Histological Comparison of the Growth of Rat Bladder Carcinoma R-4909 Observed for Two Years \textit{in Vitro} and \textit{in Vivo}\textsuperscript{1}

Keiji Toyoshima\textsuperscript{2}, Nabil Abaza, and Joseph Leighton\textsuperscript{3}

Cancer Bioassay Laboratory, Department of Pathology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

SUMMARY
A transplantable bladder tumor (Chapman R-4909) of the rat, when first received in our laboratory, grew with a complex histopathology. The predominant component was transitional cell carcinoma, but there were foci of keratinization, including pearl formation, and foci of a less well-defined cystic appearance.

We report here observations made during the first 2 years of an ongoing study on the divergent histopathology of R-4909 under several conditions of propagation. During the entire period, the tumor has been maintained by serial passage in rats (Fischer 344) and by serial passage \textit{in vitro}. At intervals, cells of the tissue culture series were inoculated into rats to compare the histopathology of animal- and culture-passed strains. We obtained several clones from the stock cultures and these also were maintained continuously \textit{in vitro}. At intervals, cells from two of these lineages, clone A and clone B, were inoculated into rats.

After 2 years, cells maintained in stock culture, on injection into new rats, produced growths similar to the original in that all three epithelial patterns, transitional, squamous, and adenomatous, were perpetuated. In contrast, the tumor passed exclusively \textit{in vivo} lost its squamous component completely. It became anaplastic, with tissue architecture almost entirely adenomatous and cystic. Unlike the stock tissue culture line, the clonal isolates following prolonged culture produced adenomatous tumors only.

In a related preliminary study, we inoculated into rats R-4909 cells that had been cultivated for up to 2 months under aerobic and anaerobic conditions. Tumors grew in most of the animals, and those of the aerobic group were more cystic than the others.

INTRODUCTION
Neoplasms arising in urothelium appear variously as transitional cell carcinoma, squamous cell carcinoma, or adenocarcinoma. In the United States, most bladder cancers are of the papillary transitional cell type; in Egypt, where bladder cancer is the most common tumor of men, most are squamous cell carcinomas. Adenocarcinomas are least frequent and often have, conceptually and clinically, an association with abnormalities of development (1). Tumors that are predominantly transitional cell carcinoma may have foci of squamous features or of gland-like structures, or both. Identification of the developmental mechanisms whereby such histotypic aberrations arise may be an academic exercise, since these foci are not now believed to be clinically significant.

For controlled laboratory study, transplantable urothelial carcinomas of laboratory animals and continuous cell lines derived from rodent and human bladder carcinomas provide the closest available approximation to bladder cancer in the patient. We have found that a squamous cell carcinoma of the rat bladder, NBT-II, produced abundant keratin pearls in control medium, but grew with complete inhibition of keratin formation in the presence of low concentrations of vitamin A (6). At the time the vitamin A studies were initiated, we undertook some baseline studies of a microscopically more complex transplantable rat tumor, the Chapman R-4909. This tumor, when first received from Dr. Chapman, was predominantly of the transitional cell type, but with foci of keratinization, including pearl formation, and foci of a less well-defined cystic appearance. The tumor grows readily either on transplantation in Fischer 344 rats or on cultivation \textit{in vitro}. It can be easily propagated indefinitely in culture and in laboratory rats.

We have undertaken a series of studies on R-4909 in order to understand better the mechanisms responsible for, and the significance of the several morphological patterns of, the tumor. In this first report, we describe the morphological drift, over a 2-year period, of Chapman R-4909 in 2 situations: (a) continuous passage in rats and (b) consecutive passage in culture. Cultured cells were evaluated by inoculating them into rats and studying the histopathology of the resulting tumors. We also explored the possibility that divergence in histopathology might appear when inocula in rats were selected from the aerobic and anaerobic parts of meniscus-gradient cultures after 2 months \textit{in vitro}.

MATERIALS AND METHODS

**Animals.** Fischer 344 male rats, weighing approximately 150 g each, were obtained from the NIH. All rats were placed
Cancer Cells (R-4909) In the Rat. Dr. Warren H. Chapman (Department of Urology, University of Washington, Seattle, Wash.) graciously provided us, in February 1973, with carcinoma line R-4909 (7) as a s.c. tumor growing in a Fischer 344 rat. Since then, the tumor has been maintained continuously in the same strain of rats. About every 1 to 3 months, excised tumor tissue was finely minced and injected s.c. into new rats.

Cancer Cells in Culture. Continuous passage in tissue culture was initiated from a s.c. transplant in the rat in April 1973. The medium used for the initial tissue culture passages consisted of 30% fetal calf serum in Waymouth's MB 752/1 medium (Microbiological Associates, Inc., Bethesda, Md.) containing L-glutamine (2 mm), kanamycin (50 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and Fungizone (1 μg/ml). About 6 months later, the basic commercial medium was changed to Eagle's MEM4 (Microbiological Associates, Inc., Bethesda, Md.), since we found that the quality and rate of growth were the same with either one. As a further economy, we reduced the concentration of serum in 2 steps using, in the initial preparation of subcultures, 20% fetal calf serum in Eagle's MEM immediately after suspension with EDTA-trypsin (Grand Island Biological Co., Grand Island, N. Y.). Once adhesion to glass had been observed in the subculture bottles, we used 5% fetal calf serum in Eagle's MEM to support further growth. During the transition period, we saw minimal differences in the appearance of the cells between those fed on Waymouth's and Eagle's MEM.

Assay of Cultured Cells. From time to time, a cell suspension of about 3 x 10^4 cells in 1 ml of serum-free medium was inoculated s.c. into rats. After several weeks, when a tumor nodule of sufficient size had formed, the rat was killed and the tumor was prepared for histology. Tumor tissue was fixed in 10% formalin, and multiple paraffin sections were cut into the aerobic and anaerobic parts, each about 7 mm high. Each segment was inoculated s.c. into the back of a rat. In the controls, the horizontal cultures, the Gelfilm was placed with 2 ml of fresh medium, as required by changes in pH. In the vertical cultures, 10 mm of the Gelfilm protruded above the surface of the medium, and 15 mm were immersed. The Gelfilm coverslips were removed and prepared for inoculation into rats at 4, 6, and 8 weeks. One or 2 cultures from each group at each of the 3 intervals were fixed in formalin and stained with hematoxylin and eosin.

Cell Cloning. A 2-step cloning method was devised. Plastic coverslips, 0.5 x 3.0 cm, (LUX Scientific Corp., Thousand Oaks, Calif., distributed by Microbiological Associates, Inc., Bethesda, Md.) were cut aseptically with sterile pinking shears to produce serrations on both edges of the plastic rectangle. Cultures were dispersed with EDTA-trypsin, and the cells were suspended in 20% fetal calf serum-Eagle's MEM at a concentration of approximately 10^4 cells/ml. One ml of this dilute cell suspension was inoculated into each of a series of small Leighton tubes containing a serrated plastic coverslip. The tubes were incubated overnight, and the coverslips were then transferred to fresh tubes with fresh medium. The serrated coverslips were carefully examined with an inverted microscope with phase optics to identify single cells attached in complete isolation from others on the surface of one of the points. These were carefully mapped. Colony formation starting from single cells was observed daily under the microscope.

As the 2nd step in the procedure, a segment of the cover-

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4 The abbreviation used is: MEM, minimal essential medium.
RESULTS

Tumor as Received from Dr. Chapman. The tumor, located s.c. in the back of the rat, appeared to be surrounded by a thin fibrous capsule but was growing invasively. No metastases were found. The tumor was composed primarily of many elongated masses of transitional cell carcinoma in which the cancer cells were in no particular pattern. Necrosis was scanty. The cells at the periphery of the masses had a palisade arrangement. Stroma separating the islands of tumor cells was cellular, and newly formed fine collagen was prominent. Some of the solid masses of tumor cells had foci of squamous features, including keratinization. In other areas, but very infrequent, were cystic formations. Typical well-defined adenomatous patterns were not observed (Fig. 1).

Appearance in Tissue Culture. The tumor taken from the rat grew in vitro in a predominantly epithelial pattern. During 2 years of growth in continuous culture, we observed the consistent presence of randomly distributed mononucleated giant cells and the infrequent occurrence of multicellular epithelial hemicystic structures. The latter resembled structures seen previously in a dog kidney cell line (2). Hemicysts were observed with greatest frequency in 1 clonal population, clone B. Details on the appearance of the cultures through time in the parent line and in the clones, and, in particular, the conditions of cultivation in which bladder carcinoma hemicysts were seen with high frequency, are described in the 2nd paper of this series (5). In clone B, a coincidence of hemicysts in vitro and of adenomatous features in vivo was observed as time passed. We think that they are related.

Appearance after Serial Passage in Rats. There were no discernible changes when the original tumor was compared with growth after the 1st few passages. After 18 months (the 12th passage), the tumor cords, columns, and masses were larger and more irregular than in the donor tumor. Squamous foci were very infrequent, but cystic changes had increased. These changes were observed in many masses of tumor cells with and without accompanying necrosis. Distinct adenomatous changes were also found.

After 28 months and 17 passages, the histopathology of the tumor was further removed from the donor tumor. Squamous changes could not be identified. Much of the tumor consisted of a relatively haphazard arrangement of atypical and anaplastic transitional cells. Zones of tumor necrosis were large and numerous. Adenomatous foci were very frequent, and some were highly organoid (Fig. 2; Table 1).

Inoculation into Rats of Cells from Horizontal Monolayer Cultures. When cells that had been in culture for 3 months were inoculated in rats, the tumors that developed were predominantly of transitional cell type. Distinct foci of squamous epithelium with keratinization and of adenomatous growth were also found. After 5 months, the 3 patterns of the tumor appeared in equal amounts. After longer periods, the squamous pattern was more prominent and mature keratin became a regular feature. This was evident after 17 months and even more so after 26 months (Fig. 3; Table 2).

The tumors produced following injection of clonal isolates differed from those seen following injection of the stock strain, especially after 2 years. Clone A and clone B, injected in rats 2 months after their isolation, produced tumors of mixed morphology similar to those seen from the

### Table 1

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<th>Histological pattern</th>
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<td>+</td>
<td>±</td>
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<tr>
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<td>-</td>
<td>±</td>
<td>±</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>+</td>
<td>++</td>
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<td>++</td>
<td>++</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
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*a, ++, prominent; +, present; ±, rare; −, none.

### Table 2

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\(^a\) Clones A and B each arose from the parent line when the latter had been in culture 17 months.

\(^b\) See explanation of symbols in Table 1.
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stock line. After 9 months in culture following cloning, a total of 26 months in vitro, tumors produced in rats by the cloned lines were almost exclusively adenomatous (Fig. 4; Table 2).

Segments of Meniscus-Gradient Cultures on Gelfilm Inoculated in Rats. In fixed stained preparations of horizontal and vertical Gelfilm cultures, tumor giant cells were distributed in a random pattern. Even after 8 weeks no concentration of giant cells just below the gas phase was seen on the gelatin substrate, in contrast to our consistent observation of such concentrations near the gas phase when the cells grew on glass coverslips.

Tumors grew in most of the inoculated animals. 26 of 31 from the aerobic segments and 17 of 28 from the anaerobic. The tumors were allowed to reach a large size, to the point of ulceration of the skin, before the animals were killed and the tumors examined. In gross examination of cross-sections of the tumors, areas of necrosis and foci of fluid-filled cysts were seen. No metastases were found either on gross examination or on histological examination of various organs.

Microscopically, the tumors from the gradient were different from the original donor tumor. They showed a decrease in the predominance of the transitional cell pattern, a slight increase in squamous areas, and a greater increase in cystic glandular areas. Cells from the aerobic and anaerobic ends of the gradient gave rise to tumors with similar mixtures of the 3 patterns, except that there was a greater proportion of cystic foci in the tumors derived from aerobic inocula of 8-week-old gradient cultures.

DISCUSSION

The data presented here indicate that the Chapman tumor is a worthy candidate for study of the determinants and regulators of histopathological identity of bladder cancer. Four populations of carcinoma cells, all derived from a single mass of transplantable tumor, were maintained independently for more than 2 years. Divergence of pattern was observed after long intervals when rats received inocula from the original donor tumor. They showed a decrease in the predominance of the transitional cell pattern, a slight increase in squamous areas, and a greater increase in cystic glandular areas. Cells from the aerobic and anaerobic ends of the gradient gave rise to tumors with similar mixtures of the 3 patterns, except that there was a greater proportion of cystic foci in the tumors derived from aerobic inocula of 8-week-old gradient cultures.

REFERENCES


Fig. 1. The appearance of the R-4909 tumor obtained from Dr. Chapman. The pattern was almost completely that of a transitional cell carcinoma in which there were rare foci of keratinization and of cyst formation. The predominant transitional cell pattern is seen in most of the figure. Cystic areas are seen at the bottom. H & E, × 112.

Fig. 2. The appearance of the tumor after serial passage in rats for over 2 years. The pattern is one of anaplastic tumor cells arranged as a papillary adenocarcinoma. No areas of squamous appearance were found. H & E, × 112.

Fig. 3. The appearance of the tumor that developed in the rat following inoculation of Chapman tumor cells that had been passed in vitro for over 2 years. There is abundant squamous cell carcinoma and keratinization interspersed with cystic cavities lined by a neoplastic glandular epithelium. H & E, × 112.

Fig. 4. The appearance of the tumor that developed in the rat following inoculation of clone B, 9 months after clonal isolation. The tumor is composed exclusively of adenocarcinoma arranged as many small neoplastic glands. H & E, × 112.
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