ABSTRACT

Human bladder cancer from cystoscopic biopsies and from established cell lines was transplanted into mice that were immune suppressed by thymectomy plus sequential treatment with 1-ß-D-arabinofuranosylcytosine and whole-body irradiation. Each of four established human bladder cancer cell lines generated transplantable tumors in these mice, and some of the mice developed pulmonary metastases. Eight of 33 cystoscopically obtained biopsies of transitional cell carcinoma and one from a metastatic site led to xenografts that grew progressively, and some of these have been transplanted and/or have generated cell lines in vitro. Xenografts grew after a lag period of 0 to 32 weeks and had doubling times of 9 to 30 days. All of those examined histologically were consistent with transitional cell carcinoma, but some of the xenografts became more or less well differentiated in first and subsequent passages. The immune-deprived mouse is an alternative host to the nude mouse for generation of human tumor xenografts and may be a useful model for study of biological properties and therapeutic response of human bladder cancer.

INTRODUCTION

Xenografts of human tumors may provide a useful model for study of their biology and for selection and testing of anticancer drugs. Congenitally athymic "nude" mice have been the most frequent recipients of xenografts of human solid tumors, and the success of xenografting depends on the type of tumor, the strain and state of health of the nude mice, and the criteria used to describe a successful transplant. Recently, Steel et al. (20) and others (1, 2, 4, 10, 18) have reported the successful xenografting of several types of human tumor into immune-deprived conventional CBA mice. One method of immune depri- vation has used thymectomy, followed by sequential treatment with ara-C and whole-body irradiation. Immune-deprived mice were reported to be sturdier than nude mice, to allow a higher rate of acceptance of human tumor xenografts, and to allow the development of metastases (4, 20).

Bladder cancer is increasing in incidence, and optimal treatment at several stages of disease is controversial. However, there have been few attempts to xenograft human bladder cancer despite ready access to biopsy specimens obtained through the cystoscope. Using nude mice, 3 studies have shown 20 to 40% incidence of tumor growth (but no metastases) after implantation of biopsies of transitional cell carcinoma (16, 22), while in another study there was growth of 3 established cell lines of human transitional cell carcinoma and 2 of them metastasized to lymph nodes and lungs (12). Franks et al. (5) have also reported growth and transplantation of a single human bladder tumor in immune-deprived conventional mice. A less expensive and more easily maintained alternative to the nude mouse for xenografting of human bladder cancer might facilitate study of the biology of the disease in its different stages and might allow insight into its response to radiation and anticancer drugs. We therefore report a study of trans- plantation of human bladder cancer into immune-deprived conventional mice.

MATERIALS AND METHODS

Immune Deprivation of Mice. Male CBA/CAJ mice were obtained at 3 to 4 weeks of age from The Jackson Laboratory and were thymectomized 1 week later. Mice were anesthetized with Avertin (300 mg tribromoethanol per kg), and the thymus was removed through a small suprasternal incision using a Pasteur pipet attached to a suction pump. Approximately 100 mice could be thymectomized by 2 investigators in 4 hr with an average mortality of 10%. Three to 6 weeks later, mice were treated with ara-C (200 mg/kg, i.p.) followed by 8.5 Gy whole-body irradiation (137Cs; dose rate, 0.75 Gy/min). Pretreatment with ara-C protected the mice from an otherwise lethal dose of radiation (14). Less than 1% of mice died from this procedure. Immune-suppressed animals were housed in conventional shoebox cages, with sterilized bedding, and received acidified water and standard (unsteri- lized) food.

In a few experiments, we used athymic RNC-nu/nu and C57BL/6-nu/nu mice which were bred in our animal colony (6). These mice were maintained in shoebox cages with paper covers within laminar air flow rooms and received sterilized food.

Established Cell Lines. While establishing techniques for xenografting of primary bladder cancer, we experimented with 4 cell lines of human bladder cancer that have been adapted for growth on plastic. The cell lines (RT-4, T-24, MGH-U1, and MGH-U2) were kindly supplied by Dr. G. Prout and his colleagues, Urology Research Laboratory, Massachusetts General Hospital. Characteristics of all of the cell lines have been described elsewhere (7, 9, 11, 13, 17). In our laboratory, they were maintained as monolayers in plastic flasks in a-medium supplemented with antibiotics and 10% fetal calf serum (21). We have obtained karyotypes of all 4 lines and confirmed their human origin; however, all are aneuploid and have chromosomal abnormalities. Electron microscopic examination of the cell lines failed to reveal the presence of viruses.

For implantation, cells were harvested with 0.05% trypsin and 0.02% EDTA, and 0.1 ml of serial dilutions (10^4 to 10^6 cells) was injected into the right thigh muscle of immune-deprived or nude mice. Suspensions containing less than 2 x 10^6 cells were admixed with 10^6 lethally irradiated cells of the same type prior to i.m. injection. Suspensions of the cell lines containing 10^6 cells were also injected i.v. into tail veins of immune-deprived mice in an attempt to generate metastatic tumors (23).

Transplantation of Tumor Biopsies. We obtained permission to use biopsies from patients with bladder carcinoma who underwent diagnostic or therapeutic cystoscopy in the Princess Margaret, Wellesley, or Women's College Hospitals, Toronto, Canada. Part of each biopsy was fixed in formalin for histopathology, and the remainder was placed in sterile 0.9% NaCl solution and was divided for s.c. implantation of pieces and for production of a cell suspension. For implantation of

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1 Supported by Research Grant CA 29526 from the National Cancer Institute through the National Bladder Cancer Project.
2 To whom requests for reprints should be addressed.
3 The abbreviation used is: ara-C, 1-ß-D-arabinofuranosylcytosine.
Received November 23, 1981; accepted June 3, 1982.
tumor pieces, biopsies were cut into 2- to 3-mm cubes and inserted through small dorsal midline incisions into one or both flanks (depending on amount of tissue available) of 5 to 6 anesthetized immune-deprived mice. Tissue was processed as soon as possible after removal from the patient and was never stored overnight. When adequate tissue was obtained for production of a single-cell suspension, at least $10^6$ cells were implanted i.m. into 5 to 6 mice, and cells were also assessed for in vitro growth in semisolid medium by Dr. R. N. Buick (3).

For generation of a suspension of single cells, the biopsy was placed in medium + 10% fetal calf serum, teased apart with needles, and then passed through successive smaller bore needles. If cell clumps were still evident, these were separated from the single cells by allowing them to fall through 7% bovine serum albumen. The cell suspension was then retrieved from the top of the bovine serum albumen, washed and resuspended in medium to a concentration of $10^8$ to $5 \times 10^5$/0.1 ml, and injected into the right thigh of immune-deprived mice.

Estimation of Tumor Volume. Following transplantation of biopsies from primary tumors, all mice were observed weekly, and animals without tumors were kept for at least 1 year. Animals implanted with established cell lines were observed until their tumors grew to a mean diameter of 1.5 cm or for at least 4 months in the absence of tumors.

Animals bearing tumors on their flanks were shaved, and perpendicular diameters were measured with calipers at weekly intervals. To avoid bias, measurements were made without knowledge of previous estimates. Tumor volume was estimated from the formula

$$V = \frac{1}{6 \pi} (d_1 d_2)^{3/2}.$$ 

The diameter of i.m. tumors growing in the leg was estimated by passing the leg through graduated circular holes drilled in Lucite. Tumor weight was then estimated from an empirically derived calibration curve which related tumor weight with diameter of the tumor-bearing leg.

Examination and Transplantation of Xenografts. Mice were killed when their xenografts attained a volume of 1.5 to 2.0 cu cm. They were examined for presence of metastases, and pieces of lung, liver, spleen, and the tumor were fixed in formalin. Thin paraffin sections were then cut and stained with hematoxylin and eosin for histological analysis. Pieces of tumor were also transplanted into other immune-deprived mice, and cell suspensions were prepared for i.m. implantation and for in vitro cloning in semisolid media.

RESULTS

Transplantation of Cell Lines. Each of the 4 cell lines grew as xenografts in immune-deprived mice (Table 1). Transplantation of $10^6$ cells gave close to 100% incidence of tumors, and all cell lines grew exponentially with doubling times that were usually in the range of 1 to 2 weeks (Chart 1). The number of cells required to give 50% tumor "takes" was about $10^4$ (RT-4), $5 \times 10^4$ (T-24 and MGH-U1), and $<5 \times 10^5$ (MGH-U2). The mortality of immune-deprived mice was less than 1% during the 4-month period of observation.

Three of the cell lines were also implanted into 2 strains of nude mice. Up to $2 \times 10^5$ RT-4 or T-24 cells were implanted in a total of 4 groups each containing 6 to 7 RNC-nu/nu mice. Fifty % of the mice died at 3 to 6 weeks after tumor implantation, and there was no tumor growth in the survivors. We also implanted $10^5$ RT-4 or MGH-U1 cells into 3 groups containing 5 to 8 C57BL/6-nu/nu mice. There was no mortality, and tumors were generated in 50% of the mice.

Histological examination of xenografts from all 4 cell lines revealed anaplastic carcinomas (Fig. 1A). One of the lines (RT-4) led to pulmonary metastases following i.m. implantation in immune-deprived mice (Fig. 1B), and it is noteworthy that the same cell line did not give metastases in another study using nude mice (12).

The cells of the i.m. implants and lung metastases are similar

![Chart 1. Growth curves for xenografts of 4 established cell lines of human bladder cancer after i.m. implantation of $10^6$ cells. For each cell line, arrows indicate 10 days after implantation. Points, means for groups of 5 mice; bars, S.E.](image-url)

### Table 1

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>Cell no. implanted</th>
<th>Groups of mice with xenografts</th>
<th>Lag period (wk)</th>
<th>Doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Established cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-4</td>
<td>$\geq 2 \times 10^3$</td>
<td>16</td>
<td>1-3</td>
<td>10-20</td>
</tr>
<tr>
<td>T-24</td>
<td>$\geq 10^6$</td>
<td>7</td>
<td>1-2</td>
<td>7-10</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>$\geq 10^6$</td>
<td>6</td>
<td>1-2</td>
<td>7-10</td>
</tr>
<tr>
<td>MGH-U2</td>
<td>$&gt;5 \times 10^5$</td>
<td>4</td>
<td>1-2</td>
<td>7-10</td>
</tr>
<tr>
<td>Pieces from cystoscopic biopsies</td>
<td></td>
<td>33</td>
<td>0-32</td>
<td>9-30</td>
</tr>
<tr>
<td>Biopsy from metastatic lesion</td>
<td></td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Cell suspensions from cystoscopic biopsies</td>
<td></td>
<td>13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>First-generation xenografts</td>
<td></td>
<td>6</td>
<td>2-4</td>
<td>12-20</td>
</tr>
<tr>
<td>Second-generation xenografts</td>
<td></td>
<td>2</td>
<td>1-2</td>
<td>10-20</td>
</tr>
</tbody>
</table>

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Each group contained 5 to 6 mice initially.

After implant of $10^6$ cells.
Table 2
Characteristics of 8 xenografts generated from cystoscopically obtained biopsies

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Original tumor</th>
<th>Pathological Stage*</th>
<th>Pathological Grade*</th>
<th>Clinical course of patient</th>
<th>Tumor takes in mice</th>
<th>Lag period (wk)</th>
<th>Doubling time (days)</th>
<th>Histological grade of xenograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single</td>
<td>T1</td>
<td>I</td>
<td>No recurrence (&gt;1 yr)</td>
<td>1/4</td>
<td>13</td>
<td>14</td>
<td>I, III</td>
</tr>
<tr>
<td>2</td>
<td>Multiple</td>
<td>T1</td>
<td>II</td>
<td>Recurrent T1 lesions</td>
<td>1/5</td>
<td>18</td>
<td>9</td>
<td>III</td>
</tr>
<tr>
<td>3</td>
<td>Single</td>
<td>Carcinoma in situ</td>
<td>I</td>
<td>No recurrence (&gt;1 yr)</td>
<td>4/6</td>
<td>2-7</td>
<td>10-20</td>
<td>II</td>
</tr>
<tr>
<td>4</td>
<td>Multiple</td>
<td>Ta</td>
<td>II</td>
<td>Recurrent Ta lesions</td>
<td>1/4</td>
<td>6</td>
<td>20</td>
<td>II</td>
</tr>
<tr>
<td>5a</td>
<td>Multiple</td>
<td>Ta</td>
<td>I</td>
<td>Recurrent Ta lesions</td>
<td>1/5</td>
<td>8</td>
<td>17</td>
<td>III</td>
</tr>
<tr>
<td>5b</td>
<td>Multiple</td>
<td>Ta</td>
<td>I</td>
<td></td>
<td>1/5</td>
<td>32</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Multiple</td>
<td>T3</td>
<td>III</td>
<td>Died, metastases</td>
<td>2/5</td>
<td>0</td>
<td>10-20</td>
<td>II, III</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>T3</td>
<td>III</td>
<td>Alive, metastases</td>
<td>2/6</td>
<td>8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Single</td>
<td>T3</td>
<td>III</td>
<td></td>
<td>1/1</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* Stage and grade as per Unió Internationale Contra Cancrum TNM Classification (8): Ta, superficial papillary tumor; T1, tumor not extending beyond lamina propria; T3, tumor with invasion of deep muscle; I, well differentiated; II, moderately well differentiated; III, poorly differentiated.

In appearance (Fig. 1); they are moderately large and anaplastic with pleomorphic hyperchromatic nuclei and scanty cytoplasm. Mitotic figures were seen in the primary implants and in the lung metastases.

Injection i.v. of $10^6$ cells from 4 cell lines into our mice also led to macroscopic pulmonary nodules 6 to 8 weeks later (Fig. 2). Histologically, these nodules showed anaplastic tumors, and we have also shown that they will generate tumors after s.c. implantation into immune-deprived mice.

Transplantation of Tumor Biopsies. Results of transplantation of biopsies from human bladder cancer into immune-deprived mice are summarized in Tables 1 and 2. Xenografting was considered successful if there was progressive growth of tumor in at least one mouse to a mean diameter of more than 1 cm. Any tumor which showed transient growth followed by regression was excluded.

Eight of 33 biopsies obtained cystoscopically and one from a metastatic site have generated xenografts from tumor pieces, and microscopic examination has confirmed the presence of transitional cell carcinoma in the 5 xenografts that have been examined histologically. Three of the xenografts have been karyotyped, and all had human chromosomes. Other tumors continue to grow as first-generation xenografts. Fifty % of the biopsies were implanted unilaterally on the right flank and 7 of 17 generated tumors. Two of 17 tumors grew after bilateral implantation. Only in 3 of 9 successful implantations was there growth of tumor in more than one animal. No growth was observed from i.m. implants of cell suspensions in 13 groups of mice. Failure to generate xenografts was not due to high animal mortality. Deaths were observed in only 6 of 47 groups of mice despite the prolonged observation period.

Representative growth curves of first-generation xenografts are shown in Chart 2. There was a wide range in lag period (0 to 32 weeks) before growth was evident, emphasizing the need to observe animals for a long period. Thereafter, tumors grew progressively, and at a tumor volume of ~0.5 cu cm, doubling times were in the range of 9 to 30 days.

Three xenografts have been successfully transplanted, and following transplantation of 3 others, there was initial growth followed by subsequent regression. Cell lines consisting of 4 distinct clones from a single xenograft have been derived and are being maintained in culture. Chart 3 depicts growth curves for first-, second-, and third-generation xenografts for Patient 6. In the second and third generations of transplants, there was a shorter lag period before growth was observed but little change in growth rate following this lag period.

Pathology of Xenografts. Eight of the first-generation xenografts grew as solid encapsulated tumors without local inva-
sion and the ninth developed liquid necrosis. Blood vessels were seen to penetrate the tumors, but all of them contained necrotic tissue at time of excision.

Patients donating the biopsies had transitional cell carcinoma, and the grade and stage of disease is shown in Table 2. Histologically, all of the xenografts were transitional cell carcinomas, but in some of them there was a change from the primary towards a greater or lesser degree of differentiation. In Patient 2, the first- and second-generation xenografts became progressively more anaplastic compared with the original tumor (Fig. 3). Of particular interest were xenografts derived from an anaplastic transitional cell carcinoma in Patient 6 (Fig. 4). A first-generation xenograft in one mouse remained anaplastic and was not successfully transplanted thereafter; in contrast, a first-generation transplant in another mouse was moderately well differentiated, and the degree of differentiation increased further on second passage but decreased in the third (Fig. 4).

**Influence of Clinical Characteristics.** We were able to examine the charts of 28 patients who donated biopsies on 31 occasions. Eight of these biopsies led to successful xenografts. Three of the xenografts were derived from patients with superficial papillary tumors, one from carcinoma in situ, 2 from nonpapillary tumors without muscle invasion, and one each from a locally invasive tumor and from a s.c. metastasis. Our study of the growth of xenografts revealed variable and sometimes long lag periods observed for first-generation xenografts might be explained by a low and variable fraction of clonogenic cells in the original biopsies.

Our failure to generate xenografts from cell suspensions prepared from fresh biopsies might reflect damage to cells during preparation or loss of biologically important cell-cell interactions. However, 17 cell suspensions prepared from the same biopsies were cultured in vivo in semisolid medium, and 9 of these generated clones of cells. In vitro plating efficiency was low ($10^{-6}$), and there may have been too few clonogenic cells to initiate growth of xenografts.

All xenografts that have been examined histologically were recognizable as transitional cell carcinomas, although in some of them there was a change toward more or less well-differentiated tumors. Xenografts from tumors of different origin may also show divergence in pathology (1, 19). Our observation that an anaplastic tumor could give rise to one anaplastic and one well-differentiated xenograft in different mice suggests the presence of clonogenic cells with different characteristics in the original tumor and some selection of these cells during xenografting.

There are both advantages and disadvantages to the use of immune-deprived mice. Nude mice do not require manipulation before transplantation, although with practice, thymectomy and treatment with ara-C and radiation can be performed rapidly and with low mortality. Immune-deprived mice are inexpensive and sturdy. Residual immunity (e.g., natural killer cells and macrophages) may be present in both types of animal but returning T-cell immunity in immune-deprived mice that require a long lag period. Our success rate with bladder cancer biopsies was within the same range as reported in 3 studies using nude mice (15, 16, 22), and implantation of established cell lines suggested that the immune-deprived mice were equal or better hosts than 2 strains of nude mice maintained in our colony. However, the high mortality among nude mice might suggest the presence of pathogens, and it is known that the presence of occult viral infection may adversely influence the success of xenografting. Others have reported that immune-deprived mice will allow metastases (20), whereas metastases are rarely reported in adult nude mice (12).

We were able to generate metastases from i.m. implantation of a cell line but not from s.c. implanted tumor biopsies. We were also able to generate macroscopic pulmonary nodules after i.v. implantation of 4 human bladder cancer cell lines.

In conclusion, we have demonstrated that xenografts of human bladder cancer will grow in immune-deprived mice that can be maintained in a conventional animal colony. These xenografts have potential for study of biological properties and response to treatment of human bladder cancer.

**ACKNOWLEDGMENTS**

We thank Dr. M. Platts for consultation about histopathology, Drs. J. Heddle and C. Norman for assistance with karyotyping, Dr. A. Howatson for performing

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*R. N. Buick, personal communication.*
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electron microscopy to rule out viral contamination, and Drs. J. Connolly, M. Jewett, and A. Keresteci for providing biopsy material. We are also grateful to Dr. R. Buick for his consultation and collaboration and Mrs. D. Vidgen for technical assistance.

REFERENCES

Fig. 1. An i.m. tumor (A) and pulmonary metastases (B) in a mouse that received an i.m. implant of RT-4 cells. × 1600.
Fig. 2. Pulmonary nodules observed 6 weeks after i.v. implantation of MGH-U1 cells.
Fig. 3. Original biopsy (A) and first- and second-generation xenografts (B and C, respectively) from Patient 2. Note decrease in differentiation on successive transplants. × 350.
Fig. 4. Original biopsy (A) and first-, second-, and third-generation xenografts (B, C, and D, respectively) from Patient 6. Note increase in differentiation in first- and second-generations. × 350.
Xenografts of Human Bladder Cancer in Immune-Deprived Mice

Asya Kovnat, Margaret Armitage and Ian Tannock


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