EFFECTS OF THREE SWEETENERS ON RAT URINARY BLADDER CARCINOGENESIS INITIATED BY N-BUTYL-N-(4-HYDROXYBUTYL)-NITROSAMINE

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The effects of three sweeteners, sodium saccharin, aspartame and stevioside, on urinary bladder carcinogenesis in rats initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) were evaluated. Male F344 rats were given 0.01% BBN in their drinking water for 4 weeks and then the test sweeteners in their diet for 32 weeks. All surviving rats were sacrificed after 36 weeks, and examined histologically. Treatment with sodium saccharin significantly increased the incidence and extent of preneoplastic lesions, papillary or nodular (PN) hyperplasia, in rats treated with BBN for 4 weeks. Administration of 5% aspartame or 5% stevioside in the diet did not, however, affect the incidence or extent of PN hyperplasia in BBN-treated rats. No preneoplastic or neoplastic lesions of the urinary bladder were observed in rats treated with the test sweeteners only. The results with sodium saccharin were consistent with those in our previous experiments. The data also suggest that aspartame and stevioside do not promote bladder carcinogenesis.

Key words: Bladder promoter — Sweetener — N-Butyl-N-(4-hydroxybutyl)nitrosamine — F344 rat

It is uncertain from epidemiological studies whether artificial sweeteners cause cancer in humans. However, there is some evidence that saccharin increases the incidence of bladder tumors in male rats, when exposure is started in utero. 2, 29) Recently, Fukushima et al. found that ACI rats given 5% sodium saccharin in the diet for up to 52 weeks developed urinary bladder cancer. 6) Moreover, Hicks et al. reported that saccharin and cyclamate promoted urinary bladder carcinogenesis initiated by N-methyl-N-nitrosourea (MNU). 14) Saccharin has also been found to promote urinary bladder carcinogenesis initiated by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT). 4) Furthermore, we demonstrated that saccharin acts as a promoter of urinary bladder carcinogenesis in rats initiated by BBN 8, 10, 15, 23-26) or N-2-fluorenylacetamide (2-FAA). 23) A dose-dependent effect 23) of saccharin as a promoter have been shown in rat urinary bladder carcinogenesis initiated by BBN. There are reports that saccharin induces slight epithelial hyperplasia in rat urinary bladder, 7, 22) which is believed to a characteristic effect of promoters. 3, 28)

There have been many reports on preneoplastic and neoplastic lesions in rat urinary bladder carcinogenesis, and there is strong evidence of a correlation between PN hyperplasia and cancer. 12, 18, 19) Thus PN hyperplasia has been used as a marker in our relatively short-term studies on the two-stage process of urinary bladder carcinogenesis. 8-10, 15, 23-26)

In the present study, the effects of two artificial sweeteners (sodium saccharin and aspartame) and a natural sweetener (stevioside) as promoters of bladder carcinogenesis in our BBN-F344 rat model were examined.
MATERIALS AND METHODS

Chemicals  BBN (Tokyo Kasei Kogyo Co. Ltd., Tokyo) was used as an initiator. Sodium saccharin (Sherwin-Williams Co., U.S.A.), aspartame (Ajinomoto Co. Ltd., Tokyo) and stevioside (Nikken Kagaku Co. Ltd., Tokyo) were tested as promoters. BBN was given at a concentration of 0.01% in the drinking water, and test sweeteners were added to the basal diet, Oriental M (Oriental Yeast Co., Tokyo) on a weight/weight basis.

Animals and Maintenance  Male F344/DuCrj rats were obtained from Charles River Japan, Inc., Kanagawa. The rats, which were about 6 weeks old at the beginning of the experiment, were housed five to a plastic cage with hard-wood chips for bedding. The room temperature was kept at 21±2°C and the humidity at 55±10% with a 12-hr light/dark cycle. A positive air pressure was maintained with 15 air changes/hr. Water and the appropriate diet were available ad libitum.

Experimental Procedure  Three subgroups of rats were used for tests on each sweetener. Rats were given drinking water with (subgroups 1 and 2) or without (subgroup 3) BBN for 4 weeks and then were maintained on diet containing one of the sweeteners (subgroups 1 and 3) or no addition (subgroup 2) for 32 weeks. The animals were observed daily for abnormalities and were weighed every two weeks. Food and water consumption were measured over a 2-day period before each time of weighing.

In week 36, fresh urine samples were obtained from at least five rats in each group. The pH of the samples was measured with bromothymol blue (pH 6.2—7.8) or methyl red (pH 5.4—7.0) test paper (Toyo Roshi Co., Ltd., Tokyo) and the osmolality (Osmette A, Precision System, Inc., Mass.) was recorded. Urine samples collected over a 4-hr period were centrifuged and the precipitate was examined for epithelial cells, red blood cells (RBC), white blood cells (WBC), crystals and casts.

After 36 weeks, surviving animals were deprived of food, but not water, overnight and then killed under ether anesthesia by exsanguination from the aorta. The liver and kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. The urinary bladder was fixed by filling it, and then immersing it in 10% phosphate-buffered formalin. After fixation, the surfaces of the bladder were examined under a dissecting microscope. The bladder was then cut into 8 strips. Bladder tissue was embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined histologically. For quantitative analysis, urinary bladder lesions were counted by light microscopy, the total length of the basement membrane was measured with a color video image processor (VIP-21CH; Olympus-Ikegami Tsushin Co., Tokyo), and numbers of lesions per 10 cm of basement membrane were recorded. The liver and kidneys were also examined histologically.

RESULTS

No clinical abnormality or mortality related to the test sweeteners was apparent in any of the rats during the 36-week experiment. Body weight gain was slightly decreased or retarded in rats treated with 5% sodium saccharin, 5% aspartame or 5% stevioside with or without BBN from week 5 to 36 of the experiment. Data on water and food intakes are shown in Table I. During the initiation period (0—4 weeks), there were no differences between the subgroups in their average water intakes. The total BBN intakes of rats in subgroups 1 and 2 (receiving 0.01% BBN in the drinking water) were similar. In the promotion period (weeks 5—36), groups given 5% sodium saccharin with or without BBN drank more water than control subgroup 2. The food consumptions of all the subgroups were essentially the same throughout the experiment.

Results of urinalysis in week 36 are presented in Table II. In week 36, the pH of the urines of animals given 5% sodium saccharin or 5% aspartame with or without BBN increased slightly. Crystals, casts, epithelial cells, WBC and RBC in the urinary sediment were examined, but only the crystal count of rats given 5% sodium saccharin exceeded that of controls. Other parameters, such as the protein, glucose, ketone bodies, bilirubin, occult blood and urobilinogen, examined in week 36 were within the ranges for normal rats of this strain of the same age in our laboratory. No significant differences (P<0.05) in the liver or kidney weight relative to the body weight were found in any
### Table I. Average Intakes of Water, Total Test Sweetener, and Food

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>5–36 wks Av. water intake (g/rat/day)</th>
<th>Total test sweetener intake (g/kg body wt.)</th>
<th>5–36 wks Av. food consumption (g/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Saccharin</td>
<td>25</td>
<td>26.9</td>
<td>537.5</td>
<td>15.3</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>25</td>
<td>21.0</td>
<td>0</td>
<td>16.1</td>
</tr>
<tr>
<td>- Saccharin</td>
<td>25</td>
<td>27.0</td>
<td>550.6</td>
<td>15.9</td>
</tr>
<tr>
<td>+ Aspartame</td>
<td>30</td>
<td>18.5</td>
<td>400.6</td>
<td>14.1</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>30</td>
<td>NE</td>
<td>0</td>
<td>14.1</td>
</tr>
<tr>
<td>- Aspartame</td>
<td>30</td>
<td>18.1</td>
<td>395.7</td>
<td>14.5</td>
</tr>
<tr>
<td>+ Stevioside</td>
<td>25</td>
<td>22.4</td>
<td>483.5</td>
<td>15.5</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>25</td>
<td>21.8</td>
<td>0</td>
<td>15.2</td>
</tr>
<tr>
<td>- Stevioside</td>
<td>25</td>
<td>22.6</td>
<td>486.6</td>
<td>15.6</td>
</tr>
</tbody>
</table>

NE, not examined.

### Table II. Urinalysis in Week 36

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats examined</th>
<th>pH</th>
<th>Osmolality (mOsm/kg H₂O)</th>
<th>Urinary sedimentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Saccharin</td>
<td>10</td>
<td>6.9</td>
<td>1237 ± 391</td>
<td>++</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>10</td>
<td>6.6</td>
<td>1243 ± 297</td>
<td>±</td>
</tr>
<tr>
<td>- Saccharin</td>
<td>10</td>
<td>7.1</td>
<td>1777 ± 264</td>
<td>++</td>
</tr>
<tr>
<td>+ Aspartame</td>
<td>5</td>
<td>7.2</td>
<td>2313 ± 457</td>
<td>+</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>5</td>
<td>6.3</td>
<td>2030 ± 470</td>
<td>+</td>
</tr>
<tr>
<td>- Aspartame</td>
<td>5</td>
<td>6.7</td>
<td>1534 ± 590</td>
<td>+</td>
</tr>
<tr>
<td>+ Stevioside</td>
<td>10</td>
<td>6.5</td>
<td>1731 ± 296</td>
<td>+</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>10</td>
<td>6.6</td>
<td>1674 ± 565</td>
<td>±</td>
</tr>
<tr>
<td>- Stevioside</td>
<td>10</td>
<td>6.6</td>
<td>1541 ± 420</td>
<td>±</td>
</tr>
</tbody>
</table>

±, very slight; +, slight; ++, moderate; +++ marked.

### Table III. Histological Findings in the Urinary Bladder

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats examined</th>
<th>PN hyperplasia Incidence No. (%)</th>
<th>Density No./10 cm BM</th>
<th>Papilloma Incidence No. (%)</th>
<th>Density No./10 cm BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Saccharin</td>
<td>25</td>
<td>23 (92.0)*** 2.5±2.9***</td>
<td>5 (20.0) 0.3±0.6</td>
<td>+ Saccharin</td>
<td>25</td>
</tr>
<tr>
<td>+ Basal diet</td>
<td>25</td>
<td>23 (92.0)*** 2.5±2.9***</td>
<td>5 (20.0) 0.3±0.6</td>
<td>+ Basal diet</td>
<td>25</td>
</tr>
<tr>
<td>+ Aspartame</td>
<td>28</td>
<td>8 (28.6) 0.5±1.1</td>
<td>11 (39.3) 0.5±0.7</td>
<td>+ Aspartame</td>
<td>28</td>
</tr>
<tr>
<td>+ Stevioside</td>
<td>25</td>
<td>4 (16.0) 0.2±0.5</td>
<td>4 (16.0) 0.2±0.4</td>
<td>+ Stevioside</td>
<td>25</td>
</tr>
</tbody>
</table>

BM, basement membrane.

**P<0.01, ***P<0.001.
group. The histological appearance of these organs was also normal.

Quantitative data on histopathological changes of the urinary bladder are summarized in Table III. Animals that died or became moribund during the experiment were excluded from effective numbers. The lesions observed in the urinary bladder mucosa were classified as PN hyperplasia, papilloma and cancer on the basis of the criteria described previously. 12, 18, 19)

The incidence of PN hyperplasia was significantly higher than that in the appropriate control (subgroup 2) only in the group given 0.01% BBN and 5% sodium saccharin. Quantitative analysis showed that the number of areas of PN hyperplasia per 10 cm of basement membrane was also significantly increased in this group. However, there were no significant differences between the experimental and control groups in the incidences and densities of papillomas. Cancer was not found in any group. Rats given a sweetener without BBN (subgroup 3) had no bladder lesions (including epithelial hyperplasia).

**DISCUSSION**

To prevent urinary bladder cancers in man, environmental promoters, especially in dietary constituents and nutritional factors, must be detected and eliminated. However, promoters that are non-genotoxic cannot be detected in short-term in vitro tests, such as Ames’ test. Moreover, although long-term toxicity studies are necessary to detect carcinogenic chemicals, such experiments are very expensive and time-consuming. Thus, we developed a relatively short-term test using relatively few animals which is based on the development of preneoplastic lesions. There is much evidence that PN hyperplasia is a preneoplastic lesion of the urinary bladder that develops before the induction of papilloma or cancer. 12, 18, 19)

Recently several chemicals were found by this method to act as promoters in bladder carcinogenesis, namely sodium saccharin, 8, 23, 25, 26) DL-tryptophan, 8) phenacetin, 24) butylated hydroxyanisole, 15) butylated hydroxytoluene, 15) sodium L-ascorbate, 8, 10) ethoxyquin, 11) and sodium erythorbate. 11) In contrast, 4,4'-diaminodiphenylmethane acted as an inhibitor of bladder carcinogenesis. 9) The present study was undertaken to determine whether other sweeteners besides 5% sodium saccharin are promoters of urinary bladder carcinogenesis in rats.

Saccharin, a noncaloric sweetener, is often used by diabetics as a sugar substitute, but its safety has been questioned, and there is evidence that it induces bladder carcinogenesis 2, 6, 29) and has promoting activity 4, 8, 13, 14, 23, 25, 26) in animals. In the present study, incorporation of 5% sodium saccharin into the diet increased urinary crystals and epithelial cells, and the incidence of PN hyperplasia in the urinary bladder of rats pretreated with BBN. The urinary crystals were probably not related to the promoting activity, since acetazolamide, which also causes formation of urinary crystals, does not act as a promoter. 8) These results are consistent with our previous finding that saccharin acts as a promoter after BBN treatment. 8, 23, 25, 26)

These results are also consistent with the findings that saccharin has promoting activity in urinary bladder carcinogenesis initiated by MNU, 13, 14) FANFT 4) and 2-FAA. 23)

Aspartame, a low calorie sweetener and flavor enhancer, is the methyl ester of a dipeptide of phenylalanine and aspartic acid, L-aspartyl-L-phenylalanine, and usually contains about 1% of its decomposition product (diketopiperazine). 5, 16, 20) Ishii et al. reported that Wistar rats maintained on diet containing up to 11.2% aspartame (4 g/kg body weight) showed a dose-related increase in urinary calcium and a dose-dependent increase in incidence of focal mineralization of the renal pelvis, but these increases were not related to epithelial hyperplasia. 17) In the present study, aspartame caused no significant increase in the incidence or density.
of PN hyperplasia or papilloma of the urinary bladder of rats initiated by BBN. The occurrence of renal pelvic mineralization was similar in the aspartame-treated and control groups. No hyperplastic lesions of the urinary bladder or kidneys were found in rats treated with aspartame alone.

Stevioside is a very sweet (1'→2) linked disaccharide-containing substance found in the leaves of a small shrub, Stevia rebaudiana Bertoni, that grows wild in Paraguay. The pure compound can be obtained in 6% yield from the dried leaves and is 300 times sweeter than sucrose. It has been reported that stevioside does not show mutagenicity, subchronic toxicity or teratogenicity, However, no data are available on its carcinogenicity in laboratory animals. In our study, stevioside showed no promoting activity after initiation with 0.01% BBN. Moreover, no urinary tract lesions were observed in rats treated with stevioside without initiation with BBN.

Acknowledgments

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