Lack of Enhancing Effect of Mucosal Regeneration following Ulcération of the Urinary Bladder on N-Butyl-N-(4-Hydroxybutyl)nitrosamine Carcinogenesis in Rats

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ABSTRACT

The potential enhancing effects of the regeneration response of the urothelium following ulceration of the bladder mucosa on N-butyl-N-(4-hydroxybutyl)nitrosamine carcinogenesis were examined in male Fischer rats. The carcinogen was administered in the drinking water at a concentration of 0.05% for 2 weeks. Ulcération was performed by a freezing technique 24 hr before the administration or 24 hr or 8 weeks after the discontinuation of N-butyl-N-(4-hydroxybutyl)nitrosamine. No enhancing effects by ulceration were observed. Ulcération prior to carcinogen treatment decreased rather than increased the induction of bladder tumors to approximately one-half the incidence of the control. Ulcération after N-butyl-N-(4-hydroxybutyl)nitrosamine did not change the frequency of tumor induction. While the inhibitory effect of the prior ulceration may result from the exposure of fewer mature mucosal cells that are capable of activating the carcinogen, the ineffectiveness of the subsequent ulcerations suggests that a single wave of regeneration does not enhance the tumorigenic response of bladder mucosa.

INTRODUCTION

It is generally accepted that the enhanced DNA replication of a tissue may contribute to the tumorigenic response of that tissue (9, 38). Early evidence in support of this concept was obtained by Friedewald and Rous (11), who noted that wounding of rabbit ears enhanced the incidence of sar-induced tumors, and by Frei and Ritchie (10), who demonstrated a higher yield of skin tumors following treatment with 7,12-dimethylbenz(a)anthracene if the carcinogen was given at the time of the greatest mitotic frequency. Partial hepatectomy has been widely studied and is known to have a dramatic effect on the response of liver to initiation by many carcinogenic compounds. Numerous publications have reported the induction of hepatocellular carcinomas as a result of exposure of the dividing liver cells to a stimulus of carcinogen which was ineffective in control animals (2, 6, 19, 26, 37). Moreover, the combination of partial hepatectomy and a second stimulus, such as phenobarbital or some other compounds, further increases the potential for hepatocarcinogenesis (25, 32, 34, 36). Partial pancreatectomy or unilateral nephrectomy has also been shown to have similar enhancing effects on the carcinogenic response of these organs (7, 15).

In spite of numerous studies of experimental bladder carcinogenesis by BBN (1, 13, 14, 18) and N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (4, 8), there have been as yet no definitive investigations as to the relationship between cell regeneration and carcinogenesis in the urinary bladder.

Recently, Shirai et al. (30, 31) presented a rapid reproducible method for the production of ulcers and reversible regenerative hyperplasia of the urinary bladder of rat by freezing. Autoradiographs of the bladder by means of [3H]thymidine incorporation into the nuclei of the urothelium demonstrated labeling indexes of approximately 27, 20, and 11% in the hyperplastic area after 24, 48, and 72 hr, respectively. This method has the advantage of avoiding other factors during regeneration, such as the presence of a foreign body or the effects of chemicals such as cyclophosphamide which, in addition to its cytotoxicity, has numerous effects (e.g., carcinogenic and immunosuppressive) on the urothelium (5, 17, 27, 28). Furthermore, the proliferative response may be amplified by treatment with a carcinogen. Therefore, ulceration may serve as an experimental model in which the carcinogenic effects of chemicals or the carcinogenic process may be accelerated via stimulation of DNA synthesis. The present study explored the possibility that the ulceration of the urinary bladder by freezing might increase the induction of bladder tumors by BBN.

MATERIALS AND METHODS

Male Fischer 344 rats (purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were 40 days old and weighed approximately 93 ± 6 (S.D.) g at the beginning of the experiment. Housed 3 or 4 per cage, they were maintained at 24° C on a 12-hr light-dark cycle and were given food (Wayne Lab Blox, Allied Mills, Inc., Chicago, Ill.) and water ad libitum. The rats were divided into 5 groups as shown in Table 1. BBN (Izumi Chemical Co., Yokohama, Japan) was given as a 0.05% solution in distilled water for 2 weeks. The BBN consumption in each group was measured.

Freezing ulceration of the urinary bladder was performed by the method described by Shirai et al. (30). The lower abdominal wall was opened through a midline incision to expose the bladder under anesthesia with ethyl ether. While holding the dome of the bladder with forceps, a stainless steel rod, 5 mm in diameter, kept in dry ice-acetone (—78°) was applied to the serosal surface of the anterior wall of the bladder for 2 seconds.
twice, with a 5-sec interval between each exposure, during which time the rod was reimmersed in dry ice-acetone. The abdominal incision was immediately closed. The freezing procedure was performed 24 hr before the beginning of BBN administration in Group 1, 24 hr after the discontinuation of BBN in Group 2, and at Week 10 in Group 3. Groups 4 and 5 were the corresponding controls and were treated only with BBN or with ulceration at the start of the experiment, respectively. All rats were killed at Week 58 and, after careful macroscopical examination, the urinary bladders were inflated with 10% buffered formalin and placed in the same fixative. The urinary bladders were cut in half; then the number and the size of the visible tumors were scored, and the tissues were processed to the paraffin sections. Sections (5 µm thick) were stained with hematoxylin and eosin.

RESULTS

The rats in all groups gained weight at the same rate (Table 1). One week after the freezing procedure, the body weights were depressed by less than 4%, but the normal growth rate was recovered quickly. The estimated average cumulative consumption of BBN is shown in Table 1 as g/rat/total time of the administration. This figure represents a maximum estimate since spillage was not taken into account. There was no definite difference in the consumption between each group. One rat in Group 3 which had a large bladder tumor with many small tumors accompanied by numerous stones was omitted from the effective number, since bladder calculi have been considered to be tumorigenic to the urinary bladder epithelium and to enhance the bladder carcinogenesis by carcinogens in rats and mice (3, 35). No stones were observed in other animals.

All bladders ulcerated by freezing were slightly contracted around the midportion resulting in an alteration in shape, and there were some adhesions between the serosal side of the bladder and the abdominal wall and/or the surrounding fibrofatty tissue. The size distribution of visible tumors failed to reveal any characteristic pattern among Groups 1 through 4. There were no visible tumors in Group 5.

Histological bladder lesions observed in each group were classified into 4 types: simple hyperplasia; nodular or papillary hyperplasia; papilloma; and carcinoma (21) (Table 2). Each rat is tabulated in the column of the most advanced lesions found in the bladder. A diagnosis of cancer was based on the loss of differentiation, the presence of nuclear pleomorphism, and the presence of mitoses. Furthermore, carcinomas were subdivided according to the presence or absence of invasion and into Grades 1 to 3 on the basis of the degree of their cellular atypism and structural atypism as usual. As described by Squire et al. (33), subepithelial invasion was scored as an invasive lesion if individual epithelial cells or loose clusters of cells without a well-defined boundary were observed below the presumed location of the basal lamina of the tumors.

Rats subjected only to the ulceration procedure (Group 5) had no epithelial lesions of the urinary bladder except for occasional simple hyperplasia. This finding agreed with the results of a previous experiment in which proliferative epithelial lesions resulting from the regeneration induced by freezing were reversible (30). The incidences of papilloma and carcinoma of rats in Group 1 were clearly suppressed as compared to those in Group 4 in which rats were treated with only BBN. Although the incidence of carcinoma in Group 1 was not significantly different from Group 4, the difference in the incidence of papillomas plus carcinomas between these 2 groups (i.e., 10 rats with papilloma or carcinoma in Group 1, and 25 rats with these tumors in Group 4) was significant (by χ² test, p < 0.001). However, there was no effect in the incidence of papilloma or carcinoma by ulceration in Groups 2 and 3 as compared with the incidence in Group 4.

Calcification was observed on the serosal side of the bladder at various degrees in almost all rats in Groups 1, 2, 3, and 5, which were given the freezing ulceration. No persistent ulcers in the bladders of those rats were detected.

The total number of papillomas and carcinomas including presence of invasion and grade of malignancy are summarized in Table 3. No evidence was found that ulceration increases either the number of tumors or their malignancy. On the contrary, the number of papillomas and carcinomas per rat in Groups 1, 2, and 3 were smaller than those in the control (Group 4), especially those in Group 1 which were significantly less than the control values (0.4 ± 0.7 versus 1.0 ± 0.9; p < 0.01). Incidences of the bladder tumors (papilloma plus carcinoma) at the site of the ulceration were 2 of 14 in Group 1, 6 of 26 in Group 2 and 3 of 17 in Group 3. All papillomas and carcinomas were of a transitional cell type; squamous cell metaplasias were occasionally seen in the tumors.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats at start</th>
<th>Effective no. of rats</th>
<th>Av. cumulative dose/organic rat (g)</th>
<th>Final body wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ulcer → BBN</td>
<td>34</td>
<td>33</td>
<td>0.111</td>
<td>440 ± 34*</td>
</tr>
<tr>
<td>2. BBN → ulcer</td>
<td>34</td>
<td>32</td>
<td>0.122</td>
<td>438 ± 41</td>
</tr>
<tr>
<td>3. BBN → ulcer</td>
<td>24</td>
<td>22</td>
<td>0.119</td>
<td>430 ± 26</td>
</tr>
<tr>
<td>4. BBN</td>
<td>34</td>
<td>34</td>
<td>0.122</td>
<td>440 ± 33</td>
</tr>
<tr>
<td>5. Ulcer</td>
<td>24</td>
<td>24</td>
<td>0.212</td>
<td>443 ± 21</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective no. of rats</th>
<th>Normal</th>
<th>Simple hyperplasia</th>
<th>Nodular or papillary hyperplasia</th>
<th>Papilloma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ulcer → BBN</td>
<td>33</td>
<td>5 (15)*</td>
<td>12 (36)</td>
<td>6 (18)</td>
<td>3 (9)</td>
<td>7 (21)</td>
</tr>
<tr>
<td>2. BBN → ulcer</td>
<td>32</td>
<td>1 (3)</td>
<td>2 (6)</td>
<td>0 (6)</td>
<td>9 (28)</td>
<td>12 (38)</td>
</tr>
<tr>
<td>3. BBN → ulcer</td>
<td>22</td>
<td>0</td>
<td>3 (14)</td>
<td>4 (15)</td>
<td>8 (36)</td>
<td>14 (41)</td>
</tr>
<tr>
<td>4. BBN</td>
<td>34</td>
<td>5 (15)</td>
<td>2 (6)</td>
<td>0 (6)</td>
<td>11 (32)</td>
<td>14 (41)</td>
</tr>
<tr>
<td>5. Ulcer</td>
<td>24</td>
<td>20 (83)</td>
<td>4 (17)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
DISCUSSION

While the concept of the 2-stage process of carcinogenesis, initiation and promotion, is firmly accepted, the mechanisms of the 2-stage process are not yet well understood. It appears that the mechanisms of initiation and promotion differ biologically and biochemically. Investigations of the epidermal and hepatic system have revealed that initiation is irreversible while promotion is reversible (25, 29, 39). The initiation step is likely that the initiation step can be more effective when the carcinogen treatment decreased rather than increased the replication of the altered DNA (9, 38).

This investigation was conducted to determine whether the incidence of bladder tumors by BBN would be increased in response to the stimulated DNA replication in the urothelium after the production of an ulcer by freezing. Contrary to expectations, bladder tumor induction was inhibited if the bladder was frozen 24 hr before the administration of BBN. The incidence of the bladder tumors was approximately one-half of that in the unulcerated control group. If, however, the ulceration occurred after BBN treatment, the incidence of bladder tumors was not affected. The reason(s) why ulcer formation prior to the carcinogen treatment decreased rather than increased the induction of the bladder tumors is not clear from the present study.

The difference in tumor incidence does not seem to have resulted from the amount of BBN consumed. Therefore, it is reasonable to assume that the interaction between BBN or its metabolite(s) with DNA or other macromolecules of the bladder epithelium was reduced in the ulcerated rats. This phenomenon could come from a decrease in the final metabolic activation of BBN or its metabolite and/or contact of carcinogen with the bladder epithelium through the urine. Okada et al. (22, 23) have shown that a major urinary metabolite of BBN is BCPN and have suggested that BCPN is a proximate carcinogen. Under certain conditions, further metabolic activation of BCPN in the urothelium is required for BCPN to become carcinogenic (20, 24). Oyasu et al. (24) showed that topical application of BCPN into heterotopic bladder induced bladder tumors in homotopic natural bladders but not in the heterotopic bladders. He postulated that the failure of BCPN to induce tumors in heterotopic bladders may have resulted from the inability of the epithelial cells in the heterotopic bladder to activate BCPN to an ultimate carcinogen, and the absence of urine, which could be a cofactor in BCPN carcinogenesis. Similar reasons may be applied to the present experiment.

As a result of rapid regeneration following ulceration, many areas of the bladder mucosa, including the ulcer site, are covered with immature cells. Whereas superficial cells of the normal bladder of the rat are lined with an asymmetrical membrane and have numerous fusiform vesicles (12), immature regenerated mucosa were covered with a symmetrical membrane with numerous short microvilli. In the cytoplasm of the immature mucosa, there were numerous ribosomes, an increased number of mitochondria, and only a few fusiform vesicles. This immaturity of the mucosa lasted for at least 5 days (30). In addition, a markedly reduced number of fusiform vesicles might play a role in the uptake of the carcinogen from urine into the cells (12). If the carcinogenicity of BBN is mediated through activated metabolic events that occur in mature cells of the mucosa, it is likely that the immaturity implied by the altered ultrastructure may extend to functional alterations in the abilities of those cells to metabolize xenobiotic compounds. The immature mucosa could, for example, have a decreased ability to activate BBN metabolites.

Alternatively, there could be a shortened time of contact and interaction between urothelial cells and carcinogen in the bladder, possibly due to frequent urination resulting from a physiological mechanism to protect the ulcer from exposure to urine. Ito et al. (16) showed that a combination of unilateral ureter ligation and BBN administration p.o. induced transitional cell carcinomas in the ipsilateral kidney pelvis and ureter, which are rare sites of tumor development by BBN in normal rats. These results suggest that the excreted bladder carcinogen from the kidney may require contact with urothelium for a relatively long time in order to exhibit its carcinogenicity.

The lack of enhancing effects by ulceration after BBN treatment is consistent with the concept that chronic repeated cell proliferation is more important for promotion than a single burst of cell proliferation (9). A further factor may be that ulceration after BBN treatment actually destroys a large portion of the transformed cells and consequently obscures a weak promoting activity that may have resulted from a brief promoting stimulus.

Further investigation will be required to develop an experimental model by which one will be able to detect weak potential bladder carcinogens through the amplification of their activity as a consequence of DNA replication.

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Table 3

<table>
<thead>
<tr>
<th>Carcinomas</th>
<th>Grade 1</th>
<th>Grade 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of papillomas</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Effective no. of rats</td>
<td>33</td>
<td>7</td>
</tr>
</tbody>
</table>

Mucosal Regeneration and Bladder Carcinogenesis

Total number of papillomas and carcinomas and grade of malignancy

1. Ulcer — BBN
2. BBN — ulcer
3. BBN — ulcer
4. BBN
REFERENCES


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