Increased Micronuclei in Exfoliated Bladder Cells of Individuals Who Chronically Ingest Arsenic-contaminated Water in Nevada[1]

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Abstract

It is well established that inorganic arsenic is causally associated with lung cancer via inhalation and skin cancer via ingestion. Epidemiological evidence based on studies in Taiwan suggests that ingestion of inorganic arsenic may also cause other more fatal internal cancers, with the highest relative risks reported for bladder cancer. Here, we have used a biological marker of response, the micronucleus assay in exfoliated bladder cells, to evaluate the possible genotoxic effects of chronic arsenic ingestion on the bladder. The overall objective of this study was to compare the frequency of micronucleated cells in exfoliated bladder and buccal cells between a group of 18 individuals in Nevada who chronically ingested high levels of inorganic arsenic from their well water (average level, 1,312 μg/liter) and an individually matched control group with low exposure to arsenic (average level, 16 μg/liter). A 1.8-fold increase (90% confidence interval, 1.06–2.99) was observed in the weighted mean frequency of micronucleated bladder cells in the exposed group (2.79 per 1000 cells) compared with the unexposed group (1.57 per 1000 cells). In addition, the frequency of micronucleated bladder cells was positively associated with the urinary concentration of inorganic arsenic plus its methylated metabolites (Spearman correlation = 0.33; $P = 0.03$). In contrast, there was no increase in micronucleated buccal cells associated with arsenic ingestion (frequency ratio = 1.0; 90% confidence interval, 0.65–1.53). The results of this study provide evidence that chronic ingestion of high levels of inorganic arsenic in drinking water is associated with an increased frequency of micronucleated bladder cells. These findings are consistent with a genotoxic effect of arsenic on bladder cells, but a larger study is needed to confirm them.

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Introduction

Background. Arsenic is a naturally occurring element throughout the environment. Like some metals, arsenic can form both inorganic and organic compounds. The inorganic forms are considered to be the most toxic species (1). It is well established that inorganic arsenic is causally associated with lung cancer via inhalation and with skin cancer via ingestion (2). A review of the epidemiological evidence suggests that ingestion of inorganic arsenic may also cause other more fatal internal cancers, including bladder cancer (3).

The main source of epidemiological evidence comes from studies of a large population in southwest Taiwan, where residents were exposed to high levels of inorganic arsenic in drinking water from the 1920s until an alternate water supply was provided in the 1960s (4–8). Significant dose-response increased risks for mortality from a variety of cancers were reported for inorganic arsenic concentrations ranging from 170 to 800 μg/liter (6–8). The highest risks were reported for bladder cancer, with mortality rate ratios of 5.1, 12.2, and 28.7 for males, and 11.9, 25.1, and 65.4 for females (6). Additional evidence for an association between arsenic ingestion and bladder cancer is provided by a study of persons who ingested medicinal arsenicals (9).

The results of risk extrapolation from the Taiwanese findings suggest that the relative risk of bladder cancer arising from lifetime consumption of 1 liter of drinking water per day at the current U.S. MCL[3] for arsenic (50 μg/liter) could be about 2.6 for females and 1.4 for males, when compared to ingestion of water containing background levels of arsenic (<5 μg/liter) (10).

Although estimates of human cancer risk exceed that estimated for other chemicals regulated by the Safe Drinking Water Act, the association between arsenic ingestion and internal cancers has not been investigated in the U.S. until recently. It has been estimated that more than 300,000 people have drinking water supplies with arsenic levels in excess of the MCL (11). Many of these people reside in less populated parts of the western U.S. and are frequently served by private water supply wells.

In this study, we used a biological marker of response, the micronucleus assay in exfoliated bladder cells, to evaluate the possible carcinogenic effects of chronic arsenic ingestion on the bladder. Micronuclei are extranuclear bodies in the cytoplasm of a cell that form when acentric chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis (12). The presence of micronuclei in a population of cells is an indication that chromosome damage has occurred as a result of

[3] The abbreviations used are: MCL, maximum contaminant level; MNC, micronucleated cells; CI, confidence interval; MMA, methylarsonic acid; InAs, inorganic arsenic; DMA, dimethylarsinic acid; FR, frequency.

exposure to a genotoxic agent that causes either clastogenic effects or spindle dysfunction (13).

The mechanism of arsenic carcinogenicity is not well understood; however, the results of short-term tests indicate that arsenic does not induce point mutations but rather acts as a clastogen, inducing the formation of chromosomal aberrations and micronuclei in human cells (14–16). Thus, arsenic is an appropriate agent to be evaluated using the micronucleus assay. Additional support for examining micronuclei in bladder cells derives from the fact that cigarette smoking and schistomiasis, both associated with increased bladder cancer risks, have been found to increase the frequency of micronuclei in bladder cells (17).

Exfoliated bladder cells are epithelial cells sloughed from the surface of the genitourinary tract. Epithelial cells turnover in the bladder every 1 to 2 weeks and micronucleated cells do not accumulate over time (18). Because epithelial cells are derived from basal cells, damage induced by a genotoxic agent that acts as a clastogen at the basal cell layer should theoretically be reflected in the frequency of micronuclei in exfoliated cells (12). The presence of micronuclei in exfoliated bladder cells may serve as a way to measure the extent to which arsenic is associated with DNA damage to the bladder and thus potentially provide evidence for increased risk of bladder cancer development (19).

Purpose of Study. To test the hypothesis that chronic ingestion of high levels of inorganic arsenic is associated with a detectable increase in the frequency of micronuclei in exfoliated bladder cells, a cross-sectional study was conducted in a small county in Nevada. According to historical water data maintained by the state of Nevada, Division of Water Planning, inorganic arsenic concentrations measured in private wells in that area have ranged from <10 to over 3000 μg/liter and have remained generally consistent within a given well. The high levels of arsenic in the ground water of this county are derived from the solubilization of arsenic-bearing minerals in geological formations (20). Some of these drinking water arsenic levels exceed those measured in Taiwan and are perhaps some of the highest reported in the world.

The overall objective of this study was to compare the frequency of micronucleated cells in exfoliated bladder and buccal cells between a group of individuals who chronically ingest high levels of inorganic arsenic and a matched control group with little exposure to arsenic. Bladder cells were selected because they represent cells from the target organ of concern, the bladder. Buccal cells were also collected for comparability with other studies. An increase in micronucleated buccal cells was not expected, however, because there is no epidemiological evidence that arsenic is associated with oral cancer.

Materials and Methods

Selection of Study Subjects. The study population included residents of a county in Nevada with private water supply wells. For this study, exposed subjects were defined as individuals with well water arsenic levels ≥500 μg/liter As, more than 10 times the U.S. MCL. Unexposed subjects were defined as individuals with well water arsenic levels ≤10 μg/liter As. To ensure chronic exposure, the study was restricted to individuals who had resided in their home for at least 1 year and used no additional water source such as filtered or bottled water.

A letter describing the study and the participation criteria was first sent to the 57 households in the county whose well water reportedly was ≥500 μg/liter As. Follow-up contact was made via telephone. Of the 13 eligible households identified, 11 agreed to participate (85%) and information about the ages and sex of household members was obtained. The primary reason for ineligibility of households was consumption of filtered or bottled water at home. The final exposed group consisted of 18 subjects from 11 households with well water arsenic levels over 500 μg/liter.

To reduce the potential for confounding, each subject in the exposed group was individually matched on age (±4 years), sex, and smoking status to a similarly identified subject in the unexposed group. A letter describing the study and the participation criteria was sent to 70 households in the community whose well water reportedly was ≤10 μg/liter As. Follow-up contact was made via telephone and at that time information about the ages and gender of household members was obtained. If a household member matched an exposed subject profile, that member was invited to participate in the study. All 18 eligible subjects agreed to participate (100%). Thus, the final study population comprised 18 matched pairs of exposed and unexposed participants.

Exposure Assessment. After giving written informed consent, each study subject was interviewed at home using a questionnaire regarding demographic information, daily fluid consumption, occupation, smoking status, and recent diet. To confirm the well water arsenic levels from the historical data, a sample of tap water was collected from each home and analyzed for arsenic by Environmental Protection Agency Method 6010 (21). As an additional measure of arsenic exposure, a spot urine sample was collected from each subject and analyzed for inorganic arsenic and its urinary metabolites, methylarsonic acid and dimethylarsinic acid.

The method used for arsenic speciation was a modification of the method of Crecelius (22) and has been previously reported (23). Inorganic and methylated arsenic species in urine were converted to their respective arsines by treatment with sodium borohydride under acidic conditions and were then collected by sparging and cryogenic trapping. Following the collection of arsine vapors, the trap was allowed to warm and the arsine species were sequentially volatilized and detected by atomic absorption spectroscopy using a microburner combustion cell. Detection limits for each of the arsenic species were 1–5 μg/liter with coefficients of variation of 0.1–0.15.

Exfoliated Cell Collection. After the interview, buccal cell samples were obtained by gently rubbing the inside of the subject's cheek with a premoistened wooden tongue depressor dipped in water. Cells were washed twice in a buffer solution of pH 7.0 that contained 0.1 M EDTA, 0.01 M Tris HCl, and 0.02 M NaCl. Gentle pipetting of cells in the buffer solution reduced clumping and lysed broken cells. Cells were dropped on a coded slide, air-dried, and fixed in 80% methanol at 0°C.

To obtain bladder cell samples, each subject was asked to provide a urine sample from the second and third void of the day. The first void of the day was not collected because exfoliated bladder cell degradation occurs when the cells have been in contact with urine overnight. Because females generally provide more cells per void than males, a total of two urine samples were obtained from females and four from males. Participants were supplied with precoded polypropylene bottles and instructions for urine collection. Bladder cells were collected from the urine specimens within 2 hours via centrifugation and the cell pellet was

washed with 0.9% NaCl. After centrifugation, cells were dropped on a coded slide, air-dried, fixed in 80% methanol at 0°C, and stored in a nitrogen atmosphere at −20°C until use for the micronucleus assay.

Micronucleus Assay. A new version of the micronucleus assay that uses the fluorescent dye propidium iodide and fluorescent *in situ* hybridization with a biotin-labeled probe for all human centromeres was used (24). This procedure is easier and more reliable than the previous method of Feulgen-Fast-Green staining and also allows for the mechanism of micronucleus formation to be determined.

Briefly, the slides were preheated for 30 min on a slide warmer at 63°C to fully attach the cells to the slide. They were then treated with 300 μg/ml pepsin for 30 min at 37°C to permeabilize the cells (25). The slides were subsequently rinsed twice in phosphate-buffered saline and fixed in buffered 4% paraformaldehyde for 20 min at 0°C. After washing, the slides were baked for 20 min at 63°C. They were then hybridized with a biotin labeled alpha-satellite probe for all human centromeres (Oncor) as described by Titenko-Holland *et al.* (24). The fluorescent dye, propidium iodide at 1 μg/ml in antifade solution, was used to counterstain the DNA.

Scoring Procedure and Criteria. All slides were first examined with low power magnification to observe the quality of the slide and the presence of polymorphonuclear leukocytes, bacteria, and fungi since heavy infections may interfere with scoring. Slides were then scored using a Nikon microscope equipped with epifluorescent illumination, a 100× oil immersion lens, and a filter for fluorscein and propidium iodide (excitation at 450–490 nm, dicroic at 510 nm, and emission at 520 nm).

Between 500 and 2,900 cells were scored for each subject. Only cells that were not smeared, clumped, or overlapped and that contained intact nuclei were included in the analysis. Cells undergoing abnormal cell division and degenerative processes such as karyorrhexis, karyolysis, nuclear fragmentation, or pyknosis (26) were recorded separately.

The frequency of micronucleated cells was estimated based on the number of normal exfoliated cells scored. Micronuclei had to: (*a*) be less than 1/3 the diameter of the main nucleus; (*b*) be in the same plane of focus; (*c*) have the same color, texture, and refraction as the main nucleus; (*d*) have a smooth oval or round shape; and (*e*) be clearly separated from the main nucleus. All micronuclei were photographed and cross-checked by two observers. Any questionable micronuclei were disregarded.

Statistical Analyses. The data were analyzed using PC SAS software (27). The analysis focused on the effect of arsenic exposure on the frequency of cells with micronuclei. All counts were, therefore, converted to frequency of MNC per 1000 normal exfoliated cells.

The mean frequency of MNC for the exposed and unexposed groups was computed with and without weighting the frequency of MNC for each subject by the number of normal cells scored. By including a weighting term, it was possible to account for the variation in the number of normal cells scored per subject and thus reduce the overall variance of the mean frequency for each group.

The general formula for computing the weighted mean frequency is:

$$F_i = \sum_{j=1}^{18} w_{ij} \times f_{ij}$$

where i = 0 for unexposed and 1 for exposed; $f_{ij} = mnc_{ij}/n_{ij}$ = frequency of MNC for subject ij; $w_{ij} = n_{ij}/N_i$ = weight factor; mnc_{ij} = micronucleated cells scored for subject ij; n_{ij} = number of normal cells scored for subject ij; and N_i = total number of normal cells scored for group i.

The frequency ratio, contrasting the mean frequency (weighted or unweighted) of MNC among the exposed and unexposed groups, was selected as the measure of effect. The frequency ratio was computed:

$$FR = F_1/F_0$$

where FR = frequency ratio; F_1 = mean frequency of MNC for exposed; and F_0 = mean frequency of MNC for unexposed. The 90% CI were calculated for the frequency ratios:

$$90\%\ CI = \exp[\ln FR \pm 1.645[\mathrm{var}(\ln FR)]^{1/2}]$$

where $\mathrm{var}(\ln FR) = [v_1/F_1^2 + v_0/F_0^2]$; and v_i = [standard error $(F_i)]^2$ (28).

Because the data were not normally distributed, statistical significance of the effect measure was assessed by the Wilcoxon sign-rank test. It was hypothesized *a priori* that arsenic exposure would be associated with an increase in the frequency of MNC, so one-tailed tests were used.

In addition to dichotomous exposure status, the following arsenic exposure indices were investigated:

(1) Exposure Index 1 (μg As/liter) = tap water arsenic (μg As/liter);
(2) Exposure Index 2 (μg As/day) = tap water arsenic (μg As/liter) × liters As-fluid consumed per day (liters/day);
(3) Exposure Index 3 (μg As/liter fluid) = Index 2 (μg As/day)/total liters fluid consumed per day (liters fluid/day);
(4) Sum of urine arsenic species (Sum) (μg/liter) = InAs + MMA + DMA;
(5) InAs in urine (μg/liter);
(6) MMA in urine (μg/liter); and
(7) DMA in urine (μg/liter).

Exposure index 1 reflects the household tap water arsenic concentration. Exposure indices 2 and 3 were estimated by incorporating fluid consumption data obtained during the interview to provide a more refined measure of individual exposure. Exposure index 2 reflects individual daily arsenic exposure. Exposure index 3 reflects individual daily arsenic exposure relative to total daily fluid consumption, thus enabling consideration of the possibility that the volume of other fluids consumed may dilute the effect of arsenic on the bladder.

The dose-response relationship between each arsenic exposure measure and frequency of MNC was assessed by regression analysis. Analysis of covariance was conducted to examine the relation between the other covariates and frequency of MNC while adjusting for arsenic exposure.

Results

Descriptive data comparing the 18 exposed and 18 unexposed subjects are summarized in Table 1. The exposed and unexposed groups were each composed of eight males and 10 females. Four of the females in each group were current smokers. All men in the study were either nonsmokers or ex-smokers. The average age of the exposed group was 37.5 years (range, 14–74 years), which was similar to that of the unexposed group (37.0 years; range,

Table 1 Descriptive characteristics of study participants

	Exposed (n = 18; 8 Males, 10 Females)			Not exposed (n = 18; 8 Males, 10 Females)		
	Mean	SD	Range	Mean	SD	Range
Age (yrs)	37.5	14.2	(14–74)	37.0	13.8	(16–70)
Education (yrs)	13	2	(8–17)	13	2	(11–17)
Duration of residence	4	3	(1–13)	5	4	(1–13)
Estimated total fluid intake (liters/day)	3.8	1.5	(2.4–6.9)	3.6	1.3	(1.7–6.4)
Vegetables[a] (servings/week)	9.2	5.2	(3–20)	13.0	6.7	(1–27)
Dark green vegetables[b] (servings/week)	1.9	2.0	(0–7)	2.1	1.8	(0–6)
Carrots (servings/week)	1.3	1.8	(0–7)	2.9	2.9	(0–10)
	Frequency (%)			Frequency (%)		
Alcohol consumption						
Any	6	(33.3)		9	(50.0)	
None	12	(66.7)		9	(50.0)	
Coffee consumption						
1+ cup/day	10	(55.6)		8	(44.4)	
<1 cup/day	8	(44.4)		10	(55.6)	
Smoking						
Current	4	(22.2)		4	(22.2)	
Ex	3	(16.7)		4	(22.2)	
Never	11	(61.1)		10	(55.6)	
Average cigarettes/day (Current smokers)	27.5			27.5		
Oral snuff use						
Current	1	(5.6)		1	(5.6)	
Never	17	(94.4)		17	(94.4)	

[a] Vegetables include broccoli, spinach, dark green lettuces, corn, tomatoes, peas, green beans, cabbage, and carrots.
[b] Dark green vegetables include broccoli, spinach, and dark green lettuces.

Table 2 Summary of arsenic exposure measures [mean (SE)][a]

	Exposure index 1 (μg As/liter)	Exposure index 2 (μg As/day)	Exposure index 3 (μg As/liter fluid)	Urine arsenic species (μg/liter)			
				Sum As	InAs	MMA	DMA
Exposed	1,310	2,260	630	750	170	190	390
(n = 18)	(350)	(570)	(160)	(150)	(47)	(45)	(66)
Not Exposed	16	36	12	68	9	14	44
(n = 18)	(7.2)	(15)	(5.6)	(23)	(2.5)	(4.8)	(16)
Mean Difference	1,296	2,224	621	683	158	177	348
P^* (one tail)[b]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] SE, standard error; Sum As = InAs + MMA + DMA; Index 1 = tap water arsenic level; Index 2 = tap water arsenic level × liters As-containing fluid consumed per day; Index 3 = tap water arsenic level × (liters As-containing fluid consumed per day/total liters fluid consumed per day).
[b] Wilcoxon Sign Rank Test.

16–70 years). The average age difference for the 18 pairs of subjects was 0.6 years. The descriptive data show that the matching criteria (age, sex, smoking status) were well met and that the exposed and unexposed groups do not differ significantly with respect to the other covariates measured. None of the subjects were occupationally exposed to genotoxic agents.

The mean, median, and range of each measure of arsenic exposure is presented for the exposed and unexposed groups in Table 2. Although we attempted to include in the unexposed group only individuals with well water arsenic levels <10 μg/liter, one individual had a level of 100 μg/liter. As a result, the average exposure level to arsenic for the unexposed group was 16 μg/liter (mean) and 5 μg/liter (median). Nonetheless, by any measure of arsenic exposure, ranging from household tap water arsenic concentration to a more refined estimate of arsenic consumption or a spot urine arsenic concentration, the exposed group is very highly exposed compared to the controls.

A comparison of the weighted mean frequencies of MNC in bladder and buccal cells between the exposed group and the unexposed group is presented in Table 3. There was a 1.8-fold increase in the weighted mean frequency of MNC in bladder cells of the exposed group compared with the unexposed group (90% CI, 1.06–2.99). The weighted mean frequency in the exposed group was 2.79/1000 cells as compared with 1.57/1000 cells in the unexposed group. In contrast, the frequency ratio for buccal cells was 1.0 (90% CI, 0.65–1.53).

In Table 4, a comparison of the weighted mean frequencies of MNC in bladder and buccal cells between the exposed and unexposed groups after stratification on sex is presented. The weighted mean frequency of MNC in blad-

Table 3 Comparison of MNC frequency in exfoliated bladder and buccal cells by arsenic exposure status

	Exfoliated bladder cells			Exfoliated buccal cells		
	MNC frequency/ 1000 cells Mean (SE)	Frequency ratio (90% CI)	*P*-Value[a] (one-tail)	MNC frequency/ 1000 cells Mean (SE)	Frequency ratio (90% CI)	*P*-Value[a] (one-tail)
Exposed	2.79 (0.73) (n = 18)	1.78 (1.06, 2.99)	0.09	2.49 (0.42) (n = 16)	1.00 (0.65, 1.53)	0.5
Not exposed	1.57 (0.28) (n = 18)			2.50 (0.50) (n = 16)		

[a] Wilcoxon Sign Rank Test. *Note:* The apparent discrepancy between a *P* value of 0.09 and a confidence interval excluding 1 is due to the test of significance being nonparametric whereas confidence limits were derived with parametric statistics.

Table 4 Comparison of MNC frequency in exfoliated bladder and buccal cells by arsenic exposure status and sex

	Exfoliated bladder cells			Exfoliated buccal cells		
	MNC frequency/ 1000 cells Mean (SE)	Frequency ratio (90% CI)	*P*-Value[a] (one tail)	MNC frequency/ 1000 cells Mean (SE)	Frequency ratio (90% CI)	*P*-Value[a] (one-tail)
Males						
Exposed (n = 8)	5.00 (1.50)	2.34 (1.27, 4.29)	0.07	1.36 (0.44)	0.89 (0.42, 1.86)	0.34
Not Exposed (n = 8)	2.14 (0.46)			1.53 (0.48)		
Females						
Exposed (n = 10)	1.82 (0.53)	1.42 (0.76, 2.65)	0.38	3.47 (0.48)	1.00 (0.66, 1.49)	0.40
Not Exposed (n = 10)	1.28 (0.31)			3.50 (0.72)		

[a] Wilcoxon Sign Rank Test.

der cells was clearly elevated among exposed males compared to unexposed males (FR = 2.34; 90% CI, 1.27–4.29); however, there was little difference between exposed females and unexposed females (FR = 1.42; 90% CI, 0.76–2.65). The frequency ratio for buccal cells remained close to one for both males (FR = 0.89; 90% CI, 0.42–1.86) and females (FR = 1.00; 90% CI, 0.66–1.49).

The unweighted results were not significantly different from the weighted results presented in Tables 3 and 4. There was a two-fold increase in the unweighted mean frequency of MNC in bladder cells of the exposed group (3.36/1000 cells) compared with the unexposed group (1.67/1000 cells). The frequency ratio for buccal cells was 1.1 (90% CI, 0.73–1.70). After stratifying on sex, the unweighted and weighted results remained similar. The unweighted mean frequency of MNC in bladder cells was elevated among exposed males compared to unexposed males (FR = 2.49; 90% CI, 1.32–4.70); but there was little difference between exposed and unexposed females (FR = 1.37; 90% CI, 0.67–2.77). The frequency ratio for buccal cells remained close to one for both males (FR = 0.83; 90% CI, 0.40–1.74) and females (FR = 1.18; 90% CI, 0.76–1.84).

Fig. 1 presents a scatter plot of the relationship between the frequency of MNC in bladder cells and the sum of arsenic species in urine for males and females. The figure suggests that an increase in arsenic exposure is accompanied by an increased frequency of micronucleated bladder cells. In addition, the apparent increase appears to be stronger for males compared to females.

Associations between each measure of arsenic exposure and the frequency of MNC in bladder cells are summarized in Table 5 with Spearman correlation coefficients and one-way *P* values. Adjusting for age, sex, and smoking status did not change the findings; therefore, only the unadjusted results are presented. Each arsenic exposure index is positively associated with the outcome, with index 3 reflecting the strongest correlation. Each urine arsenic species measure is also significantly positively associated with the outcome. In contrast, all arsenic exposure measures were neither significantly associated with the frequency of MNC in buccal cells nor were they consistent in terms of direction of association.

After adjusting for arsenic exposure and sex, no significant or suggestive associations were found between the weighted mean frequencies of MNC in bladder cells or buccal cells and the other covariates measured. There was no evidence of any association between the frequency of cells undergoing abnormal cell division and degenerative processes and arsenic exposure.

Discussion

The results of the exposure assessment portion of this study confirm that residents of the U.S. can be very highly exposed to inorganic arsenic through ingestion of arsenic-contaminated drinking water. In this study, due to the very high exposure concentrations (average level, 1312 μg/liter As), subjects consumed an average of 2260 μg As/day, much more than the 100 μg As/day estimated for individuals who consume 2 liters of water/day containing arsenic at the MCL (50 μg/liter).

The major route for inorganic arsenic detoxification involves methylation of arsenic by methyltransferase fol-

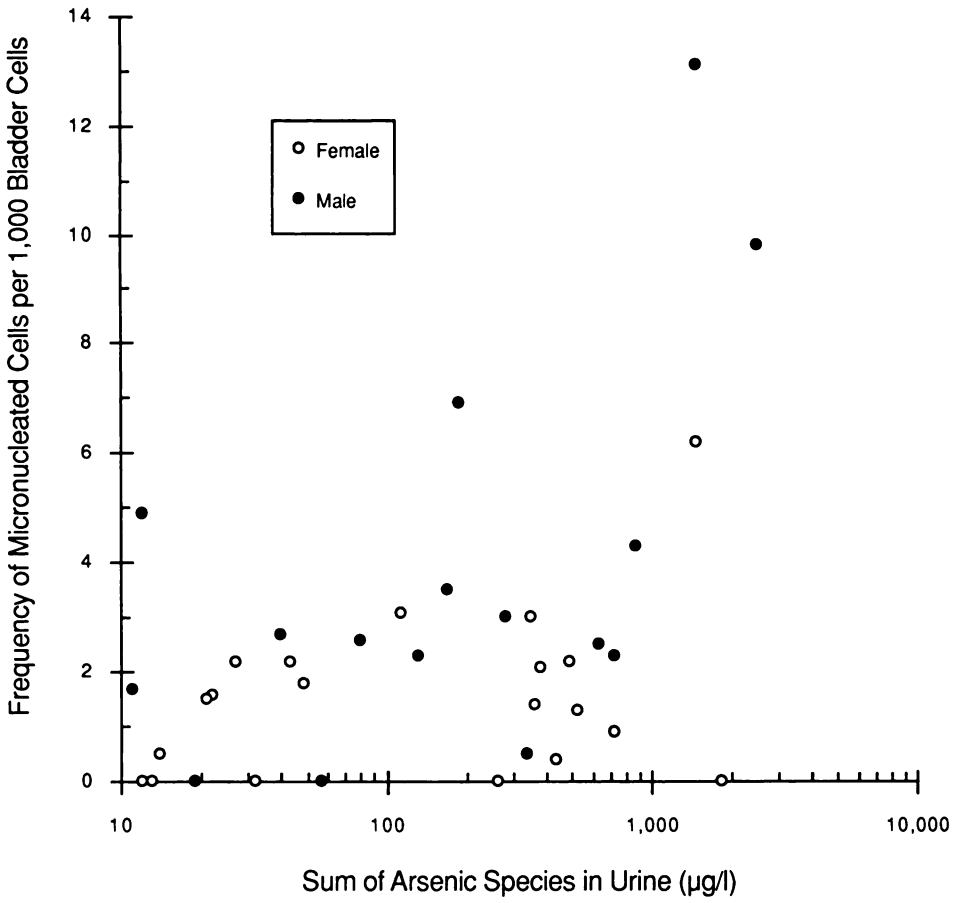

Fig. 1. Relationship between the frequency of micronucleated cells/1000 exfoliated bladder cells and sum of arsenic species in urine.

Table 5 Summary of spearman correlation coefficients for arsenic exposure indices and frequency of MNC in bladder and buccal cells[a]

	Exposure status (yes/no)	Exposure index 1 (µg As/liter)	Exposure index 2 (µg As/day)	Exposure index 3 (µg As/liter fluid)	Urine arsenic species (µg/liter)			
					Sum As	InAs	MMA	DMA
Frequency of MNC/ 1000 bladder cells	0.209	0.258	0.222	0.284	0.326	0.283	0.388	0.296
P-value (one-way)	(0.11)	(0.06)	(0.10)	(0.05)	(0.03)	(0.05)	(0.01)	(0.04)
Frequency of MNC/ 1000 buccal cells	0.068	0.106	0.083	0.089	−0.054	−0.056	−0.025	−0.075
P-value (one-way)	(0.36)	(0.28)	(0.33)	(0.32)	(0.39)	(0.38)	(0.45)	(0.34)

[a] Sum As = InAs + MMA + DMA; Index 1 = tap water arsenic level; Index 2 = tap water arsenic level × liters As-containing fluid consumed per day; Index 3 = tap water arsenic level × (liters As-containing fluid consumed per day/liters fluid consumed per day).

lowed by elimination via urinary excretion. The sum of the urinary concentration of inorganic arsenic and its methylated metabolites, MMA and DMA, is therefore considered to be a good biological measure of arsenic exposure. After exposure, inorganic arsenic and its metabolites can be measured in the urine in the following approximate proportions: InAs, 20%; MMA, 20%; and DMA, 60% (29). It has been proposed that a threshold exposure concentration exists at which arsenic methylation becomes saturated (30). Above the threshold concentration arsenic methylation activity would become saturated, and as a consequence, the proportion of methylated urinary metabolites (MMA and DMA) would decrease.

In this study, the mean proportions of InAs, MMA, and DMA measured in the urine were 19.5, 22.0, and 58.4% for the exposed group, and 18.6, 21.7, and 59.6% for the unexposed group, respectively. Urine arsenic metabolite profiles for both the exposed and unexposed groups do not differ significantly from each other or from that reported by others (31–33). This finding suggests that with chronic ex-

posure, there is no threshold exposure concentration and the ability to detoxify inorganic arsenic by methylation does not diminish even at very high exposure concentrations. The findings are consistent with data from many other population studies (29).

Although other studies have been conducted to confirm exposure to arsenic in the U.S. (34–39), only one study has utilized a biological marker of response to assess possible genotoxic effects of arsenic. No differences in the frequencies of chromosome aberrations or sister chromatid exchanges were found in lymphocytes of individuals who were exposed to 100 μg/liter arsenic in drinking water compared to unexposed controls (40). However, the population studied was exposed to much lower levels of arsenic than the current study population and arsenic has not been shown to be associated with cancer in blood-forming tissue.

The results of this study provide evidence that chronic ingestion of high levels of inorganic arsenic in drinking water is associated with an increased frequency of MNC in exfoliated bladder cells. The weighted mean frequency of MNC in bladder cells of the exposed group is increased approximately 1.8-fold compared to the unexposed group. The weighted mean frequency rate of MNC in bladder cells among the unexposed group is similar to the frequency rates reported in other studies of control populations (24, 41–43). The finding that each of the arsenic exposure indices was significantly positively associated with the frequency of MNC in bladder cells is suggestive of a dose-related effect (Table 5).

It is noteworthy that after stratification on sex (Table 4), the findings are less consistent. The frequency ratio in bladder cells remained elevated among exposed males compared to unexposed males; however, there was little difference between exposed females and unexposed females. This apparent lack of consistency may be due to one of three possible explanations. First, perhaps male bladder cells are more susceptible to genotoxic damage caused by chronic ingestion of high levels of inorganic arsenic than female bladder cells. This explanation is consistent with the approximately three-fold increase in the incidence of bladder cancer observed in males relative to females that cannot be explained entirely by differences in exposure to known risk factors for bladder cancer (44).

Second, it is possible that there is no difference in the risk of genotoxic damage to the bladder cells of males and females from chronic ingestion of high levels of inorganic arsenic, but due to the small sample size of this study, the power to detect an effect after stratification on sex is very small.

Third, the apparent lack of consistency may be due to the fact that males exfoliate almost exclusively transitional bladder cells in the urine, while females primarily exfoliate squamous cells from the trigone of the bladder and only a small percentage of transitional bladder cells in the urine (45, 46). In addition, the rate of exfoliation of squamous cells varies with the female hormonal cycle. Since arsenic exposure has been shown to be associated with transitional cell carcinoma, the exfoliation of squamous cells by females (which may not be affected by arsenic exposure) without the ability to differentiate them from transitional cells could be expected to dilute any real association between chronic ingestion of inorganic arsenic and bladder cell micronuclei. The occurrence of such bladder cell misclassification among females but not males could be expected to bias the results among females towards no effect. A larger study must be conducted to confirm these findings and determine which explanation is correct.

It is noteworthy that as hypothesized a priori, the results provide no evidence that the frequency of MNC in exfoliated buccal cells is associated with chronic arsenic ingestion. As observed for the bladder cells, the weighted mean frequency rate of MNC in buccal cells among the unexposed group falls within the range of frequency rates reported in other studies of control populations (24, 41, 47–51). In contrast to the significantly positive correlation observed between each arsenic exposure index and the frequency of micronucleated bladder cells, none of the exposure indices was associated with the frequency of micronucleated buccal cells.

In summary, this is the first study to show genotoxic effects of chronic ingestion of high levels of inorganic arsenic in the U.S. The results of this study provide evidence that chronic ingestion of high levels of inorganic arsenic is associated with an increased frequency of MNC in exfoliated bladder cells. Although there is no documented direct association between micronuclei formation and development of cancer, an increased frequency of MNC in exfoliated bladder and buccal cells has been reported in studies of populations with exposures to known risk factors for bladder cancer (41–43, 52) and oral cancer (19, 41, 48, 50, 53–55).

The significance of these findings with respect to bladder cancer risk for people ingesting high concentrations of arsenic in drinking water remains to be determined. However, it is becoming increasingly apparent that carcinogenesis requires the progressive accumulation of numerous genetic alterations in a target tissue (56, 57). An observed increase in the amount of genetic damage occurring in a tissue should be associated with an elevation in risk for development of cancer at that site. Such a situation would increase the probability of the tissue acquiring the genetic changes necessary for carcinogenesis to occur (58).

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