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Examination of *in vivo* mutagenicity of sodium arsenite and dimethylarsinic acid in *gpt* delta rats

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ABSTRACT

Arsenic is a well-known human bladder and liver carcinogen, but its exact mechanism of carcinogenicity is not fully understood. Dimethylarsinic acid (DMA^V) is a major urinary metabolite of sodium arsenite (iAs^{III}) and induces urinary bladder cancers in rats. DMA^V and iAs^{III} are negative in *in vitro* mutagenicity tests. However, their *in vivo* mutagenicities have not been determined. The purpose of present study is to evaluate the *in vivo* mutagenicities of DMA^V and iAs^{III} in rat urinary bladder epithelium and liver using *gpt* delta F344 rats. Ten-week old male *gpt* delta F344 rats were randomized into 3 groups and administered 0, 92 mg/L DMA^V, or 87 mg/L iAs^{III} (each 50 mg/L As) for 13 weeks in the drinking water. In the mutation assay, point mutations are detected in the *gpt* gene by 6-thioguanine selection (*gpt* assay) and deletion mutations are identified in the red/gam genes by Spi^- selection (Spi^- assay). Results of the *gpt* and Spi^- assays showed that DMA^V and iAs^{III} had no effects on the mutant frequencies or mutation spectrum in urinary bladder epithelium or liver. These findings indicate that DMA^V and iAs^{III} are not mutagenic in urinary bladder epithelium or liver in rats.

Introduction

Arsenic compounds continue to pose environmental public health challenges for hundreds of millions of people worldwide. Epidemiological evidence indicates that inorganic arsenic is carcinogenic to the skin, lung, and bladder (Iarc, 2012; Saint-Jacques et al., 2014).

It is well known that the toxicity of arsenic varies according to its chemical form (Dodmane et al., 2013; Hughes et al., 2011). Inorganic arsenic compounds such as arsenate (iAs^V) and arsenite (iAs^{III}) are highly toxic (Cohen et al., 2006; Styblo et al., 2000). Trivalent arsenicals exhibit a strong affinity to sulfhydryl groups, which exist in the active sites of numerous enzymes, and inhibit the activity of these enzymes (Shen et al., 2013). Consequently, trivalent organic arsenic compounds such as monomethylarsonous acid and dimethylarsinous acid (DMA^{III}), which are metabolites of iAs^{III}, have strong cytotoxicity (Goering et al., 1999; Kligerman et al., 2003; Tokar et al., 2010). In contrast, the organic arsenic compound DMA^V, which is one of the major metabolites of iAs^V, has weak cytotoxicity. However, it is known that DMA^{III} is generated during the metabolism of DMA^V, suggesting that DMA^V may play a critical role in the carcinogenicity of arsenic.

Gestational exposure to iAs^{III} induces tumors in multiple organs, including the liver and lung, in mice (Tokar et al., 2011; Waalkes et al., 2003). In rats, DMA^V induces urinary bladder

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tumors (Arnold et al., 2006; Cohen et al., 2007; Wei et al., 2002) and promotes liver carcinogenesis (Wanibuchi et al., 2004). Cohen et al. (2007) demonstrated that the mode of action for DMA^V induced rat urinary bladder tumors involved cytotoxicity of the urinary bladder epithelium, followed by regenerative proliferation, hyperplasia and ultimately urothelial tumors. However, the mutagenicity of DMA^V and iAs^{III} remains unclear.

An *in vivo* mutagenicity study using Muta™Mice demonstrated that arsenite and its metabolite, DMA^V, were not mutagenic in whole bladder (Noda et al., 2002). In addition, Basu et al. (2001) has reported that arsenic is not mutagenic in either bacterial or mammalian gene mutation assays. However, in the Muta™Mice study, effects on the urinary bladder epithelium may have been masked by the mesenchymal tissue. Therefore, isolated urinary bladder epithelium needs to be examined. Moreover, DMA^{III}, a highly reactive trivalent arsenical derived from the reduction of DMA^V, may play an important role in DMA^V-induced urinary bladder carcinogenesis in rats (Cohen et al., 2007). Therefore, we examined the mutagenicity of DMA^V and iAs^{III} in rats so that the mutagenicity of these compounds and their metabolites, such as DMA^{III}, could be evaluated. The *gpt* delta rat is a transgenic animal with reporter genes inserted into its genome; consequently, the mutagenicity of compounds administered to *gpt* delta rats reflects the *in vivo* mutagenicity of the test chemical and the test chemical's metabolites and the organ specificity of any mutagenic activity (Hayashi et al., 2003; Kanki et al., 2005).

This study examined *in vivo* mutagenicity of DMA^V and iAs^{III} in rat urinary bladder epithelium and liver using *gpt* delta F344 rats.

1. Materials and methods

1.1. Chemicals and diets

Sodium arsenite (arsenite, iAs^{III}) (purity >90%) and DMA^V (purity >99%) were purchased from Sigma-Aldrich (Tokyo, Japan). MF basal diet was provided by Oriental Yeast Co. (Tokyo, Japan).

1.2. Animals

Five-week-old male *gpt* delta Fisher 344 rats were supplied by Nihon SLC (Shizuoka, Japan). Animals were housed in plastic cages (3 rats/cage) in experimental animal rooms with a target temperature of 22 ± 2°C, a relative humidity of 50% ± 5%, and a 12-hr light/dark cycle. Diet and water were available *ad libitum* during the study. Body weights, food consumption, and water intake were measured weekly during the experimental period. The animals were acclimatized for 5 weeks prior to the beginning of the experiment. The experiment was conducted following approval of the Animal Care and Use Committee of the Osaka City University Graduate School of Medicine.

1.3. Experimental design

A total of 36 ten-week-old *gpt* delta F344 rats were randomized into 3 groups (12 rats in each group) and administered 0, 92 mg/L DMA^V, or 87 mg/L iAs^{III} (each 50 mg/L As) for 13 weeks in their drinking water. At the end of the experiment, all animals were sacrificed by exsanguination *via* transection of the abdominal aorta under deep anesthesia (50 mg/kg sodium pentobarbital intraperitoneally) and their bladder and liver were excised.

1.4. In vivo mutagenicity analysis

1.4.1. Extraction of high-molecular-weight genomic DNA from urinary bladder epithelium and liver, and in vitro packaging of lamda phage DNA

Methods used to collect rat urinary bladder epithelium have been described previously (Wei et al., 2005). Briefly, urinary bladders were excised quickly and inverted on wooden applicator sticks. After rinsing with cold DNase free PBS buffer, bladder epithelial cells were removed by swirling the inverted bladders vigorously in microcentrifuge tubes containing 50 μL cell lysis buffer (8.20 g NaCl, 0.22 g KCl, 120 g sucrose, 0.30 g EDTA, 10 mL Triton X-100, and 1.58 g Tris–HCl (pH 8.3) in 1 L dH_2O). Exfoliated urinary bladder epithelium from two bladders was collected into one tube containing 500 μL cell lysis buffer (total of 600 μL cell lysis buffer per tube). The sample was homogenized by pipetting, and the solution was centrifuged at 13,200 r/min at 4°C for 12 min. The supernatant was discarded and the nuclei were resuspended in 70 μL of digestion butter (1.75 g Na_2HPO_4, 8.0 g NaCl, 0.2 g KCl, and 20 mL 0.5 mol/L EDTA (pH 8.0) in 1 L dH_2O, pH adjusted to 8.0 with NaOH) supplemented with 2% RNaseIt cocktail and 70 μL of proteinase K solution (100 mg proteinase K in 30 mL d dH_2O supplemented with 10 mL 10% (W/V) SDS and 10 mL 0.5 mol/L EDTA (pH 7.5)). The tubes were incubated at 50°C in a water bath for 90 min with occasional and gentle mixing of the solution. After digestion, using a wide bore pipette tip, the solution was gently pipetted into a dialysis cup, which was floated on TE buffer in a beaker with a capacity of 500 mL. The buffer was slowly stirred on a magnetic stirrer overnight to obtain high-molecular-weight genomic DNA. Lamda EG10 phages were rescued from the DNA using Transpack Packaging Extract (Stratagene, La Jolla, CA).

High-molecular-weight genomic DNA was extracted from the liver using the RecoverEase DNA Isolation kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). Lamda EG10 DNA in the genomic DNA was rescued as the lamda phage by Transpack packaging extract (Stratagene).

1.4.2. gpt mutation assay

The assay was conducted according to previously published methods (Nohmi et al., 2000). All the confirmed *gpt* mutants recovered from the urinary bladder epithelium and liver were sequenced; identical mutations from the same rat were counted as one mutation. The mutation frequency of the *gpt* gene in the urinary bladder epithelium and liver were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of rescued plasmids. DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Inc., Carlsbad, CA, USA) on an Applied Biosystems PRISM 3100 Genetic Analyzer. A:T to T:A transversions in the 299th bp of the *gpt* gene is a spontaneous mutation in *gpt* delta

Table 1 – Final body weight, water intake, food consumption, and liver weight.

Treatment	Number of animals	Final body weight (g)	Average water intake (g/rat/day)	Average food consumption (g/rat/day)	Liver weight	
					Absolute (g)	Relative (%)
Control	12	360.3 ± 23.9	18.4 ± 0.7	13.9 ± 0.5	11.1 ± 1.0	3.08 ± 0.08%
92 mg/L DMA^V	12	366.9 ± 22.0	22.4 ± 2.5	14.2 ± 0.7	11.3 ± 0.8	3.07 ± 0.08%
87 mg/L iAs^{III}	12	324.7 ± 24.9 *	13.0 ± 1.9	12.7 ± 0.8	9.9 ± 1.1 *	3.06 ± 0.15%

* Significantly different from the control group ($p < 0.05$).

F344 rat regardless of the experimental treatment (Masumura and Nohmi, 2009). Therefore, A:T to T:A transversions in the 299th bp of the *gpt* gene were excluded from the mutation frequency and mutation spectra.

1.4.3. *Spi⁻ assay*

The assay was conducted according to previously published methods (Nohmi et al., 2000). Packaged phages were incubated with *Escherichia coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lamda-trypticase agar and poured onto lamda-trypticase agar plates. The next day, plaques (Spi^- candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. The Spi^- phenotype was confirmed by spotting the suspensions on three types of plates in which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with lamda-trypticase soft agar. True Spi^- mutants, which made clear plaques on all of the plates, were counted.

1.5. *Statistical analysis*

All mean values were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the Statlight program (Yukms Co., Ltd., Tokyo, Japan). Homogeneity of variance was tested by the F test between the treatment and control groups. Differences in mean values between the treatment and control groups were evaluated by the two-tailed Student's *t*-test when variance was homogeneous and the two-tailed Aspin–Welch *t*-test when variance was heterogeneous. *p* values less than 0.05 were considered significant.

2. Results

2.1. *General observations*

The body and liver weights of the rats and water intake and food consumption are shown in Table 1. Compared with the control group, the final body weights and absolute liver weights were significantly decreased in the iAs^{III} treatment group. No significant difference in final body weights, absolute liver weights, or relative liver weights were observed in the DMA^V treatment group compared with the control group.

In the DMA^V treatment group, water intake was significantly increased from week 2 compared to the control group. There was no significant difference in food consumption between these two groups (Fig. 1). In contrast, water intake and food consumption was significantly decreased in the iAs^{III}

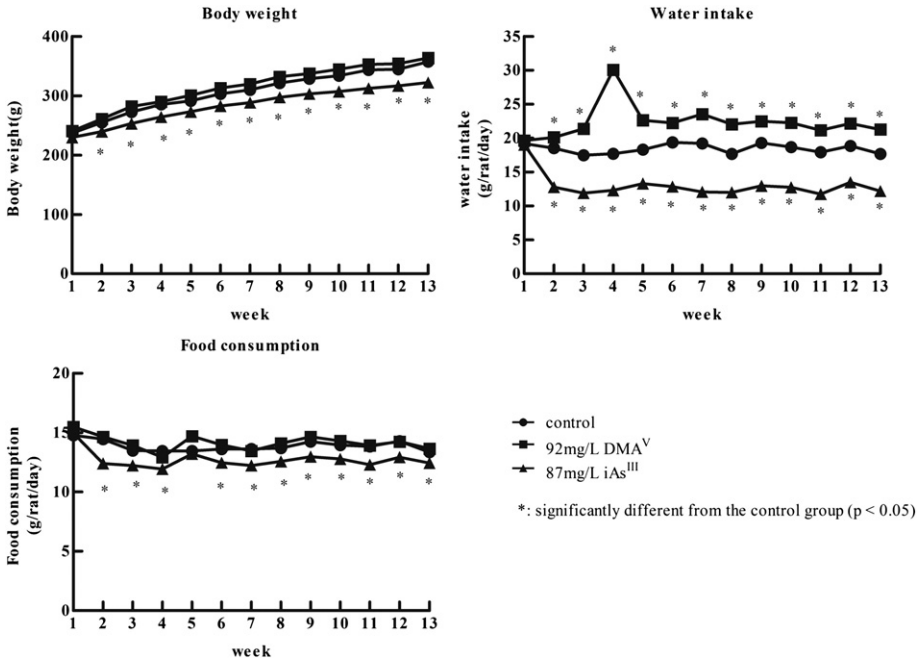

Fig. 1 – Body weight, water intake, and food consumption.

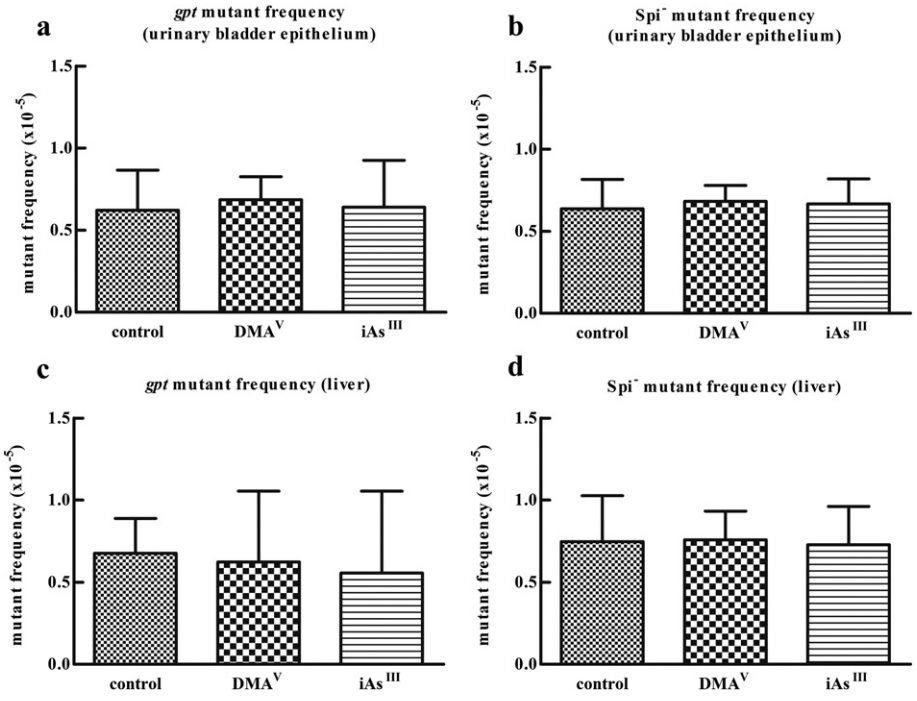

Fig. 2 – Results of *in vivo* mutagenicity analysis. (a) *gpt* mutation frequency in the *gpt* delta rat bladder epithelium. (b) Spi$^-$ mutant frequency in the *gpt* delta rat bladder epithelium. (c) *gpt* mutation frequency in the *gpt* delta rat liver. (d) Spi$^-$ mutant frequency in the *gpt* delta rat liver.

treatment group from week 2 compared to the control group (Fig. 1).

2.2. *In vivo mutagenicity assay*

The *gpt* and Spi$^-$ mutation frequencies in urinary bladder epithelium are shown in Fig. 2. In the urinary bladder epithelium, there were no differences in the *gpt* mutation frequencies in the iAs^{III} or DMA^V-treated rats compared with the controls (Fig. 2a). DMA^V and iAs^{III} had no effect on the spectrum of the *gpt* mutations in the urinary bladder epithelium (Table 2).

The frequencies of *gpt* mutations in the livers of rats treated with iAs^{III} or DMA^V were also similar to the controls (Fig. 2c). Similar to the findings in the urinary bladder epithelium, DMA^V and iAs^{III} had no effects on the spectrum of the *gpt* mutations in the liver (Table 3).

There were no significant differences in the frequency of Spi$^-$ mutations in the urinary bladder epithelium (Fig. 2b) or liver (Fig. 2d) in rats treated with DMA^V and iAs^{III} compared to the controls.

3. Discussion and conclusion

This is the first report evaluating the *in vivo* mutagenicity of DMA^V and iAs^{III} in the urinary bladder epithelium. The results of the present study demonstrate that DMA^V and iAs^{III} did not exhibit *in vivo* mutagenicity in rat urinary bladder epithelium or liver.

A number of studies have reported that arsenic is mutagenic. Inorganic arsenic induces structural and numerical chromosome changes, cell transformation, and gene amplification in mammalian cells *in vitro* (Basu et al., 2001). Furthermore, some metabolites of arsenic, such as DMA^V and DMA^{III}, have also been observed to cause numerical

Table 2 – Mutation spectrum of the *gpt* transgene in the urinary bladder epithelium of male *gpt* delta F344 rats.

Type of mutation		Control	DMA^V	iAs^{III}
Transition	G:C to A:T	4 (23.5%) [a]	2 (11.8%)	2 (13.3%)
		0.14 ± 0.20 [b]	0.07 ± 0.12	0.10 ± 0.21
	A:T to G:C	2 (11.8%)	3 (17.6%)	3 (20.0%)
		0.06 ± 0.09	0.11 ± 0.18	0.07 ± 0.11
Transversion	G:C to T:A	5 (29.4%)	7 (41.2%)	5 (33.3%)
		0.18 ± 0.15	0.26 ± 0.14	0.26 ± 0.19
	G:C to C:G	1 (5.9%)	2 (11.8%)	2 (13.3%)
		0.03 ± 0.07	0.09 ± 0.17	0.03 ± 0.08
	A:T to T:A	2 (11.8%)	1 (5.9%)	1 (6.7%)
		0.06 ± 0.10	0.03 ± 0.08	0.02 ± 0.04
	A:T to C:G	1 (5.9%)	1 (5.9%)	0 (0%)
		0.03 ± 0.07	0.07 ± 0.17	0
Deletion	Single bp	1 (5.9%)	1 (5.9%)	1 (6.7%)
		0.03 ± 0.07	0.02±0.05	0.01±0.03
	Over 2 bp	1 (5.9%)	0 (0%)	1 (6.7%)
		0.05 ± 0.13	0	0.01 ± 0.03
Insertion		0 (0%)	0 (0%)	0 (0%)
		0	0	0
Total		17 (100%)	17 (100%)	15 (100%)
		0.57 ± 0.19	0.66 ± 0.41	0.51 ± 0.30

[a] Number of mutations (%).
[b] Mutation frequency (×10^{-5}).

Table 3 – Mutation spectrum of the *gpt* transgene in the liver of male *gpt* delta F344 rats.

Type of mutation		Control	DMA^V	iAs^{III}
Transition	G:C to A:T	3 (20.0%)[a]	0 (0%)	0 (0%)
		0.14 ± 0.16[b]	0	0
	A:T to G:C	3 (20.0%)	2 (16.7%)	2 (22.2%)
		0.11 ± 0.12	0.09 ± 0.16	0.19 ± 0.38
Transversion	G:C to T:A	5 (33.3%)	6 (50.0%)	3 (33.3%)
		0.25 ± 0.16	0.32 ± 0.09	0.18 ± 0.20
	G:C to C:G	2 (13.3%)	0 (0%)	1 (11.1%)
		0.07 ± 0.11	0	0.05 ± 0.13
	A:T to T:A	0 (0%)	0 (0%)	1 (11.1%)
		0	0	0.03 ± 0.08
	A:T to C:G	1 (6.7%)	0 (0%)	0 (0%)
		0.03 ± 0.08	0	0
Deletion	Single bp	0 (0%)	3 (25.0%)	1 (11.1%)
		0	0.14 ± 0.17	0.05 ± 0.13
	Over 2 bp	1 (6.7%)	0 (0%)	1 (11.1%)
		0.08 ± 0.19	0	0.07 ± 0.17
Insertion		0 (0%)	1 (8.3%)	0 (0%)
		0	0.06 ± 0.15	0
Total		15 (100%)	12 (100%)	9 (100%)
		0.68 ± 0.21	0.62 ± 0.43	0.58 ± 0.29

[a] Number of mutations (%).
[b] Mutation frequency ($\times 10^{-5}$).

chromosome changes in mammalian cells *in vitro* (Eguchi et al., 1997; Ochi et al., 2003). In addition, *in vivo* cytogenetic assays in mice demonstrated an aneuploidogenic effect of different arsenicals (Basu et al., 2001). Finally, in humans, cytogenetic assays of arsenic exposed human populations report micronucleus formation, chromosomal aberrations, and sister chromatid exchanges (Basu et al., 2001). However, the *in vitro* studies obtained positive mutagenic effects only at high cytotoxic concentrations of the tested arsenicals; consequently, these results cannot be interpreted as demonstrating *in vivo* mutagenic capability. The *in vivo* studies also obtained positive mutagenic results only at high cytotoxic doses of the tested arsenical, and cytotoxic doses are well known to lead to false positive mutagenic results in *in vivo* studies (Cohen et al., 2016). Finally, reports of genotoxicity in arsenic exposed human populations have been called into question (Cohen et al., 2016). Our results in rat indicate that sodium arsenite and dimethylarsinic acid are not mutagenic *in vivo*.

Spi^- selection is useful for the efficient detection of intrachromosomal deletion mutations *in vivo*: The size of deletions detectable by Spi^- selection is up to 10 kb, consequently, Spi^- selection detects intrachromasomal deletions but not interchromosomal translocations or intrachromosomal megabase deletions. In this study, no long DNA fragment deletion was detected in the bladder or livers of rats treated with DMA^V or iAs^{III}; however, other approaches, including FISH analysis and microarray-based comparative genomic hybridization, might be required to characterize the entire spectrum of chromosome rearrangements caused by exposure to DMA^V and iAs^{III}. Therefore, further study is necessary to examine the *in vivo* capability of arsenic to induce chromosomal aberrations.

Several nongenotoxic modes of actions for arsenic carcinogenicity have been suggested, including cytotoxicity followed by regenerative proliferation (Cohen, 2010), oxidative stress (Kakehashi et al., 2013; Kinoshita et al., 2007a, 2007b; Kitchin and Conolly, 2010; Wei et al., 2013), activation of the nuclear receptors (Umannova et al., 2007), epigenetic abnormalities such as aberrant methylation of DNA (Reichard and Puga, 2010), and histone modifications (Ren et al., 2011; Suzuki and Nohara, 2013). Our data showing lack of *in vivo* mutagenicity of DMA^V and iAs^{III} provide indirect evidence for the importance of the above mentioned nongenotoxic mechanisms in arsenic carcinogenicity.

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