Establishment of an Animal Model to Evaluate the Biological Effects of Intramuscularly Embedded Depleted Uranium Fragments

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Contents

Introduction

Natural uranium (U) consists of three isotopes: ^{238}U (99.276%), ^{235}U (0.718%), and ^{234}U (0.0056%). During the uranium enrichment process two isotopic mixtures are produced, "enriched uranium" and "depleted uranium" (DU) with different relative ratios of the three isotopes. Enriched uranium contains a higher percentage of the fissionable isotope ^{235}U and is used for nuclear reactor fuel and nuclear weapons. DU has a lower ^{235}U content. The DU used by the U.S. military for kinetic energy penetrators is alloyed with titanium (0.75% by weight) to increase its tensile strength and to retard oxidation. Current U.S. antitank weapons contain DU penetrators, and most of the Abrams tanks are armored with DU. During Operation Desert Storm, DU munitions were fired by the Army and Air Force. Unfortunately, during this conflict, a number of U.S. military personnel were wounded by DU fragments (Daxon, 1993; Daxon and Musk, 1993; GAO Report, 1993). Many of these fragments were not removed because the surgical procedure would produce excessive tissue damage. Radiographs of injured soldiers show multiple embedded fragments ranging in size from 1 mm to over 5 mm in diameter (see figures 1 and

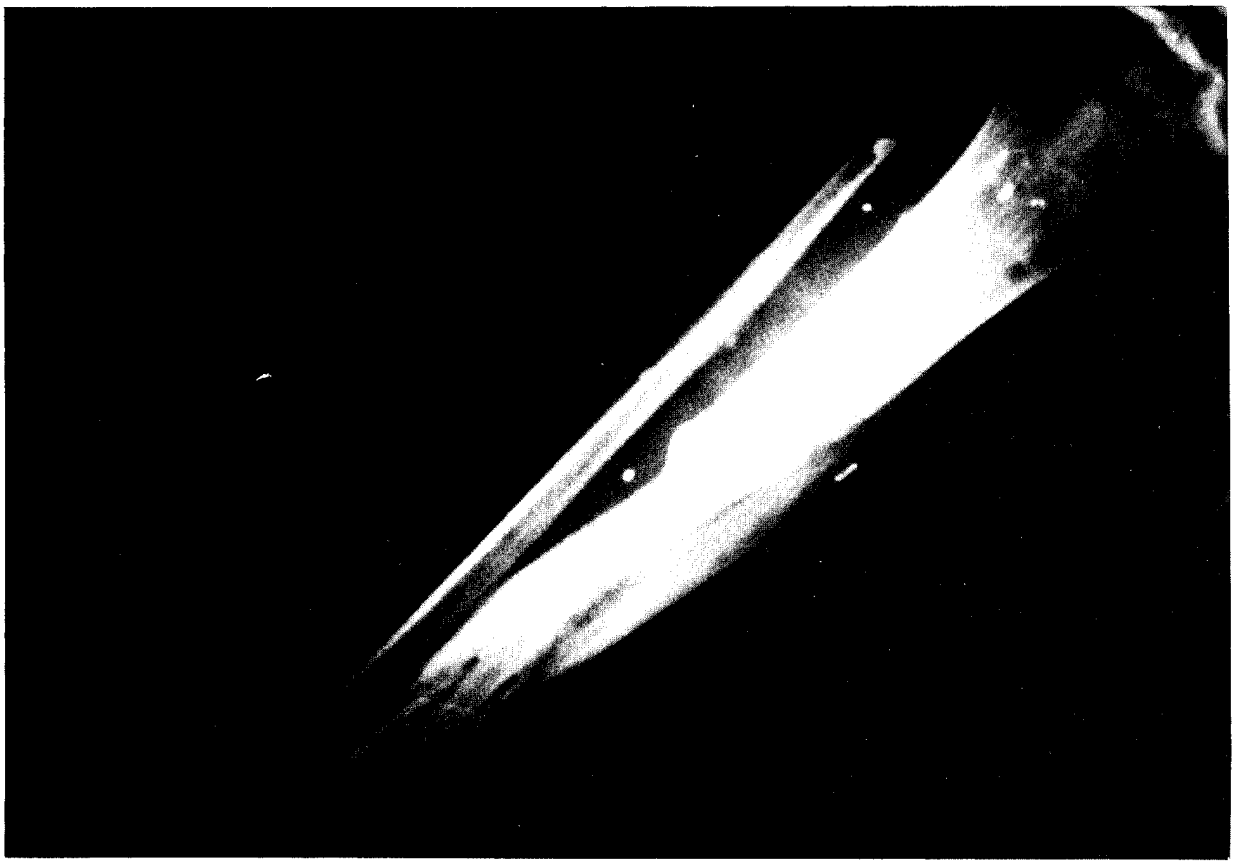

Fig. 1. Radiograph of the leg of a soldier wounded by a DU munition during the Persian Gulf War. This soldier also had DU fragments in the feet and knees of both legs.

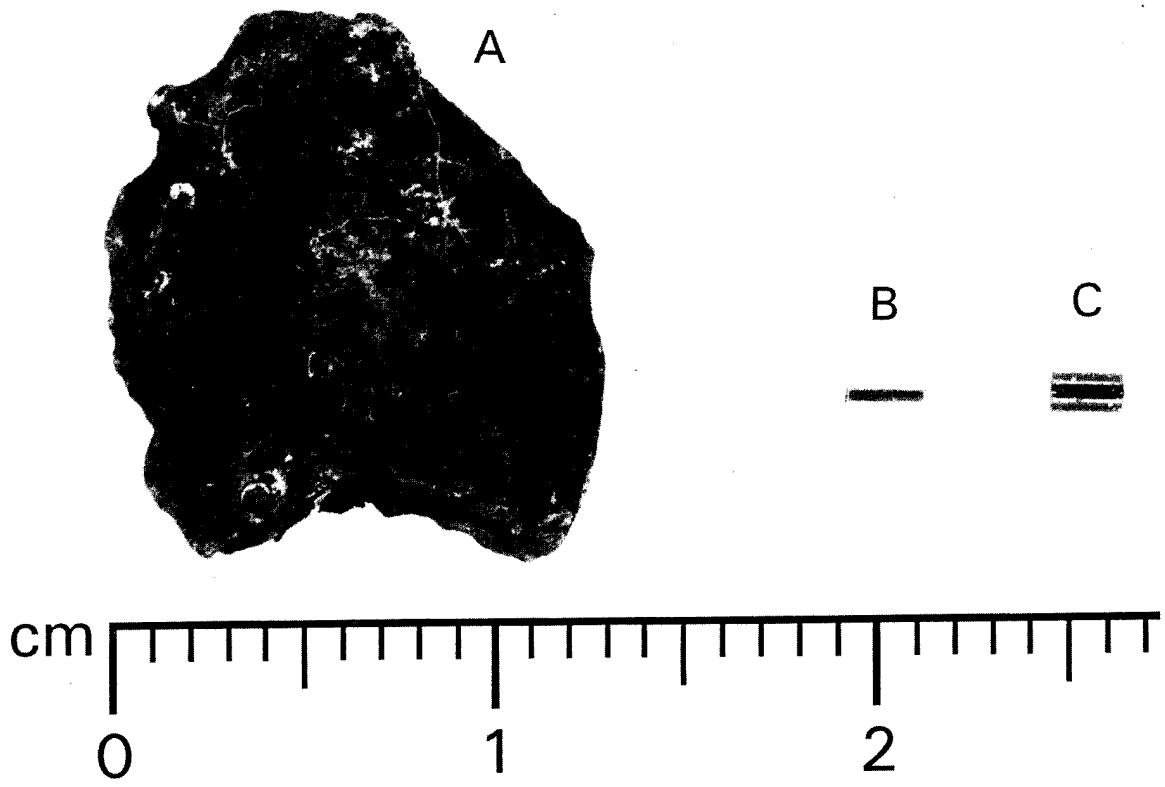

Fig. 2. (A) Photograph of an actual DU fragment removed from a soldier wounded during the Gulf War. (B) Photograph of a Ta pellet implanted in a rat. (C) Photograph of a DU pellet implanted in a rat.

2a). Indeed, fragments as large as 20 mm in diameter have been noted in other patients. Bioassays taken over a year after injury indicate that uranium was present at levels up to 30 μg U/l urine, well in excess of natural background (U.S. Army Environmental Hygiene Agency Memorandum for Office of the Surgeon General, 1994).

Although the toxicity of embedded DU is unknown, numerous studies have addressed the consequences of inhalation, ingestion, and parenteral administration of other forms of uranium (Diamond, 1989; La Touche et al., 1987; Morrow et al., 1982; Ortega et al., 1989a, b; Wrenn et al., 1989). After uranium is absorbed, it circulates in the blood as the uranyl ion, forming uranium-carbonate and uranium-albumin complexes. As the uranium-carbonate complex passes through the kidney, it is filtered rapidly by the glomeruli where 60% to 80% of the absorbed uranium is excreted in the first 24 hours after acute exposure. The uranium that is not excreted is reabsorbed by the proximal tubules where it produces significant toxic effects. Uranium also enters the bone, where it competes with calcium to form complexes with phosphate ions, thus becoming part of the bone matrix (Cabrini et al., 1984; Domingo et al., 1992; Guglielmotti et al., 1989; Neuman, 1950). This bone matrix then serves as both a long- and short-term storage site from which uranium is slowly released back into circulation (Kathren et al., 1989; Wrenn et al., 1985). The liver and muscle are other major sites of uranium deposition, with a possible long-term storage mechanism in the kidney (Kathren et al., 1989; Wrenn et al., 1985).

Acute morphological and biochemical changes of the kidney result from uranium exposure (Diamond, 1989; Kocher, 1989; Leggett, 1989; Neuman, 1950). Changes in the glomerular epithelial architecture (Kobayashi et al., 1984) and cellular necrosis in the proximal tubules near the corticomedullary junction of the kidney have been reported in experimental animals after acute uranium exposure (Brady et al., 1989; Haley et al., 1982; Haley, 1982). In addition,

polyuria, enzymuria, glucosuria, and increased excretion of amino acids have been demonstrated (Diamond, 1989; Diamond et al., 1989; Kocher, 1989; Zalups et al., 1988). Acute renal failure can indeed occur following exposure to high doses of uranium (Neuman, 1950; Ubios et al., 1994). Even acute environmental stressors such as restricted diets or changes in housing conditions have enhanced uranium toxicity significantly (Andrews and Bates, 1987; Damon et al., 1986).

Few studies have addressed the chronic toxicity of uranium, and the results available are conflicting (U.S. Department of Health and Human Services, 1990). Galibin and colleagues (1971) reported severe renal toxicity in rats that inhaled ammonium diuranate (1 or 8 mg/m^3), a slightly soluble uranium compound, for 128 days. Urine protein and blood non-protein nitrogen were elevated. In the proximal tubules, there were sloughed dead cells and abnormal regenerating cells. Although the total number of tubules was reduced and the kidney exhibited an increased amount of connective tissue, all the animals recovered. In contrast, Leach and colleagues (1970; 1973) found no renal toxicity in rats repeatedly exposed to uranium dioxide dust (5 mg/m^3) for a period of 12 months nor in dogs or monkeys exposed for 5 years. Yet uranium concentrations in the kidneys were as high as 1.1 µg U/g kidney wet weight in the rat, 8.3 µg U/g kidney weight in the dog, and 17.0 µg U/g kidney weight in the monkey. Uranium concentrations at these levels have been reported to cause acute renal toxicity (e.g., Kathren et al., 1989). Thus, the chronic effects of uranium exposure remain for the most part unresolved (Diamond, 1989).

The threshold concentration of kidney uranium levels in humans that result in kidney chemical toxicity is in dispute (Diamond, 1989; Kathren and Moore, 1986; Kocher, 1989; Stradling et al., 1988). While the Nuclear Regulatory Commission has set the level at 3.0 µg U/g kidney weight for renal damage in humans, there is evidence from both human and animal reports that this level could be considerably lower. For example, chronically exposed uranium mill workers, whose kidney uranium levels probably did not exceed 1 µg U/g kidney weight (Thun et al., 1985), showed mild renal dysfunction with increased urinary excretion of $ß_2$-microglobulin and various amino acids. In rats exposed subchronically to low doses (cumulative dose: 0.66 or 1.32 mg/kg) of uranyl fluoride, kidney uranium levels as low as 0.7 to 1.4 µg U/g wet weight kidney produced cellular and tubular necrosis of the proximal tubule, proteinuria, and enzymuria (Diamond et al., 1989). These changes in rat renal function, however, were temporary, with complete recovery occurring within 35 days of exposure. These studies are important because they indicate that renal injury can occur at kidney uranium levels well below the 3.0 µg U/g limit.

Currently, no research into the direct toxic effects of embedded DU has been reported. The toxicity data that exist for low-level chronic uranium exposure used other routes of administration, and the results are contradictory. The uranium levels in humans that result in kidney toxicity are in dispute. For these various reasons, it is necessary to determine the health risks to the soldier resulting from long-term exposure to DU fragments. The goal of this pilot study was to establish an animal model that could be used in future research to investigate the biological effects of embedded DU.

Methods

Subjects and Experimental Design

Subjects were 12 naive Sprague-Dawley male rats (8-10 weeks old) obtained from Charles River Breeding Laboratories, Raleigh, N.C. On arrival, rats were quarantined and screened for diseases and were maintained in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23). Six rats were implanted with eight DU pellets (four in each biceps femoris muscle of the lateral thigh), and six rats were implanted with eight tantalum (Ta) pellets. Rats were individually housed in plastic Micro-Isolator cages with hardwood chips as bedding; during urine collection, rats were placed in metabolic cages. Commercial rodent chow and acidified water (pH 2.5, using concentrated HCl) were provided *ad libitum*. Rats were on a 12-hour light/dark cycle.

DU and Ta Pellets

DU pellets (1 mm in diameter x 2 mm in length) were obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. (see figure 2c). The cylindrical shape was chosen because it is the geometrical average of fragments left in soldiers wounded by conventional or DU munitions. The size of the pellets was based on two considerations. First, the total DU implanted was approximately 1% of the total biceps femoris muscle volume and did not seem to cause undue discomfort to the animal. Second, the surface area of 8 DU pellets of this size should result in detectable urinary uranium levels. DU pellets consisted of 99.25% DU and 0.75% titanium by weight. The uranium isotopes in DU were ^{238}U (99.75%), ^{235}U (0.25%), and trace amounts of ^{234}U. This is the same DU alloy used in U.S. military munitions.

Ta pellets (1 mm in diameter x 2 mm in length) were obtained from Alfa Products, Ward Hill, Mass., and served as the heavy metal control (see figure 2b). Ta was selected because its density is similar to DU density, 16.6 g/cm^3 for Ta versus 18.8 g/cm^3 for DU (Radiological Health Handbook, 1970), it is relatively inert in a biological medium (Johansson et al., 1990), and it is commonly used in human orthopedic reconstructive surgery (Hockley et al., 1990).

Surgical Procedures for Pellet Implantation

Before implantation surgery, the DU and Ta pellets were cleaned by immersion in an industrial detergent, rinsed in absolute alcohol, sterilized by immersion in a 50% nitric acid solution for 3 minutes, rinsed with sterile water, and then placed in acetone to inhibit oxidation. These sterilization procedures completely remove the oxide formation from the surface of DU metal (Tonry, 1993), and the results of an abbreviated sterility test of 10 Ta pellets using either a thioglycollate medium or soybean-casein digest medium detected no microorganisms.

Rats were administered atropine (0.05 mg/kg i.m.) before being anesthetized. Anesthesia was induced with ketamine hydrochloride (50 mg/kg) in combination with xylazine hydrochloride (10 mg/kg) given i.p. in a 0.5-ml bolus, using a 25-gauge needle. These injections were administered intraperitoneally to prevent irritating the site of implantation. The surgical sites were then shaved and cleansed with Betadine. Four pellets were implanted approximately 15 mm apart in each biceps femoris muscle on the lateral side of each thigh. Using a scalpel blade, incisions were made through the skin and approximately 10 mm deep into the muscle mass. The proximal incisions were 10 mm distal to the iliac crest and were the implantation sites of the first pellets. Pellets were secured in place with absorbable sutures (Dexon 4-0) to prevent movement. Rats were closely monitored following surgery until they were ambulatory. A veterinarian or a veterinary technician examined the surgical sites for signs of inflammation, infection, and local DU toxicity daily for 2 weeks

following surgery and weekly thereafter throughout the study.

Behavioral Measurements

Locomotor activity and grip strength were assessed on days 3 and 5 before surgical implantation and on days 1, 3, 7, 14, 28, 60, and 120 after surgery. Locomotor activity was quantified using computerized Digiscan activity monitors (Omnitech Electronics, Columbus, Ohio). Each monitor used an array of infrared photodetectors spaced 2.5 cm apart to determine horizontal locomotor activity, which was expressed as total distance traveled. Activity was monitored for 1 h with measurements taken every 5 min (Landauer et al., 1988).

Immediately following locomotor activity testing, the strength of both hindlimb and forelimb grips of each animal was measured using a grip strength apparatus (San Diego Instruments, San Diego, Calif.). In this test, the animal was required to grip a rectangular wire mesh surface (12 x 7 cm) with its forepaws and was then gently pulled back along a platform until its grip was broken. The backward motion was continued until the animal's hindpaw gripped another rectangular wire mesh surface (12 x 10 cm). As with the forelimb grip, the animal was gently pulled back until the hindlimb grip was broken. Readings on three push-pull strain gauges were used to record the maximum strain required to break both forelimb and hindlimb grips. This behavioral test is used in many laboratories to assess muscular weakness (Haggerty, 1989; Meyer et al., 1979).

Urinary Sampling and Collection Procedures

Urine samples were collected following behavioral testing on days 1, 3, 7, 14, 28, 60, and 120 after surgery and analyzed for uranium levels. Sampling at these time points was necessary because signs of nephrotoxicity in laboratory animals exposed to low doses of uranium are frequently not detected until 3 to 5 days after exposure and may subside within 7 days (Diamond, 1989). Urine samples were collected from rats in individual metabolic cages (23.5 cm diameter x 12 cm high) where they had continuous access to food and water. Rats were acclimated to the metabolic cages for 5 days before the study began because naive rats exposed to these housing procedures have shown a stress-induced increase in uranium toxicity (Damon et al., 1986).

A 24-h urine sample was obtained from each rat, and the volume was recorded. In addition, each animal's body weight and food and water consumption were recorded. Care was taken to prevent contaminating the urine with food or feces. After collection, urine was filtered to remove any debris and stored in plastic containers at 4° C until analyzed. The metabolic cages were disinfected and decontaminated between each animal use. During animal-handling periods, overt signs of behavioral toxicity and the overall appearance of the rats were recorded.

Determination of Urinary Uranium Levels

Urinary uranium levels were determined by alpha spectrometric techniques (Martin Marietta Energy Systems, Inc., Oak Ridge, Tenn.). An aliquot of the sample was dissolved in nitric acid (HNO_3) and hydrogen peroxide (H_2O_2). The sample was then wet ashed, and the uranium coprecipitated with calcium oxalate. After dissolving the precipitate in HCl, the uranium was further separated by ion exchange chromatography. The uranium was then eluted from the column with a solution of dilute HCl to which titanous chloride had been added to reduce actinides that may have been in an elevated oxidation state. The final fraction of the eluate was treated first with ascorbic acid to reduce any iron and then with hydrofluoric acid. The uranium isotopes were next coprecipitated on neodymium fluoride. The neodymium was caught on a 0.1-μm filter, which was rinsed, dried, and then mounted on a planchet for alpha spectrometry. The minimum detectable activities (MDA) for uranium in urine using these procedures were 1.4×10^{-6} μg/l for ^{234}U and 0.03 μg/l for ^{238}U.

Results

Surgical Implantation

Two rats assigned to the DU group and one rat assigned to the Ta group did not survive implantation surgery. One of these rats expired during surgery, and the other two within 6 h after surgery. Necropsies indicated asphyxiation, suggesting that the animals received too much anesthetic. The other nine animals were alert and moving in the metabolic cages within 2 h after surgery. Figure 3 is a radiograph of the left rear leg of a rat implanted with four DU pellets; the right rear leg was also implanted with four DU pellets. The cylindrical shape and size of the pellets are similar to DU fragments observed in wounded soldiers (figure 1).

Locomotor Activity and Grip Strength

The locomotor activity of rats implanted with DU pellets was not significantly different from the activity of rats implanted with Ta, $p > 0.05$ (figure 4).

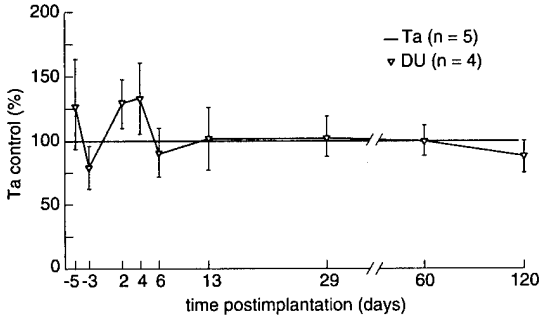

Fig. 4. Locomotor activity of rats surgically implanted with DU pellets expressed as percent of Ta control. Vertical bars represent the SEM (standard error of the mean).

Fig. 3. Radiograph of the left rear leg of a rat surgically implanted with four DU pellets (1 mm in diameter x 2 mm in length).

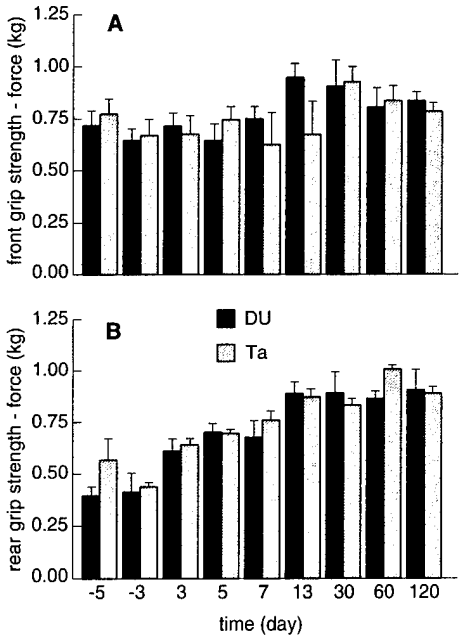

Fig. 5. (A) Forelimb grip strength of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM. (B) Hindlimb grip strength of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM.

Similarly, neither the forelimb nor the hindlimb grip strength of the two groups was different, $p > 0.05$ (figures 5a and 5b).

Body Weights, Food and Water Consumption, and Urinary Output

The body weights of the rats embedded with DU pellets were not different than the body weights of rats embedded with Ta, $p > 0.05$ (figure 6). In fact,

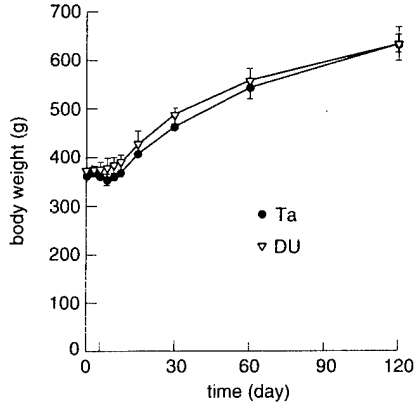

Fig. 6. Body weights of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM.

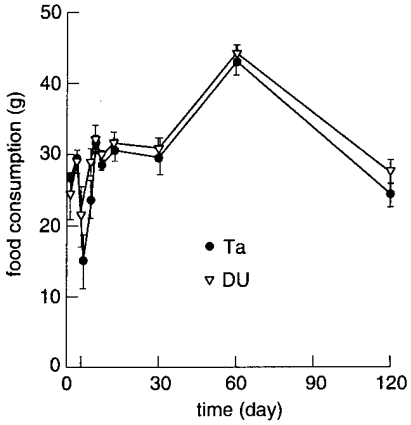

Fig. 7. Food consumption of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM.

the body weights in both groups remained relatively stable for the first week following surgery and, as expected, increased throughout the study as observed in normal rats.

The food and water consumption for the DU- and Ta-implanted rats did not differ, $p > 0.05$ (figures 7 and 8). There was, however, a trend toward a decrease in water consumption for the Ta group and an increase in water consumption for the DU group.

There was a significant difference in the volume of urinary output between the DU and Ta groups. On the day of surgery, urine output for the Ta group decreased but did not change for the DU group, p

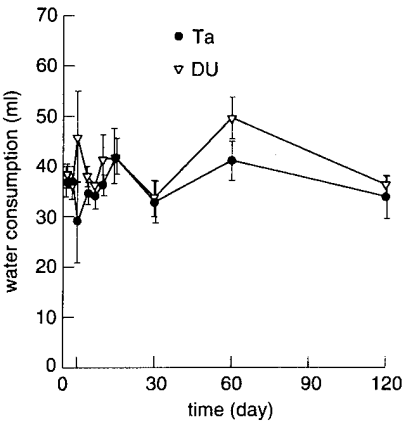

Fig. 8. Water consumption of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM.

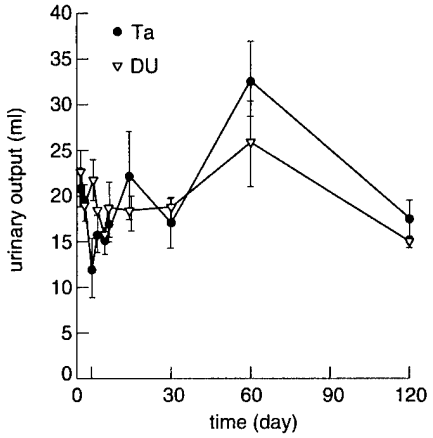

Fig. 9. Urinary output of rats before and after receiving DU or Ta implants. Vertical bars represent the SEM.

<0.05 (figure 9). This decrease in the urinary output for the Ta group, however, was temporary and returned to baseline levels by day 3 after surgery.

Urinary Uranium Levels

Figure 10 illustrates mean uranium levels in the urine of DU-implanted animals and the pooled value of the uranium analysis for Ta-implanted animals after implantation surgery. Figure 11 provides the individual urinary uranium levels of the four DU-implanted rats. As expected, only background levels of uranium were detected in the Ta control group. In contrast, significant levels of uranium were detected within 24 h of DU implantation (mean = 28.69 ± 10.00,

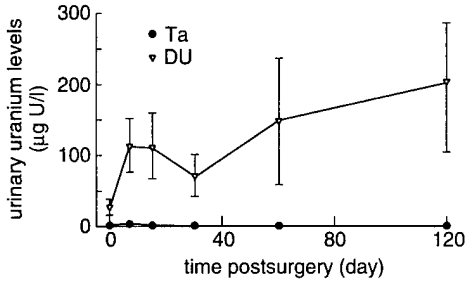

Fig. 10. Time course of uranium levels detected in the urine of rats implanted with either DU or Ta. Uranium concentration detected in the Ta group is at background levels. Vertical bars for the DU group (N = 4) represent the SEM. Urine for the Ta-implanted animals was pooled for uranium analyses.

range = 14.21 to 56.99 µg U/l). By day 7 following surgery, uranium levels had increased nearly fourfold (mean = 111.86 ± 41.05, range = 56.38 to 233.91 µg U/l) and remained elevated at day 120 (mean = 204.56 ± 99.73, range = 35.01 to 458.53 µg U/l).

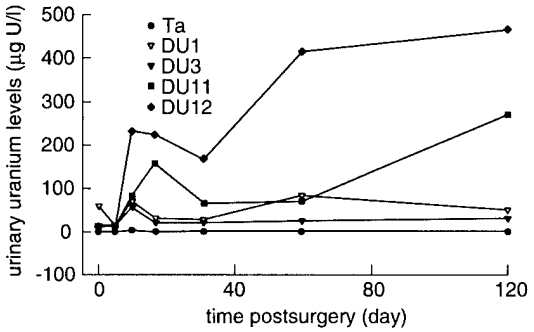

Fig. 11. Individual time courses of uranium levels detected in the urine of each rat implanted with DU. Data on Ta time courses are the same as in figure 10.

Discussion

The purpose of this study was to develop an animal model that could be used in future research to determine the health risks associated with DU fragment injuries. It was especially important to establish procedures in which DU exposure would produce urinary uranium levels comparable to those observed in soldiers wounded by DU munitions during the Persian Gulf War. Measured by these criteria, this initial study was successful. The average urinary uranium level in the rat 24 h after DU implantation was 28.69 μg U/l. This value is very close to the urinary level of 30 μg U/l reported for soldiers wounded during the Persian Gulf War and assayed 1 year after injury. Unfortunately, no bioassays were taken of any of the soldiers within the first year after DU injury so no direct time course comparisons can be made.

It should be emphasized that the urinary uranium levels in the rat did not reach asymptote until day 7 following DU implantation surgery and remained elevated throughout the study (figure 10). Although the data are preliminary, this finding has clinical significance because it indicates that soldiers with suspected DU fragment wounds should be monitored for uranium exposure for at least the first week after injury and perhaps even longer. Certainly a complete pharmacokinetic study should be conducted to definitively address this patient-monitoring issue (Daxon, 1993).

Although numerous studies have assessed the toxic effects of other forms of uranium exposure (Diamond, 1989, and Kocher, 1989, for the latest reviews of the literature), this is the first study that assessed the effects of intramuscularly embedded DU. The rat proved to be an excellent animal model for this purpose. It tolerated the surgical procedures for pellet implantation relatively well, as measured by both locomotor activity and grip strength (figures 4 and 5), both indices of quality of life for humans. Further, the lateral thigh muscle of the adult rat is large enough to implant at least four pellets (1.0 mm diameter x 2 mm length) into each leg (figure 3), with the possibility of as many as ten pellets. Moreover, the rat's lifespan of more than 18 months enables it to be used in chronic toxicity studies (Brady et al., 1989; Lang and White, 1994; Lumley et al., 1992; Lumley and Walker, 1986; Monro, 1993; Nohynek et al., 1993; Rao et al., 1990).

In conclusion, this study was successful in developing a rodent model that can be used to evaluate the biological effects of intramuscularly embedded DU fragments. However, the potential short-term and long-term health risks associated with DU exposure remain to be investigated. Certainly the behavioral, physiological, biochemical, and histological consequences of embedded DU are research areas of immediate concern. Equally important is identification of the health risks to the fetus exposed *in utero* to DU from fragments embedded in the mother before pregnancy (Angleton et al., 1988; Bosque et al., 1993; Domingo et al., 1988a, b, c; Paternain et al., 1989). This latter research area is especially significant considering that the placenta does not prevent cross-placental transfer of uranium (Durbin and Wrenn, 1976; Sikov and Mahlum, 1968). Moreover, fetal toxicity often occurs in the absence of maternal toxicity (e.g., Price et al., 1985). Regardless of the research strategy adopted, a coordinated interdisciplinary health hazard assessment is required to identify the potential medical risks that DU poses to our soldiers wounded by this unconventional munition.

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During the Persian Gulf War, 36 U.S. soldiers were wounded by depleted uranium (DU) munitions. Based on medical guidelines for conventional shrapnel injuries (nonradioactive), many DU fragments were left in soldiers. Unfortunately, health risks associated with embedded DU were unknown, and an animal model to investigate this did not exist. The purpose of this study was to develop an animal model to examine the health risks associated with DU shrapnel injuries. Twelve rats were surgically implanted intramuscularly with 8 DU pellets (1 mm in diameter x 2 mm in length) or 8 chemically inert tantalum (Ta) pellets of similar size. Urinary uranium levels were measured on days 1, 3, 7, 14, 28, 60, and 120 after implantation of DU pellets. Physiological and behavioral parameters, including locomotor activity, forelimb and hindlimb grip strength, food and water consumption, and urinary output, were measured 5 and 3 days before surgery and on days 1, 3, 7, 14, 28, 60, and 120 after surgery. Urinary uranium levels for Ta-implanted rats remained at background levels. In contrast, the average urinary uranium level for DU-implanted rats was significantly elevated on day 1 (28.69 μg U/l) after implantation and remained elevated until day 120 (204.56 μg U/l). There was no significant difference between DU- and Ta-implanted rats in any behavioral or physiological measures. Results indicate that the rat is an appropriate animal model for evaluating biological effects of embedded DU fragments.

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