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Award Number: W81XWH-05-1-0537

TITLE: Role of Myelofibrosis in Hematotoxicity of Munition RDX Environmental Degradation Product MNX

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REPORT DATE: September 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 1 Sep 2008		2. REPORT TYPE Annual		3. DATES COVERED 31 Aug 2007 – 30 Aug 2008	
4. TITLE AND SUBTITLE Role of Myelofibrosis in Hematotoxicity of Munition RDX Environmental Degradation Product MNX				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0537	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sharon A. Meyer, Ph.D. E-Mail: meyer@ulm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Louisiana at Monroe Monroe, LA 71209				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The purpose of this research is to determine mechanisms through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes persistent anemia in the rat. We have hypothesized MNX targets hematopoietic stem cells and, like other myelosuppressive chemicals, will be fibrogenic to the bone marrow. Findings of this period are: 1) detection of extramedullary hematopoiesis in support of the hypothesis that RDX is toxic to hematopoietic bone marrow stem cells, but results with MNX also suggest decreased erythropoietin may compound the myelosuppressive effect and 2) a MNX effect on the bone marrow stromal niche, which supports hematopoiesis, through expansion of one of its cell types. Collectively, these results continue to suggest an early erythroid/myeloid lineage precursor and/or the bone marrow stromal niche supporting hematopoiesis as the target of MNX and RDX. These results suggest that MNX- and RDX toxicity in the rat appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus may offer a model for study of disease progression and intervention strategies.					
15. SUBJECT TERMS hexahydro-1,3,5-trinitro-1,3,5-triazine; hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; RDX; MNX; anemia; myelosuppression					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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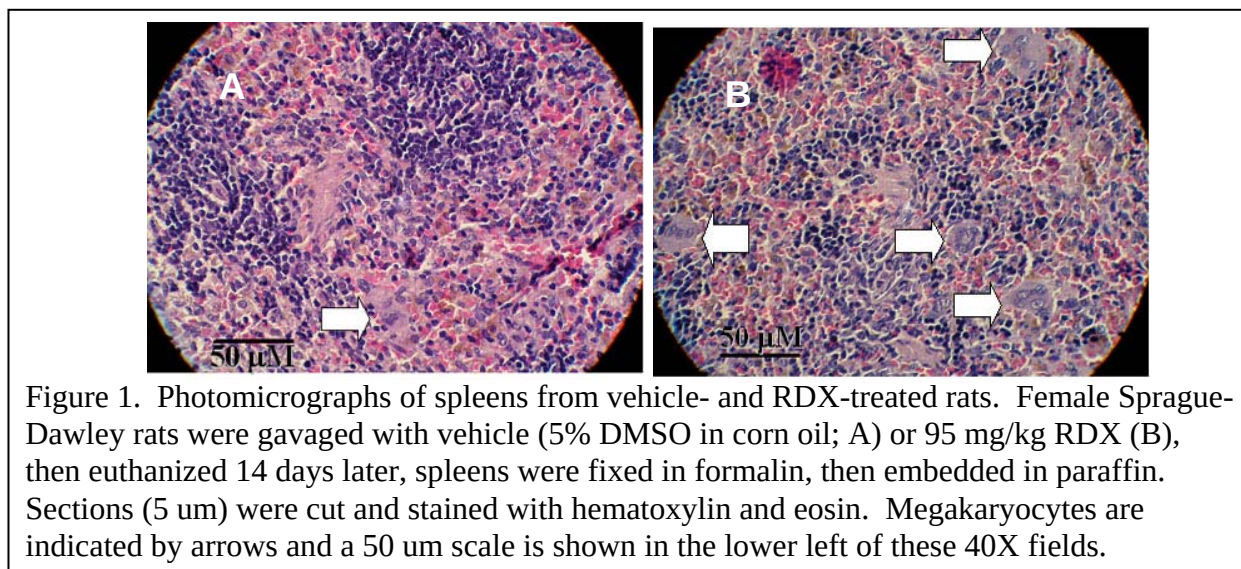
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INTRODUCTION: The subject of research supported by this grant is a determination of the mechanism through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes anemia. Anemia was detected in our previous acute toxicity studies in the rat (Meyer et al. 2005) and persisted 14 days after a single dose of MNX (NOAEL 47 mg/kg). Since anemia resulting from direct chemical destruction of intravascular erythrocytes is typically resolved within ~7 days in the rat, the 14-day persistence led us to hypothesize that MNX was cytotoxic to erythroid-lineage progenitor cells. Like other myelosuppressive chemicals, we also postulated that longer term, repeat exposure to MNX would be fibrogenic to the bone marrow microenvironment necessary for maturation of hematopoietic stem cells and hence offer an experimental model analogous to human idiopathic myelofibrosis. Further, previous studies on detection of a MNX ring cleavage product suggested this toxicity could be due to metabolism by bone marrow stromal cells. The scope of the proposed work of the overall project encompasses determination of whether: 1) MNX produces persistent bone marrow toxicity, 2) MNX and ring cleavage metabolite MDNA, as produced from metabolism by bone marrow stromal cells, accumulate in bone after acute exposure; 3) acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid lineage; 4) acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment, and 5) repeated administration of lower doses of MNX produces bone marrow toxicity including fibrosis. In addition, effects of parent RDX on selected endpoints will be assessed to provide structure-activity information relevant to mechanism of hematotoxicity and necessary for assessment of relative risk of the two nitramines in remediation of RDX-contaminated sites.

BODY:

Task 1: Determination of whether acute exposure to MNX produces persistent bone marrow toxicity to include examination of selected endpoints in RDX-exposed rats (Months 1 – 12).

One consequence of anemia in the absence of sufficient regeneration from bone marrow erythroid precursors is recruitment of extramedullatory sites of hematopoiesis (EMH). We have examined hematoxylin/eosin (H&E) stained sections (5 μ m) of paraffin-embedded, formalin-fixed spleens for evidence of EMH, which can be recognized by presence of prominent large, multinuclear megakaryocytes (Fig. 1). Qualitatively, we see frequent megakaryocytes in spleens



taken 14 d after treatment of rats with 47 and 94 mg/kg RDX. It is not uncommon to observe a few megakaryocytes within the same 40X microscopic field of treated rats, while we have yet to observe other than singlets in controls (Fig. 1).

Method, in vivo study: Female Sprague-Dawley rats (~200 g) were orally gavaged with RDX at 47 and 94 mg/kg (LD₅₀ ~ 190 mg/kg) or vehicle (5% DMSO in corn oil). After 14 days, rats were euthanized with CO₂ and exsanguination, spleens were excised and a ~2 mm piece from the medial lobe placed in neutral buffered formalin, then 24 hr later, into 70% ethanol. Fixed tissue was embedded in paraffin, then 5 um sections were cut and stained with hematoxylin and eosin by routine procedures.

Further evidence that EMH was occurring came from our collaborative toxicogenomics studies with Dr. Ed. Perkins, USACE. It has been shown by others (Rokushima et al. 2007) that hemoglobin synthetic gene expression is increased early (24 h) in EMH tissues (liver, spleen) upon treatment with chemicals subsequently exhibiting hemolytic anemia. Of those genes, we likewise see increased expression of 2 hemoglobin synthesis genes (Table 1) at 24 hr after exposure to 24 and 48 mg/kg RDX, a dose lower than that where microscopic EHM is observed. The Meyer lab contribution to this project by performing the rat exposures and tissue collections through other DoD support (W912HZ-05-P-0145).

Table 1. Toxicogenomic results of liver 24 hr after treatment with RDX.

Name	Significantly changed?	Accession No.	Protein Name	Relative change over vehicle control; ln ₂ ratios	
				24 mg/kg	48 mg/kg
Alas2	Yes (↑)	NM_013197	Aminolevulinate synthetase2,delta	0.95-fold	1.42-fold
β-globin 2	Yes (↑)	S82293	II β-globin=II beta-globin (5' region)	0.93-fold	2.19-fold

Method, Microarray assessment of gene expression was done using Whole Rat Genome Oligo Microarray Kits (Agilent, Santa Clara, CA). One color analysis was performed using an Agilent Quick Amp labeling kit to synthesize cRNA from total RNA, hybridized to arrays and detected per manufacturer's instructions (Agilent). Arrays have 41,000+ rat genes and transcripts represented, all with public domain annotations. Arrays were read and quantified with an Axon GenePix4200b microarray scanner and data normalized to spike-in controls and analyzed using GeneSpring microarray analysis package (Agilent).

Another event associated with a successful regeneration of an anemic episode is increased reticulocytes in the peripheral blood. We quantitated reticulocyte numbers on blood collected from rats treated with MNX and RDX as before. As shown in Figure 2, no increase in reticulocyte count was observed at the dose of either RDX or MNX (94 mg/kg) at which anemia had been observed, which is consistent with impairment of bone marrow erythropoiesis.

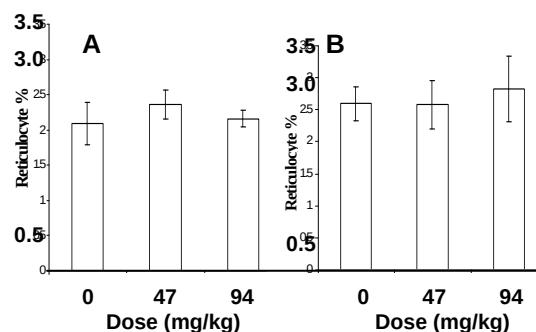
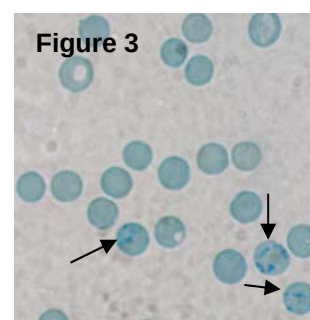


Figure 2. Reticulocyte counts of blood from RDX (A) and MNX (B) 14 d after exposure. Values are means $\pm$ SEM for n=5.

Method. Blood was incubated with new methylene blue (Sigma-Aldrich Reticulocyte Stain; Sigma-Aldrich, Inc., St. Louis, MO; Procedure No. R 4132), then was spread on microscopic slides and air dried. Reticulocytes were counted as cells with blue RNA precipitate (Fig. 3) using a 100X oil objective and were expressed as percentage of at least 2000 red blood cells counted per animal.



A potential cause of chemically-induced anemia not due to toxicity to bone marrow erythroid progenitors would be loss of erythropoietin due to renal toxicity. We did find that serum erythropoietin was decreased 14 days after 47 and 94 mg/kg MNX (Fig 4). However, the absence of effect on the classical serum marker for renal damage, blood urea nitrogen (BUN) suggested that this occurred for reasons other than a direct toxic effect of MNX on kidney. Although this result, i.e., loss of erythropoietin, could contribute to the chemically induced anemia we observe, it could not explain our previous observation of a loss in erythroid precursor (BFU-Es) of bone assayed in colony forming assays provided with optimal exogenous erythropoietin (reported year 2).

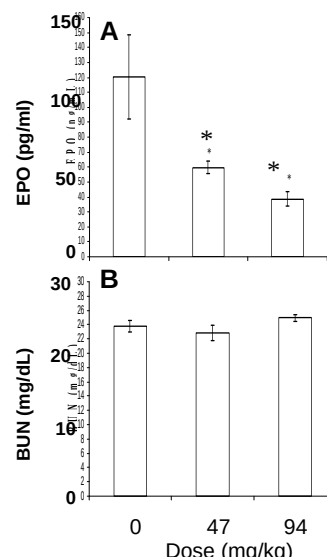


Figure 4. Serum erythropoietin (A) and blood urea nitrogen (B) of rats 14 d after treatment with MNX. Values are means $\pm$ SEM for n=5.

To summarize Task #1 accomplishments for year 3, we have provided further evidence supporting the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow. In particular, histopathological and

toxicogenomics evidence that hematopoiesis is induced in spleen and liver and absence of reticulocytosis suggest that bone marrow hematopoiesis is insufficient to reverse RDX- and MNX-induced anemia. An evaluation of an alternate hypothesis that renal toxicity with consequential loss of erythropoietin was not supported by direct determination of MNX effects on BUN, although a decrease in blood erythropoietin was noted.

Task 2: Determination of whether MNX and metabolite MDNA accumulate in bone after acute exposure and whether bone marrow stromal cells are metabolically capable of converting MNX to MDNA. (Months 13 – 36).

A manuscript describing previous collaborative work with U.S. Army Corps of Engineers colleagues (MacMillan, Denise, now at US Environmental Protection Agency) on disposition and metabolism of RDX and MNX has been peer-reviewed for publication. The manuscript is currently in revision for resubmission. The Meyer lab contribution to this project was funded through other DoD support (DACA42-02-P-0035). One observation from that project salient to the present report is that of detection of ring cleavage product MDNA in liver of MNX-exposed rats. This further justifies future pursuit of Task #2 determination of whether bone marrow stromal cells are able to similarly metabolize MDNA.

Dr. MacMillan has left our collaborators' laboratory at USACE in Vicksburg, MS; however, we are currently preparing a contract for pursuit of these studies with Dr. Tony Bednar who is continuing Dr. MacMillan's work. Previous ULM graduate student, Mitchell Wilbanks (now a contractor with USACE in Vicksburg, MS), has successfully cultured bone marrow stromal cells from female Sprague-Dawley rats and immunohistochemically stained these cells for vimentin, an intermediate filament protein specific for mesenchymal cells. These results demonstrated that there are 2 classes of vimentin-positive cells in this preparation, those with a flat fibroblast morphology with vimentin-positive intracellular cables and an overlying small cell, most likely vascular-derived endothelial cells with paranuclear staining (Fig. 5).

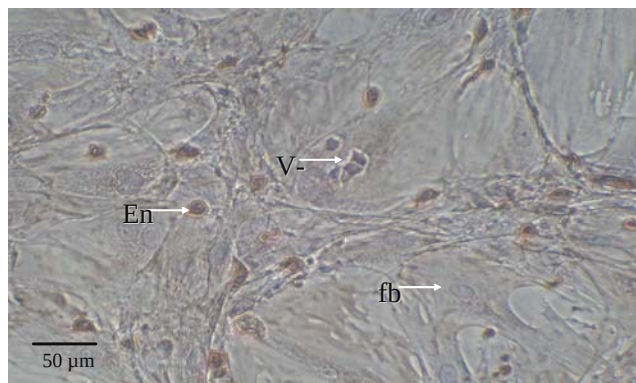


Figure 5. Photomicrograph of cultured bone marrow stromal cells from Sprague-Dawley rats immunohistochemically stained for vimentin. Vimentin-positive cells with fibroblast morphology (fb) and small overlying cells (En) are indicated. A small overlying vimentin-negative cell (V-) was also evident.

We have also prepared microsomes from these cells. We were able to generate two 150 mm² plates of confluent cells from 7-8 rats after 3 months in culture with two passages. Upon harvest, we were obtained ~75 ug of microsomal protein. We have since repeated the procedure to obtain a combined ~150 mg protein. Although we will attempt to determine whether MNX is metabolized by this material, we may be limited by amount and will need to collect and process bone marrow cells from additional rats.

Method.

Cell culture: Nucleated bone marrow cells (NBMCs) were isolated by centrifugation of total marrow cells from femurs over Histopaque-1077 (Sigma). Cells were counted with a hemacytometer and 4×10^5 cells/mL at a total volume of 5 mL medium (DMEM supplemented with 7.5% fetal calf serum, 7.5% horse serum, 0.29 mg/mL L-glutamine, 10^{-4} M 2-mercaptoethanol, and antibiotic/antimycotic (1 mL/100 mL medium) were plated in a T25 tissue culture flask. Cells were cultured in a 5% CO₂ incubator at 37°C and adherent cells were refed with 50% replacement of medium after 3 days. Old medium was removed and centrifuged at $200 \times g$ for 10 min to remove non-adherent cells and ½ volume was added to 2.5 mL fresh medium. One-half medium changes were done every 3-5 days until cultures were 80% confluent. Cells were then passed and plated into a T75 flask with a total volume of 15 mL and refed every 3-5 days with the same procedure until 80% confluent. Cells were passed and plated onto two 150 mm culture dish at a volume of 17 mL each. One mL of cells were plated onto each of three 35 mm culture dishes for immunohistochemical staining. Medium was changed every 3-5 days as before until approximately 100% confluency was reached. Microsomes were prepared in 1.15% KCl in 50 mM potassium phosphate, pH 7.5, 0.1mM EDTA by centrifugation of homogenate at $10,000 \times g$, 15 min and supernatant at $100,000 \times g$ for 60 min. Pellet was resuspended in 75 µL of storage buffer (0.25 M sucrose in 50 mM potassium phosphate, pH 7.5, 0.1 mM EDTA and stored at -80°C.

Immunohistochemistry: Cultured adherent bone marrow stromal cells were fixed in methanol at -20°C. Cells were quenched with 3% hydrogen peroxide, then washed with Tris buffered saline (0.05M, pH 7.6)/Tween 20 (0.5% v/v) and blocked with goat serum. Samples were incubated with anti-vimentin antibody (Clone V9, DAKO N. America, Carpinteria, CA) diluted 1:80 in TBS/Tween 20 buffer then secondary biotinylated antibody, goat anti-mouse (Southern Biotechnology Associates Incorporated, Birmingham, AL). After washing with buffer, samples were incubated with the Vectastain Elite ABC Reagent (Vector Laboratories Inc., Burlingame, CA), then incubated with peroxidase and diaminobenzidine. Cells were lightly counterstained with hematoxylin, rinsed with tap water, “blued” in 1% v/v sodium bicarbonate, rinsed in tap water, and mounted using aqueous mounting medium (Crystal Mount, Biomed Corporation, Foster City, CA).

Task 3: Determination of whether acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid and myeloid lineages. (Months 18 – 36) – done years 1 & 2; no further work

Task 4: Determination of whether acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment. (mos. 12 – 30)

We have approached this by quantitating the number of stromal colony forming units (CFU-Fs) in nucleated bone marrow cells (NBMCs) of rats 14 days after treatment with vehicle or 47 and 94 mg/kg MNX. These are determined as outgrowths from plated NBMCs that adhere to tissue culture plastic. In general, these include the fibroblasts, endothelial cells and macrophages, but not hematopoietic progenitor cells, of bone marrow. After 14 days in culture, cultures were fixed and stained with Wright-Giemsa stain. The appearance of colonies magnified with a dissection photomicroscope is shown in Figure 6. Results from counting of these colonies are shown in Figure 7 in which a statistically significant increase is observed at 94

mg/kg. Although we anticipated stromal toxicity would be manifested as a decrease in CFU-Fs, these results may instead indicate an inflammatory response with increased macrophages or a potential fibrogenic response from an increase in fibroblasts.

Figure 6. Colonies of bone marrow stromal cells (CFU-Fs) from rats treated with vehicle (A) and 94 mg/kg MNX (B). A 2 mm scale is shown in the upper right.

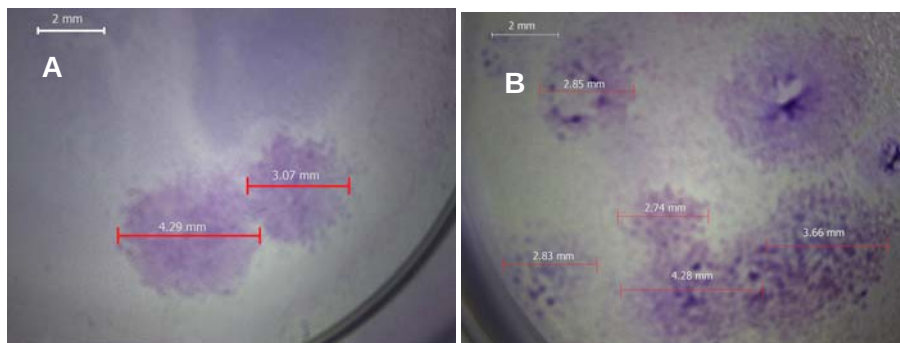
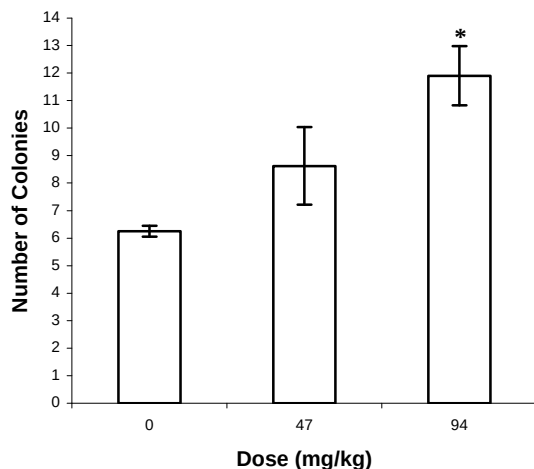


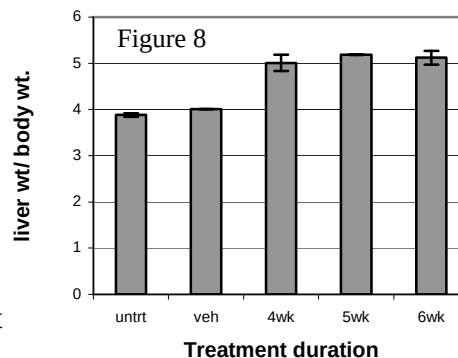
Figure 7. CFU-F colony counts from bone marrow cells harvested 14 days after treatment of rats with vehicle or 47 and 94 mg/kg MNX. Values are means $\pm$ SEM. *, $p < 0.05$.



Method. Bone marrow stromal cells were isolated and cultured as above for preparation of microsomes except plating densities were increased. Cells were seeded in duplicate at a concentration of 2×10^6 cells/mL onto a 12 well plate. The volume plated in each well was 0.5 mL. Cells were fixed and stained after 14 days.

Task 5: Determination of whether repeated administration of lower doses of MNX produces bone marrow toxicity, especially fibrosis. (mos. 24 – 36).

Graduate student Sindhura Ramasahayam, who started in the Meyer lab January 2007, has begun studies addressing Task 5. We have divided the study into two phases to accommodate the effort required for daily gavage for several weeks. Phase 1 was conducted with $n=2$ (of the planned $n=5$) rats dosed daily for 4, 5 and 6 weeks with 47 mg/kg MNX ($1/4$ LD₅₀). Thus far, animal exposures have been completed and tissues collected and archived. Blood analyzed for clinical pathology has indicated no effects on parameters diagnostic of liver (AST, alkaline phosphatase, total bilirubin) or renal (BUN, creatinine) toxicity. Average weekly body weight gains were also unaffected, however, it appeared that relative liver weights were increased by all treatment durations (Fig. 8) indicating that



systemic delivery of administered dose did occur. Histological processing of archived tibias to include Gomori silver staining for reticulin are planned.

Statistics: Effects of MNX and RDX on endpoints with normally distributed data (EPO, BUN, liver weights) were determined by ANOVA with post-hoc comparisons of treatment means against vehicle control done with Dunnett's test. CFU-F count data were transformed to the square root with a 3/8 continuity factor, then analyzed with ANOVA and Dunnett's. Results were considered statistically significant with $p < 0.05$. Data were statistically analyzed using JMP 4.0.4 software (SAS Institute Inc.).

KEY RESEARCH ACCOMPLISHMENTS:

Studies thus far have demonstrated:

- detection of EMH and absence of reticulocytosis in support for the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow.
- methods were developed for culture of bone marrow stromal cells, both clonally and in mass culture. Results from clonal CFU-Fs indicated an increase in a bone marrow stromal cell upon treatment with MNX.

REPORTABLE OUTCOMES:

1. Abstract presented at 2008 annual meeting of the Society of Toxicology, Seattle, WA

Wilbanks, MS Reeves, SL, Escalon, BL, Perkins, EJ and Meyer, SA. Comparative Hematotoxicity of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) vs. Mono- and Tri-Nitroso Environmental Degradation Products MNX and TNX. *The Toxicologist*. 2008; 102 (S-1): 26 (abst. #130).

2. July 2008 submission of grant proposal "Myelosuppression from Munitions RDX and Product MNX: Mediation through Bone Marrow Inflammation", Meyer, SA, PI to the 2009 Peer-Review Medical Research Program (PRMRP) of the Congressionally-directed Medical Research Program (CDMRP). Preliminary data presented in the proposal was funded by this grant.

3. Mitchell S. Wilbanks, graduate student whose stipend was paid from this grant, has successfully defended his M.S. thesis and will graduate December 2008. He will begin working as a contractor in October with the US Army Corps of Engineers, Vicksburg, MS.

CONCLUSION: Results of these studies on EMH further support the hypothesis that munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is toxic to hematopoietic bone marrow stem cells, but also suggest decreased erythropoietin by environmental degradation product hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) may compound the myelosuppressive effect. Data suggest a MNX effect on the bone marrow stromal niche, which supports hematopoiesis, through expansion of one of its cell types. Thus, previously observed peripheral blood disorders in

MNX-treated rats appear to be the consequence, in part, of loss in replenishing stem cell populations. As such, this system appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus offer a model for study of possible mechanisms of disease progression and development of intervention strategies. In addition to the adverse hematological effects we have documented, these results suggest additional functional consequences with respect to host resistance to infection, inflammation and tissue trauma.

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Rokushima M., K. Omi, K. Araki, Y. Kyokawa, N. Furukawa, F. Itoh, K. Imura, K. Takeuchi, M. Okada, I. Kato I and J. Ishizaki. A toxicogenomic approach revealed hepatic gene expression changes mechanistically linked to drug-induced hemolytic anemia. *Toxicol. Sci.* 95:474-484, 2007.

APPENDICES: None

SUPPORTING DATA: None