Isolation and Characterization of a Cloned Cell Line R3327H-G8-A₁
Derived from the Dunning R3327H Rat Adenocarcinoma

Mary Anne Sestili,¹ James S. Norris,² and Roy G. Smith³

Department of Urology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

A cloned cell line (R3327H-G8-A₁) has been isolated from the Dunning R3327H adenocarcinoma. Light and electron microscopic studies showed that the cell line possessed features common to secretory epithelial cells. These cells, which grow in monolayer culture, produced s.c. hind flank tumors when inoculated into Copenhagen × Fischer F₁ rats. Chromosomal karyotype analysis confirmed that the cell line is distinctly that of the Rattus norvegicus genus and species. The cells specifically bind testosterone and dexamethasone with equilibrium dissociation constants (Kᵦ) of 0.49 and 0.8 nm, respectively. The numbers of saturable binding sites per cell are 10,000 for testosterone and 60,000 for dexamethasone. The cells also have 5α-reductase activity. These properties are characteristic of the prostate and of the Dunning tumor from which the cells are derived. Cell growth in vitro was stimulated by androgens and inhibited by glucocorticoids at concentrations of 10⁻⁶ M. An intriguing finding was that estradiol and progestins dramatically stimulated growth in the apparent absence of receptors for these hormones. Finally, comparisons between the G8-A₁ cells and the tumor induced by the G8-A₁ clone and a second generation of cells from this G8-A₁-induced tumor showed that the cloned cells retained their properties following passaging in the animal.

INTRODUCTION

The prostate adenocarcinoma of the Copenhagen rat (R3327), first described by Dunning (6), is recognized as a suitable model for human prostate carcinoma because it mimics the characteristics of human prostate cancer (30). The tumor has appeared spontaneously in aged animals, and its histological, biochemical, and metastatic properties are all similar to those of the human disease. Furthermore, the tumor is able to transform from a hormone-dependent to a hormone-independent state resulting in insensitivity to castration and/or hormone treatment. Its usefulness as a model system is exemplified by the fact that it has been used to investigate the effect of chemotherapeutic agents (1, 17, 28) and to study the role of the immune response on retardation of tumor growth (4, 12, 13).

Initially, Dunning showed that, when pieces of the hormone-dependent well-differentiated adenocarcinoma were inoculated s.c. into Copenhagen × Fischer F₁ rats, the growth rate of tumors was greater in intact males than it was in castrated male or female animals. Enzyme profiles of this hormone-dependent tumor showed that it possessed 5α-reductase activity (33) and high levels of acid phosphatase (8). Steroid receptor assays showed the presence of saturable high-affinity receptors for androgen and estrogen, but not progesterone (7, 15).

Other studies have shown that there are several tumor variants which originate from the hormone-dependent R3327H type. Two examples of these which have received detailed studies are the R3327-H₁, a slow-growing hormone-insensitive adenocarcinoma, and the R3327-AT, a rapidly growing anaplastic, hormone-insensitive tumor (8). These variants have altered growth properties growing as well in intact males as in castrated and female Copenhagen × Fischer F₁ rats (34). Their enzyme content (9) and steroid receptor profiles (16) are also altered. It is not known whether the hormone-sensitive cells convert to a hormone-insensitive condition. It has been suggested, however, that the initial inoculum contains at least 10 to 30% of hormone-insensitive cells and that in the model tumor system there are at least 2 cell types with different steroid response capabilities existing simultaneously (8, 30).

A fundamental approach to the study of this prostate carcinoma is to clone the cell populations of the tumor and to investigate their individual biochemical properties and growth characteristics. Through the use of these clones, which would lend themselves to hormonal and chemical manipulations, many aspects of the tumor’s basic biology could then be investigated. The isolation and characterization of a cloned cell line from the Dunning R3327H tumor is described herein. This new cloned cell line has been designated the R3327H-G8-A₁ clone (24).

MATERIALS AND METHODS

Materials. Radioinert steroids used included testosterone, DHT,¹ 17β-estradiol, DES, progesterone, and cortisol which were obtained from Sigma Chemical Co., St. Louis, Mo. Radiolabeled steroids [1,2,6-³H(N)]testosterone (98.8 Ci/mmol), [1,2,5,6,7-³H(N)]DHT (131 Ci/ mmol), 17β-[2,4,6,7-³H(N)]estradiol (101 Ci/mmol), [1,2-³H(N)]progesterone (55 Ci/mmol), and [6,7-³H(N)]dexamethasone (47.5 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Phenylmethylsulfonyl fluoride was purchased from Boehringer Mannheim, Mannheim, West Germany. All reagents for tissue culture including DME, HBSS, trypsin (0.25% in HBSS), trypan blue, horse serum, penicillin, and streptomycin were obtained from Grand Island Biological Co., Grand Island, N. Y. Thin-layer silica gel chromatography plates were purchased from E. M. Laborato-

¹The abbreviations used are: DHT, dihydrotestosterone; DES, diethylstilbestrol; DME, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ balanced salt solution; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid) monosodium salt; PBS, Dulbecco’s phosphate buffered saline (0.9% NaCl).
a Copenhagen × Fischer F, hybrid was obtained from Dr. Norman Altman in December 1976. The tumor was removed and explanted into culture as described previously. The medium used to establish the cell line consisted of Ham’s F-12 with 10% fetal bovine serum, 1% penicillin-streptomycin, 10⁻⁸ m testosterone and 10⁻⁹ m 17β-estradiol. Culture medium was changed every 2 days following the initial explant procedure.

**Cloning**. Cells isolated from localized regions of growth in the explant cultures were cloned in Falcon Microtest II plates following the method of Kohler et al. (10). Cells isolated by this technique were further cloned in soft agar as described by MacPherson (14). The agar bed contained 5 x 10⁻⁸ m DHT, 5 x 10⁻⁶ m 17β-estradiol, and 5 x 10⁻⁴ m triamcinolone acetonide to enhance hormone-dependent growth. When colony formation was observed, they were removed using a wide-tip Pasteur pipet and placed into monolayer culture. The R3327H-G8-A, colony was the largest colony observed and was used throughout this study.

**Cell Culture Conditions**. R3327H-G8-A cells were routinely grown and maintained in 75-cm² flasks (Falcon Plastics) with DME supplemented with 10% (v/v) horse serum and penicillin-streptomycin (100 units/ml) in a humidified 5% CO₂-air incubator at 37°. At confluence, cells were subcultured using trypsin (0.25% in HBSS), washed with HBSS, and resuspended in complete medium. Cells were passaged by splitting at approximately 1:3 ratio at 7-day intervals.

Cells were counted using a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.), and their doubling time was determined as described by Norris et al. (19). For storage, cultured cells were suspended in normal growth medium containing 10% dimethyl sulfoxide at a cell concentration of 2 x 10⁶/ml. One-ml aliquots were dispensed into screw-cap polymer vials which were gradually cooled for 3 hr before transferring the vials into liquid nitrogen.

**Animals**. Twenty male and ten female Copenhagen × Fischer F, rats were obtained from the Papanicolaou Cancer Research Institute at Miami Inc., Miami, Fla., at the fourth week of birth. At 5 weeks, 10 of the 20 male rats were castrated via the scrotal route using ether anesthesia. At 7 weeks, animals were inoculated s.c. with 2 x 10⁵ R3327H-G8-A, cells in 0.2 ml HBSS in the right hind flank using a 25-gauge needle. Cell inocula were prepared from trypsin harvested cells washed with DME and resuspended in HBSS. Cell suspensions contained 95% viable cells as determined by the trypan blue exclusion test. Three months from the time of inoculation and when the tumors were palpable, they were surgically removed, weighed and measured. Tumors were divided and processed as described for testosterone, 17β-estradiol, and progesterone one steroid receptor assays (see below). Tumors were also minced and treated enzymatically for tissue culture to provide monolayers from which subsequent testosterone receptor assays were conducted.

**Histoilogy of Tumors**. Tumors produced by R3327H-G8-A, cell inoculations were fixed in 10% buffered formalin, processed by standard histological techniques, and stained with hematoxylin and eosi.

**Chromosomal Analysis**. The modal chromosome number and the karyotypic analysis of the R3327H-G8-A, cloned cells were performed according to Morris et al. (19). Chromosomes were arranged as described by Schnied and Schnied (23).

**Mycoplasma Screening**. Evidence of Mycoplasma contamination was assayed using the Hoescht Compound No. 33258 fluorescent staining technique. Cells were grown on 12-mm glass coverslips, but were not permitted to reach confluency in order to ensure the best visualization area around the cells. Cells were fixed in acetic acid:methanol (3:1), stained for 10 min with Hoescht fluorescent stain (1%), and examined under fluorescent light.

**Electron Microscopy**. For electron microscopy, cells were grown in a Petri dish to approximately 80% confluence in complete medium. Medium was aspirated and cells rinsed 3 times with 0.1 m PIPES buffer (pH 7.2) and fixed in 2.4% glutaraldehyde:0.1 m PIPES at room temperature for 45 min. The samples were then rinsed 3 times in 0.1 m PIPES buffer, stained using 1% osmium tetroxide in 0.1 m PIPES at room temperature for 30 to 60 min, rinsed with distilled water, and then dehydrated using 30, 50, 70, and 90% ethanol for 5 min each. This was then followed by 15-min exposures to 100% ethanol and then infiltration with 1:1,100% 30, 50, 70, and 90% ethanol.

**Striped Serum**. Serum steroids were removed by the addition of 2% (w/v) charcoal (Norit A; Fisher) to horse serum followed by heating at 55° for 1 hr. To ensure the removal of all steroids, the charcoal treatment was repeated. Charcoal was removed from suspension by centrifugation at 2000 x g for 20 min. Serum was sterilized by filtration through a 0.22 Millipore filter. Testosterone was not detectable in the striped serum by radioimmunoassay. The within and between coefficients of variation of the testosterone assay using control serum specimens were 5% and 9%, respectively.

**Whole Cell Uptake of Steroids by R3327H-G8-A, Cells**. For whole-cell steroid-receptor assays, approximately 6 x 10⁶ cells were plated into four 60-mm Petri dishes per assay point. The cells were permitted to reach confluency which occurred 48 to 72 hr after plating. Twenty-four hours prior to assay, the media was aspirated, monolayers were gently washed with HBSS, and the medium was replaced with DME:1% stripped horse serum. At the time of assay, medium was aspirated and cells were washed once with HBSS and once with serum-free DME. Varying concentrations of radioabeled steroids [³H]testosterone (0.25 to 3 nm), [³H]DHT (0.5 to 6 nm), 17β-[³H]estradiol (0.25 to 10 nm), [³H]progesterone (0.4 to 20 nm), and [³H]dexamethasone (0.25 to 10 nm) were prepared in serum-free DME. To correct for nonspecific binding, 100x excess radioinert steroid, prepared in DME, was added simultaneously to 2 of the 4 dishes/point. DES was used to correct for nonspecific binding in measuring estrogen receptors. Petri dishes were placed in a humidified 5% CO₂,95% air incubator at 37° for 1 hr. Upon completion of incubation, Petri dishes were placed on a tray of ice, the medium was removed by aspiration, and cells were washed 3 times with 3 ml each of ice-cold PBS. Monolayers were dispersed in 3 ml of PBS with a rubber policeman and centrifuged at 1000 x g for 10 min in a Sorval RC-5 refrigerated centrifuge, and the supernatant was discarded. Absolute ethanol (3 ml) was added to each pellet, vortex mixed, and permitted to extract for 24 hr at room temperature. The tubes were then centrifuged at 1000 x g for 10 min, and the ethanolic extract was added to 10 ml ScintiVerse to quantitate the incorporation of radioactivity. Pellets were retained, washed twice with 0.9% NaCl solution, centrifuged, and stored in 1 ml 0.9% NaCl solution at -20°C until assayed for DNA content according to the procedure of Burton (2).

**Competition Binding Assay**. For all competition assays, cells were prepared in culture dishes as above. Radioabeled steroid solutions (1.5 nm) were prepared in DME with the radioinert competitors of testosterone, DHT, 17β-estradiol, DES, progesterone, and cortisol used at a 10x excess concentration. Dishes were incubated at 37° for 1 hr and then placed on ice. Incorporation of label was measured as described above. The specificity of [³H]dexamethasone binding was determined similarly using a 10x excess of radioinert dexamethasone, corticosterone, testosterone, DHT, 17β-estradiol, and progesterone.

**α-Reductase Activity: Conversion of [³H]Testosterone to [³H]DHT**. Three types of samples were prepared and used in this assay: R3327H-G8-A, cells, slices of R3327H adenocarcinoma (from tumor-bearing rats obtained from Dr. Altman) and normal rat ventral prostates. The 2 latter samples were used as controls in this assay. Cultures of G8-A, cells were prepared in exactly the same manner as above for whole cell uptake, with the exception that approximately 2 x 10⁶ cells were plated in 25-cm² flasks with 5 ml complete medium. At cell confluency, the medium was replaced with DME plus 1% stripped horse serum after which cells were incubated for 24 hr. On the following day, monolayers, as well as slices from the R3327H tumor and ventral prostate, from a 24-hr castrated male rat were prepared for assay. Each tissue slice was minced in a separate Petri dish and to these samples as well as the cell monolayers were added 5 ml of a 5 nm [³H]testosterone solution. All samples were incubated at the same time and for 1 or 6 hr at 37°. At

100% ethanol: Epon 812 (Electron Microscopy Sciences, Fort Washington, Pa.) for 1 hr. Finally, Epon 812 was added and allowed to polymerize overnight. Initially, 1-μm section was made with an ultramicrotome. Sections were stained in uranyl acetate (saturated aqueous) followed by Reynolds lead citrate and viewed in a Siemens 102 electron microscope at 60 kV.
the end of the incubation period, monolayers were washed, dispersed, and centrifuged. The medium and tissue sections from the tumor or normal prostate were transferred into 12- × 75-mm borosilicate tubes and centrifuged, and the pellets were washed twice with ice-cold PBS to remove free $[^3H]$testosterone. Finally, all samples were extracted with ethanol. The ethanol extracts were evaporated to dryness under $N_2$ pressure and redisolved in 0.1 ml methylene chloride. Aliquots (10 $\mu$l) were removed to determine total $^3$H incorporation, and the remainder of the samples were spotted onto an oven-dried precoated thin-layer chromatography plate using a 10-$\mu$l micropipet. Chromatography was performed on a 20- × 20-cm glass backed thin-layer silica gel plate using chloroform:ether (90:10 v/v) as eluant. Steroids were visualized under iodine vapor, and 5-mm fractions were scraped off, suctioned into pipets with glass wool plugs, and eluted with 10 to 15 ml ethyl acetate. Glass wool plugs retained <1% of the total radioactivity. Eluants were evaporated to dryness and counted in minivials with 5 ml Scinti-Vers in a scintillation counter. The percentage of $[^3H]$testosterone (R, 0.25) converted to $[^3H]DHT$ (R, 0.38) was calculated.

Preparation of Cytosol and Steroid Binding Assays in the G8-A, Induced Tumor. Tumors which had been induced by injecting G8-A, cells into intact males, castrated males, and females were kept separately, but tumors of each group were pooled. Tumors were rinsed of excess blood in cold 0.9% NaCl solution, minced, and homogenized at 4$^\circ$ in 4 volumes per g wet weight of tissue of cold buffer (10 mM Tris: 1.5 mM EDTA: 12 mM thiolycerol: 500 $\mu$M phenylmethylsulfonyl fluoride and 10% glycerol, pH 7.4) with a Brinkmann polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.). The homogenate was first centrifuged at 800 $\times$ g for 10 min in a Sorvall refrigerated centrifuge and finally ultracentrifuged at 105,000 $\times$ g for 1 hr with a Beckman (Berkeley, Ca.) L5-65 ultracentrifuge. The lipid layer was aspirated by suction, and cytosol (0.2 ml) was added to glass tubes which contained various concentrations of $[^3H]$testosterone (0.2 to 4 nm), 17$\beta$-estradiol (0.2 to 10 nm), and $[^3H]$progesterone (0.4 to 20 nm) with or without 100 $\times$ excess of the corresponding radioinert steroid. Culture tubes were incubated at 4$^\circ$ for 24 hr, and bound and free testosterone were separated by a dextran-coated charcoal assay (29).

Preparation of a Monolayer Culture from the G8-A, Induced Tumor. The G8-A, induced tumor was removed under sterile conditions, washed in HBSS plus 1% penicillin:streptomycin, transferred to DME plus 1% penicillin:streptomycin and minced. This solution was permitted to settle, and the supernatant was aspirated. Enzymatic tissue digestion was completed on the free cell fraction with a solution containing collagenase (0.1%), trypsin (0.1%), and chick serum (1%) and agitated for 15 to 20 min in a water bath. The undigested tissue fraction was discarded, and fetal calf serum (10%) was added to inactivate the trypsin. The cell suspension was then centrifuged at 800 $\times$ g for 10 min. The pellet was resuspended in DME plus 10% horse serum and incubated in flasks (25 sq cm) at 37$^\circ$.

RESULTS

Morphology of Cells. A considerable period of time elapsed (4 months) from when the initial culture was explanted until cell colonies were evident. The reason for the slow adaptation is unknown although this type of phenomenon has been seen in other attempts to culture prostate. Eventually, however, cells were available in sufficient quantities for cloning and serial passage. Generation times during these early passages were never measured but appeared to be of long duration. Cloning efficiencies were always greater than 85% except in soft agar where 0.01 to 0.05% viability was common.

When viewed by phase-contrast microscopy, cells of the R3327H-G8-A, cell line appear as broad, flat, polygonal epithelial cells which are joined one to another on all sides without any apparent intercellular spaces. These cells grow in a continuous monolayer (Fig. 1A). When the cultures are left undisturbed, except for medium changes, monolayers become confluent and the cells form domes as seen in Fig. 1B. The doubling time of the cells was found to be 35 hr. There was no evidence of Mycoplasma contamination when cells were examined by fluorescent microscopy.

Electron micrographs showed that the G8-A, have properties consistent with secretory epithelial cells (Figs. 2 and 3). Each cell contains an abundance of ribosomes and polyosomes contributing to the electron density of the cytoplasm. The large Golgi complex appears active with membrane-bound granules; microvilli project from the peripheral cell membrane (Fig. 2). The mitochondria contain lamellar type crystae, and the cells are connected by junctional complexes (Fig. 3). There was no evidence of viral particles within the cells.

Karyotype Analysis. The chromosome count distributes around a modal number of 70 with a second minor component at 80. The karyotypes in Figs. 4 and 5 are representative of the 2 peaks. These data indicate that a certain degree of instability exists in the mitotic process during replication of R3327H-G8-A, cells in vitro, a characteristic of cancer cells in general. Specifically, karyotypic analysis of the cells reveals numerous abnormalities. Many of the No. 1 chromosomes have short arms and are fragmented. The No. 3 chromosomes are also missing part of the arm and exist as fragmented multiple copies. Other chromosomes such as chromosomes 11 and 12 have short
arms. The Y chromosomes appear to be less densely stained distally, and numerous marker chromosomes exist.

Steroid Binding Studies. To characterize the cells biochemically, steroid binding assays were performed using [3H]testosterone, [3H]dexamethasone, 17β-[3H]estradiol, and [3H]progesterone. These cells were deprived of steroids for 24 hr by maintaining them in DME containing charcoal-stripped 1% horse serum. The confluent monolayers of cells were then incubated with each of the above steroids at various concentrations for 1 hr at 37° using the procedure described by Norris et al. (19).

When R3327H-G8-A1 cells (passage 5) were incubated with increasing concentrations of [3H]testosterone with or without 100X excess of radioinert testosterone, the results showed a single high-affinity component that was saturable at 0.25 to 3 nM concentration. In Chart 1a, the specific binding is calculated as the difference between the total radioactive ligand bound and the nonspecific bound. The plot given in Chart 1b represents the Scatchard analysis of data shown in Chart 1a. The single straight line is indicative of a single class of high-affinity binding sites with an equilibrium dissociation constant (Kd) of 0.49 nM. Extrapolation of this line to the abscissa gives the concentration of receptor sites as approximately 10,000 sites/cell. This procedure was repeated using passages 30 and 90. Kd values of the other 2 testosterone receptor assays were 4.7 × 10⁻¹⁰ M and 4.8 × 10⁻¹⁰ M, respectively. Receptor sites per cell were 10,000 and 12,000, respectively.

To confirm that testosterone binding by the cells after 1 hr was specific for androgens, competition studies were completed by simultaneously adding to the cells a 1.5 nM concentration of [3H]testosterone in the presence of a 10X excess (15 nM) of unlabeled testosterone, DHT, 17β-estradiol, progesterone, or cortisol. Results are presented as the amount of [3H]testosterone that was bound by the cells in the presence of the competitor. Chart 2 shows that the androgenic compounds testosterone and DHT displace 62 and 63% of the [3H]testosterone, respectively, whereas the other steroids displace none or very little [3H]testosterone, DES (<1%), 17β-estradiol (15%), progesterone (6%) and cortisol (9%).

In a manner similar to that described above, glucocorticoid binding was measured using [3H]dexamethasone (0.25 to 10 nm) with or without a 100-fold excess of radioinert dexamethasone. Scatchard analysis of the binding gave a straight line with a Kd of 8.6 × 10⁻¹⁰ M and 60,000 binding sites per cell (Chart 3). Competition studies revealed that displacement of [3H]dexamethasone could be accomplished with a 10-fold excess of radioinert dexamethasone or corticosterone but not by a tenfold excess of testosterone or 17β-estradiol.

Attempts were made to measure specific 17β-[3H]estradiol binding by the R3327H-G8-A1, cloned cells. The first assays to measure 17β-estradiol receptor were performed on approximately 1 × 10⁶ cells/Petri dish (60 mm) with 17β-[3H]estradiol at a concentration of 0.25 to 10 nm with and without 100X excess of DES to correct for nonspecific binding. These assays resulted in no specific binding since the radioactive incorporation represented by nonspecific binding equaled that of total binding. Since the estrogen receptors might be present in low concentrations, the assays were repeated using a greater number of cells (2 × 10⁶). Even though these data from the second experiment show a slight displacement of total binding by excess DES, they do not exhibit saturable binding in the 0.25 to 10 nm concentration range. When these data are combined with DNA assays, it is estimated that there are fewer than 500 high-affinity estrogen binding sites/cell.

Tritiated progesterone (0.4 to 20 nm) was added to confluent monolayers of cells with and without a 100-fold excess of radioinert progesterone. A high concentration of progesterone-displaceable binding sites was evident. However, when these data were plotted according to Scatchard, no saturable binding was observed.

Conversion of [3H]Testosterone to [3H]DHT by G8-A1 Cells. In rat prostate gland studies in vivo (16), there is a marked
conversion of testosterone to the principal metabolite DHT. Studies were therefore conducted to determine whether the G8-A, cells possessed 5α-reductase activity. Control tissues, the R3327H tumor, and the rat ventral prostate were included for comparative purposes. Table 1 shows that when 5 nm \( [3H] \)testosterone was incubated with the G8-A, after 1 hr only 9% of the macromolecular bound androgen was DHT compared to 86 and 39% DHT for the ventral prostate and R3327H tumor, respectively. After 6 hr of exposure of the G8-A, cells to \( [3H] \)testosterone, 42% of the macromolecular bound androgen was DHT, and the remainder was testosterone. This was again lower than that observed for the ventral prostate (71%) but similar to that observed for the R3327 tumor (48%).

**Effect of Steroids on Growth of the G8-A, Clone In Vitro.**

To study the effect of various steroids on the growth rate of the G8-A, clone, it was first determined that the minimum serum requirement for maintenance of a quiescent but healthy cell population was 0.5% charcoal-stripped serum. DME medium (2 ml) containing 0.5% penicillin:streptomycin and 5000 cells/ml was added to 60-mm plastic tissue culture dishes on Day 1. After 24 hr, the medium was changed and \( 10^{-8} \) M steroids were added to triplicate groups (Table 2). Subsequently, the medium was changed every 24 hr for 10 days. Cells were then trypsinized and counted in a Coulter Counter. Table 2 shows that the glucocorticoids dexamethasone and in particular triamcinolone acetonide dramatically inhibited cell growth. Surprisingly, estradiol and progesterone increased the growth rate of the cells more effectively than either testosterone or DHT.

**Growth Characteristics In Vivo and Histological Characterization of G8-A,–induced Tumor.** Approximately 8 weeks after cells (2 \( \times 10^5 \)) of the epithelial G8-A, clone were inoculated into rats, small s.c. tumors were palpable in intact male rats only. However, by 13 weeks, tumors were growing rapidly (1 to 2 cm) in all animals. After 13 weeks, tumors had appeared in 10 of 10 intact males with an average tumor weight of 4 g and 9 of 10 castrated males with average tumor weight of 3.8 g; and 6 of 10 females had tumors with an average weight of 2.8 g. Histologically, the tumors appeared undifferentiated, possessing densely packed cells which were elongated and spindle shaped with considerable nuclear and cellular pleomorphism and anaplasia. It is possible that the early arising tumors in male animals may have originally been hormone sensitive, reverting later to a hormone-insensitive state. On gross inspection, the tumors had remained localized and encapsulated within the s.c. tissue, showing no evidence of invasion of skin or underlying muscle.

**Steroid Hormone Binding Profile of G8-A,–induced Rat Tumor.** Cytosol from intact male, castrated male, and female rats was obtained and analyzed separately for testosterone, 17β-estradiol, and progesterone receptor sites by the dextran-coated charcoal procedure. For testosterone receptor assays, cytosols were titrated with increasing concentrations of \( [3H] \)testosterone (0.2 to 4 nm) with and without 100X excess radioinert testosterone. Scatchard analysis of these data show a linear plot consistent with a single class of binding sites in all cytosol groups with a \( K_d \) of 0.69 ± 0.26 nm. When these same cytosol preparations were incubated with either 17β-[\( ^{3}H \)]estradiol (0.2 to 10 nm) or \( [3H] \)progesterone (0.4 to 20 nm), no high-affinity saturable binding was observed. Both triitated hormones were displacable by a 100-fold excess of their respective radioinert ligands; however the binding was nonsaturable.

\( [3H] \)Testosterone Binding by Cultured Cells Derived From G8-A,–induced Tumor. A portion of the G8-A,–induced rat tumor was treated enzymatically to furnish a cell suspension and put into culture to determine if changes in cell morphology and steroid binding profiles had occurred during the in vivo growth period. At the fourth passage of these “second generation” tumor cells, \( [3H] \)testosterone whole-cell uptake studies were conducted in exactly the same manner as described earlier for the original G8-A, cells. The testosterone binding to cell monolayers was measured by incubating with increasing concentrations of \( [3H] \)testosterone. Then, to determine binding specificity, assays were performed using \( ^{3}H \) and unlabelled testosterone, DHT, 17β-estradiol, progesterone, and cortisol. Scatchard analysis of these data showed a single class of high-affinity binding with a \( K_d \) of 1 nm and the binding sites somewhat fewer than in the original clone being approximately 6000 sites/cell. A 69% displacement of \( [3H] \)testosterone is seen with 10X of unlabelled testosterone and 65% displacement with radioinert DHT, whereas 17β-estradiol shows a displacement of only 27% and no displacement is evident in the presence of progesterone and cortisol consonant with our earlier observations.

**DISCUSSION**

There has been considerable interest in the development of models for prostate carcinoma since cancer of the prostate is the second most common cancer in American males (27). Various models, such as the Nobel (18), the Pollard Germ-Free Wistar Rat (21), the AXC Rat (25), and the Dunning (1) have been investigated. We have selected the Dunning tumor for our studies since certain aspects of this model are very well defined; fur-
thermore, its biochemical, morphological and growth characteristics are similar to those of the human tumor (8, 13, 30).

The rat Dunning tumor in common with the human prostate tumor appears to be composed of at least 2 distinctive cell types. One type is dependent upon androgens for growth, while the other grows equally well in the absence of androgens. In an attempt to study these individual cell types, we have isolated and subcultured cloned cells from the original Dunning R3327H tumor. In this report, we have described the characterization of one of these cell types, the R3327H-G8-A, clone.

The G8-A, clone has been characterized histologically by both phase-contrast and electron microscopy. In culture, the cells form continuous sheets of flat, broad cells, adhering to one another with little intercellular space. Normally, they grow as monolayers, but when confluent they form domes. This condition has been reported in other epithelial cell systems including the MDCK kidney cell line (11), human breast carcinoma (31), cultures of normal glandular tissue from mice (20), and cell lines from mouse and rat ventral prostate (35). Electron microscope studies confirm the secretory epithelial nature of these cells due to the presence of microvilli, active Golgi complex, numerous mitochondria, and junctional complexes.

Evidence to suggest that this cell line is neoplastic includes its chromosome analysis, its ability to produce tumors when inoculated into Copenhagen × Fischer F1 rats, and its characteristic growth in soft agar. Normally the chromosome number for Rattus norvegicus is 2n = 42 (5), whereas cells of this cell line are characterized as heteroploid male rat in the hypotetraploid range almost 100% aneuploid with one modal number at 70 and a second minor modal at 80. The 2 karyotypes represented are characterized by intercalated chromosome segments in the hypotetraploid range and they demonstrate several abnormal marker chromosomes.

Biochemical characterization of the G8-A, cells demonstrated major similarities to both the Dunning tumor from which the cells were cloned and to the rat prostate. The cells also contained high-affinity limited-capacity binding sites for testosterone and dexamethasone. The steroid binding specificities, the equilibrium dissociation constants, and the number of binding sites per cell are all characteristic of steroid hormone receptors. However, we have not yet defined these as receptors since a more complete characterization is necessary.

The G8-A, cells contain 5α-reductase activity. The lower activity observed after 1 hr compared to activity in the prostate and the R3327H tumor (Table 1) may be explained by the fact that the cells were removed from androgen stimulation 24 hr prior to their assay. Within 6 hr of exposure of the cells to testosterone, 42% macromolecular bound steroid was DHT, which is similar to that observed in similar experiments with the parent tumor. These results imply that the enzyme activity might be inducible; however, further studies are necessary to prove such an induction. A similar testosterone-inducible 5α-reductase system has been reported previously by Shimazaki et al. (26). The particularly high level of conversion of testosterone to DHT is surprising, since the G8-A, tumor is not dependent upon androgens for growth and previous studies with the Dunning tumor system have indicated that the hormone-insensitive tumors have low levels of 5α-reductase activity (9).

A characterization of the poorly differentiated tumors produced by inoculation of G8-A, cells into the hind flank of Copenhagen × Fischer F1 rats was also performed. The tumors demonstrated no hormonal sensitivity since they grow equally well in females and in intact or castrated male rats. It is not known whether a hormone-insensitive cell was selected in cloning or if the cell was originally hormone sensitive but converted to an insensitive state. In spite of the fact that the tumors are insensitive to androgens, they possess high-affinity saturable binding sites for androgens. The tumors also contained progesterone-displaceable binding which is not receptor-like since the binding did not approach saturation at 0.2 to 20 nM concentrations of progesterone. In agreement with the results obtained from the cells in culture, the tumor contained no detectable estrogen receptors. When monolayer cultures were obtained from the tumors, the cells were shown to contain high-affinity saturable binding for testosterone similar to that observed in the original G8-A, monolayer cultures.

The steroid binding properties and enzymatic activities of the G8-A, cells and the resulting tumor are different to those described in other variants of the R3327 tumor (13, 33). This new cell line retains the enzyme markers acid phosphatase (24) and 5α-reductase and possesses specific high-affinity saturable binding for testosterone, while at the same time it grows in an anaplastic, androgen-insensitive condition in all animals. These properties are intermediate between those of the well-differentiated R3327H tumor and the anaplastic R3327-AT tumor. Thus, the G8-A, clone may represent a state of transition from an androgen-sensitive to an androgen-insensitive state. This suggests that the anaplastic tumor results from a gradual dedifferentiation of the well-differentiated androgen-dependent cells.

The growth studies in Table 2 are intriguing since cell growth was dramatically enhanced in the presence of estradiol and progestins yet no evidence could be found for the presence of classical intracellular receptors for those 2 hormones. Furthermore, since whole-cell uptake studies revealed no high-affinity (10⁻¹¹ to 10⁻⁹ M) specific binding for these hormones (at least <500 sites/cell), in addition to excluding binding to classical receptors, we can also exclude high affinity (Kd 10⁻¹¹ to 10⁻⁸ M) binding to cell membranes or the nuclear matrix. We cannot, however, exclude the presence of nuclear type II binding proteins for estradiol which have been associated with growth and bind estradiol with lower affinity (3). Alternatively, growth might be mediated via low affinity binding sites existing on the plasma membrane of these cells. Recently, such sites have been reported for estradiol (Kd 10⁻¹⁰ M) in the chick oviduct (32) and for the synthetic progestin, R5020 (Kd 10⁻⁸ M), on the surface of Xenopus oocytes (22). In the case of the latter, the binding of either R5020 or progesterone to the plasma membrane was associated with maturation of the oocyte. Our future studies are designed to look for such low-affinity binding proteins in the G8-A, clone in an attempt to explain the growth effects of estrogen and progesterone on the cells. Interestingly, the growth of the cells was inhibited by glucocorticoids.

In summary, several characteristics of this cell line are significant. It is one of a few animal culture systems which has characteristics of prostate carcinoma and which has maintained biochemical properties in common with the tumor from which it was derived. It has been characterized both for its morphology and biochemical properties and has been shown by karyotypic analysis to be an uncontaminated Rattus norvegicus cell line which possesses 5α-reductase activity and high-affinity testosterone and dexamethasone binding. Because it has been cloned, it originates from a common cell type and common genome. This cell line can be subcultured, frozen, and rethawed and, since it grows in a culture system, it affords the advantages that the system can be manipulated and will lend itself quite readily to...
studies that require quantification. The fact that these cells are not dependent upon androgens for growth does not detract from their usefulness as a model. It is well known that although human prostate carcinoma is initially responsive to hormones, as the disease progresses it changes, and becomes hormone insensitive with the tumor changing from an adenocarcinoma to an anaplastic form. It is our belief that the R3327H-G8-A1 cloned cell will prove valuable in the investigation of the cellular mechanisms associated with prostate neoplasia.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Rosemary Zuna, University of Arkansas for Medical Science, Little Rock, Arkansas, for performing the karyotype analysis. We also thank Dr. Norman Altman and associates at the Papanicolaou Cancer Research Institute at Miami, Inc., for their invaluable assistance in providing the R3327H tumor and Copenhagen × Fischer F1 rats.

REFERENCES

Fig. 2. Electron photomicrograph of a R3327H-G8-A, cell. Arrow, microvilli projecting from the cell membrane; Go, large active-looking Golgi complex. × 12,000.

Fig. 3. Electron photomicrograph of a R3327H-G8-A, cell. Arrows, junctional complexes; M, typical mitochondria containing lamellar type crystals; Nu, nucleus of the cell. × 42,000.
Fig. 4. Karyotype analysis showing G banding of R3327H-G8-A1 cells with a modal chromosome number of 70. A number of marker chromosomes are observed.

Fig. 5. Karyotype analysis showing G banding of R3327H-G8-A1 cells with a chromosome number of 80. A large number of marker chromosomes can be observed.
Isolation and Characterization of a Cloned Cell Line R3327H-G8-A Derived from the Dunning R3327H Rat Adenocarcinoma

Mary Anne Sestili, James S. Norris and Roy G. Smith


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/5/2167

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/43/5/2167. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.