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Effects of dietary dimethylarsinic acid on the urine and urothelium of rats

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Dimethylarsinic acid (DMA), fed to rats for 2 years, produced bladder hyperplasia and tumors at doses of 40 and 100 p.p.m., more in females than males. No urothelial proliferation was seen in mice. Our objectives were to investigate the mode of action of bladder tumor formation, evaluate the dose–response and the role of diet and to determine if the urothelial effects were reversible. The study included groups of female F344 rats fed DMA in Purina 5002 diet at doses of 0, 2, 10, 40 or 100 p.p.m. for 10 weeks; two groups of females fed DMA (0 and 100 p.p.m.) in Altromin 1321 for 10 weeks; two groups of males fed DMA (0 and 100 p.p.m.) in Purina 5002 for 10 weeks; a female high-dose recovery group (100 p.p.m. in Purina 5002 diet for 10 weeks followed by control diet for 10 weeks); and two female groups (0 and 100 p.p.m.) in Purina diet for 20 weeks. Urothelial toxicity and hyperplasia were detected by light and scanning electron microscopy (SEM), and the bromodeoxyuridine labeling index was increased in the female 40 and 100 p.p.m. groups. The effects were less in males, but were similar in females fed DMA in Altromin 1321. SEM detected no abnormal urinary solids related to treatment in any group. Urinary calcium was increased in the females fed 40 and 100 p.p.m. in Purina diet, despite overall urinary dilution. Calcification was increased in kidneys of female rats fed Purina diet. The urothelial effects of DMA were reversible. The findings support a non-DNA reactive mechanism for DMA rat bladder carcinogenicity related to urothelial toxicity and regeneration. The toxicity is probably not due to urinary solids. The toxicity and regeneration are produced in a dose-responsive manner in female rats, are greater in female than in male rats, and are reversible.

Introduction

Dimethylarsinic acid (DMA, cacodylic acid) was found to produce an increased incidence of bladder tumors and hyperplastic lesions in F344 rats when fed in the diet in a 2 year bioassay at doses of 40 or 100 p.p.m. (1), but no significant incidences of bladder lesions were observed at doses of 2 or 10 p.p.m.. There was a higher incidence of lesions in females than in males. No bladder or other treatment-related tumors were produced in male or female $B6C3F_1$ mice in a 2 year bioassay (1). Fukushima and colleagues recently completed a 2 year bioassay in male F344 rats and observed an increased incidence of bladder tumors at doses of 50 and 200 p.p.m. in the drinking water, but no proliferative lesions at doses of 12.5 p.p.m. (2). Previously, they had shown an increased incidence of bladder tumors in male rats when DMA was administered after treatment with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN), with positive results at 25, 50 and 100 p.p.m., a borderline response at 10 p.p.m. and no response at 2 p.p.m. in the drinking water (3).

Various *in vitro* and *in vivo* investigations of DMA as well as other arsenicals suggest that they do not act by binding to DNA, so that a DNA-reactive mechanism for carcinogenicity is unlikely (4–9). An alternative mechanism involves induction of increased cell proliferation, either by direct mitogenesis or by toxicity and consequent regeneration.

Most non-DNA-reactive chemicals that produce bladder cancer in rats do so by directly or indirectly inducing urothelial toxicity and consequent regeneration (10). Mechanisms involved in producing urothelial toxicity include the production of solids (such as calculi, microcrystals and amorphous precipitate) in the urine, or toxicity due to the chemical and/or a metabolite(s). Other indirect processes, such as alterations in urinary pH, volume, osmolality, calcium or other changes in urinary chemistry have also been suggested (10,11).

The intent of this study was to define the mode of action for the formation of the bladder tumors induced by DMA in the rat to determine if toxicity and/or increased proliferation was produced within a relatively short time (10 weeks.), preceding the appearance of tumors. Subsequent examination of kidneys from the rats treated in the 2 year bioassay (1) suggested the presence of increased renal calcification (unpublished observations), but evaluation for urinary solids was not performed. The present study was designed to evaluate the urine of rats fed DMA for the formation of urinary solids (calculi, microcrystals and/or precipitate) and to analyse the urine chemically for urinary substituents that might be related to their formation, such as pH, calcium, phosphorus and magnesium. To evaluate toxicity and proliferation we used more sensitive morphological techniques, such as scanning electron microscopy (SEM) with attached X-ray energy dispersive spectroscopy, and bromodeoxyuridine (BrdU) labeling index, in addition to standard histopathology to evaluate toxicity and proliferation (12). We also included techniques to investigate tissue calcification (von Kossa stain for calcium).

We also investigated the dose–response for the effects of feeding DMA on the urine and urinary bladder and the potential reversibility of the urothelial lesions produced. This study was performed in female rats since they were more susceptible than males to the bladder proliferative effects following feeding of DMA (1). Male groups were included only at the highest dose and in controls. DMA-containing diet was fed to rats for

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BrdU, bromodeoxyuridine; DMA, dimethylarsinic acid; SEM, scanning electron microscopy.

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Table I. Study design

Group	Sex	Treatment	Basal diet
1a	F	0 p.p.m. DMA; 20 weeks	Purina 5002
1b	F	0 p.p.m. DMA; 10 weeks	Purina 5002
1c	F	0 p.p.m. DMA; 8 weeks	Purina 5002
2	F	2 p.p.m. DMA; 10 weeks	Purina 5002
3	F	10 p.p.m. DMA; 10 weeks	Purina 5002
4	F	40 p.p.m. DMA; 10 weeks	Purina 5002
5a	F	100 p.p.m. DMA; 20 weeks	Purina 5002
5b	F	100 p.p.m. DMA; 10 weeks 0 p.p.m. DMA; 10 weeks	Purina 5002
5c	F	100 p.p.m. DMA; 10 weeks	Purina 5002
5d	F	100 p.p.m. DMA; 8 weeks	Purina 5002
6	F	0 p.p.m. DMA; 10 weeks	Altromin 1321
7	F	100 p.p.m. DMA; 10 weeks	Altromin 1321
8	M	0 p.p.m. DMA; 10 weeks	Purina 5002
9	M	100 p.p.m. DMA; 10 weeks	Purina 5002

10 weeks, because it was anticipated, based on studies with other chemicals (10,12–16) that proliferative lesions would be produced by then, and that removal of DMA from the diet after 10 weeks should result in reversibility of these proliferative lesions. The reversibility phase of the study lasted for an additional 10 weeks.

In the 2 year rat bioassay (1), Altromin diet (commonly used in Europe) was used. However, Altromin diet is known to produce a markedly alkaline urine in rats (17), in contrast to the neutral urine produced in rats fed Purina diet, which is the standard diet used in the USA (18). Urinary pH is known to frequently affect the urothelial response to bladder toxicants (11,18,19). Therefore, Purina diet was used in the present study, and Altromin diet was fed to two groups for the purpose of bridging with the 2 year bioassay.

Materials and methods

Chemical and diets

DMA was received from Luxembourg Industries (Pamol; Tel-Aviv, Israel). The purity of the test material was documented by Luxembourg Industries and confirmed by NMR at our facility at the University of Nebraska Medical Center to be pure DMA. DMA was fed in the diet at levels of 0, 2, 10, 40 and 100 p.p.m.. These doses are the same as those previously fed in the chronic 2 year bioassay (1). DMA was mixed in the respective diets at the designated doses by Dyets (Bethlehem, PA) and stored at –20°C. Samples were taken from different portions of the mix to test for homogeneity and at different times to evaluate chemical stability. Samples were analyzed by inductively coupled plasma mass spectrometry (by Dr William Cullen, University of British Columbia, BC, Canada) after water:methanol (1:1) extraction. Diet homogeneity and stability were found to be within acceptable limits. Fresh diets were prepared every 5 weeks and fed within 10 weeks of preparation. Fresh diet was provided weekly.

Test animals and experimental design

A total of 120 female and 20 male F344 rats were purchased from Charles River Breeding Laboratories (Raleigh, NC). One day after arrival the rats were randomized into 12 female and two male groups of 10 each (Table I). Two groups of female rats were started on pelleted Altromin 1321 diet, and the rest of the groups were started on pelleted Purina Mills Certified Rodent Lab Chow 5002 (St Louis, MO). The rats were housed five per cage in polycarbonate cages with dry corn-cob bedding in a room with a targeted temperature of 22°C, humidity of 50% and a 12 h light/dark cycle. Food and tap water were available *ad libitum* at all times during the study. After 7 days of quarantine, the appropriate amounts of test material were added to the basal diets. After 8 weeks of treatment, the rats of Groups 1c (controls) and 5d (highest dose) were killed to check if the expected effects on the bladder could be detected. The rest of the rats, except those of Groups 1a (controls), 5a and 5b (both at the highest dose) were killed after 10 weeks of treatment. The latter three groups remained on the study for an additional period of 10 weeks to investigate potential recovery. During the additional 10 weeks, Group 1a continued to receive control diet, Group 5a continued to receive DMA and Group 5b was reversed from DMA to control diet to examine the reversibility of the urinary and urothelial effects.

Experimental procedures

Water and food consumptions were measured during study weeks 2, 6 and 10 of the experiment and during weeks 4 and 8 of the recovery phase (weeks 15 and 19 of the study). Body weights of all rats were measured the day after arrival, on study day 0, on the last day of each period during which food and water consumptions were determined, before and after placement in metabolism cages and on the day of killing. Detailed clinical observations of each animal were done on day 0 and on the last day of each consumption period. Fresh voided urine was collected between the hours of 0700 and 0900 from the first five animals in each group on study days 22 and 60, from the last five animals per group on days 25 and 61 and from all animals in groups 1a, 5a and 5b during study week 17. Urinary pH was measured, a 100 µl aliquot of each urine sample was centrifuged and the supernatant removed. The pellet was speed vacuumed and then resuspended in water, vacuum filtered through a 0.22 µm Millipore filter and air dried (13). The filters were examined by SEM and X-ray energy dispersive spectroscopy for the presence of calculi, microcrystals and/or precipitate. After 48 h acclimation to metabolism cages with food and water available *ad libitum*, two consecutive 24 h urine samples were collected from the first five animals in groups 1a, 2–4, 5a and 6–9 during study days 28 and 29, and from the last five animals in these groups during days 32 and 33. Urine was collected with nylon netting in the funnel to prevent contamination by the diet or feces. Volume was measured, osmolality was determined with a Model 3MO osmometer (Advanced Instruments, Needham Heights, MA) and sodium, creatinine, calcium, magnesium and phosphorus were determined on the Vitros 250 Chemistry Analyzer (Johnson and Johnson Clinical Diagnostics, Rochester, NY).

Rats were killed by an overdose of Nembutal (50 mg/kg of body weight) 1 h ± 1 min. after an i.p. injection of 100 mg/kg body weight of BrdU (14). The bladder was inflated *in situ* with Bouin's fixative and removed. One-half of the bladder was processed for SEM and classified as described previously (12). The other half of the bladder was cut longitudinally into strips and, with a slice of the stomach (to serve as a positive control for BrdU labeling), was embedded in paraffin, stained with hematoxylin and eosin (H&E) and examined histopathologically (12,20). Bladders of all animals killed after 10 and 20 weeks were evaluated by light microscopy, SEM and BrdU labeling index of the bladder epithelium. Kidneys (fixed in 10% phosphate-buffered formalin) and stomachs (fixed in Bouin's solution) of all of these rats were examined by light microscopy after paraffin embedding and H&E staining. Calcium in the bladders and kidneys of these animals was assessed by the von Kossa stain (21).

Results

Body weight

The body weights of the different treatment groups fed Purina 5002 were comparable for each sex throughout the study (Table II). Body weights in the two female groups fed Altromin 1321 diet were significantly decreased compared with the Purina 5002 groups after the 1 week quarantine, but this decrease did not persist throughout the study for the control rats. However, the weights of rats fed 100 p.p.m. DMA in the Altromin diet remained lower than the weights of the rats fed 100 p.p.m. DMA in Purina diet, although not statistically significantly.

Water and food consumptions

Water consumption was increased in female and male rats fed Purina diet in a dose-responsive manner, although the increase was not significantly different at all time points (Table II). The increase was smaller in the female rats fed Altromin diet than in the females fed Purina diet. Water consumption relative to body weight was increased in all groups fed DMA regardless of sex or basal diet, although not significantly at all time points. The increase in water consumption tended to decrease in the rats over the course of the study. Food consumption relative to body weight was similar in all treatment groups when compared with their respective controls, but it was increased in the Altromin-fed groups compared with the Purina-

Table II. Body weights, water and food consumptions

Group	Treatment	Sex	Diet	Body Weight (g)				Water Consumption[a]	Food Consumption[a]
				d.0 (*n*)	d.14 (*n*)	d.42 (*n*)	d.70 (*n*)	g/rat/day (week 10)	g/rat/day(week 10)
1	0 p.p.m. DMA	F	Purina 5002	78 ± 1 (30)	116 ± 1 (30)	155 ± 1 (30)	168 ± 2 (20)	19 ± 0	10.3 ± 0.1
2	2 p.p.m. DMA	F	Purina 5002	79 ± 1 (10)	116 ± 2 (10)	158 ± 2 (10)	175 ± 1 (10)	21 ± 1	11.1 ± 0.3
3	10 p.p.m. DMA	F	Purina 5002	78 ± 1 (10)	114 ± 1 (10)	155 ± 2 (10)	172 ± 3 (10)	22 ± 1	11.2 ± 0.0[b]
4	40 p.p.m. DMA	F	Purina 5002	79 ± 1 (10)	117 ± 2 (10)	155 ± 3 (10)	169 ± 3 (10)	23 ± 2[b]	11.0 ± 0.5
5	100 p.p.m. DMA	F	Purina 5002	79 ± 1 (40)	115 ± 1 (40)	155 ± 1 (40)	171 ± 1 (30)	24 ± 1[b]	11.1 ± 0.2[b]
6	0 p.p.m. DMA	F	Altromin 1321	74 ± 1[b] (10)	113 ± 2 (10)	153 ± 1 (10)	168 ± 2 (10)	21 ± 2	12.0 ± 0.3[b]
7	100 p.p.m. DMA	F	Altromin 1321	75 ± 1 (10)	109 ± 2 (10)	150 ± 2 (10)	165 ± 2 (10)	24 ± 2	12.3 ± 0.3
8	0 p.p.m. DMA	M	Purina 5002	91 ± 1[b] (10)	154 ± 3[b] (10)	239 ± 5[b] (10)	285 ± 4[b] (10)	24 ± 0[b]	16.9 ± 0.3[b]
9	100 p.p.m. DMA	M	Purina 5002	93 ± 1 (10)	162 ± 3[c] (10)	234 ± 3 (10)	281 ± 3 (10)	29 ± 1[c]	17.2 ± 0.2

[a]Representative of values observed at weeks 2, 6 and 10 of the experiment.
[b]Significantly different from female Purina 0 p.p.m. group, $P < 0.05$.
[c]Significantly different from male Purina 0 p.p.m. group, $P < 0.05$.

fed groups (TableII). Food consumption decreased in all groups over the course of the study.

Urinary pH

At week 9, the pH was significantly increased in the female Purina 2, 40 and 100 p.p.m. groups and in the males compared with their respective controls. The pH was similar in the Altromin high-dose group compared with Altromin controls, and higher than rats fed Purina diet (Table III). In all groups fed DMA regardless of sex or basal diet, the pH at week 9 was increased compared with the pH of the group at week 4. At week 4 urinary pH was comparable in all female Purina groups compared with the female Purina control. The pH was significantly decreased in the Altromin high-dose group and slightly decreased in the male high-dose group when compared with their respective controls.

Urinary volume and chemistries

Urinary volume was significantly increased in the 40 and 100 p.p.m. female Purina groups and in the male 100 p.p.m. groups compared with their respective control groups, with a corresponding decrease in the concentration of most of the constituents that were analyzed (Table III). The most notable exception was calcium concentration which was increased in the female 40 and 100 p.p.m. groups. When the calcium concentrations are normalized for creatinine concentration, the result is statistically significant compared with controls; the level in the high-dose group was almost twice as high as the level in the control group. Calcium concentration normalized for creatinine concentration was also significantly increased in the male high-dose group compared with the male control group, but the increase was much less than that seen in the female high-dose group. There was no difference between the urine volumes of the two groups fed Altromin diet, but the concentrations of all the constituents analyzed, except phosphorus, were slightly decreased in the high-dose Altromin group compared with the Altromin control.

Urinary particulate matter

Examination of urinary filters prepared from fresh voided urine collected during weeks 4 and 9 showed no urinary solids in the urine of the treated animals attributable to the treatment.

Organ weights

The bladder weights in the female groups fed DMA in Purina at the two highest doses were increased (Table IV) compared with controls in both actual weight and relative to body weight, although the increase was statistically significant only at 40 p.p.m.. The bladder weights in the male high-dose group were significantly increased compared with the male controls. In the Altromin groups, bladder weights of the high-dose group were similar to those of the Altromin controls and to those of the Purina high-dose group. Kidney weights, both actual and relative to body weights, were significantly increased at the 40 and 100 p.p.m. dose when compared with controls (Table V). The kidney weights in the male high-dose group were significantly increased compared with the male controls. In the Altromin groups, kidney weights of the high-dose group were similar to those of the Altromin controls and to those of the Purina controls.

Histopathology

There was a significant increase in the incidence of simple hyperplasia (Figures 1 and 2) in the bladder epithelium in the female high-dose Purina groups treated for 8 and 10 weeks compared with controls. The increase in the female high-dose Altromin group or the male high-dose group was not signficantly different when compared with their respective controls (Table IV). In the females fed high-dose DMA in Altromin diet, the increase was not statistically significant because the controls fed Altromin diet (Group 6) had evidence of mild simple hyperplasia in four of 10 rats, whereas the DMA-treated group showed hyperplasia in six of 10 rats by histopathology. Vacuolization was focally present (Figure 2) in the urothelium of the bladder of female rats fed 100 p.p.m. DMA, and occasionally there was erosion of the epithelium and blood in the lumen (Figure 3). No calcification of the bladder epithelium was detected by routine histopathology with H&E staining or with von Kossa stain for calcium.

Examination of H&E-stained renal tissue from rats treated for 10 weeks showed calcification at the corticomedullary junction in the kidneys of female rats in all groups fed Purina diet and in the cortex of the kidneys from the groups of male rats fed Purina diet. Although present in most female rats at all doses, the quantity was more at the higher doses. There was little calcification noted in the renal tissue from rats in the groups fed the Altromin diet. Evaluation of calcification in the renal tissue by von Kossa staining (Figure 4), which is specific for tissue calcification, showed a dose-responsive increase in the amount of calcification in the female rats fed Purina diet (Table V).

There was no abnormal histopathology noted in stomach tissue from any group.

Table III. Twenty-four hour urine chemistries in week 5 and urine pH in weeks 4 and 9

Group	Treatment	Fresh void pH		Volume	Creatinine	Sodium	Potassium	Calcium	Calcium/ Creatinine	Osmolality	Chloride	Magnesium	Phosphorus	Protein
		week 4	week 9	(ml)	(mg/dl)	(mEq/l)	(mEq/l)	(mg/dl)	(mg/mg)	(mOSm/l)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/ml)
1	0 p.p.m. DMA-Purina	6.7 ± 0.1	6.8 ± 0.1	6 ± 0	68 ± 3	155 ± 7	328 ± 15	15.6 ± 1.3	0.25 ± 0.03	1707 ± 70	213 ± 9	38 ± 4	149 ± 13	0.3 ± 0.0
2	2 p.p.m. DMA-Purina	6.6 ± 0.1	7.2 ± 0.1[a]	7 ± 1	66 ± 6	133 ± 8[a]	295 ± 21	15.3 ± 0.9	0.26 ± 0.02	1554 ± 73	180 ± 10[a]	38 ± 5	164 ± 17	0.3 ± 0.0
3	10 p.p.m. DMA-Purina	6.6 ± 0.1	7.0 ± 0.2	7 ± 0	65 ± 4	147 ± 9	314 ± 18	15.5 ± 0.9	0.25 ± 0.02	1682 ± 92	198 ± 11	42 ± 4	173 ± 10	0.3 ± 0.0
4	40 p.p.m. DMA-Purina	6.5 ± 0.1	7.3 ± 0.2[a]	9 ± 1[a]	52 ± 2[a]	120 ± 5[a]	252 ± 9[a]	17.7 ± 1.4	0.35 ± 0.03[a]	1373 ± 49[a]	159 ± 6[a]	32 ± 4	142 ± 9	0.2 ± 0.0[a]
5	100 p.p.m. DMA-Purina	6.4 ± 0.1[a]	7.2 ± 0.1[a]	10 ± 1[a]	48 ± 2[a]	117 ± 5[a]	247 ± 9[a]	21.5 ± 2.0[a]	0.47 ± 0.05[a]	1420 ± 43[a]	157 ± 6[a]	32 ± 5	132 ± 9	0.3 ± 0.0
6	0 p.p.m. DMA-Altromin	7.9 ± 0.1[a]	8.0 ± 0.1[a]	7 ± 1	49 ± 2[a]	130 ± 7[a]	325 ± 18	26.3 ± 1.3[a]	0.55 ± 0.03[a]	1603 ± 87	173 ± 9[a]	61 ± 4[a]	3 ± 1[a]	0.4 ± 0.0
7	100 p.p.m. DMA-Altromin	7.5 ± 0.1[b]	7.9 ± 0.0	8 ± 0	44 ± 2	121 ± 5	291 ± 10	24.9 ± 1.4	0.58 ± 0.04	1419 ± 45[b]	157 ± 5	58 ± 3	13 ± 9	0.3 ± 0.0
8	0 p.p.m. DMA-Purina	6.4 ± 0.2[a]	6.5 ± 0.2	8 ± 1	89 ± 4[a]	189 ± 7[a]	420 ± 18[a]	15.7 ± 0.5	0.18 ± 0.01	2042 ± 80[a]	255 ± 10[a]	48 ± 3[a]	221 ± 10[a]	2.4 ± 0.1[a]
9	100 p.p.m. DMA-Purina	6.2 ± 0.1	7.0 ± 0.1[c]	12 ± 1[c]	59 ± 4[c]	130 ± 7[c]	266 ± 13[c]	14.7 ± 2.0	0.29 ± 0.05[e]	1405 ± 66[c]	175 ± 9[c]	24 ± 3[c]	144 ± 12[c]	1.4 ± 0.1[c]

[a]Significantly different from female Purina 0 p.p.m. group, $P < 0.05$.
[b]Significantly different from female Altromin 0 p.p.m. group, $P < 0.05$.
[c]Significantly different from male Purina 0 p.p.m. group, $P < 0.05$.

Table IV. Effects of DMA treatment on the urinary bladder

Group	Treatment	Bladder weight (g)	Bladder histology		Labeling index (*n*)	SEM classification				
		(mean ± SE)	Normal	Simple hyperplasia	(mean ± SE)	1	2	3	4	5
	8 Weeks									
1	0 p.p.m. DMA-Purina	0.073 ± 0.004	10	0		–	7	3	–	–
5	100 p.p.m. DMA-Purina	0.090 ± 0.004[a]	3	7[a]		–	–	–	2	8[b]
	10 Weeks									
1	0 p.p.m. DMA-Purina	0.071 ± 0.005	9	1	0.22 ± 0.05 (8)	5	5	–	–	–
2	2 p.p.m. DMA-Purina	0.070 ± 0.005	10	0	0.20 ± 0.03 (9)	–	4	5	1	–
3	10 p.p.m. DMA-Purina	0.075 ± 0.005	10	0	0.33 ± 0.08(10)	–	2	5	3	–
4	40 p.p.m. DMA-Purina	0.097 ± 0.009[c]	6	4	0.95 ± 0.15 (8)[c]	–	5	3	2	–
5	100 p.p.m. DMA-Purina	0.086 ± 0.005	1	9[c]	0.93 ± 0.11 (7)[c]	–	–	–	4	6[d]
6	0 p.p.m. DMA-Altromin	0.085 ± 0.009	6	4	0.19 ± 0.03 (10)	–	4	4	2	-
7	100 p.p.m. DMA-Altromin	0.084 ± 0.007	4	6	0.87 ± 0.09 (10)[e]	–	–	1	3	6[f]
8	0 p.p.m. DMA-Purina	0.099 ± 0.007[c]	10	0	0.23 ± 0.03 (10)	–	3	7	–	–
9	100 p.p.m. DMA-Purina	0.122 ± 0.008[g]	8	2	0.95 ± 0.05 (10)[g]	–	1	4	5	–

[a]Significantly different from week 8 female Purina 0 p.p.m. group, $P < 0.05$.
[b]Number of class 5 bladders significantly different from week 8 female Purina 0 p.p.m. group, $P < 0.05$.
[c]Significantly different from week 10 female Purina 0 p.p.m. group, $P < 0.05$.
[d]Number of class 5 bladders significantly different from week 10 female Purina 0 p.p.m. group, $P < 0.05$.
[e]Significantly different from female Altromin 0 p.p.m. group, $P < 0.05$.
[f]Number of class 5 bladders significantly different from female Altromin 0 p.p.m. group, $P < 0.05$.
[g]Significantly different from male Purina 0 p.p.m. group.

Table V. Effects of DMA treatment for 10 weeks on the kidney

Group	Sex	Treatment	Diet	Kidney wt (g) (mean ± SE)	Corticomedullary junction calcification[a]				
					Negative	+1	+2	+3	+4
1b	F	0 p.p.m. DMA	Purina 5002	1.11 ± 0.01	4	4	2		
2	F	2 p.p.m. DMA	Purina 5002	1.17 ± 0.01[b]	7	3			
3	F	10 p.p.m. DMA	Purina 5002	1.16 ± 0.02	5	4	1		
4	F	40 p.p.m. DMA	Purina 5002	1.24 ± 0.03[b]			9	1	
5c	F	100 p.p.m. DMA	Purina 5002	1.32 ± 0.03[b]		1	4	4	1
6	F	0 p.p.m. DMA	Altromin 1321	1.10 ± 0.01	10				
7	F	100 p.p.m. DMA	Altromin 1321	1.11 ± 0.02	9				
8	M	0 p.p.m. DMA	Purina 5002	1.89 ± 0.04[b]	8	2			
9	M	100 p.p.m. DMA	Purina 5002	2.01 ± 0.04[c]	8	1			

[a]No statistical analysis of results.
[b]Significantly different from female Purina 0 p.p.m. group, $P < 0.05$.
[c]Significantly different from male Purina 0 p.p.m. group, $P < 0.05$.

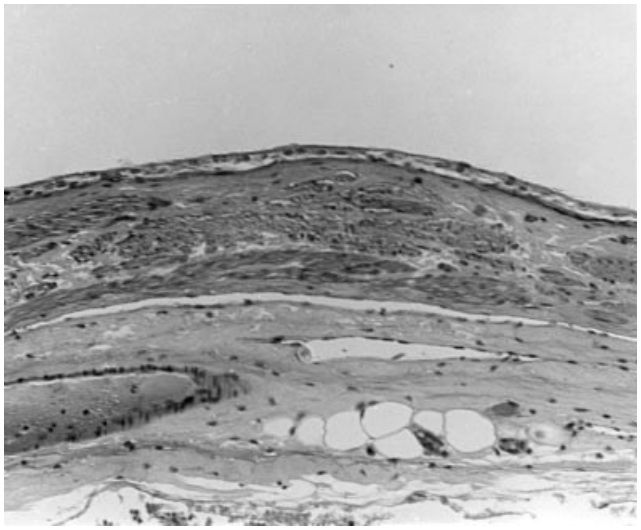

Fig. 1. Normal bladder from a control rat. ×400

Scanning electron microscopy

SEM examination of the bladder urothelium of rats fed high doses of DMA showed extensive necrosis, with exfoliation of the large, flat polygonal cells normally present (Figure 5) on the epithelial surface (Figure 6), and piling up of small round cells, some of which had pleomorphic microvilli (Figure 7). The classification of the bladders, based on previously established criteria (12), is tabulated for rats treated for 8 and 10 weeks (Table IV). There was clearly a dose–response relationship with evidence of toxicity and proliferation (classes 4 and 5) in the female rats fed 40 and 100 p.p.m. DMA in Purina diet. The effects in female rats fed DMA in Altromin diet were similar to those seen with Purina diet. The male rats showed less effect than did the females. Like other examples of bladder urothelial toxicity (10,12,22), the earliest and most extensive changes of necrosis are seen in the dome of the bladder, especially ventrally (Figure 7). This presumably occurs because the dome has the greatest contact with the urine, due to the horizontal nature of the quadruped rat. Interpretation of the SEM observations is difficult because of a relatively high level

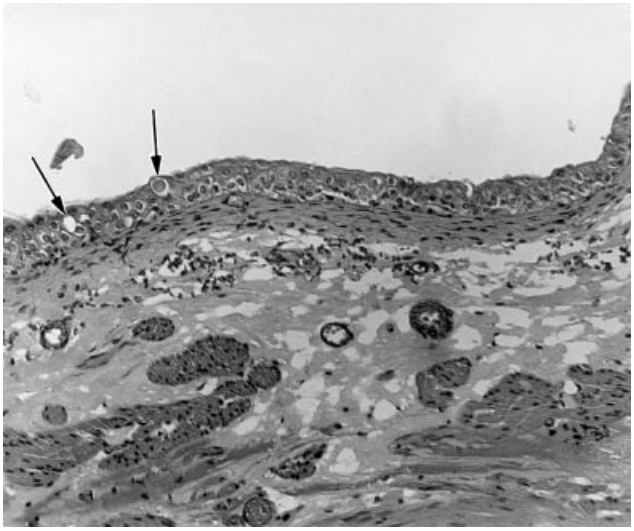

Fig. 2. Bladder urothelial hyperplasia with focal vacuolization (arrows), some of which contain red cells; from a female rat fed 100 p.p.m. DMA in Purina diet for 10 weeks; ×400.

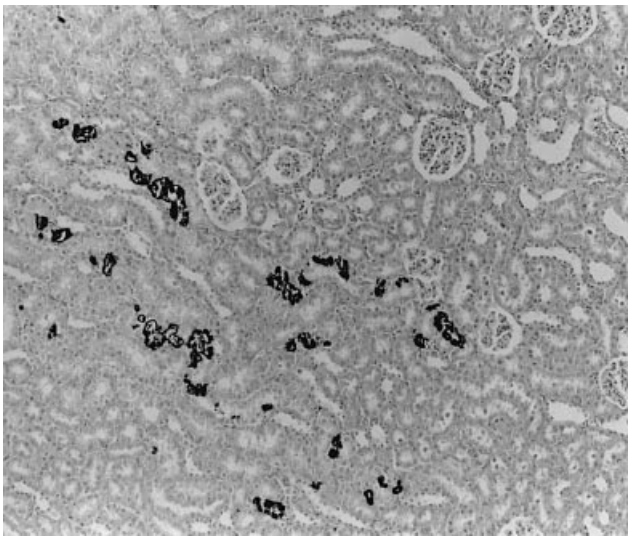

Fig. 4. Kidney showing extensive calcification at the corticomedullary junction; from a female rat fed 100 p.p.m. DMA in Purina diet for 10 weeks von Kossa stain; ×100.

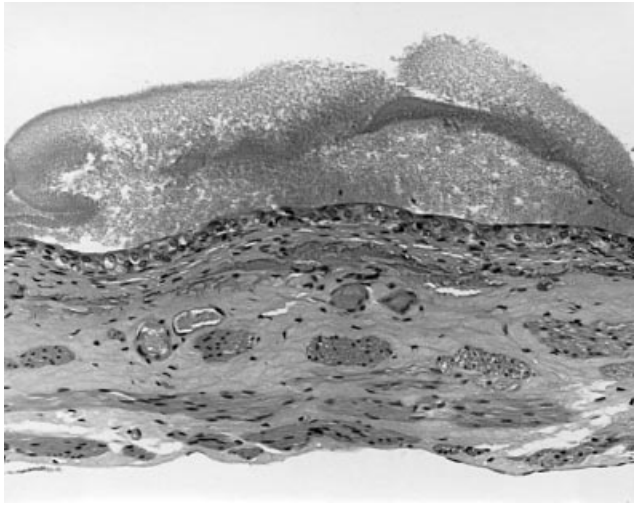

Fig. 3. Blood on the bladder surface and in the mucosa in a rat fed 100 p.p.m. DMA in Purina diet for 10 weeks; ×400

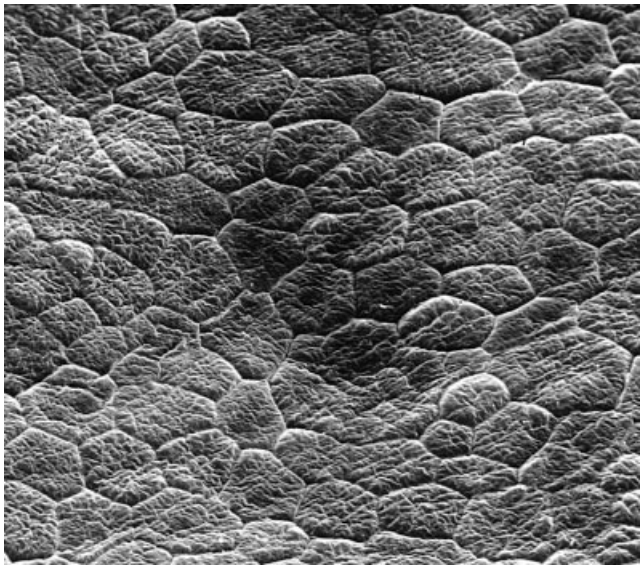

Fig. 5. Normal bladder surface as observed by scanning electron microscopy; from a control rat; ×300

of changes seen in the control rats. We have observed this phenomenon occasionally in previous studies (22).

No arsenic was detected in the bladder epithelium or the pelvis of the kidney by SEM with X-ray energy dispersive spectroscopy.

BrdU labeling index

The BrdU labeling index was significantly increased in the bladder epithelium of the female Purina-fed groups treated with 40 and 100 p.p.m. DMA, when compared with controls (Table IV). Labeling indices in the high-dose Altromin group and high-dose male groups were also increased when compared with their respective controls.

Recovery period

During the recovery period, body weights were comparable in the control and recovery groups, but higher in the continued high-dose group (187 ± 3, 187 ± 2 and 194 ± 2 g, respectively, at the end of the experiment). The differences between the weights of the continued group compared with those of the other groups are small but statistically significant. Water consumption was higher in both the 100 p.p.m. group and recovery group compared with controls (25 ± 1, 23 ± 1 and 21 ± 1 g/day, respectively, during week 19). Food consumption was similar in all groups.

Urinary pH measured on fresh voided urine collected during week 17 was comparable in all groups. There were no abnormal solids detected in the urines collected during week 17 when examined by SEM and X-ray energy dispersive spectroscopy.

Bladder tissue weights were similar in the control and recovery groups but significantly higher in the high-dose group compared with either the control or recovery group (Table VI). Kidney weights were significantly increased in both the high-dose and recovery groups compared with controls (1.44 ± 0.03, 1.37 ± 0.03 and 1.25 ± 0.02 g, respectively).

Examination of the bladder epithelium by light microscopy showed no evidence of simple hyperplasia in the recovery group at the end of the recovery phase (Table VI). Four animals in the high-dose group had simple hyperplasia of the

Table VI. Effects of DMA treatment and reversibility of effects on the urinary bladder

Group	Treatment	Bladder weight (g) (mean ± SE)	Bladder histology		Labeling index (*n*) (mean ± SE)	SEM classification				
			Normal	Simple hyperplasia		1	2	3	4	5
1a	0 p.p.m. DMA; 20 weeks	0.063 ± 0.004	9	1	0.25 ± 0.03 (10)	6	4	–	–	–
5a	100 p.p.m. DMA; 20 weeks	0.085 ± 0.006[a]	6	4	0.97 ± 0.11 (10)[a]	–	–	3	6	1
5b	100 p.p.m. DMA; 10 weeks 0 p.p.m. DMA; 10 weeks	0.065 ± 0.002[b]	10	0	0.21 ± 0.04 (9)[b]	–	–	6	4	–

[a]Significantly different from 0 p.p.m. group; 20 weeks, $P < 0.05$.
[b]Significantly different from 100 p.p.m. group; 20 weeks, $P < 0.05$.

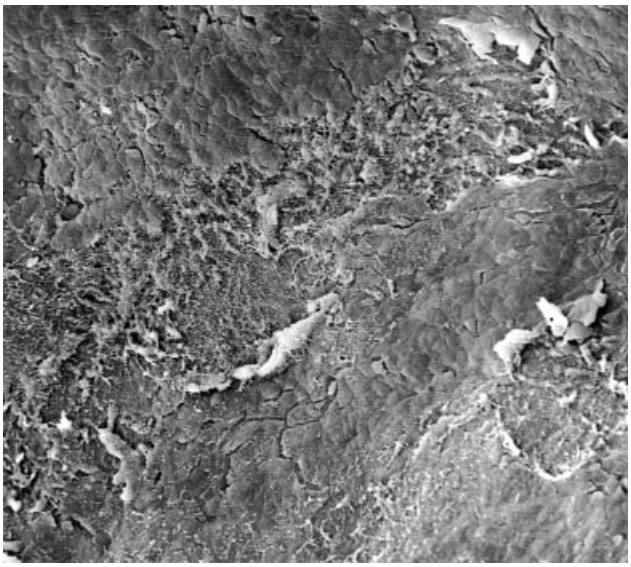

Fig. 6. Bladder surface from a female rat fed 100 p.p.m. DMA in Purina diet for 10 weeks showing necrosis and exfoliation; ×106.

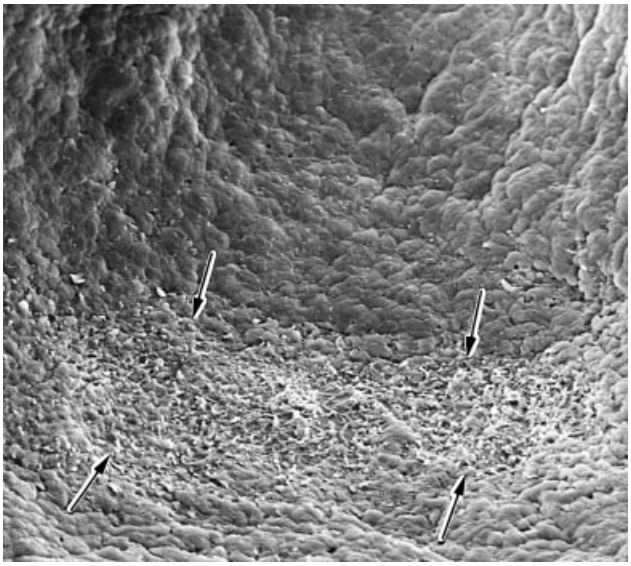

Fig. 7. Dome of a bladder from a female rat fed 100 p.p.m. DMA in Purina diet for 10 weeks showing a localized area (arrows) of superficial necrosis surrounded by hyperplastic epithelium (piling up of round cells); ×65

urothelium compared with one in the control group. No increased calcification was observed by light microscopy using routine H&E staining or the von Kossa stain. The histopathology was normal in all stomach tissue from all groups.

The classification of the appearance of the bladders by SEM in the recovery group was somewhat higher compared with the controls and somewhat lower than the group treated for 20 weeks, but the presence of changes observed in controls earlier in the experiment makes interpretation difficult (Table VI). BrdU labeling index in the recovery group was similar to the labeling index in the control group (Table VI). The labeling index in the high-dose group was significantly higher when compared with either the control or recovery group. The bladder epithelial changes secondary to exposure to DMA, including the histopathology and labeling index, appear to be reversible when followed by 10 weeks of control diet. The group fed DMA continuously for 20 weeks showed effects similar to those seen after 8 and 10 weeks of treatment.

Discussion

Based on the present experiment, dietary administration of DMA to rats causes cytotoxicity of the urinary bladder epithelium with a consequent regenerative hyperplasia. The effect is greater in female than in male rats. It appears to occur clearly at doses of 40 and 100 p.p.m. but not at lower doses. Urothelial changes were observed in the control groups which made the interpretation of the lower dose groups difficult. There did not appear to be much difference in the toxicity or regeneration when DMA was administered in Purina diet or in Altromin diet, suggesting that the diet does not have a significant role in the urothelial effects seen in this experiment, even with the significant differences in urinary pH and chemistries produced by the two diets.

Careful examination of the urine did not suggest any evidence of formation of precipitates, abnormal or increased microcrystalluria, or calculus formation in the urine of the rats fed DMA in the diet. It is unlikely that formation of solid materials in the urine is the cause of the cytotoxicity observed following the administration of DMA in the diet.

There was a dose-related increase in urinary volume, possibly secondary to increased water consumption. The increased urine volume was associated with decreased urinary osmolality and urinary creatinine concentrations. Most of the urinary constituents examined showed decreased concentrations, as expected for diluted urine secondary to increased urinary volume (11). However, there was a striking increase in the calcium concentration in the urine, particularly in the female rats fed DMA in Purina diet. When this was normalized to creatinine concentration, as an indicator of the extent of urinary dilution and increased volume, the results indicated that the absolute amount of calcium being excreted was markedly

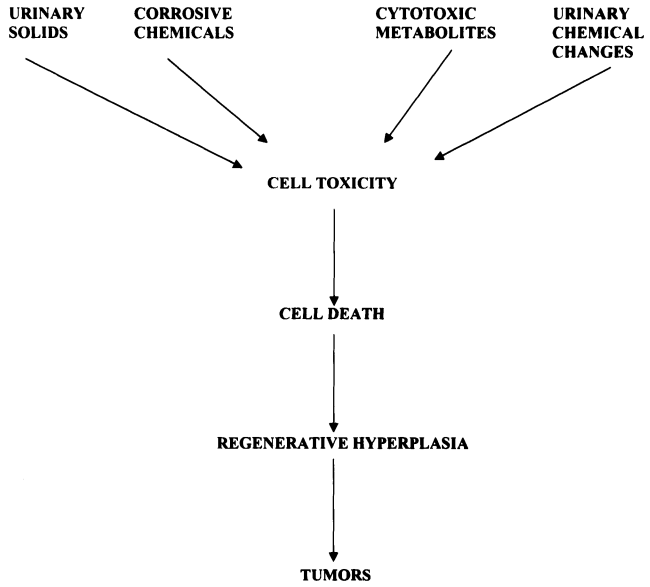

Fig. 8. Possible pathways leading to urothelial toxicity and consequent hyperplasia and tumors.

increased in the rats administered DMA in the diet. This occurred to a statistically significant extent in female rats fed DMA in Purina diet at doses of 40 and 100 p.p.m., but not at 2 or 10 p.p.m. compared with controls. The increased excretion of calcium in the urine was clearly reflected in the marked increased calcium deposition in the kidney of the rats administered DMA in Purina diet, particularly at the higher doses and particularly in females. The urinary concentration of calcium was not increased in the male rats, although the amount normalized to creatinine concentrations was increased.

The effects on the urinary bladder following administration of high doses of DMA in the diet appear to be reversible when the chemical was administered to rats for 10 weeks followed by 10 weeks of control diet.

Since no abnormal production of urinary solids occurred, the urothelial cytotoxicity following administration of DMA in the diet to rats is likely due to either an alteration in calcium metabolism, including marked changes in urinary calcium concentrations, or due to DMA itself or one of its metabolites (10,11). The fact that the urinary calcium levels and the extent of renal calcification were lower in males than in females, like urothelial cytotoxicity and tumor formation, supports the possibility that the changes in calcium metabolism induced by DMA have a role in its urothelial effects in rats. However, those changes in urinary calcium did not occur in the female rats fed DMA in Altromin diet, although these rats also showed similar urothelial cytotoxicity and regeneration.

Multiple studies of DMA have provided strong evidence that it does not react with DNA (4–8). Thus, a DNA-reactive mechanism for the effects of DMA on the rat bladder is highly unlikely. Rather, an increased proliferative effect is the likely mechanism leading to the eventual increased incidence of bladder tumors. The increased bladder urothelial proliferation was demonstrated in the present study to be due to regenerative hyperplasia following urothelial toxicity and necrosis.

Although the most studied mechanisms leading to urothelial toxicity and regeneration are related to the formation of urinary solids, such as calculi, microcrystals or amorphous precipitate, other mechanisms of toxicity have also been demonstrated (Figure 8) (10). For example, severe toxicity with necrosis of the full thickness of the urothelium leading to ulceration can be produced by direct contact with highly corrosive chemicals such as tributyl phosphate, acetic acid or formalin, with consequent extensive hyperplasia, including papillomatosis. This is similar to the toxicity and regeneration seen with urinary calculi. If the urothelial toxicity is more superficial, there is less regenerative hyperplasia and the incidence of bladder tumors is also lower, as seen with ortho-phenylphenol as well as with chemicals producing a calcium phosphate-containing urinary precipitate (e.g. sodium saccharin and sodium ascorbate). As with other forms of cytotoxicity and necrosis, all of these chemicals, acting through a variety of mechanisms, produce cell death followed by regeneration that eventually leads to formation of tumors, at relatively high doses with a highly non-linear dose–response relationship. A similar non-linear dose–response relationship is expected and observed for the bladder effects of DMA in the rat since it is non-DNA-reactive and produces cytotoxicity, necrosis and consequent regenerative hyperplasia.

There is no evidence that DMA is carcinogenic in humans (8,9). There actually is extensive evidence that the methylation of inorganic arsenicals in humans may represent a detoxification pathway (23); DMA is fully methylated. Also, because there are extensive differences in the metabolism of arsenicals in rats compared with other species, particularly humans, several investigators have suggested that the rat is an inappropriate animal model for examining the toxic or carcinogenic effects of arsenicals for humans (9,23). Such differences in metabolism need to be considered for risk assessment purposes.

The dose–response relationship that was seen in the present experiment is similar to that observed in the long-term bioassay in rats with neoplasia as the endpoint (1). In the 2 year bioassay, the female rat was more affected than the male rat, and tumors were only seen in increased incidences at the 40 and 100 p.p.m. level. These doses are considerably higher than any human exposures to DMA.

The dose–response results in the present experiment are also similar to those reported by Fukushima and colleagues in their studies on DMA in rats, primarily evaluating the tumorigenic effects when the chemical is administered after pretreatment with BBN (3). In his experiments, the DMA was administered in the drinking water, but the total daily consumption on a per kg basis was similar compared with that achieved when the chemical was administered in the diet. In a recent 2 year bioassay in male rats, Fukushima has observed that there is a significant increase in bladder tumors in male rats at doses of 50 and 200 p.p.m. in the drinking water but not at 12.5 p.p.m. (2). This is in keeping with the bioassay findings when DMA was administered in the diet for 2 years to F344 rats (1).

The present experiment provides strong support for a non-linear mode of action for rat bladder tumor formation following DMA treatment. The carcinogenic effect in the rat bladder resulting from long-term exposure is likely a consequence of the regenerative hyperplasia which is secondary to cytotoxicity to the bladder epithelium. The lack of DNA reactivity of DMA provides additional support for the non-linearity of the carcinogenic effects (9,24). This provides a mechanistic basis for a non-linear approach to the dose–response of DMA carcinogenesis in the rat, providing a basis for approaching a risk extrapolation to humans based on a margin of exposure as suggested in the revised EPA Guidelines for Cancer Risk Assessment of 1996.

In summary, our results demonstrate that DMA administered

in the diet produces urothelial cytotoxicity and regenerative hyperplasia in F344 rats, with changes observed at 40 and 100 p.p.m.. The cytotoxicity is unlikely to be due to the formation of particulate matter in the urine. Abnormalities in calcium metabolism and excretion were observed and might contribute to the process. The cytotoxicity and regeneration were greater in female than in male rats and they were reversible. The findings strongly support a non-DNA reactive, increased cell proliferation mechanism for the carcinogenicity of DMA in rats.

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