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Maintenance by Saccharin of Membrane Alterations of Rat Bladder Cells Induced by Subcarcinogenic Treatment with Bladder Carcinogens[1]

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ABSTRACT

Saccharin is known to have a tumor-promoting effect on bladder cancer in rats, but its mechanism of action is unknown. We demonstrated that the increased agglutinability of isolated epithelial cells of the bladder in the presence of concanavalin A caused by a subcarcinogenic dose of bladder carcinogens disappeared shortly after the end of their administration. However, saccharin maintained the increased agglutinability when given continuously after administration of carcinogen. Moreover, the agglutinability of bladder cells previously exposed to a subcarcinogenic dose of bladder carcinogens increased again when saccharin was given after the agglutinability had disappeared completely.

INTRODUCTION

Skin tumor production in mice can be divided into 2 stages; a rapid, irreversible process called initiation, caused by a single application of a subthreshold dose of 7,12-dimethylbenz[a]-anthracene, and a process called promotion, caused by repeated applications of a noncarcinogenic agent such as croton oil or 12-*O*-tetradecanoylphorbol-13-acetate (1, 2, 8). Similar 2-stage carcinogenesis of the urinary bladder was reported in rats; a subcarcinogenic dose of methyl-*N*-nitrosourea followed by saccharin (9, 10), or a subcarcinogenic or weakly carcinogenic dose of FANFT[3] (6) followed by saccharin (4, 7), induced a high incidence of bladder cancer. Saccharin, considered a weak bladder carcinogen (3, 19), may actually work as a tumor promoter in bladder carcinogenesis in rats. Plant lectins such as Con A are useful in studying membrane alterations of transformed cells (20). We previously demonstrated that agglutination of isolated epithelial cells of the bladder by Con A increased shortly after *in vivo* treatment of rats with various bladder carcinogens (13, 15). This phenomenon was specific to bladder carcinogens and was dependent on the time and dose of carcinogen (14, 15). This experimental model provided a novel system for study of cancer promotion *in vivo*, and we have now demonstrated that the membrane alteration of bladder cells induced by treatment with a subcarcinogenic dose of carcinogen can be maintained or reinstated by administration of saccharin.

In a pilot study, we observed that the increased agglutinability induced by administration of a bladder carcinogen BBN (5, 11) at a concentration of 0.05% for 1 week disappeared 6 weeks after discontinuation of carcinogen treatment. Similarly, the increased agglutinability caused by administration of a 0.2% FANFT diet for 4 weeks disappeared 6 weeks after changing to a normal diet. These carcinogen treatments were reported to be subcarcinogenic (7, 12).

The agglutinability of bladder cells by Con A increased with the period of BBN administration. Isolated bladder cancer cells induced by long-term administration of BBN also showed increased agglutinability by Con A. From these observations, we postulated that administration of saccharin might result in maintenance of early membrane changes caused by subcarcinogenic treatment with BBN or FANFT and may lead to formation of bladder cancer.

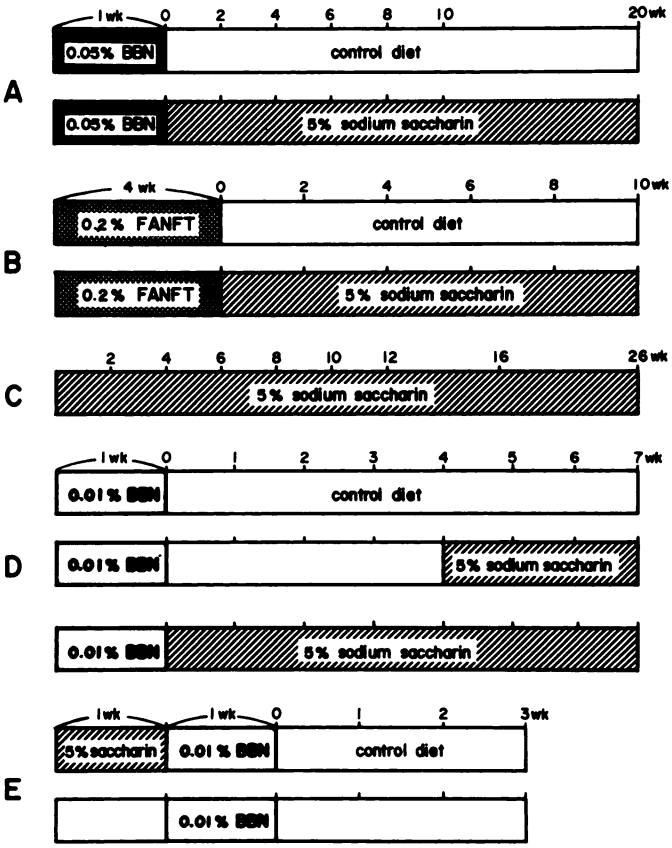

Chart 1. Experimental protocol. *A*, combination of 0.05% BBN in the drinking water for 1 week and 5% sodium saccharin diet. *B*, combination of 0.2% FANFT diet for 4 weeks and 5% sodium saccharin diet. *C*, 5% sodium saccharin diet alone. *D*, combination of 0.01% BBN solution for 1 week and 5% sodium saccharin diet. One group was given saccharin diet from Week 4 after BBN administration, when agglutination disappeared completely. Another group was given saccharin diet immediately after the end of BBN administration. In *E*, the order of administration of 0.01% BBN solution and 5% sodium saccharin diet was reversed.

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[3] The abbreviations used are: FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; Con A, concanavalin A; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

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MATERIALS AND METHODS

Six-week-old male Wistar rats were used throughout. They were purchased from Nihon Rat Co., Urawa, Japan, and kept 5 in a cage. Chemicals were purchased from the following sources: BBN, Izumi Chemicals Co., Yokohama, Japan; FANFT, Saber Laboratories, Morton Grove, Ill.; sodium saccharin, Aisan Chemical Co., Aichi, Japan; Con A and α-methyl mannoside, Sigma Chemical Co., Saint Louis, Mo.

The experimental protocol shown in Chart 1 was designed, and the Con A agglutination of bladder cells of the animals was studied. As bladder carcinogens, BBN added to the drinking water at a concentration of 0.05 or 0.01% and FANFT mixed in the powder diet (CE-2; CLEA, Tokyo, Japan) at a concentration of 0.2% were used. Sodium saccharin was given in the powder diet at a level of 5%. The purity of sodium saccharin was found to be 99% at the National Institute for Hygienic Sciences, Tokyo, Japan.

Five experiments (Experiments A to E), were performed as follows. In Experiment A, 65 animals were given 0.05% BBN solution for 1 week and 5 animals were then killed; the other 60 animals were shifted to tap water and divided into 2 groups; one group was given 5% sodium saccharin diet; 5 animals from each group were killed every 2 weeks. Agglutination assays were performed on bladder cells from the 5 animals sacrificed at each time. Experiment B was similar to Experiment A, except that a 0.2% FANFT diet was given for 4 weeks. In Experiment C, 40 rats were given 5% sodium saccharin diet throughout; 5 animals were killed after 2, 4, 6, 8, 10, 12, 16, and 26 weeks for agglutination assay. In Experiment D, 85 rats were given 0.01% BBN solution for 1 week and then shifted to tap water and divided into 3 groups; one group was given control diet throughout; another group was given 5% sodium saccharin diet from Week 4 after BBN administration; a third group was given 5% sodium saccharin diet throughout. In Experiment E, the order of administration of 0.01% BBN solution and 5% sodium saccharin diet was reversed.

As a negative control, 5% sodium chloride included in the diet was also studied; after administration of 0.01% BBN in the drinking water for 1 week, 5% sodium chloride diet was given for 4 weeks and then agglutination was assayed.

Agglutination was examined as reported previously (15). Briefly, the urinary bladder was removed, everted, and incubated in 2.0 ml of 5 mM EDTA in 0.15 M NaCl for 15 min at room temperature. Epithelial cells were separated by sonicating the bladder mucosa for 5 sec at room temperature and then squashing the mucosal surface firmly against the side of the tube to scrape off the cells. The cells isolated from the 5 animals in each group were combined and collected by centrifugation, first at 600 rpm for 3 min to remove tissue fragments and then at 3000 rpm for 5 min. Agglutination was assayed in a final volume of 40 μl of phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) containing 2 to 5 x 10^6 cells/ml, and 200 or 400 μg of Con A per ml with or without 100 μg of α-methyl mannoside per ml. After gentle shaking on a micromixer

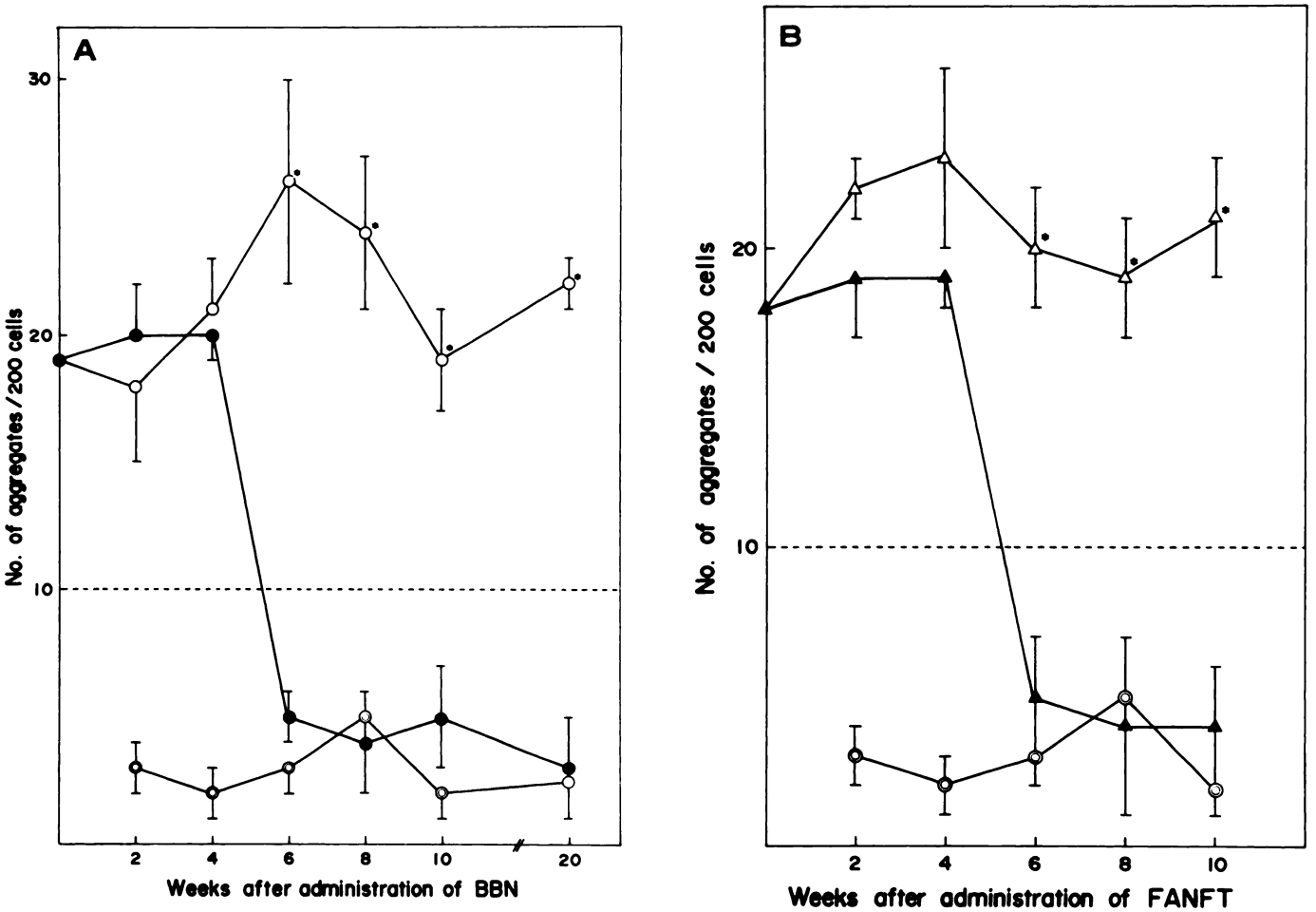

Chart 2. *A*, change of agglutinability of bladder cells by Con A after treatment with 0.05% BBN for 1 week. ○, 5% sodium saccharin diet; ●, control diet; ⊙, 5% sodium saccharin diet alone (Con A, 200 μg/ml) (Chart 1*A*). Means are for 6 different visual fields in 2 independent experiments; *bars*, S.D. •, difference from the value for the group on control diet significant ($p < 0.001$) by Student's *t* test. *B*, change of agglutinability of bladder cells by Con A after treatment with 0.2% FANFT for 4 weeks. △, 5% sodium saccharin diet; ▲, control diet; ⊙, 5% sodium saccharin diet alone (Con A, 200 μg/ml). (Chart 1*B*). Means are for 3 different visual fields; *bars*, S.D. •, $p < 0.001$. Long-term administration of 5% sodium saccharin diet alone did not induce increased agglutinability of bladder cells by Con A within 26 weeks.

for 30 min at 37°, the number of aggregates of 3 or more cells per 200 single cells or aggregates was counted in a hemocytometer. One cell aggregate was scored as 1, regardless of the number of cells in it.

RESULTS

Chart 2*A* shows that the increased agglutination of bladder cells by Con A after administration of 0.05% BBN for 1 week (Chart 1*A*) decreased 6 weeks after treatment and remained low for at least 20 weeks when rats were kept on control diet. However, when the animals were maintained on 5% sodium saccharin diet after administration of BBN (Chart 1*A*), the increased agglutinability persisted for at least 20 weeks. Chart 2*B* shows that the agglutination of bladder cells by Con A was also increased by administration of the 0.2% FANFT diet for 4 weeks (Chart 1*B*) and disappeared 6 weeks after the end of FANFT treatment, but that it was maintained when 5% sodium saccharin was given continuously in the diet (Chart 1*B*). Long-term administration of 5% sodium saccharin diet alone (Chart 1*C*) did not induce increased agglutinability of bladder cells by Con A within 26 weeks (Chart 2). Administration of BBN, even at a lower concentration of 0.01% for 1 week (Chart 1*D*) resulted in increased agglutinability of bladder cells by Con A. However, the increased agglutinability disappeared 3 weeks after the change to control diet (Chart 3). When the 5% sodium saccharin diet was started in Week 4 after the end of BBN administration (Chart 1*D*), the agglutinability of bladder cells increased again and remained high until at least Week 7 (Chart 3). Administration of 5% sodium saccharin from immediately after administration of 0.01% BBN for 1 week (Chart 1*D*) maintained a high level of agglutination until Week 7, as observed on its administration after 0.05% BBN or 0.2% FANFT (Chart 3). Reversal of the order of administration of BBN and sodium saccharin (Chart 1*E*) did not result in maintenance of agglutination induced by BBN. The increased agglutinability caused by treatment with 0.01% BBN for 1 week disappeared in 3 weeks, with or without pretreatment with 5% sodium saccharin for 1 week (data not shown).

The increased agglutinability did not persist when 5% sodium chloride diet was given after administration of 0.01% BBN for 1 week. The number of aggregates at the fourth week after the end of BBN administration in the control diet group was 5 ± 2 (S.D.), while that in the 5% sodium chloride diet group was 3 ± 1 (S.D.).

The above results were obtained with Con A at 200 μg/ml. Con A at 400 μg/ml gave similar results, although the number of aggregates was generally higher. The increased agglutinability of bladder cells by Con A was completely inhibited by α-methyl mannoside, a specific inhibitor of Con A binding.

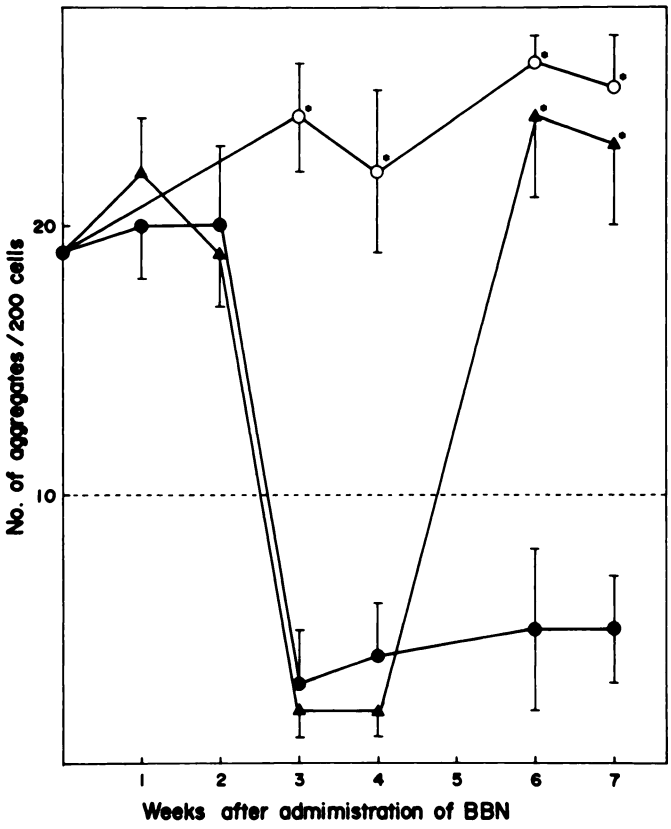

Chart 3. Reappearance of agglutinability of bladder cells by Con A after treatment with 0.01% BBN for 1 week (Chart 1*D*). ○, continuous administration of 5% sodium saccharin after BBN treatment; ▲, administration of 5% sodium saccharin from Week 4 to Week 7; ●, control diet. Note the reappearance of agglutination when 5% sodium saccharin diet was started after the agglutination had disappeared. Means are for 6 different visual fields in 2 independent experiments; *bars*, S.D. •, $p < 0.001$.

DISCUSSION

Administration of the 0.2% FANFT diet for 4 weeks, followed by continuous administration of 5% sodium saccharin for 2 years, induced a high incidence of bladder cancer in rats (7). The results of agglutination assay with Con A seem to reflect these results on carcinogenesis. No long-term carcinogenesis experiment has been performed with a combination of BBN and sodium saccharin, but administration of 0.01% BBN for 4 weeks, followed by 5% sodium saccharin for 32 weeks, induced a significantly high incidence of nodular hyperplasia, which is regarded as a preneoplastic change of the bladder in rats (18).

In the absence of saccharin, the increased agglutinability of bladder cells induced by administration of 0.05 or 0.01% BBN for 1 week, or 0.2% FANFT for 4 weeks, soon disappeared, probably owing to mechanisms of the cells for repair of the damage caused by carcinogens. Saccharin may interact directly with the cellular membrane or give some signal to DNA that causes membrane alterations, resulting in maintenance of increased agglutinability by Con A and reappearance of agglutinability in dormant cells.

As a negative control, 5% sodium chloride mixed in the diet was administered to examine whether 5% sodium chloride additive instead of saccharin may have any effect on the maintenance of agglutination. However, administration of 5% sodium chloride diet did not maintain the increased agglutinability caused by BBN. As a positive control, we had previously examined the effect of 2% DL-tryptophan, which is another established bladder promoter in the rats (4) by this assay system. Tryptophan also maintained the increased agglutinability when given continuously after administration of BBN or FANFT (16).

Reinstatement of agglutinability by saccharin seems very important, because it indicates that bladder cells previously exposed to a subcarcinogenic dose of bladder carcinogens

keep the memory. This phenomenon suggests the important relevance of tumor promoters to human bladder carcinogenesis. People who were exposed to a subcarcinogenic dose of bladder carcinogens may develop bladder cancer when exposed to tumor promoters later. In this respect, identification of tumor promoters of human bladder cancer appears extremely important.

Recent investigations suggest that 12-*O*-tetradecanoylphorbol-13-acetate and related phorbol esters act by binding to specific high-affinity cell surface membrane receptors (21, 23). The critical target of the phorbol ester tumor promoters appears to be cell membranes. Saccharin may act similarly on the cell surface membrane of the bladder. There are other interesting reports on saccharin; it may act as a tumor promoter by inhibiting metabolic cooperation between cells (22). Saccharin also enhanced the *in vitro* transformation of mouse fibroblasts (17).

This system appears useful in elucidating the mechanism of action of tumor promoters involved in bladder carcinogenesis.

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