

## Low Susceptibility of Long-Evans Cinnamon Rats to *N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced Urinary Bladder Carcinogenesis and Inhibitory Effect of Urinary Copper

Yoshifumi Chone,<sup>1</sup> Takemi Kinouchi,<sup>2</sup> Takamasa Yamada,<sup>3</sup> Yasuo Suzuki,<sup>4</sup> Keisuke Kitaura,<sup>1,6</sup> Zhongxian Jiao,<sup>1</sup> Takanori Minami,<sup>1</sup> Yoshimi Bando,<sup>1</sup> Hisanori Uehara,<sup>1</sup> Masataka Mochizuki,<sup>5</sup> Yoshinari Ohnishi<sup>2</sup> and Keisuke Izumi<sup>1,7</sup>

<sup>1</sup>Second Department of Pathology, <sup>2</sup>Department of Bacteriology, <sup>3</sup>Institute for Animal Experimentation and <sup>4</sup>Department of Hygiene, The University of Tokushima School of Medicine, 3-18-15 Kuramoto-cho, Tokushima 770-8503 and <sup>5</sup>Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512

We studied the susceptibilities to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced urinary bladder carcinogenesis of male Long-Evans Cinnamon (LEC), F344 and Long-Evans Agouti (LEA) rats. Male rats ( $n=21$ ) were given 0.1% BBN in their drinking water from week 6, 8 and 10 for one week, and killed in week 56. The incidences of transitional cell tumors (papillomas plus carcinomas) in BBN-treated LEC and F344 rats were 12% and 76%, respectively ( $P<0.001$ , experiment 1), and those in LEC and LEA rats were 11% and 95%, respectively ( $P<0.001$ , experiment 2). When male LEC and F344 rats were given 0.1% BBN in their drinking water for 7 days, the intake of BBN and the urinary concentration of its active metabolite, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN), were higher in the LEC rats ( $P<0.01$ ). The urinary pHs of untreated LEC and F344 rats were similar between week 6 and 30. The urinary copper concentration was lower in LEC rats before jaundice than in F344 rats, but its concentrations in 28- and 50-week-old LEC rats were 1.7 and 2.3 times those in F344 rats. In a two-stage carcinogenesis study using F344 rats, i.p. injections of cupric nitrilotriacetate increased urinary copper excretion, and inhibited BBN-induced bladder carcinogenesis. In a two-stage carcinogenesis study using LEC rats, oral administration of D-penicillamine decreased urinary copper excretion, and increased BBN-induced bladder cancer, although the difference was not significant. These data show that LEC rats are resistant to bladder carcinogenesis and suggest that urinary copper has a significant role in their resistance.

Key words: LEC rat — Urinary bladder — *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine — Copper — Nitrilotriacetate

Several epidemiological studies have suggested that arylamine *N*-acetyltransferase-related slow *N*-acetylation increases the risk of bladder cancer in workers exposed to aromatic amines, although this is controversial.<sup>1,2</sup> Familial bladder cancer has been reported recently,<sup>3</sup> but the genetic background of bladder cancer risk is not well known. In rats, strain differences in BBN-induced bladder carcino-

genesis and in susceptibility to the tumor promoter sodium ascorbate have been reported.<sup>4,5</sup> The albino Sprague-Dawley rats are susceptible to BBN, and F344 and Wistar rats are resistant, although the cause of this strain difference is unknown.

The LEC rat is an inbred strain showing abnormally high copper accumulation in the liver,<sup>6</sup> and has a deletion in the copper-transporting ATPase gene (*Atp7b*)<sup>7</sup> homologous to that in human Wilson's disease. This mutant rat strain was isolated from a closed colony of Long-Evans rats.<sup>8</sup> LEC rats develop necrotizing hepatitis with jaundice 4 to 6 months after birth and this hepatitis is inherited in an autosomally recessive manner.<sup>9</sup> About 10–20% of male, and 40–50% of female rats die of fulminant hepatitis, and hepatic and renal cancer develops in rats of 12 months old or more that recover from liver injury.<sup>10–12</sup>

An *in vivo* short-term assay study suggested that LEC rats are highly susceptible to *N*-diethylnitrosamine,<sup>13</sup> and that susceptibility to hepatocarcinogens is genetically inde-

<sup>6</sup> Present address: Tokushima Research Institute, Otsuka Pharmaceutical Co., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192.

<sup>7</sup> To whom correspondence should be addressed.

E-mail: izumi@basic.med.tokushima-u.ac.jp  
Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; LEC, Long-Evans Cinnamon; LEA, Long-Evans Agouti; BCPN, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine; NTA, nitrilotriacetate disodium salt; Cu-NTA, cupric nitrilotriacetate; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

pendent of copper accumulation in the liver.<sup>14)</sup> To our knowledge, the susceptibilities of LEC rats to other carcinogens have not been investigated. Moreover, LEC rats have been suggested to be sensitive to radiation,<sup>15)</sup> but its effect on carcinogenesis is not known.

In the present study, we examined, 1) the carcinogenicity of BBN in LEC, F344 and LEA rats, 2) the urinary excretion of BCPN and changes of urinary pH and metal concentrations in LEC and F344 rats, 3) metallothionein in LEC and F344 rats by western blotting, and 4) the modifying effect of copper on BBN-induced carcinogenesis in F344 rats, and the effect of D-penicillamine on BBN-induced carcinogenesis in LEC rats, in order to elucidate the reason for the strain difference in BBN-induced carcinogenesis.

## MATERIALS AND METHODS

**Animals** LEC/Tj rats and LEA/Tj rats, a sibling line of the LEC rats, were bred in the Institute for Animal Experimentation of the University of Tokushima, Tokushima, in specific pathogen-free conditions. F344/DuCrj rats were obtained from Charles River Japan, Inc. (Kanagawa). Animals were housed three to a plastic cage with sterilized woodchips for bedding in an air-conditioned room at  $23\pm 2^\circ\text{C}$  and  $55\pm 10\%$  humidity with a 12 h light/dark cycle, and given pellet diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

**Carcinogenicity studies of BBN** In experiment 1, male LEC and F344 rats (21 rats in each group) were given 0 or 0.1% BBN (Tokyo Chemical Industry Co., Tokyo) in their drinking water from week 6, 8 and 10 for one week (Fig. 1). Body weight was recorded once a week, and all surviving animals were killed in week 56 under ether anesthesia. Bladders were inflated with 10% buffered formalin, cut into eight pieces, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination. Other major organs were also examined histologically. Histological types of bladder tumors were classified as reported.<sup>16)</sup>

In experiment 2, male LEC and LEA rats ( $n=21$ ) were given 0.1% BBN as in experiment 1, and killed in week 56. Bladders with tumors were weighed after fixation.

**Analysis of urinary BCPN** Nine-week-old male LEC and F344 rats ( $n=6$ ) were given 0.1% BBN in their drinking water for 7 days. Then the rats were housed individually in metabolic cages and 48-h urine samples were collected in ice-cold plastic tubes. These urine samples were filtered through a cellulose acetate membrane (0.45  $\mu\text{m}$ , Toyo Roshi Co., Tokyo) and stored at  $-80^\circ\text{C}$  until use.

The concentration of BCPN in urine was analyzed by HPLC.<sup>17)</sup> Briefly, diluted urine samples (2.5 ml) were adjusted to pH 1.0 with 12 N hydrochloric acid and

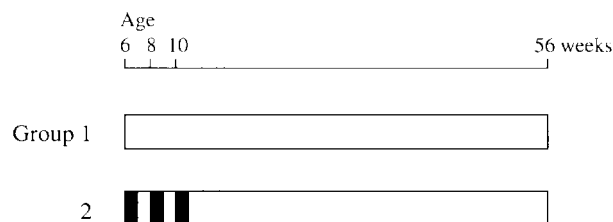


Fig. 1. Experimental design for BBN-induced bladder carcinogenesis (experiments 1 and 2). ■ 0.1% BBN in drinking water.

extracted three times with ethyl acetate. The ethyl acetate layer was separated by centrifugation and dehydrated with anhydrous sodium sulfate. The organic layer was then evaporated to dryness *in vacuo*, and dissolved in 100  $\mu\text{l}$  of dimethyl sulfoxide for HPLC analysis (Shimadzu LC5A, Shimadzu Co., Kyoto). A 10  $\mu\text{l}$  aliquot was injected into a HPLC column (Chemcosorb 5-ODS-H,  $4.6\times 250$  mm, Chemco Scientific Co., Osaka) and eluted with 30% methanol/1 mM acetic acid at a flow rate of 1 ml/min. The detection wavelength was 254 nm and the concentration of BCPN was calculated from a standard curve and the peak area of the sample.

**Urinary pH** Fresh urine samples of 6-, 12- and 30-week-old untreated male LEC and F344 rats ( $n=5$ ) were collected in 1.5 ml plastic tubes between 8:30 and 9:30 a.m. by forced urination. The urinary pH was determined immediately using a flat-type electrode (6261-10C, Horiba, Kyoto). The average pH of each rat over three consecutive days was used.

**Analyses of metal concentrations** Samples of 48-h urine of untreated male LEC and F344 rats ( $n=5$  to 7) were collected from 8-, 28-, and 50-week-old animals, filtered and stored at  $-80^\circ\text{C}$ . Their copper, iron and zinc concentrations were determined in an atomic absorption spectrophotometer (AA-782, Nippon Jarrel Ash Co., Kyoto).

**Western blot analysis** Urinary bladders of 6-, 15-, 24- and 40-week-old LEC and F344 rats ( $n=2$ ) were homogenized in 20 mM phosphate buffer (pH 7.4) containing 1 mM PMSF, 1 mM *N*-ethylmaleimide, 0.01 mM pepstatin A, 0.15 M NaCl and 1% Triton X-100. The homogenates were centrifuged at 10000g for 20 min, and the resulting supernatants were subjected to western blot analysis. Protein concentration was determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Samples of 50  $\mu\text{g}$  protein were subjected to sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and the proteins separated on the gel were transferred electrophoretically to a nitrocellulose membrane (Millipore Co., Bedford, MA), as described previously.<sup>18)</sup> Blots were blocked with PBS (pH 7.0) containing 2% bovine serum albumin and 0.05%

Tween 80 and incubated with anti-metallothionein antibody (DAKO Co., Carpinteria, CA). Then they were washed three times with PBS containing 0.05% Tween 80, and immune complexes were detected with ProtoBlot Western Blot AP systems (Promega Co., Madison, WI).

**Two-stage carcinogenesis study** In experiment 3, 63 male F344 rats were given 0.1% BBN in their drinking water from week 6, 8, 10 and 12 for one week, and divided into three groups (Fig. 2). A mixture of 0.4% CuSO<sub>4</sub>·5H<sub>2</sub>O (Wako Pure Chemical Industries, Osaka) in 0.1 M nitrilotriacetate disodium salt (Nacalai Tesque, Kyoto) was prepared before use and adjusted to pH 7.4 with sodium bicarbonate (Cu-NTA). Rats in group 1 (*n*=21) were given weekly i.p. injections of Cu-NTA (0.2 ml/100 g body weight) from week 14–42. Rats in group 2 were given injections of 0.1 M NTA only (pH 7.4, 0.2 ml/100 g body weight), and rats in group 3 were untreated after initiation. In week 26, 24-h urine samples were collected immediately after the injection from six rats in each group for determination of their metal concentrations. All

surviving animals were killed in week 46. Bladders and other major organs were examined histologically.

In experiment 4, male LEC and F344 rats (*n*=21) were given 0.1% BBN from week 6, 8 and 10 for one week (group 1) as shown in Fig. 3. They were given 0.05% D-penicillamine (Tokyo Chemical Industry Co.) in their drinking water from week 5–55 except in the administration period of BBN, and all surviving animals were killed in week 55. Rats in group 2 were given BBN only and rats in group 3 were given D-penicillamine only. In week 15, 30 and 45, 24-h urine samples were collected.

**Statistical analyses** The incidences of tumors were analyzed by using Fisher's exact probability test and other data were analyzed by means of Student's *t* test.

**RESULTS**

**BBN-induced carcinogenesis** In experiment 1, the average intake of BBN/kg body weight of LEC rats was 1.3–1.5 times that of F344 rats. Body weights in treated

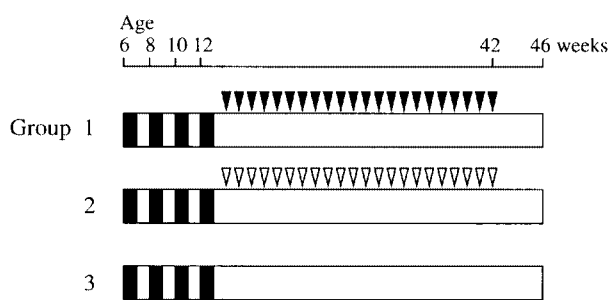


Fig. 2. Experimental design for two-stage carcinogenesis study (experiment 3). ■ 0.1% BBN in drinking water, ▼ Cu-NTA, i.p., ▽ NTA, i.p.

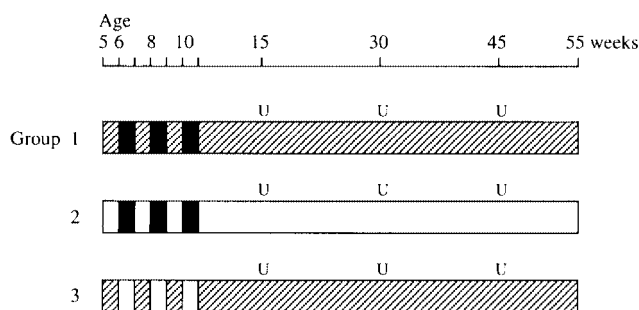


Fig. 3. Experimental design for two-stage carcinogenesis study (experiment 4). ■ 0.1% BBN in drinking water, ▨ 0.05% D-penicillamine in drinking water, U, collection of 24-h urine.

Table I. Incidences of Bladder, Liver and Kidney Tumors in Male LEC and F344 Rats Treated with BBN (Experiment 1)

Treatment	Strain	Effective no. of rats	Body weight (g)	Bladder			Liver			Kidney
				Transitional cell tumor	Papilloma	Transitional cell carcinoma	Hepato-cellular tumor	Hepato-cellular adenoma	Hepato-cellular carcinoma	Renal cell adenoma
none	LEC	16 <sup>a)</sup>	347±34 <sup>c)</sup>	0	0	0	1 (6%)	1 (6%)	0	1 (6%)
	F344	21	493±21	0	0	0	0	0	0	0
BBN	LEC	17 <sup>b)</sup>	351±19	2 (12%) <sup>e)</sup>	2 (12%) <sup>d)</sup>	0 <sup>e)</sup>	7 (41%) <sup>d)</sup>	6 (35%) <sup>d)</sup>	1 (6%)	1 (6%)
	F344	21	491±29	16 (76%)	13 (62%)	10 (48%) <sup>f)</sup>	0	0	0	0

a) Five rats died of hepatic injury at 19–21 weeks old.

b) Four rats died of hepatic injury at 17–26 weeks old.

c) Mean±SD.

d, e) Significantly different from BBN-treated F344 rats by Fisher's exact probability test; d) *P*<0.005, e) *P*<0.001.

f) One showed squamous differentiation.

Table II. Incidences of Bladder and Liver Tumors in Male LEC and LEA Rats Treated with BBN (Experiment 2)

Strain	Effective no. of rats	Body weight (g)	Bladder			Liver	
			Bladder/body weight (%)	Transitional cell tumor	Papilloma	Transitional cell carcinoma	Hepatocellular adenoma
LEC	18 <sup>a)</sup>	343±22 <sup>d)</sup>	0.04±0.01 <sup>e)</sup>	2 (11%) <sup>c)</sup>	2 (11%) <sup>f)</sup>	0 <sup>c)</sup>	6 (33%) <sup>b)</sup>
LEA	21	544±47	0.15±0.12	20 (95%)	7 (33%)	20 (95%) <sup>g)</sup>	0

a) Three rats died of hepatic injury at 20–27 weeks old.

b, c) Significantly different from DEN-treated LEA rats by Fisher's exact probability test; b)  $P < 0.01$ , c)  $P < 0.001$ .

d) Mean±SD.

e) Significantly different from BBN-treated LEA rats at  $P < 0.001$  by Student's *t* test.

f) One showed squamous differentiation.

g) Five showed squamous differentiation, and one showed osseous differentiation.

groups increased similarly to those in the control groups. Table I shows the incidences of tumors in LEC and F344 rats. The incidence of transitional cell tumors (papillomas plus carcinomas) in BBN-treated LEC rats was significantly lower than that in F344 rats ( $P < 0.001$ ). BBN treatment enhanced the development of hepatocellular tumors in LEC rats.

In experiment 2, the average intake of BBN/kg body weight of LEC rats was 1.1–1.3 times that of LEA rats. Table II shows the bladder weights and incidences of tumors in LEC and LEA rats. The incidence of transitional cell tumors in BBN-treated LEC rats was also lower than that in LEA rats ( $P < 0.001$ ). Fig. 4 shows the gross appearances of bladder tumors in LEC and LEA rats. The tumors in LEA rats were larger and more numerous than those in LEC rats.

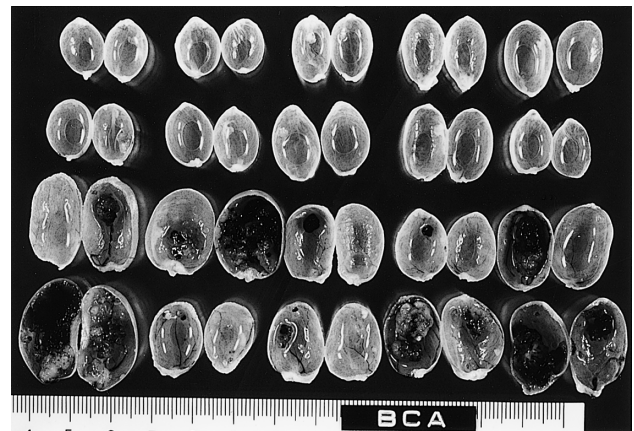


Fig. 4. Gross appearance of bladder tumors in consecutive cases of LEC (upper two lines) and LEA rats (lower two lines, experiment 2).

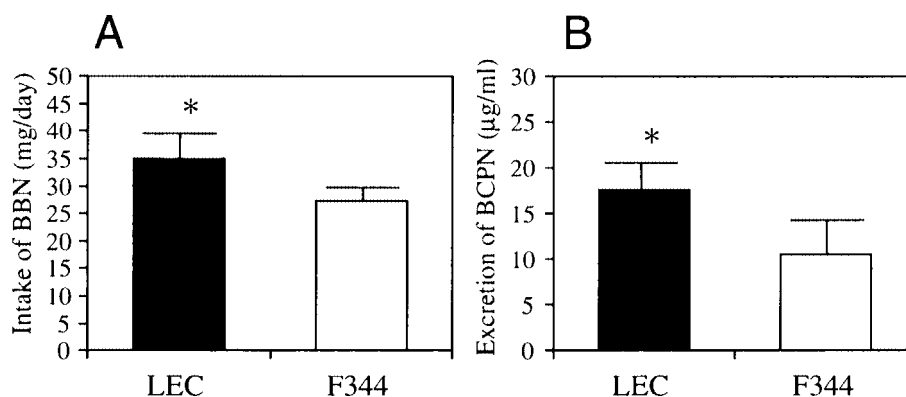


Fig. 5. Average intakes of BBN (A), and concentrations of BCPN in urine (B) in 9-week-old male LEC and F344 rats ( $n=6$ ) given 0.1% BBN in their drinking water for 7 days. Differences are significant at \*  $P < 0.01$  by Student's *t* test. Bars, SD.

The incidence of squamous differentiation of transitional cell carcinomas in LEA rats in experiment 2 was higher than that in F344 rats in experiment 1.

**Urinary excretion of BCPN** The average intake of BBN of LEC rats was 1.3 times that of F344 rats ( $P < 0.01$ ), and the concentration of BCPN in the urine of LEC rats was 1.7 times that of F344 rats ( $P < 0.01$ , Fig. 5).

**Urinary pH** The average urinary pH of LEC rats was 7.52–7.64, and that of F344 rats was 7.40–7.45 at 6, 12 and 30 weeks old, the difference not being statistically significant (Fig. 6).

**Urinary excretions of copper, iron and zinc in untreated rats** Changes of metal concentrations in the urine of untreated LEC and F344 rats are shown in Fig. 7.

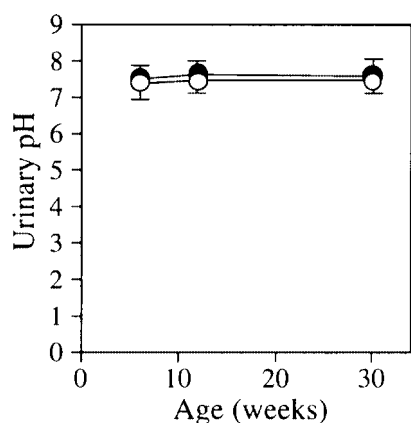


Fig. 6. Urinary pH values of untreated 6-, 12- and 30-week-old LEC and F344 rats ( $n=5$ ). ● LEC, ○ F344. Bars, SD.

In 8-week-old rats before jaundice developed, the urinary concentrations of these metals were lower in LEC rats than in F344 rats. However, the copper concentrations of 28- and 50-week-old LEC rats were 1.7 and 2.3 times those of F344 rats. The urinary iron and zinc concentrations were similar in these strains.

**Metallothionein level of the bladder wall** The metallothionein level of the bladder wall of LEC rats showed a peak at 24 weeks old, the time of onset of necrotizing hepatitis (Fig. 8). Its level in LEC rats was lower than that in F344 rats between 15 and 40 weeks.

**Inhibitory effect of cupric sulfate on bladder carcinogenesis** In week 26, the urinary copper concentration in group 1 was significantly higher than those in groups 2 and 3 (Fig. 9). NTA treatment in groups 1 and 2 increased the urinary zinc, but not the iron concentrations. In week 46, numbers of gross bladder tumors of more than 2 mm in diameter per rat in group 1 were less than those in group 2. The incidence of transitional cell carcinomas in group 1 was lower than that in group 2, although the dif-

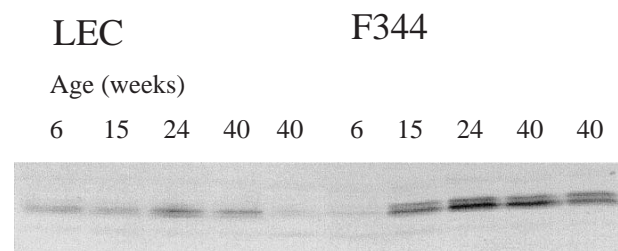


Fig. 8. Metallothionein expression in the bladder wall of LEC and F344 rats.

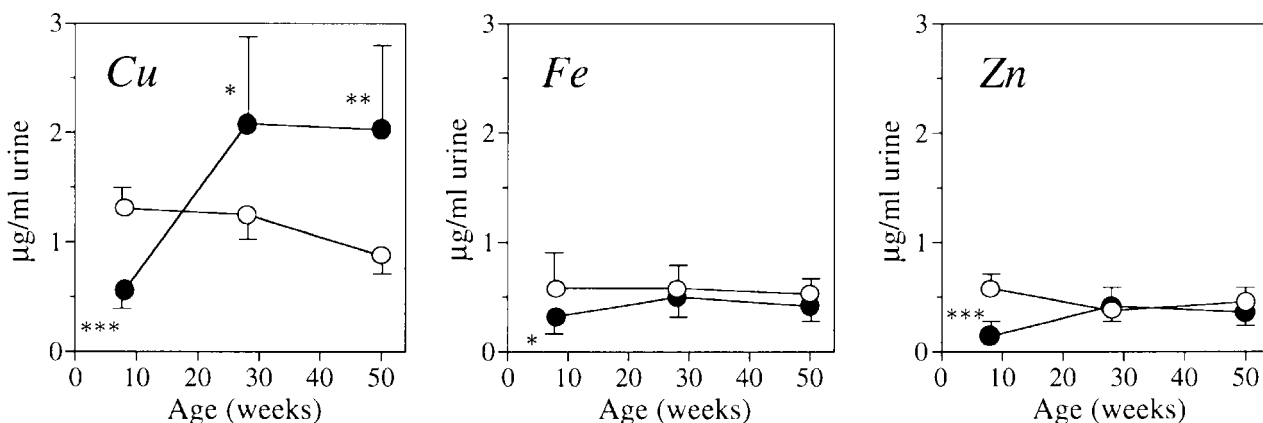


Fig. 7. Copper, iron and zinc concentrations in urine samples of untreated 8-, 28- and 50-week-old LEC and F344 rats ( $n=5$  to 7). Differences are significant at \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  by Student's  $t$  test. ● LEC, ○ F344. Bars, SD.

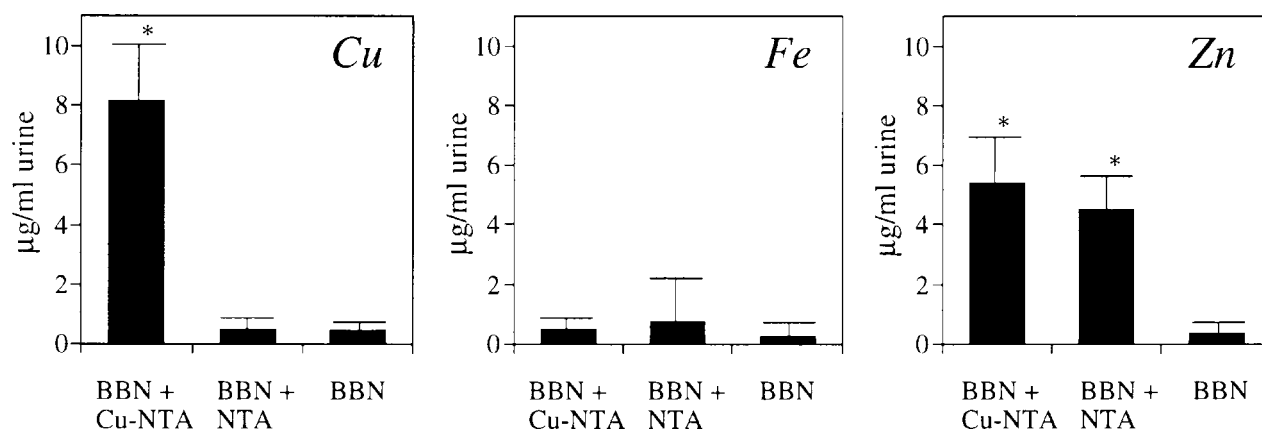


Fig. 9. Urinary metal concentrations in F344 rats ( $n=6$ ) given BBN plus Cu-NTA (group 1), BBN plus NTA (group 2) or BBN (group 3). In week 26, 24-h urine samples were collected. Differences are significant at \*  $P<0.001$  from control groups by Student's  $t$  test. Bars, SD.

Table III. Incidences of Bladder Tumors in Male F344 Rats Treated with BBN and Cu-NTA (Experiment 3)

Group	Treatment	Effective no. of rats	Body weight (g)	Bladder				
				No. of gross tumors/rat		Transitional cell tumor	Papilloma	Transitional cell carcinoma
				( $\geq 1$ mm)	( $\geq 2$ mm)			
1	BBN+Cu-NTA	19 <sup>a)</sup>	394 $\pm$ 17 <sup>c, e)</sup>	1.16 $\pm$ 1.07	0.74 $\pm$ 0.87 <sup>d)</sup>	15 (79%)	11 (58%)	9 (47%)
2	BBN+NTA	20 <sup>b)</sup>	404 $\pm$ 16 <sup>f)</sup>	1.80 $\pm$ 1.28	1.40 $\pm$ 1.10	16 (80%)	10 (50%)	16 (80%)
3	BBN	21	420 $\pm$ 21	1.86 $\pm$ 1.39	1.24 $\pm$ 1.09	19 (90%)	15 (71%) <sup>g)</sup>	14 (67%) <sup>h)</sup>

a) One rat died of carcinoma of the prostate at 28 weeks old, and one died of unknown cause at 34 weeks old.

b) One rat died accidentally at 16 weeks old.

c) Mean $\pm$ SD.

d, e) Significantly different from group 2 by Student's  $t$  test; d)  $P<0.05$ , e)  $P<0.001$ .

f) Significantly different from group 3 by Student's  $t$  test;  $P<0.01$ .

g) Two papillomas showed squamous differentiation.

h) One transitional cell carcinoma showed squamous differentiation.

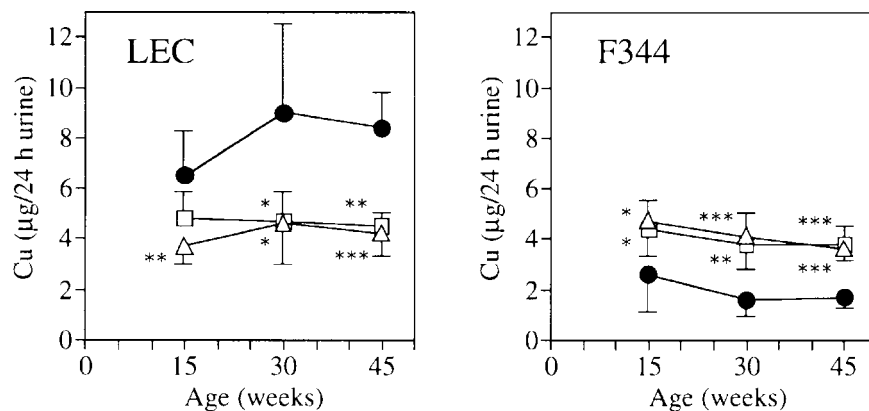


Fig. 10. Urinary copper contents in LEC and F344 rats ( $n=6$ ) given BBN plus D-penicillamine (group 1,  $\Delta$ ), BBN (group 2,  $\bullet$ ) or D-penicillamine (group 3,  $\square$ ). Differences are significant at \*  $P<0.05$ , \*\*  $P<0.01$  and \*\*\*  $P<0.001$  from group 2 by Student's  $t$  test. Bars, SD.

Table IV. Incidences of Bladder and Liver Tumors in Male LEC and F344 Rats Treated with BBN and D-Penicillamine (Experiment 4)

Strain	Treatment	Effective no. of rats	Body weight (g)	Bladder			Liver	
				No. of gross tumors/rat ( $\geq 1$ mm)	Transitional cell tumor	Papilloma	Transitional cell carcinoma	Hepatocellular adenoma
LEC	BBN+D-penicillamine	21	340 $\pm$ 18 <sup>b)</sup>	0.33 $\pm$ 0.48	7 (33%)	5 (24%)	2 (10%)	0
	BBN	17 <sup>a)</sup>	338 $\pm$ 24	0.18 $\pm$ 0.39	3 (18%)	2 (12%)	1 (6%)	2 (12%)
	D-Penicillamine	21	356 $\pm$ 23	0	0	0	0	0
F344	BBN+D-penicillamine	21	445 $\pm$ 21	1.85 $\pm$ 1.42	17 (81%)	15 (71%)	7 (33%)	0
	BBN	21	456 $\pm$ 24	1.95 $\pm$ 1.85	14 (67%)	12 (57%)	11 (52%)	0
	D-Penicillamine	21	455 $\pm$ 22	0	0	0	0	0

a) Four rats died of hepatic injury at 19–22 weeks old.

b) Mean $\pm$ SD.

ference was not statistically significant (Table III). Some bladder tumors in group 3 showed squamous differentiation. No liver or kidney tumors developed in any group.

**Effect of D-penicillamine on bladder carcinogenesis**

Long-term oral administration of D-penicillamine decreased the urinary copper content (Fig. 10) and increased BBN-induced bladder cancer in LEC rats, although the difference was not significant (Table IV). In F344 rats it increased urinary copper excretion and had no effect on bladder carcinogenesis.

**DISCUSSION**

This is the first report that LEC rats are resistant to BBN-induced bladder carcinogenesis. LEC rats were more resistant than F344 rats, which are known to be a resistant strain.<sup>4)</sup>

BBN is a urinary bladder-specific carcinogen, and in rats, approximately 40% of orally administered BBN is excreted as BCPN in the urine.<sup>19)</sup> BCPN is known to be a major active metabolite of BBN because, 1) intravesical instillation of BCPN induces bladder cancer in female rats,<sup>20)</sup> 2) BCPN is able to transform normal bladder cells of rats *in vitro*,<sup>21)</sup> and 3) BCPN is mutagenic in TA 1535 without S-9 mix.<sup>22)</sup> However, the susceptibility to BBN-induced bladder carcinogenesis seems to be independent of urinary excretion of BCPN.<sup>4,19)</sup> In the present study, the urinary concentration of BCPN in resistant LEC rats was higher than that in more susceptible F344 rats. The concentrations of BCPN in the bladder wall of LEC and F344 rats given 0.1% BBN for 8 days and tap water for one day were similar (data not shown). We conclude therefore that the low susceptibility to BBN-induced carcinogenesis of LEC rats could not be explained by the urinary excretion of BCPN.

It has been shown that a high urinary pH is related to promotion of rat bladder carcinogenesis.<sup>23,24)</sup> LEC rats show polyuria, and because of low osmolarity, their urinary sodium concentration at 8 and 28 weeks old was

lower than that of F344 rats (data not shown). However, the urinary pHs of LEC and F344 rats were similar in the present study.

Copper is a potent generator of reactive oxygen species *in vivo*,<sup>25)</sup> and reactive oxygen species induce DNA damage. In LEC rats, the amounts of copper and 8-OHdG in DNA are increased in the liver and kidneys.<sup>26)</sup> We found that kidney tumors developed spontaneously<sup>12)</sup> and showed that copper accumulated in renal tubular cells might be the major cause of spontaneous renal carcinogenesis in LEC rats.<sup>27)</sup> In contrast, the present two-stage model studies on bladder carcinogenesis demonstrated that a high copper level in the urine might have a slight inhibitory effect, unlike in parenchymal organs such as the liver and kidneys of LEC rats. The role of urinary trace metals such as copper, iron and zinc in urothelial carcinogenesis is not yet known,<sup>24)</sup> and thus further investigations are necessary to establish the role of copper.

Metallothionein is a metal-binding protein, but the role of endogenous metallothionein in chemical carcinogenesis is not clearly understood. Recently, endogenous metallothionein was reported to inhibit apoptosis<sup>28)</sup> and carcinogenesis.<sup>29,30)</sup> In the present study, we found that the metallothionein level of the bladder wall in the resistant LEC strain was rather lower than that in F344 rats.

Addition of trisodium nitrilotriacetate monohydrate to the diet increases urinary zinc excretion, increases urinary pH, induces kidney and bladder neoplasms, and promotes BBN-induced bladder carcinogenesis.<sup>31,32)</sup> In our experiment, the pH of the solution of NTA was adjusted to 7.4 and i.p. injection of NTA itself did not enhance bladder carcinogenesis.

LEC rats have alterations of drug-metabolizing enzymes, such as lowered liver cytochrome P450 and lowered alcohol dehydrogenase.<sup>33,34)</sup> Airoidi *et al.* reported that O<sup>6</sup>-butylguanine was formed in urothelial cell DNA of rats given BBN.<sup>35)</sup> Therefore, it is necessary to search for metabolite(s) of BBN<sup>36)</sup> which may act as ultimate carcinogen(s). Linkage analysis to identify bladder-cancer

susceptibility/resistance gene(s) would also be useful. LEC rats are a unique and useful strain for such studies.

The present study showed that LEC rats are resistant to BBN-induced urinary bladder carcinogenesis. Their low susceptibility to bladder cancer may be explained partly by their high urinary copper concentration, but not urinary BCPN concentration, urinary pH alteration or the metallothionein level in the bladder.

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