[CANCER RESEARCH 40, 2006–2009, June 1980]
0008-5472/80/0040-0000$02.00

Increased Agglutinability of Bladder Cells by Concanavalin A after Administration of Carcinogens[1]

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ABSTRACT

The agglutination by concanavalin A of isolated epithelial cells of the rat bladder was examined after *in vivo* treatment of rats with various bladder carcinogens for one week. The carcinogens tested were *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, dibutylnitrosamine, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, 2-acetylaminofluorene, 2-naphthylamine, benzidine, *N*-methyl-*N*-nitrosourea, and cyclophosphamide, and they were given to male Wistar rats p.o., s.c., intravesically, or i.p. As negative controls, the effects of administration of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, dimethylnitrosamine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and surgical implantation of glass beads in the bladder were also tested. One week after the start of treatment, epithelial cells were isolated from the bladder by sonication, and agglutination of the isolated cells with concanavalin A was assayed. The observed agglutinabilities of isolated cells were found to be closely correlated with the reported bladder carcinogenicities of these chemicals in rats. Thus, concanavalin A agglutination of bladder cells should be a useful rapid *in vivo* mammalian system for screening bladder carcinogens.

INTRODUCTION

The plant lectin Con A[3] has been used extensively as a probe in studies on changes of the cell surface of transformed cells in culture, hematopoietic cells, and ascites cells (21). Available data suggest that transformed cells are agglutinated by lectins more easily than are normal cells. Previously, we developed a method for isolating epithelial cells from rat bladder (15) and found that the agglutinability of isolated cells by Con A increased within 5 days (17) of treatment of rats with BBN, a potent bladder carcinogen. Moreover, among the derivatives of BBN tested, only those with bladder carcinogenicity in rats caused increased agglutination of bladder cells after 1 week (16). In the present investigation, epithelial cells of the urinary bladder of rats treated with a wide variety of known carcinogens or noncarcinogens of the urinary bladder were tested for Con A agglutinability at 1 week after the start of treatment. A close correlation was found between the increased agglutinabilities of the bladder cells by Con A and the bladder carcinogenicity of the chemicals tested.

MATERIALS AND METHODS

Eight bladder carcinogens were given to 6-week-old male Wistar rats (Nihon Rat Co., Urawa, Japan) by various routes, following the conditions reported for carcinogenesis experiments: BBN, DBN, FANFT, AAF, 2-naphthylamine, benzidine, MNU, and cyclophosphamide were given p.o., s.c., intravesically, or i.p. As negative controls, the effects of administration of AF-2, DMN, and MNNG were studied, since AF-2, DMN, and MNNG are reported to be carcinogens with no bladder carcinogenicity. In addition, glass beads were implanted intravesically by opening the bladder surgically to study the effect of mechanical irritation on agglutination of urinary bladder cells by Con A. Groups of 5 animals were subjected to each treatment and killed by cervical dislocation 1 week after the beginning of treatment.

Chemicals that showed negative agglutination at 1 week, such as AF-2, DMN, and MNNG, were also given for 2 weeks to test whether agglutination would be increased by the longer period of administration.

Isolation of cells and assay of their agglutination were performed as described previously (15, 16), with slight modifications. The urinary bladder was removed surgically, washed in 10 ml of phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.46 mM KH_2PO_4, pH 7.4), everted, and incubated in 2.0 ml of 5 mM EDTA in 0.15 M NaCl (pH 4.5) for 15 min at room temperature. Epithelial cells were separated by sonicating the bladder mucosa for 10 sec in an ultrasonic cuvet washer (Fujiwara Scientific Co., Tokyo, Japan) 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 1 min to remove tissue fragments and then at 3000 rpm for 5 min at 4°. Contaminating RBC were lysed by hypotonic shock, and bladder cells were again centrifuged at 3000 rpm for 5 min. Agglutination was assayed in a final volume of 40 μl of phosphate-buffered saline containing 2 to 5 × 10^6 cells/ml, and Con A (200 or 400 μg/ml) with or without α-MM (100 μg/ml). After gentle shaking on a micromixer for 30 min at 37°, the number of aggregates was counted in a hemocytometer. An aggregate of more than 3 cells was scored as 1, regardless of the number of cells in it, and numbers of these aggregates per 200 single cells or aggregates were determined. As 4 to 5 aggregates were formed by bladder cells from rats without any carcinogen, a score of more than 10 cell aggregates was considered to indicate increased agglutination.

[1] Supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare, Japan.

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[3] The abbreviations used are: Con A, concanavalin A; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; DBN, dibutylnitrosamine; FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; AAF, 2-acetylaminofluorene; MNU, *N*-methyl-*N*-nitrosourea; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; DMN, dimethylnitrosamine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; α-MM, α-methyl mannoside; i.g., intragastrically.

Received June 29, 1979; accepted February 26, 1980.

Table 1

Agglutination of bladder cells by Con A after administration of carcinogens

Correlation between bladder carcinogenicities of various chemicals in rats and Con A agglutination of isolated bladder cells after 1-week treatment with the chemicals. Groups of five 6-week-old male Wistar rats were given one injection of test chemical s.c. (DBN or benzidine), i.p. (cyclophosphamide), i.g. (2-naphthylamine), or intravesically (MNU or glass beads) or were treated continuously with the chemical in the drinking water (BBN, DBN, or MNNG), or in the powder diet (FANFT, AAF, AF-2, DMN, or 2-naphthylamine). Animals were killed by cervical dislocation 1 week after the beginning of treatment. Cells were isolated and their agglutination with Con A was assayed as described in the text. A score of more than 10 cell aggregates was considered to indicate increased agglutination. For some chemicals, 2-week treatment was also performed to test whether agglutination might increase, depending upon the period of administration.

Carcinogen	Route of administration	Dose	Agglutination: Con A (200 μg/ml) Without α-MM	Con A (200 μg/ml) With α-MM	Con A (400 μg/ml) Without α-MM	Con A (400 μg/ml) With α-MM	Bladder carcinogenicity in rats	Ref.
BBN	Drinking water	0.05%	37 ± 11[a,b]	7 ± 2	44 ± 6[b]	7 ± 2	+	12
		0.01%	28 ± 9[b]	7 ± 1	36 ± 1[b]	4 ± 1	+	12
		0.005%	17 ± 5[b]	5 ± 0	25 ± 4[b]	7 ± 2	+	12
		0.001%	7 ± 2	4 ± 2	8 ± 2	6 ± 1	−	12
DBN	s.c.	200 mg/kg	25 ± 13[c]	4 ± 2	24 ± 7[b]	5 ± 3	+	5
	Drinking water	0.05%	23 ± 6[b]	10 ± 1	30 ± 1[b]	8 ± 0	+	5
FANFT	Diet	0.2%	17 ± 3[b]	3 ± 2	20 ± 5[b]	5 ± 1	+	1, 6
		0.05%	12 ± 1[b]	4 ± 2	12 ± 2	6 ± 1	+	1
		0.01%	8 ± 2	5 ± 2	7 ± 2	7 ± 1	−	1
		0.01% (2)[d]	5 ± 1	3 ± 1	3 ± 2	3 ± 1	−	1
AAF	Diet	0.125%	27 ± 5[b]	7 ± 3	23 ± 13[c]	5 ± 2	+	25
		0.016%	17 ± 4[b]	3 ± 1	21 ± 8[b]	4 ± 1	+	25
		0.004%	13 ± 2[b]	3 ± 2	13 ± 5[b]	4 ± 3	+	25
		0.001%	6 ± 2	3 ± 2	8 ± 2	5 ± 1	−	25
Cyclophosphamide	i.p.	50 mg/kg	27 ± 4[b]	6 ± 2	25 ± 7[b]	6 ± 5	?[e]	19
2-Naphthylamine	Diet	1.0%	31 ± 6[b]	7 ± 1	39 ± 1[b]	9 ± 1	?	
		0.01%	4 ± 2	4 ± 0	5 ± 1	3 ± 2	−	2
		0.01% (2)	2 ± 1	2 ± 1	3 ± 1	3 ± 1	−	2
	i.g.	300 mg/kg	25 ± 3[b]	3 ± 2	32 ± 5[b]	10 ± 5	?	
Benzidine	s.c.	30 mg/head	8 ± 3	5 ± 2	7 ± 2	7 ± 2	?	
		30 mg/head (2)	3 ± 1	3 ± 1	6 ± 2	2 ± 1	?	
		15 mg/head	7 ± 3	6 ± 1	6 ± 1	7 ± 2	−	22
MNU	Intravesically	1.5 mg/head	27 ± 14[c]	6 ± 4	29 ± 6[b]	7 ± 3	+	8
AF-2	Diet	0.25%	7 ± 4	3 ± 1	7 ± 1	4 ± 1	−	23
		0.25% (2)	5 ± 0	2 ± 1	5 ± 2	3 ± 2	−	23
DMN	Diet	0.01%	4 ± 1	3 ± 1	6 ± 1	4 ± 0	−	17
		0.01% (2)	5 ± 1	2 ± 1	4 ± 1	2 ± 1	−	17
MNNG	Drinking water	83 μg/ml	4 ± 0	4 ± 1	7 ± 1	5 ± 1	−	7
		83 μg/ml (2)	4 ± 2	4 ± 1	3 ± 1	3 ± 1	−	7
Glass bead	Intravesically	1 bead/bladder	6 ± 1	4 ± 1	8 ± 1	4 ± 0		18[f]
Control			4 ± 1	3 ± 1	5 ± 1	3 ± 1		

[a] Average ± S.D. of 2 to 4 independent experiments.
[b] $p < 0.001$.
[c] $p < 0.02$.
[d] Numbers in parentheses, week of administration of the chemical.
[e] ?, the bladder carcinogenicity of the compound has not been reported in the literature.
[f] Ref. 18 describes results in an experiment on mice.

Chemicals were purchased from the following sources: BBN, Izumi Chemicals Co., Yokohama, Japan; DBN and DMN, Eastman Organic Chemicals, Rochester, N. Y.; AAF, Nakarai Chemicals Co., Kyoto, Japan; 2-naphthylamine, Tokyo Kasei Organic Chemicals, Tokyo, Japan; benzidine, E. Merck, Darmstadt, West Germany; MNU, Iwai Kagaku Yakuhin Co., Tokyo, Japan; cyclophosphamide, Shionogi and Co., Ltd., Tokyo, Japan; MNNG, Aldrich Chemical Co., Milwaukee, Wis.; Con A and α-MM, Sigma Chemical Co., St. Louis, Mo. FANFT was kindly provided by Dr. N. Ito, Nagoya City University Medical College, and AF-2 was obtained through the distribution program of the Ministry of Health and Welfare, Japan.

Statistical analysis was done by Student's *t* test to compare the results of treated animals and control.

RESULTS AND DISCUSSION

Administration of a wide variety of known carcinogens for rat bladder resulted in increased agglutinability of bladder cells by Con A at 1 week after the beginning of treatment (Table 1). The increased agglutination was inhibited by inclusion of α-MM, a competitive inhibitor to Con A binding (11). BBN and AAF had dose-dependent effects. Administration of 0.001% BBN in the drinking water or 0.001% AAF in the diet did not

induce increased Con A agglutination of bladder cells, whereas administration of 0.005% BBN or 0.004% AAF led to increased agglutination of the cells. These results were compatible with those obtained in carcinogenesis experiments: concentrations of 0.005% BBN (13) in the drinking water and 0.004% AAF (26) in the diet were reported to be the lowest concentrations required for induction of bladder cancer in rats. DBN (5), FANFT (6), and MNU (9), potent bladder carcinogens with unrelated chemical structures, caused increased agglutination, irrespective of their route of administration.

2-Naphthylamine is a potent bladder carcinogen in humans, dogs, and hamsters (20), but the bladder carcinogenicity of this chemical at a concentration of 0.01% (2) in the diet has not been demonstrated in rats. Consistent with this observation, p.o. intake of 0.01% 2-naphthylamine did not result in increased agglutination of bladder cells at both 1 and 2 weeks.

Administration of 1% 2-naphthylamine in the diet induced bladder cancer in hamsters (22), but there are no reports on the effect of intake of 1% 2-naphthylamine on the development of bladder cancer in rats. In the present study in rats, 1% 2-naphthylamine caused abnormalities, such as edema, hemorrhage, and thickening of the wall of bladder at 1 week, and increased cell agglutination by Con A. These results suggest that it should induce bladder cancer in male Wistar rats when administered in the diet at this concentration. The same is probably true of cyclophosphamide. Extensive epithelial necrosis and increased agglutination were observed 1 week after a single i.p. injection of 50 mg of cyclophosphamide per kg body weight. The bladder carcinogenicity of cyclophosphamide has not been established in rats (20), although treatment of patients with cyclophosphamide has been suggested to be associated with bladder cancer (3, 7, 25). If extensive carcinogenesis experiments are carried out, bladder carcinogenicity of cyclophosphamide may be proved in rats. Benzidine (23), which is supposed to be a human bladder carcinogen, did not induce bladder cancer in rats. No Con A agglutination was induced by a dose as high as 15 mg/head or even by twice this dose for 2 weeks. Thus, if rats are used as detector animals for human bladder carcinogens, since they are insensitive to a carcinoger such as benzidine, these compounds cannot be detected as bladder carcinogens by this assay method. This problem might be overcome by using various species of animals such as rats, mice, and hamsters for screening of bladder carcinogenicity of unknown chemicals. It does not reduce the effectiveness of this assay system, since no known animal is sensitive to all kinds of carcinogens.

Administration of AF-2, DMN, or MNNG to rats induced mammary tumors (24), hepatoma and kidney tumors (18), and gastric carcinomas (8), respectively, but these chemicals did not induce bladder cancer. Administrations of these chemicals to rats did not lead to increased agglutination of bladder cells by Con A at both 1 and 2 weeks. Intravesicular glass beads induced severe inflammatory bladder changes associated with small bladder stones after 1 week but did not cause increased Con A agglutination of the cells. Thus, inflammation induced by simple mechanical irritation did not result in increased agglutinability of the cells by Con A.

Although the precise mechanism of increased agglutinability of bladder cells remains obscure, it seems that similar membrane alterations to those observed in transformed cells may take place as early as 1 week after the start of treatment with bladder carcinogens. Bladder cancer cells induced by administration of BBN showed strongly increased agglutination by Con A (data not shown).

The appearance of pleomorphic microvilli or focal hyperplasia was regarded as an early preneoplastic change in rat bladder when rats were treated with FANFT or BBN for 8 weeks (12, 14). The appearance of a glycocalyx was also reported as a specific preneoplastic and neoplastic marker induced by MNU, BBN, or 4-ethylsulfonylnaphthalene-1-sulfonamide (10). Since increased agglutinability of bladder cells with Con A appears as early as 1 week and the assay method is quite simple and easy to perform, Con A agglutination looks more useful as a primary screening of bladder carcinogens than do other morphological markers. Although the visual counting of cell aggregates appears subjective, the results of 2 independent countings by at least 2 observers agreed within the ranges of error of 10%.

Human bladder cancer has been considered to result from exposure of the bladder mucosa to carcinogens in the urine. Carcinogenesis experiments for identification of unknown carcinogens in the human environment are expensive in terms of time and manpower. As the influence of environmental factors on the incidence of cancer appears to be increasing (4, 27), rapid organ-specific methods are required for screening carcinogens *in vivo* in mammals. The assay reported here may be useful for rapid screening of bladder carcinogens.

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