Effects of Co-administration of Antioxidants and Arsenicals on the Rat Urinary Bladder Epithelium

Min Wei,*† Lora Arnold,* Martin Cano,* and Samuel M. Cohen*†

*Department of Pathology and Microbiology and Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska 68198-3135; and †Department of Pathology, Osaka City University Medical School, Osaka 545-8585, Japan

Received October 6, 2004; accepted November 5, 2004

Oxidative stress has been increasingly recognized as a possible mechanism in the toxicity and carcinogenicity of various chemicals, including arsenic. Therefore, treatment with antioxidants may afford a protective effect against arsenic-induced cytotoxicity and carcinogenesis. Dimethylarsinic acid (DMA\textsuperscript{V}) has been shown to be a bladder carcinogen in rats when administered at high doses (100 ppm) in the diet or in the drinking water. The main purpose of the present study was to evaluate the effects of co-administration of antioxidants with arsenicals on the rat urinary bladder epithelium in vitro and in vivo. In a previous experiment, treatment with 1000 ppm melatonin for two weeks did not inhibit cell proliferation induced in the rat urothelium by 100 ppm DMA\textsuperscript{V}. In the current study, we examined the effects of five antioxidants that act via different mechanisms, on the in vitro cytotoxicity of various arsenicals, for the purpose of determining which antioxidants might have protective effects against arsenic-induced cytotoxicity. The antioxidants that inhibited cytotoxicity in vitro were then studied also in vivo. Melatonin showed slight inhibition of the cytotoxicity of arsenite, but had no effect on the other arsenicals. N-acetylcysteine (NAC) inhibited the cytotoxicity of monomethylarsenous acid (MMA\textsuperscript{V}), DMA\textsuperscript{V}, dimethylarsinous acid (DMA\textsuperscript{III}), and trimethylarsine oxide (TMAO). Vitamin C inhibited cytotoxicity induced by arsenate, arsenite, MMA\textsuperscript{III}, and DMA\textsuperscript{III}. Tiron and Trolox had no effect on the cytotoxicity of any arsenical. The in vitro inhibitory effects of NAC and vitamin C on DMA\textsuperscript{V} and on DMA\textsuperscript{III}, suggested that these antioxidants might afford preventive effects on DMA\textsuperscript{V}-induced bladder cytotoxicity and carcinogenesis in rats. To test this hypothesis, a 10-week rat bioassay was conducted. Melatonin was also included to clarify the results of the previous two-week experiment. The sodium salt of vitamin C (Na-Asc), but not melatonin or NAC, inhibited the proliferative effects of DMA\textsuperscript{V} on the bladder epithelium in rats. These results suggest that oxidative stress is at least in part involved in DMA\textsuperscript{V}-induced rat bladder toxicity and proliferation, and therefore, vitamin C may afford inhibitory effects in DMA\textsuperscript{V}-induced bladder carcinogenesis in rats. Microarray analysis of DMA\textsuperscript{V}-responsive genes revealed that DMA\textsuperscript{V} did not have a consistent modifying effect on gene expression in the rat bladder epithelium, suggesting that proteins and/or lipids may be the targets of damage by DMA\textsuperscript{V}-induced oxidative stress.

Key Words: dimethylarsinic acid; arsenicals; antioxidants; carcinogenesis; rat urinary bladder epithelium.

Inorganic arsenic (arsenate and arsenite) is a known human carcinogen of the skin, lung, and urinary bladder (Environmental Protection Agency, 2001; National Research Council, 1999). The metabolism of inorganic arsenic compounds plays an important role in their toxic and carcinogenic effects. It has been proposed that inorganic arsenic is metabolized in humans in a stepwise manner through alternating steps of reduction of the pentavalent arsenicals to the trivalent arsenicals and oxidative methylation of the trivalent arsenicals to pentavalent metabolites (Cullen et al., 1984), as follows: $\text{As}^{\scriptscriptstyle V} \rightarrow \text{As}^{\scriptscriptstyle III} \rightarrow \text{monomethylarsonic acid (MMA}^{\scriptscriptstyle V} \rightarrow \text{monomethylarsonic acid (MMA}^{\scriptscriptstyle III} \rightarrow \text{dimethylarsonic acid (DMA}^{\scriptscriptstyle V} \rightarrow \text{dimethylarsinous acid (DMA}^{\scriptscriptstyle III} \rightarrow \text{trimethylarsine oxide (TMAO).}$

DMA\textsuperscript{V} has been shown to be a bladder carcinogen in rats when administered in the diet (van Gemert and Eldan, 1998) or in the drinking water (Wei et al., 1999). Our previous studies demonstrated that the mechanism of DMA\textsuperscript{V}-induced rat bladder carcinogenesis involves induction of necrosis and sustained increased cell proliferation in the bladder epithelium (Arnold et al., 1999; Cohen et al., 2001). In those studies, DMA\textsuperscript{V} caused necrosis in the rat bladder epithelium as early as 6 h after the start of treatment and regeneration followed necrosis with a significant increase in cell proliferative activity within 3–7 days of treatment (Cohen et al., 2001). These findings suggest that DMA\textsuperscript{V}-induced urinary bladder carcinogenesis is a multistage process. We also demonstrated that urine of DMA\textsuperscript{V}-treated rats contains DMA\textsuperscript{III} at levels that cause cytotoxicity in vitro, which suggests that DMA\textsuperscript{III} contributes to the cytotoxicity, possibly through generation of oxidative stress (Cohen et al., 2002a).

Oxidative stress has been proposed as a possible mechanism for arsenic-induced cytotoxicity and carcinogenesis (Hughes, 2002; Kitchin and Ahmad, 2003). Several in vivo studies have provided evidence of induction of oxidative DNA damage by arsenicals. Matsui et al. (1999) measured 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the most commonly measured biomarkers of oxidative stress, in human skin neoplasms
and reported that the frequency of 8-OHdG positive cases was significantly higher in human skin neoplasms associated with arsenic exposure than in cases that were not related to arsenic exposure. Also, 8-OHdG has been reported to be elevated in the liver (Wanibuchi et al., 1997), kidneys (Vijayaraghavan et al., 2001), and bladder (Wei et al., 2002) of rats treated with DMA\textsuperscript{V} in the drinking water, although tumors associated with exposure to DMA\textsuperscript{V} were found only in the rat bladder. In vitro, reactive oxygen species (ROS) have been detected in human-hamster hybrid cells after exposure to arsenite (Liu et al., 2001). There is also in vitro evidence that DMA\textsuperscript{III} may indirectly increase the level of ROS by release of cellular iron that can then lead to the production of ROS (Ahmad et al., 2002). It is reasonable to hypothesize that antioxidants may afford protection against arsenic-induced carcinogenesis. However, the in vivo effect of antioxidants on arsenic-induced carcinogenesis has not been investigated.

Investigation of the effects of antioxidants would also facilitate our understanding of the mechanism of the carcinogenic effects of arsenic compounds, because various antioxidants react via different mechanisms. For example, melatonin is a general antioxidant and an effective free radical scavenger that does not undergo redox cycling and therefore acts as a terminal antioxidant (Tan et al., 2003). N-acetylcysteine (NAC) has been demonstrated to reduce free radical species thereby protecting cells against oxidative damage (De Flora et al., 2001). NAC is also the precursor of intracellular cysteine and reduced glutathione. Additionally, it has a sulfhydryl group, which can bind trivalent arsenic. Tiron is a cell membrane permeable scavenger of superoxide anion (Krishna et al., 1992). Trolox is a vitamin E analogue, which is also a scavenger of oxygen radicals (Walker et al., 1998). Vitamin C is a general antioxidant and an effective scavenger of free radicals (Buettner and Jurkiewicz, 1996; Chou and Khan, 1983). Vitamin C can also regenerate other small-molecular antioxidants such as vitamin E (Chan, 1993).

Recently, Nesnow et al. (2002) reported the in vitro protective effects of the antioxidants melatonin, Tiron, and Trolox against DNA damage induced by MMA\textsuperscript{III} and DMA\textsuperscript{III} in a DNA nicking assay. However, in that study, the concentrations of MMA\textsuperscript{III} and DMA\textsuperscript{III} causing the oxidative damage were much higher than the concentrations causing in vitro or in vivo cytotoxicity.

The first objective of the present study was to evaluate the effects of co-administration of antioxidants with arsenicals on the rat urinary bladder epithelium. Antioxidants used were melatonin, N-acetylcysteine (NAC), Tiron, Trolox, and vitamin C. To determine which antioxidants might inhibit the cytotoxic and proliferative effects induced by DMA\textsuperscript{V} administration in female F344 rats, we evaluated the effects of antioxidants on the cytotoxicity of various arsenicals in MYP3 rat bladder epithelial cells in vitro. The antioxidants that inhibited cytotoxicity in vitro were then examined in vivo in rats treated with DMA\textsuperscript{V}.

Studies with DMA\textsuperscript{V}-induced rat urinary bladder tumors have shown diverse genetic alterations such as downregulation of p27, and overexpression of cyclin D1 and cyclooxygenase-2 (Wei et al., 2002). It is not clear whether the changes in gene expression levels are early or late events in tumor development induced by DMA\textsuperscript{V}. To determine whether gene expression may be related to cytotoxic changes and the malignant transformation of urinary epithelial cells, we used microarray analysis to identify alterations in gene expression in the urothelium of rats treated with DMA\textsuperscript{V}.

**MATERIALS AND METHODS**

**Chemicals.** Sodium arsenite (purity, 99.7%) and sodium arsenate (purity, 99.7%) were purchased from Sigma (St. Louis, MO). MMA\textsuperscript{V} (purity, 99.5%) and DMA\textsuperscript{V} (purity, 99.5%) were provided by Luxembourg Industries Ltd. (Tel-Aviv, Israel). MMA\textsuperscript{III} (supplied as diiodide), DMA\textsuperscript{III} (supplied as monoiiodide), and TMAO were synthesized by Dr. William Cullen (University of British Columbia, Vancouver, Canada). Anti-BrdU was obtained from Chemicon International (Temecula, CA). Melatonin, bromoexoyuridine (BrDU), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), NAC, Vitamin C, sodium ascorbate (Na-Asc), Tiron, and Trolox were obtained from Sigma (St. Louis, MO).

**Diets.** Basal diet (Certified Rodent Diet 5002, PMI Nutrition International, Inc., St. Louis, MO) and the diets containing test chemicals were pelleted by Dyets, Inc. (Bethlehem, PA) 10 weeks or less, before used. All diets were stored at −20°C throughout the study.

**Animals.** Female F344 rats, four weeks old, were purchased from Charles River Breeding Laboratories (Raleigh, NC). On arrival, the animals were placed in a level-4 barrier facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), in a room with a targeted temperature of 22°C, humidity of 50%, and a 12-h light/dark cycle (0600/1800). 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 1986). The animals were housed five/cage in polycarbonate cages, on dry corn cob bedding and fed basal diet. Food and tap water were available ad libitum throughout the study. Fresh diet was supplied to the rats at least once weekly. Rats were approximately five weeks of age at the beginning of treatment.

**In Vitro Studies**

**Cell line.** MYP3 rat urinary bladder epithelial cells provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL) were used in an in vitro viability assay (Kawamata et al., 1993). 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). 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. MYP3 cells were grown as monolayers in Ham’s F-12 medium 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-BRL, Grand Island, NY) and 250 mg/ml dextrose and 1 mg/ml hydrocortisone (both from Sigma, St. Louis, MO) in an atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C.

**Treatment and MTT assay.** The effect of an antioxidant on the in vitro cytotoxicity of various arsenicals was assessed by determining the viability of MYP3 cells using the MTT assay. Briefly, MYP3 cells (5000/well) in 200 μl medium were seeded into 96-well plates. At least six wells were set up for each treatment, and five wells containing the culture medium without cells were set up as background wells for each plate.

Stock solutions of arsenate, arsenite, MMA\textsuperscript{V}, DMA\textsuperscript{V}, and TMAO were prepared by dissolving the arsenicals in Dulbecco’s PBS buffer (Gibco, Grand...
Co-administration of Antioxidants and Arsenicals

Island, NY). Stock solutions of MMAIII and DMAIII were prepared by dissolving them in 200 and 500 µl of absolute ethanol, respectively, and then diluting them in Dulbecco’s PBS buffer. All arsenicals were diluted to the appropriate working concentrations with culture medium before addition to the cells. Arsenical stock solutions were stored in the dark at 4°C, and used within one week. NAC, vitamin C, and Tiron were dissolved in Dulbecco’s PBS, whereas melatonin and Trolox were dissolved in absolute ethanol. All antioxidant solutions were prepared and diluted to the working concentration with medium immediately prior to use. One, three, and seven days after seeding, the medium was removed and replaced by fresh medium with or without test chemicals. Twenty-four hours after the last addition of test chemicals, the medium was removed and replaced by fresh medium. Twenty µl of MTT (5 mg/ml) was added to each well. After incubation for 5 h at 37°C, the culture medium supernatant was aspirated from the wells without disturbing the formazan precipitate. The formazan crystals were dissolved by addition of 200 µl of 100% DMSO and incubation for 5 min at 37°C. The absorbance was measured at 570 nm with reference wavelength of 650 nm using a Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Menlo Park, CA). The optical density (O.D.) of all control and treated wells was corrected by subtracting the average O.D. of the background wells. The percent viability of each well was calculated relative to the O.D. of the control wells by the following formula: viability (%) = (O.D. of treated well/mean O.D. of control wells) × 100.

In Vivo Studies

Experiment 1. Forty rats were randomized into four groups of 10 rats each, using a weight stratification method (Martin et al., 1986). Group 1 was fed basal diet only, group 2 was fed a diet containing 1000 ppm melatonin, group 3 was fed a diet containing 100 ppm DMAV, and group 4 was fed a diet containing 100 ppm DMAV and 1000 ppm melatonin. The use of 100 ppm DMAV in the diet was based on our previous studies that demonstrated that DMAV at this dose causes urothelial effects as early as 6 h after the start of treatment and that cell proliferation reached a maximum after two weeks (Cohen et al., 2001). The dose level of melatonin for this study was selected based on the results of a preliminary study in which rats were fed with melatonin at doses ranging from 200 ppm to 1000 ppm in the diet. There was no apparent systemic toxicity in terms of mortality, body weight decrease, clinical signs or histopathology up to a dose of 1000 ppm (data not shown). Water and food consumption were measured during week 1 of the study, and body weights were measured weekly. All rats were sacrificed after two weeks of treatment to determine the possible effects of melatonin on DMAV-induced cell proliferation. One hour prior to sacrifice, all rats were injected ip with 100 mg BrdU/kg body weight. All rats were sacrificed by an overdose of Nembutal (50 mg/kg of body weight, ip) at each of four time points: 24 h, 2, 10, and 26 weeks after start of treatment. At sacrifice, urinary bladders were excised quickly from the rats under Nembutal anesthesia, and inverted on wooden applicator sticks. After rinsing with cold RNase free PBS buffer, bladder epithelial cells were removed by swirling the inverted bladders vigorously in microcentrifuge tubes containing RLT solution from the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). The solution containing the urinary bladder epithelial cells was vortexed and homogenized using a 25 gauge needle attached to a syringe. The solution was frozen and kept on dry ice until RNA isolation within 2 h. To confirm the removal of the urothelium, the remaining bladder tissue was fixed in Bouin’s fixative, processed for paraffin embedding, stained with hematoxylin and eosin and examined by light microscopy. Total RNAs were isolated from the RLT solution containing the urinary bladder epithelial cells using RNeasy Total RNA Isolation Kit, and stored at –80°C until microarray analysis. The integrity of the total RNA was confirmed by agarose gel analysis.

Gene Expression Studies

Microarray analysis procedure and data analysis. Ten mg of total RNA from urothelial cells from a control rat and a DMAV-treated rat was labeled with Cy3 and Cy5, respectively, using SuperScript Indirect cDNA Labeling System (Invitrogen, CA). Labeled probes were simultaneously hybridized to a Rat 10K chip (MWG Biotech Inc.) containing 10,000 genes. Fluorescent array images were collected for both Cy3 and Cy5 by using a GenePix 4000A fluorescent scanner (Axon Instrument, Foster City, CA), and the intensity of each hybridization signal was evaluated by GenePix Pro 4.0. To identify genes that consistently changed during the DMAV treatment, two methods were employed. One-sample t-test was performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html) to identify genes that showed significant changes at each time point. To limit the number of false positives, only genes whose p-value was less than 0.001 and that were induced or suppressed by ≥2 fold were considered as possibly significantly changed. Genes that significantly changed over time were determined by the F-test, and then used for cluster analysis. Cluster analysis was performed by Self Organizing Maps (SOMs) analysis using the freely available software GENECLEMENT 2.0 (www.gene.wi.mit.edu/cancer/software/genecluster2/2c.html) (Tamayo et al., 1999). SOMs focuses attention on the shape of expression pattern rather than on absolute levels of expression (Tamayo et al., 1999), and is therefore suited for clustering and analysis of genes that behave in the same way over time.

TaqMan real-time quantitative PCR. The expression levels of five genes that might be involved in carcinogenesis and that showed consistent increases feeding this dose resulted in an approximate 10-fold increase in concentrations of urinary ascorbic acid compared to the control group, with only a slight elevation of urinary pH and with no effects on the urothelium (Fukushima et al., 1983, 1986). NAC was administered in the drinking water at a dose of 0.4 mg/kg body weight/day based on a report indicating that this dose was nontoxic (Balansky et al., 2002). According to the literature, NAC is stable in the drinking water for at least two days (Tamayo et al., 1999). Water containing NAC was prepared three times a week based on the results of the in vitro studies, in which consistent inhibitory effects of NAC were observed when NAC-containing medium was prepared every three days. Freshly voided urine was collected by forced urination between 0700–0900 h during week 10; pH was immediately measured using a microelectrode. During the experiment, body weight and water consumption for animals in the NAC-treated groups (Groups 4 and 8) were measured weekly, and in the other groups over a seven-day period during weeks 1 and 8, when diet consumption was also measured for all groups. Final body weights for all rats were determined at the time of sacrifice. Ten weeks after the start of the treatment all rats were injected ip with 100 mg BrdU/kg body weight 1 h prior to sacrifice. Urinary bladders were processed for histopathological examination and BrdU immunohistochemistry under the same conditions as in the Experiment 1.

Experiment 3. Forty female F344 rats were randomized into two groups of 20 rats each and fed 0 or 100 ppm DMAV in the diet. Five rats from each group were sacrificed by an overdose of Nembutal (50 mg/kg of body weight, ip) at each of four time points: 24 h, 2, 10, and 26 weeks after start of treatment. At sacrifice, urinary bladders were excised quickly from the rats under Nembutal anesthesia, and inverted on wooden applicator sticks. After rinsing with cold RNase free PBS buffer, bladder epithelial cells were removed by swirling the inverted bladders vigorously in microcentrifuge tubes containing RLT solution from the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). The solution containing the urinary bladder epithelial cells was vortexed and homogenized using a 25 gauge needle attached to a syringe. The solution was frozen and kept on dry ice until RNA isolation within 2 h. To confirm the removal of the urothelium, the remaining bladder tissue was fixed in Bouin’s fixative, processed for paraffin embedding, stained with hematoxylin and eosin and examined by light microscopy. Total RNAs were isolated from the RLT solution containing the urinary bladder epithelial cells using RNeasy Total RNA Isolation Kit, and stored at –80°C until microarray analysis. The integrity of the total RNA was confirmed by agarose gel analysis.

Microarray analysis procedure and data analysis. Ten mg of total RNA from urothelial cells from a control rat and a DMAV-treated rat was labeled with Cy3 and Cy5, respectively, using SuperScript Indirect cDNA Labeling System (Invitrogen, CA). Labeled probes were simultaneously hybridized to a Rat 10K chip (MWG Biotech Inc.) containing 10,000 genes. Fluorescent array images were collected for both Cy3 and Cy5 by using a GenePix 4000A fluorescent scanner (Axon Instrument, Foster City, CA), and the intensity of each hybridization signal was evaluated by GenePix Pro 4.0. To identify genes that consistently changed during the DMAV treatment, two methods were employed. One-sample t-test was performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html) to identify genes that showed significant changes at each time point. To limit the number of false positives, only genes whose p-value was less than 0.001 and that were induced or suppressed by ≥2 fold were considered as possibly significantly changed. Genes that significantly changed over time were determined by the F-test, and then used for cluster analysis. Cluster analysis was performed by Self Organizing Maps (SOMs) analysis using the freely available software GENECLEMENT 2.0 (www.gene.wi.mit.edu/cancer/software/genecluster2/2c.html) (Tamayo et al., 1999). SOMs focuses attention on the shape of expression pattern rather than on absolute levels of expression (Tamayo et al., 1999), and is therefore suited for clustering and analysis of genes that behave in the same way over time.

TaqMan real-time quantitative PCR. The expression levels of five genes that might be involved in carcinogenesis and that showed consistent increases...
during treatment in expression levels by microarray analysis were validated by TaqMan real-time quantitative PCR using the same RNA samples as those used for microarray analysis. These genes were phosphoribosyl pyrophosphate synthetase-associated protein 2, cytochrome p-450 4A2, prostaglandin I2 synthase, RAB4A, and glyceraldehyde 3. We also determined the expression levels of Cyt19, Uroplakin1A and 1B. These genes were not included on the microarray chip but have been suggested to be involved in bladder carcinogenesis. Sequence-specific primers and probes were designed using Primer Express software (Applied Biosystems, Inc.) and synthesized in the core laboratory at the Eppley Institute for Cancer Research, University of Nebraska Medical Center (Omaha, NE). Sequences of PCR primers and fluorogenic probes can be obtained by e-mail request to Dr. Samuel M. Cohen.

Statistics. All mean values were reported as the mean ± SE. Group means for body and tissue weights, pH, and the labeling indices in the rat bioassays 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. Calculations were performed using SAS software (SAS Institute, Cary, NC). Mean values for viability in the in vitro studies were evaluated by Dunnett analysis using StatView J-5.0 software (Abacus Concepts, Berkeley). P values of less than 0.05 were considered significant.

RESULTS
In Vitro Viability Assay
The maximum nontoxic effect doses were first determined for the antioxidants used. Maximum nontoxic doses were: for melatonin–0.2 mM, NAC–1 mM, vitamin C–0.8 mM, Tiron–0.1 mM, and Trolox–0.2 mM. Doses higher than these killed or delayed the growth of cells during treatment. Melatonin inhibited arsenite-induced cytotoxicity, but had no effect on the cytotoxic effects of other arsenicals. NAC showed inhibitory effects on the cytotoxicity of MMA III, DMA V, DMA III, and TMAO (Fig. 1). Vitamin C inhibited cytotoxicity induced by arsenate, arsenite, MMA III, and DMA III (Fig. 2). Tiron and Trolox had no effect on the cytotoxicity of any arsenical.

![Graph A](https://example.com/graphA.png)

![Graph B](https://example.com/graphB.png)

![Graph C](https://example.com/graphC.png)

![Graph D](https://example.com/graphD.png)

**FIG. 1.** Inhibitory effects of NAC on cytotoxicities of MMA III (A), DMA V (B), DMA III (C), and TMAO (D) in MYP3 cells. *Significantly different from respective arsenical alone group. Bar indicates SE.
Two-Week Rat Bioassay

DMA\textsuperscript{V}, melatonin, or the combination of the two, had no effect on the final body weight after two weeks of treatment. Bladder weights were also comparable among groups. Histo-pathologically, the bladder epithelium was normal in all rats in all groups by light microscopy. There was no significant difference in food or water consumption among groups. The BrdU labeling index was significantly increased in the DMA\textsubscript{V}-treated rats with or without melatonin, compared to untreated rats, but there was no significant difference between the group treated with DMA\textsubscript{V} alone and the group treated with DMA\textsubscript{V} and melatonin.

Ten-Week Rat Bioassay

The in vitro inhibitory effects of NAC and vitamin C on the cytotoxicity of DMA\textsuperscript{V} or DMA\textsubscript{III} suggested that these antioxidants might afford a protective effect against DMA\textsuperscript{V}-induced bladder carcinogenesis in rats. To test the hypothesis, a 10-week rat bioassay was conducted. Melatonin was also included to clarify the results of the previous two-week experiment. Ten weeks treatment with antioxidants or DMA\textsuperscript{V} alone had no effect on the body weight or bladder weight. However, co-administration of DMA\textsuperscript{V} and melatonin significantly decreased body weight, compared to the control group. Absolute and relative bladder weights were significantly increased in the group co-administered DMA\textsuperscript{V} and NAC compared to control. Urinary pH in the NAC treated-groups was significantly lower than the control. Na-Asc or DMA\textsuperscript{V} had no effects on the urine pH. However, co-administration of Na-Asc and DMA\textsuperscript{V} significantly increased the urinary pH when compared to the control group or the groups treated with Na-Asc or DMA\textsuperscript{V} alone. Melatonin had no effect on urinary pH. Histopathologically, mild simple hyperplasia was found in all DMA\textsuperscript{V}-treated groups. Mild simple hyperplasia was also observed in 1 of 10 rats treated with melatonin alone and 3 of 10 rats treated with NAC alone. There was no significant difference in the incidence of simple hyperplasia among the groups. The BrdU labeling index was significantly increased in all DMA\textsuperscript{V}-treated groups compared to the control group. Co-administration of sodium ascorbate significantly decreased the BrdU labeling index compared to the group administered DMA\textsuperscript{V} alone. Melatonin and NAC had no effects on the BrdU labeling index when co-administered with DMA\textsuperscript{V}.

FIG. 2. Inhibitory effects of vitamin C on cytotoxicities of arsenate (A), arsenite (B), MMA\textsubscript{III} (C), and DMA\textsubscript{III} (D) in MYP3 cells. *Significantly different from respective arsenical alone group. Bar indicates SE.
Microarray Analysis and Real-time RT-PCR

The purpose of the microarray analysis was to identify potential target genes consistently involved in the carcinogenicity of DMA\textsuperscript{V} and to find potential markers for use in risk assessment. After 24 h, 2, 10, and 26 weeks of treatment with DMA\textsuperscript{V} there were 1, 6, 0, and 4 genes respectively, with known function significantly down- or up-regulated as determined by the one-sample \textit{t}-test. However, none of the examined genes showed consistent changes at all four time points (Table 1). Furthermore, 107 genes with known function had significant changes over the four time points (F-tests, \( p \leq 0.001 \)). These genes were grouped into nine clusters based on similar expression pattern using SOMs analysis. Although SOMs analysis showed five genes that might be involved in carcinogenesis due to consistent increases in expression levels compared to controls during DMA treatment, none of these changes were validated by real-time RT-PCR (data not shown). This result is consistent with the result from the one-sample \textit{t}-test, suggesting that DMA\textsuperscript{V}-induced rat urinary bladder carcinogenesis is a multi-stage process. Furthermore, DMA\textsuperscript{V} had no effect on the expression level of Cyt19, uroplakin1A or 1B at any time point.

DISCUSSION

DMA\textsuperscript{V} rat bladder carcinogenesis shows a clear dose response, with a no-effect level somewhere between 2–10 ppm of dietary administration (van Gemert and Eldan, 1998). Research by Fukushima and colleagues has demonstrated a similar dose response curve following administration in the drinking water (Wei \textit{et al}., 1999). The overall mode of action of DMA\textsuperscript{V} rat bladder carcinogenesis appears to be cytotoxicity and necrosis followed by sustained urothelial cell regeneration (Cohen \textit{et al}., 2001), ultimately leading to the development of a relatively low incidence of bladder tumors in a two-year bioassay. DMA\textsuperscript{V} is excreted predominantly in the urine, and the major metabolite in the rat is TMAO (Cohen \textit{et al}., 2002a; Wei \textit{et al}., 2002). However, the highly reactive metabolite DMA\textsuperscript{III} is found at urinary concentrations comparable to those which were cytotoxic in an \textit{in vitro} urothelial cytotoxicity study, i.e., 0.5 to 1.5 \( \mu \text{M} \) (Cohen \textit{et al}., 2002a).

The DMA\textsuperscript{V} rat bladder carcinogenesis mode of action appears to have the following sequence of events: (1) ingestion of large amounts of DMA\textsuperscript{V}; (2) absorption of DMA\textsuperscript{V} into the cells; (3) metabolism of DMA\textsuperscript{V} to DMA\textsuperscript{III} and TMAO (and possibly other metabolites); (4) excretion of the metabolites and the remainder of the parent compound in the urine; (5) urothelial cytotoxicity and cell death; (6) sustained regenerative proliferation; and (7) tumor formation. Obviously, the key to understanding the mechanism of carcinogenicity of DMA\textsuperscript{V} in the rat bladder is to identify the specific mechanism(s) involved in the production of urothelial cytotoxicity (step no. 5).

There are several possible specific cellular and molecular mechanisms that could account for the cytotoxicity in response to DMA\textsuperscript{V} administration: (1) direct damage to DNA (DNA reactivity); (2) indirect genotoxicity, including possibly inhibition of DNA repair, reaction with the mitotic apparatus (especially tubulin), or oxidative injury to the DNA; (3) oxidative damage to non-DNA components of the cell; (4) reaction with sulfhydryl groups of proteins that are critical to cell function; or (5) depletion of glutathione (Kitchin, 2001). Evidence suggests that direct damage to DNA does not occur; rather, indirect damage, such as through generation of peroxy radicals and oxidative damage can be produced, at least in \textit{in vitro} systems.

### Table 1

<table>
<thead>
<tr>
<th>Genes Up-regulated or Down-regulated in Bladder Epithelial Cells of Female F344 Rats after DMA\textsuperscript{V} Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GeneBank accession #</strong></td>
</tr>
<tr>
<td>24 h Down-regulated</td>
</tr>
<tr>
<td>Week 2 Up-regulated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Down-regulated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Week 10&lt;sup&gt;a&lt;/sup&gt; Up-regulated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Down-regulated</td>
</tr>
</tbody>
</table>

<sup>a</sup>No gene up or down-regulated.
CO-ADMINISTRATION OF ANTIOXIDANTS AND ARSENICALS

(Kitchin and Ahmad, 2003). More than one of the others may be operative as they are not mutually exclusive.

Cytotoxicity secondary to inhibition of DNA repair or interaction with the mitotic apparatus appears an unlikely mechanism for DMA\textsuperscript{V}-induced effects in the rat bladder, since the mammalian bladder urothelium is a slowly turning over tissue (average life span of 100–200 days) (Cohen \textit{et al.}, 2002b), and the cytotoxicity is seen morphologically within 6 h or less after administration of DMA\textsuperscript{V} in the diet to rats (Cohen \textit{et al.}, 2001). Other forms of indirect genotoxicity also appear unlikely as the cause of the cytotoxicity, although they may contribute to the formation of somatic mutations later in the carcinogenic process.

In this article, we present data in support of a role for oxidative damage in the cytotoxic process in the urothelium that is produced following administration of DMA\textsuperscript{V} to the rat. Evidence for this includes results in both \textit{in vitro} and \textit{in vivo} studies. In the \textit{in vitro} studies, the inhibitory effect of co-administration of specific antioxidants with various forms of arsenic was identified primarily for trivalent arsenicals, not with the pentavalent arsenicals. This is in keeping with the much higher reactivity of trivalent species compared to the pentavalent species. Although cytotoxicity is seen with the pentavalent arsenicals in this \textit{in vitro} assay, it is at much higher concentrations, usually in the millimolar range in contrast to micromolar concentrations and below for the trivalent arsenicals.

The lack of consistent findings with the different antioxidants, may serve as a tool for the investigation and understanding of the specific mechanism for the cytotoxicity. The different antioxidants have different reactivities for specific oxidative species, such as hydroxyl radicals, singlet oxygen, hydrogen peroxide, peroxyl radicals, or superoxide anion. Understanding how each of them reacts may lead to the understanding of the factor causing the cytotoxicity.

Vitamin C has a preponderant scavenging ability for the hydroxyl radical, peroxy radical, superoxide anion and singlet oxygen (Buettner and Jurkiewicz, 1996; Chou and Khan, 1983), whereas the other antioxidants scavenge other or more limited types of oxidative agents (Chan, 1993; Greenstock and Miller, 1975; Moldeus \textit{et al.}, 1986; Russel \textit{et al.}, 2002; Tan \textit{et al.}, 2002; Walker \textit{et al.}, 1998). Surprisingly, even at extremely high concentrations we saw no inhibitory activity for melatonin in the \textit{in vitro} assays except for a slight inhibition of arsenite cytotoxicity.

NAC showed inhibitory activity in the \textit{in vitro} studies, but it is unclear whether this is due entirely to an antioxidant effect, or whether it could be due to interaction of the arsenical reactive species with the thiol group of NAC. Trivalent arsenicals are known to be reactive with molecules containing thiol groups such as glutathione (Scott \textit{et al.}, 1993) and 2,3-dimercaptopropane-1-sulfonic acid (Aposhian \textit{et al.}, 1984; Delnomdedieu \textit{et al.}, 1993).

Although some of the antioxidants showed inhibitory activity in the \textit{in vitro} assay, only vitamin C showed inhibitory activity in the \textit{in vivo} bioassay. With regard to vitamin C, there are several limitations as to why greater inhibition was not seen. To begin with, ascorbic acid is not a vitamin in the rat in the sense that the rat produces its own ascorbic acid and is not dependent upon exogenous chemicals as a source. Thus, even with massive amounts of vitamin C in the diet (1% of the total diet), there is only an increase of approximately ten-fold in the urinary levels of ascorbic acid and dehydroascorbic acid. Nevertheless, the amounts present in urine are substantially higher in the rats administered the vitamin C at this dietary concentration than in the controls, and we did see evidence of an inhibitory effect.

For a chemical to have inhibitory activity for DMA\textsuperscript{V} cytotoxicity of the urothelium, it must be excreted at substantial concentrations in the urine. Although we have not determined the amount of free NAC in the rat urine, low bioavailability of NAC may provide an explanation for the lack of inhibitory activity with NAC \textit{in vivo} studies. The reason for the lack of activity for melatonin \textit{in vitro} and \textit{in vivo} is unclear. The limitation regarding urinary excretion of melatonin \textit{in vivo} is one possibility, but that does not explain the lack of inhibitory activity in the \textit{in vitro} setting, where the concentration of melatonin was extremely high.

We also examined gene expression, both by microarray analysis and by RT-PCR, but did not find consistent changes. This may be due to several factors. The changes may not be consistent over time, and this is most likely true since even morphologically different events are occurring at different times. Thus, early in the process there is extensive cell death, whereas after three days there is both cell death and regeneration. In addition, the changes are focal and did not generally involve the entire urothelium of the bladder. Thus, the change in expression may not be detectable with our current assays since they examine the entire urothelium of an individual rat rather than the specific foci that are being affected.

The results of these experiments provide supportive evidence for a role for oxidative damage as part of the process of DMA\textsuperscript{V}-induced cytotoxicity, both \textit{in vitro} and \textit{in vivo}, regarding the rat urothelium. Other investigators have also obtained evidence in support of a role for oxidative damage including identifying increases in 8-OHdG (Vijayaraghavan \textit{et al.}, 2001; Wanibuchi \textit{et al.}, 1997; Wei \textit{et al.}, 2002). Kitchin and his colleagues have demonstrated evidence of oxidative damage, although the concentrations used in their studies were substantially higher than the 1 \(\mu\)M or less that appear to be necessary for cytotoxicity \textit{in vitro} (Kitchin and Ahmad, 2003). Whether the changes they identified also occur at these low concentrations and whether they occur \textit{in vivo} has yet to be reported.

Overall, although there is a significant amount of information accumulating supporting the role for oxidative damage in arsenic-induced cytotoxicity, and therefore, probably carcinogenicity, the evidence is not complete. Since arsenicals have numerous affects on the cell, including reaction with a wide variety of sulfhydryl groups, multiple effects on cellular constituents leading to cytotoxicity and carcinogenicity are quite
likely. In addition, although the evidence supports a role for oxidative damage, it is unclear whether it is a cause of the cytotoxicity or a result of the cytotoxicity.

The carcinogenicity of inorganic arsenic in humans has been well documented (Environmental Protection Agency, 2001; National Research Council, 1999). Although administration of DMA\textsuperscript{V} to rats in the diet or drinking water is carcinogenic to the bladder (van Gemert and Eldan, 1998; Wei et al., 1999), the implications of this model to humans exposed to DMA\textsuperscript{V} need to be carefully evaluated. Metabolism and toxicokinetics of arsenicals in rats are significantly different than in other rodents, and even more different than in humans. In addition, the DMA\textsuperscript{V} doses administered to rats in these experiments are extraordinarily high in comparison to human exposures to arsenicals in general and formation of methylated arsenicals differs following exposure of humans to inorganic arsenic compared to exposure to already methylated organic arsenicals. Thus, although this DMA rat bladder cancer model may contribute to our further understanding of some of the specific details of the mechanism of action of arsenic carcinogenicity, these and other factors need to be taken into account in an overall assessment of human applicability.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. James Eudy, Director of the DNA Microarray Core Facility at the University of Nebraska Medical Center for the performance of the microarray analyses, Dr. James Lynch and Lynnette M. Smith for assistance in the statistical evaluation of the microarray results, and Earline Titsworth for assistance in the preparation of this manuscript. The DNA Microarray Core Facility is partially supported by NIH Grant Number 1 P20 RR16469 from the BRIN Program of the National Center for Research Resources.

REFERENCES


