Paper

PRECONCEPTIONAL PATERNAL EXPOSURE TO DEPLETED URANIUM: TRANSMISSION OF GENETIC DAMAGE TO OFFSPRING

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Abstract—**Depleted uranium (DU) is an alpha particle emitter and radioactive heavy metal used in military applications. Due to internalization of DU during military operations and the ensuing chronic internal exposure to DU, there are concerns regarding its potential health effects. Preconceptional paternal irradiation has been implicated as a causal factor in childhood cancer and it has been suggested that this paternal exposure to radiation may play a role in the occurrence of leukemia and other cancers to offspring. Similarly, in vivo heavy metal studies have demonstrated that carcinogenic effects can occur in unexposed offspring. Using a transgenic mouse system employing a λ shuttle vector allowing mutations (in the *lacI* gene) to be analyzed in vitro, we have investigated the possibility that chronic preconceptional paternal DU exposure can lead to transgenerational transmission of genomic instability. The mutation frequencies in vector recovered from the bone marrow cells of the F1 offspring of male parents exposed to low, medium, and high doses of internalized DU for 7 mo were evaluated and compared to control, tantalum, nickel, and gamma radiation F1 samples. Results demonstrate that as paternal DU-dose increased there was a trend towards higher mutation frequency in vector recovered from the DNA obtained from bone marrow of F1 progeny; medium and high dose DU exposure to P1 fathers resulted in a significant increase in mutation frequency in F1 offspring (3.57 ± 0.37 and $4.81 \pm 0.43 \times 10^{-5}$; $p < 0.001$) in comparison to control ($2.28 \pm 0.31 \times 10^{-5}$). The mutation frequencies from F1 offspring of low dose DU, Ta- or Ni-implanted fathers (2.71 ± 0.35, 2.38 ± 0.35, and $2.93 \pm 0.39 \times 10^{-5}$, respectively) were not significantly different than control levels ($2.28 \pm 0.31 \times 10^{-5}$). Offspring from ^{60}Co (4 Gy) irradiated fathers did demonstrate an increased *lacI* mutation frequency ($4.69 \pm 0.48 \times 10^{-5}$) as had been shown previously. To evaluate the role of radiation involved in the observed DU effects, males were exposed to equal concentrations (50 mg U L^{-1}) of either enriched uranium or DU in their drinking water for 2 mo prior to breeding. A comparison of these offspring indicated that there was a specific-activity dependent increase in offspring bone marrow mutation frequency. Taken together these uranyl nitrate data support earlier results in other model systems showing that radiation can play a role in DU-induced biological effects in vitro. However, since the *lacI* mutation model measures point mutations and cannot measure large deletions that are characteristic of radiation damage, the role of DU chemical effects in the observed offspring mutation frequency increase may also be significant. Regardless of the question of DU-radiation vs. DU-chemical effects, the data indicate that there exists a route for transgenerational transmission of factor(s) leading to genomic instability in F1 progeny from DU-exposed fathers.**

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INTRODUCTION

DEPLETED URANIUM (DU) is a dense heavy metal and an alpha particle emitter used in military applications. It has been used in military conflicts in Iraq, Bosnia, and Kosovo and the technology has been established for future use by multiple nations. Exposure can occur via wounding, ingestion, or inhalation. During the 1991 Gulf War and the recent Iraq War, several soldiers were wounded during friendly fire accidents and now have chronic internal exposure to DU; additionally, the extent of DU inhalation by soldiers and civilians during previous military operations is difficult to verify.

Recent studies have investigated the potential health effects of this unique heavy metal which is also a radioactive heavy metal (Miller and McClain 2007; McClain and Miller 2006). These in vitro investigations have not only demonstrated the neoplastic transforming ability, the mutagenicity, and the genotoxicity of DU, but also that DU exposure can induce genomic instability in a human cell model (Miller and McClain 2007; McClain and Miller 2006). Furthermore, some results demonstrated that alpha particle radiation is responsible for some of the cellular damage induced by DU (Miller et al. 2002a, 2007) while others suggested that chemical damage is responsible for DU effects (Miller 2002; Stearns et

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al. 2005). In vivo studies of DU implants in rodent models have demonstrated the carcinogenicity (Hahn et al. 2002), neurotoxicity (Pellmar et al. 1999), and leukemogenic effect (Miller 2005; Miller et al. 2009) of chronic long-term internal exposure to embedded DU. Renal dysfunction following long-term chronic exposure has been observed as well (Zhu et al. 2009).

Inhalation studies in vivo have also demonstrated that inhaled DU is genotoxic (Monleau et al. 2006), inhibits vitamin metabolism (Tissandie et al. 2006), accumulates in brain (Houpert et al. 2007), and adversely affects rodent behavior (Monleau et al. 2005). The Capstone DU Aerosol Study was conducted to characterize aerosols generated from DU munitions fired into combat vehicles (Guilmette et al. 2009; Hahn et al. 2009). The report concluded that radiological risk for cancer mortality from inhaled DU aerosols is low although the risks should still be further reduced using ventilation systems and decreased exposure times.

It is unknown as to whether chronic internal exposure to DU will affect the health of unexposed offspring. Studies have demonstrated that parental preconceptional exposure (PPE) to radiation or metal can induce cancer in unexposed offspring. Surprisingly, PPE to a diverse spectrum of agents including radiation, chemicals, and heavy metals can induce adverse effects in unexposed offspring (Gardner et al. 1990; Luke et al. 1997; Lord et al. 1998; Lord 1999; Yu et al. 1999). These adverse responses include developmental abnormalities, tumor induction, or offspring tumor susceptibility. While recent studies have indicated that short-term ($<$120 d) exposure to internalized DU may not be a significant reproductive or developmental hazard (Arfsten et al. 2009), the authors suggest that adverse effects to offspring are not fully characterized, nor are longer-term DU exposures, so conclusions regarding offspring effects cannot be made (Arfsten et al. 2009). Preliminary data from our laboratory have shown an increased frequency of DU-induced germline mutations suggesting that further offspring studies are warranted (Merlot and Miller in press). Heavy metal and radiation exposure studies have already demonstrated that the possibility exists that transmissible alterations might be imprinted in germ cells for the future development of cancer in the postnatal environment (Nomura 2003; Dubrova et al. 2000). Furthermore, in vitro data from our laboratory indicated that DU exposure can induce genomic instability in cultured cells (Miller et al. 2003); therefore, the transmission of genetic damage to offspring is a possibility that should be explored.

The purpose of this study was to determine whether genetic instability can arise de novo in cells of hematopoietic tissues of F1 generation offspring of male parents chronically exposed to internalized DU or orally administered DU. We have used a mutation system (Kohler et al. 1991) employing a λ shuttle vector carried by cells of a transgenic mouse (Stratagene Big Blue) that carries the target *lacI* gene. Big Blue male mice were implanted with DU, mated with unexposed non-transgenic females and DNA was recovered from bone marrow of F1 offspring. Packaged λ vector was then assayed in vitro for mutations and detected in viral plaques by the blue color resulting from metabolism of 5-bromo-4-chloro-3-indoyl-1,3-galactopyranoside (X-gal). This type of a λ shuttle system is efficient for detecting point mutations and small deletions. However, the mutations recovered in this system are restricted by λ packaging machinery and large deletions arising from high linear energy transfer (LET) radiation such as alpha particles are difficult to detect in this system. This model will, however, enable us to determine whether preconceptional paternal DU exposure can cause a transmission of genetic damage to unexposed offspring. Secondly, heavy metals have been shown to induce base substitutions (McBride 1999; Stearns et al. 2005), so the model is appropriate to assess mutations associated with heavy metal exposure. The data presented demonstrate that transmission of genetic damage from DU-exposed fathers to unexposed offspring is possible.

MATERIALS AND METHODS

Mice

Male transgenic mice (Big Blue strain C57BL/6 hemizygous mouse containing 40 copies of a γ shuttle vector *lacI* transgene) and non-transgenic females C57BL/6 were used. This transgenic model has been used before to study radiation- and chemically-induced transmission of genetic damage to offspring. The λ phage shuttle vector contained the bacterial *lacI* gene which encodes the Lac repressor protein that controls β-galactosidase expression. P1-Big Blue and P1-non-transgenic females (10–12 wk of age) were maintained in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Animals* (NRC 1996). Upon arrival, mice were quarantined and screened for diseases. Except during urine collection, all animals were housed in plastic micro-isolator rat cages with hardwood chips as bedding. Mice were fed a certified diet and acidified water was provided ad libitum and they were housed in quarantine under conditions of constant temperature (25°C) and a regular 12 h light/dark regimen. The resulting F1 progeny were housed initially with their mothers and, after weaning at 4–5 wk, were housed as described above.

Metal pellets

DU pellets (Oak Ridge National Laboratories, Oak Ridge, TN) consist of 99.25% DU and 0.75% titanium by weight with the uranium isotopes ^{238}U (99.75%), ^{235}U (0.20%), and trace levels of ^{234}U. Nickel pellets consist of 100% Ni. The pellets were prepared from metal remaining from manufacture of U.S. military munitions. Ta pellets (Alfa Products, Ward Hill, MA) were used as controls because the metal has a mass similar to that of uranium, is biologically inert (Pellmar et al. 1999), and is supported by its frequent use in human prostheses (Pellmar et al. 1999). Each pellet (DU, Ni, and Ta) was a cylinder 1 mm in diameter and 2 mm long.

Pellet implantation surgery

Pellets were cleaned and chemically sterilized prior to implantation as previously described (Miller and McClain 2007). Anesthesia was induced with ketamine hydrochloride (80 mg kg^{-1}) in combination with xylazine hydrochloride (4 mg kg^{-1}) given by intraperitoneal (i.p.) injection. Following cleaning of the surgical site with betadine, pellets were implanted in the gastrocnemius muscle of each leg, spaced approximately 4–5 mm apart. Scalpel incisions were made through the skin and pellets inserted into the muscle with a 16-gauge needle with plunger. Incisions were closed with absorbable sutures and mice were closely monitored following surgery until ambulatory.

Uranyl nitrate exposure

Male transgenic mice (Big Blue strain C57BL/6 hemizygous mouse containing 40 copies of a γ shuttle vector *lacI* transgene) were housed as above. The male mice were contaminated by acidified water supplemented with either enriched uranium (95.74% ^{238}U, 4.24% ^{235}U, 0.02% ^{234}U, specific activity 67.0 kBq g^{-1}) or depleted uranium (99.74% ^{238}U, 0.26% ^{235}U, 0.001% ^{234}U, specific activity 14.7 kBq g^{-1}) incorporated as nitrate at a concentration of 50 mg U L^{-1} (approximately 1 mg d^{-1} per mouse). The control animals and females drank non-contaminated acidified water.

Heavy metal/radiation exposure and post-exposure mating

For pellet implant studies, seven treatment groups were established. Three doses (low, medium, and high) of DU were studied in comparison to Ni (high dose), Ta (high dose), ^{60}Co gamma radiation, and un-implanted/un-irradiated controls. Pellets doses were as follows: low dose (2 DU pellets + 4 Ta pellets); medium dose, (4 DU pellets + 2 Ta pellets); high dose, (6 DU, Ni, or Ta pellets). Pellets remained in place for 7 mo. At 7 mo post-pellet implantation, 1 male transgenic mouse/group was mated to 2 non-transgenic females (C57BL/6). Appropriate un-implanted transgenic males were mated in parallel. For the gamma radiation comparison, male transgenic mice were exposed to ^{60}Co (4 Gy; 0.60 Gy min^{-1}) and subsequently mated (at 30 d post-radiation) with un-irradiated non-transgenic females.

Screening of litters

The experiments (exposures and matings) were conducted three times per treatment group. In all, 105 hemizygous transgenic male parents (five per treatment group per experiment) were mated with 525 non-transgenic females (35 per mating group/experiment). The resulting F1 progeny were grown to maturity (~4–5 wk) and the presence of the transgene (hemizygosity) was determined by genotyping of the offspring. Offspring carrying the *lacI* transgene were identified by polymerase chain reaction (PCR) analysis. Genomic DNA was isolated from a tail-snip tissue sample of each offspring and prepared according to procedures described by the manufacturer of the QiAmp tissue kit (QiAgen, Valencia, CA). PCR analysis was conducted as instructed by the Stratagene Cloning Systems, La Jolla, CA, using standard PCR techniques.

Extraction of genomic DNA

Following screening of offspring for the presence of the transgene, shuttle vector was recovered from offspring bone marrow tissue. A single femur was dissected free from muscle and fat and the cells aspirated with a minimal amount of Fischer's medium containing penicillin (50 IU mL^{-1}), streptomycin (50 μg mL^{-1}) and 2 mM glutamine using a 1-mL syringe with a 23-gauge needle. The resulting cell suspensions were flash-frozen in liquid nitrogen for 1–2 min and stored at −70°C. Genomic DNA was prepared from bone marrow cells using a standard protocol of proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The amount and purity of DNA was assessed by spectrophotometry and the final concentration adjusted to 0.5 mg mL^{-1}.

Uranium analyses

Uranium determinations in wet-ashed tissues were performed by inductively coupled plasma mass spectroscopy (ICP-MS) as previously described (Pellmar et al. 1999; Miller et al. 2007). Sample preparation involved dry-ashing the tissue sample at 450°C followed by wet-ashing with a series of additions of concentrated nitric acid and 30% hydrogen peroxide. The ash was dissolved in 1M nitric acid, and the intensity of ^{235}U and ^{238}U ions was measured by ICP-MS.

In vitro mutagenesis assay

The λ shuttle vector (containing the *lacI* target gene and a *lacZ* reporter gene) was recovered from genomic DNA using λ bacteriophage packaging extract (Transpack©, Stratagene, San Diego, CA). One packaging reaction was performed per sample. The *lacI* gene (1080 bp of coding region) encodes for the *lac* repressor protein. Mutations in the *lacI* target gene inactivate the repressor gene, thereby allowing expression of a *lacZ* reporter gene which then complements the *lacZ* in the bacterial host cell (Escherichia colt SCS-8; Stratagene). The functional protein product, (3-galactosidase), then cleaves the chromogenic substrate X-gal present in the plating agar to produce a blue-colored plaque. Non-mutated phage produces colorless plaques. The ratio of blue to total plaques is a measure of the mutation frequency. In order to determine induced mutation frequency, at least 3×10^5 plaques were recovered from each animal tissue. Density of plaques was limited to 15,000 per assay tray to ensure accuracy in detection of mutants (i.e., at least 20 assay trays were used per sample).

Colony assays

Hematopoietic stem cells were assayed as 10-d spleen colony-forming units (CFU-S) in BDF1 recipients as previously described (Miller et al. 2009). Ten recipient mice per donor were irradiated with 15 Gy ^{60}Co (0.6 Gy min^{-1}) before intravenous (i.v.) injection of 7×10^4 bone marrow cells obtained from femora of control, DU-, TA-, Ni-, ^{60}Co (4 Gy)-offspring donor mice. Ten days after transplantation recipients were euthanized, spleens excised, and cells were fixed for colony counting. In vitro colony-forming cell (CFC) assays for committed hematopoietic progenitors were performed by culturing 5×10^4 bone marrow cells in soft agar (Miller et al. 2009). Cultures were established and granulocyte/macrophage colonies were counted after seven days. Blood counts were obtained with an electronic blood counter.

Statistical methods

In each experiment the data are expressed as the mean ± S.E.M. (standard error of mean) and the values were analyzed by analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. Differences were considered to be significant if $p < 0.05$.

RESULTS

Uranium tissue concentration after 2 or 7 mo exposure

Within 1 d of DU exposure via implantation or drinking water, male animals (P1) showed a significant uranium accumulation in kidneys, testes, and femur (data not shown). At 2 mo (oral U administration) or 7 mo post-pellet implantation, uranium content was measured with ICP-MS. Uranium content in kidneys, testes, and femur (right) for each male animal was measured and is shown in Table 1. Uranium concentration in testes tissue of DU-implanted males (low, medium, and high dose) was 456 ± 98, 529.2 ± 110, and 618 ± 141 ng U g^{-1} tissue, respectively. Uranium content in testes tissue from control-, Ta-, Ni-, or ^{60}Co-exposed males ranged from 1.2 ± 0.5 to 3.6 ± 1.4 ng U g^{-1} tissue. Testes tissues from DU-implanted mice (low, medium, and high dose) were all significantly elevated in comparison to control mice. Uranium content in kidney and femurs obtained from DU-implanted males was significantly increased above unexposed controls as expected and is shown for comparison (Table 1).

Uranium concentration in testes tissue of males contaminated with DU- or enriched-uranyl nitrate was 539 ± 37 and 518 ± 141 ng U g^{-1} tissue, respectively. These increased uranium concentrations were significantly elevated in comparison to control mice (2.9 ± 0.32 ng U g^{-1} tissue). Uranium content in kidney and femurs obtained from males exposed to DU or enriched uranium was significantly increased above unexposed controls as expected and is shown for comparison (Table 1).

Litter assessment and genotyping of offspring for transmission of *lacI* transgene

For the implanted DU studies, three experiments were performed with a total of 105 *lacI* transgenic males (±exposure) and 525 control non-transgenic females

Table 1. Tissue uranium content after two or seven months exposure (ng U g^{-1} tissue).[a]

Tissues	Control 7 mo	TA 7 mo	DU implants 2 DU pellets "Low" 7 mo	DU implants 4 DU pellets "Med" 7 mo	DU implants 6 DU pellets "High" 7 mo	Control 2 mo	Depleted uranyl nitrate (50 mg U/L) 2 mo	Enriched uranyl nitrate (50 mg U/L) 2 mo
Kidneys	2.7 (0.4)	1.8 (0.3)	410[b] (39.2)	689[b] (65.5)	881[b] (77.3)	3.1 (0.33)	619[b] (55.7)	634[b] (59.2)
Testes	1.8 (0.3)	2.5 (0.4)	456[b] (98)	529[b] (49)	618[b] (77)	2.9 (0.32)	539[b] (37)	518[b] (48)
Femur	1.2 (0.3)	1.1 (0.3)	321[b] (29.2)	477[b] (48.3)	559[b] (54.1)	1.3 (0.34)	429[b] (38.5)	451[b] (48.8)

[a] Tissues were collected at 2 (oral DU- or enriched-uranium exposure) or 7 (DU- or TA-implants) months post exposure initiation. The data expressed as ng U g^{-1} tissue are presented as means with S.E.M. in parentheses, $n = 10$; ($p < 0.05$).
[b] The data are statistically significant in comparison to control values ($p < 0.05$).

resulting in a total of 1,097 offspring examined at weaning and genotyped for presence of the *lacI* transgene (Table 2). Big Blue males implanted with DU (high, medium, low doses) were mated to non-transgenic un-implanted females seven months after DU implantation. Seven months was chosen since this time frame would approximate an adult male age similar to the ages of the DU-wounded soldiers. A comparison to control (un-implanted), Ta (high dose), Ni (high dose), and ^{60}Co gamma radiation (4 Gy) was done. In comparison to litter sizes from control un-implanted fathers (5.5 ± 0.4 pups/litter), litters from DU-embedded fathers were smaller and the decrease in litter size followed a DU-dose-dependence. Litters from DU-embedded fathers (low, medium, and high dose) demonstrated an average of 5.0 ± 0.4 pups/litter, 4.1 ± 0.4 pups/litter, and 3.8 ± 0.5 pups/litter, respectively. In comparison to controls, litters from Ta- and Ni-exposed fathers did not result in a significant decrease in litter size (5.1 ± 0.6 and 5.2 ± 0.5 pups/litter, respectively). In contrast, ^{60}Co radiation (4 Gy) did result in a statistically significant decrease in litter size (4.2 ± 0.4 pups/litter; $p < 0.05$). There were no observed morphological fetal malformations and no significant increase in offspring hematopoietic malignancies in weaned offspring was determined.

For the uranyl nitrate-drinking water studies, three experiments were performed with a total of 60 *lacI* transgenic males (± exposure) and 121 control non-transgenic females resulting in a total of 484 offspring examined at weaning and genotyped for presence of the *lacI* transgene. Big Blue males were given either depleted- or enriched-uranyl nitrate in their drinking water for two months prior to mating with non-transgenic females. Two months was chosen since earlier gastrointestinal (GI) toxicity studies demonstrated that longer times of enriched-uranium exposure through drinking water resulted in GI toxicity to the males (data not shown). Litter sizes from fathers given equal concentrations (50 mg L^{-1}) of depleted- or enriched-uranyl nitrate in their drinking water showed a statistically significant difference in comparison to control (age-matched) fathers. In comparison to litter sizes from control fathers (5.9 ± 0.4 pups/litter), litters from depleted- or enriched-uranium fathers exposed were smaller; litters from depleted- and enriched-uranium-exposed fathers demonstrated an average of 4.3. ± 0.4 pups/litter and 4.1 ± 0.4 pups/litter, respectively. There was no statistical difference in litter sizes from fathers exposed to either depleted or enriched uranium. There was, however, a statistical difference in litter size between uranium-exposed fathers and the age-matched controls ($p > 0.05$).

Genomic DNA was isolated from tail-tip samples of all offspring and screened by PCR analysis to identify carriers of the *lacI* gene. The transmission rate of the *lacI* gene for offspring derived from each treatment group was determined and is shown in Table 2. The overall transmission rate for all groups was 47.9% (Table 2), which is statistically similar to the expected Mendelian rate of 50% transmission for a hemizygous gene. While this rate was not significantly different ($p > 0.05$) from the Mendelian expectation, the actual *lacI* copy number transmitted to offspring was not determined.

Mutation frequencies in offspring bone marrow from fathers exposed to DU, Ni, Ta, or ^{60}Co radiation

Bone marrow DNA from offspring identified as *lacI* carriers in treated and control groups was screened for mutations in the *lacI* transgene. Table 3 summarizes the mutation frequencies found in the hemizygous F1 offspring of male parents exposed to DU, Ta, Ni, or ^{60}Co radiation from the three experiments conducted. Mutation frequency in shuttle vector recovered from bone marrow of F1 offspring of control males was 2.28 ± 0.31 × 10^{-5}. The mutation frequencies from F1 offspring of Ta- or Ni-implanted fathers (2.38 ± 0.35 and 2.93 ± 0.39 × 10^{-5}) were not significantly different than control levels (2.28 ± 0.31 × 10^{-5}). The mutation frequencies from F1 offspring of DU-implanted fathers demonstrated a dose-dependent increase in comparison to control F1 offspring; medium and high dose DU exposure to P1 fathers resulted in a significant increase in mutation frequency in F1 offspring (3.57 ± 0.37 and 4.81 ± 0.43 × 10^{-5}; $p < 0.001$). A *t* test was used to determine

Table 2. Genotyping of offspring for transmission of the *lacI* gene.

Treatment	Control	TA	DU implants DU pellets "Low"	DU implants DU pellets "Low"	DU implants DU pellets "Low"	Ni implants "High"	^{60}Co	Depleted uranyl nitrate (50 mg U/L)	Enriched uranyl nitrate (50 mg U/L)
No. screened	188	151	148	171	133	140	166	145	132
No. *lacI* carriers	89	77	69	85	66	71	86	74	64
Percent *lacI* carriers	47.3[a]	50.9[a]	46.6[a]	49.7[a]	49.6[a]	50.7[a]	51.8[a]	51[a]	48.5[a]

[a] Based on the expected 50% transmittal rate, the observed rates do not differ significantly ($p > 0.05$). Furthermore, there were no significant differences between the ratios of male to female carrier offspring.

Table 3. Summary of mean mutation frequencies in shuttle vector (*lacI* gene) recovered from bone marrow of F1 offspring.

Treatment	Control	TA	DU implants DU pellets "Low"	DU implants DU pellets "Med"	DU implants DU pellets "High"	Ni implants "High"	^{60}Co	Depleted uranyl nitrate (50 mg U/L)	Enriched uranyl nitrate (50 mg U/L)
Mean	2.28[a]	2.38[a]	2.71[a]	3.57[a,c]	4.81[a,c]	2.93[a]	4.69[a,c]	3.3[a,c]	4.55[a,c]
Mutation	0.31	±0.35	±0.37	±0.37	±0.43	0.39	0.48	±0.29	0.41
Frequency	(2.00–2.61)[b]	(2.01–2.71)[b]	(2.29–3.25)[b]	(3.10–3.97)[b]	(4.33–5.20)[b]	(2.41–3.49)[b]	(4.16–5.20)[b]	(3.0–3.71)[b]	(4.02–5.2)[b]

[a] Mean values ($\times 10^{-5}$) S.E.M.
[b] Range of mutational frequencies for offspring within each exposed group.
[c] Significant when compared to control value, $p < 0.05$.

statistical significance and demonstrated that fathers chronically exposed to medium and high dose DU resulted in offspring with increased *lacI* mutations in their bone marrow. A comparison to nickel and ^{60}Co gamma radiation was done since both exposures had previously shown preconceptional paternal exposure could induce genomic susceptibility and genomic instability in offspring (Prows et al. 2003; Luke et al. 1997). While offspring from Ni-exposed fathers did not show an increased *lacI* mutation frequency, offspring from ^{60}Co (4 Gy) did demonstrate an increased *lacI* mutation frequency as had been shown previously by others (Luke et al. 1997). A graphic comparison of mutation frequency from offspring from control, Ni-, DU (high)-, and ^{60}Co-exposed fathers is illustrated in Fig. 1.

Since DU can cause both radiation and chemical effects (Miller 2002; Miller et al. 2002a, 2007), an evaluation whether radiotoxicity from DU could contribute to the DU-induced effect on offspring bone marrow mutations was conducted. Uranyl nitrate compounds at equal concentration but with significantly different specific activities were used. The results are shown in Table 3. Results show that the offspring from fathers that had drinking water-exposure to equal uranium concentrations (50 mg U L^{-1}) but differing U specific activities had a specific-activity dependence in their bone marrow mutation frequencies. Fathers that had DU in their drinking water produced offspring with a mean mutation frequency of 3.3 ± 0. 29 while fathers with enriched U in their drinking water produced offspring with a mean mutation frequency of 4.55 ± 0.41. These data demonstrate a statistical difference ($p < 0.05$), which indicates that there is a specific activity-dependent effect on offspring bone marrow mutation frequency under experimental conditions where the uranium concentration in each uranyl nitrate compound was the same. The statistically significant difference ($p < 0.05$) between mutation frequencies in offspring from DU-enriched vs. U-exposed fathers (differing specific activities with equal chemical effect) suggests that the difference in the mutation frequency was due to the increased radioactivity in the uranium compound tested.

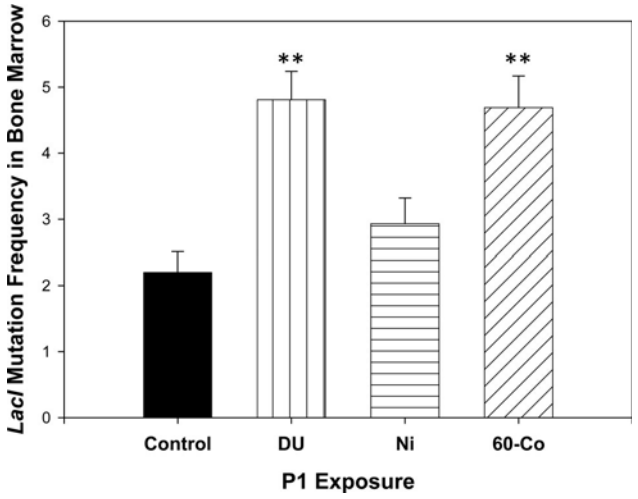

Fig. 1. Comparison of mutation frequency in *lacI* transgene from bone marrow of offspring from DU-, Ni-, and ^{60}Co-exposed fathers to control. Mutation frequencies were determined in *lacI* transgene from bone marrow of offspring from fathers exposed to high dose DU (vertical pattern), high dose Ni (horizontal pattern), and ^{60}Co (4 Gy) (diagonal pattern). A comparison to controls (solid pattern) was done. Statistical significance is shown by the double asterisk symbols**.

DISCUSSION

The results of our study indicate the possibility of transmission of genomic instability from DU-exposed fathers to the somatic cells of unexposed offspring. While it has been previously shown by others that gamma radiation can cause transmission of genetic damage to offspring, this is the first report demonstrating that DU can cause genetic transmission of a mutation in a transgene to an unexposed progeny. The data show that the mutation frequency in shuttle vector recovered from bone marrow of F1 offspring is significantly increased in comparison to controls after the male parent has had a chronic internal exposure to DU (medium or high doses) for seven months. A similar exposure to low doses

of DU did not increase the *lacI* bone marrow mutation frequency. These results suggest that there is a DU dose-dependence on the induction of *lacI* mutation in offspring bone marrow. It appears that transmission of factors via spermatozoa between male parent and offspring has occurred leading to the increased mutation frequency in bone marrow, possibly as a result of genomic instability transmitted to the somatic cells of unexposed progeny.

The increase in bone marrow *lacI* gene mutation frequency cannot be explained by a simplistic "jackpot effect." A "jackpot" mutation occurs when the elevated mutation frequency is derived from the matings of one parent. In this study the mutation frequencies were derived from multiple fathers and mothers as indicated in the results section.

A statistical analysis suggests that a direct germline mutational mechanism is not responsible for the observed effects. Consider that if only one copy of the 40 viral copies of present per carrier spermatozoa were mutated, every somatic cell of the resulting F1 offspring would be similarly affected. Using calculations, the mutant plaque frequency should be at least 375 per plate, because 15,000 viral plaques (assayed per plate) divided by 40 (number of viral particles per hemizygous cell) equals 375. None of the plates in this study contained more than several (4–9 plaques) mutant plaques; therefore, it is unlikely that we are detecting germline mutations. Regardless, studies investigating effects of DU exposure on germ cell mutagenesis and direct DNA damage to sperm are being completed.

Litter size was affected by increasing DU exposure, so it is possible that dominant lethal mutations were induced at the medium and high DU doses resulting in decreased litter sizes, but the induction of dominant lethal mutations was not measured in this study so we cannot conclude that these types of mutations actually occurred in exposed males. Significant decreases in the number of litters produced and litter size are consistent with high dose uranium exposures (Domingo 2001; Arfsten et al. 2009). In contrast, a study using a rat model and DU pellet implantation indicated that chronic internalized DU exposure (20–150 d) did not have an adverse impact on male reproductive success, sperm concentration, or sperm velocity (Arfsten et al. 2005, 2009). Our data demonstrate a DU dose-dependent effect on litter size; it is possible that a DU exposure time-dependent effect on litter size may also occur and may explain why the data from Afsten and colleagues (at 150 d exposure) are different from ours (210 d). Furthermore, the differences between the rodent models (rat vs. mouse) may also explain the different litter size results. It is possible that chronic long-term exposure to DU caused a reduction in sperm count or motility. These possibilities are being furthered studied and will be presented in a future report. The ongoing studies in our laboratory examining DU-induced germ cell damage will provide further clarification of the litter size effects induced by DU.

An assessment of the hematopoietic status of the offspring was done. Offspring of control, Ta-, Ni-, and gamma-irradiated fathers were hematologically normal, as were the offspring from fathers exposed to the low and medium dose of DU. In contrast, the offspring from fathers exposed to high dose DU were not hematologically normal. While these offspring did exhibit normal peripheral blood profiles, the stem cell population (CFU-S) was elevated (data not shown). In our study the significance of this finding is not clear. Previous studies evaluating transgenerational exposure to radiation or ^{55}Fe have demonstrated that an induction of stem cell cycling in offspring increased their sensitivity to a chemical leukemogen and thus may have implications for genetic instability (Hoyes et al. 2001; Lord 1999). Since the kinetics of hematopoiesis depends on the quality of the regulatory microenvironment, the development of a high level of CFU-S in offspring from high-DU exposed fathers suggests a degree of hyper-proliferation during fetal and postnatal development or a defective microenvironment that cannot properly limit CFU-S proliferation.

It is still not fully known as to whether the observed elevation in the *lacI* gene mutation frequency in offspring from DU-exposed fathers is due to DU's chemical, radiation, or combined chemo-radiotoxic effects to the exposed father. The discussion is also highly speculative since the analysis of the data regarding sperm effects in these fathers has not been completed and so it cannot be referenced here.

The *lacI* mutation model used in this study measures small deletions and point mutation/base substitutions, which would suggest that chemical effects are predominantly responsible for the observed effects. On the other hand, the data from fathers exposed to either enriched or depleted uranyl nitrate at equal chemical concentrations would suggest that radiation has played a role in the observed effects. Previously it was thought that DU caused its effects through its chemical toxicity alone since the radioactive contribution was calculated to be low. However, several studies have demonstrated that cellular damage and neoplastic transformation associated with DU exposure in vitro involves, at least partially, radiation toxicity (Miller et al. 1998a and b, 2000, 2001, 2002b, 2003, 2004; Miller and McClain 2007). The current results presented here would tend to agree with those studies demonstrating that DU exposure does provide some radiation contribution. In contrast, other DU in vitro studies have confirmed that DU can cause cellular effects even at negligible radiation doses (Miller et al. 2003;

Stearns et al. 2005). Although the data indicate that radiation is involved in DU effects in vitro, several questions remain unanswered. We know neither the extent to which radiation contributes to the effects exerted by DU nor understand its mechanism(s). Furthermore, we can only speculate as to whether the radiation and chemical effects are synergistic. Limited studies have shown that a nonradioactive metal like cadmium combined with gamma radiation can result in a synergistic response in vivo (Prise et al. 1998). It is also intriguing to speculate whether radiation actually plays a significant role in DU cellular effects, perhaps through nontargeted effects of radiation exposure. Several recent radiation studies have demonstrated the important role that bystander effects have in cellular radiation response by causing damage in unirradiated neighboring cells (Prise et al. 1998; Zhou et al. 2000; Belyakov et al. 2001; Sawant et al. 2001). In the case of DU, cells not traversed by an alpha particle may be vulnerable to radiation-induced effects as well as chemically-induced effects. While the data presented here do not fully and definitively answer the question as to the contribution of radiation-induced damage in DU cellular effects, they do provide additional evidence of radiation involvement in the cellular effects of DU and, therefore, potentially in DU-associated health effects.

The transmission of effects to F1 offspring from paternal exposure to radiation has been documented (Luke et al. 1997; Dubrova et al. 2000; Nomura 2003; Baulch et al. 2005). These effects include protein signaling, cell proliferation alterations, and genomic instability. The effects of maternal heavy metal exposure on offspring have been explored showing significant gestational effects of cadmium, chromium, and nickel (Antilla and Sallmen 1995; Tian et al. 2009; Bailey et al. 2006). Similarly, this report is the first demonstrating that preconceptional paternal exposure to DU can cause genetic transmission of a mutation in a transgene to an unexposed progeny. Our results indicate that there exists a route for transgenerational transmission of a genetic factor(s) leading to genomic instability in F1 offspring resulting from preconceptional paternal chronic internal exposure to DU or preconceptional paternal contamination with uranium (enriched and depleted).

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