Genetic Instability Coupled to Clonal Selection as a Mechanism for Tumor Progression in the Dunning R-3327 Rat Prostatic Adenocarcinoma System

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ABSTRACT

The androgen-sensitive Dunning R-3327-H prostatic adenocarcinoma has been maintained by continuous serial passage in intact male rats for many years. While it has been possible to maintain the original characteristics of the well-differentiated H tumor over 16 years, there have evolved spontaneously, however, more aberrant sublines from this tumor at several subpassages in intact male rats. Serial passage of these individual sublines has established five additional R-3327 tumors each with distinct phenotypes and each more aberrant than the parent H tumor. In addition, it has been possible by passage of the H tumor in castrated male rats to obtain a well-differentiated slow-growing androgen-insensitive tumor termed the HI-S tumor. The continuous serial passage of this HI-S tumor has likewise resulted in the emergence of three new types of Dunning tumors. The results from the biochemical and chromosomal studies presented demonstrate that there is a consistent association in each of these tumor progressions between the expression of genetic instability, which results in the addition of phenotypically new clones of cells to the tumor population, and the subsequent selection of these newly developed clones. These results suggest that the process of genetic instability coupled to clonal selection is one mechanism for the change in tumor phenotype characteristically associated with tumor progression within this system of prostatic tumors.

INTRODUCTION

In 1961, Dunning (3) observed at necropsy a tumor of the dorsal lobe of the prostate of a 22-month-old male Copenhagen rat. Pathological examination of this spontaneous primary tumor revealed a well-differentiated prostatic adenocarcinoma composed of distinct well-formed acini and glandular formations, including secretory material, corresponding to the dorsal type lobe of the rat prostate. Fortunately, Dr. Dunning was successful in serially passaging the original tumor s.c. into both pure Copenhagen and the Fischer × Copenhagen F1 rats. This serially transplantable tumor, originally termed the R-3327, was further shown by Drs. Voigt and Dunning to be a slow-growing, androgen-sensitive, well-differentiated adenocarcinoma having both the high-affinity androgen-specific receptor and the ability via 5α-reductase to metabolize testosterone to dihydrotestosterone (18, 19).

This parent Dunning tumor received the suffix -H (R-3327-H) to denote its hormone sensitivity and has been shown to be an important model for human prostatic cancer since it mimics many of the properties of the human disease (8). One of its important similarities is its response to androgen ablation. If intact adult male rats bearing an exponentially slow-growing (20-day tumor-doubling time) H tumor are castrated, the tumor appears to stop growing for 50 to 60 days. This initial response to castration is inevitably followed by a subsequent relapse in which the tumor again demonstrates growth. In this regard, the H tumor mimics very closely the clinical situation in Stage D human prostatic cancer, where also the initial response to androgen ablation therapy is almost universally followed by subsequent relapse that is resistant to hormonal control. The relapse of the H tumor to androgen ablation has been demonstrated to be dependent upon the initial heterogeneity of the H tumor; the tumor is composed of a mixture of preexisting clones of both androgen-dependent and -independent tumor cells (7). Following castration, only the androgen-independent tumor cells continue to proliferate; the androgen-dependent tumor cells die. It has been possible, therefore, to clone in vivo by passage in castrated male hosts the androgen-independent tumor cells from the heterogeneous H tumor. These well-differentiated androgen-independent tumor cells, once cloned, are completely androgen insensitive, having an identically slow growth rate (20-day tumor-doubling time) in both intact and castrated male hosts. The hormone-insensitive tumor, which the androgen-independent tumor cells produce, has been termed the R-3327 HI-S tumor. This HI-S tumor, while being distinguishable by its lack of androgen dependence for growth, is phenotypically, however, very similar to the parent androgen-sensitive H tumor. Both H and HI-S tumors possess androgen receptor, 5α-reductase, and prostatic-specific secretory acid phosphatase, and both tumors are slow growing and well differentiated (9).

Since 1975, the androgen-sensitive H tumor has been maintained at Johns Hopkins by continuous serial passage in intact male rats. While it has been possible to maintain the original characteristics of the H tumor over this period, there have evolved spontaneously, however, more aberrant sublines from the tumor at several subpassages. Subsequent serial passage of these individual sublines has allowed the development of 5 additional R-3327 tumors each with distinct biological characteristics and each more aberrant than the parent H tumor. The continuous serial passage of the androgen-insensitive HI-S tumor over the last 2 years likewise has resulted in the emergence of 3 new, more aberrant types of Dunning tumors. The present study, therefore, was undertaken to examine the mechanism(s) responsible for the progression of prostatic cancer wherein a tumor continuously evolves to an ever-increasingly aberrant phenotype using the H and HI-S tumor as prototypes.
tumor cells are inoculated into castrated male rats, a tumor differentiated prostatic adenocarcinoma (Fig. 1). The growth of as described previously (8). The DMA content per cell was determined previously (7, 8). Whole homogenate 5α-reductase assays were performed by assaying an aliquot of whole homogenate for total DMA by the method of Coffey et al. (2) and counting the total number of nuclei by determining the tumor volume-doubling time are as reported previ-ously (7, 8). Inson FACS-II instrument according to the technique of Vindelov (17). By division of the total DMA by the total number of nuclei present in an identical homogenate aliquot with the aid of a hemocy-tometer. By division of the total DNA by the total number of nuclei present, the pg of DNA per nucleus were determined for all tumor sublines. Flow cytometric analysis was performed on a Becton Dick-inson FACS-II instrument according to the technique of Vindelov (17). Chromosomal number and karyotype analysis was performed by sequen-tially staining cells with conventional Giemsa and then quinacrine mustard (Q-banding) according to the method of Wake et al. (20).

RESULTS

Spontaneous Progression of the H Tumor. When 1.5 x 10⁶ viable H tumor cells are injected s.c. into intact adult male rats, a tumor becomes palpable after 40 to 50 days. It requires 135 ± 16 (S.E.) days for such s.c. H tumor cell inoculations to produce 1-cu cm tumors. During the exponential phase of the H tumor growth in intact males, the tumor volume doubles with a constant 21 ± 6-day period. Histological examination of the H tumor during its exponential growth reveals a uniformly well-differentiated prostatic adenocarcinoma (Fig. 1). The growth of the H tumor is androgen sensitive as demonstrated by its response to castration. If a similar batch of 1.5 x 10⁶ viable H tumor cells are inoculated into castrated male rats, a tumor does not become palpable until 90 to 100 days and does not reach the 1-cu cm size until 240 ± 25 days. In addition, if 1.5 x 10⁶ viable H cells are inoculated into intact male rats, the tumor is allowed to grow exponentially for 150 days, and then the rats are castrated, the tumor abruptly stops its exponential growth. For 50 to 60 days, the tumor does not increase in size.

DNA analysis of the H tumor, growing in intact males, reveals that the DNA content per cell for the H tumor is identical with that of the normal adult dorsal prostate, which is the tissue of origin for the original spontaneous R-3327 tumor. Both H tumor and the normal dorsal prostatic cells have a diploid amount of DNA (Table 1). In addition to the biochemical analysis, flow cytometric analysis of the H tumor revealed a single G₁ peak with a channel number identical to that observed with normal prostatic tissue (Chart 1). Chromosomal studies further revealed that the modal number of chromosomes for the H tumor is diploid at 42 (Chart 2). Further analysis demonstrated that none of the cells from the H tumor which were karyotyped had any demonstrable chromosomal abnormalities.³

In order to maintain over the years the original characteristics of the H tumor (i.e., its androgen sensitivity and slow growth rate), it has been routinely necessary to constantly passage only the slowest growing H tumor from each passage. If this is not done, tumors with increasing growth rates are eventually obtained. Recently, we have taken advantage of this fact to establish a new fast-growing subline from the parent slow-growing H tumor. By passage of the fastest-growing H tumor from an initial transplantation of the slow-growing H tumor and then by continuous selection of only the fastest-growing tumor produced for each subsequent passage, it has been possible to develop within 10 passages, a tumor which now has a doubling time of 5.4 ± 2 days as opposed to 21 days. This tumor, termed H-F, is histologically less well differentiated than the parent slow-growing H tumor, having less tumor stroma and smaller tumor acini (Fig. 2). The H-F tumor is, however, still a moderately well-differentiated tumor with no areas of anaplastic cells. Biochemically, the H-F is also less differentiated in that the level of 5α-reductase in this tumor is less than one-fifth that seen in the H tumor (Table 1). In addition to these histological and biochemical changes, the H-F is no longer androgen sensitive; it grows equally well in intact or castrated male rats. This loss of androgen sensitivity occurred even though the tumor was always passaged in intact male rats.

Chromosomal analysis of H-F tumor cells revealed that this tumor has a diploid modal chromosomal number of 42 with a

### Table 1

Comparative summary of biological characteristics of normal dorsal prostate and Dunning tumor sublines

| R-3327 tumor sub-lines | Histology               | Growth rate (tumor-doubling time (days)) | Androgen sensitivity | Metastatic potential | DNA content/cell (pg/cell) | 5α-Reductase activity
<table>
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<tr>
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<tbody>
<tr>
<td>Normal adult prostate</td>
<td>Well-differentiated</td>
<td>Static</td>
<td>Yes</td>
<td>Metastatic</td>
<td>8.7 ± 1.3</td>
<td>85.5 ± 8.5</td>
</tr>
<tr>
<td>H tumor</td>
<td>Well-differentiated</td>
<td>21 ± 6</td>
<td>Yes</td>
<td>Low</td>
<td>10.0 ± 2.1</td>
<td>17.0 ± 5.4</td>
</tr>
<tr>
<td>H tumor derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-F</td>
<td>Moderately well-differentiated</td>
<td>5.4 ± 2.0</td>
<td>No</td>
<td>Low</td>
<td>8.9 ± 1.3</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>AT-1</td>
<td>Anaplastic</td>
<td>2.5 ± 0.2</td>
<td>No</td>
<td>Low</td>
<td>15.5 ± 1.0</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>Anaplastic</td>
<td>1.5 ± 0.1</td>
<td>No</td>
<td>High</td>
<td>14.3 ± 1.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MAT-Lu</td>
<td>Anaplastic</td>
<td>2.7 ± 0.3</td>
<td>No</td>
<td>High</td>
<td>14.5 ± 1.5</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>AT-2</td>
<td>Anaplastic</td>
<td>2.5 ± 0.2</td>
<td>No</td>
<td>Low to moderate</td>
<td>16.1 ± 2.5</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>HI-S</td>
<td>Well-differentiated</td>
<td>24 ± 5</td>
<td>No</td>
<td>Low</td>
<td>9.5 ± 0.5</td>
<td>12.1 ± 3.0</td>
</tr>
<tr>
<td>HI-S tumor derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HI-M</td>
<td>Moderately well-differentiated</td>
<td>9.0 ± 0.8</td>
<td>No</td>
<td>Low</td>
<td>9.3 ± 1.3</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>HI-F</td>
<td>Moderately well-differentiated</td>
<td>4.8 ± 1.8</td>
<td>No</td>
<td>Low</td>
<td>8.5 ± 1.9</td>
<td>1.5 ± 0.4</td>
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<tr>
<td>AT-3</td>
<td>Anaplastic</td>
<td>1.8 ± 0.2</td>
<td>No</td>
<td>High</td>
<td>13.8 ± 0.5</td>
<td>1.2 ± 0.1</td>
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³ N. Wake, J. T. Isaacs, and A. A. Sandberg. Chromosomal changes associated with the progression of the Dunning R-3327 rat prostatic adenocarcinoma system, manuscript in preparation.

MATERIALS AND METHODS

The H tumor was originally obtained from the Mason Research Institute. All Dunning tumors described in this paper are passaged in adult male syngeneic Fischer × Copenhagen F₁ rats (>60 days old). Surgical treatment, methods of serial s.c. tumor transplantation, and calculation of the tumor volume-doubling time are as reported previ-ously (7, 8). Whole homogenate 5α-reductase assays were performed as described previously (8). The DNA content per cell was determined by assaying an aliquot of whole homogenate for total DNA by the method of Coffey et al. (2) and counting the total number of nuclei present in an identical homogenate aliquot with the aid of a hemocy-tometer. By division of the total DNA by the total number of nuclei present, the pg of DNA per nucleus were determined for all tumor sublines. Flow cytometric analysis was performed on a Becton Dick-inson FACS-II instrument according to the technique of Vindelov (17). Chromosomal number and karyotype analysis was performed by sequen-tially staining cells with conventional Giemsa and then quinacrine mustard (Q-banding) according to the method of Wake et al. (20).

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Mechanisms for Tumor Progression

Chart 1. DNA histograms of normal dorsal prostate, H tumor, and HI-S tumor as determined by flow cytofluorometry. Each histogram is based upon analysis of about 50,000 cells. Ordinate, cells/channel; abscissa, channel number (relative fluorescence intensity). 2C and 4C, diploid and tetraploid DNA content, respectively.

Table 1. DNA histograms of normal dorsal prostate, H tumor, and HI-S tumor as determined by flow cytofluorometry. Each histogram is based upon analysis of about 50,000 cells. Ordinate, cells/channel; abscissa, channel number (relative fluorescence intensity). 2C and 4C, diploid and tetraploid DNA content, respectively.

Chart 2. Chromosomal number histogram of the H, H-F, MAT-LyLu, MAT-Lu, AT-2 (fifth passage), and AT-2 (tenth passage) tumors. The following number of cells was examined for each tumor: H, 95; H-F, 79; MAT-LyLu, 54; MAT-Lu, 39; AT-2 (fifth passage), 86; and AT-2 (tenth passage), 37.

Anaplastic cells (Fig. 3). When such heterogeneous tumors were passaged, the subsequent tumors uniformly became palpable as early as 10 days after inoculation instead of the usual 40- to 50-day period for normal H tumors. These unusually fast-growing tumors often grew to the size of the host rat within 60 days. Histological examination of these unusually fast-growing tumors revealed uniformly anaplastic tumors with no indication of any areas of well-differentiated tumor cells. These fast-growing anaplastic tumors were thus termed AT tumors.

The first of these anaplastic tumors to be maintained in serial passage was termed the AT-1 tumor. Once developed, the AT-1 was completely androgen insensitive with regard to its growth; it grew equally well in intact or castrated male hosts with a tumor volume-doubling time of 2.5 ± 0.2 days. This is a growth rate 9-fold faster than the parent H tumor. In the earlier serial passages, the AT-1 tumor had a very low rate of distant metastases (<5%). The average survival time of 51 ± 6 days following injection of 1.5 x 10⁶ viable AT-1 tumor cells into intact male rats, however, is less than one-seventh of the total survival time found for male rats inoculated with H tumor cells at a comparable dose (mean H tumor survival time, 352 ± 58 days) demonstrating the increased malignancy of the AT-1 tumor. Biochemically, as well as histologically, the AT-1 tumor was also much less differentiated than was the parent H tumor. The AT-1 tumor had no detectable levels of either high-affinity androgen-specific receptor or prostatic-specific acid phosphatase, and the levels of 5α-reductase were only about 10% of those seen for the H tumor (Table 1).

After 60 continuous serial passages in the Hopkins laboratories, the AT-1 tumor eventually became highly metastatic, spreading to both the lymph nodes and lungs in nearly 100% of all inoculated animals (10). This tumor was thus termed the MAT-LyLu tumor to denote its metastatic site specificity. Continuous serial passage of the AT-1 tumor in Dr. W. D. W. Heston’s laboratory at Washington University, St. Louis, Mo., also eventually led to a highly metastatic tumor; however, this

range of 34 to 45 (Chart 2). Further analysis demonstrated that greater than 75% of cells from the H-F tumor which were karyotyped had marker chromosomes. All of these abnormal cells had lost the Y and one of the No. 1 chromosomes, and had a t(4;7) and t(1;2) translocation. None of these types of chromosomal abnormalities were observed in karyotypes from the parent slow-growing H tumor, confirming that the progression of the H tumor to the less-differentiated fast-growing HF tumor is associated with definitive chromosomal changes.

In contrast to progression of the slow-growing H tumor to the fast-growing HF tumor which developed gradually over several passages, additional progressions have occurred within a single passage of the H tumor. At several distinct passages during the last 6 years, a few random H tumors growing in intact males began to grow at rates 8 to 10 times faster than normal. In these animals, the tumor volume-doubling times increased from approximately 20 to less than 3 days. Histological examination of these unusually fast-growing tumors at a time when they are still less than 5 cm revealed heterogeneous tumors composed of distinct areas of well-differentiated glandular acini and areas of poorly differentiated
tumor metastasizes almost exclusively to the lung and was thus termed the MAT-Lu tumor (11). Unfortunately, the original AT-1 before it became highly metastatic was not karyotyped. Direct analysis of the DNA content per cell, determined by biochemical assay, did reveal, however, that the AT tumor before becoming highly metastatic was not diploid but polyploid (Table 1). Chromosomal analysis performed on the MAT-LyLu and MAT-Lu tumors demonstrated that both of these anaplastic tumors are hypotetraploid. For the MAT-LyLu tumor, the modal chromosomal number is 66 with a range of 51 to 71 (Chart 2); in addition, there were numerous chromosomal markers including loss and gain of chromosomes and structural abnormalities. The consistent abnormalities were loss of the X chromosomes and 5 kinds of structural abnormalities which include a t(1;4) and t(4;X) translocation. For the MAT-Lu tumor, the modal chromosomal number is 67 to 69 with a range of 61 to 72 (Chart 2); again, there was both gain and loss of chromosomes and chromosomal structural abnormalities. The consistent abnormalities for the MAT-Lu tumor were the loss of a Y chromosome and 5 kinds of structural rearrangements including the t(4;7) translocation also seen in the HF tumor. There is no common karyotypic abnormality characterizing the MAT-LyLu and MAT-Lu tumors, however, other than the facts that both tumors are hypotetraploid and both tumors are chromosomally distinct from the slow-growing H tumor.

During the last year, an additional anaplastic tumor spontaneously developed within one passage of the slow-growing H tumor. This anaplastic tumor too was completely androgen insensitive, even though it was always passaged in intact male rats. This anaplastic tumor, termed AT-2 to distinguish it from the previous AT-1 tumor, like the latter tumor was fast growing (doubling time, 2.2 ± 0.2 days) and in its early passage had a low metastatic potential (<5%). Flow cytometric analysis of the AT-2 tumor, in its fifth serial passage in intact male rats, demonstrated cells of both diploid (2C) and tetraploid (4C) amounts of DNA (Chart 3). If the AT-2 tumor cells are cultured in vitro, however, only the tetraploid (4C) cells are obtained, suggesting that the diploid (2C) component of the AT-2 tumors seen in vivo are non-tumor-supporting cells. This is further supported by the fact that the tetraploid AT-2 cells grown in culture, when inoculated back into rats, produce tumors with characteristics identical with those of the noncultured AT tumors (e.g., identical histology, growth rates, etc.). In addition, chromosomal analysis of the AT tumor at its fifth passage demonstrated the tumor to be tetraploid with a modal chromosomal number of 84 and a range from 77 to 85 (Chart 2). Karyotypes of cells from the fifth passage showed tetrasomy (4 chromosomes) in almost all chromosomal groups, although deviation from tetrasomy (gain or loss) was observed in a few chromosomes; in addition, there were 2 unidentified minute chromosomes. These results demonstrate that the H → AT-2 tumor progression, like all the previously described H tumor progressions, was clearly associated with definitive chromosomal changes. Further chromosomal changes continued to develop with subsequent serial passage of the AT-2 tumor. By its tenth passage, aneuploidization from tetraploidy (i.e., loss of chromosomes) and the development of structural abnormalities were observed in the AT-2 tumor; the modal chromosomal number decreased to a value of 75 with a range of 67 to 80 (Chart 2).3

**Spontaneous Progression of the HI-S Tumor.** The H tumor is heterogeneously composed of both androgen-dependent and androgen-independent, slow-growing, well-differentiated tumor cells (7). By serial passage of the H tumor in castrated male rats, it has been possible to select in vivo for only the androgen-insensitive, slow-growing clone of tumor cells. The tumor which such selection produced is termed the HI-S tumor, and it is completely androgen insensitive, as demonstrated by its identical slow growth rate (doubling time, 24 ± 5 days) when passaged in intact rats, castrated rats, or castrated rats treated with exogenous testosterone. In its slow growth rate, the HI-S tumor is essentially identical with the parent H tumor. Following injection of 1.5 × 10^6 viable HI-S tumor cells, 140 ± 20 days are required for the tumor to reach a size of 1 cm regardless of the androgen status of the host. Histologically, the HI-S tumor (Fig. 4) is also very similar to the H tumor, both being well-differentiated prostatic adenocarcinomas. Biochemically, the HI-S tumor has 5α-reductase levels just slightly lower than those of the H tumor (Table 1).

DNA analysis of the HI-S tumor growing in castrated males demonstrates that the DNA content per cell for the HI-S tumor is identical with both the normal adult dorsal prostate and the H tumor (Table 1). The diploid nature of the DNA content of the
HI-S tumor is further supported by flow cytometric analysis (Chart 1), which demonstrates a single G1 peak with the channel number identical to that for the diploid prostate. Unfortunately, chromosomal analysis of the HI-S tumor in its earliest passage was not performed. Chromosomal analysis of the HI-S tumor from cells of the fifth serial passage, however, revealed a diploid modal chromosomal number of 42 with a range of 33 to 42 (Chart 4). Detailed chromosomal examination of the HI-S tumor at its fifth serial passage reveals that, although 80% of the cells examined had a completely normal diploid karyotype, approximately 20% of the examined cells had lost the Y chromosome and had a t(4;7) translocation marker. This t(4;7) translocation marker was identical to that observed in the HF tumor. Histologically (Fig. 5A), biochemically, and by growth rate, the HI-S tumor at its fifth passage was very similar to the first-passage HI-S tumor. Upon continuous passage of the HI-S tumor, however, these parameters began to change. Between the fifth and seventh passages, the growth rate nearly doubled from a doubling time of over 20 days to one of only 9.0 ± 0.8 days. This 2-fold increase in growth rate was paralleled by decreases in 5α-reductase activities; 5α-reductase activity at the fifth and seventh passages were, respectively, 11.3 ± 2.0 versus 3.1 ± 0.4 x 10^5 molecules of testosterone reduced per hr per cell. Histologically, the epithelial cells of the tumor acini of these latter passages are less well differentiated, and they begin to pile up into the acinar lumen (Fig. 5B). The HI-S, after its seventh passage, was thus termed the HI-M tumor, first passage, to denote its moderately fast (10-day doubling time) growth rate and its changed phenotype. Chromosomal analysis of the HI-M tumor demonstrated a modal number of 41 to 42 with a range of 37 to 84 (Chart 4). The frequency of cells with abnormal karyotypes was increased to 50% in this tumor. Comparison of the karyotypes in these abnormal cells demonstrated the presence of at least 3 separate clones: one clone, accounting for 58% of the abnormal cells, had the t(4;7) translocation and a ring No. 1 marker chromosome; the second clone, accounting for 25% of the abnormal clones, had the t(4;7) and t(1;2) translocation markers; and the third clone, accounting for 17% of the abnormal cells, had only the t(4;7) translocation marker. The t(4;7) marker, seen in all the abnormal cells of the HI-M tumor, was also observed in all of the abnormal cells of the HI-S tumor (fifth passage), demonstrating the cellular continuity between these 2 tumors.

At the second serial passage of the HI-M tumor, 10 castrated male rats were inoculated with 1.5 x 10^6 viable HI-M tumor cells, and in all 10 rats a primary tumor grew. Nine of the 10 rats had tumors which doubled at a rate of 9.8 ± 0.8 days; however, one tumor grew with a doubling time of only 4.5 days. Passage of one of the 9 slower-growing second-passage HI-M tumors (doubling time, 9.2 days) into 5 castrated male rats produced third-passage tumors which grew with a doubling time of 8.8 ± 1.2 days. In contrast, passage of the one exceptionally fast-growing second-passage HI-M tumor (termed HI-F, first passage, to denote its increased growth rate) into 5 castrated males produced tumors, all of which had a fast doubling time of 4.8 ± 1.8 days. Serial passage of these fast-growing tumors established the HI-F tumor.

Histologically, the HI-F (Fig. 5C), is less well differentiated than is the HI-M tumor (Fig. 5B) and possesses even lower 5α-reductase levels; 5α-reductase activities of HI-M and HI-F were, respectively, 3.1 ± 6.4 versus 1.3 ± 0.4 x 10^5 molecules of testosterone reduced per hr per cell. There are, however, no areas of completely anaplastic tumor cells within the HI-F tumors. Chromosomal analysis revealed that the HI-F tumor had a modal number in the diploid range (Chart 4). The frequency of cells with abnormal karyotypes was increased in the HI-F tumor. One hundred % of all the cells karyotyped had the addition of a No. 4 chromosome and had the t(4;7) translocation markers, and 80% of the cells also had a ring No. 1 chromosome. The morphology of the t(4;7) and ring No. 1 markers were completely identical with those of the major clone of the HI-M tumor, thus demonstrating that the progression of the HI-M to HI-F tumors involved clonal selection.

At the eighth passage of the HI-F tumor, a single rat of 4 within the passage developed a completely anaplastic tumor (Fig. 5D). This anaplastic tumor was serially passaged and termed the AT-3 tumor. This AT-3, besides being anaplastic, is completely androgen insensitive and is even faster growing than the HI-F tumor. The doubling time for the AT-3 tumor is 1.8 ± 0.2 days regardless of the androgen status of the host. Even in its earliest passage (first to fifth), this AT-3 tumor has had a moderately high incidence of spontaneous metastases (i.e., over 50% of rats inoculated developed axillary lymph node and lung metastases). DNA analysis of the AT-3 tumor demonstrated that it had more than the diploid amount of DNA per cell (Table 1). Chromosomal analysis further revealed that the AT-3 tumor, at its third passage, has a modal number, 65, in the hypotetraploid range (Chart 4). This deviation from tetraploidy involved the loss of the Y chromosome and the gain of 5 kinds of structural chromosomal abnormalities including a t(1;4) and t(4;X) translocation marker. Thus, the progression of the HI-F to the AT-3, like all previous progressions, was associated with development of definitive chromosomal change.

**DISCUSSION**

That tumors often progress to more aberrant phenotypes with time is a well-established principle of tumor biology (5). Indeed, such tumor progression has been repeatedly demonstrated with the use of a variety of serially transplantable animal tumors including the Dunning R-3327 system as presented in this paper. The exact mechanism responsible for these altera-
tions of tumor phenotype have not been completely resolved, however, and could involve either genetic and/or epigenetic mechanisms (14). In this regard, it is highly relevant that, as demonstrated in this paper, each time that progression occurred within the Dunning R-3327 system, chromosomal changes could be concomitantly detected. Unfortunately, the association of demonstrable chromosomal changes and tumor progression does not in itself allow a decision as to the mechanism for these phenotypic changes (i.e., genetic or epigenetic). Indeed, these chromosomal changes may or may not be causally related to the phenotypic changes involved in these tumor progressions. These chromosomal changes, however, do make it clear that the Dunning tumor cells, unlike normal prostatic cells, are genetically unstable under certain conditions. Numerous studies have demonstrated that normal mammalian tissues consist essentially of genetically homogeneous cell populations which upon cellular proliferation produce genetically identical progeny (4, 6, 12). In direct contrast to this consistent genetic stability of normal cells, Dunning tumor cells can begin to produce novel tumor cell progeny that are genetically different from their parental cell precursors. That definitive genetic differences do exist between parental and progeny Dunning tumor cells when such genetic instability develops has been consistently demonstrated in the present study by comparison of the various karyotypes.

The observation that the H tumor, from which all the other tumor sublines described in this paper were derived, can give rise to genetically novel clones of tumor cells raises the question of whether the H tumor is constantly genetically unstable or whether this genetic instability is a dormant characteristic which is expressed only sporadically under certain conditions. It is possible, for example, that the H tumor is constantly unstable, but the effects of this instability are expressed only when the random chromosomal changes result in a specific rearrangement which confers to the new cell a selective growth advantage (i.e., androgen independence, increased growth rate, etc.). In such an explanation, the expression of genetic instability would be a stochastic event. It is also possible, however, that the H tumor is not constantly genetically unstable but instead that this instability is a dormant characteristic which is expressed only under certain conditions. In this latter explanation, the expression of genetic instability could develop at various times as an inducible event. While the studies presented in this paper do not resolve whether the expression of the genetic instability of the H tumor is a stochastic or inducible process, one point is clear, however; not every H tumor at each passage expresses this instability. Indeed, it has been through this selective expression of genetic instability that it has been possible not only to generate new Dunning tumor sublines but also to maintain the properties (i.e., diploid chromosomal number, slow growth rate, androgen sensitivity, and well-differentiated morphology) of the original H tumor for over 16 years in serial passage.

Regardless of whether the expression of the genetic instability of the Dunning tumor cells is a stochastic or inducible event, an important consequence of the expression of this genetic instability is that new clones of tumor cells are capable of being added to the tumor population at any time. These additions, when they occur, increase the heterogeneous nature of the initial tumor. For example, in the progression of the HI-S to the HI-M tumor, 2 new clones of tumor cells developed producing a heterogeneous tumor with at least 3 distinct but highly related tumor cell subpopulations. Once established, the heterogeneous nature of a tumor can remain stable for years as long as all the clones within a heterogeneous tumor have similar doubling times. This type of stability can be seen with the H tumor which is a heterogeneous tumor composed of both well-differentiated androgen-dependent and androgen-independent tumor cells with identical slow growth rates (7). As long as there are no exogenous selective pressure (i.e., androgen ablation) and no further expression of genetic instability, this H tumor remains heterogeneous and highly stable as documented by its ability to be maintained in continuous passage for over 16 years.

In contrast to the relative stability of heterogeneous tumors composed of clones with identical doubling times, heterogeneous tumors having clones with differing growth rates are markedly unstable since eventually any clone(s) with a definitive growth advantage will selectively outgrow the other slower-growing clones initially present. This process, termed clonal selection, thus reduces the heterogeneous nature of tumors. Clonal selection has been consistently demonstrated in all of the progressions of the various Dunning sublines examined in the present study. In all of these progressions, a particular clone of cells within a heterogeneous tumor outgrew the other slower-growing clones producing a more homogeneous tumor. In this manner, the heterogeneous versus homogeneous nature of a tumor, at any moment, is determined by the dynamic relationship between genetic instability increasing and clonal selection decreasing the number of tumor cell clones present within a tumor.

In conclusion, the present study suggests that the process of genetic instability, which results in the addition of phenotypically new clones of cells to the tumor population, coupled with the process of clonal selection of these newly developed cells is at least one mechanism for the progression of prostatic cancer. Indeed, the idea that tumor progression involves such a coupled series of events has been proposed by Nowell (16) as a general model for all tumor progressions. Whether such a process is the only mechanism for tumor progression is not yet clear. In support of such a concept, however, there are a series of previous reports that, like the progression of the Dunning tumors, progression in other tumor systems is likewise associated with chromosomal changes and clonal selection. For example, both Mark (13), studying the mouse, and Mittelman (15), studying the rat, demonstrated that during the serial transplantation of Rous sarcoma virus-induced tumors there is a sequential change in the chromosomal makeup of the tumor which is associated with the progression of these tumors to a more malignant and less differentiated state. The studies of Mittelman (15) are particularly relevant since they demonstrated that the change in chromosomal number upon serial transplantation of rat sarcomas was typically associated with the progressive loss of differentiation, as determined both histologically and by decreased collagen production. In addition to these studies, Al-Saadi and Beierwaltes (1) have demonstrated that the serial transplantation of thyroid tumors in the rat is likewise associated with sequential chromosomal changes which are correlated with the progression of these tumors from a large degree of dependence on iodine deficiency and thyroidectomy for growth, to anaplastic, fast-growing, and metastatic tumors independent of hormonal modulation.
REFERENCES


Fig. 1. Histology of the R-3327-H tumor. A, × 40. B, × 400. H&E.
Fig. 2. Histology of the R-3327-HF tumor. H&E, × 40.
Fig. 3. Histology of the "unusually fast growing" H tumor. A, × 40. B, × 200. H&E.
Fig. 4. Histology of the R-3327-HI-S tumor. H&E, × 40.
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Fig. 5. A, histology of R-3327-HI-S, fifth passage; B, histology of R-3327-HI-M; C, histology of the R-3327-HI-F; D, histology of the R-3327-AT-3. H&E. × 140.
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