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Studies on the Generation and Application of Monoclonal Antibodies against Tumor Marker Antigen
I. Production and Characterization of Monoclonal Antibodies against Placental Alkaline Phosphatase

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Human placental alkaline phosphatase (PLAP), one of the oncofetal antigen was purified from placentas through the procedures including butanol extraction, concanavalin A-Sepharose, DEAE-cellulose and Sephadex G-200 gel chromatography. Monoclonal antibodies (MAbs) against human PLAP were produced by hybridizing SP 2/0-Ag 14 mouse myeloma cells with spleen cells of Balb/c mice immunized with PLAP. Six stable monoclones were obtained by cloning twice in serial dilutions, and the monoclonal specificity of these MAbs was confirmed by biochemical and immunological criteria.

KEY WORDS: Tumor marker antigen, Alkaline phosphatase, Monoclonal antibodies

Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is a group of membrane-associated glycoprotein widely distributed in almost all animal and human tissues, which catalyze the hydrolysis of various monophosphate esters *in vitro* (McComb *et al.*, 1979). Although the membrane functions of alkaline phospahtase like possible roles in transport of phosphate and/or nutrients across the cell membrane has been suggested, the precise biological functions of this enzyme are still obscure (McComb *et al.*, 1979; Shirazi *et al.*, 1981).

In human tissues, there exist at least three different isoenzymes called placental, intestinal and liver/bone/kidney type, encoded by one of multigene family (McKenna *et al.*, 1979; Seargeant & Stinson, 1979; Lehmann, 1980). Of these, placental alkaline phosphatase (PLAP) is of more interest because of its high degree of genetic polymorphism (Fishman, 1974; Slaughter *et al.*, 1983), and its use as a tumor marker owing to its ectopic expression in certain cancer tissues and tumor cell lines (Fishman & Stolbach, 1979; Waharen *et al.*, 1979; Lange *et al.*, 1982; Strigbrand, 1984). In fact, high levels of PLAP activity are often detected in the serum of cancer patients, and therefore, PLAP levels have been measured and used for the clinical management of testicular and ovalian tumors (Jeppson *et al.*, 1983; McDicken *et al.*, 1985; Neuwen *et al.*, 1985; Pollet *et al.*, 1985).

A major difficulty in detecting with polyclonal antibodies has always been cross reactivity of these antibodies with the common epitopes of genetic variants of PLAP itself and of intestinal alkaline phosphatase (Seargeant & Stinson, 1979;

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Abbreviations: DMEM, Dulbeco's modified Eagle's medium; MAb, monoclonal antibody; PEG, polyethylene glycol; PLAP, placental alkaline phosphatase; HAT, hypoxanthine-aminopterine-thymidine; SDS, sodium dodecyl sulfate

Chang *et al.*, 1980; Slaughter *et al.*, 1981; Mabon *et al.*, 1984). Hybridoma technology has allowed the production of monoclonal antibodies (MAbs) which are very specific and sensitive to changes in conformation and/or protein sequence (Kohler and Milstein, 1975). Several groups are now using different MAbs to characterize some of the structural aspects and genetic polymorphism of ALPs (Slaughter *et al.*, 1980; 1983; Millan & Strigbrand, 1982; Loose *et al.*, 1984; McLaughlin *et al.*, 1984; Wray & Harris, 1984; Pollet *et al.*, 1985). More recently, cDNA clones for placental and other ALPs have been isolated and partially sequenced, which has provided primary structural data on this protein (Kam *et al.*, 1985; Millan, 1986; Ovitt *et al.*, 1986; Weiss *et al.*, 1986; Fishman, 1987; Terao & Mintz, 1987). Hence, the possible biological roles of PLAP which are still a matter of speculation will be elucidated.

In order to assess the potential application of anti-PLAP MAbs to elucidate the structure-function relationship and to establish a sensitive immunoassay method for tumor marker, we have purified the PLAP from human placenta and produced stable monoclones secreting anti-PLAP antibody by hybridoma technique.

Materials and Methods

Materials

An unselected series of term placenta was collected and stored at −40°C until use.

Bovine serum albumin (BSA), alkaline phosphatase (Type XXIV, from human placenta), α-D-methyl mannoside, *p*-nitrophenyl phosphate, Fast blue RR, concanavalin A, 2,6,10,14-tetramethylpentadecane (pristane), polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma chemical Co. (St. Louis, USA). Polyethylene glycol (PEG) of Mr ~1,500 was obtained from Baker (Phillipsberg, USA). Ampholine, protein A-Sepharose, CNBr-activated Sepharose and other Sephadex products were ordered from Pharmacia Fine Chemicals (Uppsala, Sweden); DE-52 from Whatman (Clifton, USA); rabbit or goat anti-mouse immunoglobulins (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA & IgM) from Nordic Immunochemicals (El Toro, USA); rabbit anti-mouse *k*- and λ-chain antisera from Cappel Lab (West Chester, USA); $Na^{125}I$ (1 mCi) from Amersham Co. (Arlington, USA). All other chemicals were of the highest purity available. Most of the cell culture media and equipments were obtained from Flow Lab (Worth Lyde, Australia) or from GIBCO Co. (Grand Island, USA).

Purification of Placental Alkaline Phosphatase

Placental alkaline phosphatase was isolated from placentas showing the type 1-1 electrophoretic phenotype using a four-step procedure by Slaughter *et al.* (1983) with slight modifications.

(1) Butanol extraction: The placental material was homogenized in an equal volume of water and one-half volume of butanol with a VirTis homogenizer. The homogenate was incubated for 1 hr at 37°C and then centrifuged at 20,000 x g for 30 min. The aqueous phase that contained the enzyme was then separated.

(2) Concanavalin A affinity chromatography: The aqueous phase from the butanol extract was dialyzed extensively against 0.1 M acetate buffer, pH 6.5, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.01% thimerosal. Concanavalin A was coupled to CNBr-activated Sepharose 4B (10 mg Con A/ml gel) according to procedures by manufacturer (Pharmacia), and the above preparation was applied to Con A-Sepharose column (2 x 35 cm) equilibrated with the same buffer. Most of the enzyme activity was retained on the column. Of the activity, 60-70% could subsequently be recovered by repeated cycles of elution with 10% α-D-methyl mannoside in the buffer.

(3) Anion-exchange chromatography: The PLAP-containing preparation from the affinity column was dialyzed against 0.01 M Tris-HCl buffer (pH 7.0) containing 2 mM $MgCl_2$, and was applied to a column (3 x 45 cm) of DE-52 equilibrated with the same buffer. The enzyme activity was retained and eluted with a linear salt gradient of 0-0.2 M NaCl (500 ml) in the same buffer. Virtually 100% of the enzyme activity was recovered in fractions centering at 0.08 M NaCl. The active fractions were pooled and concentrated for gel filtration.

(4) Gel filtration: This was carried out on a col-

umn (2 x 60 cm) of Sephadex G-200 equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 3 mM $MgCl_2$, and eluted with the same buffer. No enzyme activity was lost during the gel filtration, but to maximize the yield of material of the highest specific enzyme activity, two to three gel filtration runs were performed in sucession. The entire procedure yielded about 900-fold or greater purification and produced up to 15 mg of PLAP from single placenta.

The PLAP activity was assayed using 5.0 mM *p*-nitrophenyl phosphate as a substrate at pH 10.0 as described previously (Kang & Park, 1982). Detection and purity of PLAP in each purification step was examined by typical alkaline polyacrylamide gel electrophoresis (Davis, 1964), immunoelectrophoresis (Scheidegger, 1955) and SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Immunization Schedule

Eight-week old female Balb/c mice (Animal Breeding Laboratories, Seoul National University) were immunized with 100 μg of PLAP emulsified 1:1 in complete Freund's adjuvant (Difco) 4 to 5 times at 2 week intervals. Serum was collcted 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 50 μg PLAP in 0.1 ml of sterile PBS.

Production of Monoclonal Antibodies

Immune spleeen cells prepared by breaking the tissues with sterile mess were fused with plasmacytoma cells of the 8-azaguanine resistant mouse cell line, SP 2/0-Ag 14, according to the method of Kohler and Milstein (1975). Briefly, 3.25×10^8 spleen cells were mixed with 3.3×10^7 myeloma cells in serum-free normal Dulbeco's modified Eagle's medium (DMEM) supplimented with streptomycin (100 μg/ml) and penicilin (100 units/ml). The cells were spun down, and 1 ml of 50% PEG was added slowly (1 min) with gentle mixing of the cells. After another 1 min, the cell suspension was diluted by addition of 1 ml DMEM, kept for 4 min, and then diluted further with gentle mixing of the cells. The cells were spun down, and washed twice with normal DMEM supplemented with 10% fetal calf serum (FCS). The fused mixture were suspended in 50 ml of selective HAT-medium (hypoxanthine, 1×10^{-4} M; aminopterine, 4×10^{-7} M; thymidine, 1.6×10^{-5} M), and distributed in 72 wells of three 24-well microculture plates and incubated at 37°C in a 5% CO_2 humid atmosphere. The hybrid cells were selected during 2 weeks in HAT-medium and the following week in HT-medium. Cultures of antibody-secreting hybridoma were screened by enzyme-linked immunosorbent assay (ELISA) using biotin-avidin system (Zhu *et al.*, 1987), and were subcultured in normal DMEM medium.

Hybridomas found to secrete anti-PLAP antibody were cloned and recloned by limiting dilution in 96-well microculture plates in the presence of 1.0×10^5 mouse peritoneal cells or normal mouse spleen cells as feeder layer (Goding, 1980).

Unique monoclonal hybridoma clone was expanded as ascite tumors in pristane-primed syngenic mice by injecting 1.0×10^6 cells into peritoneal cavity.

Radioimmunoassay

The affinity of the antibody and its concentration was determined by radioimmunoassay using ^{125}I-labeled PLAP, which was prepared by chloramine T method (Hunter, 1978).

One hundred μl of monoclonal antibody or conventional antiserum diluted to bind 50% of PLAP was incubated with 100 μl of ^{125}I-labeled PLAP, 100 μl of purified PLAP (0.1-10 ng/ml) of sample and 200 μl of 0.05 M phosphate-buffered saline of pH 7.4 containing 10 mM EDTA (PBS) overnight at room temperature. The bound fraction was precipitated by addition of 400 μl of 5% horse serum in PBS and 800 μl of polyethylene glycol-6,000 in the same buffer, followed by vortex mixing. After further incubation for 4 hr at room temperature, the precipitate was spun down by centrifugation at 5,000xg for 30 min. The supernatant was then carefully removed, and the precipitate at the bottom of each tube was counted. Duplicate tubes without antibody served as background controls. About 5% of the added radioactivity was precipitated non-specifically in these tubes. The ratio of bound/free antigen and the concentration of bound antigen was calculated for Scatchard analyses (1949). From the

slope of Scatchard plot, the affinity constant of each monoclonal antibody was calculated.

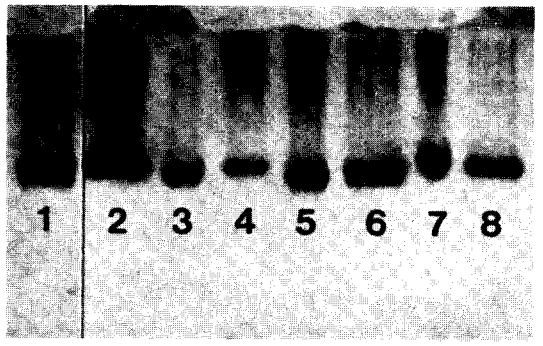


Fig. 1. Alkaline polyacrylamide slab gel electrophoresis of placental extract. Protein (50 μl) samples, diluted to 0.2 IU/ml, were applied on each slot, and electrophoresis was carried out in 9.5% polyacrylamide gel of 0.05 M Tris-HCl buffer of pH 7.4 at 40 mA for 4 hrs. Migration was downward. β-Naphthyl phosphate and fast blue RR were used for enzyme staining. Slot 1: PLAP from Sigma; 2-7: butanol extract of 6 placentas; 8: PLAP (type 1).

Other Methods

Typing of PLAP was carried out either by starch gel electrophoresis or by 9.5% alkaline acrylamide gel electrophoresis (Harris & Hopkins, 1974; Gogolin *et al.*, 1982).

Antisera against human placental extract, human PLAP were raised in rabbits as reported elsewhere (Tung *et al.*, 1983). Immunoglobulin subclass characterization of each MAb was performed either by double gel immunodiffusion analysis or by ELISA using subclass-specific rabbit anti-mouse immunoglobulins.

The protein concentration was assayed by a dye-binding method of Bradford (1976) using bovine serum albumin as standard.

Results

Isolation of Placental Alkaline Phosphatase

From the typing of seven term placental extracts, it was found that three of them showed two enzyme bands (heterozygotes), while three of

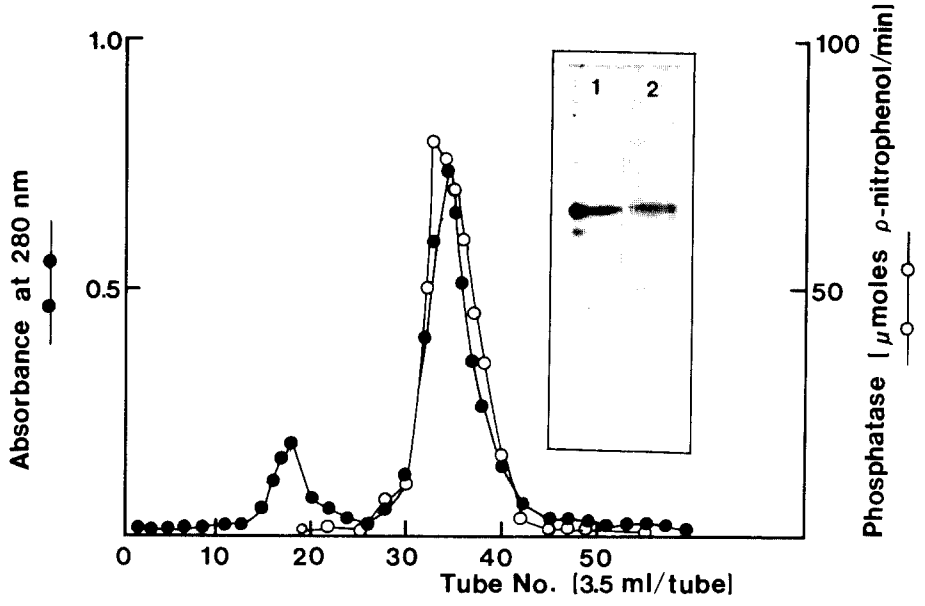
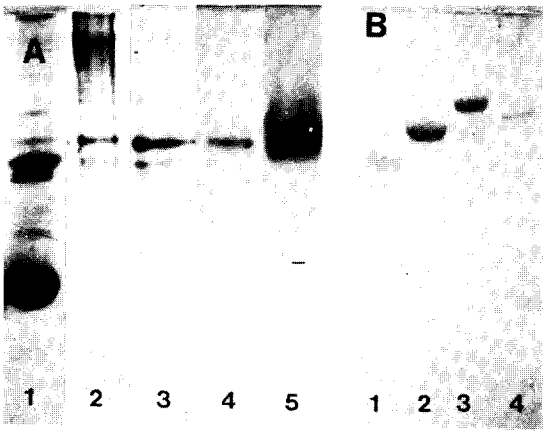


Fig. 2. Gel filtration of human alkaline phosphatase preparation after DE-52 ion exchange chromatography on a Sephadex G-200 column (2x60 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 3 mM $MgCl_2$. (Insert 1: PLAP preparation after DE-52; 2: purified PLAP after Sephadex G-200 chromatography)



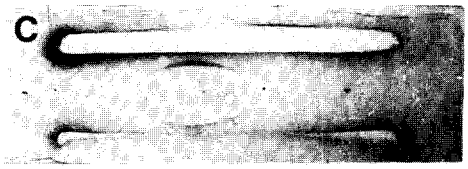
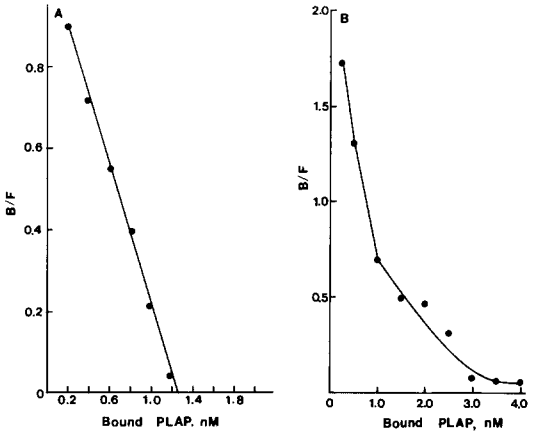


Fig. 3. (A) Alkaline polyacrylamide gel electrophoretic pattern of PLAP preparation after each purification step. Electrophoresis was performed in 7.5% polyacrylamide gel of 0.05 M Tris-HCl buffer, pH 8.8, at 40 mA for 4 hrs. Coomassie blue G in 2% perchloric acid stained. Slot 1: Butanol extract; 2: PLAP preparation after Con A-Sepharose; 3: PLAP preparation after DE-52; 4: PLAP-preparation after Sephadex G-200 chromatography; 5: PLAP preparation after Sephadex G-200 (enzyme staining).
(B) SDS-polyacrylamide gel (10%) electrophoresis of purified PLAP with molecular weight marker proteins. Slot 1: egg albumin (Mr, 45,000); 2: human fetal albumin (Mr, 55,000); 3: human serum albumin (Mr, 68,000) 4: purifted PLAP. (C) Immunoelectrophoretic pattern of purified PLAP. Migration to the left (anode) and Amido black 10B stained. Upper through: rabbit anti-PLAP; lower through: rabbit anti-placental extract; antigen hole: purified PLAP.



Fiig. 4. Scatchard analyses of monoclonal antibody and immune serum for human placental alkaline phosphatase. The equilibrium solution direct binding assays were done as described under "Materials and Methods". A: monoclonal antibody, AP-5; B: conventional immune serum, whose spleen was used for fusion. The affinity constant calculated from the slope of the line was $0.84 \times 10^9\ M^{-1}$. Curvature of the plot B represents heterogeneity of antibody affinity.

them revealed a single band in 9.5% polyacrylamide gel electrophoresis (Fig. 1). Therefore, we chose the placental extract showing a single band (type 1-1) for isolation as explained in "Materials and Methods".

The PLAP preparation after DE-52 ion-exchange chromatography appeared to have trace amount of other placental proteins. To remove these contaminations, the Sephadex G-200 gel filtration was perfomed for the final purification (Fig. 2).

The final product showed a single band both on alkaline polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis (Fig. 3A & B). As shown in Fig. 3C, the PLAP-preparation also displayed a single precipitin arc against anti-human placental extract and anti-human PLAP on immunoelectrophoresis. These results indicate that purified PLAP preparation is homogeneous.

Production of Monoclonal Antibodies

From the 4th immunization of PLAP, mouse serum antibody reacted with PLAP and goat anti-mouse IgG, which indicates that mouse spleen cells synthesize and secrete immunoglobulins. Fifty μg of PLAP in sterile PBS was boosted intravenously, and 3 days later the mouse was sacrificed to obtain spleen cells for fusion according to the protocol descibed in "Materials and Methods".

The culture supernatants of HAT-selected hybrid cells were screened for the presence of antibodies to PLAP by ELISA. Twelve wells out of 58 hybridoma-growing wells were found to have antibodies that bind to PLAP molecule. Two of

Table 1. Summary of monoclonal anti-PLAP antibodies

Clone	Ig Subclass	Scatchard plot	Affinity for PLAP($M^{-1}x10^9$)
AP-1	$IgG_1(k)$	Linear	1.33
AP-2	$IgG_1(k)$	Linear	1.25
AP-3	IgA (k)	Linear	1.07
AP-4	$IgG_1(k)$	Linear	1.31
AP-5	$IgG_1(k)$	Linear	0.84
AP-6	$IgG_1(k)$	Linear	0.52

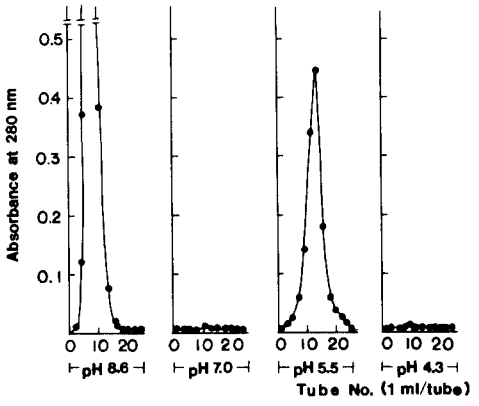


Fig. 5. Protein A-Sepharose 4B column chromatography of the ascitic fluid preparation. The precipitate of ascitic fluid by ammonium sulfate was dissolved in 0.05 M Tris-HCl buffer of pH 8.6 containing 0.15 M NaCl, and then applied on a Protein A-Sepharose column (1x15 cm) with the same buffer at a flow rate of 3 ml/h. The adsorbed monoclonal antibody was eluted out by stepwise pH gradient elution with 0.05 M phosphate buffer-0.15 M NaCl, pH 7.0, 0.05 M citrate buffer-0.15 M NaCl, pH 5.5, and 0.05 M acetate buffer-0.15 M NaCl, pH 4.3. The fractions at pH 5.5 buffer was pooled and lyophilized.

the anti-PLAP producing hybridomas were selected at random and cloned. Six stable clones (AP-1~AP-6) appeared to produce monoclonal anti-PLAP antibodies. These six stable monoclones were subjected to multiplications in clutures for characterization and storage in liquid N_2.

Characterization of Monoclonal Antibodies

Scatchard plot was carried out using the data obtained by equilibrium solution radiobinding assay of culture supernatant of our monoclone along with that of immune serum from one of the

Fig. 6. SDS-polyacrylamide gel electrophoresis of purified monoclonal antibody preparation (AP-1). The samples were reduced prior to electrophoresis on 0.1% SDS-12.5% polyacrylamide gel. Migration downward. Coomassie blue R stained. Slot 1: polyclonal mouse IgG; 2: purified MAb.

mice, whose spleen cells were used for fusion (Fig. 4). Our monoclonal antibody produced linear plot, while the immune serum antibody produced a curved concave-up plot. All the other monoclonal antibody showed a linear Scatchard plot and the calculated affinity constant of each MAb was shown in Table 1. Our MAbs have the affinity constant in the range of 0.5-1.5 x 10^9 M^{-1}. The isotype of 5 MAbs appeared to be $IgG_1(K)$, and that of the other was IgA(K).

In order to isolate antibody, each of the two clones (AP-1 & AP-2) having high titer was subcultured and expanded as a peritoneal tumor in Balb/c mice. Ascitic fluid was 50% saturated with ammonium sulfate, and the precipitate was dissolved in 0.05 M Tris-HCl buffer (pH 8.6) containing 0.15 M NaCl. The antibody preparation was then applied on a Protein A-Sepharose column equilibrated with the same buffer and the adsorbed antibody was eluted by stepwise pH gradient as shown in Fig. 5. The anti-PLAP fractions eluted at pH 5.5 were pooled and lyophilized.

The purified antibody showed a single band on SDS-polyacrylamide gel under non-reducing condition having molecular weight of about 150,000

dalton and the reduced anti-PLAP antibody showed a homogeneous heavy and light chain compared to those of mouse IgG (Fig. 6). The purified antibody also displayed two bands comprising a major and a minor bands in isoelectrofocusing gel in the range of pH 3-10 (data not shown).

Discussion

The placental alkaline phosphatase is a dimeric enzyme composed of identical subunits (Mr = 65,000; 513 residues), which is present on the plasma membrane of the microvilli of the syncytioblast from 10 to 16 week throughout pregnancy (Fishman, 1974; McComb *et al.*, 1979).

The ectopic expression of PLAP in adult serum and certain tissues often signals several malignant diseases, particulary testicular and ovarian cancers (Waharen *et al.*, 1979; Lange *et al.*, 1982; Moss *et al.*, 1982; McLaulin *et al.*, 1983; Stigbrand, 1984; McDicken *et al.*, 1985). Therefore, PLAP has been regarded as one of remarkable oncodevelopmental proteins and several investigators have confirmed the potentiality of this molecule as a tumor marker. A major difficulty in detecting PLAP with conventional antisera has always been the cross reactivity of these antibodies with common epitopes of genetic variants of PLAP itself, and of intestinal alkaline phosphatase (Chang *et al.*, 1980; Millan & Stigbrand, 1981; Mabon *et al.*, 1984). Monoclonal antibodies which have the advantage of high specificity to its corresponding antigenic determinant of antigen molecule should be extremely useful in detecting tumor marker antigen (Kohler & Milstein, 1975).

We have purified PLAP from placentas (Fig. 2 & 3), and established several monoclonal cell lines secreting specific MAb to human PLAP using hybridoma techniques (Table 1). Theoretically, monoclonal antibody should have a single affinity to a single antigenic determinant of PLAP molecule and should thus yields a linear Scatchard plot (Scatchard, 1949). Affinity chromatographycally purified anti-PLAP antibodies from immune serum, even if they are all specific for same determinant, will be heterogeneous in affinity and yield a curved, concave-up Scatchard plot. As shown in Fig. 4, our monoclonal antibody produced a straight line in Scatchard plot, whereas the polyclonal anti-PLAP antibody did not. These results indicate that our antibody is the product of monoclone having a single affinity for a single determinant of PLAP molecule. The typing for a unique heavy- and a unique light-chain subclass provided further evidence for homogeneity (Table 1).

Two of the monoclones were expanded and MAbs were isolated (Fig. 5). The possible applications of these highly specific antibodies to make diagnostic and therapeutic immunoreagents are in progress.

Since human PLAP is known to be highly polymorphic (Slaughter *et al.*, 1981, 1983), the binding differences of our MAbs to allelic varients of this enzyme should be checked, which are scheduled to be investigated.

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Tumor Marker 항원에 대한 단일 클론항체의 생성과 활용에 대한 연구. I. 태반형 Alkaline phosphatase에 대한 모노클론항체의 생산과 분석

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Tumor marker의 하나인 태반형 alkaline phosphatase(PLAP)에 대한 단일 클론항체의 생산과 분석을 위하여, 태반조직을 재료로 butanol 추출법 및 concanavaline A-Sepharose, DEAE-cellulose, Sephadex G-200 gel 크로마토그라피법에 의하여 PLAP를 순수 분리하였다. 이를 항원으로 하여 하이브리도마 방법에 의해 항-PLAP 단일 클론항체를 생산 분비하는 안정된 6 클론세포를 얻었으며 생화학적 및 면역학적 분석방법으로 이들의 단일 클론성을 확인하였다.