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The Mutagenic Activity of Anti-Cancer Drugs and the Urine of Rats Given These Drugs

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Summary. Twenty-one anti-cancer drugs have been tested for their ability to cause mutations in Salmonella typhimurium test strains in the Salmonella/microsome mutagenicity test. Nine of the 21 anti-cancer drugs showed this ability: cyclophosphamide, nitromin, thio-tepa, busulfan, 6-mercaptopurine, neocarzinostatin, daunomycin, adriamycin and estramustine phosphate. Seven of these 9 mutagenic drugs were injected continuously into the jugular veins of rats. Urine was collected through a cystostomy tube and tested for mutagenicity. The urine from rats treated with 6 of these 7 drugs was mutagenic. These were cyclophosphamide, nitromin, thio-tepa, neocarzinostatin, adriamycin and daunomycin.

Key words: Anti-cancer drug - Carcinogen - Mutagen - Salmonella/microsome test - Rat urine.

Several anti-cancer drugs have been reported to be carcinogenic and some of these are suspected of being carcinogenic in man (3, 6, 12, 16, 18, 22). A high correlation between the mutagenicity and carcinogenicity of anti-cancer drugs has been reported(2, 10).

Clinically, bladder tumours have been described in patients receiving long term treatment with cyclophosphamide and following treatment with chlornaphazine (19, 20). Therefore, it is very important to determine the presence of mutagens in the urine of patients treated with these drugs. Minnich et al. have shown that the urine of patients receiving certain anti-cancer drugs is mutagenic in the Salmonella system (13). In our clinic, the urine of 35 patients receiving anti-cancer drugs was not mutagenic (15). There are some problems in the detection of mutagens in human urine in the Salmonella system (9). Prior to the investigation of human urine, a preliminary experiment was carried out with rats.

This paper reports on the mutagenic activities of the anti-cancer drugs used in Japan and the mutagenic activity of urine from rats treated with some of these drugs.

MATERIALS AND METHODS

1. Chemicals

The anti-cancer drugs tested in this study are listed in Table 1.

2. Bacteria

Salmonella typhimurium strains TA 98 and TA 100 were kindly supplied by Dr. Minako Nagao, Biochemistry Division, National Cancer Center, Tokyo, Japan. TA 98 is a histidine-requiring, frameshift mutant and TA 100 is a histidine-requiring, base-change mutant (11). Overnight cultures of these strains in nutrient broth were used in mutation test.

3. Preparation of S-9 Mix

Liver S-9 fraction prepared from male Sprague-Dawley rats killed 5 days after an intraperitoneal injection of PCB at a dosage of 50 mg/100 g body weight was supplied by Eisai Co., Ltd., Tokyo, Japan. The S-9 mix consisted of 50 μmols of sodium phosphate buffer (pH 7.4), 4 μmols of $MgCl_2$ and 2.5 μmols of NADPH in a total volume of 0.5 ml (22).

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Table 1. Anti-cancer drugs tested

1. Alkylating agents
1) Cyclophosphamide
2) Nitromin
3) Thio-tepa
4) Carboquone
5) Busulfan
2. Antimetabolites
1) Methotrexate
2) 6-mercaptopurine
3) 5-fluorouracil
4) FT-207: N-(2-tetrahydrofuryl)-5-fluorouracil
5) Cytosine arabinoside
3. Antibiotics
1) Neocarzinostatin
2) Daunomycin
3) Adriamycin
4) Bleomycin
5) Toyomycin
4. Alkaloids
1) Vincristine
2) Vinblastine
5. Enzymes
1) ℓ-asparaginase
6. Miscellaneous
1) Estramustine phosphate
2) Stilboestrol diphosphate
3) Cobalt protoporphyrine

4. Animals and Preparation of Urine Samples

Female Wistar rats, weighing about 200 g, were obtained from the Institute of Laboratory Animals, Kyoto University, Kyoto, Japan. Under ether anaesthesia, a 50 cm long polyethylene catheter, 0.8 mm in diameter (Igarashi Ika Kogyo Co., Ltd., Tokyo, Japan), was inserted into one jugular vein and fixed. A 20 cm long silicone catheter, 2.5 mm in diameter (Fuji Systems Co., Ltd., Tokyo, Japan) was used for the cystostomy tube (Fig. 1). Rats were immobilised in a plastic holder.

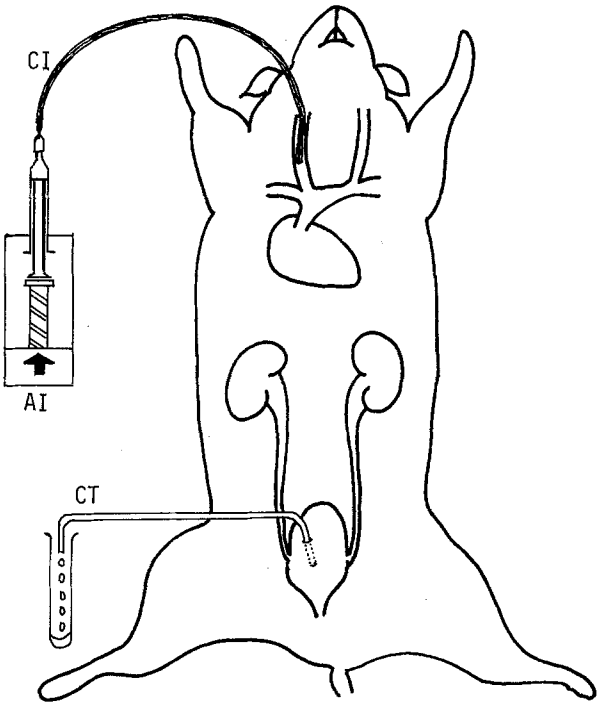

Fig. 1. Schematic drawing of experimental arrangement (AI: Auto-injector; CI: catheter for continuous installation; CT: cystostomy tube)

At first, 20-30 ml of saline was instilled continuously into the jugular vein at a speed of 11.4 ml/h by an auto-injector (Nakagawa-Seikodo, Tokyo, Japan) to produce enough urine. The urine collected through the cystostomy tube during this period was assayed and used for control values. Immediately after this collection, an anti-cancer drug, dissolved or diluted with saline to a total volume of 10-20 ml, was infused continuously at the same speed for 1-2 hours, and the urine produced during this period was assayed. Both control and test urines were centrifuged at once and their supernatants were stored in a freezer at -20°C. Mutation assays were performed during the following two or three days.

5. Assay of Anti-Cancer Drugs

The assay was carried out as described by Ames et al. with some modifications (1, 23). The anti-cancer drugs were dissolved in water or dimethyl sulfoxide (DMSO), and dilutions were made with the same solvent. 0.1 ml of each dilution was tested. In tests with metabolic activation the drugs were preincubated with 0.5 ml of the S-9 mix and 0.1 ml of bacterial culture (1×10^8 cells) at 37°C for 20 min; then 2 ml of soft agar was added, and the mixture was poured over 25 ml of minimal glucose-agar containing 0.1 μmol of L-histidine in a Petri dish. In tests without metabolic activation, 0.5 ml

Table 2. Drugs mutagenic in the Salmonella test

Anti-cancer drugs	μg/plate	Revertant colonies/plate[a] TA 98 no S-9	TA 98 S-9	TA 100 no S-9	TA 100 S-9
Cyclophosphamide	1000	27	31	185	434
	100	31	35	196	238
	10	23	29	157	146
Nitromin	500	73		511	
	50	68		151	
	5	67		128	
Thio-tepa	1000	24		582	
	500	27		667	
	100	28		176	
Busulfan[b]	5000	17		1380	
	500	29		630	
	50	31		327	
6-Mercaptopurine[b]	5000	36		263	
	500	56		503	
	50	51		445	
	5	24		266	
Neocarzinostatin	50	9548	46	210	283
	20	97	40	274	211
	10	88	42	214	205
Daunomycin	10	4347		3334	
Adriamycin	1	3405		2840	
Estramustine phosphate	10000	399		145	
	1000	275		145	
	100	21		139	
Control (buffer)		39	29	172	198
Control (DMSO)		36		181	

[a] Average of 3 determinations

[b] Dissolved in dimethyl sulfoxide (DMSO)

Table 3. Drugs not mutagenic in the Salmonella test

Anti-cancer drugs	μg/plate	Revertant colonies/plate[a] TA 98 no S-9	TA 98 S-9	TA 100 no S-9	TA 100 S-9
Carboquone	20	38	24	284	189
	2	29	25	238	117
	0.2	22	33	184	131
Methotrexate	100	35	41	155	118
	10	34	36	156	117
	1	40	40	134	122
5-Fluorouracil	500	0		20	
	50	1		22	
	5	15		115	
	0.5	36		127	
FT-207	400	5		130	
	40	21		145	
	4	36		137	
	0.4	32		145	
Cytosine arabinoside	2000	36	59	136	140
	200	34	44	113	144
	20	48	39	138	148
	2	34	20	180	140
Bleomycin	3	32		100	
	0.3	40		117	
	0.03	37		139	
Toyomycin	25	15		89	
	2.5	44		133	
	0.25	44		129	
Vincristine	10	24	31	139	173
	1	30	32	187	249
	0.1	39	32	95	198
Vinblastine	100	17	26	149	119
	10	19	25	124	108
	1	38	26	113	116
ℓ-Asparaginase	500[b]	22		77	
	50[b]	26		75	
	5[b]	32		66	
Stilboestrol diphosphate	5000	56		208	
	500	41		230	
	50	59		249	
	5	62		238	
Cobalt protoporphyrine	125	11	66	78	97
	12.5	23	81	97	140
	1.25	29	37	144	138
Control (buffer)		39	29	172	198

[a] Average of 3 determinations

[b] Unit/Plate

Table 4. Mutation assay for rat urine treated with anti-cancer drugs

Anti-cancer drugs	Dose per hour	Urine volume (ml)	Revertant colonies per plate[a]	
			TA 98	TA 100
Cyclophosphamide	300mg/2hr	13	26	541
Control		8	33	215
Nitromin	50mg/lhr	7	30	1485
Control		10	30	102
Thio-tepa	20mg/2hr	26	44	316
Control		10	30	117
Neocarzinostatin	10mg/2hr	12.5	7429	450
Control		7.5	54	210
Daunomycin	20mg/2hr	11	0	273
D_1^b			89	214
D_2^c			188	174
Control		7	26	198
Adriamycin	20mg/2hr	3.5	0	0
D_1^b			2839	10300
D_2^c			2478	149
Control		4	38	94
Estramustine phosphate	100mg/1hr	6	33	159
Control		9	28	134
Control (buffer)			39	172

[a] Average of 3 determinations

[b] Urine diluted with buffer (1:10)

[c] Urine diluted with buffer (1:20)

of 0.1 M sodium phosphate buffer (pH 7.4) was used instead of the S-9 mix.

6. Assay of Urine Samples

Urine samples were sterilised by filtration through 0.45 μm membranes. Then, 0.3 ml of urine or urine diluted with buffer was tested. Neither β-glucuronidase nor S-9 mix was added to the urine.

RESULTS

1. Drugs Mutagenic in the Salmonella Test

Table 2 lists the drugs found to be mutagenic in the Salmonella system. Strain TA 98, sensitive to chemicals causing frame-shift, was reverted by neocarzinostatin, daunomycin, adriamycin and estramustin phosphate. Strain TA 100, sensitive to chemicals causing base-pair substitution, was reverted by cyclophosphamide, nitromin, thiotepa, busulfan, 6-mercaptopurine, daunomycin and adriamycin. Cyclophosphamide was active only in the presence of S-9 mix.

2. Drugs Not Mutagenic in the Salmonella Test

Carboquone, methotrexate, 5-fluorouracil, FT-207, vinblastine, vincristine, cytosine arabinoside, bleomycin, toyomycin, ℓ-asparaginase, stilboestrol diphosphate and cobalt protoporphyrine were not mutagenic for Salmonella. Each drug was tested at several concentrations over a 100-fold range in which no plate was significantly higher than in the controls (Table 3).

3. Urine Samples

Seven of the 9 mutagenic drugs were chosen because they were soluble in water. Each drug was administered in as high a dose as possible in order to keep it or its metabolites in high concentration in the urine. In this system, the mean urine volumes were: control urine 4.7 ml/h, test urine 6.6 ml/h. The control urine was always negative. Except for the urine from rats treated with estramustin phosphate, all were positive. Urine from rats treated with cyclophosphamide was positive in the absence of S-9 mix.

DISCUSSION

Our studies show that 9 of the 21 anti-cancer drugs commonly used in Japan are mutagenic in the Salmonella/microsome system. Some of these 9 drugs are carcinogenic in animal studies (3, 7, 12, 16, 17, 18, 22).

In studying carcinoma of the urinary tract caused by anti-cancer drugs in man, it is very important to assay the mutagenic activity in the urine. However, there are numerous problems, e.g., metabolites appear in the urine in several conjugated forms, and the concentration of the metabolites in the urine may be relatively low (9). It has been reported that the urine of patients receiving cyclophosphamide or 5-fluorouracil is mutagenic in the Salmonella system (13). In our clinic, the urines of 35 patients receiving various anti-cancer drugs were tested without the use of β-glucuronidase or concentration of the urine and in these cases we could not demonstrate any mutagens in the urine (15). Histidine in human urine and the low concentration of metabolites in the urine may interfere with the test (9).

It has been reported that the urine of rats fed N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide

(FANFT) and 2-acetyl-aminofluorene, which induce bladder tumours, is mutagenic in the Salmonella system (4, 9, 21). N-Butyl-N-(3-carboxypropyl) nitrosamine (BCPN), reported to be the main urinary metabolite of N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) and to induce bladder tumours, is also mutagenic (14). In an effort to demonstrate the possibility of mutagens in human urine, we tested the urine of rats fed anti-cancer drugs.

In the collection of rat urine, rats are usually kept in metabolic cages so that the urine can be separated from the faeces (4, 8, 21). However, with this method, the urine is contaminated with faeces and food, so it can be further metabolised by bacteria. To avoid this contamination, we placed an indwelling cystostomy tube in the bladder. To provide diuresis and to keep metabolites in a high concentration in the urine, a catheter for continuous instillation of saline and drugs was inserted in the jugular vein. Thus, it was possible to measure accurately the dosage of administered drugs and the urine volume.

There are a few problems with this model. Water insoluble drugs such as busulfan and 6-mercaptopurine cannot be tested in this model. The speed of excretion of drugs into the urine is important with short-term urine sampling. Neocarzinostatin, which was negative at 50 μg/plate in the presence of S-9 mix, was positive in urine from rats treated with a high dose of 10 mg of neocarzinostatin. The data suggest that excess neocarzinostatin was excreted into the urine without being metabolised and that this was detected as a mutagen in the urine. Neocarzinostatin is excreted rapidly in the urine if given to the rat i.v. (5).

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