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Delayed myelosuppression with acute exposure to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and environmental degradation product hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) in rats

Sridhar Jaligama [a], Vijay M. Kale [a,1], Mitchell S. Wilbanks [a,2], Edward J. Perkins [b], Sharon A. Meyer [a,*]

[a] Department of Toxicology, College of Pharmacy, University of Louisiana at Monroe, Monroe, LA 71209, USA
[b] US Army Engineer Research and Development Center, Vicksburg, MS 39180, USA

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ABSTRACT

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a widely used munitions compound, and hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), its *N*-nitroso product of anaerobic microbial nitroreduction, are contaminants of military sites. Previous studies have shown MNX to be the most acutely toxic among the nitroreduced degradation products of RDX and to cause mild anemia at high dose. The present study compares hematotoxicity with acute oral exposure to MNX with parent RDX. Both RDX and MNX caused a modest decrease in blood hemoglobin and ~50% loss of granulocytes (NOAELs = 47 mg/kg) in female Sprague–Dawley rats observed 14 days post-exposure. We explored the possibility that blood cell loss observed after 14 days was delayed in onset because of toxicity to bone marrow (BM) progenitors. RDX and MNX decreased granulocyte/macrophage-colony forming cells (GM-CFCs) at 14, but not 7, days (NOAELs = 24 mg/kg). The earliest observed time at which MNX decreased GM-CFCs was 10 days post-exposure. RDX and MNX likewise decreased BM burst-forming units-erythroid (BFU-Es) at 14, but not 7, days. Granulocyte–erythrocyte–monocyte–megakaryocyte (GEMM)-CFCs were unaffected by RDX and MNX at 7 days suggesting precursor depletion did not account for GM-CFC and BFU-E loss. MNX added to the culture media was without effect on GM-CFC formation indicating no direct inhibition. Flow cytometry showed no differential loss of BM multilineage progenitors ($Thy1.1^+$) or erythroid ($CD71^+$) precursors with MNX suggesting myeloid and erythroid lineages were comparably affected. Collectively, these data indicate that acute exposure to both RDX and MNX caused delayed suppression of myelo- and erythropoiesis with subsequent decrease of peripheral granulocytes and erythrocytes.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine/royal demolition explosive (RDX, Fig. 1) is a highly energetic compound widely used as an explosive, often in a plasticized form called C-4 (Yinon, 1990). Munitions manufacture and usage has resulted in RDX contamination of surface and ground water and soil of numerous firing ranges and retired production facilities (Agency for Toxic Substances and Disease Registry (ATSDR), 2010). The U.S. Department of Defense (DoD) has recently identified approximately 2000 current or formerly active sites with munitions contamination (Defense Environmental Network and Information eXchange (DENIX), 2010) and the U.S. Environmental Protection Agency (EPA) lists RDX as a contaminant of at least 31of 1699 current and formerly identified sites of the National Priority List (ATSDR, 2010). RDX has been designated by the DoD as an emerging contaminant (Murnyak et al., 2011), a classification for chemicals of importance to the military mission that are without adequate human health standards or for which science and regulatory status is evolving.

In the environment, RDX undergoes anaerobic microbial metabolism by nitroreductases to form *N*-nitroso derivatives hexahydro-1-nitroso-3,5-dinitro-1,3,5- triazine (MNX, Fig. 1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (Crocker et al., 2006; McCormick et al., 1981). The *N*-nitroso degradation products of RDX are found as intermediates of bioremediation (Hawari et al., 2000) and as contaminants in ground water of RDX contaminated sites, with MNX present in highest concentrations (Beller and Tiemeier, 2002). Mammalian metabolism of RDX to nitroreduced products is also indicated by the detection of trace amounts of MNX and DNX in plasma of miniature pigs and gastrointestinal content of deer mice orally treated with RDX (Major et al., 2007; Pan et al., 2007).

Understanding the toxicity of degradation products relative to parent RDX is important to estimate whether these compounds present an additional risk of adverse health effects associated with remediation

* Corresponding author at: Department of Toxicology, College of Pharmacy, University of Louisiana at Monroe, 1800 Bienville Dr, Monroe, LA 71201, USA. Fax: +1 318 342 3037.
E-mail address: meyer@ulm.edu (S.A. Meyer).
[1] Present address: College of Pharmacy, Roseman University of Health Sciences, South Jordan, UT 84095, USA.
[2] Present address: US Army Engineer Research and Development Center, Vicksburg, MS 39180, USA.

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activities. Neurotoxicity is a commonly observed early-onset effect of acute exposure to RDX in several species. Seizures have been reported within hours of human acute exposures from prior wartime weapons production before adoption of modern occupational safety standards (Stone et al., 1969; Testud et al., 1996; Woody et al., 1986). Intentional and accidental consumption of C-4 has produced similar effect, with recovery within days (Davies et al., 2007; Goldberg et al., 1992; Harrell-Bruder and Hutchins, 1995; Hollander and Colbach, 1969; Kasuske et al., 2009; Kucukardali et al., 2003). Coincident mild anemia has been less frequently reported (Kucukardali et al., 2003), but for one case with high dose exposure, persisted for weeks (Stone et al., 1969). Seizures are also observed shortly after acute exposure to RDX in rats (Burdette et al., 1988; Schneider et al., 1978; Williams et al., 2010) and dogs (Bruchim et al., 2005).

Very little is known about the toxicity of RDX degradation products. In our study that directly compared acute toxicity of RDX with its degradation products in rats, lethality and neurotoxicity of MNX were comparable to that of RDX and of greater potency than DNX and TNX (Meyer et al., 2005). Similar results have been reported for deer mice (*Peromyscus maniculatus*) (Smith et al., 2007). In addition, hematotoxic effects of MNX were seen in rats surviving 14 days after single oral exposure as evidenced by decreased blood hemoglobin and splenic hemosiderosis (Meyer et al., 2005). Because anemia resulting from direct chemical destruction of intravascular erythrocytes typically resolves within ~7 days in the rat (Berger, 1985a, 1985b; Harrison and Jollow, 1986), the 14-day persistence led us to hypothesize that MNX was cytotoxic to bone marrow (BM) progenitor cells. Incidence of acquired BM failure from non-therapeutic toxicants has been estimated at ~30% (Montane et al., 2008). Notable environmental myelosuppressants include benzo(a)pyrene, 7,12-dimethylbenz(a) anthrecene (DMBA) and benzene (Cronkite et al., 1989; Galvan et al., 2006; Gasiewicz et al., 2010; Snyder et al., 1980). Toxicant effects on proliferation, differentiation and apoptosis of hematopoietic stem and lineage-committed progenitor cells (Wang et al., 2012; Yoon et al., 2001) result in subsequent loss of their derived mature cells in blood. Reductive activation of *C*-nitroso 1,2,4-benzotriazine 1,4-dioxide prodrugs in hypoxic niches of BM associated with hematopoietic stem cell loss (Parmar et al., 2007) suggests a similar mechanism could apply to *N*-nitroso MNX (Uchimiya et al., 2010).

Toxicity of MNX compared to RDX on BM hematopoietic progenitor cells of treated rats with time after single exposure is described here. Erythroid and myeloid lineage responses of BM were evaluated with colony forming assays (Pessina et al., 2003; Rich and Hall, 2005) and correlated with levels of mature blood cells. Results demonstrated suppression of BM hematopoiesis by both RDX and MNX. Loss of both myeloid and erythroid lineages occurred and RDX was of comparable potency to MNX. Onset of these BM effects was delayed until after 7 day-post treatment suggesting development of preceding events are necessary to drive the suppressive outcome at the level of the BM that is then apparent at 14 days as loss of mature blood cells.

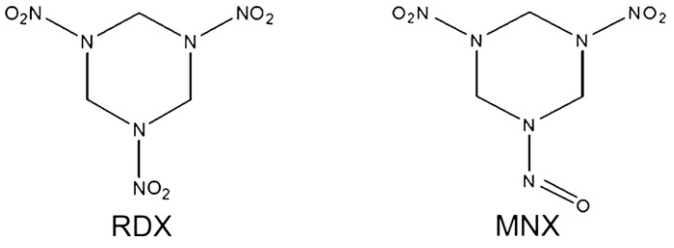

Fig. 1. Structure of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and its environmental degradation product, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX).

Materials and methods

Materials. RDX (>99%) was obtained from Stan Caulder (Naval Surface Warfare Center, Indianhead, MD) and stored under absolute ethanol. MNX was obtained from Dr. Ron Spanggord (SRI Intl., Menlo Park, CA). Purity of MNX as determined by HPLC with UV detection was greater than 98.4% with ~1.2% RDX contamination. Both compounds were used without any additional purification. Assay kits for colony formation of granulocyte/macrophage-colony forming cells (GM-CFC; catalog no. K1-GM2-1R, now renamed KCO-GM2-1R), granulocyte–erythrocyte–monocyte–megakaryocyte-CFCs (CFC-GEMM, KCO-GEMM2-1R) and burst-forming units-erythroids (BFU-E, KCO-B2-1R) were the methylcellulose HALO platform (now relabeled CAMEO-96) from Hemogenix, Inc. (Colorado Springs, CO). Iscove's modified Dulbecco medium (IMDM) and antibiotic/antimycotic solution were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) and Histopaque-1077 were purchased from Sigma (St. Louis, MO). Antibodies for flow cytometry were mouse anti-rat CD32 (Rat Fc block [FcγIII/II], mAB D-34-485), phycoerythrin (PE)-conjugated IgG_1 κ-isotype control antibody, fluorescein isothiocyanate (FITC)-conjugated IgG_{2a} κ-isotype control antibody, PE-conjugated antibody to rat Thy1.1 (mAB; OX-7) and FITC-conjugated mouse monoclonal antibody to rat CD71 (mAB OX-26) purchased from BD Pharmingen (San Jose, CA). All other reagents were of analytical grade and purchased from commercially available sources.

Animals and treatment. Female Sprague–Dawley (SD) rats (210–240 g) were obtained from the in-house breeding colony of University of Louisiana at Monroe and housed individually with a 12-h light/dark cycle, controlled temperature (21 ± 1 °C) and humidity (50 ± 10%), and free access to water and rodent chow (Harlan Teklad rat chow No.7001, Madison, WI). Rats were allowed to acclimate in polycarbonate cages for one week prior to study. All animal handling and husbandry were in accordance with the Guide for Use and Care of Animals (National Research Council, 2011) and all the protocols were pre-approved by the Institutional Animal Care and Use Committee. Food was withdrawn the night before treatments. Treatments were randomly assigned to groups of rats ($n = 3–5$) and orally administered between 9:00 and 10:00 AM. Treatments were RDX (0–94 mg/kg) or MNX (0–94 mg/kg) in 5% DMSO (v/v) in corn oil administered as a single oral dose (10 ml/kg). High doses were equal to half the RDX and MNX LD_{50}s (Meyer et al., 2005). Rats were frequently observed for convulsions over the first 8 h and were euthanized with CO_2 if moribund according to OECD criteria (Organisation for Economic Co-operation and Development (OECD), 2000). Survivors were euthanized with CO_2 at different time points ranging from 7 to 14 days and blood was collected by cardiac puncture for hematological assessment. Both femurs were excised and immediately processed for BM cell isolation.

Hematology. Blood was collected by cardiac puncture into heparinized syringes and transferred to EDTA-containing vacutainer tubes (Becton, Dickinson and Co.). The hematological parameters hemoglobin, erythrocyte, leukocyte and platelet counts and leukocyte differentials were determined with a CELL-DYN Sapphire System (Abbott Laboratories, Abbott Park, IL) (Fairbanks and Klee, 1986). Hematocrit was derived from measured red blood cell size and number. Hemoglobin was measured as absorbance at 540 nm after erythrocyte lysis and conversion to hemoglobin-hydroxylamine. Granulocyte count was determined by summing eosinophil, basophil and neutrophil counts.

BM cell isolation. Marrow was extracted from both femurs of each rat. Bones were disarticulated from the pelvis, excised and the proximal and distal heads of each were cutoff with bone sheers. Marrow was flushed by inserting 18 ½-gauge needles with 3 ml ice-cold IMDM plus 0.2% BSA and antibiotic/antimycotic (1 ml/100 ml medium) through one end of the bone shaft. Residual fluid in the bones was