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Quantitative Relationship between Arsenic Exposure and AP-1 Activity in Mouse Urinary Bladder Epithelium

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Because of the potential of arsenic for causing cancer in humans, and of the fact of widespread environmental and occupational exposure, deriving acceptable human-limit values has been of major concern to industry as well as to regulatory agencies. Based upon epidemiological evidence and mechanistic studies, it has been argued that a non-linear dose-response model at low-level exposures is more appropriate for calculating risk than the more commonly employed linear-response models. In the present studies, dose-response relationships and recovery studies employing a cancer precursor marker, i.e., activating protein (AP)-1 DNA-binding activity, were examined in bladders of mice exposed to arsenic in drinking water and compared to histopathological changes and arsenic tissue levels in the same tissue. While AP-1 is a functionally pleomorphic transcription factor regulating diverse gene activities, numerous studies have indicated that activation of the MAP kinase pathway and subsequently increased AP-1 binding activities, is a precursor for arsenic-induced cancers of internal organs as well as the skin. We observed previously that within 8 weeks of exposure AP-1 activation occurs in urinary bladder tissue of mice exposed to arsenic in the drinking water. In the present studies, C57BL/6 mice were exposed to sodium arsenite at various concentrations in the drinking water for 8 consecutive weeks. Minimal but observable AP-1 activity occurred in bladder tissue at exposure levels below which histopathological changes or arsenic tissue accumulation was detected. Marked AP-1 DNA-binding activity only occurred at exposure levels of sodium arsenite above 20 μg/ml, where histopathological changes and accumulation of arsenic in the urinary bladder epithelium occurred. Although the experimental design did not allow statistical modeling of the entire dose-response curve, the general shape of the dose-response curve is not inconsistent with the previously proposed hypothesis that arsenic-induced cancer follows a non-linear dose-response model.

Key Words: **cancer causes; risk assessment; EPA levels; arsenic; cancer; mechanism.**

Chronic arsenic exposure, which occurs primarily through contaminated drinking water and in the environment of workers in the agricultural and mining industries, has been related to the development of vascular disease, skin cancer, and internal cancers. Regarding internal cancers, the association between arsenic ingestion and cancers of the bladder have been well documented in Taiwan (Chiou *et al*., 1995), Argentina (Hopenhayn-Rich *et al*., 1996), Chile (Smith *et al*., 1998), and Japan (Tsuda *et al*., 1995). Subsequently, arsenic has been ranked highest in priority on a list of the top 20 hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR) and the U.S. EPA (ATSDR, 1997). Epidemiological studies in areas of the United States are underway to investigate the quantitative association between exposure and increased incidences of bladder cancer (Karagas *et al*., 1998; Lewis *et al*., 1999). At the present U.S. EPA arsenic water standard of 50 μg/l, the internal cancer risk may be comparable to those of environmental tobacco smoke and radon in homes, with risk estimates of around 1 per 1000 (IARC, 1987; Smith *et al*., 1992). It has been estimated that over 350,000 people in the U.S. consume drinking water containing over 50 μg/l of arsenic, and more than 2.5 million people use water containing more than 25 μg/l (Karagas *et al*., 1998). Subsequently, there is significant regulatory pressure to lower the acceptable levels. However, results from epidemiological studies where exposure levels have been collected suggest that the current EPA cancer slope factor (CSF) for arsenic may actually over-predict cases at relatively low exposure levels (Valberg *et al*., 1998). This may be due to the fact that the CSF was calculated assuming a standard linear dose-response relationship, while a non-linear or sublinear dose response may be more appropriate. Epidemiological data are available providing empirical evidence supporting both linear and non-linear associations between excess cancer and arsenic exposure, and appropriate animal models for use in risk assessment have yet to be developed.

Understanding the mechanism of action for arsenic carcinogenicity can be an important factor in establishing the shape of the dose-response curve and assessing cancer risk, particularly at low levels of exposure. The mechanism by which arsenic causes cancer has been under intense investigation, and progress has been made in elucidating this process. Although arsenic itself is not mutagenic at doses that are not cytotoxic,

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some deleterious effects on DNA have been observed including inhibition of DNA repair, potentiation of DNA damage by other agents, sister chromatid exchange, and gene amplification (Lee *et al*., 1988; Lerda, 1994; Li and Rossman, 1989). These effects do not adequately explain arsenic′s carcinogenic properties, and epigenetic mechanisms have been proposed. Central to an epigenetic process is evidence indicating that arsenic stimulates cell proliferation by affecting specific cell-signal-transduction pathways. Specifically, arsenic has been shown to activate the mitogen-activated protein kinase (MAPK) cascade (Chen *et al*., 1998; Huang *et al*., 1999; Liu *et al*., 1996; Trouba *et al*., 2000), ultimately resulting in the activation of transcription factors, such as the activating protein (AP)-1 family (Burleson *et al*., 1996; Cavigelli *et al*., 1996; Simeonova *et al*., 2000). AP-1, which is one of several transcription factors that helps regulate the expression of diverse genes, is responsible for many of the biological effects of tumor promoters, including induction of transforming oncoproteins and growth-factor expression (Angel *et al*., 1991). A consequence of chronic cell proliferation would be an increased likelihood of neoplasia, by providing a microenvironment for increased proliferation of mutated cells. Consistent with the hypothesis are studies demonstrating increased numbers of papillomas in Tg:AC transgenic mice given sodium arsenite (Germolec *et al*., 1998). Similarly, bladder tumors have been reported in diethynitrosamine-treated rats provided dimethyarsenic acid (DMA) in their drinking water (Yamamoto *et al*., 1995).

On the assumption that AP-1 activation represents a "precursor" marker for arsenic-induced bladder cancer, its DNA binding activity was semi-quantified in urinary bladders of mice exposed to control and arsenic-containing drinking water in order to help define dose-response characteristics. Changes in AP-1 binding activity were compared to histopathological changes and arsenic accumulation in bladder tissue.

MATERIALS AND METHODS

Experimental animals. Female C57BL/6 mice were obtained from Charles River, Portage, MI. All animals were housed at NIOSH facilities in compliance with AAALAC-approved guidelines for the humane treatment of laboratory animals. Animals were maintained on a 12-h light/dark cycle and were provided chow and water *ad libitum.* Groups of 8-week-old mice were provided concentrations of sodium arsenite ranging from 0 to 100 μg/ml (Sigma Chemical Co., St Louis, MO) in drinking water for 8 weeks. No arsenic was detected in control drinking water as measured by ICP-MS (detection limit 0.01 μg/ml). In recovery studies, mice were provided drinking water containing 50 μg/ml for 8 weeks and then placed on normal drinking water for indicated times. Mice were sacrificed by CO_2 asphyxia and the urinary bladders were collected under aseptic conditions. Mice receiving the higher concentrations of arsenic-treated water demonstrated a moderate reduction in water consumption. Previous findings in this laboratory, using paired-water studies, indicated that this reduction was not sufficient to cause any significant clinical or pathological effects (Simeonova *et al*., 2000).

Histology. Bladders were removed and fixed by immersion in 10% neutral-buffered formalin and processed for paraffin embedding. Each paraffin block was step-sectioned and stained with hematoxylin and eosin. Pathological assessments were performed in a blind fashion. The samples were fixed for transmission electron microscopy as previously described (Simeonova *et al*., 2000). Ultrathin sections were prepared and stained with uranyl acetate and lead acetate and examined by electron microscopy.

Arsenic determination in tissues. Urinary bladders from control or arsenic-treated mice were quick-frozen in acid-free vials and stored at –70°C. The tissue samples were digested by addition of 6 N HCl at 80°C for 16 h in a specially designed reaction vessel. Analyses of arsenic tissue levels were performed by Battelle Marine Sciences Lab (Seqium, WA) using a complex atomic absorption method (Crecelius, 1998) as previously described (Simeonova *et al*., 2000). Quality control was established through calibration and testing of the hydride generation, purging, and detection systems.

Nuclear extracts and electrophoresis mobility-shift assay (EMSA). Nuclear proteins were prepared from aliquots of 1×10^7 cells or frozen samples of bladder tissue pooled from 3 identically treated mice as previously described (Schreiber *et al*., 1989). DNA binding reactions and EMSAs were performed as described previously (Simeonova *et al*., 1997, 2000). Briefly, the 5′ ends of the double-stranded oligonucleotides were labeled with γ^{32}P-ATP (New England Nuclear/Dupont, Boston, MA), using 6–10 U of T4 polynucleotide kinase (USB/Amersham, Cleveland, OH). Binding reactions (30 μl) were performed on ice for 30 min in reaction mixtures containing 10 μg of nuclear proteins, 20 mM Tris–HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 2 μg of poly(dI-dC).poly(dI-dC), 10% glycerol and approximately 0.1 ng (2×10^5 cpm) of specified probe. For detection of AP-1 DNA-binding activity, an oligonucleotide was obtained from Santa Cruz (Santa Cruz, CA) containing an AP-1 consensus sequence: 5′-CGC TTG ATG ACT CAG CCG GAA-3′. Protein-DNA complexes were separated on a 5% non-denaturing polyacrylamide gel. Gels were electrophoresed at 125 V in 50 mM Tris-50 mM boric acid/1 mM EDTA, dried, and autoradiographed overnight. The autoradiograms were scanned with a computerized laser densitometer (Eagle Eye II Image Analysis System, Stratagene, La Jolla, CA) and the results were examined using the One Dscan gel-analysis software and the NIH Image 1.54 analysis software. The data are presented graphically as a ratio of the mean control to experimental values.

Statistical analysis. All experiments were replicated and representative findings are shown. Analyses were conducted using JMP software (SAS Institute, Cary, NC). One-way analysis of variance was performed on the data and linear contrasts were determined using the least-squares means. The Jonckheere-Terpstra test was used to establish dose-response trends using one-side alternatives, which gives *a priori* hypothesis for the direction of the response.

RESULTS AND DISCUSSION

Animal models for arsenic carcinogenicity have been difficult to establish. Thus, we quantified AP-1 DNA-binding activity in the urinary bladder of mice administered concentrations of sodium arsenite in their drinking water, assuming this represents a cancer precursor marker. Similar to classical tumor promoters such as phorbol esters, okadaic acid, and UV radiation, arsenic activates several transcription factors, including AP-1, to a similar degree, resulting in the induction of immediate early genes such as c-*fos*, c-*jun*, *EGR-1*, and c-*myc* (Burleson *et al.*, 1996; Cavigelli *et al.*, 1996; Ishikawa *et al*., 1999; Simeonova *et al.*, 2000) involved putatively in cell growth. This appears to occur through the ability of arsenic to activate upstream events including the MAP kinase pathway (Huang *et al.*, 1999; Liu *et al.*, 1996; Trouba *et al.*, 2000). While these studies were conducted exclusively *in vitro*, we demonstrated recently that AP-1 activation occurs in urinary

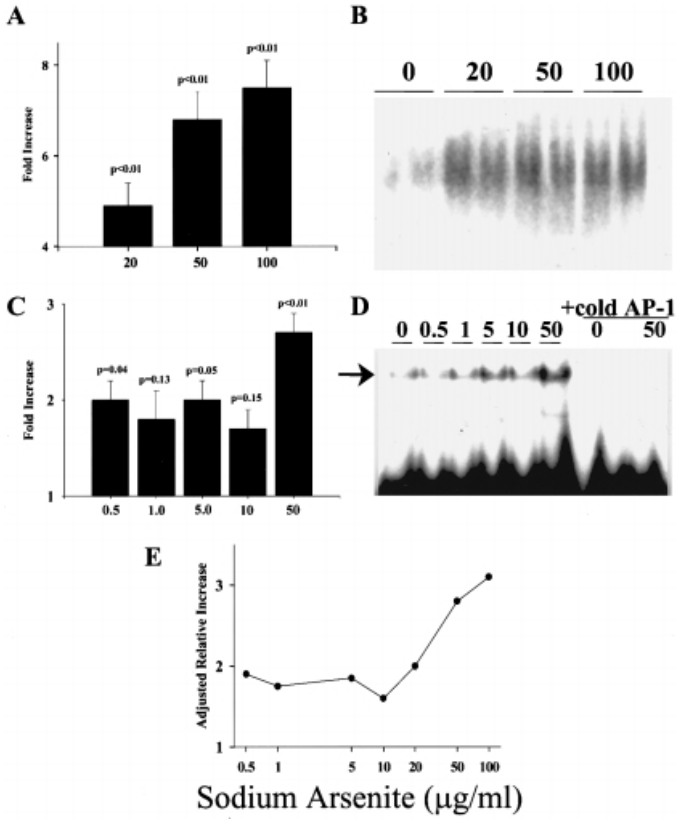

FIG. 1. AP-1 DNA binding activity in mouse urinary bladders as measured by EMSA. Mice were given drinking water containing various amounts of sodium arsenite for 8 weeks. Nuclear protein extracts were isolated from the urinary bladder and probed using a ^{32}P-labeled double-stranded oligonucleotide containing the AP-1 consensus sequence as described in the Methods and Discussion section. The gels were autoradiographed and scanned using a computerized laser densitometer. Data are presented as fold-change from animals that received untreated drinking water. A and C are representative data from 2 experiments employing identical experimental designs, except different dose levels. E shows adjusted values from combining results from experiments A and B; B and D autoradiographs of gels from A and B, respectively showing, in some instances, duplicate samples. D also shows nuclear extracts from the control and 50-μg/ml treated groups pre-incubated with a 50-fold excess of the unlabeled probe to help establish specificity. *Arrow,* AP-1 band. Each value represents a mean ± SE of 3 samples, where a sample represents a pool of 3 bladders.

bladder epithelium of mice and rats, following exposure to sodium arsenite in the drinking water or in similarly exposed transgenic mice possessing an AP-1 luciferase reporter construct (Simeonova *et al.*, 2000). In the present studies we observed a marked increase in AP-1 DNA binding activity within 8 weeks in mice receiving sodium arsenite at exposure levels ranging between 20 and 100 μg/ ml (Figs. 1A and 1D).

In order to better define the dose response, a second study was conducted under identical exposure conditions, except with lower doses of arsenic (Figs. 1B and 1E). The 50-μg/ml exposure level was selected as the high dose to be examined, as AP-1 activity was consistently increased at this concentration. Due to the complex nature of the assay, the relative percent increase in the AP-1 response at comparable exposures varied between the 2 experiments (compare Figs. 1A and 1B). As would be expected from the results observed in the first study, AP-1 activity in the 50-μg/ml dose group was significantly increased over controls. However, AP-1 was only slightly increased in mice that received 10 μg/ml of sodium arsenite or less, and only values from the 0.5- and 10-μg/ml-dosed groups were statistically different from controls. There was no statistical indication that any of these groups within this range differed from one another.

When the data from 0.5 to 10 μg/ml exposure groups were pooled for comparison with the control group, the average AP-1 activity was significantly different from controls ($p = 0.03$). In an attempt to provide a more thorough examination of the dose-response relationship between arsenic exposure and AP-1 activity, the AP-1 values from the 2 experiments were normalized to the 50-μg/ml dose groups and then presented as the percent change from controls (Fig 1E). Since this represented pooled data, the response curve was not statistically modeled. However, features reflected in the combined dose-response curve suggest that measurements at and below 10 μg/ml may have a much shallower slope than measures above 10 μg/ml, and thus, reflect a non-linear response curve. Alternatively, the measurement error for the EMSA's in this dose range may be relatively high compared to the real impact of arsenic on induction of AP-1 activity enough so as to bias the dose-response curve to reflect a linear-type relationship. In any case, at arsenic exposure levels between 10 and 100 μg/ml, a dose-responsive, monotonic increase in AP-1 activity is noted.

Rodents subchronically administered sodium arsenite (Simeonova *et al.*, 2000) or DMA (Arnold *et al.*, 1999; Yamamoto *et al.*, 1995) in the drinking water develop hyperplasia of the urinary bladder epithelium, manifested by structural irregularities and thickening of the transitional cell layer. In the present studies, multiple histopathological changes in the bladder epithelium were also evident in mice administered exposure levels of 50 and 100 μg/ml (Fig. 2B) of sodium arsenite in the drinking water. The changes in the 50 and 100 μg/ml exposure groups were indistinguishable, consisting of simple hyperplasia and the appearance of eosinophilic, cytoplasmic inclusions. The latter reflect pathological degenerative changes. Urothelial cells from hyperplastic bladders did not form papillary structures but progressed toward the lumen of the bladder. Occasional squamous metaplasia, without keratinization, was observed in some hyperplastic areas. Transmission electron microscopy indicated the presence of pleomorphic projections representing microvilli formation on the intercellular surface (Fig. 2D). There was no evidence of histopathological changes in mice exposed to levels below 50 μg/ml (data not shown), nor was there evidence of microcrystalluria, calculi, or amorphous precipitates in any of the tissues examined.

Previously, we demonstrated that arsenite was the predominant form of arsenic contained within the bladder tissue of

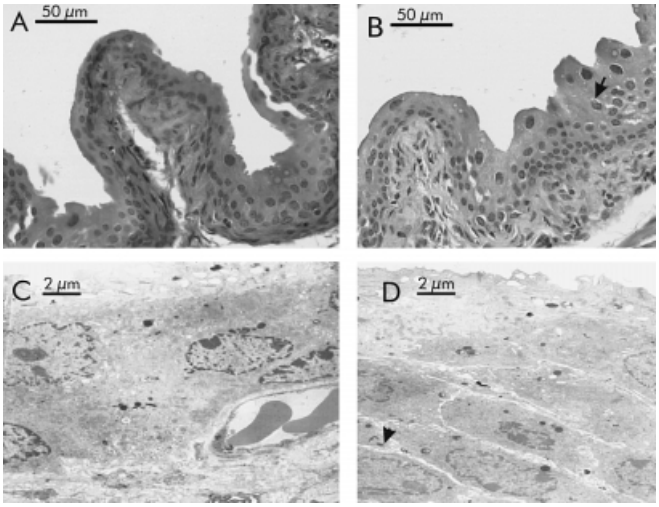

FIG. 2. Histopathology of representative urinary bladder tissue from mice provided either untreated drinking water (A, C) or 50 μg/ml (B, D) of sodium arsenite-treated drinking water for 8 weeks. Full-thickness bladder sections were cut, and 6-μm sections were prepared and stained with H&E (A, B). The magnification is $\times$40. *Arrows*, note mild transitional cell hyperplasia and apparent eosinophilic inclusions. Transmission electron microscopy of a bladder of a mouse administered either vehicle (C) or 50 μg/ml sodium arsenite in its drinking water (D). The magnification is $\times$3000. Bladders from treated animals showed microvilli (arrow) consistent with hypermetabolic activity.

rodents exposed to sodium arsenite in the drinking water, representing more than twice as much DMA (Simeonova *et al.*, 2000). This is in contrast to urine where the predominant form is DMA. Only trace amounts of other metabolites were detected in the urine or the bladder epithelium. To examine the relationship between exposure levels and arsenic accumulation, total concentrations of arsenic in the bladder epithelium were assessed, and to establish experimental error variability, were followed by pairwise treatment contrasts (Fig.3). The mean arsenic tissue concentration in the 50 μg/ml group was significantly greater than all other group means ($p < 0.0001$). No other pairwise contrasts were significant.

Based upon indirect evidence from *in vitro* genotoxicity studies, and more recent albeit controversial, epidemiological finding, a sublinear or threshold relationship has been proposed to characterize the relationship between arsenic exposure and skin and internal cancers (Rudel *et al*., 1996). This response relationship is in contrast to a linear dose-response relationship, which is used historically by U.S. regulatory agencies for cancer relationships and which reflects a more conservative approach. The latter originates from the "one-hit" notion that one molecule has some probability of interacting with DNA to produce a mutation that may lead to cancer. Based upon what many believe is the mechanism by which arsenic induces cancer, a sublinear or threshold model might be more appropriate, and is dependent upon the assumption that arsenic does not induce cancer through initiation, i.e., a genotoxic event, but rather through epigenetic processes such as altered cell growth (i.e., promoter activities). These biological processes would inherently proceed with a supralinear dose-response, where effects at lower doses are disproportionately lower than at higher doses, or with a supralinear dose-response, where effects at lower doses are disproportionately higher than at higher doses. A supralinear model would also be consistent if physiological saturation of detoxification activities occurred, as has been proposed to occur from high-dose or chronic arsenic exposure (Thompson, 1993). Arsenite is enzymatically methylated in the liver to monomethyarsonic acid (MMA) and then to DMA, resulting in urinary excretion and detoxification. This biotransformation process involves glutathione (GSH) as a cofactor, methyltransferase for methylation, and cofactor S-adenosyl-methionine (SAM) as the methyl donor. Thus, assuming inorganic arsenite is the toxic metabolite, a linear response would only occur when methylation (detoxification processes) are exhausted, while a different dose-response curve might be observed prior to saturation.

A series of experiments were also conducted to help establish time-to-recovery. Groups of mice were provided drinking water containing 50 μg/ml of sodium arsenite for 8 weeks, and then allowed untreated water for varying periods. AP-1 DNA binding activity and arsenic bladder concentrations were examined at 2, 4, and 8 weeks following exposure cessation, and values were adjusted relative to their respective controls (Fig. 4). Mice treated for 8 weeks at the 50 μg/ml level served as the 0-time group. Pairwise contrasts indicated that arsenic levels remained statistically elevated until week 8 following removal of arsenic from the drinking water, at which time there was a significant decrease from time 0 ($p < 0.03$). A *t*-test between animals never exposed to arsenic and those allowed to recover from exposure for 8 weeks indicated that a significant amount of arsenic, however, remained in the tissue at this time ($p = .003$). AP-1 levels at each time point were also compared to the 0-time recovery point. AP-1 activity at 2 and 4 weeks remained

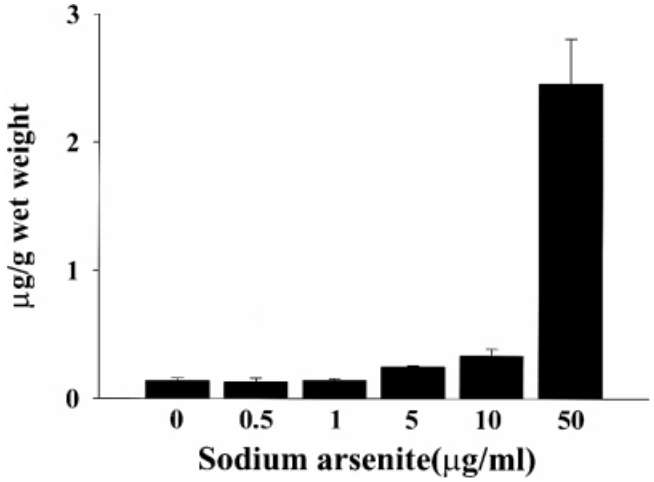

FIG. 3. Urinary bladder tissue levels of arsenic. Mice were exposed to concentrations of sodium arsenite in the drinking water for 8 weeks and total arsenic determined from bladder epithelium as described in Materials and Methods. Each value represents the mean $\pm$ SE of 3 animals.

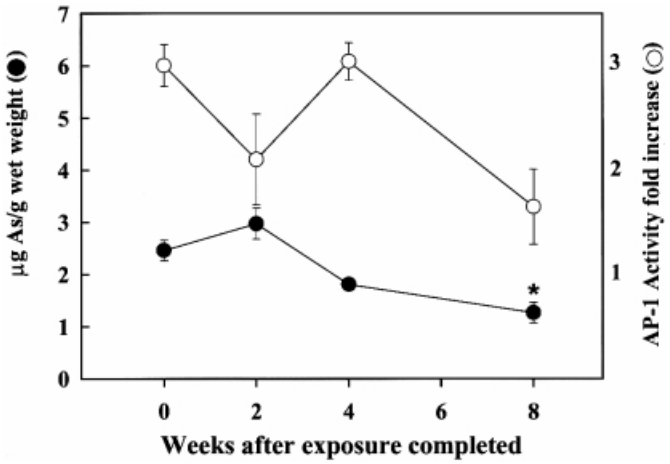

FIG. 4. AP-1 DNA binding activity and arsenic levels in urinary bladder tissue in mice that received 50 μg/ml of sodium arsenite in the drinking water for 8 weeks and then allowed to consume untreated water. Details of the methodology are described in the Materials and Methods section. AP-1 activity is presented as fold-change from control mice that received untreated water for the entire period. Each value for AP-1 activity represents a mean ± of 3 samples, where a sample represents a pool of 3 bladders. Each value for arsenic tissue levels represents mean ± SE of 3 animals. *Significantly different from time-0 at $p < 0.05$.

elevated, being similar to the 0 time. A slight, but not significant difference ($p = 0.07$) between the 0-time group and the 8-week recovery time point was observed. There was a statistically significant dose-response decrease in arsenic tissue levels as a function of time ($p = 0.037$) but not in AP-1 activity. This would suggest that the arsenic bladder effects described in this study are fairly long lasting although eventually reversible.

In summary, dose-response relationships between a precursor marker for bladder cancer (AP-1 DNA binding) and arsenic exposure in mice are presented. Although AP-1 activation can reflect activities other than cancer, its involvement in arsenic-induced cancers is likely, as *c-jun* and c-*fos* expression are consistently increased by arsenic (Burleson *et al.*, 1996; Cavigelli *et al.*, 1996; Chen *et al.*, 1998; Huang *et al.*, 1999; Liu *et al.*, 1996; Simeonova *et al.*, 2000; Trouba *et al.*, 2000). The AP-1 transcription factor is commonly composed of Jun and Fos heterodimers and c-*jun* expression is a concomitant factor associated with urinary bladder transitional carcinomas (Tiniakos *et al*., 1994). Because arsenic has been linked mainly to development of typical transitional carcinomas without any unique clinical or histopathological features (Chow *et al*., 1997), it can be expected that genes induced by arsenic are typical of molecules involved in bladder carcinogenesis. Although the EMSA assay is only semiquantitative in nature, we report that AP–1 DNA-binding activity is a very sensitive indicator for arsenic exposure in the urinary bladder. While AP-1 activity appeared increased at all exposure levels tested, only at exposures above 50 μg/ml were there notable increases in activities that were accompanied by histological changes or a measurable accumulation of arsenic in bladder tissue. As AP-1 activity is associated with normal physiological processes (e.g., cell turnover) as well as pathological conditions, it could be argued that a no-observable-adverse-effect level (NOAEL) was observed in the 20 μg/ml exposure range. Since AP-1 activity in the bladder epithelium occurs even at levels of exposure less than 10 μg/ml, an impact of arsenic above zero-exposure levels may reflect effects leading to increased risk of cancer or it may reflect repair processes. It is possible that these low-level effects are of a more "shallow" nature than dose-response relations above 10 μg/ml. To clearly establish this, however, better characterization of this response would be required, focusing on laboratory methods and study designs that would minimize error in this dose range.

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