High-Affinity Monoclonal Antibodies Specific for Human Prostatic Acid Phosphatase
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We produced two monoclonal hybridoma cell lines that secrete IgG, immunoglobulins with high affinities (Ke = 7.3 to 8.0 × 10⁻¹¹ mol/L, Ke = 1.25 to 1.37 × 10⁻¹⁰ (mol/L) for 125I-labeled human prostatic acid phosphatase (PAP), and that specifically bind this enzyme from human serum. The antibodies were produced in high titers in murine ascitic fluid (700 mg/L) and in cell-culture media (30 mg/L) and were further purified to homogeneity by affinity chromatography on PAP- and Protein A-Sepharose CL-4B. After purification they were shown to be homogeneous by liquid chromatography. Both of these monoclonal antibodies exhibit strict specificity for PAP as determined by radioimmunoassay and by immunofluorescence studies of human pancreas, kidney, prostate, and leukocytes. The antibodies react only with the native form of the enzyme, as shown by the slot-immunoblotting method.

Additional Keyphrases: monoclonal antibodies · immunofluorescence · F(ab')₂ fragments · immunoblot technique · cancer

The human prostate gland secretes prostatic acid phosphatase (EC 3.1.3.2; PAP), a 100 000-Da glycoprotein consisting of two 48 000-Da polypeptide subunits (1). This enzyme is under androgen regulation (2). We have previously purified PAP to homogeneity (3) and described a specific and sensitive radioimmunoassay in which monoclonal antibodies are used to measure concentrations of PAP in serum in the diagnosis and follow-up of prostatic-cancer patients (4, 5). PAP may possess antigenic properties that are present in the other acid phosphatases that originate from the pancreas, spleen, kidney, placenta, and neutrophil and eosinophil granulocytes (6–13). However, by using monoclonal antibodies and subunits of PAP it has also been shown that PAP has an antigenically unique region, not present in acid phosphatases from other sources (14). Our purpose here was to produce monoclonal antibodies with high affinity and specificity for human PAP, to be used in studies on in vitro and in vivo diagnosis of prostatic cancer.

Materials and Methods
Preparation of Mouse Hybridoma Cells
Six nine-week-old BALB/c mice were immunized with 100 μg of human PAP isolated from fresh hyperplastic prostatic tissue (3). The antigen was dissolved in 100 μL of 20 mmol/L phosphate-buffered saline (PBS) and 100 μL of Freund's complete adjuvant. A booster injection of 50 μg of immuno- gen in PBS buffer was given intraperitoneally five weeks later and a second booster injection two weeks later. Two weeks later, mouse-serum specimens were tested for antibodies against PAP by radioimmunoassay (4) and the two mice showing the highest immunoresponses were given 50 μg of PAP intravenously. On the fourth day after the last injection, the spleens were removed and the lymphocytes were used for fusion with mouse myeloma cells (P3/Ne 1/1-Ag4-1) as previously described (15–17). Briefly, the myeloma cells and spleen cells (1:10 mixture) were washed twice with serum-free RPMI-1640 medium containing 20 mmol/L HEPES buffer (Gibco Europe, U.K.) and collected by centrifugation. Then 0.5 mL of an equivalent mixture of RPMI-1640 medium and polyethylene glycol (Mf, 1500; BDH, Poole, U.K.) was slowly added to the pellet, with gentle agitation. RPMI-1640 medium was then slowly added, initially dropwise, to dilute the polyethylene glycol further. After centrifugation of this mixture, the cell pellet was resuspended in Dulbecco's modified Eagle's medium (Gibco Europe) supplemented with, per liter, 200 mL of fetal calf serum (Flow Laboratories, Ayshire, Scotland; lot no. 29112132), 2 mmol of L-glutamine, 0.1 mmol of hypoxanthine, 0.4 μmol of aminopterin, 16 μmol of thymidine ("HAT" medium, Flow), 100 000 int. units of penicillin, and 100 mg of streptomycin (KC Biologicals, Lenexa, KS 66215). The cells (100 000 cells per well) were cultured in 96-well flat-bottom microcritter plates (Flow) with mouse peritoneal cells used as a feeder layer (16, 17). The wells showing growth in about two to three weeks were screened for antibody production by radioimmunoassay (4). These cells producing antibodies to PAP were cloned by the dilution method (17) in 96-well flat-bottom plates.

Antibody Production
Anti-PAP antibodies were isolated from ascitic fluid or cell-culture medium. Ascitic fluid was produced in BALB/c mice primed with "Pristane" (2,6,10,14-tetramethylpentadecane; Aldrich Chemie, Steinheim, F.R.G.), which were then injected intraperitoneally two to five days after priming with 5 × 10⁶ hybrid cells. Ascitic fluid was collected after two weeks and centrifuged. When the ascitic-fluid samples were stored frozen, 100 μL of apronin (Medica, Helsinki, 00101 Finland) and 40 μL of N-ethylmaleimide (Sigma Chemical Co., St. Louis, MO 63176) were added to them. Before purification, ascitic fluid was filtered through an 0.8-μm Millex-AA cellulose ester membrane (Millipore Corp., Bedford, MA 01730) and the pH was adjusted to 8.0.

Hybrid cells were grown in tissue-culture flasks or roller bottles in Dulbecco's Modified Eagle's medium containing 4500 mg of glucose (KC Biologicals) per liter, supplemented with 2 mmol of glutamine, 10⁵ int. units of penicillin, 100 mg of streptomycin, and 100 mL of fetal bovine serum (KC Biologicals) per liter. Medium in roller bottles was additionally supplemented with 25 mmol of HEPES buffer (KC Biologicals) per liter. Medium was collected and centrifuged at 3000 × g to remove cells and concentrated 10-fold with a
Millipore Minitan Ultrafiltration System including "PTHK" plates (Millipore Corp.), the nominal $M_r$ limit being 100,000. The pH of the concentrate was adjusted to 8.0.

Antibody Purification

Ascitic fluid or culture medium was applied to a column of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS (20 mmol/L, pH 8.0). The column effluent was monitored by measuring the absorbance at 280 nm. After application of ascites or culture medium, the column was washed with PBS until the $A_{280}$ returned to baseline. Antibodies of subclass IgG1 were eluted with sodium citrate buffer (0.1 mol/L, pH 5.5). The elution of different isotypes of IgG from ascitic fluid was also tested with 0.1 mol/L citrate buffer at pH 4 and pH 6. The column was washed with 0.1 mol/L glycine HCl, pH 3.0, to remove any other bound immunoglobulins. Eluted IgG1 was dialyzed against more of the pH 8 PBS.

Antibodies against PAP were also purified from ascitic fluid on PAP-Sepharose CL-4B gel, prepared according to the instructions of the manufacturer (Pharmacia). Antibodies were eluted with the glycine HCl buffer.

Characterization of Antibodies by Radioimmunoassay

The monoclonal antibodies were screened and characterized by a modification of our RIA method previously described (4). The monoclonal antibodies, either in culture media or in ascites fluid, were diluted with sodium phosphate buffer (100 mmol/L, pH 6.6) containing 20 mmol of EDTA, 800 mg of NaN₃, and 1 mL of rabbit serum per liter. The contents of the tubes were preincubated for 4 h at 20 °C and then approximately 50,000 cpm of $^{125}$I-labeled PAP (about 1.5 to 2.0 ng per milliliter of enzyme-protein solution) was added in 0.2 mL of sodium phosphate buffer (50 mmol/L, pH 6.6) containing, per liter, 1 mL of rabbit serum, 10 mmol of EDTA, 500 mg of NaN₃, 50 mmol of (l-)-tartrate, and 1 g of bovine serum albumin (BSA). The tubes were then incubated overnight at 20 °C. We then used, for precipitation, 0.6 mL of a 220 g/L solution of polyethylene glycol ($M_r$, 6000; Fluka) with ascites fluid or a 100 g/L solution of IgG with culture media, the solution being in either case in sodium phosphate buffer (50 mmol/L, pH 7.6, containing 150 mmol of NaCl, 3 mmol of NaN₃, and 10 mL of Tween 20 surfactant per liter). The tubes were incubated for 60 min at 20 °C, then centrifuged (300 × g, 15 min, 20 °C). The radioactivity of the pellets was counted in a Megagamma counter (LKB Wallac, Turku, Finland).

Other Procedures

Immunoaffinity. We established the antibody class of the monoclonal antibodies by double immunodiffusion on 10 g/L agar plates, using commercial antibodies against various mouse Ig classes. Anti IgA, IgE, IgM, IgG, IgG₁, IgG₂a, IgG₂b, and IgG₃ (from Miles-Yeda Ltd., Israel, and Miles Laboratories Inc., Indianapolis, IN 466515) were tested.

Purification of non-prostatic acid phosphatases. Acid phosphatases from leukocytes, erythrocytes, and thrombocytes were purified for use in cross-reactivity tests as previously described (4).

Immunoaffinity. Binding of monoclonal PAP antibodies to histological sections of human prostatic hyperplastic tissue and prostatic cancer tissue was investigated by using FITC-conjugated goat antibody to mouse IgG (Hybritech Inc., San Diego, CA 92121). The sections were blocked with BSA, 10 g/L in PBS, for 30 min, incubated with purified antibodies (500 μg/mL, 50 μL per slide) for 120 min, and washed three times with PBS for 5 min. Incubation with FITC-conjugated antibodies for 60 min was followed by washes with PBS, after which the sections were dried and covered with cover slips. Fluorescence was studied by using an epiluminaire fluorescence microscope (Dialuz 20; Ernst Leitz Wetzlar GmbH, D-6330 Wetzlar, F.R.G.).

For immunofluorescence studies of pancreas, kidney, and leukocytes, we tested affinity-purified (on Protein A-Sepharose CL-4B) immunoreactive Fab(α')₂ fragments of monoclonal antibodies (18). FITC-conjugated rabbit anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA 94080) was also in the form of F(ab')₂ fragments to avoid nonspecific Fc-receptor binding. For the positive control of leukocytes, we used monoclonal antiserum against anti-Leu-3A(T₁) (Becton Dickinson Monoclonal Center, Mountain View, CA 94043).

Enzyme activity assays. The catalytic activity of PAP was measured as described previously (3). Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of reduced and non-reduced purified monoclonal antibodies was done according to Laemmli (19).

For liquid-chromatographic analysis of the purified monoclonal antibodies we used a TSK 3000 SW (LKB-Wallac, Turku, Finland) gel filtration column, with phosphate buffer (0.1 mol/L, pH 6.5) as eluent. The affinity of purified monoclonal antibodies was determined by Scatchard analysis (20).

Immunoblotting. We studied immunoreactivity of monoclonal antibodies by slot-blotting of 0.6, 2.4, 9.7, 39, 156, 625, and 2500 ng of native and denatured PAP, using the Bio-Rad dot blot apparatus according to the instructions of the manufacturer.

Results

The newly fused cells were cultured in 420 wells on 96-well plates. Of these, 19 were positive for antibody production to human PAP as measured by the RIA. Hybridomas

![Fig. 1. Binding of the monoclonal antibodies 4B4C (○-○) and 4B4C (●-●) to human PAP](attachment:fig1.png)
from two different wells with the highest antibody titers were cloned and re-cloned on 96-well plates. Two clones were selected for further studies on the basis of high titers of antibodies in cell-culture medium. They were all of IgG₁ isotype. The selected clones produced monoclonal antibodies with high titers (Figure 1), in concentrations of 700 μg per milliliter of ascitic fluid and 30 μg per milliliter of cell-culture medium.

Both monoclonal antibodies gave strong immunofluorescence staining of the epithelium of hyperplastic and cancerous prostatic tissue (Figure 2).

Monoclonal antibodies from cell-culture medium could be purified by affinity chromatography on Protein A-Sepharose CL-4B and were eluted with the pH 5.5 citrate buffer, appearing as a single protein peak of IgG₁ isotype. From ascitic fluid, IgG₁ was eluted at pH 5.5–6.0. IgG₃ was eluted at pH 4.0. Glycine HCl (0.1 mol/L, pH 3.0) eluted isotypes of IgG₁, IgG₂, and IgG₃. Monoclonal antibodies purified from culture medium, from both clones, had the same characteristics as those purified from ascitic fluid by enzyme affinity chromatography on PAP-Sepharose CL-4B and were shown to be pure by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and liquid chromatography (Figure 3). The affinity of the monoclonal antibodies, determined by Scatchard plot analysis, indicated the presence of a single class of saturable high-affinity binding sites, in both cases, with dissociation rate constants (K_d) of 8 × 10⁻¹¹ and 7.3 × 10⁻¹¹ mol/L (Figure 4). Slot-blotting revealed that the monoclonal antibodies recognized only active (i.e., native) PAP (Figure 5). PAP-affinity-purified polyclonal rabbit antibodies to PAP

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Fig. 2. Immunofluorescence staining of the epithelium of prostatic tissue (left) and the control (right) 35 μg of purified monoclonal antibodies was used per tissue selection.

Fig. 3. Liquid-chromatographic analysis of purified monoclonal antibodies (peak I). 1 μg of antibodies was injected. Elution buffer was 0.1 mol/L phosphate buffer, pH 6.5. The second peak (peak II) indicates elution of sodium citrate buffer.

Fig. 4. Scatchard analyses of the monoclonal antibodies 4B4C (left) (K_d = 7.3 × 10⁻¹¹ mol/L) and 4B4G (right) (K_d = 8.0 × 10⁻¹¹ mol/L) binding to human PAP.

Fig. 5. Slot blot of PAP (2500–0.6 μg) binding to monoclonal antibody under native and denatured (1% sodium dodecyl sulfate) conditions. Antibody concentration, 10 μg/mL.
reacted to a somewhat larger proportion of $^{125}$I-labeled PAP than did the purified monoclonal antibodies (Table 1). The molar ratio of polyclonal anti-PAP/$^{125}$I-PAP was 33 and that of monoclonal anti-PAP/$^{125}$I-PAP was 97 at 33% binding.

The monoclonal antibodies had no effect on the catalytic activity of purified PAP when used in concentrations as great as 10 μg/mL in the reaction mixture. In RIA, the monoclonal antibodies were not cross reactive with acid phosphatases purified from erythrocytes, leukocytes, or thrombocytes at concentrations of 1 mg of purified enzyme per liter.

In immunofluorescence studies no cross reactivity was

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<th>Table 1. Binding Capacities of Purified Poly- and Monoclonal Antibodies</th>
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<td>Molar ratio of anti-PAP/$^{125}$I-labeled PAP</td>
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seen in the tissue sections of pancreas or kidney, or in the peripheral leukocytes used in this study (Figure 6, a–g).

**Discussion**

Here we have used established cell-fusion and cloning techniques to produce two monoclonal hybridoma cell lines that secrete antibodies with high affinity and specificity for prostatic acid phosphatase. These antibodies were produced for use in radioimaging of prostatic cancer metastases. For such purposes the affinity constant of a useful monoclonal antibody should be at least $10^6$ L/mol, and for highly specific preparations this constant can be as high as $10^{10}$ L/mol (21). The monoclonal antibodies produced here had a $K_a$ as high as $10^{10}$ L/mol, which is higher than previously described for monoclonal antibodies against PAP ($K_a = 10^8$–$10^9$ L/mol, 14, 22). Our selected hybridoma cell lines produced high concentrations of monoclonal antibodies in cell-culture medium (30 μg/mL). The polyclonal antibodies against PAP we previously produced by using an unusual procedure consisting of repeated booster injections (200 μg of the antigen) of the rabbit every two weeks for a year have resulted in polyclonal antibodies with high affinity and specificity (4). However, this is not a procedure suitable for the large-scale production of homogeneous antibodies that is needed for in vivo techniques (radioimaging, therapy) intended to be used in association with prostatic cancer. Our monoclonal and polyclonal antibodies have both been able to reveal prostatic cancer and its metastases after intravenous injection of their derivatives labeled with $^{99}$mTc or $^{111}$In (23, 24).

![Fig. 6. Immunofluorescence staining of pancreas (a) monoclonal antibodies against PAP and (b) control; of kidney (c) monoclonal antibodies against PAP and (d) control; of leukocytes (e) monoclonal antibodies against PAP and (f) control; and (g) positive control with monoclonal anti-Leu-3a(T)](image)
Results concerning the presence of human prostatic acid phosphatase in different human tissues depend on the antisera against PAP that is used. Polyclonal antisera against PAP was shown to precipitate acid phosphatase activity in leukocytes, kidney, spleen, and placenta: 2, 56–63, 32, and 13% of their total acid phosphatase activity, respectively (7). Shaw et al. (9), using a peroxidase–antiperoxidase technique, studied specimens from 24 normal human tissues and 16 different types of human tumors and detected slight staining of PAP only in the lumen of distal tubules and collecting ducts and in some small blood vessels. They proposed that the PAP they detected originated from serum and was excreted in the urine. In addition, they measured immunoreactive PAP concentrations by RIA in different tissue and cell extracts and detected PAP in leukocytes, lung, spleen, and kidney at 2.9 × 10^{-6}, 0.021, 0.014, and 0.055%, respectively, of the PAP concentration in prostatic tissue. Other authors (7) have measured trastate-inhibitable acid phosphatase activities in specimens precipitated by polyclonal antisera against PAP from spleen and kidney and obtained values corresponding to concentrations of 0.12% and 0.77% of the PAP concentrations in prostatic tissue, amounts more than 10-fold those reported by Shaw et al. (9).

Our monoclonal antibodies did not detect any cross reactivity with acid phosphatases from leukocytes, erythrocytes, thrombocytes, pancreas, or kidney. These results are supported by the findings of Liljehøj et al. (14) and Lee et al. (25), suggesting that there may be an antigenically unique region of PAP. On the other hand, Lee et al. (25) showed that goat anti-PAP xenoantibodies cross reacted with nonprostatic acid phosphatases, in contrast to IgG1 and IgM. Further information is needed concerning the number, nature, and location of the specific antigenic determinants on the prostatic acid phosphatase molecule, or the antigenic determinants that are responsible for cross reaction with other acid phosphatases.

We further propose that preparative isoelectric focusing after affinity chromatography on 1(+)–tartrate–AH-Sepharose 4B in our purification system (3) effectively separates PAP from other acid phosphatase isoenzymes in the prostate, which are suggested (17) to be lysosomal and to share antigenic determinants with leukocyte-derived acid phosphatase. Our monoclonal antibodies do not interact with denatured PAP, and this implies a high degree of recognition of the tertiary structure of the molecule rather than a particular sequence of amino acids, which could also be one reason for the specificity of our monoclonal antibodies.

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References