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TITLE: 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

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14. ABSTRACT

The Mustards Consortium has utilized both in vivo and in vitro models simultaneously to continue to elucidate mustard gas pathophysiology. In previous work done by the MC it was found that CEES, the mustard analogue, induced oxidative stress and was its primary mechanism of action. Consequently, NAC (N-acetyl cystiene) was found to be protective as a prophylaxis and treatment. A combination of a water and fat soluble antioxidant encapsulated in a liposome (STIMAL) was found to have the best ameliorative effect against CEES. We have initiated development of next generation STIMAL, in order to optimize its ameliorative effect. The mechanism of action of the antioxidants is suspected to be primarily by their effect on redox regulated pathways. In an effort to elucidate the mechanism of action of the antioxidants and the pathophysiology of mustards, profiles are being developed for: gene expression and antioxidant levels, as well as biochemical pathways. The first known histological comparison between CEES and sulfur mustard was carried out. Two new rat lung models were developed for the administration of sulfur mustards in preparation for efficacy testing of STIMAL against sulfur mustards. Pulmonary fibrosis was demonstrated in both guinea pig and rat lung models.

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

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

Introduction and Body

An overview of the Mustard Consortium work 2004 -year 1 of 2

See the Mind Map for graphical overview of the results of this phase of the work (appendix).

Two major problems are faced by the threat of the use of mustard gas on the battlefield: 1) the absence of an antidote; and 2) the inability to detect sulfur mustard at subclinical levels.

One of the primary organs affected by exposure are the lungs. Known complications are: ARDS, pulmonary fibrosis, stenosis of the bronchial tree. The absence of an ameliorative agent for mustards is compounded by the inability to diagnose exposure in the person who is asymptomatic or showing minimal symptoms. Subclinical exposures may have long term complications (e.g. Gulf War syndrome (?)). Tests have been developed for detection of SM specifically, but none exists for testing for multitude of agents rapidly. In this new body of work we are providing the foundation for a better understanding of the pathophysiology sulfur mustard and its analogue CEES (2-chloroethyl ethyl sulfur); as well as potential treatment and diagnostic capability.

Antioxidants have been found to be an ameliorative agent for CEES, the mustard analogue. Liposome encapsulation of the antioxidants (STIMAL) significantly enhances the ameliorative effect.

In this phase of the research we have begun preparation for efficacy testing of STIMAL in the animal models with sulfur mustards. Dr. Sciuto has developed an aerosol inhalation model, which simulates the inhalation of SM under physiological conditions (similar to what would occur on the battle field).

The LCt10 at 1250ug of SM was determined. An LCt50 has not been achieved as yet. Protein levels increased 4 fold at 0.5 to 24 h at the 1250 ug dose, suggesting a breach of the air/blood barrier. Unforeseen difficulties have arisen which will impede the progress of the work for one or more months.

Mr. Anderson has used the experimental design that was developed by Dr. Ward, which is the instillation of the agent into the deep lung. In the Anderson studies it was determined the SM was equivalent to the CEES dosage (approximately 1:6 ratio) that was used by Dr. Ward in the rat lung model. This information will increase the relevancy of the work done by civilian investigators that are restricted to the use of CEES. Rat spleen, kidney, and liver that had been exposed to SM (in vivo) were exported to Doctors Stone and Crawford.

Dr. Stone's lab will serves as a core facility for the consortium that will perform analysis of oxidative stress reactions in tissues. Glutathione levels were assayed in tissues exported from

Mr. Anderson's laboratory. In the CEES exposed organs GSH was decreased. In contrast, the SM exposure resulted in a decrease in the splenic GSH, but essentially no change in the liver and kidney. The decreased GSH levels would imply that oxidative stress is occurring in these tissues. There was essentially no change in the tocopherol levels. These early results would imply that CEES produces systemic oxidative stress at a greater level than does SM.

Dr. Stone is in the process of optimizing a several formulations of STIMAL. Out of the several formulations that are developed, a few will be tested in the efficacy studies that will be performed by Dr. Sciuto and Mr. Anderson. Dr. Stone has determined that polymyxin B (an antibiotic) can inhibit the exacerbating effects LPS in macrophages exposed to CEES. This finding may be significant in the treatment of skin that is exposed to SM. It is unknown if polymyxin B will have the same inhibitory effect on SM that it has shown for CEES.

Dr. Ward determined in the rat lung model, that the bronchoalveolar lavage fluid (BAL) contained inflammatory mediators that increased after CEES administration. IL- 2 β peaked at 2 hours; whereas TNF- α, MP-2, CINC-1 peaked at 4 hours. Liver enzyme release, an indicator of injury, peaked at 4-6 hours; there was essentially no change in kidney function. Selected genes associated with apoptosis (BAX, Egr1, Hspb1, HSP90-Rik, Nos2, Ccl2) were increased at 4 hours. Pulmonary fibrosis, a known complication of SM, was examined in this phase of the study. It was found that lung collagen levels are increased within 3-4 hours of CEES exposure. The question of whether CEES can compromise the innate immunity of the lung has also begun to be examined.

Dr. Das has continued to elucidate a biochemical pathway in the guinea pig lung model that may be one of the contributing factors to the complication of ARDS. Cholinephosphotransferase (CPT) gene expression and enzyme activity were decreased. There is an inverse correlation between ceramide production and CPT gene expression. The same inverse correlation exists for ceramide and the CPT enzyme activity. Selected gene expression was examined. Increases were note for IL-α, EOTAXIN, MP1 γ, IFN-γ, TNF-α, NFkB (Light), PDGF-BB, FGF7, IGFBP-I. Pulmonary fibrosis occurred at 7 days.

Dr. Crawford has developed one of the two core facilities (Dr. Stone being the other). His core is responsible for the genomic and proteomic analysis of samples sent from the other members of the group. The data from this analysis will be made available to the group members via a secure web site that has been developed. A toxic gene micro array for rat and mouse has been developed that will assist in the identification of gene expression for potentially several chemical weapons. A protocol is being developed for the isolation of mRNA from blood, spleen, and lung. It is anticipated that obtaining this base line data will result in a new robust diagnostic tool that could diagnose several chemical WMD.

(Net) Key Research Accomplishments

- Animal models to test the efficacy of STIMAL against sulfur mustard have been developed; two different methods of delivery of STIMAL will be utilized along with two different methods of sulfur mustard delivery.
- Development of the next generation of STIMAL.

- Sulfur mustard and CEES equivalent doses have been determined (about a 1:6 ratio); histological comparisons were done. Glutathione and tocopherol levels were assayed in kidney, lung and liver.
- Observation of pulmonary fibrosis in rat and guinea pig lung models.
- Observation of BAL inflammatory mediators.
- Gene expression in guinea pig and rat lung.
- Development of tox gene micro array and web access for group data.

Reportable Outcomes

- See the individual investigator reports.
- A Bioscience presentation was made on the role of oxidative stress in CEES pathophysiology.
- A Mustard Consortium meeting was held during the Bioscience meeting- 2004.
- Presentations were given by all of the members of the group that facilitated an understanding of the individual projects and how they fit it to the overall research effort.

Conclusions

The biological effects at the cell, organ and systemic levels, are being described for sulfur mustard (SM) as well as the protective effects of liposome encapsulated antioxidants (STIMAL). STIMAL is able to protect against the deleterious effects of SM on the cellular and organ redox balances, thereby attenuating the injurious and destructive biological impact of SM. Current studies also suggest that STIMAL can be protective even after lung exposure to SM.

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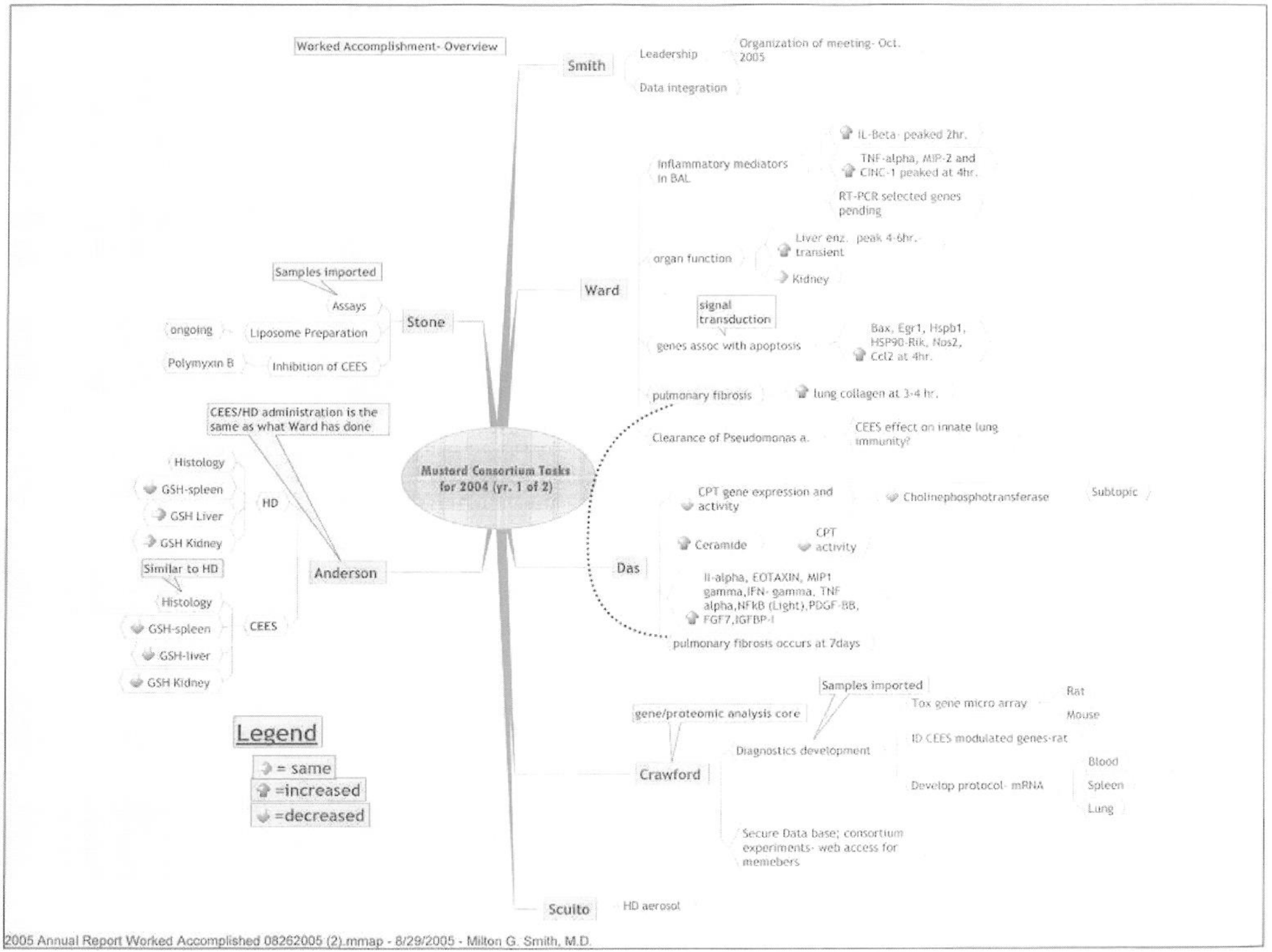
See individual reports

Appendices

Mind Map for graphical overview of the results of this phase of work.

APPENDIX

Milton G. Smith, M.D.


Worked Accomplishment- Overview
Mustard Consortium Tasks for 2004 (yr. 1 of 2)
Smith
Leadership
Organization of meeting- Oct. 2005
Data integration
Ward
Inflammatory mediators in BAL
IL-Beta- peaked 2hr.
TNF-alpha, MIP-2 and CINC-1 peaked at 4hr.
RT-PCR selected genes pending
organ function
Liver enz. peak 4-6hr. transient
Kidney
signal transduction
genes assoc with apoptosis
Bax, Egr1, Hspb1, HSP90-Rik, Nos2, Ccl2 at 4hr.
pulmonary fibrosis
lung collagen at 3-4 hr.
Clearance of Pseudomonas a.
CEES effect on innate lung immunity?
Stone
Samples imported
Assays
ongoing
Liposome Preparation
Polymyxin B
Inhibition of CEES
CEES/HD administration is the same as what Ward has done
Anderson
HD
Histology
GSH-spleen
GSH Liver
GSH Kidney
Similar to HD
CEES
Histology
GSH-spleen
GSH-liver
GSH Kidney
Das
CPT gene expression and activity
Cholinephosphotransferase
Subtopic
Ceramide
CPT activity
Il-alpha, EOTAXIN, MIP1 gamma,IFN- gamma, TNF alpha,NFkB (Light),PDGF-BB, FGF7,IGFBP-I
pulmonary fibrosis occurs at 7days
Crawford
gene/proteomic analysis core
Samples imported
Rat
Mouse
Tox gene micro array
ID CEES modulated genes-rat
Diagnostics development
Blood
Spleen
Lung
Develop protocol- mRNA
Secure Data base; consortium experiments- web access for memebers
Sculto
HD aerosol
Legend
= same
=increased
=decreased

SECTION 2: Peter A. Ward, M.D., University of Michigan Medical School

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

Abstract

Acute lung injury in rats following airway delivery of CEES is associated with loss of distal lung barrier function (resulting in alveolar flooring) and an intense inflammatory response, which is lung-damaging. These acute lung injury parameters are attenuated by neutrophil depletion or complement blockade. Injuring the redox balance in lung after exposure to CEES by administration into lung of liposomes containing antioxidant compounds is highly protective even when delivery of liposomes is delayed by at least 1 hr. CEES-induced lung injury is progressive, as manifested by development of interstitial fibrosis which seems to peak at three weeks. Whether STIMAL will attenuate development of lung fibrosis is currently unknown.

Introduction

As is well known, mustard gas [bis (2-chloroethyl ethyl) sulfide], also known as sulfur mustard (HD), has long been known to be a vesicant in humans and, when inhaled, causes extremely lung damaging reactions (1-3). In human survivors, progressive lung dysfunction due to pulmonary fibrosis is well documented (4). Not unexpectedly, HD is radiomimetic, teratogenic and mutagenic (5,6). Currently, there is no effective therapy for either the vesicant-inducing properties of HD or for the outcomes that can lead to acute and progressive lung injury and death.

2-chloroethyl ethyl sulfide (CEES) is less toxic than HD and can be used in the absence of facilities required for HD studies. In rats CEES has been shown to induce acute lung injury in a dose-dependent and time-dependent manner (7). CEES-induced acute lung injury is complement and neutrophil-dependent, suggesting that some of the CEES-induced injury is due to engagement of the inflammatory response in lung in an unknown manner (7). Furthermore, lung injury is attenuated after intravenous treatment with the anti-oxidant, N-acetylcysteine (NAC), or airway delivery of anti-oxidants or anti-oxidant enzymes (7). These data have suggested that CEES compromises the redox potential in lung, putting it at risk of oxidant-mediated injury.

Liposomal delivery of drugs or chemical compounds is a way to achieve high intracellular levels of a desired compound in tissue macrophages (8-10). In lung, airway delivery of liposomes results in macrophage uptake of liposomes by a phagocytic pathway (11-13). As far as is known, liposomes are not internalized by any other lung cells. Our recent studies suggest that intrapulmonary delivery of liposomes containing anti-oxidants are strongly protective of CEES-induced acute lung injury, even though little is currently known about how CEES produces acute and progressive lung injury.

Body

Experiments performed over the past year (Aug 04 – Aug 05) have provided evidence that depletion of the complement system as well as the intratracheal instillation of liposomes containing anti-oxidants or reducing agents, or liposomes containing the combination of both, results in greatly reduced lung injury. Histological analyses following airway instillation of CEES have revealed intra-alveolar hemorrhage, edema and intra-alveolar accumulates of

macrophages, neutrophils and mononuclear cells in lung by 6 hour post injury. Increased fibrin and collagen deposition in alveolar walls, as defined by trichrome staining of tissue sections, was seen as early as 24 hours after instillation and by 3 weeks resulted in dense deposits of fibrin and extensive confluent collagen deposits together with collapse of alveolar structures. This resulted in the histologic appearance of "honeycombing" of the lung, indicating lung fibrosis and alveolar collapse. A manuscript entitled "*Attenuation of Half Sulfur Mustard Gas–induced Acute Lung Injury in Rats*" has been accepted, pending revision, for publication in the *Journal of Applied Toxicology*.

Continuing studies involve four different areas:

1. Patterns of inflammatory mediators after CEES-induced lung injury. Bronchoalveolar lavage fluid (BALF) levels of pro-inflammatory cytokines/chemokines are assessed by ELISA. IL-1β was found to peak 2 hours after lung exposure to CEES, while TNFα, MIP-2 and CINC-1 levels peaked at 4 hours. A time course of lung inflammatory mediators involving superarray analysis is currently underway. RT-PCR validation of selected genes is also in progress.
2. Long-term effects (fibrosis) after CEES injury. Using biochemical assays (hydroxyproline content), lung collagen content is significantly increased 3 and 4 weeks after CEES exposure. This appears to mimic what happens in humans exposed to mustard gas. Possible beneficial effects of liposomes containing reducing agents (tocopherol and NAC) will be assessed in the CEES model, in conjunction with Dr. W. Smith (E. Tennessee State U.) and Dr. S. Das (Mehary Medical School).
3. Pulmonary clearance of *Pseudomonas aeruginosa* in CEES treated rats. It is not known if exposure to CEES compromises the ability of the lung to clear bacteria. In order to simulate a clinical situation (bacterial superinfection in the ICU), *P. aeruginosa* will be administered intratracheally at certain time points after CEES administration to induce acute bacterial pneumonia. Bacterial clearance will be assessed by content of colony forming units (CFU) in lung homogenates and in whole blood. Possible beneficial effects of liposomes containing reducing agents (NAC/GSH) will be determined.
4. Systemic effects of intratracheal application of CEES on organ function. Preliminary data suggest that liver enzymes (AST, ALT) peak in the serum 4 and 6 hours after lung exposure to CEES, with return to normal values within 48 hours, indicating transient liver damage after airway delivery of CEES. In contrast, renal parameters (CREA, BUN) were unchanged. Possible protective effects of airway instillation or intravenous injection of liposomes containing reducing agents (NAC/GSH) are in progress.

Key Research Accomplishments (Aug 04 – Aug 05)

- Ability of STIMAL (antioxidant liposomes) to greatly attenuate acute lung injury after CEES, even when liposomal administration is delayed following exposure to CEES.
- Appearance in BAL fluids of cytokines and chemokines (IL-1b, TNFa, MIP-2, CINC-1) after lung exposure to CEES.
- Evidence that lung exposure to CEES also includes acute liver injury.
- Biochemical evidence that lung exposure to CEES results in progressive pulmonary fibrosis based on histopathology and biochemical evidence.

Reportable Outcomes

1. "Systemic Effects of CEES (Half Sulfur Mustard Gas) after Intratracheal Instillation", L.M. Hoesel, A.D. Nielerbichler, S.D. McClintock, J.V. Sarma, P.A. Ward, presented at the SHOCK Society Meeting (San Marcos Island, Florida, June 05)
2. "Protective Effects of Anti-Oxidant Liposomes on Acute Lung Injury after CEES", presented by P.A. Ward at Bioscience 2004, Hunt Valley, Maryland May 18-20, 2004.
3. "Protective Effects of Antioxidant Liposomes in Lung Injury", poster presented by P.A. Ward and G.O. Till at the Mustard Gas Consortium, Hunt Valley, Maryland, May 19, 2004.
4. "Protective Lung Effects of STIMAL", presented by P.A. Ward to Congressional Staffers at Summit Meeting, October 18, 2004, Capital Building, Washington DC.
5. Discussion of STIMAL Strategy for Protection Against CEES in Induced Acute Lung Injury, by P.A. Ward and other consortium members in Plenary Session at Bioscience 2004, Hunt Valley, Maryland, May 19, 2004.
6. Attenuation of Half Sulfur Mustard Gas – Induced Acute Lung Injury in Rats, S.D. McClintock, L.M. Hoesel, S.K. Das, G.O. Till, T. Neff, R.G. Kunkel, M.G. Smith, P.A. Ward. 2005 Accepted, pending manuscript revision.

Conclusions

Administration of CEES into rat lung produces both acute and progressive lung injury. The former is characterized by an acute inflammatory response associated with a large lung leak (alveolar flooding with plasma components) and accumulation of neutrophils and mononuclear cells. These changes are associated with the appearance of chemokines and cytokines. Airway instillation of STIMAL (liposomes containing antioxidants) together with CEES results in greatly attenuated lung injury, even if STIMAL intervention is delayed for 1 hr after instillation of CEES. CEES also causes progressive lung injury (pulmonary fibrosis), although the causes for this are not known, and it is not known if STIMAL will prevent this complication.

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Appendices

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Protection from Half-mustard-gas-induced Acute Lung Injury in the Rat

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Key words: Half-mustard gas; lung injury; complement; neutrophils; antioxidants; *N*-acetyl-L-cysteine.

The chemical warfare agent analog, 2-chloroethyl ethyl sulfide, known as 'half-mustard gas' (HMG), is less toxic and less of an environmental hazard than the full molecule and has been shown to produce an acute lung injury in rats when instilled via intrapulmonary injection. This injury is characterized by massive, localized hemorrhage and edema into the alveolar compartment and can be quantitated by measuring extravasation of ^{125}I-bovine serum albumin into the extravascular compartment. Employing this rat model of HMG-induced lung injury, we observed significant attenuation of the pulmonary injury when experimental animals were complement or neutrophil depleted prior to HMG challenge. Significant protection also was provided by the use of antioxidants such as catalase, dimethyl sulfoxide, dimethyl thiourea, resveratrol and *N*-acetyl-L-cysteine (NAC). The last compound showed protection from lung injury as high as 70% and was still effective even when given up to 90 min after exposure of the lungs to HMG. These data suggest that acute lung injury caused by exposure to HMG may be related partially to complement mediated pathways and the generation by neutrophils of toxic oxygen species The data indicate that NAC is an effective antidote against HMG-induced acute lung injury in the rat. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Mustard gas [bis (2-chloroethyl) sulfide, also known as sulfur mustard (HD)] was first synthesized in the early to mid-1800s.[1] It is an oily liquid and is a lipophilic alkylating agent that, when absorbed, causes chemical reactions with cellular components, resulting in cytotoxic effects.[1–6] Mustard agents are most commonly described as vesicating or blistering agents, owing to the fact that the wounds most often seen in HD exposure resemble burns and blisters. Mustard agents can be described more accurately as vesicating and tissue-damaging agents because of the severe widespread damage to the lungs,[7,8] internal organs[9–12] and eyes,[13] as well as to the skin.[14,15] Mustard gas also has been described as radiomimetic, carcinogenic, teratogenic and mutagenic.[16–19] It has been used recently in chemical attacks against the Iranians during the Gulf War of 1984–1985 and by the Iraqis in 1988 against their own Kurdish population.[6,20] Unfortunately, there is no effective treatment for HD intoxication. Decontamination of HD immediately after contact is still the recommended treatment.[1,6]

In addition to skin, the lungs and respiratory tract are among the most commonly affected organs, with effects that can be both acute (owing to its vesicating action) and long term (including airway hyperreactivity, chronic bronchitis, asthma, bronchiectasis and pulmonary fibrosis).[17,18] Complications due to HD exposure also can include hemorrhagic inflammation, erosion and effects on the lung parenchyma.[17,18,21–23] There has been only a limited number of animal studies investigating HD-induced lung injury. Calvet *et al.*[21–23] have described the development of peribronchial edema and bronchoconstriction in guinea pigs, whereas Vijayaraghavan *et al.*[19,24] have studied the changes in breathing patterns caused by inhaled or percutaneous exposure to HD in mice and rats.

A rat lung injury model has been established in our laboratory to study the effectiveness of complement and neutrophil depletion as well as treatment with antioxidants in attenuating injury caused by intrapulmonary instillation of half-mustard gas (HMG). Based on our long-standing experience with experimental pulmonary injury models in the rat,[25–27] we have chosen this species to study the pathogenesis of HMG (2-chloroethyl ethyl sulfide)-induced acute lung injury. Half-mustard gas is the standard agent employed for studies in order to avoid the need for high-level containment facilities.

MATERIALS AND METHODS

Chemicals

Except where noted, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

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Animals

Adult male (275–325 g) specific-pathogen-free Long-Evans rats (Harlan Co., Indianapolis, IN) were used in these studies. Intraperitoneal ketamine (100 mg kg^{-1} body weight) (Fort Dodge Animal Health, Fort Dodge, IA) was used for continuous anesthesia and sedation (4 h). After induction of anesthesia, ^{125}I-labeled bovine serum albumin ([^{125}I]BSA, 0.5 μCi per rat) was injected intravenously as a quantitative marker for vascular permeability. The trachea then was surgically exposed and a slightly curved catheter was inserted into the trachea past the bifurcation to facilitate a unilateral, left-lung injury. A small volume of HMG (2 μl per rat) was solubilized in ethanol (58 μl per rat) and then added to a syringe containing Dulbecco's phosphate-buffered saline (DPBS) (340 μl per rat). This solution was injected via the intratracheal catheter, causing a unilateral lung injury. Studies not requiring the use of a radiolabeled marker proceeded identically, substituting DPBS for the radioactive injection. For all studies, except the time response experiment, animals were sacrificed 4 h later, the pulmonary circulation was flushed with 10 ml of cold DPBS, the lungs were surgically dissected and placed in counting vessels and the amount of radioactivity ([^{125}I]BSA) was determined by gamma counting. For calculations of the permeability index, the amount of radioactivity ([^{125}I]BSA) remaining in the lungs in which the vasculature was perfused with saline was divided by the amount of radioactivity present in 1.0 ml of blood obtained from the posterior vena cava at the time of sacrifice.

All animal experiments were in accordance with the standards in 'The Guide for the Care and Use of Laboratory Animals' and were supervised by veterinarians from the Unit for Laboratory and Animal Care of the University of Michigan Medical School.

Neutrophil depletion

Neutrophil depletion was induced by intraperitoneal injection of 1.0 ml of rabbit anti-serum to rat polymorphonuclear neutrophils PMNs (Accurate, Westbury, NY). Twenty-four hours later, peripheral venous blood was obtained from the tail vein and analyzed for total neutrophil counts. The antibody reduced the number of neutrophils in peripheral blood by >90%.[25]

Complement depletion

Cobra venom factor (CVF) was purified from crude, lyophilized cobra venom (*Naja atra*) by ion exchange chromatography.[28] Complement depletion of experimental animals was achieved by intraperitoneal injections of 25 units of CVF per rat at time zero and 24 h later, resulting in undetectable levels of serum hemolytic complement activity as confirmed by CH50 assay.[26] Experiments were performed 24 h after the second CVF injection.

Antioxidants

The dosing of the antioxidant compounds used in the present study has proved effective in other rat models of acute lung injury that were also dependent on blood neutrophils and complement.[25,29,30] The following antioxidant compounds were tested separately and given intraperitoneally 10 min prior to intrapulmonary instillation of HMG: dimethyl thiourea (DMTU) (1000 mg kg^{-1} body wt. in 1.0 ml of sterile DPBS), dimethyl sulfoxide (DMSO) (1.5 ml kg^{-1} body wt in 1.0 ml of sterile DPBS); catalase (2 50 000 units per rat in 2.0 ml of sterile DPBS); and resveratrol (50 mg kg^{-1} body wt in 0.5 ml of propylene glycol).

N-Acetyl-L-cysteine (NAC) was administered intravenously in varying concentrations and at different time points throughout the course of the HMG injury to establish both time and dose response data. The iron chelator—2,3-dihydroxybenzoic acid (100 mg kg^{-1} body wt in 0.5 ml of DPBS) or deferoxamine mesylate (15 mg kg^{-1} body wt in 0.5 ml of DPBS)—was injected intravenously 10 min prior to HMG instillation. As mentioned above, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

Results are presented as mean ± SEM in the text and figures. Groups ($n = 5$) were subjected to one-way analysis of variance and when significance was found Student's *t*-test with the Bonferroni correction for multiple comparisons was applied. A value of $P < 0.05$ was considered significant.

RESULTS

Dose and time dependency of HMG- induced lung injury

A lung injury model was developed in the rat using HMG in our laboratory (for details, see above). Extravasation of [^{125}I] BSA was used as a measure of tissue damage in the lung 4 h after HMG injection. A dose response experiment was run over a period of 4 h. Animals treated included a group of controls and groups of animals treated intrapulmonary with 3, 6, 9 or 12 mg HGM kg^{-1} body wt. The results are shown in Fig. 1. The control group (intrapulmonary injection of the vehicle only) produced a background permeability value of 0.25 ± 0.03. Treatments with 3, 6, 9 or 12 mg of HMG yielded lung permeability indices of 0.97 ± 0.17, 1.92 ± 0.17, 2.00 ± 0.31 and 2.23 ± 0.16, respectively. From the dose response studies, the dosage of 6 mg kg^{-1} (about 2 mg of HMG per rat) was selected. All the subsequent experiments used this concentration of HMG.

A time dependency experiment was run after determining the optimal dosage of HMG. When lung injury values were determined at 2, 4 and 6 h after HMG instillation into the lungs, injury values of 0.76 ± 0.19, 1.92 ± 0.17 and 4.08 ± 0.19, respectively, were obtained (Fig. 2). These studies indicated an almost linear increase in injury during the first 4 h of injury and confirmed the suitability of the 4-h time point for our studies. Unless otherwise noted, the 4-h time point following exposure to HMG was used, because previous studies with acute lung injury employed the same time point.

Protective effects of neutrophil depletion

Blood neutrophil depletion (<250 neutrophils μl^{-1} blood) was induced by the injection of a rabbit anti-rat PMN

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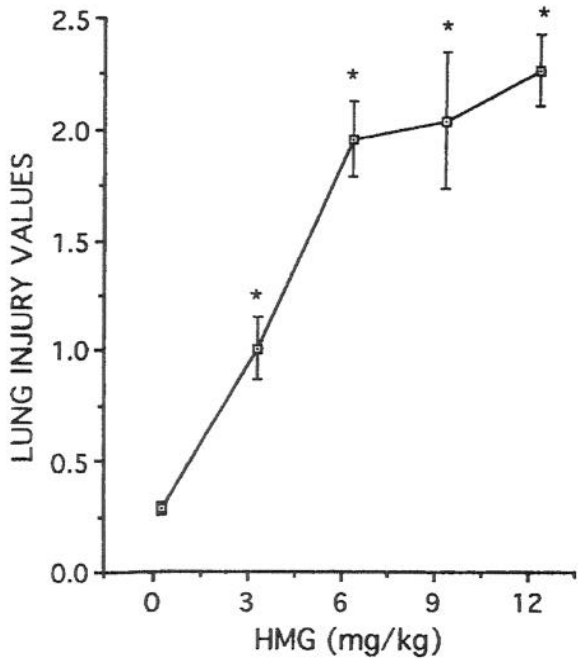


Figure 1. Dose-dependent increases in acute pulmonary injury (lung injury values defined by the permeability index) in the rat caused by HMG. Lung injury was determined at 4 h after intrapulmonary HMG injection. Based on these observations, 6 mg HMG kg^{-1} body wt was chosen for all subsequent experiments. *Significant changes compared with reference value and $n = 5$ for each bar, for this and all subsequent figures.

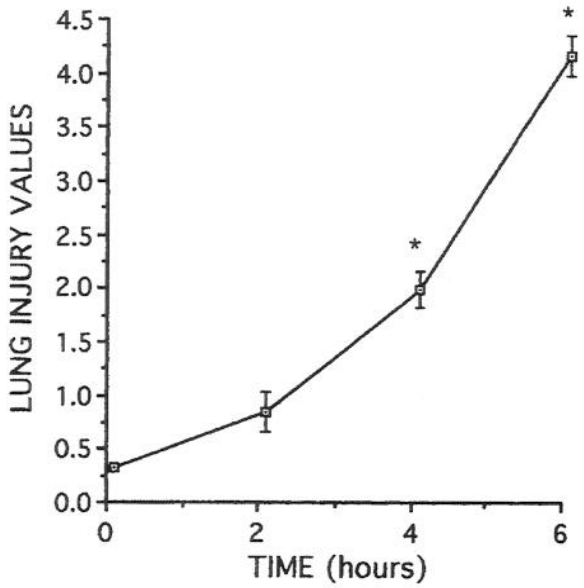


Figure 2. Time-dependent increases in lung injury values following intrapulmonary HMG (6 mg kg^{-1}) injection into rats. Based on these observations, lung injury was determined in subsequent studies at 4 h post-HMG injection.

antibody as described above. ^{125}I-Labeled BSA extravasation was used as a measure of tissue damage in the lung 4 h after the HMG instillation. Protection was calculated after subtracting the negative control values from the positive controls and lung injury values obtained from experimental animals. The results of neutrophil depletion on HMG-induced lung injury are shown in Fig. 3. As can be seen, neutrophil depletion was associated with a 62% reduction in HMG-induced lung injury (neutrophil-depleted rats showed a mean permeability index of 0.88 ± 0.07, compared with a value of 1.92 ± 0.17 for non-neutropenic rats). This indicates that full development of the HMG-induced lung injury requires the availability of neutrophils.

Protective effects of complement depletion

Complement depletion was achieved by the serial intraperitoneal administration of CVF as described above. The results of complement depletion on vascular permeability are illustrated in Fig. 3. Depletion of complement resulted in a 43% reduction in lung injury (complement-depleted rats had a permeability index of 1.21 ± 0.05, compared with a value of 1.92 ± 0.17 for non-depleted rats). These observations indicate that full development of the HMG-induced lung injury is, at least in part, complement dependent.

Protection by antioxidants

The dosing of the antioxidant compounds used in the present study (see above) has proved effective in other rat models of acute lung injury that were also dependent on blood neutrophils and complement.[25–27] The results of these interventional studies in the HMG model are depicted in Fig. 4. The DMSO treatment resulted in a 51% reduction of the observed positive control injury (DMSO administration showed a lung injury value of 1.07 ± 0.013, compared with a value of 1.92 ± 0.17 for non-treated rats). The DMTU afforded a 38% protective effect (mean lung injury value $= 1.29 \pm 0.02$). Catalase treatment resulted in a 47% protective effect (lung injury value $= 1.13 \pm 0.14$). Treatment with resveratrol—a phytoalexin and one of several antioxidants found in wine[31,32]—resulted in a 61% reduction in HMG-induced lung injury (mean lung injury value $= 0.91 \pm 0.03$). The iron chelators 2,3 dihydroxybenzoic acid (DHBA) and deferoxamine mesylate (desferal) showed no significant protection.

In summary, significant reductions in lung injury were seen when catalase, the hydroxyl radical scavengers (DMSO and DMTU) and resveratrol were given. Superoxide dismutase (SOD), 2,3-dihydroxybenzoic acid (DHBA)

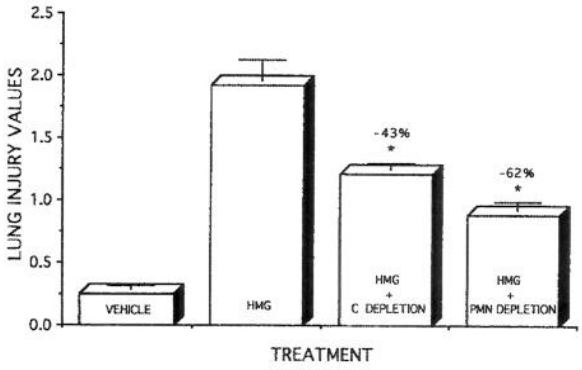


Figure 3. Intrapulmonary HMG injection into complement (C)-depleted or neutrophil (PMN)-depleted rats.

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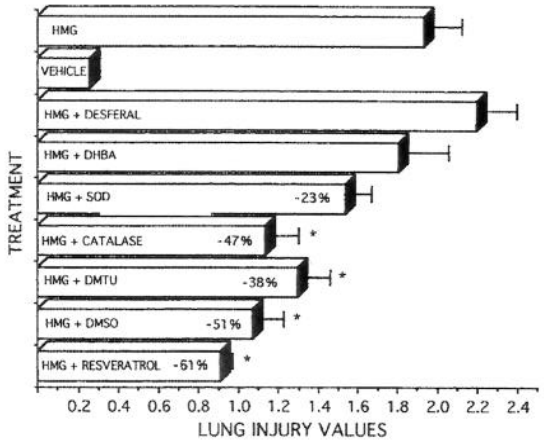


Figure 4. Effect of various antioxidants on HMG-induced acute lung injury. Test compounds included iron chelators, [desferal and 2,3-dihydroxybenzoic acid (DHBA)], antioxidant enzymes [catalase and superoxide dismutase (SOD)], hydroxyl radical scavengers [dimethyl thiourea (DMTU) and dimethyl sulfoxide (DMSO)] and a phytoalexin (resveratrol), which can also display antioxidative effects. For further details, see text.

and desferal showed no protective effects (Fig. 4). These observations support the concept of toxic oxygen species in the pathogenesis of HMG-induced acute lung injury.

N-Acetyl-L-cysteine (NAC) dose responses and time dependency

N-Acetyl-L-cysteine was also tested and demonstrated highly protective effects against HMG-induced lung injury (see data below). A dose response for intravenously administered NAC was generated for the 4-h HMG injury. All doses of NAC were given 10 min prior to instillation of the HMG. The NAC was used at doses of 5, 10, 20, 30 and 40 mg kg^{-1} body wt. The treatment groups at 4 h showed lung injury values of 1.53 ± 0.06 at 5 mg kg^{-1}, 1.18 ± 0.09 at 10 mg kg^{-1}, 0.75 ± 0.10 at 20 mg kg^{-1}, 1.06 ± 0.06 at 30 mg kg^{-1} and 0.99 ± 0.08 at 40 mg kg^{-1} (Fig. 5). These data showed significant protection for all NAC doses tested, the highest being 70% for a NAC dose of 20 mg kg^{-1} (Fig. 5). From this experiment the optimum intravenous dosage of NAC was determined to be 20 mg kg^{-1} body wt.

The time course studies for the effectiveness of NAC treatments used the optimum dosing (20 mg kg^{-1} body wt i.v.) administered at various time points throughout the course of the injury. The treatment time points included the previously used 10 min prior to injury as well as administration of NAC 10, 30, 60, 90, 120 and 180 min after the instillation of HMG (Fig. 6). The 4-h lung injury values after HMG instillation were 1.23 ± 0.02 when NAC was given at 10 min, 0.99 ± 0.02 at 30 min, 1.11 ± 0.10 at 60 min, 1.02 ± 0.15 at 90 min, 1.64 ± 0.14 at 120 min and 2.04 ± 0.30 at 180 min. These lung injury values translated into a 42% protection when NAC was given 10 min after HMG and rose to 56% and 49% protection for the 30-min and 60-min time points, respectively. Even when NAC treatment was administered as late as 90 min after HMG exposure, there was still a 54% reduction in lung injury. No significant changes were observed when NAC treatment was delayed by >90 min (Fig. 6).

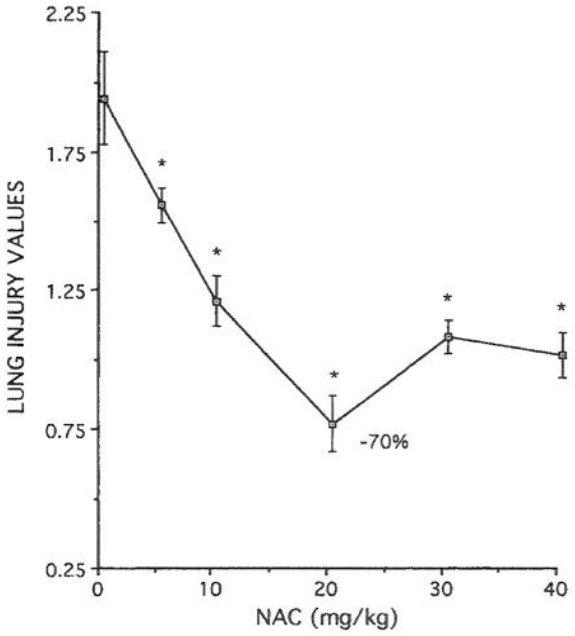


Figure 5. Dose-dependent protective effects of NAC on HMG-induced lung injury; NAC was injected 10 min prior to HMG. Lung injury values were determined at 4 h post-HMG injection.

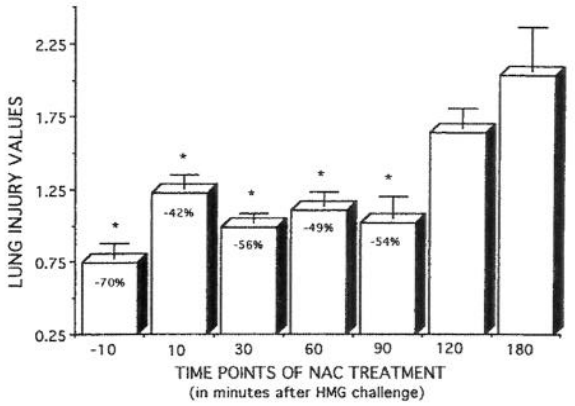


Figure 6. Effect of delayed NAC (20 mg kg^{-1}) treatment of HMG-injected rats.

DISCUSSION

To elucidate the pathogenic mechanisms involved in the development of HM-induced lung injury and to assess promising therapeutic interventions, a new rat model of HMG-induced acute pulmonary injury has been developed. Employing this model, experimental data have been obtained that strongly suggest that the HMG-induced acute (4-h) lung injury requires a fully functioning complement system and the availability of blood neutrophils. Complement or neutrophil depletion of experimental animals prior to HMG challenge resulted in 40% and 60% protection from lung injury, respectively. These are new findings for this type of acute lung injury. Anderson *et al.* found increased numbers of neutrophils in bronchoalveolar lavage fluids of HD-treated rats.[33] In

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chronic lung injury of patients exposed to mustard gas, neutrophilic alveolitis was described as a predominant feature.[34] The early appearance of neutrophils in HD-induced skin lesions is well established.[35] Although serum complement consumption by HD *in vitro* was reported in 1946,[36] and increased *in vitro* binding of Clq to HD-treated keratinocytes has been reported recently,[37] the pathogenic role of complement in HD-induced organ injury remains a matter of speculation. It is assumed that complement activation products may participate in the pathogenesis of HMG-induced lung injury by stimulating neutrophils and other blood and organ cells.

Because other animal models of neutrophil- and complement-dependent acute lung injury have shown that, at least in part, toxic oxygen species are participating in the pathogenesis,[25,29,38] the effect on HMG-induced lung injury of various antioxidants was investigated. As shown in Fig. 4, catalase, the hydroxyl radical scavengers (dimethyl thiourea and dimethyl sulfoxide) and the phytoalexin (resveratrol) all exhibited pronounced protection from HMG lung injury. Superoxide dismutase (SOD) reduced lung injury by 23%, but these changes were not significant. Eldad *et al.*,[39] using a guinea pig model of HD-induced skin burns, saw significant reductions in the skin lesions when SOD was given intraperitoneally or intralesionally 1 or 3 h in advance of wound infliction. No protection was seen when SOD was given 1 h after skin exposure. We also tested iron chelators, which have shown strong protection in other models of oxidant-induced lung injury.[30,40] Why desferal (deferoxamine) and 2,3-dihydroxybenzoic acid (DHBA) did not show any protective effect remains unclear at present. Nevertheless, oxidants appear to play a role in HMG-induced lung injury. Our observations are corroborated by a recently published study by Kumar *et al.*,[7] who demonstrated that antioxidants such as vitamin E, quercetin and reduced glutathione (GSH) could enhance survival time and protect lungs from oxidative damage in mice exposed to HD.

Additional support for an involvement of oxidants in the pathogenesis of HMG-induced acute lung injury was provided by the dramatic effects seen after treatment of experimental animals with NAC. Not only did NAC demonstrate the most pronounced protection from lung injury (Fig. 5), but it also provided significant protection even when given as late as 90 min after intrapulmonary injection of HMG (Fig. 6). This protection after onset of the injury was not seen with any of the other antioxidants used in our study (data not shown). Others have observed decreased numbers of neutrophils in bronchoalveolar lavage fluids obtained from HD-injured lungs of rats that were treated intraperitoneally with NAC at the beginning of the experiment.[41] The mechanisms of NAC-mediated protection from mustard-induced lung injury are not clear. Recent data by Atkins and colleagues[42] seem to suggest that NAC protects from HD-induced apoptotic endothelial cell death by enhancing the synthesis of reduced glutathione, which in turn may scavenge HD and also prevent oxidative activation of the transcription factor NF-kappa-B, which is usually upregulated by stress signals.

In summary, our observations suggest that the development of HMG-induced lung injury is dependent on the availability of both neutrophils and complement, and is largely mediated by toxic oxygen metabolites. The latter is supported by our findings that NAC, a well-known antioxidant, has powerful protective effects related to the dose administered and the time of its administration.

Acknowledgement

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APPENDIX

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ATTENUATION OF HALF SULFUR MUSTARD GAS - INDUCED ACUTE LUNG INJURY IN RATS

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ABSTRACT

Airway instillation into rats of 2-chloroethyl ethyl sulfide (CEES), the half molecule of sulfur mustard compound, results in acute lung injury, as measured by the leak of plasma albumin into lung. Morphologically, early changes in lung include alveolar hemorrhage and fibrin deposition and the influx of neutrophils. Following lung contact with CEES, progressive accumulation of collagen occurred in the lung, followed by parenchymal collapse. The co-instillation with CEES of liposomes containing pegylated (PEG)-catalase (CAT), PEG-superoxide dismutase (SOD), or the combination, greatly attenuated development of lung injury. Likewise, the co-instillation of liposomes containing the reducing agents, N-acetylcysteine (NAC), glutathione (GSH), or resveratrol (RES), significantly reduced acute lung injury. The combination of complement depletion and airway instillation of liposomes containing anti-oxidant compounds maximally attenuated CEES-induced lung injury, by nearly 80%. Delayed airway instillation of anti-oxidant-containing liposomes (containing NAC or GSH, or the combination) significantly diminished lung injury even when instillation was delayed as long as one hour after lung exposure to CEES. These data indicate that CEES-induced injury of rat lungs can be substantially diminished by the presence of reducing agents or anti-oxidant enzymes delivered via liposomes.

Keywords: CEES, anti-oxidant liposomes, neutrophils, fibrosis, macrophages

INTRODUCTION

As is well known, mustard gas [bis (2-chloroethyl ethyl) sulfide], also known as sulfur mustard (HD), has long been known to be a vesicant in humans and, when inhaled, causes extremely lung damaging reactions (Eisenmenger *et al.*, 1991; Khateri *et al.*, 2003; Lakshmana Rao *et al.*, 1999). In human survivors, progressive lung dysfunction due to pulmonary fibrosis is well documented (Emad *et al.*, 1999). Not unexpectedly, HD is radiomimetic, teratogenic and mutagenic (Angelov *et al.*, 1996; Dube *et al.*, 1998). Currently, there is no effective therapy for either the vesicant-inducing properties of HD or for the outcomes that can lead to acute and progressive lung injury and death.

2-chloroethyl ethyl sulfide (CEES) is less toxic than HD and can be used in the absence of facilities required for HD studies. In rats CEES has been shown to induce acute lung injury in a dose-dependent and time-dependent manner (McClintock *et al.*, 2002). CEES-induced acute lung injury is complement and neutrophil-dependent, suggesting that some of the CEES-induced injury is due to engagement of the inflammatory response in lung in an unknown manner (McClintock *et al.*, 2002). Furthermore, lung injury is attenuated after intravenous treatment with the anti-oxidant, N-acetylcysteine (NAC), or airway delivery of anti-oxidants or anti-oxidant enzymes (McClintock *et al.*, 2002). These data have suggested that CEES compromises the redox potential in lung, putting it at risk of oxidant-mediated injury.

Liposomal delivery of drugs or chemical compounds is a way to achieve high tissue levels of a desired compound (Fan *et al.*, 2000; Freeman *et al.*, 1985; Suntres *et al.*, 1996). In lung, airway delivery of liposomes results in macrophage uptake of liposomes by a phagocytic pathway (Gonzalez-Rothi *et al.*, 1991; Shephard *et al.*, 1981; Sone *et al.*, 1980). As far as is known, liposomes are not internalized by any other lung cells. In the current studies, we demonstrate that liposomes containing anti-oxidants or anti-oxidant enzymes cause reduction in acute lung injury in rats following airway delivery of CEES. Furthermore, delivery of such liposomes, when delayed 1 hour after CEES administration, still provides significant attenuation of acute lung injury. These findings may have important therapeutic implications for HD-induced acute lung injury in humans.

MATERIALS AND METHODS

Chemicals:

Except where noted, all chemicals and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO)

Animal Model:

Adult male (275-325 g) specific pathogen-free Long-Evans rats (Harlan Co., Indianapolis, IN) were used in these studies. Intraperitoneal ketamine (100 mg/kg body weight) (Fort Dodge Animal Health, Fort Dodge, IA) was used for anesthesia and intraperitoneal xylazine (13 mg/kg body weight) (Bayer Corp. Shawnee Mission, KS)

was used for sedation when required (delayed time point liposome administration). The experimental procedure for CEES-induced lung injury in rats has been described previously (McClintock *et al.*, 2002). Briefly, after induction of anesthesia, ^{125}I-labeled bovine serum albumin (^{125}I-BSA, 0.5 μCi /rat) was injected intravenously as a quantitative marker for vascular leakage. The trachea was then surgically exposed and a slightly curved P50 catheter was inserted into the trachea past the bifurcation such as to facilitate a unilateral, left-lung injury. A small volume of CEES (2 μl/rat; about 6 mg/kg) was solubilized in ethanol (58 μl/rat) and then added to a syringe containing Dulbecco's phosphate buffered saline (DPBS) (340 μl/rat). This solution was injected via the intratracheal catheter, into the left lung main stem bronchus. Studies, not requiring the usage of a radio-labeled marker, proceeded identically substituting DPBS for the radioactive injection. For all studies, except the time response experiment, animals were sacrificed 4 hours later, the pulmonary arterial circulation was flushed with 10 ml of cold DPBS, the lungs were surgically dissected, placed in counting vessels, and the amount of radioactivity (^{125}I-labeled BSA) determined by gamma counting. For calculations of the permeability index, the amount of radioactivity (^{125}I-labeled BSA) remaining in the lungs in which the vasculature was perfused with saline was divided by the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice as described elsewhere (McClintock *et al.*, 2002). ^{125}I-BSA present in lung after thorough flushing of the vasculature is a quantitative measure of the degree of vascular endothelial and alveolar epithelial damage, in which much of the ^{125}I-BSA can be lavaged from the distal airway compartment, indicating loss of the vascular and epithelial barriers (Johnson *et al.*, 1974).

All animal experiments were in accordance with the standards in The Guide for the Care and Use of Laboratory Animals, and were supervised by veterinarians from the Unit for Laboratory and Animal Care of the University of Michigan Medical School.

Complement Depletion:

Cobra venom factor (CVF) was purified from crude, lyophilized cobra venom (*Naja atra*) by ion exchange chromatography (Ballow *et al.*, 1969). Complement depletion of experimental animals was achieved by intraperitoneal injections of 25 units of CVF/rat at time zero and 24 hours later, resulting in undetectable levels of serum hemolytic complement activity as confirmed by CH50 assay (Mayer, 1961). Experiments were performed 24 hours after the second CVF injection.

Liposome Preparation:

Dipalmitoylphosphotidylcholine (DPPC, Avanti Polar Lipids) was dissolved 20 mg/ml in a 2:1 v/v chloroform/ methanol solution. When α-tocopherol (αT) was also included in the liposomes, it was added just after the chloroform/ methanol solvent to provide a 7:3 molar ratio (DPCC:α-T) after first being carefully dissolved in a small volume of ethanol. The DPPC or (DPPC:α-T) solution was then dried under a thin stream of nitrogen in a round bottom flask to form a thin lipid film on the walls of the tube. Once the film had been dried, the tube was then placed on a vacuum for at least one hour to further dry and remove any excess organic compounds from the lipid film.

The compounds being encapsulated in the liposomes were exclusively prepared in Dulbecco's phosphate buffered saline (DPBS), pH adjusted to 7.4 and then added to the lipid film. The tube was then vortexed to free the lipid film from the walls of the tube, and then placed in a heated water bath (41°C). When sizing the liposomes, it is necessary to keep them at a temperature above their transition phase. The transition phase temperature for DPPC is 41°C. Vortexing the liposomes once they are above the transition phase temperature results in large multilamellar vesicles. To reduce the size of the vesicles and to produce uniform small unilamellar vesicles, the lipid suspension was then passed ten times through polycarbonate membrane filters in a Liposofast Basic mini extruder available from Avestin, Inc. (Ottawa, Ontario). The resulting liposomes were uniform in size measuring 100nm in diameter. According to the manufacturer, the use of an extruder is an efficient method for producing liposomes that are of relatively uniform size. Liposomes were checked via light microscopy for uniformity and size. Liposomes were injected intratracheally in a volume of 100 µl per rat through the same catheter setup used for CEES instillation at the time point designated by each individual experimental protocol.

Morphological Assessment of Lung Injury:

To morphologically assess lung injury, lungs were fixed by intratracheal instillation of 10 ml buffered (pH 7.2) formalin (10%) at the indicated time points following airway instillation of CEES. Lung sections were then obtained for histological examination by staining with hematoxylin and eosin. In addition, lung sections were stained with trichrome in order to assess deposition of fibrin and collagen (Luna, 1968).

Statistical Analysis:

Results are presented as mean ± SEM in the text and Figures. Groups ($n \geq 5$) were subjected to one-way analysis of variance and when significance was found, Student's *t*-test with the Bonferroni correction for multiple comparisons was applied. A value of $p<0.05$ was considered significant.

RESULTS

Histopathologic Features of Lung Response to CEES

Following airway instillation of CEES into rat lungs, tissues were obtained at times 0, 6, 12 and 24 hr as well 3 and 6 days and 6 weeks after exposure to CEES. Lung sections were stained with trichrome stain to evaluate lung deposition of fibrin and collagen. Composite results are shown in Figure 1 (frames A-I). At time 0, trichrome stains revealed the usual perivascular and septal evidence of collagen (frames A, B). As early as 6 hr, increased evidence of trichrome stained deposition in alveolar walls was likely related to fibrin deposition (frame C). By 24 hr after lung instillation of CEES, dense interstitial and intra-alveolar accumulations trichrome positive (blue dye) were evident throughout the affected lungs suggestive of increased deposition of fibrin and collagen fibers (frame D). Intra-alveolar hemorrhage, edema and intra-alveolar accumulation of macrophages and mononuclear cells were found at 24 hr (frame E). By 3 days, dense interstitial deposits of fibrin and collagen occurred (frame F). By day 6,

extensive confluent collagen deposits were found in lung, together with collapse of alveolar structures and appearance of honeycombing (frames G, H). By the third week, little recognizable lung structure remained in the face of dense collagen deposits, and parenchymal collapse, together with numerous interstitial macrophages and mononuclear cells (frame I).

Attenuation of CEES-Induced Acute Lung Injury by Anti-Oxidant Enzymes in Liposomes

As shown in Figure 2, the airway instillation of CEES together with unloaded liposomes resulted 4 hours later in approximately a 10-fold increase in lung injury, as defined by the leakage of ^{125}I-albumin from blood into lung. When instilled into lung immediately after CEES, polyethyleneglycol (PEG)-linked catalase-containing liposomes (LIP-PEG-CAT) attenuated injury by 40%. Liposomes containing PEG-superoxide dismutase (PEG-SOD) diminished injury by 57%. The combination of PEG-SOD and PEG-CAT in liposomes further reduced injury by 71%. With the combination of PEG-SOD and PEG-CAT liposomes given to complement-depleted animals, the injury was reduced by 86%. These data indicate that anti-oxidant enzymes have powerful attenuative effects on CEES-induced acute lung injury. Since, as described above, airway delivery of liposomes results in their phagocytosis by lung macrophages, it seems likely that the attenuative effects of liposomes containing anti-oxidant enzymes are due to the bolstering of anti-oxidant defenses in lung macrophages.

Attenuative Effects of Liposomes Containing Reducing Agents

In an additional set of experiments (shown in Figure 3), there was approximately a 10-fold increase in leakage of albumin from the circulation into lungs of animals receiving airway instillation of CEES 4 hour earlier together with unloaded liposomes. When liposomes containing NAC (Lip-NAC) were instilled immediately after CEES, injury was attenuated by 60%. Liposomes containing glutathione (GSH) led to a 48% reduction in lung injury. Liposomes containing α-tocopherol (αT) reduced injury by 37%. Liposomes containing the reducing agent present in red wine, resveratrol (RES), reduced injury by 48%, while liposomes containing PEG-CAT reduced injury by 44%. These data indicate that reducing agents presented in liposomes have significantly attenuative effects against CEES-induced acute lung injury. The data also indicate that the non-derivatized form of catalase (CAT) also has attenuative effects when given within liposomes.

Additive Effects of Complement Depletion and Liposomes Containing Reducing Agents.

Previous studies in our laboratory have shown that complement depletion resulted in a 43% reduction of lung injury (McClintock *et al.*, 2002). As shown in Figure 4, approximately a 10-fold increase in the leakage of ^{125}I-albumin into lungs occurred following instillation of CEES together with unloaded liposomes. When animals were complement (C) depleted, the instillation of liposomes containing NAC reduced injury by 79%, those containing GSH reduced injury by 72% in complement-depleted rats, liposomes containing the combination of NAC and GSH reduced injury by 78% in complement-depleted rats. Complement-depleted animals receiving liposomes containing

αT together with GSH showed an 82% reduction in lung injury as measured by leakage of albumin from the blood. Thus, the combination of complement depletion and anti-oxidant liposomes seems to significantly attenuate CEES-induced acute lung injury in an additive manner.

Effects of Delayed Lung Instillation of Anti-Oxidant Liposomes

As shown in Figure 5, in CEES treated animals instillation of liposomes containing reducing agents was done either 10 min before the airway instillation of CEES or at 30, 60 and, in one case, 90 min following the airway instillation of CEES. As shown in Figure 4, over the course of the first 60 min after instillation of CEES, there were significant attenuative effects of liposomes containing NAC or GSH, or the combination. Under these circumstances, injury was reduced between 55% and 77%, respectively. In the case of liposomes containing the combination of NAC and GSH, even when delivery was delayed until 90 min following instillation of CEES, there was a 55% reduction in development of acute lung injury. These data indicate that delayed airway administration of anti-oxidant-containing liposomes results in significant reduction of CEES-induced lung injury, even when delivery is delayed by at least an hour following exposure of lungs to CEES.

DISCUSSION

The data described in this report indicate that CEES instillation into the lung produces acute lung injury in a manner that seems related to loss of the redox balance in lung, although this has not been directly demonstrated. This conclusion is based on the attenuative effects of reducing agents (NAC, GSH, αT, resveratrol) or anti-oxidant enzymes (SOD, CAT) or various combinations, all presented in liposomes alone or in combination. Since it is well known that liposomes given into the airways are phagocytized by macrophages and internalized (Gonzalez-Rothi *et al.*, 1991; Lentsch *et al.*, 1999; Shephard *et al.*, 1981), the implications from the current studies are that liposomal delivery selectively enhances a reducing environment in lung macrophages, which may be compromised when these cells came into contact with CEES.

The morphological features described in this report are consistent with our earlier report of an accumulation of myeloperoxidase (MPO) in lung after CEES instillation (McClintock *et al.*, 2002). The presence of alveolar hemorrhage and edema implies a severe disruption of vascular and distal airway barrier. These changes are consistent with the concept that CEES induces an acute lung-damaging inflammatory response that is complement-dependent. Beneficial effects of neutrophil and complement depletion as demonstrated previously indicate that the inflammatory response to CEES contributes to the development of lung injury (McClintock *et al.*, 2002). Masson's Trichrome staining revealed an accumulation of fibrin and/or collagen within alveolar spaces. Deposition of fibrin reflects a non-specific reaction to tissue damage. It remains to be shown that CEES-exposure causes rapid development of interstitial fibrosis, as confirmed biochemically by collagen accumulation. We hypothesize that epithelial and endothelial damage following CEES-exposure results in disruption of tissues, resulting in collagen accumulation in the interstitial and alveolar spaces. It appears likely that following lung exposure to CEES collagen deposition occurs in a widespread manner, resulting in

parenchymal collapse and the honeycombing changes that occur in humans with pulmonary fibrosis. Numerous macrophages and mononuclear cells in areas of collagen deposition in lung may be associated with the release of mediators (such as TGF_{β}) that promote lung production of collagen.

How CEES functions as a powerful oxidant and what lung cells are targets of CEES is unclear. The extensive leakage of albumin into the lung after exposure to airway administration of CEES infers that the blood-gas barrier has been seriously compromised, causing functional impairment (or destruction) of both vascular endothelial and alveolar epithelial cells. The subsequent alveolar flooding with plasma components leaking into the distal airway compartment could seriously compromise blood-gas exchange, resulting in hypoxia.

The permeability index after instillation of CEES and empty liposomes was found to be similar to CEES instillation alone (as reported in [McClintock *et al.*, 2002]) implying that empty liposomes do not cause any lung damage by themselves. The fact that the combination of complement depletion and liposomal delivery of GSH or NAC enhances the attenuation when compared to the use of either types of liposomes given to complement-intact rats (Figures 3 and 4) suggests that it may be both the loss of reducing potential in lung as well as engagement of complement activation products (e.g., C5a) that leads to intense acute lung injury. Whether complement activation products are directly responsible for lung injury or are functioning to enhance cytokine and chemokine expression remains to be determined. In a recent study we showed that neutrophil depletion prior to CEES delivery was also capable of reducing CEES-induced lung injury (McClintock *et al.*, 2002), suggesting that activated neutrophils enter into the sequence of destructive events after CEES instillation into lung.

A matter of considerable interest is that delayed delivery (for as long as 60 min) of liposomes containing NAC or GSH, or the combination, into lungs after CEES instillation still provides substantial attenuation from the massive leak of albumin into lung. It should be noted that there is not much increased albumin leak into lung in the first 60 min after administration of CEES (McClintock *et al.*, 2002). In fact, compared to permeability index values at 1 hour, 2, 4 and 6 hour after instillation of CEES rose 2 fold, 2.9 fold, 7.7 fold and 16.2 fold, respectively, when compared to uninjured lung values. Accordingly, development of extensive lung injury after airway instillation of CEES requires considerable time for full development of lung injury. This would be in accord with the concept that CEES triggers in lung an acute inflammatory response, which itself serves to cause lung damage. Since this sequence requires several hours before the large increases in lung permeability (albumin leak into lung) are seen, this may explain why delayed administration of anti-oxidant liposomes can still bring about significant attenuative effects. Understanding more fully the molecular events that lead to CEES-induced intense acute lung injury may provide even better strategies for effective therapeutic intervention after exposure of lung to HD and related compounds.

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LEGENDS TO FIGURES

Figure 1. Tissue sections of lungs with trichrome stain. Lungs were obtained after airway instillation of CEES at time 0 (A, B, 10x and 40x); 6 hr (C, 40x); 24 hr (D, E, 10x and 40x); 3 days (F, 10x), 6 days (G, 10x and H, 40x); and 3 weeks (I, 40x). All tissue sections were reacted with trichrome stain.

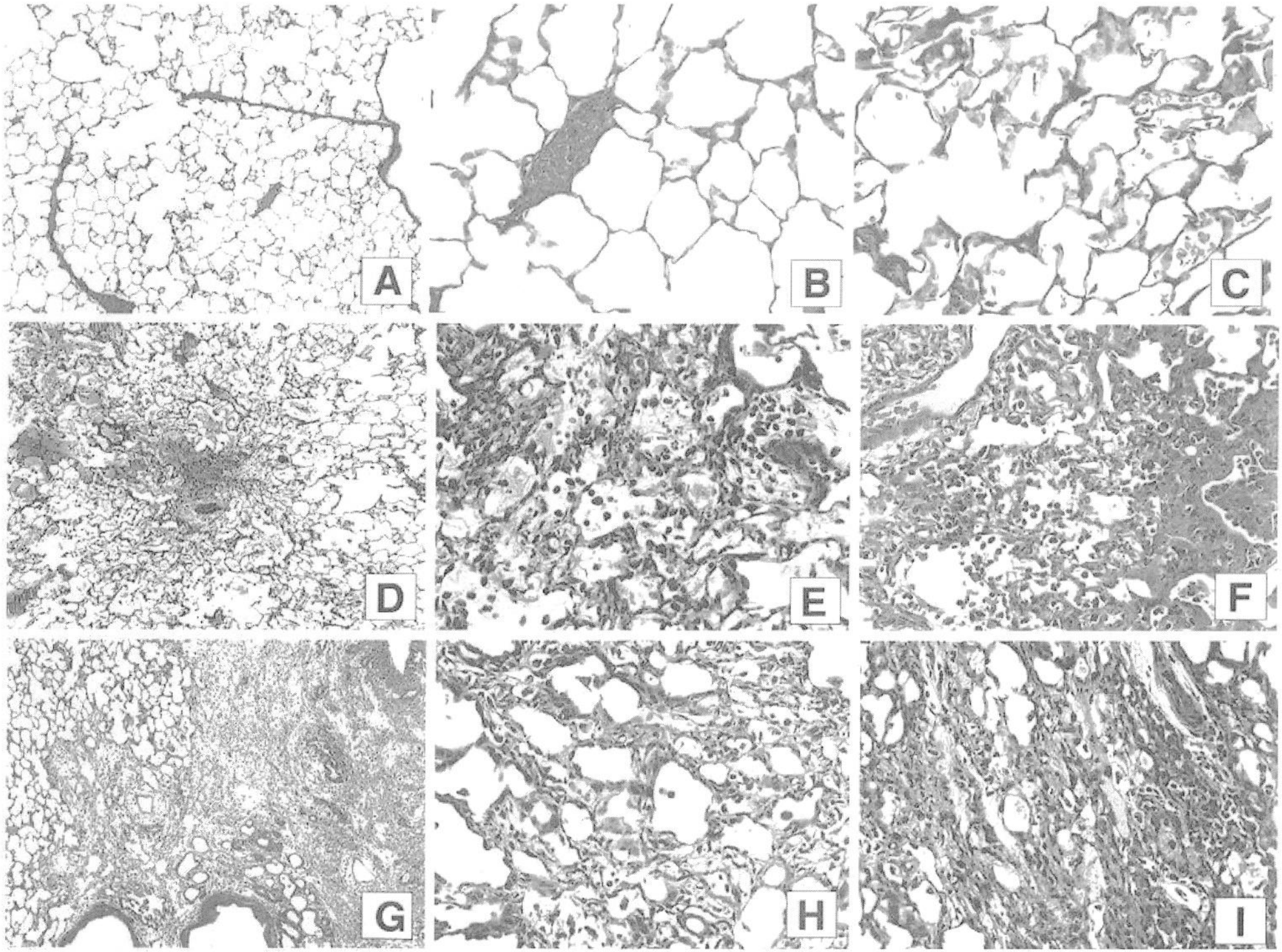
Figure 2. Attenuative effects of liposomes loaded with anti-oxidant enzymes. Rats received either saline followed by unloaded liposomes (negative control), CEES followed by airway delivery of unloaded liposomes (positive control), or CEES with liposomes containing either pegylated (PEG)-CAT or PEG-SOD, both or the combination, or the combination of liposomes in complement depleted animals induced by the earlier intraperitoneal injection of purified cobra venom factor. Liposomes were administered immediately after CEES instillation. For each bar $n \geq 6$. Lung injury values are represented by the leak of ^{125}I-albumin from the vascular compartment into the airway compartment 4 hour after airway delivery of CEES (see text). * represents p values of $<.05$ when compared to the positive control group.

Figure 3. Attenuative effects of liposomes loaded with reducing agents in CEES lung injury. The positive and negative controls are similar to those described in Figure 1. When used, liposomes were injected intratracheally immediately after the airway instillation of CEES. Lung injury was determined by the permeability index. αT, α-tocopherol.

Figure 4. Enhanced effects of anti-oxidant containing liposomes in complement depleted rats. The negative and positive control groups are similar to those described in Figure 1. Complement depletion was induced in four groups of animals by the prior intraperitoneal injection of purified cobra venom factor (CVF). Liposomes containing the various anti-oxidant compounds were given immediately after airway instillation of CEES. For each group, $n \geq 6$.

Figure 5. Attenuative effects of anti-oxidant containing liposomes when airway instillation was given at various time points before or after airway delivery of CEES. The dotted line near the top represents the positive control value. Lung injury values (permeability indices) in the negative control are not shown but were < 0.5, as described in Figures 1-3.

Figure 1



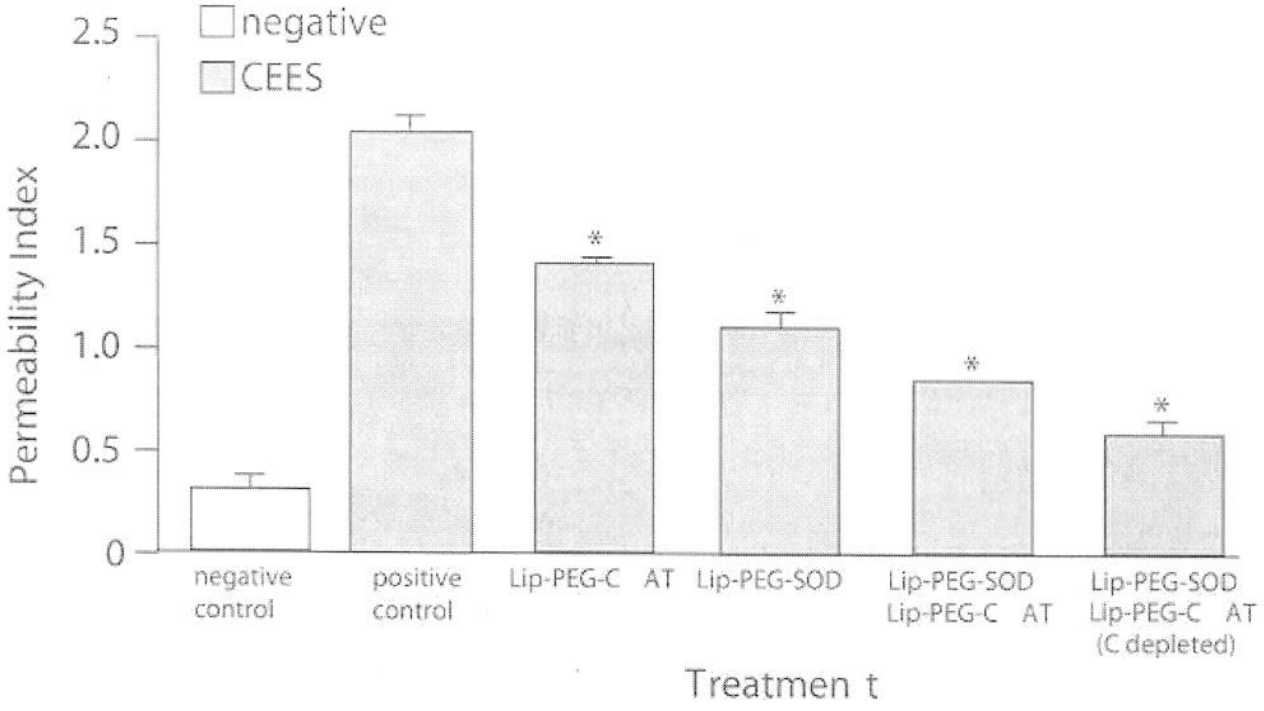

negative
CEES
Permeability Index
2.5
2.0
1.5
1.0
0.5
0
*
*
*
*
negative control
positive control
Lip-PEG-C AT
Lip-PEG-SOD
Lip-PEG-SOD Lip-PEG-C AT
Lip-PEG-SOD Lip-PEG-C AT (C depleted)
Treatmen t

Fig 2

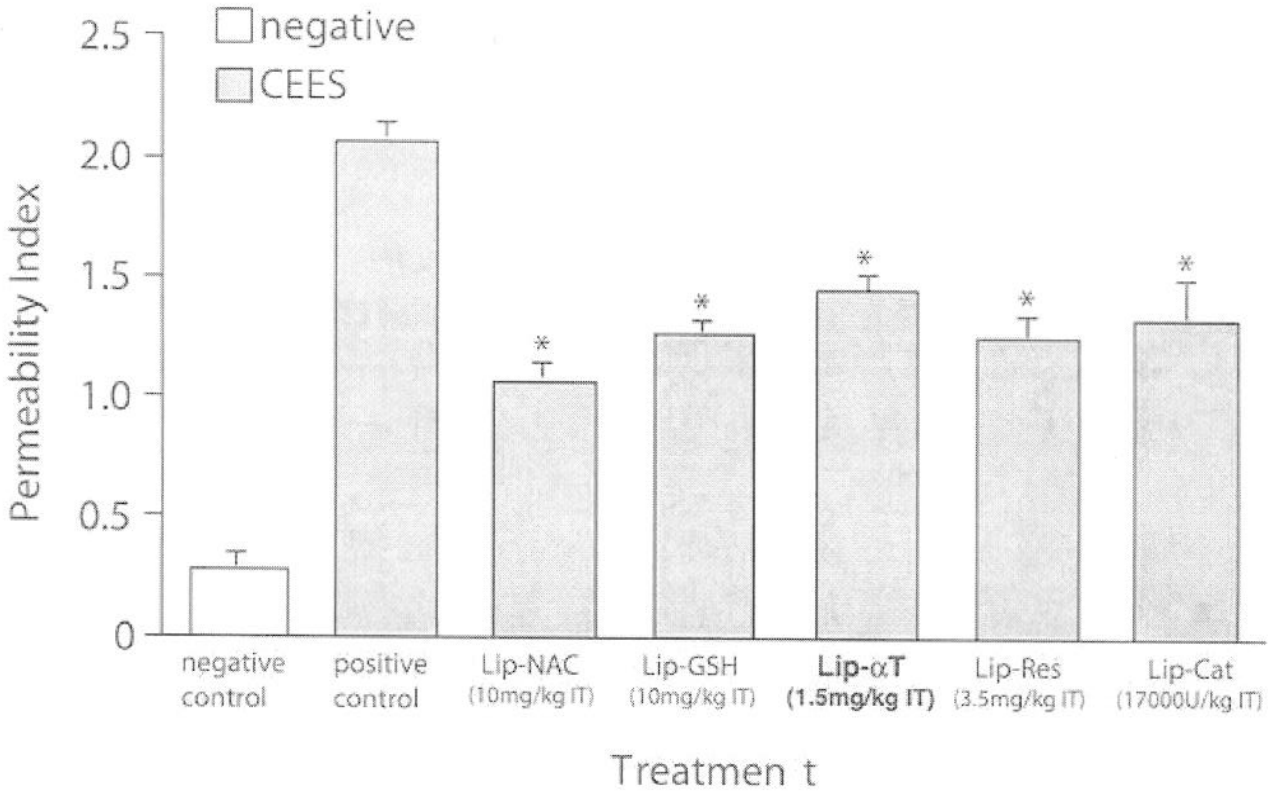

negative
CEES
Permeability Index
2.5
2.0
1.5
1.0
0.5
0
*
*
*
*
*
negative control
positive control
Lip-NAC (10mg/kg IT)
Lip-GSH (10mg/kg IT)
Lip-αT (1.5mg/kg IT)
Lip-Res (3.5mg/kg IT)
Lip-Cat (17000U/kg IT)
Treatmen t

Fig 3

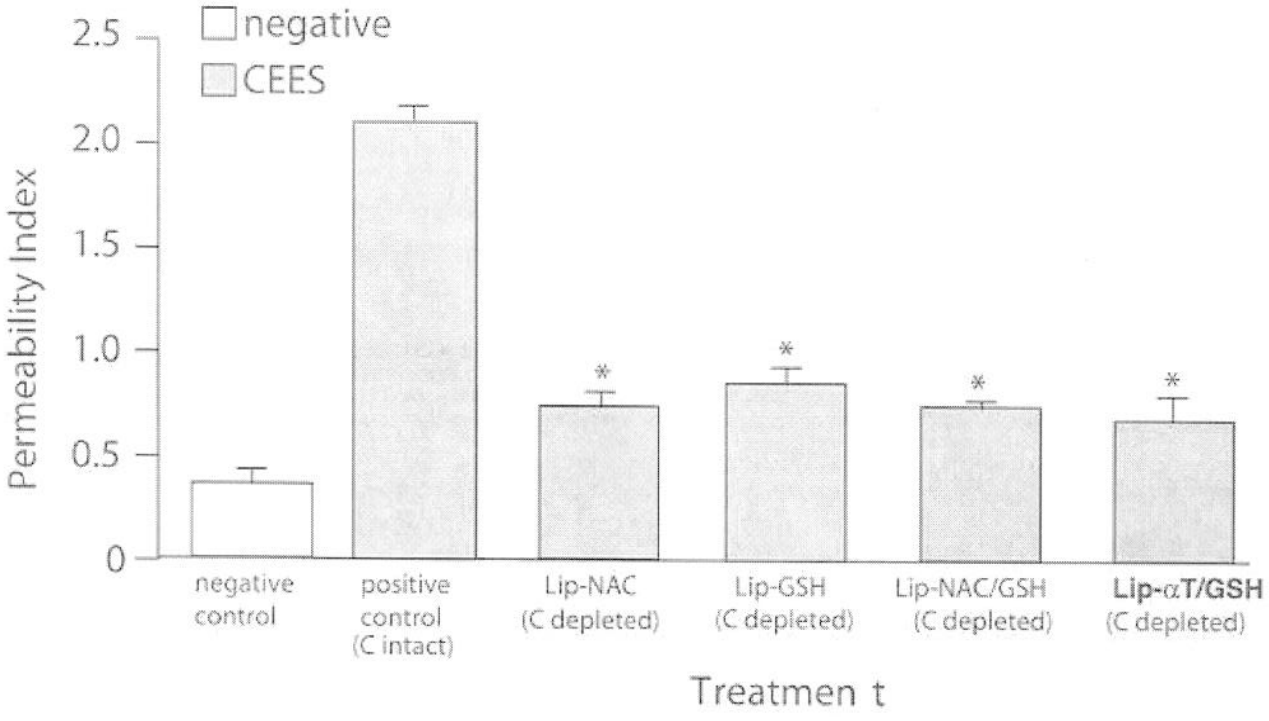

negative
CEES
Permeability Index
2.5
2.0
1.5
1.0
0.5
0
*
*
*
*
negative control
positive control (C intact)
Lip-NAC (C depleted)
Lip-GSH (C depleted)
Lip-NAC/GSH (C depleted)
Lip-αT/GSH (C depleted)
Treatmen t

Fig 4

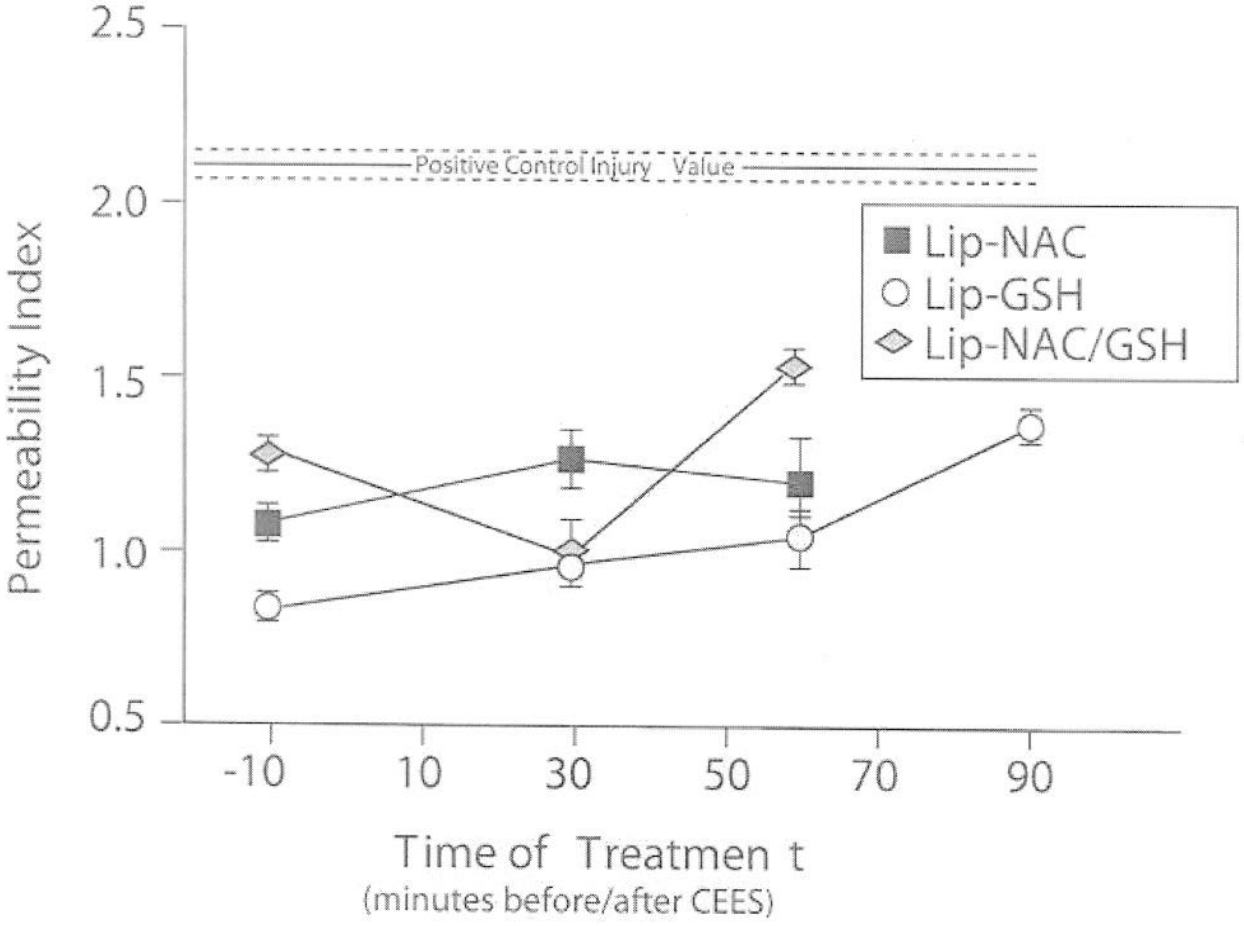

2.5
2.0
1.5
1.0
0.5
Permeability Index
Positive Control Injury Value
Lip-NAC
Lip-GSH
Lip-NAC/GSH
-10
10
30
50
70
90
Time of Treatmen t
(minutes before/after CEES)

fig 5

"Systemic effects of CEES (Half Sulfur Mustard Gas) after intratracheal instillation"

L.M. Hoesel, A.D. Niederbichler, S.D. McClintock, J.V. Sarma, P.A.Ward
Shock, Vol 23, Suppl 3, 2005

SYSTEMIC EFFECTS OF CEES (HALF SULFUR MUSTARD GAS) AFTER INTRATRACHEAL INSTILLATION. L.M. Hoesel, A.D. Niederbichler*, S.D. McClintock, J.V. Sarma, P.A.Ward Departments of Pathology and Surgery*, University of Michigan Medical School, Ann Arbor, MI 48109

Objective: Airway instillation into rats of 2-chlorethyl ethyl sulfide (CEES, half sulfur mustard compound), results in acute lung injury similar to what has been found in conflicts. Little is known about systemic effects of CEES after intratracheal instillation. **Methods**: Serum AST/ALT (parameters of liver function) and BUN/CREA (parameters of renal function) were measured in rats that underwent airway instillation of either CEES (2µl in 15% ethanol/PBS) or 15% ethanol/PBS as controls. **Results:** Both groups showed an increase in ALT and AST with a peak 6 hours after instillation and a return to normal values within 48 hours. However, CEES-treated rats displayed higher values of ALT and AST (1.6fold increase) at 4 and 6 hours after instillation, suggesting liver damage caused by CEES. In contrast, kidney parameters BUN and CREA did not change during the observation period of 24 hours in either group. **Conclusion:** These results show for the first time that intratracheal instillation of CEES may access the systemic blood circulation and exert harmful effects on liver function, but not on renal function. The administration of reducing agents, such as N-acetylcysteine (NAC) and glutathione (GSH), which have been shown to attenuate lung injury, may prevent evidence of liver damage. Possible protective impacts of intratracheal or intravenous administration of NAC/GSH on distal organ function and implications for future investigations will be discussed.

SECTION 3: William L. Stone, Ph.D., East Tennessee State University

Optimization of Antioxidant Liposomes for Treating 2-Chlorethyl Sulfide (CEES) Toxicity

Abstract

We are exploring the hypotheses that oxidative stress contributes to mustard gas and CEES toxicity and that antioxidant liposomes are a potential countermeasure. Unilamellar antioxidant liposomes containing RRR-alpha-tocopherol have been prepared and characterized by measuring particle size distribution and tocopherol content. Our data indicates that the vitamin E content was stable and the liposome preparation remained unilamellar over a three week period (at 4 deg C). In our previous published work, we reported that lipopolysaccharide (LPS) was capable of enhancing the cytotoxic effects of CEES. We have found that murine RAW264.7 macrophages treated with LPS and CEES show a cytotoxicity that is partially blocked by Polymyxin B, an antibiotic capable of binding and blocking the action of LPS. Similar results showed that LPS induced production of nitric oxide in RAW264.7 macrophages could be blocked by polymyxin B pretreatment. These data suggest that polymyxin B could be useful in minimizing the toxicity of mustard gas.

3. Oxidative stress associated with CEES toxicity in Stimulated Macrophages. In further support of the hypothesis that oxidative stress is important in CEES toxicity, RAW264.7 macrophages treated with CEES (500 □M) and LPS (10 ng/ml)) were found to have significantly elevated levels of protein carbonyls (see Figure 1). CEES alone or was not sufficient to cause an elevation in carbonyl content. Protein carbonyls are an excellent and stable indicator of oxidative stress. Similarly, only the combination of LPS with CEES was sufficient to reduce the levels of thiols in RAW264.7 macrophages (data not shown).

Introduction and Body

We are exploring the hypotheses that: (1) oxidative stress contributes to mustard gas and CEES toxicity and; (2) antioxidant liposomes are a potential countermeasure. Two manuscripts describing the use of antioxidant liposomes and their use in preventing CEES toxicity have been published (see Stone, W.L., Smith, M.: Therapeutic Uses of Antioxidant Liposomes, Molecular Biotechnology, 27: 217-230 (2004) and Stone, WL, , Qui, M, , Yang, H, and Smith, M: Lipopolysaccharide Enhances the Cytotoxicity of 2-Chloroethyl Ethyl Sulfide, Bioscience 2004 Proceedings, US Army Medical Defense, Chapter 236, pages 1-9. (2004)).

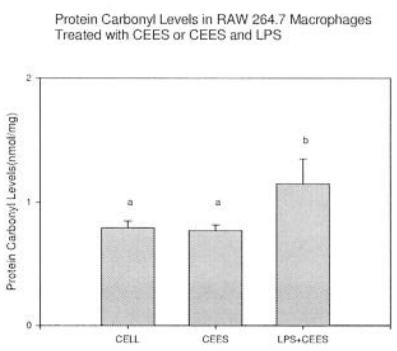
Key Research Accomplishments

1. Preparation and characterization of antioxidant liposomes. We have developed the ability to prepare and characterize antioxidant liposomes in quantities sufficient to supply other members of the Mustard Consortium. A Model M-110L Microfluidics instrument was used for the preparation of unilamellar antioxidant liposomes containing 6.6 mole percent RRR-alpha-tocopherol (as well as 66.6, 26.5 and 0.66 mole percent of soy lecithin, cholesterol and phosphatidyl serine, respectively). The liposomes were characterized by measuring: 1) particle size distribution with a dynamic light scattering Model 380 Nicomp particle analyzer; 2) tocopherol content. Our data indicates that the vitamin E content was stable over a three week storage (at 4 deg C) period and did not diminish with up to five passes through the Microfluidics instrument. The mean liposome diameter decreased with increasing passes (as expected) through the fluidizer. The liposome preparation remained unilamellar over a three week period (at 4 deg C). In summary, we can now prepare large (100 ml) batches of antioxidant liposomes that have

sufficient stability for shipping and further testing. We are now in the process of preparing a liposome preparation for Dr. Peter Ward's group for *in vivo* testing.

2. Polymyxin B Inhibits CEES toxicity in stimulated macrophages. In our previous published work, we reported that lipopolysaccharide (LPS) was capable of enhancing the cytotoxic effects of CEES. In preliminary results, we have found that murine RAW264.7 macrophages treated with LPS and CEES show a cytotoxicity that is partially blocked by Polymyxin B, an antibiotic capable of binding and blocking the action of LPS. Similar results showed that LPS induced production of nitric oxide in RAW264.7 macrophages could be blocked by polymyxin B pretreatment. These data suggest that polymyxin B could be useful in minimizing the toxicity of mustard gas.

Figure 1



3. Oxidative stress associated with CEES toxicity in Stimulated Macrophages. In further support of the hypothesis that oxidative stress is important in CEES toxicity, RAW264.7 macrophages treated with CEES (500 μM) and LPS (10 ng/ml)) were found to have significantly elevated levels of protein carbonyls (see Figure 1). CEES alone or was not sufficient to cause an elevation in carbonyl content. Protein carbonyls are an excellent and stable indicator of oxidative stress. Similarly, only the combination of LPS with CEES was sufficient to reduce the levels of thiols in RAW264.7 macrophages (data not shown).

Reportable Outcomes

See above.

Conclusions

See above.

References

None.

Appendices

1. Therapeutic Uses of Antioxidant Liposomes, Review
2. Liver Tocopherol Levels

APPENDIX

William L. Stone, Ph.D.

REVIEW

Therapeutic Uses of Antioxidant Liposomes

William L. Stone,[1,] and Milton Smith[2]*

Abstract

This review will focus on the therapeutic uses of antioxidant liposomes. Antioxidant liposomes have a unique ability to deliver both lipid- and water-soluble antioxidants to tissues. This review will detail the varieties of antioxidants which have been incorporated into liposomes, their modes of administration, and the clinical conditions in which antioxidant liposomes could play an important therapeutic role. Antioxidant liposomes should be particularly useful for treating diseases or conditions in which oxidative stress plays a significant pathophysiological role because this technology has been shown to suppress oxidative stress. These diseases and conditions include cancer, trauma, irradiation, retinotherapy or prematurity, respiratory distress syndrome, chemical weapon exposure, and pulmonary infections.

Index Entries: Antioxidants; α-tocopherol; γ-tocopherol; liposomes; respiratory distress syndrome pulmonary infections.

1. Introduction

This review focuses on the use of antioxidant liposomes in the general area of free radical biology and medicine. The term *antioxidant liposome* is relatively new and refers to liposomes containing lipid-soluble chemical antioxidants, water-soluble chemical antioxidants, enzymatic antioxidants, or combinations of these various antioxidants. The role of antioxidants in health and disease has been extensively discussed, and many excellent reviews and books are available ***(1–6)***.

Antioxidant liposomes hold great promise in the treatment of many diseases in which oxidative stress plays a prominent role. Oxidative stress is a physiological condition in which the production of damaging free radicals exceeds the in vivo capacity of antioxidant protection mechanisms to prevent pathophysiology. Free radicals are molecules with unpaired electrons, which are often highly reactive and damaging to biological systems. The biological membranes of subcellular organelles are major sites for free radical damage but proteins and deoxyribonucleic acid (DNA) are also significant targets. Moreover, free radicals can alter cellular signal-transduction pathways and stimulate the synthesis of inflammatory cytokines ***(7–9)***. Oxygen radicals and other reactive oxygen species (ROS) arise from the single electron reductions of oxygen.

$$O_2 + e- O_2^{*-} \quad (1)$$

$$O_2^{*-} + e^- + 2H+ \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + e^- + H+ \rightarrow H_2O + OH^* \quad (3)$$

$$OH^* + e^- + H+ \rightarrow H_2O \quad (4)$$

$$O_2^{*-} + NO \rightarrow ONOO^- \quad (5)$$

In addition, the superoxide radical (O2*) can react rapidly with nitric oxide to yield peroxynitrite as shown in **Eq. 5**. Peroxynitrite is a reactive nitrogen oxide species (RNOS) that can also cause damage to deoxyribonucleic acid (DNA), proteins, and lipid–protein complexes (i.e., biomembranes and lipoproteins). Moreover, ONOO– is likely to be generated during inflammation and the killing of bacteria. Free radicals are generated in both the aqueous and lipid compartments of cells, and to minimize their damaging effects requires both lipid- and water-soluble antioxidants. Nevertheless, the

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potential clinical use of such bifunctional liposomes has been extremely limited *(10)*.

A primary use of antioxidant liposomes has been to define the molecular mechanism of action for various antioxidants or pro-oxidants *(11–21)*. Antioxidants, such as butylated hydroxytoluene (BHT) and α-tocopherol (α-TOH), have also been used to prevent the oxidation of unsaturated fatty acid moieties in the phospholipids of liposomes during storage *(22)* or sonication *(23)*. This chapter, however, focuses on the potential therapeutic uses of antioxidant liposomes. This is a rapidly evolving area of medical research that has not been extensively reviewed. Most of the research to date has been accomplished using in vitro cell culture systems or animal models. Very few clinical trials have been attempted, yet obvious medical situations exist (e.g., protection against influenza infection and adult respiratory distress syndrome as discussed later) in which antioxidant liposomes have enormous health-related significance. The preparation of antioxidant liposomes that can be targeted to specific sites in the body is also a promising area but awaits further research. Most chemical antioxidants are phytoceuticals whose properties have already been extensively studied and are generally regarded as nontoxic and safe for human consumption *(24)*.

In the following sections, we first review the varieties of antioxidants that have either been used in antioxidant liposomes or hold the promise of such utilization. We then focus on issues relating to the modes of administration and lastly describe the clinical uses of antioxidant liposomes for diseases in which oxidative stress plays a major role. Major emphasis is placed on the use of antioxidant liposomes for pulmonary diseases.

2. Lipid-Soluble Antioxidants

The lipid-soluble antioxidants that can be incorporated into liposomes include vitamin E (TOHs and tocotrienols) *(25)*, ubiquinones *(26)*, retinoids *(27–29)*, carotenoids (e.g., lutein, β-carotene, lycopene, astaxanthin, and peridinin *[30–33]*, lipid-soluble flavonoids (e.g., quercetin, hesperetin, naringenin) *(34)*, soy isoflavones (genistein and daidzein) *(35)*, tamoxifen *(36,37)*, as well as synthetic lipid-soluble antioxidants such as BHT, tertiary-butyl hydroquinone (TBHQ), and probucol. Nitric oxide can also be incorporated into liposomes where it can inhibit free radical-mediated cholesterol peroxidation *(38)*.

TOHs can readily be incorporated into both monolayers of unilamellar liposomes in a monomeric form *(25)*. Furthermore, TOH in liposomes can undergo spontaneous intermembrane transfer to an acceptor membrane without the fusion of the TOH liposome *(25)*. This intermembrane transfer is more pronounced when the TOH liposome contains polyunsaturated fatty acids *(25)*. RRR-α-TOH and RRR-α-tocotrienol are forms of vitamin E that have the same aromatic chromanol head group but differ in the structure of their hydrocarbon tails. RRR-α-tocotrienol is, however, a better peroxyl radical scavenger than RRR-α-TOH in phosphatidylcholine liposomes *(39)*.

β-Carotene (a carotinoid) can be incorporated into liposomes to a maximum of about 0.5*M* (based on phospholipid), whereas TOH can be incorporated at levels as high as 30 mol%. The ability of β-carotene in liposomes to inhibit free radical-mediated lipid peroxidation appears, however, to be much lower than that of α-TOH *(40)*. Indeed, Chen and Djuric *(41)* found that carotenoid-containing liposomes were very sensitive to degradation by free radicals generated from iron and 2,2'-azobis(2-amidinopropane) dihydrochloride but were not protective against lipid peroxidation. β-Carotene at 0.45 mol% (of phospholipid) is, however, a more powerful inhibitor of singlet oxygen-mediated lipid peroxidation than α-TOH at 0.45 mol% *(42)*. α-TOH at 4.5*M* is, however, also effective at inhibiting both free radical lipid peroxidation as well as singlet oxygen mediated lipid peroxidation *(42)*. Singlet oxygen can be generated by photosensitizes and this ROS may contribute to light-induced skin toxicity as well as the aging of skin. Antioxidant liposomes have been proposed as a tool for optimizing photochemotherapy *(43)*.

The lipids used in the preparation of antioxidant liposomes also provide an opportunity to introduce antioxidant capacity into liposomes. For example, plasmalogens (1-alkenyl, 2-acyl-) phospholipids

are thought to have antioxidant properties *(44,45)*. Liposomes constructed with ethanolamine plasmalogen inhibit both iron- and copper-dependent peroxidation in the presence of preformed lipid hydroperoxides *(46)*. Sindelar et al. *(47)* have show that plasmalogens protect polyunsaturated fatty acids from oxidative damage and that the vinyl ether function of plasmalogens is consumed simultaneously. Koga et al. have synthesized a novel phospholipid containing a chromanol structure as its polar head group *(18,48)*. This phosphatidyl derivative of vitamin E is almost as effective an antioxidant as α-TOH in unilamellar liposomes subjected to free radicals generated in the lipid phase. The potential therapeutic value of liposomes with antioxidant phospholipids has not been explored, but this is an obvious area for future research.

A major advantage of antioxidant liposomes is their ability to simultaneously contain (and deliver) both water- and lipid-soluble antioxidants *(32)*. This is particularly important in the case of liposomes with both TOH and ascorbate (Asc) because it has been demonstrated that ascorbate can regenerate TOH from the tocopheroxyl radical (TO*) *(49)*.

$$TO^* + Asc \rightarrow TOH + Asc^* \qquad (6)$$

Junghans et al. *(32)* have used unilamellar liposomes to investigate the interaction of GSH with the lutein, β-carotene and lycopene in preventing lipid peroxidation. This group found that GSH and carotenoids interacted to improve the resistance of biological membranes toward lipid peroxidation, but the optimal level for protection varied between the different carotenoids *(32)*.

3. Water-Soluble Antioxidants

The water-soluble antioxidants that can be used in antioxidant liposomes include ascorbate (vitamin C), urate, glutathione, *N*-acetylcysteine (NAC), lipoic acid (or dihydrolipoic acid, which is its reduced form), pro-cysteine, and water-soluble flavonoids (as in pycnogenol). Dihydrolipoic acid is somewhat unique because it can quench peroxyl radicals generated both in the aqueous phase and in membranes *(50)*. Chemical antioxidants generally act by donating an electron to a free radical (thereby quenching the free radical) or by serving as a substrate for an antioxidant enzyme. Glutathione, for example, is itself an antioxidant *(12)* and can also function as a substrate for glutathione peroxidase, a key (selenium-containing) antioxidant enzyme that converts lipid hydroperoxides (LOOHs) or H_2O_2 into the corresponding lipid alcohols (LOHs) or H_2O. Chemical antioxidants can also be chelators of transition metal ions that catalyze lipid peroxidation reactions. Urate, which is present at very high concentrations in human plasma, is an excellent antioxidant that can both chelate transition metal ions and also quench aqueous free radicals *(51–53)*.

4. Entrapped Antioxidant Enzymes

The application of antioxidant liposomes to problems of medical interest has primarily been with liposomes containing entrapped antioxidant enzymes. Recombinant biotechnology has provided the means to obtain large (i.e., commercial) quantities of human antioxidant enzyme, but these enzymes do not normally penetrate the plasma membrane of cells and have a short half-life when introduced into the body by intravenous injection. Turrens has reviewed the potential of antioxidant enzymes as in vivo pharmacological agents *(54)*. The attachment of polyethylene glycol (PEG) to antioxidant enzymes increases their in vivo half-lives and their effectiveness in preventing pulmonary oxygen toxicity in rats *(55)*. The various procedures for preparing liposomes with entrapped antioxidant enzymes have been evaluated by Aoki et al. *(56)*. This group and others *(57)* have found that positively charged liposomes have a superior trapping efficiency for superoxide dismutase (which has a negative charge).

Early work by Freeman et al. *(58)* has shown that porcine aortic endothelial cells treated with liposomes with entrapped superoxide dismutase (SOD) liposomes can dramatically increase their cellular SOD levels and thereby protect the cells from oxygen-induced injury. In a key paper, Beckman et al. *(59)* found that endothelial cells treated with liposomes containing entrapped SOD and catalase (SOD + CAT liposomes) can increase

the cellular-specific activity of these enzymes by at least 40-fold within 2 h. These results are particularly important because endothelial cells are major sites for oxidative damage. Moreover, intravenous antioxidant liposomes would certainly make contact with vascular endothelial cells under in vivo conditions.

5. Modes of Administration

Antioxidant liposomes can be administered topically, intratracheally, intravenously, by inhalation in an aerosol form, subcutaneously, or by intramuscular injection. Topical administration can certainly be long term and is of considerable interest to the cosmetic industry in treating specific skin disorders such as psoriasis. α-Tocopheryl acetate in liposomes has been found to have a better dermal absorption than free α-tocopheryl acetate ***(60)***. Topical administration of antioxidant liposomes could also be useful in situations where individuals were exposed to toxic substances causing skin damage by free radical mechanisms (e.g., chemical warfare agents). Inhalation and intratracheal administration can be useful for those situations in which pulmonary tissues are subjected to oxidative stress, such as with influenza infection or inhalation of toxic substances such as paraquat, which is quaternary nitrogen herbicide ***(2,10)***.

Intravenous administration would primarily be limited to situations in which oxidative stress is a component of an acute trauma or disease. The intravenous use of antioxidant liposomes has the potential for rapidly increasing the plasma and tissue concentration of antioxidants far beyond what oral administration could achieve. Moreover, the proteolytic and bioselective processes of the gastrointestinal tract do not limit the types of antioxidants that can be administered via intravenous antioxidant liposomes. For example, it is known that plasma levels of α-TOH are about 10 times higher than the levels of γ-TOH despite the fact that dietary levels of γ-TOH are at least two times that of α-TOH. Nevertheless, γ-TOH has a unique chemical ability to detoxify peroxynitrite that is not shared with α-TOH ***(61)***. Peroxynitrite is a powerful oxidant formed by the reaction of nitric oxide with superoxide radicals (*see* **Eq. 5**) and may be an important mediator of acute oxidant tissue damage. It is reasonable to suspect, therefore, that medical situations could arise in which it would be desirable to rapidly increase plasma (and tissue) levels of γ-TOH. The poor bioavailability of orally administered γ-TOH makes this very difficult to accomplish. This limitation could, however, be overcome by the intravenous administration of liposomes containing γ-TOH.

Vitamin E used in oral supplements is often in the form of a tocopheryl ester such as tocopheryl acetate or tocopheryl succinate. Tocopheryl esters are not, however, absorbed and must first be acted on by intestinal esterases to liberate the unesterified TOH. It is interesting, therefore, that α-tocopheryl succinate, but not α-TOH, has been found to inhibit the activation of nuclear factor κB (NFκB) in cultured macrophages ***(62)***.

NFκB is a key transcription factor that regulates the expression of many inflammatory cytokines. α-Tocopheryl succinate can be incorporated into liposomes and intravenous injection would deliver this form of vitamin E to phagocytic cells ***(63)***. Oral administration of tocopheryl succinate would not, however, be expected to deliver this form of vitamin E to cells.

It is very significant that Cu,ZnSOD liposomes administered by intravenous injection can penetrate the blood–brain barrier and significantly elevate brain levels of SOD activity within 24 h ***(64,65)***. Moreover, the intravenous administration of Cu,ZnSOD liposomes to rats can reduce cerebral infarction caused by ischemia ***(65)*** and also inhibit learning dysfunction caused by a low dose of total body irradiation ***(66)***. Surprisingly, intraperitoneal injection of SOD liposomes has also been found to increase the brain levels of SOD in gerbils and to inhibit ischemia/reperfusion oxidative stress ***(67)***.

A major problem with conventional liposomes is that they are recognized by the immune system as foreign substances and are rapidly removed from circulation by the phagocytic cells of the reticuloendothelial system. The Kupffer cells of the liver are the most abundant population of phagocytic cells in the body. In some circumstances,

however, the uptake of conventional liposomes by hepatic Kupffer cells can actually be an advantage. Carbon tetrachloride (CCl_4), for example, is known to induce hepatotoxicity by a free-radical-mediated mechanism. Yao et al. ***(63)*** found that intravenous administration of liposomes containing vitamin E (TOH liposomes) was very effective in decreasing mortality in mice given a lethal dose of $CC1_4$. The TOH liposomes were found to primarily accumulate in the Kupffer cells of the liver.

In recent years considerable advances have been made in the design of stealth liposomes that are not recognized by the immune system and, therefore, have a much longer half-life in circulation than conventional liposomes. Stealth technology employs liposomes with a polymer coating of polyethylene glycol-phosphatidylethanolamine (PEG liposomes). Recently, the preparation of pH-sensitive stealth liposomes has been described ***(68)***. These liposomes have a prolonged circulation in vivo and destabilize at mildly acidic pH thereby being particularly efficient at delivering a water-soluble compound into a cell's cytoplasm. The use of stealth antioxidant liposomes is very new with an increasing commercial interest in their potential therapeutic applications.

Corvo et al. ***(69)*** have studied the practical aspects of subcutaneous SOD–PEG liposomal delivery with the aim of maximizing their therapeutic activity in a rat model of chronic arthritis. Rheumatoid arthritis is an autoimmune disease affecting the joints and involving the generation of damaging ROS. Antioxidants have, therefore, been proposed as potential therapeutic agents ***(70)***. Liposome size was found be the most important factor influencing the rate and extent of drainage of liposomes from the subcutaneous injection site as well as uptake by an arthritic site. Small-sized SOD–PEG liposomes(110 nm) were much more effective at targeting the arthritic site than large-sized SOD–PEG-liposomes (450 nm) ***(70)***.

In addition to encapsulating SOD (or other antioxidant enzymes) within liposomes, it is also possible to create liposomes in which these enzymes present their enzymatic activity on the external surface of liposomes. Gaspar et al. ***(71)*** have termed these liposomes with surface exposed enzymes *enzymosomes*. This group covalently linked fatty acid chains to the accessible epsilon-amino groups of the SOD (Ac-SOD). The resulting Ac-SOD was incorporated in conventional and long-circulating liposomes (Ac-SOD liposomes), which presented SOD activity on their external surfaces ***(71)***. Enzymosomes may provide a novel therapeutic tool in which enzyme release from the aqueous liposomal compartment is not required.

The ability to target liposomes to specific tissues has been the topic of considerable research. In highly imaginative work, Galovi-Rengel et al. ***(72)*** have encapsulated SOD into mucoadhesive chitosan-coated liposomes to increase their releasing time and to facilitate their cellular penetration. Chitosan is a natural aminopolysaccharide product derived from chitin, which is found in the exoskeleton of shellfish such as shrimp or crabs. Chitosan has mucoadhesive properties that have been exploited in targeting drug delivery to mucosal tissues.

This type of antioxidant-chitosan liposome could prove useful for preventing radiation damage to the esophageal lining during chemoradiotherapy for non-small-cell lung carcinoma or for protecting the esophageal lining from mustard gas toxicity.

6. Antioxidant Liposomes and Oxidative Stress

Increasing evidence suggests that oxidative stress is an important factor in the aging process and in the etiology of many chronic diseases, such as atherosclerosis, ischemic heart disease ***(73)***, rheumatoid arthritis ***(70)***, and cancer ***(74,75)***. Schwartz et al. ***(76)*** at the Harvard School of Dental Medicine have used the hamster cheek pouch tumor model to explore the potential anticancer use of various antioxidants. This group found that β-carotene liposomes injected into the oral squamous cell carcinoma of the hamster caused a lysis of the tumor cells but not of normal cells ***(76)***. Retinoids have also been shown to be clinically effective in treating diverse premalignant and malignant conditions, such as cutaneous T-cell lymphomas, leukoplakia, squamous cell carcinomas of the skin, and basal cell carcinomas ***(77,78)***.

Several investigators have documented dramatic improvement in patients with acute promyelocytic leukemia after treatment with all-*trans*-retinoic acid *(79–81)*. However, the side effects of oral all-*trans*-retinoic acid therapy are similar to effects seen with vitamin A: headaches, other central nervous system problems, and dryness of mucosal tissues, erythema, and desquamation of skin. When incorporated in liposomes, all-*trans*-retinoic acid-associated toxicity is markedly reduced, whereas the antitumor properties (i.e., growth inhibition and differentiation induction) of all-*trans*-retinoic acid are maintained or even enhanced *[82,83]*). Phase I and phase II clinical studies found that plasma levels of all-*trans*-retinoic acid were maintained at high concentrations even after prolonged treatment of patients with all-*trans*-retinoic acid liposomes *(84)*. In general, the use of retinoids is safe and induces complete remission in 80 to 90% of acute promyelocytic leukemia patients. However, chronic oral administration results in reduced plasma levels associated with disease relapse in the majority of patients; this can be circumvented by using all-*trans*-retinoic acid liposomes.

Oxidative stress also contributes to the pathology observed in acute medical problems, such as heart attack *(73,85–88)*, respiratory distress syndrome *(89)*, trauma *(90)*, irradiation *(66)*, cold injury *(91)*, and certain types of infectious diseases such as influenza and HIV infection. Evidence suggests that trauma to the brain results in the overproduction of superoxide radicals that may contribute to edema *(92,93)*. Antioxidant liposomes containing SOD have been used effectively to treat posttraumatic brain edema *(92,93)* and neurological dysfunctions in rats *(94)*.

Retinopathy of prematurity is a leading cause of blindness in premature and low-birthweight infants who are often treated with high levels of oxygen owing to surfactant deficiency. Considerable evidence *(95–97)* indicates that oxidative stress is a major contributor to this disease. In an animal model, Niesman et al. *(98)* found that intraperitoneal administration of SOD–PEG liposomes resulted in a significant increase in retinal SOD activity and an improved tolerance to high oxygen levels. Despite the enormous health-related significance, there are no clinical trials testing the efficacy of antioxidant liposomes to treat retinopathy of prematurity.

7. Pulmonary Applications of Antioxidant Liposomes

7.1. Potential Clinical Applications

Premature children often suffer from respiratory distress syndrome because they lack the capacity to synthesize pulmonary surfactant *(99)*. Surfactant is necessary to maintain proper expansion of the small air sacs in the lungs. If surfactant levels are low, the small air sacs in the lungs collapse resulting in poor oxygen delivery (hypoxia) to tissues. Infants deficient in surfactant therefore require treatment with high levels of oxygen to prevent damage to their vital organs. Unfortunately, premature infants are often deficient in antioxidants that are necessary to protect organs from injury caused by high concentrations of oxygen. The combination of surfactant deficiency and the presence of oxygen free radicals promote the development of chronic lung disease (bronchopulmonary dysplasia or BPD). BPD is a major cause of morbidity and mortality in premature infants. An estimated 50% of all neonatal deaths result from BPD or its complications. In the adult form of respiratory distress syndrome (ARDS), antioxidants such as *N*-acetylcysteine are recognized for their role in reducing the duration of acute lung injury *(100,101)*. The rationale for using antioxidant liposomes to treat respiratory distress in premature infants or adults is certainly compelling and supported by the animal models detailed below. However, almost no clinical trials have been initiated.

7.2. Animal Models

Shek et al. *(102)* have discussed the general application of liposomes for improved drug delivery to pulmonary tissues. These authors point out that the delivery of drugs to the lung via liposomes is particularly useful because it can minimize extrapulmonary side effects and potentially result in increased drug retention time. In addition (as discussed previously), liposomes for delivery by inhalation or instillation can encapsulate enzyme

and/or chemical substances that cannot be delivered by an oral route. Smith and Anderson *(103)* demonstrated that intratracheally administered liposomes (with phosphatidyl choline, cholesterol, and stearylamine) have a long retention time (more than 5 d) in the mouse lung. Liposomes with entrapped Cu,Zn SOD and CAT (Cu,ZnSOD + CAT) liposomes were intratracheally instilled in rabbits and the alveolar distribution of the antioxidants measured after 4 and 24 h *(104)*. The results indicate that Cu1nSOD + CAT liposomes could increase both SOD and CAT activities in distal lung cells, including alveolar type I, alveolar type II cells, and macrophages. More recent studies by Walther et al. *(105)* have shown that intratracheal administration of CuZn–CAT liposomes to premature rabbits can increase the lung SOD activity and protect against hyperoxic lung injury. Moreover, intratracheal delivery of SOD liposomes or CAT liposomes does not down-regulate mRNA synthesis of these enzymes in the premature rabbit lung *(106)*.

Archer et al. *(107)* have made effective use of the isolated perfused rat lung to study the role of oxygen radicals in modulating pulmonary vascular tone. This group showed that the generation of oxygen radicals (from xanthine–xanthine oxidase) decreased pulmonary vascular presser response to alveolar hypoxia. Either pretreatment of the lung with desferrioxamine or a mixture of superoxide and CAT liposomes inhibited decreases in pulmonary vascular reactivity. SOD administered free in solution or combined with CAT in liposomes, increased the normoxic pulmonary arterial pressure and enhanced vascular reactivity to angiotensin 11 and hypoxia *(107)*.

In a rat model, Freeman et al. *(108)* have shown that intravenous injection of SOD liposomes or CAT liposomes can increase (two- to fourfold) the lung-associated specific activity of these antioxidant enzymes and also provide resistance to oxygen injury. Intravenous injection of nonentrapped (i.e., free) SOD or CAT (in the absence or presence of control liposomes) neither increased the specific lung activities of these enzymes nor provided resistance to oxygen toxicity. Similarly, intratracheal administration of SOD liposomes or CAT liposomes (negatively charged and multilamellar) to rats resulted in a significant elevation of lung SOD or CAT activity as well as resistance to pulmonary oxygen toxicity *(109)*.

Bamard et al. *(110)* have demonstrated that instillation of cationic SOD + CAT liposomes in a rabbit model was effective in preventing the increase in pulmonary filtration coefficient (a sensitive index of microvascular permeability) owing to free-radical-initiated lung injury. Repair of lung injury was inhibited by inhalation of elevated oxygen concentrations. This is of particular importance to the preterm human infant who may be exposed to elevated oxygen concentrations for weeks or months that could result in the chronic pneumopathy known as bronchopulmonary dysplasia. Treatment with liposome-encapsulated SOD and CAT conferred protection against the cytotoxic effects of 50 and 95% oxygen *(111,112)* and also protection against cell death *(113)*.

Briscoe et al. *(114)* have evaluated the delivery of SOD to cultured fetal rat pulmonary epithelial cells via pH-sensitive liposomes. A fivefold increase in cellular SOD activity was observed after incubating the cultured cells with the pH-sensitive SOD liposomes *(114)*. Fetal pulmonary epithelial cells express a high affinity receptor for surfactant protein A (SP-A). This receptor can be used to target liposome delivery to these cells by incorporating SP-A during the preparation of the SOD liposomes *(114,115)*. The presence of SP-A in the SOD liposomes facilitates their uptake by pulmonary epithelial cells *(114,115)*.

Considerable evidence suggests that oxidative injury to lung tissues can be mediated by neutrophils *(116)*. Phorbol myristate acetate (PMA) has often been used to induce neutrophil-mediated lung injury in animal models, It is significant, therefore, that liposomes (dipalmitoylphosphatidylcholine) with α-TOH are able to counteract some PMA-induced lung injury in a rat model *(116)*. In contrast, rats pretreated with blank liposomes (no α-TOH) showed no protection from PMA-induced lung injury *(116)*.

Paraquat has also been used to induce oxidative lung injuries in animal models *(2,10)*. Suntres and Shek *(10)* have compared the ability of α-

TOH liposomes or liposomes with both α-TOH and glutathione (TOH + GSH liposome) to inhibit paraquat-induced lung damage in a rat model. Lung damage was assessed by increases in lung weight (caused by edema) and decreases in lung activities of angiotensin-converting enzyme (ACE) that reflects damage to endothelial and alveolar type II epithelial cells. These investigators found that both TOH liposomes and TOH + GSH liposomes were equally effective in preventing loss of lung ACE activity but that TOH + GSH liposomes were more effective in preventing injury to alveolar type II epithelial cells *(10)*. Interestingly, neither antioxidant liposome was effective in preventing lung edema *(10)*.

Liposomes encapsulated with CAT have also been found to be efficacious in preventing chronic pulmonary oxygen toxicity in young rats *(117)*. In this work, rats were treated with 100% oxygen for 8 d and also given daily intratracheal injections of the CAT liposomes (with 160 U of CAT) that prevented chronic lung toxicity. Liposomes encapsulated with SOD or with lower levels of CAT (50 or 70 U) did not prevent the chronic lung changes. SOD + CAT liposomes are also effective in protecting lung tissues from bleomycin-induced injury as evidenced by decreased levels of lipid peroxidation products *(118)*.

Muzykantov *(119,120)* has pointed out that pulmonary vascular endothelial cells are not readily accessible from the airways and protecting them from oxidative stress is better accomplished through the circulatory system. To improve both the targeting and intracellular delivery to endothelium, this investigator has used a strategy in which antioxidant enzymes are first conjugated with antibodies against endothelial antigens, such as ACE, or adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or platelet endothelial cell adhesion molecule-1 (PECAM-1). Muzykantov reports that these immunoconjugates accumulate in the pulmonary vasculature of intact animals, enter endothelium, and increase antioxidant defense capacity *(120)*. Moreover, the ICAM-1 and PECAM-1 immunoconjugates could potentially decrease inflammatory processes in the lung by decreasing the infiltration of leukocytes *(120)*.

7.3. Antioxidant Liposomes for the Treatment of Pulmonary Infections

As detailed above, there is an increasing body of information on the role of antioxidant liposomes in modulating pathophysiological processes in the lung. It is not surprising, therefore, that various researchers have explored the potential role of antioxidant liposomes in preventing pulmonary damage during lung infections. Suntres et al. *(121,122)* have used lipopolysaccharide (LPS)-induced lung injuries as a model in which to study the potential prophylactic role of antioxidant liposomes. This is a very reasonable model because Gram-negative bacteria have LPS as a component of their cell wall, and it is a potent stimulus for the generation of ROS and RNOS by phagocytic cells. LPS-induced lung injury is an excellent model for acute respiratory distress syndrome caused by sepsis. Suntres and Shek *(121)* found that pretreating Sprague-Dawley rats with α-TOH liposome, by intravenous administration, could significantly reduce LPS-induced lung injury. This group also found that liposome-entrapped dexamethasone was effective in preventing LPS-induced lung injury in rats *(122)*.

8. Future Directions

Influenza is a viral disease that affects the respiratory tract. The three types of influenza viruses are designated A, B, and C, with the A and B types primarily being responsible for the yearly winter epidemics. The influenza viruses continually mutate over time causing antigenic drift, which can result in large populations of people being devoid of antibody protection. The resulting periodic pandemics can cause large numbers of deaths. For example, the 1918–1919 influenza pandemic resulted in the death of approx 20 million people worldwide. Increasing evidence suggests that free radical production and lipid peroxidation play a major role in the damage caused by influenza infection *(123–125)*. In particular, influenza infection is accompanied by an increase in the production of superoxide radicals and decrease in the activity of

SOD that removes superoxide radicals *(126)*. It has been suggested that the high production of ROS in the lung during influenza infection could inactivate protease inhibitors resulting in a damaging increase in protease activity *(127)*. Administration of SOD has been found to be very effective in preventing mortality owing to influenza infection in animal models *(128)*. It has also been suggested that antioxidants along with protease inhibitors could be useful in the treatment of severe influenza infection *(129)*. It is surprising, therefore, that antioxidant liposomes have not yet been used for the treatment of severe influenza infection. This is certainly an area requiring further investigation considering its enormous health-related significance. Also of interest is that the influenza virus causes apoptosis in macrophages that can be prevented by the antioxidants NAC or pyrrolidine dithiocarbamate *(130)*. Liposomes are readily phagocytized by macrophages, and hence this cell type is a natural target for antioxidant liposomes. Similarly, antioxidant liposomes are very likely to be effective in ameliorating the pathophysiology associated with severe acute respiratory syndrome (SARS), which is caused by an atypical coronavirus. SARS has an extremely high mortality rate (currently up to 15–19%) and has expanded to over 25 countries.

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Liver Tocopherol Levels for CEES Reasearch 05/11/2005

Concentration of Tocol	**47.46ng/ml**
Responese Factor of alpha-Tocopherol	**2.79 relative to Tocol**
Responese Factor of gamma-Tocopherol	**1.236 relative to Tocol**
MW of alpha-Tocopherol	**430.69**
MW of gamma-Tocopherol	**416.66**

Note: Content means ng of tocopherols per mg of tissue

Sample		Sample Weight		Tocol				alpha-Tocopherol				gamma-Tocopherol			
				Volume	Conc.	Weight	Area	Area	Content			Area	Content		
ID		mg	ul	ul	ng/µl	ng			µg	ng/mg	Avg (ng/mg)		µg	µg/mg	Avg (ng/mg)
LIVER SAMPLE															
Rat1ETOH 03/10/05	1A	101.1	500	10	47.46	474.600	25319	57221	2.993	29.600	**30.005**	9804	0.227	2.247	**2.357**
Rat1ETOH 03/10/05	1A	101.1	500	10	47.46	474.600	24963	57961	3.074	30.410		10614	0.249	2.467	
Rat1B 04/13/05	1B	106.2	500	10	47.46	474.600	36059	75116	2.758	25.973	**26.720**	10993	0.179	1.684	**1.688**
Rat1B 04/13/05	1B	106.2	500	10	47.46	474.600	34085	75088	2.917	27.467		10437	0.180	1.691	
Rat1ETOH/DPBS3/10/05	1C	97.4	500	10	47.46	474.600	31990	76826	3.180	32.649	**32.485**	11823	0.217	2.226	**2.154**
Rat1ETOH/DPBS3/10/05	1C	97.4	500	10	47.46	474.600	31574	75065	3.148	32.321		10914	0.203	2.082	
Rat10.7mg/kgHD03/30/05	1D	106.8	500	10	47.46	474.600	34806	75018	2.854	26.722	**26.252**	8906	0.150	1.405	**1.389**
Rat10.7mg/kgHD03/30/05	1D	106.8	500	10	47.46	474.600	34694	72143	2.753	25.781		8667	0.147	1.372	
Rat11.4mg/kgHD03/24/05	1E	97.1	500	10	47.46	474.600	37203	64087	2.281	23.491	**24.349**	7609	0.120	1.236	**1.236**
Rat11.4mg/kgHD03/24/05	1E	97.1	500	10	47.46	474.600	33690	62274	2.448	25.207		6891	0.120	1.236	
Rat11.4mg/kgHD04/11/05	1F	108.2	500	10	47.46	474.600	33450	69105	2.736	25.282	**25.022**	6583	0.115	1.067	**1.019**
Rat11.4mg/kgHD04/11/05	1F	108.2	500	10	47.46	474.600	34905	70625	2.679	24.761		6248	0.105	0.970	
Rat2 CEES02/17/05	2A	107.0	500	10	47.46	474.600	36286	80211	2.927	27.355	**27.301**	9502	0.154	1.436	**1.332**
Rat2 CEES02/17/05	2A	107.0	500	10	47.46	474.600	35728	78663	2.915	27.246		8009	0.131	1.229	
Rat2 6mg/kgCEES03/9/05	2B	106.9	500	10	47.46	474.600	33959	65299	2.546	23.818	**23.296**	7565	0.131	1.222	**1.067**
Rat2 6mg/kgCEES03/9/05	2B	106.9	500	10	47.46	474.600	34060	62623	2.435	22.774		5652	0.097	0.911	
Rat2 6mg/kgCEES03/10/05	2C	106.2	500	10	47.46	474.600	33691	80010	3.145	29.610	**30.025**	12339	0.215	2.023	**1.867**
Rat2 6mg/kgCEES03/10/05	2C	106.2	500	10	47.46	474.600	33249	81172	3.233	30.439		10299	0.182	1.711	
Rat2 4mg/kgCEES03/23/05	2D	103.7	500	10	47.46	474.600	31761	55559	2.316	22.336	**22.449**	7861	0.145	1.400	**1.377**
Rat2 4mg/kgCEES03/23/05	2D	103.7	500	10	47.46	474.600	32108	56734	2.340	22.562		7680	0.140	1.353	

Rat2 0.7mg/kgHD03/30/05	2E	105.5	500	10	47.46	474.600	32487	53971	2.200	20.851	**21.741**	5487	0.099	0.939	**0.999**
Rat2 0.7mg/kgHD03/30/05	2E	105.5	500	10	47.46	474.600	31588	56956	2.388	22.631		6017	0.112	1.059	
Rat2 0.7mg/kgHD04/11/05	2F	104.9	500	10	47.46	474.600	24283	64275	3.505	33.412	**33.420**	7800	0.188	1.796	**1.844**
Rat2 0.7mg/kgHD04/11/05	2F	104.9	500	10	47.46	474.600	25114	66507	3.507	33.428		8493	0.198	1.891	
Rat2 1.4mg/kgHD04/13/05	2G	102.6	500	10	47.46	474.600	28608	44842	2.076	20.229	**20.306**	5751	0.118	1.149	**1.125**
Rat2 1.4mg/kgHD04/13/05	2G	102.6	500	10	47.46	474.600	29493	46580	2.091	20.383		5678	0.113	1.101	
Rat2 HD 04/24/05	2H	106.2	500	10	47.46	474.600	30336	61609	2.689	25.322	**25.920**	7565	0.146	1.377	**1.317**
Rat2 HD 04/24/05	2H	106.2	500	10	47.46	474.600	28305	60202	2.816	26.519		6439	0.133	1.257	
Rat3 B 04/24/05	3A	108.5	500	10	47.46	474.600	32239	50520	2.075	19.124	**19.073**	13371	0.243	2.242	**1.818**
Rat3 B 04/24/05	3A	108.5	500	10	47.46	474.600	31383	48916	2.064	19.022		8086	0.151	1.393	
Rat3 6mg/kgCEES03/09/05	3B	105.7	500	10	47.46	474.600	30799	61444	2.642	24.992	**24.458**	8271	0.158	1.490	**1.489**
Rat3 6mg/kgCEES03/09/05	3B	105.7	500	10	47.46	474.600	31290	59754	2.529	23.923		8386	0.157	1.487	
Rat3 6mg/kgCEES03/23/05	3C	103.2	500	10	47.46	474.600	30482	45184	1.963	19.019	**18.847**	7505	0.144	1.400	**1.359**
Rat3 6mg/kgCEES03/23/05	3C	103.2	500	10	47.46	474.600	30778	44796	1.927	18.675		7134	0.136	1.318	
Rat3 4mg/kgCEES03/10/05	3D	102.4	500	10	47.46	474.600	31018	50837	2.170	21.193	**21.293**	7230	0.137	1.335	**1.385**
Rat3 4mg/kgCEES03/10/05	3D	102.4	500	10	47.46	474.600	30732	50840	2.191	21.392		7696	0.147	1.435	
Rat3 1.4mg/kgHD03/24/05	3E	104.6	500	10	47.46	474.600	29419	57742	2.599	24.846	**24.937**	9459	0.189	1.803	**1.788**
Rat3 1.4mg/kgHD03/24/05	3E	104.6	500	10	47.46	474.600	30374	60052	2.618	25.028		9604	0.185	1.773	
Rat3 1.4mg/kgHD03/30/05	3F	100.7	500	10	47.46	474.600	25945	44184	2.255	22.393	**22.588**	6813	0.154	1.530	**1.446**
Rat3 1.4mg/kgHD03/30/05	3F	100.7	500	10	47.46	474.600	26614	46113	2.294	22.783		6223	0.137	1.362	
Rat3 1.4mg/kgHD04/13/05	3G	74.1	500	10	47.46	474.600	23430	35818	2.024	27.318	**26.633**	5087	0.127	1.719	**1.815**
Rat3 1.4mg/kgHD04/13/05	3G	74.1	500	10	47.46	474.600	24278	35254	1.923	25.948		5860	0.142	1.911	
Rat3 0.7mg/kgHD04/11/05	3H	106.4	500	10	47.46	474.600	33129	50219	2.007	18.865	**19.111**	5272	0.093	0.877	**0.908**
Rat3 0.7mg/kgHD04/11/05	3H	106.4	500	10	47.46	474.600	32239	50146	2.060	19.357		5487	0.100	0.938	
Rat4 4mg/kgCEES03/23/05	4B	107.9	500	10	47.46	474.600	27902	64861	3.078	28.527	**29.104**	11565	0.243	2.253	**2.357**
Rat4 4mg/kgCEES03/23/05	4B	107.9	500	10	47.46	474.600	26321	63661	3.203	29.681		11917	0.266	2.461	
Rat4 6mg/kgCEES03/10/05	4C	105.2	500	10	47.46	474.600	35019	58525	2.213	21.036	**20.730**	5530	0.093	0.881	**0.934**
Rat4 6mg/kgCEES03/10/05	4C	105.2	500	10	47.46	474.600	35282	57252	2.149	20.425		6254	0.104	0.988	
Rat4 0.7mg/kgHD03/24/05	4D	105.7	500	10	47.46	474.600	31399	59190	2.496	23.615	**23.463**	5075	0.095	0.897	**0.898**
Rat4 0.7mg/kgHD03/24/05	4D	105.7	500	10	47.46	474.600	30782	57278	2.464	23.310		4989	0.095	0.899	
Rat4 0.7mg/kgHD04/11/05	4E	107.9	500	10	47.46	474.600	30985	57098	2.440	22.614	**22.983**	8858	0.168	1.554	**1.523**
Rat4 0.7mg/kgHD04/11/05	4E	107.9	500	10	47.46	474.600	29541	56212	2.520	23.351		8105	0.161	1.492	
Rat4 A 04/13/05	4F	105.6	500	10	47.46	474.600	24930	58661	3.116	29.505	**29.262**	9582	0.225	2.135	**2.190**
Rat4 A 04/13/05	4F	105.6	500	10	47.46	474.600	23751	54967	3.064	29.019		9601	0.237	2.246	

SECTION 4: Salil Das, DSc, Meharry Medical College

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

Abstract

The exact mechanism by which mustard gas exposure causes ARDS is not well known. The present study indicates that CEES, a mustard gas analog causes lung injury and significantly decreases expression and activity.of cholinephosphotransferase (CPT), the terminal enzyme in CDP-choline pathway for phosphatidylcholine synthesis. This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating the expression of CPT. We have previously shown that exposure of CEES to guinea pigs causes an increase in the levels of TNF-α and NF-κB in the lung within an hour. However, NF-κB disappeared after 2 hours indicating an intricate interplay of pro- and anti- apoptotic inflammatory cytokines. In the present study, we utilized a state of the art cytokine array technology to identify other cytokines affected by CEES exposure. The array of cytokine induction within an hour of CEES exposure and dynamic changes in cytokine profile by one day post CEES exposure reveals that following an initial damage, the lung tissue tries to recover and prevent further damage through self defense mechanisms mediated through various classes of cytokines.

Introduction and Body

Mustard gas is a poisonous chemical agent that exerts a local action on eyes, skin and respiratory tissue followed by impairment of nervous, cardiac and digestive system in humans and laboratory animals [1-4]. Sulfur mustard disrupts and impairs a variety of cellular activities. Inhalation of mustard gas causes hemorrhagic inflammation to the tracheobronchial tree with severe pulmonary complications including adult respiratory distress syndrome (ARDS) [5]. Most deaths are due to secondary respiratory infections. Besides its use in World war I and World war II, sulfur mustard has been used on Iranian soldiers, on civilians during the Gulf war and on the Iranian-occupied village of Halabja as a vesicant chemical warfare agent resulting in many civilian casualties [6,7]. Mustard agents are also harmful in long-term exposure at low doses. Long term exposure of mustard gas may lead to lung cancer as indicated by the studies on Japanese who worked in poison gas factories [8]. Unfortunately, the molecular mechanisms of carcinogenesis in former poison gas workers remains unclear [9], and the attempts to seek confirmatory and substantial evidence in laboratory animals for links between mustard gas exposure and cancer have not yielded consistent results [10].

Several studies in rats and mice have shown that the mechanism of mustard gas action on lung, skin or other organs includes DNA alkylation; cross linking of DNA [11]; activation of proteases resulting in proteolysis of several vital intracellular enzymes and structural proteins [12]; production of free radicals and free radical-mediated oxidative stress [13,14]; inflammation [15]; and activation of tumor necrosis factor (TNF-α), a part of the inflammatory cytokine cascade [16,17]. It appears that the initiation of free radical-mediated TNF-α cascade is the major pathway in the mustard gas mediated ARDS.

We have established that structurally and functionally, guinea pig lungs are more alike to human lungs in comparison to other animal species [18]. Therefore, we developed the guinea pig model to understand the mechanisms of mustard gas mediated lung injury with particular emphasis on the down stream signal transduction events [19,20]. Our results clearly demonstrate a complex signal transduction pathway in mustard gas mediated lung injury. After intratracheal injection of CEES to guinea pigs, TNF-α level increased sharply within one hour of exposure.

TNF-α level started declining after one hour and returned to basal levels within 24 hours. After the accumulation of TNF-α, both acid and neutral sphingomyelinase activities were stimulated, and both peaked within 4 to 6 hours after CEES exposure. Though both the acid and neutral sphingomyelinase activities were stimulated, the level of acid sphingomyelinase was found to be much higher after CEES exposure. In comparison to lung tissue, lung macrophages contain higher levels of TNF-α and sphingomyelinases and this may be due to fact that lung tissue consists of several types of cells not all of which are responsive to CEES.

As the sphingomyelinase activity increased, there was an accumulation of ceramides. Ceramide levels increased within one hour of CEES exposure. However, there was a slight fall in the ceramide level between 3-6 hours; it increased again at high level even up to 14 days after CEES exposure. It is not known at this time what is the physiological significance of this 14 days elevation of ceramides. The slight drop in the ceramide level between 3 and 6 hours might be due to the activation of NF-kB, which showed a sharp transient activation at 1 to 2 hours after CEES exposure. The activation of NF-kB coincided with the increase of TNF-α in lung tissue.

It is well known that TNF-α is proapoptotic [21] and NF-κB acts as antiapoptotic by opposing the TNF-α induced apoptosis [22-24]. Here, we observed a biphasic effect of CEES on lung. After the initial damage by TNF-α, there was some recovery due to activation of NF-κB within 2 hours. This biphasic pattern was also observed in caspases activation. Significant but small activation of caspase 2, caspase 3, caspase 8 and caspase 9 were observed within 1 hour of CEES exposure. This activation of caspases declined thereafter and reappeared in between 4-6 hours, initiating cell apoptosis in lung as observed by light as well as electron microscopy (paper communicated). This second phase of caspase activation disappeared within 24 hours and we could not observe any further activation of any of the above caspases. This type of biphasic action has been observed in mustard gas induced skin lesions also [25], where an initial phase of injury after 1 hr is followed by a delayed phase which becomes evident after 8 hr of exposure. Our results explain this biphasic action of mustard gas and delineate the events leading to cell death after mustard gas exposure.

Our study thus indicates that CEES exposure causes accumulation of TNF-α which thereby activates sphingomyelinases resulting in the production of ceramides and simultaneous activation of caspases, and finally apoptosis. Ceramides are known to cause apoptosis via activation of caspases (26-28). The present study revealed that there was some initial damage of the lung tissue when exposed to CEES but self-defense mechanism/s of lung tried to recover from the damage and prevent from further damage. The balance between these two opposite effects determines the extent of damage to the tissue. Furthermore, the present investigation enhances our understanding of mustard gas mediated pro-apoptotic signaling pathways and characterizes the events of mustard gas induced lung dysfunction. The results presented here provide a molecular and cellular basis for developing strategies for pharmacological intervention, with potential of clinical application. As the effects of CEES is dose-dependent, it will be beneficial to design the drugs which not only block CEES induced intracellular signal transduction events, but which also directly reduce the contact of CEES on lung surface. We are in progress of developing some aerosols containing a mixture of drugs which will not only prevent the CEES induced signaling events, but also chemically inactivate/modify CEES within lung before it reaches and interacts with the lung cells.

It is known that skin injured by mustard gas exposure can be treated by rapidly decontaminating or detoxifying the contaminated sites and applying ointment to the surface. However, those kinds of treatments are not possible for lung injury and no prophylactic treatment has been available for pulmonary injury by mustards. Due to this lack of a prophylactic

treatment, the battlefield soldier is at risk for pulmonary injury from mustards. We have tested several antioxidants and decided to study NAC in details as that was found to be most effective [20]. A single dose of NAC just before the exposure could not prevent any of the CEES mediated signal transduction events, but pretreatment of animals for 3 days prior to CEES exposure was highly effective in preventing the early signaling steps of CEES-mediated lung injury. Treatment for a longer period (30 days) with NAC provided additional protection. Although the oral administration of NAC could not block all of the CEES mediated signal transduction events, inhibition was sufficient to prevent the ultimate lung damage as observed by histochemical studies. The failure of single dose of NAC just before CEES exposure was not unexpected since systemic levels of NAC were insufficient. This delay offered ample time for CEES to initiate signal transduction and progress of the lung injury.

Protection by NAC from half-mustard gas-induced acute lung injury has also been demonstrated recently in rats by McClintock *et al.* [29]. However, in those studies NAC was administered by liposome encapsulation directly into the lung, as a method of treatment for acute exposure to mustard gas. The mechanism of protection was not elucidated in the studies by McClintock *et al.* [29]. In our study, we have demonstrated that NAC inhibits the production of NF-*k*B. The protection of lung injury thus may be etiologically related to the inhibition of oxidative activation of the transcription factor NF-*k*B, which is usually upregulated by stress signals. In fact, Atkins *et al.* [30] have suggested that NAC protects from sulfur mustard induced apoptotic endothelial cell death by enhancing the synthesis of reduced glutathione, which in turn may scavenge sulfur mustard and also prevent activation of NF-*k*B.

In summary, our study clearly suggests that NAC, a well-known antagonist, can be used as an effective antidote against CEES–induced lung injury. Work is under progress to develop devices to deliver this drug directly into lung even immediate after CEES exposure. Oral administration of NAC, as a prophylactic treatment, for three days or greater has shown significant protection against CEES. Prior to this work there has been no means of prophylaxis against mustards. It would appear that NAC is an excellent candidate prophylactic agent that is inexpensive, non-addicting, safe, and readily obtainable. It is important to note here that there is no other known example of the down regulation of the activity of NF-*k*B that has been shown by the oral administration of an antioxidant. This is important in terms of possible systemic inflammatory pathologic reactions – this point deserves further investigation.

It needs to be emphasized that there were no long-term studies to mimic the clinical or battle field conditions of mustard gas exposure. Therefore, it is necessary that we continue our efforts to study also long-term effects of mustard gas exposure on lungs.

Key Research Accomplishments

Study 1. **Inhibition of Cholinephosphotransferase Activity in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analog**

Exposure to mustard gas causes inflammatory lung diseases, including acute respiratory distress syndrome (ARDS). A defect in the lung surfactant system has been implicated as a cause of ARDS. A major component of lung surfactant is dipalmitoyl phosphatidylcholine (DPPC) and the major pathway for its synthesis is the cytidine diphosphocholine (CDP choline) pathway. It is not known whether the ARDS induced by mustard gas is mediated by its direct effects on some of the enzymes in the CDP-choline pathway. In the present study, we investigated whether mustard gas exposure modulates the activity of cholinephosphotransferase (CPT), the terminal enzyme by CDP-choline pathway.

Adult guinea pigs were intratracheally infused with single doses of 2-chloroethyl ethyl sulfide (CEES) (0.5 mg/kg b. wt. in ethanol). Control animals were injected with vehicles only. The animals were sacrificed at different time and the lungs were removed after perfusion with physiological saline.

CPT activity increased steadily up to 4h and then decreased at 6h and stabilized at 7 days in both mitochondria and microsomes (Fig. 1).

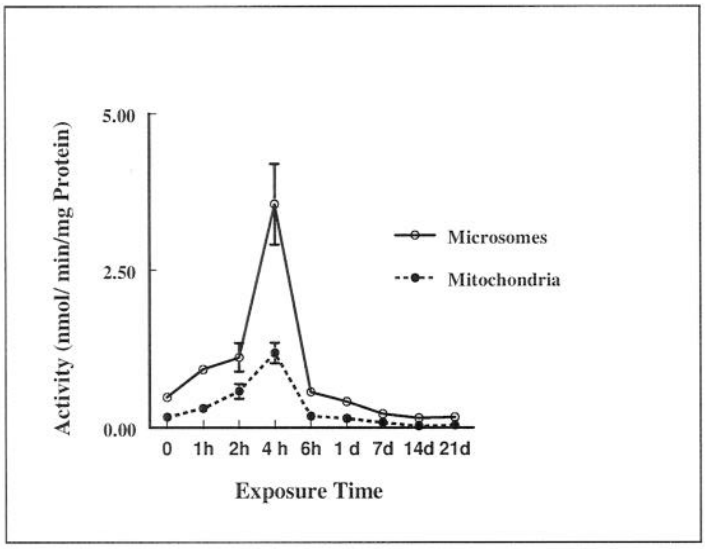
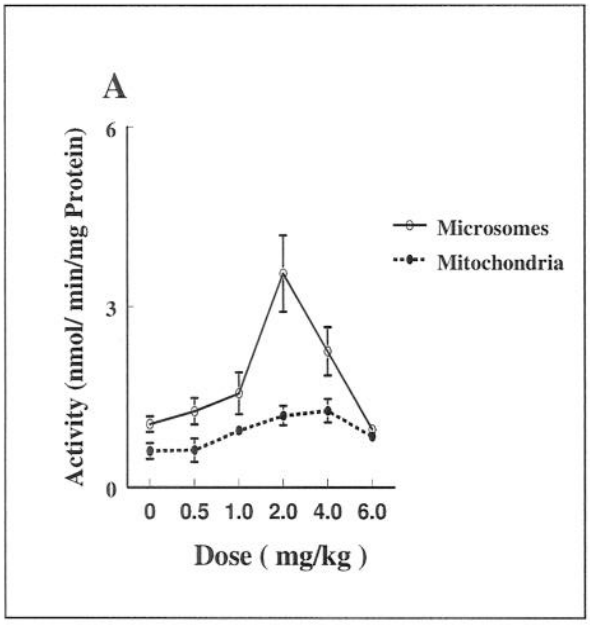


Figure 1. Time dependent effect of 0.5 mg/ kg body weight CEES treatment on mitochondrial and microsomal CPT activity. N=3

To determine the dose-dependent effect of CEES on CPT activity, we varied the dose of CEES (0.5–6.0 mg/kg b. wt.) and sacrificed the animals at 1 h and 4h. CPT activity showed a dose-dependent increase up to 2.0 mg/kg b. wt. of CEES in both mitochondria and microsomes, .and then decreased at 4.0 mg/kg b. wt. (Fig. 2A for 1 h, 2B for 4 h)



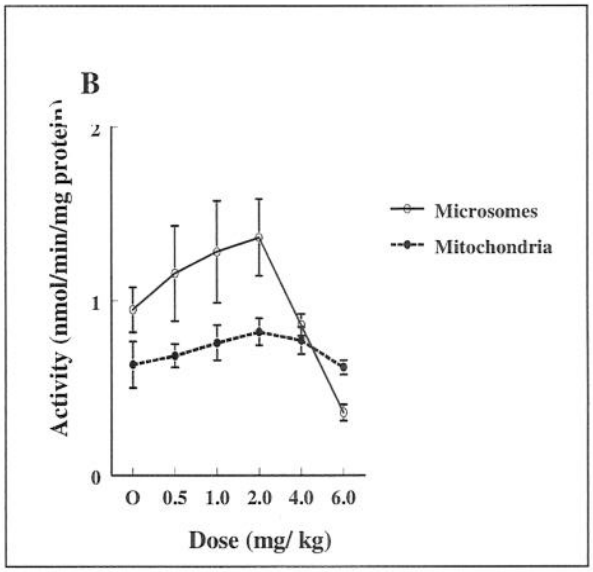


Figure 2. Dose dependent effect of CEES on cholinephosphotransferase activity in mitochondria and microsomes. (A) after 1 h CEES treatment and (B) after 4 h CEES treatment. N=3

For further studies we used a fixed single dose of CEES (2.0 mg/kg b. wt.) and fixed exposure time (7 days). Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue and expressed as the permeability index (Fig. 3).

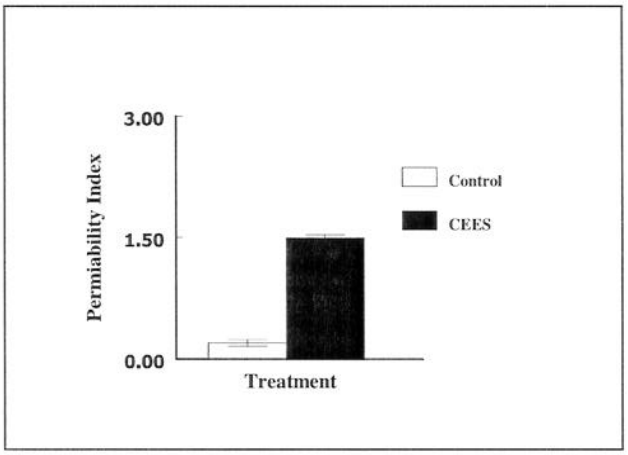


Figure 3. Lung injury measured by leakage of ^{125}I from lung. Animals were exposed to 2 mg/kg body weight CEES for 1 h. $p \leq 0.05$, N=3

CEES exposure (2.0 mg/kg b. wt. for 7 days) caused a significant decrease of both CPT gene expression (~1.7 fold, Fig. 4) and activity (~1.5 fold, Fig. 5) in lung. This decrease in CPT activity was not associated with any mutation of the CPT gene.

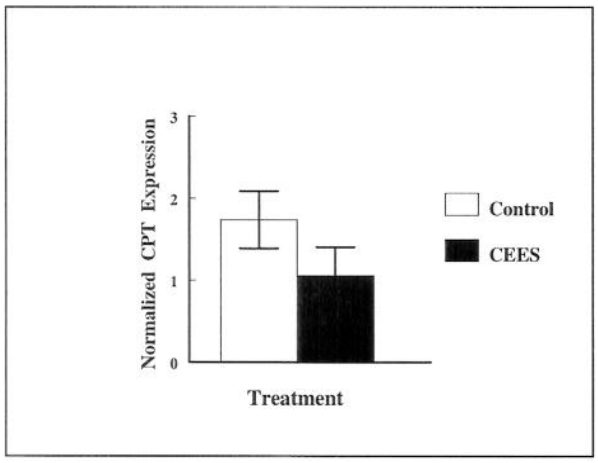


Figure 4. Northern blot analysis for the expression of the CPT gene. Graph showing down-regulation of CPT expression as the result of mustard gas treatment normalized with GAPDH. N =3

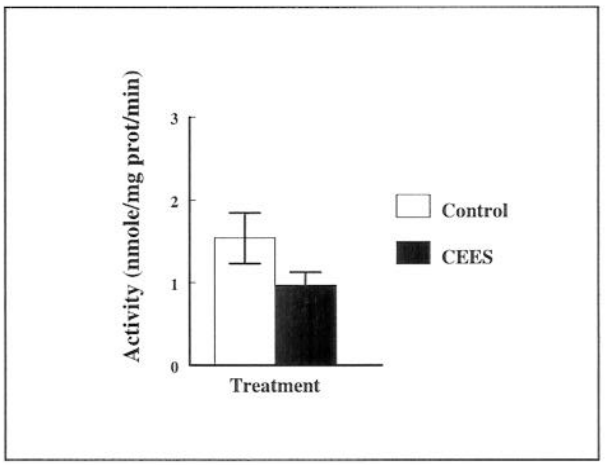


Figure 5. CPT enzyme activity in the lung microsomal fraction of 2 mg/kg body weight CEES (for 7 days) treated lung as compared to the control (only vehicle) showing significant decrease in activity. $p \leq 0.05$, N=5

Previously we reported that CEES infusion increased the production of ceramides, which are known to modulate PC synthesis. To determine whether ceramides affect microsomal CPT activity, the lung microsomal fraction was incubated with different concentrations of C_2-ceramide prior to CPT assay. CPT activity decreased significantly with increasing dose and time (Fig. 6).

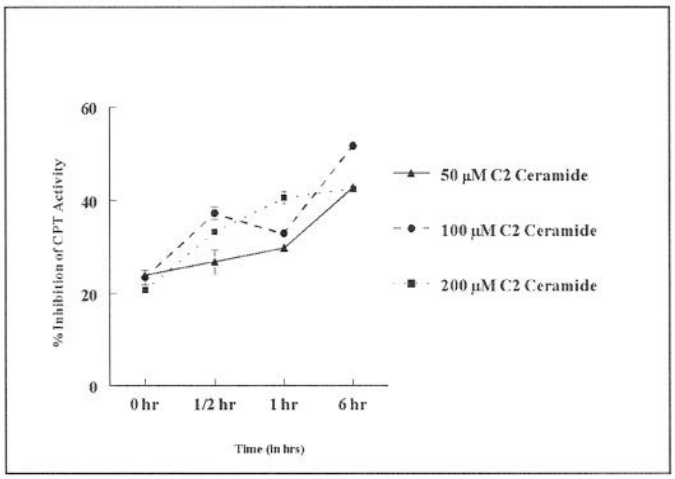


Figure 6. Percent inhibition of the CPT enzyme activity as the result of C_2 ceramide treatment of the lung microsomal fraction. N=3

The present study indicates that CEES causes lung injury and significantly decreases CPT gene expression and activity. This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating CPT enzyme(Fig. 7) .

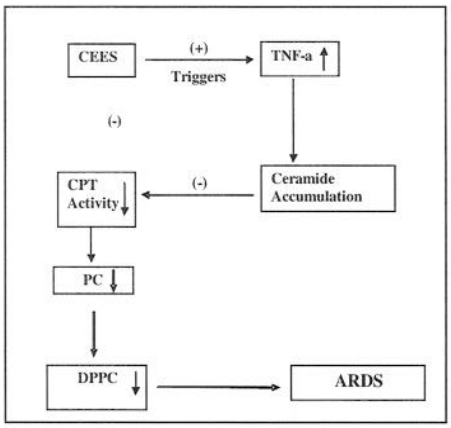


Figure 7. Proposed pathway for induction of ARDS by CEES.

Study 2. Array of Cytokine Induction in Early Lung Injury Response to 2-Chloroethyl Ethyl Sulfide, A Mustard Gas Analog.

We have previously shown that exposure of 2-chloroethyl ethyl sulfide (CEES), a mustard gas to guinea pigs causes an increase in the levels of TNF-α and NF-κB in the lung within an hour. However,

NF-κB disappeared after 2 hours indicating an intricate interplay of pro- and anti- apoptotic inflammatory cytokines.

Elucidating the early signaling events initiated in response to mustard gas mediated lung injury, would help us to design early intervention and /or protection against severe lung injury. With this in mind, we utilized the state of the art cytokine array technology to identify cytokines induced in response to mustard gas exposure.

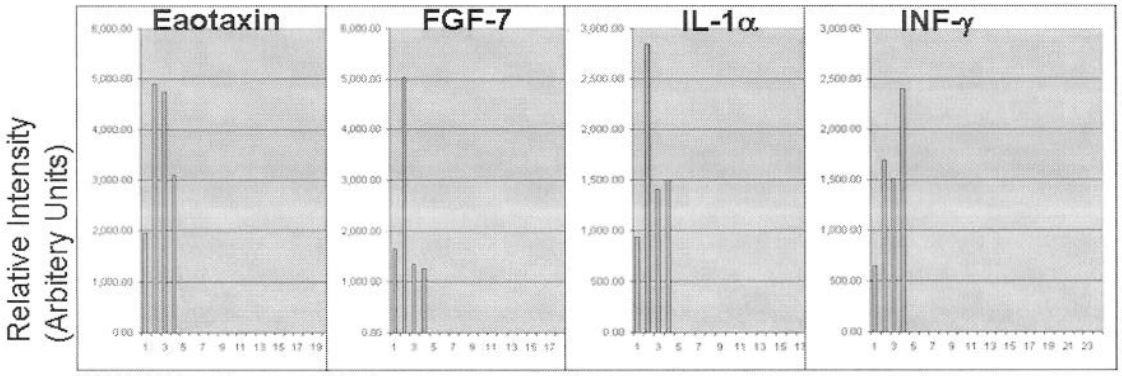


Figure 8. Induction Profile of Selected Cytokines: Columns in graphs indicate (left to right) cytokine induction levels from ; **1**- 1HR Control, **2**- 1HR SMG exposed, **3**- Day1 Control, **4**- Day1-SMG exposed lungs (3 lungs per treatment)

Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs. Among these, 9 cytokines with known or predicted functions in cellular injury and defense signal (IL-1α, EOTAXIN, MIP-1γ), macrophage activation (IFN-γ), inflammatory response (TNF-α), apoptosis (TNF-α), activation of NF-κB (LIGHT), cell proliferation and wound healing (PDGF-BB, FGF-7 and IGFBP-I) were all induced at higher levels with a minimum cut-off point of 2x above the levels of the control lungs (eg; Figure.8). Eotaxin regulated by both TNF-α and IL-1α is also known to be induced in response to radiation. We extended our evaluation to additional 60 cytokines (for a total of 120) at one hour and post mustard gas exposure which identified up-regulation (> 1.5x) of several growthfactors (FGF2) chemoattractant proteins (MCP-3) and cytokines involved in extra-cellular (TSP) remodeling (uPAR and TIMPs). To further understand the dynamics of cytokine induction profile we also evaluated the changes in the levels of these 120 cytokines by one-day post mustard gas exposure.

The array of cytokine induction within an hour of CEES exposure and dynamic changes in cytokine profile by one day post mustard gas exposure reveals that following an initial damage, the lung tissue tries to recover and prevent further damage through self defense mechanisms mediated through various classes of cytokines.

Study 3. Pulmonary Fibrosis in Guinea Pig Induced by 2-Chloroethyl Ethyl Sulfide.

Cross sectional clinical study on veterans with single heavy exposure to sulfur mustard gas (SMG) revealed that inhalation of SMG can lead to the development of series of chronic destructive pulmonary sequelae such as chronic bronchitis, pulmonary fibrosis (PF), and bronchiectasis. To understand the mechanism by which SMG exposure causes PF, we have used 2-chloroethyl ethyl sulfide (CEES) as a SMG analog to induce lung injury in guinea pigs. Our initial electronmicroscopic study revealed that intratracheal exposure of single dose of CEES (0.5 mg/kg b.wt.) developed PF within 7 days (Fig. 9).

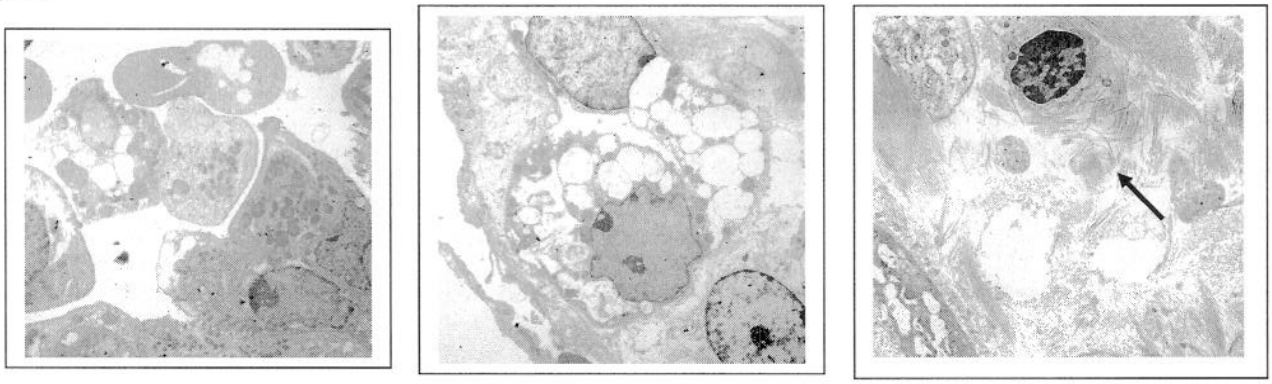


Fig. 9 Electronmicrograph of CEES induced pulmonary fibrosis. Magnification (X 4200)

Macrophage, eosinophils and neutrophils were the predominant cell types in bronchiolar lavage fluid (BALF) as observed in SMG-induced PF patients. The present ultrastructural studies on lung of guinea pigs exposed to CEES indicate evidence of interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen. CEES exposure caused oxidative stress in a time and dose dependent manner in both lung tissue and cells of BALF. Furthermore, there was a significant time dependent increase in MMP-9 (92 Kda gelatinase) activity in cytosol (Fig. 10) and a decrease in vascular endothelial growth factor in BALF (Fig. 11).

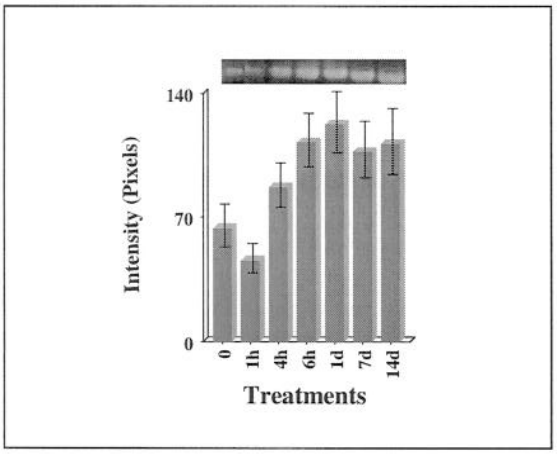


Fig. 10 Time dependent effect of CEES on MMP-9 activity in Cytosol. N=4

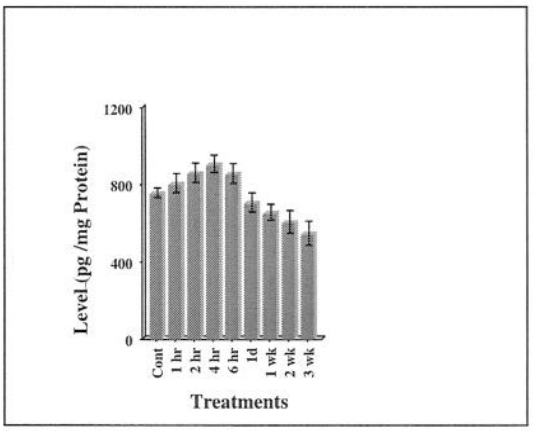


Fig. 11 Time dependent effect of CEES on VEGF level in BALF. N=4

Summary of Key Research Accomplishments

- ❖ CEES exposure significantly decreases gene expression and activity of a key enzyme in phospholipid biosynthesis, Cholinephosphotransferase (CPT). This decrease in CPT activity was not associated with any mutation of the CPT gene and is probably mediated by accumulation of ceramides. CEES induced ceramide accumulation may thus play an important role in the development of ARDS by modulating CPT enzyme.
- ❖ We utilized the state of the art cytokine array technology to identify cytokines induced in response to mustard gas exposure. Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs. Among these, 9 cytokines with known or predicted functions in cellular injury and defense signal (IL-1α, EOTAXIN, MIP-1γ), macrophage activation (IFN-γ), inflammatory response (TNF-α), apoptosis (TNF-α), activation of NF-κB (LIGHT), cell proliferation and wound healing (PDGF-BB, FGF-7 and IGFBP-I) were all induced at higher levels with a minimum cut-off point of 2x above the levels of the control lungs.
- ❖ CEES exposure causes interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen without granulation formation. Furthermore, there was a significant time dependent increase in MMP-9 (92 Kda gelatinase) activity and a decrease in vascular endothelial growth factor in BALF.

Reportable Outcomes

Abstracts

1 Mukherjee S and Das SK. Pulmonary Fibrosis in Guinea Pig Induced by 2-Chloroethyl Ethyl Sulfide. FASEB J., Vol. 19, A280, 2005, FASEB Meeting, April 2-6, 2005, San Diego, CA.

2 Rajaratnam VS and Das SK. Array of Cytokine Induction in Early Lung Injury Response to 2-Chloroethyl Ethyl Sulfide, A Mustard Gas Analog. FASEB J., Vol. 19, A852, 2005, FASEB Meeting, April 2-6, 2005, San Diego, CA.

Manuscripts

1. Chatterjee, D., Mukherjee, S. and Das, S.K. Mustard Gas Induced Pulmonary Complications in Guinea Pigs. In: Recent Advances in Molecular Medicine, Allergy and Immunology (Editors: B. Pilo, M.P.Nair, M.S.Patel, C.N.Ramchand), Allied Publisher, Chenai, India, p. 105-116, 2004. (Book Chapter)
2. Chatterjee, D., Mukherjee, S., Smith, M. G. and Das, S. K. Role of Sphingomyelinase in the Environmental Toxin Induced Apoptosis of Pulmonary Cells, in Press, LIPIDS: Sphingomyelin Metabolizing Enzymes" (Volume Editors: D. Haldar and S. K. Das), Research Signpost Publishers; Trivandrum, p. 117-139, (ISBN: 81-7736-229-1), 2004. (Book Chapter).
3. Chatterjee, D., Mukherjee, S., and Das, S. K. Evidence of Hair Loss After Sub-Acute Exposure to 2-Chloroethyl Ethyl Sulfide, A Mustard Analog and Beneficial Effects of N-Acetyl Cysteine (NAC), J. Biochem. Mol. Toxicol. 18: 150-153, 2004.
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7. Sinha Roy S, Mukherjee S, Kabir S, Rajaratnam V, Smith M and Das, S. K. Inhibition of Cholinephosphotransferase Activity in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analog, In Press, J. Biochem. Mol. Toxicol., 2005.

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.

Conclusions

- CEES induced ceramide accumulation may play an important role in the development of ARDS by modulating CPT enzyme.
- A state of the art cytokine array technology may be successfully used to identify cytokines induced in response to mustard gas exposure. Our initial analysis of 60 cytokines showed 16 cytokines up-regulated within 1 hr. in the mustard gas exposed lungs, 1.5-fold above the control vehicle treated lungs.
- CEES exposure causes interstitial pulmonitis with varying degrees of interstitial fibrosis, neutrophilic alveolities and increased amount of visualized collagen without granulation formation.

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SECTION 5: Keith Crawford, M.D., Ph.D., Center for Blood Research

Utilization of Gene Expression Signatures to Diagnosis Acute Exposure to Genotoxic Agent, 2-Chloroethyl Sulphide (CEES)

Abstract

Our previous work involved the in vitro and in silico screening of transcripts, which are selectively expressed by CEES-exposed circulating blood dendritic cells and monocytes. These transcripts serve as the template for the development of toxicology-base gene expression microarray (ToxArray). Consortium member will have access to these arrays for the evaluation of HD induced cellular pathways. Gene expression signatures generated from these studies will serve as the foundation for the development genomic- and protein-based methods of diagnosing exposure to various chemical weapons.

Introduction

Dendritic cells (DC) and monocytes (Mo), principle immune cell residents of the lung, are intimately involved in the maintenance of lung physiology. When exposed to pathogens or chemical agents, these immune cells become activated and release various inflammatory mediators. In addition, these activated immune cells migrate into the circulation and modulate the function of circulating DC/Mo. In extreme cases and depending on the inhaled substance, these lung DC/Mo may induce circulatory collapse (i.e. shock). Because of these unique properties of DC/Mo, our project aim is to identify those genes, which are modulated in human DC/Mo after exposure to CEES. These genes will serve as the foundation for the development of microarrays and the subsequent identification of diagnostic indicators of exposure from HD and other chemical warfare agents. A further goal in conjunction with consortium members is to design rat and mouse microarrays and QPCR reagents, which will be used to assess chemical-specific gene expression in mouse and rat experimental models.

Body

The experiments performed during the reporting period (Aug 04 – Aug 05) have focused on the development of protocols for the enrichment of mRNA and proteins from human DC/Mo. In vitro studies evaluating the effect of CEES, ammonia, hypochlorous acid and azide clearly document agent-specific alterations in protein profiles. Total RNA has been isolated from the treated samples and gene expression analysis of these samples is presently underway. The total RNA from these samples will undergo assessment with full human genome microarrays. Data generated form these samples will be evaluated and affected genes that were not included in our earlier panel will be added to the pool of genes, which will makeup our final ToxArray panel. Following the completion of the above study, the human gene chips will be printed and tested.

Completed project areas

1. Identify CEES-modulated genes and cellular pathways.
2. Development of a tox-gene template for microarray printing .
3. Tissue bank containing tissue samples from HD- or CEES- exposed rats

Continuing studies involve four different areas:

5. Analysis of HD and CEES exposed tissue by gene microarray and protein profiling.
6. Construction of rat and mouse tox-panels.
7. Development of a secure consortium database (DB): Experimental data generated from consortium experiments will be stored in a secure DB. This DB not only allows storage of experimental data, but also will allow viewing and analysis of gene expression data with data mining software.
8. Gene valiadation of unique genes by QPCR.

Key Research Accomplishments (Aug 04 – Aug 05)

- Identification of the cellular pathways, which are responsible for CEES-induced pathogensis. Experimental results suggested that DNA damage induced by DNA adduct formation causes imbalances in the redox state of cells and tissue. This redox imbalance or increase in oxidant levels initiates the caspase pathways.
- Interference of caspase pathways with Pan Caspase inhibitors protects against CEES-induced apoptosis.
- N-Acetylcyteine is more protective against CEES exposure than Pan Caspase inhibitors.
- CEES suppresses proinflammatory cytokine production in by DC/Mo but increases secretion of nitric oxide.
- Development of a panel of tox genes, which allows for the identification of genes, which may serve as biomarkers for exposure to various chemical weapons.

Reportable Outcomes

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8. Turnquist, S., Fluckiger, R., Smith M., Crawford, K. 2004. CEES-induced Apoptosis in Myeloid Cells. (manuscript in preparation).
9. "APC biosensor: Use of biomarkers for early detection of exposure to chemical or biological agents", presented by K. D. Crawford to Congressional Staffers at Summit Meeting, , Capital Building, Washington DC Oct 18, 2004.
10. APC-biosensor: Use of Dendritic cells and Monocytes for early detection of Exposure to Toxic Substances. 2005 In vitro Biology Meeting, Baltimore, Maryland, June 5-7 2005.

Conclusions

See above.

References

None.

Appendices

1. Hunt Valley Poster 2004.
2. Consortium Core Microarray Gene List

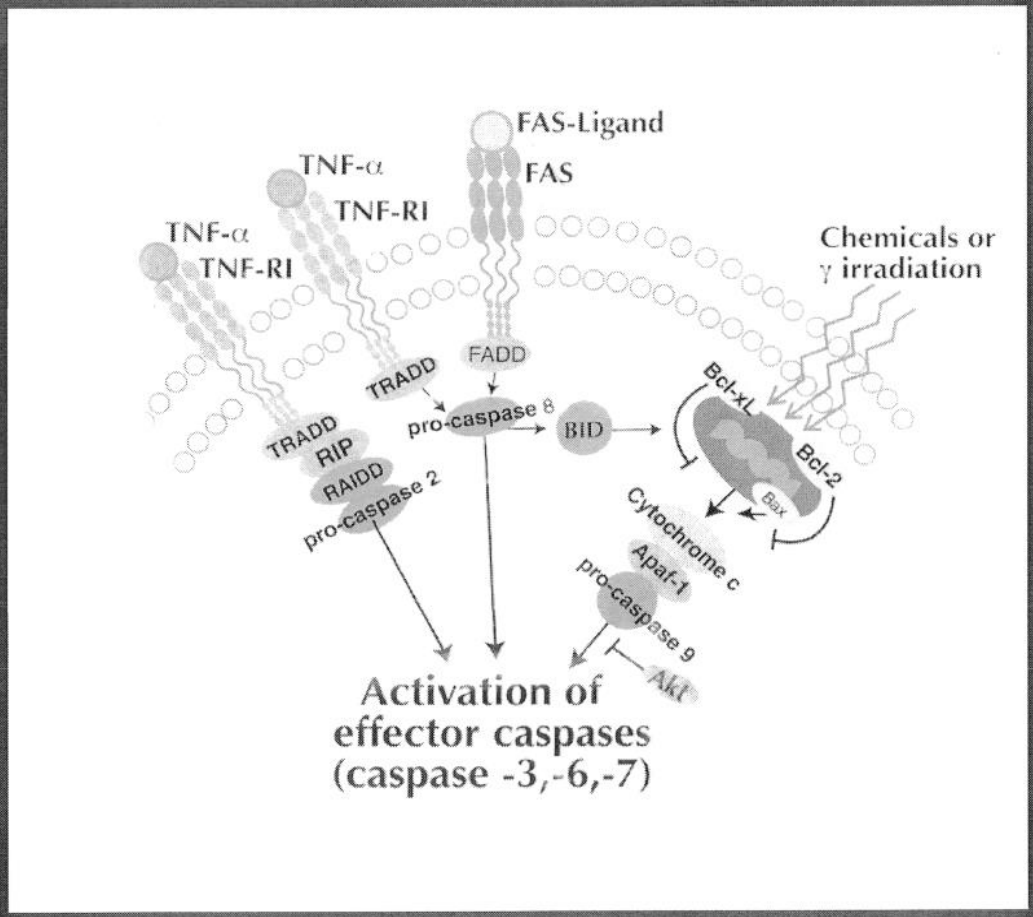
APPENDIX

Keith Crawford, M.D., Ph.D.

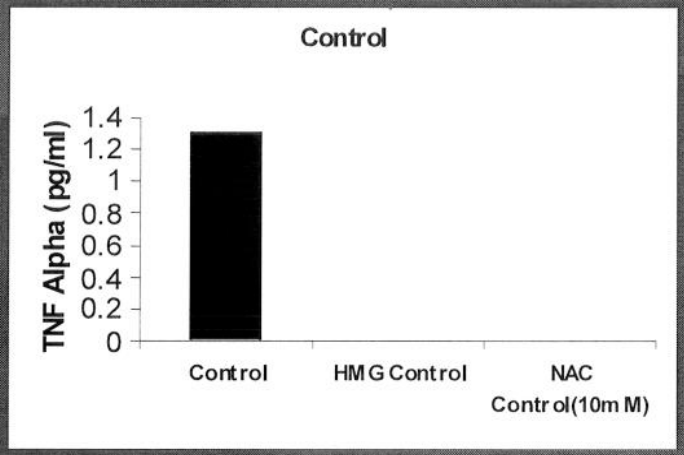
The Effect of Sulfur Mustard on Primary Macrophages

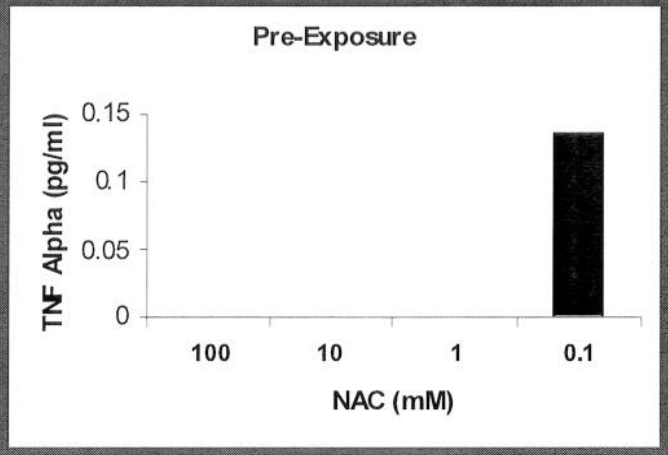
Shawn Turnquist[1], Milton Smith[2], and Keith Crawford[1]

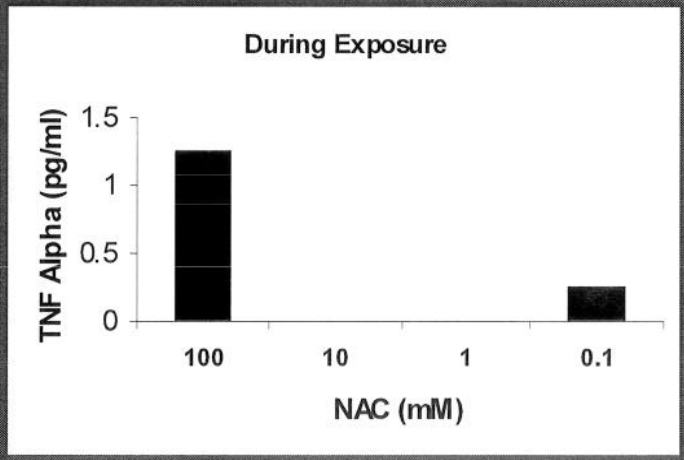
Apoptotic Pathways

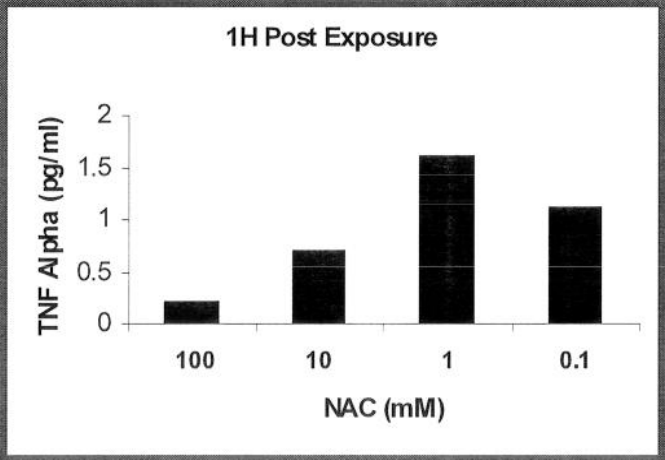


Effect of CEES on TNFα Production

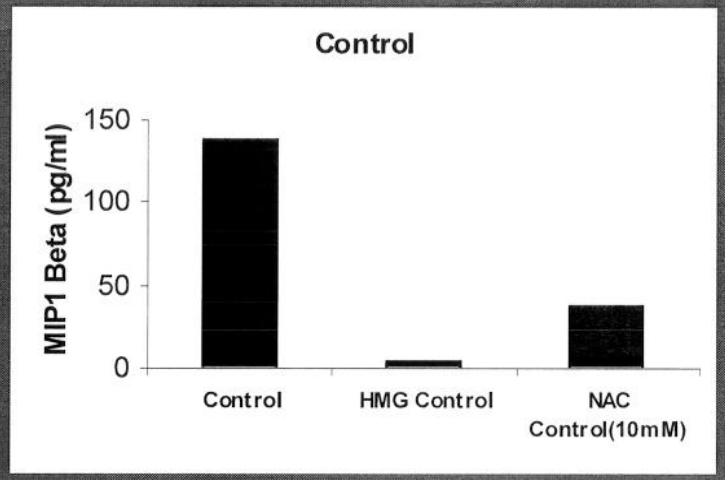


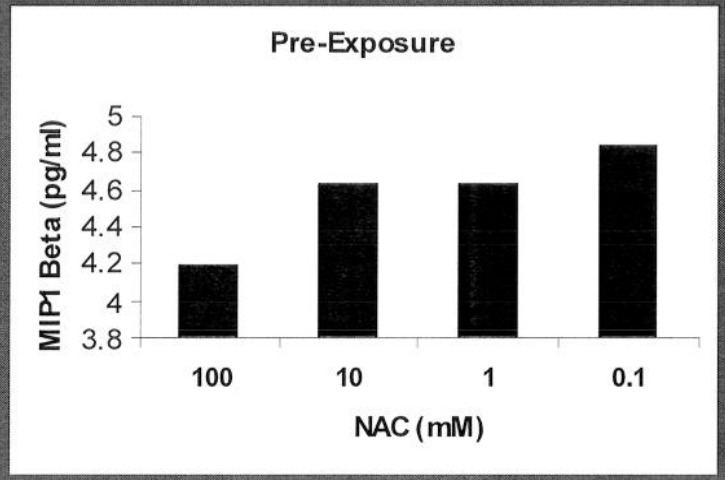


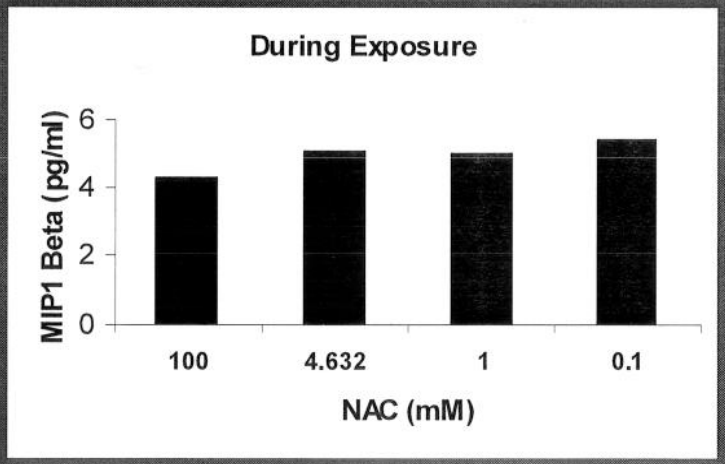


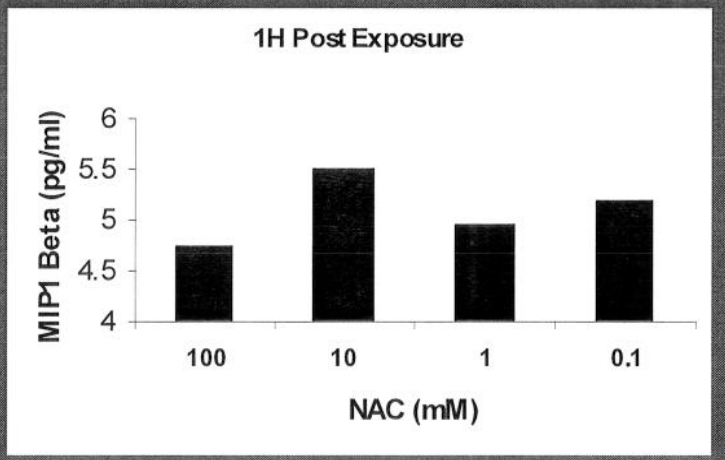


Effect of CEES on MIP-1β Production

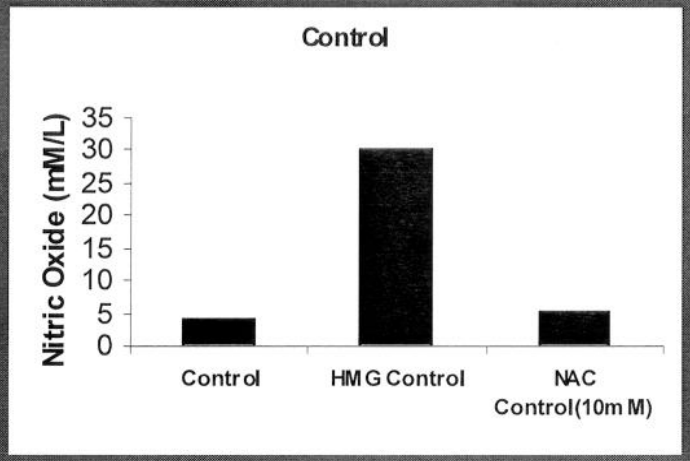


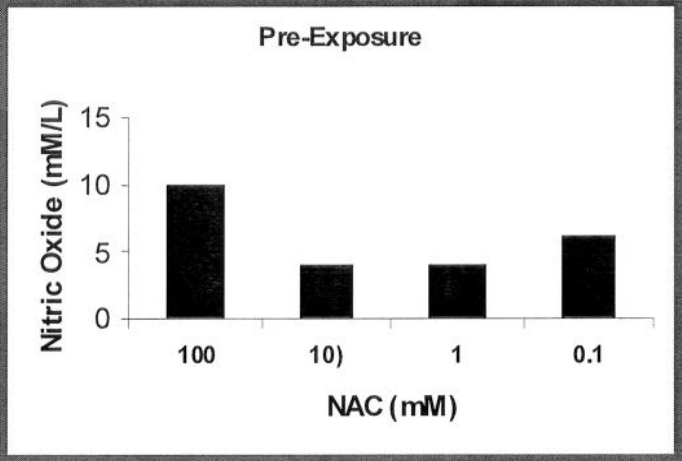


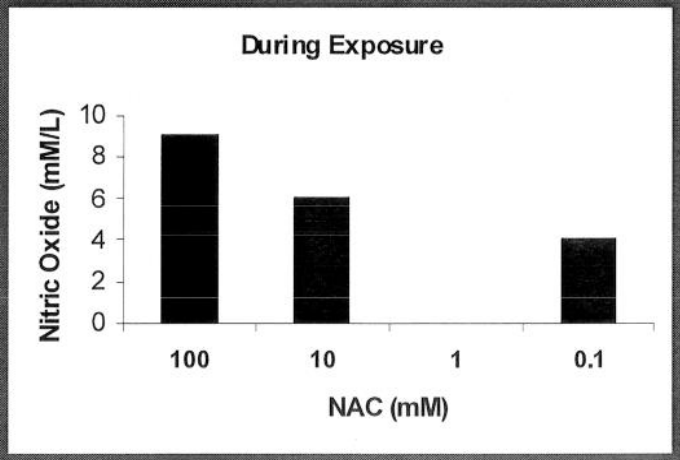


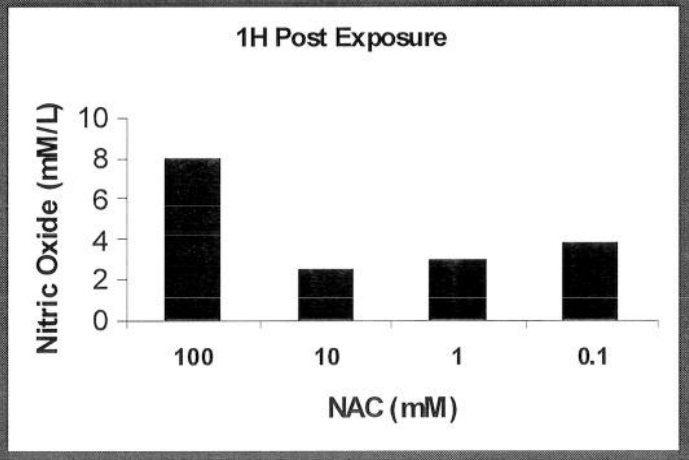


Effect of CEES on Nitric Oxide Production

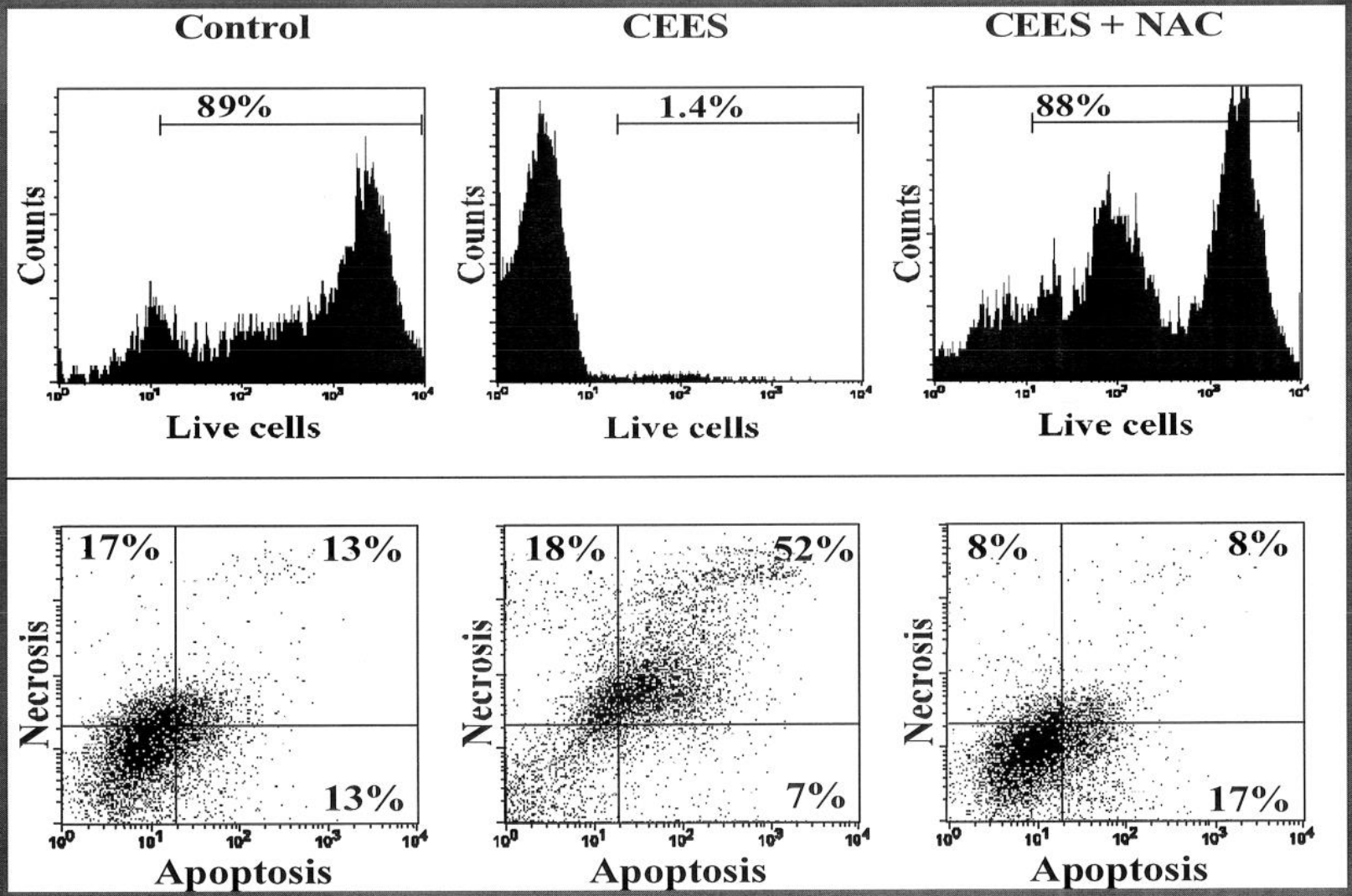




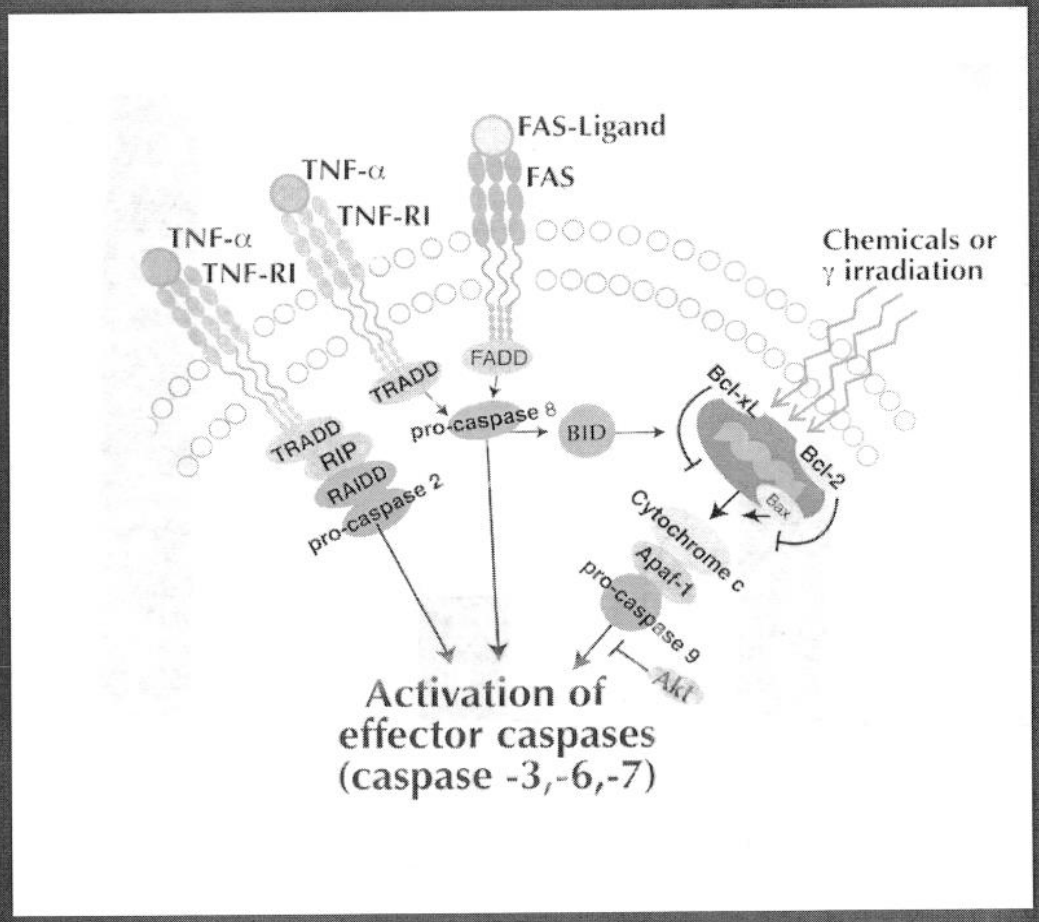




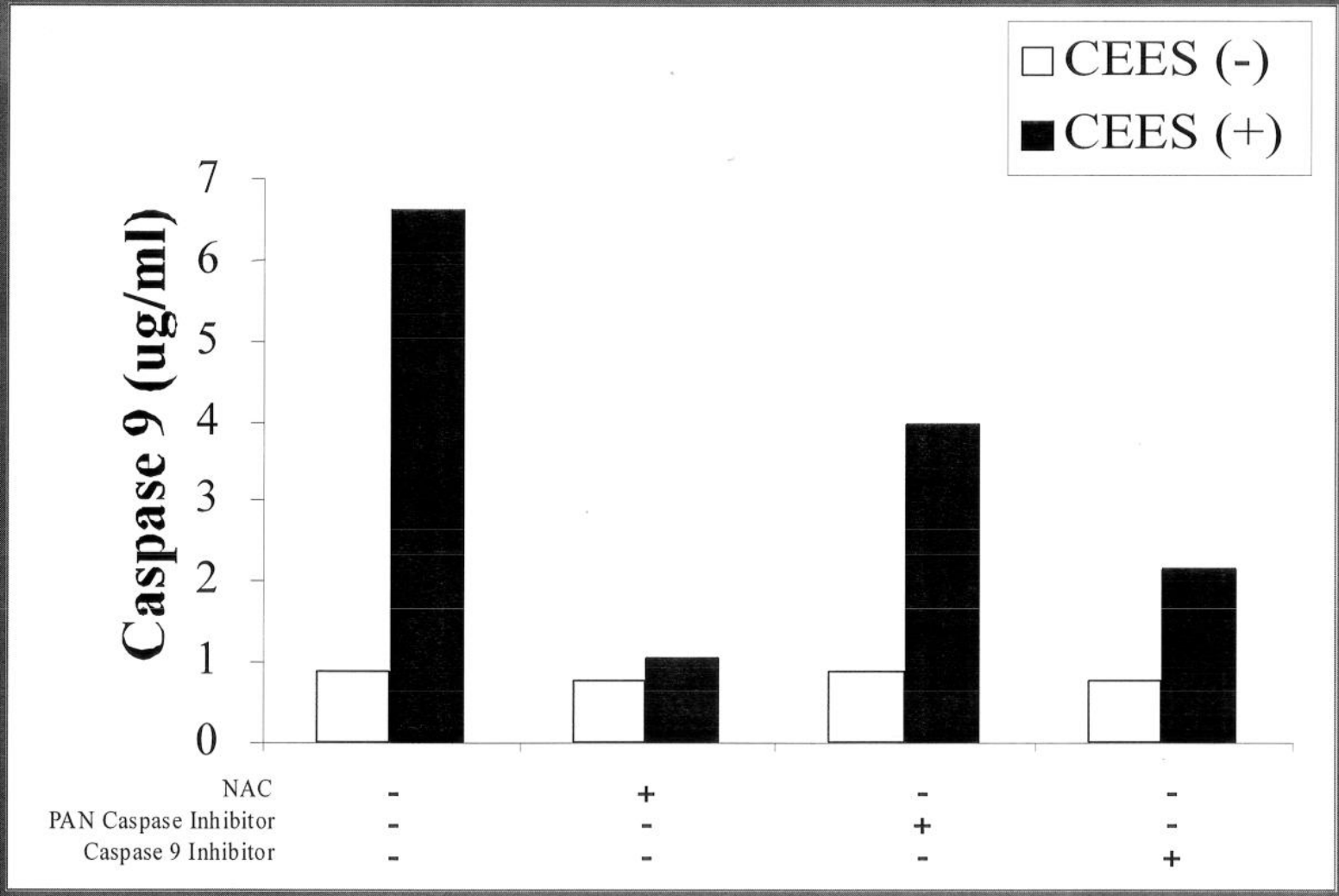
NAC Protects Against CEES-induced Death



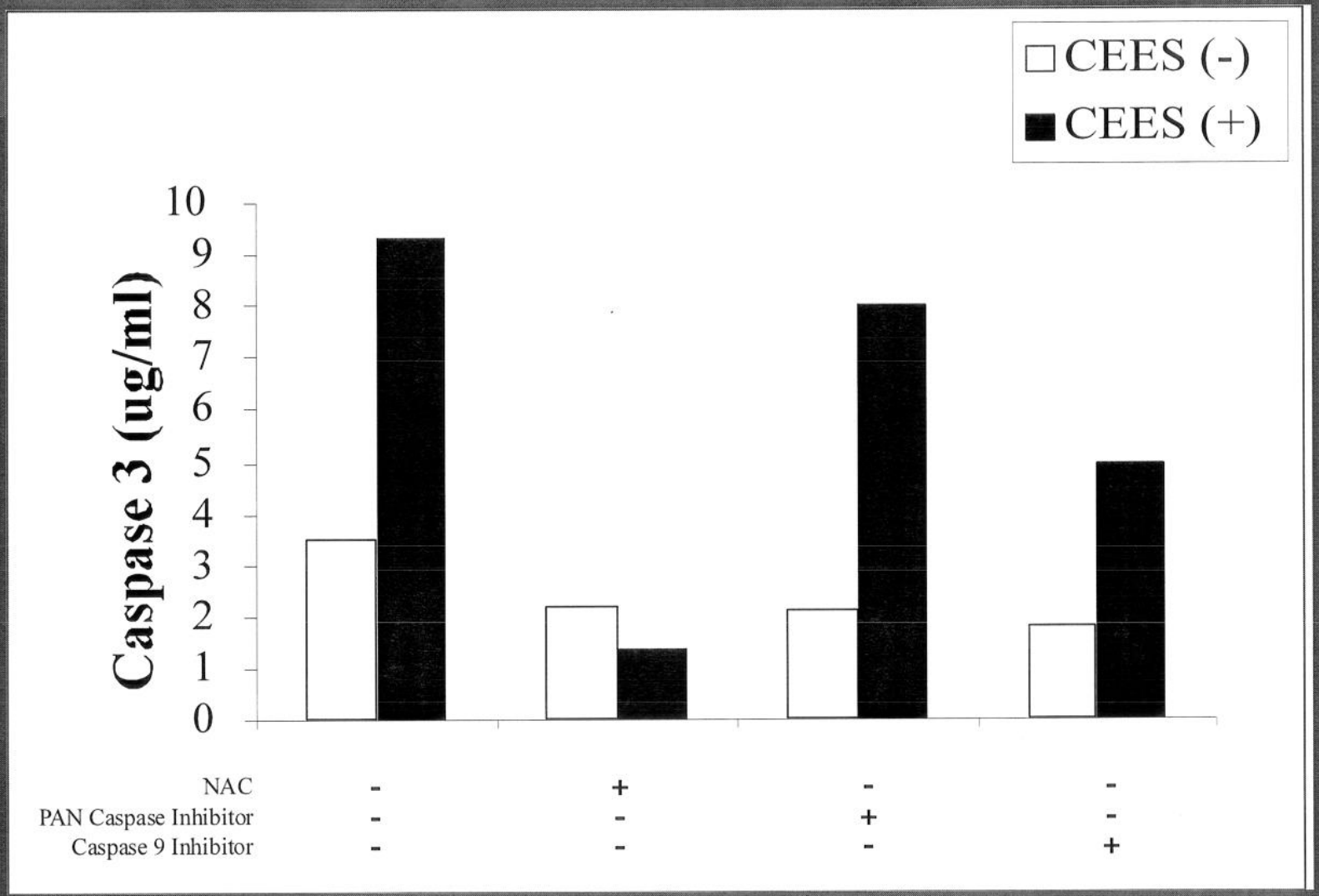
Apoptotic Pathways



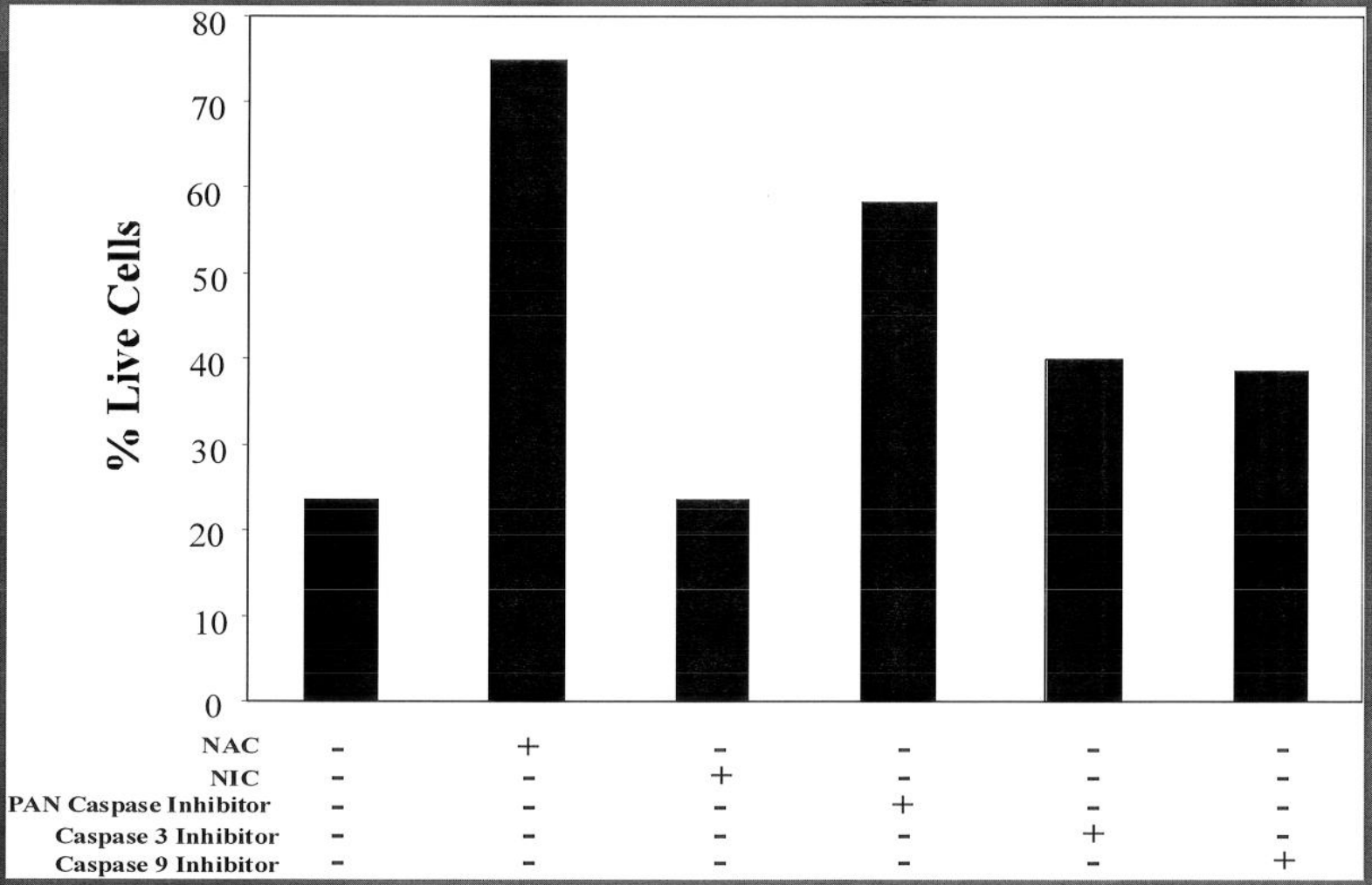
CEES Increases Caspase 9 Activity



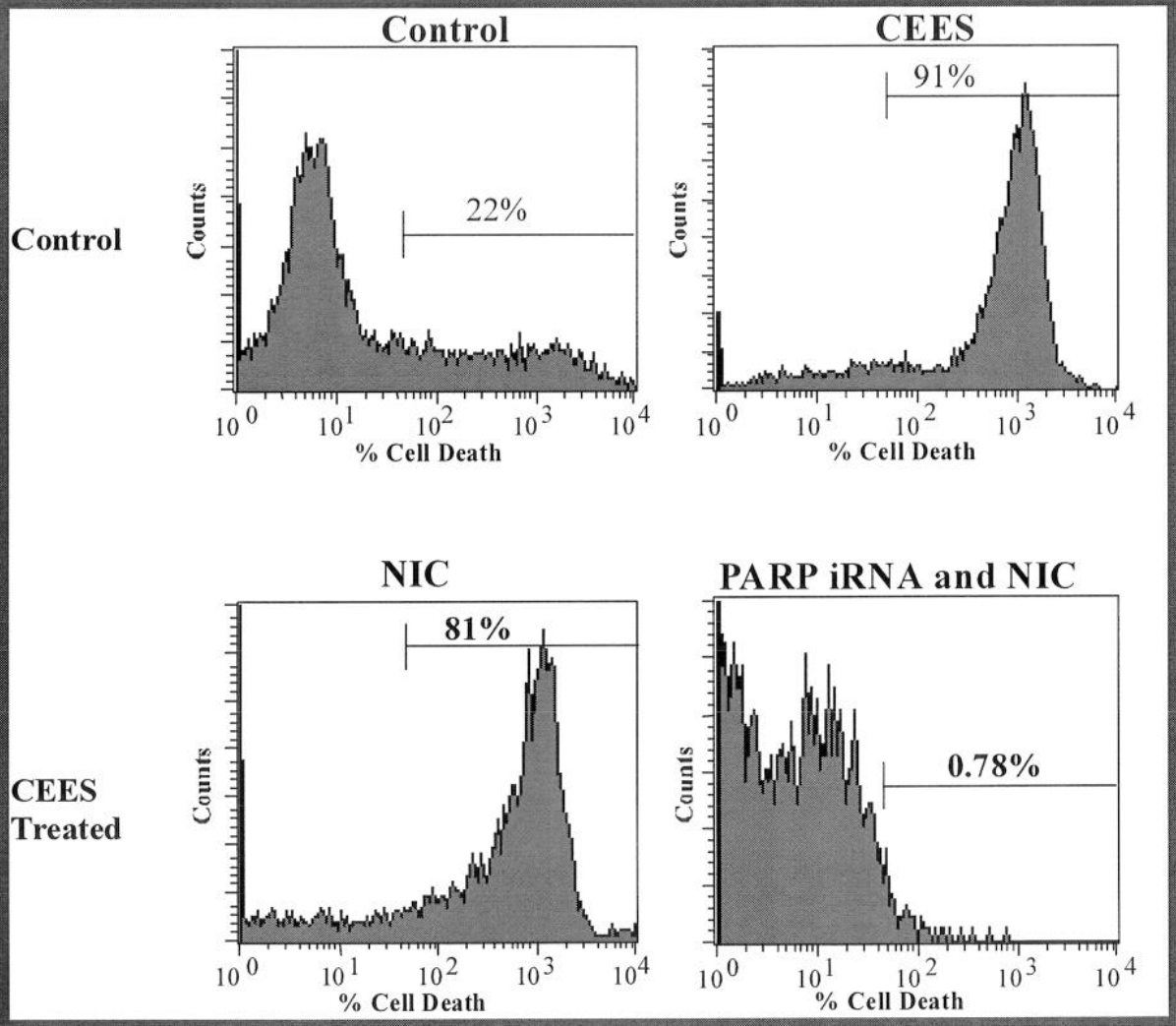
CEES Increases Caspase 3 Activity



Comparison of Apoptotic Pathway Inhibitors



Both PARP iRNA and Nicotinamide are Required for Protection Against CEES



Our future studies will focus on the use of high-density microarrays to detect chemical-specific gene signatures in various tissues

Construction of High Density Microarray of Genes from the Following Categories

1. Apoptotic pathway genes (1)
2. Apoptotic pathway genes (2)
3. P53 genes
4. Stress and Toxicity Genes
5. DNA damage genes
6. JAK/STAT signaling pathway
7. MAP Kinase Signaling Pathways Gene
8. Inflammatory cytokine genes

RAT LUNG TRANSCRIPTS SULFUR MUSTARD (Dana Anderson)

Up-Regulated

Heme Oxygenase-2
NAD(P)H:menadione oxidoreductase
Integrin beta-1 subunit
Extracellular Response Kinase 1
Protein Phosphatase 2A
alpha4 phosphoprotein
Bcl-2-related protein (Mcl-1)
inhibitor of apoptosis protein 2

RAT LUNG TRANSCRIPTS SULFUR MUSTARD (Dana Anderson)

Down-Regulated

Agrin (extracellular adhesion protein)
Clathrin (endocytosis)
Clathrin assembly protein
Microtubule-actin crosslinking factor (Macf)

Rat and Mouse transcripts
(Peter Ward)

SOCS3

JUN

FOS

AKT1

iNOS

Integrins (a1-a10, b1-b6)

Selectins (E, L and P)

ICAM, VCAM, PECAM

Metaloproteinases (MMP 1-15)

TIMPS

Rat and Mouse transcripts (Peter Ward)

Cytokines: CSF1 (MCSF), CSF2 (GM-CSF), CSF3 (GCSF), FGF2, IFNA1, IFNB1, IFNG, IL1A, IL1B, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12A, IL12B, IL13, IL14, IL15, IL16, IL17, IL18, IL19, KITLG, PDGFB, TNF.

Cytokine Receptors: IL2RA, IL4R, IL5RA, IL8RA, IL8RB, IL12RB1, IL12RB2, IL13RA1, IL13RA2, IL18R1.

Chemokines: CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL17, CCL18, CCL19, CCL20, CCL22, CCL24,CX3CL1, CXCL1, CXCL2, CXCL3, XCL1.

Chemokine Receptors: CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CX3CR1, GPR2, XCR1.

Th2 Cytokine Signaling Pathway: FOS, GATA3, JAK1, JAK3, STAT6, TBX21, TGFB1, TNFSF6,

Rat and Mouse transcripts (Peter Ward)

Complement component 2
Complement component 3
Complement component 4
Complement component 5
Complement component 6
Complement component 7
Complement component 8
Complement component 9
Complement component 1q
Complement component 1s
Factor B
Properdin
Clusterin (complement lysis inhibitor)
Complement component receptor 1
Complement component receptor
MASP1
MASP2
MBL

Consortium Core Microarray Library

Description	Gene Name	Group (related to)
Apoptotic protease activating factor	APAF1	Apoptosis
Apoptosis-associated speck-like protein	Asc	Apoptosis
Ataxia telangiectasia mutated	ATM	Apoptosis
BCL2-antagonist of cell death	Bad	Apoptosis
BCL2-antagonist/killer 1	BAK1	Apoptosis
BCL2-associated X protein	BAX	Apoptosis
B-cell CLL/lymphoma 10	Bcl-10/HuE10	Apoptosis
B-cell CLL/lymphoma 2	BCL2	Apoptosis
BCL2-related protein A1	BCL2A1	Apoptosis
BCL2-like 1	BCL2L1	Apoptosis
BCL2-like 11 (apoptosis facilitator)		Apoptosis
BCL2-like 2	Bcl-w	Apoptosis
BCL2-interacting killer (apoptosis-inducing)	Bik	Apoptosis
Baculoviral IAP repeat-containing 1	NAIP	Apoptosis
Baculoviral IAP repeat-containing 2	CIAP1	Apoptosis
Baculoviral IAP repeat-containing 3	MIHC/cIAP2	Apoptosis
Baculoviral IAP repeat-containing 4	XIAP/API3	Apoptosis
Baculoviral IAP repeat-containing 5 (survivin)	Survivin/API4	Apoptosis
Baculoviral IAP repeat-containing 6 (apollon)	Apollon/Bruce	Apoptosis
B lymphoid tyrosine kinase	Blk	Apoptosis
BCL2/adenovirus E1B 19kDa interacting protein 3	Nip3	Apoptosis
BCL2-related ovarian killer	BCL2L9	Apoptosis
Caspase 1, (interleukin 1, beta, convertase)	ICE	Apoptosis
Caspase 10, apoptosis-related cysteine protease	MCH4/FLICE2	Apoptosis
Caspase 13, apoptosis-related cysteine protease	Caspase 13	Apoptosis
Caspase 14, apoptosis-related cysteine protease	Caspase 14	Apoptosis
Caspase 2, apoptosis-related cysteine protease	ICH1	Apoptosis
Caspase 3, apoptosis-related cysteine protease	CPP32	Apoptosis
Caspase 4, apoptosis-related cysteine protease	ICH-2	Apoptosis
Caspase 5, apoptosis-related cysteine protease	Caspase-5	Apoptosis
Caspase 6, apoptosis-related cysteine protease	Mch2	Apoptosis
Caspase 7, apoptosis-related cysteine protease	Mch3	Apoptosis
Caspase 8, apoptosis-related cysteine protease	FLICE	Apoptosis
CASP8 associated protein 2	Flash	Apoptosis
Caspase 9, apoptosis-related cysteine protease	MCH6/APAF3	Apoptosis
CASP8 and FADD-like apoptosis regulator	CASPER/FLIP	Apoptosis
CHK1 checkpoint homolog (S. pombe)	Chk1	Apoptosis
Cell death-inducing DFFA-like effector a	CIDE-A	Apoptosis
Cell death-inducing DFFA-like effector b	CIDE-B	Apoptosis
CASP2 and RIPK1 domain	CRADD	Apoptosis
Death-associated protein kinase 2	DAP-kinase 2	Apoptosis
DNA fragmentation factor, 45kDa, alpha polypeptide	DFFA	Apoptosis
DNA fragmentation factor (caspase-activated DNase)	DFF40/CPAN	Apoptosis
Fas (TNFRSF6)-associated via death domain	FADD	Apoptosis
Growth arrest and DNA-damage-inducible, alpha	GADD45	Apoptosis
Harakiri, BCL2-interacting protein	HRK	Apoptosis
HUS1 checkpoint homolog (S. pombe)	Hus1	Apoptosis

Bifunctional apoptosis regulator	Bar	Apoptosis
Lymphotoxin alpha (TNF superfamily, member 1)	TNFB/LT	Apoptosis
Lymphotoxin beta (TNF superfamily, member 3)	LT-b	Apoptosis
Lymphotoxin beta receptor	LTbR	Apoptosis
Myeloid cell leukemia sequence 1 (BCL2-related)	MCL-1	Apoptosis
Mdm2	Mdm2	Apoptosis
Myeloid differentiation primary response gene (88)	MyD88	Apoptosis
Caspase recruitment domain family, member 4	Nod/CARD4	Apoptosis
Nucleolar protein 3	Nop30	Apoptosis
Cytoskeleton-associated protein 4	p63	Apoptosis
CHK2 checkpoint homolog (S. pombe)	CHK2/RAD53	Apoptosis
Receptor (TNFRSF)-interacting serine-threonine kinase 1	RIP	Apoptosis
Receptor-interacting serine-threonine kinase 2	Cardiac/Rip2	Apoptosis
Replication protein A3, 14kDa	RPA3	Apoptosis
TRAF family member-associated NFKB activator	I-TRAF	Apoptosis
Tumor necrosis factor (TNF superfamily, member 2)	TNFA	Apoptosis
Tumor necrosis factor receptor superfamily, member 10a	TRAIL-R/DR4	Apoptosis
Tumor necrosis factor receptor superfamily, member 10b	KILLER/DR5/TRAILR2	Apoptosis
Tumor necrosis factor receptor superfamily, member 10c	TRAIL-R3/DcR1	Apoptosis
Tumor necrosis factor receptor superfamily, member 10d	TRAIL-R4/DcR2	Apoptosis
Tumor necrosis factor receptor superfamily, member 25	DR3/Apo3	Apoptosis
Tumor necrosis factor receptor superfamily	TNFRSF14	Apoptosis
Tumor necrosis factor receptor superfamily, member 1A	TNFR1	Apoptosis
Tumor necrosis factor receptor superfamily, member 1B	TNFR2/p75	Apoptosis
H.sapiens mRNA for OX40 homologue	OX40	Apoptosis
Tumor necrosis factor receptor superfamily, member 5	CD40	Apoptosis
Tumor necrosis factor receptor superfamily, member 6	Fas/Apo-1/CD95	Apoptosis
Tumor necrosis factor receptor superfamily, member 7	CD27	Apoptosis
Tumor necrosis factor receptor superfamily, member 8	CD30	Apoptosis
Tumor necrosis factor receptor superfamily, member 9	4-1BB	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 10	TRAIL	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 11	TRANCE	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 12	TNFSF12/APO3L	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 13	April	Apoptosis
Homo sapiens TNF member (LIGHT mRNA)	HVEM-L	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 4	OX40L	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 5	CD40L/CD154/TRAP	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 6	Fas ligand	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 7	CD27L/CD70	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 8	CD30L/CD153	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 9	4-1BB-L	Apoptosis
Tumor protein p53 (Li-Fraumeni syndrome)	p53	Apoptosis
TNF receptor-associated factor 1	TRAF1	Apoptosis
TNF receptor-associated factor 2	TRAP3	Apoptosis
TNF receptor-associated factor 3	CRAF1	Apoptosis
TNF receptor-associated factor 4	TRAF-4	Apoptosis
TNF receptor-associated factor 5	TRAF5	Apoptosis
TNF receptor-associated factor 6	TRAF6	Apoptosis
TRAF interacting protein	Trip	Apoptosis
Annexin A5	Annexin V	Apoptosis
Ataxia telangiectasia mutated	ATM	Apoptosis

BCL2-associated X protein	Bax	Apoptosis
BCL2-like 1	Bcl-x	Apoptosis
BCL2-like 2	Bcl-w	Apoptosis
Caspase 1, (interleukin 1, beta, convertase)	ICE	Apoptosis
Caspase 10, apoptosis-related cysteine protease	MCH4/FLICE2	Apoptosis
Caspase 8, apoptosis-related cysteine protease	FLICE	Apoptosis
Catalase	Catalase	Apoptosis
Cyclin C	Cyclin C	Apoptosis
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1	Apoptosis
Cyclin G1	Cyclin G	Apoptosis
Cyclin-Dependent Kinase Inhibitor 1A	P21/Waf1/CIP1	Apoptosis
CHK2 checkpoint homolog (S. pombe)	CHK2/RAD53	Apoptosis
Crystallin, alpha B	Cryab	Apoptosis
Colony stimulating factor 2 (granulocyte-macrophage)	GM-CSF	Apoptosis
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	Apoptosis
Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	Apoptosis
Cytochrome P450, family 2, subfamily E, polypeptide 1	CYP2E	Apoptosis
Cytochrome P450, family 7, subfamily A, polypeptide 1	CYP7A1	Apoptosis
Cytochrome P450, subfamily VIIB	CYP7B1	Apoptosis
Damage-specific DNA binding protein 1, 127kDa	DDB1	Apoptosis
DNA-damage-inducible transcript 3	GADD153(CHOP)	Apoptosis
DnaJ (Hsp40) homolog, subfamily A, member 1	HSJ2	Apoptosis
DnaJ (Hsp40) homolog, subfamily B, member 4	HLJ1	Apoptosis
E2F transcription factor 1	E2F	Apoptosis
Early growth response 1	Krox-24	Apoptosis
Epoxide hydrolase 2, cytoplasmic	EPHX2	Apoptosis
Excision repair rodent repair deficiency, group 1	ERCC1	Apoptosis
Excision repair rodent repair deficiency group 3	XPB	Apoptosis
Excision repair rodent repair deficiency group 4	XPF	Apoptosis
Excision repair rodent repair deficiency, group 5	XPG	Apoptosis
Flavin containing monooxygenase 1	FMO1	Apoptosis
Flavin containing monooxygenase 5	FMO5	Apoptosis
Growth arrest and DNA-damage-inducible, alpha	GADD45	Apoptosis
Growth arrest and DNA-damage-inducible, beta	GADD45 b	Apoptosis
Glutathione peroxidase 1	GPX1	Apoptosis
Glutathione reductase	GSR	Apoptosis
Glutathione S-transferase M3 (brain)	GSTM3	Apoptosis
Heme oxygenase (decycling) 1	HO-1/HEME1	Apoptosis
Heme oxygenase (decycling) 2	HEME2	Apoptosis
Heat shock transcription factor 1	Tcf5	Apoptosis
Heat shock 105kDa/110kDa protein 1	HSP105B	Apoptosis
Heat shock 70kDa protein 1A	HSP70-1A	Apoptosis
Heat shock 70kDa protein 1B	HSPA1B	Apoptosis
Heat shock 70kDa protein 1-like	HSPA1L	Apoptosis
Heat shock 70kDa protein 2	HSPA2	Apoptosis
Heat shock 70kDa protein 4	Hsp70	Apoptosis
Heat shock 70kDa protein 5	Grp78	Apoptosis
Heat shock 70kDa protein 6 (HSP70B')	HSP70B	Apoptosis
Heat shock 70kDa protein 8	HSPA8	Apoptosis
Heat shock 70kDa protein 9B (mortalin-2)	Mortalin-2	Apoptosis
Heat shock 27kDa protein 1	HSP28/HSP27/Hsp25	Apoptosis

Heat shock 90kDa protein 1, alpha	HSP90A/HSP86	Apoptosis
Heat shock 90kDa protein 1, beta	HSP90 b	Apoptosis
Heat shock 60kDa protein 1 (chaperonin)	Chaperonin (HSPD1)	Apoptosis
Heat shock 10kDa protein 1 (chaperonin 10)	Chaperonin 10 (HSPE1)	Apoptosis
Insulin-like growth factor binding protein 6	IGFBP-6	Apoptosis
Interleukin 18 (interferon-gamma-inducing factor)	IL-18	Apoptosis
Interleukin 1, alpha	IL-1a	Apoptosis
Interleukin 1, beta	IL-1b	Apoptosis
Interleukin 6 (interferon, beta 2)	IL-6	Apoptosis
Lymphotoxin alpha (TNF superfamily, member 1)	TNFB/LT	Apoptosis
Mdm2, transformed 3T3 cell double minute 2	Mdm2	Apoptosis
Macrophage migration inhibitory factor	MIF	Apoptosis
Metallothionein 1A (functional)	MT1A	Apoptosis
Metallothionein 1H	metallothionein 1H	Apoptosis
Metallothionein 2A	Metallothionein 2A	Apoptosis
NFk light polypeptide gene enhancer (p105)	KBF1	Apoptosis
NFk light polypeptide gene enhancer, alpha	IKBA/MAD-3	Apoptosis
Homo sapiens inducible nitric oxide synthase	iNOS	Apoptosis
Proliferating cell nuclear antigen	PCNA	Apoptosis
Prostate differentiation factor	PLAB	Apoptosis
P450 (cytochrome) oxidoreductase	CYPOR	Apoptosis
Prostaglandin-endoperoxide synthase 2	Cox-2	Apoptosis
RAD23 homolog A (S. cerevisiae)	HHR23A	Apoptosis
RAD50 (S. cerevisiae) homolog	RAD50	Apoptosis
Chemokine (C-C motif) ligand 21	SCYA21	Apoptosis
Chemokine (C-C motif) ligand 3	MIP-1a/SCYA3	Apoptosis
Chemokine (C-C motif) ligand 4	MIP-1b	Apoptosis
Chemokine (C-X-C motif) ligand 10	SCYB10/IP 10	Apoptosis
Serine (or cysteine) proteinase inhibitor, clade E	PAI-1	Apoptosis
Superoxide dismutase 1	Cu/ZnSOD	Apoptosis
Superoxide dismutase 2, mitochondrial	IPO-B/MNSOD	Apoptosis
Tumor necrosis factor (TNF superfamily, member 2)	TNFA	Apoptosis
Tumor necrosis factor receptor superfamily, member 1A	TNFR1	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 10	TRAIL	Apoptosis
Tumor necrosis factor (ligand) superfamily, member 6	Fas ligand	Apoptosis
Tumor protein p53 (Li-Fraumeni syndrome)	p53	Apoptosis
TNFRSF1A-associated via death domain	TRADD	Apoptosis
UDP glycosyltransferase 1 family, polypeptide A9	UGT1A9	Apoptosis
Uracil-DNA glycosylase	UNG	Apoptosis
X-ray repair complementing defective repair in CHO 1	XRCC1	Apoptosis
X-ray repair complementing defective repair in CHO 2	XRCC2	Apoptosis
X-ray repair complementing defective repair in CHO 4	XRCC4	Apoptosis
X-ray repair complementing defective repair in CHO 5	KU80	Apoptosis
ATP-binding cassette	PGY1,MDR1	P53
Actin, alpha 1, skeletal muscle	ACTA1	P53
TRAF and TNF receptor associated protein	AD022	P53
Apoptotic protease activating factor	Apaf-1	P53
APEX nuclease (multifunctional DNA repair enzyme) 1	APEX/Ref-1	P53
Apoptosis related protein APR-3	3-Apr	P53
Ataxia telangiectasia mutated	ATM	P53
Ataxia telangiectasia and Rad3 related	ATR	P53

Brain-specific angiogenesis inhibitor 1	BAI1	P53
BRCA1 associated protein-1	BAP1	P53
BCL2-associated X protein	Bax	P53
BCL2 binding component 3	PUMA	P53
B-cell CLL/lymphoma 2	Bcl-2	P53
BRCA1 associated protein	BRAP	P53
Breast cancer 1, early onset	BRCA1	P53
Benzodiazapine receptor (peripheral)	BZRP	P53
Caspase 9, apoptosis-related cysteine protease	MCH6/APAF3	P53
Cyclin H	Cyclin H	P53
Cell division cycle 2, G1 to S and G2 to M	Cdk1	P53
Cyclin-dependent kinase 7	CDK7	P53
Cyclin-Dependent Kinase Inhibitor 1A	P21/Waf1/CIP1	P53
Cyclin-dependent kinase inhibitor 2A	p16INK4	P53
CHK1 checkpoint homolog	Chk1	P53
CHK2 checkpoint homolog	CHK2/RAD53	P53
CREB binding protein (Rubinstein-Taybi syndrome)	CBP	P53
Casein kinase 1, alpha 1	CSNK1A1	P53
Casein kinase 2, alpha 1 polypeptide	CK-II alpha	P53
Casein kinase 2, alpha prime polypeptide	CSNK2A2	P53
Casein kinase 2, beta polypeptide	CK-II beta	P53
Cathepsin D (lysosomal aspartyl protease)	Cathepsin D	P53
Polyposis locus protein 1	D5S346	P53
Death-associated protein 6	DAXX	P53
E1B-55kDa-associated protein 5	E1B-AP5	P53
E2F transcription factor 1	E2F	P53
E1A binding protein p300	p300	P53
Estrogen receptor 1	ER alpha	P53
Fas (TNFRSF6)-associated via death domain	FADD	P53
Fas (TNFRSF6) associated factor 1	FAF1	P53
Growth arrest and DNA-damage-inducible, alpha	GADD45	P53
G-2 and S-phase expressed 1	B99	P53
Hypoxia-inducible factor 1, alpha subunit	HIF1A	P53
Homeodomain interacting protein kinase 2	HIPK2	P53
Heat shock 70kDa protein 4	Hsp70	P53
DNA-dependent PK catalytic subunit-interacting protein 2	KIP2	P53
DNA-dependent PK catalytic subunit-interacting protein 3	KIP3	P53
Leucine-rich and death domain containing	PIDD	P53
Mitogen-activated protein kinase kinase 4	Mkk4/JNKK1	P53
Mitogen-activated protein kinase kinase 7	JNKK2/MKK7	P53
Microtubule-associated protein 4	MAP4	P53
Mitogen-activated protein kinase 8 interacting protein 2	JIP2	P53
Mdm2, transformed 3T3 cell double minute 2	Mdm2	P53
Mdm2, transformed 3T3 cell double minute 2	MTBP	P53
V-myc myelocytomatosis viral oncogene homolog (avian)	c-Myc	P53
N-myc downstream regulated gene 1	NDRG	P53
NF kappa light polypeptide gene enhancer (p105)	KBF1	P53
Numb homolog (Drosophila)	NUMB	P53
P53-regulated apoptosis-inducing protein 1	P53AIP1	P53
P300/CBP-associated factor	PCAF	P53
Etoposide induced 2.4 mRNA	Pig8	P53

Phorbol-12-myristate-13-acetate-induced protein 1	NoxA	P53
Promyelocytic leukemia	PML	P53
Peripheral myelin protein 22	PMP22	P53
Protein kinase C, alpha	PKC alpha	P53
Protein kinase C, beta 1	PKC beta	P53
Protein kinase C, gamma	PRKCG	P53
Protein kinase C, theta	PRKCQ	P53
Protein kinase, DNA-activated, catalytic polypeptide	DNA-PK	P53
RAS p21 protein activator (GTPase activating protein) 1	RasGAP	P53
Retinoblastoma 1 (including osteosarcoma)	Rb	P53
Reticuloendotheliosis Viral Oncogene Homolog A	NFKB3	P53
Candidate mediator of the p53-dependent G2 arrest	REPRIMO	P53
Ribonucleotide reductase M2 B (TP53 inducible)	p53R2	P53
Serine proteinase inhibitor, clade B, member 5	Maspin	P53
Stratifin	14-3-3	P53
Sirtuin 1 (S. cerevisiae)	SIRT1	P53
Sp1 transcription factor	Sp1	P53
Signal transducer and activator of transcription 5A	MGF	P53
TATA box binding protein	TBP	P53
TEA domain family member 1	TEAD1	P53
Thrombospondin 1	TSP1	P53
Thyroid hormone receptor, alpha	c-ErbA	P53
Tumor necrosis factor (TNF superfamily, member 2)	TNFA	P53
Tumor necrosis factor, alpha-induced protein 1	TNFAIP1	P53
Tumor necrosis factor receptor superfamily, member 10b	KILLER/DR5/TRAILR2	P53
Tumor necrosis factor (ligand) superfamily, member 6	Fas ligand	P53
Tumor protein p53 (Li-Fraumeni syndrome)	p53	P53
Tumor protein p53 binding protein, 2	ASPP2	P53
TP53 activated protein 1	TP53TG1	P53
Tumor protein 63 kDa with strong homology to p53	p51B(TP63)	P53
Homo sapiens tumor protein p73 (TP73)	p73	P53
TNF receptor-associated factor 1	TRAF1	P53
TNF receptor-associated factor 4	TRAF-4	P53
TNF receptor-associated factor 5	TRAF5	P53
P53 target zinc finger protein	WIG1	P53
Werner syndrome	WRN	P53
Wilms tumor 1	WT1	P53
Annexin A5	Annexin V	Stress/Toxicity
Ataxia telangiectasia mutated	ATM	Stress/Toxicity
BCL2-associated X protein	Bax	Stress/Toxicity
BCL2-like 1	Bcl-x	Stress/Toxicity
BCL2-like 2	Bcl-w	Stress/Toxicity
Caspase 1, (interleukin 1, beta, convertase)	ICE	Stress/Toxicity
Caspase 10, apoptosis-related cysteine protease	MCH4/FLICE2	Stress/Toxicity
Caspase 8, apoptosis-related cysteine protease	FLICE	Stress/Toxicity
Catalase	Catalase	Stress/Toxicity
Cyclin C	Cyclin C	Stress/Toxicity
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1	Stress/Toxicity
Cyclin G1	Cyclin G	Stress/Toxicity
Cyclin-Dependent Kinase Inhibitor 1A	P21/Waf1/CIP1	Stress/Toxicity
CHK2 checkpoint homolog (S. pombe)	CHK2/RAD53	Stress/Toxicity

Damage-specific DNA binding protein 1, 127kDa	DDB1	DNA damage
Damage-specific DNA binding protein 2, 48kDa	DDB2	DNA damage
DNA-damage-inducible transcript 3	GADD153(CHOP)	DNA damage
Ubiquitin carrier protein	E2-EPF	DNA damage
Exostoses (multiple) 1	EXT1	DNA damage
Flap structure-specific endonuclease 1	RAD2	DNA damage
Forkhead box O3A	FKHRL1	DNA damage
Growth arrest and DNA-damage-inducible, alpha	GADD45	DNA damage
Growth arrest and DNA-damage-inducible, beta	GADD45 b	DNA damage
Growth arrest and DNA-damage-inducible, gamma	GADD45gamma	DNA damage
G-2 and S-phase expressed 1	B99	DNA damage
Histone 1, H2ac	H2AFL	DNA damage
HUS1 checkpoint homolog (S. pombe)	Hus1	DNA damage
Kinesin family member 23	MKLP-1	DNA damage
Kinesin family member 2C	MCAK	DNA damage
Legumain	LGMN	DNA damage
Leucine-rich and death domain containing	PIDD	DNA damage
Mdm2, transformed 3T3 cell double minute 2	Mdm2	DNA damage
MutL Homolog 1	MLH1	DNA damage
N-methylpurine-DNA glycosylase	MDG	DNA damage
MRE11 meiotic recombination 11 homolog A	MRE11A	DNA damage
MutS homolog 2, colon cancer, nonpolyposis type 1	MSH2	DNA damage
MutS homolog 3 (E. coli)	MSH3	DNA damage
MutS homolog 6 (E. coli)	MSH6	DNA damage
Nijmegen breakage syndrome 1 (nibrin)	Nibrin	DNA damage
P53-regulated apoptosis-inducing protein 1	P53AIP1	DNA damage
Sestrin 1	PA26	DNA damage
Proliferating cell nuclear antigen	PCNA	DNA damage
Protein (peptidyl-prolyl cis/trans isomerase)	PIN1	DNA damage
PMS1 postmeiotic segregation increased 1	PMS1	DNA damage
PMS2 postmeiotic segregation increased 2	PMS2	DNA damage
Protein phosphatase 1D magnesium-dependent	Wip1	DNA damage
Protein kinase, DNA-activated, catalytic polypeptide	DNA-PK	DNA damage
Protein tyrosine phosphatase	LPAP	DNA damage
Purine-rich element binding protein A	PURA	DNA damage
RAD1 homolog (S. pombe)	RAD1	DNA damage
RAD17 homolog (S. pombe)	RAD17	DNA damage
RAD23 homolog A (S. cerevisiae)	HHR23A	DNA damage
RAD23 homolog B (S. cerevisiae)	RAD23B	DNA damage
RAD50 (S. cerevisiae) homolog	RAD50	DNA damage
RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	RAD51	DNA damage
RAD52 homolog (S. cerevisiae)	RAD52	DNA damage
RAD9 homolog A (S. pombe)	RAD9	DNA damage
Candidate mediator of the p53-dependent G2 arrest	REPRIMO	DNA damage
Replication protein A3, 14kDa	RPA3	DNA damage
Ribonucleotide reductase M2 B (TP53 inducible)	p53R2	DNA damage
Stratifin	14-3-3	DNA damage
Tumor necrosis factor (TNF superfamily, member 2)	TNFA	DNA damage
Tumor necrosis factor receptor superfamily, member 10b	KILLER/DR5/TRAILR2	DNA damage
H.sapiens mRNA for OX40 homologue	OX40	DNA damage
Tumor necrosis factor receptor superfamily, member 6	Fas/Apo-1/CD95	DNA damage

Jun B proto-oncogene	Jun-B	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 1	Smad1	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 2	Smad2	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 3	SMAD3	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 4	Smad4/DPC4	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 5	Smad5	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 6	Smad6	JAK/STAT signaling
MAD (mothers against decapentaplegic) homolog 7	Smad7	JAK/STAT signaling
MAD, mothers against decapentaplegic homolog 9	Smad9	JAK/STAT signaling
V-maf musculoaponeurotic fibrosarcoma oncogene	c-Maf	JAK/STAT signaling
MCM5 minichromosome maintenance deficient 5	CDC46	JAK/STAT signaling
MHC class II transactivator	CIITA	JAK/STAT signaling
Msx-interacting-zinc finger	PIASX-BETA	JAK/STAT signaling
Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	TRANSIN	JAK/STAT signaling
Myeloproliferative leukemia virus oncogene	TPOR	JAK/STAT signaling
V-myc myelocytomatosis viral oncogene homolog (avian)	c-Myc	JAK/STAT signaling
Nuclear receptor coactivator 1	NCOA1	JAK/STAT signaling
N-myc (and STAT) interactor	NMI	JAK/STAT signaling
Nitric oxide synthase 2A (inducible, hepatocytes)	NOS	JAK/STAT signaling
2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	JAK/STAT signaling
Oncostatin M	OSM	JAK/STAT signaling
Protein inhibitor of activated STAT, 1	DDXBP1	JAK/STAT signaling
Protein inhibitor of activated STAT3	PIAS3	JAK/STAT signaling
Protein inhibitor of activated STAT protein PIASy	PIASy	JAK/STAT signaling
Pim-1 oncogene	Pim-1	JAK/STAT signaling
Protein tyrosine phosphatase, non-receptor type 1	PTP-1B	JAK/STAT signaling
Protein tyrosine phosphatase, receptor type, C	Cd45	JAK/STAT signaling
SH2-B homolog	SH2B	JAK/STAT signaling
Suppressor of cytokine signaling 1	SSI-1/Cish1	JAK/STAT signaling
Suppressor of cytokine signaling 2	STATI2	JAK/STAT signaling
Suppressor of cytokine signaling 3	SSI-3	JAK/STAT signaling
Suppressor of cytokine signaling 4	SOCS6/CIS4	JAK/STAT signaling
Suppressor of cytokine signaling 5	SOCS5	JAK/STAT signaling
Suppressor of cytokine signaling 7	SOCS4	JAK/STAT signaling
Sp1 transcription factor	Sp1	JAK/STAT signaling
Spleen focus forming virus	PU.1	JAK/STAT signaling
V-src sarcoma	c-Src	JAK/STAT signaling
Signal transducing adaptor molecule 1	STAM	JAK/STAT signaling
Signal transducer and activator of transcription 1, 91kDa	Stat1	JAK/STAT signaling
Signal transducer and activator of transcription 2, 113kDa	ISGF-3	JAK/STAT signaling
Signal transducer and activator of transcription 3	Stat3	JAK/STAT signaling
Signal transducer and activator of transcription 4	STAT 4	JAK/STAT signaling
Signal transducer and activator of transcription 5A	MGF	JAK/STAT signaling
Signal transducer and activator of transcription 5B	STAT5	JAK/STAT signaling
Signal transducer and activator of transcription 6	STAT6	JAK/STAT signaling
STIP1 homology and U-Box containing protein 1	STUB1	JAK/STAT signaling
Tumor necrosis factor receptor superfamily, member 6	Fas/Apo-1/CD95	JAK/STAT signaling
Tyrosine kinase 2	Tyk2	JAK/STAT signaling
Upstream transcription factor 1	USF1	JAK/STAT signaling
YY1 transcription factor	YY1	JAK/STAT signaling
Peroxiredoxin 6	AOP2	MAP Kinase Signaling

V-raf murine sarcoma 3611 viral oncogene homolog 1	A-raf	MAP Kinase Signaling
Activating transcription factor 2	Creb-2	MAP Kinase Signaling
V-raf murine sarcoma viral oncogene homolog B1	B-raf	MAP Kinase Signaling
Cyclin A1	Cyclin A1	MAP Kinase Signaling
Cyclin A2	Cyclin A	MAP Kinase Signaling
Cyclin B1	Cyclin B	MAP Kinase Signaling
Cyclin B2	Cyclin B2	MAP Kinase Signaling
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1	MAP Kinase Signaling
Cyclin D2	Cyclin D2	MAP Kinase Signaling
Cyclin D3	Cyclin D3	MAP Kinase Signaling
Cyclin E1	Cyclin E1	MAP Kinase Signaling
Cyclin E2	Cyclin E2	MAP Kinase Signaling
Cell division cycle 42 (GTP binding protein, 25kDa)	Cdc42	MAP Kinase Signaling
Cyclin-dependent kinase 2	Cdk2	MAP Kinase Signaling
Cyclin-dependent kinase 4	Cdk4	MAP Kinase Signaling
Cyclin-dependent kinase 6	Cdk6	MAP Kinase Signaling
Cyclin-Dependent Kinase Inhibitor 1A	P21/Waf1/CIP1	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	p27Kip1	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	P57/Kip2	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 2A	p16INK4	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	MTS2/TP15/INK4B	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	p18/INK4C	MAP Kinase Signaling
Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	p19-INK4D	MAP Kinase Signaling
Conserved helix-loop-helix ubiquitous kinase	IKKA/IKK1	MAP Kinase Signaling
Collagen, type I, alpha 1	COL1A1	MAP Kinase Signaling
CAMP responsive element binding protein 1	CREB	MAP Kinase Signaling
CREB binding protein (Rubinstein-Taybi syndrome)	CBP	MAP Kinase Signaling
Delta-like 1 homolog (Drosophila)	DLK	MAP Kinase Signaling
Dual specificity phosphatase 1	MKP-1	MAP Kinase Signaling
E2F transcription factor 1	E2F	MAP Kinase Signaling
Epidermal growth factor receptor	EGFR	MAP Kinase Signaling
Early growth response 1	Krox-24	MAP Kinase Signaling
ELK1, member of ETS oncogene family	Elk1	MAP Kinase Signaling
ELK4, ETS-domain protein (SRF accessory protein 1)	SAP1	MAP Kinase Signaling
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ATX	MAP Kinase Signaling
V-ets erythroblastosis virus E26 oncogene homolog 1	Ets1	MAP Kinase Signaling
V-Ets erythroblastosis virus E26 oncogene homolog 2	c-Ets2	MAP Kinase Signaling
V-fos FBJ murine osteosarcoma viral oncogene homolog	c-Fos	MAP Kinase Signaling
Growth factor receptor-bound protein 2	GRB2	MAP Kinase Signaling
V-Ha-ras Harvey rat sarcoma viral oncogene homolog	H-ras	MAP Kinase Signaling
Heat shock 70kDa protein 5	Grp78	MAP Kinase Signaling
Heat shock 27kDa protein 1	HSP28/HSP27/Hsp25	MAP Kinase Signaling
V-jun sarcoma virus 17 oncogene homolog (avian)	V-jun	MAP Kinase Signaling
V-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	KI-RAS	MAP Kinase Signaling
Kinase suppressor of ras	Ksr	MAP Kinase Signaling
MAD, mothers against decapentaplegic homolog 4	Smad4/DPC4	MAP Kinase Signaling
Mitogen-activated protein kinase kinase 1	MEK1	MAP Kinase Signaling
Mitogen-activated protein kinase kinase 1	MP1	MAP Kinase Signaling
Mitogen-activated protein kinase kinase 2	MEK2	MAP Kinase Signaling
Mitogen-activated protein kinase kinase 3	MKK3	MAP Kinase Signaling
Mitogen-activated protein kinase kinase 4	Mkk4/JNKK1	MAP Kinase Signaling

SECTION 6: Alfred Sciuto, PhD, US Army Medical Research Institute of Chemical Defense

In vivo model assessment of the effects of nebulized sulfur mustard (SM) and CEES in the anesthetized and ventilated rat.

Abstract

This study is designed to establish the following: (1) develop an inhalation model to determine the LCt50 of nebulized mustard and CEES agents in a dose-response manner (2) assess toxicological, biochemical and physiological markers of injury at 0.5, 1, 3, 6, or 24 h post exposure (3) markers of interest include but ae limited to wet/dry lung weight ratio, survival at 24 h and out to 14 days, bronchioloar lavage fluid (BALF) protein and LDH levels. Once these common endpoints are established then antioxidant liposome countermeasures will be administered.

Introduction and Body

1. Model development. The inhalation exposure glovebox became fully operational for animal use in February 1005. Currently we have studied the following nebulized doses of dilute sulfur mustard 0, 250, 500, 750, 1250, or 1750 μg total delivered amount over 10 min via a nebulized stream in the ventilated and anesthetized male rat (260-300 g). XCSM (exempt chemical surety material) HD was diluted from a stock solution into a 75% saline/25% ETOH solution. Nebulized particles were in the respirable range of 1-2 μm. The concentration of HD was verified using the MIN Chemical Agent Monitor (MINICAMS).
2. Determination of markers of exposure. We have not yet achieved a LCt50 for SM. However, we have reached an approximate LCt10 at 1250 μg. Therefore, we will need to complete studies at concentrations that approach 2000 μg (200 μg/10 min exposure). We have determined basic markers of injury at 0, 0.5, 1, 3, 6, and at 24 h postexposure. To this point we have completed only three rats at 1750 μg. There has been relatively little change in wet/dry wt ratios across all time points and doses. BALF protein levels have not shown any marked changes at the lower doses. We did begin to see temporal increases at the 1250 μg level. Protein levels increased 4-fold from 0.5 h to 24 h suggesting a breach in the air/blood barrier is developing over time. Increased BALF LDH levels appear to be dose-dependent at 0.5, 1 and 3 h, but remain inconclusive at 6 and 24 h post exposure. At 1250 μg there is an increased time-dependent trend that is in general agreement with protein levels. Taken together the data thus far suggests that we see some degree of early HD-induced cellular injury 0.5-3 h, but HD-induced distal lung effects as measured by protein levels begin as early as 1 h postexposure and continue to increase out to 24 h. Observationally, we see hemorrhagic lung tissue at necropsy especially in the upper cranial lobes. We have collected unperfused lung, liver, and brain tissues for future analysis. Because we have been diligently investigating the effects of HD, we have not had the time to do similar work with CEES.
3. We have since run into unforeseen difficulties such as senior tech is on continued medical leave, junior tech is pregnant and cannot work with agent, the MINICAMS has been returned to the factory for repairs, and the exposure glove box is down due to cracked glass casing causing pressure fluctuations. It could be a month before we resume studies.

Key Research Accomplishments

See above.

Reportable Outcomes

See above.

Conclusions

See above.

References

None.

SECTION 7: Dana Anderson, US Army Medical Research Institute of Chemical Defense

Comparison Of Antioxidant Liposome Treatment Of Sulfur Mustard or 2-Chloroethyl Ethyl Sulfide Induced Lung Injury

Abstract

Several investigators in the Mustard Consortium have documented protection against 2-chloroethyl-ethyl sulfide (CEES) induced lung injury using n-Acetyl Cysteine (NAC) alone or encapsulated in liposomes. In order to provide additional information for the assessment of liposome encapsulated antioxidants as a treatment for sulfur mustard (SM) induced lung injury we have proposed to do a direct comparison between SM and CEES. Once a dose of each compound is established the subsequent evaluation of the antioxidant in liposomes will begin.

Introduction and Body

1. Identify doses of CEES and SM, which yield comparable injury. In the current work, we used the dose of CEES (6 mg/mg) and have determined, via light microscopy, a SM dose to induce a comparable lung injury. To accomplish this anesthetized rats were tracheally intubated and placed them on their backs on an incline. A piece of PE-50 tubing was introduced through the endotracheal tube into the left bronchus, and one hundred microliters of either diluted CEES or SM was slowly infused into the left lung. To prepare the 6 mg/kg dose of CEES, 14.3 ul of CEES was solubilized in 85.7 ul of absolute EtOH and further diluted in 900 ul of Dulbecco's Phosphate Buffered Saline (DPBS). Initially, two doses of SM, 0.7 and 1.4 mg/kg, were evaluated. The stock SM (9.4 mg/ml) was made up in EtOH and then further diluted in EtOH to the final concentration. A new SM stock (8 mg/ml) was made up 50/50 in EtOH and DPBS and further diluted in DPBS to allow a dose of 1.2 mg/kg. At 24 hrs postexposure lungs were removed and formalin fixed for H&E staining and evaluation. All lung lobes (left lung, right cranial, right medial, right caudal, and right accessory) and the trachea were each assessed for extent of damage based on the following parameters: Trachea/chondrocyte necrosis, bronchiolar infiltrates, bronchiolar epithelial necrosis, bronchiolar lymphoid tissue necrosis, alveolar fibrin/edema, alveolar hemorrhage, alveolar cellular infiltrates, alveolar epithelial necrosis, pulmonary congestion, perivascular fibrin/edema, and perivascular cellular infiltrates. Each parameter was given a score of 0 thru 4. (0 being normal.. 1 = minimal; present in 1-10 % of the section. 2=scattered changes; present in 11-25% of the section. 3=moderate; present in 25-45% of the section. 4=severe; present in greater than 45 % of the section.). The exposed lobe and trachea were typically the only sections showing any injury and these data are shown below. A small number of lungs are currently being assessed by electron microscopy. Based on pathologist's light microscopy assessment of the SM exposed lungs, the lung injury was qualitatively indistinguishable from that induce by CEES.

24 hr H&E Pathology comparison between CEES and HD following left lung instillation.

	Lung				Trachea		
	0.7 HD	1.2 HD	1.4 HD	6 CEES	0.7 HD	1.4 HD	6 CEES
Alveolar epithelial necrosis	1.6	1.5	1.7	2.0	*	*	*
Alveolar exudates	2.0	2.3	2.6	2.3	*	*	*
Alveolar hemorrhage	1.4	1.3	1.7	1.7	*	*	*
Alveolar PMN infiltrates	0.3	0.5	0.0	1.0	*	*	*
BALT necrosis	1.2	1.3	2.0	0.7	2.0	2.3	1
Bronchiolar epithelial necrosis	2.1	1.0	2.9	2.3	*	*	*
Bronchiolar exudates	2.1	2.0	2.7	2.2	*	*	*
Bronchiolar PMN infiltrates	0.7	1.0	0.1	0.8	*	*	*
Cartilage necrosis	*		*	*	1.9	1.8	1.6
Perivascular edema	2.0	1.3	1.9	2.3	*	*	*
Tracheal epithelial necrosis	*	*	*	*	1.6	1.3	0.4

A caveat in the interpretation of the table is that the pathology is scored with a categorical score, not on a continuum, so these "average scores" are not true averages. However, they do give a feel for the data and are provided for that purpose only.

2. Evaluation of liposome encapsulated antioxidant. In an effort to prepare for the liposome evaluation studies, a blood sample was taken from animals used in the dose ranging work above, and rats were then perfused with PBS/EDTA to remove remaining blood from tissues. Samples of liver, kidney, spleen and plasma were flash frozen in liquid nitrogen and sent to Drs. Keith Crawford (Brigham and Womens Hospital) and William Stone (East Tennessee State Univ) for analysis. This gave us the opportunity to establish all the methods that will be used. Dr. Stone's group determined the GSH concentration levels in tissues, and also completed alpha and gamma tocopherol determinations on tissue and plasma samples.

It is shown in the graphs that GSH levels detected in the spleen decreased in response to CEES or SM. Interestingly, in both kidney and liver, GSH levels were stable (kidney) or rose (liver) in response to SM but decreased in response to CEES intoxication. There were only 1 or 2 animals in the 1.2 SM GSH group and this data was quite variable. There appeared to be no changes in tocopherol values due to CEES or SM.

Future Studies: Currently awaiting Drs. Stone and Ward selection of the liposome preps that we will be evaluating in this model.

Key Research Accomplishments

See above.

Reportable Outcomes

See above.

Conclusions

See above.

References

None.