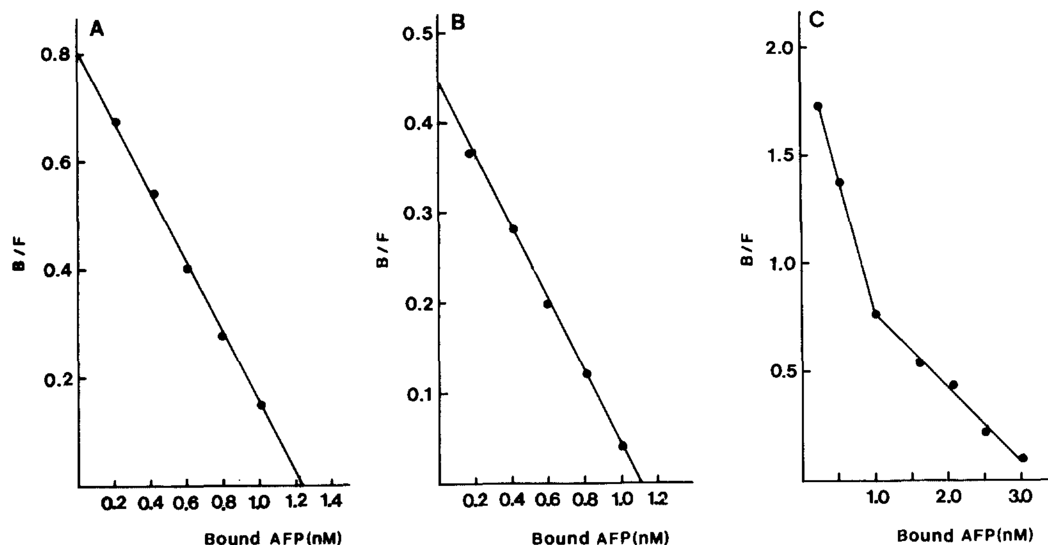

Fig. 3. Scatchard analysis of monoclonal antibodies and immune serum for human alpha-fetoprotein. The equilibrium solution direct binding assays carried out as described 'Material and Methods'. A: MAb, E-6; B: MAb, D-6; C: conventional immune serum, whose spleen was used for fusion. The affinity constant of E-6 and D-6 MAb calculated from the slopes of the line was 4.2 × 10^{-8}/M and 6.4 × 10^{-8}/M, respectively. Curvature of the plot in C represents heterogeneity of antibody affinity.

10^{-8} M^{-1}, respectively. And the isotype of D-6 and E-6 MAb appeared to be IgG_1 (κ) and IgG_{2b} (κ), respectively. Affinity chromatographically purified antibodies from immune serum, even if they all specific for same determinant, will be heterogenous in affinity and yield a curved, concaving-up plot. Thus, our results indicate that D-6 and E-2 MAb is the product of monoclone having a single affinity for a single determinant of AFP molecule, respectively. The isotyping for a unique heavy- and a unique a light-chain subclass provides further evidence for homogeneity.

In order to isolate antibody, each of the two clones (D-6 & E-6) having high titer was subcultured and expanded as a peritoneal tumor in Balb/c mice. Ascitic fluid was saturated 50% ammonium sulfate, and the precipitate was dissolved in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The antibody preparation was then applied on a protein A-Sepharose column equilibrated with same buffer and the adsorbed antibody was eluted by stepwise pH gradient. The anti-AFP fraction, D-6 and E-6, adsorbed to protein A-Sepharose was eluted at pH 5.5 and at pH 3.0, respectively.

The purified antibody showed a single band on SDS-poly-acrylamide gel under non-reducing condition having molecular weight of about 150 kDa and the reduced anti-AFP antibody showed homogeneous heavy chain and light chain compared to those of mouse IgG (Fig. 4). The purified antibody also displayed two bands comprising a major and a minor band in isoelectric focusing gel in the range of pH 3-10 (data not shown).

From the dose-response titration of alkaline phosphatase-conjugated anti-AFP, it was found that our conjugate showed proper enzyme activity at the dilution of 1:50 to 1:6,250 when 50 ng/ml~1 μg/ml of AFP was used for coating (Fig 4A). A typical standard curve was obtained (Fig. 5B), which shows that our conjugate can be used as an excellent assay reagents for enzyme immunoassay for AFP. Although the sensitivity of our reagents is similar to that of conventional EIA kit, the accuracy and specificity by using MAbs are of prime importance.

One of our goals is to delineate the epitopic

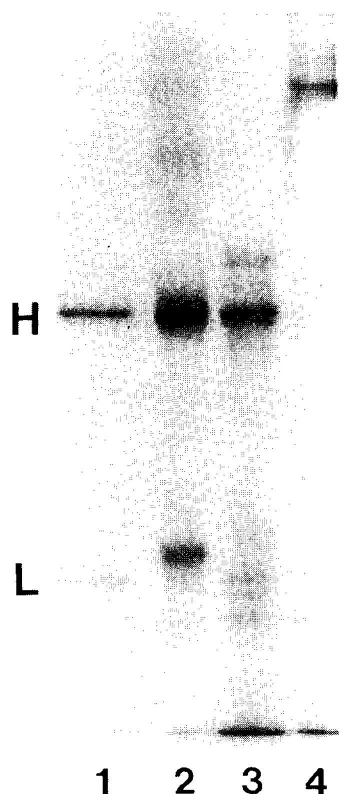

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified monoclonal antibody preparation (E-6). The samples (1, 2 and 3) were dissolved in 62.5 mM Tris-HCl buffer (pH6.8) containing 0.1% SDS and 1% 2-mercaptoethanol and heated on boiling water bath for 2 min prior to electrophoresis on 0.1% SDS-12.5% polyacryamide gel. Migration downward and Coomassie blue stained. Slot 1: purified monoclonal antibody; 2: human IgG; 3: mouse IgG; 4: non-reduced monoclonal antibody.

sites in AFP molecule as done for other proteins (Zue *et al.*, 1987). In a given protein the epitopic sites may be predominantly of sequence-specific or conformation-specific type, though both types of sites may be present in some proteins (Atassi, 1984). Monoclonal antibodies which have the advantage of high affinity and specificity to its corresponding antigen molecules will be extreamly useful for known polypeptide sequence. Recently, it has been reported that AFP is associated to a developing immune system (Laan-Putsep, *et al.*, 1991; Esteban, *et al.*, 1992). And several workers are carrying out to make immunotherapeutic reagents using MAbs for killing tumor cells by immunotoxin (Konno, *et al.*, 1987; Tsukada, *et al.*, 1987; Wawrzyaczak, *et al.*, 1990) or by mononuclear phagocytes through antibody-dependent cell mediated cytotoxicity (Mufson *et al.*, 1989; Woodhouse and Morgan, 1989).

The availability of pure AFP and anti-AFP MAbs is the limiting factor for these applications as described above. The identification of antigenic structure of AFP and the possible application of these anti-AFP MAbs as immunotherapeutic reagents for AFP-associted disorders are under investigations.

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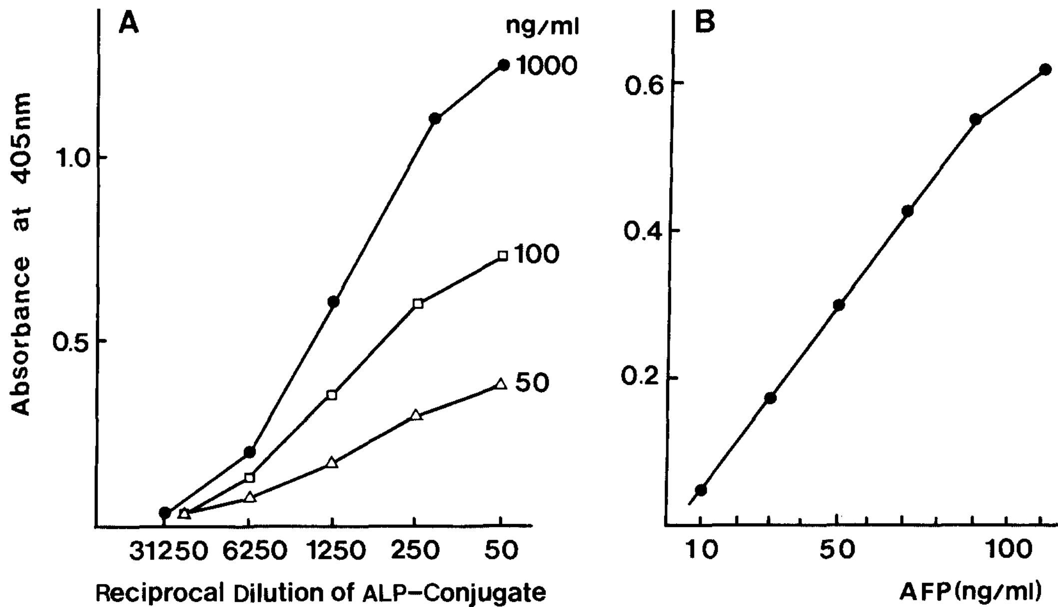

Fig. 5. (A) Titration curve of various concentrations of alpha-fetoprotein for coating, and various dilutions of ALP-conjugated anti-AFP (E-6). (B) Standard curve for the enzyme linked immunosorbent assay of alpha-fetoprotein. See 'Materials and Methods' for details.

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사람 alpha-fetoprotein에 대한 단일클론 항체의 생산 및 분석

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암-연관 항원의 하나인 alpha-fetoprotein(AFP)으로 면역시킨 Balb/c 마우스의 비장세포와 SP 2/0-Ag 14 마우스 미에로마세포를 하이브리도마 방법으로 융합시켜 항-AFP를 생성 분비하는 단일클론들을 얻었다. 이들 중 역가가 높은 2 클론세포 (D-6 & E-6)를 각각 마우스에 주사하여 복수암을 유발시켜 복수를 채취한 다음, 면역글로블린을 분리하였다. 분리된 면역글로블린은 생화학적 및 면역학적 분석에 의해 단일클론 항체임이 확인되었고, D-6와 E-6 항체의 AFP에 대한 결합상수는 4.4×10^{-8} 및 6.4×10^{-8} M^{-1}로 산출되었다. 이들 모노클론항체를 이용한 민감하고 특이성 높은 ELISA 방법을 정립하였다.