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

PRINCIPAL INVESTIGATOR: Sharon A. Meyer, Ph.D.

CONTRACTING ORGANIZATION: University of Louisiana at Monroe
Monroe, LA 71209

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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 munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes anemia after acute exposure in the rat and examine whether similar effects were elicited by subchronic exposure. We have hypothesized MNX targets hematopoietic stem cells and, like other myelosuppressive chemicals, will be fibrogenic to bone marrow. During this reporting period, results from subchronic exposure in which rats were treated orally with ¼ LD50 MNX daily for 4 or 6 weeks were: 1) significant increases in granulocytes and platelets, but not erythrocytes, at both times, 2) an increase in serum K and decreases in Na, Cl, glucose, and creatinine levels in the absence of effect on body weight gain and serum albumin, 3) increased liver weights and 4) increased megakaryocytes in bone marrow, but not fibrosis as indicated by methenamine silver stain for reticulin fibers. Collectively, these results continue to support a bone marrow effect of MNX upon subchronic exposure that is consistent with impaired hematopoiesis and identify additional non-marrow targets. These results suggest that MNX toxicity in the rat may resemble the prefibrotic phase of the myeloproliferative disorder, idiopathic myelofibrosis, and thus may offer a model for study of disease progression and intervention strategies.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	8
References.....	9
Appendices.....	N/A

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 after acute exposure and whether similar toxic effect is elicited by subchronic exposure. Anemia was detected in our previous acute toxicity studies in the rat (Meyer et al. 2005) at 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 hematopoietic 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).

No further work on this task in year 4.

Previous findings relevant to this task include: 1) hemoglobin, hematocrit and granulocytes were dose-dependently decreased 14 days after treatment of rats with a single dose of MNX or RDX. RDX was more potent for these endpoints with a NOAEL of 12 mg/kg for decreased granulocytes vs. 47 mg/kg for MNX (reported year 1). 2) An alternate mechanism for MNX-induced anemia, i.e., through formation of methemoglobin, was shown not to occur either in vivo or in vitro (report year 2). 3) Histopathological evidence for splenic extramedullary hematopoiesis 14 d after treatment with 47 and 94 mg/kg RDX was observed and was consistent with an increase in expression of genes involved with hemoglobin synthesis in liver determined by toxicogenomics (report year 3).

Collectively, these data suggest a bone marrow target for the hematotoxicity observed with acute exposure to RDX and MNX with the former being more potent.

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).

We have now cultured 2 sets of bone marrow stromal cells from female Sprague-Dawley rats and prepared microsomes in sufficient quantities to yield 2 lots of ~75 mg microsomal protein

each. These microsomes are stored at -70°C and are available for determination of their ability to produce MNX metabolites.

Task 3: Determination of whether acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid and myeloid lineages. (Months 18 – 36)

Previous work has demonstrated dose-dependent decreases in bone marrow progenitors for erythroid (BFU-E) and myeloid (CFU-GM) lineages at 14 d after acute exposure to RDX and MNX. CFU-GMs were more affected than BFU-Es and both compounds were comparable in magnitude of effect (report year 2). Either no effect or an increase (CFU-GMs with RDX) in erythroid and myeloid stem cells was observed at 7 d after treatment. These results were suggested that hematotoxicity to these compounds required a time-dependent development and we suggested this might development of inflammation. A role for inflammation would be mediated, in part, through the stromal microenvironment, as examined in Task 4.

In an attempt to understand a mechanism through which these compounds exert this effect, a search for interventional strategies was initiated. Through these studies we have identified an ethanolic extract from the herbal supplement *Echinacea* that rescues CFU-GMs from MNX toxicity. The extract also independently stimulates CFU-GMs, so that prophylactic use of this material offsets loss due MNX toxicity. Since *Echinacea* has several anti-inflammatory activities, we suggest this is supportive of an inflammatory mechanism for RDX and MNX myelotoxicity.

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

A procedure for culturing bone marrow stromal colony forming units (CFU-Fs) was developed and described in reporting year 3. Importantly, a doubling of CFU-F colonies was observed from culturing bone marrow from rats treated 14 d earlier with 94 mg/kg MNX. These results were consistent with a treatment-related inflammatory response with increased macrophages or a potential fibrogenic response from an increase in fibroblasts.

We now have cultured bone marrow stromal cells fixed in methanol and archived for further cell-type characterization. We have previously identified two morphologically distinct cell types that are positive for vimentin, an intermediate filament protein specific for mesenchymal cells, and plan further staining for endothelial cell-specific lectin BSL I-B4 and macrophage fluoride-inhibitable nonspecific esterase.

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, has now completed the exposure phase of the full repeat-dose subchronic exposure studies. In the previous reporting period, we described results of a pilot study with $1/4\text{LD}_{50}$ MNX (45 mg/kg, $n=2$) administered daily for 4, 5 and 6 wk. We had observed an increase in relative liver weights and serum clinical chemistry results in which no evidence for liver or kidney toxicity was observed. We had encountered a problem in assessing hematology for the pilot study because the hospital clinical lab reported back that the blood samples had clotted precluding analysis. At the time we thought that result was a laboratory error.

Data from the complete study (n=5) are consistent with the previous pilot study in that increased relative liver weights were found and no effect on liver biomarkers of toxicity (ALT, AST and alkaline phosphatase) or blood urea nitrogen (kidney) were observed. Contrary to what is observed with renal toxicity, serum creatinine was decreased. Body weight gain was unaffected (11.4 ± 1.0 , 11.5 ± 3.1 and 10.5 ± 1.4 gm/wk for vehicle control, MNX-4wk and MNX-6wk, resp.). We did observe altered electrolytes in that serum K was elevated and Na and Cl were decreased. Glucose was decreased at both time points, while albumin were unchanged (Table 1).

Table 1. Selected endpoints from clinical chemistry assessment of MNX repeat dose study.

Treatment	Potassium (mmol/L)	Sodium (mmol/L)	Chlorine (mmol/L)	Glucose (mg/dL)	Creatinine (mg/dL)	Albumin (g/dL)
Veh 6wk	7.7 ± 0.4	141 ± 1.5	99.2 ± 1	235 ± 8	0.46 ± 0.02	1.6 ± 0.04
MNX 4 wk	8.6 ± 0.4	$136 \pm 1.5^*$	$95.2 \pm 0.5^*$	$171 \pm 7^{**}$	0.42 ± 0.02	1.5 ± 0.03
MNX 6 wk	$9.6 \pm 0.2^*$	$134 \pm 0.9^*$	$94.8 \pm 0.7^*$	$153 \pm 5^{**}$	$0.40 \pm 0.01^*$	1.6 ± 0.03

We were successful in analyzing hematological parameters in the full study and, consistent with the observed clotting in the pilot study, blood platelets were increased at both time points (Table 2). Likewise, blood granulocytes were increased to twice that of vehicle, but lymphocytes were unaffected. Unlike 14 d after a single acute exposure to 95 mg/kg MNX, hemoglobin (HGB) and erythrocyte number were unchanged.

Table 2. Selected endpoints from hematology assessment of MNX repeat dose study.

Treatment	Granulocytes ($10^3/\mu\text{L}$)	Lymphocytes ($10^3/\mu\text{L}$)	Erythrocytes ($10^6/\mu\text{L}$)	HGB (g/dL)	Platelets (mg/dL)
Veh 6wk	0.43 ± 0.12	3.24 ± 0.37	7.18 ± 0.16	13.1 ± 0.29	830 ± 20
MNX 4 wk	$1.04 \pm 0.21^*$	4.10 ± 0.97	7.20 ± 0.13	12.9 ± 0.34	$981 \pm 37^*$
MNX 6 wk	0.96 ± 0.15	4.07 ± 0.38	7.57 ± 0.20	13.7 ± 0.26	$1021 \pm 18^*$

Consistent with the hematological assessment, microscopic observation of Wright's stained preparations of blood smears from rats treated repeatedly with MNX exhibited large aggregates of platelets (Figure 1).

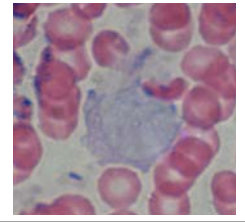
Overall, the increased serum K supports the observation of thrombocytosis and platelet aggregation. Decreases in serum Na, Cl and glucose may indicate gastrointestinal toxicity, while lower creatinine may indicate loss of



Figure 1. Platelet aggregate (arrows) in a Wright's stained blood smear from rat treated 6 wk with 47 mg/kg MNX per day

muscle mass (Hall and Everds, 2008). Further, increased blood granulocytes in the absence of blood lymphocyte change, as would occur with stress or excitement, suggests a systemic inflammatory response. Consistent with this notion was the frequent observation of reactive lymphocytes in Wright-stained blood smears (Figure 2)

Figure 2. Reactive lymphocyte in a Wright-stained blood smear from a rat treated daily with 47 mg/kg MNX per day.



Histopathological observations on bone marrow suggested that the observed peripheral thrombocytosis was a consequence of increased megakaryocytes in the bone marrow. In the hematoxylin and eosin-stained sections below, numerous pleiotrophic megakaryocytes are typically observed in a field of marrow from rats treated subchronically with MNX, while few populate that of vehicle treated control (Figure 3).

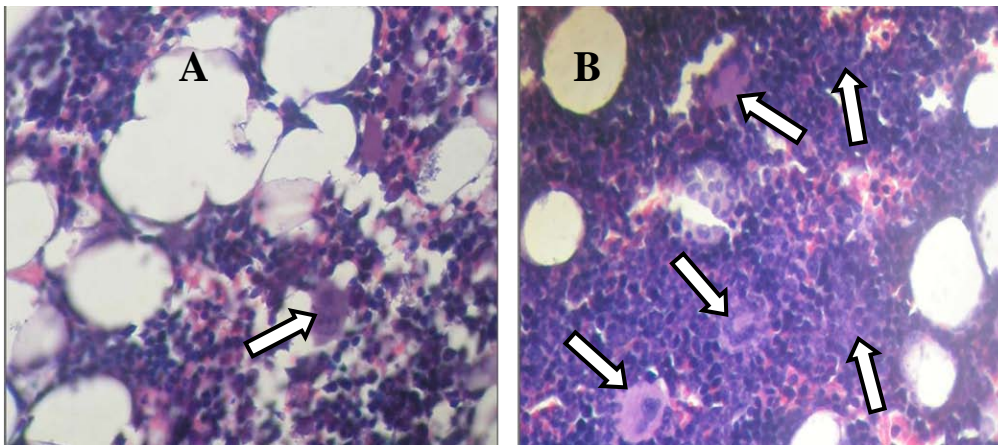
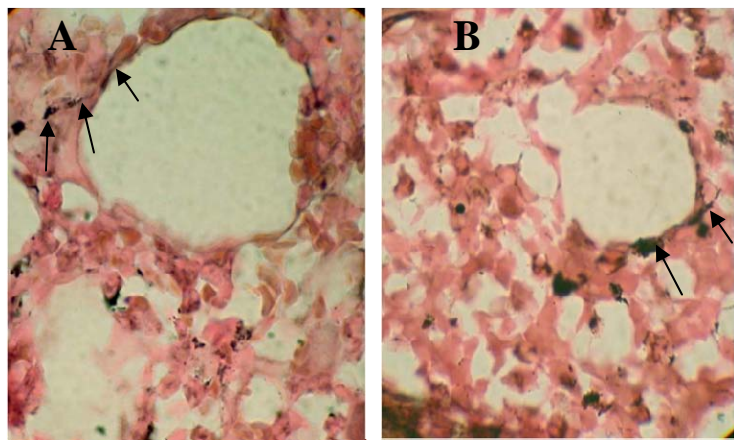


Figure 3. H&E stained ileum from rats treated with vehicle (A) and 47 mg/kg MNX daily for 6 wk. Bone was fixed in neutral buffered formalin, decalcified with EDTA, then embedded in paraffin. Sections (5 μ m) were cut and processed with routine methods for H&E staining. Megakaryocytes are indicated by arrows.

However, increased bone marrow megakaryocytes was not associated with fibrosis. As illustrated in Figure 4, no additional or enlarged reticulin fibers were seen.

Figure 4. Sections (5 μ m) of ileum from rats treated with vehicle (A) and 47 mg/kg MNX daily for 6 wk stained for reticulin with Grocott methenamine silver stain. Examples of reticulin fibers are indicated by arrows.



Statistics: Effects of MNX and RDX on endpoints with normally distributed data (clinical chemistry and hematological endpoints, liver weights) were determined by ANOVA with post-hoc comparisons of treatment means against vehicle control done with Dunnett's test. 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 of this thus far have demonstrated:

- Conduct of the labor intensive repeat dose exposure to 1/4LD₅₀ MNX.
- Development of methods for Grocott methenamine silver stain for reticulin fibers in bone marrow.

REPORTABLE OUTCOMES:

1. Abstract presented at 2009 annual meeting of the Society of Toxicology, Seattle, WA
Ramasahayam, S, Baraka, HN, Abdel Bar, FM, El Sayed, K and Meyer, SA. Effects of chemically characterized fractions from a commercial *Echinacea* herbal product and aerial parts of *Echinacea purpurea* and *angustifolia* plants on myelopoiesis. The Toxicologist. 2009; 108:165 (abst #798).
2. September 2009 submission of grant pre-proposal "Myelosuppression by MNX, Environmental Degradation Product of Munitions RDX Mediated through Bone Marrow Inflammation", Meyer, SA, PI to the 2009 Bone Marrow Failure Research Program (BMFRP) Synergistic Idea Award of the Congressionally-directed Medical Research Program (CDMRP). Preliminary data presented in the proposal was funded by this grant. Not selected for full application.

CONCLUSION: Results of these studies on effects of subchronic exposure to MNX at reduced levels from those of the earlier acute studies continue to implicate bone marrow as a target site for MNX toxicity. In particular, the clinical chemistry and hematology in conjunction with histopathology of the bone marrow suggest a thrombocytosis induced by repeated exposure to MNX. This peripheral blood effect is accompanied by megakaryocyte proliferation of bone marrow, but the bone marrow shows no evidence of fibrosis, the hallmark of the myeloproliferative disease, idiopathic fibrosis. However, the progression of this disease includes an early prefibrotic phase marked by peripheral thrombocytosis and megakaryocytic hyperplasia (Wojciech 2008). As such, this system may mimic the early clinical progression of idiopathic myelofibrosis and thus offer a model for study of possible mechanisms of disease progression and development of early intervention strategies. In addition to the adverse hematological effects we have documented, these results suggest additional functional consequences with respect to inflammation and possible adaptive changes of the liver.

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Meyer, S.A., A.J. Marchand, J.L. Hight, G.H. Roberts, L.S. Inouye, L.B. Escalon and D.K. MacMillan. Up-and-down procedure (UDP) determination of acute toxicity of nitroso degradation products of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). J. Appl. Toxicol. 25:427-434, 2005

Wojciech, G. (2008) Cytogenetics, FISH and Molecular Testing in Hematologic Malignancies, Informa Health Care, p. 207.

APPENDICES: None

SUPPORTING DATA: None