ABSTRACT

The immunoperoxidase technique was used to study the localization and distribution of antigens reactive with two monoclonal antibodies, D83.21 and P6.2, produced against cultured prostate tumor cells, in formalin-fixed, paraffin-embedded histological sections of human tissues. Monoclonal D83.21 reacted with 11 of 19 (58%) primary prostate carcinomas and 1 of 6 (17%) metastatic tumors, whereas monoclonal P6.2 reacted with 14 of 19 (68%) primary and 4 of 6 (67%) metastatic prostate tumors. Neither antibody reacted with five primary prostate tumors and one metastatic prostate tumor. In some tumor cells, the antigens recognized by these monoclonals were localized in either the cytoplasm or cell membrane, while in other tumor cells, both diffuse cytoplasmic and membrane focal staining patterns were observed. In addition to the variable staining patterns, antigenic heterogeneity was also noted within most prostate tumors examined. Two types of staining variability were observed: (a) tumor cells in one area of the tissue section stained positive, but in another area they did not react with the antibody; and (b) both stained and unstained tumor cells were adjacent to each other. These results would suggest that a panel of monoclonals will be required to detect the different subpopulations of prostate tumor cells. Neither antibody reacted with 6 normal or 12 benign prostate tissues, nor any of a variety of other normal human tissues except for staining of the proximal tubules of normal kidneys. The antigen detected by P6.2 demonstrated a wider tissue distribution being found on bladder, breast, lung, and pancreatic tumors, whereas the antigen recognized by D83.21 was restricted to prostate and bladder carcinomas. These antibodies may have clinical applicability for the identification of prostate tumor cells in biopsy specimens and for immunohistopathological classification of prostate carcinomas.

INTRODUCTION

The immunoperoxidase method is proving to be an invaluable tool for assessing the specificity of monoclonal antibodies directed against human tumor antigens (1, 5, 6, 10, 12, 15). In addition to localization of TAAs in histological sections, the reactivity to normal cell types can also be determined in the medium. The immunoperoxidase assay is used to determine the distribution of the antigens recognized by monoclonal antibodies D83.21 and P6.2 in formalin-fixed, paraffin-embedded normal and malignant human tissues.

MATERIALS AND METHODS

Monoclonal Antibodies. The production of monoclonal antibodies used in this study was carried out as described previously (14). Monoclonal antibody D83.21 is an IgM antibody secreted by a hybrid obtained following the fusion of splenocytes from mice immunized with the DU145 prostate adenocarcinoma cell line (11) with the mouse myeloma cell line P3X63/Ag8. Antibody P6.2 is also of the IgM isotype and was generated from a mouse-to-mouse fusion similar for production of D83.21, except that the immunogen was the PC3 prostate tumor line (8) and the NS-1 mouse myeloma line was used for fusion. The hybridoma cell lines were cloned twice by limiting dilution. A monoclonal antibody (IgG) directed against PAP (Hybritech, Inc., La Jolla, Calif.) was used at a 1:400 dilution in all experiments.

Monoclonal antibodies were produced in serum-free medium by culturing the cloned hybrid cell lines in Dulbecco's modified Eagle's medium supplemented with 1% nonessential amino acids, 1% l-glutamine, and insulin-transferrin-selenium (Collaborative Research, Inc., Wattham, Mass.) at a concentration of 5 μg/ml for each additive. The cells were cultured in 150-ml plastic flasks or 500-ml plastic roller bottles at 37° in a 5% CO2 humidified atmosphere. When the cells reached a concentration of about 5 x 10⁶ cells/ml, the supernatant was harvested by centrifugation at 1000 rpm for 5 min. The supernatant was dialyzed against 2 changes of 20 mM Tris, pH 7.4-0.15 M NaCl buffer at 4° for 2 hr. The supernatant was then concentrated by pervaporation at 4° until the volume reached one-tenth the original volume, and the protein concentration determined by the method of Lowry et al. (9). The concentrated supernatant (1 to 3 mg/ml) was stored in 1- to 5-ml quantities at −70° and thawed only when required for the immunoperoxidase assay. Aliquots of each batch of monoclonal antibody supernatant were period-
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Globally checked for binding reactivity by RIA or IF prior to using it for the immunoperoxidase assay.

**Tissue Specimens.** Formalin-fixed, paraffin-embedded blocks of tumor tissue were obtained from the Department of Pathology, Norfolk General Hospital, Norfolk, Va., and the Department of Pathology, Veterans Administration Hospital, Hampton, Va. Some of the primary prostate and urinary bladder tissues and draining lymph nodes were obtained at surgery. Metastatic tissues of other sites were obtained at autopsy. All tissues were fixed in neutral buffered 10% formalin and embedded in paraffin.

**Immunoperoxidase Method.** The avidin-biotin immunoperoxidase assay (Vector Laboratory, Burlingame, Calif.) Vectastain ABC kit was used as described in detail by Hsu et al. (7) with slight modifications. Five-μm tissue sections were adhered to precleaned uncoated slides and heated for 30 min at 65°C. The sections were deparaffinized in xylene and rinsed in absolute ethanol followed by 95% ethanol, 70% ethanol, and finally distilled water. The slides were next immersed in methanol containing 0.3% H2O2 for 30 min at room temperature to block endogenous peroxidase activity, and the slides washed for 10 min in PBS. All subsequent reagents were diluted in PBS, containing 0.1% crystalline pure bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a protein carrier and added at 200 μl/slide. To suppress nonspecific binding of immunoglobulins to collagen and elastic fibers, the sections were incubated with 10% normal horse serum for 30 min at room temperature. After removal of the normal horse serum, the slides were incubated with 25 to 50 μg/section of monoclonal antibody. Duplicate slides (from serial sections) were incubated with P3x63Ag8 myeloma supernatant, which served as a control in all experiments. In some experiments, adjacent sections were incubated with a mouse myeloma IgM (Cappel, Cochranville, Pa.), or PBS containing 0.1% bovine serum albumin, as the primary antibody step and served as additional controls. The slides were incubated for 60 min at room temperature, after which the primary antibody was removed, and the sections were rinsed in PBS for 15 min. The sections were next incubated with biotinylated horse antimouse antisera at a 1:200 dilution for 30 min at room temperature, and the sections washed in PBS for 15 min. Avidin DH-biotinylated horseradish peroxidase H complex (ABC) was then added for 30 min at room temperature. The slides were washed in PBS for 15 min, and the peroxidase reaction was initiated by the addition of 0.05% diaminobenzidine (Litton Bionetics, Charleston, S. C.) and 0.01% H2O2. After incubation for 5 min at room temperature, the slides were washed in PBS for 15 min, and the sections were briefly (30 sec to 1 min) counterstained with hematoxylin, rinsed, dipped in ammoniapwater for 1 min, washed for 5 min in running water, mounted in glycerin jelly, cleared in xylene, and mounted under a coverslip with Permount.

**RESULTS**

**Immunoperoxidase Staining of Tissue.** To determine the localization and tissue distribution of the antigens detected by D83.21 and P6.2, the immunoperoxidase technique was used to test a variety of malignant, benign, and normal tissues. Several pathological types (i.e., well differentiated, poorly differentiated, and undifferentiated) of primary prostate carcinomas were examined. The percentage of primary prostate tumors demonstrating positive staining with D83.21 was 58% (11 of 19), while 68% (14 of 19) of the primary prostate tumors were positive with antibody P6.2 (Table 1). The reactivity of the monoclonal antibodies with the prostate tumor tissue appeared to be influenced by the state of cell differentiation. The highest percentage of positive (Table 1) and strongest staining reactions with both monoclonal antibodies were observed with poorly differentiated (Fig. 1, C and D) and undifferentiated (Fig. 1, A and B) tumor tissues. The percentage of the poorly differentiated to undifferentiated tumors reactive with D83.21 and P6.2 was 83% (10 of 12) and 92% (11 of 12), respectively, while 14% (1 of 7) and 43% (3 of 7) of the well- to moderately differentiated tumors (Fig. 2, A and B) were positive with D83.21 and P6.2, respectively. Metastatic prostate tumor cells also stained positive in regional lymph nodes (Fig. 6C) and at distal sites such as skin, liver (Fig. 6, A, B, and D), and urinary bladder. Monoclonal P6.2 reacted with 4 of 6 (67%) and D83.21 with only 1 of 6 (17%) of the metastatic tumor tissues tested.

Several patterns of staining reactivity were observed with antibodies D83.21 and P6.2 in both primary and metastatic prostate tumors. In the majority of the prostate tumors, the staining was localized in the membrane (Figs. 1D and 6B), and to a lesser degree in the cytoplasm (Figs. 1D and 6B). In other prostate tumor tissues, the tumor cells exhibited diffuse cytoplasmic staining (Figs. 1A, 1B, 4A, and 6C) with some intense focal areas of reactivity in individual tumor cells (Fig. 2B). Occasionally, localized staining of the luminal border of acinar epithelium of tumor ducts in well- to moderately differentiated prostate tumors was observed with P6.2 but not with D83.21.

Heterogeneity of antigen expression was observed in approximately 90% of the prostate tumors tested. In some tumors, stained (reactive) positive tumor cells were found next to or surrounded by unstained (nonreactive) negative tumor cells (Figs. 1, C and D, and 6, A and B). In other specimens, the tumor cells in one area would be intensely stained while the tumor cells in another area would not be stained with the identical antibody.

Some prostate tumors showed differential expression of the antigens recognized by monoclonal antibodies D83.21 and P6.2. As shown in Fig. 4, an undifferentiated prostate tumor stained positive with D83.21 (Fig. 4A) but not with P6.2 (Fig. 4B). Serial sections of 22 prostate tumors were tested with antibodies P6.2 and D83.21 to determine the range of expression of the antigens detected by these antibodies. The reactivity could be placed into 4 distinct groups (Table 2). Although some prostate tumors reacted with both antibodies (12), others reacted with neither antibody (28%). The majority (60%) of the prostate tumors reacted with one or the other monoclonal antibody, indicating a noncoordinate expression of these 2 antigens.

Both monoclonal antibodies were tested against benign prostate tumors and normal prostate tissues (Table 3). None of the 12 BPH showed positive staining with D83.21 (Fig. 3B). Similarly, no staining of the ductal epithelium or stromal elements of the BPH specimens was observed with P6.2. However, 2 specimens showed positive staining of a few cells or cellular debris in the
**Table 2**

Differential immunoperoxidase reactivity against prostate tumors with monoclonals D83.21 and P6.2

<table>
<thead>
<tr>
<th>Group</th>
<th>D83.21</th>
<th>P6.2</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

* Includes both primary (19) and metastatic (6) tumors.

**Table 3**

Immunoperoxidase staining of BPH and normal prostate tumor tissues with monoclonals D83.21 and P6.2

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Site</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH</td>
<td>Ductal epithelium</td>
<td>83.21 6.2 PAP*</td>
</tr>
<tr>
<td></td>
<td>Stromata</td>
<td>0/12 2/12 12/12</td>
</tr>
<tr>
<td>Normal</td>
<td>Ductal epithelium</td>
<td>0/6 0/6 6/6</td>
</tr>
<tr>
<td></td>
<td>Stromata</td>
<td>0/6 0/6 0/6</td>
</tr>
</tbody>
</table>

* IgG1 monoclonal antibody from Hybritech, Inc., was used at a 1:400 dilution.

**Table 4**

Immunoperoxidase staining of nonprostate tumors and normal tissues with monoclonals D83.21 and P6.2

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Type of tumor</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary bladder</td>
<td>Primary</td>
<td>1/4*          3/4</td>
</tr>
<tr>
<td>Normal</td>
<td>Benign</td>
<td>0/2 0/2</td>
</tr>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>0/3 0/3</td>
</tr>
<tr>
<td>Other tumor*</td>
<td>Primary</td>
<td>0/15 6/15</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>2/32 2/32</td>
</tr>
</tbody>
</table>

* Number of tissues showing reactivity with the monoclonal antibodies/total number tested.

**Human Prostate Carcinoma Antigens**

lumen of less than 5% of the ducts when reacted with P6.2. Monoclonal antibodies D83.21 and P6.2 were also tested against normal prostate tissues that were obtained from noncancerous males that died of other causes. These were pathologically verified to be free of benign or malignant foci. No staining was observed in the normal ductal epithelium or stroma of the 6 normal prostate tissues tested with D83.21 and P6.2 (Table 3). By contrast, a monoclonal antibody to PAP stained the ductal epithelium but not the stroma in all the BPH (Fig. 3A) and normal prostate tissues tested.

To further define the range of reactivity of monoclonal antibodies D83.21 and P6.2, several nonprostate tissues were tested. All cell types present in tissue sections obtained from normal lung, colon, heart, spleen, liver, pancreas, testes, vas deferens, and urinary bladder were negative with both D83.21 and P6.2 (Table 4). Both monoclonal antibodies showed staining reactivity in the proximal convoluted tubules of normal kidney, with the strongest staining observed with antibody P6.2 (Table 4). Several nonprostate primary tumors were also tested for reactivity including bladder carcinoma, lung carcinomas, breast carcinoma, colon carcinoma, liver carcinoma, pancreas carcinoma, kidney carcinoma, and melanoma. The only nonprostate tumor positive with D83.21 was one of the 4 urinary bladder carcinomas (Fig. 5A), whereas 3 bladder carcinomas (Fig. 5B), 3 lung carcinomas, 2 breast carcinomas, and 1 pancreatic carcinoma were positive with 6.2.

**DISCUSSION**

These studies demonstrate that 2 mouse monoclonal antibodies, D83.21 and P6.2, produced against antigens expressed on prostate adenocarcinoma cell lines reacted with these antigens in formalin-fixed, paraffin-embedded tumor tissues. The spectrum of reactivity to cultured cells (14) and tissues suggest that the TAA detected by antibody D83.21 is apparently restricted to prostate and urinary bladder tumor cells. The immunohistochemical staining of tissue specimens with D83.21 correlates with the results obtained by 3 other immunoassays (membrane immunofluorescence, RIA, and complement-dependent immunoassay) used to test the reactivity to cell lines and membrane (tissue) extracts directly or after absorption (14). The apparent restricted reactivity of D83.21 to prostate and urinary bladder tumors might suggest that this monoclonal antibody is detecting a common urogenital differentiation antigen. Embryologically, both the normal bladder uroepithelium and the ductal epithelium of the prostate gland originate from the endoderm of the urogenital sinus. Because the majority of the prostate and bladder tumors are derived from the ductal epithelium and uroepithelium, respectively, the possibility of D83.21 binding to a urogenital differentiation antigen appears feasible. However, numerous urogenital cell types and tissues from fetuses and adults will have to be tested before a definitive answer to this observation can be made. Previous studies showed that D83.21 did not bind to either of the organ-specific prostate tumor markers, PAP or prostate antigen (14). In this study, D83.21 did not react with the ductal epithelium of BPH and normal prostate tissues, whereas the ductal epithelium of all of the BPH (12 of 12) and normal prostate (6 of 6) tissue stained positive with a monoclonal antibody directed against PAP. Collectively, the data sufficiently indicated that the epitope bound by D83.21 is not related to PAP. Also the fact that PAP is not associated with the cell membrane (2) and that the D83.21 antigen is a M, 180,000 surface glycoprotein, 4 gives further support that the 2 epitopes are distinctly different.

The P6.2 monoclonal antibody directed against the PC3 prostate adenocarcinoma cell line bound strongly to primary and metastatic prostate tumors and, similar to D83.21, cross-reacted with bladder tumors but not with normal and BPH tissues. Unlike D83.21, however, antibody P6.2 reacted with 6 of 15 of the nonprostate-bladder tumor tissues tested, suggesting that the P6.2 antigen has a wider tissue distribution. Numerous tumor types and tissues will need to be examined for both antibodies to determine the range of antigen expression. Of the 32 nonprostate normal tissues tested, only the proximal convoluted tubules of normal kidneys stained positive with antibodies D83.21 and P6.2. This reaction in kidney tissues with mouse...
monoclonal antibodies has been observed by others (3, 4). The nature of this cross-reactivity remains to be determined.

Considerable antigenic heterogeneity was observed in the immunoperoxidase-stained tissue sections of prostate tumors. Some tumors were positive by one antibody and negative by the other, some were positive with both antibodies, and others were not bound by either antibody (Table 2). This heterogeneity also extended within the same tumor tissue where both positive and negative tumor cells could be observed in the same mass of tumor cells. This variable expression of TAA is similar to other reports (3, 6) and may be due to the state of tumor cell differentiation, antigens expressed at different phases of the cell cycle, subpopulations of tumor cells, antigen density (or concentration), or antigenic phenotypes (6, 12). Although it appeared in this study that both D83.21 and P6.2 antibodies reacted strongest and with a greater frequency with poorly differentiated to undifferentiated tumors, not enough prostate tumors have been tested to permit the definition of any correlations between type of staining pattern and such clinical parameters as stage of disease or histological grade.

The specificity and range of reactivity of monoclonals D83.21 and P6.2 appears to be distinct from other monoclonal antiprostate antibodies recently reported. Frankel et al. (4) described 8 monoclonal antibodies produced against cell membranes from BPH tissue. Most of these antibodies had distinct patterns of reactivity to various normal tissues and could be classified into 3 major groups: epithelium specific; polyepithelial; and stroma specific. Two antibodies, 35 and 24, were the most specific of the antibodies tested. Both antibodies react with BPH, normal prostate tissues, and malignant prostate tissues. Both of these antibodies react with the PC3 prostate tumor cell line but not with the DU145 prostate carcinoma cell line. A monoclonal antibody, designated α Pro3, produced against the PC3 prostate tumor cell line was recently described by Ware et al. (16). This antibody was shown to react to PC3 but not DU145 cells, and with normal and BPH cell extracts in addition to malignant prostate tissues. The reactivity of this antibody appears to be similar to the monoclonals (35 and 24) of Frankel’s but not to the pattern of reactivity observed with D83.21 and P6.2, because the latter antibodies do not react with normal tissues or BPH. Obviously, studies to directly compare (i.e., reciprocal binding inhibition experiments) the antiprostate monoclonal antibodies generated in different laboratories under the same experimental conditions will be necessary to determine if any of the monoclonals are detecting the same, different, or related antigens or epitopes.

Based on the results of this study, both D83.21 and P6.2 appear to be detecting 2 new membrane antigens present on prostate tumors. The differential reactivity and staining heterogeneity observed in the immunoperoxidase-stained prostate tissues would suggest that a panel of well-characterized monoclonal antibodies will be required to detect all the cell types and subpopulations of prostate tumor cells. Studies are in progress to use D83.21 and P6.2 in a panel with antibodies to PAP and prostate antigen, to determine if the presence or absence of a marker or combination of markers can be of prognostic value, especially for monitoring patients before, during, and after therapy. From these studies, perhaps a more useful immunohisto-pathological classification of prostate tumors can be developed that can better relate to the clinical status of the prostate cancer patient.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Patricia Wirth for skilful technical assistance, Lucy Krauss for dedicated secretarial services, and Dr. Ann Campbell for helpful comments in reviewing this manuscript.

REFERENCES

Fig. 1. Immunoperoxidase staining of formalin-fixed tissue sections of primary prostate carcinomas with monoclonal antibody D83.21 or P6.2. A and B, undifferentiated prostate adenocarcinoma; C and D, poorly differentiated prostate tumors. Sections A and C are stained with P6.2, and Sections C and D were reacted with D83.21. Note the stained (T) and unstained (t) tumor cells and the unstained fibromuscular stroma (N). A to C, × 200; D, × 400.
Fig. 2. Reactivity of monoclonal antibodies D83.21 and P6.2 with well- to moderately differentiated prostate carcinoma using the immunoperoxidase technique. Section A was stained with P6.2. The epithelia of all the tumor ducts are stained. Section B was reacted with D83.21. Note the focal and membrane-stained tumor cells (T) and unstained to weakly stained tumor cells (t). The fibromuscular stroma (N) is not stained. × 400.

Fig. 3. Immunoperoxidase staining of serial sections of a human benign prostatic hyperplasia tissue. Section A was reacted with a monoclonal antibody to PAP. Note diffuse cytoplasmic staining of the ductal epithelial cells (arrows) and absence of staining in the normal fibromuscular stroma (N). Serial Section B was reacted with monoclonal antibody D83.21. Note absence of staining in both the ductal epithelium and stroma (N). × 200.
Fig. 4. Differential reactivity of monoclonal antibodies D83.21 and P6.2 on serial sections of an undifferentiated prostate adenocarcinoma using the immunoperoxidase technique. Section A was reacted with D83.21. Note the diffuse cytoplasmic staining of almost all the tumor cells in the tumor mass. Section B was reacted with P6.2. Note the absence of staining of the tumor cells. The fibromuscular stroma (W) is unstained. × 400.

Fig. 5. Reactivity of monoclonal antibodies D83.21 and P6.2 with sections of urinary bladder carcinomas using the immunoperoxidase technique. Section A was reacted with D83.21. Note nest of stained tumor cells within the unstained fibromuscular stroma (W). Section B was reacted with P6.2. Note the stained tumor cells (T) that have invaded the muscle (M). The fibromuscular stroma (W) and muscle (M) is unstained. × 400.
Fig. 6. Metastatic prostate tumors stained with monoclonal antibodies DB3.21 and P6.2 using the immunoperoxidase technique. A and B, prostate tumor metastatic to the liver reacted with P6.2. Note the 2 stained (T) and 2 unstained (t) metastatic foci in Section A. A higher magnification of one of the foci shows both stained (T) and unstained (t) tumor cells within the metastatic foci. Both intense membrane and diffuse cytoplasmic staining of the tumor cells is evident. The hepatocytes (N) are unstained. C, prostate tumor metastatic to lymph node stained with DB3.21. Diffuse cytoplasmic staining of almost all the tumor cells (T) and no staining of the lymphocytes (L). The lymphocytes in this section stained a deep blue with hematoxylin, therefore, the contrast between the lymphocytes and tumor cells on the black and white photographs is not sufficient to distinguish positive from negative peroxidase-stained cells. Section D is from a prostate tumor liver metastasis of another patient showing a nest of tumor cells (T) stained with P6.2 within a blood vessel. Note that the RBC (r) and normal cells (N) are unstained. A, x 100; B, x 400; C and D, x 200.
Immunohistochemical Localization of Prostate Carcinoma-associated Antigens

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