DIRECT TISSUE LOCALIZATION OF ACID PHOSPHATASE
IN THE PROSTATE: OBSERVATIONS UTILIZING THE
PEROXIDASE-ANTI-PEROXIDASE TECHNIQUE

A Thesis
Presented in Partial Fulfillment of the Requirements
for the Degree Master of Science
by
Scott Douglas Jewell, B.S.
The Ohio State University
1980

Copyright © 1980
by Scott Douglas Jewell.
All rights reserved.

Approved by

Adviser

Department of Pathology
ACKNOWLEDGEMENTS

TO

John C. Neff, M.D.
Donald A. Senhauser, M.D.
Judith W. Noltimier, MBES.
Judith L. M. Jewell, MT ASCP.
Mary P. Grose, B.S., MT ASCP.
Susan M. Knorr
Kendra Quellhorst

I give my sincere gratitude to all involved with the development, review, and production of this research. I especially wish to thank Dr. John C. Neff, for his great interest, expertise, and guidance, for which without this research could not have been accomplished. A special note of thanks to my wife, Judy, and my co-worker and good friend, Mary P. Grose, for their patience and understanding.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................... ii
TABLE OF CONTENTS ........................................... iii
ABSTRACT ....................................................... iv
INTRODUCTION .................................................. 1
MATERIALS AND METHODS ..................................... 3
ILLUSTRATIONS .................................................. 12
RESULTS .......................................................... 17
GRAPH AND TABLES ............................................. 23
DISCUSSION ..................................................... 27
SUMMARY .......................................................... 34
REFERENCES ..................................................... 36
A quantitative peroxidase-anti-peroxidase (PAP) method was developed and applied to the analysis of formalin-fixed Paraplast embedded tissue. The PAP method was used to ascertain the amounts of prostatic specific acid phosphatase (PSAP) in the tissue from patients with benign prostatic hypertrophy (BPH) and adenocarcinoma of the prostate (ADCP). In twenty-four cases of BPH, staining for PSAP was uniform and heavy. In twelve cases of ADCP, staining for PSAP was erratic and quantitatively lower than BPH tissues. Results from ADCP-PSAP tissue also indicated the presence or absence of PSAP was unrelated to the Gleason method of histological grading of ADCP. In addition the use of tissue localization of PSAP in the evaluation of primary ADCP and its metastases is discussed.
INTRODUCTION

Elevation of the serum acid phosphatase is caused by many disorders (31). The prostate contains more acid phosphatase than any other tissue, therefore elevated serum acid phosphatase levels are seen in a wide variety of prostatic lesions. Patients with carcinomas of the prostate often, though not always, have elevated levels of serum acid phosphatase (9). Such elevations of serum acid phosphatase are thought to be related to the extent of prostatic malignancy, particularly extension of the carcinoma beyond the prostatic capsule (16).

The methods which are currently used to measure serum acid phosphatase consist of enzymatic-spectrophometric and radioimmunoassay techniques. Acid phosphatase in tissue sections is being demonstrated using routine histochemical techniques; recently immunohistochemical methods have been described as very sensitive and said to be much more organ specific.

This research project is designed to develop a method for the tissue localization of prostatic specific acid phosphatase (PSAP) in benign prostatic hypertrophy (BPH) and in adenocarcinoma of the prostate (ADCP) utilizing the peroxidase-anti-peroxidase (PAP) method. The intention of this project by the use of this method is to demonstrate the quantitative relationship of the amount of tissue prostatic specific acid phosphatase to a modern histopathological
grading system for adenocarcinoma of the prostate, the Gleason grading system (10,11). The usefulness of this technique in the diagnosis of prostatic carcinoma and the relationship of serum and tissue prostatic specific acid phosphatase is discussed.
MATERIALS AND METHODS

Tissue:

Material utilized in this study consisted of prostatic tissue bearing the diagnosis of benign prostatic hyperplasia or adenocarcinoma. All cases were 1979 accessions from the Division of Surgical Pathology at Ohio State University Hospital. Tissue from twenty-four cases of BPH and twelve cases of ADCP were assayed. Tissues were obtained from either suprapubic prostatectomies or needle biopsies of the prostate. These tissues were chosen because rapid fixation was probable and to avoid the possible change in tertiary structure caused by cauterization routinely used in transurethral resections (TUR) of prostate. The tissues were fixed in 10% neutral buffered formalin and subsequently embedded in Paraplast for sectioning.

Antiserum:

Rabbit anti-human prostatic specific acid phosphatase (PSAP) was obtained from Eureka Laboratories, Inc., Sacramento, CA, lot number PB102. Goat anti-rabbit immunoglobulins (GAR) and rabbit peroxidase-anti-peroxidase (RPAP) were obtained from Cappel Laboratories, Inc., Cochranville, PA. Goat serum was obtained from Grand Island Biological Company (GIBCO) and rabbit serum was obtained from DAKO Accurate Chemicals and Scientific Corp., Hicksville, NY.

The antigen source for PSAP was seminal fluid. The
antigen was purified by column chromatography and showed one band when electrophoresed on acid polyacrylamide gel. The antibody titer was high as determined by immunodiffusion. At a 10,000 to 20,000 dilution the antiserum is estimated to bind 50% of I\textsuperscript{125} acid phosphatase at a concentration determined by Eureka Laboratories, Inc. Sensitivity of the antiserum in radioimmunoassay is 0.2 ng/ml. The product shipped was as whole serum with 0.1% sodium azide added for preservation.

A 1:1,000 dilution of PSAP was assayed on all BMH and ADCP tissues. In addition all ADCP's were stained with a 1:200 dilution of PSAP. A 1:30 dilution of GAR and RPAP was used. All antisera were diluted in TRIS-saline buffer (pH 7.6).

\textbf{Materials:}

The materials used in this peroxidase method were:

1. ethyl alcohol (\textit{ETOH}) 100%, 95%, 70%
2. 10% Phosphate buffered formalin (pH 7.4)
4. Harris Hematoxylin stain
5. HCl
6. hydrogen peroxide (30%)
7. normal saline (0.85%)
8. Pro-Texx mounting media, Scientific Products
10. Xylenes, Mallincrodt
11. double distilled water
12. Paraplast
13. Fisher Histomatic tissue processor
14. microtome

**Staining Procedure:**

Tissues were processed for 24 hours in 10% phosphate buffered formalin (pH 7.4). After fixation the tissues were processed through the Fisher Histomatic with graded alcohols and cleared with xylenes, then embedded in Paraplast. Tissues next were sectioned at 4 microns (μ) and placed on 25 x 75 mm microscope slides. Slides were placed in holders and immersed in Tissue-Tek trays containing xylenes and graded alcohols for deparaffinization.

The entire procedure was divided into two parts:

**Part I. Deyparaffinisation:**

1. Incubate slides in two xylenes baths, five minutes each.
2. Incubate slides in one bath 1/2 xylenes and 1/2 100% ETOH for five minutes.
3. Incubate slides in two 100% ETOH baths, five minutes each.
4. Incubate slides in two 95% ETOH baths, five minutes each.
5. Incubate slides in one 70% ETOH bath, five minutes.
6. Incubate slides in two baths of double distilled 
$\text{H}_{2}\text{O}$, five minutes each.
All incubations include periodic agitation.

**Part II. Peroxidase-Anti-Peroxidase Method:**

1. Wash slides in .05 M TRIS-buffer (pH 7.6) for five 
   minutes.

2. Block endogenous peroxidase activity by incubating 
   slides in TRIS-buffer and 0.5% hydrogen peroxide 
   for thirty minutes.

3. Wash slides in TRIS-saline buffer for five minutes.
   TRIS-saline buffer is prepared by diluting one 
   part .05 M TRIS-buffer with nine parts normal 
   saline (.85%), i.e. 1:10 dilution.

4. Blocking nonspecific receptors is accomplished by 
   immersing the slides in a 1:20 dilution of goat 
   serum for ten minutes.

5. Wash in two baths of TRIS-saline buffer, five 
   minutes each.

6. Dry slides with gauze except for the tissue area. 
   Then overlay slides with a "sufficient" pool of the 
   primary antisera, PSAP, before tissue sections dry. 
   Incubate in a moisture chamber at room temperature 
   for one hour.

7. Wash slides in three baths of TRIS-saline buffer, 
   five minutes each.

8. Dry slides as above and overlay with the secondary
antibody, GAR, and incubate for thirty minutes at room temperature.

9. Wash slides in three baths of TRIS-saline for five minutes each.

10. Dry slides and overlay with RPAP and incubate for thirty minutes at room temperature.

11. Wash slides in three baths of TRIS-saline for five minutes each.

12. Chromogen reaction: Dry slides, overlay with Hanke-Yates reagent (17). Incubate for ten minutes in the dark at room temperature. The Hankers-Yates reagent is prepared by adding 7.5 - 15 mg of the powdered reagent to 10 ml of 0.05 M TRIS-buffer, then add 100 μl of 1% H₂O₂ diluted in 0.05 M TRIS-buffer and use immediately.

13. Wash slides in three baths of TRIS-saline buffer for two minutes each.

14. Counterstain with Harris Hematoxylin stain for two minutes and rinse with double distilled H₂O. The Harris Hematoxylin stain should be filtered each time before use.

15. Dehydrate the slides by reversing the deparaffinization protocol, incubating for three minutes with each step.

16. Coverslip the slides using Pro-TeXx mounting medium. The optimal color of the peroxidase chromogen reaction

7
was a granular dark brown as shown in the cytoplasm of the epithelial cells of the acini in Illustration 1.

Controls:
1. Buffer control: The buffer control substituted TRIS-saline buffer for the primary and secondary antibody applications. The expected staining reaction was negative. Any positive staining would have indicated nonspecific binding of the RPAP to the human tissue or development of endogenous peroxidase.

2. Primary antibody control: This control substituted TRIS-saline buffer for the primary antibody application. The resulting reaction was negative, indicating that in the absence of the primary antibody no reaction took place. A negative reaction also substantiated the probability that nonspecific receptors in the tissue were occupied.

3. Secondary antibody control: This control substituted TRIS-saline buffer for the secondary antibody application. The resulting reaction was negative, indicating an absence of RPAP binding to human nonspecific receptors and to the primary antibody. In addition, the disruption of the cascade insured that the positive reactions were due to the specific antibody sequencing and subsequent chromogen reaction.
4. Rabbit serum control: This control consisted of rabbit serum (1:30) in place of the primary antibody. The importance of this control rests in its ability to define the specificity of the rabbit anti-human PSAP. Primary antibody specificity is the most important aspect of this technique. The sensitivity of the reaction may be adjusted by altering the dilution of this antibody.

5. Positive control: BPH tissues were utilized as positive controls. In the presence of the primary (PSAP), secondary (GAR), and tertiary (RPAP) antibodies the positive control indicated the ability of the rabbit anti-human PSAP to recognize human prostatic acid phosphatase.

6. Specificity controls: An absorption control is most desirable to prove specificity of the primary antibody for prostatic specific acid phosphatase. However, the purified specific antigen is not easily obtainable. The following tissues, some of which contain other isoenzymes of acid phosphatase, were examined to rule out cross reactivity. All tissues were negative for intracytoplasmic staining. These tissues were:

a. liver
b. large bowel
c. small bowel
d. pancreas  
e. myelinated peripheral nerve  
f. red blood cells  
g. skeletal muscle  
h. stomach, fundus  
i. sympathetic ganglion

Grading and Immunohistochemical Quantitation Method:

I. The histological grading of prostatic neoplasms was undertaken utilizing the method of Gleason (10,11). This method depends upon the degree of glandular differentiation and growth pattern in relationship to surrounding stroma. There are five patterns, two of which have a single subtype. These patterns are based upon an evaluation of the tumor margin, gland distribution, and stromal invasion. A summary of the Gleason pattern and the relative proportion of tumors in each category is given in Table 1 (29).

II. A modification of the Chalkley method of quantitation (3) was used to measure the acid phosphatase content of the tissues utilized in this study, as follows: An AO microscope, Series Twenty-One, was fitted with an AO 1408A ocular grid (20 mm diameter disc, 10 mm square, divided into 1 mm squares). Upon viewing, the grid appeared as shown in Illustration 2. All measurements were done at a magnification of 400 times. The slide was positioned so that a representative portion of the most
glandular tissue was examined. This area was positioned under the grid such that the maximal amount of glandular tissue was contained in the calibrated square. If any portion of a 1 mm square touched a cell a count of one was given. It was therefore possible to count one cell four times, however, lumina and stroma and negatively stained cells would receive zero counts. We found this method more reproducible than a simple estimation of the amount of acid phosphatase within the tissue utilizing the zero to +4 method of quantitation, as shown in Illustration 3.
Illustration 1. Prostate tissue stained with peroxidase chromogen, Hanks'-Yates reagent. The color of the stain is dark brown as indicated by the arrows.
Illustration 2. Reproduction of the ocular grid used to score F3AP localization in tissue.
Illustration 3. The counting grid layered over the prostate tissue as seen under the microscope. The amount of acid phosphatase is easier estimated than with the zero to $+\frac{1}{4}$ method.
Illustration 4. Two adenocarcinomas with appreciably different amounts of PSAP. The top photo has more intracytoplasmic PSAP than the lower as indicated by the arrows.
Illustration 5. The cascade of the primary, secondary and tertiary antibody structures as bound to the antigen on the tissue.
RESULTS

In Tables 2-4, the prostatic tissue specimens received in the Surgical Pathology Division at Ohio State University Hospitals during 1979 are listed. Table 2 lists each tissue according to route of removal and the number from each particular route assayed in this study. It is evident from this Table that a relatively small number of needle biopsies and suprapubic resections were received. Therefore, a large number of TUR-P specimens remain for further study. Table 3 lists each tissue removed by surgical pathology diagnosis and the number of tissues with each particular diagnosis (BPH, ADCP, and Other) assayed. Thirty-three percent of the carcinomas of the prostate and more than one fourth of all prostatic tissue removed in 1979 were assayed. Table 4 lists each tissue again by route of removal; opposite each mode of removal is the frequency of diagnosis of BPH, ADCP, or Other from that site in 1979. Fifty percent of the needle biopsies were productive of a diagnosis of carcinoma, while only 24 percent of the transurethrally resected prostatic tissue revealed such a diagnosis.

In almost every tissue bearing the diagnosis of BPH, staining for intracytoplasmic PSA is uniform and heavy. As presented in Graph 1, these tissues, when treated with a 1:1000 dilution of the primary antibody, characteristically gave a high score for PSA with a median of 86, mean
of 76.4, and a standard deviation of 21.

Whether treated with a 1:200 or 1:1000 dilution of the primary antibody, adenocarcinomas of the prostate stained erratically for intracytoplasmic PSAP, as noted in Illustration 4. As presented in Graph 1, the quantitative scores for PSAP were highly variable and generally lower then for those tissues revealing BPH. Carcinomas of the prostate were classified according to Gleason grade 1, 2, 3, 4, or 5. Some prostatic tissue contained only one grade while others contained more than one grade. The erratic variability of the prostate adenocarcinomas is more clearly shown in Table 5. Lowering the dilution of the primary antibody did not necessarily result in a higher PSAP score. Table 6 shows marked variability of PSAP staining of adenocarcinomas of the prostate. The standard deviations are almost equal to the mean values.

The results of this study indicate that the amount of PSAP within prostatic adenocarcinoma appears to be independent of Gleason histological grade. However, in tumors having more than one histological grade, the higher grades had less PSAP.

Summarizing the results of this study: The PAP method for PSAP is sensitive and specific. The PSAP content of BPH is high and uniform, while the PSAP content in adenocarcinomas of the prostate is lower and tends to be very erratic. Also the quantitative PSAP scores of the carcino-
omas are independent of the primary antibody dilution. This is significant because it is the most important variable in the PAP technique. Finally, though the quantity of PSA in adenocarcinoma of the prostate appears independent of Gleason's histological grade, there is some evidence that the higher or more undifferentiated grades contain less PSA.
Graph 1: Illustrates the quantitative PSAP score of 24 cases of BPH (•) and 12 cases of carcinoma of the prostate. The carcinomas are arranged by Gleason grade (1, 2, 3, etc.). Some of the carcinomas were assigned more than one Gleason grade, hence the symbols (•, ▲, etc.). Those carcinomas with a single grade are denoted with a solid circle (●).
<table>
<thead>
<tr>
<th>%</th>
<th>Pattern</th>
<th>Margins of tumor areas</th>
<th>Gland pattern</th>
<th>Gland size</th>
<th>Gland distribution</th>
<th>Stromal invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>well defined</td>
<td>single, separate, round</td>
<td>medium</td>
<td>closely packed</td>
<td>minimal, expansile</td>
<td></td>
</tr>
<tr>
<td>24.8</td>
<td>less definite</td>
<td>single, separate, rounded, but more variable</td>
<td>medium</td>
<td>spaced up to one gland diameter, average</td>
<td>mild, in larger stromal planes</td>
<td></td>
</tr>
<tr>
<td>87.7</td>
<td>poorly defined</td>
<td>single, separate, more irregular</td>
<td>small, medium or large</td>
<td>spaced more than one gland diameter, rarely packed</td>
<td>moderate, in larger or smaller stromal planes</td>
<td></td>
</tr>
<tr>
<td>12.1</td>
<td>poorly defined</td>
<td>rounded masses of cribriform or papillary epithelium</td>
<td>medium or large</td>
<td>rounded masses with smooth sharp edges</td>
<td>expansile masses</td>
<td></td>
</tr>
<tr>
<td>22.6</td>
<td>ragged infiltrating</td>
<td>fused glandular masses or &quot;hyper-nephroid&quot;</td>
<td>small</td>
<td>fused in ragged masses</td>
<td>marked, through smaller planes</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ragged, infiltrating</td>
<td>almost absent, few tiny glands or signet ring cells</td>
<td>small</td>
<td>ragged anaplastic masses of epithelium</td>
<td>severe, between stromal fibers or destructive</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>poorly defined</td>
<td>few small lumina in rounded masses of solid epithelium + central necrosis?</td>
<td>small</td>
<td>rounded masses and cords with smooth, sharp edges</td>
<td>expansile masses</td>
<td></td>
</tr>
</tbody>
</table>

50% of prostatic carcinomas may demonstrate two different patterns.
Table 2

<table>
<thead>
<tr>
<th>Type of Tissue</th>
<th>Total Number of Specimens</th>
<th>Number of Research Specimens Assayed</th>
<th>Percent of the Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of transurethral resections</td>
<td>81</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Number of needle biopsies</td>
<td>20</td>
<td>19</td>
<td>95.0</td>
</tr>
<tr>
<td>Number of suprapubic resections</td>
<td>16</td>
<td>16</td>
<td>100.0</td>
</tr>
<tr>
<td>Others (referrals, etc.)</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total received in 1979</td>
<td>126</td>
<td>36</td>
<td>28.6</td>
</tr>
</tbody>
</table>

Table 2: Indicates the total number of prostatic tissue specimens received in Surgical Pathology in 1979. The tissues are divided according to the route of removal and how many from each site were assayed in this study.
<table>
<thead>
<tr>
<th></th>
<th>total number of specimens</th>
<th>number of research specimens assayed</th>
<th>percent of the total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of BPH diagnosis</td>
<td>82</td>
<td>24</td>
<td>29.3</td>
</tr>
<tr>
<td>Number of ADCP diagnosis</td>
<td>26</td>
<td>12</td>
<td>33.3</td>
</tr>
<tr>
<td>Others (prostatitis, transitional cell carcinoma)</td>
<td>7</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Totals received in 1979</td>
<td>126</td>
<td>36</td>
<td>28.6</td>
</tr>
</tbody>
</table>

Table 3: Indicates the diagnosis of all prostatic tissue specimens received in 1979 and how many bearing each diagnosis were assayed in this study.
### Table 4

<table>
<thead>
<tr>
<th>Total number of specimens received</th>
<th>BPH (%)</th>
<th>ADCP (%)</th>
<th>Others (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of transurethral resections</td>
<td>81</td>
<td>58 (71.6)</td>
<td>20 (24.7)</td>
</tr>
<tr>
<td>Number of needle biopsies</td>
<td>20</td>
<td>10 (50.0)</td>
<td>10 (50.0)</td>
</tr>
<tr>
<td>Number of suprapubic resections</td>
<td>16</td>
<td>13 (80.4)</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td>Others</td>
<td>9</td>
<td>1 (11.2)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Total number received in 1979</td>
<td>126</td>
<td>82 (65.1)</td>
<td>36 (28.6)</td>
</tr>
</tbody>
</table>

Table 4: Indicates the total number of prostatic tissue specimens removed in 1979, by mode of removal. Opposite the number of each mode of removal are the total of each diagnosis (BPH/ADCP/Other) for tissues removed via that mode.
<table>
<thead>
<tr>
<th>Surgical Pathology Number</th>
<th>PSAF scores using a 1:200 dilution of the primary antibody</th>
<th>PSAF scores using a 1:1000 dilution of the primary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>79-2544</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>79-2679</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>79-2680*</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>79-2971*</td>
<td>45</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>79-3408*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>68</td>
</tr>
<tr>
<td>79-3917</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>79-6062</td>
<td>63</td>
<td>84</td>
</tr>
<tr>
<td>79-6086</td>
<td>82</td>
<td>65</td>
</tr>
<tr>
<td>79-6158*</td>
<td>87</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>79-7261</td>
<td>82</td>
<td>85</td>
</tr>
<tr>
<td>79-7633*</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>72-8335</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5: Compares the quantitative PSAF scores of adenocarcinoma of the prostate utilizing primary antibody dilution of 1:200 versus 1:1000.
* ADCP having more than one pattern
Table 6: Compares the means and standard deviations of the BH prostate tissue with the Gleason grading system for ADCP tissues.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH</td>
<td>77</td>
<td>21</td>
</tr>
<tr>
<td>ADCP Gleason Grade 1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ADCP Gleason Grade 2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ADCP Gleason Grade 3</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>ADCP Gleason Grade 4</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>ADCP Gleason Grade 5</td>
<td>45</td>
<td>19</td>
</tr>
</tbody>
</table>
DISCUSSION

In recent years, tissue immunoperoxidase techniques have been reported to have a wide use in diagnostic pathology. They have been utilized for the tissue localization of such molecules as enzymes, hormones, serum proteins, viral antigens, and tumor antigens (8). The advantages of immunoperoxidase techniques over immunofluorescent techniques are several, and include a permanent slide record, superior morphologic localization of antigen, the lack of a need for specialized microscopes and other expensive equipment, the ability to use routine formalin-fixed tissue and therefore the possibility to study retrospectively tissue which was formalin-fixed and paraffin embedded years previously.

The PAP sensitivity relies on a highly specific primary antibody which binds to an antigen in the tissue. The secondary antibody is an anti-immunoglobulin acting as a link between the primary and tertiary antibody and must therefore be species specific. Followed by the binding of the second antibody to the Fc region of a third antibody complex. The tertiary antigen-antibody complex comprises two identical antibody molecules bound to two peroxidase enzyme molecules at the variable regions of the tertiary immunoglobulins. The cascade of the antibodies is presented in Illustration 5. Thus two peroxidase enzymes will be developed for each antigenic site on the tissue, causing
increased sensitivity due to an amplification effect. The peroxidase enzyme when in the presence of $H_2O_2$ and the chromogen reagent, Harker-Yates, aids in the oxidation of the chromogen reagent generating a dark brown color and $H_2O$. Due to the large amount of antibody attached to a ubiquitous antigen, a granular brown appearance is seen in the tissue. Immunoperoxidase techniques also can be adapted to electron microscopy, and are considerably more sensitive than immunofluorescent procedures (22,28). The disadvantages of immunoperoxidase techniques when compared to immunofluorescent procedures are that they are more time-consuming, a larger selection of fluorescein conjugated antibodies exists at present, and immunofluorescence may allow for the demonstration of two antigens in the same tissue if a rhodamine as well as fluorescein conjugate is utilized. Finally, diaminobenzidine (DAB) is the most common chromogen reagent used in immunoperoxidase procedures; this has been recognized as a possible carcinogen (13). However, with the introduction of P-phenylene-diamine-pyrocatechol (PPD-PC), Harkers-Yates reagent, a chromogen now exists which is thus far deemed safe for use.

Acid phosphatase has been localized in many organs and tissues (26,27,31), including bone, bone marrow, intestines, kidney, liver, leukocytes, nerve, pancreas, platelets, erythrocytes, spleen, skin, but in especially large amounts in the epithelium of the prostate gland. In
the past, acid phosphatase has been routinely measured by enzymatic methods. Recently, however, immunologic techniques have proven to be much more sensitive (1,5,5,7,9,26,30). Acid phosphatase may be found in tissue, or in serum. As with other enzymes, acid phosphatase exists in multiple molecular forms which appear to be relatively organ-specific (26,31). The prostatic isoenzyme likewise has been measured both enzymatically and immunochimically, both in serum and directly in prostatic tissue. The immunologic methods are virtually specific for the prostatic isoenzyme. This has been shown to be true for the measurement of serum acid phosphatase (4,5,9,11,30). In this study we have demonstrated the high specificity of the direct tissue localization of prostatic acid phosphatase utilizing a peroxidase-anti-peroxidase technique.

Immunoperoxidase techniques have been used to localize acid phosphatase in both primary adenocarcinoma of the prostate and its metastases (19,20,24).

Utilizing older enzymatic methods, an elevation of serum acid phosphatase was thought to indicate extension of carcinoma of the prostate beyond the capsule (14,15,18,25). Newer immunologic (RIA) techniques bring this older observation into question; using highly sensitive RIA techniques, some investigators have found significant numbers of stage A and B neoplasms to cause elevations in serum acid phosphatase (12,21). Increase in the serum value measured by
this technique may also be seen in cases of BPH, infarction of prostatic tissue, and after prostatic massage (9).

Even though prostatic carcinoma has been thought of as one major reason for the elevation of serum acid phosphatase, actual tissue levels of acid phosphatase in prostatic carcinoma have been reported to be generally lower than that seen in normal prostatic tissue or in benign prostatic hypertrophy (2,20,24). Recently, investigators have used immunoperoxidase methods to demonstrate PSAP in malignant prostatic neoplasms, both primary and metastatic (19,20,23,24). These investigators have found the amount of acid phosphatase in the tissue to be unrelated to the histologic grade of the neoplasm. We have confirmed this observation with the very sensitive PAP immunoperoxidase technique and have shown that prostatic carcinomas stain erratically and with a lower intensity than does tissue from patients with benign prostatic hypertrophy. Our observation is that the amount of acid phosphatase in a histologic section apparently has no correlation with histologic grade. Our quantitative method has shown this convincingly, although our number of cases analyzed is small. Certainly a larger study is warranted so that this impression may be confirmed.

We began our study utilizing only tissue obtained suprapubically or by needle biopsy of the prostate. This was done in order to eliminate from consideration the possible
heat alteration in tertiary structure of the enzyme and consequent loss of antigenic sites in specimens obtained via the transurethral route. During the course of the study the assay was performed on a small number of transurethrally resected specimens and excellent results were obtained. Retrospective analysis could utilize the large number of cases which are available in most pathology files.

Our findings serve to pose a number of questions, and also indicate the need for discretion in utilization of this direct tissue assay for acid phosphatase. First, adenocarcinoma of the prostate stains erratically for PSAAP and appears to contain less enzyme activity than normal prostate or benign prostatic hypertrophy. Although less acid phosphatase may be produced in adenocarcinoma of the prostate, because of necrosis and the lack of an efferent duct system, more of this acid phosphatase may diffuse into surrounding tissue and find its way into the circulation. The acid phosphatase seen in serum may not all be from the neoplastic tissue; some may be from surrounding benign prostatic tissue due to distortion of the normal glandular architecture. This is unlikely, since the highest levels are seen in patients with metastases. Alteration in structure or incompleteness of the basement membrane associated with malignant acini may allow increased access of PSAAP to the peripheral and lymphatic circulation. A final possi-
bility is that the lowered amounts in tissue may be an artifact of the method of measurement. Some cells may be producing large amounts with immediate diffusion into the circulation while other cells may produce little or none. Thus the presence of PSAP in the tissue may not be seen by staining.

Most of the antibodies which are used in immunologic studies of serum or tissue were raised against acid phosphatase antigen in normal semen. Acid phosphatase produced by prostatic carcinoma cells may be structurally different than the acid phosphatase in semen. This would seem an unlikely explanation for the low amounts seen in some malignant tissues since the same antibody is capable of measuring serum acid phosphatase in both benign and malignant conditions. The simultaneous study of serum and tissue levels of acid phosphatase, utilizing the same antibody preparation, may help to answer some of these questions. Other questions which might also be resolved by such a simultaneous study of tissue and serum prostatic specific acid phosphatase are the following: Is the amount of acid phosphatase in serum in any way related to histological grade of a neoplasm? It is our impression that it is not. Is the amount of PSAP present in serum and/or tissue related in any way to subsequent response to therapy?

Secondly, another observation which is important
follows. PAP-PSAP is a sensitive and specific method for the demonstration of acid phosphatase in tissue, however it is not a "cancer" stain. While it is true that most carcinomas of the prostate contain acid phosphatase, and that it can be stained, this reaction is erratic and the neoplasms may have less acid phosphatase in total than normal tissue. And in this research occasional neoplasms in which the cells do not stain at all have been seen. This must be borne in mind when evaluating possible prostatic primaries or their metastases. Because the stain is highly specific, a positive result is quite diagnostic; a negative result may not rule out carcinoma of the prostate. While most PAP procedures call for a relatively high dilution of primary antibody, a low dilution of primary antibody (1:200) should be utilized with this PAP technique. Although little difference is seen in staining with low or high dilutions, a low dilution utilizing this technique appears to insure maximum sensitivity. A larger study would help to clarify whether this low dilution is indeed a requirement. False negatives due to insensitivity of the procedure may thus be avoided.
SUMMARY

A comparison of the amount of tissue prostate specific acid phosphatase in benign prostatic hypertrophy and adenocarcinoma of the prostate was carried out utilizing a quantitative application of the peroxidase-anti-peroxidase method. The results indicated that the quantitative values for benign prostatic hypertrophy were consistently higher than those for adenocarcinoma of the prostate. In addition, staining of tissue from adenocarcinoma of the prostate was found to be not only less prominent than that for benign prostatic hypertrophy, but much more erratic. We found no correlation between prostate specific acid phosphatase in adenocarcinoma of the prostate and Gleason histological grade. However, there was some evidence to indicate that in tumors having more than one histological grade, there was less PSAP in the more undifferentiated component.

After evaluation of the results we feel two significant areas need further study. 1) In prostatic carcinoma, PSAP tissue levels may be lower than those noted in normal prostate or in BPH, while serum PSAP levels may be elevated. Local diffusion of the enzyme, caused by a failure to develop a normal duct system in carcinoma, or an obstruction of normal duct systems by the carcinoma, is a probable explanation for this observation. In addition increased serum PSAP levels may be attributed to altera-
tions in the basement membrane of malignant acini allowing immediate diffusion of PSAF into the circulation. Finally malignant cells may produce structurally unrecognizable PSAF in the presence of the primary antibody. This is possible but improbable since elevated serum levels in carcinoma patients is a fact. Simultaneous serum and tissue assays utilizing the same primary antibody may help in determining if there is a relationship between serum and tissue PSAF levels.

2) Discretion must be employed when this immunoperoxidase methodology is used to evaluate prostatic primaries or possible prostate metastases. A positive result lends a great deal of specificity to the diagnosis. However, negative results may not rule out adenocarcinoma of the prostate.

The number of observations in this study were small, however they indicate the need for further study. Large retrospective studies are feasible because the technique can be applied to formalin-fixed, paraffin embedded tissue, which is readily available in most pathology files.
REFERENCES


36
12. Griffiths: Clinical study of the RIANEN™ prostatic acid phosphatase 125I RIA kit, New England Nuclear Medical Diagnostics Division, N. Billerica, MA

38


