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1. Introduction

The first extensive combat use of depleted uranium (DU) armament occurred in the Gulf War. Only Coalition forces possessed DU weapons, but a small number of US military personnel were wounded by fragments of DU because of friendly fire incidents. Several continue to retain fragments in their bodies. The demonstrated effectiveness of DU munitions and armor during the Gulf War has led other nations to develop their own DU armaments. Future conflicts may therefore lead to large numbers of personnel wounded by DU. There is still little known about the long-term health consequences of this kind of internal exposure.

Chemically similar to natural uranium (National Research Council, BIER-IV Report, 1988), DU is a low specific activity heavy metal, with a density approximately 1.7 times that of lead (19 g/cm^3 versus 11.35 g/cm^3). DU differs from natural uranium in that it has been depleted of ^{235}U and ^{234}U. Consequently, the specific activity of DU is significantly less than natural uranium (0.44 μCi/g versus 0.7 μCi/g, respectively) (Danesi, 1990).

Our laboratory has used an in vitro human cell model and rodents to examine the potential late health effects of DU and surrogate metals, particularly heavy-metal tungsten alloys (HMTA). A summary of our findings is shown in Table 1 and Fig. 1. In our studies, we have demonstrated that DU and HMTA are neoplastically transforming and genotoxic in vitro. The in vivo effects of internalized DU include enhancement of urine mutagenicity, oncogene activation, and uranium redistribution to multiple organs. These data demonstrate a strong correlation between uranium levels/concentration and DU-induced effects (Fig. 1).

Unlike natural uranium, which is considered both a radiological and a chemical (heavy-metal) hazard (National Research Council, BIER-IV Report, 1988), DU is not believed to be a significant radiation hazard because of its low specific activity. Studies with DU in our laboratory demonstrated neoplastic transformation of human cells under conditions where approximately 14% of the DU-exposed cells were transformed even though less than 5% were traversed by an alpha particle (Miller et al., 1998; Miller et al., 2000; Miller et al., 2002). These findings suggest that factors other than direct or "targeted" damage to the DNA may be involved in the transformations. Chemical effects of DU and "non-targeted" effects of radiation may also play a role. Non-targeted effects can result in damage in cells not traversed by an alpha particle. The overall level of transformation observed may result from contributions by any or all of these factors.

According to conventional radiobiology principles, the biological consequences following radiation exposure occur as a result of DNA damage in directly "hit" cells, and the damage is manifested in these cells within 1{n}2 cell generations (Grosovsky, 1999). Increasing evidence shows, however, that ionizing radiation may also produce non-targeted effects; specifically, critical genetic consequences may arise in cells that receive no direct radiation exposure. These non-targeted effects include radiation-induced bystander effects and genomic instability. A bystander effect is the expression of cellular damage such as chromosomal alterations, apoptosis, transformation, or mutations in unirradiated cells neighboring an irradiated

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Table 1
Summary of findings on DU and HMTA

Metal; Type of Study	Parameters Examined	Outcome	Authors
DU, In vivo	Oncogenes, uranium levels	↑ Oncogene expression with DU	Miller et al., 1996
DU, In vitro	Neoplastic transformation	DU induced neoplastic transformation	Miller et al, 1998
DU, In vivo	Urine mutagenicity	↑ Urine mutagenicity with DU level	Miller et al., 1998
DU, In vivo	DU tissue distribution	DU redistributed to multiple sites	Pellmar et al.,1999
DU, In vivo	Neurotoxicity	Electrophysiological brain effects	Pellmar et al.1999
DU, HMTA In vitro/in vivo	Genotoxicity	↑ genotoxicity/genomic instability	Miller et al., 2000
DU, In vitro	Transformation	↑ transformation/mutagenicity	Miller et al., 2001a
HMTA, In vitro	Transformation/genotoxicity	HMTA induces transformation	Miller et al, 2001b
DU, HMTA, In vitro	Genomic instability	↑ genotoxicity/genomic instability	Miller, in press

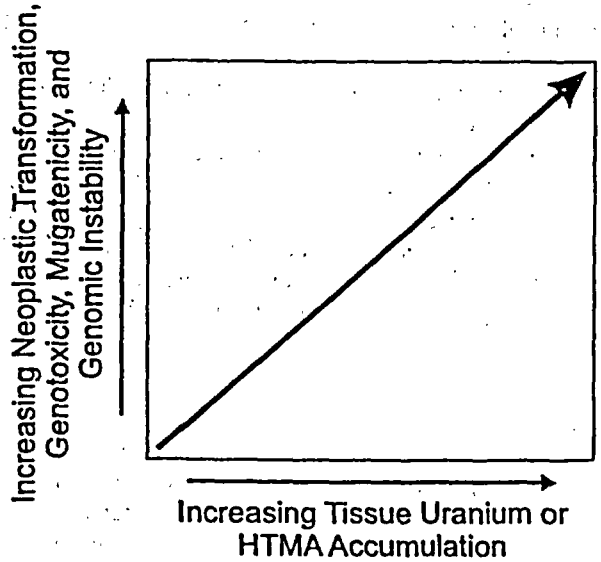

Fig. 1. Relationship between uranium levels and biological effects in previously published studies.

cell. In contrast, genomic instability is defined as the induction of a persistent instability in the genome of surviving irradiated cells. The progeny of cells exposed to ionizing radiation can exhibit transmissible genomic instability (Kadhim et al., 2001). Cellular manifestations of instability have been measured as delayed reproductive death (Gorgojo and Little, 1989), lethal mutations (Seymouret al., 1986), delayed mutations (Littleet al., 1990), and chromosomal instability (Pampfer and Streffer, 1989; Kadhim et al., 1992; Kadhim et al., 1994; Kadhim et al., 1998; Holmberg et al., 1993; Holmberg et al., 1995; Marder and Morgan, 1993; Martins et al., 1993). Considering the newly developing paradigm in radiobiology that non-targeted effects may be critical to low-dose radiation-induced carcinogenesis, an understanding of the potential involvement of these phenomena in DU-induced effects is important.

The objective of this study was to determine the relative importance of acute and delayed effects in immortalized human cells exposed to DU. We investigated the relationship between delayed reproductive death (yield of lethal events) and the yield of micronuclei produced following exposure. These endpoints were assessed up to 36 days (30 population doublings). Results were compared with yields from cells exposed to gamma radiation and Ni.

2. Materials and methods

2.1. Cell lines and culture

Human osteosarcoma cells (HOS) (TE85, clone F-5) were obtained from the American Type Culture Collection (Manassas, VA). Cell cultures were propagated

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2.2. Heavy metals and radic

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2.3. Cellular survival assay

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2.4. Micronuclei analysis

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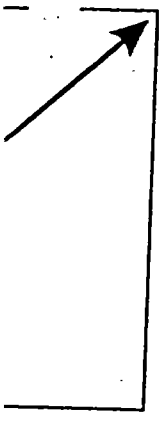

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in Dulbecco's modified Eagle medium (D-MEM) supplemented with 2 mM glutamine, 10% heat inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were tested for Mycoplasma by MycoTect Kit (Sigma) and only cells negative for Mycoplasma were used.

2.2. Heavy metals and radiation exposure

DU-UO_2NO_2 (Alfa Aesar, Ward Hill, MA) and $NiSO_4$ (Alfa Aesar) were dissolved in sterile water, then filtered to prepare concentrated stock solutions used throughout these studies. Working solutions were prepared by diluting stock solutions with complete medium immediately before experiments.

Cells were exposed to gamma radiation (bilateral, 1 Gy/min, room temperature) using the AFRRI Cobalt Facility.

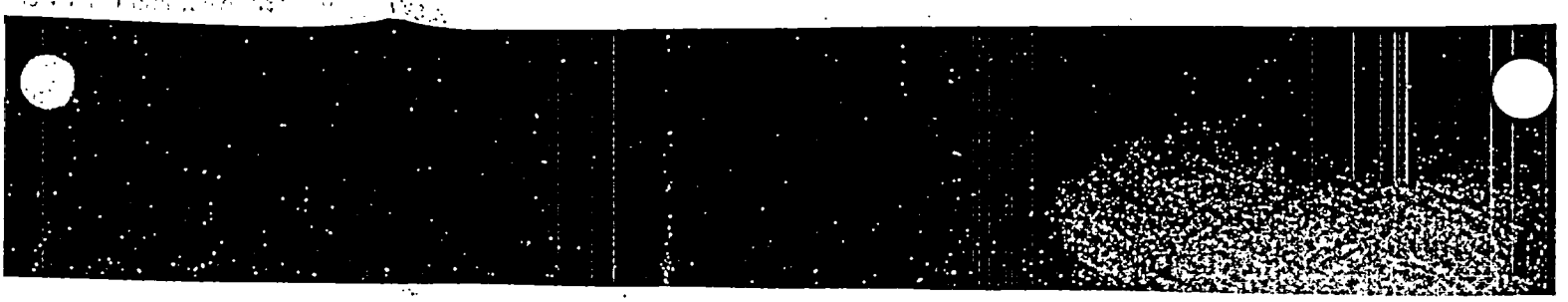
Preliminary studies determined equivalent ranges of metal concentrations and radiation doses in terms of their capacity to induce lethality, delayed micronuclei expression, and micronuclei frequency. For cell lethality and delayed micronuclei expression experiments, metal concentration of 0, 25, 50, and 100 μM were compared to 0, 2, 4, 6, and 8 Gy. For micronuclei frequency experiments, metal concentrations of 0, 10, and 25 μM were compared to 0, 2, and 3 Gy.

2.3. Cellular survival assay

Surviving fraction was assayed by measuring a reduction in plating efficiency. Exponentially growing cells were seeded at 10^4 cells/100-mm dish, using 3-dishes/treatment group. Cultures were then treated 24 hr later with increasing concentrations of metal for 24 hr or increasing doses of gamma radiation. For metal studies, cells were then rinsed with Dulbecco's phosphate-buffered saline (PBS). Immediately following metal exposure or radiation and at various times up to 36 days later, cells were harvested and counted. Fifty to 100 cells were seeded into 60-mm diameter petri dishes and returned to the incubator for ten days to allow for colony formation. Cultures were then fixed with methanol and stained with 2% (w/v) crystal violet. Plates with more than 15 colonies of more than 50 cells were counted as survivors.

2.4. Micronuclei analysis

The induction of micronuclei in control cells and cells exposed to metal powder(s) was assessed using the conventional fluorescence-plus-Giemsa harlequin staining protocol [24]. Following a 1 hr exposure, the media containing the metal powder was removed, and cells were rinsed (3× with sterile serum-free medium) and incubated again at 37 °C in complete media. Mitomycin C was used as a positive control (24-hr exposure). Cytochalasin B (6 μg/ml) was added after 24 hr to block cytokinesis. At 48 hr post-treatment, cells were dropped onto slides using a Cytospin (Shandon, St. Louis, MO; 5 min at 600 rpm). Slides were fixed with 5% Giemsa.



3. Isolation of clones from irradiated cells

Following exposure to metal or gamma radiation, cells were plated on 60-mm petri dishes and incubated for 1 week to give rise to approximately 20–25 colonies per dish. Three to five large colonies from each concentration/dose group were selected. Special care was taken to ensure that the selected colony was separate from other colonies. Each selected colony was transferred into an individual flask and grown to a total clonal progeny of more than 7×10^5 cells. Cells were then trypsinized and plating efficiency and micronuclei yield determined for each clone.

3.1. Statistics

Statistics for the micronuclei assays were performed with the chi-square (χ^2) test and the Tukey–Kramer multiple comparisons test.

4. Results

4.1. Delayed lethality following DU exposure

HOS cells were incubated with increasing concentrations of soluble DU, soluble Ni, or gamma radiation, and clonogenic survival was determined either 3, 12, 24, or 36 days after exposure. Survival curves were obtained by determining the number of viable colonies present ten days after plating at these times. Data from DU-, Ni-, and gamma-irradiated cells are shown in Fig. 2. For DU-exposed cells plated at day 3, a shouldered survival curve was obtained (Fig. 2a). Survival levels were lower than controls at all times after initial DU exposure, even at 36 days. Data obtained from gamma-irradiated or Ni-exposed cells (Fig. 2b, c) were similar to that seen with DU.

4.2. Delayed micronuclei formation

The metal concentration- or dose-dependence of micronuclei production is presented in Fig. 3. For all three exposures (DU, gamma radiation, or Ni), delayed production of micronuclei was observed at all times measured. Levels of delayed micronuclei formation were overall greater after DU exposure than after gamma radiation or Ni treatment. At 12, 24, and 36 days after DU exposure, levels of micronucleated cells being produced in the dividing cell population remained significantly greater than untreated controls (Fig. 3a). In contrast, data from gamma-irradiated or Ni-exposed cells showed that micronuclei yields, though also maximal at three days, were not significantly different from controls at 24 and 36 days post-radiation. This suggests that DU treatment results in a more prolonged effect (in terms of micronuclei yields) than was seen after the other exposures.

Acute and Delayed Survival Curve for Ni Exposure

Acute and Delayed Survival Curve for Gamma Radiation

Acute and Delayed Survival Curve for DU Exposure

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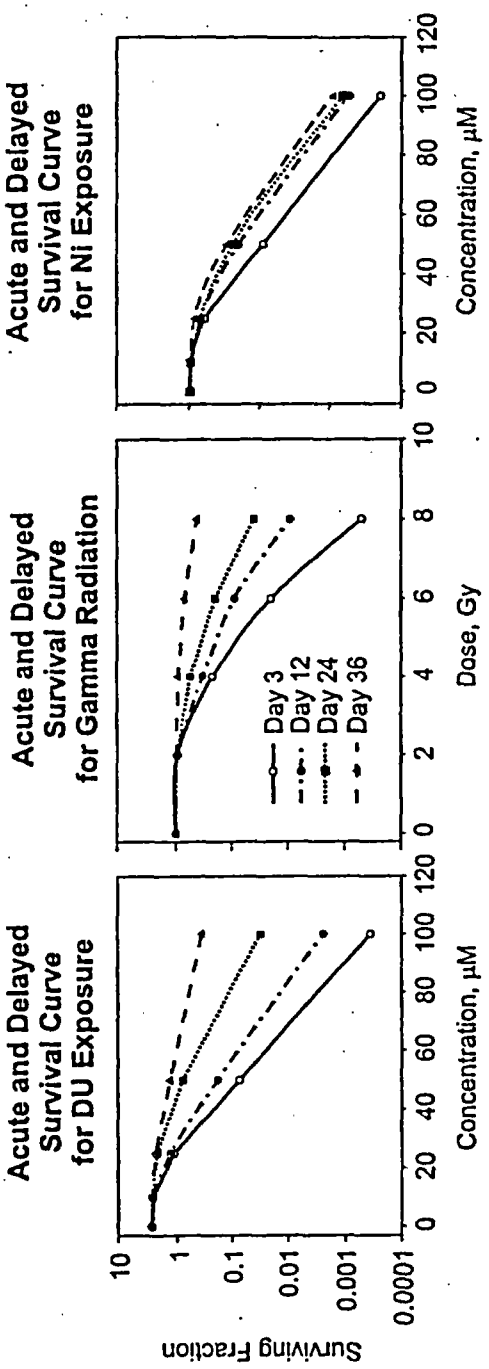

Fig. 2. Acute and delayed survival curves after exposure to DU (panel a), gamma radiation (panel b), and Ni (panel c). Cells were plated for measurement of clonal efficiency 3 (M), 12 (Φ), 24 (□) and 36 (◆) days after exposure.

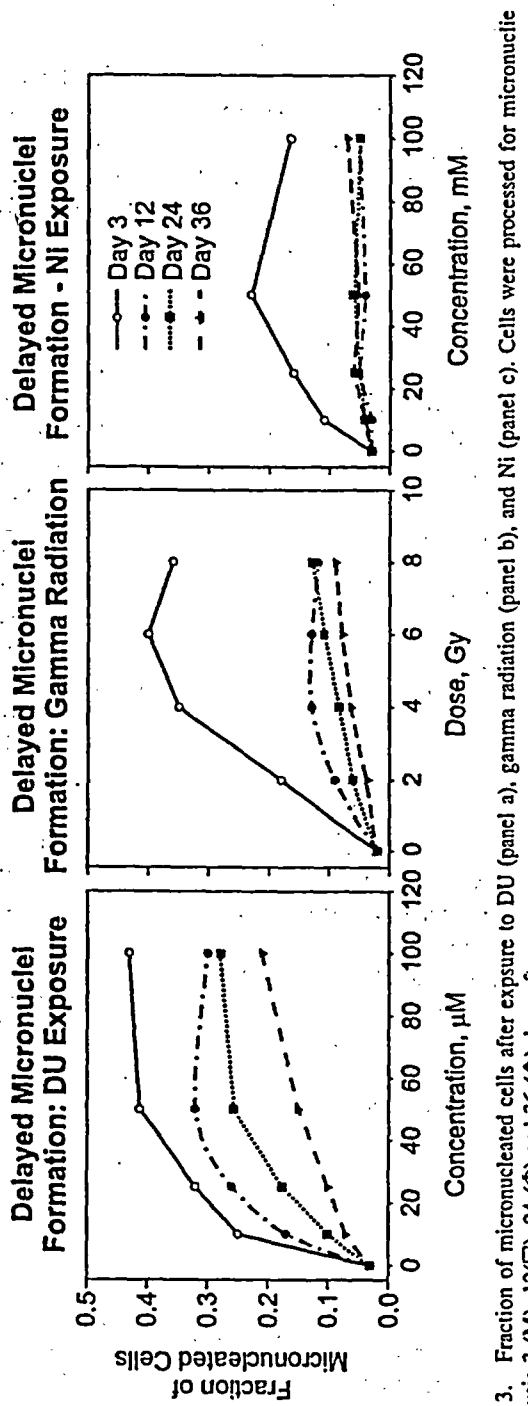

Fig. 3. Fraction of micronucleated cells after exposure to DU (panel a), gamma radiation (panel b), and Ni (panel c). Cells were processed for micronuclie analysis 3 (M), 12(□), 24 (Φ) and 36 (◆) days after exposure.

4.3. Micronuclei frequency i

The frequency of micronu DU, Ni, or gamma radiation Three to five individual clon concentrations or doses of th pared to clones arising from u strated a frequency of micro increase in the frequency of clones generated by DU-exp from 2.2 to 4.5. Interestingl observed in ten clones isolat exposure (Manti et al., 199 resulted in the generation o quency of micronuclei rangi gamma radiation, clonal de increase in micronuclei freq

5. Discussion

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Our studies demonstrate persistent effects in distant p with previous observations omic instability in vitro sir Mothersill et al., 1998). Sir ticles can induce a persister it is difficult to determine responsible for the induced microdosimetry studies wit cells that had been incuba alpha particle. Our origina probably responsible for experiments in our laborato gest that radiation may als current results do not prov but they do provide evide

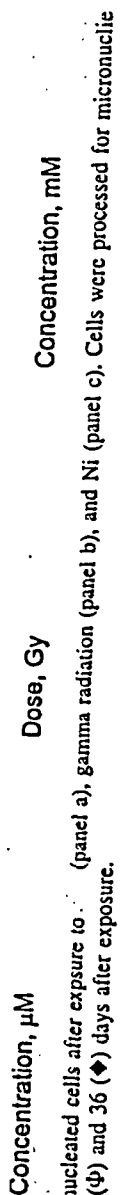

Fig. 3. Fraction of micronucleated cells after exposure to (panel a), gamma radiation (panel b), and Ni (panel c). Cells were processed for micronuclie analysis 3 (M), 12(□), 24 (Ψ) and 36 (◆) days after exposure.

4.3. Micronuclei frequency in individual clones obtained from DU-treated cells

The frequency of micronuclei in individual clones raised from cells exposed to DU, Ni, or gamma radiation was determined for each type of exposure (Fig. 4). Three to five individual clones arising from cells originally exposed to increasing concentrations or doses of the metals or gamma radiation were analyzed and compared to clones arising from untreated cells. Clonal progeny of untreated cells demonstrated a frequency of micronuclei lower than 1.8%. For DU exposure, a persistent increase in the frequency of micronuclei was observed in all clones examined. All clones generated by DU-exposed proliferating cells exhibited a frequency ranging from 2.2 to 4.5. Interestingly, the range seen with DU exposure is similar to that observed in ten clones isolated and expanded in the same way after alpha radiation exposure (Manti et al., 1997). Similar to observations with DU, gamma radiation resulted in the generation of clones that exhibited a persistent increase in the frequency of micronuclei ranging from 2.2 to 3.6. In contrast to both DU exposure and gamma radiation, clonal descendents of Ni-treated cells exhibited only a slight increase in micronuclei frequency (percentage of micronuclei, 1–2.3) (Fig. 4).

5. Discussion

This study was undertaken to assess the ability of DU to induce genomic instability in the progeny of DU-exposed cells. The endpoints used to determine this instability were delayed reproductive death and delayed micronuclei formation. It is well documented that ionizing radiation can induce a persistent instability in the genome of surviving cells. A recent study has demonstrated that heavy metals can also induce genomic instability manifested as chromosomal aberrations (Coen et al., 2001). Since both of these findings could have significant implications for risk estimates, it is important to assess whether DU exposure can also induce genomic instability.

Our studies demonstrate for the first time that cellular DU exposure results in persistent effects in distant progeny of DU-exposed cells. These studies are consistent with previous observations that heavy metals like cadmium and Ni can induce genomic instability in vitro similar to that observed with radiation (Coen et al., 2001; Mothersill et al., 1998). Since there are also extensive data showing that alpha particles can induce a persistent instability in the genome of progeny of irradiated cells, it is difficult to determine whether the alpha particle or metal component of DU is responsible for the induced genomic instability. In our previous transformation and microdosimetry studies with DU we measured sister chromatid exchanges in HOS cells that had been incubated with soluble DU but had not been traversed by an alpha particle. Our original interpretation was that the chemical effect of DU was probably responsible for that chromosomal damage. However, results of recent experiments in our laboratory measuring dicentric formation after DU exposure suggest that radiation may also play a role in DU effects (McClain et al., 2001). The current results do not prove that alpha particles are involved in DU cellular effects, but they do provide evidence that genomic instability is strongly associated with

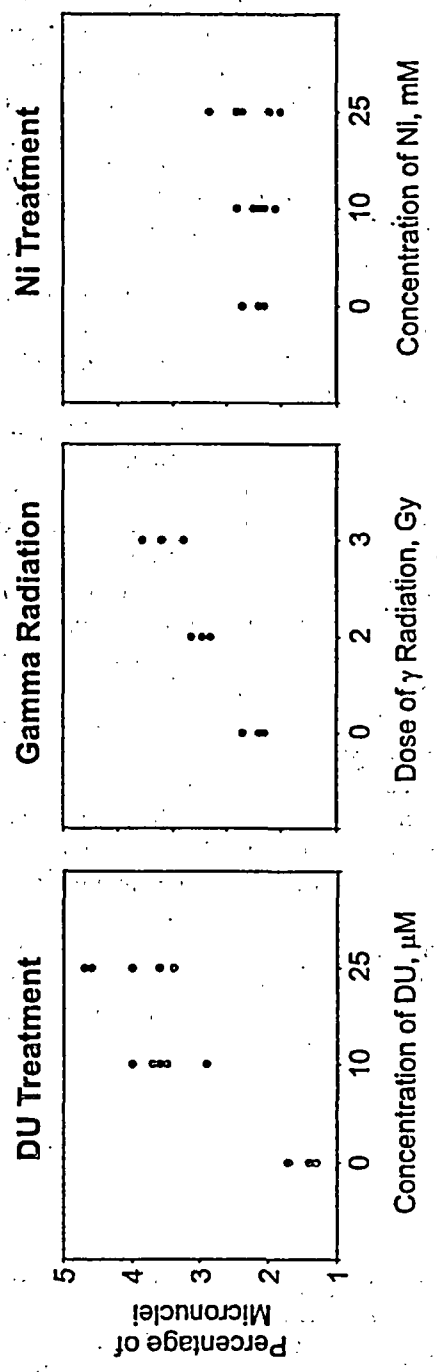

Fig. 4. Micronuclei frequency in clonal descendents. Frequency of micronuclei in clonal progeny of single cells exposed to DU (panel a), gamma radiation (panel b), or Ni (panel c) were analyzed at three doses. Concentrations tested for DU and Ni were (0, 10 uM, and 25 uM); for gamma radiation, doses were (0, 2, and 3 Gy).

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exposure to DU. A complete understanding of the role of radiation versus chemical effects awaits further investigation.

Our data indicate that delayed lethality is associated with a significant increase in micronuclei frequency after exposure to DU. The ability of the cells to regain normal plating efficiencies was similar for cells exposed to DU, gamma radiation, or Ni. The DU-associated delayed increase in micronuclei frequency exhibited a clear concentration-dependent response in the low-dose range. This is similar to what has been observed with ionizing radiation (Belyakov et al., 1999; Manti et al., 1997).

The persistent increase in the frequency of micronuclei after DU exposure is due to de novo production of micronuclei in surviving proliferating cells. In our experiments, when 10^5 cells were exposed to DU and assayed 24 hr later, cultures yielded 42% micronucleated cells (4.2×10^4 cells). Twelve days after DU exposure a frequency of 31% micronuclei was measured in 10^7 cells, indicating that the cultures contained 3.1×10^6 micronucleated cells. These calculations suggest that micronuclei were formed de novo in surviving cells.

It is interesting to note that the degree of instability for both micronuclei endpoints was similar for DU and gamma radiation exposures; however, the pattern of delayed micronuclei induction by Ni was somewhat less than DU and gamma radiation. In contrast to our present findings with Ni, studies of Ni and genomic instability conducted by Coen et al. (2001) showed that Ni exposure resulted in a pattern of expression of delayed effects that was similar to ionizing radiation (Coen et al., 2001). Differences in the kinds of Ni salts employed in each investigation (nickel sulfate vs nickel chloride) might partially explain the different results. The precise mechanism(s) by which DU induces genomic instability is unknown. Since the pattern of expression of delayed effects is similar to that of gamma radiation, we could speculate that the mechanism(s) might be similar to that for radiation. The mechanism(s) underlying radiation-induced instability remains unclear, however. Epigenetic changes have been suggested as a common underlying mechanism (Clutton, et al, 1996). Sustained oxyradical activity in the progeny of irradiated cells supports a role for epigenetic alterations. Speculations regarding mechanism are further complicated by recent data suggesting that there could be a link between bystander effects, i.e., cellular damage expressed in unirradiated cells, and genomic instability (Belyakov, et al., 2000). The potential involvement of bystander effects in DU-induced biological responses is currently being investigated by our laboratory. The involvement of bystander effects in the mechanism of DU-induced effects could mean that conventional microdosimetry assessment of the radiation dose from DU might be significantly undervalued.

In summary, we have presented data showing the production of genomic instability in the progeny of human cells exposed to DU. The findings demonstrate that DU can induce delayed cell death and genetic alterations in the form of micronuclei. Compared to gamma radiation or Ni, DU exposure resulted in a greater manifestation of genomic instability. Although animal studies are needed to address the effect of protracted DU exposure and genomic instability in vivo, results obtained from our in vitro system can play a significant role in determining risk estimates of DU exposure.

Fig. 4. Micronuclei frequency in clonal descendents. Fre[illegible]y of micronuclei in clonal progeny of single cells exposed to DU (panel a), gamma radiation (panel b), or Ni (panel c) were analyzed at three doses. Concentrations tested for DU and Ni were (0, 10 uM, and 25 uM); for gamma radiation, doses were (0, 2, and 3 Gy).

Acknowledgements

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