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# HEALTH EFFECTS OF DEPLETED URANIUM ON EXPOSED GULF WAR VETERANS: A 10-YEAR FOLLOW-UP

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*Medical surveillance of a group of U.S. Gulf War veterans who were victims of depleted uranium (DU) "friendly fire" has been carried out since the early 1990s. Findings to date reveal a persistent elevation of urine uranium, more than 10 yr after exposure, in those veterans with retained shrapnel fragments. The excretion is presumably from ongoing mobilization of DU from fragments oxidizing in situ. Other clinical outcomes related to urine uranium measures have revealed few abnormalities. Renal function is normal despite the kidney's expected involvement as the "critical" target organ of uranium toxicity. Subtle perturbations in some proximal tubular parameters may suggest early although not clinically significant effects of uranium exposure. A mixed picture of genotoxic outcomes is also observed, including an association of hypoxanthine-guanine phosphoribosyl transferase (HPRT) mutation frequency with high urine uranium levels. Findings observed in this chronically exposed cohort offer guidance for predicting future health effects in other potentially exposed populations and provide helpful data for hazard communication for future deployed personnel.*

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The first widespread use of depleted uranium (DU) by U.S. military forces in the 1991 Gulf War created an unintended consequence of exposing soldiers to this radioactive heavy metal already well known for its chemical toxicity in workers in the nuclear industry (ATSDR, 1999). Possessing almost twice the density of lead, DU is relatively low in cost and is used both as material for tank armor and in armor-piercing weapon rounds. A by-product of the uranium enrichment process, DU possesses only 60% of the radioactivity of natural uranium, as it has been "depleted" of much of the more radioactive $^{235}U$ and $^{234}U$ isotopes (Army Environmental Policy Institute, 1995).

Uranium decays primarily by high-energy emission of alpha particles, which travel short distances in tissues; thus the principal radiological hazard is to tissues in immediate contact with internalized DU small particles or fragments. The dose is a function of contact time, particle solubility, and rate of elimination (Army Environmental Policy Institute, 1995; Eckerman, 1988).

Several exposure scenarios occurred in the 1991 Gulf War conflict, the most significant involving "friendly fire" incidents during which tank crews were fired upon with DU penetrators. The majority of these exposures were of short duration and involved inhalation of aerosolized DU particles that were primarily uranium oxides. These exposures occurred in individuals on or in a tank when it was hit, or in rescuers on the scene immediately thereafter. DU particles could also have contaminated wounds or could have been ingested following coughing to clear airways. Another more unique exposure scenario has developed chronically over time, whereby DU shrapnel fragments embedded in soft tissue are oxidizing in situ and allowing systemic, ongoing uranium absorption.

Questions regarding the long-term health consequences of these exposures have fueled considerable debate regarding continued use of DU in combat. Responding to these health concerns in the early 1990s, the Department of Veterans Affairs (DVA) and the Department of Defense (DoD) initiated a medical surveillance follow-up program for veterans involved in the DU friendly fire incidents. The health effects of concern derive from both uranium's radiologic and its chemical, heavy metal characteristics. Although natural uranium and DU are radioactive, they do not appear to be highly carcinogenic. There is poor evidence for an excess cancer risk specifically of lung, bone, or kidney (the most likely targets) in occupational cohorts (ATSDR, 1999; Institute of Medicine, 2000) whose exposure intensities were greater and of longer duration than the Gulf War-exposed groups. The lung cancer excess observed in uranium miners has been well documented to be attributed to radon present in the mines (Samet et al., 1989; Samet, 1989). Radon is a more intensely radioactive constituent than natural uranium by a factor of 10,000 (Kathren & Moore, 1986; Kathren et al., 1989). Little to no decay products beyond $^{234}U$ exist in DU, as these are separated in the processing of the uranium ore. New post-$^{234}U$ decay products have not had sufficient time to form since leaving the processing plants due to the 10,000-yr half-life of thorium-230, the initial decay product of $^{234}U$ (Papastefanou, 2002). Radiation dose estimates for Gulf War veterans with shrapnel calculated from whole-body radiation counting using the ICRP 30

Biokinetic model for uranium yielded upper limits of 0.1 rem/yr (the public dose limit) and 5.3 rem/50 yr (with the annual occupational exposure limit being 5 rem/yr as a comparison) (McDiarmid et al., 2000). Therefore, uranium's chemical toxicity has been the primary focus of the surveillance of the Gulf War veterans, with emphasis on the target organs most likely affected by uranium and other heavy metals—the kidney, the central nervous system, and the reproductive system.

To date, four rounds of surveillance (1994, 1997, 1999, 2001) have been conducted on an inpatient basis at the Baltimore VA Medical Center (BVAMC). The principal finding thus far has been that mean urine uranium excretion is significantly higher in veterans with confirmed retention of metal fragments in soft tissue compared to either those DU-exposed without fragments (Hooper et al., 1998: McDiarmid et al., 2000, 2001) or a comparison population of Gulf War deployed, but not DU-exposed veterans (McDiarmid et al., 2000). Multiple smaller fragments remain in some veterans despite surgeries because the fragments are not easily accessible or due to risk of excessive surgical morbidity associated with their removal. Veterans without retained fragments possess a urine uranium concentration similar to that of the comparison population and other published normal values for urine uranium (Dang et al., 1992; Medley et al., 1994; Ting et al., 1999).

This study reports results of the 2001 clinical assessment of this cohort, a 10-yr follow-up since exposure first occurred during the Gulf War.

## MATERIALS AND METHODS

Thirty-nine Gulf War veterans who had been exposed to DU during friendly fire incidents in February 1991 were evaluated at the Baltimore VA Medical Center between April and July 2001. Thirty-one of these had been seen previously on at least one occasion. Eight were examined for the first time.

### Clinical Assessment

The clinical assessment included a detailed medical history including an extensive exposure history, a thorough physical examination, laboratory studies, and radiologic surveys for retained DU fragments. The laboratory studies included hematologic and blood clinical chemistry measures, as well as neuroendocrine, immunologic, and genotoxicologic parameters. Semen quality was also evaluated. Urine samples were obtained for measurement of total uranium excretion and clinical chemistry parameters related to renal function. Participants also underwent a battery of neurocognitive tests. New participants were also clinically evaluated for post traumatic stress disorder (PTSD) and substance abuse.

### Uranium Exposure Assessment

Twenty-four-hour urine specimens were sent to STL Richland (formerly Quanterra, Inc., and International Technology Analytic Services) in Richland,

WA, for total uranium analysis. Ashed urine specimens dissolved in dilute hydrochloric acid were passed through a base anion-exchange resin (Bio-Rad AGMP-1) from which the retained uranium was eluted with a small volume of dilute nitric acid. Uranium concentrations were determined using a kinetic phosphorescence analyzer (KPA), with a minimum detection concentration of 0.006 μg/L of urine.

The concentration of uranium in a 24-h collection of urine, expressed as micrograms per gram creatinine, is used in this study as an exposure measure in 3 forms: its natural metric, as a binary variable, and as its natural logarithm (ln), as follows:

*Urine uranium as a binary variable* Two exposure groups, high ($n=13$) versus low ($n=26$), were determined based on the individual participant's 2001 urine uranium results. High exposure was defined as urine uranium concentrations greater than 0.10 μg/g creatinine. While there is no generally accepted standard normal urine uranium value, a value was chosen intermediate between, at the low end, several estimates of mean urine uranium concentration in nonexposed populations in the literature (11–22 ng/L) (Dang et al., 1992; Medley et al., 1994; Ting et al., 1999), and at the high end, upper dietary limits due to natural uranium in soil and ground water (up to 0.35 μg/L) urine (ICRP, 1974).

*Natural logarithm of urine uranium* The natural log of the 24-h urine uranium measure was used as a continuous variable in regression equations predicting neurocognitive outcomes after controlling for intelligence and emotional factors. The natural log transformation was used to correct extreme skewness. This value was also used as a continuous variable in the analysis of the association between mutation frequency and urine uranium.

### Hematologic and Renal Toxicity Measures

Hematologic parameters, serum and urine creatinine, and serum uric acid measures were evaluated by the VA clinical laboratory using standard methodologies. Urine samples that included a first morning void were kept on ice until collected. Aliquots removed for $\beta_2$-microglobulin analysis were immediately neutralized using 0.5 *N* NaOH. $\beta_2$-Microglobulin was analyzed by microparticle enzyme immunoassay by Quest Diagnostics Laboratory. Five-milliliter aliquots removed for retinol binding protein analysis were immediately stabilized by addition of 250 μl of stabilization buffer (1 *M* imidazole, 2% Triton X-100, 20 m*M* benzamidine, 2000 U/ml aprotinin, 1% sodium azide, pH 7.0) and frozen at −70 °C until analysis. Retinol binding protein was measured using an automated nonisotopic immunoassay based on latex particle agglutination (Bernard & Lauwerys, 1983). Total protein was measured using the BCA protein assay (Pierce, Rockford, IL).

### Neurocognitive/Psychiatric Assessment

All participants were administered a neurocognitive test battery consisting of traditional (paper and pencil) and automated measures, similar to the batteries used during earlier evaluations of DU-exposed veterans (McDiarmid et al., 2001).

Traditional paper and pencil tests were used to construct a neurocognitive impairment index, the NP4. Included in the NP4 were the following tests: Digit Span, Arithmetic, Block Design, Digit Symbol, Letter–Number Sequencing, and Symbol Search from the Wechsler Adult Intelligence Scale–III; Total Score Trials 1–5 and Long Term Recall (percent retained) from the California Verbal Learning Test–II; and parts A and B from the Trail Making test.

The automated neurocognitive measures were selected tests from the Automated Neuropsychological Assessment Metrics (ANAM) (Kane & Reeves, 1997) test library. ANAM was developed by the Department of Defense for studies in chemical defense and countermeasures and to study alterations in human performance as a result of various environmental stressors and neurological insults. Three general performance indices were derived from the automated measures reflecting impairment in: response accuracy (A-IIac), median response time for correct responses (A-IIrt), and the number of correct responses per minute, or throughput (A-IItp).

Four impairment indices (NP4, A-IIac, AIIrt, and A-IItp A) were constructed. They represent the proportion of scores within each set that were greater than one standard deviation below the mean. Hence, higher impairment scores were indicative of more problematic performance on each set of neurocognitive measures.

Participants also completed measures designed to assess potential confounders (intelligence and depression) of the association of urine uranium with neurocognitive outcomes. Predeployment intellectual functioning was estimated using participants' scores on the average of Information and Similarities tests of the WAIS–III. Neither of these two measures was used to compute impairment ratings. Emotional status was assessed by the Beck Depression Inventory (BDI) (Beck et al., 1996).

### Reproductive Health Measures

*Neuroendocrine parameters* Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin were analyzed by the microparticle enzyme immunoassay (MEIA) using an Abbott AxSYM analyzer. Thyroid-stimulating hormone (TSH), free thyroxine, and total testosterone were analyzed by electrochemiluminescence immunoassay using a Roche ELECSYS 2010 analyzer. These assays were performed at the Baltimore VA clinical laboratory.

*Semen characteristics* Evaluation of semen characteristics included volume, sperm concentration, total sperm count, and functional parameters of sperm motility. All veterans were requested to abstain for 2 d prior to their arrival at BVAMC. Collected specimens were immediately transported to the laboratory, allowed to liquefy, and examined for count, motility, and motion parameters using computer-assisted semen analysis (Hobson Vision, Ltd.). Procedures for semen dilution, enzyme treatment, and mechanical disruption have been previously described (McDiarmid et al., 2001). Semen dilution to permit sperm motion studies was required in many cases ($n = 6$ low urinary uranium subjects, $n = 6$ high urinary uranium subjects), and specimens not liquefied within 25 min

at 37 °C ($n = 10$ low urinary uranium subjects, 6 high urinary uranium subjects) were subjected to enzyme treatment. One subject's sample (high urinary uranium) also required mechanical disruption after enzyme treatment to complete liquefaction of the specimen. World Health Organization (1987) criteria were used for an assessment of normality for the semen parameters measured.

### Genotoxicity Measures

*Chromosomal aberrations (CA) and sister chromatid exchange (SCE)* Peripheral blood lymphocytes were cultured for the examination of background frequencies of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs). Using standard methods, cells were cultured for 48 h for CAs and 72 h for SCEs, stained, and evaluated for the 2 conditions (Perry & Wolff, 1974; Evans & O'Riordan, 1975; Swierenga et al., 1991). Fifty cells for CAs and 25 cells for SCEs were examined from each sample. In addition to baseline measures, SCEs were also measured after challenge with two doses of bleomycin (2 μg/ml and 4 μg/ml).

*Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mutation assay* To assess mutagenic effects of DU exposure, HPRT mutations were measured in peripheral blood lymphocytes. Venous blood samples (~30 ml) were obtained in heparinized vacuum tubes in Baltimore and sent at ambient temperature by overnight airmail to Burlington, VT. On receipt, blood samples were centrifuged and the mononuclear cell fractions (containing the lymphocytes) were separated, washed, counted, and cryopreserved in liquid nitrogen. Within approximately 2 wk, mononuclear cells were thawed, added to RPMI 1640 tissue culture medium, centrifuged, counted, and assayed by standard T-cell cloning assay as described previously (O'Neill et al., 1987, 1989). The mononuclear cells were then inoculated in limiting dilution in 96-well round bottom microtiter dishes at 1, 2, and 5 cells per well in the absence of 6-thioguanine and at 1 to $3 \times 10^4$ cells per well in the presence of 6-thioguanine to select for *HPRT* mutants. Following a 10- to 16-d incubation, microtiter dishes were scored using an inverted phase contrast microscope to identify growing colonies. Cloning efficiencies in the nonselection and selection dishes were determined from the frequencies of negative wells to determine the $P_0$ class of Poisson distribution. Cloning efficiencies (CE) are equal to $(\ln P_0)/N$, where $P_0$ is the fraction of wells without cell growth and $N$ is the number of cells inoculated per well. The ratio of CE in the presence to CE in the absence of 6-thioguanine selection defined the mutant frequency.

### Immunologic Measures

Lymphocytes were prepared from ethylenediamine tetraacetic acid (EDTA)-treated whole blood using density-gradient centrifugation with lymphocyte separation medium (ICN/Cappel, Aurora, OH). Lymphocytes were diluted in 10% dimethyl sulfoxide (DMSO) and frozen in liquid nitrogen until staining. On the day of staining, lymphocytes were quickly thawed and washed twice to remove DMSO. The following anti-human antibodies purchased from

Pharmingen (San Diego, CA) were used for staining: fluorescein isothiocyanate-labeled CD4, CD8, CD14, CD16; *R*-phychoerythrin-labeled CD3, CD45RA, CD28; and Cy-Chrome-labeled CD8, CD19, CD56, CD45RO. Cells were triple labeled with appropriate antibodies or isotype control for 30 min at 4 °C, washed, and fixed. Cell staining was measured using a FACSCAN flow cytometer and data were analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Diego, CA). Lymphocyte acquisition was based on forward and side scatter properties of the cells. CD4-positive and CD8-positive T cells were acquired for analysis of CD28, CD45RA, and CD45RO expression.

Other clinically available immunologic measures were also obtained, including immunoglobulins, complement, C-reactive protein, rheumatoid factor, antinuclear antibodies (ANA), and antithyroid peroxidase. These analyses were conducted by the BVAMC clinical laboratory.

### Statistical Data Analysis

*Criterion for reporting differences* Because this is a surveillance program, it is important to follow even subtle adverse DU health effects. However, because the number of DU-exposed Gulf War Veterans in the group is small ($n = 39$), the power to detect subtle effects is low. To deal with this problem we report not only differences meeting traditional statistical significance levels ($p \leq .05$, two-sided test), but also certain differences with $.05 < p \leq .2$ These latter differences are reported if they (1) are in line with biological plausibility, (2) are in the expected direction, (3) are consistent with previous findings, or (4) if a confounder is believed to be masking an association.

*Tests of differences/associations* Differences in outcome measures between high and low urine uranium groups were examined using the Mann–Whitney *U*-test or Fisher's exact test. Further analysis was conducted if results met the criteria already detailed.

Linear regression was used to characterize associations between the outcomes of interest and the exposure variable, the natural logarithm (ln) of urine uranium, and to determine whether associations persisted after adjustment for possible confounders. Regression diagnostics were used to determine the suitability of the data for linear regression analysis. Where criteria for regression were not met, corrections were applied when possible. In one case (chromosomal aberrations, CA) in which the outcome consisted of only three values, the relationships were explored among possible confounders, predictors, and outcome values to try to obtain the clearest understanding of the relationship between CA and urine uranium.

Where outliers were found (A-IIac as a function of ln urinary uranium), robust regression (StataCorp, 2001) was used, which down-weights outliers.

Stata's fracpoly (fractional polynomial) and mfracpol (multivariable fractional polynomial) commands (StataCorp, 2001) were used to study the nonlinear relationship of the ln(HPRT mutation frequency) to the ln(urine uranium). As a result, the ln(urine uranium) was transformed to a cubic: $[\ln(\text{urine uranium}) + 6.86]^3$, where –6.86 is the minimum value of ln(urine uranium). A statistically significant

relationship was found between ln(HPRT mutation frequency) and [ln(urine uranium)+6.86]$^3$. An influence diagnostic (dfbeta) was used to investigate whether any individuals unduly influenced the relationship. The persistence of the relationship was examined after adjustment for possible confounders by entering the confounders into the equation one or two at a time.

SPSS 10.0 (Statistical Products and Service Solutions, 1999) was used for all but the regression analyses, which were done using Stata software (StataCorp, 2001).

## RESULTS

The demographic characteristics of the 39 members of this cohort are presented in Table 1. Nine of these participants are still on active duty in the U.S. Army; eight were new to the DU Follow-up Program in 2001.

### Biologic Monitoring for Uranium

The results of the 24-h total urine uranium analysis are presented in Figure 1. The dashed line in this figure depicts an occupational exposure decision level of 0.8 μg/L of urinary uranium that is used at the Department of Energy's Fernald Environmental Management Project (McDiarmid et al., 2000; Fernald Environmental Management Project, 1997) as a trigger for investigating work areas for sources of elevated uranium exposure. This decision level, which is based on

**TABLE 1.** Demographic Characteristics of the 2001 DU Follow-Up Program Participants

| | *n* | % |
|---|---|---|
| Race | | |
| African-American | 12 | 31 |
| Caucasian | 22 | 56 |
| Hispanic | 4 | 10 |
| Other | 1 | 3 |
| Education | | |
| 0–8 yr | 1 | 3 |
| 9–12 yr | 9 | 23 |
| Some college | 22 | 56 |
| College degree | 4 | 10 |
| Post college | 3 | 8 |
| Marital status[a] | | |
| Never married | 3 | 8 |
| Married | 31 | 79 |
| Divorced | 4 | 10 |
| Unknown | 1 | 3 |
| Age[b] | | 35.1 ± 0.76 |

*Note*. DU, depleted uranium; $n = 39$ (8 members of this cohort were new in 2001).
[a] At time of 2001 evaluation.
[b] Mean age at time of 2001 evaluation (± SE, standard error of the mean).

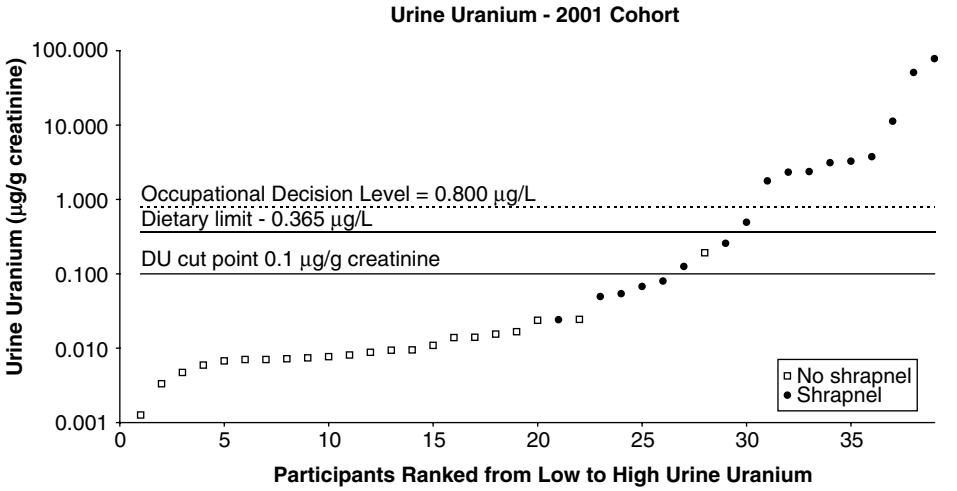


**FIGURE 1.** Twenty-four-hour urine uranium measures of returning and new DU-exposed veterans from 2001 assessment. Values are ranked from low to high. The open squares represent participants with no history of shrapnel ($n = 22$) and the circles indicate those with current shrapnel or history of shrapnel (metal type unknown) (n = 17). See text for a discussion of the values indicated by the dashed, dotted, and solid lines.

the log-normal distribution of uranium in urine from a control population, allows comparison of the DU-exposed group to measured dietary levels. The dotted line (0.365 µg/L) is an upper limit for the dietary contribution of uranium in urine for a general population from uranium in drinking water (ICRP, 1974; McDiarmid et al., 2000). This value was calculated by dividing the estimated upper limit for 24-h uranium excretion for "reference man" by 1.4 L/24 h. It is assumed that corrections per gram creatinine and per liter urine are generally equal for "reference man" and for this group of veterans with normal renal function. The solid line indicates the cut point established by the DU Follow-up Program to identify low versus high urine uranium concentrations (McDiarmid et al., 2000).

Uranium concentrations for the 2001 cohort ranged from 0.001 µg/g creatinine to 78.125 µg/g creatinine (Figure 1). All values over 0.1 µg/g creatinine except one were in participants with known retained shrapnel fragments. The only new participant who had urine uranium >0.1 µg/g creatinine also had a history of retained shrapnel. The majority of individual cohort members had very similar urine uranium values on their different visits (1994, 1997, 1999, 2001) (Figure 2). In addition, significant correlations among the cohorts of different years ranged from a low of 0.660 between the 1994 and 1997 groups to 0.993 between the 1999 and 2001 groups.

## Clinical Findings

As reported in the past (McDiarmid et al., 2000, 2001), the only significant differences in the frequency of medical problems between the low and high

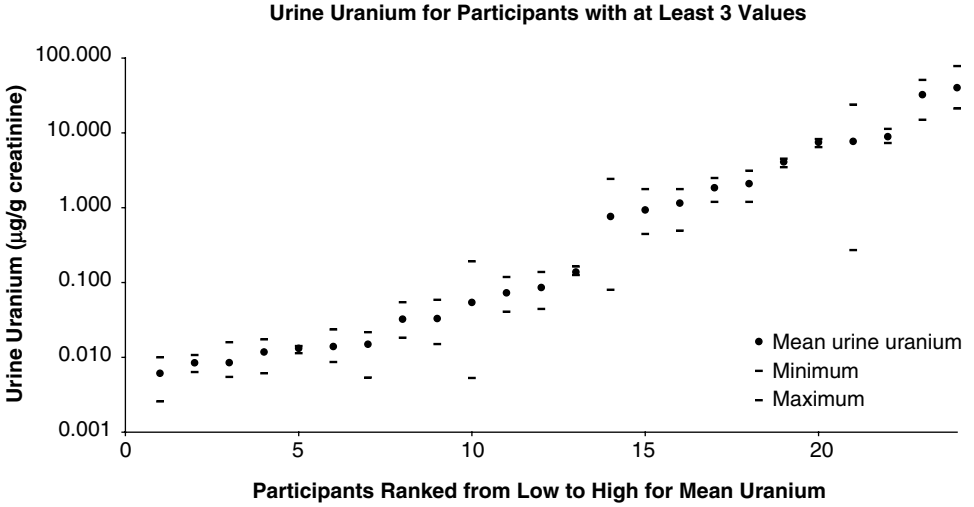


**FIGURE 2.** Individual variability in urine uranium excretion over time in Gulf War veterans. Circles represent the mean of three or four urine uranium values (reported in µg uranium/g creatinine) from the evaluations done in 1994, 1997, 1999, and 2001 for each individual. The bars represent the minimum and maximum values obtained during these three or four evaluations.

uranium groups is in the percentage of participants that suffered injuries during the friendly fire incidents. There were no differences in frequency of musculoskeletal, cardiovascular, psychiatric, nervous system, or other disorders.

*Hematologic parameters* Although there were a few statistical differences in some of the hematologic parameters, means for both the high and low uranium groups were within normal clinical limits. The high uranium group had significantly lower hematocrit (42.59% vs. 44.60%) and hemoglobin (14.79 vs. 15.40 g/dl) values than the low uranium group. These differences were not evident in either the 1997 or 1999 cohorts. There were no significant differences in any parameters of the differential white cell count.

*Renal function parameters* Table 2 indicates that there were statistically significant differences in some of the renal function parameters between the high and low uranium groups. Serum creatinine was higher in the low uranium group (0.85 vs. 0.95 mg/dl), while urine retinol binding protein (65.68 vs. 46.13 µg/g creatinine) and urine total protein (78.69 vs. 54.63 mg/g creatinine) were higher in the high uranium group. This suggestion of decreased protein reabsorption or increased glomerular filtration of proteins in the high uranium group was not observed in the 1997 or 1999 surveillance visits. These differences, although statistically significant, are within the normal clinical ranges for these parameters.

## Neurocognitive Evaluation

Consistent with previous years, there were no statistically significant differences between the high and low uranium groups for the neurocognitive parameters

**TABLE 2.** Renal Function Parameters: Comparison of Low Versus High Urine Uranium Groups

| Laboratory test (normal range) | Low uranium group[a] (mean ± SE) | High uranium group[b] (mean ± SE) | Mann–Whitney test ($p$) |
|---|---|---|---|
| Serum creatinine (0.5–1.1 mg/dl) | 0.95 ± 0.03 | 0.85 ± 0.03 | 0.03 |
| Serum uric acid (3.4–7 mg/dl) | 5.94 ± 0.23 | 5.85 ± 0.51 | 0.45 |
| Serum calcium (8.4–10.2 mg/dl) | 9.17 ± 0.006 | 9.27 ± 0.137 | 0.67 |
| Serum $PO_4$ (2.7–4.5 mg/dl) | 3.82 ± 0.101 | 3.82 ± 0.148 | 0.63 |
| Urine calcium (100–300 mg/24 h) | 183.50 ± 23.8 | 214.50 ± 26.3 | 0.35 |
| Urine $PO_4$ (0.4–1.3 g/24 h) | 1.03 ± 0.008 | 1.15 ± 0.107 | 0.40 |
| Urine beta-2 microglobulin (0–300 µg/g creatinine)[c] | 38.53 ± 6.71 | 36.42 ± 7.46 | 0.78 |
| Urine retinol binding protein (3–610 µg/g creatinine) | 46.13 ± 3.46 | 65.68 ± 11.11 | 0.06 |
| Urine creatinine (1.3–2.6 g/24 h) | 1.99 ± 0.11 | 2.14 ± 0.10 | 0.29 |
| Urine total protein (0–92.8 mg/g creatinine) | 54.63 ± 4.94 | 78.69 ± 10.52 | 0.01 |

[a] <0.1 µg/g creatinine ($n$ = 26).
[b] >0.1 µg/g creatinine ($n$ = 13).
[c] $n$ = 16 for low uranium group and $n$ = 9 for high uranium group; samples lost due to lab error.

measured (data not shown). A higher impairment score for accuracy derived from the computerized battery (AIIac) for those in the high uranium group was observed using the "low-bar" probability level of .20 or less (0.16 ± 0.04 vs. 0.27 ± 0.07). This impairment index (A-IIac) was then used as an outcome measure in a robust regression to assess its relationship with urine uranium values controlling for emotional status as assessed with the Beck Depression Inventory and general intellectual level. Results revealed a marginal association ($p$ = .069) between measured urine uranium and the accuracy index. On further analysis of individual cases, it was clear that the relationship between urine uranium and the accuracy impairment index for the automated tests was being driven by the two cases with extremely high uranium values and also persistent complications due to combat injuries.

### Reproductive Health Measures

*Neuroendocrine function* There was a statistically significant difference in free thyroxine between the high and low uranium groups, with the low uranium group having a higher level (1.66 vs. 1.08 ng/dl) than the high uranium group. These results are still within expected norms and group differences were not previously observed for this parameter. A difference approaching significance ($p$ = .06) was also seen in prolactin levels, with higher levels seen in the low uranium group (18.84 vs. 14.70 ng/ml). Neither of these findings was present in either the 1997 or 1999 evaluations. In fact, in the 1997 evaluation, the high uranium group had a higher prolactin relative to the low uranium group. No statistically significant differences were observed in FSH, LH, testosterone or TSH.

*Semen characteristics* Semen samples were obtained from a total of 35 participants in the 2001 cohort. Seven of these samples were azospermic. Five of these participants had been vasectomized and two were azospermic for other known reasons (both participants were in the low urinary uranium group). Data (days abstinence and liquefaction status) from these azospermic subjects were excluded from data analysis. One additional participant from the low urinary uranium group was excluded from the study because of an implausible days abstinence report. For the remaining 27 participants, the distributions of abstinence period, semen volume, and incidence of incomplete liquefaction were not significantly different between exposure groups. The incidences of subnormal sperm count and motility characteristics (below WHO 1987 norms) were also not significantly different between the low and high uranium exposure groups. Means of semen characteristics for subjects with high urinary uranium were generally greater than subjects in the low urinary uranium group (Table 3) however, none of these differences were statistically significant.

## Genotoxicity

Genotoxic effects of DU exposure were assessed at both the chromosome and gene level. Standard cytogenetic analyses (chromosomal aberrations [CA] and sister chromatid exchange [SCE]) were employed to examine chromosomal alterations. The HPRT assay, which assays for gene level mutations, was chosen because it is well characterized with a worldwide database and is the only well-characterized test with the potential for molecular analyses in

**TABLE 3.** Reproductive Function: Semen Characteristics. Comparison of Low Versus High Urine Uranium Groups

| Clinical parameters (normal range) | Low uranium group[a] (mean ± SE) | High uranium group[b] (mean ± SE) | Mann–Whitney test ($p$) |
|---|---|---|---|
| Days abstinence (2–5 days) | 4.8 ± 1.7 | 4.2 ± 0.9 | 0.820 |
| Semen volume (2–5 ml) | 2.6 ± 0.4 | 3.5 ± 0.6 | 0.167 |
| Sperm concentration(>20 million/ml) | 102.8 ± 28.6 | 219.1 ± 70.5 | 0.126 |
| Total sperm count (>40 million) | 241.6 ± 66.4 | 708.6 ± 215.1 | 0.061 |
| Percent motile sperm (>50%) | 57.6 ± 4.9 | 60.5 ± 6.3 | 0.639 |
| Percent progressive sperm [WHO[c] Class A and B] (>50%) | 27.3 ± 3.2 | 25.7 ±3.7 | 0.766 |
| Total Progressive Sperm [WHO Class A and B] (>20 million) | 79.9 ± 22.6 | 206.8 ± 58.3 | 0.126 |
| Percent rapid progressive sperm [WHO Class A] (>25%) | 17.6 ± 2.7 | 16.3 ± 2.5 | 0.586 |
| Total rapid progressive sperm [WHO Class A] (>10 million) | 54.9 ± 16.2 | 134.8 ± 40.5 | 0.152 |

[a] <0.1 μg/g creatinine ($n$=16).
[b] >0.1 μg/g creatinine ($n$=11).
[c] WHO, World Health Organization.

humans. In addition, the HPRT assay measures mutations in peripheral T lymphocytes that circulate throughout the body, and thus it can detect exposure to mutagens present in many different tissues (Albertini et al., 2000).

*Chromosomal aberration and sister chromatid exchange* Table 4 displays results for the genotoxicity parameters measured in this study. Baseline CAs were statistically different, with the high uranium group displaying a higher, but minimally different, CA frequency per cell. This difference was not observed in previous surveillance rounds. Due to the limited number of chromosomal aberrations observed, it was not possible to use regression to assess its relationship with ln urine uranium or a method to test for the persistence of the relationship despite the presence of confounders. However, the association between chromosomal aberrations and urine uranium observed here does not appear to be the result of smoking, exposure to mutagens, or age in this cohort, since none of these were found to be significantly associated with either average chromosomal aberrations or urinary uranium levels. Moreover, x-ray history was not significantly associated with the chromosomal aberrations observed.

The levels of SCE were not markedly lower in the high urinary uranium group (Table 4). No association between SCE and ln (urine uranium) emerged when potential confounders (age, x-rays, exposure to gene toxicants or current smoking) were included in the regression.

*HPRT mutation frequency* HPRT cloning assays were successful in samples from all 39 subjects. Nonselection cloning efficiencies (controls) ranged from a low of 0.2 to a high of 0.63, indicating good T-cell viability. The *HPRT* mutant frequencies (MFs) determined over a 4-mo period ranged from a low of $4.4\times10^{-6}$ to a high of $69.1\times10^{-6}$, with a mean value of 13.9 (SD±11.5)$\times10^{-6}$. The probability that levels of HPRT differed in the high versus low urine uranium groups was 0.105 (Table 4). However, raising the urine uranium cut point from 0.1 to 0.2 µg uranium/g creatinine results in moving only 2 cases from the high to the low uranium category and yields a $p$ value of .043.

**TABLE 4.** Genotoxicity Parameters: Comparison of Low Versus High Urine Uranium Groups

| Laboratory test | Low uranium group[a] [mean ± SE (*n*)] | High uranium group[b] [mean ± SE (*n*)] | Mann–Whitney test (*p*) |
|---|---|---|---|
| Mean aberrations/cell | 0.003 ± 0.001 (26) | 0.01 ± 0.004 (13) | 0.027 |
| Mean SCE[c] untreated | 5.07 ± 0.32 (25) | 4.39 ± 0.37 (13) | 0.199 |
| Mean SCE with bleomycin 2 µg/ml | 5.42 ± 0.32 (23) | 5.95 ± 0.71 (11) | 0.663 |
| Mean SCE with bleomycin 4 µg/ml | 6.31 ± 0.60 (20) | 5.30 ± 0.42 (11) | 0.197 |
| HPRT MF[d] | 10.97 ± 0.97 (26) | 19.84 ± 4.89 (13) | 0.105 |

[a] <0.1 µg/g creatinine.
[b] >0.1 µg/g creatinine.
[c] SCE, sister chromatid exchange.
[d] HPRT MF, hypoxanthine phosphoribosyl transferase mutation frequency.

The association of the ln(HPRT mutation frequency) and [ln(urine uranium)+ 6.83]$^3$ was significant whether or not we adjusted for any combination of cloning efficiency, age, current smoking, or exposure to genetic toxicants at home or at work. Having had an x-ray during the past year was also not associated with level of mutation frequency in a subset of cases for which data were available ($n = 32$). The portion of the preceding cubic expression that covers the range of the data shows that there is no association at the lowest levels of urine uranium (Figure 3) but that a positive association is apparent as levels of ln(urine uranium) increase, and it becomes increasingly strong above a ln (urine uranium/gm creatine) value of 0 (1 μg urine uranium/g creatinine). The graph in Figure 3 shows the unadjusted association, but the graphs of the adjusted associations are not visually different from this graph. Examination of absolute dfbetas to evaluate undue influence of individual cases to the overall association showed that with the criterion set at absolute dfbeta < 1 (Bollen & Jackman, 1990), no case would be identified as contributing unduly to the association. With the criterion set at $2/\sqrt{n}$ (Belsy et al., 1980), the 3 cases in the upper right of Figure 3 had possible undue influence. When these three cases were removed, the association was

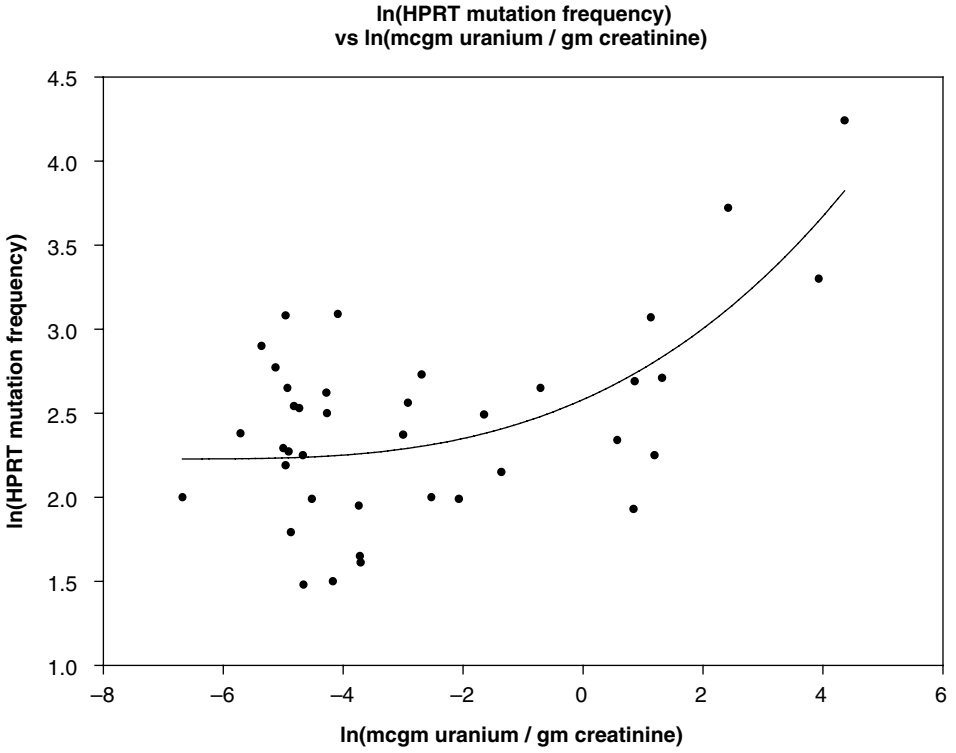


**FIGURE 3.** The relationship of ln(HPRT mutation frequency) to ln(μg uranium/g creatinine) in 39 DU-exposed Gulf War veterans.

linear with a slope not significantly different from zero. When only the top point was removed, the cubic association remained significant. These findings are consistent with a mutagenic effect of depleted uranium, at least in cases with retained body burdens.

### Immunologic Measures

The percent of cells bearing various lymphocyte or monocyte phenotypic markers determined using flow cytometric analysis revealed that the low uranium and high uranium groups differed statistically in only two of fourteen phenotypic markers studied. The percent but not the absolute number of CD4+ T cells was significantly elevated in the high compared to low uranium group (65.98% vs 60.83%), while the percent but not the absolute number of CD8+ T cells was significantly lower in the high compared to low uranium group (26.55% vs. 31.28%). Although statistical differences were observed in the percentage of CD4+ T cells and CD8+ T cells, all the values fell within published normal ranges. Similarly, the higher percent of monocytes in the high uranium group (11.22 vs. 8.21, $p = .09$, for the high vs. low uranium groups, respectively) approached significance; however, there was no significant difference in absolute numbers of monocytes between the high and low groups. In addition, the percentages of monocytes for the two groups determined by differential counting (7.94 vs. 7.79) were not markedly different. Other immunological parameters (circulating immunoglobulins, complement proteins, and C-reactive protein) were also not statistically different in the two exposure groups.

## DISCUSSION

Ten years after first exposure, a small group of Gulf War veterans wounded with depleted uranium-containing shrapnel continue to excrete elevated concentrations of uranium in their urine. Urine U concentrations in this group of soldiers are clearly above normal concentrations present in the general population, which occur from exposure to natural U through dietary and drinking sources. Reported urine uranium concentrations in unexposed persons have ranged from a geometric mean of 0.007 μg/L (CDC, 2003) to 0.0309 ± 0.0196 μg/L (Medley et al., 1994). The highest urine uranium concentrations in soldiers with fragments are similar to levels reported by Thun and coworkers (1985) for a cohort of uranium mill workers in 1975. The mean urine uranium concentration in this group was 65.2 μg/L, with a 95th percentile value of 120 μg/L.

Throughout the duration of this surveillance program, a 24-h urine uranium determination standardized per gram creatinine has provided an effective integrating dosimeter of systemic uranium exposure. The clear determinant of urine uranium concentration, the presence of retained uranium containing metal fragments in soft tissue, has been observed in all of our previous evaluations (Hooper et al., 1998; McDiarmid et al., 2000, 2001). The consistency in uranium excretion over time suggests the uranium body burden is in a steady state in both the high and low urine uranium groups. For those soldiers possessing

metal fragments, the size of these depots is sufficiently large as to not allow any appreciable decline of the uranium body burden over the 2-yr time period between medical evaluations. For the majority of the soldiers in the 2001 cohort who do not have retained metal fragments, but sustained their DU exposure through inhalation or wound contamination, any initial systemic uranium has been eliminated or transported to long-term storage sites such as bone. Consequently, their uranium burden is also in a steady state, with minimal release from body stores, as evidenced by their low urinary uranium excretion.

### Clinical Evaluations

Other than the frequency of battle injury, which is the method by which shrapnel fragments were inflicted, there is clear absence of a "signature" specific medical problem shared by this cohort of Gulf War vets.

As in previous surveillance examinations, mean values for all hematologic parameters were within the normal range. The clinical significance of the single statistical difference in hematocrit observed between groups is unclear. Over the years, various parameters have been different between the two groups, but not consistently so, and they have not been outside their normal ranges. Because multiple outcomes are being examined, there exists the risk that statistically significant findings may be observed by chance alone.

Although the kidney is the putative "critical" target organ for uranium toxicity under acute and chronic exposure conditions (Gilman et al., 1998; Leggett, 1989; Zamora et al., 1998), no evidence of renal dysfunction (glomerular or tubular) was found. The biomarkers for proximal tubule dysfunction, the presumed target of uranium (Leggett, 1989), showed minimal differences between the groups. There was a statistically significant difference in total urinary protein that was higher in the high uranium group; however, the increase was only 1.5-fold and the protein concentration values were still within normal range. The difference in urinary retinol binding protein concentration, a more specific marker of proximal tubule function, approached statistical significance and was higher in the high uranium group, but again the increase was not clinically significant. Because kidney concentrations of uranium have been shown to increase with time under chronic exposure conditions (Pellmar et al., 1999; Squibb et al., 2001), this evidence of small changes in renal proximal tubule function may be a harbinger of greater effects in the future and emphasize the need to continue surveillance of renal function in this exposed cohort.

### Reproductive Health Measures

*Neuroendocrine function* The neuroendocrine and thyroid measures were all within normal limits with the exception of serum prolactin, which demonstrated a slightly elevated level outside the normal range in the low uranium group. Although other metal exposed populations displayed neuroendocrine effects (Cullen et al., 1984; Gustafson et al., 1989), making these endpoints biologically plausible targets for uranium toxicity is at present not reliable. The experience over time is also helpful here, given that in a previous evaluation,

the opposite prolactin relationship was observed (McDiarmid et al., 2000) and in the subsequent visit, there was no difference in the prolactin levels between groups at all (McDiarmid et al., 2001). Results from future evaluations may provide some clarity as to effects taking place.

*Semen characteristics* For the parameters evaluated in this study, both uranium exposure groups have normal semen characteristics based on average values. The generally elevated values in the high uranium exposure group are not considered clinically significant for an individual's fertility, as upper limits of normal do not exist.

### Genotoxicity

*Chromosomal aberrations and sister chromatid exchanges* In two previous observations, no differences in chromosomal aberrations (CAs) were noted between the high and low uranium groups, although there was a statistically significant increase in SCE observed in the high uranium group in the last evaluation (McDiarmid et al., 2001) that was not observed in a previous evaluation (McDiarmid et al., 2000). Against this mixed picture, data show no difference in SCE baseline or bleomycin-challenged frequency, but a statistical difference in CAs (higher in the high U group). This is, however, based on close to normal absolute frequencies of CAs per cells. Our determination of HPRT frequencies, performed for the first time in this surveillance battery, showed that they are also significantly higher in the high uranium groups, even when adjusted for smoking, age and x-rays in the last year.

Only one previous study examined genotoxic endpoints in humans (uranium fuel production and enrichment workers), and findings reported were an increase in SCE, total CAs and dicentrics as a function of uranium exposure (Martin et al., 1991). Although CAs would argue for a clastogenic (likely radiologically mediated) insult, the authors discussed the low radiation exposure and ascribed their findings to uranium's chemical toxicity. Two cell culture experiments have documented uranium's genotoxicity; one was in Chinese hamster ovary (CHO) cells exposed to uranyl nitrate ($UO_2^{2+}$) and found an increased frequency of micronuclei, SCE, and CAs (Lin et al., 1993). In a human osteoblast (HOS) cell line, increased SCE and an increase in transformation to a tumorigenic phenotype were seen in DU-exposed cells in culture (Miller et al., 1998a). Miller and colleagues (1998b) also showed increased urine mutagenicity in TA98 and AMES II (TA 7001–7006) in DU-implanted animals. More recently, this group has reported genetic instability in the HOS cell line after DU exposure manifested as delayed lethality and micronuclei formation (Miller et al., 2003).

In our study, bleomycin, a radiomimetic and potent clastogen but a poor SCE inducer, was used on the SCE cultures as a provocative challenge to examine enhanced expression of SCE where such an enhancement could represent heritable genetic instability, presumably from previous genotoxic exposure (Kim et al., 1985; Lundgren & Lucier, 1985). However, such an enhancement did not occur. This approach will be pursued in future assessments.

*HPRT mutation frequency* Background *HPRT* MFs, the most widely used measure of somatic gene mutations in humans (Albertini & Hayes, 1997; Cole & Skopek, 1994; Robinson et al., 1994), reflect not only spontaneous and endogenously induced somatic mutations but also mutations induced by ubiquitous and unknown exogenous mutagen exposures. For this reason, levels of T-cell *HPRT* mutations must be correlated with known exposures to infer causation. Analysis of other factors known to affect *HPRT* MFs indicated that these were not responsible for the range of values observed. No other known mutagenic confounders appeared to influence our results. However, other unknown influences/agents may also be contributing to the measured mutation frequencies and their variability.

The lack of association between mutation frequency and urine uranium levels at low levels of urine uranium could have several causes. It may be due to a threshold effect. However, the ability to attribute *HPRT* MF exclusively to urine uranium values in this low background range, as opposed to other competing environmental mutagens, becomes increasingly difficult.

Our finding of somatic gene mutations in humans is in accord with findings of others of genotoxic effects in vitro in mammalian cells and in vivo in intact animals (Lin et al., 1993). Follow-up studies should define DU's mutagenicity at the mechanistic level, differentiating between its chemical and its low-level radiological effects. Characterization of the *HPRT* mutational spectrum in the DU-exposed *HPRT* mutant isolates should provide insights when compared to the background in vivo spectra for humans and spectra for low and high LET ionizing radiations and some chemicals (Albertini, 2001).

### Immunologic Measures

Results from clinically available measures of immune competence and a panel of phenotypic markers suggest that exposure to depleted uranium has no clinically significant effect on immune parameters. Future studies should include a clinical battery of immune competence measures to follow any effect that may occur as exposure duration continues.

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