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Depleted uranium–uranyl chloride induces apoptosis in mouse J774 macrophages

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Abstract

Depleted uranium entering the body as a result of inhalation or embedded fragments becomes associated to a great extent with macrophages. As part of our continuing studies on the health effects of internalized depleted uranium, we investigated the effect of soluble depleted uranium–uranyl chloride on the mouse macrophage cell line, J774. Using a cytochemical staining protocol specific for uranium, we found that uranium uptake by the macrophages increased in a time-dependent manner. Treatment with 1, 10, or 100 μM depleted uranium–uranyl chloride resulted in decreased viability of the J774 cells within 24 h. Flow cytometric analysis of the treated cells with annexin V showed the translocation of phosphatidylserine from the inner face of the plasma membrane to the outer surface indicating the loss of phospholipid symmetry and the beginning of the apoptotic process. Significant differences in annexin V labeling between control cells and cells treated with 100 μM depleted uranium–uranyl chloride were apparent within 2 h. Other events associated with apoptosis, including morphological changes and DNA fragmentation, were also apparent after depleted uranium–uranyl chloride treatment. These results suggest that the uptake and concentration of soluble depleted uranium by macrophages initiates events that results in the apoptotic death of these cells.

Keywords: Apoptosis; Depleted uranium; Macrophages; Programmed cell death

1. Introduction

Natural uranium consists of three isotopes in the following percentages (by weight): ^{238}U (99.283%), ^{235}U (0.711%), and ^{234}U (0.005%). The enrichment process for the production of weapon- and reactor-grade uranium results in uranium containing greater than 0.711% ^{235}U ('enriched' uranium) and less than 0.711% ^{235}U ('depleted' uranium). Depleted uranium (DU) usually contains 0.2% ^{235}U and 0.001% ^{234}U with the remainder being ^{238}U (approximately 99.8%) (Livengood, 1998). As a result, DU has half the specific activity of natural uranium, and because of this low radioactivity, is one of the few radio-

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active materials whose occupational exposure limits are based on chemical and not radiological toxicity. DU is extremely dense (1.7 times the density of lead). Because of this property, DU is used militarily in kinetic-energy penetrator munitions and commercially as aviation counterweights and radiation shielding. The expanded use of DU has led to questions about potential health effects. DU can be internalized in a variety of ways. For example, dust and particulates can be inhaled, as well as contaminate wounds and fragments can become embedded in tissues. In all these cases, the primary cell type involved in the body's response to the internalized metal, regardless of the route of internalization, is the macrophage.

Macrophages or macrophage-like cells are present in large numbers in tissues throughout the body including the lung, liver, lymph nodes, brain, kidney, skin, and spleen. They are one of the first cells (after neutrophils) to accumulate in a wound (Agaiby and Dyson, 1999) where they phagocytose debris and invading microorganisms. The macrophage is important in the transition from inflammation to wound healing through the secretion of various factors including platelet-derived growth factor, tumor necrosis factor alpha, and transforming growth factor beta (Mutsaers et al., 1997). Macrophages assist in the removal of particulate metal debris from tissues (Haynes et al., 1996; Chen et al., 1999) as well as in metal detoxification (Berry et al., 1997). Alveolar macrophages clear inhaled particulates, including metals, from the lungs (Brain, 1986; Fels and Cohn, 1986; Bowden, 1987; Lohmann-Matthes et al., 1994). Several metals, including cadmium (Labedzka et al., 1989), chromium (Gercken et al., 1988), gadolinium (Kubota et al., 2000), beryllium (Sawyer et al., 2000), and lead (Shabani and Rabbani, 2000), decrease viability and interfere with macrophage function. Because of the ubiquitous nature of macrophages, their ability to produce a wide range of biological mediators and toxic agents, and their importance in immune surveillance, we investigated the effect of soluble DU, in the form of uranyl chloride, on the mouse macrophage line J774.

2. Materials and methods

2.1. Materials

J774 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (Manassas, VA). RPMI-1640 medium, fetal bovine serum (FBS), glutamine, pencillin/streptomycin, and Hanks' balanced salt solution (HBSS) were purchased from Gibco/BRL (Gaithersburg, MD). Ethylhexadecyldimethylammonium bromide and DU–uranyl chloride were purchased from Fluka Chemical Company (Ronkonkoma, NY). Ethidium bromide, Hoechst 33258, ethylenediaminetetraacetic acid (EDTA), sodium citrate, paraformaldehyde, 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP), and 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium (MTT) were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Cell culture conditions and uranyl chloride treatments

J774 cells were grown in RPMI-1640 medium containing 10% FBS, 3 mM glutamine, and 100 U/ml penicillin/streptomycin in an atmosphere of 5% CO_2 in air at 37 °C. Cells were grown either in suspension or as a monolayer. Cells were treated with DU–uranyl chloride, prepared as sterile 1-, 10-, or 100-μM solutions in RPMI, for various times before harvesting.

2.3. Colorimetric determination of macrophage-associated uranium

J774 cells were plated in 96-well, flat-bottom plates at a concentration of 10^5 cells/well and treated with 100 μM uranyl chloride for various times. Plates were centrifuged for 5 min at 1500 rpm in a Beckman RT6000 centrifuge, supernatant removed, and cells washed twice with HBSS. Cells were then treated for 30 min at room temperature (20–22 °C) with gentle shaking ($\sim$50 rpm) with 'staining solution' (100 μM Br-PADAP, 10 mM sodium citrate, 10 mM EDTA, 0.5% (w/v) ethylhexadecyldimethylammonium bromide, 100 mM borate buffer, pH 9.5). The presence of sodium

citrate and EDTA in the staining solution prevents the binding of Br-PADAP to other metals, but still allows binding to uranium (Kalinich et al., 2000; Kalinich and McClain, 2001). Absorbance was measured at 578 nm with a SpectraMax Model 250 microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) and DU uptake determined from standard curves produced with known concentrations of DU–uranyl chloride in 96-well plates. Data are expressed as μg DU/10^5 cells.

2.4. Viability determinations

Viability was determined by trypan blue dye exclusion with direct microscopic observation or with a modification of the MTT [3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] assay (Hansen et al., 1989). Briefly, at various times post-treatment, 5×10^3 J774 cells were incubated with 125 μg/ml MTT at 37 °C. After 2 h, cells were lysed with 20% SDS in 50% dimethylformamide (pH 4.7) and absorbance determined at 570 nm. Data were normalized to control values (no DU treatment) for the same time point.

2.5. Flow cytometric analysis of annexin V binding

At various times post incubation, cells were removed from the culture medium and incubated with fluorescein isothiocyanate (FITC)-labeled annexin V using the ApoAlert Annexin V Apoptosis kit (Clontech, Palo Alto, CA). Briefly, 1×10^6 cells were washed with phosphate-buffered saline and resuspended in 200 μl of 1 × binding buffer (supplied in the assay kit). Cell suspensions were incubated with annexin V-FITC (final concentration 1 μg/ml) at room temperature for 5–15 min in the dark. Samples were analyzed by flow cytometry using a Becton Dickinson (San Jose, CA) FACSCalibur.

2.6. DNA fragmentation assay

Cells ($1–2 \times 10^6$) were collected by centrifugation, lysed with ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100, and pelleted by centrifugation ($14{,}000 \times g$, 4 °C, 20 min) to separate the intact from the fragmented DNA. The pellet was resuspended in lysis buffer and sonicated for 10 s at 4 °C. DNA in both supernatant and pellet fractions was determined by an automated fluorometric protocol and the DNA-specific fluorescent probe Hoechst 33258 (Ramakrishnan et al., 1993). Percent DNA fragmentation was calculated by dividing the amount of DNA in the supernatant by the total amount of DNA in the sample (supernatant + pellet DNA) and multiplying by 100.

2.7. Photomicroscopy

Cells were fixed in 3% formaldehyde in HBSS and stored at 4 °C. Uranium uptake was determined using a cytochemical staining protocol and the pyridylazo indicator dye Br-PADAP (Kalinich and McClain, 2001). Briefly, J774 cells (5×10^5) were plated on sterile, glass coverslips in six-well tissue culture plates and treated with various DU–uranyl chloride concentrations for a variety of times. The medium was removed and the cells washed twice with HBSS before fixation with 3% formaldehyde in HBSS. The cells were washed with water then stained with 100 μM Br-PADAP in a solution of 10 mM sodium citrate, 10 mM EDTA, 0.5% (w/v) ethylhexadecyldimethylammonium bromide, and 100 mM borate buffer for 30 min at a pH of 9.5 at room temperature (20–22 °C) with gentle shaking (50 rpm). After washing with water, the cells were examined microscopically.

For morphological examination and apoptotic assessment, cells were stained with 50 μg/ml ethidium bromide (McClain et al., 1995). All photomicroscopy was performed with an Olympus (Melville, NY) AHBT3 Research Microscope with Nomarski-type differential interference contrast and reflected-light fluorescence and photographed with a Kodak (Rochester, NY) Professional DCS 420 digital camera.

2.8. Statistical analysis

Data were analyzed with a one-way ANOVA to assess the effect of DU treatment. If a significant effect was observed, Dunnett's test was used to

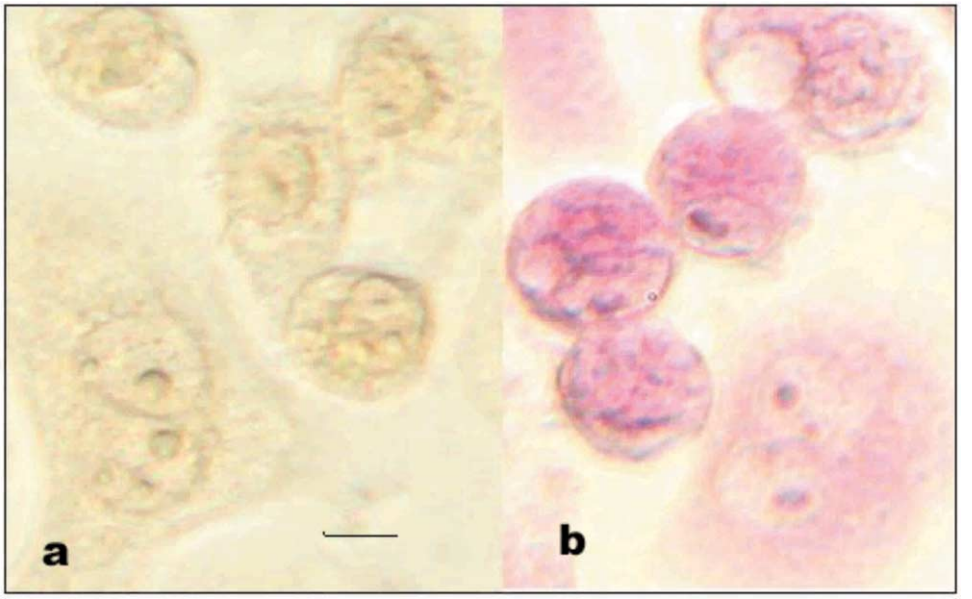

Fig. 1. Uptake of DU by mouse J774 macrophages. Cells (5×10^5) were plated on microscope coverslips in six-well tissue culture plates. Panel a: No DU–uranyl chloride; panel b: DU–uranyl chloride added to a final concentration of 100 μM. After 24 h medium was removed and cells processed and stained as described in Section 2. Scale bar, 10 μM.

determine which DU-treatment groups were significantly different from control. In all cases, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Uptake of DU by J774 macrophages

When J774 macrophages were cultured in medium containing soluble DU–uranyl chloride, the DU was internalized and concentrated by the cells. Intracellular DU in these cells was detected by staining with the pyridylazo dye, 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP). Cells not treated with DU did not stain, while cells that were cultured with DU present in the medium were stained reddish-purple (Fig. 1). By slightly modifying the staining procedure, the cellular levels of DU were quantitated spectrophotometrically. As seen in Fig. 2, J774 cells internalized DU in a time-dependent manner

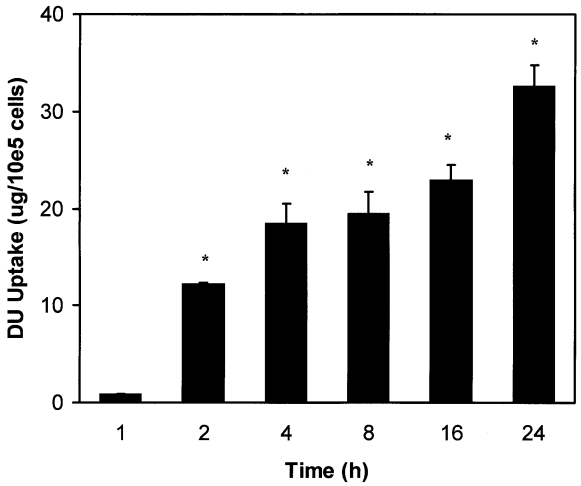

Fig. 2. Colorimetric quantitation of DU uptake by mouse J774 macrophages. Cells (1×10^5 cells/well) were plated on 96-well plates and treated with 100 μM DU–uranyl chloride for various times before processed as detailed in Section 2. Data are expressed as μg DU/10^5 cells and represent mean of eight independent experiments. Error bars represent standard deviation. Asterisks indicate difference from control is statistically significant ($P < 0.05$).

over a 24 h period. After culturing in medium containing 100 μM DU for 24 h, each cell contained approximately 0.3 ng of DU.

3.2. Effect of DU on macrophage viability

The effect of DU exposure on macrophage viability, assessed by the trypan blue dye exclusion method, is summarized in Table 1. Incubation of J774 cells in medium containing 1 or 10 μM DU–uranyl chloride for times ranging from 2–24 h had no significant effect on the viability of the cells. However, when J774 cells were treated with 100 μM DU–uranyl chloride, small, but statistically significant ($P < 0.05$), decreases in viability were seen after as little as 2 h of exposure. In addition, viability of these cells continued to decrease as the exposure time to DU increased.

Determination of cell viability using trypan blue relies upon the plasma membrane of the badly damaged, dying, or dead cell becoming permeable to the dye molecule. However, cells can be metabolically dead, yet still exclude trypan blue, and thus appear viable. A more sensitive measure of viability is metabolic viability, determined by the ability of mitochondrially located enzymes to reduce MTT to an insoluble form. Fig. 3 shows the results of DU–uranyl chloride exposure on the ability of J774 cells to metabolize MTT as compared to control cells. With all concentrations tested, DU–uranyl chloride treatment for 24 h resulted in a significant ($P < 0.05$) decrease in the ability of the cells to metabolize MTT. As seen in Fig. 4, this decrease in viability also correlated with an increase in cellular DU.

Table 1
Percent viability of DU–uranyl chloride-treated J774 cells. trypan blue dye exclusion assay

Time (h)	0 μM	1 μM	10 μM	100 μM
2	98.6±0.6	96.2±0.8	97.1±0.5	95.5±0.6*
4	98.9±0.5	99.2±0.5	96.9±1.0	94.6±0.7*
8	98.4±0.8	99.6±0.4	97.7±0.7	94.1±1.0*
24	98.6±0.4	97.6±0.5	98.7±0.3	93.6±0.7*

Data are given as percentage of viable cells and represent the mean of five independent experiments. Errors are given as standard error of the mean. Asterisks indicate difference from control is statistically significant ($P < 0.05$).

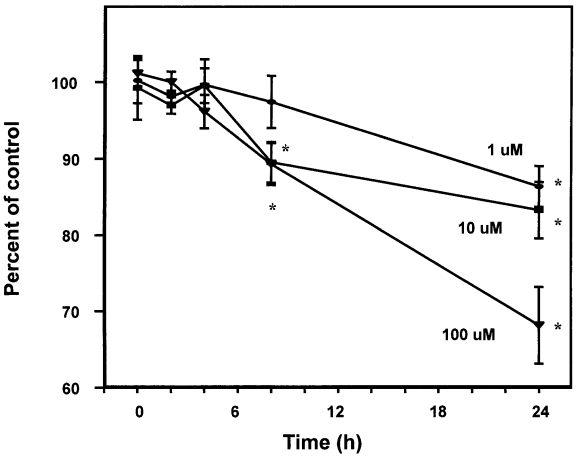

Fig. 3. Cell viability determined by MTT assay. Cells were treated with DU–uranyl chloride for various times before viability was assessed using the MTT assay as described in Section 2. Data are normalized to control values (0 μM uranyl chloride) for same time point. Error bars represent standard deviation and asterisks indicate difference from control is statistically significant ($P < 0.05$).

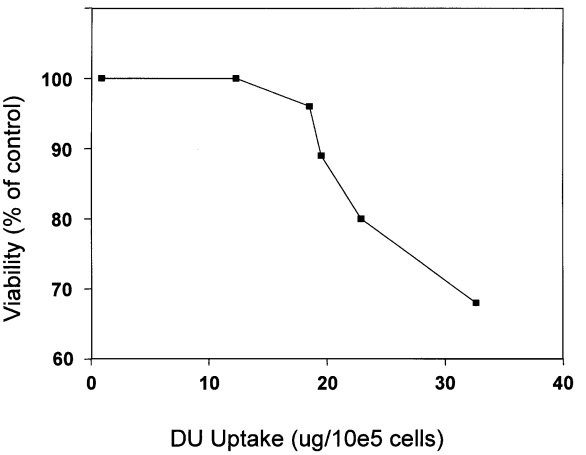

Fig. 4. Correlation of cell viability with DU uptake. Data represent cellular viability after 100 μM DU–uranyl chloride treatment, as determined by the MTT assay, versus cellular DU levels after the same treatment.

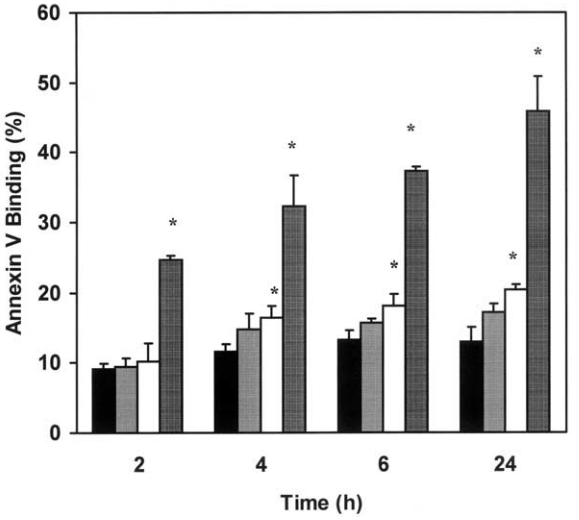

Fig. 5. Histogram of flow cytometric analysis of annexin V binding to control and uranyl chloride-treated J774 cells. Cells were treated with 1, 10, or 100 μM DU–uranyl chloride for various times and flow cytometric analysis conducted as described in Section 2. Data are represented as percentage of cells binding annexin V at various times post-treatment and are the mean of three individual experiments. Error bars represent standard deviation. Asterisks indicate difference from control is statistically significant ($P < 0.05$).

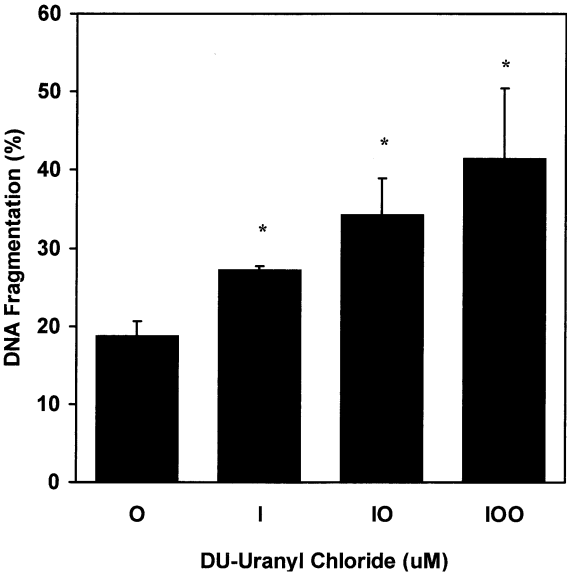

Fig. 6. DNA fragmentation in J774 cells after treatment with DU–uranyl chloride. Cells were treated with DU–uranyl chloride (0, 1, 10, 100 μM) for various times and the percentage of fragmented DNA determined as described in Section 2. Data are given as percentage DNA fragmentation and represent the mean of three independent experiments. Error bars represent standard deviation. Asterisks indicate difference from control is statistically significant ($P < 0.05$).

3.3. Induction of apoptosis in J774 cells by DU treatment

The treatment of J774 cells with DU–uranyl chloride also resulted in apoptotic cell death. The binding of annexin V to phosphatidylserine was used to assess apoptosis. As seen in Fig. 5, J774 cells treated with 100 μM DU–uranyl chloride showed a significant increase ($P < 0.05$) in annexin V after only 2 h. The binding of annexin V binding to these cells increased over time. Treatment with 10 μM DU–uranyl chloride also resulted in an increase in the amount of phosphatidylserine in the plasma membrane outer leaflet, but annexin V binding was not significantly different from control until after 4 h of DU exposure, and binding did not reach the levels attained by treatment with 100 μM DU–uranyl chloride. Annexin V binding to J774 cells treated with 1 μM DU–uranyl chloride was not significantly different than control values at all exposure times tested.

The process of apoptosis involves the orderly self-destruction of the cell into small 'pieces' known as apoptotic bodies. One portion of this destruction pathway is the fragmentation of the nuclear DNA. Fig. 6 shows the extent of DNA fragmentation following treatment of J774 cells with different concentrations of DU–uranyl chloride. Because of the phagocytic ability of the J774 cells, DNA fragmentation over time was found to peak and then decline, as the remaining cells phagocytized the apoptotic bodies of the dead cells. Therefore, the results in Fig. 6 indicate the maximum extent of DNA fragmentation observed in control and DU-treated cells. For untreated control cells, maximum DNA fragmentation occurred at 72 h after the start of the experiment (the maximum time measured). For cells treated with 1 μM of DU–uranyl chloride, maximum DNA fragmentation occurred after 48 h of treatment, while treatment with 10 μM DU–uranyl chloride resulted in maximum DNA fragmentation in J774

cells after 24 h. Treatment of J774 cells with 100 μM DU–uranyl chloride resulted in maximum DNA fragmentation after only 8 h of exposure. In all treatment cases, the extent of DNA fragmentation was significantly greater than control.

Morphological changes occur as cells undergo apoptotic death. The morphological changes that occur as a result of treatment of J774 cells with 100 μM DU–uranyl chloride for 24 h are shown in Fig. 7. Incubation of cells in DU resulted in cell shrinkage, chromatin condensation, and formation of apoptotic bodies in the treated cells, indicating that DU–uranyl chloride exposure resulted in the apoptotic death of J774 cells.

4. Discussion

The expanded use of munitions containing DU in both the Gulf War and the Balkans conflict has raised questions as to its effect on human health. DU can enter the body in a variety of ways: shrapnel can be embedded in the body, particulates can be inhaled, and wounds suffered on the battlefield can be contaminated with dust and dirt containing DU. One of the first cells involved in the body's response to these types of insults is the macrophage. As part of our broad study of DU health effects, we believed it was important to determine what, if any, affect DU had on this important cell type. We used a cultured mouse macrophage cell line, J774, and soluble DU, in the form of uranyl chloride, to address this question.

In the rat, both soluble aerosols and insoluble particles of a variety of metals concentrate in the alveolar macrophages after inhalation (Berry et al., 1997). An initial question was whether cultured macrophages would function similarly. This proved to be the case. J774 cells internalized and concentrated soluble DU from the extracellular

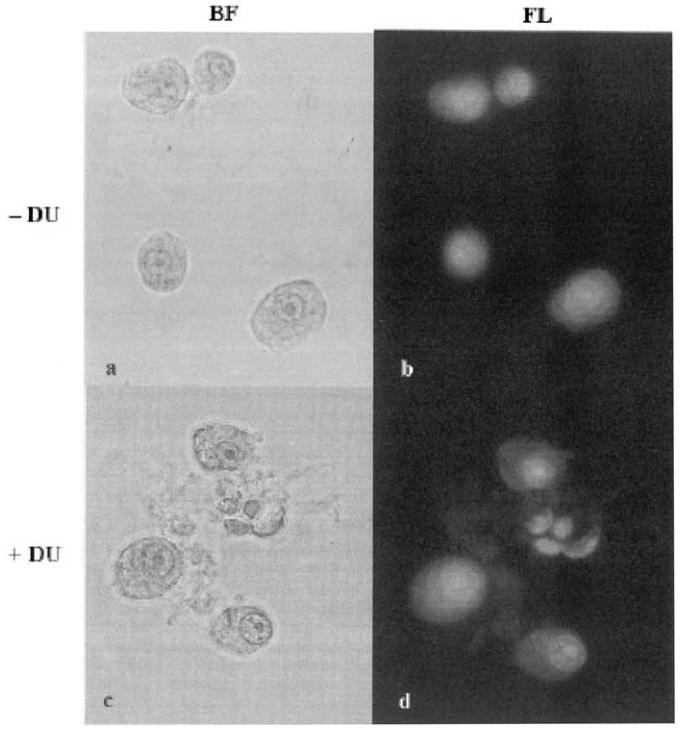

Fig. 7. Bright-field and fluorescent photomicrographs of J774 cells cultured in medium without and with DU–uranyl chloride. Cells were cultured in medium without or with 100 μM DU–uranyl chloride for 24 h then processed for microscopy as described in Section 2. Panels a and b are bright-field and fluorescent images of control cells, respectively. Panels c and d are bright-field and fluorescent images of cells treated for 24 h with DU–uranyl chloride, respectively.

medium. The intracellular localization of DU was shown with a colorimetric staining procedure utilizing the pyridylazo dye, Br-PADAP. Br-PADAP will bind to a number of metals, including copper, zinc, cobalt, and nickel (Sumi et al., 1983a); however, its specificity can be controlled through the use of 'masking agents' (Sumi et al., 1983b). The stain can be made specific for uranium by including sodium citrate and EDTA in the staining mixture. These compounds prevent the Br-PADAP from binding to metals other than uranium (Kalinich et al., 2000; Kalinich and McClain, 2001). The amount of DU in cells can also be quantitated spectrophotometrically using the same pyridylazo dye and a slightly modified staining protocol with cells in 96-well plates. The J774 cells internalized DU in a time-dependent manner, with DU levels reaching a peak after 24 h of exposure. Interestingly, internalization of soluble DU was not observed with MOLT-4 cells (human T-cell leukemia line) or REH cells (human B-cell line) when using either of these methods (data not shown). Thus, uptake and concentration of soluble DU appears to be a macrophage-dependent phenomenon.

Once internalized, DU induced a concentration- and time-dependent decrease in the viability of J774 cells. Using the trypan blue dye exclusion technique, a decrease in viability over time was observed only with the 100 μM uranyl chloride treatment. Although this decrease was small, it was statistically significant. However, employing a more sensitive test of metabolic viability (MTT assay), it was found that, after 24 h, DU exposure resulted in decreased viability of J774 cells at all concentrations tested. Viability of cells exposed to DU–uranyl chloride concentrations of 10 and 100 μM for 8 h was also significantly lower than control cells.

When the viability of J774 cells treated with 100 μM DU–uranyl chloride is compared to cellular DU uptake over the same treatment times, there appeared to be an intracellular DU concentration threshold below which cell viability was not affected. However, when this level was exceeded, viability decreased as the intracellular DU levels increased. It is unknown at this time why this occurs. It may be possible that increased incorporation of DU into phagolysosomal vesicles also results in increased oxidative stress within the cell as it attempts to 'kill' and dispose of this foreign material using reactive oxygen and nitrogen species, as well as the degradative enzymes in the lysosomes. Since the DU would become insoluble as a result of the action of the acid phosphatases in the phagolysosomal vesicle (Berry et al., 1997), the cell would never succeed in disposing of the internalized DU. As time went on or as the intracellular levels of DU increased, the antioxidative defenses of the cell would eventually be exhausted and the resulting increase in oxidative stress/damage would lead to the death of the cell.

DU–uranyl chloride treatment of J774 cells also resulted in apoptotic death. One of the earliest indicators of apoptosis is the translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane. The reason for this movement is not clear; however, it may serve as a signal for removal of dead cells by the macrophages (Fadok et al., 1992, 2001). Phosphatidylserine can be detected using a flow cytometric protocol with fluorescently labeled annexin V. J774 cells treated with 100 μM of uranyl chloride exhibited a significant increase in annexin V binding after as little as 2 h of exposure. The amount of annexin V bound to these cells increased in a time-dependent manner. Treatment with 10 μM of uranyl chloride resulted in an increase in annexin V binding but to a far lesser extent than seen with 100 μM uranyl chloride. Despite the decrease in metabolic viability, 1 μM uranyl chloride treatment did not result in a significant increase in annexin V binding. This suggested that low concentrations of DU affected the metabolic ability of J774 without immediately initiating apoptosis.

The process of apoptosis involves the programmed breakdown of the cell, including condensation and fragmentation of nuclear DNA. Treatment of J774 cells with DU–uranyl chloride also resulted in increased DNA fragmentation at all concentrations tested. The phagocytic ability of the J774 cells had to be considered when designing and interpreting these experiments. It was found that as the cells apoptosed, they were partially cleared from the culture by the surviving phago-

cytic cells. Therefore, the results shown in Fig. 6 represent the maximum amount of DNA fragmentation observed. This occurred after 8 h of treatment with 100 μM uranyl chloride, after 24 h of treatment with 10 μM uranyl chloride, after 48 h with 1 μM uranyl chloride, and after 72 h with untreated control cells. It is inconsistent that 1 μM uranyl chloride-treated J774 cells showed significantly greater DNA fragmentation (a late indicator of apoptotic death) than did control cells, yet were not significantly different with respect to annexin V binding (an early indicator of apoptotic death). It may be possible that with up to 24 h of 1 μM DU incubation there is little induction of apoptosis in the treated J774 cells, although there is a significant decrease in metabolic viability. However, after that time, the cells begin to die by apoptosis as a result of accumulated damage due to DU–uranyl chloride exposure.

Morphological changes indicative of apoptotic death are also seen in J774 cells treated with DU–uranyl chloride. Cell shrinkage, chromatin condensation, cell blebbing, and formation of apoptotic bodies are seen in cells treated with 100 μM DU–uranyl chloride for 24 h (Fig. 7).

These changes were not apparent at earlier treatment times with 100 μM DU–uranyl chloride, nor were they seen with lower concentrations of DU–uranyl chloride. Since these morphological changes are normally seen very late in the apoptotic process, we hypothesize that most apoptotic cells were engulfed by their neighbors prior to the appearance of these changes. Treatment with 100 μM DU–uranyl chloride for 24 h may overwhelm the ability of the surviving cells in the culture to 'clean out' the apoptotic cells simply by increasing the number of dying cells in the culture. Treatment for shorter time periods or with lower concentrations of DU–uranyl chloride probably resulted in a smaller number of dead/dying cells that are phagocytosed by the surviving cells in the culture.

In conclusion, we demonstrated that soluble DU, in the form of uranyl chloride, is taken up and concentrated by J774 cells, a mouse macrophage cell line. DU uptake is time dependent and results in the decreased viability of the cells. Eventually cells incorporating DU die by apoptosis. Our results raise several questions, which are under investigation, with regard to both in vitro and in vivo model systems. First, will similar results be seen with insoluble DU compounds? Second, are macrophage functions, such as phagocytotic and cell-kill ability, impaired as a result of DU exposure? Third, what is the mechanism of damage that leads to apoptosis as a result of DU–uranyl chloride treatment? And finally, are the mechanisms similar for both in vitro and in vivo model systems? As discussed earlier, macrophages have a key role in the body's response to DU internalization regardless of the route of entry. A thorough investigation of the effect of both soluble and insoluble DU compounds on macrophages will add significantly to our understanding of the risks, if any, that are associated with DU exposure.

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