ABSTRACT

Since the sodium salt of ascorbic acid (AA) promoted two-stage urinary bladder carcinogenesis in rats, whereas AA itself did not, the roles of the urinary sodium ion concentration and pH on urinary bladder carcinogenesis were investigated. Male F344 rats were given 0.05% N-butyl-N-(4-hydroxybutyl)nitrosoamine in their drinking water for 4 weeks and then treated with basal diet containing 5% AA plus 3% sodium bicarbonate (NaHCO₃), 5% AA, 3% NaHCO₃ or 5% sodium L-ascorbate (SA), 5% SA plus 1% ammonium chloride (NH₄Cl) or 1% NH₄Cl, or no added chemical for 32 weeks. NaHCO₃ significantly increased the induction of neoplastic and preneoplastic lesions of the urinary bladder. Like SA, AA plus NaHCO₃ induced high incidences of neoplastic and preneoplastic lesions of the urinary bladder, whereas AA alone did not. NH₄Cl reduced the promoting activity of SA in urinary bladder carcinogenesis. These results suggest important roles for urinary sodium ion concentration and pH in modulating urinary bladder carcinogenesis. Moreover, AA was found to act as a co promoter under conditions of increased urinary pH and sodium ion concentration.

INTRODUCTION

The two-stage model of chemical carcinogenesis in the urinary bladder, consisting of initiation and promotion, has been demonstrated by several investigators (1-6). Recently, we examined the promoting activities of various chemicals in two-stage urinary bladder carcinogenesis in rats, using BBN, a strong urinary bladder carcinogen, as an initiator (7-12). Administration of 5% SA in the diet promoted urinary bladder carcinogenesis in rats (7), whereas administration of 5% AA in the diet did not (9). Urinary analysis showed that SA increased the urinary pH and sodium ion concentration, whereas AA did not (9). These results suggested that sodium ion has a key role in the promoting activity of AA and that elevation of the pH induced by sodium ingested as SA might be related to promotion of urinary bladder carcinogenesis.

In the present study, we examined the effects of AA plus NaHCO₃ and of SA plus NH₄Cl to determine the roles of urinary sodium ion concentration and pH on promotion by AA in two-stage urinary bladder carcinogenesis in rats.

MATERIALS AND METHODS

Animals. A total of 182 male 6-week-old F344 rats (Charles River Japan, Inc., Atsugi, Japan) were used. The rats were housed 5 per plastic cage with wood chips for bedding in an animal room with a 12-h light, 12-h dark cycle at 22 ± 2°C (SD) and 55 ± 10% relative humidity. Body weights, food consumption, and water intake were measured weekly up to week 14 and every other week from weeks 16 to 36. The amounts of food and water consumed in 2 consecutive days of a week were measured on a per cage basis.

Chemicals. BBN was from Tokyo Kasei Co., Tokyo, Japan. Food additive grade SA (Wako Pure Chemical Ind., Osaka, Japan), AA (Tanabe Seiyaku Co., Osaka, Japan), NaHCO₃ (Wako Pure Chemical Ind.), and NH₄Cl (Wako Pure Chemical Ind.) were used in experiments 1 and 2.

Experiment 1. Rats were randomly divided into 7 groups of 20 rats each. In the first 4 weeks they were given drinking water with 0.05% BBN, and then for 32 weeks they were given powdered basal diet (Oriental M; Oriental Yeast Co., Tokyo, Japan) containing 5% AA plus 3% NaHCO₃ (group 1), 5% AA (group 2), 3% NaHCO₃ (group 3), 5% SA (group 4), 5% SA plus 1% NH₄Cl (group 5), 1% NH₄Cl (group 6), or no added chemical (group 7, control group). The total observation period was 36 weeks.

For urine examination, fresh urine samples were obtained from 10 rats in each group in weeks 12, 24, and 36 by forced urination in the early morning. The pH was measured with a pH meter (Hitachi-Horiba pH meter, model F-7DE, Tokyo, Japan). In addition, 10 rats in each group were housed individually in metabolic cages without food or water for 4 h in the early morning for collections of urine samples. The osmolality was measured with an Osmette A instrument (Precision System, Inc., Natick, MA), and other parameters such as protein and occult blood were measured. Volumes of 2 ml of the remainder of samples were concentrated for microscopic examination of the urinary sediment.

In week 37, the rats were killed and their liver and kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. The urinary bladder was inflated by intraluminal injection of 10% phosphate-buffered formalin solution and then cut into 8 strips for histological examination. 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, Japan), and the number of lesions per 10 cm of basement membrane was calculated. The liver and kidneys were also examined histologically.

Experiment 2. Rats were randomly divided into 7 groups of 6 rats each. The animals were given powdered diet (Oriental M) containing a test chemical as in experiment 1 but without BBN treatment for 4 weeks.

In week 4, for urinary electrolyte analysis, samples of the urine were obtained from 6 rats in each group. For collection of these samples, rats were housed individually in metabolic cages without food or water for 4 h in the early morning. Sodium, potassium, calcium, chloride, phosphorus, magnesium, copper, and iron were analyzed in the Chu-nichi Clinic Center, Ohgaki, Japan. For measurement of the AA content of the urine, 4 rats in each group were placed in separated metabolic cages with glass collections tubes surrounded by ice and were given no food or water for 4 h in the early morning of different days in week 4. The urine was stored in a freezer. The following day, the contents of total AA and DA in the urine were measured by the 2,4-dinitrophenylhydrazine calorimetric method at the Japan Food Research Laboratories, Tokyo, Japan.

RESULTS

Experiment 1. Rats in test groups showed no toxic symptoms due to the chemicals, but several rats given AA plus NaHCO₃ (group 1) or SA (group 4) had hematuria in later stages of the experiment. Data on the weights of the body and urinary

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TABLE 1
Average body and urinary bladder weights and food and water consumptions (weeks 5–36) of rats in experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemicals</th>
<th>No. of rats</th>
<th>Body wt (g)</th>
<th>Urinary bladder wt</th>
<th>Av. food consumption (g/rat/day)</th>
<th>Av. water consumption (g/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA + NaHCO₃</td>
<td>20</td>
<td>Initial: 132.6 ± 5.2*</td>
<td>394.0 ± 17.6*</td>
<td>0.85 ± 0.92*</td>
<td>16.0 ± 0.25*</td>
</tr>
<tr>
<td>2</td>
<td>AA</td>
<td>20</td>
<td>Initial: 133.4 ± 4.6</td>
<td>424.4 ± 18.4*</td>
<td>0.16 ± 0.03</td>
<td>18.0 ± 0.04*</td>
</tr>
<tr>
<td>3</td>
<td>NaHCO₃</td>
<td>20</td>
<td>Initial: 132.8 ± 5.6</td>
<td>413.9 ± 25.1*</td>
<td>0.25 ± 0.15*</td>
<td>15.3 ± 0.06*</td>
</tr>
<tr>
<td>4</td>
<td>SA</td>
<td>20</td>
<td>Initial: 132.1 ± 6.6</td>
<td>405.6 ± 26.8*</td>
<td>0.51 ± 0.69*</td>
<td>14.7 ± 0.15*</td>
</tr>
<tr>
<td>5</td>
<td>SA + NH₄Cl</td>
<td>20</td>
<td>Initial: 133.8 ± 5.5</td>
<td>395.0 ± 18.3*</td>
<td>0.20 ± 0.06</td>
<td>18.7 ± 0.02*</td>
</tr>
<tr>
<td>6</td>
<td>NH₄Cl</td>
<td>20</td>
<td>Initial: 133.5 ± 6.7</td>
<td>416.7 ± 20.7*</td>
<td>0.15 ± 0.02</td>
<td>18.3 ± 0.01*</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>20</td>
<td>Initial: 133.6 ± 4.4</td>
<td>438.1 ± 18.4*</td>
<td>0.17 ± 0.04</td>
<td>15.0 ± 0.01*</td>
</tr>
</tbody>
</table>

Mean ± SD.

Values for the following groups were significantly different: groups 1 and 2, * P < 0.01; groups 1 and 3, * P < 0.001; groups 1 and 4, * P < 0.01; groups 1 and 7, * P < 0.01; groups 2 and 7, * P < 0.05; groups 3 and 7, * P < 0.01; groups 4 and 7, * P < 0.01; groups 5 and 7, * P < 0.01; groups 6 and 7, * P < 0.01.

TABLE 2
Induction of urinary bladder lesions in rats treated with BBN followed by test chemicals

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemicals</th>
<th>Effective no. of rats</th>
<th>Incidence of simple hyperplasia (%)</th>
<th>Incidence of papillary or nodular hyperplasia (%)</th>
<th>Papilloma</th>
<th>Incidence (%)</th>
<th>No./10 cm of BM</th>
<th>Carcinoma</th>
<th>Incidence (%)</th>
<th>No./10 cm of BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA + NaHCO₃</td>
<td>20</td>
<td>18 (90)*</td>
<td>18 (90)*</td>
<td>2.27 ± 1.35*</td>
<td>19 (95)*</td>
<td>2.76 ± 1.44*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AA</td>
<td>20</td>
<td>19 (95)*</td>
<td>20 (100)*</td>
<td>0.46 ± 0.70</td>
<td>8 (40)</td>
<td>0.18 ± 0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NaHCO₃</td>
<td>20</td>
<td>19 (95)*</td>
<td>20 (100)*</td>
<td>0.94 ± 0.82</td>
<td>15 (75)</td>
<td>1.03 ± 0.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SA</td>
<td>20</td>
<td>19 (95)*</td>
<td>20 (100)*</td>
<td>1.75 ± 1.02*</td>
<td>17 (85)*</td>
<td>1.93 ± 1.18*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>SA + NH₄Cl</td>
<td>20</td>
<td>19 (90)*</td>
<td>19 (95)*</td>
<td>0.93 ± 0.97</td>
<td>9 (45)</td>
<td>0.69 ± 1.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NH₄Cl</td>
<td>20</td>
<td>11 (55)</td>
<td>11 (55)</td>
<td>0.37 ± 0.66</td>
<td>5 (25)</td>
<td>0.20 ± 0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BM = basement membrane.

Values for the following groups were significantly different: groups 1 and 2, * P < 0.01; groups 1 and 3, * P < 0.001; groups 1 and 4, * P < 0.01; groups 1 and 7, * P < 0.05; * P < 0.01; groups 2 and 7, * P < 0.05; groups 3 and 7, * P < 0.01; groups 4 and 7, * P < 0.01; groups 5 and 7, * P < 0.01; groups 6 and 7, * P < 0.01.

Mean ± SD.

TABLE 3
Urinary analysis of rats treated with BBN followed by test chemicals (data in week 36)

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemicals</th>
<th>No. of rats examined</th>
<th>pH</th>
<th>Osmolality (mOsmol/kg H₂O)</th>
<th>Crystals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA + NaHCO₃</td>
<td>10</td>
<td>7.25 ± 0.32*</td>
<td>1130 ± 204*</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>AA</td>
<td>10</td>
<td>6.12 ± 0.11*</td>
<td>2102 ± 349</td>
<td>±</td>
</tr>
<tr>
<td>3</td>
<td>NaHCO₃</td>
<td>10</td>
<td>7.89 ± 0.51*</td>
<td>1743 ± 325*</td>
<td>~ + +</td>
</tr>
<tr>
<td>4</td>
<td>SA</td>
<td>10</td>
<td>7.48 ± 0.23*</td>
<td>1425 ± 344*</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>SA + NH₄Cl</td>
<td>10</td>
<td>6.49 ± 0.30</td>
<td>2041 ± 264</td>
<td>~ + +</td>
</tr>
<tr>
<td>6</td>
<td>NH₄Cl</td>
<td>10</td>
<td>5.78 ± 0.07*</td>
<td>2254 ± 126</td>
<td>±</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>10</td>
<td>6.69 ± 0.35</td>
<td>2099 ± 379</td>
<td>±</td>
</tr>
</tbody>
</table>

Mean ± SD.

* Significantly different from group 7 at P < 0.05.

Mean ± SD.
controls and were thus different from those of groups 1, 2, 4, and 6. Similar results were also observed in the present study, that the values in groups 3 and 6 were similar to those of group 7, while the values in group 3 were significantly lower than those in group 7. However, it is considered that the values of the contents of total AA and DA in rats given NaHCO3 were similar to those of controls, although significantly different. The promoting activity of AA plus NaHCO3 on urinary bladder carcinogenesis was greater than that of NaHCO3 alone. Therefore, AA acts as a copromoter under conditions of increased urinary pH and sodium ion concentration in urinary bladder carcinogenesis and the promoting activity of SA is nonspecific. A high intracellular concentration of AA or its metabolites might be induced in the observed changes in composition of the urine. Further investigations are needed on this point.

The membrane potential of the epithelium in the early stage of urinary bladder carcinogenesis is significantly increased by BBN or sodium saccharin treatment (14). Since the apical membrane potential of the cell depends largely on the permeability of the sodium ion, it reflects the activity of the sodium ion channel, which is essential in sodium ion transport across the urinary bladder epithelium. Therefore, it seems likely that a high concentration of sodium ion in the urine produces high levels of intracellular sodium ion in the urinary bladder epithelium and these may induce elevation of the intracellular pH. In general, a high intracellular concentration of sodium ion is thought to be related to cellular proliferation (15, 16). In addition, there is a good correlation between increase in the intracellular pH and DNA synthesis in cells (17). Increases in the sodium ion content of the urine result in proliferation of the urinary bladder epithelial cells (18, 19) and renal pelvic epithelial cells (20). This phenomenon may be correlated with the promoting activity of sodium ion in urinary bladder carcinogenesis.

In the present study, treatment with NH4Cl reduced the promoting activity of SA on urinary bladder carcinogenesis and did not cause an increase in the urinary pH, although SA increased the sodium ion concentration of the urine. Moreover, previously we found that sodium hippurate did not show promoting activity in urinary bladder carcinogenesis, whereas AA does not. Sodium o-phenylphenate, sodium erythorbate, and sodium saccharin increased the urinary pH and sodium ion concentration of the urine. Therefore, increases of the sodium ion concentration and pH of the urine are important factors for urinary bladder carcinogenesis.

Urinary analyses showed differences in the contents of total AA and DA of groups treated with AA plus NaHCO3 and NaHCO3 alone, but not in other parameters, such as the pH, osmolality, and electrolytes. It is considered that the values of the contents of total AA and DA in rats given NaHCO3 were similar to those of controls, although significantly different. The promoting activity of AA plus NaHCO3 on urinary bladder carcinogenesis was greater than that of NaHCO3 alone. Therefore, AA acts as a copromoter under conditions of increased urinary pH and sodium ion concentration in urinary bladder carcinogenesis and the promoting activity of SA is nonspecific. A high intracellular concentration of AA or its metabolites might be induced in the observed changes in composition of the urine. Further investigations are needed on this point.

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genesis. Recently it was reported that proliferative alteration of the bladder epithelium induced by sodium saccharin is correlated with changes in urinary sodium ion and urinary pH (22). Calcium ion is also known to be important in the proliferation of bladder epithelium cells (23). However, in the present study no increase in the calcium ion content of the urine was observed in any test group. Treatment with AA plus NaHCO₃, NaHCO₃, or SA induced low urinary osmolality as the results of polyuria. However, this change probably did not influence promoting activity in urinary bladder carcinogenesis, because acetazolamide and α-phenylphenol also induced very low osmolality of the urine.

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Roles of Urinary Sodium Ion Concentration and pH in Promotion by Ascorbic Acid of Urinary Bladder Carcinogenesis in Rats

Shoji Fukushima, Masa-Aki Shibata, Tomoyuki Shirai, et al.


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