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Summation Effects of Uracil and Other Promoters on Epithelial Lesion Development in the F344 Rat Urinary Bladder Initiated by N-Butyl-N-(4-hydroxybutyl)nitrosamine

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Five non-genotoxic chemicals previously demonstrated to be bladder cancer promoters in 36-week *in vivo* assays for carcinogenesis were reevaluated in a 20-week experiment in order to assess the summation influence of dietary uracil, a component of RNA, on the development of (pre)neoplastic lesions. The test chemicals, sodium bicarbonate, sodium L-ascorbate, sodium citrate, butylated hydroxytoluene and ethoxyquin, were mixed into the diet at concentrations of 3%, 5%, 5%, 1% and 0.8%, respectively, and administered to male F344 rats after initiation with 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water for 4 weeks. The test chemicals were given from the 4th to the 8th and the 11th to 20th experimental weeks, uracil being administered at the level of 3% in the diet during the intervening period. Rats in the control group received only BBN and uracil. All animals were killed at week 20 and the bladders were evaluated for the occurrence of putative preneoplastic papillary or nodular (PN) hyperplasia and tumors. Significant increase in the occurrence of PN hyperplasia was observed in all groups initiated with BBN and fed uracil and test chemicals. Quantitative values for papillomas were also significantly increased except in the ethoxyquin-treated group. The results confirm that uracil given in the middle of the post-initiation stage enhances the promoting activity of chemicals and suggest that the use of this chemical might be useful to reduce the duration of current bioassays for bladder chemical carcinogens.

Key words: Bladder cancer promoter — Uracil — Medium-term assay

The two-step model of chemical carcinogenesis, comprising initiation and promotion stages, has also been found applicable to the urinary bladder.[1-3] Our laboratory has examined the promoting potential of several chemicals using a 36-week two-stage protocol in rats with BBN,[4] a strong urinary bladder carcinogen, as the initiating agent.[4-7] However, consideration of cost and the number of chemicals which require testing suggests that a shorter-term assay would be of great advantage for evaluating the bladder carcinogenic potential of new compounds.

Recently it was demonstrated that provision of uracil, a component of RNA, at the level of 3% in the diet during the post-initiation stage of rat bladder carcinogenesis markedly enhanced the carcinogenic activity of a low dose of the carcinogen EHBN and the promoting influence of BHA, in a 20-week study.[8] Uracil is known to induce reversible urolithiasis and papillomatosis in the urinary bladder of F344 rats.[9,10] Moreover, administration of 10% NaCl with uracil prevented induction of urolithiasis and papillomatosis, although the urinary content of uracil was similar to that on administration of uracil alone (unpublished data). This fact means that prolonged stimulation by uracil-induced urolithiasis results in cell proliferation of urinary bladder epithelium. These uracil-dependent lesions seem to be associated with promoting activity after initiation with BBN.[11] It was therefore suggested[8] that the administration of uracil would be a simple and effective way to induce cell proliferation and to make the urinary bladder epithelium more sensitive to the modifying potential of other test chemicals given for a relatively short period. The present experiment was performed in order to confirm the applicability of such a shorter protocol using five chemicals previously demonstrated to be unequivocal bladder promoters in 36-week studies.

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[4] The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; EHBN, N-ethyl-N-(4-hydroxybutyl)nitrosamine; BHA, butylated hydroxyanisole; Na-AsA, sodium L-ascorbate; Na-Ci, sodium citrate; BHT, butylated hydroxytoluene; ETQ, ethoxyquin; PN, papillary or nodular.

MATERIALS AND METHODS

Animals and chemicals Male 6-week-old F344 rats (Charles River Japan, Inc., Kanagawa) were housed 5 or 6 per plastic cage on wood chips for bedding, in an air-conditioned room with a 12 h light-dark cycle at 22±2°C and 60% relative humidity. Chemicals used in the experiment were BBN (Tokyo Kasei Co., Tokyo), uracil (Yamasa Shoyu Co., Choshi), food additive grade Na-AsA, Na-Ci, $NaHCO_3$, and BHT (all purchased from Wako Pure Chemical Co., Osaka), and ETQ (Tokyo Kasei Co., Tokyo).

Experimental design Rats were randomly divided into eleven groups (Groups 1A to 1E, Group 2, Groups 3A to 3E). There were no differences in body weight among the groups at the beginning of the experiment. Animals were given free access to water and food. Body weights were measured weekly. Food ingestion and water consumption were measured on a per cage basis during two consecutive days of the 4th, 11th, and 20th experimental weeks. For the first 4 weeks the animals were given drinking water with 0.05% BBN (Groups 1A to 1E and Group 2) or without BBN (Groups 3A to 3E). Then, from experimental weeks 5 to 8 and from weeks 12 to 20, they were fed powdered basal diet (Oriental MF; Oriental Yeast Co., Tokyo) containing the different test chemicals as follows: Groups 1A and 3A=$NaHCO_3$ 3%; Groups 1B and 3B=Na-AsA 5%; Groups 1C and 3C=Na-Ci 5%; Groups 1D and 3D=BHT 1% (supplemented with 7 ppm of vitamin K in the drinking water to avoid hemorrhagic complications); Groups 1E and 3E=ETQ 0.8%. Group 2 was fed plain basal pelleted diet. From weeks 9 to 11 all groups were fed the basal diet containing 3% uracil. At the end of the 20th week all rats were killed.

The pH of the urine was measured within the last week of uracil treatment (11th week) and in the week before killing (20th week), with a Hitachi-Horiba pH meter (Model F-9DP, with a 6210 electrode). For this procedure five rats were randomly chosen in each group and the urinary samples obtained by forced micturition in the early morning.

At the time of autopsy the urinary bladders were inflated by intraluminal injection of 10% phosphate-buffered formalin solution. After fixation they were cut into eight strips for histological examination. Urinary bladder lesions were classified as PN hyperplasia, papilloma and carcinoma, as previously described.[12, 13] For quantitative analysis, bladder lesions were counted under the light microscope and the number of lesions calculated per linear centimeter of basement membrane was measured with a color video image processor (VIP-21C; Olympus-Ikegami Tsushin Co., Tokyo).

Statistical analyses The results were analyzed by comparing each of the groups submitted to the complete sequence of treatments (Groups 1A to 1E) to the control group (Group 2) and to each of the corresponding groups fed the same test chemical but not initiated by BBN (Groups 3A to 3E). Data for lesion incidences were analyzed for statistical significance by using the Fisher exact probablity test (two-tailed) and the other data were analyzed by using Student's t test.

RESULTS

Data for initial and final mean body weights, mean daily food ingestion, water consumption and urinary pH are summarized in Table I. The results suggest that the animals were homogeneously initiated by BBN (approximately the same amount of water consumption at the 4th week) and exposed to uracil (approximately the same amount of food ingestion at the 11th week). The groups fed test chemicals failed to gain body weight to the same extent as the controls (Group 2) although they ate about the same amounts of food. At the 20th week all groups fed sodium salts, but not the antioxidant type of promoters, demonstrated significant increase of the urinary pH when compared to the BBN-plus-uracil-treated group (Group 2). Only ETQ significantly decreased the mean pH value, as shown in Group 1E.

The mean urinary bladder weights of all groups treated with uracil and test chemicals (Groups 1A to 1E; Groups 3A to 3E) showed a tendency to be higher than those of the controls (Group 2), regardless of previous treatment with BBN (Table II). However, significant differences and higher values were seen more frequently in the BBN-treated groups. Stones were not observed in the urinary bladders of any of the groups.

Uracil given after BBN treatment (Group 2) induced a 50% incidence of PN hyperplasia, with a density of 0.06±0.07 lesions per linear centimeter of basement membrane. No carcinomas were seen in this control group, although papillomas occurred in 20% of the animals with a density of 0.02±0.03 lesion per linear centimeter. On the other hand, all groups given uracil and test chemical after initiation with BBN (Groups 1A to 1E) demonstrated significant increases in the incidence and density of PN hyperplastic lesions when compared to Group 2. PN hyperplasia also occurred in the groups not initiated with BBN and treated with uracil and test chemicals (Groups 3A to 3E) but at densities much lower than those registered in the groups submitted to the complete sequence of treatments (Groups 1A to 1E). The incidence and density of papillomas were higher in groups 1A to 1E than in Group 2; only the group treated with ETQ did not show a significantly higher incidence and/or density of papillomas at the end of the experi-

Table I. Average Body Weights, Daily Food Ingestion, Water Consumption and Urinary pH of Rats Administered Test Chemicals with or without Prior Exposure to BBN

Group	Treatment			Effective number of rats	Final body weight[a] (g)	Average food ingestion (g/rat/day)		Average water consumption (ml/rat/day)		Urinary pH[d]	
	BBN	Uracil	Test chemical			11th week[b]	20th week	4th week[c]	20th week	11th week[a,b]	20th week
1A	+	+	$NaHCO_3$ 3%	16	320±12[e]	10.6	14.6	28.0	23.7	6.7±0.2	8.3±0.1[e]
1B	+	+	Na-ASA 5%	17	313±21[e,h]	11.3	12.6	27.6	25.6	6.9±0.2	7.6±0.1[e]
1C	+	+	Na-Citrate 5%	15	322±19[e]	10.3	13.7	27.0	25.8	6.8±0.1	8.2±0.1[e]
1D	+	+	BHT 1%	16	272±8[e,g]	11.7	13.0	25.6	24.6	6.7±0.2	6.6±0.4
1E	+	+	ETQ 0.8%	15	288±10[e,h]	9.7	13.6	25.4	22.9	6.8±0.2	6.2±0.2[f]
2	+	+	−	10	354±16	9.5	12.8	26.2	22.6	6.5±0.3	6.8±0.2
3A	−	+	$NaHCO_3$ 3%	10	325±16	11.0	14.7	22.5	23.8	7.0±0.1	8.1±0.3
3B	−	+	Na-ASA 5%	10	329±12	9.5	13.6	24.0	25.6	6.9±0.2	7.6±0.2
3C	−	+	Na-Citrate 5%	10	326±8	9.5	13.8	23.5	23.9	7.1±0.3	8.2±0.1
3D	−	+	BHT 1%	10	284±10	10.0	13.0	23.5	24.6	6.6±0.3	6.6±0.3
3E	−	+	ETQ 0.8%	10	298±14	10.0	13.7	24.0	22.8	6.8±0.2	6.3±0.3

a) Numbers are mean±SD.
b) Measured within the last week of uracil feeding.
c) Measured within the last week of BBN treatment.
d) Values obtained from 5 animals/group.
e) Significantly different from Group 2 at $P<0.001$ or *f*) at $P<0.01$.
g) Significantly different from the corresponding Group 3 at $P<0.01$, or *h*) at $P<0.05$.

Table II. Mean Urinary Bladder Weights and Histopathological Findings in the Bladder Mucosa of Rats Administered Test Chemicals with or without Prior Exposure to BBN

Group	Treatment			Effective number of rats	Bladder weight[a] (g)	Papillary or nodular hyperplasia		Papillomas		Incidence of carcinomas (%)
	BBN	Uracil	Test chemical			Incidence (%)	Density[a,b]	Incidence (%)	Density[a,b]	
1A	+	+	$NaHCO_3$ 3%	16	0.24±0.02[c,e]	16 (100)[d]	2.04±0.58[c,e]	16 (100)[c,e]	0.44±0.16[c,e]	5 (31)
1B	+	+	Na-ASA 5%	17	0.21±0.04[c,g]	17 (100)[d]	2.23±0.92[c,e]	15 (88)[c,e]	0.25±0.16[c,e]	2 (12)
1C	+	+	Na-Citrate 5%	15	0.27±0.05[c,e]	15 (100)[d,g]	3.89±1.89[c,e]	15 (100)[c,e]	0.63±0.33[c,e]	1 (6)
1D	+	+	BHT 1%	16	0.17±0.04	16 (100)[d,e]	1.38±0.53[c,e]	16 (100)[c,e]	0.15±0.11[c,e]	1 (6)
1E	+	+	ETQ 0.8%	15	0.19±0.04[d]	15 (100)[d,e]	1.95±0.80[c,e]	4 (40)	0.04±0.05[f]	1 (6)
2	+	+	−	10	0.14±0.04	5 (50)	0.06±0.07	2 (20)	0.02±0.03	0
3A	−	+	$NaHCO_3$ 3%	10	0.18±0.03	9 (90)	0.32±0.36	0	0	0
3B	−	+	Na-ASA 5%	10	0.17±0.03	8 (80)	0.25±0.25	0	0	0
3C	−	+	Na-Citrate 5%	10	0.18±0.03	6 (60)	0.28±0.37	0	0	0
3D	−	+	BHT 1%	10	0.17±0.03	2 (20)	0.08±0.18	0	0	0
3E	−	+	ETQ 0.8%	10	0.19±0.05	1 (10)	0.07±0.21	0	0	0

a) Mean±SD.
b) Number of lesions per cm of basement membrane.
c) Significantly different from Group 2 at $P<0.01$ or *d*) at $P<0.01$.
e) Significantly different from the corresponding Group 3 at $P<0.001$, or *f*) at $P<0.01$, or *g*) at $P<0.02$.

ment. In every group submitted to the complete sequence of treatment (BBN-uracil-test chemicals), at least one animal developed transitional cell carcinoma but such lesions were not observed in Groups 2 or 3. In fact, in the groups not initiated with BBN (Groups 3A to 3E) even papillomas were not found within the time period adopted.

DISCUSSION

All chemicals tested in the present study had already been identified as bladder cancer promoters in 36-week experiments.[1, 4–7] Histological lesions quantified in the urinary bladder including PN hyperplasia, considered as a form of preneoplastic alteration of the urothelium,[12, 13] papillomas and carcinomas, were all more frequent in the groups submitted to the complete sequence of treatment (Group 1A to 1E) (Table II). Therefore, the present results demonstrated that the promoting potential of test chemicals can be recognized within 20 weeks when uracil is applied as an enhancing factor.

A tentative classification of classes of promoters of urinary bladder carcinogenesis would include sodium salt compounds, urolithiasis-inducing chemicals and antioxidants, among others.[3] It seems that all classes of urinary bladder promoters and carcinogens share the ability to induce cell proliferation and characteristic cell surface alterations which are discernible under scanning electron microscopy.[14, 15] However, a considerable variation in the urinary conditions and in the mechanisms associated with these changes has been registered for different groups of agents.[3] For example, sodium salt promoters such as Na-AsA, $NaHCO_3$ and Na-Ci increase the urinary pH and Na^+ concentration.[6, 7, 14–16] It seems likely that a high concentration of Na ions in the urine produces high levels of intracellular Na ion concentration and elevation of intracellular pH, which are thought to influence DNA synthesis and cell proliferation.[3, 16, 17] Urolithiasis-inducing chemicals, such as uracil and diphenyl, do not influence the urinary pH or Na ion concentration[16, 18] and their tumor-promoting activity seems to be caused by chronic increase in cell proliferation within the urothelium, associated with the irritant effects of calculi or crystals.[9, 10, 15, 18] However, at least one report has suggested that urinary crystals might not have direct significance as a promoting factor for urinary bladder carcinogenesis in the rat and the possibility exists that different kinds of crystals might have different effects on the urothelium.[18] Antioxidant type of promoters, such as BHT, BHA, and ETQ, do not show any specific relation between their potency as antioxidants, urinary characteristics and their promoting capability.[14, 15] The precise mechanisms underlying their promoting influence remain unclear at present.

In general, the results herein presented are in good agreement with the above findings from the literature. The sodium-containing test chemicals induced higher values of urinary pH at the end of the experiment (Table I); when the animals were initiated by BBN this was correlated with a quite clear promoting activity, as evidenced by the increased incidence and density of PN hyperplasias and papillomas (Table II). The antioxidant type of promoters (BHT and ETQ) did not increase the pH; in fact, ETQ significantly decreased the urinary pH in animals fed this compound (Table I). Consideration of the incidences and densities of PN hyperplasias and of papillomas, however, did reveal that these antioxidants were also efficient as promoters under the conditions of the assay.

Enhancement of cell proliferation is an important event for promotion of bladder chemical carcinogenesis.[10, 11, 15, 17, 19, 20] Proliferative epithelial lesions such as simple and PN hyperplasia are seen two weeks after starting provision of 3% uracil in the diet although in the absence of any other influence the lesions begin to disappear as soon as three weeks after cessation of uracil treatment.[10] The reversibility of these lesions was the most critical and advantageous point to justify the design of the present experimental protocol. The lesions caused by uracil are time- and dose-dependent; no epithelial lesions were observed after 15 weeks when the concentration of uracil in the diet was only 1% but PN hyperplasia did appear when the experiment was extended to 30 weeks.[9] Moreover, even when provided after BBN treatment, 0.8% uracil in the diet did not exert a promoting effect on the bladder mucosa.[18] Under the present experimental conditions uracil itself may be considered a weak promoter for bladder epithelium as revealed by the incidences and densities of PN hyperplasia and papillomas in Group 2 (Table II). However, it would not be surprising if additional long-term studies demonstrate a carcinogenic potential of uracil, even without a previous initiating procedure, because the sustained cell proliferation induced by this chemical might provide an adequate environment for carcinogenesis to occur since the rat urine itself contains carcinogenic factors.[21] Our data indicate, similarly to previous findings,[8] that a cumulative effect occurred between uracil and each one of the other promoters investigated so that the cells initiated by BBN and expanded by uracil were stimulated to develop into (pre)-neoplastic lesions.

Some animals in the groups not initiated by BBN also demonstrated PN hyperplasia, although at a density level much lower than in the groups previously treated with BBN. PN hyperplasia has not been observed in animals without BBN initiation when fed bladder promoters such as Na-AsA, BHT, Na-Ci and BHA for periods of 24 to 32 weeks.[4, 7, 14] Therefore, in the present case its occur-

rence in Groups 3A to 3E was presumably caused by the association of those chemicals with uracil.

Since in the present study the induction of bladder (pre)neoplastic lesions was clearly enhanced within 20 weeks, this was a clear improvement over the protocol currently used in this laboratory which requires 36 weeks. The introduction of this modified assay appears warranted for the assessment of the influence of suspected chemicals on urinary bladder carcinogenesis. In fact, this model has already allowed early detection of promoting activity for $NaHCO_3$ and K_2CO_3 with or without L-ascorbic acid[22] and clofibrate,[23] underlining its usefulness as a bioassay for carcinogens and promoters acting on the urinary bladder.

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