Establishment and Characterization of an in Vitro Clonogenic Cell Assay for the R-3327-At Copenhagen Rat Prostatic Tumor

B. Ramanath Rao, Alexander Nakeff, Charles Eaton, and Warren D. W. Heston

Section of Cancer Biology, Division of Radiation Oncology, Mallinckrodt Institute of Radiology (B. R. R., A. N., C. E.) and Division of Urology, Department of Surgery (W. D. W. H.), Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

We have established an in vitro clonogenic cell assay for a class of anaplastic rat prostatic tumor cells (R-3327-At). We used collagenase to isolate these cells from transplanted solid tumors growing in the flanks of mature male Copenhagen rats and cultured them in α-minimal essential medium supplemented with 10% fetal calf serum. A linear relationship was obtained between the number of cells plated and the number of colonies formed. The addition of conditioned media (CM) prepared from R-3327-At tumor cell cultures to low density R-3327-At cell cultures produced larger colonies compared to those cultures to which we added either no CM or CM from Copenhagen rat skin fibroblasts. These data indicated that the growth-promoting activity present in the CM of R-3327-At cultures can increase the extent of proliferation, although it is not essential for its initiation. The addition of sera from rats bearing either the R-3327-At or the hormone-dependent (R-3327-H) tumor was "cytotoxic" to R-3327-At clonogenic cells. Not only were fewer colonies formed, but also individual colonies contained fewer cells when compared to control cultures to which normal rat serum was added. These findings are potentially important since they may reflect a host immune response to the tumor.

INTRODUCTION

The Dunning rat prostatic tumor (R-3327) is increasingly being considered the most relevant experimental tumor model for the study of human prostatic disease (3, 10, 11, 14, 15). It has been used to study the effect of hormones (1, 7, 10), and immunological factors (2, 5, 6) on prostatic tumor growth; in none of these reports, however, were responses directly on those cells responsible for the proliferative growth of the tumors. In addition to strong histological, biochemical, and histochemical similarities to human prostatic carcinoma, the hormone-dependent rat adenocarcinoma (R-3327-H) also has an anaplastic variant (R-3327-At) that corresponds to the rapidly growing tumor cell type in human prostatic disease. Smolev et al. (11) have demonstrated that R-3327-At is a hormone-independent tumor by showing that the tumor line grows equally well in male, female, and castrated male rats. In addition to strong histological, biochemical, and histocytological differences, this information has limited application to the understanding of the basic biology of the various subpopulations since it does not deal with the underlying complex interaction of proliferating, "resting," and dying tumor cells which accounts for the grossly different changes in tumor volume. Furthermore, it is imperative to "dissect" out the various populations of cells from a particular tumor type if one wishes to interpret and optimize the efficacy of tumoricidal therapies that operate at the cellular level. Of particular importance in this respect is the ability to measure the effectiveness of various therapeutic modalities directed primarily against those cells responsible for the growth of the tumor by cellular proliferation, i.e., the clonogenic cell population. For these reasons we have directed our efforts to first establishing optimal culture conditions that will permit us to assay for those cell progenitors capable of growing colonies of tumor cells in vitro from the R-3327-At tumor.

In this publication we report (a) the establishment of a linear assay for clonogenic R-3327-At tumor cells in liquid culture; (b) the successful retransplantation of R-3327-At cultured cells through their 30th passage in vitro into normal male Copenhagen rats; (c) the effects of CM from cultured R-3327-At tumor cells and Copenhagen rat skin fibroblasts on the clonogenicity of R-3327-At tumor cells; and (d) the effects of sera from normal rats and rats bearing either the R-3327-At or R-3327-H tumor on in vitro colony formation of R-3327-At tumor cells.

The abbreviations used are: CM, conditioned medium; α-MEM, minimal essential medium; α-modification; α-MOPS, α-minimal essential medium containing 2-N-morpholinopropane sulfonic acid; α-MEM-10, α-minimal essential medium containing 10% fetal calf serum; FCS, fetal calf serum.

1 This investigation was supported by Grant SPO1CA13053-05 and awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

Received March 27, 1978; accepted August 31, 1978.
MATERIALS AND METHODS

Animals

We used inbred Copenhagen rats that were either bred in our animal facility or obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, Md.

Tumors

The R-3327-At and R-3327-H tumors were originally obtained from Dr. D. S. Coffey (Johns Hopkins School of Medicine, Baltimore, Md.) and have been passaged in Copenhagen rats as a cell suspension and by trochar for the past 2 years in our facilities.

Preparation of Monodispersed Cell Suspensions

Mechanical Dispersion of Cells. Tumors were grown in the flanks of mature male Copenhagen rats and used when they were approximately 1 cm in diameter as measured by either a template (Rapidesign 2040) or calipers. Each rat was anesthetized [sodium pentobarbitol (60 mg/kg) i.p.,] and the tumor area was shaven and rinsed with 70% ethanol. The tumor was excised aseptically and rinsed free of blood with α-MEM (12). It was then cut into several pieces and examined for necrosis; only tumors free of necrosis were used. The tumor pieces were minced with scissors over stainless steel cloth (mesh 120; Ludlow-Fay
tor, St. Louis, Mo.) and washed thoroughly with α-MOPS, pH 7.2 (Nutritional Biochemicals Corp., Cleveland, Ohio). The bulk of the fibrous connective tissue was left behind on the screen. The resulting cell suspension was then further monodispersed by gentle pipetting, and the total and viable cells were counted.

Collagenase Treatment. Tumors were easily and rapidly cut into uniform 1-mm-thick sections with a polyacrylamide gel slicer (Hoefer Scientific Instruments, San Francisco, Calif.). The tissue sections were placed in α-MOPS containing various types and concentrations of collagenase (see “Results”) and incubated on a shaker platform (Eberbach Corp., Ann Arbor, Mich.) at 37° for 30 min. Following gentle dispersions by pipetting, the cells were decanted and washed in α-MOPS by centrifugation at 350 × g for 10 min at room temperature. Both total and viable cell counts were obtained.

Cell Counting

We counted the total and viable cells on an electronic particle counter (Celloscope; Particle Data, Chicago, Ill.) with the cetrimide-pronase technique (13). Briefly, to obtain the total number of nucleated cells, we added 25 μl of cell suspension to 10 ml of cetrimide solution [hexadecyltrimethylammonium bromide (30 mg/ml); Baker] which is a plasmacytolytic agent that rapidly yields a suspension of cell nuclei. For counting the number of viable nucleated cells, 25 μl of cell suspension were added to 1 ml of pronase (2 mg/ml) plus 1 ml of 0.9% NaCl solution and incubated for 10 min at 37° to digest nonviable cells. Following the addition of 8 ml of cetrimide, the cell nuclei were counted.
washed in citrate-phosphate buffer (pH 5.8) for 1 min, followed by a 2-min rinse in running tap water, then air-dried and mounted in Eukitt.

**RESULTS**

**Histology of R-3327-At Tumor.** The R-3327-At is an encapsulated tumor, and the blood supply is restricted to an area immediately beneath the capsule. Capillaries in the center of this tumor are sparse. Histology is anaplastic (Fig. 1) with no acini that is characteristic of both normal prostate and R-3327-H tumor. The cells are densely packed with round nuclei frequently with more than one nucleol. Recently, Issacs *et al.* (4), on the basis of enzyme profiles, concluded that R-3327-H and R-3327-At tumor lines are of prostatic origin and the tumor cells are of epithelial nature.

**Tumor Monodispersion by Collagenase.** Since no data existed to our knowledge concerning the enzymatic digestion of the R-3327-At tumor and its behavior in cell culture, we undertook a preliminary experiment prior to establishing the clonogenic assay to optimize the use of collagenase and to characterize its effects on the growth, morphology, and tumorigenicity of the cultured tumor cells. As can be seen from Table 1, the total number of cells decreased with increasing concentration of collagenase, regardless of its source. The number of viable cells determined by the pronase-cetrimide technique also decreased with increasing collagenase concentration, although not to as great an extent as total cells, so that the percentage of viability increased 2- to 3-fold for the 3 collagenase solutions tested. We chose Sigma type 5 collagenase at a concentration of 0.1 mg/ml for subsequent experiments since this treatment resulted in no growth of fibroblasts. It was subsequently found that 30 min of incubation was optimal since extending the incubation period beyond that time resulted in fewer total and viable cells.

**Growth of the R-3327-At Tumor Cells in Culture.** Cells obtained by collagenase treatment of tumors (as described previously) were plated in 5 ml of α-MEM-10 in 60-mm culture dishes at cell concentrations of from 2 to 9 x 10^6 total cells. After various periods of incubation, dishes were removed and rinsed with α-MEM, and the cells were collected by trypsinization for counting. The growth curves presented in Chart 1 indicate a rapid period of cell proliferation commencing 1 to 2 days after the initial plating, with a cell doubling time of about 24 hr; confluency was attained by 7 to 10 days in culture at saturation densities of between 2 to 9 x 10^6 cells per culture. The morphology of the cells remained similar to that observed after initial monodispersion of the tumor (Fig. 2).

**Induction of Tumors in Vivo by Cultured R-3327-At Cells.** Weekly increases of approximately 100- to 200-fold were observed in R-3327-At cell cultures initially plated at 1 x 10^6 cells/10 ml/flask. To date we have carried this line through to the 30th passage with no change in the rate of cell proliferation *in vitro*. Each week when cultures were passaged, aliquots of 1 x 10^6 cultured cells were injected s.c. into the flanks of male Copenhagen rats (one injection per rat), and the time for the resultant tumor to grow to 1 cm diameter was noted. Tumors from early passage cultures (<10 passages) were palpable within 5 to 8 days and grew to 1 cm diameter in all recipient rats within 10 to 15 days (Chart 2). When later passage cells were injected, there was an increase in the time required to form a 1-cm tumor so that by the 30th passage, it took 28 days for all the recipients to attain a tumor of this size. Histology of all the tumors was similar.

**Comparison of R-3327-At Cells and Copenhagen Rat Skin Fibroblasts.** For demonstration that the cells prepared and cultured from R-3327-At tumors were of tumor origin and were not fibroblasts that may be present in the tumor cell population, we established a line of Copenhagen rat skin fibroblasts from the flank dermis for comparison with R-3327-At tumor cells in terms of their morphology, sensitivity to collagenase treatment, growth kinetics in liquid and plasma cultures, and “tumorigenicity” when retransplanted to Copenhagen rats.

On the basis of morphology, the 2 cell lines were readily distinguished from each other (Fig. 3). The tumor cells were

---

**Table 1**

Preparation of R-3327-At cells with collagenase

<table>
<thead>
<tr>
<th>Type of collagenase</th>
<th>Concentration (mg/ml)</th>
<th>Cells/g tissue (×10^-4)</th>
<th>Total</th>
<th>Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worthington (CLS-2EA)</td>
<td>0.01</td>
<td>11.8</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.9</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.8</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Sigma type 1 (C-0130)</td>
<td>0.01</td>
<td>6.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.6</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Sigma type 5 (C-9763)</td>
<td>0.01</td>
<td>11.3</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6.7</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.2</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>
B. R. Rao et al.

that were distinct from those of skin fibroblasts.

**In Vitro Clonogenic Assay for R-3327-At Tumor Cells.**

For demonstration of the relationship between the number of viable cells plated and the number of colonies formed in cultures, R-3327-At cells were plated at low cell density in α-MEM-10 in 60-mm culture dishes. Following 7 days of incubation, methanol fixation, and staining with hematoxylin, dishes were scored for colony formation (>50 cells were considered to constitute a cell colony). As shown in Chart 3, there was a linear relationship between the number of viable cells initially plated and the number of colonies formed after 7 days in culture. Figure 4 is a photomicrograph showing the actual distribution of colonies on a stained dish for the highest and lowest cell doses. Upon examination at higher magnification, we confirmed that the colonies were composed of cells morphologically identical with those shown in Fig. 2.

**R-3327-At Tumor Cell Colonies in Plasma Culture.** The clonogenic assay described previously was also applied to the growth of clonogenic cells in semisolid plasma cultures and resulted in an extensive degree of proliferation. The ability of the R-3327-At cells to form colonies in plasma culture with a plating efficiency similar to that determined in liquid cultures demonstrated that these clonogenic cells did not require attachment to a surface to proliferate.

**Effect of CM on R-3327-At Colonies.** It was clear from the broad range of colony sizes that there existed a spectrum of proliferative activities among clonogenic cells; colonies ranged from less than 50 cells to greater than 500 cells per colony. Therefore, we examined the effect of adding CM obtained from cultures of R-3327-At cells (R-3327-At-CM) or rat skin fibroblasts (RSF-CM) to a final concentration of 10% to increasing concentrations of R-3327-At cells. Although we obtained identical cell dose-response curves regardless of the type of CM used, the distribution of colony sizes was quite different, as shown in Chart 4. The addition of 10% R-3327-At-CM resulted in larger colonies, especially

[Chart 2: Tumorigenicity of passaged R-3327-At cells as a function of the number of weekly passages in culture and measured in terms of the time taken to form 1-cm tumors when 10⁶ cells were retransplanted s.c. into the flanks of normal male Copenhagen recipients. Number of in vitro passages: primary tumor (●); 1-5 (○); 5-10 (△); 15 (▲); 20 (□); 25 (▲); 30 (▲). Numbers in parentheses, total number of tumors transplanted in each group.]

[Chart 3: Relationship between the number of viable R-3327-At cells plated and the resultant number of cell colonies (>50 cells) formed after 7 days in culture. Points, average of 4 dishes; bars, ± S.E.]
in those cultures initially seeded with larger numbers of cells. When either RSF-CM or no CM was added, fewer larger colonies were observed with the majority of colonies being either small or medium sized.

**Effect of Rat Serum on the Growth of R-3327-At Colonies.** R-3327-At tumor cells were plated at a concentration of $1 \times 10^3$ cells in 5 ml $\alpha$-MEM-10 in 60-mm culture dishes. This medium was then further supplemented with 10% serum from normal male rats (N serum) or rats bearing either the R-3327-At tumor (At serum) or the R-3327-H tumor (H serum). Addition of either At or H serum completely suppressed colony growth (Figure 5) whereas $\alpha$-MEM-10 plus 10% N serum supported prolific colony growth that was reflected in both a larger number of colonies as well as colonies that contained more cells than that observed previously when FCS was the sole source of serum. A similar difference was observed when the sera were used in the absence of FCS and in concentrations as low as 2%.

**DISCUSSION**

The development and use of a clonogenic cell assay in *vitro* in a number of experimental solid tumor models has contributed significantly to our understanding of the cellular kinetics of tumor growth and, in particular, the response to anticancer agents of those cells capable of extensive proliferation. This study represents the first report of an *in vitro* clonogenic cell assay established for an experimental prostatic tumor model, the anaplastic R-3327-At tumor in Copenhagen rats. It indicates that a linear relationship exists between the number of colonies (clonogenic cells) obtained after 7 days of culture and the number of tumor cells initially plated. In addition, the ease of establishing and analyzing for colony formation makes this assay an attractive approach to the measurement of the proliferative activity of the R-3327-At tumor and constitutes the first demonstration of a direct, cellular assay for a subpopulation of clonogenic cells responsible for the proliferative growth of the tumor.

There are several factors that merit discussion as to the applicability and interpretation of this assay when applied to R-3327-At cells that are capable of extensive proliferation. First, although the R-3327-At is a rapidly growing tumor, only a small fraction of cells from the tumor were clonogenic. This may to some extent be due to the method used in preparing the monodispersed cells, which has been shown to be an important consideration in a number of other transplantable tumors, as discussed by Noel et al. (9). It is conceivable that the judicious use of other enzymes, such as trypsin or pronase, or some optimal combination of these agents with collagenase may result in a higher plating efficiency and thus a larger fraction of clonogenic cells than reported here. Of course, this limitation is circumvented in assays in which the same dispersal technique is used, and only relative changes in the number of clonogenic cells are of importance.

Second, it was imperative to demonstrate that the colonies cultured were composed of tumor cells and not fibroblasts resident in the tumor that might also be able to grow under our culture conditions. For exclusion of such a possibility, several different parameters were measured to distinguish tumor cells from fibroblasts. Morphological differences between the R-3327-At cells cultured in *vitro* and skin fibroblasts from normal male Copenhagen rats were readily apparent. Furthermore, collagenase treatment similar to that used to monodisperse the R-3327-At cells prevented subsequent *in vitro* growth of primary skin fibroblasts from fresh explants. In addition, if one assumes that skin and tumor fibroblasts proliferate at the same rate, then they both do so at a much lower rate (a 10- to 20-fold increase in 7 days) than that characteristic of the R-3327-At cells, which proliferated 100- to 200-fold in 7 days. It would not be surprising if fibroblasts present in the tumor had growth kinetics and morphological characteristics different from those displayed by normal skin fibroblasts; however, at this time it seems reasonable to compare the kinetics of tumor cells and skin fibroblasts in primary cultures. Nevertheless, in long-term cultures one must consider the possibility of spontaneous transformation of the fibroblasts with each cell passage and a corresponding change in their cell kinetic properties. Furthermore, our attempts to grow skin fibroblasts in plasma culture have been unsuccessful, indicating an additional difference between fibroblasts and tumor cells since the latter proliferated well in the absence of FCS and in concentrations as low as 2%.
of surface adherence.

Finally, R-3327-At cells passed in vitro were capable of forming a tumor when retransplanted into animals. Skin fibroblasts failed to form a tumor, even when maintained in vitro by weekly passages for a sufficiently long time during which an increasing possibility of spontaneous transformation would be expected. From the previous differences, we feel that the R-3327-At cell cultures probably do not contain a significant fibroblast component.

Having established both a long-term culture of the R-3327-At cells and the clonogenic assay, we felt it important to demonstrate the following: (a) the ability of cultured cells to form anaplastic tumors when retransplanted in vivo; and (b) the pattern of tumor regrowth as a function of time in culture, since no data to our knowledge existed in the literature concerning the relationship between tumor formation by cultured R-3327-At cells and the number of passages they had undergone in vitro. Although our long-term culture is in its 30th passage, it is still capable of forming a 1-cm tumor in 100% of cases in normal male Copenhagen rats injected with $1 \times 10^6$ cultured cells. However, the distinctly slower pattern of tumor growth from the tenth passage on, indicates that cells passed in vitro ultimately may lose their tumorigenicity. This may reflect a host-immune response directed against those cells that may be altered by prolonged exposure in culture in such a way as to inhibit their growth and correspondingly prolong the time to tumor "take." Regardless of the mechanism responsible, these data clearly indicate that R-3327-At cells derived from long-term cultures have "tumorigenic" properties that are different from those exhibited by the original transplantable tumors and that these altered properties are manifested as early as the tenth weekly passage in vitro. A study of long-term cultures of R-3327-At cells in the absence of in vivo data on their tumorigenicity is of limited value; such a study may even be misleading if one attempts to extrapolate in vitro data to predict tumor growth in vivo. However, our demonstration that R-3327-At cells can be cultured for up to 10 weekly passages without appreciable loss of tumorigenicity is important, for it shows that this particular tumor can be passaged back and forth between the host and the Petri dish. This characteristic makes it an extremely attractive tumor cell model system for investigating the mechanism of action of various antitumor therapies, since it permits cellular quantitation of tumor proliferation under 2 quite distinct growth conditions.

The addition of R-3327-At-CM (in contrast to either no addition of CM or addition of RSF-CM) resulted in a significant shift to larger numbers of cells per colony; this may signify that R-3327-At cells produce a factor(s) in culture that increases the number of cell divisions. The factor(s) is responsible, these data clearly indicate that R-3327-At cells passaged in vitro by weekly passages for a sufficiently long time during which an increasing possibility of spontaneous transformation would be expected. From the previous differences, we feel that the R-3327-At cell cultures probably do not contain a significant fibroblast component.

Hofstein et al. have shown that increases the number of cell divisions. The factor(s) is responsible, these data clearly indicate that R-3327-At cells passaged in vitro by weekly passages for a sufficiently long time during which an increasing possibility of spontaneous transformation would be expected. From the previous differences, we feel that the R-3327-At cell cultures probably do not contain a significant fibroblast component.

REFERENCES


Clonogenic Prostate Cell Assay


---

Fig. 1. Histology of R-3327-At shows the anaplastic nature of the tumor. Sections were 10 μm. H & E, × 63.

Fig. 2. Photomicrograph of R-3327-At cells cultured for 8 days. Hematoxylin, × 400.
Fig. 3. Photomicrograph comparing the morphology of the R-3327-At tumor cells (a) and the Copenhagen rat skin fibroblasts (b) in culture. Hematoxylin, x 250.
Fig. 4. Photomicrograph showing 7-day-old colonies of R-3327-At cells. Left, 313 viable cells initially plated; right, 5000 cells.

Fig. 5. Effect of the addition of 10% of either N-serum (left) or At-serum (right) on the ability of R-3327-At cell cultures (10⁶ cells in 5 ml α-10) to form cell colonies after 8 days in culture (see text for details).
Establishment and Characterization of an *in Vitro* Clonogenic Cell Assay for the R-3327-At Copenhagen Rat Prostatic Tumor
