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ABSTRACT

Depleted uranium (DU) is a high density heavy metal that has been used in military munitions since the 1991 Gulf War. DU is weakly radioactive and chemically toxic. Long term exposure can cause adverse health effects. This study assessed genotoxic effects in DU exposed Gulf War I veterans as a function of uranium (U) body burden. Levels of urine U were used to categorize the cohort into low and high exposure groups. Exposure to DU occurred during friendly fire incidents in 1991 involving DU munitions resulting in inhalation and ingestion exposure to small particles of DU and soft tissue DU fragments from traumatic injuries. All of these Veterans are enrolled in a long term health surveillance program at the Baltimore Veterans Administration Medical Center. Blood was drawn from 35 exposed male veterans aged 36–59 years, then cultured and evaluated for micronuclei (MN) using the cytokinesis block method. The participants were divided into two exposure groups, low and high, based on their mean urine uranium (uU) concentrations. Poisson regression analyses with mean urine U concentrations, current smoking, X-rays in the past year and donor age as dependent variables revealed no significant relationships with MN frequencies. Our results indicate that on-going systemic exposure to DU occurring in Gulf War I Veterans with DU embedded fragments does not induce significant increases in MN in peripheral blood lymphocytes compared to MN frequencies in Veterans with normal U body burdens.

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1. Introduction

Depleted uranium (DU) is a heavy metal that is both radioactive and hazardous due to its chemical properties. Natural U is composed of three U isotopes (U^{234}, U^{235} and U^{238}). DU is a by-product of the U enrichment process that natural uranium undergoes in order to extract the U^{235} isotope for use in nuclear weapons and nuclear fuel production. Thus, DU has a lower U^{235}/U^{238} ratio and is approximately 40% less radioactive than natural U. DU metal is used for military applications due to its high density, high pyrophoricity, tensile strength that is similar to steel, high availability, and low cost. It is ideal for use in armor piercing munitions because it has self-sharpening properties upon impact allowing it to penetrate armor more effectively than other metals. DU dust is formed upon impact of the projectiles, which is one source of internal inhalation exposure to DU in the battlefield. The other military application of DU is for protective tank armor, which can increase inhalation and ingestion exposures to DU dust if a munition pierces DU armor dur-

ing battle [1]. Under normal conditions, occupants of tanks with DU armor are also exposed to increased amounts of DU-derived radiation. However, this additional radiation dose is very small and does not constitute a significant health risk [2]. The heavy metal toxicity of DU is generally considered to present a greater health hazard than its radioactivity.

External exposure to DU does not present a significant health hazard, but internal exposure via dust particle inhalation and embedded DU fragments may lead to adverse health effects due to both chemical and radiological toxicity. *In vitro* studies in human cell lines treated with soluble DU compounds show increased transformation to tumorigenic phenotypes; several bacterial strains show increased mutagenic activity after DU exposure and animal models implanted with DU pellets developed local tumors [3]. These results suggest that DU may increase the risk of cancer [4]. However, in contrast to the animal and *in vitro* studies, humans exposed to high internal and external doses of DU do not appear to suffer measurable health effects [5,6]. For 18 years, the Department of Veterans Affairs (VA) has been monitoring the health of Gulf War I veterans that were exposed to DU during friendly fire incidents. In addition to inhalation and ingestion exposures, about forty percent of this cohort sustained traumatic injury resulting in embedded fragments of DU being retained in soft tissue. During biennial visits to the Baltimore VA Medical Center, the veterans

Table 1
Demographic characteristics of the DU follow-up program 2009 medical surveillance visit participants.

	2009 cohort ($n = 35$)	
	n	%
Gender (% males)	35	100
Race		
African American	12	34%
Asian American	1	3%
Caucasian	20	57%
Hispanic	2	6%
Participants with embedded DU fragments	15	43%
Age[a]	43.62 ± 5.35	

[a] Mean age at time of 2009 evaluation (+ standard deviation).

are monitored for the concentration of DU in their urine, for clinical chemistry measures that assess organ system function with a focus on biomarkers of adverse effects on the renal and reproductive systems, and for hematological, neuroendocrine and bone metabolism parameters. Additional tests have included measures of chromosomal aberrations and HPRT mutation frequency in blood lymphocytes. To date, no clinically significant DU-related health effects have been observed, even in subjects with the highest urine uranium concentrations [7–14].

Enumeration of micronuclei (MN) in peripheral blood lymphocytes (PBLs) is a well-established cytogenetic method for detecting chromosome damage caused by radiation and chemical exposures in humans [15]. Here we used the cytokinesis-blocked MN assay to measure the number of MN found in the PBLs of DU-exposed Gulf War I veterans enrolled in the VA monitoring program. The goal of this study was to determine whether DU exposure as measured by urine U concentration results leads to detectable levels of cytogenetic damage. The results indicate that chronic systemic exposure to DU in Gulf War I Veterans with embedded DU fragments does not result in elevated frequencies of MN in peripheral blood lymphocytes compared to the frequencies of MN in Veterans with normal U body burdens.

2. Materials and methods

2.1. Recruitment of subjects

The number of micronuclei present in peripheral blood lymphocytes was measured in blood samples collected from 35 members of the Veteran Administration (VA)'s DU-exposed Gulf War I veteran cohort who participated in the 4-day medical surveillance visit at the Baltimore VA Medical Center (Baltimore, MD) between April and June 2009. Although all 79 members of this cohort were invited to participate in this surveillance visit, only about half of the total cohort accepted the invitation due to personal, employment or military service schedule constraints. One participant was excluded from this examination of micronuclei because he had previously received Cobalt radiation therapy. Demographics for the group of 35 veterans that were examined for micronuclei in blood lymphocytes are shown in Table 1. Approximately 43% of the veterans in this group had evidence of embedded fragments when examined by X-ray.

2.2. Blood collection, cell culturing, slide preparation and staining, and micronuclei analysis

Blood was drawn using 6 mL green topped vacutainer tubes containing heparin. The tubes were kept on a tilt shaker at low speed until they were prepared for shipping (within 2 h). The blood samples were shipped overnight from the Baltimore VA Medical Center to Wayne State University with ice packs to remain cold (approximately 4 °C). Samples were stored at 4 °C upon arrival for 1–2 h before culturing.

Lymphocytes were isolated by carefully layering 2 mL of blood diluted with 2 mL Hank's Balanced Salt Solution (HBSS) over 3 mL Lymphocyte Separation Medium (Mediatech, Inc.), then centrifuged at $400 \times g$ at room temperature for 30 min. The mononuclear cell layer was transferred to a new tube and mixed with 4 mL HBSS, then centrifuged for 10 min at $260 \times g$ at room temperature. The cell pellet was washed twice with 4 mL HBSS and centrifuged for 10 min at $260 \times g$ at room temperature. The pellet was then re-suspended in 1 mL RPMI 1640 medium (Hyclone), supplemented with 15% Fetal Bovine Serum (Atlanta Bio-

logicals), penicillin–streptomycin (100 units/mL penicillin G sodium, 100 μg/mL streptomycin in 0.85% saline, Gibco), 0.02 mg/mL PHA (Gibco) and 2 mM L-glutamine (Gibco). A cell count of the 1 mL cell suspension was obtained and cultures were seeded at a concentration of approximately 500,000 cells/mL. Cells were incubated in a fully humidified incubator with 5% CO_2 at 37 °C in T25 suspension culture flasks (Corning) for 44 h, then treated with Cytochalasin B (Sigma–Aldrich) (6 μg/mL final concentration) and cultured for an additional 28 h for a total culture time of 72 h. Cells were re-suspended in their culture medium with a transfer pipette to break up cell clumps. The cells were then spun onto pre-cleaned microscope slides using a cytocentrifuge (Stat-Spin) for 4 min at 1300 RPM. The slides were air dried and fixed in 100% methanol for 15 min, then dried and stored at room temperature until staining. Slides were stained with 10% Giemsa Solution in Sorenson's buffer (67 mM Na_2HPO_4, 67 mM KH_2PO_4 pH 6.8) for 15 min, rinsed briefly in distilled H_2O, air-dried and then mounted with Permount (Fisher Scientific) and a glass coverslip.

All blood samples were coded prior to shipping to the cytogenetics laboratory. The code was not broken until all the slide scoring had been completed. A total of 2000 binucleated cells was scored from each donor under a light microscope (Nikon Eclipse E200) by 2 trained individuals each of whom scored 1000 cells. Only intact, binucleated cells with clearly distinct nuclei were scored [16]; the number of micronuclei (MN) per sample as well as the number of cells with 0, 1, 2, 3, 4, and 5 or more MN were recorded. Binucleated cells containing nucleoplasmic bridges were excluded from scoring. The Nuclear Division Index (average number of nuclei per cell) was also determined for each sample.

2.3. Urine uranium analysis

At each biennial health surveillance visit, twenty-four hour urine samples are collected from each subject and shipped to the Armed Forces Institute of Pathology, Department of Environmental Toxicologic Pathology (Washington, DC) for analysis of total uranium using a previously described inductively coupled plasma-dynamic reaction cell-mass spectrometer (ICP-DR-MS) method [10,17]. Urine U concentrations are standardized on the basis of urine creatinine concentrations to obtain micrograms of U per gram of creatinine to account for urine dilution due to water intake and/or dehydration [12,18].

2.4. Uranium exposure metric

A mean urine U (uU) exposure metric for each participant in this study was calculated using all the uU concentrations obtained for a participant each time they had participated in a surveillance visit at the Baltimore VA between 1994 and 2007. This U exposure metric, labeled mean uU 2007, was used to determine whether a relationship exists between mean uU exposure over the past 18 years and the presence of micronuclei in blood lymphocytes.

2.5. Statistical analysis

The Mann–Whitney U test was used to test for the significance of differences observed between High versus Low U exposed groups established based on each participant's mean uU 2007 value. Historically, the Baltimore VA DU health surveillance program has used a cut-off value of 0.1 μg U/g creatinine for dividing High from Low exposed individuals [8]. This cut-off point was chosen because it was between the 95th percentile reported by [19] for creatinine-adjusted urine U concentrations in U.S. populations with normal exposure to natural U through their diet and drinking water (0.034 μg/g creatinine) and 0.35 μg/L, a value reported as a uU upper limit in populations living in areas where natural U is elevated in water and food [20]. Differences were considered statistically significant when calculated p values were < 0.05.

Data were also analyzed using regression analysis. Since micronuclei frequency is a discrete variable created by a count, Poisson or Negative Binomial probability distribution is indicated for multivariate analysis [21]. Poisson distribution can be used when the mean equals the variance but the negative binomial is indicated when this assumption is violated (e.g., by over-dispersion). The use of the Poisson regression to estimate the association of micronuclei abnormality frequency with uranium adjusting for age, current smoking and X-ray exposure during the past year was examined using the statistical package STATA version 11 (StataCorp, College Station, TX). Because of over-dispersion of the data, the results of the equivalent negative binomial distribution are reported.

3. Results

A total of 35 veterans were evaluated for the formation of MN as a function of their urine U concentrations. All subjects were adult males, ranging from 36 to 59 years of age. Table 2 shows the ages of each subject, the mean urine uranium concentrations calculated for each individual (mean uU 2007) and the MN data obtained from each subject. Cells with 0, 1, 2, 3, 4, 5 and >5 MN were observed.

To examine the relationship between MN frequency and U body burden, the participants in this cohort were divided into two

Table 2
Urine uranium concentrations and micronuclei data by donor and exposure group.

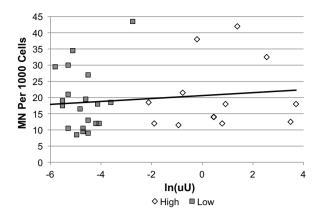| Donor code	Urine [U] (μg U/g cre)	Donor age	Number of normal cells	Number of cells with MN	Mean MN per 1000 cells	Nuclear Division Index (mean # of nuclei per cell)
High exposure group (>0.1 μg U/g cre)						
102	0.81	38.59	1948	52	38.0	1.31
104	32.61	51.47	1976	24	12.5	1.28
105	40.40	41.74	1967	33	18.0	1.48
115	0.12	41.83	1967	33	18.5	1.72
116	0.46	50.49	1963	37	21.5	1.34
122	1.58	45.9	1978	22	14.0	1.39
123	1.56	39.18	1975	25	14.0	1.74
129	2.19	44.03	1980	20	12.0	1.46
145	12.7	56.06	1947	53	32.5	1.15
193	3.98	45.86	1937	63	42.0	1.47
199	2.5	44.81	1971	29	18.0	1.66
201	0.15	40.3	1983	17	12.0	1.62
202	0.39	40.96	1980	20	11.5	1.85
Mean	7.65	44.71	1967	32.92	20.35	1.50
S.D.	13.32	5.24	14.59	14.59	10.42	0.21
Low exposure group (<0.1 μg U/g cre)						
101	0.064	41.78	1927	73	43.5	1.45
110	0.007	42.49	1989	11	8.5	1.48
119	0.005	48.84	1966	34	21.0	1.54
126	0.016	38.54	1972	28	18.0	1.57
130	0.010	40.37	1963	37	19.5	1.55
134	0.005	42.66	1984	16	10.5	1.63
143	0.027	42.6	1968	32	18.5	1.34
174	0.011	36.73	1976	24	13.0	1.48
176	0.004	52.49	1972	28	17.5	1.51
179	0.011	41.69	1982	18	9.0	1.51
183	0.005	59.83	1952	48	30.0	1.33
186	0.003	41.74	1949	51	29.5	1.48
187	0.002	39.49	1974	26	13.5	1.64
188	0.008	41.71	1977	23	16.5	1.31
190	0.015	44.33	1980	20	12.0	1.45
194	0.011	49.58	1957	43	27.0	1.51
200	0.009	43.85	1982	18	9.5	1.18
204	0.004	38.87	1970	30	19.0	1.44
205	0.006	38.55	1936	64	34.5	1.31
207	0.017	38.19	1978	22	12.0	1.37
210	0.002	42.13	1963	37	19.0	1.34
212	0.009	38.62	1980	20	10.5	1.33
Mean	0.011	42.96	1968	31.95	18.73	1.44
S.D.	0.013	5.43	15.7	15.72	9.15	0.12

groups based on their mean uU2007 exposure metric, with the Low group consisting of all individuals with mean urine U concentrations below 0.1 μg U/g creatinine and the individuals in the High group having concentrations equal to or above 0.1 μg/g creatinine. The value of 0.1 μg/g creatinine was chosen as the cut-point between the high and low groups based on its close proximity to the 95th percentile upper limit value reported by NHANES (2003) for the concentration of natural U in urine the U.S. population (0.043 μg/g creatinine). Twenty-two (22) samples were placed in the Low group with mean urine [U] ranging from 0.002 μg U/g creatinine to 0.064 μg U/g creatinine while 13 samples in the High group ranged from 0.12 μg U/g creatinine to 40.41 μg U/g creatinine. The effect of a high U burden on two MN outcome measures, (1) the number of cells with MN, or (2) the total number of MN per 2000 cells, was determined by comparing the mean values for these two parameters in the Low versus High groups. The mean number of cells with MN was 32.9 in the High group versus 32.0 in the Low group; while the average number of MN/1000 binucleated cells in the Low group was 18.7 versus 20.4 for the High group. Statistical analysis of the data using the Mann Whitney test of significant difference indicated that the means of the Low versus High groups were not significantly different at the 0.05 level for either parameter.

Because micronuclei results are discrete variables created by a count, a more acceptable analysis for examining continuous rela-

tionships between data is the Poisson or Negative Binomial analysis [21]. No significant relationships were observed between the mean uU2007 exposure metric and MN frequency when mean uU2007 was examined by itself (Fig. 1) or when covariates (current smoking, X-rays in the past year and age) were included in the analysis (data not shown). The relationship between the number of cells with MN and age was also examined and is shown in Fig. 2. There was no significant relationship between MN frequency and age when examined separately or when controlled for uU concentrations, smoking and X-rays in the past year (data not shown).

4. Discussion

The results of our study show no difference in MN frequency in the high versus low urine U groups. This conclusion is consistent with other results from the DU surveillance program which has been monitoring the health of these veterans since the 1991 Gulf War. The surveillance program has not detected any significant health effects that can be attributed to DU exposure, even in those veterans with DU shrapnel in their bodies [8]. Urine Uranium levels in most of the veterans in the DU exposed cohort are similar to the general U.S. population and only remain elevated in veterans with embedded DU fragments [5]. In a previous assessment of this DU-exposed 'friendly-fire' cohort, a group of Gulf War I deployed, but non-DU-exposed controls was evaluated [12]. The

Fig. 1. Relationship between micronuclei frequency (MN/1000 cells) and the natural log of urine uranium concentrations of Gulf War I veterans exposed to DU. No significant relationship exists between urine U excretion and the frequency of micronuclei in blood lymphocytes. Members of the cohort were separated into Low versus High uU groups based on the mean of their past urine U concentrations (Low uU concentrations are <0.1 μg U/g creatinine; High uU concentrations are >0.1 μg U/g creatinine).

urine U distribution of the non-DU exposed veterans was found to be similar to the cohort of veterans that are DU-exposed but without embedded fragments; these values were also within the normal range for the U.S. population. Thus, using uU as a measure of U body burden, the low uU group within the friendly-fire cohort of DU exposed veterans provides an appropriate comparison group for the veterans with embedded DU fragments who have high uU concentrations.

We hypothesized that veterans with substantial numbers of DU fragments embedded in their bodies may have elevated MN frequencies due to the prolonged and constant systemic DU exposure they experience. DU causes radiation exposure because it is a radioactive metal which naturally emits alpha particles along with beta and gamma radiation during the decay process [2]. The alpha particles are high energy but have low penetrating power and they can be a potential internal hazard when DU is inhaled, ingested or found in wounds. The beta and gamma radiation are generally external exposure hazards, however, the overall radioactivity of DU is very low [4]. Existing experimental evidence also suggests that DU may be genotoxic based on its chemical characteristics [22–24].
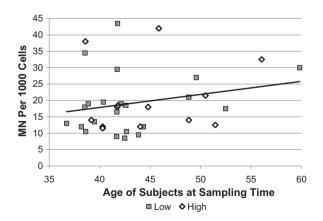

Fig. 2. Relationship between micronuclei frequency (MN/1000 cells) and the age of each veteran at the time of sampling. No significant relationship exists between age and the frequency of micronuclei in blood lymphocytes. Members of the cohort were separated into Low versus High uU groups based on the mean of their past urine U concentrations (Low uU concentrations are <0.1 μg U/g creatinine; High uU concentrations are >0.1 μg U/g creatinine).

These data do not support our original hypothesis that MN may be elevated in veterans with a chronically elevated U body burden due to embedded DU fragment retention. A possible physical explanation for this finding is that the embedded fragments are not close enough to the blood supply to cause any visible damage to the passing lymphocytes, either by emission of poorly penetrating alpha particles or by chemical toxicity. If many DU fragments are located close to large blood vessels, it would be more likely that radiation or chemical toxicity effects could be seen in the lymphocytes. The cells in the tissue surrounding the shrapnel would suffer the most damage from DU, and these effects would diminish with distance from the metal. Detailed information about the size, locations, and number of fragments embedded in each individual is not available.

Although no significant relationship between elevated MN and uU concentration was observed in this study, DU exposure may lead to small increases in micronucleus frequencies in some individuals but these increases are too low to be detectable. The effect of DU exposure could be masked by factors that are known to influence MN frequencies, such as age and cigarette smoking. MN frequencies are known to increase with age [15,25], yet we do not see an age effect present in our results, probably due to the relatively small sample size. Cigarette smoking can also be a factor in heavy smokers. The Human MicroNucleus Project examined the effects of smoking on MN frequencies in multiple studies involving nearly 6000 subjects. These analyses indicate that smokers do not have more MN than non-smokers, and even show a small decrease in MN frequencies. However, heavy smokers (at least 30 cigarettes per day) do show increases which can only be observed in people who are not occupationally exposed to genotoxic agents [26]. We controlled for current smoking (yes/no) in our regression analysis, but did not have information on the number of cigarettes smoked per day for each individual.

Exposure to ionizing radiation can also lead to increased MN frequencies, thus we controlled for past exposure to x-rays as reported by the veterans. Many received multiple X-rays or other diagnostic procedures following their injuries, however controlling for number of X-rays in the past year did not alter the relationship between MN and mean uU concentrations.

Only one other study has used micronuclei to evaluate individuals with environmental exposure to DU. Krunic et al. [27] recruited individuals from areas of Bosnia and Herzegovina where DU munitions were used during the Balkan conflict. A control population was recruited from West Herzegovina which was not impacted by war activities. Results of this study showed a small increase in the MN frequency in the exposed group compared to controls. However, there was no control for exposure to other genotoxic chemicals, and DU exposure was not directly measured in individual subjects but was assumed based on the presence of DU in environmental matrices [27]. Other important differences also exist between their study design and the one we report here. Krunic et al. evaluated individuals thought to be exposed to DU dust through direct contact and aerosol inhalation, but did not include subjects with embedded shrapnel. Their control group consisted of unexposed individuals while our Low group consisted of individuals with previous exposure to DU but low urine U concentrations.

Depleted uranium exposure can be external (skin contact) and internal (ingestion, inhalation and embedded fragments) and present both radiological and chemical hazards. However, the levels of exposure that occurred during the 1991 Gulf War did not lead to significant increases in body burdens above non-exposed populations [2] except in the cases involving embedded DU fragments. This study concurs with others showing that the DU exposure that occurred in Gulf War I veterans with embedded DU fragments does not appear to increase biomarkers of genotoxic damage despite an ongoing elevation of their U body burdens for over 18 years.

Conflicts of interest

Acknowledgments

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