Inhibition of N-n-Butyl-N-(4-hydroxybutyl)nitrosamine-induced Urinary Bladder Cancer in Rats by Administration of Disulfiram in the Diet

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ABSTRACT

The objective of this study was to determine if disulfiram would influence the induction of urinary bladder cancer in rats given N-n-butyl-N-(4-hydroxybutyl)nitrosamine (BHBN). Adult male Wistar rats were divided into: Group 1, control diet, 30 rats; Group 2, control diet plus 0.025% BHBN in the drinking water, 60 rats; Group 3, control diet containing 0.5% disulfiram, 30 rats; and Group 4, control diet containing 0.5% disulfiram plus 0.025% BHBN in the drinking water, 60 rats. The animals were kept on these regimens for 15 weeks and then were transferred to and maintained on control diet. The average total intake of BHBN was 1.21 g/rat for Group 2 and 1.23 g/rat for Group 4. The cumulative incidences of bladder cancer at 25 weeks after initial exposure to BHBN were: Group 1, 0 of 9; Group 2, 27 of 27; Group 3, 0 of 9; and Group 4, 0 of 27. At termination of the experiment (32 to 42 weeks), the final bladder cancer incidences were: Group 1, 0 of 9; Group 2, 57 of 57 (100%); Group 3, 0 of 24 (0%); and Group 4, 7 of 27 (25%). Except for a carcinoma of the renal pelvis in one rat in Group 2 and the bladder tumors in Groups 2 and 4, tumors were not detected in other organs of any of these rats. It was concluded that disulfiram significantly inhibited the induction of bladder cancer in rats exposed to BHBN. The mechanism of action of disulfiram in this process is under investigation.

INTRODUCTION

In 1964, Druckrey et al. (4) reported the selective action of BHBN, a metabolite of di-n-butylnitrosamine, in the induction of bladder cancer in rats. Since that time, BHBN has been widely used as a model bladder carcinogen (1, 2, 10, 13, 17). The principal urinary metabolite of BHBN in rats has been identified as BCPN, formed by oxidation of the alcoholic group of BHBN to the carboxyl group (19). BCPN was later shown to also be a selective bladder carcinogen in the rat (11), and BHBN, which is not commercially available, has also been improved.

N-n-Butyl-4-aminobutanol. A mixture of 167 g (1.5 mol) of 4-chlorobutanol (23) and 309 g (4.2 mol) of n-butylamine in 835 ml of toluene was refluxed for 48 hr. After the mixture cooled to room temperature, 80 ml of 50% NaOH were added, and toluene and excess butylamine were removed under reduced pressure. The residual aqueous solution was extracted several times with ether. The combined ether extracts were dried with anhydrous sodium carbonate and the solvent was removed under reduced pressure. The final product was obtained by distillation (b.p. 72—75° at 0.2 to 0.3 mm Hg).

BHBN. N-n-Butyl-4-aminobutanol (58 g, 0.4 mol) was dissolved in 120 ml of ether; then 40 g of ice and a solution of 130 g (1.9 mol) of sodium nitrite in 200 ml of water were added. Without stirring, a mixture of 86 ml of concentrated nitric acid in 120 g of ice was added over a period of 1.5 to 2 hr, keeping the reaction mixture at 10—15°. The ether layer was removed, washed with cold water, then washed with cold saturated sodium carbonate until neutral, and again washed with cold water. After drying with anhydrous sodium carbonate, the ether was stripped, yielding 112 g (0.8 mol, 50%) of N-n-butyl-4-aminobutanol which was purified by distillation (b.p. 72—75° at 0.2 to 0.3 mm Hg).

MATERIALS AND METHODS

Synthesis of BHBN. The methods in the literature for the synthesis of BHBN (2, 4) gave very low overall yields, which we have been able to improve considerably. The major reason for the low yields is the formation of the nitrite ester of BHBN in the nitrosation of N-n-butyl-4-aminobutanol and the difficulty in hydrolyzing this nitrite ester (2, 4, 21). We have found that the nitrite ester of BHBN is rather easily converted to BHBN by solvolysis in methanol. The synthesis of N-n-butyl-4-aminobutanol, which is not commercially available, has also been improved.

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Disulfiram was added to the diet (5.0 g disulfiram per kg diet) by dissolving the compound in the corn oil before blending and pelleting. Animals were divided into: Group 1, control diet, 30 rats; Group 2, control diet plus 0.025% BHBN in the drinking water, 60 rats; Group 3, control diet containing 0.5% disulfiram, 30 rats; and Group 4, control diet containing 0.5% disulfiram plus 0.025% BHBN in the drinking water, 60 rats. Diets and drinking water were provided ad libitum, and the amount of water or 0.025% BHBN solution consumed was recorded. For the animals in Group 4, the disulfiram-containing diet was started 2 days after BHBN administration. The animals were kept on the regimens indicated for 15 weeks and then were transferred to and maintained on control diet. For Groups 3 and 4, the disulfiram was removed from the diet 4 days after BHBN was discontinued in Group 4. For necropsy, the animals were killed in a CO₂ chamber, and the urinary bladders were inflated with 0.5 ml of Carson's fixative (phosphate-buffered formalin) and placed in this fixative. Other organs were examined grossly, and samples of liver and the entire esophagus were also taken for histological examination. Sections were cut at 3 to 4 μm and stained with hematoxylin and eosin.

RESULTS

General Observations. Survival of animals during the 15-week period on the diets of disulfiram and BHBN was very good (Table 1). In all groups, only 2 of 180 (1%) animals died during this period. Administration of BHBN in the drinking water did not have any significant effect on either survival or weight gain at 15 weeks. The rats fed the disulfiram diet (Groups 3 and 4) for 15 weeks showed significantly less weight gain compared to those receiving the control diet [Groups 1 and 2 (Table 1)], but otherwise the animals appeared quite healthy. Most of the discrepancy in weight gain was due to an initial weight loss when the animals were first put on the diets containing disulfiram (Chart 1). After 2 to 3 weeks, the rate of weight gain of rats on the disulfiram diet was almost parallel to that of rats on the control diet (Chart 1), and at the termination of the experiment there were no significant differences in body weights.

There was no difference in consumption of water or the 0.025% BHBN solution between rats in the 4 experimental groups. Mean (±S.E.) intakes (ml/day/rat) for animals in Groups 1, 2, 3, and 4 were 47 ± 3.3, 46 ± 3.3, 44 ± 1.8, and 47 ± 1.5, respectively. Calculated intakes of BHBN per animal for Groups 2 and 4 were 11.50 mg/day (1.21 g, total) and 11.75 mg/day (1.23 g, total), respectively.

Effect of disulfiram on bladder cancer induction in rats by administration of BHBN

Animals in each group were treated as described in Table 1. Small numbers of animals in each group were serially sacrificed at 15 to 25 weeks after the beginning of the experiment. Group 2 was terminated at 32 weeks, since all the rats in that group had developed bladder cancer by that time. The experiment was terminated at 42 weeks.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body wt (g)</th>
<th>Survival at 15 wk (no. alive/no. started)</th>
<th>Wt gain at 15 wk (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>298 ± 2.6</td>
<td>30/30</td>
<td>193 ± 8.9</td>
</tr>
<tr>
<td>BHBN</td>
<td>296 ± 2.2</td>
<td>59/60</td>
<td>194 ± 4.9</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>300 ± 2.2</td>
<td>29/30</td>
<td>87 ± 4.7</td>
</tr>
<tr>
<td>BHBN + disulfiram</td>
<td>301 ± 1.9</td>
<td>60/60</td>
<td>84 ± 2.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>15 wk</th>
<th>20 wk</th>
<th>25 wk</th>
<th>32 wk</th>
<th>42 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0/3</td>
<td>0/6</td>
<td>0/9</td>
<td>0/30</td>
<td></td>
</tr>
<tr>
<td>2. BHBN</td>
<td>9/9</td>
<td>18/18</td>
<td>27/27</td>
<td>57/57</td>
<td></td>
</tr>
<tr>
<td>3. Disulfiram</td>
<td>0/3</td>
<td>0/6</td>
<td>0/9</td>
<td>0/24</td>
<td></td>
</tr>
<tr>
<td>4. BHBN + disulfiram</td>
<td>0/9</td>
<td>0/18</td>
<td>0/27</td>
<td>1/39</td>
<td></td>
</tr>
</tbody>
</table>

Table 2

| Effect of disulfiram on bladder cancer induction in rats by administration of BHBN |
|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Group                           | 15 wk  | 20 wk  | 25 wk  | 32 wk  | 42 wk  |
| 1. Control                      | 0/3    | 0/6    | 0/9    | 0/30   |        |
| 2. BHBN                         | 9/9    | 18/18  | 27/27  | 57/57  |        |
| 3. Disulfiram                   | 0/3    | 0/6    | 0/9    | 0/24   |        |
| 4. BHBN + disulfiram            | 0/9    | 0/18   | 0/27   | 1/39   |        |

Disulfiram Inhibition of Bladder Cancer Induction

Cumulative bladder cancer incidence at 15 weeks:

- Control: 0/3 rats developed bladder cancer (Table 1).
- BHBN: 9/9 rats developed bladder cancer (Table 1).
- Disulfiram: 0/3 rats developed bladder cancer (Table 1).
- BHBN + disulfiram: 0/9 rats developed bladder cancer (Table 1).

For Groups 2 and 4 were 11.50 mg/day (1.21 g, total) and 11.75 mg/day (1.23 g, total), respectively.

Tumor Incidence. At the time the BHBN was discontinued (15 weeks), the urinary bladders of small numbers of animals in each group were examined. Three of 9 rats examined in Group 2 (BHBN only) had gross bladder lesions, histologically diagnosed as transitional cell carcinoma. Histological examination of the other 6 bladders in this group of rats revealed that they all had bladder lesions (2 papillomas, 2 carcinoma in situ, and 2 transitional cell carcinoma). Although the papillomas were histologically benign lesions, it is clear from other studies (14) that these lesions progress to transitional cell carcinoma. There were no histological changes seen in the bladders of control rats (Groups 1 and 3). Four of 9 bladders examined from rats in Group 4 (BHBN plus disulfiram) showed slight dysplasia, and the other 5 bladders were histologically normal.

By 20 weeks (5 weeks after disulfiram and BHBN were discontinued), all rats examined in Group 2 (BHBN; 9 of 9 rats) had gross bladder cancer, with lesions ranging in size from 1 to 4 mm in diameter (Table 2). Bladders of control rats in Groups 1 and 3 were histologically normal, as were 8 of 9 bladders of rats from Group 4 (BHBN plus disulfiram). The bladder epithelium of 1 rat in Group 4 showed some focal hyperplasia.

At 25 weeks, the findings were essentially the same as those found at 20 weeks (Table 2). The bladder cancers of rats from Group 2 (BHBN) were larger (up to 10 mm in diameter).
One of 9 rats in Group 4 (BHBN plus disulfiram) had some focal hyperplasia, and 2 of 9 rats in this group showed some dysplasia of the urothelium.

All 30 rats remaining in Group 2 (BHBN) were killed at 32 weeks, and all of these had bladder cancer. Many of the tumors filled the lumen of the bladder by this time and most were transitional cell carcinoma, Grade III, Stage B or C. Except for carcinoma of the renal pelvis in 1 rat, no other tumors were seen in these rats. These findings are consistent with the results of others (8, 14). At the time the rats in Group 2 were killed, 12 animals in Group 4 (BHBN and disulfiram) were also killed. Except for a small bladder tumor 2 mm in diameter, diagnosed as a transitional cell carcinoma (Stage 0, Grade II), there were no other pathological changes seen in these animals (Table 2).

The experiment was terminated at 42 weeks. No pathological changes were observed in the remaining animals in Group 1 (control) or Group 3 (disulfiram; Table 2). Four of 16 rats in Group 4 (BHBN and disulfiram) had small macroscopic bladder tumors 1 to 2 mm in diameter. All of these were transitional cell carcinoma, Stage 0, Grade I or II. Histological examination of the remaining bladders in Group 4 turned up 2 additional lesions, 1 papilloma and 1 transitional cell carcinoma (Stage 0, Grade II).

Bladder Stone Formation. Wistar rats are prone to develop bladder stones, but no data could be found in the literature on the incidence of bladder stones in this strain of rat. Consequently, we carefully examined the contents of each urinary bladder at necropsy. Stones, ranging in size from 1 mm in diameter to as large as 3 x 10 mm, were found in animals in all groups. The total incidence of bladder stones was 59 of 166 (36%) and there were no significant differences in stone incidences between any of the groups (Table 3). Likewise, there was no relationship between total bladder cancer and the presence of stones; 30 of 30 (100%) of the rats with bladder cancer and 33 of 102 (32%) rats with no bladder cancer had stones ($\chi^2 = 0.84$, no significant difference). Stone formation did not appear to be involved as a promoting agent in the induction of bladder cancer by BHBN in the rats protected with disulfiram (Group 4); in this group, 3 of 7 (43%) rats with bladder cancer (Table 3) had stones, while 14 of 48 (29%) rats with no bladder cancer had stones ($\chi^2 = 1.37$, no significant difference).

DISCUSSION

These findings demonstrate that concurrent administration of disulfiram in the diet with BHBN markedly inhibits the induction of urinary bladder cancer in Wistar rats by BHBN. Disulfiram has been shown previously by Wattenberg (24) to inhibit carcinogen-induced neoplasia in the forestomach of mice treated with benzo(a)pyrene, mammary glands of rats given 7,12-dimethylbenz(a)anthracene, and the large intestine of mice given s.c. injections of 1,2-dimethylhydrazine or azoxymethane. Schmähl et al. (22) reported that disulfiram had no effect on total tumor incidences of rats given dimethylhydrazine or diethylnitrosamine but found that disulfiram did change the organotrophy of these 2 nitrosamines. Liver cancer induced by dimethylhydrazine or diethylnitrosamine was significantly reduced by giving disulfiram by stomach tube 2 hr prior to p.o. administration of the nitrosamines. However, carcinoma of the esophagus was enhanced in rats given diethylnitrosamine plus disulfiram, and the incidence of tumors of the paranasal sinuses was elevated in those rats given dimethylhydrazine plus disulfiram. In our studies with BHBN, change in organotrophy did not accompany the marked reduction of bladder cancer observed by treatment with disulfiram.

The mechanism by which disulfiram inhibits carcinogenesis in other model systems has been most extensively studied for the induction of colon cancer with 1,2-dimethylhydrazine (5, 6). Disulfiram inhibits the N-oxidation of azoxymethane (formed by oxidation of dimethylhydrazine) to azoxymethane, an essential step in the metabolic activation of dimethylhydrazine (5, 6). The observation that disulfiram partially suppresses the neoplastic effects of azoxymethane itself suggests that it inhibits more than one oxidative step (24). It has been postulated that methylazoxymethanol, a metabolite of azoxymethane, is converted to a reactive aldehyde (9) or carboxylic acid (7) by an NAD$^+$-dependent dehydrogenase, similar to alcohol dehydrogenase, and that disulfiram inhibits formation of the postulated reactive acid derivative of methylazoxymethanol. From these studies and previous work by other investigators (12, 15), it is clear that disulfiram depresses microsomal mixed-function oxidase activity. This may be due to a decrease in hepatic cytochrome P-450 content (15).

We do not yet know the mechanism by which disulfiram inhibits the induction of bladder cancer by BHBN, but it seems most likely to be due to some alteration in the metabolism of BHBN. Disulfiram is well known as an inhibitor of aldehyde dehydrogenases (3) and is, of course, widely used for this purpose in the treatment of alcoholism. Disulfiram may inhibit the oxidation of BHBN to BCNP, the major urinary metabolite of BHBN, which is postulated to be a proximate carcinogenic metabolite of BHBN (17, 18). This possibility is being investigated. In this connection, Ito et al. (13) found that pretreatment of rats with α-naphthylisothiocyanate significantly decreased the induction of bladder cancer in rats given BHBN. They suggested that the hepatotoxicity produced by α-naphthylisothiocyanate decreased the oxidation of BHBN to BCNP, but this has not been tested experimentally. 13-cis-Retinoic acid also diminishes the number and severity of bladder cancers induced by BHBN in rats (10) and mice (1), but the mechanism of action of 13-cis-retinoic acid does not involve effects on the metabolism of BHBN since the retinoid was not started until 7 days after the last exposure to BHBN.

There are other sites at which disulfiram might influence the metabolic fate of BHBN. Hepatic UDP-glucuronoltransferase activity and several other enzymes associated with the α-glucuronic acid pathway are increased after treatment of rats with disulfiram (16), which raises the possibility that there might be...
increased detoxification of BHBN or its metabolites by glucuronidation reactions.

REFERENCES


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