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TITLE: An Overview of the Continuation of the Work of the Mustard Consortium for the Use of the Free and Liposome Encapsulated Antioxidants as a Counter Measure to Mustards

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SECTION 1: Milton G. Smith, M.D., Amaox, Ltd.

An Over View of The Continuation Of The Work of the Mustard Consortium For The Use Of The Free and Liposome Encapsulated Antioxidants as A Counter Measure to Mustards

Introduction

The Mustard Consortium (MC) consists of Amaox, Ltd., University of Michigan, and Center for Blood Research, East Tennessee State University, Meharry Medical College and Institute for Chemical Defense. It was developed in order to take advantage of the core competencies of each investigator and institution; as well as creating a learning organization. The creation of this type of organization has allowed us to have an unparallel insight into the pathophysiology of mustards; as well as redox regulated mediated pathways that have much larger implication in the pathogenesis of disease.

The MC has used a systemic approach for the elucidation of the pathophysiology of the mustard analogue CEES (2-chloroethyl ethyl sulfur); and the development of methods of intervention (potential treatments). Both in vitro and in vivo models were used by the various investigators. One investigator used human primary myeloid cells for his investigation. The success of this approach will be evident to the reader in examination of the methods of interventions that have been developed, and will continue to evolve. It is anticipated that of these several methods of experimental intervention a few will be developed into treatment regimen.

Body

Although mustard gas was invented over one hundred years ago, it remains a threat to our modern day soldiers since there are no commercially available antidotes. It is more so a debilitating agent rather than life threatening. It is well known to affect all major organs, but primarily the eyes, lungs and skin [Papirmeister et al., 1985]. Most of the research done on mustards has been on its effects on the skin. There have been multiple theories regarding the true pathogenesis of mustards such as: alkylation of DNA, NAD depletion, inhibition of glycolysis, and PARP activity [Lakshmana Rao et al., 1999; Papirmeister et al., 1985]. Recently, oxidative stress would appear to be a predominant mechanism of action in lung injury [Chatterjee et al., 2003; McClintock et al., 2002]. Significant protection against lung injury has been achieved with the use of antioxidants in animal models [McClintock et al., 2002]
**Key Research accomplishments (group)**

**Specific Aim 1- Integration of data for the entire Consortium**

The doses of CEES that the civilian counterpart of the Consortium have been using was found to be comparative to the doses of sulfur mustard that were being used be secure facilities (e.g. ICD). This most recent finding places a greater value on the work that has been done by the civilian counterpart use of CEES in the in vivo and in vitro experiments.

CEES exposure to the lung exposure induces an intense inflammatory reaction, in which myeloid cells participate. The genomics of human myeloid cells exposure to CEES has been previously unknown. In the last year, genomic expression analysis was performed. Genes that were modulated by CEES were elucidated. Three patterns were defined and deemed to be most important: apoptotic pathway, stress/ toxicity, and inflammatory cytokines.

In the guinea pig lung model it was observed that one of the primary enzymatic antioxidant defenses SOD, particularly the major isomer (SOD3) in guinea pig lung is diminished after exposure. LPS (lipopolysaccharide- a bacterial endotoxin) a ubiquitous finding in the environment, has been found to greatly enhance oxidative stress in tissues exposed to CEES by many fold.

**Specific Aim 2- Chaired meetings**

Chaired the meeting of the Mustard Consortium members- May 2004. Experimental results were reviewed as well as future experiments were discussed.

**Reportable Outcomes**

Methods of intervention (potential treatments)

1. Polymyxin B, an antibiotic, binds specifically with the lipid A toxophore of LPS. Polymyxin B was found to effectively block the exacerbating effects that LPS have on CEES.

2. Liposome encapsulated superoxide dismutase and catalase were found to greatly attenuate the lesion induced by CEES. Similarly, if liposome encapsulated non-enzymatic antioxidants were used 30, 60 and 90 minutes after exposure to CEES damage to the lung could be ameliorated (respectively 57, 55 and 38% protection).

3. PARP iRNA, nictotinamide and NAC were all protective in the cellular models.
4. Complement depletion is protective

See specific aims of the individual investigators

See individual Section reports.

Abstracts

1. Smith MG, Stone W, Ward PA, Till GO, Crawford K, Das S
   An overview of the work of the Mustard Consortium in the elucidation and
development of countermeasures to CEES

2. William L. Stone, Min Qui, Hongsong Yang, and Milton Smith, Lipopolysaccharide
   Enhances the Cytotoxicity of 2-Chloroethyl Ethyl Sulfide, U.S. Army Medical

Manuscripts

1. Chatterjee, D., Mukherjee, S., Smith, M. G., and Das, S. K. Signal Transduction
   Events in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide (CEES), A Mustard

2. Das, S. K., Mukherjee, S., Smith M., and Chatterjee, D. Prophylactic Protection by N-
   Acetylcysteine Against the Pulmonary Injury Induced By 2-Chloroethyl Ethyl Sulfide

   Antioxidant Liposomes – A Promising New Treatment for Mustard Gas With The
Potential To Substantially Reduce The Threat Posed By Chemical, Biological And

4. Shanon D, McClintock, Gerd O. Till, Smith MG and Ward PA
   Protection from Half-mustard-gas-induced Acute Lung Injury in the Rat.

5. Shanon D, McClintock, Till GO, Smith MG and Ward PA
   Protective Effects of Antioxidant Liposomes in CEES-induced Acute Lung Injury in
Rats. To be submitted.

6. Stone, W.L., Qui, M and Smith, M.: Lipopolysaccharide Enhances the Cytotoxicity of

7. Hongsong, Y., Qui, M., Smith, M. and Stone, W.L: Inhibition of Inducible Nitric
   Oxide Synthase by a Mustard Gas Analog, BMC Cell Biology, to be submitted.
Conferences

- All investigators attended the Bioscience Conference, Hunt Valley, Maryland, 16-21 May 2004.

Presentation

- Bioscience (2004) workshop – Novel antidotes to WMD?

Important Accomplishments

Amaox, Ltd. - M. Smith; Director
- Consistent and frequent contact concerning experimental outcomes and design were discussed with all of the collaborators over the entire one year period
- Focal point for information flow between the four experimental investigators
- There will be an expansion of the Mustard Consortium to several other universities that will expand the application of STIMAL to biological weapons and cancers
- Preliminary talks have been held with the FDA in regards to the use of the Amaox technology, STIMAL (antioxidant liposomes) as a potential antidote for the inhalation of mustards. A pre-IND meeting will be scheduled if the technology is proven to be efficacious in animal lung models with mustards to be done at ICD within the next six months.

ICD (Institute for Chemical Defense) -
- A pilot study was done by Dr. Fred Cowan- In vitro (human lung epithelial cell lines) studies showed that the commonly used dosage of sulfur mustards, 100 μM, is equivalent to 400 μM of CEES.

University of Michigan; PA Ward and GO Till- rat lung model
- CEE activates an inflammatory response. If neutrophils or complement were depleted it was protective against the lesion produced by CEES.
- High levels of protection were achieved using either liposome encapsulated enzymatic or non-enzymatic antioxidants. If the non enzymatic antioxidants
- It was demonstrated with the non-enzymatic antioxidants that they could be administered as a “rescue” agent. That is they were protective at 57%, 55% and 38% (respectively 30, 60, and 90 minutes) after the intratracheal administration of CEES.
• It was demonstrated that oxidative stress is the primary mechanism of action of CEES

**Center for Blood Research** - K. Crawford; primary human myeloid cells
• cDNA library of genes for human primary myeloid cells
  o modulated genes were grouped into three categories:
    1. apoptotic
    2. stress and toxicity
    3. inflammatory cytokines
• CEES does not break or cleave DNA, but does in induce warping
  o Induces the activation of PARP with subsequent consumption of NAD^+
• Cellular protection was achieved by the use of PARP iRNA, Pan Caspase inhibitors, NAC or nicotinamide
• CEES induces increased production of IL-8 and IL-6
  o Cells treated with NAC significantly reduce IL 8 and 6 close to basal levels.

**Meharry Medical College** - S. Das; Guinea pig lung model
• CEES increases the activity of SOD1 and 2; whereas the predominant isomer SOD3 is markedly decreased.
• Defects in beta-adrenergic receptors are thought to be contributory to the development of ARDS (a known occurrence in the exposure to mustards). Both high and low affinity receptors were examined. There was a significant decrease the binding capacity of the low affinity receptors.
• Tissues samples were prepared by Dr. Das and sent to Dr. Ward. Transmission electron microscopy was used to examine the samples. Lung tissue harvested from animal sacrificed at day 7, 14, and 21 showed interstitial fibrosis. At 4 and 6 hours after exposure there was evidence of apoptosis.

**East Tennessee State University** - W. Stone; RAW macrophages
• CEES alone induces death of by apoptosis. CEES in the presence of LPS enhances caspase activity
• CEE reduces glutathione levels. CEES + LPS exacerbates the loss of glutathione more than CEES alone
• Polymyxin B protects against the enhanced lethality to cells by LPS in the presence of CEES
• Liposome encapsulated glutathione or vitamin E were protective against the effects of CEES and LPS
Collaborative Arrangements

All investigators were contacted about once or more per week to discuss experimental outcomes of a particular experiment set or design. If the discussion had bearing on the work of other investigators then they were also contacted regarding their relevant information and how it can be expanded, redirected. Experimental agendas were mutually agreed upon at the outset of the funding period.

Conclusion

The finding that polymyxin B, a common topical antibiotic, is able to decrease damage done by CEES in the presence of LPS may have a significant impact on the care of skin wounds. It is evident from the current, and previous work that was done that significant protection is achieved by the diminution of oxidative stress. We have gained further experience with antioxidant liposomes which effectively reduces oxidative stress. Demonstration of achieving protection against CEES at various time intervals after exposure is a significant milestone.

In upcoming experiments, the finding that complement depletion is protective will be expanded upon, by the use of complement blockade using C5a antibodies (the net effect is similar). The STIMAL technology (liposome encapsulated antioxidants) is a potentially new treatment that suppresses oxidative stress. Manipulation of the dosing and formulation will have to be done over time to determine the optimal dosages and scheduling. It is anticipated that the STIMAL could be administered intravenously and or by aerosol.

There are now several modalities that can be potentially utilized as treatments for mustard gas exposure. They could be applied topically (e.g. skin or the eyes) or for either as a prophylaxis or a treatment for an inhalation injury. We will continue to build on these early promising methods of intervention.

References


SECTION 2: Keith Crawford, M.D., Ph.D., Center for Blood Research

Normalized cDNA Library From 2-chloroedthyl ethyl sulphide exposes myeloid cells.

Abstract

As part of the Mustard Gas Consortium, these studies focused on improving our understanding of the underlying pathophysiology and subsequent tissue death associated with chloroethyl-ethyl-sulfide (CEES) exposure. The principal objective of this project is to apply genetic strategies to investigate the underlying pathophysiology associated with mustards gas exposure. The exposure of primary human myeloid cells to CEES induces significant death. Preliminary data reveals significant suppression of cellular function and protein production. However, intervention with N acetylcysteine (NAC), a potent antioxidant, decreases the amount of immune cell death. To better understand the mechanism(s) by CEES induces cell death in these lung immune cells, a cDNA library will be created. This cDNA library will allow the construction of microarrays, which will be used by Mustard Consortium members to further characterize apoptotic (cell death), anti-apoptotic, and signal transduction pathways involved in CEES induced tissue injury.
Introduction

Inhaled sulphur mustard 2-chloroethylethyl sulphide (CEES), a vesicant, induces lung damage by alkylating with biological molecules found in lung tissue and lung myeloid cells [LMC (dendritic cells and monocytes)] and the subsequent activation of the immune system. The pathway(s) involved in CEES-induced direct and/or indirect tissue damage is poorly understood. To improve our understanding of the pathophysiology associated with CEES exposure, we used cellular and molecular strategies to investigate the mechanism(s) by which CEES induces LMC death. Furthermore, data generated from these results will allow the development of a cellular and silico-based cDNA library, which will be used to construct microarrays. These global microarrays will be used to improve our characterization of apoptotic (cell death), anti-apoptotic, and signal transduction pathways involved in CEES exposure. Thus, allowing the development of more focused chemical detection arrays capable of further assisting in the diagnosis of not only CEES exposure but also other chemicals, which may serve as a WMD. Collectively, the results will increase our understanding of the pathways involved in CEES-induced tissue damage, allow the development of novel biologic markers, and the development of novel countermeasure therapies.

Body

The specific objectives of our studies involved the following. First, cDNA libraries will be constructed from mRNA obtained from unexposed and CEES-exposed LMC. These libraries will provide the need information to investigate various cellular pathways, which are modulated during CEES exposure. Second, cDNA microarrays will be designed and constructed which will serves as tools to better understand the role of CEES in cellular apoptosis but also provide additional information detailing the various cellular pathways by which STIMAL confers protection following exposure to CEES. Third, cDNA microarrays will be designed and developed, which will allow the investigation of human tissue, as well as, mouse and rat tissues.
**Objective One:**

Unexposed and CEES-exposed primary human myeloid cells experimental studies have been completed. Messenger (m)RNA and cellular proteins were collected from the experimental samples. The mRNA from various studies was pooled and used to synthesize cDNA. The pooled cDNA not only served as the source of cDNA for the cDNA library but also served as the source of cDNA for gene expression studies. Gene expression and silico-base studies revealed that CEES modulated the expression of a variety of myeloid genes. These modulated genes were grouped into three categories, (1) Apoptotic, (2) Stress and Toxicity, and (3) Inflammatory Cytokine. An abbreviate list of the genes are provided in Tables 1-3.

<table>
<thead>
<tr>
<th>Description</th>
<th>Gene Name</th>
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<tbody>
<tr>
<td>Apoptotic protease activating factor</td>
<td>Apaf-1</td>
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<td>Apoptosis-associated speck-like protein CARD</td>
<td>Asc</td>
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<tr>
<td>BCL2-antagonist of cell death</td>
<td>Bad</td>
</tr>
<tr>
<td>BCL2-antagonist/killer 1</td>
<td>Bak</td>
</tr>
<tr>
<td>BCL2-associated X protein</td>
<td>Bax</td>
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<tr>
<td>B-cell CLL/lymphoma 2</td>
<td>Bcl-2</td>
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<tr>
<td>BCL2-related protein A1</td>
<td>BFL1</td>
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<tr>
<td>BCL2-like 1</td>
<td>Bcl-x</td>
</tr>
<tr>
<td>BCL2-like 11 (apoptosis facilitator)</td>
<td>BinL</td>
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<tr>
<td>BCL2-like 2</td>
<td>Bcl-w</td>
</tr>
<tr>
<td>BCL2-interacting killer (apoptosis-inducing)</td>
<td>Bik</td>
</tr>
<tr>
<td>Caspase 1, apoptosis-related cysteine protease</td>
<td>ICE</td>
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<tr>
<td>Caspase 10, apoptosis-related cysteine protease</td>
<td>MCH4/FLICE2</td>
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<td>Caspase 13, apoptosis-related cysteine protease</td>
<td>Caspase 13</td>
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<td>Caspase 14, apoptosis-related cysteine protease</td>
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<td>Caspase 7, apoptosis-related cysteine protease</td>
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<td>Caspase 8, apoptosis-related cysteine protease</td>
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<td>CASP8 associated protein 2</td>
<td>Flash</td>
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<td>Caspase 9, apoptosis-related cysteine protease</td>
<td>MCH6/APAF3</td>
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<td>CASP8 and FADD-like apoptosis regulator</td>
<td>CASPER/FLIP</td>
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<td>Description</td>
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<td>Annexin V</td>
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<td>Catalase</td>
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<td>Cyclin C</td>
<td>Cyclin C</td>
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<tr>
<td>Cyclin D1 (PRAD1: parathyroid adenomatosis 1)</td>
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<td>Cyclin G1</td>
<td>Cyclin G</td>
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<td>Cyclin-Dependent Kinase Inhibitor 1A</td>
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<td>Crystallin, alpha B</td>
<td>Cryab</td>
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<tr>
<td>Colony stimulating factor 2 (granulocyte-macrophage)</td>
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<tr>
<td>Cytochrome P450</td>
<td>CYP1A1</td>
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<td>Damage-specific DNA binding protein 1, 127kDa</td>
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<td>DNA-damage-inducible transcript 3</td>
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<td>E2F transcription factor 1</td>
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<td>Early growth response 1</td>
<td>Krox-24</td>
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<td>Epoxide hydrolase 2, cytoplasmic</td>
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<td>Excision repair cross-complementing rodent repair deficiency</td>
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<td>Flavin containing monoxygenase 5</td>
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<td>Glutathione peroxidase 1</td>
<td>GPX1</td>
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<td>Glutathione reductase</td>
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<td>Glutathione S-transferase M3 (brain)</td>
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<td>Heme oxygenase (decycling) 1</td>
<td>HO-1/HEME1</td>
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<td>Heme oxygenase (decycling) 2</td>
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<td>Heat shock transcription factor 1</td>
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<td>Heat shock 105kDa/110kDa protein 1</td>
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In addition to the gene expression studies, cellular and flow cytometric studies revealed the possible pathway by which CEES induces cellular damage in the myeloid cells as well as in other tissue. Our finding demonstrate that CEES does not break or cleave DNA immediately following exposure (data not shown), but does alter the structure of DNA and causes the activation of poly (ADP-ribose) polymerase (PARP). Once activated, PARP begins the DNA repair process and competes with the mitochondria for cellular NAD+. Because PARP has a greater affinity for NAD+ than the mitochondria, ATP production decreases and mitochondrial oxidant load increases. It is this inability of the mitochondria to handle the increasing levels of oxidants, which initiates the onset of the apoptotic cascade. To explore this theory, we exposed LMC with CEES for 1 hour and treated samples with nicotinamide (NA) alone or with PARP iRNA (Figure 1). Our finding demonstrated that NA alone did not confer protection against CEES exposure but a combination of PARP iRNA and NA significantly prevent CEES – induced LMC death.

Fig 1. Both Nicotinamide and PARP iRNA are required to prevent CEES induced myeloid cell death.

**Figure 2.** CEES-induced cytochrome c release from LMC mitochondria.
Elevated levels of oxidants cause changes in membrane permeability and the subsequent release of cytochrome c into cytoplasm (Figure 2). Cytochrome c interacts with the inactive form of caspase 9 producing a cleaved or active form of caspase 9 (data not shown), which in turn activate caspase 3 (Figure 3).

Caspase 3 is a focal protein in the cellular apoptotic pathway and is instrumental in the final stages of DNA cleavages and apoptosis. Our studies have demonstrated that blocking PARP activation, caspase activation, or oxidant production confers protection against CEES exposure.

Figure 3. CEES-induced Caspase 3 activation in myeloid cells.

Of the various pathway inhibitor uses, Nicotinamide, PARP iRNA, Pan Caspase inhibitors, or NAC, only NAC consistently provided greater than 60% protective effect against CEES-induced apoptosis.

In addition to direct tissue damage by CEES, previous studies have demonstrated that CEES initiates the inflammatory immune response. It is lose of immune regulation in exposed tissue which is responsible for the indirect tissue damage. Our previous findings revealed that CEES (400 μM) suppressed the ability of primary myeloid cells to secret IL-1β, IL-10, IL-12, and TNFα cytokines, but increased the levels of only nitric oxide. Additional studies using flow cytometric analysis confirmed our previous finding but also revealed increases in two additional cytokines, IL-8 (8,272 pg/ml) and IL-6 (435 pg/ml). When CEES-exposed samples were treated with NAC, IL-8 and IL-6 cytokine levels decreased significantly to 78 pg/ml and 0 pg/ml respectively, and resembled levels similar to the untreated or samples (Figure 4). Similar results were found with samples treated with various caspase inhibitors.
Figure 5. N-acetylcysteine prevents release of IL-6 and IL-8 cytokines.

These findings are in contrast to those generated from previous experiments using 1mM concentrations of CEES, which demonstrated inhibition of all of the inflammatory cytokines except nitric oxide. Collectively, these findings further demonstrate that the antioxidant properties of NAC confer protection against apoptosis induced by CEES.

**Key Research Accomplishments**

**CEES induce myeloid cell apoptosis**

Our studies demonstrate that CEES induced apoptosis in LMC involves direct DNA damage by CEES and the elevation of oxidants in the LMC mitochondria. The inability of the LMC to manage this increase in oxidants is responsible for initiation of cellular apoptosis via cytochrome c release and caspase 9 and 3 activation. By determining the pathway which CEES-induces cell death will allow the development of new therapeutic countermeasure for our troops in the field.

**Development of a Gene-based Toxicology Panel**

The experimental data generated from analysis of CEES-exposed LMC has allowed the development of gene-based toxicology panel. Next, we will construct DNA microarrays, which will be used to evaluate gene expression in various tissues exposed to CEES.

**Reportable Outcomes**

**Abstracts**

Manuscripts


2. The ¹CBR Institute for Biomedical Research, Boston, Massachusetts, ²Amaox Ltd Pawpaw, Michigan, and ³Meharry Medical School, Nashville Tennessee

Collaborative Arrangements

1. Dr. Milton G. Smith: direct interaction involving experimental design and direction; in addition to weekly journal club and research article review sessions.

2. Dr. Peter Ward: information provided regarding effects of CEES on myeloid and myeloid-derived cells.

3. Dr William Stone: direct interaction involving the appropriate CEES and NAC concentration involving comparison of primary myeloid cells vs. monocyte/macrophage cell-line.

4. Dr. Salil Das: direct interaction involving comparison of pre and post CEES-exposed lung specimens.

5. Dana Anderson: direct interaction involving the development of CEES toxicology cDNA microarray.

6. Harvard Medical Genomics Core

Conclusions

The cellular pathways involved in CEES-induced cytopathology and the subsequent LMC death are poorly understood. By improving our understanding of these pathways, we can further develop countermeasures that better protect our soldiers. We have demonstrated that CEES initiates cellular apoptosis by altering the primary structure of DNA; thus, activating the PAPR-mediated repair process. Since DNA repair requires adequate levels of NAD+ for efficient PARP function while the mitochondria requires NAD+ for efficient ATP production, any cellular insult, which disrupts this NAD+ homeostasis and causes a shift in NAD+ utilization from the ATP production to PARP mediated DNA repair will initiate cellular apoptosis. This decrease in NAD+ availability and the increase in mitochondrial oxidant load are principal factors responsible for CEES induced cytopathology. Our research demonstrates that direct DNA damage, increases oxidant load, and caspase activation are the principal mechanisms involved in CEES-
induced apoptosis. Furthermore, we demonstrate that interference of these pathways with NAC, nicotinamide, or caspase inhibitors confers a level of protection from CEES exposure. However, only NAC provided the consistent level of protection needed for potential medical countermeasure.

References

N/A

Primary Investigation References

1. Dr. Peter A. Ward, University of Michigan Medical School
2. Dr. Milton Smith, Amaox, Ltd., Paw Paw, Michigan
3. Dr. William Stone, East Tennessee State University
4. Dr. Gerd O. Till, University of Michigan Medical School
5. Dr. Salil Das, Meharry Medical College.
SECTION 3: William L. Stone, Ph.D., East Tennessee State University

Inflammatory Amplification of Mustard Gas Toxicity

Abstract

Mustard gas toxicity is associated with increased generation of damaging free radical production. Antioxidant liposomes may provide a unique therapeutic strategy for mustard gas exposure. The long-term goals of this two-year project are to further define the mode of action of mustard gas and to develop a therapy using liposomes containing both lipid and water soluble antioxidants. The bacterial endotoxin, lipopolysaccharide (LPS), is an inflammatory factor found in the cell wall of Gram-negative bacteria. Very low levels of LPS (10 ng/ml) dramatically enhance the toxicity of sulfur mustard analog 2-chloroethyl ethyl sulfide (CEES or ClCH2CH2SCH2CH3). The specific aims of this proposal are to determine: (1) if the inflammatory amplification of CEES cytotoxicity caused by LPS occurs by necrosis or apoptosis; (2) if oxidative stress is associated with inflammatory amplification of CEES cytotoxicity; (3) the molecular mechanisms whereby inflammatory agents amplify the toxicity of CEES; (4) if antioxidant-liposomes can prevent CEES toxicity. We found that LPS dramatically enhances apoptosis in CEES treated cells; an effect associated with loss of intracellular glutathione (GSH). Treating cells with N-acetyl cysteine has been shown to prevent GSH loss and decrease CEES toxicity. The antibiotic, polymyxin B was found to inhibit LPS enhanced CEES toxicity and this may have direct clinical application in minimizing mustard gas toxicity.
Introduction

Considerable evidence suggests that mustard gas toxicity is associated with an increased generation of damaging free radical production (1-4). Antioxidant liposomes may provide a unique therapeutic strategy for mustard gas exposure because: (a) the antioxidants are nontoxic and could, therefore, be used at the earliest stages of toxicity; (b) liposomes themselves are composed of nontoxic, biodegradable and reusable phospholipids; (c) liposomes are preferentially taken up by the reticuloendothelial system which is an early target of mustard gas toxicity; (d) chemical antioxidants are relatively inexpensive and a wide range of nontoxic commercial antioxidants are available. The long term goals of this two year project are to further define the mode of action of mustard gas and to develop a therapy using liposomes containing both lipid and water soluble antioxidants. These goals are consistent with the USAMRMC Medical Chemical Defense Research Program interests in the area of defense against chemical agents.

The bacterial endotoxin, lipopolysaccharide (LPS), is a well-characterized inflammatory factor found in the cell wall of Gram-negative bacteria. We have found that very low levels of LPS (10 ng/ml) dramatically enhances the toxicity of 2-chloroethyl ethyl sulfide (CEES or CICH2CH2SCH2CH3). CEES is a sulfur vesicating agent and is an analog of 2, 2'-dichlorodiethyl sulfide (sulfur mustard).

The specific aims of this proposal are to determine: (1) if the inflammatory amplification of CEES cytotoxicity caused by LPS occurs by necrosis or apoptosis; (2) if oxidative stress is associated with inflammatory amplification of CEES cytotoxicity; (3) the molecular mechanisms whereby inflammatory agents amplify the toxicity of CEES; (4) if antioxidant-liposomes can prevent CEES toxicity.
The most important accomplishments for each specific aim are described below.

**Specific Aim 1:** (1) determine if the inflammatory amplification of CEES cytotoxicity caused by LPS occurs by necrosis or apoptosis.

![Caspase 3 Activity after Exposure to LPS (50 ng/ml) and CEES for 18 Hours (Normalized to Viability)](image)

Apoptosis in RAW264.7 macrophages was determined by measuring the activity of caspase 3. Members of the caspase (CED-3/ICE) family of proteases are key mediators of the complex biochemical events associated with apoptosis. In particular, the activation of caspase 3 is important for the initiation of apoptosis.

Figure-1 shows the activity of caspase 3 normalized to number of live RAW264.7 macrophages (measured by the MTT assay). These data show that CEES alone is able to induce apoptosis in a dose dependent manner. In the presence of LPS (50 ng/ml), however, we noted a dramatic enhancement of apoptosis. For example, at 750 µM the caspase 3 activity was increased about 15-fold in the presence of LPS. LPS by itself did not induce significant apoptosis but staurosporine, the positive control, did show enhanced apoptosis as expected. In summary, apoptosis is clearly a key mechanism for CEES induced cell death and LPS enhances the apoptotic process.
**Specific Aim 2**: Determine if oxidative stress is associated with inflammatory amplification of CEES cytotoxicity.

Figure 2 shows that glutathione (GSH), a key intracellular antioxidant, is somewhat reduced in macrophages treated with 500 μM CEES alone but that the combination of both LPS and CEES dramatically reduces intracellular GSH. Moreover, the addition of N-acetyl cysteine (NAC) is effective at preventing the loss of GSH induced by LPS+CEES. These data show that oxidative stress (as measured by GSH loss) is, indeed, associated with LPS enhanced CEES cytotoxicity.
Specific Aim 3: The molecular mechanisms whereby inflammatory agents amplify the toxicity of CEES.

These studies are still in progress but we have initiated experiments with polymyxin B, a cationic cyclic lipopeptide antibiotic which specifically binds to the lipid A toxophore of LPS. LPS is known to activate transcription factor NFkappaB which then induces the expression of inducible nitric oxide synthase (iNOS). Figure 3 demonstrates that polymyxin B can block the expression of iNOS induced by 10 ng/ml LPS. Figure 3 also shows that neither polymyxin B (at levels of 10 μg/ml or 20 μg/ml) nor CEES induced nitric oxide (NO) production. However, CEES did inhibit NO production in LPS stimulated macrophages. These data show that polymyxin B effectively blocks the action of LPS.
We then wanted to determine if polymyxin B could prevent LPS enhancement of CEES cytotoxicity. Figure 4 shows that polymyxin B (at levels of 10 µg/ml or 20 µg/ml) had only a small effect on cell viability as measured by the MTT assay. Significantly, polymyxin B protected against the enhanced cell death induced by LPS (10 ng/ml) in the presence of 500 µM CEES. Although polymyxin B did not fully protect cells, it did increase survivability by a factor of 6 which could be of clinical significance. It was concluded that LPS enhancement of cell death reflects interactions of the macrophage membrane with the lipid A region of LPS.

**Specific Aim 4:** Determine if antioxidant-liposomes can prevent CEES toxicity.

Liposomes (large unilamellar vesicles or LUVs) were prepared in RPMI-1640 medium by sequential extrusion using an Avanti Mini-Extruder with a 0.1 µm polycarbonate membrane. The liposomes contained glutathione (GSH) or alpha-tocopherol (alpha-TOH). The GSH liposomes contained 95% egg phosphatidyl choline, 5% cholesterol (mol%) and the alpha-tocopherol liposomes contained 90% egg phosphatidyl choline, 5% cholesterol and 5% alpha-tocopherol.

As shown in Figure 5, we found that liposomes encapsulated with 5 mM (but not 1 mM) glutathione (GSH) were effective at preventing CEES cytotoxicity to LPS-stimulate macrophages as measured by the MTT assay.
Figure 6 shows alpha-tocopherol containing liposomes (at a concentration of either 13.5 μM or 27 μM alpha-tocopherol in the culture medium) are also effective in preventing CEES toxicity to stimulated macrophages.

**Key Research Accomplishments**

- Apoptosis plays a key role in CEES induced cell death and LPS dramatically enhances this effect
• CEES inhibits nitric oxide production in stimulated macrophages
• Polymyxin B effectively blocks LPS enhancement of CEES cytotoxicity.
• Oxidative stress is associated with LPS amplification of CEES cytotoxicity and the antioxidant N-acetyl cysteine blocks this effect.
• Antioxidant liposomes are effective in preventing CEES toxicity in the presence of LPS.

**Reportable Outcomes**

**Abstracts**


**Manuscripts**


2. Hongsong, Y., Qui, M., Smith, M. and Stone, W.L: Inhibition of Inducible Nitric Oxide Synthase by a Mustard Gas Analog, BMC Cell Biology, to be submitted.

**Collaborative Arrangements** (within Mustard Gas Consortium)

• Direct interactions with Dr. Peter Ward (University of Michigan Medical School) and Dr. Salil Das (Meharry Medical College) to advise on the most protective forms of anti-oxidant-containing liposomes, based on *in vitro* studies using cell lines exposed to CEES.
• Patterns in gene expression in cells after CEES/LPS exposure in collaboration with Dr. Keith Crawford (Brigham and Women’s Hospital, Boston, MA).
• Dr. Milton G. Smith (Amaox) consulted in the formulation of the studies.

**Conclusions**

The *in vitro* results reported here and elsewhere support a role for inflammatory cytokines in the mechanism and kinetics of CEES toxicity. LPS is ubiquitous and is present in serum, tap water, and dust. Military and civilian personnel would always have some degree of exposure to environmental LPS, which could increase the toxicity of sulfur mustard. Our mechanistic studies show that LPS dramatically enhances apoptosis in CEES treated cells and this effect is associated with a loss of intracellular glutathione. Treating cells with NAC has been shown to prevent the loss of glutathione and to decrease CEES toxicity. The antibiotic, polymyxin B was found to inhibit LPS enhanced CEES toxicity and this may have direct clinical application in minimizing mustard gas toxicity. The data in Figures 5 and 6 indicate that antioxidant liposomes are effective in preventing CEES toxicity to macrophages.
References


Can Antioxidant Liposomes Protect Lungs from Deleterious Effects of Mustard Gas Exposure

Abstract

The exact mechanism by which mustard gas exposure causes lung injury including ARDS is not well known. Since human volunteers cannot be used for this type of study, we have developed a guinea pig model. The results clearly demonstrated the involvement of superoxide dismutase in the mustard gas (CEES) mediated lung injury. CEES-induced acute lung injury occurs, at least in part, by decreasing the activity of extracellular superoxide dismutase (SOD3), the predominant SOD in lung. It is also interesting that there was a differential effect of CEES exposure on the expression of SOD1 between lung and liver. Expression of SOD1 was significantly increased in lung whereas it was decreased in liver. SOD1 is the predominant form of SOD in liver and a minor form in lung. CEES-induced lung injury is associated with a decrease in the binding capacity of β-adrenergic receptors in lung. Thus, ARDS induced by mustard gas exposure may be mediated by hypofunction of β-adrenergic receptors in lung. Furthermore, CEES exposure induces apoptosis of cells in the lung and that at 4 and 6 hours there is evidence of endothelial cell damage with extensive blebbing. At 7, 14 and 21 days, there is clear evidence of interstitial fibrosis. Therefore, long-term exposure of CEES potentially may lead to pulmonary adenoma. This guinea pig model holds promise for development of drugs to combat lung cancer.
Introduction

Mustard gas exposure causes pulmonary complications, including Adult Respiratory Distress Syndrome (ARDS) (1). The exact mechanism is not well understood. However, many inflammatory diseases including ARDS are associated with free radical accumulation in lung (2). Recently, mustard gas exposure to human monocytic cell line has been shown to activate alpha-human tumor necrosis factor (TNF-alpha) (3). Since TNF is known to play a role in the generation of free radicals (4), it is possible that mustard gas-induced ARDS is associated with the induction of TNF-alpha and accumulation of free radicals. TNF activity has been suggested to be associated with a 'Trypsin-like' serine protease involved in cell lysis (3). Furthermore, it is known that TNF-alpha activates sphingomyelinase causing accumulation of ceramides which can result in cell cycle arrest (5). It has been demonstrated that free radicals cause desensitization of beta-receptors (6). Since Beta-adrenergic receptors play a major role in the secretion of surfactant by the alveolar type II cells (7), it is important to elaborate whether ARDS development due to mustard gas exposure is associated with free radicals induced hypofunction of beta-adrenergic receptors. It is also important that we know whether mustard gas exposure damages the alveolar type II cells which are the primary cell types involved in the synthesis, storage and secretion of pulmonary surfactant. The purpose of the current study is to better understand the nature of lung damage and associated free radicals induced hypofunction of beta-adrenergic receptors in guinea pig lung following exposure to chloroethyl ethyl sulfide (CEES).

Body

Study 1. Modulation of the Expression of Superoxide Dismutase Gene in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide (CEES), A Mustard Analog
Mustard gas exposure causes inflammatory lung diseases. Many inflammatory lung diseases are associated with oxidative stress. We have recently reported that mustard gas exposure decreases the overall activity of superoxide dismutase (SOD). In the present study, we investigated the effects on each of the three isozymes [SOD-1 (Cu/Zn), SOD-2 (Mn) and SOD-3 (extracellular)]. Adult guinea pigs were intratracheally injected single doses of CEES (4 mg/kg body weight) in ethanol. Control animals were injected with ethanol in the same way. The animals were sacrificed after 7 days and lungs were removed after perfusion with physiological saline. Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue (Fig. 1).

Mustard gas exposure causes a significant increase in the activity of SOD-1 and SOD-2 (Fig. 2). However, the SOD-3 activity which is the predominant type in lung was significantly decreased (Fig.2).
Northern blot analysis indicated 5-fold increased expression of SOD-1 in mustard gas-exposed lung (Fig.3). However, at the same time, mustard gas exposure caused a 2.8-fold decrease in the expression of SOD-1 in liver (Fig. 3). The modulation of the expression of SOD-1 gene by mustard gas exposure did not cause any mutation. We are currently investigating the effects of mustard gas on the expression of the other two forms of SOD.

**Fig. 3. Differential Effect of Mustard Gas on SOD-1 Gene Expression in Lung and Liver**

![Image of gel with bands labeled SOD-1 and GAPDH]

Lane 1: Control lung
Lane 2: Experimental lung
Lane 3: Control liver
Lane 4: Experimental liver

**Study 2.** Desensitization of β-Adrenergic Receptors in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide (CEES), A Mustard Analog

Mustard gas exposure causes inflammatory lung diseases, including Adult Respiratory Distress Syndrome (ARDS). Many inflammatory lung diseases are associated with oxidative stress. Oxidative stress has been implicated in the desensitization of beta-adrenergic receptors (β-ARs) which play a major role in the secretion of surfactant by the alveolar type II cells. A defect in the β-ARs system has been thought to be a contributing factor in the development of respiratory diseases. The objective of this study was to investigate whether lung injury induced by mustard gas exposure causes desensitization of β-ARs. Adult guinea pigs were intratracheally injected single doses of CEES (4 mg/kg body weight) in ethanol. Control animals were injected with ethanol in the same way. The animals were sacrificed after 7 days and lungs were removed after perfusion with physiological saline. Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue (Fig. 1). Receptor binding characteristics were determined by
<table>
<thead>
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<th>Samples</th>
<th>High Affinity</th>
<th>Low Affinity</th>
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<tbody>
<tr>
<td></td>
<td>Kd (nM)</td>
<td>B&lt;sub&gt;max&lt;/sub&gt; (fmol/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>0.68 ± 0.17</td>
<td>221.2 ± 56.9</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.62 ± 0.15</td>
<td>203.8 ± 69.0</td>
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*B<sub>max</sub> at low affinity sites is significantly reduced by mustard gas exposure (p = 0.03) (n = 3 for each group)

Table 1. Effects of mustard gas exposure on binding characteristics of β-adrenoreceptors in Lung

measuring the binding of [³⁵H]-dihydroalprenolol (0.5-24 nM) to membrane fraction in the presence and absence of DL-propranolol (10 μM). Both high (0.6-0.7 nM Kd) and low affinity (2.9-6.4 nM Kd) β-ARs were identified in lung. Binding capacity was significantly higher in low affinity site in both control and experimental groups. Mustard gas exposure caused a significant decrease in B<sub>max</sub> at the low affinity site (Table 1). However, there was no mutation of the β-ARs gene due to mustard gas exposure. We are currently investigating whether the decrease in B<sub>max</sub> is associated with transcriptional and/or translational inhibition of the β-ARs gene.

**Study 3.** Assessment of Light and Transmission Electron Microscopy Structural Changes in Lungs Exposed to CEES.

This study is currently being collaborated with Dr. Peter Ward (University of Michigan School of Medicine). Based upon what we have seen so far from the TEM’s we conclude that there is evidence of apoptosis of cells in the lung and that at 4 and 6 hours there is evidence of endothelial cell damage with extensive blebbing. At 7, 14 and 21 days, there is clear evidence of interstitial fibrosis. It should be noted that in all TEM sections both from control and CEES exposed lungs, the type II alveolar epithelial cells have lost their lamellar bodies. Most probably this is a processing artifact. We are in the process of further evaluating guinea pig lungs exposed to CEES in the presence and absence of N-acetyl cysteine.

**Key Research Accomplishments**

- CEES-induced acute lung injury occurs, at least in part, by decreasing the activity of extracellular superoxide dismutase (SOD3), the predominant SOD in lung. Northern blot analysis indicated 5-fold increased expression of SOD-1 in CEES-exposed lung.
However, at the same time, CEES exposure caused a 2.8-fold decrease in the expression of SOD-1 in liver. It is to be noted that SOD1 is the predominant SOD in liver. Thus, there was a differential expression of SOD1 between lung and liver.

- CEES-induced lung injury is associated with a decrease in the binding capacity of β-adrenergic receptors in lung. Thus, ARDS induced by mustard gas exposure may be mediated by hypofunction of β-adrenergic receptors in lung.

- Based upon what we have seen so far from the TEM's, we conclude that CEES exposure induces apoptosis of cells in the lung and that at 4 and 6 hours there is evidence of endothelial cell damage with extensive blebbing. At 7, 14 and 21 days, there is clear evidence of interstitial fibrosis.

**Reportable Outcomes**

**Abstracts**


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**Collaborative Arrangements** (within Mustard Gas Consortium)

- Direct interactions with Dr. Peter Ward (University of Michigan) to assess by light and transmission electron microscopy structural changes in lungs exposed to CEES.

- Direct interactions with Dr. William Stone (East Tennessee State University) to determine the most protective forms of anti-oxidant liposomes, based on Dr. Stone's *in vitro* studies using cell lines exposed to CEES.

- Direct interactions with Dr. Keith Crawford (Brigham and Women’s Hospital, Boston, <A>) to assess patterns of gene expression in lungs of guinea pigs after CEES exposure.

- Direct interactions with Dr. Milton G. Smith (Amaox) as a consultant in the formulation of the studies.

**Conclusions**

The results presented here clearly demonstrated the involvement of superoxide dismutase in the mustard gas mediated lung injury. CEES-induced acute lung injury occurs, at least in part, by decreasing the activity of extracellular superoxide dismutase (SOD3), the predominant SOD in lung. CEES-induced lung injury is associated with a decrease in the binding capacity of β-adrenergic receptors in lung. Thus, ARDS induced by mustard gas exposure may be mediated by hypofunction of β-adrenergic receptors in lung. Furthermore, CEES exposure induces apoptosis of cells in the lung and that at 4 and 6 hours there is evidence of endothelial cell damage with extensive blebbing. At 7, 14 and 21 days, there is clear evidence of interstitial fibrosis. Therefore, long-term exposure of CEES potentially may lead to pulmonary adenoma. This guinea pig model holds promise for development of drugs to combat lung cancer.

**References**


Section 5: Peter A. Ward, M.D., University of Michigan Medical School

Protective Effects of Anti-Oxidant Liposomes in CEES-induced Acute Lung Injury

Abstract

As part of the Mustard Gas Consortium, these studies deal with the induction and prevention of acute lung injury following airway instillation of chloroethyl ethyl sulfide (CEES). The induction of injury is linked to triggering of an anti-inflammatory response, since depletion of either complement or neutrophils reduces the intensity of injury. Preliminary data also reveal that liposomes containing either anti-oxidant enzymes (superoxide dismutase or catalase) or reducing agents (glutathione, N-acetyl cysteine) confer high degrees of protection. Interventions with anti-oxidant liposomes are even protective when airway delivery of liposomes is delayed after CEES instillation. The current studies will define which liposomes are most protective and the mechanisms of the protective effects. These studies will involve collaboration with all other members of the Consortium.
**Introduction**

The purpose of the current studies is to better understand the nature of lung damage and altered innate immune responses of rodent (rat) lungs following exposure to chloroethyl ethyl sulfide (CEES). We are also interested in the ability of liposomes containing anti-oxidants to protect lungs from CEES-induced acute injury. There is a special emphasis to pursue evidence that delayed administration of anti-oxidant liposomes into lung after exposure to CEES still affords significantly protective effects.

**Body**

The most important accomplishments are described in the attached graphs and text (see below).

1. **Complement depletion** (induced by infusion of purified cobra venom factor) or **neutrophil depletion** (induced by infusion of monoclonal antibody to rat neutrophils) prior to airway administration of CEES results in reduced acute lung injury by 43% and 62%, respectively. Injury was measured by the leakage of $^{131}$I-albumin from blood into the interstitial and alveolar compartments of lung and was expressed as a ratio of radioactivity in lung to that present in blood (the permeability index) (1). These findings indicate that CEES induces injury in lung by activating components of the inflammatory system. In other words, the inflammatory response is triggered by CEES, and products of the inflammatory system contribute to the developing acute lung injury.

![Figure 1](image-url)  
**Figure 1.** Protective effects of complement depletion and neutrophil depletion in acute lung injury of rats induced by CEES.

2. **Antioxidant containing liposomes are protective against CES-induced acute lung injury in rats**. As summarized in Figure 2, liposomes were loaded with polyethylene glycol-catalase (PEG-CAT), PEG-superoxide dismutase (PEG-SOD), the combination (PEG-SOD/PEG-CAT) or the combination in conjunction with complement depletion (following infusion of purified cobra venom factor). Liposomes were given intratracheally immediately after airway administration of CEES. When compared to the permeability index (lung injury) values in the positive controls, which received unloaded liposomes (LIP), the lung injury values of animals
receiving loaded liposomes were reduced by 40%, 57%, 71%, and 82%, respectively. These data indicate that the use of liposomes containing anti-oxidant enzymes results in very substantial protection against CEES-induced lung injury in rats. Because it is known that liposomes are taken up into lung macrophages (2), these data suggest that maintaining strong anti-oxidant defenses within macrophages is key to the protective effects of anti-oxidant enzymes.

![Figure 2. Protective effects of liposomes containing anti-oxidant enzymes in CEES-induced acute lung injury.](image)

3. Delayed administration of the anti-oxidant liposomes still conveys substantial protective effects against CEES-induced lung injury. As shown in Figure 3, liposomes containing the combination of glutathione (GSH) and N-acetylcysteine (NAC) were remarkably protective against CEES-induced acute lung injury, even when administration of liposomes via the airways was substantially delayed. As shown in Figure 3, administration 10 minutes before CEES or thereafter (at 30, 60, or 90 min.) conveyed remarkable protection as determined by albumin leak into lung. The degrees of protection were 62%, 57%, 55% and 38%, respectively (p values being <0.05 in each case when compared to the unprotected positive controls).

![Figure 3. Ability of liposomes containing GSH and NAC to protect against CEES-induced lung injury even after delayed airway administration. The open circle indicates delivery of LipNAC/GSH into normal lung.](image)
Key Research Accomplishments

- CEES-induced acute lung injury occurs, at least in part, by engaging inflammatory mediator systems (complement and neutrophils).
- Liposomes containing anti-oxidants confer substantial protection from CEES-induced acute lung injury.
- Delayed airway delivery of anti-oxidant liposomes after CEES exposure provides substantial protective effects against acute lung injury.

Reportable Outcomes

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Collaborative Arrangements(within Mustard Gas Consortium)

- Direct interactions with Dr. William Stone (E. Tenn. State University) to determine the most protective forms of anti-oxidant-containing liposomes, based on Dr. Stone’s in vitro studies using cell lines exposed to CEES.
- Direct interactions with Dr. Salil Das (Meharry University School of Medicine) to assess by light and transmission electron microscopy structural changes in lungs exposed to CEES.
- Patterns in gene expression in lungs of rodents after CEES exposure in collaboration with Dr. Keith Crawford (Brigham and Women’s Hospital, Boston, MA).
Conclusions
Although the data are not complete, we have determined (as described above) that exposure of rat lungs to CEES results in acute lung injury that is related to triggering of an inflammatory response and after a flood of oxidants in lung. These conclusions draw from the fact that complement blockade or neutrophil depletion is protective. Furthermore, anti-oxidant-containing liposomes were highly protective, implying that lung exposure to CEES results in oxidant generation causing derangement in the redox balance in lung. Very importantly, delayed administration of liposomes containing anti-oxidants after lung exposure to CEES still conferred substantial protection. These studies suggest that anti-oxidants and anti-oxidant liposomes may be useful therapeutically to protect lung from acute injury following exposure to products of mustard gas.

References

Abstracts


Manuscripts


Primary Investigator References

1. Dr. William Stone, East Tennessee State University
2. Dr. Salil Das, Meherry University
3. Dr. Keith Crawford, Brigham and Women’s Hospital, Boston, Massachusetts
4. Dr. Peter A. Ward, University of Michigan Medical School
5. Dr. Milton G. Smith, Amaox, Ltd., Paw Paw, Michigan
6. Dr. Gerd O. Till, University of Michigan Medical School