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# Promotion by Sodium L-Ascorbate in Rat Two-stage Urinary Bladder Carcinogenesis is Dependent on the Interval of Administration

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**In our two-stage model of rat urinary bladder carcinogenesis employing *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) as the initiator, sodium L-ascorbate (Na-AsA) exhibits dose-dependent promotion. In the present study, in order to assess the possible reversibility of the promoting effects, we investigated how different administration periods of Na-AsA influence its promoting activity. In experiment 1, rats were treated with 5% Na-AsA for different administration periods with or without withdrawal and injected with 5-bromo-2′-deoxyuridine (BrdU) to allow determination of the cell proliferation status. Replicative DNA synthesis in the urinary bladder epithelium was shown to return to normal after removal of the promoting stimulus. In experiment 2, rats were initially given BBN for 4 weeks and subsequently received 16 weeks of Na-AsA, alternating with basal diet, at intervals of 4, 8 or 16 weeks, within a total 32-week period. The longer the continuous exposure to Na-AsA, the greater the yield of papillomas and carcinomas in the urinary bladder. In experiment 3, Na-AsA was given for 4 or 8 weeks after BBN initiation and the animals were killed at weeks 8 and 12. Both promotion of lesion development and increase of DNA synthesis in the urinary bladder epithelium were dependent on the length of exposure to Na-AsA and the total period of exposure. The results indicate that the promoting effects of Na-AsA in urinary bladder carcinogenesis are reversible to a certain extent after its withdrawal, and the existence of a cumulative exposure time threshold seems likely.**

Key words: Urinary bladder cancer — BBN — Sodium L-ascorbate— Accumulated time threshold

The environment contains many carcinogens to which human beings are continuously or intermittently exposed. It is well known that carcinogens can be generally classified into genotoxic and non-genotoxic types.[1, 2] Because of their complex effects, it is difficult to determine exact carcinogenic mechanisms but there appears to be a major difference between those which cause DNA alterations, for which no dose threshold exists, and those which act by promoting tumor development, generally by stimulating cell proliferation.[3, 4] The latter seem generally to demonstrate threshold effects regarding the dose.[5–7]

The division of chemical carcinogenesis into initiation and promotion stages has allowed the development of models to identify promoters, for example of urinary bladder neoplasia.[8–11] These have been classified into several types,[12, 13] and a good example of the Na salt group is Na-AsA.[14] A rise in the pH and Na ion concentration of the urine plays an important role in its promoting action,[15–18] which shows a positive correlation with the Na salt and L-ascorbic acid doses.[14, 19, 20]

The importance of the time factor for long-term, low-dose carcinogenicity in safety evaluation or human risk assessment has been established,[21] and the action of non-genotoxic chemicals such as promoters is understood to be reversible to a large extent.[8] Consequently, the period of exposure to a promoter would be expected to be important for promoting action. For example, in the case of the mouse skin tumor promoter TPA, the tumor occurrence depends on the length of the treatment period and the treatment schedule.[12] However, in the case of rat two-stage urinary bladder carcinogenesis, studies on the time period dependence of promoter action have not been reported.

One of the definitive biological characteristics of promoters is their ability to increase cell proliferation.[22, 23] For instance, TPA causes epithelial hyperplasia in mouse skin.[24] Similarly, administration of Na-AsA and other promoters of urinary bladder carcinogenesis such as sodium saccharin and butylated hydroxyanisole is associated with enhanced DNA synthesis in the urinary bladder epithelium, resulting in epithelial hyperplasia.[22, 25–27] Recently we found cyclin D1 to be overexpressed in association with

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The abbreviations used are: BBN, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine; Na-AsA, sodium L-ascorbate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BrdU, 5-bromo-2′-deoxyuridine; BD, basal diet; PN, papillary or nodular.

cell proliferation in rat two-stage urinary bladder carcinogenesis induced by BBN followed by Na-AsA.[28]

We earlier demonstrated a dose-response relation for promotion with Na-AsA in rat two-stage urinary bladder carcinogenesis.[17, 20] In this model, the time factor also appears to be important. In the present study, the model was used to examine the effects of various administration periods of Na-AsA and to assess whether there is a threshold length of exposure for tumor promotion, particularly from the viewpoint of cell proliferation and tumor promotion.

## MATERIALS AND METHODS

**Animals** A total of 138 male, 6-week-old, F344 rats (Charles River Japan Inc., Hino, Shiga) was used. The rats were housed five per plastic cage with wood chips for bedding in an animal room with a 12 h light/dark cycle at 22±2°C and 44±5% relative humidity. Powdered diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) and drinking water were given *ad libitum*. Body weights and food consumption were measured weekly during the experiment. Water consumption was measured weekly from the beginning to the 4th week in experiments 1, 2 and 3. The amounts of food and water consumed on two consecutive days (one 48 h interval) of a week were measured on a per cage basis, and then calculated on a per rat basis.

**Chemicals** BBN was obtained from Tokyo Kasei Co., Osaka. Food additive grade Na-AsA (Wako Pure Chemical Industries, Tokyo) was used in experiments 1, 2 and 3.

**Experiment 1** Twenty-four rats were randomly divided into 4 groups. They were given powder basal diet (Oriental MF) with and without 5% Na-AsA for different periods (Fig. 1). Group 1 was given basal diet for 8 weeks as a control group. Group 2 was alternately administered diet containing Na-AsA for 2 weeks and then basal diet for 2 weeks, this cycle being repeated for a total of 8 weeks. Group 3 received the promoter for 4 weeks and then basal diet for 4 weeks. Group 4 was given the Na-AsA diet for the entire 8 weeks. At the end of this period, the rats were injected i.p. with 100 mg/kg body weight of BrdU (Sigma Chemical Co., St. Louis, MO) 1 h before being killed.[29]

**Experiment 2** The experimental protocol is shown in Fig. 2. Sixty-four rats were randomly divided into 4 groups. All of them were given 0.05% BBN in drinking water for the initial 4 weeks and then powdered basal diet (group 1) as a control or diet containing 5% Na-AsA (groups 2 to 4) for a total of 16 weeks within a 32-week promotion period. Group 2 received four cycles of 4 weeks Na-AsA, 4 weeks basal diet, and groups 3 and 4 were given the promoter twice for 8 weeks and once for 16 weeks, respectively.

**Experiment 3** Fifty rats were randomly divided into 5 groups and administered drinking water containing 0.05% BBN for the initial 4 weeks (Fig. 3). After BBN treatment, groups 1 and 4 were given basal diet for 8 and 4 weeks, respectively. Groups 2, 3 and 5 received 5% Na-AsA in the diet for 4, 8 and 4 weeks respectively. In group 2, basal diet was again administered from the 8th to the 12th week. All rats received a single i.p. injection of BrdU at a dose of 100 mg/kg body weight, 1 h before being killed.

**Pathological examination** The rats were killed under ether anesthesia, and gross examinations were performed. The urinary bladder was inflated and fixed in 10% phosphate-buffered formalin, routinely cut into 8 strips, embedded in paraffin and sectioned for H & E staining and light microscopic examination. Epithelial lesions of

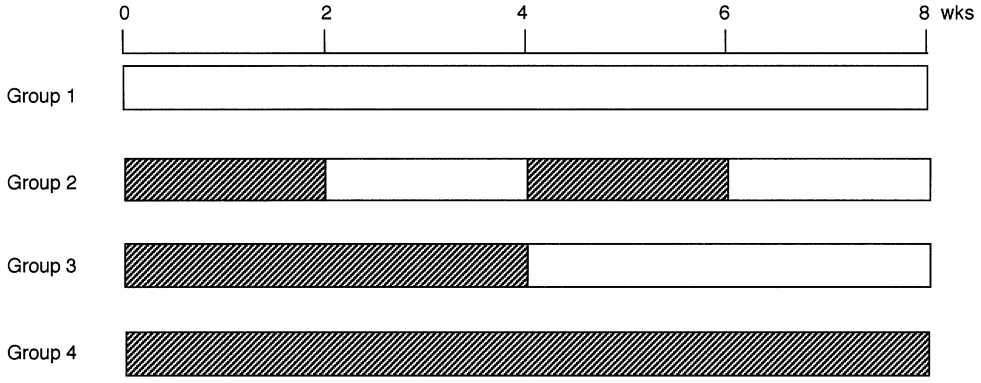


Fig. 1. The design of experiment 1. Group 1 was given basal diet for 8 weeks as the control. Group 2 received 5% Na-AsA for 2 weeks at 2-week intervals twice, group 3 received 5% Na-AsA diet for 4 weeks and then basal diet for 4 weeks, and group 4 received 5% Na-AsA diet throughout. The total experimental period was 8 weeks. One hour before being killed, all rats were given BrdU 100 mg/kg body weight i.p. ▨, 5% Na-AsA in diet; □, basal diet.

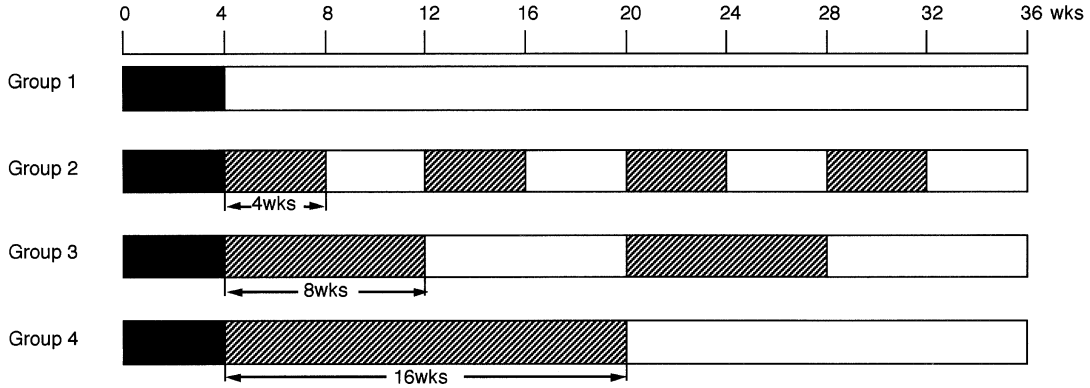


Fig. 2. The design of experiment 2. All rats were given 0.05% BBN in the drinking water for 4 weeks initially. Group 1 was then given basal diet for 32 weeks as the control. Group 2 received 5% Na-AsA diet for 4 weeks at intervals of 4 weeks, a total of 4 times. Group 3 was given 5% Na-AsA diet for 8 weeks twice and group 4 received a single 16-week exposure. At the end of week 36, all of the rats were killed. ■, 0.05% BBN in drinking water; ▨, 5% Na-AsA in diet; □, basal diet.

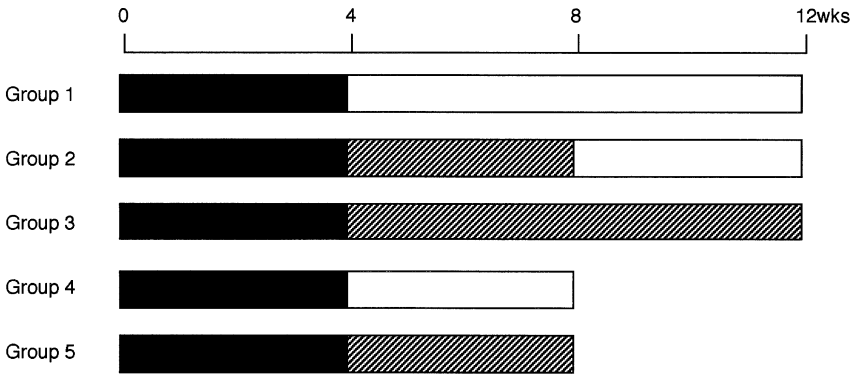


Fig. 3. The design of experiment 3. All rats were given 0.05% BBN in drinking water for 4 weeks and then groups 1 and 4 received basel diet for 8 or 4 weeks as controls. Groups 2, 3 and 4 were given 5% Na-AsA diet for 4, 8 and 4 weeks, respectively, with group 2 then receiving basal diet from weeks 8 to 12. ■, 0.05% BBN in drinking water; ▨, 5% Na-AsA in diet; □, basal diet.

the urinary bladder were histopathologically classified into simple hyperplasia, papillary or nodular hyperplasia, papilloma and carcinoma as previously described.[30]

In experiment 1, the BrdU labeling index was examined by immunohistological staining with a monoclonal anti-BrdU antibody (Dako Japan Co., Ltd., Tokyo) using the ABC method. The numbers of cells incorporating BrdU into the DNA per 1,000 urothelial cells were counted.

**Statistical analysis** For comparison of the incidences of tumors, mean numbers of tumors and BrdU labeling indices, Fisher's exact probability test and Student's *t* test (StatView-J4.02) were applied.

## RESULTS

**Experiment 1** Simple hyperplasia of the urinary bladder was observed only in group 4. As presented in Fig. 4, the BrdU labeling indices of urothelial cells in group 4 were significantly increased ($P<0.05$) as compared to the group 1 to 3 values. The value in group 3 was slightly higher than those of groups 1 and 2, although the differences were not significant.

**Experiment 2** There were no significant intergroup differences in total BBN and Na-AsA intakes and final body weights (data not shown). Percentage incidence and multiplicity data for urinary bladder papillomas and carcinomas in groups 1 to 4 are summarized in Table I, significant increases as compared to group 1 being observed for one or more parameters in all the Na-AsA treatment groups. In particular, numbers of tumors (papilloma and carcinoma) were significantly different in group 4 than in groups 2 and 3; the longer the period of continuous administration of Na-AsA, the greater the yield of urinary bladder tumors.

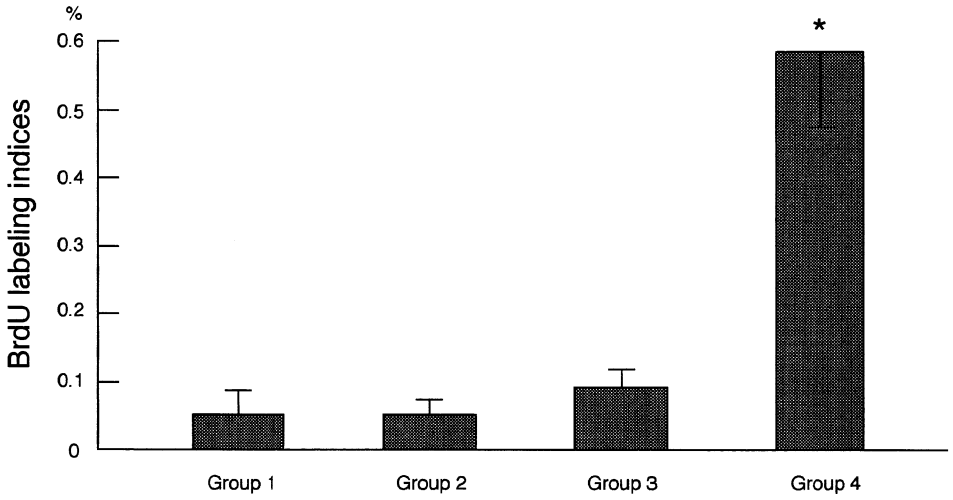


Fig. 4. BrdU labeling indices for experiment 1. ∗$P$<0.05 vs. groups 1 and 2 (Student's $t$ test).

Table I. Quantitative Data for Tumors Induced in the Urinary Bladders of Rats Initiated with BBN for 4 Weeks in Experiment 2

| G | Post-initiation | BBN treatment (wks) | No.of rats | Papillomas | | Carcinomas | | No.of tumors/rat |
|---|---|---|---|---|---|---|---|---|
| | | | | Incidence (%) | No./rat | Incidence (%) | No./rat | |
| 1 | BD 32 wks | 4 | 16 | 3 (19) | 0.31±0.48[a)] | 2 (13) | 0.13±0.34 | 0.44±0.53 |
| 2 | (Na-AsA 4 wks→BD 4 wks)×4 | 4 | 15 | 6 (40) | 0.60±0.83 | 6 (40) | 0.20±0.41 | 0.83±0.87 |
| 3 | (Na-AsA 8 wks→BD 8 wks)×2 | 4 | 12 | 7 (58) | 0.42±0.52 | 10 (83) | 0.92±0.79[b)] | 1.09±0.83 |
| 4 | Na-AsA 16 wks→BD 16 wks | 4 | 16 | 3 (19)[c)] | 0.67±0.87[c)] | 10 (63) | 1.13±0.96[b)] | 1.41±0.75[b)] |

Tumor includes papilloma and carcinoma.
*a*) Mean±SD.
*b*) $P$<0.05 vs. group 2 (Student's $t$ test).
*c*) $P$<0.05 vs. group 3 (Student's $t$ test).

**Experiment 3** Data from experiment 3 are summarized in Table II. No significant differences were found regarding final body weights within groups 1 to 3 (12 weeks experimental period) and groups 4 and 5 (8 weeks experimental period). The number of PN hyperplasias was increased in groups 2 and 3, significantly so in group 3. Tumor induction was observed in all Na-AsA treatment groups, with a significant increase in group 3 when compared to groups 1 and 2 (Table II). Induction of PN hyperplasias differed significantly between groups 4 and 5.

The BrdU labeling indices of non-neoplastic areas in the urinary bladder epithelium are shown in Table II. As compared with the control groups 1 and 4, groups 3 and 5 (5% Na-AsA diet) showed significant increases. The BrdU labeling index tended to be decreased in group 2 as compared to group 3.

## DISCUSSION

Unlike uracil, which promotes urinary bladder carcinogenesis because of a mechanical stimulus, Na-AsA causes alkalization of the urine with elevation of the $Na^+$ and total ascorbic acid concentrations.[16, 17)] These urinary conditions, which depend on the Na-AsA exposure dose or time, appear to play an important role in the cell proliferation and promotion effects in the urinary bladder. In the present study, we examined the time relationship between the promoting and proliferation activities. We found that the cell proliferation effect was largely dependent on the continued application of Na-AsA (experiments 1 and 3). In experiment 2, the longer the continuous exposure to Na-AsA, the greater the yield of papillomas and carcinomas in the urinary bladder. Therefore, it is clear that after a short treatment, persistence of promoting action is limited, whereas after a long treatment, the return to the nor-

Table II. Numbers of Urinary Bladder Tumors and BrdU Labeling Indices in Non-neoplastic Area of the Urinary Bladder in Experiment 3

| G | Total experimental period (wks) | BBN treatment | Post initiation | No. of rats | PN hyperplasia | | Papillomas | | Carcinomas | | BrdU labeling index |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Incidence (%) | No./rat | Incidence (%) | No./rat | Incidence (%) | No./rat | |
| 1 | 12 | 4 wks | BD 8 wks | 10 | 3 (30) | 0.5±0.9[a)] | 0 | 0 | 0 | 0 | 0.44±0.37 |
| 2 | 12 | 4 wks | Na-AsA 4 wks→BD 4 wks | 10 | 5 (50) | 1.2±1.9 | 4 (40) | 0.4±0.5[b)] | 1 (10) | 0.1±0.3 | 1.09±1.13 |
| 3 | 12 | 4 wks | Na-AsA 8 wks | 10 | 8 (80) | 3.4±2.5[b)] | 6 (60)[b)] | 1.3±1.0[b,c)] | 2 (20) | 0.3±0.4 | 2.08±1.49[b)] |
| 4 | 8 | 4 wks | BD 4 wks | 10 | 2 (20) | 0.2±0.4 | 0 | 0 | 0 | 0 | 0.68±0.41 |
| 5 | 8 | 4 wks | Na-AsA 4 wks | 10 | 10 (100)[d)] | 3.6±1.6[d)] | 1 (10) | 0.1±0.1 | 0 | 0 | 2.60±1.41[d)] |

*a*) Mean±SD.
*b*) $P<0.05$ vs. group 1(Student's *t* test).
*c*) $P<0.05$ vs. group 2 (Student's *t* test).
*d*) $P<0.05$ vs. group 4 (Student's *t* test).

Table III. The Ratios of Incidences of PN Hyperplasia, Papilloma, Carcinoma and BrdU Labeling Indices in Experiment 3

| | PN hyperplasia | Papilloma | Carcinoma | BrdU labeling index |
|---|---|---|---|---|
| Group 2/Group 1 | 2.4 | — | — | 2.5 |
| Group 3/Group 1 | 6.8 | — | — | 4.7 |
| Group 3/Group 2 | 2.8 | 3.3 | 3 | 1.9 |
| Group 5/Group 4 | 18 | — | — | 3.8 |

mal state takes an appreciable time, and promoting activity persists.

Since promoters are dose-dependent in their promoting action,[17)] it is very important to be aware of how this relates to the time-dependence of cancer induction. With regard to genotoxic carcinogens, a clear correlation between tumor yield and exposure time can be expected, because the genotoxic effects are considered to be irreversible and therefore they accumulate. However, there is no clear evidence concerning minimum effective exposure to promoters, whose action is reversible and which are usually categorized as non-genotoxic carcinogens. With regard to the reversibility of promoting action, we speculate that if Na-AsA treatment is stopped, the effects will be reduced and eventually disappear. If the first exposure period is short and the tissue status returns to the normal level before the second exposure, two exposures with an intervening withdrawal period should have no cumulative action, because of the interruption. When the first exposure is long, however, the promoting effect does not completely disappear after the withdrawal so that a cumulative effect can occur with a second exposure.

In experiment 1, the BrdU labeling index in group 3 was slightly higher than in groups 1 and 2. After 4 weeks of Na-ASA treatment and 4 weeks of withdrawal, the cell proliferation activity did not completely return to normal. When the promoter was reapplied within this period the promoting action was strengthened. However, constant treatment with Na-AsA for 16 weeks exerted stronger promoting activity than divided exposure when the total treatment period was kept constant. Thus, continuous promotion pressure is more effective. Since the BrdU labeling index in group 3 of experiment 1 was only slightly higher than that for group 2, we chose the 4 week-4 week regimen of Na-AsA treatment in experiments 2 and 3 to examine minor effects of promoting activity. However, further study is required to decide "no-effect levels" with regard to the cumulative time threshold of promoting action.

The BrdU labeling index was used here to check the effects of the promoter on cell proliferation. We compared the ratios between incidences of PN hyperplasia, papilloma, carcinoma and the BrdU labeling index (Table III), and found wide intergroup variation for the PN hyperplasia cases. Moreover, in experiment 3, induction of PN hyperplasia was reduced in group 3 as compared to group 5. This result provides support for the existence of 2 types, reversible and irreversible, of PN hyperplasia as suggested previously.[29)]

We have recently established that the cyclin family expression, especially of cyclin D1, bears a close relationship to urinary bladder cancer development in human, rat

and mouse.[28, 31, 32] Cyclin D1-positive PN hyperplasias appear to progress to cyclin D1-positive papillomas or carcinomas.[28] Further studies of their relevance to irreversibility are necessary.

In conclusion, the exposure period to Na-AsA is very important for urinary bladder carcinogenesis in the present model. Thus, there may be cumulative exposure time threshold for the promoting activity of Na-AsA with intermittent exposure.

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