**Biochemical Methods for Predicting Metastatic Ability of Prostatic Cancer Utilizing the Dunning R-3327 Rat Prostatic Adenocarcinoma System as a Model**

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**ABSTRACT**

At present, there is no established diagnostic method by which the metastatic ability of an individual prostatic cancer can be accurately predicted. Metastasis is a multistep process, the first critical step of which is invasion. Tumor invasion has been suggested to involve a variety of hydrolytic enzyme activities; therefore, the tumor levels of these activities might be indicative of the overall metastatic ability of the cancer. In order to evaluate if the quantitative levels of hydrolytic enzymes can be used to predict the metastatic ability of individual prostatic cancers, five different Dunning R-3327 rat prostatic adenocarcinoma sublines, with widely varying metastatic abilities, were assayed for the respective levels of a variety of hydrolytic enzyme activities (collagenase, trypsin-like, cathepsin B, neutral protease, N-acetyl-β-glucosaminidase, chymotrypsin-like, leucine aminopeptidase, elastase, and plasminogen activator). These studies demonstrated that most hydrolytic activities are not elevated when going from normal prostate to prostatic cancer. In addition, only the levels of elastase and chymotrypsin-like activity were found to be consistently higher in highly metastatic prostatic cancers than in either the normal prostate or low-metastatic prostatic cancers. It was found that, by combining the relative activities of elastase and chymotrypsin-like activity and then dividing by the relative activities of N-acetyl-β-glucosaminidase, a biochemical metastatic index could be constructed which accurately reflected the respective metastatic ability of the Dunning sublines.

**INTRODUCTION**

Adenocarcinoma of the prostate varies widely in its clinical aggressiveness. In some patients, prostatic cancer metastasizes rapidly and kills the patient in less than 1 year, while other patients may live for many years with localized disease without apparent metastases (3). If indeed the cancer is truly localized (Stage B1 and B2), then radical prostatectomy offers a real possibility for cure for such patients (38). However, if the cancer appears clinically only to be localized but has in fact already produced metastases, then radical surgery alone will not be curative and alternative systemic therapy would be required. Unfortunately, at present, there is no established diagnostic method by which the metastatic ability of an individual primary prostatic cancer can be accurately predicted. This inability is partially due to the limited knowledge concerning what constitutes the unique phenotypic characteristics which allow a prostatic cancer cell to be metastatic. It is known, however, that the pathogenesis of cancer metastasis is a multistep process (4, 10-12, 29, 32, 39). The basic process following the initial development of the primary cancer involves 5 major steps: (a) dislodgment of cancer cells from the primary tumor, invasion into surrounding tissues and penetration of the blood and/or lymph vessels; (b) release of tumor cell emboli into the general circulation; (c) arrest of tumor cell emboli in small vessels of distant organs; (d) tumor cell invasion of the wall of the arresting vessel, infiltration into adjacent tissues, and proliferation; and (e) growth of the new blood vessels into the new tumor (41).

The process of metastasis is thus complex; however, the first critical step in the metastatic process is invasion, because without invasion metastasis cannot occur (41). The release by the primary tumor cells of tissue-destructive enzymes, particularly hydrolases and proteases, has been postulated to be important in tumor invasion (27). A positive correlation between elevated levels of various hydrolytic enzymes with increased malignancy and/or increased metastatic potential has been made in several human and animal tumor systems. These enzymes include plasminogen activator (20, 31), collagenase (8, 40) (especially type IV) (23-25), N-acetyl-β-glucosaminidase (7), neutral protease (19), elastase (13), thiol protease (28), and cathepsin B (34-36).

Hart and Fidler (12) suggested that it was probably naive to ascribe the complex process of invasion to the elevation of a single enzyme. Indeed, the metastatic process may involve the coordinated elevation of several of these and other hydrolytic activities. It therefore might be possible to predict individually which prostatic cancers are likely to be highly metastatic versus nonmetastatic at the time of initial treatment by comparing the quantitative levels of a series of different hydrolytic enzyme activities determined on a representative biopsy of the primary prostatic cancer. If this could be done accurately, then those patients having a cancer with high likelihood of being metastatic could be identified and immediately started on intensive systemic therapy.

In order to determine experimentally if the quantitative levels of hydrolytic enzymes can be used to predict the metastatic abilities of various prostatic cancers, some type of well-characterized system of prostatic cancers with widely varying metastatic ability is required. Fortunately, such a system presently exists in the form of the Dunning R-3327 system of serially transplantable rat prostatic adenocarcinomas (17). This R-3327 system of tumors has been derived over the years from the parental R-3327 tumor, initially described by W. F. Dunning in 1961 as a spontaneous tumor originating in the prostate gland of an aged male Copenhagen rat (9). The parental H tumor is a slow-growing, well-differentiated, androgen-sensitive prostatic adenocarcinoma which has only a very low rate (<0.1%) of
distant metastasis. While it has been possible to maintain these distinct characteristics of the H tumor in continuous serial pas-

sage in male Copenhagen rats for over 20 years, there have evolved spontaneously, however, completely anaplastic sublines from the originally well-differentiated tumor at several subpas-

sages in our laboratory and others (15, 17, 18, 21). Unlike the parental H tumor, several of these anaplastic sublines have very high metastatic abilities. In the present study, 4 of these anap-

astic sublines were extensively characterized with regard to their respective incidence and site of metastasis. Once biologi-

cally characterized, these anaplastic sublines were assayed for their respective levels of a variety of hydrolytic enzyme activities in order to determine if any of these biochemical parameters could be used to predict the metastatic ability of primary prostatic cancers.

MATERIALS AND METHODS

Animal Studies. The history of the development of the various Dun-

ning R-3327 rat prostatic adenocarcinoma sublines used in this study has been described in detail elsewhere (17). For the present study, the H tumor and the 4 anaplastic sublines, AT-2, AT-3, MAT-LyLu, and MAT-

Lu, were used. Each of these sublines was routinely passaged by inoculating intact adult male Copenhagen rats (Harlan Sprague-Dawley, Indianapolis, IN) s.c. in the flank with a 25-mg trocar piece of the respective tumor subline as described earlier (17). The animals, unless otherwise stated, were then allowed to go untreated until death. During this time, tumor volume measurements were performed for each of the sublines at routine intervals as described previously (15). From these measurements, the individual tumor volume-doubling time for each subline was calculated as described previously (15). The time to death for each of the tumor-bearing rats was recorded. At death, each animal underwent complete gross and microscopic autopsy; this permitted determination of the overall incidence and exact localization and distri-

bution of any metastases. From these data, the overall host survival time and metastatic ability of each subline was determined.

Androgen sensitivity of each subline was determined by separately inoculating 10 intact rats with 25-mg trocar pieces each of 5 different sublines and allowing the tumors to grow to 1 cu cm. During this initial growth to 1 cu cm, the volume-doubling times for each subline were determined as described above; when the tumor reached 1 cu cm, the host rats were castrated. If castration resulted in a decrease or cessation of the continuous growth of the tumor subline, then it was classified as androgen sensitive. If no change in tumor volume-doubling time occurred following castration, then the subline was classified as androgen insen-
sitive.

Where indicated, groups of animals were separately inoculated s.c. in the lower leg with 25-mg trocar pieces of each of the 4 anaplastic sublines. These primary tumors were allowed to grow for various times, and then one-half of each group was operated upon to remove the entire leg bearing the established primary tumor where it reached 0.5 to 1 cu cm in size. These leg amputations were performed as follows. Animals were anesthetized with Metafane (methoxyflurane; Pittman-Moore, Washington Crossing, NJ), and skin incisions made at the level of the hip; the leg muscle at the hip was then surgically divided with a scalpel, and an electric cautery was used to coagulate the vasculature to prevent bleeding; the femur was then disarticulated at the hip, and the entire leg was removed. Remnants of the gluteal superflcialis muscle were then sutured to the external abdominal muscle, and the skin was closed with metal wound clips.

Biochemical Studies. Each of the 5 Dunning tumor sublines described above was assayed for the quantitation of the specific activity of 9 different hydrolytic enzymes. This was done for each subline by rapidly removing individual tumors from rats at a time when they were 2 to 3 cu cm in total size (2 to 3 weeks post-tumor inoculation for the fast-growing anaplastic sublines and 150 days for the slow-growing H tumor). This time was chosen because the tumors were growing exponentially and had only minimal central tumor necrosis. Following removal, each tumor was rapidly divided into a series of aliquots of known tissue amounts which were then rapidly frozen and stored in liquid nitrogen. In preliminary studies, rapid freezing and storage in liquid nitrogen were found to have no effect (i.e., >10% change) on the subsequent assayability of the 9 enzymes studied when compared to the respective activities assayed in fresh tissue. By using frozen tissue, it was possible to collect all the various tumors and then subsequently to assay all of these tumors at one time for each activity as follows. Separate frozen aliquots from 3 different tumors for each of the 5 sublines were homogenized at a ratio of 1:10 (w/v) in 0.01 m phosphate buffer, pH 7.0, using an all-glass conical homogenizer. These homogenates were used to assay all 9 enzymes described below for each of the individual tumors of the respective tumor sublines. In addition, normal dorsolateral prostate, the normal tissue of origin for the original Dunning tumor (9), was also assayed for the 9 enzymatic activities. Since only about 200 mg of dorsolateral prostate are obtained from a single non-tumor-bearing Co-

penhagen rat, 20 Copenhagen rats were killed, and their dorsolateral prostates were pooled to give approximately 4 g of starting tissue. This pooled dorsolateral prostatic tissue was then divided into aliquots of known weight, rapidly frozen, and stored in liquid nitrogen. Three sepa-

rate aliquots of this pooled dorsolateral prostatic tissue were then homogenized and assayed simultaneously with the other tumor samples for each of the 9 enzymes described below.

All assays were standardized such that they were linear with respect to both time and tissue amount. Of the 9 enzyme assays performed for each tumor subline, 7 were assayed with fluorometric substrates ob-

tained from Vega Biochemicals (Tucson, AZ) using a Turner Model 112

fluorometer coupled to an Omniscure (Houston Instruments) chart re-
corder. The fluorometric assays were used for the determination of plasminogen activator, trypsin-like activity, chymotryptsin-like activity, elastase, cathepsin B (thiol protease), N-acetyl-β-glucosaminidase, and leucine aminopeptidase activities. Plasminogen activator was assayed using the synthetic fluorogenic substrate, α-N-carboxybenzoyl-γ-glycyglycyl-

l-arginine-4-methylcoumarinyl-7-amide, as described by Zimmerman et al. (43). The use of this synthetic substrate has been shown by Zimmer-

man et al. (43) to correlate well with the standard 125I-labeled fibrin plate assay. Trypsin-like activity was assayed using the synthetic fluorogenic substrate, α-N-carboxybenzoyl-l-arginine-4-methylcoumarinyl-7-amide accor-
ding to the method of Zimmerman et al. (42). Zimmerman et al. (42) have shown that this synthetic substrate is highly specific for trypsin-like enzymes with no detectable activity with chymotrypsin or elastase. Chymotrypsin-like activity was assayed using the synthetic fluorogenic substrate, glutaryl-β-phenylalanine-4-methylcoumarinyl-7-amide according to the method of Zimmerman et al. (42). Zimmerman et al. (42) have shown that this synthetic substrate is highly specific for chymotrypsin-like enzymes with no detectable activity with trypsin or elastase. Elastase was assayed using the synthetic fluorogenic substrate, methoxyacetyl-β-

l-alamyl-l-alamyl-l-prolyl-l-valine-4-methylcoumarinyl-7-amide, accord-
ing to the method of Castillo et al. (2). Castillo et al. (2) have shown that this substrate is highly specific for elastase, does not react with cathepsin G, and is only slowly hydrolyzed by chymotrypsin. Cathepsin B was assayed using the synthetic fluorogenic substrate l-arginyl-l-arginine-4-
methylcoumarinyl-7-amide in the assay of McDonald and Ellis (26). McDonald and Ellis (26) have shown that synthetic substrates with an arylamide bound to 2 arginyl groups instead of one are good substrates for cathepsin B but are much less active as substrates for trypsin and other trypsin-like enzymes. N-Acetyl-β-glucosaminidase was assayed using the synthetic fluorogenic substrate, 4-methylumbelliferyl-2-aceta-
mido-2-deoxy-β-D-glucopyranoside, according to the method of Lea-

back and Walker (22). Leucine aminopeptidase was assayed using the synthetic fluorogenic substrate, l-leucine-4-methylcoumarinyl-7-amide, according to the method of Saifuku et al. (33). Saifuku et al. (33) have shown that the values obtained with this fluorogenic substrate are well correlated with those obtained by the conventional l-leucine-β-naph-
thylamide method.

Collagenase activity was assayed using a commercially available kit from New England Nuclear (Boston, MA). This assay was performed according to manufacturer’s instructions and is based upon the method of Hu et al. (14) using collagen, 3H-labeled rat tail tendon type I, as the substrate. Activity was measured in cpm above background and expressed as tadpole collagenase equivalents according to kit instructions. Neutral protease activity was measured using methyl[14C]-α-casein (New England Nuclear) with α-casein (Sigma Chemical Co., St. Louis, MO) as the diluent. The methodology was as described by Reimeides and Klostermeyer (30). The activity was measured in cpm above background.

The data from each of the 9 enzymatic assays were initially expressed as the amount of product formed per min per g, wet weight, of tissue. However, preliminary studies demonstrated that there are substantial differences in the number of cells per g, wet weight, of tissue for each of the various tumor sublines. Therefore, in order to directly compare the quantitative levels of each enzymatic activity between the individual sublines, each of the activities was normalized on a per cell basis. This was done by dividing the activity per g, wet weight, of tissue by the number of cells per g of tissue. The number of cells per g for each of these tumors per respective sublines was individually determined by homogenizing an aliquot of known starting wet weight in 0.25 M sucrose: 1 mM MgCl₂; 0.1% Triton X-100 and then counting the number of total nuclei with the aid of a hemocytometer using a Zeiss phase-contrast microscope. Using this direct counting method, the number of cells per g (S.D.) weight was determined to be: (a) for the dorsolateral prostate, 2.1 ± 0.3 x 10⁶; (b) for the H tumor, 5.2 ± 0.7 x 10⁶; (c) for the AT-2 tumor, 4.2 ± 0.5 x 10⁶; (d) for the AT-3 tumor, 3.7 ± 0.6 x 10⁶; (e) for the MAT-LyLu tumor, 4.2 ± 0.5 x 10⁶; and (f) for the MAT-Lu tumor, 3.6 ± 0.4 x 10⁶. The validity of this direct counting method for determining the number of cells per g, wet weight, of tissue was preliminarily documented for each of the Dunning tumor sublines and the dorsolateral prostate using an indirect method based upon DNA analysis as follows: (a) aliquots from 3 separate tumors for each subline and 3 separate pooled tissue samples of the dorsolateral prostate were each homogenized in 0.25 M sucrose: 1 mM MgCl₂; 0.1% Triton X-100; (b) an aliquot of this initial homogenate was then treated with propidium iodide by the method of Vindekel (37) to stain the DNA of whole nuclei; (c) the propidium iodide-stained aliquot was then processed through a Becton-Dickinson fluorescence-activated cell sorter (FACS-II) programed using both perpendicular light scatter and propidium iodide fluorescence as discriminatory factors, to quantitate and collect whole cell nuclei; (d) since this machine counts the total number of nuclei collected during the separation, an aliquot of a known number of nuclei from the separation (i.e., approximately 5 x 10⁸ nuclei) was assayed for its total DNA content by the method of Coffey et al. (5) to determine the pg DNA per individual cell nuclei for each of the 3 samples for each tumor subline and the dorsolateral prostate [i.e., pg DNA/individual cell nuclei was determined to be: (a) for dorsolateral prostate, 8.7 ± 1.3; (b) for the H tumor, 10.5 ± 1.2; (c) for the AT-2 tumor, 12.1 ± 1.5; (d) for the AT-3 tumor, 16.7 ± 0.9; (e) for the MAT-LyLu tumor, 18.0 ± 1.1; and (f) for the MAT-Lu tumor, 15.7 ± 2.1]; (g) an aliquot of the original 0.25 M sucrose: 1 mM MgCl₂; 0.1% Triton X-100 homogenate, corresponding to a known wet weight of tissue for each sample, was then assayed for its DNA content to determine the total DNA/g, wet weight, for each sample [i.e., mg DNA/g, wet weight, of tissue was determined to be: (a) for dorsolateral prostate, 2.0 ± 0.2; (b) for the H tumor, 5.9 ± 0.6; (c) for the AT-2 tumor, 4.9 ± 1.0; (d) for the AT-3 tumor, 5.5 ± 0.6; (e) for the MAT-LyLu tumor, 7.4 ± 0.8; and (f) for the MAT-Lu tumor, 5.3 ± 0.2]; (f) dividing the total DNA content per g, wet weight, of tissue by the total DNA content per individual cell nuclei for each sample, the number of cells per g, wet weight, was determined for each of these separate tumors per tumor subline and for the dorsolateral prostate. By this indirect DNA method, the number of cells per g, wet weight, was determined to be: (a) for the dorsolateral prostate, 2.3 ± 0.4 x 10⁶; (b) for the H tumor, 5.6 ± 0.5 x 10⁶; (c) for the AT-2 tumor, 4.0 ± 0.8 x 10⁶; (d) for the AT-3 tumor, 3.3 ± 0.4 x 10⁶; (e) for the MAT-LyLu tumor, 4.8 ± 0.51 x 10⁶; and (f) for the MAT-Lu tumor, 3.4 ± 0.2 x 10⁸. These results demonstrated that there were no statistically significant differences between the number of cells per g, wet weight, of tissue determined using the direct counting method or the indirect method based upon DNA measurement for any of the tumor sublines or the dorsolateral prostate. Therefore, the direct counting method was used for routine analysis of the number of cells per g, wet weight, for each of the tumor samples used for enzymatic analysis.

**Statistical Analysis.** All data are presented as the mean ± S.E. Statistical analyses of significance were made by Student’s t test.

### RESULTS

**Characterization of the Sublines.** The parental R-3327 H tumor, from which the other sublines were developed, has been well described in the past (17). It is an androgen-sensitive, slow-growing tumor with a doubling time of 21 days (Table 1) and is histologically a uniform well-differentiated adenocarcinoma, being composed of prominent well-developed secretory acini surrounded by well-developed stromal elements (Fig. 1). In contrast to the H tumor, the other 4 Dunning sublines used in this study were all androgen insensitive, fast growing, and histologically anaplastic, being composed of sheets of tumor cells with no indication of glandular structure or secretory activity (Fig. 2).

Tumor volume-doubling times of the 4 anaplastic sublines varied from 1.5 to 1.7 days (Table 1), which are values 7 to 14 times faster than that of the H tumor. The greatly increased growth rates for each of the 4 anaplastic tumor sublines are directly reflected in the reduced survival time of the respective tumor-bearing hosts as compared to H tumor-bearing rats (Table 1).

The H tumor took approximately 1 year (352 ± 58 days) to kill its hosts animal. Chart 1 illustrates the survival curves for the 4 anaplastic tumors. The AT-2-bearing animals survived for approximately 2 months (63 ± 2.7 days), which was twice as long as for the animals bearing AT-3, MAT-Lu, and MAT-LyLu tumors. Indeed, following the inoculation of equal sized trocar pieces, all of the AT-3, MAT-LyLu, and MAT-Lu-bearing hosts were dead before any of the AT-2-bearing rats had died. The MAT-LyLu animals had the shortest survival time, only 25.8 ± 0.8 days (p < 0.01 as compared to each of the other sublines). The AT-3 and MAT-Lu animals survived for approximately 1 month, which was statistically different from the survival times of H-, AT-2-, and MAT-LyLu-bearing hosts (p < 0.01) but not from each other.

While each of the Dunning tumor sublines studied has the ability, when placed s.c. in the flank, to grow continuously between the skin and the abdominal muscle wall, eventually producing massive local tumors, only rarely do these tumors invade through the abdominal wall. In contrast to the rather similar abilities of the various sublines to expand locally, the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of Dunning R-3327 sublines used in the present study</th>
</tr>
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<tbody>
<tr>
<td><strong>Dunning R-3327</strong></td>
<td><strong>Histology</strong></td>
</tr>
<tr>
<td>H</td>
<td>Well differentiated</td>
</tr>
<tr>
<td>AT-2</td>
<td>Anaplastic</td>
</tr>
<tr>
<td>AT-3</td>
<td>Anaplastic</td>
</tr>
<tr>
<td>MAT-Lu</td>
<td>Anaplastic</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>Anaplastic</td>
</tr>
</tbody>
</table>

* Determined by changes in growth rate of 1-cm established tumors, growing in intact rats, to subsequent castration.

** Day post-s.c. inoculation with 25-mg trocar pieces of respective tumor.

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Predicting Metastatic Ability of Prostatic Cancer

Two of the anaplastic sublines were previously named to reflect their characteristic patterns of metastasis: Metastatic Anaplastic Tumor able to disseminate to Lung [MAT-Lu (21)] or to Lymph nodes and Lung [MAT-LyLu (18)]. The MAT-Lu tumors metastasized 100% of the time and only to the lungs; no lymph node metastases were ever found. The MAT-LyLu tumor also metastasized 100% of the time, with 94% of the animals having the characteristic pattern of both axillary lymph node and lung metastases. The AT-3 tumor had a distribution pattern of metastases similar to that of the MAT-LyLu; 87.5% of the animals had both axillary lymph node and lung metastases.

In order to determine how early in the course of the growth of the primary tumor metastasis is occurring for each of the anaplastic sublines, the following experiment was performed. Ten animals were separately inoculated s.c. in the lower leg with 25-mg trocar pieces of each of the AT-2, AT-3, MAT-Lu, and MAT-LyLu tumors. Half of the animals of each tumor group were allowed to go until death with the exception of the surgically treated AT-2-bearing rats, which were killed 150 days postsurgery. At death, each rat was autopsied for the presence of metastasis. The results of these experiments are presented in Table 3, and they illustrate that, even if the primary prostatic cancer is completely removed as early as 13 days post-tumor inoculation, the AT-3, MAT-LyLu, and MAT-Lu tumors have already metastasized. In fact, for these 3 highly metastatic sublines, surgical removal of primary cancer sites does not substantially increase overall survival as compared to their respective surgically untreated controls. These results demonstrate that, for these highly metastatic lines, it is the metastases which kill the host animals, not the primary tumor. In direct contrast to the situation for the highly metastatic anaplastic sublines, early surgical removal of the primary low-metastatic AT-2 tumor produced cures in all rats, demonstrating that it is the growth of the primary tumor alone which leads to death in AT-2-bearing animals. These results demonstrate that the AT-2 tumor is biologically quite different from the other highly meta-

Table 2
Table 3

<table>
<thead>
<tr>
<th>Anaplastic Dunning R-3327 sublines</th>
<th>No. of tumor-bearing rats autopsied</th>
<th>% with metastasis at death</th>
<th>% with lung metastases only at death</th>
<th>% of axillary lymph node metastasis only at death</th>
<th>% with both axillary lymph node and lung metastasis at death</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-2</td>
<td>13 (22)</td>
<td>15.4</td>
<td>15.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AT-3</td>
<td>24</td>
<td>100</td>
<td>0</td>
<td>12.5</td>
<td>87.5</td>
</tr>
<tr>
<td>MAT-Lu</td>
<td>19</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>16</td>
<td>100</td>
<td>100</td>
<td>6.3</td>
<td>93.7</td>
</tr>
</tbody>
</table>

* Nine animals were sacrificed at Day 42; none had metastasis, 13 were followed until death.

Various sublines differ greatly in their ability to produce distant metastases. The H tumor very rarely metastasizes; during the last 6 years, over 500 animals have been inoculated with the H tumor, but only 2 of these rats developed distant metastasis. Both of these H tumor metastases were to the lungs. Therefore, the H tumor is essentially a nonmetastatic tumor. The AT-2 tumor also has a low metastatic ability, only 15%, compared to 100% for the other 3 anaplastic lines. This low metastatic ability is the major differentiating factor for the AT-2 tumor as compared to the other anaplastic AT-3, MAT-Lu, and MAT-LyLu tumors. The organ site distribution of metastasis was determined for each of the anaplastic sublines (Table 2). The AT-2 tumor, when it did metastasize, did so exclusively to the lungs and only very late in its growth as compared to the other anaplastic lines. This was illustrated by the following experiment. At 42 days post-tumor implantation, a time when all the animals implanted with the other anaplastic sublines had already died with distant metastases (Chart 1), 9 AT-2-bearing rats were autopsied, and none had any evidence of metastases either macroscopically or microscopically. The occasional metastasis of the AT-2 tumor occurred only in animals whose primary tumor weighed more than 150 gm, a weight equal to that of the hosts.

<table>
<thead>
<tr>
<th>Tumor sublines inoculated into the leg</th>
<th>Survival (days post-tumor inoculation)</th>
<th>% with metastases</th>
<th>Complete surgical removal of primary tumor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-2</td>
<td>63 ± 3 (a)</td>
<td>10</td>
<td>Curved</td>
</tr>
<tr>
<td>AT-3</td>
<td>32 ± 1</td>
<td>100</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>MAT-Lu</td>
<td>35 ± 1</td>
<td>100</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>28 ± 1</td>
<td>100</td>
<td>31 ± 2</td>
</tr>
</tbody>
</table>

* Legs bearing established 0.5- to 1-cm primary tumors were removed at Day 15 postinoculation for the AT-2 group and at Day 13 for the AT-3, MAT-Lu, and MAT-LyLu groups.  
1. Mean ± S.E.  
2. Rats were allowed to live 150 days postsurgery before being sacrificed and autopsied. At this time, there was no indication of any tumor.

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static anaplastic sublines. These results also demonstrate that for the AT-3, MAT-Lu, and MAT-LyLu tumors metastasis is an early event in their growth occurring before the tumors reach 1 cm.

**Biochemical Analysis.** In an attempt to explain the biological differences in metastatic ability between the various Dunning sublines, each of the sublines was assayed to determine the quantitative levels of 9 different tissue-degradative enzymes, each of which has been associated in other studies with increased malignancy and metastatic potential. Dorsolateral prostate, the tissue of origin of the R-3327 tumors, was also assayed for comparison. Tumors between 2 and 3 cm were used for these enzymatic analyses. This size tumor was selected for several reasons. Primarily, because the leg amputation experiments had shown that the highly metastatic sublines (AT-3, MAT-LyLu, and MAT-Lu) had already developed metastases by the time they were less than 1 cm, while no metastases had occurred by this time for the AT-2 tumor. In addition, at this size, the tumors were still on the exponential part of their growth curves, as well as having only minimal central necrosis. Also, histological examination of the tumors when they were 2 to 3 cm (e.g., Figs. 1 and 2) revealed that there was little, if any, infiltration of the tumors by host lymphocytes or macrophages. This is important since several normal host cell types, particularly macrophages, are known to produce many of the hydrolytic enzymes assayed. This complication was thus minimized by using 2- to 3-cm tumors. The results of these comparative biochemical studies are presented in Table 4; these data demonstrate that the elevation of hydrolytic enzymatic activities is not a generalized characteristic of these cancers. Indeed, the activities of all 9 of the hydrolytic enzymes were consistently higher in the dorsolateral prostate than in any of the 5 Dunning sublines studied. In fact, the activities of 5 of the 9 hydrolytic enzymes (collagenase, trypsin-like, cathepsin B, neutral protease, and N-acetyl-β-glucosaminidase) were statistically higher in the dorsolateral prostate than in any of the 5 Dunning sublines studied.

Of the 9 enzymes assayed, only elastase activity was consistently increased (p < 0.05) in all the highly metastatic sublines compared to either the normal dorsolateral prostate or the low-metastatic H and AT-2 tumors. For all the highly metastatic sublines, elastase was increased between 3- and 6-fold as compared to the normal dorsolateral tumor, 7- to 17-fold compared to the parental H tumor, and 2.5- to 5.5-fold compared to the AT-2 tumor. In addition, the chymotrypsin-like activity of the 3 highly metastatic anaplastic sublines was also statistically increased (p < 0.05) 1.4- to 1.7-fold compared to the dorsolateral prostate or the H tumor. The chymotrypsin-like activity of the 3 highly metastatic anaplastic sublines was also 1.3 to 1.5 greater than that of the low-metastatic AT-2 tumor. Interestingly, plasminogen activator activity was significantly increased (p < 0.01) by 3.3-fold in the AT-2 tumor as compared to normal prostate. No other subline had any increase in this activity. Indeed, in the other sublines, plasminogen activator levels were statistically decreased (p < 0.05) to between 13 and 75% of the value found for the dorsolateral prostate. The activity of N-acetyl-β-glucosaminidase was also consistently statistically lower in all 5 of the tumor sublines compared to the normal prostate. In addition, all of the highly metastatic anaplastic sublines had statistically lower (p < 0.05) levels of this activity than did the low-metastatic AT-2 tumor; however, the low-metastatic H tumor was also statistically lower than the AT-2 tumor. The collagenase, trypsin-like, cathepsin B, and neutral protease activities were all lower for all 5 sublines compared to the normal prostate. Besides this general decrease, the only trend within the tumor sublines for these 4 activities was a tendency for the anaplastic sublines, as a group, to have higher levels of these activities than does the H tumor. The level of leucine aminopeptidase activity was also not consistently associated with metastatic ability. The level of activity for this enzyme was significantly (p < 0.05) increased in the AT-2, AT-3, and MAT-Lu but not in either the MAT-LyLu or H tumors.

**Development of a Biochemical Metastatic Index.** In an earlier study using a variety of Dunning tumor sublines, it was found that, by assaying each subline for a series of specific enzyme activities and then mathematically combining the relative levels of those activities, it was possible to construct an enzyme index which accurately predicted the respective androgen sensitivity of each subline (16). Therefore, a similar approach was undertaken to construct an enzyme index which would accurately predict metastatic ability using several of the hydrolytic activities presented in Table 4.

As a preliminary step in constructing such a biochemical metastatic index, every activity for each individual tumor was converted to a relative activity value. Relative activities for each enzyme were calculated by dividing each absolute activity by the mean absolute activity for the corresponding enzyme in the

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**Table 4**

Hydrolytic activities of various tissues

<table>
<thead>
<tr>
<th>Hydrolytic activities assayed</th>
<th>Normal dorsolateral prostate</th>
<th>Low metastatic</th>
<th>High metastatic</th>
<th>Enzyme units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elasticase</td>
<td>23 ± 4</td>
<td>9 ± 4</td>
<td>28 ± 6</td>
<td>154 ± 21</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>45 ± 4</td>
<td>46 ± 5</td>
<td>50 ± 8</td>
<td>77 ± 12</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>46 ± 4</td>
<td>6 ± 2</td>
<td>154 ± 31</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>238 ± 7</td>
<td>58 ± 4</td>
<td>104 ± 8</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>Collagenase</td>
<td>1033 ± 94</td>
<td>252 ± 7</td>
<td>506 ± 16</td>
<td>512 ± 72</td>
</tr>
<tr>
<td>Trypsin</td>
<td>14.5 ± 2.0</td>
<td>1.9 ± 1.6</td>
<td>4.2 ± 0.2</td>
<td>3.7 ± 1.6</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>46 ± 5</td>
<td>9 ± 1.5</td>
<td>17 ± 4.2</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>Neutral protease</td>
<td>6.5 ± 0.4</td>
<td>1.0 ± 0.5</td>
<td>5.2 ± 1.8</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>437 ± 19</td>
<td>387 ± 113</td>
<td>677 ± 139</td>
<td>767 ± 134</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Statistically lower (p < 0.05) compared to dorsolateral prostate.
* Statistically higher (p < 0.05) compared to dorsolateral prostate.
* a, pmol/min/10⁶ cells; b, pg tadpole collagenase equivalents/10⁶ cells; c, cpm/min/10⁶ cells.

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dorsolateral prostate. Thus, all activities were normalized to the dorsolateral prostate (mean activity of each enzyme in the dorsolateral equals 1). Various mathematical combinations of these relative activity (RA) values were used to construct empirically a series of indices, each of which was then individually tested for its respective ability to reflect the known metastatic ability of the various sublines. The index which best reflected this ability was found to be defined by the equation

\[
\text{RA (elastase)} \times \text{RA (chymotrypsin)} \\
\text{RA (N-acetyl-\beta-glucosaminidase)}
\]

Using this biochemical metastatic index as a discriminatory factor, one can clearly separate the nonmetastatic H and the low-metastatic AT-2 tumors from the highly metastatic AT-3, MAT-Lu, and MAT-LyLu tumors (Table 5).

**DISCUSSION**

The purpose of the present study was to determine if biochemical parameters could be used to predict the metastatic ability of individual prostate cancers. As a model system to examine this question, the Dunning R-3327 rat prostatic adenocarcinoma system of serially transplantable sublines was utilized, since among these sublines there is a wide range of metastatic ability. In particular, 4 R-3327 anaplastic sublines (AT-2, AT-3, MAT-Lu, and MAT-LyLu) were selected for detailed study because, while there were marked differences among these sublines with regard to their respective metastatic ability, each of these sublines has a similar histology, androgen insensitivity, and rapid growth rate.

Previous studies have demonstrated that elevation in various single hydrolytic enzyme activities (e.g., neutral protease, collagenase, elastase, cathepsin B, plasminogen activator, and N-acetyl-\(\beta\)-glucosaminidase) are correlated with increased metastatic ability in other types of cancer (7, 8, 13, 19, 20, 23-25, 28, 34-36, 40). In order to examine if elevations of these same or other hydrolytic enzymes (e.g., chymotrypsin-like activity, trypsin-like activity, and leucine aminopeptidase) are correlated with increased metastatic ability for prostate cancer, these 9 hydrolytic enzymes were determined and compared for the various Dunning R-3327 prostatic cancer sublines.

These studies demonstrated that elevated levels of hydrolytic enzyme activities are not a general characteristic of all prostatic cancers. For example, the activity levels of 5 of the 9 hydrolytic enzymes assayed were lower in all prostatic tumor sublines than in the normal dorsolateral prostate. In addition, the levels of the remaining 4 hydrolytic enzymes were always lower in the H tumor than in normal prostate. Elevated levels of elastase were, however, a consistent marker differentiating (\(p < 0.05\)) the highly metastatic tumors (AT-3, MAT-Lu, MAT-LyLu) from the nonmetastatic H tumor and the low-metastatic AT-2 tumor. However, elevation of elastase activity did not separate the AT-2 and H from the normal dorsolateral prostate. Chymotrypsin-like activity also demonstrated this same consistent trend of increasing hydrolytic activity with increasing metastatic ability, although with a smaller absolute increase in magnitude than that for elastase.

Since the levels of activities of neither elastase nor chymotrypsin-like activity alone provided a completely consistent and continuous discriminatory factor going from the normal prostate to the nonmetastatic H tumor to the low-metastatic AT-2 tumor and then to the highly metastatic AT-3, MAT-Lu, and MAT-LyLu tumors, these activities were mathematically combined with the activities for N-acetyl-\(\beta\)-glucosaminidase to create a biochemical metastatic index. This index did provide a consistent discriminator for metastatic ability. An advantage of this biochemical metastatic index approach is that it could be scaled down and applied to needle biopsy samples and multiple assays could be performed with a high degree of reliability. Further studies are needed to validate this approach for human prostatic cancers.

Conceivably, a biochemical analysis, such as the biochemical metastatic index, could be used on biopsy specimens to differentiate accurately and prospectively those cancers that will metastasize from those with low metastatic potential. This could be helpful in determining the treatment needs for those patients with Stage A2, B1, B2, and C prostate cancer. Walsh and Jewett (38) demonstrated that radical prostatectomy can cure Stage B prostate cancer. In those patients, standard pathological analysis had been unable to predict accurately the good responders from the poor responders; however, Diamond et al. (6) recently reported that quantitative pathological analysis for the nuclear roundness factor could accurately predict retrospectively the good responders to radical prostatectomy. Recent studies by Benson et al. (1) have shown that flow cytometric analysis of cell and nuclear size and shape can be useful to predict tumor aggressiveness in the Dunning model system. It is conceivable that the biochemical metastatic index and nuclear roundness factor and/or cell cytometric analysis might be used in concert to increase the discriminatory value of any prognostic indicators.

**REFERENCES**

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Fig. 1. Histology of the parental R-3327-H tumor. a, × 100; b, × 1000.
Fig. 2. Histology of the various anaplastic Dunning R-3327 sublines. a, AT-2 tumor; b, AT-3 tumor; c, MAT-Lu tumor; d, MAT-LyLu tumor. × 1000.
Biochemical Methods for Predicting Metastatic Ability of Prostatic Cancer Utilizing the Dunning R-3327 Rat Prostatic Adenocarcinoma System as a Model

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