ABSTRACT

The role of urine as a tumor-enhancing agent in urinary bladder carcinogenesis was investigated by using the heterotopically transplanted rat urinary bladder. Bladders removed from rats initiated with the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine in drinking water for 4 or 10 weeks were heterotransplanted to syngeneic rats. Those heterotopically transplanted bladders receiving repeated instillations of normal rat urine subsequent to transplantation had a higher incidence of carcinoma than did those receiving 0.9% NaCl solution. These results suggest that normal urine may contain tumor promoters.

INTRODUCTION

Experimental evidence suggests that urine plays a vital role in urinary bladder carcinogenesis as a carrier of carcinogen(s) (5, 12). Whether urine may contain some endogenous or exogenous substance(s) which could act as tumor promoters remains unclear. The study of Chapman et al. (2) suggested presence of such substances.

To study the role of urine in bladder carcinogenesis, a urinary bladder totally free from urine flow is needed. To meet this requirement, a HTB was separated from the ureters was created in a syngeneic rat in our laboratory (8). In an earlier study, we have shown that repeated instillations of N-methyl-N-nitrosourea into HTB induce the development of transitional cell carcinomas (7) resembling those that usually develop in rats and humans. The present experiment was designed to investigate the role of normal rat urine in urinary bladder carcinogenesis.

MATERIALS AND METHODS

The experimental design is shown in Chart 1. Two hundred twenty-two male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 130 to 180 g received tap water ad libitum which contained 0.05% BBN (Izumi Chemical Co., Yokohama, Japan) and were fed regular Purina 5012 laboratory chow (Ralston Purina Co., St. Louis, Mo.) for 4 and 10 weeks. All bladders exhibited an orderly and diffuse increase in the thickness of the epithelium (simple hyperplasia) at 4 weeks and nodulopapillary hyperplasia at 10 weeks. These bladders were aseptically transplanted into the gluteal muscle of male syngeneic rats (8) which likewise had been treated with 0.05% BBN for the identical period of time. Two weeks after transplantation, 94 recipients of HTB’s which were free from either cicatricial contracture or evidence of infection were divided into 2 groups, the first group receiving pooled normal rat urine and the second, 0.9% NaCl solution. The control groups consisted of 28 normal rats carrying HTB’s from donors which did not receive carcinogen. The urine sample to be instilled was prepared as follows: a 24-hr urine was collected from fasted rats in metabolism cages (Acme Products, Chicago, Ill.) into a 50-ml centrifuge tube chilled in ice water and containing 0.2 ml of mineral oil. The animals used were the same as those used for subsequent heterotransplantation, and the collection had been made prior to carcinogen treatment. The urine was filtered through Whatman no. 1 filter paper (Whatman, Inc., Clifton, N. J.), the pH being adjusted between 6.8 and 7.0 by adding sodium hydroxide and the osmolality adjusted to 800 mOsmol by adding distilled water, filtered through 0.45-µm Nalgene filter (Sybron Corp., Rochester, N. Y.), and stored in 5-ml portions at −20° until use. Instillation of the urine into the HTB via the attached Ommaya reservoir (850-1274; Heyer-Schulte Corp., Goleta, Calif.) was begun 2 weeks after transplantation. The fluid which accumulated following transplantation of HTB (0.8 to 1.0 ml) was removed by percutaneous needle aspiration and replaced with pooled normal rat urine (0.8 to 1.0 ml) or 0.9% NaCl solution. The procedure was continued once a week until the end of the experiment; animals were killed 38 and 44 weeks after the beginning of the experiment. The HTB was separated from the reservoir and dissected with 10% buffered neutral formalin. On the following day, it was cut longitudinally along the midline, and the mucosa was inspected under a dissecting microscope. Tumors which were more than 2 mm in size were counted, and their major and minor principal axes were measured with a vernier caliper. The criteria utilized for classification of bladder cancer are those previously described (7). The nodulopapillary hyperplasia was considered as preneoplastic atypical hyperplasia (1).

RESULTS

Rats of all groups gained weight progressively with no significant difference in weight among them. The 4- and 10-week p.o. treatment of rats with 0.05% BBN in drinking water was carcinogenic to the natural bladders when examined at 38 weeks; the incidence of cancer was 75% (13 of 18 rats) and 100% (28 rats), respectively (Table 1). The incidence of cancer in the 2 groups of HTB animals treated with weekly instillation of urine was significantly higher than that of the corresponding 0.9% NaCl solution groups (p < 0.001 for animals treated with BBN for 4 weeks and 0.05 for those treated with BBN for 10 weeks) (Fig. 1) but did not differ significantly from that of the natural bladders. The incidence of tumors in HTB’s at 44 weeks was also higher in the urine-treated group than in the 0.9% NaCl solution control (p < 0.05).
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>HTB’s</th>
<th>Natural bladders</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBN (4) → 0.9% NaCl solution (38)</td>
<td>9</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>BBN (4) → 0.9% NaCl solution (44)</td>
<td>13</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>BBN (4) → urine (38)</td>
<td>9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>BBN (4) → urine (44)</td>
<td>14</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BBN (10) → 0.9% NaCl solution (38)</td>
<td>13</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>BBN (10) → urine (38)</td>
<td>15</td>
<td>14′</td>
<td>15</td>
</tr>
<tr>
<td>No BBN (4) → 0.9% NaCl solution (38)</td>
<td>120</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No BBN (4) → urine (38)</td>
<td>12′</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses at end of entry, total experimental period (weeks).

p < 0.001 (1-tailed) when compared to the respective 0.9% NaCl solution group by Fisher’s exact test.

Four rats were removed from study because of fibrosis around the connector tip or evidence of infection involving HTB.

In 18 HTB’s pretreated with BBN for 10 weeks, fibrous obliteration between transplant and reservoir developed, and weekly instillation of fluids was discontinued after 3 to 12 doses. Rats carrying such HTB’s were nevertheless allowed to live until the time of scheduled termination of experiments. The incidence of tumors in these HTB’s was 5 of nine 0.9% NaCl solution-treated HTB’s and 9 of 9 for the urine-treated HTB’s, and the incidence did not differ from those of the corresponding HTB’s where weekly instillation of fluids was carried out without interruption. In this group, the incidence of cancer in natural bladders was 100% (18 rats). Fibrosis around the connector tip was rare and was observed in only 4 rats in the groups of HTB’s treated with BBN for 4 weeks prior to transplantation.

The average numbers of tumors per bladder and the average volume of the largest bladder tumors relative to the posttransplant treatment are shown in Table 2. The average number of tumors in urine-treated HTB’s was significantly greater ($p < 0.001$) than that of 0.9% NaCl solution-treated HTB’s whether urine treatment was completed or discontinued. Likewise, the average number of tumors per natural bladder was significantly greater ($p < 0.005$) than that in HTB’s treated with 0.9% NaCl solution. The average number of tumors in HTB’s with discontinued urine administration was not significantly different from that in HTB’s with urine administration completed as scheduled. There was no significant difference in the average size of the largest tumors among the various groups. The tumors, however, tended to be larger in HTB’s than in the natural bladders.

There was no difference in tumor histology between the HTB’s and natural bladders nor between the urine- and 0.9% NaCl solution-treated HTB’s. They were nodulopapillary growths lined by transitional epithelium and supported by mast cell-rich fibrovascular stroma (Figs. 2 to 7). Focal squamous metaplasia was common. All tumors were at Stage 0 (no invasion of lamina propria) except for 4 bladders in the 10-week BBN pretreatment groups; one HTB treated with urine showed Stage A invasion (extension to lamina propria), and 3 natural bladders exhibited Stage A (2 bladders) and Stage C (1 bladder) invasion (extension beyond tunica muscularis). All tumors which developed in HTB’s receiving 0.9% NaCl solution were classified as Grade 1 tumors while 6 of 14 HTB’s in the urine group contained at least one Grade 2 tumor. The difference was not significantly different from that in the HTB treated with urine.

The urine treatment did not induce tumors in HTB’s which were not previously exposed to the carcinogen.

DISCUSSION

The present investigation was designed to investigate the role of normal rat urine in urinary bladder carcinogenesis. As
an initiator, 0.05% BBN in drinking water was given ad libitum for 4 and 10 weeks. These doses proved to be carcinogenic to the natural bladders. Weekly instillation of pooled normal rat urine into HTB’s resulted in tumor incidence comparable with that of the natural bladders of the hosts while weekly instillation of 0.9% NaCl solution yielded a low tumor incidence. Urine treatment also produced more tumors per bladder than did 0.9% NaCl solution treatment. Thus, under the experimental conditions outlined in “Materials and Methods,” pooled normal rat urine enhances tumorigenesis in the bladder previously treated with BBN.

The urine instilled into the HTB diffuses out slowly, and its initially high osmolality declines progressively to the level isosmotic to the serum within 48 hr (4). The urea concentration in the urine maintains a high level until the experiment. Thus, its stimulatory effect in the present experiment may be limited. Nevertheless, its effect appeared similar to that of the normal fresh urine on the natural bladders.

In this study, we used a commercial rat chow as animal food for collection of the urine. The urines collected from rats fed a chemically defined diet and the commercial diet were tested for mutagenicity by the Ames assay as well as for ornithine decarboxylase inducibility which parallels with most tumor promoters (6). No difference was found in the enzyme activities between the urines, and neither urine was mutagenic.

Normal urine contains growth-stimulating factors such as epidermal growth factor (13), polyamines (10), and colony-stimulating factor (9). Other recent observations that urine may act as a tumor promoter in bladder (11) and colon (3) carcinogenesis as well as an agent capable of inducing hyperplasia in bladder epithelium (4) suggest that such actions may be due to specific substance(s) contained in normal urine.

### ACKNOWLEDGMENTS

We thank Dr. Joan Chmiel and Vadreuv Subbarao for performing the statistical analysis and the Ames assay of rat urine, respectively.

### REFERENCES


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### Table 2

<table>
<thead>
<tr>
<th>Subsequent treatment $^b$</th>
<th>No. of rats</th>
<th>No. of tumors $^c$</th>
<th>Size of largest tumor $^d$</th>
<th>No. of tumors $^c$</th>
<th>Size of largest tumor $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution (38 wk) uninterrupted $^a$</td>
<td>13</td>
<td>$0.7 \pm 1.1$</td>
<td>$188 \pm 278$</td>
<td>$4.4 \pm 2.8^e$</td>
<td>$354 \pm 348$</td>
</tr>
<tr>
<td>Urine (38 wk) uninterrupted</td>
<td>15</td>
<td>$6.8 \pm 3.8^e$</td>
<td>$840 \pm 950$</td>
<td>$5.3 \pm 2.4^e$</td>
<td>$256 \pm 245$</td>
</tr>
<tr>
<td>Urine (38 wk) interrupted</td>
<td>9</td>
<td>$4.6 \pm 2.9^e$</td>
<td>$609 \pm 403$</td>
<td>$4.4 \pm 1.4^e$</td>
<td>$257 \pm 262$</td>
</tr>
</tbody>
</table>

$^a$ The volume (cu mm) of the largest tumor. The volume was calculated as $\frac{4}{3}xab^2$, where a and b are the major and minor axes of the tumor.

$^b$ Following BBN pretreatment (10 weeks).

$^c$ Mean $\pm$ S.D.

$^d$ In the "interrupted" group, weekly instillation of urine was discontinued after 3 to 12 doses, but such animals were allowed to live until the scheduled termination of experiment.

$^e$ $p < 0.005$ (2-tailed) when compared to the paired 0.9% NaCl solution-injected HTB group by the Wilcoxon signed-rank test.

$^f$ $p < 0.001$ (2-tailed) when compared to the 0.9% NaCl solution-injected group by the Wilcoxon 2-sample rank sum test.

$^g$ $p < 0.01$ (2-tailed) when compared to the 0.9% NaCl solution-injected group by the Wilcoxon 2-sample rank sum test.

$^h$ $p < 0.005$ (2-tailed) when compared to the 0.9% NaCl solution-injected group by the Wilcoxon 2-sample rank sum test.

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$^9$ NaCl solution (38 wk) uninterrupted.

$^6$ NaCl solution (38 wk) interrupted.

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$^R$ Oyasu, Y. Hirao, and K. Izumi, unpublished data.
Figs. 2 and 3. Grade 1 and Stage 0 transitional cell carcinoma observed in a natural bladder of the rat which received BBN p.o. for 10 weeks followed by a normal rat chow for 28 weeks. It is a papillonodular tumor lined by a well-differentiated transitional epithelium. The stroma is loose, fibrous, and vascular. A large number of mast cells infiltrate the stroma. Fig. 2, H & E, x 46. Fig. 3, H & E, x 185.

Figs. 4 and 5. Grade 1 and Stage 0 transitional cell carcinoma observed in a HTB from a donor rat which received BBN p.o. for 10 weeks. Following transplantation, this HTB received weekly instillation of pooled normal rat urine for 26 weeks. The tumor is a papillonodular mass lined by a well-differentiated transitional epithelium and has a fibrovascular stroma. Note resemblance with tumor grown in a natural bladder after BBN treatment as shown in Figs. 2 and 3. Fig. 4, H & E, x 46. Fig. 5, H & E, x 185.

Figs. 6 and 7. Grade 1 and Stage 0 transitional cell carcinoma observed in a HTB from a donor rat which received BBN p.o. for 10 weeks. Following transplantation, this HTB received weekly instillation of 0.9% NaCl solution for 26 weeks. This tumor albeit smaller is microscopically indistinguishable from those shown in Figs. 2 through 5. Fig. 6, H & E, x 46. Fig. 7, H & E, x 185.
Enhancement by Urine of Urinary Bladder Carcinogenesis

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