Monoclonal antibodies to human prostatic acid phosphatase: Probes for antigenic study

(antigenic mapping/prostatic acid phosphatase fragment/antigenic specificity/immunoassay/immunohistology)

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ABSTRACT Hybrid cell lines producing monoclonal antibodies against human prostatic acid phosphatase [PAPase; orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] were prepared by the fusion of mouse myeloma cells with the spleen cells of PAPase-immunized BALB/c mice. Approximately 23% of the hybrid cells initially plated after cell fusion produced specific antibodies: 34 microcultures were cloned, and 8 eventually yielded stable cell lines. The monoclonal antibodies produced by these eight hybridomas were characterized for their isotypes, isoelectric points, concentrations, and affinities. All of the eight monoclonal antibodies exhibited strict specificity for PAPase as determined by radioimmunoassay and immunohistochemical methods. These antibodies were used as probes for the antigenic mapping of this enzyme, and three nonoverlapping determinants were recognized. Further binding studies with PAPase fragments, generated by cleavage with a submaxillary protease, showed that those three determinants are clustered on one fragment of PAPase. These monoclonal antibodies may be useful in refinement of clinical immunoassays of PAPase or immunohistological study of PAPase-synthesizing cells.

Since Gutman *et al.* reported a correlation between acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] levels in the serum and prostatic cancer over 40 years ago, prostatic acid phosphatase (PAPase) has been regarded as a marker for prostatic adenocarcinoma (1, 2). Acid phosphatases in serum include lysosomal enzymes of diverse cellular and tissue origins. Diseases other than prostatic cancer are known to cause an elevation of acid phosphatase levels in sera (for review, see refs. 1 and 2). Therefore, antigenic studies of PAPase became important in the development of clinically useful PAPase immunoassays.

Acid phosphatase in humans occurs in a number of different molecular forms: erythrocyte acid phosphatase is distinct from other isoenzymes in its size and kinetic properties (3). Nearly all differentiated cells synthesize and store acid phosphatase in lysosomes, and this enzyme has been regarded as one of the markers for these organelles (4). Structural genes for erythrocyte acid phosphatase and for lysosomal isoenzyme have been localized on chromosomes 2 (3) and 11 (5), respectively.

The human prostate gland secretes PAPase that is a glycoprotein of 100,000 daltons consisting of two identical polypeptide subunits of 48,000 daltons (6). The PAPase is similar to lysosomal acid phosphatase (LAPase) in its molecular size and kinetic properties (2, 7). However, the two isoenzymes show distinct antigenicities (8-10) and function. Ultrastructural and cytochemical studies of prostatic epithelial cells revealed that they produce two separate forms of acid phosphatase, lysosomal

and secretory. The secretory acid phosphatase, PAPase, is regulated by androgen and estrogen and is decreased by orchiectomy or hypophysectomy (11). Although the antigenic and functional differences between the two isoenzymes suggest genetic nonidentity, the chromosomal gene location of PAPase is not yet known.

In the light of these findings, it is important to elucidate the structural and antigenic differences between the two isoenzymes by the use of monoclonal antibodies that recognize different determinants on the PAPase and LAPase molecules. Using the method of Köhler and Milstein (12), we fused spleen cells from PAPase-immunized mice with mouse myeloma cells and obtained several hybrids producing antibodies reacting specifically with PAPase. This paper describes some properties of these monoclonal antibodies and preliminary antigenic mapping studies with the use of these monoclonal antibodies. A preliminary account of some of this work has appeared (13).

MATERIALS AND METHODS

Human PAPase and LAPase. Methods of purification and physicochemical and immunochemical evaluations for the purity of PAPase have been described (14, 15). Details of procedures for the submaxillary protease digestion of PAPase, tryptic peptide mappings, and affinity labeling of the active site will be described elsewhere. The procedure for LAPase purification has been described (26).

Cell Cultures. The azaguanine-resistant mouse myeloma cell lines, P3-X63-Ag8 and P3-NS1-Ag4-1 (16), and X63-Ag8.653, a nonsecreting subclone of P3-X63-Ag8 (17), were grown in Iscove-modified Dulbecco's minimal essential medium (GIBCO) supplied with 10% fetal calf serum and 50 µg of gentamycin and 20 µg of 8-azaguanine per ml (Sigma) (18).

The following human cell lines were used for the immunofluorescence study: fibroblasts (WI-38), epithelial cells (HeLa, MCF-7, DU-145, and PC-3), and lymphoid cells (Daudi) that had been cultivated in Eagle's minimum essential medium (GIBCO) or RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum.

Immunization and Fusion Protocol. BALB/c mice, each 3-4 months old (Jackson Laboratory), were immunized by multiple injections of 50-80 µg of PAPase. The primary immunization was initiated by subcutaneous injection of antigen in complete Freund's adjuvant. The second injections were administered subcutaneously with incomplete Freund's adjuvant a month

Abbreviations: PAPase, prostatic acid phosphatase; LAPase, lysosomal acid phosphatase; Sp1, Sp2, and Sp3, PAPase fragments 1, 2, and 3 generated by submaxillary protease cleavage; HAT, hypoxanthine/aminopterin/thymidine.

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later. A third booster was given in 0.5 ml of saline containing 0.3 ml of pertussis vaccine (a gift of Eli Lilly) 1 month after the second immunization. Three days after the last injection, spleens were removed aseptically, and fusion was performed. The fusion protocol used was essentially that described by Galfré *et al.* (19). Hybrid cells were selected by hypoxanthine/aminopterin/thymidine (HAT) medium of Littlefield (20) and maintained according to the feeding schedule described by Galfré *et al.* (19). Culture supernates were assayed for antibodies against PAPase by the radioimmunoassay (14) and immunoenzyme assay (15), and culture medium was changed to HT and then HAT-free Iscove medium. When positive mass cultures contained $\approx 10^5$ cells per ml, most of the cells were frozen. The remaining cells were serially diluted, and $\approx 10^2$ to 10^3 cells were plated in soft agar containing 0.32% agar, complete Iscove media, and 20% fetal calf serum. After 2–3 wk of incubation, well-isolated clones were subcultured either for the *in vitro* cultures or for the inoculation of mice.

Determination of Affinity Constant. The double antibody radioimmunoassay was used to measure the capacity of antibodies from medium and ascites as described (14, 15). To calculate K_a , saturation curves obtained at equilibrium were transformed by Langmuir double reciprocal plot of 1/antigen bound as a function of 1/antigen free as described (21).

Isotype Determination. The isotype of the monoclonal antibodies was determined by double immunodiffusion with the use of purified monoclonal antibodies (25 μ g per well) and goat antiserum against mouse Igs. Goat antisera specific for mouse IgM, IgG₁, IgG₂, and IgA were purchased (Meloy, Springfield, VA).

Immunofluorescence. Binding of monoclonal anti-PAPase antibodies to tissue culture cells and histological sections of human tissues was investigated by indirect immunofluorescence as described (15). The F(ab')₂ fragment was prepared from anti-mouse Ig antibodies and labeled with fluorescein by the procedure described by Forni (22).

Polyacrylamide Gel Electrophoresis. For the identification of subunit polypeptides of hybridoma antibodies and of antigens recognized by monoclonal antibodies, samples were analyzed by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis and the Tris/glycine buffer system of Laemmli (23).

Isoelectric Focusing. For the identification of the clonotype pattern of monoclonal antibodies, analytical isoelectric focusing of specific antibodies was carried out in a 5% polyacrylamide slab gel (pH gradient, 5.0–9.0; LKB Producer AB, Stockholm, Sweden) as described by the manufacturer. For the bulk purification of monoclonal antibodies, preparative isoelectric focusing was performed in a horizontal layer of Sephadex G-75 with the LKB focusing apparatus as described by Schalch and Braun (24).

Epitope Saturation Experiment. Monoclonal antibodies harvested from each cell culture flask were first precipitated by (NH₄)₂SO₄ at 50% saturation at 0°C, and antibodies were further purified by immunoabsorbent affinity chromatography. An affinity column matrix was prepared by conjugation of PAPase to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Purified monoclonal antibodies were radioiodinated with ¹²⁵I by the chloramine-T method (25). Specific radioactivities of each labeled monoclonal antibody preparation varied from 2.5×10^5 to 1.2×10^6 cpm per μ g of protein.

For epitope saturation experiments, 100 ng of PAPase were incubated with 500 ng of each unlabeled monoclonal antibody for 3 days at 4°C to achieve maximal binding of monoclonal antibodies to antigenic determinants. Then ¹²⁵I-labeled monoclonal antibodies (100 ng) were added to each reaction mixture and allowed to react for 1 hr at 4°C. At the end of incubation,

immune complexes were precipitated by rabbit anti-mouse Ig serum, and the precipitated radioactivities were determined.

Limited Digestion of PAPase by Submaxillary Protease. Ten milligrams of PAPase was incubated with 200 units of submaxillary protease (Pierce) in 2 M urea/1% NaHCO₃, pH 8.0, overnight at 4°C. The digest was then passed through a Sephadex G-75 (superfine; Pharmacia, Uppsala, Sweden) column. Three polypeptide fragments designated as Sp1, -2, and -3 have been generated from this limited proteolysis procedure, and details will be described elsewhere. Submaxillary protease cleavage fragments Sp1, Sp2, and Sp3 (100 μ g each) were radioiodinated with ¹²⁵I by the chloramine-T method (25) for use in binding assays. Specific radioactivities of PAPase fragments ranged from 4×10^5 to 10^6 cpm per μ g of protein.

RESULTS

Monoclonal Anti-PAPase Antibodies. Ten fusions of mouse myeloma (NS-1 or Ag8.653) cells with spleen cells from mice immunized with a crude PAPase preparation yielded 240 microcultures containing anti-PAPase antibodies. Of the 240 positive cultures, 12 were cloned in soft agar, and 8 hybridoma clones were maintained as mouse ascites tumors and tissue culture lines for 6–16 months.

The affinity constants of the eight monoclonal antibodies derived from ascites and tissue cultures were demonstrated by inhibition curves obtained with increasing concentrations of unlabeled PAPase and a trace amount of ¹²⁵I-labeled PAPase.

The hybridoma antibodies were analyzed also by analytical isoelectric focusing in polyacrylamide gel. All hybridoma antibodies were found to have sharply restricted isoelectric points. Even though the hybridomas originated from different fusions, isoelectric points (pI) of their antibodies were remarkably similar. Isotypes, pIs, antibody concentrations, and affinity constants of the eight monoclonal antibodies are summarized in Table 1.

Specificity of Monoclonal Antibodies. The specificity of the monoclonal anti-PAPase antibodies described in Table 1 was tested by determining the concentrations of unlabeled LAPase of different tissue origins needed to compete for binding to antibodies in the presence of ¹²⁵I-labeled PAPase. The binding of ¹²⁵I-labeled PAPase to these monoclonal anti-PAPase antibodies was competitively inhibited by unlabeled PAPase, whereas none of the LAPase preparations exhibited measurable competitive inhibition of binding (Fig. 1). The specificity of monoclonal antibodies for PAPase was further demonstrated by immunohistological examination of various tissue culture cells and tissue sections for the cells synthesizing PAPase. The tissues examined included normal liver and peripheral blood lymphocytes. PAPase-specific immunofluorescence was observed only among the epithelial cells of prostatic origin (Fig. 2).

Preliminary Antigenic Mapping by Monoclonal Antibodies. To investigate the arrangement of individual determinants on

Table 1. Hybridoma lines secreting antibodies to human PAPase

Hybrid lines	Myeloma	IgG subclass	pIs of Igs	Secreted antibody, μ g/ml	$K_a \times 10^{-7}$ liters/mol
G215	653	IgM	6.3	0.3	4.7
H34	NS-1	IgG ₁	6.1	1.0	10
I23	NS-1	IgG ₁	6.2	0.8	2.6
R119	NS-1	IgG ₁	6.0	0.4	35
R317	NS-1	IgG ₁	6.1	0.6	2.9
R511	NS-1	IgG ₁	6.2	0.6	3.1
S24	NS-1	IgG ₁	6.0	0.7	8.4
S26	NS-1	IgG ₁	6.1	0.3	4.4

FIG. 1. Competitive inhibition of binding of monoclonal antibody H34 to ^{125}I -labeled PAPase (^{125}I -PAPase) in the presence of various concentrations of unlabeled PAPase (■) or LAPase from fibroblasts, WI-38 (□) from normal human liver (○), and from pancreas (●).

the PAPase molecule, we have used two techniques—one involving topological mapping by an epitope saturation assay and the other involving direct binding assays through the use of PAPase fragments generated by submaxillary digestion.

In the epitope saturation assays, PAPase was mixed with an excess of unlabeled monoclonal antibodies, then ^{125}I -labeled monoclonal antibodies were briefly reacted for the competitive binding. Epitope saturation assays were performed between each of eight ^{125}I -labeled prototype monoclonal antibodies and

the nonlabeled antibodies from a panel of eight hybridomas. The distinction of three binding groups is demonstrated by the experiments described in Fig. 3.

By controlled digestion of the native enzyme with submaxillary protease, we obtained three large antigenically active fragments, Sp1, Sp2, and Sp3 (ref. 26; unpublished data). For the identification of structural domains of PAPase that are recognized by monoclonal antibodies, direct binding assays were performed by the use of separated Sp1, Sp2, and Sp3 fragment preparations. The results (Table 2) show that the monoclonal antibodies bound exclusively to the Sp1 fragment. Therefore, it was concluded that all three antigenic determinants recognized by monoclonal antibodies were clustered on the Sp1 domain. Because the Sp3 fragment was found to regain enzymatic activity after the interaction with heterogeneous rabbit anti-PAPase antibodies, it was regarded as the catalytic site (ref. 26; unpublished data). None of these monoclonal antibodies reacted with Sp3.

DISCUSSION

Human PAPase is antigenically different from acid phosphatases of other tissues. Nevertheless, a small degree of antigenic crossreactivity between PAPase and other lysosomal acid phosphatase(s) has been suspected. In order to resolve this question, we have adopted two approaches—one involving topological mapping through the use of uniquely defined monoclonal antibodies and the second using peptide fragments generated by limited proteolytic digestion.

In order to estimate the number of antigenic determinants on the PAPase, the enzyme was allowed to react with unlabeled monoclonal antibody in antibody excess, and then ^{125}I -labeled monoclonal antibody was added to investigate the competitive binding for the same determinant. Clear from the binding molar ratio of PAPase and ^{125}I -labeled antibodies of each group, there

FIG. 2. Indirect immunofluorescence for PAPase-secreting cells and tissues with monoclonal antibody H34. (Left) PC-3 cells, a prostatic adenocarcinoma line. (Right) Prostatic adenocarcinoma, a biopsy.

FIG. 3. Antigenic determinants on PAPase molecules determined by the epitope saturation experiment. The radioactivities associated with the homologous pair of monoclonal antibodies ranged from 2% to 4% of the input ^{125}I -labeled antibodies, which represented simple displacement of unlabeled antibodies from epitopes (antigenic determinants) by homologous ^{125}I -labeled antibodies. The radioactivities associated with the nonidentical pair ranged from 45% to 60% of the input ^{125}I -labeled antibodies. Binding of ^{125}I -labeled antibodies to PAPase reached 50–70% of the input during a 1-hr incubation at 4°C in the absence of competing unlabeled antibodies. Unlabeled antibodies: A, G215; B, R119, R317, S26, I23, and R11; C, S24 and H34. The corresponding ^{125}I -labeled monoclonal antibodies: A*, B*, C*. Bars show the standard deviation from three determinations.

were discrepancies of binding ratios among three groups (Fig. 3). It was reasoned that, if two determinants were overlapping on the PAPase molecule, the corresponding ^{125}I -labeled monoclonal antibodies would show an antigen-to-antibody molar ratio that would be significantly smaller than unity as a result of steric hindrance. On the other hand, if the two discrete determinants were located at a sufficient distance, their corresponding antibodies would not exhibit any such interference in binding. Because the binding ratios of three groups of monoclonal antibodies to PAPase did not seem to indicate steric hindrance that would result from closely spaced determinants, three nonidentical and nonoverlapping antigenic determinants were tentatively assigned to the PAPase molecule.

Table 2. Binding of monoclonal antibodies to ^{125}I -labeled PAPase fragments Sp1, Sp2, and Sp3

Antibodies	% Binding		
	Sp1	Sp2	Sp3
G215	45	2.8	2.3
R119	55	3.0	2.8
R317	30	2.5	3.0
S26	40	2.8	3.1
I23	75	2.5	2.8
R511	44	3.0	2.5
S24	55	3.2	3.0
H34	75	3.0	2.8

To each tube containing monoclonal antibodies (0.25–0.5 μg) ^{125}I -labeled submaxillary protease fragments Sp1, Sp2, and Sp3 (50–100 ng) were added and incubated at 4°C . After 3 days, goat antiserum to mouse Ig was added to precipitate the primary immune complexes. After washing by centrifugation, radioactivity of the pellets was determined. Each value is an average of triplicate assays, and nonspecific bindings were 3.5%.

FIG. 4. Provisional antigenic map of human PAPase molecules. Three antigenic determinants defined by monoclonal antibodies are designated a (G215), b (R119, R317, S26, I23, and R11), and c (H34 and S24). The crossreactive determinant recognized by heterologous rabbit anti-PAPase antibodies (R22) and by fractionated anti-LAPase antibody (R37) is designated d (26). CHO, carbohydrate moiety.

tatively assigned to the PAPase molecule.

During structural studies of prostatic acid phosphatase, we found that limited digestion of the PAPase with submaxillary protease yielded three antigenically active fragments—Sp1, Sp2, and Sp3. The data presented in Table 2 indicate that the three determinants found by these eight monoclonal antibodies are localized only on the Sp1 fragment. Therefore, the antigenic specificity of PAPase may be attributable to the Sp1 domain of the molecule.

Previously, we reported the phenomenon of antibody-mediated restoration of catalytic activity for PAPase (10). We now have confirmed the antibody-mediated restoration of catalytic activity with Sp3 fragments and subunit polypeptides by the use of rabbit anti-PAPase antibodies and rabbit anti-LAPase antibodies. From these observations, we suggest that the Sp3 region of the PAPase molecule contains the enzymatically active site and a "crossreactive" antigenic determinant (26). With the collective data from structural and antigenic studies, an antigenic map of PAPase has been proposed (Fig. 4). This map is compatible with the findings that none of the eight monoclonal antibodies was able to restore the catalytic activity of the Sp3 fragment. Monoclonal antibodies described in this paper will be useful for the study of genetic variants of PAPase and the study of tissue distribution of human acid phosphatase.

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