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Possible Role of Dimethylarsinous Acid in Dimethylarsinic Acid-Induced Urothelial Toxicity and Regeneration in the Rat

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Dimethylarsinic acid (DMA^V) is carcinogenic to the rat urinary bladder when administered at high doses in the diet or drinking water. At a dietary dose of 100 ppm (μg/g), it produces cytotoxicity within 6 h and increased proliferation (hyperplasia) by 7 days of administration. We hypothesize that formation of the reactive organic intermediate dimethylarsinous acid (DMA^{III}) is involved in the induction of the cytotoxicity. To evaluate the possibility that DMA^V administration produces urothelial toxicity and regeneration by the formation of trivalent arsenicals, 2,3-dimercaptopropane-1-sulfonic acid (DMPS, 5600 ppm), a chelator of trivalent arsenicals, was co-administered with DMA^V (100 ppm) for 2 weeks to groups of female Fischer F344 rats. Based on light and scanning electron microscopy, and bromodeoxyuridine labeling index, DMA^V produced cytotoxicity and regenerative hyperplasia of the urothelium which was inhibited by co-administration with DMPS. The major forms of arsenic in the 24-h urine of rats administered DMA^V were high concentrations of DMA^V (66.4 ± 2.7 μM) itself and the pentavalent organic arsenical trimethylarsine oxide (TMAO) (73.2 ± 9.5 μM). Co-administration with DMPS led to an increase in DMA^V (507 ± 31 μM) with a decrease in TMAO (2.8 ± 0.4 μM) excretion. The formation of TMAO from DMA^V mechanistically suggests formation of the intermediate trivalent metabolite, DMA^{III}. In a second experiment evaluating fresh void urines collected on study days 1, 71, and 175, we detected DMA^{III} in the urine of DMA^V and DMA^V plus DMPS-treated rats at approximately micromolar concentrations. Using rat (MYP3) and human (1T1) urothelial cells, cytotoxicity for trivalent arsenicals, sodium arsenite, monomethylarsonous acid (MMA^{III}), and DMA^{III} was demonstrated at 0.4−4.8 μM concentrations, whereas MMA^V, DMA^V, and TMAO were cytotoxic at millimolar concentrations. The presence of DMA^{III} at micromolar concentrations in the urine of rats fed 100 ppm DMA^V suggests that DMA^{III} produced in vivo may be involved in the toxic effects in the rat urinary bladder after dietary administration of DMA^V.

Introduction

Arsenic is a known human carcinogen with skin, lung, and bladder as target organs (*1*, *2*). There is some evidence suggesting that other tissues might also be involved. A major exposure for humans to arsenic occurs through the ingestion of drinking water contaminated with inorganic arsenic. The inorganic arsenic is then methylated to the organic pentavalent arsenicals, monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V), which are more easily execreted in the urine. The proposed pathway for the methylation of arsenic involves reduction of the pentavalent species to the trivalent species of arsenic and subsequent methylation of the trivalent species. The presence of the intermediate organic trivalent species monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) has only recently been confirmed in the urine of humans exposed to arsenic (*3*). The methylation pathway has long been considered to be a method of detoxification, but recent reports have shown that MMA^{III} and DMA^{III} are more cytotoxic in vitro than the inorganic arsenicals (*4*−*6*).

There has been little progress in understanding the mechanism of action of arsenic as a carcinogen, partly because of the lack of animal models. Recently, DMA^V was demonstrated to be a bladder carcinogen in rats when administered at high doses either in the diet (100 ppm) (*7*) or in the drinking water (50 and 200 ppm) (*8*). Relatively high doses were required to produce either hyperplasia or bladder tumors, and the effect appeared to be greater in female than in male rats. Also, DMA^V administered in the drinking water to male rats after a known genotoxic bladder carcinogen, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), enhanced the incidence of bladder tumors in a dose-dependent manner (*9*). In

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contrast, DMA^{V} was not carcinogenic toward the urinary bladder or other organs in mice when administered in the diet in a chronic bioassay (*7*), and no effect was produced in the urinary tract of hamsters fed DMA^{V} at 100 ppm of the diet for 10 weeks (*31*). Although the rat clears arsenic slower than humans and other laboratory rodents because of storage of dimethylated arsenic in red blood cells, the rat does methylate arsenic in the same manner as other species, thereby providing an animal model for the study of the effects of the various metabolites.

Most evidence suggests that DMA^{V}, like other arsenicals, does not bind directly to DNA, and it is therefore unlikely to be a DNA-reactive carcinogen (*2*, *10*−*13*). Although it has recently been reported that DMA^{III} reacts with DNA in vitro, this occurred only at concentrations of 150 μM or higher (*14*). These concentrations are much higher than the reported in vitro LC_{50} for DMA^{III} in various cell types and at least 10 times the highest reported LC_{50} for cultured human bladder cells (*4*, *5*). Other genetic effects have been demonstrated, such as clastogenicity and chromosomal aberrations, but these effects also occurred predominantly at high doses or could have been produced by cytotoxicity or other indirect mechanisms rather than by direct DNA reactivity (*2*, *10*−*13*).

Since it is unlikely that DMA^{V} is acting as a DNA-reactive carcinogen, increased cell proliferation is a plausible alternative mode of action (*11*, *12*, *15*). We demonstrated that DMA^{V} administered in the diet at the same doses as used in the 2-year bioassay (2, 10, 40, and 100 ppm) produced urothelial cytotoxicity and consequent regeneration at the two highest doses (*15*). Cytotoxicity was evident as early as 6 h following the beginning of oral administration of DMA^{V}, whereas increased urothelial proliferation occurred sometime between 3 and 7 days of administration (*16*). By 2 weeks of administration, there was evidence of significant superficial urothelial necrosis and regeneration. The necrosis involved the superficial cell layer and was best observed by scanning electron microscopy (SEM). Increased labeling index following a 1-h pulse of bromodeoxyuridine (BrdU) demonstrated increased proliferation along with light microscopic and SEM evidence of hyperplasia. We hypothesize that formation of the reactive intermediate DMA^{III} is involved in the induction of the urothelial cytotoxicity produced in rats administered DMA^{V}. This 2-week model provides a short-term surrogate marker system for evaluating DMA^{V}-induced urothelial effects without having to perform a full 2-year bioassay for each experiment.

2,3-Dimercaptopropane-1-sulfonic acid (DMPS), a vicinal dithiol, has been used in the treatment of arseniasis in humans (*17*, *18*). Studies have shown that dithiols are capable of reducing pentavalent arsenicals to the trivalent state and binding to the trivalent species (*19*). Current evidence indicates that DMPS competes with other endogenous binding agents to bind the trivalent organic species, especially to MMA^{III}, leading to its rapid excretion in the urine (*18*). We hypothesized that DMPS could bind to DMA^{III} and inhibit the urothelial effects of DMA^{V} in the rat. This possibility was evaluated using the 2-week model system.

To investigate the possibility that DMA^{III} is the reactive species of arsenic that produces the cytotoxic effects seen in DMA^{V}-treated rats, it was necessary to develop the qualitative and quantitative analytical techniques necessary to determine the urinary concentrations of DMA^{V} and its various metabolites following DMA^{V} administration using the 2-week model. These results were then compared to the levels identified following the administration of DMPS with DMA^{V}.

The second series of experiments involved an evaluation of the cytotoxic effects of the different arsenic species in rat and human bladder epithelial cell culture systems. The intent was to identify the concentrations at which metabolites of DMA^{V} are cytotoxic and to determine if those cytotoxic concentrations are attained in the urine of rats administered cytotoxic and carcinogenic doses of DMA^{V}.

These experiments demonstrated that DMPS inhibited the cytotoxic and proliferative effects of orally administered DMA^{V} in female rats and that trivalent arsenic, whether as arsenite, monomethylarsonous acid (MMA^{III}), or DMA^{III}, was cytotoxic in vitro at concentrations 3−4 orders of magnitude lower than seen with the pentavalent, methylated arsenicals, DMA^{V}, MMA^{V}, or trimethylarsine oxide (TMAO).

Materials and Methods

Animal Studies. (A) Chemicals and Diets. The DMA^{V} was provided by Luxembourg Industries (Pamol; Tel-Aviv, Israel). The purity of the test article was documented by Luxembourg Industries to be at least 99.5%, and these results were confirmed by NMR at our facility at the University of Nebraska Medical Center. The level of DMA^{V} fed in the diet was 100 ppm (μg/g) based on previous experiments showing urothelial cytotoxicity and regeneration after 2 weeks of treatment at this dose (*16*). DMPS was purchased from Heyltex Corp. (Houston, TX). The purity of the chemical was determined by the company to be 100% and was accepted without further testing. The level of DMPS fed in the diet was 5600 ppm based on results from a preliminary dose range finding study (unpublished observations) to determine the maximum tolerated dose in the rat. The dose was determined to be nontoxic based on a less than 5% decrease in the body weights of the treated animals compared to control animals and the lack of any clinical signs of distress in the animals during the treatment period. The diets were prepared and pelleted at Dyets, Inc. (Bethlehem, PA). Chemcials were mixed into the diet on a weight to weight basis, 0.1 g of DMA^{V} per 1000 g of diet and 5.6 g of DMPS per 1000 g of diet, prior to pelleting and were fed within 10 weeks of pelleting. The diets were stored at approximately −20 °C until fed to the animals, and the diets were provided fresh at least weekly.

(B) Test Animals and Experimental Design. Forty female F344 rats, 4 weeks old, were purchased from Charles River Breeding Laboratories (Raleigh, NC) for the first experiment, and 30 female F344 rats, 4 weeks old, were purchased from the same vendor for the second experiment. All rats were quarantined for at least 7 days. On arrival they were placed in a level-4 barrier facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1996). The animals were housed in polycarbonate cages (5/cage) on dry corncob bedding in a room with a targeted temperature of 22 °C, humidity of 50%, and a 12-h light/dark cycle (0600/1800). They were fed pelleted Certified Rodent Diet 5002 (PMI Nutrition International, Inc., St. Louis, MO). Food and water were available ad libitum throughout the study. Following quarantine, rats were randomized into the appropriate treatment groups using a weight stratification method (*20*). In the first experiment, rats were randomized into 4 groups of 10 rats each: group 1 was fed basal diet only, group 2 was fed a diet

containing 100 ppm DMA^V, group 3 was fed a diet containing 5600 ppm DMPS, and group 4 was fed a diet containing 100 ppm DMA^V + 5600 ppm DMPS. All animals were sacrificed after 2 weeks of treatment. In the second experiment, rats were randomized into 3 groups of 10 each: group 1 was fed basal diet, group 2 was fed 100 ppm DMA^V in the diet, and group 3 was fed 100 ppm DMA^V + 5600 ppm DMPS in the diet. All rats in each group were sacrificed after 26 weeks of treatment to determine the urothelial effects.

(C) Experimental Procedures. Water and food consumption were measured over a 7-day period starting on study day 0 (the first day of treatment) in Experiment 1 and at 6-week intervals starting at week 4 in Experiment 2. Body weights of all rats were measured the day after arrival, on study day 0, on the last day of the consumption period(s), before and after placement in metabolism cages, and on the day of sacrifice. Detailed clinical observations of each animal were conducted on day 0, at the end of the consumption period(s) and just prior to sacrifice.

In Experiment 1, the animals were acclimated to metabolism cages for 48 h before collection of two consecutive 24-h urines during study week 2. Food and water were available ad libitum while the rats were in the metabolism cages. The specimens were collected on ice and over nylon netting to decrease contamination of the urine with diet or feces. Total volume was determined, and calcium and creatinine were measured on the Vitros 250 Chemistry Analyzer (Johnson and Johnson Clinical Diagnostics, Rochester, NY). In Experiment 2, fresh void urines were collected from 10 rats in each group between 0700 and 0900, approximately 18 h after treatment began, and again on study days 71 and 175. All urines were immediately frozen in liquid nitrogen, and samples were analyzed for the arsenic metabolite DMA^{III} within 48 h of collection.

The speciation and quantitation of arsenical metabolites present in the urine were performed using ion pair chromatographic separation with hydride generation atomic fluorescence (HPLC−HGAFS) detection (*3*). Briefly, an aliquot of a sample was filtered through a 0.45-μm membrane prior to being subjected to HPLC−HGAFS analysis. A reversed-phase C18 column (ODS-3, 150 × 4.6 mm, 3-μm particle size, Phenomenex, Torrance, CA) with a mobile phase solution (pH 5.9) containing 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol was used for separation of arsenite (As^{III}), MMA^{III}, MMA^V, DMA^V, DMA^{III}, and arsenate (As^V). The flow rate of the mobile phase was 1.2 mL/min. For HGAFS detection, hydrochloric acid (1.2 M, 10 mL/min) and sodium borohydride (1.3%, 3 mL/min) were used for hydride generation. Arsines generated were carried by a continuous flow of argon (250 mL/min) to an atomic fluorescence detector for quantitation. TMAO coeluted with arsenite under these chromatographic conditions.

An anion exchange column (PRP ×100, 140 × 4.1 mm, Hamilton, Reno, NV) was used to separate TMAO from arsenite with a mobile phase containing 5 mM phosphate (pH 8.2) and 5% methanol. The flow rate of the mobile phase was 1.0 mL/min. The HGAFS detector was used for detection.

One hour prior to sacrifice, all rats were injected ip with BrdU, 100 mg/kg body weight. All rats were sacrificed by an overdose of Nembutal (50 mg/kg of body weight, i.p.) between 1000 and 1200 h to avoid diurnal variations in the labeling index (*21*). The urinary bladder and stomach were inflated in situ with Bouin's fixative, removed, and placed in the same fixative. The kidneys were removed from all animals in each experiment, weighed, and placed in formalin. Half of the bladder from each animal was processed for examination by SEM and classified as previously described (*22*). Briefly, class 1 bladders show flat, polygonal superficial urothelial cells; class 2 bladders show occasional small foci of superficial urothelial necrosis; class 3 bladders show numerous small foci of superficial urothelial necrosis; class 4 bladders show extensive superficial urothelial necrosis, especially in the dome of bladder; and class 5 bladders show necrosis and piling up of rounded urothelial cells. Normal bladders are usually class 1 or 2, but occasionally class 3.

The other half of the bladder was cut longitudinally into strips, and with a slice of stomach, was embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined histopathologically (*22*, *23*). The diagnosis of simple hyperplasia was made when the number of cell layers in the bladder epithelium was increased above 3. The kidneys were also embedded in paraffin, stained with H&E, and examined histopathologically.

Unstained slides of the bladder and stomach tissue were used for immunohistochemical detection of incorporation of bromodeoxyuridine (BrdU) into the urothelial cells. The stomach served as a positive control for determination of the BrdU labeling index. Anti-BrdU (Chemicon International, Temecula, CA) was used at a dilution of 1:50. The number of BrdU-labeled cells in at least 3000 urothelial cells was counted to determine a labeling index.

In Vitro Cytotoxicity Studies. (A) Cell Lines. Two urinary bladder cell lines were provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL). The MYP3 cell line was obtained from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with *N*-methyl-*N*-nitrosourea (MNU) (*24*). The cell line has retained the characteristics of epithelial cells in culture, expresses keratin 5 mRNA, does not exhibit anchorage-independent growth, and does not cause development of tumors in nude mice. The 1T1 cell line is a human epithelial cell line derived from benign ureter tissue obtained during a radical nephrectomy due to renal carcinoma in a 71-year-old male (*25*). The cells were immortalized by transfection with the human papillomavirus type 16 E6 and E7 genes. MYP3 cells were grown in Ham's F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10 μM nonessential amino acids, 10 ng/mL EGF, 10 μg/mL insulin, 5 μg/mL transferrin, 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Gibco) and 250 mg/mL dextrose and 1 mg/mL hydrocortisone (both from Sigma, St. Louis, MO). 1T1 cells were grown in keratinocyte serum-free medium supplemented with 50 μg/mL bovine pituitary extract, 5 ng/mL EGF, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). All cells were grown in an atmosphere of 95% air and 5% CO_2 at 37 °C.

(B) Chemicals. Sodium arsenite ($NaAs^{III}O_2$) and sodium arsenate ($Na_2HAs^VO_4 \cdot 7H_2O$) were purchased from Sigma. The purity determined by Sigma was 99.7% for both chemicals. The DMA^V used was from the same batch as used in the animal studies. MMA^V was also provided by Pamol, and purity was determined at Pamol to be 99.5%. MMA^{III}, DMA^{III}, and TMAO were synthesized by Dr. William Cullen (University of British Columbia, Vancouver, Canada). MMA^{III} and DMA^{III} were supplied as the diiodide and monoiodide, respectively. NMR analysis at the University of British Columbia confirmed the identity of the synthesized chemicals and that the purity of each chemical was at least 99%.

(C) Experimental Design. Cells were seeded at a concentration of (0.7−1.0) × 10^5 cells/25 cm^2 flask. Twenty-four hours later, the cells were treated with various concentrations of each arsenical, and treatment lasted for 7 days. Each concentration was tested in triplicate, and fresh chemical in the appropriate medium was applied every 3 days during the treatment period. Cell viability was determined by staining with 0.4% trypan blue and counting in a hemocytometer. The percent survivability was calculated as the ratio of cell number in the arsenical-treated cell culture to that in the control culture. The data were graphed with the known concentration of arsenical on the *x* axis and the percent survivability at that concentration on the *y* axis. The LC_{50} was calculated using linear regression analysis of the data in Microsoft Excel.

Statistics. Group means for continuous data, tissue weights, and the labeling indices were evaluated using analysis of variance followed by Duncan's multiple range test for group-wise comparisons. Histopathology was compared using the 2-tail, Fisher's exact test. SEM data were analyzed using 1-way nonparametric procedures followed by a chi square test. *P* values

Table 1. Body Weight, Food Consumption, and Water Consumption[a]

	body weight (g)		food consumption g/kg BW/day		water consumption g/kg BW/day	
	Experiment 1		Experiment 1	Experiment 2	Experiment 1	Experiment 2
treatment	Wk 1	Wk 2	Wk 1	Wk 4	Wk 1	Wk 4
control	94 ± 1	104 ± 2	116[b]	84 ± 1	198 ± 1	139 ± 2
100 ppm DMA^{V}	96 ± 1	106 ± 2	117 ± 3	90 ± 4	188 ± 3	239 ± 2[c]
5600 ppm DMPS	91 ± 1[c]	102 ± 2	116 ± 1	–	225 ± 6	–
100 ppm DMA^{V} + 5600 ppm DMPS	92 ± 1[d]	102 ± 2	107 ± 1	86 ± 1	223 ± 12[d]	159 ± 3[c,d]

[a] Values expressed as the mean ± SE. [b] Data used from only one cage. [c] Statistically significantly different from control, $p < 0.05$. [d] Statistically significantly different from 100 ppm DMA^{V} group, $p < 0.05$.

Table 2. Measurement of Urinary Volume and Quantitation of Creatinine and Arsenic Metabolites in the Urine of Female Rats Treated with DMA^{V}, DMPS, or DMA^{V} + DMPS Collected during Week 2 of Treatment (Experiment 1)[a]

treatment	volume (mL)	creatinine (mg/dL)	arsenite (μM)	arsenate (μM)	MMA^{V} (μM)	DMA^{V} (μM)	TMAO (μM)
control	9.1 ± 0.3	41.7 ± 1.5	0.04 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.2 ± 0.0	0.3 ± 0.1
100 ppm DMA^{V}	11.1 ± 0.4[b]	37.5 ± 1.2	ND[f]	ND	ND	66.4 ± 2.7[b]	73.2 ± 9.5[b]
5600 ppm DMPS	8.4 ± 0.6	46.3 ± 2.2	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.2 ± 0.0	0.1 ± 0.0
100 ppm DMA^{V} + 5600 ppm DMPS	7.6 ± 0.4[b,c]	49.0 ± 2.0[b,c]	0.11 ± 0.01[b,d]	DL[e]	5.35 ± 0.53[b,d]	506.8 ± 31.1[b,c,d]	2.8 ± 0.4[c]

[a] Values expressed as the mean ± SD. [b] Statistically significantly different from control group, $p < 0.05$. [c] Statistically significantly different from 100 ppm DMA^{V} group, $p < 0.05$. [d] Statistically significantly different from 5600 ppm DMPS group, $p < 0.05$. [e] DL: below detection limit. Detection limits: As^{III}, 0.007 μM; As^{V}, 0.014 μM; MMA^{V}, 0.007 μM; DMA^{V}, 0.014 μM; TMAO, 0.1 μM. [f] ND: not determined.

less than 0.05 were considered significant. All statistical analyses were performed using a SAS computer program (*26*).

Results

Animal Studies. Body weight gains were decreased in Experiment 1 in the groups fed DMPS alone or DMPS with DMA^{V} when compared to the control group and the 100 ppm DMA^{V} group, respectively (Table 1). However, the weight gain over the 2 weeks of the study was similar in all groups. Body weights were similar in all treatment groups throughout Experiment 2 (data not shown). There was a statistically significant decrease in food consumption, measured as g/rat/day, in the group co-administered 100 ppm DMA^{V} plus 5600 ppm DMPS compared to the control group or to the group fed 100 ppm DMA^{V} (Table 1). Food consumption in the group co-administered 100 ppm DMA^{V} and 5600 ppm DMPS in Experiment 1 was slightly decreased compared to control, but consumption in all other groups in both experiments was similar to the respective control group (Table 1). Water consumption in Experiment 1 was increased in the group fed 5600 ppm DMPS and the group fed 100 ppm DMA^{V} plus 5600 ppm DMPS (Table 1). The increase was statistically significant only when the group co-administered DMA^{V} and DMPS was compared to the group fed 100 ppm DMA^{V}. Water consumption in Experiment 2 was significantly increased in the groups fed 100 ppm DMA^{V} and 100 ppm DMA^{V} plus 5600 ppm DMPS compared to control (Table 1). The increase in water consumption was significantly less in the group co-administered DMA^{V} and DMPS compared to the DMA^{V}-treated group.

The volume of 24-h urine collected in Experiment 1 from the group fed 100 ppm DMA^{V} was significantly increased compared to control (Table 2). The volume of urine in the group co-administered 100 ppm DMA^{V} plus 5600 ppm DMPS was significantly decreased compared to the control group and to the group fed 100 ppm DMA^{V}. The creatinine concentration in the group fed 100 ppm DMA^{V} was slightly lower than the control. The creatinine concentration in the groups fed 5600 ppm DMPS and 100 ppm DMA^{V} plus 5600 ppm DMPS was increased compared to the control group, but the increase was statistically significant only in the group co-administered DMA^{V} and DMPS. The creatinine in the group co-administered 100 ppm DMA^{V} plus 5600 ppm DMPS was also statistically significantly increased compared to the creatinine concentration in the group fed 100 ppm DMA^{V}.

Identification and quantitation of the arsenicals present in the 24-h urine specimens showed that there were submicromolar amounts of arsenite, arsenate, MMA^{V}, DMA^{V}, and TMAO in the urines collected from the control animals and the animals treated with 5600 ppm DMPS (Table 2). Treatment with 100 ppm DMA^{V} led to very high levels of DMA^{V} and TMAO in the urine. There was an approximately 10-fold increase in the urinary concentration of DMA^{V} in the group fed 100 ppm DMA^{V} with 5600 ppm DMPS compared to the group treated with 100 ppm DMA^{V}, but this was accompanied by a greater than 10-fold decrease in the concentration of TMAO. The concentrations of arsenite and MMA^{V} were also increased in the urine of rats co-administered 5600 ppm DMPS with 100 ppm DMA^{V} compared to the control group and the group administered 5600 DMPS. MMA^{III} was detected (0.21 ± 0.03 μM) only in the urine of rats fed 100 ppm DMA^{V} with 5600 ppm DMPS. Two unidentified peaks were present in the chromatograms from the analysis of urines in the group fed 100 ppm DMA^{V} and in some of the urines in the group fed 100 ppm DMA^{V} with 5600 ppm DMPS. One of these unidentified peaks eluted between DMA^{V} and MMA^{V}, and the other between MMA^{V} and arsenate. It was confirmed that neither peak was free DMA^{III} or MMA^{III}. Their retention time did not correspond to those of known arsenic standards. Their concentrations were generally below 0.5 μM when quantified against the DMA^{V} standard.

DMA^{III} was not detected in any of the 24-h urine samples in any group in Experiment 1. These samples were analyzed for arsenic species 4 weeks after collection. It is possible that DMA^{III} was oxidized to DMA^{V} during sample storage because DMA^{III} is highly reactive and readily oxidized (*27*). To minimize the possibility that DMA^{III} was oxidized to DMA^{V} during the 24-h collection

Table 3. Concentration of DMA^{III} (μM) in Fresh Void Urine Samples Collected from Female Rats under Treatment for 1, 71, and 175 Days (Experiment 2)[a]

treatment	day 1	day 71	day 175
control	DL[d]	DL	DL
100 ppm DMA^V	1.38 ± 0.44[b]	5.05 ± 1.19[b]	0.80 ± 0.31[b]
100 ppm DMA^V + 5600 ppm DMPS	0.92 ± 0.25[b]	3.05 ± 0.21[b,c]	0.67 ± 0.07[b]

[a] Values expressed as the mean ± SE from triplicate analyses of each urine sample collected from 6−9 animals in each group. [b] Statistically significantly different from control group, $p < 0.05$. [c] Statistically significantly different from 100 ppm DMA^V group, $p < 0.05$. [d] DL: below detection limit of 0.026 μM for DMA^{III}.

period, a second experiment was performed in which fresh void urine was collected instead of 24-h urine. The urine samples were collected from the animals between 0700 and 0900, frozen immediately in liquid nitrogen, and analyzed within 48 h. This procedure of urine sample collection and analysis was repeated 3 times during day 1, day 71, and day 175 from rats fed control diet and the diet supplemented with DMA^V or DMA^V plus DMPS. DMA^{III} was present in all fresh void urines collected from animals treated with 100 ppm DMA^V and animals co-administered 100 ppm DMA^V with 5600 ppm DMPS (Table 3).

Bladder weights were increased in the group fed 100 ppm DMA^V compared to the control in both experiments, but the increase was statistically significant only in Experiment 2 (Tables 4 and 5). The bladder weights in the group co-administered 100 ppm DMA^V plus 5600 ppm DMPS were similar to control in Experiment 1 (Table 4) and slightly increased compared to the control in Experiment 2 (Table 5).

Examination of the bladder epithelium by light microscopy in Experiment 1 showed one rat with simple hyperplasia in the group fed 100 ppm DMA^V and two rats with simple hyperplasia in the group fed 5600 ppm DMPS (Table 4). Hyperplasia generally cannot be detected by light microscopy by 2 weeks of administration (*15*, *16*). The bladder epithelium was normal in all animals co-administered 100 ppm DMA^V plus 5600 ppm DMPS. Light microscopic examination of the bladder epithelium after 26 weeks of treatment detected hyperplasia in 4 of 9 rats fed DMA^V and in 1 of 10 rats co-administered 100 ppm DMA^V plus 5600 ppm DMPS (Table 5). Examination of the bladder epithelium by SEM showed a statistically significant increase in the number of class 5 bladders (Figures 1 and 2) in the group fed 100 ppm DMA^V in Experiment 1 (Table 4). There were 3 bladders classified as class 4 and 3 bladders classified as class 5 in the group fed 5600 ppm DMPS. One bladder in the group co-administered 100 ppm DMA^V plus 5600 ppm DMPS was classified as class 4, and one bladder was classified as class 5. The remaining 8 bladders in this group were classified as class 2 (Figure 3), which is considered normal. All bladders in all groups in Experiment 2 were classified as class 1 or 2. There was a statistically significant increase in the BrdU labeling index in the group fed 100 ppm DMA^V compared to the control and to the group fed 100 ppm DMA^V plus 5600 ppm DMPS in both experiments (Tables 4 and 5). The labeling index was similar in the rats fed DMPS alone, DMA^V plus DMPS, or control diet in Experiment 1 and in the rats fed DMA^V plus DMPS or control diet in Experiment 2.

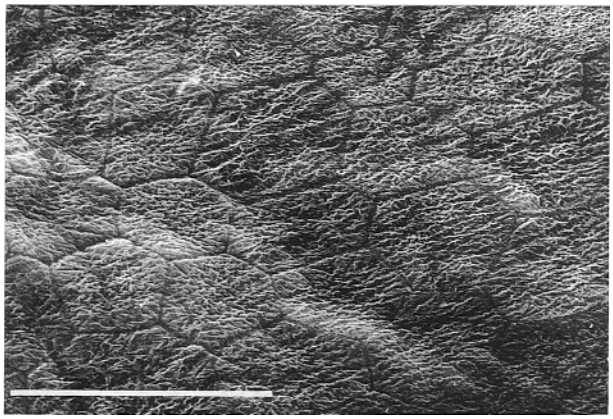

Figure 1. Normal bladder epithelium (class 1) showing large, flat, polygonal cells. Bar = 0.1 mm.

Table 4. Effects of DMA^V, DMPS, and DMA^V + DMPS on the Bladder in the Female Rat after 2 Weeks of Treatment (Experiment 1)

treatment	bladder weight (g/kg BW) (mean ± SE)	bladder histology		labeling index [mean ± SE (*n*)]	SEM classification				
		normal	simple hyperplasia		1	2	3	4	5
control	0.67 ± 0.03	10	0	0.16 ± 0.02 (9)	7	3	−	−	−
100 ppm DMA^V[a]	0.75 ± 0.04	9	1	0.63 ± 0.10 (10)[b]	−	2	2	−	6
5600 ppm DMPS[a]	0.57 ± 0.03	8	2	0.12 ± 0.02 (9)	−	2	2	3	3
100 ppm DMA^V + 5600 ppm DMPS[a,c,d]	0.63 ± 0.04[e]	10	0	0.09 ± 0.01 (8)[e]	−	8	−	1	1

[a] SEM classification statistically significantly different from control, $p < 0.05$. [b] Statistically significantly different from control, $p < 0.05$. [c] SEM classification statistically significantly different from 100 ppm DMA^V group, $p < 0.05$. [d] SEM classification statistically significantly different from 5600 ppm DMPS group, $p < 0.05$. [e] Statistically significantly different from 100 ppm DMA^V group, $p < 0.05$.

Table 5. Effects of DMA^V, DMPS, and DMA^V + DMPS on the Bladder in the Female Rat after 26 Weeks of Treatment (Experiment 2)

treatment	bladder weight (g/kg BW) (mean ± SE)	bladder histology		labeling index [mean ± SE (*n*)]	SEM classification				
		normal	simple hyperplasia		1	2	3	4	5
control	0.33 ± 0.02	10	0	0.13 ± 0.02 (10)	6	4	−	−	−
100 ppm DMA^V[a]	0.43 ± 0.03[b]	5	4[b]	0.21 ± 0.03 (9)[b]	3	6	−	−	−
100 ppm DMA^V + 5600 ppm DMPS[c]	0.39 ± 0.02	9	1	0.11 ± 0.01 (9)[d]	3	5	−	−	−

[a] One animal found dead on study day 23 due to unknown cause. [b] Statisically significantly different from control group, $p < 0.05$. [c] Unable to examine 2 bladders by SEM; 1 bladder collapsed during processing, 1 bladder inadvertently lost. [d] Statistically significantly different from 100 ppm DMA^V group, $p < 0.05$.

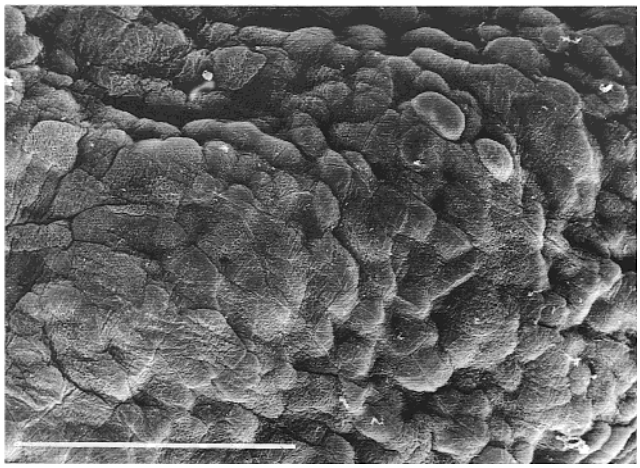

Figure 2. Bladder epithelium from a female rat treated with 100 ppm DMA^{V} for 2 weeks showing piling up of round cells indicative of hyperplasia (class 5). Bar = 0.1 mm.

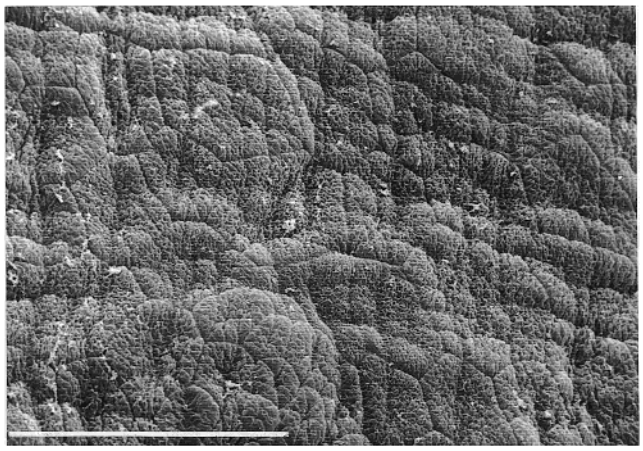

Figure 3. Bladder epithelium from a female rat treated with 100 ppm DMA^{V} + 5600 ppm DMPS for 2 weeks showing a normal appearance (class 2) of polygonal urothelial cells. Bar = 0.1 mm.

In Vitro Cytotoxicity Studies. The LC_{50} for DMA^{V}, MMA^{V}, and TMAO was determined to be in the millimolar range for the rat (MYP3) and the human cell lines (1T1) (Table 6). In contrast, the LC_{50} for MMA^{III} and DMA^{III} was found to be in the micromolar range. The LC_{50} for arsenate and arsenite was also in the micromolar range for both cell lines, but the LC_{50} for arsenite was greater than 10 times lower than that for arsenate for the rat cell line and more than 5 times lower for the human cell line. For the human cell line, the LC_{50} for MMA^{III} and DMA^{III} was at a concentration 4−6 times lower than that of arsenite, but the LC_{50} for all 3 trivalent arsenicals was similar for the rat cell line. The LC_{50} curves for all arsenicals in both cell lines were very steep.

Discussion

DMA^{V} is carcinogenic to the rat bladder when administered at high doses in either the diet (100 ppm) (*7*) or the drinking water (50 and 200 ppm) (*8*). Currently available data indicate that human exposure to naturally occurring DMA^{V} in the environment is in the micromolar range (*1*, *2*). Therefore, the doses used in the rat studies are 3−4 orders of magnitude higher than those to which humans usually are exposed. The high doses of DMA^{V} used in the rat studies rapidly induced urothelial cytotoxicity in the rat followed by regeneration a few days later (*15*, *16*). DMA^{V} is not DNA-reactive in various in vitro and in vivo assay systems (*1*, *2*, *10*−*13*). At the present time, the likely mode of action for DMA^{V} carcinogenesis in the rat bladder is induction of necrosis of the superficial layer of the urothelium followed by increased proliferation, leading eventually to the production of a relatively low incidence of bladder tumors in a 2-year bioassay (*7*, *8*, *15*, *16*).

The present experiments demonstrate that DMPS, an agent capable of chelating arsenic in the +3 oxidation state (*18*), inhibits the cytotoxic and proliferative effects of DMA^{V} in the female rat in a relatively short-term bioassay system that we developed to evaluate these effects. The majority of the bladders in the rats co-administered DMPS with DMA^{V} did not show the typical DMA^{V}-induced cytotoxic effects as examined either by light microscopy or by SEM. Administration of 5600 ppm DMPS alone caused some urothelial cytotoxicity as seen by SEM, and it may have contributed to the minor changes noted by SEM in 2 of the 10 rats administered 100 ppm DMA + 5600 ppm DMPS. However, the BrdU labeling index in the bladder epithelium was at control levels in the rats co-administered DMPS with the DMA^{V} in contrast to the significantly increased proliferation seen in the DMA^{V}-treated rats.

In the 2 cell lines evaluated, the organic arsenicals containing trivalent arsenic (MMA^{III} and DMA^{III}) were cytotoxic at concentrations at least 3 orders of magnitude lower than the corresponding pentavalent organic arsenic species (MMA^{V}, DMA^{V}, and TMAO) and at concentrations similar to or lower than arsenite. Thus, arsenite, arsenate, MMA^{III}, and DMA^{III} were cytotoxic in vitro at micromolar doses and, in contrast, MMA^{V}, DMA^{V}, and TMAO were cytotoxic at approximately millimolar concentrations. These findings for rat and human urothelial cells are similar to those observed for arsenic species in other cell systems (*4*, *6*, *14*). Importantly, they indicate what concentration levels are likely to be necessary in the urine for cytotoxicity of the urothelium to occur in the intact rat. The in vitro results, however, can only serve as an approximation of the in vivo effects because the cultured cells do not achieve terminal differentiation.

In control rats and in those treated with DMPS, extremely low concentrations of the various arsenic species were present, as expected. Following DMA^{V} administration as 100 ppm of the diet, the principal arsenic species in the urine were the pentavalent organic species, DMA^{V} and TMAO. Also present were two unknown arsenic-containing metabolites. Li et al. (*28*) previously reported the presence of two unknown metabolites detected in urine from rats administered DMA^{V} in the drinking water at comparable doses. It is clear that

Table 6. In Vitro Cytotoxicity of Various Arsenicals

	LC_{50}						
cell line	arsenite	arsenate	MMA^{III}	MMA^{V}	DMA^{III}	DMA^{V}	TMAO
MYP3 (rat)	0.4 μM	5.3 μM	0.8 μM	1.7 mM	0.5 μM	1.1 mM	4.5 mM
1T1 (human)	4.8 μM	31.3 μM	1.0 μM	1.7 mM	0.8 μM	0.5 mM	1.7 mM

these unknown arsenicals are not free MMA^{III}, DMA^{III}, or any of the other metabolites that we evaluated. Additional research is ongoing to identify these unknown arsenic-containing metabolites. Preconcentration, separation, and mass spectrometry techniques are necessary for this purpose.

It is apparent from the quantitative results in the DMA^{V}-treated rats that the principal forms of arsenic present in the urine are DMA^{V} and TMAO. Methylation of arsenic is a common metabolic process by which animals convert inorganic arsenite to MMA, MMA to DMA, and DMA to TMAO (*29*). It has long been presumed that for methylation to occur, the arsenic must first be reduced to the +3 oxidation state (*3*, *18*). This implied the existence of MMA^{III} and DMA^{III}, and recent reports have confirmed the presence of these two species of arsenic in human urine (*3*, *18*). Like inorganic arsenite, methylated trivalent arsenicals are highly reactive and cytotoxic.

The observation that TMAO was a primary metabolite of DMA^{V} in the urine from DMA^{V}-treated rats implied that it was formed through a DMA^{III} intermediate. In Experiment 1, we did not detect DMA^{III}, presumably because of possible oxidation of DMA^{III} to DMA^{V} during sample storage over 4 weeks. In Experiment 2, fresh void urine specimens were obtained, immediately frozen, and rapidly analyzed. Under such circumstances, we detected DMA^{III}, and it was present in the urine of all DMA^{V}-treated rats at concentrations of approximately 1 μM. Importantly, this concentration is greater than the LC_{50} for cytotoxicity for DMA^{III} for rat and human urothelial cells in vitro. The other trivalent arsenicals, arsenite and MMA^{III}, were present in the urine of DMA^{V}-fed rats at concentrations considerably lower than those necessary for cytotoxicity in vitro.

Mass et al. (*14*) recently observed damage to naked DNA secondary to exposure to MMA^{III} and DMA^{III}, but this occurred at concentrations of 30 mM MMA^{III} and 150 μM DMA^{III}, respectively, and higher in an in vitro assay. Arsenite was negative in this assay. Based on studies with arsenite and the chemistry of MMA^{III} and DMA^{III}, it is possible that this DNA damage occurs through an indirect process rather than due to direct chemical reactivity with DNA (*1*, *2*, *11*−*13*) and may be an artifact of the extremely high concentrations. Nevertheless, this potential genotoxic effect requires further evaluation in determining a mechanism for arsenic carcinogenesis.

Although the concentration of DMA^{V} in the urine following administration of 100 ppm DMA^{V} is elevated, possibly at concentrations that could be anticipated to be cytotoxic, DMA^{V} is not considered by us to be the cytotoxic, reactive metabolite in the urine. The reason for this is the observation that with DMPS co-administration, the DMA^{V} concentration in the urine actually increased by a factor of approximately 8 rather than decreased, in contrast to the biological effects; cytotoxicity and proliferation were inhibited by co-administration of DMPS with DMA^{V}. TMAO was present at quite high concentrations in the urine of DMA^{V}-fed rats, and the concentration was significantly reduced in the rats co-administered DMPS. However, the concentration of TMAO in either group of rats was considerably lower than the concentration that appears to be required for cytotoxicity, at least based on the in vitro findings. Our results on the decrease in TMAO concentration and the increase in DMA^{V} after administration of DMPS to the rats are consistent with previous findings from human administration of DMPS, except that no TMAO was detected in human urine (*18*). In humans administered DMPS, the urinary concentration of DMA^{V} was reduced and MMA^{V} was increased (*18*), suggesting that DMPS probably inhibits the methylation of arsenic.

Based on our experiments, we hypothesize that the critical reactive species in the rat urine that produces urothelial cytotoxicity and consequent regeneration following feeding of DMA^{V} is DMA^{III}. As DMPS interferes with the methylation of DMA^{V} to TMAO in rats, it may also decrease the formation of DMA^{III}, the intermediate metabolite between DMA^{V} and TMAO. However, our experiments examined the urine following administration of DMA^{V} at only one very high dose. Alternatively, one of the unknown metabolites could be acting as the cytotoxic form of arsenic in the urine, or TMAO might be contributing to the cytotoxicity.

The implications of this animal model with respect to human arsenic-induced carcinogenesis remain unclear. To begin with, the dose of DMA^{V} that was administered is several orders of magnitude greater than anticipated human exposures (*1*, *2*). Also, high concentrations of TMAO were found in the urine of DMA^{V}-treated rats, and although TMAO has been reported in the urine of a human administered high doses of DMA^{V}, it has not generally been identified as a metabolite in human urine following exposure to usual environmental levels of arsenic (*3*, *30*). There are also differences in the rate of clearance of arsenic between the rat and humans due to the storage of dimethylated arsenic in the rat red blood cells (*29*). Adminstration of DMA^{V} in the diet to the mouse (500 ppm) (*7*) and to the hamster (100 ppm) (*31*), which metabolize arsenicals in a manner more similar to humans than the rat, had no effect on the urinary bladder in either rodent species. Nevertheless, DMA^{III} has been identified in human urine (*3*, *32*, *33*).

In summary, DMA^{V} administered to female rats at 100 ppm of the diet produced cytotoxicity and regeneration of the urothelial lining of the bladder. When DMPS was co-administered with DMA^{V} in the diet for 2 weeks, the cytotoxic and regenerative effects were inhibited. The principal forms of arsenic present in the urine of rats administered DMA^{V} were DMA^{V} and TMAO. Following co-administration with DMPS, the concentration of DMA^{V} increased and TMAO decreased. DMA^{III} was identified at micromolar concentrations in the urine of rats in a separate experiment that were treated in the same manner. Urothelial cytotoxicity is produced in vitro by arsenicals in the +3 oxidative state at micromolar or lower concentrations, in contrast to millimolar concentrations required for organic pentavalent arsenicals. Where the DMA^{III} is produced remains unknown. Additional research will be required to delineate the details related to this hypothesis and to determine the relevance of this animal model to the human situation. Based on what is known so far, it is unlikely that DMA^{V} is a carcinogen for humans at levels of usual exposure, but this animal model might prove useful based on the reactivity of a trivalent-containing metabolite present at adequate concentrations to be cytotoxic. It is possible that trivalent arsenic in any form, whether arsenite, MMA^{III}, or DMA^{III}, has the potential to be cytotoxic at sufficiently high concentrations and therefore to produce a carcinogenic effect following long-term exposure. In this DMA^{V}-rat model, the reactive trivalent species appears to be DMA^{III}. In humans, because of differences in metabolism

and exposures, arsenite appears to be the critical relevant trivalent arsenical although MMA^{III} and DMA^{III} may also contribute (*10*, *11*).

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