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Synergism of Environmental Carcinogens and Promoters on Bladder Cancer Development Initiated by N-Butyl-N-(4-hydroxybutyl)nitrosamine in F344 Rats

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Synergistic or additive effects of combined treatments with carcinogens or promoters on N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-initiated rat bladder carcinogenesis were examined. Male F344 rats were given BBN as an initiator followed by low doses of 3 sodium salts (sodium bicarbonate, sodium L-ascorbate and sodium citrate) and/or 3 antioxidants (butylated hydroxyanisole, butylated hydroxytoluene and tertiary butylhydroxyquinone). Combined treatments with 3 sodium salts or 3 antioxidants, and especially all 6 chemicals together promoted bladder carcinogenesis. In addition, these combined treatments were associated with increased DNA synthesis of the bladder epithelium. Combined administration of the carcinogens, *o*-anisidine, *p*-cresidine, and 4-chloro-*o*-phenylenediamine at low doses also enhanced BBN-initiated bladder carcinogenesis. These results indicate that environmental carcinogens or promoters can exert synergistic or additive actions on bladder cancer induction.

Key words: Rat bladder carcinogenesis — Synergism — Bladder cancer promoter — Bladder carcinogen — Tumor promotion

A high proportion of human cancers has been attributed to environmental factors.[1-3] In particular, dietary components have been regarded as major extrinsic factors for the genesis of human cancers.[4,5] On the other hand, since the first recognition of a relationship between human bladder cancer and occupation,[6] a great deal of evidence has accumulated to indicate environmental involvement in the development of bladder cancer. Many chemicals have been demonstrated to be carcinogenic for the bladder of experimental animals.[7,8] Among these various bladder carcinogens, BBN[4] exhibits the greatest potency.[9,10]

The concept of two-stage chemical carcinogenesis was first proposed on the basis of studies on the development of skin tumors and is now well established for many organs. Recently, this theory has been extended to encompass a multistage nature of the carcinogenic process, including initiation, promotion and progression. Much attention has been paid to the possible potential of environmental chemicals acting on the promotion and/or progression stages of bladder carcinogenesis.[11-16] We have also examined the promoting activities of various chemicals on the development of bladder cancer in rats and demonstrated that several kinds of environmental agents exert promoting activity.[17-22]

Humans are exposed to numerous environmental chemicals during their life span and the chemicals may act in combination, positively or antagonistically, to affect cancer production. Synergistic and additive effects by two or more chemicals on carcinogenesis were demonstrated in previous experiments.[23-25] Concerning bladder carcinogenesis in rats, we reported synergistic and summational enhancing effects of carcinogens[26,27] and promoters.[28] However, data obtained from experimental animals are still insufficient for the prediction of risk for humans.

In the present studies, we examined the synergistic and additive effects of combined treatments with bladder carcinogens or promoters on BBN-initiated rat bladder carcinogenesis.

MATERIALS AND METHODS

Animals A total of 365 male 6-week-old F344 rats (Charles River Japan, Inc., Atsugi) 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 60±10% relative humidity. Body weights, food consumption and water intake were measured every 4 weeks during the experiment. The amounts of food and water consumed on two consecutive days of a week were measured on a per cage basis.

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[4] The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; Na-AsA, sodium L-ascorbate; Na-Cit, sodium citrate; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, tertiary butylhydroxyquinone; COPD, 4-chloro-*o*-phenylenediamine; BrdU, 5-bromo-2′-deoxyuridine; PGE_2, prostaglandin E_2; BM, basement membrane; PN, papillary or nodular.

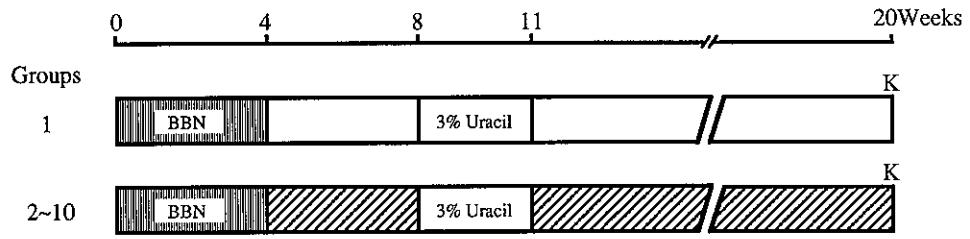

Fig. 1. Experimental protocol for rat bladder carcinogenesis. ▥, BBN, 0.05% in drinking water; ▨, test chemicals in diet; □, no treatment; 3% uracil, in diet; K, rats were killed.

Chemicals BBN was obtained from Tokyo Kasei Co., Tokyo. The compounds administered to evaluate the combined effects on the promotion stage of bladder carcinogenesis were as follows: $NaHCO_3$, Na-AsA, Na-Cit, and BHA (purchased from Wako Pure Chemical Ind., Osaka), BHT and TBHQ (Tokyo Kasei Co.) in Experiments 1 and 2, and *o*-anisidine, *p*-cresidine (Tokyo Kasei Co.), COPD (Wako Pure Chemical Ind.), and uracil (Yamasa Shoyu Co., Chiba) in Experiment 3.

Experiment 1 One hundred and fifty rats were divided into 10 groups of 15 rats each. The experimental protocol, which was recently developed and is acceptable for the detection of bladder carcinogens or promoters with suitably short duration, is shown in Fig. 1.[29-31] For the first 4 weeks all rats were given drinking water containing 0.05% BBN. During experimental weeks 5 to 8 (4 weeks) and weeks 12 to 20 (9 weeks), rats were fed powdered basal diet (Oriental MF, Oriental Yeast Co., Tokyo) without chemicals as a control (group 1), with 1.0% $NaHCO_3$ (group 2), 1.7% Na-AsA (group 3), 1.7% Na-Cit (group 4), a combination of 1.0% $NaHCO_3$, 1.7% Na-AsA and 1.7% Na-Cit (group 5), 0.7% BHA (group 6), 0.3% BHT (group 7), 0.7% TBHQ (group 8), a combination of 0.7% BHA, 0.3% BHT, and 0.7% TBHQ (group 9), or a combination of all 6 chemicals at these doses (group 10). The doses of the chemicals were one-third of those used in previous experiments[17-19, 32-34] to demonstrate bladder tumor-promoting activity. During weeks 9 to 11 (3 weeks), the rats of all groups were fed the basal diet containing 3% uracil to amplify cell proliferation. The total experimental period was 20 weeks and all animals had free access to food and water.

For urine examinations, in week 20, fresh urine samples were obtained from five rats in each group by forced urination in the morning (8:00–9:00 a.m.). The pH was measured with a pH meter (pH meter model F-7DE; Hitachi-Horiba, Tokyo). For urinary sodium ion analysis, samples of the urine were obtained from five rats in each group without any treatment of the urine. For collection of these samples, rats were housed individually in metal metabolic cages without food or water for 4 h in the morning (8:00–12:00 a.m.). Sodium ion concentrations were analyzed by flame photometry. The remaining portions of the samples were used for urinary sediment examinations.

At the end of the experiment, the rats were killed under ether anesthesia. The urinary bladders were inflated by intraluminal injection of 10% phosphate-buffered formalin solution (pH 7.4). After fixation they were divided sagittally and weighed, then each half was cut into four strips and processed for histological examination. For quantitative analysis, urinary bladder lesions were counted by light microscopy. The total length of BM was measured with a color video image processor (VIP-21CH: Olympus-Ikegami Tsushin Co., Tokyo), and numbers of lesions per 10 cm of BM were calculated.

Experiment 2 One hundred male rats were divided into 10 groups of 10 each. The animals were given powdered basal diet (Oriental MF) containing various chemicals and their combinations (group 1, no chemical; groups 2 to 10, treatments the same as in Experiment 1) for 8 weeks.

To investigate DNA synthesis of the bladder epithelium, 5 rats in each group received a single i.p. injection of BrdU (Sigma Chemical Co., St. Louis, MO) at a dose of 150 mg/kg body weight 1 h before being killed. The animals were killed under ether anesthesia. The bladders were excised, inflated, and fixed in 10% phosphate-buffered formalin solution and embedded in paraffin. Epithelial cells incorporating BrdU were demonstrated in histological sections by the avidin-biotin-peroxidase complex immunohistochemical method with anti-BrdU monoclonal antibody (purchased from Dako Japan Co., Ltd., Tokyo) as previously described.[35] Numbers of labeled cells per 1000 cells were counted by light microscopy and labeling indices were expressed as percentages.

To determine PGE_2 content in bladder tissue, the bladders were immediately removed from another five rats

in each group under ether anesthesia and bisected sagittally, then each half quickly weighed and minced on an ice-cold plastic plate. Measurement of PGE_2 content was done by radioimmunoassay as described previously.[35,36]

Experiment 3 One hundred and fifteen rats were divided into 9 groups of 10 or 15 each. Rats in groups 1 to 5, 15 rats in each, were given drinking water containing 0.05% BBN for 4 weeks and then fed basal diet (Oriental MF) with or without chemical supplements for 32 weeks. Groups 6 to 9, 10 rats in each, were treated without BBN for 4 weeks and then fed basal diet with the chemical supplements for 32 weeks. The chemicals given to each group were none (group 1, control), *o*-anisidine (groups 2 and 6), *p*-cresidine (groups 3 and 7), COPD (groups 4 and 8), and *o*-anisidine, *p*-cresidine plus COPD (groups 5 and 9). Doses of the chemicals were 1700 ppm of *o*-anisidine during the first 2 weeks and then 425 ppm, and 3300 ppm of *p*-cresidine and COPD during the first 2 weeks and then 825 ppm. The total observation period was 36 weeks. At the end of the experiment the rats were killed and the bladders were histologically examined as in Experiment 1.

Statistical analysis Data concerning incidences of lesions were analyzed for statistical significance by using the two-sided Fisher's exact probability test. Other data were analyzed by using Student's *t* test.

RESULTS

Experiment 1 Data on final average body and relative average bladder weights, and average food consumptions are summarized in Table I. The final body weights of rats in groups 2 and 5–10 were significantly lower than that of control group 1. The relative average bladder weights in groups 5, 9 and 10 were significantly higher than that of group 1. Average food consumptions in each group, except group 2, were almost the same as for group 1.

Macroscopically, bladders of rats in groups 5 (combined treatment with sodium salts) and 9 (combined treatment with antioxidants), and particularly in group 10 (combined treatment with all chemicals), had multiple tumors. No stone formation was observed in any of the groups.

Incidences of the bladder epithelial lesions are summarized in Table II. Incidences and numbers per 10 cm of BM of carcinomas and papillomas in groups 2 to 4 were similar to those of group 1, whereas their incidences in group 5 were significantly higher than those of groups 1 to 4. Incidences and numbers of papillomas in groups 6 to 8 were not significantly different compared to group 1, but incidences and numbers of papillomas were significantly higher in group 9 than in groups 1 and 6 to 8. Incidence of carcinomas and number of papillomas in group 10 were significantly higher than those in groups 1 to 9.

Table I. Average Final Body and Bladder Weights and Food Consumption (Weeks 5–8 and 12–20) of Rats Treated with BBN in Experiment 1

Group	Chemical	Final av. body wt. (g)	Av. relative bladder wt. (% of body wt.)	Av. food consumption (g/rat/day)
1	—	375±16[a]	0.039±0.007[a]	16.7
2	$NaHCO_3$	353±17[d]	0.040±0.004	14.9
3	Na-AsA	365±14	0.039±0.005	15.8
4	Na-Cit	369±19	0.040±0.006	16.1
5	A[b]	359±18[c]	0.066±0.008[d]	16.1
6	BHA	352±17[d]	0.040±0.005	15.9
7	BHT	354±15[d]	0.058±0.065	16.1
8	TBHQ	328±13[d]	0.046±0.006	15.2
9	B[b]	316±12[d]	0.060±0.009[d]	17.5
10	A+B	309±12[d]	0.093±0.006[d]	17.4

a) Mean±SD.
b) A, $NaHCO_3$, Na-AsA plus Na-Cit; B, BHA, BHT plus TBHQ.
c) $P<0.05$ (significantly different from group 1, Student's *t* test).
d) $P<0.01$ (significantly different from group 1, Student's *t* test).

Number of carcinomas was significantly higher in group 10 than in groups 1 to 9, except group 5. There were no significant differences in incidences of the putative preneoplastic lesion, PN hyperplasia,[37] among all groups. Moreover, the incidences and numbers of bladder tumors (papillomas plus carcinomas) in groups 5 and 9 were significantly higher than in groups 1 and 2 to 4 or 6 to 8, respectively. Numbers of tumors in group 10 were significantly higher than in groups 1 to 9. As shown in Fig. 2, numbers of bladder tumors in combined treatments with sodium salts (group 5, A) antioxidants (group 9, B) and all chemicals (group 10, A+B) exceeded the value gained by simple addition of the values over control level in each group; there was a synergistic interaction. The number of tumors in group 10 (A+B) was additive of groups 5 (A) and 9 (B).

Results of urine analyses in experiment 1 at week 20 are shown in Table III. The urinary pH was increased in groups 2 to 5 and 8 to 10 compared to group 1. Among groups 2 to 5, however, there were no consistent, significant differences, although sporadically there were significant differences in groups 3 and 5. Urinary pH in group 10 was significantly higher than in group 9 but not group 5. The sodium ion concentrations of the urine were significantly higher in groups 2 to 5, 9 and 10 than in group 1. There were, however, no significant differences in groups 3 and 4 compared to group 5, although there was a significant difference between groups 2 and 5. In

Table II. Induction of Neoplastic Lesions in the Bladder of Rats Treated with BBN Followed by Test Chemicals (Experiment 1)

Group	Chemical	No. of rats examined	Papilloma		Carcinoma		Papilloma + Carcinoma	
			Incidence (%)	No./10 cm BM[a]	Incidence (%)	No./10 cm BM	Incidence (%)	No./10 cm BM
1	—	15	4 (26)	0.3±0.6	1 (7)	0.1±0.2	4 (26)	0.4±0.7
2	$NaHCO_3$	15	9 (60)	0.6±0.6	1 (7)	0.1±0.2	9 (60)	0.7±0.6
3	Na-AsA	15	5 (33)	0.4±0.6	1 (7)	0.1±0.2	5 (33)	0.4±0.7
4	Na-Cit	15	5 (33)	0.4±0.6	1 (7)	0.1±0.3	6 (40)	0.4±0.6
5	A[b]	15	15 (100)[c]	3.4±1.7[c]	6 (40)[c]	0.4±0.6[c]	15 (100)[c]	3.8±1.4[c]
6	BHA	15	5 (33)	0.4±0.6	2 (13)	0.2±0.6	6 (40)	0.5±0.7
7	BHT	15	1 (7)	0.1±0.3	2 (13)	0.1±0.3	2 (13)	0.2±0.5
8	TBHQ	15	8 (53)	0.7±0.8	0	0	8 (53)	0.7±0.8
9	B[b]	15	14 (93)[d]	2.3±1.1[d]	2 (13)	0.1±0.3	14 (93)[d]	2.5±1.2[d]
10	A+B	15	15 (100)	5.9±1.2[e]	12 (80)[e]	0.9±0.7[f]	15 (100)	6.8±1.3[e]

a) BM, basement membrane.
b) A, $NaHCO_3$, Na-AsA plus Na-Cit; B, BHA, BHT plus TBHQ.
c) $P<0.01$ (significantly different from groups 1, 2, 3, and 4, respectivety, Fisher's exact probability test and Student's *t* test).
d) $P<0.01$ (significantly different from groups 1, 6, 7 and 8, respectivety, Fisher's exact probability test and Student's *t* test).
e) $P<0.01$ (significantly different from groups 1 to 9, respectivety, Fisher's exact probability test and Student's *t* test).
f) $P<0.01$ (significantly different from groups 1 to 9 except 5, respectively, Student's *t* test).

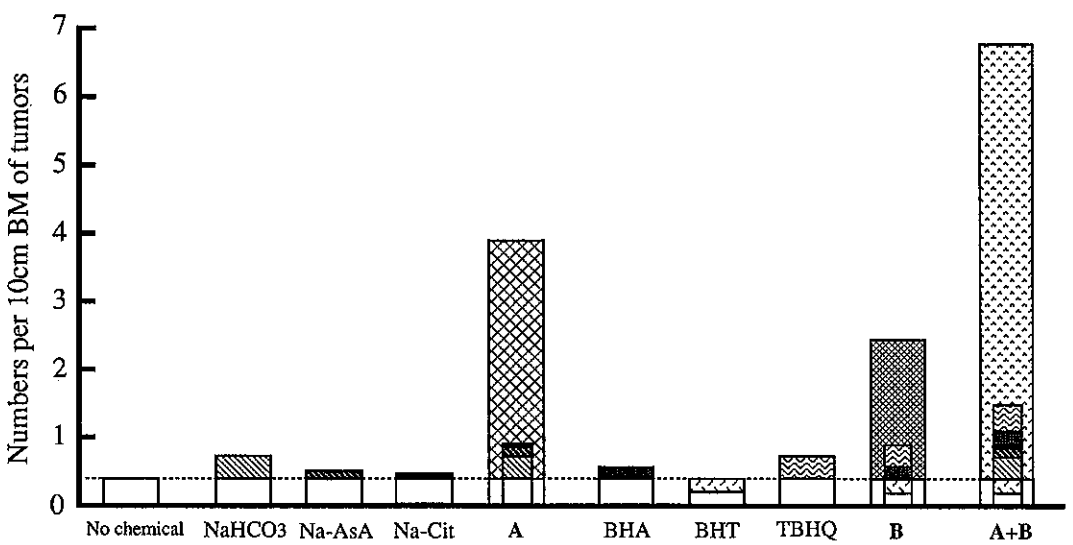

Fig. 2. Synergism in the induction of bladder tumors of BBN-initiated rats. Comparison of tumors among groups. Values are the amounts minus the control level. BM, basement membrane; A, $NaHCO_3$ + Na-AsA + Na-Cit; B, BHA + BHT + TBHQ.

addition, the sodium ion concentration in group 10 was significantly different from that in group 8 or 9. No increase of $MgNH_4PO_4$ crystals was observed in the urinary sediment in groups 2 to 10.

Experiment 2 Labeling indices (%) of the bladder epithelium as assessed by incorporation of BrdU into DNA in chemical-treated and control rats are shown in Table IV. Group 10 (combined treatment with all 6

Table III. Urine Analyses of Rats Treated with BBN Followed by Test Chemicals in Experiment 1 (Data at Week 20)

Group	Chemical	No. of rats examined	pH	Na (mEq/liter)	Crystals[h]		
					±	+	++
1	—	5	6.60±0.18[a]	107.0±39.8	0	4	1
2	$NaHCO_3$	5	7.66±0.41[d]	175.0±24.7[c, e]	1	4	0
3	Na-AsA	5	7.40±0.26[d, e]	173.0±33.5[c]	2	2	1
4	Na-Cit	5	7.92±0.18[d]	276.0±63.8[d]	1	2	2
5	A[b]	5	8.04±0.14[d]	348.0±83.3[d]	4	0	1
6	BHA	5	6.69±0.41[f]	128.0±20.8	3	2	0
7	BHT	5	6.75±0.24[f]	130.0±36.8	2	3	0
8	TBHQ	5	7.02±0.33[c]	110.0±32.1[f]	2	3	0
9	B[b]	5	7.22±0.26[d, g]	151.0±13.9[c, g]	1	3	1
10	A+B	5	8.24±0.10[d]	279.0±28.3[d]	1	4	0

a) Mean±SD.
b) A, $NaHCO_3$, Na-AsA plus Na-Cit; B, BHA, BHT plus TBHQ.
c) $P<0.05$ (significantly different from group 1, Student's *t* test).
d) $P<0.01$ (significantly different from group 1, Student's *t* test).
e) $P<0.01$ (significantly different from group 5, Student's *t* test).
f) $P<0.05$ (significantly different from group 9, Student's *t* test).
g) $P<0.01$ (significantly different from group 10, Student's *t* test).
h) ±, very slight; +, slight; ++ moderate.

Table IV. BrdU Labeling Index and PGE_2 Content in the Bladder of Rats Treated with Test Chemicals (Experiment 2)

Group	Chemical	No. of rats	BrdU labeling index (%)	PGE_2 (pg/ml)
1	—	5	0.06±0.09[a]	157.2±70.1[a]
2	$NaHCO_3$	5	0.36±0.48	213.6±92.6
3	Na-AsA	5	0.65±0.96	163.8±71.7
4	Na-Cit	5	0.32±0.39	292.2±107.7
5	A[b]	5	0.93±0.17	287.4±338.4
6	BHA	5	0.10±0.12	271.0±112.3
7	BHT	5	0.82±0.86	256.6±145.4
8	TBHQ	5	1.50±1.19	439.4±209.1
9	B[b]	5	1.70±2.62	253.6±82.3
10	A+B	5	4.03±1.02[c]	1219.2±1025.2[d]

a) Mean±SD.
b) A, $NaHCO_3$, Na-AsA plus Na-Cit; B, BHA, BHT plus TBHQ.
c) $P<0.05$ (significantly different from groups 1–9, Student's *t* test).
d) $P<0.01$ (significantly different from groups 1–3 and 5, Student's *t* test).

chemicals) had a significant increase of BrdU labeling index compared to those of groups 1 to 9. The labeling index of BrdU in group 5, which received the combined treatment with sodium salts, was higher (but not significantly) than in groups 2 to 4. Those of group 9, which received the combined treatment with antioxidants, was increased (but not significantly) compared to the values of groups 6 and 7, though the value in group 8 was similar to that in group 9.

The results of PGE_2 measurements in the bladder are also presented in Table IV. A significant increase of PGE_2 content was noted in group 10 compared to groups 1 to 3 and 5, but no significant increases in PGE_2 values were obtained in groups 5 and 9.

Experiment 3 Final average body weights in groups 2, 3, and 5 were significantly reduced compared to group 1, the controls (data not shown). Average food consumptions in groups 2 to 5 of rats treated with BBN were 12.5–15.6 g/day/rat (13.6 g/day/rat in group 1).

Histological findings of the lesions of the bladder epithelium are summarized in Table V. Incidences of PN hyperplasia in groups 2 to 5 were significantly higher than in group 1, and numbers of PN hyperplasia in groups 2, 3 and 5 were significantly more than in group 1. The number of PN hyperplasia in group 5 was significantly increased compared to groups 2, 3 and 4. In addition, the number of PN hyperplasia in group 5 exceeded the value gained by simple addition of the values over the control level in groups 2, 3 and 4. For papillomas, the incidence in group 4 and the numbers in groups 4 and 5 were significantly higher than in group 1. Incidence of papillomas in group 5 showed an increasing tendency compared to group 1.

In groups of rats without BBN treatment, no bladder lesions were found.

Table V. Induction of Preneoplastic and Neoplastic Lesions in the Bladder of Rats Treated with BBN Followed by Test Chemicals (Experiment 3)

Group	Treatment		No. of rats examined	PN hyperplasia		Papilloma		Carcinoma	
	BBN	Chemical		Incidence (%)	No./10 cm BM	Incidence (%)	No./10 cm BM	Incidence (%)	No./10 cm BM
1	+	—	13	2 (15)	0.4±0.9[a]	0	0	0	0
2	+	*o*-anisidine	16	13 (81)[d]	1.5±1.3[c]	3 (19)	0.2±0.4	2 (13)	0.1±0.3
3	+	*p*-cresidine	16	11 (69)[c]	1.4±1.2[c]	1 (6)	0.1±0.3	0	0
4	+	COPD	15	9 (56)[c]	1.3±1.5	6 (40)[c]	0.4±0.7[c]	0	0
5	+	A[b]	15	15 (100)[d, e]	5.4±1.8[d, f]	5 (33)	0.5±0.7[c]	1 (7)	0.1±0.3
6	−	*o*-anisidine	10	0	0	0	0	0	0
7	−	*p*-cresidine	9	0	0	0	0	0	0
8	−	COPD	10	0	0	0	0	0	0
9	−	A	10	0	0	0	0	0	0

a) Mean±SD.
b) A, *o*-anisidine, *p*-cresidine plus COPD.
c) $P<0.05$ (significantly different from group 1, Fisher's exact probability test and Student's *t* test).
d) $P<0.01$ (significantly different from group 1, Fisher's exact probability test and Student's *t* test).
e) $P<0.05$ (significantly different from group 4, Fisher's exact probability test).
f) $P<0.01$ (significantly different from group 2, 3 and 4, respectively, Student's *t* test).

DISCUSSION

In the present study, several promoters and genotoxic carcinogens were simultaneously administered to rats during the promotion stage after BBN initiation, and preneoplastic and neoplastic lesions of the bladder were quantitatively examined. Their synergistic or additive effects were evaluated.

In Experiment 1, mixtures of 3 sodium salts or 3 antioxidants at low doses enhanced bladder carcinogenesis initiated with BBN, although individual treatment with each chemical was insufficient to produce detectable effects. As shown in Fig. 2, since the cumulative numbers of bladder tumors in the combined treatment groups of sodium salts or antioxidants exceeded the value gained by simple addition of the increments over control levels for each chemical-treated group, their effects were synergistic rather than simply additive. The mixture of sodium salts and antioxidants additively enhanced bladder carcinogenesis.

In experiment 2, feeding of mixtures of sodium salts or antioxidants (although not statistically significant), and their combination (significantly different from groups 1 to 9), produced increased DNA synthesis. It is of interest that the increased induction of bladder tumors in rats treated with sodium salts and antioxidants in combination correlated well with increased DNA synthesis. In general, increased cell proliferation is involved in not only the promoting activities but also the carcinogenicities of these chemicals.[38, 39] Increased DNA synthesis and hyperplasia of the epithelium have a key role in promoting activity in 2-stage bladder carcinogenesis. For example, all bladder tumor promoters increase DNA synthesis and induce simple hyperplasia.[20] In addition, decreasing the extent of DNA synthesis and simple hyperplasia in the bladder epithelium correlated with reduced promoting activity of bladder carcinogenesis.[20, 32, 35] This relationship is also clear in other organs.[40] Therefore, it seems that increased DNA synthesis in the urothelium of rats fed the combination of chemicals in our studies is a major factor in the apparent synergistic effects of these promoters. It has also been reported that in forestomach 2-stage carcinogenesis, BHA dose-dependently promoted the induction of carcinomas and induced epithelial hyperplasia.[41, 42]

Previously we demonstrated significant increases in PGE_2 levels in bladder tissue of rats treated with the promoters, Na-AsA and BHA, suggesting a role for prostaglandin synthesis in bladder tumor promotion.[43] Earlier *in vivo* and *in vitro* studies also supported the significance of PGE_2 in bladder carcinogenesis.[44–46] However, in the present study, increased PGE_2 content in bladder tissue was not clearly related to proliferative effects in the rats fed the combinations of chemicals. Obviously further investigation of this relationship is required.

These studies concerning the synergistic action of chemicals during the promotion stage used environmental promoters and carcinogens. $NaHCO_3$, Na-AsA and Na-Cit are food additives, and these sodium salts show strong promoting activity in 2-stage bladder carcinogenesis of rats.[17, 18, 20, 32] Recent studies demonstrate

that a dose-dependent increase in both urinary pH and Na^+ concentration correlated with a dose-dependent promotion of bladder carcinogenesis.[47] In the present studies with sodium salts, there was no particular difference in the values of urinary pH or Na^+ concentration between groups with a single sodium salt or with the combination of sodium salts. However, the combination of sodium salts promoted bladder carcinogenesis, whereas the single treatment groups did not. Therefore, other factors besides increased urinary pH and Na^+ concentration are likely to have a key role in Na salt promotion of bladder carcinogenesis. To confirm this possibility further urinary examinations are required, since the examinations were done only at one time in the present study and only evaluated a limited range of variables. It has been reported recently that there is a relation between bladder carcinogenesis promotion due to sodium saccharin and α2u-globulin in the urine.[38,48] Other variables, such as other ions, volume, and silicates might also be related.

o-Anisidine, *p*-cresidine and COPD are aromatic amines which are used as dyes or intermediates in dye production. High incidences of bladder cancer were reported among dye manufacturing industry workers, and aromatic amines are one class of chemicals which might account for the increased cancer risk in this industry. All of these aromatic amines employed in the present study were carcinogenic for the bladder in rats when given orally for 2 years.[49] The results of this experiment are obviously important for the human situation, which involves exposure to multiple carcinogens present in the environment at low doses.[50] However, the doses in the present studies were still higher than those to which humans are exposed. Nevertheless, the results have important implications for risk assessment for humans. Similarly, Takayama *et al.*[51] reported that the simultaneous oral administration of 40 carcinogens at doses of the TD 50 for 2 years induced liver and thyroid tumors in rats. Clearly, additional studies are required for evaluating the carcinogenicity of mixtures of chemicals, especially at low doses.

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