

Effects of Rat Urine Fractionated by Molecular Weight on Urinary Bladder Carcinogenesis¹

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ABSTRACT

Experimental evidence suggests that rat urine plays a dual role in urinary bladder carcinogenesis, as a carrier of carcinogen(s) and an enhancer (or promoter) of carcinogenesis in carcinogen-initiated cells. The present experiment was conducted to determine whether tumor-enhancing activity exhibited by the whole urine resides in a specific fraction(s) when separated by molecular weight. Normal rat urine after preliminary filtration to remove gross debris was filtered through Amicon Diaflo membranes to obtain two types of filtrate, one at an exclusion level of M.W. 1,000 and the second at an exclusion level of M.W. 50,000. These filtrates were tested for tumor-enhancing activity in the heterotopically transplanted rat urinary bladders (HTBs) removed from rats pretreated with the carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in drinking water for 4 or 10 weeks. The incidences of cancer in HTBs receiving repeated instillations of the filtrate at M.W. 50,000 did not differ significantly from those of the HTBs receiving whole urine but were significantly higher than those of the HTBs receiving the filtrate at M.W. 1,000. In the HTBs, which had been exposed to the carcinogen for a longer period of time (10 weeks), the filtrate at M.W. 1000, enhanced the bladder tumorigenesis over that of the osmolality control. The results indicate that there may be two classes of tumor-enhancing activity: the first and major one being in the fraction above M.W. 1000; and a second one being below M.W. 1000. The latter fraction appears to exert its effects only on the cells which are in an advanced stage of carcinogenesis.

INTRODUCTION

Experimental evidence suggests that urine plays a dual role in urinary bladder carcinogenesis: (a) as a carrier of carcinogen(s) (4, 10); and (b) as an enhancer (or promoter) of carcinogenic process in initiated cells (5, 7, 9). Using the HTB³ model, we have shown that the absence of urine in the HTBs the epithelial cells of which were initiated either *in situ* by a directly acting carcinogen, *N*-methyl-*N*-nitrosourea (5), or by a p.o. carcinogen, BHBN, before heterotopic transplantation (7) results in a lower incidence of tumors. It appears, therefore, that normal urine enhances or, conversely, the absence of urine may suppress or delay bladder tumorigenesis. This apparent tumor enhancement could be due to nonspecific irritation by urine and/or to a specific substance(s) contained in

urine. The present investigation was conducted to determine whether tumor-enhancing activity exhibited by the whole urine resides in a specific fraction(s) when separated by molecular weight. The results indicate that the tumor-enhancing effect of urine primarily resides in the fraction above M.W. 1000 and suggests that tumor enhancement may be due to a specific substance(s).

MATERIALS AND METHODS

Experimental Animals. The experimental groups are shown in Table 1. A total of 500 male Fischer 344 rats weighing approximately 130 to 150 g were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. They were fed regular laboratory chow (Purina 5012; Ralston Purina Co., St. Louis, Mo.). Three hundred sixty rats received tap water *ad libitum* which contained 0.05% BHBN (Izumi Chemical Co., Yokohama, Japan) for 4 or 10 weeks, and the remaining rats received tap water *ad libitum*.

HTB Carcinogenesis Model System. After BHBN treatment, one-half of the rats treated with BHBN and one-half of those receiving no BHBN served as bladder donors. Bladders were aseptically transplanted into the gluteal muscle of the remaining one-half of the rats, which likewise had been treated with 0.05% BHBN or with tap water for the identical period of time. The technique for transplantation that was described previously (8) was used with the following modification. Immediately after transplantation, the HTBs received 0.5 to 0.8 ml of 0.9% NaCl solution containing gentamicin (0.1 mg/ml) and at the same time recipients received once 0.5 mg of gentamicin i.m. One week after transplantation, the reservoir bladder content was completely aspirated by percutaneous puncture and replaced by 1 ml of 0.9% NaCl solution. The aspirate was routinely cultured in a thioglycollate-containing tube. Approximately 3 to 5% of HTBs grew bacteria; the organisms commonly found were *Staphylococcus epidermidis*, *Streptococcus faecalis*, and, rarely, *Pseudomonas* species. Such bladders received an additional dose of gentamicin (0.1 mg/ml 0.9% NaCl solution) once a week for no more than 2 doses. Four weeks after transplantation, 229 recipients of bladders which were free from infection, as proven by another routine culture of the aspirate or from evidence of ischemic contracture, were divided into 12 groups (Table 1). Each group consisted of 20 recipients except in Groups 5 to 8 to which fewer animals (from 17 to 18 animals/group) were assigned, due to an immediate postoperative loss of a large number of animals from an undetermined cause. Groups 1, 5, and 9 received Urine A (see below); Groups 2, 6, and 10, received Urine B; Groups 3, 7, and 11, received Urine C; and Groups 4, 8, and 12, received 2.1% NaCl solution.

Preparation of Urine Samples. The urine samples to be instilled into HTBs were prepared as follows. A 24-hr urine sample was collected from normal rats in an ice-cold flask containing 0.5 ml of mineral oil. The animals used were those subsequently used as controls. The urine was filtered through Whatman no. 1 filter paper (Whatman Inc., Clifton, N. J.), the pH was adjusted to a range of 6.8 to 7.0 by adding sodium hydroxide, and osmolality was adjusted to 800 mOsmol by adding distilled water. The sample was then filtered through a 0.45- μ m Nalgen filter (Sybron Corp., Rochester, N. Y.) (Urine A). This preliminary filtration was necessary to avoid plugging the filter membrane used for

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³ The abbreviations used are: HTB, heterotopically transplanted rat bladder; BHBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; ODC, ornithine decarboxylase. Received June 29, 1981; accepted October 1, 1981.

Table 1

Effect of weekly instillation of urine filtrates into HTB on the tumor induction

All animals were killed after the total experimental period of 38 weeks, except for Groups 5 to 8 which were terminated after the total experimental period of 30 weeks. See the text for statistical analysis.

Group Treatment	Own bladders			HTBs			No. of carcinomas/HTB ^c
	No. of rats ^a	Hyperplasia ^b	Carcinoma ^b	Hyperplasia ^b	Carcinoma ^b	No. %	
		No. %	No. %	No. %	No. %		
1 BHBN (4 wk) → Urine A ^d	18	10 56	5 28	14 78	15 83	2.22 ± 0.44 ^e	
2 BHBN (4 wk) → Urine B ^d	17	14 82	5 29	16 94	12 71	1.71 ± 0.39	
3 BHBN (4 wk) → Urine C ^d	18	12 67	6 33	16 89	5 28	0.39 ± 0.18	
4 BHBN (4 wk) → 2.1% NaCl	19	14 74	5 26	16 84	3 16	0.21 ± 0.10	
5 BHBN (10 wk) → Urine A	16	16 100	16 100	16 100	16 100	6.19 ± 0.52	
6 BHBN (10 wk) → Urine B	18	18 100	18 100	18 100	18 100	6.11 ± 0.60	
7 BHBN (10 wk) → Urine C	16	16 100	16 100	16 100	11 69	1.63 ± 0.43	
8 BHBN (10 wk) → 2.1% NaCl	15	15 100	15 100	15 100	4 27	0.61 ± 0.29	
9 No BHBN → Urine A	17	0	0	1 6	0	0	
10 No BHBN → Urine B	18	0	0	1 6	0	0	
11 No BHBN → Urine C	17	0	0	1 6	0	0	
12 No BHBN → 2.1% NaCl	17	0	0	1 6	0	0	

^a Effective number of rats available for final evaluation.^b Hyperplasia refers to nodulopapillary hyperplastic foci which are regarded as preneoplastic hyperplasia(1). ^c The average number of tumors (larger than 1 mm) per HTB.^d Urine A refers to normal whole-rat urine with pH ranging from 6.8 to 7.0 and osmolality of 800 mOsmol. Urines B and C were obtained, respectively by filtering Urine A through an Amicon Diaflo membrane filter, XM 50 (exclusion level of M.W. 50,000) and UM 2 (M.W. 1,000). For details see the text.^e Mean ± S.E.

the subsequent sterilization of samples. Urines B (795 mOsmol) and C (790 mOsmol), respectively, were obtained by filtering Urine A through an Amicon Diaflo membrane filter XM 50 (exclusion level, M.W. 50,000) and UM2 (exclusion level, M.W. 1000) (Amicon Corp., Lexington, Mass.)⁴ Finally, each urine sample was passed through a 0.2- μ m Nalgen filter and stored in 30-ml portions at -80° until use. In this study, 2.1% NaCl solution instead of 0.9% NaCl solution was used as the control to maintain the osmolality at about 800 mOsmol.

Instillation of test urines into HTBs via the attached reservoir (Intermittent Infusion Set No. 4721; Abbott Laboratories, Inc., North Chicago, Ill.) was begun 4 weeks after transplantation. The fluid accumulated in an HTB was removed by percutaneous needle aspiration and replaced with 0.5 ml of urine or 2.1% of NaCl solution. The procedure was continued once a week until the end of the experiment.

Animals were killed 38 weeks after the beginning of the experiment except for the rats of Groups 5 to 8, which were killed at 30 weeks. The procedure for submitting urinary bladders (5) and the criteria for histological classification (8) were those described previously. Visible bladders tumors (>1 mm) in HTBs were counted, and every effort was made to demonstrate all of these tumors in microscopic sections to establish histological diagnosis.

RESULTS

Animals of all groups gained weight progressively with no significant difference in body weight among them. Altogether, 206 rats were available for histological examination. The remaining 23 rats were excluded because of loss of connection between the reservoir and the transplant, evidence of infection, or death from undetermined cause.

⁴ The decision to use these exclusion levels was made after the following preliminary study. When the filtrate through the membrane with exclusion level of a M.W. of 500 was administered to HTBs, acute hemorrhagic ulcer of the mucosa occurred in all HTBs within 1 week and persisted as weekly instillations of the filtrate continued. Microscopic examination revealed multiple ulcers of the mucosa, and active chronic inflammation, and foci of submucosal calcification. However, the filtrate through the membrane with an exclusion level of a M.W. of 1000 did not induce such complication.

Overall comparison of the 4-week BHBN treatment groups, and similarly of the 10-week groups, indicated highly statistically significant differences in tumor incidence among the groups ($p < 0.00005$, χ^2 test with 3 d.f.). Among the 4-week BHBN treatment groups (Table 1 and Chart 1), the tumor incidence in HTBs receiving Urine C (Group 3) (filtrate exclusion level of M.W. 1000) was significantly lower than that in HTBs receiving Urine A (Group 1) (whole urine) ($p < 0.005$, 2-tailed, by χ^2 test corrected for continuity) or Urine B (Group 2) (filtrate, exclusion level of M.W. 50,000) ($p < 0.05$) but was not significantly different from that of HTBs receiving 2.1% NaCl solution. Among the 10-week BHBN treatment groups, a similar trend was observed; the tumor incidence in HTBs receiving Urine C (Group 7) was significantly lower than that of the HTBs receiving whole urine (Group 5) or Urine B (Group 6) ($p < 0.03$ and 0.02 , respectively, one-tailed, by Fisher's exact test). Unlike the results from the 4-week BHBN treatment, the tumor incidence in Group 7 (Urine C) was significantly higher than that of the osmolality control group (Group 8) ($p < 0.05$); the results indicating that Urine C also has a tumor-enhancing effect over that of the osmolality control. Treatment with urine as well as with 2.1% NaCl solution with no prior carcinogen treatment did not result in tumor formation in HTBs (Groups 9 to 12).

The incidence of tumors in the natural bladder of HTB-recipients was quite uniform among the various study groups, being approximately 30% in 4-week BHBN-treatment groups and 100% in 10-week BHBN-treatment groups. Comparison of HTB with the paired natural bladder was done using McNemar's exact paired binomial test (one-tailed). The tumor incidence in the HTBs of Group 1 (Urine A after 4-week BHBN treatment) or Group 2 (Urine B after 4 week BHBN treatment) was higher than that of the paired natural bladders ($p < 0.01$ and 0.02 , respectively). The tumor incidence in the HTBs of Group 7 (Urine C after 10-week BHBN treatment) or Group 8 (2.1%

NaCl solution after 10-week BHBN treatment) was significantly lower than that of the paired natural bladders ($p < 0.04$ and 0.001 , respectively).

The average numbers of tumors per HTB among different treatment groups and their levels of statistical significance for pairwise comparisons are shown in Table 1 and Chart 2. Among the 4-week BHBN groups, the average number of tumors per HTB receiving Urine C was significantly lower than that of the group receiving Urine A or Urine B ($p < 0.001$ and 0.005 , respectively, 2-tailed, by Wilcoxon 2-sample rank sum test) but was not different from that of the group receiving 2.1% NaCl solution. Among the 10-week BHBN groups, statistical significance in average number of tumors was again demonstrated between the Urine C group (Group 7) and the whole-urine group (Group 5) ($p < 0.0001$) and between Group 7 and Group 6 (Urine B group) ($p < 0.0001$). Although the tumor incidence in Group 7 was significantly higher than that in Group B ($p < 0.05$; see above), interestingly, the average number of tumors per HTB was not significantly different between these 2 groups.

Microscopic examination revealed that bladder tumors observed in the natural bladders as well as in the HTBs were mostly Grade I and II transitional cell carcinomas in Stage 0 (no invasion of the lamina propria). Approximately 20 to 25% of tumor-bearing bladders contained tumors invasive to the lamina propria (Stage A). No significant difference was ob-

served in grade, stage, and type of tumors by difference in treatment.

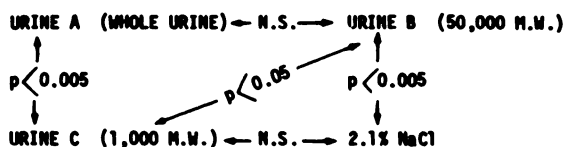
DISCUSSION

Our laboratory demonstrated previously that normal rat urine plays an enhancing role in urinary bladder carcinogenesis (5, 7). Conversely, the absence of urine within bladder lumen could conceivably delay bladder tumorigenesis (9). It was hypothesized that this apparent tumor enhancement might be due to a specific substance(s) contained in urine.

In the murine skin carcinogenesis system, a good correlation has been demonstrated between the promoter activities of phorbol diesters in mouse skin and their inducibility of ODC in murine epidermal cells (6). Recently, our laboratory has demonstrated that rat urine as well as 12-*O*-tetradecanoylphorbol-13-acetate, a potent promoter of murine skin carcinogenesis, are good inducers of ODC activity when tested with a rat bladder cancer line (804 G cells) (3). In that study, filtration of urine through an Amicon filter with exclusion levels of M.W. 500, 1,000, 10,000, and 50,000 lowered the ODC inducibility to approximately 20, 40, 40, and 80%, respectively, of that of the unfiltered urine (2, 3). Therefore, as a step to test our hypothesis, urine was fractionated by molecular weight of constituents, and the fractions were tested for tumor-enhancing role. The results suggest that tumor enhancement by normal rat urine is principally exhibited by the fractions of urine where urinary constituents are over M.W. 1000 and that tumor enhancement might be due to a specific substance(s). If indeed tumor enhancement, as observed in our study system, is due to a specific urinary component, its effect appears to be shown best in the bladders which had a low carcinogen exposure (4 weeks of BHBN treatment). Results obtained from the 10-week BHBN groups also indicate that the urine fraction under M.W. 1000 also exhibits some tumor-enhancing activity which may be manifested only in the bladders in a more advanced stage of carcinogenesis (10 weeks of BHBN treatment).

Two additional points worthy of being mentioned are: (a) that tumor enhancement by urine is not due to its high osmolality; and (b) that the ODC inducibility in a bladder cancer cell line (804G) by urine fractions (2, 3) correlated well with the tumor-enhancing activities. This suggests that determinations of ODC inducibility might be a useful measure in predicting tumor-enhancing activity exhibited by urinary components.

4-WEEK BHBN-PRETREATMENT:



10-WEEK BHBN-PRETREATMENT:

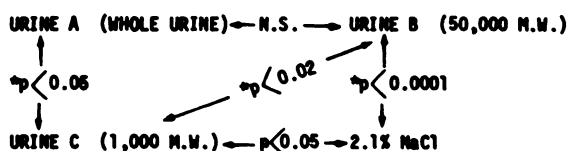
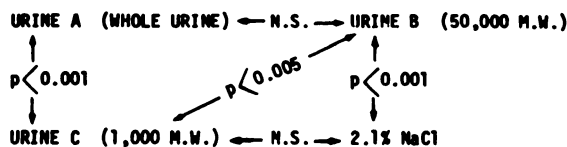


Chart 1. Tumor incidence in HTBS. Levels of statistical significance for data shown in Table 1. Two-tailed p values are reported except where indicated by *, which are one-tailed. N.S., not significant.

4-WEEK BHBN-PRETREATMENT:



10-WEEK BHBN-PRETREATMENT:

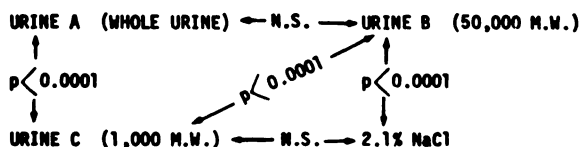


Chart 2. Average number of tumors per HTB. Levels of statistical significance for data shown in Table 1. Two-tailed p values are reported from Wilcoxon 2-sample rank sum test. N.S., not significant.

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