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Human prostate specific and shared differentiation antigens defined by monoclonal antibodies

(membrane preparations/fluorescence-activated cell sorter/epithelial antigens/stromal antigens/immunodiagnosis)

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ABSTRACT **Splenic lymphocytes of BALB/c mice immunized with membrane-enriched fractions of human benign prostatic hyperplasia tissues were fused with the NS-1 light chain-secreting murine myeloma cell line. This generated hybridoma cultures that secreted immunoglobulins reactive in solid-phase radioimmunoassays with membrane preparations of prostatic tissues but not with membrane preparations of apparently normal human liver, spleen, thymus, or erythrocytes. After further screening of immunoglobulin reactivities and cloning of cultures, eight monoclonal antibodies were chosen that demonstrated reactivity with human prostate tissues. These monoclonal antibodies could be placed into at least three major groups—epithelium-specific, polyepithelial, and stroma-specific—on the basis of differential binding to the surfaces of various component cells in the prostate and other epithelia. Two antibodies defined unique protein antigens specific for prostate epithelia that were not crossreactive with prostatic acid phosphatase or the recently described "prostatic antigen." These antibodies also detected antigens on malignant prostate tissues as well as other malignant tissues. Four antibodies defined three unique polyepithelial protein antigens (two of the antibodies were different isotypes defining the same protein). Each of the polyepithelial antigens was expressed on a different spectrum of normal epithelial tissues. Two displayed brain tissue crossreactivity, one was present on pancreas, and one was present on platelets. The two antibodies that detected prostatic stromal protein antigens showed different spectra of reactivities. One antibody reacted with apparently all prostatic stromal cells as well as endothelial cells in the prostate and other organs. The other antibody apparently reacted with all prostatic stromal cells as well as myoepithelial and muscle cells in other organs.**

The prostate gland consists of a fibromuscular stroma enclosing tubuloalveolar glands which empty their secretions into the posterior urethra. The prostrate contributes components to the semen. Its secretions include citrate, zinc, and acid phosphatase. The cell surface antigens of the prostate epithelium have received little attention.

Recently, with the use of monoclonal antibody technology, cell surface antigens specific for T lymphocytes, B lymphocytes, some colonic carcinomas, renal cell carcinomas, breast carcinomas, and malignant melanomas have been described (1–6). The most studied differentiation antigens are those found on the surface membrane of T lymphocytes. Some of these antigens, including leu1, leu5, and HTA-1, are found on both developing thymocytes and T lymphocytes and malignant T cells. Hence the development and characterization of monoclonal antibodies to these cell surface antigens have been useful both for biological study of T cell function and for clinical development of diagnostic and therapeutic tools (7).

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The application of monoclonal antibody technology to analysis of normal and malignant prostate epithelium began only recently. All of the currently described monoclonal antibodies have been made to surface antigens on cultured prostate carcinoma cell lines—DU145 and PC-3 (8, 9).

We have used a novel method (10) for preparing membrane antigens from fresh surgical material. Nonmalignant human prostate tissue was used as a source for the membrane immunogen. The screening method for hybridoma supernatants used a membrane radioimmunoassay (RIA) (10), the fluorescence activated cell sorter (FACS) (11), and immunoperoxidase-stained frozen sections of human tissues. By using these multiple assays we produced and selected a series of monoclonal antibodies to antigens expressed on normal and malignant human prostate cells. This communication deals with the generation and characterization of these monoclonal antibodies. Seven new antigens have been defined with these antibodies.

MATERIALS AND METHODS

Immunizations. Membrane-enriched cell extracts were from benign prostatic hyperplasia (BPH) tissue obtained from transurethral resections of prostates, snap-frozen in isotonic saline by using acetone/dry ice, and stored at −70°C. When needed, extracts ready were prepared by a modification of the method of Mehdi (10) in the presence of the protease inhibitors phenylmethylsulfonyl fluoride, pepstatin A, and aprotinin. The final membrane preparation, at 6–18 mg of protein per ml (Lowry assay) was stored at −70°C.

For immunizations, a volume of the membrane-enriched fraction of BPH containing 100 μg of protein was inoculated intraperitoneally into 4-week-old BALB/c mice. At 1 month the mice were given another dose of 100 μg of membrane extract, and 3 days later the spleens were removed for cell fusion.

Hybridoma Methods. Somatic cell hybrids were prepared by using the method of Herzenberg *et al.* (11). All hybridoma cell lines were cloned, and three of the hybridomas were cloned twice. All hybridoma supernatant fluids were assayed for antibody in a solid phase RIA with 0.2 mg of membrane preparation per ml as described (10). Isotyping of the antibodies was performed as described (11). Chain structure determination with [^{35}S]methionine-labeled supernatants was performed as described with two-dimensional gels (11). Competitive blocking with ^{125}I-labeled (IODO-GEN) antibodies purified from ascites was by the technique of Oi (12).

Tissue Section Immunoperoxidase Staining. Portions of various normal and neoplastic human and rat tissues were obtained, frozen, cut, and stained as described (11). Undiluted hybridoma culture supernatants were used for staining.

Abbreviations: BPH, benign prostatic hyperplasia; RIA, radioimmunoassay; FACS, fluorescence-activated cell sorter.

FACS Analysis. Peripheral blood lymphocytes and thymocytes were screened for binding of hybridoma supernatants as described (11).

Enzymatic Treatments. For studies of enzyme sensitivity of the antigenic determinants recognized by the monoclonal antibodies, membrane extracts containing 0.6 mg of protein were incubated in phosphate-buffered saline at 37°C in the presence of various enzymes as indicated in *Results;* then the membranes were washed by Airfuge (Beckman) centrifugation and processed for solid-phase RIA as described (10). Enzymes tested were Pronase, neuraminidase, α-chymotrypsin, trypsin, and mixed glycosidases (Miles).

Immunoprecipitations. A membrane preparation (1800 μg) from BPH was divided into three equal portions, pelleted in an Airfuge, redissolved in phosphate-buffered saline, and labeled separately with ^{125}I by using IODO-GEN, lactoperoxidase, and chloramine-T in standard methods (13–15). After labeling, the membrane preparations were treated with 0.5% Nonidet P-40 and chromatographed on Sephadex G-25 columns. The protein-bound ^{125}I was isolated from each column and cleared with 0.25 vol of 10% staphylococcal protein A (Pansorbin, Calbiochem). The materials were then divided into 14 equal portions for each type of labeled membrane and treated with hybridoma supernatants followed by rabbit anti-mouse Ig bound to protein A as described (12). The protein A was then pelleted for each of the 42 samples, washed three times, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with low molecular weight markers.

RESULTS

Generation and Screening of Monoclonal Antibodies. Mice were immunized with membrane-enriched fractions of human BPH and their spleens were fused with NS-1 myeloma cells to generate 580 primary hybridoma cultures. Supernatant fluid from these cultures were screened in a solid-phase RIA for the presence of immunoglobulin reactive with membrane preparations of BPH, liver, spleen, thymus, and erythrocytes. Many cultures (193/580) contained immunoglobulin reactive with BPH membranes, but only 80 cultures contained immunoglobulin reactive only with BPH membrane preparations. After these well cultures were expanded into flasks, 40 cultures continued to produce immunoglobulin reactive with BPH membranes. These supernatants were screened on BPH frozen sections and breast carcinoma frozen sections by immunoperoxidase staining. Thirteen cultures gave interesting staining patterns and were selected and cloned by FACS sterile cloning (12). The monoclonal immunoglobulins from these 13 hybridoma cell lines were studied further. The isotypes of the 13 antibodies were: IgG2a, 5; IgM, 5; and IgG1, 3 (Table 1).

Table 1. Isotypes of monoclonal antibodies to BPH membranes

Antibody	Type
35	IgG2a
24	IgM
67	IgM
44	IgG2a
17	IgM
54	IgM
45	IgM
38	IgG1
48	IgG1
77	IgG2a
75	IgG2a
20	IgG1
11	IgG2a

The chain structures of the hybridoma antibodies were determined by metabolic [^{35}S]methionine labeling of hybridoma cultures. The supernatant was precleared with protein A, immunoprecipitated with rabbit anti-mouse Ig bound to protein A, and subjected to two-dimensional gel electrophoresis. All the antibodies showed only one heavy chain and two light chains, one representing the NS-1 light chain (Fig. 1).

Competitive blocking experiments required purification of monoclonal antibodies from malignant ascites of BALB/c mice by using either protein A-Sepharose (Pharmacia) for IgGa and IgG1 or by dialysis at 4°C against distilled water for IgM. The purified antibodies were labeled with ^{125}I and incubated with BPH membranes in the solid-phase RIA after preincubation with each of the hybridoma supernatants. Blocking produced a reduction in binding of >90% (Table 2). Antibodies 44 and 11 cross-blocked each other. Similarly, antibodies 67, 17, 54, 77, 75, and 20 cross-blocked each other. The remaining five antibodies were unique. Thus, seven discretely reactive monoclonal antibodies were identified.

Characterization of Monoclonal Antibodies. The solid-phase RIA screen was used next with an antibody from each of the seven non-cross-blocking sets of antibodies. Sixteen different membrane preparations were examined (Table 3). Two of the antibodies—35 and 24—appeared to be prostate specific except for the kidney reactivity of 35 and the salivary gland reactivity of 24. The remaining five antibodies showed distinct patterns of reactivity with the various normal human tissues: 44 and 45

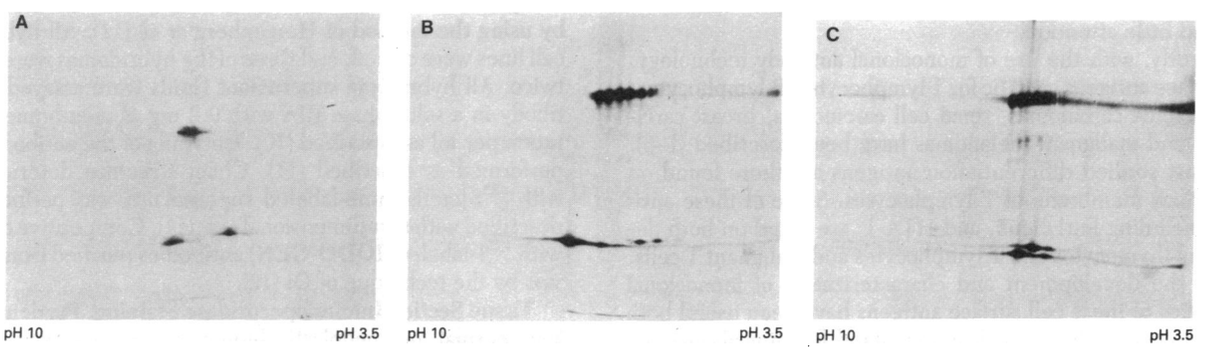

FIG. 1. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled hybridoma antibody. The hybridoma cells were incubated with 0.5 mCi of [^{35}S]methionine in 1 ml of methionine-free RPMI-1640 with 10% dialyzed fetal calf serum. After 5 hr, the supernatant was mixed with rabbit anti-mouse Ig bound to protein A and immunoprecipitated. The precipitate was mixed with urea and lysis buffer and electrophoresed as described (19). Horizontal: nonequilibrium isoelectric focusing between pH 3.5 and pH 10. Vertical: electrophoresis from top (170,000 daltons) to bottom (10,000 daltons). (*A*) Antibody 35. (*B*) Antibody 24. (*C*) Antibody 67.

Table 2. Competitive blocking of ^{125}I-labeled monoclonal antibodies by hybridoma supernatants

^{125}I-Labeled antibody	% blocking with blocking antibody:												
	35	24	67	44	17	54	45	38	48	77	75	20	11
35	95	0	0	0	0	0	0	0	0	0	0	0	0
24	0	95	0	0	0	0	0	0	0	0	0	0	0
77	0	0	95	0	90	90	0	0	0	92	99	95	0
75	0	0	95	0	90	90	0	0	0	90	97	95	0
54	0	0	92	0	92	90	0	0	0	90	95	90	0
45	0	0	0	0	0	0	97	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	90	0	0	0	0
11	0	0	0	97	0	0	0	0	0	0	0	0	95

Table 4. Reactivity of monoclonal antibodies measured by using the tissue section immunoperoxidase technique

Antibody	BPH		Breast		Salivary gland		Liver	
	Epi	Str	Epi	Str	Epi	Str	Epi	Str
35	+	−	+	−	+/−	−	−	−
24	+	−	−	−	+	−	−	−
67	+	−	+	−	+	−	−	−
44	+	−	+	−	+	−	ND	ND
45	+	−	+/−	−	+/−	−	−	−
38	−	+	−	+	−	+	ND	ND
48	−	+	−	+	−	+	ND	ND

Epi, epithelium; Str, stroma; ND, not determined.

reacted with components of brain, 67 reacted with human pancreas, and 48 reacted with human heart and skeletal muscle.

Immunoperoxidase-stained frozen sections gave results complementary to those obtained by membrane RIA with the same seven antibodies (Table 4; Fig. 2). The antibodies could be grouped into three categories—prostate epithelium-specific (35 and 24), polyepithelial (67, 44, and 45), and stroma-specific (38 and 48). Outside of the prostate, antibody 38 reacted only with endothelial cells and weakly with colonic smooth muscle cells, 48 reacted only with muscle and cells with the distribution of myoepithelial cells in the breast, 45 reacted with mononuclear cells in the rim of germinal centers of lymph nodes, and 44 reacted with a subpopulation of thymocytes. In addition, most of the antibodies showed an irregular staining of apparent lymph node dendritic cells and Hassal's corpuscles in the thymus. Most of the antibodies gave uneven staining of some of the kidney tubules, similar in pattern to that observed with several other monoclonal antibodies (unpublished observations). The antibodies that reacted with epithelium were tested on frozen sections of prostate and breast carcinoma. All of these antibodies (both apparent prostate-specific and polyepithelial groups) stained both types of carcinoma.

Preliminary experiments assaying monoclonal antibody binding to five cell lines were done by J. Starling of Eastern Virginia Medical College (personal communication). The cell lines were prostate carcinoma lines DU145 and PC-3, bladder carcinoma line T24, lung carcinoma line CALU, and embryonic lung cell line CMV-HEL. Each antibody showed a distinct pattern: 38 and 48 were not reactive with any of the cell lines, 45 and 24 reacted with PC-3, 35 reacted with PC-3 and T24, 67 reacted with DU145, PC-3, T24, and CALU, and 44 reacted with all five cell lines.

Fetal human prostate tissue was obtained from 4- to 6-month fetuses and used to prepare membrane extracts. Antibody-binding studies using the solid-phase RIA revealed binding of all seven antibodies. In contrast, mature rat prostate tissue membranes showed reactivity only with 48.

FACS analysis of peripheral blood cells obtained from a Ficoll-Paque (Pharmacia) gradient revealed no binding of 35, 24, 38, or 48 to peripheral blood monocytes, lymphocytes, or platelets. Antibody 67 showed binding to a low-density antigen on 20% of lymphocytes, 44 showed high-density binding to platelets and monocytes and low-density binding to 40% of lymphocytes, and 45 showed low-density binding to B lymphocytes and high-density binding to 15% of T lymphocytes including 50% of suppressor cells and 8% of helper cells. Only 44 showed high-density binding to a fraction, 60%, of thymocytes.

Antigen Characterization. Enzymatic pretreatment of the BPH membrane preparations followed by the solid-phase RIA revealed sensitivity of all the antigens to Pronase, trypsin, and chymotrypsin. Only antigen 24 was sensitive to neuraminidase as well. None was sensitive to mixed glycosidases.

Immunoprecipitation was carried out on the membrane preparations labeled with ^{125}I by the chloramine-T, IODOGEN, and lactoperoxidase methods. By all three methods the 25,000-dalton protein containing antigen 44 and the 90,000- and 150,000-dalton proteins containing antigen 38 were labeled (Fig. 3).

Preliminary studies using a two-step immunofluorescence technique identical to that required for FACS analysis but performed on glass slides revealed binding of antibodies 35 and 44 to PC-3 monolayers. There was slight reactivity of antibodies 67, 24, and 45.

DISCUSSION

Here we report the production of seven monoclonal antibodies against distinct antigenic determinants in human BPH tissue. The antigens are of three classes: prostate specific (defined by antibodies 35 and 24), polyepithelial (44, 67, and 45), and stroma specific (defined by 38 and 48). Each antibody displays a unique

Table 3. Solid-phase membrane RIA of monoclonal antibodies

Antibody	Tissue, cpm bound × 10^{-2}/well															
	BPH	Prostate	ProCa	Liver	Spleen	Thymus	Brain	RBC	Thyroid	Adrenal	Pancreas	Heart	Testis	Kidney	Bladder	Lung
35	13	8	16	2	2	2	2	2	4	2	3	2	3	20	2	3
24	13	8	4	2	3	2	2	4	2	2	2	2	3	3	2	3
67	15	10	7	6	5	4	2	2	5	7	13	7	8	10	3	4
44	24	30	9	6	8	9	12	3	13	7	2	9	14	16	11	8
45	12	7	2	1	2	4	12	2	2	2	2	2	3	10	2	2
38	13	20	2	5	5	4	2	2	5	4	14	6	12	13	14	12
48	8	15	2	1	4	4	2	2	2	2	2	12	10	3	8	2
ab2	10	10	7	8	19	20	2	2	6	3	9	10	10	16	6	10
Medium	2	2	2	2	2	3	2	2	2	2	2	2	3	2	1	2

ProCa, prostatic carcinoma; RBC, erythrocytes.

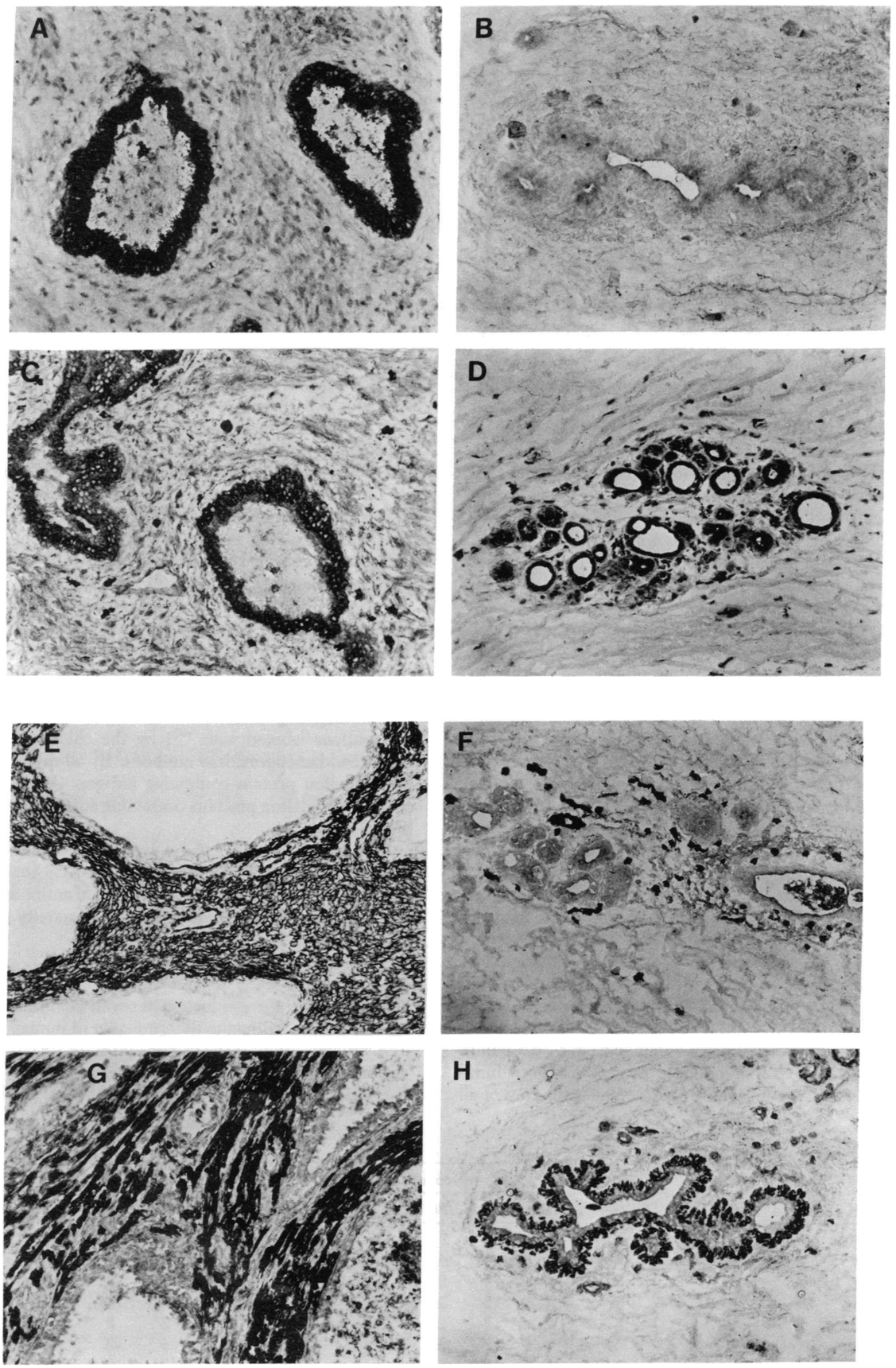

FIG. 2. Immunoperoxidase staining of BPH and breast tissue sections. (×200.) (*A*) BPH with antibody 35. (*B*) Breast with 35. (*C*) BPH with 67. (*D*) Breast with 67. (*E*) BPH with 38. (*F*) Breast with 38. (*G*) BPH with 48. (*H*) Breast with 48.

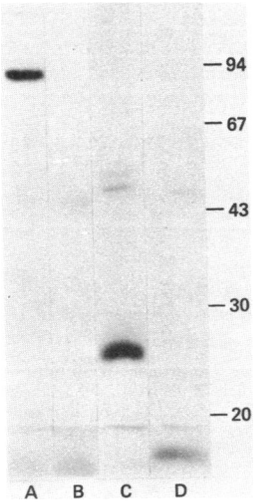

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled membrane protein immunoprecipitates. Lactoperoxidase labeled membrane vesicles were lysed with 0.5% Nonidet P-40, immunoprecipitated, and subjected to electrophoresis. Lanes A, antibody 38; B, 48; C, 44; D, 24. Molecular weight is shown ($\times 10^{-3}$) at the right.

pattern of tissue binding (Tables 3 and 4). The classification is based on the membrane RIA, immunoperoxidase studies, and live-cell RIA of various normal tissues. Neoplastic cell surfaces appear to display more complex and variable patterns of these antigens (unpublished data). The presence of all these antigens in human fetal prostate and least one antigen, that defined by antibody 48, on rat prostate, suggests interesting experiments in prostate ontogeny and phylogeny. The protease sensitivity of the antigens suggests that it should be possible to isolate and characterize the membrane proteins possessing these antigens as was done for 44 and 38.

The production of monoclonal antibodies against prostatic carcinoma cell lines reveals striking similarities. Ware *et al.* (8) described three antibodies against the prostate cancer cell line PC-3. These antibodies, anti-Pro1, -2, and -3 are found on normal prostate, BPH, and, to a lesser degree prostate cancer tissues but on no other normal human tissues. They resemble antibodies 35 and 24. However, we have not been able to immunoprecipitate 35 and 24, and further comparisons are difficult at this time. Wright *et al.* (9) described one monoclonal antibody against DU145, a prostate cancer cell line. This antibody, DU83.21, shows no reactivity with normal cell lines or tumors other than prostate. Thus, it recognizes an antigen distinct from any of ours. The generation of monoclonal antibodies by immunizing with membrane preparations of human normal or neoplastic tissue as described here appears to be at least as good as, and possibly superior to using cultured tumor cells which may have lost or modified their antigenic makeup in culture. Furthermore, a greater diversity of antigens may be accessible by our method which would be difficult with the limited number of prostate cell lines.

Using xenoantisera to prostate tissue extracts, Wang *et al.* (16) found a new intracellular protein, "prostatic antigen," which was unique to prostate tissue. With prostatic antigen generously provided by T. M. Chu (Roswell Park Memorial Institute) at 30 μg/ml as the coating material in the solid-phase RIA, no binding of our monoclonal antibodies was seen, but xenoantisera to prostatic antigen reacted readily in the assay (unpublished data). Furthermore, the xenoantiserum to prostatic antigen failed to bind membrane extracts in the solid-phase RIA, suggesting its intracellular location. Hence, although the prostatic antigen appears to be useful diagnostically, because of its tissue specificity, it has limited application in studying live cell function or in therapy because of its absence from surface membranes.

The other well-characterized human prostate protein is prostatic acid phosphatase (17). Antibody to human prostatic acid phosphatase does not block binding of our antibodies in the membrane RIA (unpublished observations).

The functions of the antigens described here are unknown. We present BPH antigens that are not prostate specific but have unique distribution among human tissues. These stromal and polyepithelial antigens may be of value in studying the physiology of various epithelial tissues, muscle, and endothelial cells.

We see both biological and clinical applications for these reagents. Normal prostate differentiation can be studied by immunofluorescent antibody staining of growing prostate epithelial monolayers. Ontogenic, phylogenetic, and early neoplastic events may be studied in human and other mammalian prostates. Clinically, we can investigate occult prostatic cancer and varying Gleason grades as to presence of these antigens. With $F(ab)_2$ fragments of the prostate-specific antibodies it may be possible to localize metastases. Furthermore, the prostate-specific monoclonal antibody of IgG2a isotype, antibody 35, could be used directly to treat prostatic carcinoma patients refractory to conventional therapy, as suggested by the studies of Miller *et al.* (7).

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