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14. 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.

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Statement of Work

Our focus has been to understand the molecular mechanisms by which CEES induces both acute and progressive (fibrotic) lung injury, and how such injury may be treated. With respect to mechanisms, we now know that within hours after lung contact with CEES, there is an acute inflammatory response with buildup in lung of PMNs and macrophages. With this is a surge of proinflammatory cytokines (IL-1α, TNFα, IL-6) and chemokines (MIP-2, MIP-1α, etc.). Oxidant injury of the lung also occurs, chiefly due to the intense inflammatory response. These events lead to severe damage of the lung vascular and alveolar epithelial barriers, resulting in alveolar flooding. We also know that the inflammatory response and resulting lung injury can be attenuated by PMN depletion or complement depletion prior to lung contact with CEES. Our preliminary evidence suggests that all of these events are correlated with a surge within lung of proinflammatory mediators (described above).

It is now well established by our work that antioxidant liposomes are highly protective against acute lung injury. Liposomes containing NAC, GSH or antioxidant enzymes (catalase, superoxide dismutase) are highly protective against the acute phase of injury after CEES, even if their airway administration of liposomes is delayed by 60 min. The existence of the Advanced Medical Countermeasures Consortium has been invaluable for these studies, especially through collaborations with Dr. William Stone (East Tennessee University), who has provided many of the liposome preparations, and with Dr. Milton Smith (Amaox, Ltd.) who has provided guidance and coordination to the entire consortium.

We intend to further pursue these observations according to the following questions:

1) What is the spectrum of proinflammatory cytokines and chemokines produced in lung after exposure to CEES?

2) Do protective antioxidant liposomes prevent *in vivo* activation of the transcription factor NFκB in lung tissues and in lung macrophages? Do these liposomes also prevent the appearance of cytokines and chemokines in lung after exposure to CEES?

3) Using lung macrophages, does *in vitro* cell exposure to LPS or CEES induce production of cytokines and chemokines, does this result in NFκB activation, and does the presence of antioxidant liposomes prevent such mediator production and NFκB activation?

4) Will liposomes containing α,γ tocopherol prevent the pulmonary fibrotic response to CEES?

Introduction

For several years we have been studying mechanisms by which 2-chloroethyl ethyl sulfide (CEES) inflicts damage on rodent (rat) lung. Our earlier publication demonstrated that CEES induces an acute lung-damaging inflammatory response characterized by intense infiltration of neutrophils (PMNs) and activation of lung macrophages (1). PMN depletion or complement depletion was protective in this model. We also showed that antioxidant liposomes were protective against acute lung injury (ALI) induced by exposure to CEES (1,2). We have recently found that CEES causes progressive pulmonary fibrosis by three weeks (2), the etiology of which is unknown.

Body

Reference 2 is a 2006 publication that describes more recent progress (see attached). The accomplishments can be summarized as follows:

1. Protection against ALI following lung exposure to CEES was substantially attenuated when liposomes (containing PEG-catalase, PEG-superoxide dismutase, N-acetyl cysteine (NAC) or glutathione (GSH)) were instilled into the left lung immediately following instillation of CEES.

2. Protective effects were found with liposomes containing NAC, GSH or the combination. Even when these same liposomes were instilled into lung as late as 60 min. after exposure of lung to CEES, substantial protective effects were noted.

3. While the antioxidant liposomes (noted above) were protective against CEES-induced ALI, they did not reduce the extent of pulmonary fibrosis (as quantitated by lung content of hydroxy-proline) at 3 weeks. However, we now have preliminary data suggesting that liposomes containing α,γ tocopherol (provided by Dr. William Stone, E. Tennessee State University) are protective against the development of pulmonary fibrosis (to be submitted for publication).

Key Research Accomplishment

See items 1-3, above. See reprints 1-3, included in Appendix.

Reportable Outcomes

Presentations:

1) NIH/NIAID Radiation Combined Injury Workshop, March 26-27, 2007, Bethesda, MD: "Factors affecting the outcomes of sepsis."
2) Advanced Medical Countermeasures Consortial Meeting, June 28, 2007, Crystal City, VA: "Protective effects of anti-oxidant liposomes in acute and progressive lung injury after CEES."

3)   BARDA 2007 Industry Day Conference, August 3, 2007, Washington, DC: "Protective effects of anti-oxidant liposomes."

Manuscripts (published during the past year of funding):
1)   McClintock, S.D., Hoesel, L.M., Das, S.K., Till, G.O., Neff, T., Kunkel, R.G., Smith, M.G., and **Ward, P.A**.: Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 2006, 26:126-311.
2)   Guo, R.F. and **Ward, P.A.:** Role of oxidants in lung injury during sepsis. Antioxidants and Redox Signaling 2007, 9:1-12.
3)   Book Chapter: Vesicants and Oxidative Stress in Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology and Therapeutics, 2nd edition. Set for publication December, 2007.

Conclusion

It is now clear that CEES-induced lung injury is associated with both an acute phase (over the first few days following lung exposure to CEES) and a late phase (by 3 weeks) associated with pulmonary fibrosis. The acute phase of injury is an oxidant-mediated process that is associated with intensive inflammatory injury, causing a breach in the vascular and alveolar epithelial barrier. This results in a buildup of inflammatory cells and oxidants in the distal lung, resulting in alveolar flooding and hemorrhage. Liposomes containing antioxidants (GSH, NAC) or antioxidant enzymes (catalase, SOD) are highly protective in this setting, even if their delivery is delayed for 60 min. after exposure to CEES. Very little is known about the late complication (pulmonary fibrosis), even though this complication is well established to occur in humans subjected to inhalation of mustard gas (3,4).   The proposed studies will further evaluate how the lung fibrotic phase can be ameliorated after exposure of rat lung to CEES.

References for Text
1.   McClintock, S.D., Till, G.O., Smith, M.G., and **Ward, P.A**.: Protection from half-mustard-gas-induced acute lung injury in the rat. J Appl Toxicol. 2002, 22:257-262.
2.   McClintock, S.D., Hoesel, L.M., Das, S.K., Till, G.O., Neff, T., Kunkel, R.G., Smith, M.G., and **Ward, P.A**.: Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 2006, 26:126-311.
3.   Eisenmenger, W., Drasch, G., von Clarmann, M., Kretschmer, E., Roider, G.: Clinical and morphological findings on mustard gas [bis(2-chloroethyl)sulfide] poisoning. J. Forensic Sci. 1991, 36:1688-1698.
4.   Khateri, S., Ghanei, M., Keshavarz, S., Soroush, M., Haines, D.: Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard gas. J. Occup. Environ. Med. 2003, 45:1136-1143.

Appendices (available as PDF files)
1.   McClintock Paper 2002
2.   McClintock Paper 2006
3.   Guo and Ward Review Chapter 2007

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

Shannon D. McClintock,[1] Gerd O. Till,[1]* Milton G. Smith[2] and Peter A. Ward[1]

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[2] Amaox Ltd, Silver Springs, MD, USA

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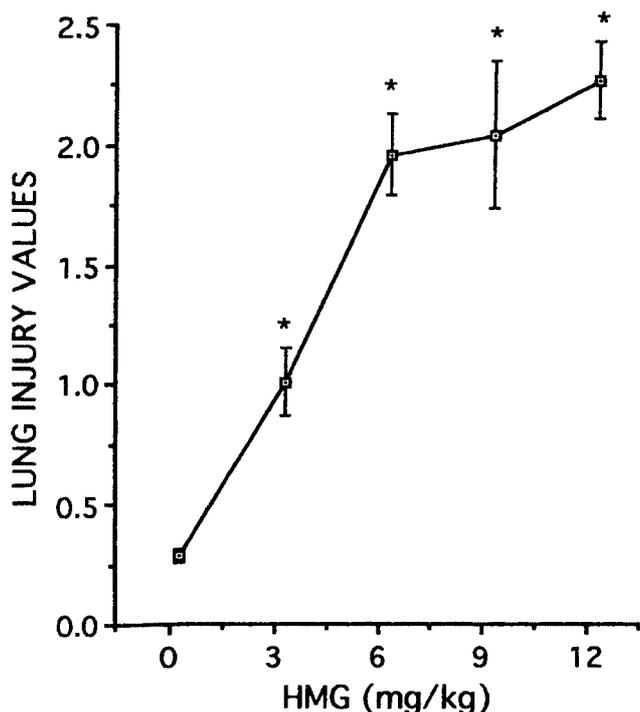
Figure 1. Dose-dependent increases in acute pulmonary injury (lung injury values defined by the permeability index) in the rat caused by HMG. Lung injury was determined at 4 h after intrapulmonary HMG injection. Based on these observations, 6 mg HMG kg^{-1} body wt was chosen for all subsequent experiments.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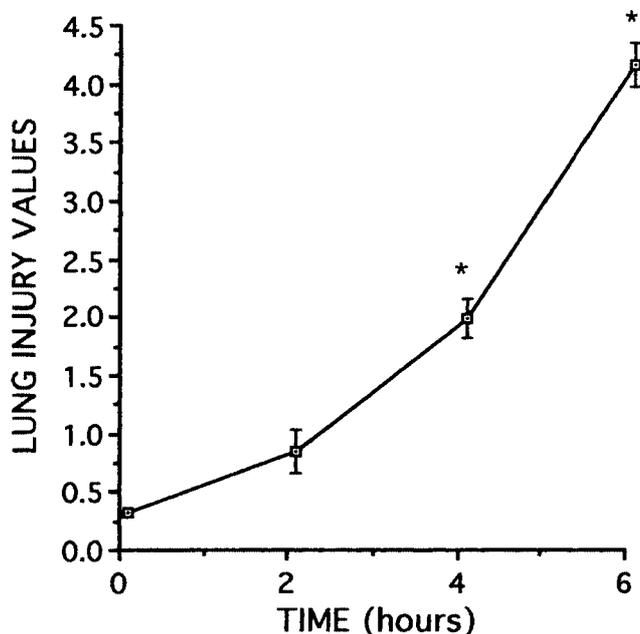

Figure 2. Time-dependent increases in lung injury values following intrapulmonary HMG (6 mg kg^{-1}) injection into rats. Based on these observations, lung injury was determined in subsequent studies at 4 h post-HMG injection.

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 ± 0.02). Catalase treatment resulted in a 47% protective effect (lung injury value = 1.13 ± 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 ± 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)
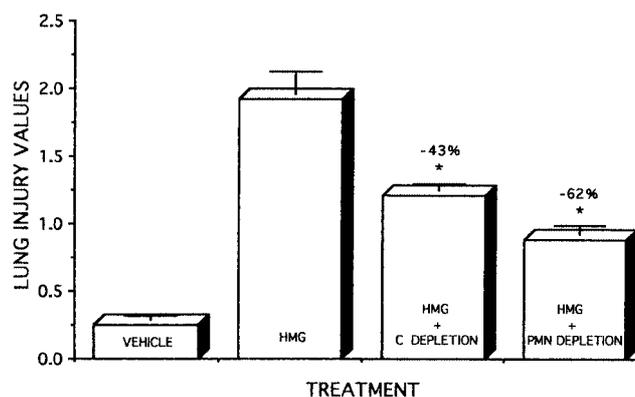

Figure 3. Intrapulmonary HMG injection into complement (C)-depleted or neutrophil (PMN)-depleted rats.
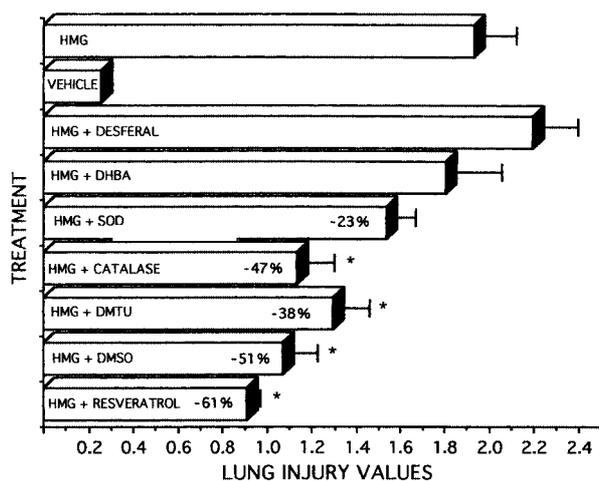
Figure 4. Effect of various antioxidants on HMG-induced acute lung injury. Test compounds included iron chelators, [desferal and 2,3-dihydroxybenzoic acid (DHBA)], antioxidant enzymes [catalase and superoxide dismutase (SOD)], hydroxyl radical scavengers [dimethyl thiourea (DMTU) and dimethyl sulfoxide (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).
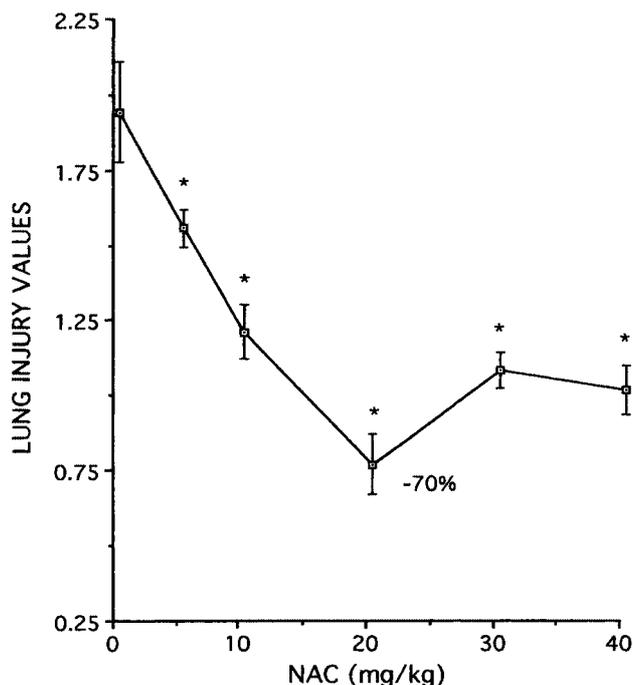

Figure 5. Dose-dependent protective effects of NAC on HMG-induced lung injury; NAC was injected 10 min prior to HMG. Lung injury values were determined at 4 h post-HMG injection.
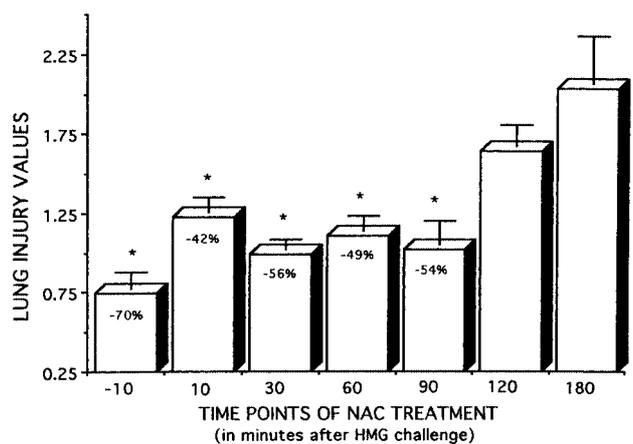

Figure 6. Effect of delayed NAC (20 mg kg^{-1}) treatment of HMG-injected rats.

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

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

REFERENCES

1. Reutter S. Hazards of chemical weapons release during war: new perspectives. *Environ. Health Perspect.* 1999; **107**: 985–990.

2. Dacre JC, Goldman M. Toxicology and pharmacology of the warfare agent sulfur mustard. *Pharmacol. Rev.* 1996; **48**: 289–296.

3. Lardot C, Dubois V, Lison D. Sulfur mustard upregulates the expression of interleukin-8 in cultured human keratinocytes. *Toxicol. Lett.* 1999; **110**: 29–33.

4. Arroyo CM, Von Tersch RL, Broomfield CA. Activation of alpha-human tumour necrosis factor (TNF-α) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulphide (H-MG). *Hum. Exp. Toxicol.* 1995; **14**: 547–553.

5. Sawyer TW, Hamilton MG. Effect of intracellular calcium modulation on sulfur mustard cytotoxicity in cultured human neonatal keratinocytes. *Toxicol. In Vitro* 2000; **14**: 149–157.

6. Watson AP, Griffin GD. Toxicity of vesicant agents scheduled for destruction by the Chemical Stockpile Disposal Program. *Environ. Health Perspect.* 1992; **98**: 259–280.

7. Kumar O, Sugendran K, Vijayaraghavan R. Protective effect of various antioxidants on the toxicity of sulphur mustard administered to mice by inhalation or percutaneous routes. *Chem. Biol. Interact.* 2001; **134**: 1–12.

8. Lakshmana Rao PV, Vijayaraghavan R, Bhaskar ASB. Sulphur mustard induced DNA damage in mice after dermal and inhalational exposure. *Toxicology* 1999; **139**: 39–51.

9. Maisonneuve A, Callebat I, Debordes L, Coppet L. Biological fate of sulphur mustard in rat: toxicokinetics and disposition. *Xenobiotica* 1993; **23**: 771–780.

10. Maisonneuve A, Callebat I, Debordes L, Coppet L. Distribution of [^{14}C]sulfur mustard in rats after intravenous exposure. *Toxicol. Appl. Pharmacol* 1994; **125**: 281–287.

11. Mozier NM, Hoffman JL. Biosynthesis and urinary excretion of methyl sulfonium derivatives of the sulfur mustard analog, 2-chloroethyl ethyl sulfide, and other thioethers. *FASEB J.* 1990; **4**: 3329–3333.

12. Tripathi DN, Suendran K, Malhotra RC, Bhattacharya A, Das Gupta S. Studies on urine and tissues of rats, guineapigs and mice to sulphur mustard using mass spectrometry. *J. Biosci.* 1995; **20**: 29–33.

13. Varma SD, Devamanoharan PS, Ali AH, Brozetti J, Petrali J, Lehnert E, Weir A. Half mustard (CEES) induced damage to rabbit cornea: attenuating effect of taurine-pyruvate-alpha-ketoglutarate-pantothenate mixture. *J. Ocul. Pharmacol. Ther.* 1998; **14**: 423–428.

14. Brown RFR, Rice P. Histopathological changes in Yucatan minipig skin following challenge with sulfur mustard. A sequential study of the first 24 hours following challenge. *Int. J. Exp. Pathol.* 1997; **78**: 9–20.

15. Eldad A, Ben Meir P, Breiterman S, Chaouat M, Shafran A, Ben-Bassat H. Superoxide dismutase (SOD) for mustard gas burns. *Burns* 1998; **24**: 114–119.

16. Anguelov A, Belchen L, Angelov G. Experimental sulfur mustard gas poisoning and protective effect of different medicines in rats and rabbits. *Indian Vet. J.* 1996; **73**: 546–551.

17. Emad A, Rezaian GR. Immunoglobulins and cellular constituents of the BAL fluid of patients with sulfur mustard gas-induced pulmonary fibrosis. *Chest* 1999; **115**: 1346–1351.

18. Emad A, Rezaian GR. Characteristics of bronchoalveolar lavage fluid in patients with sulfur mustard gas-induced asthma or chronic bronchitis. *Am. J. Med.* 1999; **106**: 625–628.

19. Dube SN, Husain K, Sugendran K, Vijayaraghavan R, Somani SM. Dose response of sulphur mustard: behavioral and toxic signs in rats. *Indian J. Physiol. Pharmacol.* 1998; **42**: 389–394.

20. Conley J, Hunter K, Lundy P, Hamilton M, Sawyer TW. Domestic swine model for the assessment of chemical warfare agent-anesthetic interactions: some effects of sulfur mustard. *Mil. Med.* 2000; **165**: 573–578.

21. Calvet JH, Jarreau JH, Levame M, D'Ortho MP, Lorino H, Harf A, Macquin-Mavier I. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pig. *J. Appl. Physiol.* 1994; **76**: 681–688.

22. Calvet JH, D'Ortho MP, Jarreau JH, Levame M, Harf A, Macquin-Mavier I. Glucocorticoids inhibit sulfur mustard-induced airway muscle hyperresponsiveness to substance P. *J. Appl. Physiol.* 1994; **77**: 2325–2332.

23. Calvet JH, Planus E, Rouet P, Pezet S, Levame M, Lafuma C, Harf A, D'Ortho MP. Matrix metalloproteinase gelatinases in sulfur mustard-induced acute airway injury in guinea pigs. *Am. J. Physiol.* 1999; **276**: L754–762.

24. Vijayaraghavan R. Modifications of breathing pattern induced by inhaled sulphur mustard in mice. *Arch. Toxicol.* 1997; **71**: 157–164.

25. Till GO, Johnson KJ, Kunkel R, Ward PA. Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. *J. Clin. Invest.* 1982; **69**: 1126–1135.

26. Mulligan MS, Till GO, Smith CW, Anderson DC, Miyasaka M, Tamatani T, Todd RF 3rd, Issekutz TB, Ward PA. Role of leukocyte adhesion molecules in lung and dermal vascular injury after thermal trauma of skin. *Am. J. Pathol.* 1994; **144**: 1008–1015.

27. Shanley TP, Foreback JL, Remick DG, Ulich TR, Kunkel SL, Ward PA. Regulatory effects of interleukin-6 in immunoglobulin G immune-complex-induced lung injury. *Am. J. Pathol.* 1997; **151**: 193–203.

28. Ballow M, Cochrane CG. Two Anti-complementary factors in cobra venom. Hemolysis of guinea pig erythrocytes by one of them. *J. Immunol.* 1969; **103**: 944–952.

29. Till GO, Beauchamp C, Menapace D, Tourtellotte W Jr, Kunkel R, Johnson KJ, Ward PA. Oxygen radical dependent lung damage following thermal injury of rat skin. *J. Trauma* 1983; **23**: 269–277.

30. Ward PA, Till GO, Kunkel R, Beauchamp C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. *J. Clin. Invest.* 1983; **72**: 789–801.

31. Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. *Lancet* 1993; **341**: 1103–1104.

32. Stojanovic S, Sprinz H, Brede O. Efficiency and mechanism of the antioxidant action of trans-resveratrol and its analogues in the radical liposome oxidation. *Arch. Biochem. Biophys.* 2001; **391**: 79–89.

33. Anderson DR, Byers SL, Vesely KR. Treatment of sulfur mustard (HD)-induced lung injury. *J. Appl. Toxicol.* 2000; **21**: S129–S132.

34. Emad A, Rezaian GR. Immunoglobulins and cellular constituents of the BAL fluid of patients with sulfur mustard gas-induced pulmonary fibrosis. *Chest* 1999; **115**: 1346–1351.

35. Dannenberg AM, Pula PJ, Liu LH, Harada S, Tanaka F, Vogt RF, Kajiki A, Higuchi K. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. *Am. J. Pathol.* 1985; **121**: 15–27.

36. Boursnell JC, Francis GE, Wormall A. Studies on mustard gas (beta-beta′-dichlorodiethyl sulfide) and some related compounds. 8. The action of mustard gas, divinyl sulphone and beta-beta-dichlorodiethyl sulphone on complement. *Biochem. J.* 1946; **40**: 774–778.

37. Cowan FM, Broomfield CA, Smith WJ. Exposure of human epidermal keratinocyte cell cultures to sulfur mustard promotes binding of complement C1q: implications for toxicity and medical countermeasures. *J. Appl. Toxicol.* 2000; **20**: S77–S80.

38. Johnson KJ, Ward PA. Role of oxygen metabolites in immune complex injury of lungs. *J. Immunol.* 1981; **103**: 2365–2369.

39. Eldad A, Ben Meir P, Breiterman S, Chaouat M, Shafran A, Ben-Bassat H. Superoxide dismutase (SOD) for mustard gas burns. *Burns* 1998; **24**: 114–119.

40. Baldwin SR, Simon RH, Boxer LA, Till GO, Kunkel RG. Attenuation by 2,3-dihydroxybenzoic acid of acute lung injury induced by cobra venom factor in the rat. *Am. Rev. Respir. Dis.* 1985; **132**: 1288–1293.

41. Anderson DR, Byers SL, Vesely KR. Treatment of sulfur mustard (HD)-induced lung injury. *J. Appl. Toxicol.* 2000; **20**: S129–S132.

42. Atkins KB, Lodhi IJ, Hurley LL, Hinshaw DB. Nacetylcysteine and endothelial cell injury by sulfur mustard. *J. Appl. Toxicol.* 2000; **20**: S125–S128.

Forum Review

Role of Oxidants in Lung Injury During Sepsis

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ABSTRACT

The role of oxidative stress has been well appreciated in the development of sepsis-induced acute lung injury (ALI). Oxidative stress in sepsis-induced ALI is believed to be initiated by products of activated lung macrophages and infiltrated neutrophils, promptly propagating to lung epithelial and endothelial cells. This leads to tissue damage and organ dysfunction. On stimulation, neutrophils (PMNs) enable their migration machinery. The lung undergoes changes favoring adhesion and transmigration of PMNs, resulting in PMN accumulation in lung, which is a characteristic of sepsis-induced ALI. Oxidative stress turns on the redox-sensitive transcription factors (NF-κB, AP-1), resulting in a large output of proinflammatory cytokines and chemokines, which further aggravate inflammation and oxidative stress. During the process, transcription factor nuclear factor-erythroid 2-p45–related factor 2 (Nrf2) and heme oxygenase (HO) appear to play the counterbalancing roles to limit the propagation of oxidative stress and inflammatory responses in lung. Many antioxidants have been tested to treat sepsis-induced ALI in animal models and in patients with sepsis. However, the results are inconclusive. In this article, we focus on the current understanding of the pathogenesis of sepsis-induced ALI and novel antioxidant strategies for therapeutic purposes. *Antioxid. Redox Signal.* 9, 0000—0000.

INTRODUCTION

DESPITE TECHNICAL DEVELOPMENTS in intensive care units (ICUs) and advanced supportive treatment, the death rate in septic patients remains high, with a range of lethality ranging from 30 to 50% (3). The acute respiratory distress syndrome (ARDS) has been defined as a severe form of acute lung injury (ALI), featuring pulmonary inflammation and increased capillary leak (98). ARDS may arise in a number of clinical situations, especially in patients with sepsis. As a common complication of sepsis, it has been considered a leading cause for death in sepsis. A well-described pathophysiologic model of ARDS is one form of acute lung inflammation mediated by inflammatory cells and mediators as well as oxidative stress (22).

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the biologic scavenger system, which can readily detoxify the reactive oxygen intermediates under normal physiologic conditions. Oxidative-induced damage has been considered to be one of the underlying mechanisms that contribute to multiple organ failure in sepsis (7, 35, 102). Because of the natural oxidizing nature of the atmosphere and the direct exposure to many atmospheric pollutants, the lung is at high risk of oxidative injury. It has been recognized that oxidative stress can not only directly cause tissue damage, but it also can affect the molecular mechanisms that control lung inflammation. In the healthy lung, airway lining fluids and extracellular spaces are maintained in a highly reduced state (with millimolar levels of reduced glutathione, GSH) to preserve normal physiologic functions. Under normal conditions, the levels of antioxidants and oxidants in lung are balanced in favor of a reducing state. Decreases in antioxidants or increases in oxidants can disrupt this equilibrium and can cause oxidative stress. An imbalance in the oxidant–antioxidant system has been recognized as one of the first events that ultimately lead to inflammatory reactions in the lung (17).

Oxidative stress has been found to occur in many forms of lung disorders, such as pneumonia, ARDS, idiopathic pulmonary fibrosis, lung transplantation, chronic obstructive pul-

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monary disease, cystic fibrosis, bronchiectasis ischemia–reperfusion, and lung cancer (10, 18, 79). Based on composition, oxidants can be divided into two main categories, ROS and reactive nitrogen species (RNS). Superoxide, hydroxyl radicals, and hydrogen peroxide are generally classified as ROS in contrast to nitric oxide and peroxynitrite, which are nitrogen based. These molecules naturally function as neurotransmitters, second messengers, and as a part of the chemical host defense against infection. It is only when their concentrations become excessive, especially extracellularly, that the potential for adverse responses can occur. High ROS/RNS levels lead to alterations in normal cell function and eventually compromise local tissue and systemic homeostatic mechanisms. In this review, we focus on the understanding of the roles of oxidative stress in sepsis-induced ALI and potential therapeutic strategies by using antioxidants.

DETECTION OF OXIDATIVE STRESS IN LUNG

It is clear that a better understanding of the oxidative state in the lung is important for the diagnosis and treatment of lung diseases. Many methods have been developed for detection of free radicals from oxygen, ROS, RNS, and their byproducts to assess the presence of oxidative stress. The techniques include established standard protocols and advanced methods using high-performance liquid chromatography (HPLC), mass spectrometry, and electron paramagnetic resonance. Described later are the most frequently used methods for evaluation of lung oxidative status.

Monitoring oxidative stress in live cells

ROS in live cells can be detected by using a fluorogenic marker for ROS and observing under fluorescence microscopy. One frequently used marker is carboxy-H_2DCFDA, a cell-permeable fluorogenic marker, which is oxidized during oxidative stress in live cells and emits bright green fluorescence (97). $NO^\cdot$ production by lung cells can be measured by using the $NO^\cdot$ fluorescent indicator, DAF-2 (89). On reaction with an active intermediate (N_2O_3) formed during the oxidation of $NO^\cdot$ to nitrite, DAF-2 is converted to its fluorescent triazole form, which can be measured by fluorescent plate readers at excitation and emission wavelengths of 485 and 538 nm, respectively..

Hydrogen peroxide (H_2O_2) and superoxide products in bronchoalveolar fluids

H_2O_2 fluids in bronchoalveolar fluids (BALs) can be measured by the simple assay for detecting the presence of peroxides in both aqueous and lipid environments. The basis of these assays is the complexing of ferrous ion (Fe^{2+}) by H_2O_2 in the presence of xylenol orange. Peroxides will oxidize Fe^{2+} to Fe^{3+}, and Fe^{3+} will form a colored complex with xylenol orange that can be read at 560 nm (46, 47). Superoxide radical generation can be estimated by nitroblue-tetrazolium reduction assay (60).

Antioxidant status in lung

The antioxidant status in the lungs can be evaluated by lung levels of superoxide dismutase (SOD) and catalase (CAT) and their activities. SOD activity can be assessed by the OxyScan SOD-525 assay, which measures the activity of all forms of SOD. The method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzôfluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Catalase (CAT) activity can be determined by a two-step reaction scheme (catalase-520 assay). First, catalase reacts with a known quantity of H_2O_2 to generate H_2O and $O_2^\cdot$. In the presence of horseradish peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a chromophore with a color intensity. Lipid peroxidation levels in lung can be measured by thiobarbutiric acid–reactive substances assay (25). The GSH/GSSG ratio, a useful measure of oxidative stress, can be determined by a colorimetric method by using Bioxtech GSH/GSSG-412.

Protein oxidation and lipid peroxidation in lung

The levels of protein carbonyls and nitrotyrosine are widely used for the detection of oxidative modification of proteins. Protein carbonyls are considered to be one of the most reliable methods to evelute the protein damage mediated by oxidative stress. Nitotyrosine levels in tissues and BAL fluids correlate with oxidant stress in animal and human studies (33). Protein carbonyls can be measured through the reaction with dinitrophenylhydrazine (DNPH) (57). The proteins are first precipitated by the addition of 20% trichloroacetic acid and then redissolved in DNPH. Nitrotyrosine is often determined by enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemistry with specific antibodies. Lipid peroxidation, oxidized lipids, and lipid mediators are believed to play an important role in lung inflammatory diseases. The detection of products of lipid peroxidation has been widely used to estimate the overall status of oxidative stress in lung. Among them, thiobarbituric acid reactants (TBARs) and malondialdehyde (MDA) are commonly used indicators (16).

DNA damage

The most common type of damage caused by reactive oxygen species in the body is oxidative damage to DNA. Hydroxydeoxyguanosine (8-OHdG), a product of this type of DNA damage, is used as a biomarker for oxidative stress. It can be measured by the immunohistochemical procedure and an HPLC system equipped with an electrochemical detector (HPLC-ECD) (63). More recently, it has been reported that capillary electrophoresis-mass spectrometry (CE/MS) can be also used for the analysis 8-OHdG to study oxidative stress (101).

Analysis of expired air for oxidation products

Studies have shown that expired $NO^\cdot$ and CO can serve as biomarkers for oxidative stress, whereas ethane can serve as a marker of lipid peroxidation (73). CO can be detected electrochemically, and it can also be measured by a laser spectrophotometer and near-infrared CO analyzers. The levels of exhaled

NO· can be assessed by chemiluminescence. Ethane content can be detected by using gas chromatography. Oxidative stress in lung appears to be an important factor in predicting or assessing (or both) lung injury. Methods for detection of oxidative stress have a broad range from well-established protocols to newly developed technologies. It is very difficult to measure oxygen free radicals directly because of their short half-lives and reactivity with other molecules. Radical spin-trapping agents have been used to form stable radical adducts, which can be detected by electron paramagnetic resonance spectroscopy (EPR). Trapping agents are generally nitrone- or nitroso-containing molecules, such as 5,5-dimethyl-1-pyrroline-n-oxide (DMPO), that react with oxygen free radicals to form stable nitroxide free radicals (72). The sampling procedure to collect exhaled breath condensate can be used to measure H_2O_2, leukotrienes, isoprostanes, and 3-nitrotyrosine in lung inflammation (73). Given the nature of complexity and importance of lung biology, it is essential to develop sensitive and reliable tools to monitor the status of lung oxidative stress. A standardized protocol is also required for clinical application.

EVIDENCE OF OXIDATIVE STRESS IN SEPSIS-INDUCED ALI

Low antioxidant and high plasma levels of oxygen free radicals have been well documented in patients with sepsis and in animal models of sepsis (30, 35, 96). Many oxidative indicators have been reported in lungs from septic patients and animals. In patients with sepsis, protein carbonyls were significantly elevated in both blood and BAL fluids during the initial phase of sepsis, decreasing within a few days but remaining above control values (102). In the same study, myeloperoxidase (MPO) activity was also markedly increased in BAL fluids from septic patients. Strong correlations were found between carbonyl concentrations in BAL fluid and plasma, when compared with protein carbonyls, TBARS, and MPO in lung, suggesting that neutrophil oxidants might be chiefly responsible for oxidative stress in lungs during sepsis. All patients with ARDS had higher levels of hypoxanthine, a prooxidant substrate for xanthine oxidase (75). Hypoxanthine levels were 2 times higher in nonsurvivors than those in survivors. Nonsurvivor ARDS patients appeared to have higher levels of oxidative stress and damage when compared with survivors. Surprisingly, hypoxanthine levels were normal in patients in intensive care with sepsis but no lung injury. These data suggest that oxidative stress plays an essential role in the pathogenesis of ALI in sepsis, and directly contributes to the bad outcome.

Oxidative stress in animal models of sepsis has been also well described. In rodent sepsis models induced by cecal ligation and puncture (CLP), the activities of enzymatic antioxidants, including SOD, CAT, and glutathione peroxidase (GSH-PX), in lung were significantly decreased during the early- and late-sepsis phages (21), indicating that sepsis sets up an environment favorable for oxidative stress in lung. As expected, MDA concentrations and nitrate (NO_3^-)/nitrite (NO_2^-) levels were also elevated in the septic lung. In addition, MPO activity was found to be enhanced, and a large number of neutrophil infiltrates were observed by histology in lungs from septic animals. Many other oxidative indicators in lung, including 8-isoprostane, exhaled NO·, superoxide anion, glutathione, protein carbonyls, and TBARS, were all changed in favor of an oxidative-stress status during experimental sepsis (2, 19, 51, 59, 85, 86, 96).

NEUTROPHIL ACCUMULATION IN LUNG DURING SEPSIS

The pathophysiology of the sepsis-induced ALI/ARDS is very complicated and not completely defined. However, increased production of ROS/RNS from PMNs has been proposed as one of the significant mechanisms underlying the development of lung inflammation. Additionally, the contribution by lung macrophages may be an important source for ROS/RNS. Sepsis-induced ALI is characterized by the activation of a variety of cells, including inflammatory cells such as PMNs and macrophages, and increased levels of inflammatory mediators. PMN infiltrates occur in lungs from both humans and animals with sepsis. The number of neutrophils in BAL fluids from patients with ARDS is significantly increased and associated with poor survival (1, 61, 100). Circulating PMNs infiltrate and accumulate in the lung *via* transmigration through the endothelium, interstitium, and alveolar epithelium to enter the alveolar compartment or be sequestered in lung capillaries. Upregulation of chemoattractant molecules (chemokines) occurs, establishing a concentration gradient that directs the neutrophils into the lung. Adhesion molecules, including integrins, selectins, and ICAMs, are also involved in the migration process (40). These events are described in Fig. 1.
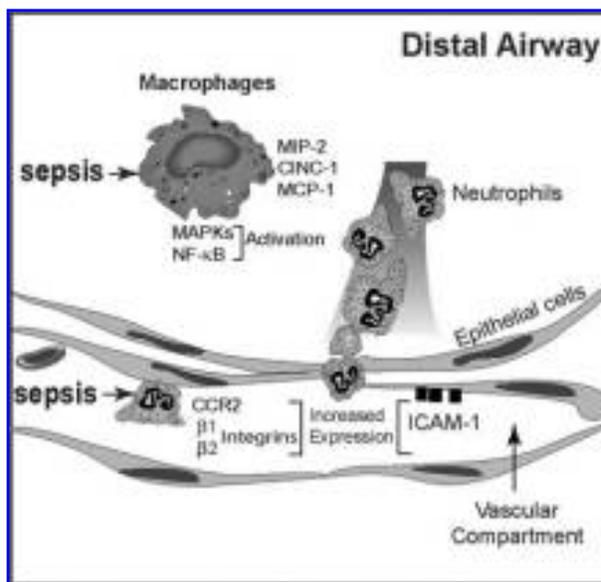

FIG. 1. The mechanism of PMN accumulation in the lung during sepsis. In rat and mouse models of sepsis, activated alveolar macrophages release MIP-2 (rat), CINC-1 (rat), and MCP-1 (mouse), which chemoattract PMNs into the lung. Increased levels of CCR2 as well as β_1 and β_2 integrins on PMNs, together with enhanced expression of ICAM-1 on endothelial cells, facilitate PMN adhesion and migration.

Local production of CXC chemokines is essential for the accumulation of PMNs in the lung under conditions of acute inflammation. For PMN chemoattractants, although sufficient local concentrations of CXC chemokines are necessary for PMN recruitment, the dictating factor is the ratio of lung-to-blood chemokine concentrations (11). In experimental sepsis, lung production of two important CXC chemokines, MIP-2 and CINC-1, increased but lagged behind in comparison with the levels of MIP-2 and CINC in blood (38). As a result, lung levels of CXC chemokines increased when blood levels of those decreased. The discrepancy might be important for CXC chemokines to create a gradient difference between the two compartments (vascular and alveolar), which is necessary for initiation of PMN migration. CXC chemokines have been implicated in all steps in the extravasation process of leukocytes, including rolling, adhesion, and transmigration *in vivo* (104). Thus, the role of blood CXC chemokines may primarily focus on the activation of PMNs and endothelial cells, setting the stage for PMN migration, whereas local CXC chemokines chiefly function chemotactically during sepsis. In addition, blood CXC chemokines produced in the early stage of experimental sepsis may provide vital signals for PMN survival, given the fact that CXC chemokines and C5a reduce PMN apoptosis (23, 39). Under such conditions, the greatly prolonged life span of PMNs may be related to the development of the symptoms of sepsis. The reason for higher BAL levels of CXC chemokines is likely the activation of lung macrophages. It is clear that a phase exists in which alveolar macrophages are prone to activation during the early stage of sepsis, and then may go into a refractory (deactivation) stage (38, 82). Conversely, PMNs quickly enter into this refractory phase, and their capability for producing CXC chemokines and other inflammatory mediators is greatly compromised. It was found that CXC chemokine macrophage-inflammatory protein-2 (MIP-2) production in both alveolar macrophages and PMNs was dependent on mitogen-activated protein (MAP) kinases (p38 and p42/p44) and NF-κB pathways (38). Interestingly, alveolar macrophages still maintain, and even increase, their capacity for activation of p38 and p42/p44 MAPKs during sepsis, whereas septic blood PMNs become nonresponsive to stimulations. Paralysis of signaling pathways in PMNs is likely caused, at least in part, by overproduction of C5a in sepsis. In addition, the CC chemokine, MCP-1, seems to also play an important role in attracting PMNs into lung (89). PMNs in sepsis used a novel migration pathway that is CC chemokine receptor CCR2 dependent. Ordinarily, PMNs do not express CCR2, but in sepsis, this receptor is clearly expressed (88).

Sepsis involves widespread upregulation of both PMN and endothelial adhesion molecules (74). In a rat model of sepsis, it was observed that the content of β_1 and β_2 integrins on circulating PMNs was elevated after CLP. The increased expression of β_1 integrin on blood PMNs followed β_2 integrin elevation, which was seen as early as 3 h after CLP. Rapid elevation of β_2 integrin may represent an important role in host defense by directing PMNs into inflamed organs. Several lines of evidence support such a role for β_2 integrin in the mobilization of PMNs into tissues. Patients with inherited deficiencies of β_2 integrin are much more susceptible to bacterial infection (41). In a canine model of lung inflammation (i.v. infusion of TNF-α), anti-CD11b treatment reduced PMN accumulation early (within

the first 24 h), but not later (>24 h) (74), suggesting that leukocyte trafficking may differ between the early and late stages of inflammation. Expression of β_1 integrin in PMN migration is amplified in sepsis. Fully activated β_1 integrin at the later stage of sepsis may alter the balance of integrin cooperativity. It was previously shown that blood PMNs from septic, but not control, patients expressed $\alpha_4\beta_1$ integrin, which caused increased adhesiveness to immobilized VCAM-1 (43). Anti-β_1 integrin antibody interferes with cell motility of septic PMNs from CLP rats, indicating that sepsis alters the trafficking of PMNs into the lung by engaging a β_1 integrin–dependent pathway (37). The adhesion molecule, ICAM-1, is also involved in PMN migration during sepsis. Absence or blockade of this molecule impairs the ability of PMNs to migrate into organ tissues and reduces consequent secondary organ damage, resulting in improved clinical indicators and overall survival (42). We previously demonstrated that PMN migration into lung during sepsis is ICAM-1 dependent, but not VCAM-1 dependent, perhaps related to VCAM-1 shedding that occurs during the course of sepsis development (54).

Figure 1 illustrates a simplified version of pathways for PMN migration in the lung during sepsis. CCR2 and β_1 and β_2 adhesion molecules on PMNs are upregulated during the course of sepsis, whereas PMN capability to produce CXC chemokines is reduced. In lung, activated macrophages release a large amount of PMN chemoattractants and other inflammatory cytokines, whereas ICAM-1 is activated on endothelial cells, setting the stage for PMN migration. These processes are tightly regulated by MAPKs and NF-κB pathways.
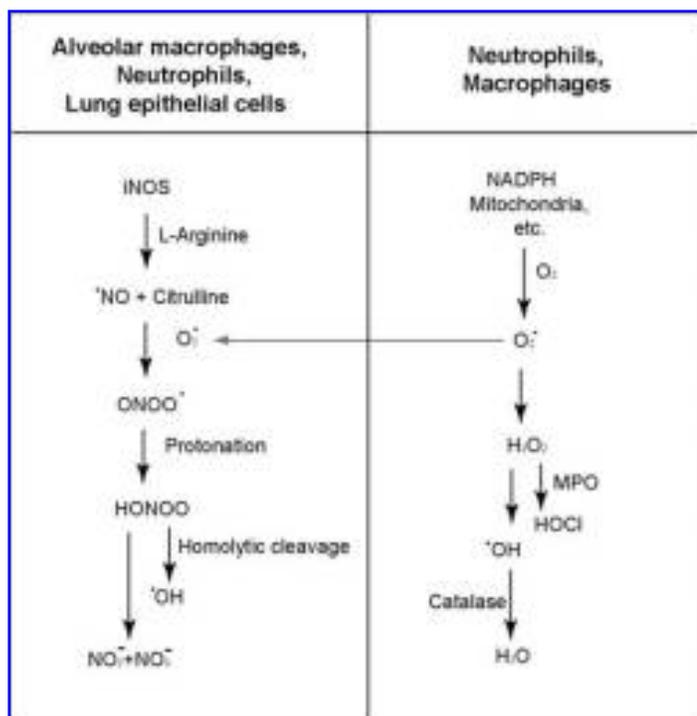
ROLE OF OXIDANTS IN SEPSIS-INDUCED ALI

Inflammation occurs after activation of PMNs and macrophages, resulting in ROS/RNS generation and the release of lysosomal enzymes and cationic proteins. Oxidative stress is initiated by ROS such as superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). These oxidants are not very harmful *per se*, but can be converted into more dangerous oxidants, causing harmful reactions in tissues (16). Generation of superoxide-derived products continues to be the main pathway responsible for the production of ROS. $O_2^{\cdot}$ can be generated through various enzymatic systems, including the mitochondrial respiratory chain, xanthine oxidase, cyclooxygenase, and NADPH oxidase (16). The NADPH oxidase pathway is well defined in phagocytic cells. Activation of the oxidase can be initiated by a variety of inflammatory mediators and is likely to be a major source of oxidant generation in sepsis-induced lung injury. Translocation of cytosolic subunits of NADPH oxidase components from the cytoplasm to the cell membrane occurs in PMNs, macrophages, and monocytes on cell stimulation, thus representing a potential therapeutic target in the treatment of ROS-mediated lung injury during sepsis. NADPH oxidase catalyzes the transfer of an additional electron to molecular oxygen (O_2) to form the $O_2^{\cdot}$ anion. Because NADPH oxidase adds single electrons to O_2, oxygen-derived intermediates are also produced. Reduction of Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^{+} by superoxide in the Fenton re-

action facilitates the generation of hydroxyl radicals (OH^-). In the presence of Fe^{2+}, superoxide is reduced to H_2O_2, which can then be metabolized in the presence of transition metals and chloride to form hypochlorous acid (HOCl). This reaction is catalyzed by MPO and provides a practical marker for PMN accumulation in tissue. Although it is not a free radical, H_2O_2 can permeate cellular membranes, thus extending the damage beyond the originating cell. These products injure cells of the lung and airway and interfere with gas exchange.

NO⋅ is an abundant signaling molecule. Like H_2O_2, it is able to cross cell membranes to alter various physiologic processes through binding and activation of guanylate cyclase. *Via* this intermediate, NO⋅ functions as a secondary messenger in the maintenance of systemic vascular tone. NO⋅ also affects platelet aggregation and can stimulate immune responses, activate genes, and cause apoptosis (32, 50). It is fairly unreactive with bioorganic molecules; however, it does react with aromatic amino acids to form stable nitrotyrosine adducts in proteins and peptides, which may impair cell function. NO⋅ is synthesized by three nitric oxide synthase (NOS) isozymes, so named by the origin of the cell in which they were originally discovered. Two of the three forms (nNOS and eNOS) are constitutively expressed and generate small amounts of NO⋅, which generally are not sufficient to cause cellular damage. Binding to calcium and calmodulin is required for NOS activation, serving as factors that regulate NOS activity. In contrast, the third isoform (iNOS) is an inducible calcium-independent enzyme that produces copious amounts of NO⋅ for several hours to even days after induction. It is during circumstances that initiate induction of this third NOS form that the physiologic roles of NO⋅ are superseded by its implication as the culprit behind various injurious inflammatory responses and potentially cytotoxic events.

In the setting of lung, all three NOS isozymes are present. Various forms of NO⋅ can be observed as *S*-nitrosothiol, nitrate, and nitrite in exhaled air and bronchoalveolar lavage from human lungs (24, 95). Upregulation of iNOS amplifies the conversion of L-arginine to L-citrulline and NO⋅ formation. In the presence of superoxide, NO⋅ is converted to peroxynitrite ($ONOO^-$), which is then protonated to form an unstable species, peroxynitrous acid (ONOOH). The presence of iNOS has been reported in alveolar macrophages, PMNs, and endothelial and lung epithelial cells (99). Accordingly, these cells are all potential sources of peroxynitrite. $ONOO^-$ reacts with protein thiols and is thought to be the predominant mechanism by which NO⋅ production leads to cytotoxicity. Peroxynitrous acid is degraded to form the hydroxy radical, NO_3^- and NO_2^- by hemolytic cleavage, or it can react with CO_2 to form peroxycarboxylate ($ONOOCO_2^-$). Detection of NO_3^- and nitrite NO_2^- serves as a convenient experimental marker for NO⋅ production. Activation of infiltrating and resident phagocytes can cause an increase in both NO⋅ and superoxide, resulting in apoptosis. On a molecular level, the resulting peryoxynitrite interacts with DNA to cause DNA fragmentation, and with membrane lipids to cause peroxidation of the endothelial or alveolar epithelial cell plasma membranes. Low concentrations of NO⋅ suppress peroxidation, but as superoxide levels increase, cell membrane injury becomes intensified. Reduced amounts of NO⋅ serve to decrease endothelial cell membrane permeability, thus limiting PMN transmigration from the vascular compartment into the lung tissue. NO⋅ is an endogenous inhibitor of leukocyte adhesion to endothelial cells, but this phenomenon is reversed by increasing amounts of superoxide anion. The potential pathways that generate oxidants in sepsis-induced ALI are illustrated in Fig. 2.

FIG. 2. Mechanisms for production of reactive oxygen species in sepsis-induced ALI.

Role of NO˙ in sepsis-induced ALI

Sepsis and ALI are associated with a high level of NO˙ production after activation of iNOS. NO˙ has been implicated in the pathophysiology of ALI in humans and animal models of ALI. However, NOS inhibition with nonselective inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), in animals with sepsis and ALI has resulted in worsened outcomes (65, 71), suggesting complex roles of NO˙ in the pathogenesis of sepsis. The results obtained from iNOS-deficient mice have been controversial. In the CLP-induced sepsis model, no pulmonary protein leak developed in iNOS-deficient mice, despite increased pulmonary MPO activity (27). By using the same model, it has been demonstrated that pulmonary oxidant stress is completely iNOS dependent and is associated with tyrosine nitration, and that pulmonary oxidant stress and nitrosative stress are dependent on the presence of iNOS in inflammatory cells (macrophages and PMNs), with no apparent contribution of iNOS in pulmonary parenchymal cells (80, 81). Therefore, the authors proposal that iNOS inhibition targeted specifically to inflammatory cells may be an effective therapeutic approach in sepsis and acute lung injury. In the bacterial lipopolysaccharide (LPS) model of ALI, lung-injury parameters such as MPO levels and albumin leak into lung were not affected by eNOS deficiency, but substantially were intensified in mice with iNOS deficiency. In LPS-indued lung injury in iNOS-deficient mice, BAL levels of CXC chemokines (MIP-2, KC) did not show any difference when compared with wild-type (WT) mice, but CC chemokines (MCP-1, MCP-3) were enhanced. Blockade of MCP-1 in iNOS-deficient mice reduced lung MPO to the levels present in WT mice. Thus, iNOS appears to play a protective role in this ALI model by limiting PMN accumulation in lung (89). Reasons for the discrepancy for iNOS-deficient effects from two ALI models are not clear. Tissue-specific KO conditions and reliable specific inhibitors for iNOS may help clarify the role of NO˙ in sepsis-induced ALI, thereby assisting in the design of future clinical trials. Nevertheless, the role of NO˙ in oxidative stress in lung is indisputable.

Role of $O_2^˙$ in sepsis-induced ALI

Generation of $O_2^˙$ by the NADPH oxidase complex of PMNs is crucial for host defense responses, and it is essential for killing invading microorganisms. However, $O_2^˙$ may also exert harmful effect in tissues. NADPH oxidase is a multisubunit complex in which gp91^{phox} and p47^{phox} are essential for $O_2^˙$ generation. $O_2^˙$ can work as a "double-edged sword," so it is not surprising that the results from p47$^{phox-/-}$ and gp91$^{phox-/-}$ mice are perplexing in the setting of sepsis (31). More PMN infiltrates and higher bacterial loads were seen in lungs from p47$^{phox-/-}$ and gp91$^{phox-/-}$ mice compared with WT mice after live *Escherichia coli* challenge, whereas lung microvascular injury was prevented in these mice. Thus, PMN infiltration in lung tissue did not result in overt lung microvascular injury, when the $O_2^˙$ generation was impaired. In the same study, it was found that increased bacterial load in NADPH-deficient mice was a critical factor for activating the release of chemokines, which subsequently enhanced PMN sequestration and migration into lung tissue. A selective SOD mimetic, M40401, was found to be protective in a live *E. coli* model of

sepsis, but it improved survival only in severe sepsis and was less effective and even harmful with less-severe sepsis (19). Another SOD mimetic, "Tempol," has been reported to improve survival in CLP-induced septic rats, reduce the plasma levels of NO˙ and IL-1β, and decrease the levels of organ $O_2^˙$ and tissue injury (59). It seems that $O_2^˙$ is indispensable for killing bacteria, but also leads to tissue damage during sepsis.

NO˙ and $O_2^˙$ appear to play dual roles in the pathogenesis of sepsis-induced ALI. They may function by totally different mechanisms at different stages of sepsis. Thus, in clinical trials that involve antioxidant inhibitors, a compelling need exists to monitor closely the bacterial load and oxidative status. For example, it might be reasonable to scavenge $O_2^˙$ at a stage when the systemic bacterial load is partially contained during sepsis.

OXIDANT-RELATED MOLECULAR EVENTS IN SEPSIS-INDUCED ALI

NF-κB and activator protein-1 (AP-1)

NF-κB and AP-1 are two well-defined redox-sensitive transcription factors. Oxidative stress activates multiple stress kinase pathways and transcription factors (NF-κB, AP-1) by modifying cysteine residues subsequently regulating gene expression for proinflammatory cytokines as well as the protective antioxidant molecules (76). Activation of NF-κB occurs in lung macrophages and in lung tissue during sepsis (28, 45, 62). NF-κB is a heteromeric dimer composed of a complex of proteins from the RelA family. NF-κB is constitutively relegated to the cytosolic compartment. The dimer most commonly comprises p50 (NF-κB1) and p65 (RelA) subunits bound to members of the inhibitor κB (IκB) family. Activation of NF-κB occurs in response to an appropriate stimulus. After phosphorylation and ubiquitination of an IκB subunit, it is subsequently degraded by a 28S proteasome. This allows the heterodimer complex to translocate to the nucleus and bind to specific DNA promotor sequences. Access to DNA is dependent on the degree of histone coiling, which is in turn regulated by the degree of acetylation of histone core residues. As the DNA becomes more acetylated, it unwinds, thus allowing binding of transcription factors, initiating gene transcription. H_2O_2 and TNF-α have both been shown to increase histone acetylation, providing a potential mechanism for oxidant-mediated inflammation (56). This effect may be antagonized by NO˙, which has been shown to be capable of maintaining levels of IκB, thus hindering NF-κB activation (95). Whether this is achieved by decreasing IκB degradation or increasing its synthesis has yet to be determined. Depletion of glutathione leads to ubiquitination of NF-κB and subsequent activation (77, 78). As glutathione levels increase, IκB degradation is inhibited so that NF-κB does not become activated. NF-κB also regulates iNOS and the inducible form of cyclooxygenase (COX-2). Thus, regulation of the inflammatory response may proceed through a negative-feedback loop *via* NF-κB and nitric oxide. An endogenous protease inhibitor, secretory leukocyte protease inhibitor (SLPI), inhibits IκB degradation, suppressing NF-κB activation. PMN accumulation, ROS activity, and levels of IL-1 and IL-8 were shown to decrease in LPS-treated rabbits that in-

haled NO· (48). These decreases correlated with a concomitant decrease in NF-κB activation. Depletion of lung macrophages in rats by airway instillation of liposome-encapsulated dichloromethylene diphosphonate suppressed activation of NF-κB and resultant BAL levels of TNF-α and MIP-2 (55). PMN accumulation and vascular permeability were also decreased, suggesting that activation of alveolar macrophages served as the source of an initial inflammatory stimulus. Activated alveolar macrophages and infiltrated PMNs generate ROS and cytokines, propagating the cycle of oxidant stress and inflammation. This scenario is likely to be the key event that drives pulmonary oxidant stress and inflammation during sepsis-induced ALI.

The promoter regions of various cytokines and chemokines contain binding sites for AP-1, suggesting that AP-1 plays a critical role in coordinating the gene expression of various inflammatory mediators. Like NF-κB, AP-1 activation occurs in lung during sepsis and likely modulates inflammation (4). AP-1 is a complex multisubunit protein composed of members of the Jun and Fos families. Much is known regarding its role in events regulating cell proliferation, transformation, differentiation, and apoptosis (49). AP-1 activation was found to be detectable in alveolar macrophages as well as in whole-lung lysates. Macrophage depletion or anti–TNF-α treatment significantly decreased the level of AP-1 activation in lung after IgG immune complex deposition, but complement depletion had no effect (36). AP-1 also responds to oxidative and cellular stress, DNA damage resulting from UV irradiation, and exposure to proinflammatory cytokines (TNF-α, IFN-γ, and TGF-β). TNF-α has been shown to activate AP-1 upstream of MAPK kinase pathways. Activation of AP-1 appears to occur concurrent with activation of other transcription factors, including Elk-1, ATF-2, and CEBP (58, 78). The AP-1 family of transcription factors has been found to play a critical role in regulating the stress-inducible protein heme oxygenase-1 (HO-1) gene after LPS treatment in rat lung (12), suggesting that AP-1 may also participate in the negative regulatory loop of the inflammatory chain.

Nuclear factor-erythroid 2-p45–related factor 2 (Nrf2)

Nrf2 is a transcription factor that is expressed in many organs, including lung. Nrf2 is directly involved in transcriptional activation of ARE-driven redox-related genes including GST, NADPH/quinine reductase, UDP-glucosyltransferases, epoxide hydrolase, heme oxygenase-1, glutathione peroxidase-2, peroxiredoxins, and glutathione reductase (GSSG-R). Therefore, it appears to be an important modulator in regulation of redox status in cells. Nrf2 has been reported as a critical intracellular molecule for regulation of the innate immune response and survival in mouse models of sepsis. Disruption of Nrf2 dramatically worsened the survival of mice in response to endotoxin and CLP-induced septic shock. Inflammation in these Nrf2-deficient mice was greatly intensified after LPS challenge. In response to LPS, Nrf2-deficient cells showed greater activation of NF-κB, which appeared to be regulated *via* the modulation of the oxidant–antioxidant system (93). In addition, these mice have been shown to be more susceptible to hyperoxic lung injury and lung inflammation induced by the oxidant butylated hydroxytoluene (BHT) (14, 15). Activation of Nrf2 by a chem-

ical agonist, CDDO-Im, attenuated LPS-induced inflammatory responses and oxidative stress in lung, and decreased mortality in Nrf2-sufficient mice (94). Nrf2 appears to be an important transcription factor that limits progression of oxidative stress during sepsis-induced ALI. Therefore, activation or overexpression of this molecule in lung appears to be an attractive strategy for antioxidative defense.

Heme oxygenase-1 (HO-1)

Cumulative evidence has demonstrated that the stress-inducible protein, HO-1, is an auxiliary antioxidant molecule, closely involved in the regulation of lung oxidative status and inflammatory responses. LPS induces high mRNA levels of HO-1 expression in the rat lung, which correlates with increased HO-1 protein levels and enzyme activity (12, 13). Redox-sensitive transcription factor AP-1 plays a critical role in regulating HO-1 gene activation after LPS exposure. In a murine model of sepsis, mice treated with a lethal dose of LPS and subsequently exposed to the HO-1 enzymatic product, CO, had significantly improved survival and lower serum IL-6 and IL-1β levels than controls. The same effect was obtained when endogenous CO was induced through overexpression of HO-1 (68). Interestingly, AP-1 binding was decreased by CO exposure. CO, ferrous iron, and biliverdin are main enzymatic products of HO activation. With the rat model of CLP-induced sepsis, biliverdin treatment offered a potent defense against lethal endotoxemia, as well as in the lungs, and effectively abrogated the lung inflammatory response. Biliverdin administration before a lethal dose of LPS led to a significant improvement in long-term survival, reduced lung permeability and lung alveolitis, and decreased proinflammatory cytokine IL-6. In the same case, augmentation of IL-10, a potent antiinflammatory cytokine in lung injury, occurred (84). Iron (Fe) released as a result of HO-1 activation returns to a transient chelatable pool, where it may potentially promote oxidative stress and inflammation. However, this pathway can be effectively inhibited by ferritin generated through HO-1. Apparently, HO-1 works as a strong negative regulator in the development of oxidative stress and lung inflammatory responses during sepsis-induced ALI. Endogenous CO indeed increased in patients with severe sepsis (103), suggesting that the HO-1 pathway has been activated, and provides a protective role in patients with sepsis.

Other important molecular events in sepsis-induced ALI

Cellular oxidative stress regulates cell function from many perspectives, including receptor function, enzymatic activity, transcription factor activation, and gene expression. As described earlier, it has been documented that protein modifications frequently occur during sepsis. Oxidation-involved molecular events such as tyrosine phosphorylation, activation of MAP kinases, protein kinase C, phospholipase A$_2$, are found in the lung during sepsis and are likely contribute to the pathogenesis of sepsis (45, 53, 69, 90). Other oxidation-involved events such as protein carbonylation, tyrosine chlorination, and tyrosine nitration are also reported in sepsis (20, 66). How these events regulate the development of sepsis-induced ALI has yet to be defined.

As depicted in Fig. 3, oxidative stress in the lung activates NF-κB and AP-1 pathways during sepsis-induced ALI, which in turn leads to amplified inflammatory responses. AP-1 and Nrf2 activation result in HO-1 expression, leading to CO production in the lung during sepsis. CO together with enzymatic antioxidants generated from Nrf2 activation may play an anti-inflammatory role in sepsis-induced ALI.

ANTIOXIDANT TREATMENT IN SEPSIS-INDUCED ALI

In an earlier study in patients with sepsis-induced ALI, it was found that oral intake of an antioxidant mixture reduced lung microvascular permeability, improved oxygenation and cardiopulmonary function, and reduced proinflammatory eicosanoid synthesis and lung inflammation (29). These findings provide hope for antioxidant strategies in the treatment of sepsis.

Both enzymatic antioxidants and nonenzymatic antioxidants have been widely tested in humans and animals with sepsis. *N*-acetylcysteine (NAC), a nonenzymatic antioxidant, is one of the most extensively tested antioxidants. NAC possesses powerful antioxidative roles by directly scavenging oxygen radicals (H_2O_2, OH , HOCl) and indirectly replenishing the cellular glutathione system. In the models of sepsis-induced ALI, NAC has been shown to be highly protective in lung inflammatory responses by reducing the levels of inflammatory mediators, inhibiting PMN activation and sequestration, suppressing the prothrombotic state, and preventing hypoxic pulmonary vasoconstriction (HPV) (6, 26). However, clinical trails have resulted in controversial findings. Although NAC improves the degree of organ-failure indexes in patients with septic shock, it reduces cardiac performance and tissue oxygenation (5). It was even harmful when initiation of NAC treatment occurred >24 h after hospital admission (67). A later clinical trial suggested
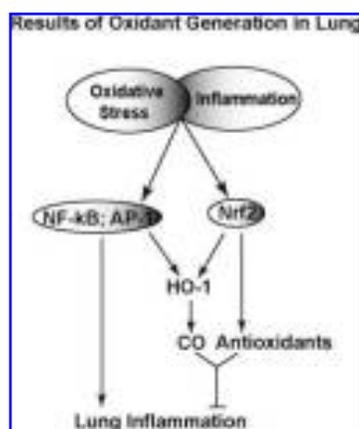

FIG. 3. Molecular events in sepsis-induced ALI. Oxidative stress and inflammation in lung activate the transcription factors, NF-κB and AP-1, leading to amplified inflammatory responses. AP-1 and Nrf2 activation result in HO-1 expression, resulting in CO production. CO, together with enzymatic antioxidants generated from Nrf2 activation, results in an antiinflammatory outcome in sepsis-induced ALI.

that NAC treatment aggravated sepsis-induced organ failure, in particular cardiovascular failure (87). In a recent preclinical trial study using a porcine model of endotoxemia, NAC failed to improve any of the variables of the systemic, pulmonary, or hepatosplanchnic hemodynamics, gas exchange, and metabolism, although it significantly elevated glutathione levels (96). More experimental and clinical studies with new management approaches are required to take advantage of the potential therapeutic utility of NAC.

Tocopherol (vitamin E) is another nonenzymatic antioxidant that has potential for treatment of sepsis-induced ALI (83, 92). Tocopherol can directly scavenge ROS and upregulate the activities of antioxidant enzymes. It can terminate the chain reaction of lipid peroxidation by scavenging lipid peroxyl radicals. In the mouse model of endotoxin-induced ALI, liposomal tocopherol administration significantly decreased the number of PMNs in airspaces and reduced lung injury, as evidenced by decreased lactate dehydrogenase activity in airways and reduced lung edema. Tocopherol failed to inhibit NF-κB and AP-1 activation, as well as the endotoxin-induced expression of proinflammatory cytokines in lung tissue. In patients with ARDS, the antioxidative system is severely compromised, as evidenced by decreased plasma levels of α-tocopherol, ascorbate, β-carotene, and selenium (64). The early administration of α-tocopherol and ascorbic acid (vitamin C) in humans reduced the incidence of organ failure and shortened the ICU length of stay (70). Vitamin C is known to scavenge $O_2^{\cdot}$ by forming the semi-dehydroascorbate free radical that is subsequently reduced by GSH. Thus, oral intake of combined antioxidants appears to be beneficial in the setting of sepsis-induced ALI.

The antioxidant enzyme SOD, with a biologic function of dismutating $O_2^{\cdot}$ to H_2O_2, holds promise for the treatment of sepsis-induced ALI. Extracellular (EC)-SOD-deficient mice showed increased evidence of ALI that occurs after hemorrhagic shock, accompanied by increased lung PMN accumulation and MPO activity (8). Overexpression of pulmonary EC-SOD in the mouse lung significantly attenuated lung injury that occurs after hemorrhagic shock (9). Overexpression of EC-SOD in lung also attenuated influenza-induced lung injury by both ameliorating inflammation and attenuating oxidative stress (91). Unlike nonenzymatic antioxidants, antioxidant enzymes such SOD and CAT have high affinities and rates of reaction, which can effectively detoxify ROS in numerous cycles, representing a highly efficient mechanism in detoxification. Similar to SOD, CAT provided impressive protection against acute lung injury induced in experimental animals after administration of LPS. EUK-8, a low-molecular-weight salen-manganese complex that exhibits both SOD-like and CAT-like activities *in vitro*, significantly attenuated several features of lung dysfunction caused by endotoxin, including arterial hypoxemia, pulmonary hypertension, decreased dynamic pulmonary compliance, and pulmonary edema (34). Because of the obvious gap between human diseases and animals models, further clinical data are needed to assess the role of the SOD/CAT approach in sepsis-induced ALI.

Other antioxidants that have shown therapeutic merits in the animal models of sepsis-induced ALI include methylene blue (21), resveratrol (52), tempol (59), β-glucan (85), M4041 (19), melatonin (86), and AT1-receptor inhibitor (44). However,

these compounds have been tested only in animals. More experimental and clinical data are expected in the coming years to validate these compounds by using various animal models and eventually in humans with sepsis.

SUMMARY

Sepsis and sepsis-induced ALI represent unsolved clinical problems due to the extremely complicated pathogenesis, which involves an imbalance of the pro- and antiinflammatory networks, complement activation, endothelial cell activation, PMN and macrophage activation, oxidative stress, and transcription factor activation. Blockade of only one of the inflammatory mediators has not resulted in a satisfactory outcome in human clinical trials, perhaps because of the complexity of the inflammatory network and the redundancy of inflammatory mediators. As oxidative stress works through the initiation and progression phases in the development of sepsis, the importance of oxidant stress in sepsis-induced ALI in humans has been appreciated. The disappointing results of NAC clinical trials may reflect the inability to reestablish a redox balance in the setting of sepsis in patients. It has been realized that severity of sepsis alters the effects of $O_2^{.}$ inhibition in sepsis (19). Further improvement of antioxidant interventions requires better understanding of the mechanisms and characteristics of oxidant stress in the specific setting of a disease condition and the development of more-effective delivery strategies. In addition, the redox-sensitive transcription factor, NF-κB, seems to be a logical target for therapy in ARDS patients. However, blockade of NF-κB inevitably turns off some gene expression, which may lead to immunosuppression. To this end, a local inhibition of lung NF-κB activation may be a less detrimental therapeutic strategy. Therapeutic strategies should be directed at the improvement of net proinflammatory, prooxidant, and cytotoxic imbalances that develop in ARDS.

ACKNOWLEDGMENTS

ABBREVIATIONS

ALI, acute lung injury; AP-1, activator protein-1; BAL, bronchoalveolar fluid; BHT, butylated hydroxytoluene; CAT, catalase; CE/MS, capillary electrophoresis–mass spectrometry; CLP, cecal ligation and puncture; COX, cyclooxygenase; DMPO, 5,5-dimethyl-1-pyrroline-n-oxide; DNPH, dinitrophenylhydrazine; EPR, electron paramagnetic resonance spectroscopy; Fe^{2+}, ferrous ion; GSH-PX, glutathione peroxidase; GSSG-R, glutathione reductase; HO-1, heme oxygenase-1;HPLC, high-performance liquid chromatography; HPV, hypoxic pulmonary vasoconstriction; HRP, horseradish peroxidase; H$_2$O$_2$, hydrogen peroxide; HOCl, hypochlorous acid; L-NMMA, NO$_3^{-}$ nitrate; LPS, lipopolysaccharide; MIP-2, mac-rophage-inflammatory protein-2; MAP, mitogen-activated protein; MPO, myeloperoxidase; MDA, malondialdehyde; NAC, N-acetylcysteine; NO$_2^{-}$, nitrite; N^{G}-monomethyl-L-arginine; NOS, nitric oxide synthase; Nrf2, nuclear factor-erythroid 2-p45–related factor 2; O$_2^{.}$, superoxide; OH^{-}, hydroxyl radical; 8-OHdG, hydroxydeoxyguanosine; ONOO^{-}, peroxynitrite; ONOOH, peroxynitrous acid; ONOOCO$_2^{-}$, peroxycarboxylate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SLPI, secretory leukocyte protease inhibitor; TBARS, thiobarbituric acid-reactive substances; vitamin C, ascorbic acid; vitamin E, tocopherol.

REFERENCES

1. Aggarwal A, Baker CS, Evans TW, and Haslam PL. G-CSF and IL-8 but not GM-CSF correlate with severity of pulmonary neutrophilia in acute respiratory distress syndrome. *Eur Respir J* 15: 895–901, 2000.
2. Andrades M, Ritter C, Moreira JC, and Dal-Pizzol F. Oxidative parameters differences during non-lethal and lethal sepsis development. *J Surg Res* 125: 68–72, 2005.
3. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, and Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 29: 1303–1310, 2001.
4. Armstead VE, Opentanova IL, Minchenko AG, and Lefer AM. Tissue factor expression in vital organs during murine traumatic shock: role of transcription factors AP-1 and NF-kappaB. *Anesthesiology* 91: 1844–1852, 1999.
5. Atkinson MC. The use of N-acetylcysteine in intensive care. *Crit Care Resusc* 4: 21–27, 2002.
6. Baboolal HA, Ichinose F, Ullrich R, Kawai N, Bloch KD, and Zapol WM. Reactive oxygen species scavengers attenuate endotoxin-induced impairment of hypoxic pulmonary vasoconstriction in mice. *Anesthesiology* 97: 1227–1233, 2002.
7. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, and Suter PM. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. *Crit Care Med* 24: 392–397, 1996.
8. Bowler RP, Arcaroli J, Abraham E, Patel M, Chang LY, and Crapo JD. Evidence for extracellular superoxide dismutase as a mediator of hemorrhage-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 284: L680–L687, 2003.
9. Bowler RP, Arcaroli J, Crapo JD, Ross A, Slot JW, and Abraham E. Extracellular superoxide dismutase attenuates lung injury after hemorrhage. *Am J Respir Crit Care Med* 164: 290–294, 2001.
10. Bowler RP and Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 110: 349–356, 2002.
11. Call DR, Nemzek JA, Ebong SJ, Bolgos GL, Newcomb DE, and Remick DG. Ratio of local to systemic chemokine concentrations regulates neutrophil recruitment. *Am J Pathol* 158: 715–721, 2001.
12. Camhi SL, Alam J, Otterbein L, Sylvester SL, and Choi AM. Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am J Respir Cell Mol Biol* 13: 387–398, 1995.
13. Camhi SL, Alam J, Wiegand GW, Chin BY, and Choi AM. Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5′ distal enhancers: role of reactive oxygen intermediates and AP-1. *Am J Respir Cell Mol Biol* 18: 226–234, 1998.
14. Chan K and Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci U S A* 96: 12731–12736, 1999.
15. Cho HY, Jedlicka AE, Reddy SP, Zhang LY, Kensler TW, and Kleeberger SR. Linkage analysis of susceptibility to hyperoxia. Nrf2 is a candidate gene. *Am J Respir Cell Mol Biol* 26: 42–51, 2002.

16. Christofidou-Solomidou M and Muzykantov VR. Antioxidant strategies in respiratory medicine. *Treat Respir Med* 5: 47–78, 2006.

17. Crapo JD. Oxidative stress as an initiator of cytokine release and cell damage. *Eur Respir J Suppl* 44: 4s–6s, 2003.

18. Crapo JD. Redox active agents in inflammatory lung injury. *Am J Respir Crit Care Med* 168: 1027–1028, 2003.

19. Cui X, Parent C, Macarthur H, Ochs SD, Gerstenberg E, Solomon S, Fitz Y, Danner RL, Banks SM, Natanson C, Salvemini D, and Eichacker PQ. Severity of sepsis alters the effects of superoxide anion inhibition in a rat sepsis model. *J Appl Physiol* 97: 1349–1357, 2004.

20. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, and Milzani A. Protein carbonylation in human diseases. *Trends Mol Med* 9: 169–176, 2003.

21. Demirbilek S, Sizanli E, Karadag N, Karaman A, Bayraktar N, Turkmen E, and Ersoy MO. The effects of methylene blue on lung injury in septic rats. *Eur Surg Res* 38: 35–41, 2006.

22. Demling RH. The modern version of adult respiratory distress syndrome. *Annu Rev Med* 46: 193–202, 1995.

23. Dunican AL, Leuenroth SJ, Ayala A, and Simms HH. CXC chemokine suppression of polymorphonuclear leukocytes apoptosis and preservation of function is oxidative stress independent. *Shock* 13: 244–250, 2000.

24. Dweik RA, Laskowski D, Abu-Soud HM, Kaneko F, Hutte R, Stuehr DJ, and Erzurum SC. Nitric oxide synthesis in the lung: regulation by oxygen through a kinetic mechanism. *J Clin Invest* 101: 660–666, 1998.

25. Erdincler DS, Seven A, Inci F, Beger T, and Candan G. Lipid peroxidation and antioxidant status in experimental animals: effects of aging and hypercholesterolemic diet. *Clin Chim Acta* 265: 77–84, 1997.

26. Fan J, Shek PN, Suntres ZE, Li YH, Oreopoulos GD, and Rotstein OD. Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome. *Surgery* 128: 332–338, 2000.

27. Farley KS, Wang LF, Razavi HM, Law C, Rohan M, McCormack DG, and Mehta S. Effects of macrophage inducible nitric oxide synthase in murine septic lung injury. *Am J Physiol Lung Cell Mol Physiol* 290: L1164–L1172, 2006.

28. Feng X, Yan W, Liu X, Duan M, Zhang X, and Xu J. Effects of hydroxyethyl starch 130/0.4 on pulmonary capillary leakage and cytokines production and NF-kappaB activation in CLP-induced sepsis in rats. *J Surg Res* 135: 129–136, 2006.

29. Gadek JE, DeMichele SJ, Karlstad MD, Pacht ER, Donahoe M, Albertson TE, Van Hoozen C, Wennberg AK, Nelson JL, and Noursalehi M. Effect of enteral feeding with eicosapentaenoic acid, gamma-linolenic acid, and antioxidants in patients with acute respiratory distress syndrome. Enteral Nutrition in ARDS Study Group. *Crit Care Med* 27: 1409–1420, 1999.

30. Galley HF, Howdle PD, Walker BE, and Webster NR. The effects of intravenous antioxidants in patients with septic shock. *Free Radic Biol Med* 23: 768–774, 1997.

31. Gao XP, Standiford TJ, Rahman A, Newstead M, Holland SM, Dinauer MC, Liu QH, and Malik AB. Role of NADPH oxidase in the mechanism of lung neutrophil sequestration and microvessel injury induced by Gram-negative sepsis: studies in p47phox-/- and gp91phox-/- mice. *J Immunol* 168: 3974–3982, 2002.

32. Gaston B, Drazen JM, Loscalzo J, and Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 149: 538–551, 1994.

33. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF 3rd, Finkel B, Lanken PN, and Ischiropoulos H. Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 278: L961–L967, 2000.

34. Gonzalez PK, Zhuang J, Doctrow SR, Malfroy B, Benson PF, Menconi MJ, and Fink MP. Role of oxidant stress in the adult respiratory distress syndrome: evaluation of a novel antioxidant strategy in a porcine model of endotoxin-induced acute lung injury. *Shock* 6(suppl 1): S23–S26, 1996.

35. Goode HF, Cowley HC, Walker BE, Howdle PD, and Webster NR. Decreased antioxidant status and increased lipid peroxida-

tion in patients with septic shock and secondary organ dysfunction. *Crit Care Med* 23: 646–651, 1995.

36. Guo RF, Lentsch AB, Sarma JV, Sun L, Riedemann NC, McClintock SD, McGuire SR, Van Rooijen N, and Ward PA. Activator protein-1 activation in acute lung injury. *Am J Pathol* 161: 275–282, 2002.

37. Guo RF, Riedemann NC, Laudes IJ, Sarma VJ, Kunkel RG, Dilley KA, Paulauskis JD, and Ward PA. Altered neutrophil trafficking during sepsis. *J Immunol* 169: 307–314, 2002.

38. Guo RF, Riedemann NC, Sun L, Gao H, Shi KX, Reuben JS, Sarma VJ, Zetoune FS, and Ward PA. Divergent signaling pathways in phagocytic cells during sepsis. *J Immunol* 177: 1306–1313, 2006.

39. Guo RF, Sun L, Gao H, Shi KX, Rittirsch D, Sarma VJ, Zetoune FS, and Ward PA. In vivo regulation of neutrophil apoptosis by C5a during sepsis. *J Leukoc Biol* 80: 1575–1583, 2006.

40. Guo RF and Ward PA. Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. *Free Radic Biol Med* 33: 303–310, 2002.

41. Hawkins HK, Heffelfinger SC, and Anderson DC. Leukocyte adhesion deficiency: clinical and postmortem observations. *Pediatr Pathol* 12: 119–130, 1992.

42. Hildebrand F, Pape HC, Harwood P, Muller K, Hoevel P, Putz C, Siemann A, Krettek C, and van Griensven M. Role of adhesion molecule ICAM in the pathogenesis of polymicrobial sepsis. *Exp Toxicol Pathol* 56: 281–290, 2005.

43. Ibbotson GC, Doig C, Kaur J, Gill V, Ostrovsky L, Fairhead T, and Kubes P. Functional alpha4-integrin: a newly identified pathway of neutrophil recruitment in critically ill septic patients. *Nat Med* 7: 465–470, 2001.

44. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, Yang P, Sarao R, Wada T, Leong-Poi H, Crackower MA, Fukamizu A, Hui CC, Hein L, Uhlig S, Slutsky AS, Jiang C, and Penninger JM. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 436: 112–116, 2005.

45. Jarrar D, Kuebler JF, Rue LW, 3rd, Matalon S, Wang P, Bland KI, and Chaudry IH. Alveolar macrophage activation after trauma-hemorrhage and sepsis is dependent on NF-kappaB and MAPK/ERK mechanisms. *Am J Physiol Lung Cell Mol Physiol* 283: L799–L805, 2002.

46. Jiang ZY, Hunt JV, and Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal Biochem* 202: 384–389, 1992.

47. Jiang ZY, Woollard AC, and Wolff SP. Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orange: comparison with the TBA assay and an iodometric method. *Lipids* 26: 853–856, 1991.

48. Kang JL, Park W, Pack IS, Lee HS, Kim MJ, Lim CM, and Koh Y. Inhaled nitric oxide attenuates acute lung injury via inhibition of nuclear factor-kappa B and inflammation. *J Appl Physiol* 92: 795–801, 2002.

49. Karamouzis MV, Konstantinopoulos PA, and Papavassiliou AG. The activator protein-1 transcription factor in respiratory epithelium carcinogenesis. *Mol Cancer Res* 5: 109–120, 2007.

50. Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, and Stamler JS. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 9: 371–377, 1993.

51. Koksal GM, Sayilgan C, Aydin S, Oz H, and Uzun H. Correlation of plasma and tissue oxidative stresses in intra-abdominal sepsis. *J Surg Res* 122: 180–183, 2004.

52. Kolgazi M, Sener G, Cetinel S, Gedik N, and Alican I. Resveratrol reduces renal and lung injury caused by sepsis in rats. *J Surg Res* 134: 315–321, 2006.

53. Kuklin V, Kirov M, Sovershaev M, Andreasen T, Ingebretsen OC, Ytrehus K, and Bjertnaes L. Tezosentan-induced attenuation of lung injury in endotoxemic sheep is associated with reduced activation of protein kinase C. *Crit Care* 9: R211–R217, 2005.

54. Laudes IJ, Guo RF, Riedemann NC, Speyer C, Craig R, Sarma JV, and Ward PA. Disturbed homeostasis of lung intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 during sepsis. *Am J Pathol* 164: 1435–1445, 2004.

55. Lentsch AB, Czermak BJ, Bless NM, Van Rooijen N, and Ward PA. Essential role of alveolar macrophages in intrapulmonary activation of NF-kappaB. *Am J Respir Cell Mol Biol* 20: 692–698, 1999.

56. Lentsch AB and Ward PA. Activation and regulation of NFkappaB during acute inflammation. *Clin Chem Lab Med* 37: 205–208, 1999.

57. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, and Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186: 464–478, 1990.

58. Li QJ, Vaingankar S, Sladek FM, and Martins-Green M. Novel nuclear target for thrombin: activation of the Elk1 transcription factor leads to chemokine gene expression. *Blood* 96: 3696–3706, 2000.

59. Liaw WJ, Chen TH, Lai ZZ, Chen SJ, Chen A, Tzao C, Wu JY, and Wu CC. Effects of a membrane-permeable radical scavenger, Tempol, on intraperitoneal sepsis-induced organ injury in rats. *Shock* 23: 88–96, 2005.

60. Libon C, Forestier F, Cotte-Laffitte J, Labarre C, and Quero AM. Effect of acute oral administration of alcohol on superoxide anion production from mouse alveolar macrophages. *J Leukoc Biol* 53: 93–98, 1993.

61. Martin TR, Pistorese BP, Hudson LD, and Maunder RJ. The function of lung and blood neutrophils in patients with the adult respiratory distress syndrome: implications for the pathogenesis of lung infections. *Am Rev Respir Dis* 144: 254–262, 1991.

62. Meduri GU, Muthiah MP, Carratu P, Eltorky M, and Chrousos GP. Nuclear factor-kappaB- and glucocorticoid receptor alpha-mediated mechanisms in the regulation of systemic and pulmonary inflammation during sepsis and acute respiratory distress syndrome: evidence for inflammation-induced target tissue resistance to glucocorticoids. *Neuroimmunomodulation* 12: 321–338, 2005.

63. Mei N, Tamae K, Kunugita N, Hirano T, and Kasai H. Analysis of 8-hydroxydeoxyguanosine 5′-monophosphate (8-OH-dGMP) as a reliable marker of cellular oxidative DNA damage after gamma-irradiation. *Environ Mol Mutagen* 41: 332–338, 2003.

64. Metnitz PG, Bartens C, Fischer M, Fridrich P, Steltzer H, and Druml W. Antioxidant status in patients with acute respiratory distress syndrome. *Intensive Care Med* 25: 180–185, 1999.

65. Minnard EA, Shou J, Naama H, Cech A, Gallagher H, and Daly JM. Inhibition of nitric oxide synthesis is detrimental during endotoxemia. *Arch Surg* 129: 142–147; discussion 147–148, 1994.

66. Mohiuddin I, Chai H, Lin PH, Lumsden AB, Yao Q, and Chen C. Nitrotyrosine and chlorotyrosine: clinical significance and biological functions in the vascular system. *J Surg Res* 133: 143–149, 2006.

67. Molnar Z, Shearer E, and Lowe D. N-Acetylcysteine treatment to prevent the progression of multisystem organ failure: a prospective, randomized, placebo-controlled study. *Crit Care Med* 27: 1100–1104, 1999.

68. Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, and Choi AM. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 278: 36993–36998, 2003.

69. Nagase T, Uozumi N, Aoki-Nagase T, Terawaki K, Ishii S, Tomita T, Yamamoto H, Hashizume K, Ouchi Y, and Shimizu T. A potent inhibitor of cytosolic phospholipase A2, arachidonyl trifluoromethyl ketone, attenuates LPS-induced lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 284: L720–L726, 2003.

70. Nathens AB, Neff MJ, Jurkovich GJ, Klotz P, Farver K, Ruzinski JT, Radella F, Garcia I, and Maier RV. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* 236: 814–822, 2002.

71. Nava E, Palmer RM, and Moncada S. Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? *Lancet* 338: 1555–1557, 1991.

72. Olea-Azar C, Rigol C, Mendizabal F, Morello A, Maya JD, Moncada C, Cabrera E, Di Maio R, Gonzalez M, and Cerecetto H. ESR spin trapping studies of free radicals generated from nitrofuran derivative analogues of nifurtimox by electrochemical and *Trypanosoma cruzi* reduction. *Free Radic Res* 37: 993–1001, 2003.

73. Paredi P, Kharitonov SA, and Barnes PJ. Analysis of expired air for oxidation products. *Am J Respir Crit Care Med* 166: S31–S37, 2002.

74. Parent C and Eichacker PQ. Neutrophil and endothelial cell interactions in sepsis: the role of adhesion molecules. *Infect Dis Clin North Am* 13: 427–447, 1999.

75. Quinlan GJ, Lamb NJ, Tilley R, Evans TW, and Gutteridge JM. Plasma hypoxanthine levels in ARDS: implications for oxidative stress, morbidity, and mortality. *Am J Respir Crit Care Med* 155: 479–484, 1997.

76. Rahman I, Biswas SK, Jimenez LA, Torres M, and Forman HJ. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid Redox Signal* 7: 42–59, 2005.

77. Rahman I, Gilmour PS, Jimenez LA, Biswas SK, Antonicelli F, and Aruoma OI. Ergothioneine inhibits oxidative stress- and TNF-alpha-induced NF-kappa B activation and interleukin-8 release in alveolar epithelial cells. *Biochem Biophys Res Commun* 302: 860–864, 2003.

78. Rahman I, Gilmour PS, Jimenez LA, and MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 234–235: 239-248, 2002.

79. Rahman I and Kelly F. Biomarkers in breath condensate: a promising new non-invasive technique in free radical research. *Free Radic Res* 37: 1253–1266, 2003.

80. Razavi HM, Wang L, Weicker S, Quinlan GJ, Mumby S, McCormack DG, and Mehta S. Pulmonary oxidant stress in murine sepsis is due to inflammatory cell nitric oxide. *Crit Care Med* 33: 1333–1339, 2005.

81. Razavi HM, Wang le F, Weicker S, Rohan M, Law C, McCormack DG, and Mehta S. Pulmonary neutrophil infiltration in murine sepsis: role of inducible nitric oxide synthase. *Am J Respir Crit Care Med* 170: 227–233, 2004.

82. Reddy RC, Chen GH, Newstead MW, Moore T, Zeng X, Tateda K, and Standiford TJ. Alveolar macrophage deactivation in murine septic peritonitis: role of interleukin 10. *Infect Immun* 69: 1394–1401, 2001.

83. Rocksen D, Ekstrand-Hammarstrom B, Johansson L, and Bucht A. Vitamin E reduces transendothelial migration of neutrophils and prevents lung injury in endotoxin-induced airway inflammation. *Am J Respir Cell Mol Biol* 28: 199–207, 2003.

84. Sarady-Andrews JK, Liu F, Gallo D, Nakao A, Overhaus M, Ollinger R, Choi AM, and Otterbein LE. Biliverdin administration protects against endotoxin-induced acute lung injury in rats. *Am J Physiol Lung Cell Mol Physiol* 289: L1131–1137, 2005.

85. Sener G, Toklu H, Ercan F, and Erkanli G. Protective effect of beta-glucan against oxidative organ injury in a rat model of sepsis. *Int Immunopharmacol* 5: 1387–1396, 2005.

86. Sener G, Toklu H, Kapucu C, Ercan F, Erkanli G, Kacmaz A, Tilki M, and Yegen BC. Melatonin protects against oxidative organ injury in a rat model of sepsis. *Surg Today* 35: 52–59, 2005.

87. Spapen HD, Diltoer MW, Nguyen DN, Hendrickx I, and Huyghens LP. Effects of N-acetylcysteine on microalbuminuria and organ failure in acute severe sepsis: results of a pilot study. *Chest* 127: 1413–1419, 2005.

88. Speyer CL, Gao H, Rancilio NJ, Neff TA, Huffnagle GB, Sarma JV, and Ward PA. Novel chemokine responsiveness and mobilization of neutrophils during sepsis. *Am J Pathol* 165: 2187–2196, 2004.

89. Speyer CL, Neff TA, Warner RL, Guo RF, Sarma JV, Riedemann NC, Murphy ME, Murphy HS, and Ward PA. Regulatory effects of iNOS on acute lung inflammatory responses in mice. *Am J Pathol* 163: 2319–2328, 2003.

90. Spitzer JA and Zhang P. Protein tyrosine kinase activity and the influence of gender in phagocytosis and tumor necrosis factor secretion in alveolar macrophages and lung-recruited neutrophils. *Shock* 6: 426–433, 1996.

91. Suliman HB, Ryan LK, Bishop L, and Folz RJ. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. *Am J Physiol Lung Cell Mol Physiol* 280: L69–L78, 2001.

92. Suntres ZE and Shek PN. The pulmonary uptake of intravenously administered liposomal alpha-tocopherol is augmented in acute lung injury. *J Drug Target* 4: 151–159, 1996.

93. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, and Biswal S. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 116: 984–995, 2006.

94. Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT, Sporn MB, Yamamoto M, Kensler TW, and Biswal S. Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide. *Biochem Biophys Res Commun* 351: 883–889, 2006.

95. Thomassen MJ and Kavuru MS. Human alveolar macrophages and monocytes as a source and target for nitric oxide. *Int Immunopharmacol* 1: 1479–1490, 2001.

96. Vassilev D, Hauser B, Bracht H, Ivanyi Z, Schoaff M, Asfar P, Vogt J, Wachter U, Schelzig H, Georgieff M, Bruckner UB, Radermacher P, and Froba G. Systemic, pulmonary, and hepatosplanchnic effects of N-acetylcysteine during long-term porcine endotoxemia. *Crit Care Med* 32: 525–532, 2004.

97. Wan XS, Zhou Z, Ware JH, and Kennedy AR. Standardization of a fluorometric assay for measuring oxidative stress in irradiated cells. *Radiat Res* 163: 232–240, 2005.

98. Ware LB and Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 342: 1334–1349, 2000.

99. Warner RL, Paine R 3rd, Christensen PJ, Marletta MA, Richards MK, Wilcoxen SE, and Ward PA. Lung sources and cytokine requirements for in vivo expression of inducible nitric oxide synthase. *Am J Respir Cell Mol Biol* 12: 649–661, 1995.

100. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, and Gadek JE. Lung neutrophils in the adult respiratory distress syndrome: clinical and pathophysiologic significance. *Am Rev Respir Dis* 133: 218–225, 1986.

101. Weiss DJ and Lunte CE. Detection of a urinary biomarker for oxidative DNA damage 8-hydroxydeoxyguanosine by capillary electrophoresis with electrochemical detection. *Electrophoresis* 21: 2080–2085, 2000.

102. Winterbourn CC, Buss IH, Chan TP, Plank LD, Clark MA, and Windsor JA. Protein carbonyl measurements show evidence of early oxidative stress in critically ill patients. *Crit Care Med* 28: 143–149, 2000.

103. Zegdi R, Perrin D, Burdin M, Boiteau R, and Tenaillon A. Increased endogenous carbon monoxide production in severe sepsis. *Intens Care Med* 28: 793–796, 2002.

104. Zhang XW, Liu Q, Wang Y, and Thorlacius H. CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration in vivo. *Br J Pharmacol* 133: 413–421, 2001.

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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 the lung. Morphologically, early changes in the 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 the 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 1 h 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. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: 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 extreme 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 and Rezaian, 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 the 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 and Shek, 1996). In the 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. The current studies demonstrated that liposomes containing anti-oxidants or anti-oxidant enzymes cause a reduction in acute lung injury in rats following airway delivery of CEES. Furthermore, delivery of such liposomes, when delayed 1 h after CEES administration, still provides significant attenuation of acute lung injury.

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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^{-1} body weight) (Fort Dodge Animal Health, Fort Dodge, IA) was used for anesthesia and intraperitoneal xylazine (13 mg kg^{-1} 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 per 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 so as to facilitate a unilateral, left-lung injury. A small volume of CEES (2 μl per rat; about 6 mg kg^{-1}) 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, into the left lung main stem bronchus. Studies, not requiring the usage of a radiolabeled marker, proceeded identically substituting DPBS for the radioactive injection. For all studies, except the time response experiment, animals were killed 4 h 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 killing as described elsewhere (McClintock *et al*., 2002). ^{125}I-BSA present in the 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 the loss of the vascular and epithelial barriers (Johnson and Ward, 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 and Cochrane, 1969). 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 (Mayer, 1961). Experiments were performed 24 h after the second CVF injection.

Liposome Preparation

Dipalmitoylphosphatidylcholine (DPPC, Avanti Polar Lipids) was dissolved 20 mg ml^{-1} 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 1 h 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 100 nm 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

In order morphologically to 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 the deposition of fibrin and collagen (Luna, 1968).

Statistical Analysis

The 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 0, 6, 12 and 24 h 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 Fig. 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 h, increased evidence of trichrome stained deposition in alveolar walls was likely related to fibrin deposition (frame C). By 24 h after lung instillation of CEES, dense interstitial and intra-alveolar accumulations of 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 h (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 the lung, together with a collapse of alveolar
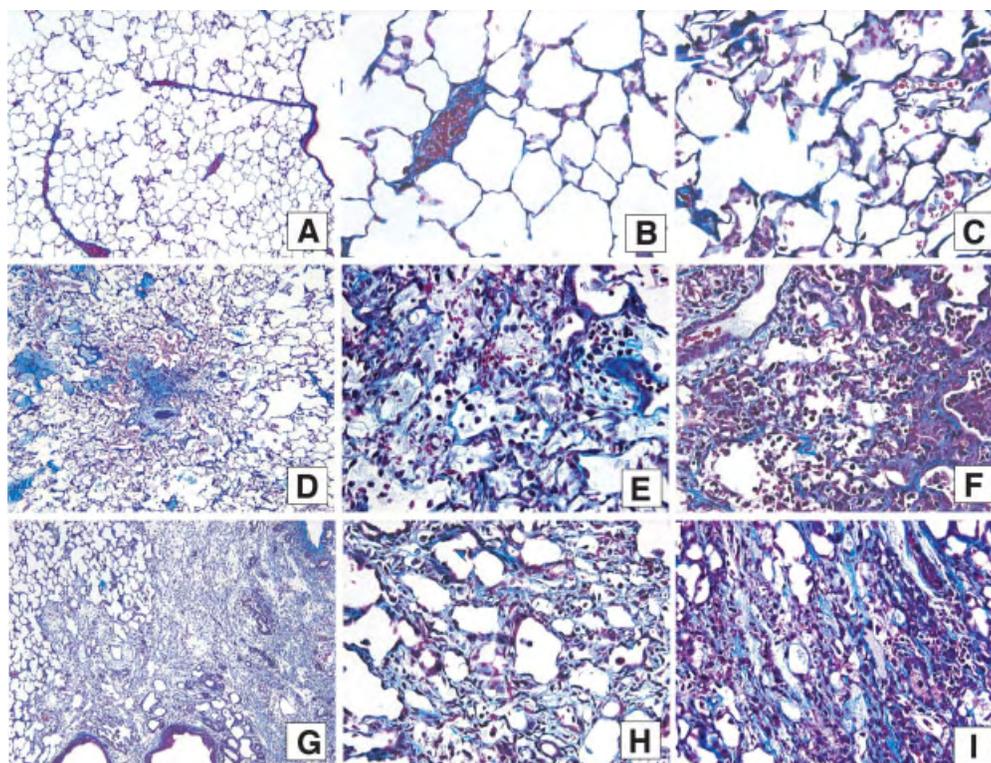

Figure 1. Tissue sections of lungs with trichrome stain. Lungs were obtained after airway instillation of CEES at time 0 (A, B, 10× and 40×); 6 h (C, 40×); 24 h (D, E, 10× and 40×); 3 days (F, 10×), 6 days (G, 10× and H, 40×); and 3 weeks (I, 40×). All tissue sections were reacted with trichrome stain
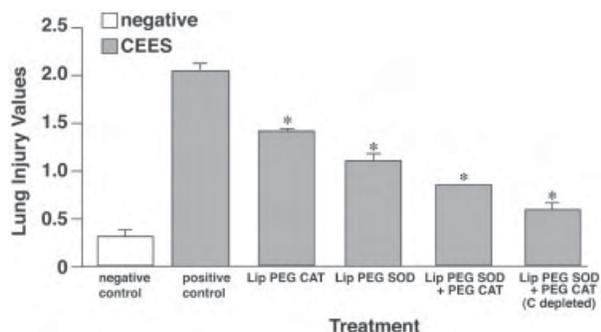
Figure 2. Attenuative effects of liposomes loaded with anti-oxidant enzymes. Rats received either saline followed by unloaded liposomes (negative control), CEES followed by airway delivery of unloaded liposomes (positive control), or CEES with liposomes containing either pegylated (PEG)-CAT or PEG-SOD, individually 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 h after airway delivery of CEES (see text). * Represents P values of <0.05 when compared with the positive control group

structures and the appearance of honeycombing (frames G, H). By week 3, 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 Fig. 2, the airway instillation of CEES together with unloaded liposomes resulted 4 h later in approximately a 10-fold increase in lung injury, as defined by the leakage of ^{125}I-albumin from blood into the lung. When instilled into the 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
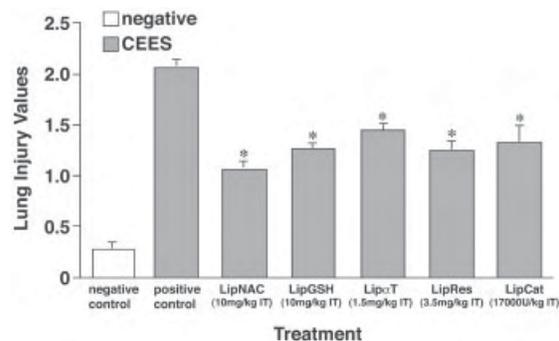

Figure 3. Attenuative effects of liposomes loaded with reducing agents in CEES lung injury. The positive and negative controls are similar to those described in Fig. 1. When used, liposomes were injected intratracheally immediately after the airway instillation of CEES. Lung injury was determined by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

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 Fig. 3), there was approximately a 10-fold increase in leakage of albumin from the circulation into the lungs of animals receiving airway instillation of CEES 4 h 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 Fig. 4, approximately a 10-fold increase in the leakage of ^{125}I-albumin into the lungs occurred following instillation of CEES together with unloaded liposomes. When the animals were complement (C) depleted, the instillation
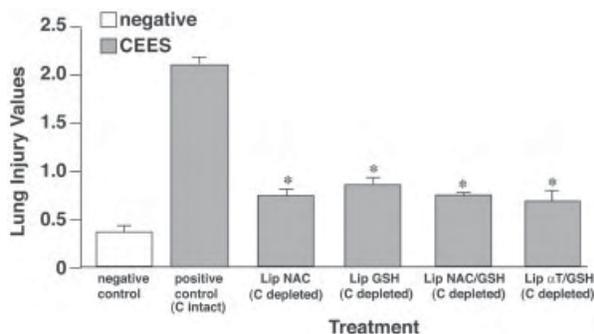
Figure 4. Enhanced effects of anti-oxidant containing liposomes in complement depleted rats. The negative and positive control groups are similar to those described in Fig. 1. Complement depletion was induced in four groups of animals by the prior intraperitoneal injection of purified cobra venom factor (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 Figs 1–3

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 significantly to attenuate CEES-induced acute lung injury in an additive manner.

Effects of Delayed Lung Instillation of Anti-Oxidant Liposomes

As shown in Fig. 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 Fig. 5, 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 the 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.
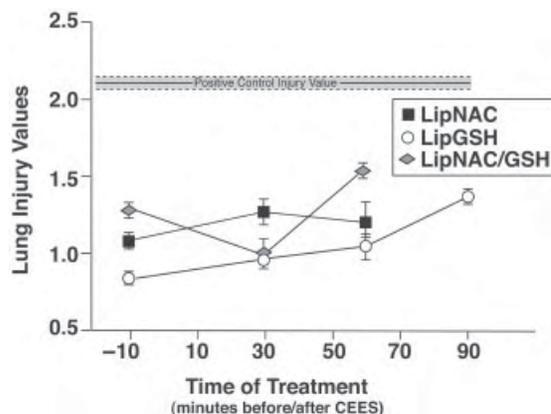
Discussion

The data described in this report indicate that CEES instillation into the lung produces acute lung injury in a manner that seems related to the loss of the redox balance in the lung, although this has not been demonstrated directly. 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 the 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 the alveolar spaces. Deposition of fibrin reflects a nonspecific reaction to tissue damage. It remains to be shown

that CEES-exposure causes rapid development of interstitial fibrosis, as confirmed biochemically by collagen accumulation. It is hypothesized 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 the lung may be associated with the release of mediators (such as TGF$_\beta$) 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 a 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 with the use of either type of liposomes given to complement-intact rats (Figs 3 and 4) suggests that it may be both the loss of reducing potential in the lung as well as an 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 it was shown 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 the 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 the lung. It should be noted that there is not much increased albumin leak into the lung in the first 60 min after administration of CEES (McClintock et al., 2002). In fact, compared with values at 1 h, the permeability index at 2, 4 and 6 h after instillation of CEES rose 2 fold, 2.9 fold, 7.7 fold and 16.2 fold, respectively, when compared with uninjured lung values. Accordingly, the 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 the 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.

References

Angelov A, Belchen L, Angelov G. 1996. Experimental sulfur mustard gas poisoning and protective effect of different medicines in rats and rabbits. *Indian Vet. J.* **73**: 546–551.

Ballow M, Cochrane CG. 1969. Two anticomplementary factors in cobra venom: hemolysis of guinea pig erythrocytes by one of them. *J. Immunol.* **103**: 944–952.

Dube SN, Husain K, Sugendran K, Vijayaraghavan R, Somani SM. 1998. Dose response of sulphur mustard: behavioral and toxic signs in rats. *Indian J. Physiol. Pharmacol.* **42**: 389–394.

Eisenmenger W, Drasch G, von Clarmann M, Kretschmer E, Roider G. 1991. Clinical and morphological findings on mustard gas [bis(2-chloroethyl)sulfide] poisoning. *J. Forensic Sci.* **36**: 1688–1698.

Emad A, Rezaian GR. 1999. Immunoglobulins and cellular constituents of the BAL fluid of patients with sulfur mustard gas-induced pulmonary fibrosis. *Chest* **115**: 1346–1351.

Fan J, Shek PN, Suntres ZE, Li YH, Oreopoulos GD, Rotstein OD. 2000. Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome. *Surgery* **128**: 332–338.

Freeman BA, Turrens JF, Mirza Z, Crapo JD, Young SL. 1985. Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase. *Fed. Proc.* **44**: 2591–2595.

Gonzalez-Rothi RJ, Straub L, Cacace JL, Schreier H. 1991. Liposomes and pulmonary alveolar macrophages: functional and morphologic interactions. *Exp. Lung Res.* **17**: 687–705.

Johnson KJ, Ward PA. 1974. Acute immunologic pulmonary alveolitis. *J. Clin. Invest.* **54**: 349–357.

Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. 2003. Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. *J. Occup. Environ. Med.* **45**: 1136–1143.

Lakshmana Rao PV, Vijayaraghavan R, Bhaskar AS. 1999. Sulphur mustard induced DNA damage in mice after dermal and inhalation exposure. *Toxicology* **139**: 39–51.

Lentsch AB, Czermak BJ, Bless NM, Van Rooijen N, Ward PA. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-kappaB. *Am. J. Respir. Cell Mol. Biol.* **20**: 692–698.

Luna L. 1968. *Manual of Histologic Staining Methods of the AFIP*, 3rd edn. McGraw-Hill: New York.

Mayer MM. 1961. Complement and complement fixation. In *Experimental Immunochemistry*, Kabat EA, Mayer MM (eds). Springfield: Thomas, 133–240.

McClintock SD, Till GO, Smith MG, Ward PA. 2002. Protection from half-mustard-gas-induced acute lung injury in the rat. *J. Appl. Toxicol.* **22**: 257–262.

Shephard EG, Joubert JR, Finkelstein MC, Kuhn SH. 1981. Phagocytosis of liposomes by human alveolar macrophages. *Life Sci.* **29**: 2691–2698.

Sone S, Poste G, Fidler IJ. 1980. Rat alveolar macrophages are susceptible to activation by free and liposome-encapsulated lymphokines. *J. Immunol.* **124**: 2197–2202.

Suntres ZE, Shek PN. 1996. Treatment of LPS-induced tissue injury: role of liposomal antioxidants. *Shock* **6** (Suppl 1): S57–S64.

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W81XWH-06-2-0044

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Annual Report-2007

The Development of Therapeutic and Diagnostic Countermeasures to WMD by the Advanced Medical Countermeasures Consortium

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Introduction

Two significant vulnerabilities exist in biological and chemical weapons research: 1) the absence of a multithreat treatment countermeasure; and 2) the capability of presymptomatic real-time diagnostics. The Advanced Medical Countermeasures Consortium (AMCC) has focused its efforts on the development of two technologies that we believe will begin to address these significant vulnerabilities in WMD defense. These technologies are the APC Biosensor and STIMAL.

APC Biosensor

The APC Biosensor uses antigen presenting cells (APCs) that can be harvested from an animal or human. APCs naturally sample all body compartments, consequently it has been theorized that they will detect changes such as inflammation, infectious and chemical agents, prior to "clinical onset" (or symptoms) of disease. The pathological occurrences in the various body compartments induce changes in the APCs that result in specific protein and gene expression (or signatures). Creation of a catalogue of these types of protein/gene changes will allow us to create a library that can be used as a reference for comparison of known samples against unknown samples (one that would be obtained from a soldier for instance); and then deriving the probability of a match-the diagnosis.

STIMAL

STIMAL or (antioxidant liposomes) are composed of liposomes and antioxidants. Liposomes are preferentially up taken by the reticuloendothelial system. The antioxidants are known to modulate redox regulated gene expression, as well as being able to quench oxidants. The production of oxidants, proinflammatory cytokines, and apoptosis are consequences of the acute inflammatory host response; and is a subset of the acute inflammation termed oxidative stress (OS). Exposures to weapons of mass destruction such as radiation, chemical or biological agents result in OS. STIMAL has proven to reduce oxidative stress associated with CEES (2-chloroethyl ethyl sulfur) several years ago. It has been hypothesized by our group that a reduction of OS is a viable means to ameliorate the inflammation that is associated with many WMD.

Body

At the beginning of the present funding cycle, as well as in other years, Dr. Smith held discussions with each of the investigators to mutually agree on the particular research project. In the background of the discussion with the individual investigators was the knowledge that each of the projects was components of the "sum total" research effort. All of the projects compose the sum of all of the projects. That sum total research effort has been and will continue to develop a multithreat diagnostic and treatment countermeasure.

Summary of the components of the projects
> Details of the individual projects can be gleaned from the individual investigators annual reports.

Diagnostics- APC Biosensor

Dr. Keith Crawford

His efforts have been dedicated to development of the APC Biosensor. There has been significant progress in this work. We have also enlisted the consultation of Dr. Winston Kuo for his expertise in bioinformatics. Mr. Jin Joo assisted in the networking of computers in Dr. Crawford's laboratory.

Two essential pieces of equipment were purchased, the Biotrove chip reader and the LightCycler (Roche), both of which facilitate quantitative PCR, albeit on different formats. A comparison of the experimental results will be used to validate the APC Biosensor technology in the two different formats.

The most salient findings from the recent experiments were the differential gene expression in the APCs that were exposed to different pathogens. Differences could also be discerned between encapsulated and unencapsulated Stern Bacillus anthracis. APCs harvested from asymptomatic animals demonstrated that there were differences in the proteomic expression for virus (vaccinia) and bacteria (Listeria M.). In two separate but related experiments we have seen that there is differential gene and protein expression that provides early evidence that the naturally occurring biosensor function of the APCs can be exploited for the purposes of a diagnostic tool.

Dr. Crawford has also received samples of tissues from the various laboratories and is examining them for gene expression changes under the different experimental conditions.

Treatment

STIMAL

Dr. Zacharias Suntres-safety studies

STIMAL has shown significant promise in the protection of the rat lung models exposed to CEES (2-cholorethyl ethyl sulfur). The liposome preparation was protective in acute injury and in the prevention of pulmonary fibrosis (sees Dr. Ward's section). The liposomes were also successful in the enhancement of macrophage function in the eradication of anthrax. In both of the previous examples where STIMAL showed an ameliorative effect for a chemical weapon and for a biological weapon is evidence of a nascent multithreat treatment countermeasure. Some may view it premature to begin safety studies on STIMAL; our thoughts were that it was quite timely to initiate these types of studies. Dr. Suntres was tasked with determining the safety of the antioxidant liposome (STIMAL). Thus far no toxicities have been found even in doses of N-acetyl cystiene that are three times higher than that which has been used in human studies (150 mg/kg).

Dr. William Stone- Liposome Development/Production; Redox Proteomics
Dr. Stone has developed the formulations for antioxidant liposomes that are used by Doctors Das, Rest, and Ward. Several iterations have occurred since the early production of STIMAL. To date the most effective liposome formulation is the one which contained both fat and water soluble antioxidants (See the report by Dr. Ward, and Das).

He is also examining the effect of CEES on mast cell (rat RBL-2H3) degranulation. CEES alone actually inhibits degranulation, if low levels of CEES (0.02 to 0.2mM) were present along with the Ca-inophore A23187 toxicity to the cells were enhanced. In the next quarter there will be an investigation as to mechanism of action of CEES inhibiting mast cell degranulation.

Sample of tissue that have been exposed to CEES or sulfur mustard are being analyzed for modulation of protein expression. The LTQ mass spectrometer has been set up and training of the laboratory personnel is currently in progress.

Dr. Peter Ward (Rat lung model) - STIMAL and a Chemical Agent
Dr. Ward has been using the in vivo rat lung model developed by his group for CEES exposure. STIMAL has been extensively tested in this model. He has been able to demonstrate that there is significant diminution of the production of inflammatory cytokines and protection of lung tissue in the acute inflammatory model. Another variation of original model has been developed for demonstration of pulmonary lung fibrosis, a known complication of the exposure to mustard gas. The most recent formulation of the antioxidant liposomes, produced by Dr. Stone, α / γ/ NAC liposomes provides protection against pulmonary fibrosis. STIMAL's effect on the proinflammatory cytokines and pulmonary fibrosis are very promising findings which could impact not only the inhalation of mustard gas but also other pulmonary irritants such as phosgene. The ability of STIMAL to reduce oxidants and proinflammatory cytokines would suggest that its ameliorative action is due to modulation of redox regulated gene expression, hence the reduction of oxidative stress. Other weapons of mass destruction that induce OS is radiation (Kang, Zhang et al. 2006), bacterial infections (e.g. anthrax (Hanna, Kruskal et al. 1994; Kuhn, Hoerth et al. 2006)), viral infections (e.g. influenza (Ghezzi and Ungheri 2004)), chemical weapons (e.g. mustard gas (Pant, Vijayaraghavan et al. 2000)) and toxins (e.g. ricin (Kumar, Sugendran et al. 2003; Suntres, Stone et al. 2005)) exposures.

Dr. Salil Das (guinea pig lung model) –
In the guinea pig model it has been shown that oxidative stress induces TNF-alpha cascade after exposure to CEES. He has begun to elucidate the role of the transcription factors in CEES pathophysiology. It has been found that there is activation/phosphorylation of p38 protein. There was also increased expression of c-fos, Fos-B, Fra-2 and Jun-D proteins.

Dr. Rick Rest- STIMAL and a biological agent

NAC (N-acetyl cystiene) is a well known drug and glutathione precursor. It has been previously used in an aerosol form as a mucolytic (for loosening mucous) from the pulmonary system although this use has largely fallen out of favor. It is primarily used today as an antidote against chemical hepatitis that induced by an overdose of acetaminophen. Surprisingly it has been found that NAC exhibits sporocidal activity. NAC encapsulated in liposomes (antioxidant liposomes) also have a fascinating effect on macrophages that have been infected with anthrax. The antioxidant liposomes have a dual effect on anthrax infected macrophages. Initially it accelerates the growth of anthrax spore development to vegetative bacteria; secondarily the same liposomes induce the macrophages to eradicate anthrax more efficiently.

Dr. Alibek

Dr. Wu, an associate of Dr. Alibek, found that anthrax in the presence of polymorphonuclear cells (PMNs) induces lysis of red blood cells (10). If lethal factor or edema factor are use individually or in combination they were incapable of lysing red blood cells. Several other bacteria besides anthrax also induce hemolysis as part of their pathophysiology- Escherichia coli, Clostridium perfringens, B. cereus, Vibrio cholerae, Listeria Monocytogenes, Streptococcus pneumoniae, Staphylococcus aureus, Helicobacter pylori, and Streptococcus pyogenes. The aim of these experiments is to examine if STIMAL is able to inhibit anthrax induced hemolysis. (See comment on administrative difficulties). There have been some technical difficulties reproducing the work done by Wu. They have been able to demonstrate that lethal toxin does induce macrophages to produce reactive oxygen species.

Key Research Accomplishments (summarized)

Diagnostics-
The host pathogen response exhibited by antigen presenting cells demonstrates gene expression which appears to be specific for a species and genus of bacteria. These early findings bode well for the eventual use of the technology as a diagnostic tool. The use of the antigen presenting cell as a method of sampling all body compartments is unique and believed to be unparalleled.

Treatment-
STIMAL has show to have an ameliorative effect in vivo for CEES a chemical agent. Protection is achieved in both acute inflammatory and fibrosis lung models. In macrophages that are infected with anthrax and treated with antioxidants spore germination is limited as well as outgrowth. It has been demonstrated that anthrax spores, in the absence of macrophages and treated with N-acetyl cystiene, inhibits the germination of the spores.

Reportable Outcomes

Presentations:

1)   Advanced Medical Countermeasures Consortium Meeting, June 28, 2007, Crystal City, VA: An overview of the work that the consortium has done and future directions". All of the members of the Consortium met and gave presentation summarizing their work. Al Graziano, from DTRA was also in attendance at the meeting. Slides of the presentation were also forwarded to Mr. Graziano at his request so that they could be review by the DTRA staff.

2)   BARDA (Biomedical Advanced Research Development Authority) 2007 Industry Day Conference, August 3, 2007, Washington, DC:
a. Three abstracts were submitted and three were accepted for presentations (see enclosure).

Meetings:

Gil Scott and Dr. Smith met with Dr. Platoff and Dr. Jet on March 9th (2007) to discuss what are current findings were in the Consortium effort. We explored other funding opportunities as well as what potential interest there may be in the technologies that were being developed. At that time there was no funding for diagnostic technologies or multithreat treatment countermeasures in the NIAID biochemical defense programs.

Manuscripts (published during the past year of funding):

McClintock, S.D., Hoesel, L.M., Das, S.K., Till, G.O., Neff, T., Kunkel, R.G., Smith, M.G., and **Ward, P.A**.: Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 2006, 26:126-311.

Manuscripts submitted for publication:

Hoesel, L.M., Flierl, M., Niederbichler, A.D.Rittirsch, McClintock, S.D., Reuben, J. S., Pianko, M.,J., Stone, W., Yang, H., Smith, M. and Ward, P.; Antioxidant and Redox Signaling
Ability of antioxidant liposome to prevent acute and progressive pulmonary injury

Book Chapter: Vesicants and Oxidative Stress in Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology and Therapeutics, 2nd edition. (see enclosure of first page of the chapter). Set for publication December, 2007.

Paromov, Victor, Suntres, Smith, M., and Stone, W.L.
J. of Burns and Wounds; August 20, 2007
Sulfur Mustard Toxicity Following Dermal Exposure-Role of Oxidative Stress, and Antioxidant Therapy

Conclusion

Although the goals of the efforts of the Consortium appear lofty, there has been excellent progress made in all of the projects. Both STIMAL and the APC Biosensor are in the early stages of their development show promise as both a multithreat treatment and diagnostic tool respectively. A significant body of information has been generated to allow the investigators to be competitive for grants from other agencies.

References for Text
1. McClintock, S.D., Till, G.O., Smith, M.G., and **Ward, P.A**.: Protection from half-mustard-gas-induced acute lung injury in the rat. J Appl Toxicol. 2002, 22:257-262.

2. McClintock, S.D., Hoesel, L.M., Das, S.K., Till, G.O., Neff, T., Kunkel, R.G., Smith, M.G., and **Ward, P.A**.: Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 2006, 26:126-311.

3. Kang, K. A., R. Zhang, et al. (2006). "Protective effect of triphlorethol-A from Ecklonia cava against ionizing radiation in vitro." J Radiat Res (Tokyo) **47**(1): 61-8.

4. Hanna, P. C., B. A. Kruskal, et al. (1994). "Role of macrophage oxidative burst in the action of anthrax lethal toxin." Mol Med **1**(1): 7-18.

5. Kuhn, J. F., P. Hoerth, et al. (2006). "Proteomics study of anthrax lethal toxin-treated murine macrophages." Electrophoresis **27**(8): 1584-97.

6. Ghezzi, P. and D. Ungheri (2004). "Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model." Int J Immunopathol Pharmacol **17**(1): 99-102.

7. Pant, S. C., R. Vijayaraghavan, et al. (2000). "Sulphur mustard induced oxidative stress and its prevention by sodium 2,3-dimercapto propane sulphonic acid (DMPS) in mice." Biomed Environ Sci **13**(3): 225-32.

8. Kumar, O., K. Sugendran, et al. (2003). "Oxidative stress associated hepatic and renal toxicity induced by Ricin in mice." Toxicon **41**(3): 333-8.

9. Suntres, Z., W. Stone, et al. (2005). "Ricin -Induced Tissue Toxicity: The Role of Oxidative Stress." J. Med CBR Def **3**.

10. Wu, A. G., D. Alibek, et al. (2003). "Anthrax toxin induces hemolysis: an indirect effect through polymorphonuclear cells." J Infect Dis **188**(8): 1138-41.

11. Bhakdi, S., H. Bayley, et al. (1996). "Staphylococcal alpha-toxin, streptolysin-O, and Escherichia coli hemolysin: prototypes of pore-forming bacterial cytolysins." Arch Microbiol **165**(2): 73-9.

Appendices (available as PDF files)
- Book chapter- Vesicant and Oxidative Stress
- The Ability of Antioxidant Liposomes to Prevent Acute and Progress Pulmonary Injury
- Sulfur Mustard Toxicity Following Dermal Exposure-Role of Oxidative Stress and Antioxidant Therapy

Appendices

Biomedical Advanced Research Development Authority
August 3rd, 2007
Abstracts

1.
Title: The use of STIMAL as countermeasure to mustard gas and potentially other chemical weapons.
Category: Therapeutic
TRL 1-3
Milton Smith[1], Peter Ward[2], Dana Anderson[3], William Stone[4], Keith Crawford[5], Zach Suntres[6]
1 Amaox. Ltd., Melbourne, FL, 2 Ann Arbor, MI; 3 Aberdeen Proving Ground, MD, 4 Johnson City, TN; 5 Boston, MA;
6 Thunderbay, Canada
Background
Mustard gas and other vesicants that were invented circa 1900s continue to be a threat today since there are no antidotes. Oxidative stress is a common property of vesicants (mustard gas, chlorine and phosgene) and other chemical weapons (e.g. cyanide). In vivo and in vitro testing has shown that the diminution of oxidative stress using STIMAL (liposome encapsulated antioxidants) has an ameliorative effect on the pathogenesis of CEES (2-chloroethyl ethyl sulfur) induced tissue damage. Tissues exposed to mustard gas reveal oxidized proteins and modulation of gene expression. The exact mechanism of the action of STIMAL has yet to be determined, but is likely to modify proteins containing thiol groups and modulate redox regulated gene expression. In studies with anthrax

STIMAL significantly inhibits growth of the bacteria.

Methods

An interdisciplinary group will be tasked with further improvement of STIMAL. Genomic and proteomic analysis would be utilized to achieve a better understanding of the mechanism of action of STIMAL as well as the pathogenesis of sulfur mustards. It is anticipated that specific gene/protein expression could be blocked and could prove to be protective.

Results

A second generation STIMAL has already shown a significant improvement in CEES skin models. Similar improvements are expected in vivo models. In vivo animal models and human immune cells show specific biomarkers after treatment with STIMAL.

Conclusions

STIMAL is the first, and most effective antidote to CEES, a mustard gas analogue. Preliminary testing in sulfur mustards has determined a modest ameliorative effect, although statistically insignificant. A second generation of STIMAL may yield a significant improvement in vivo inhalation/skin sulfur mustard animal models; and also prove to be the first multithreat treatment countermeasure.

2.

STIMAL (Liposome-Encapsulated N-Acetylcysteine) Increases the Ability of Human Macrophages to Kill *Bacillus anthracis*

R F Rest,1 Mariana Bernui,1 Milton Smith,2 William Stone3
1Drexel University College of Medicine, Phila, PA, 2Amaox, Ltd., Melbourne, FL, 3East Tennessee State University, Johnson City, TN

Category: B. Therapeutics

TRL1 / TRL2

Background STIMAL is a non-toxic anti-oxidant and anti-inflammatory therapeutic based on liposome-encapsulated NAC. We have previously shown that STIMAL ameliorates the pathophysiology associated with *in vivo* and *in vitro* administration of CEES, a mustard gas analogue. During inhalation anthrax, *B. anthracis* (BA) interacts directly with alveolar macrophages, and infection and disease is initiated following this critical interaction. In the present studies, we investigated whether STIMAL might help human macrophages limit the *in vitro* growth of BA, and thus limit disease progression in the host.

Methods We treated BA spores or vegetative cells with various concentrations of NAC *in vitro* in the presence or absence of human macrophage monolayers, and quantified BA and macrophage viability and functions. Proper negative and positive controls were included.

Results (i) NAC accelerates BA sporulation. (ii) NAC completely delays / inhibits BA germination (in the absence of macrophages). (iii) NAC, in a

dose and time-dependent manner, stimulates the ability of macrophages to kill intracellular BA. (iv) NAC is not directly toxic to vegetative BA or to BA spores.

Conclusions NAC has dramatic protective activities regarding macrophage killing of BA. In case of an anthrax attack, NAC could be used as an immediate therapeutic aid to help prevent the initial intraalveolar events of BA-macrophage interactions in humans. STIMAL, and its active component NAC, which is approved for human use, could be an ancillary therapeutic agent, as the first multithread treatment countermeasure in conjunction with vaccines and antimicrobials.

3.
B. *Anthracis*- and Y. *Pestis*-Specific Gene Expression in Antigen Presenting Cells
Cat: C
TRL 2

Keith Crawford[1,2], Jinsoo Joo[1], Rudolf Fluckiger[1,2], and Winston P. Kuo[2,3]
Harvard Medical School[1], Brigham &Women's Hospital[2], Harvard School of Dental Medicine[3], Boston, MA,
USA

Background
On the surface of most pathogens are conserved pathogen-associated molecular patterns (PAMP). PAMPs interact with pattern recognition receptors on the surface of antigen presenting cells (APCs), dendritic cells (DC) and monocytes. APCs are key immune cells found in all tissues (e.g. lung) of the body and are responsible for surveying these tissues for evidence of infection. The recent completion of the human genome sequence has improved our understanding of the molecular processes involved in cell survival, growth and differentiation in healthy and diseased states. Use of microarray and qPCR technologies has provided a means of monitoring the expression of large numbers of genes in relationship to stimuli.

Methods
DC were rapidly isolated from human blood by immunomagnetic-bead selection and exposed to B. *anthracis* (Ba) (Sterne pOX+), B. *anthracis* (Sterne pOX-) and Y. *pestis* (Ype). Cells were harvested at 2h, 4h, 8h, and 12h. RNA was isolated and prepared for DNA microarray hybridization. Following hybrization on microarrays, samples were analyzed for differential gene expression.

Results
Fig. 1. Hierarchical cluster analysis of DC exposed to pOX- (Ba-), pOX+ (Ba+), and Ype (Ype)
Ba- Ba+ Ype
Ype induces the upregulation of groups of genes in DC, which are not clustered in Ba-treated samples. In addition, different strains of Ba also demonstrate unique clustering patterns.
Fig. 2. Difference in gene expression in DC exposed to Ba and Ype. High expression (red/dark) and low expression (green/light).

Conclusions
Our findings suggest that DCs when exposed to Ba and Ype express unique gene patterns. In addition, DC exposed to similar strains, which differ by a single gene, also express unique gene patterns (not shown). Monitoring the APC response to pathogens can serve as a means of detecting presymptomatic disease states as they would occur after exposure to bio-weapons.

Using high through-put genomic or proteomic techniques to monitor the APC host response can allow the screening of large numbers of victims (40,000/day). Ba- Ba+ Ype Ba- Ba+ Ype
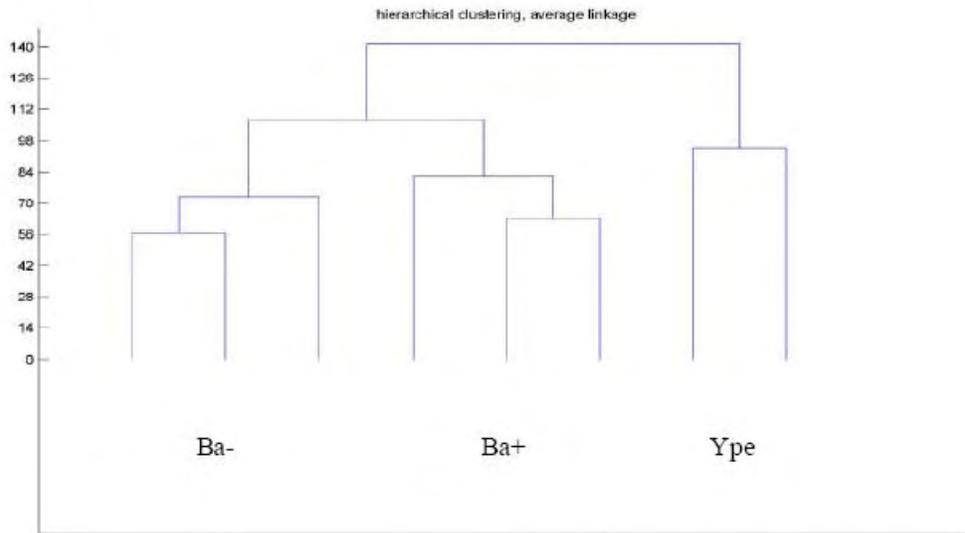

Fig. 1. Hierarchical cluster analysis of DC exposed to pOX- (Ba-), pOX+ (Ba+), and Ype (Ype)

Ype induces the upregulation of groups of genes in DC, which are not clustered in Ba-treated samples. In addition, different strains of Ba also demonstrate unique clustering patterns.

Fig. 2. Difference in gene expression in DC exposed to Ba and Ype. High expression (red/dark) and low expression (green/light).

Sulfur Mustard Toxicity Following Dermal Exposure

Role of Oxidative Stress, and Antioxidant Therapy

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Objective: Sulfur mustard (bis-2-(chloroethyl) sulfide) is a chemical warfare agent (military code: HD) causing extensive skin injury. The mechanisms underlying HD-induced skin damage are not fully elucidated. This review will critically evaluate the evidence showing that oxidative stress is an important factor in HD skin toxicity. Oxidative stress results when the production of reactive oxygen (ROS) and/or reactive nitrogen oxide species (RNOS) exceeds the capacity of antioxidant defense mechanisms. **Methods:** This review will discuss the role of oxidative stress in the pathophysiology of HD skin toxicity in both in vivo and in vitro model systems with emphasis on the limitations of the various model systems. Evidence supporting the therapeutic potential of antioxidants and antioxidant liposomes will be evaluated. Antioxidant liposomes are effective vehicles for delivering both lipophilic (incorporated into the lipid bilayers) and water-soluble (encapsulated in the aqueous inner-spaces) antioxidants to skin. The molecular mechanisms interconnecting oxidative stress to HD skin toxicity are also detailed. **Results:** DNA repair and inflammation, in association with oxidative stress, induce intracellular events leading to apoptosis or to a programmable form of necrosis. The free radical, nitric oxide (NO), is of considerable interest with respect to the mechanisms of HD toxicity. NO signaling pathways are important in modulating inflammation, cell death, and wound healing in skin cells. **Conclusions:** Potential future directions are summarized with emphasis on a systems biology approach to studying sulfur mustard toxicity to skin as well as the newly emerging area of redox proteomics.

SULFUR MUSTARD: A CENTURY OF THREAT

Sulfur mustard (SM) or mustard gas (bis-2-(chloroethyl) sulfide, military code: HD) is a chemical warfare agent classified as a weapon of mass destruction. Mustard gas was one of the first chemical weapons deployed against troops on a battlefield during World War I, almost hundred years ago. Since then, the military use of mustard gas has been documented in a number of situations. In 1988, HD was used with devastating results

The first page from the article that was written for the Journal of Burns and Wounds.

12 Vesicants and Oxidative Stress

*Milton G. Smith, William Stone, Ren-Feng Guo,
Peter A. Ward, Zacharias Suntres, Shyamali Mukherjee,
and Salil K. Das*

CONTENTS

The first page of the chapter that was written by several members of the Cosortium for the book- Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology and Therapeutics; edited by James Romano. Publication is scheduled for December 2007.

ORIGINAL RESEARCH ARTICLE

ABILITY OF ANTI-OXIDANT LIPOSOMES TO PREVENT ACUTE AND

PROGRESSIVE PULMONARY INJURY

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Running title: Anti-oxidant liposomes in CEES lung injury

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1

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W81XWH-06-2-0044

Pages 22

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<u>Award Number:</u>
Department of Army Cooperative Agreement No. W81XWH-06-02-0044
Subcontract No, 3000619434

<u>TITLE</u>
The Development of Therapeutic and Diagnostic Countermeasures to WMD
**Project 7 – Antioxidant Liposome as a new Adjuvant Therapy for inhalational
Anthrax**

<u>PRINCIPAL INVESTIGATOR:</u>
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INTRODUCTION

The purpose of this proposal is to verify the principal of a novel antioxidant therapy in addition to conventional antibiotic treatment (ciprofloxacin) for inhalational anthrax. To achieve this goal, numerous in vitro and in vivo experiments are described and will be performed on a highly expedited schedule by AFG Biosolutions, Inc.

Infection with *Bacillus anthracis* spores through intradermal inoculation, ingestion or inhalation results in three different distinct clinical presentations: cutaneous anthrax, gastro-intestinal anthrax and inhalational anthrax. Inhalational anthrax is the most fatal form of infection and therefore is the most realistic scenario for a bioterrorist attack. Victims of anthrax succumb to septicemia, toxemia, systemic inflammatory response syndrome (SIRS), septic shock, and multiple organ dysfunction syndromes (MODS) within one to seven days post-exposure (Dixon et al, 1999). During the initial infectious process, macrophages play a pivotal role as cellular mediators of infection, particularly in inhalational anthrax.

The involvement of intracellular reduction-oxidation (redox) in the development of sepsis and septic shock has been well-defined. Sufficient evidence indicates that tissue injury associated with severe sepsis and MODS are mediated in part by excessive production of reactive oxygen intermediates (ROIs). Recent studies have shown that ROIs actively contribute to the pathogenesis of anthrax infection in several aspects as described below. Depending on the stage of the infection, oxidative species can: 1). Facilitate germination of engulfed B. anthracis spores in macrophages; 2). Alter function of infected cells by oxidizing intracellular and membrane-bound molecules including DNA, proteins, and membrane lipids; 3). Induce over expression of inflammatory mediators by activating transcription factor NFκB; 4). Coordinate with anthrax LeTx to lyse macrophages and induce apoptosis of a variety of immune cells. Considering the aforementioned, blocking reactive oxygen species may ameliorate the inflammation associated with anthrax infection. In addition, modulating oxidative stress-related molecules, such as redox-sensitive transcriptional factors, may be useful for the regulation of these inflammatory responses.

Recently we have successfully developed a signal transduction inhibition antioxidant liposome (STIMAL) which can efficiently remove the overproduced ROIs both in vitro and in vivo. In this study, we propose to test protective effects of STIMAL in the event of anthrax infection.

In order to fulfill goals of this proposal, the three following specific aims were established as stated in the application:

Aim 1: To determine the protective effect of signal transduction inhibition antioxidant liposomes (STIMAL) on human PBMC from apoptosis and murine macrophages from cytolysis after infection with *B. anthracis* spores or treatment with LeTx.

Aim 2: To determine if STIMAL is able to block or diminish hemolysis and production of inflammatory mediators caused by anthrax infection.

Aim 3: To evaluate the protective efficacy of antioxidant liposome in murine anthrax model.

These aims have not been modified since the original submission.

BODY OF REPORT

Aim 1: To determine the protective effect of signal transduction inhibition antioxidant liposomes (STIMAL) on human PBMC from apoptosis and murine macrophages from cytolysis after infection with *B. anthracis* spores or treatment with LeTx.

Suitable method to measure reactive oxidative species (ROS) production in cells is necessary to assess the role of lethal toxin on the production of ROS. Our preliminary studies suggest that different cell populations such as human neutrophils and monocytes might have different oxidative metabolism that require different methods to measure the generation of ROS.

Neutrophils and monocytes were isolated from human blood that was purchased from Seracare Diagnostics. The blood was shipped and stored at 4^0C; blood components were isolated within 2 days after collection. Human peripheral blood mononuclear cells (PBMCs), monocytes, and neutrophils were isolated before use in assay.

PBMCs were isolated by centrifuging for 30 minutes at 900g on Fico/Lite-LymphoH (Atlanta Biologicals, GA). The buffy coat containing PBMCs were collected and resuspended in DMEM + 10% FBS. Monocytes were purified from PBMCs using adherence method. Monocytes were collected and resuspended in DMEM + 10% FBS. Neutrophils were purified from the pellet after ficolling of the blood. The pellet was resuspended with equal amount of PBS and 3% dextran, and then incubated for 30 minutes at room temperature. The neutrophil-rich part were removed and residual red blood cells were lysed with 0.2% cold NaCl for 30 seconds, followed by adding 1.6% NaCl to resume the osmotic pressure.

The amount of ROS produced by human monocytes and neutrophils were measured using DCF-diacetate (DCFDA). This DCFDA is reported to be the most common reagent to probe for cellular ROS (Halliwell and Whiteman, 2004). Cells are usually loaded with DCFDA and the dye is diffuse into the cytoplasm where it is deacetylated by esterase to DCFH. The non fluorescent DCFH is converted to highly fluorescent DCH by ROS. Briefly, monocytes/neutrophils were resuspended to final concentration of 10^6cells/ml in Hank's balanced salt solution (HBSS) with 10mM HEPES. 10^6 cells were then incubated with LeTx (PA500ng/ml:LF 100ng/ml) for 30min, 60min, or 120min. After centrifugation (125g, 5min), the cells were resuspended in Krebs-Hensleit buffer supplemented with 12.5mM HEPES and 5µM DCFDA. The cells were incubated at 370C for 15 min, and then analyzed by flow cytometer.

Figure 1 shows that neutrophils challenged with LeTx produced slightly more ROS compared to untreated at 30 minutes after challenge but not at 60 or 120 minutes after challenge. Figure 2 shows that monocytes treated with both LPS and LeTx did not produce ROS. We used the same method in an attempt to measure ROS in human monocytes but were not able to measure significant increase of ROS when cells were treated with LeTx or LPS, which serves as positive control. This result concurs with a study done by Robinson et al, which proposes that the mechanism of intracellular processing of DCFDA in monocytes differs from that in neutrophils (Robinson et al, 1988). Thus, different methods to measure ROS in cells in response to lethal toxin challenge were investigated. Experiments were conducted in murine macrophage cell line RAW264.7.

We examined DCFDA for the assessment of ROS generation in RAW 264.7 challenged with LeTx. An improved product of DCFDA, the carboxy derivative of fluorescein (carboxy-DCFDA), was used in the assay because it carries additional negative charges that improve its retention in cells compared to noncarboxylated forms.

RAW 264.7 cells were seeded in 6 well plates at a concentration of 1 x 10^6 cells/well and incubated for 2-3 hours to allow attachment to the plate. The cells were washed twice with PBS and treated with 500ng/ml PA + 100ng/ml LF or 2µM PMA for 60, 75, 90, 105 and 120mins. The cells were loaded with 5µM of carboxy-DCFDA (Invitrogen) 30mins before harvesting. Cells were dislodged from the plate and immediately analyzed by flow cytometry. The cells were gated by forward scatter (FSC) and side scatter (SSC) and the fluorescence intensity monitored by FL1 channel. A minimum of 10,000 events were collected in order to achieve statistical significance.

Lethal toxin-treated cells showed an increase in the fluorescence intensity when probed with carboxy-DCFDA. Among all the various time point tested, only cells treated with lethal toxin for 120mins (Figure 3) showed an increase in the FL1 intensity. This time point coincides with toxin-induced cell death as observed by microscope before cell harvesting. This demonstrates that the toxin-treated cells induced the production of ROS in RAW 264.7 cells.

Alternative method using a fluorescence plate reader instead of flow cytometry to measure fluorescence intensity was also investigated. Briefly, RAW 264.7 cells were seeded at 7.5 x 10^4 cells per well (96 well plate) in DMEM + 5% FBS and incubated at 37°C for 5 hours to reduce ROS production to basal level. The cells were washed twice with Krebs Ringer buffer and incubated with 1000ng/ml PA + 200ng/ml LF, 500ng/ml PA + 100ng/ml LF, 250ng/ml PA + 50ng/ml LF, 100ng/ml PA + 100ng/ml LF and 1.2µM PMA at 37°C for 3 hours. Carboxy-DCFDA was added at a final concentration of 25µg/ml 1hr after treatment initiation and fluorescence intensity were measured with a FluoroSkan plate reader of excitation 485nm and emission 527nm.

Figure 4 shows that there is a significant increase of fluorescence intensity in LeTx treated cells compared to untreated. The level of fluorescence in lethal toxin treated cells is 20-60% higher than control and 70% increase for PMA treated cells. We observed that the level of fluorescence correlates with the amount of LeTx concentration used to treat cells. This suggests that the toxin-treated cells induced the production of ROS in RAW 264.7 cells and ROS generation is toxin dose-dependent.

We tested additional methods to measure ROS in RAW264.7 cells such as superoxide dismutase (SOD) inhibitable reduction of cytochrome c and Oxyburst green H2HFF to confirm DCFDA results.

Superoxide (O2-) generation was assayed as superoxide dismutase (SOD) inhibitable reduction of cytochrome-c (Hanna et al, 1994). Briefly, RAW 264.7 cells were seeded at 2 x 10^6 cells per well and incubated at 37°C for 5 hours to normalize ROS production to background levels. Cells were then washed and treated with 500ng/ml PA and 100ng/ml LF in HBSS + 2.2mg/ml of glucose containing 160µM of acetylated cytochrome c (Sigma) for 2hrs. Positive control cells were stimulated with 1.2µM PMA and negative control with PBS. Another set of cells were treated the same manner with the addition of 100U/ml of SOD. The amount of superoxide anion produced was calculated from SOD-inhibitable reduction of cytochrome c.

Lethal toxin treated cells resulted in up to 2.5 fold increase in the amount of cytochrome c reduction after 2 hours incubation (Figure 5A). However, as most of the reduction is not inhibited by the presence of SOD, the reduction of cytochrome c by lethal toxin cannot be affirmatively attributed to increase in superoxide anion production. The amount of superoxide anion production for lethal toxin treated cells is 33% higher than control vs 400% increase for PMA stimulated cells (Figure 5B).

The assay was repeated many times and similar results were obtained. Potency of lethal toxin was verified by microscopic observation of cell lysis and the assay was shown to be

working by the significant increase of SOD-inhibitable reduction of cytochrome c reduction when cells were treated with PMA.

Other methods of measuring ROS production were explored in order to corroborate cytochrome c reduction results. Oxyburst green H2HFF (Invitrogen) is also used for detecting release of oxidative products and is reported to be up to 1000 times more sensitive than cytochrome c reduction. It consists of BSA conjugated to dihydro-2′, 4,5,6,7,7′-hexafluorofluorescein (H2HFF) and the oxidation of the compound result in the formation of highly fluorescent product which can be detected by spectrofluorometer.

RAW 264.7 cells were seeded in 96 well plate at 2.5 x 10^4 cells per well in DMEM + 10% FBS and incubated for 24 hours. The media were aspirated and replaced with fresh DMEM+5% FBS containing either 12.5mM or 25mM NAC and incubated for 16 hours. Control cells were replaced with just fresh DMEM+5% FBS. RAW 264.7 cells were subsequently treated with 500ng/ml PA + 100ng/ml LF or 2μM PMA as positive control. Final concentration of 20μg/ml of oxyburst green H2HFF was added immediately and fluorescence intensity was monitored by excitation 485nm and emission 527nm.

We obtained similar results as cytochrome c reduction; Figure 6 shows that cells that were treated with lethal toxin did not increase the amount of fluorescence intensity as measured by oxyburst green H2HFF oxidation. However, there is a dose-dependent reduction in the fluorescence intensity for cells that were pre-treated with N-acetyl-L-cysteine (NAC) for 16 hours. The assay is verified by the increase in fluorescence intensity for cells treated with PMA. The oxyburst green H2HFF assay was repeated using different concentrations of LeTx challenge. The results show that cells that were treated with lethal toxin have lower fluorescence intensity compared to untreated cells (data not shown).

In summary, DCFH-diacetate seems to be the suitable method to measure reactive oxidative species in RAW 264.7 cells treated with lethal toxin. It is unclear why the production of ROS in RAW 264.7 can only be observed with DCFDA probe and not with cytochrome c and oxyburst green H2HFF. The amount of reactive oxidative species produced by human monocytes and neutrophils will also be measured using the assays mentioned above to determine the most appropriate assay for each cell population.

Subsequent studies will be conducted to determine whether STIMAL has a neutralizing effect against ROS produced by RAW264.7. Preliminary studies in human neutrophils suggest that treatment with N-acetyl-L-cysteine- (NAC-) and Glutathione- (GSH-) liposomes (University of Tennessee) at 7.5mM reduced the amount of ROS produced by neutrophils at all time points (Figure 1). We will investigate the best STIMAL dose and treatment method (simultaneous vs preincubation), which gives the best protection against ROS due to LeTx challenge.

We have developed cytolytic assay using murine macrophage cell line RAW 264.7 to assess the protective effect of STIMAL against LeTx or *B. anthracis* sterne spores induced cell lysis. The assays include traditional cell counting after Trypan Blue staining and colorimetric cell viability assays i.e. Cell Counting Kit-8 (CCK-8, Dojindo), and CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Preliminary experiments were conducted to establish and optimize the cytolytic assays mentioned above.

Trypan blue cell counting and CCK-8 methods confer similar results. When the murine macrophage cells were treated with LeTx (PA 500ng/ml:LF 100ng/ml), the cells were lysed (>90% cell death) within 3 hours . Cell death was confirmed by microscopy using trypan blue staining. The cells that were treated with PA alone, LF alone, and non-treated did not show any cell lysis by both methods (data not shown).

B. anthracis sterne spores was prepared in a liquid media and the culture was allowed to progress to sporulation. Spores were then purified by gradient centrifugation. RAW 264.7

cells were infected with *B. anthracis* sterne spores and monitored for cell lysis. After 12 hours, we noted that 90% of cells were lysed. Percent of cell viability was measured by cell counting and microscopy using trypan blue staining. This result was confirmed by CytoTox 96 assay, which measures lactate dehydrogenase (LDH) release upon cell lysis.

We conducted experiments to determine the optimal concentration of antioxidant liposome that shows protective effect against LeTx induced cell lysis. CytoTox 96 assay was used in the experiments because of its reproducibility and compatibility with the use of spores in subsequent experiments.

Different concentrations of antioxidant liposomes starting with 7.5mM were tested to determine whether it protects or mitigates the cytolytic action of lethal toxin. Briefly, RAW 264.7 cells were seeded in a 96 well plate at a density of 2.5×10^4 cells per well in DMEM supplemented with 5% FBS without antibiotic. For the assay, LeTx and described amounts of antioxidant liposome were added simultaneously to cells. Three hours after treatment, cell cytotoxicity was determined by CytoTox 96 assay. Percent cytotoxicity was determined using the following formula: (Macrophage treated-macrophage LDH spontaneous release)/ (macrophage maximum LDH release-macrophage LDH spontaneous release)

Figure 7 shows that antioxidant liposome both NAC- and GSH-liposomes protect cells from LeTx induced cytotoxicity. NAC- and GSH- liposome improves survivability of LeTx treated cells by 12% (vs blank liposome) and 30% (vs untreated). NAC-liposome does not show cell cytotoxicity while GSH-liposome shows some level of cell toxicity in a dose-dependent manner (Figure 8).

Since the protective effect of antioxidant liposomes seems to be dose-dependent, we will assess the effect of higher concentration of antioxidant liposomes on cell lysis. We will also investigate the best treatment method (simultaneous vs preincubation), which gives the best protection against cell lysis due to LeTx challenge.

Aim 2: To determine if STIMAL is able to block or diminish hemolysis and production of inflammatory mediators caused by anthrax infection.

Previous studies by Wu et al. showed that anthrax lethal toxin can induce hemolysis in the presence of polymorphonuclear cells (Wu et al, 2003).

The hemolysis experiment was conducted following methods described by Wu et al. Briefly, 1 ml human blood was transferred into 5ml glass tube, and treated with PA alone, LF alone, or in combination. The blood cells were monitored every 10 minutes for visual sign of hemolysis and incubated for up to 16 hours at 37^0C. Plasma and supernatants were collected for measurement of hemoglobin concentration. The concentration of hemoglobin was determined by a Quantichrom Hemoglobin Assay Kit (Bioassay systems).

Human blood was purchased from Seracare Diagnostics. The blood was shipped and stored at 4^0C, and used within 2 days after collection. Fresh human blood was also obtained from donors and used immediately for hemolysis assay (less than 2 hours after blood collection) to eliminate working with non-functional polymorphonuclear cells (PMNs).

The components of lethal toxin (PA and LF) were purchased from List Biologicals, Inc. or collected from Dr. Steve Leppla (NIH). Activity of lethal toxin was tested with the murine macrophage RAW 264.7, and most cells were lysed (>90% cell death) within 3 hours.

Human blood was challenged with PA alone, LF alone, or PA and LF combination at various concentrations. The highest concentration of PA tested was 1000ng/ml and lowest concentration was 100ng/ml. The highest concentration of LF tested was 200ng/ml and lowest concentration was 10ng/ml. However, we are not able to reproduce hemolysis

induced by lethal toxin in human blood. Table 1 shows that there are no significance difference in hemoglobin concentration between control and treatment group.

Hemolysis assay was repeated many times with various modifications to eliminate possible variables. However, none of the efforts were successful. The modifications include using different anticoagulant for blood collection (Na citrate, heparin, or EDTA), performing the assay on a 48 well plate instead of glass tubes, or increasing LeTx concentration. Human blood was also collected in a 50ml polypropylene conical tube with heparin at 100U/ml as anti-coagulant. The use of polypropylene tube is to investigate whether silicone coating vacutainer used in blood collection may inhibit hemolysis. We suspect that silicone coating might have an effect in inhibiting reactive oxygen species (Suzuki et al, 2003).

In recent experiments, we preincubated blood cells with *B. anthracis* cell wall at 1ug/ml for 30 minutes at 370C before adding PA, LF or LeTx. We do not observe hemolysis in any of the treatment groups. We also investigated the effects of LeTx to human neutrophils and red blood cells instead of whole human blood. Neutrophils and red blood cells were collected from the pellet after ficolling of the blood. The pellet was resuspended with equal amount of PBS. The suspension was then used in hemolysis assay. No hemolysis was observed within hours or up to 16 hours incubation.

Experiments to measure production of inflammatory cytokines from human PBMC and RAW 264.7 after treatment with lethal toxin have been initiated. Human PBMCs were collected and cultured as described. After treatment with LeTx (PA 500ng/ml:LF 100ng/ml or PA 100ng/ml: LF 20ng/ml), the media was collected at 2 hours and 3 hours post treatment and analyzed using BD Pharmingen CBA kit. Level of cytokines such as IFN-g, TNF-a, IL-10, IL-4, IL-2, and IL-5 were measured by flow cytometry.

Figure 9 shows that level of IFN-g, IL-5, and IL-4 slightly increase at LeTx (PA100ng/ml:LF20ng/ml) group compared to control at 3 hours post treatment. Amount of TNF-a, IL-10, and IL-2 was similar to control. We currently are working on finding the optimal concentration of LeTx and appropriate time points for production of inflammatory cytokines.

Aim 3: To evaluate the protective efficacy of antioxidant liposome in murine anthrax model.

We have received approval for the OLAW animal welfare assurance and successfully passed the USDA inspection. Internal Animal Program has been developed and IACUC has been established in house. Animal protocols have been reviewed and approved. Current renovation of the HVAC systems in our animal facility will be completed in September followed by animal experiments under this Aim.

KEY RESEARCH ACCOMPLISHMENTS

- Preliminary results suggest that antioxidant liposome neutralize ROS production in cells and partially protect cells from cytotoxic effect of lethal toxin.

- The protective role of antioxidant liposome has been demonstrated in our *in vitro* assays and that could be used as a basis to proceed with the testing of STIMAL in an *in vivo* model.

REPORTABLE OUTCOMES

None during this reporting period

CONCLUSIONS

The results obtained so far suggest that lethal toxin induced the production of ROS as measured by the oxidation of carboxy-DCFDA. This overproduction can be partially neutralized by the presence of exogenous antioxidant and was also observed to confer some protection to the cells from the cytotoxic effect of lethal toxin. It is believed that the overproduction of ROS is responsible for the cell lysis in lethal toxin-treated cells (Hanna et al, 1994).

Although it is too early to draw a conclusion at this stage, the protective role of antioxidant as shown in our in vitro assays could be used as a basis to proceed with the testing of STIMAL in an in vivo model. The *in vivo* results are crucial in substantiating the claims of the protective action of STIMAL against lethal toxin/anthrax treated cells. BALB/c mice pretreated with N-acetyl-L-cysteine NAC showed partial protection against lethal toxin (Hanna et al, 1994). STIMAL hold more promising results due to several advantages over the conventional antioxidant in solution. STIMAL maintains the pH of the solution compared to NAC alone, which creates less stress to the body; STIMAL does not degrade as rapidly as antioxidant in solution; STIMAL stores both lipid and water soluble antioxidant. Moreover, STIMAL allows for the use for treating both chemical and biological agents.

In summary, these preliminary results and background work are important for the continuing progress towards the goal of the study, which is to explore the possibility of antioxidant liposome as a new adjuvant therapy for inhalational anthrax.

REFERENCES

Dixon TC, Meselson M, Guillemin J, Hanna PC. Anthrax. N Engl J Med. 1999, 341(11):815-26.

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British Journal of Pharmacology. 2004, 142, 231-255.

Hanna PC, Kruskal BA, Ezekowitz RA, Bloom BR, Collier RJ. Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol Med. 1994, 1:7-18.

Robinson JP, Bruner LH, Bassoe CF, Hudson JL, Ward PA, Phan SH. Measurement of intracellular fluorescence of human monocytes relative to oxidative metabolism. Journal of Leukocyte Biology. 1988, 43: 304-310.

Wu AG, Alibek D, Li YL, Bradburne C, Bailey CL, Alibek K. Anthrax toxin induces hemolysis: an indirect effect through polymorphonuclear cells. J Infect Dis. 2003,15; 188 (8):1138-41.

Suzuki R, Muyco C, McKittrick J, Frangos JA. Reactive oxygen species inhibited by titanium oxide coatings, J Biomed Mater Res A. 2003 Aug 1;66(2):396-402.
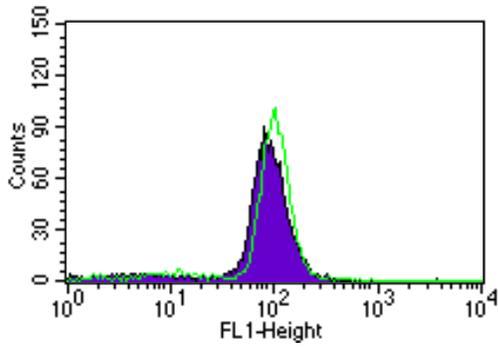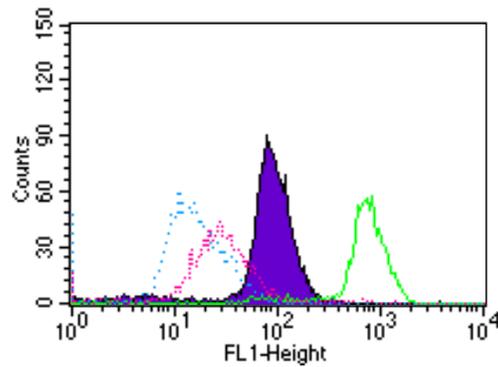
APPENDICES

A.

Human neutrophils treated with LeTx - 30mins
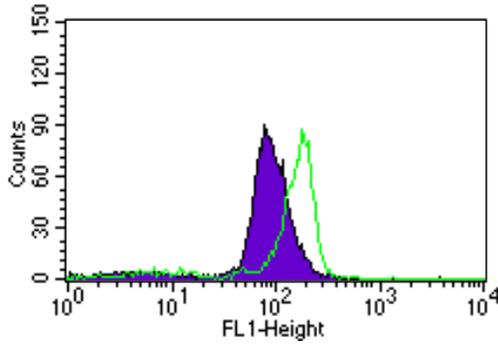
Key	Name	Parameter	Gate
■	30 mins -ve control .001	FL1-H	G1
—	30 mins LPS.002	FL1-H	G1

Key	Name	Parameter	Gate
■	30 mins -ve control .001	FL1-H	G1
—	30 mins LeTx.003	FL1-H	G1

Key	Name	Parameter	Gate
■	30 mins -ve control .001	FL1-H	G1
—	30 mins LeTx + blank.004	FL1-H	G1
····	30 mins LeTx + GSH.005	FL1-H	G1
····	30 mins LeTx + NAC.006	FL1-H	G1

B.

Human neutrophils treated with LeTx - 60mins
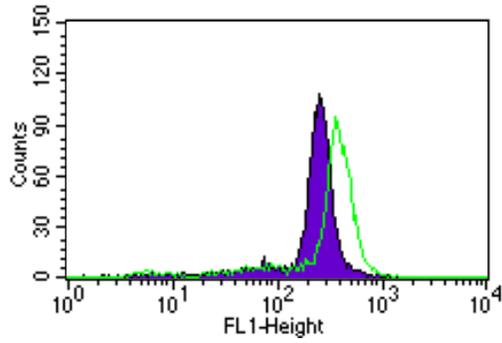
Key	Name	Parameter	Gate
■	60 mins -ve control.007	FL1-H	G1
—	60 mins LPS.008	FL1-H	G1
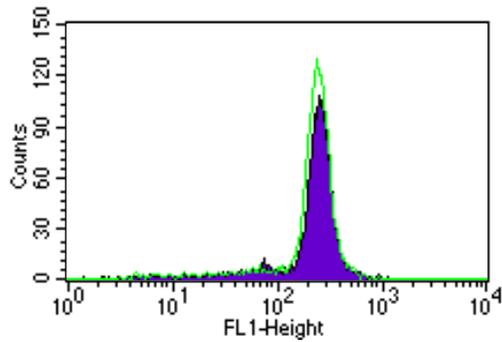
Key	Name	Parameter	Gate
■	60 mins -ve control.007	FL1-H	G1
—	60 mins LeTx .009	FL1-H	G1
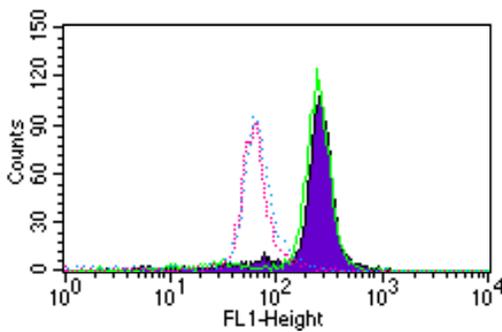
Key	Name	Parameter	Gate
■	60 mins -ve control.007	FL1-H	G1
—	60 mins LeTx + Blank.010	FL1-H	G1
......	60 mins LeTx + GSH.011	FL1-H	G1
....	60 mins LeTx + NAC.012	FL1-H	G1

C.

Human neutrophils treated with LeTx - 120mins

Key	Name	Parameter	Gate
■	120 mins -ve control.013	FL1-H	G1
—	120 mins LPS.014	FL1-H	G1

Key	Name	Parameter	Gate
■	120 mins -ve control.013	FL1-H	G1
—	120 mins LeTx.015	FL1-H	G1

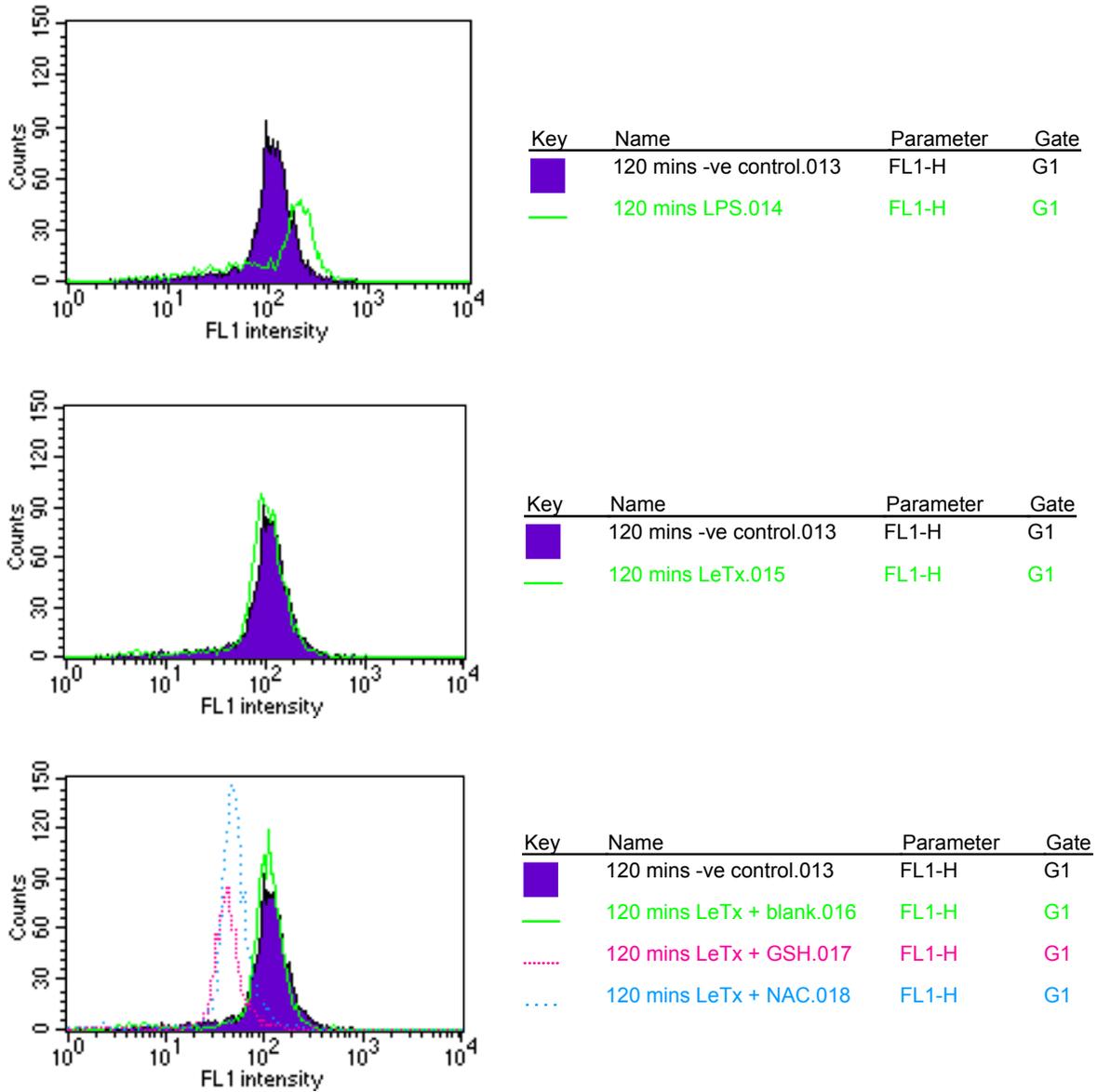| Key	Name	Parameter	Gate
■	120 mins -ve control.013	FL1-H	G1
—	120 mins LeTx + blank.016	FL1-H	G1
....	120 mins LeTx + GSH.017	FL1-H	G1
....	120 mins LeTx + NAC.018	FL1-H	G1

Figure 1. Reactive oxidative species (ROS) produced by neutrophils after challenged with LeTx and treated with antioxidant liposomes (7.5mM NAC-liposome or 7.5mM GSH-liposome).LPS treatment serves as positive control. The highly fluorescent 2',7'-dichlorofluorescein was measured post challenge at 30 min (A), 60 min (B), and 120 min (C) by flow cytometer.
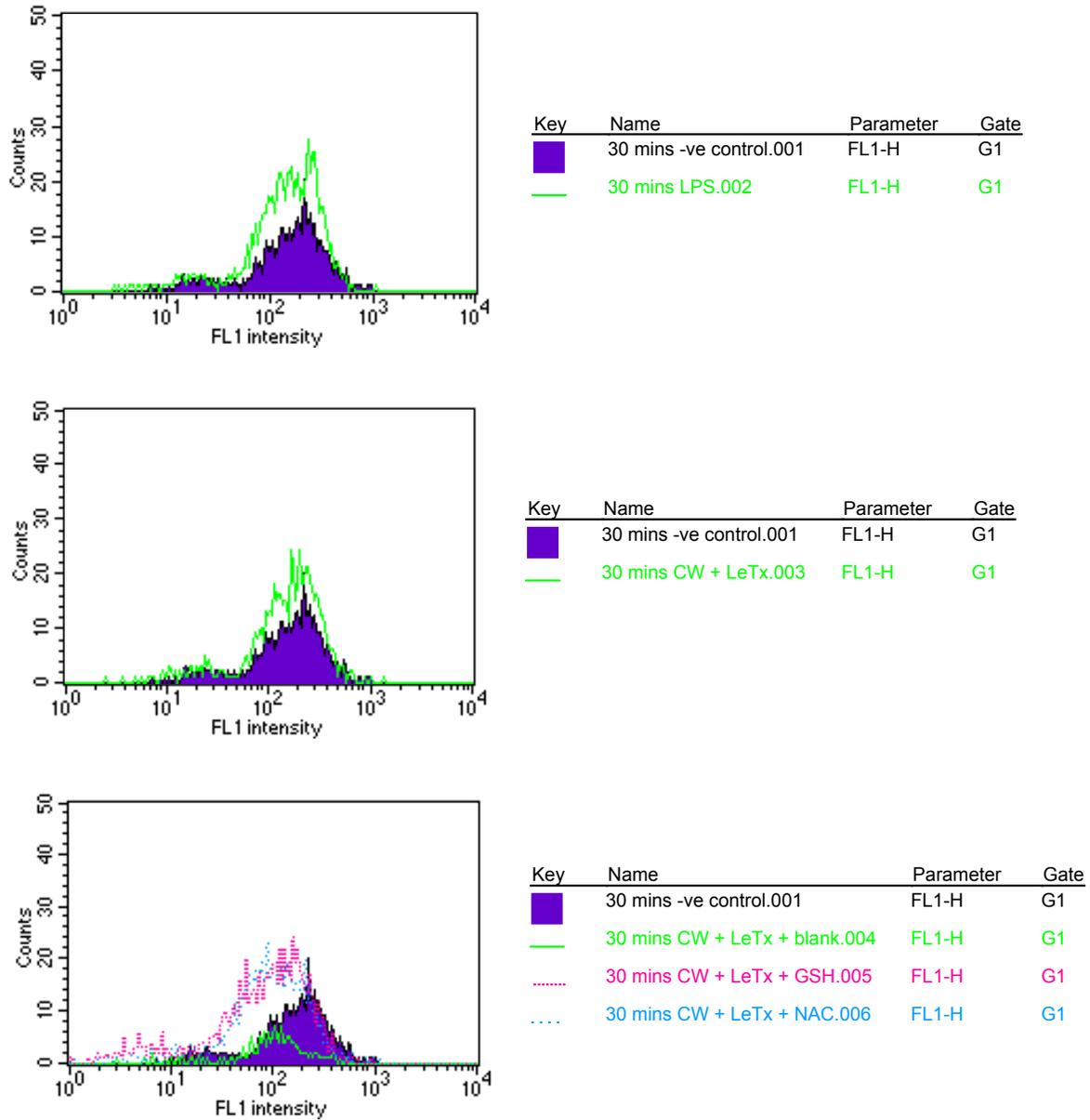
Human monocytes treated with LeTx - 30mins

Key	Name	Parameter	Gate
■	30 mins -ve control.001	FL1-H	G1
—	30 mins LPS.002	FL1-H	G1

Key	Name	Parameter	Gate
■	30 mins -ve control.001	FL1-H	G1
—	30 mins CW + LeTx.003	FL1-H	G1

Key	Name	Parameter	Gate
■	30 mins -ve control.001	FL1-H	G1
—	30 mins CW + LeTx + blank.004	FL1-H	G1
·······	30 mins CW + LeTx + GSH.005	FL1-H	G1
····	30 mins CW + LeTx + NAC.006	FL1-H	G1

Figure 2. Reactive oxidative species (ROS) produced by monocytes after challenged with *B. anthracis* cell wall and LeTx and treated with antioxidant liposomes (7.5mM NAC-liposome or 7.5mM GSH-liposome). LPS at 1ug/ml was used as positive control. The highly fluorescent 2',7'-dichlorofluorescein was measured by flow cytometer.
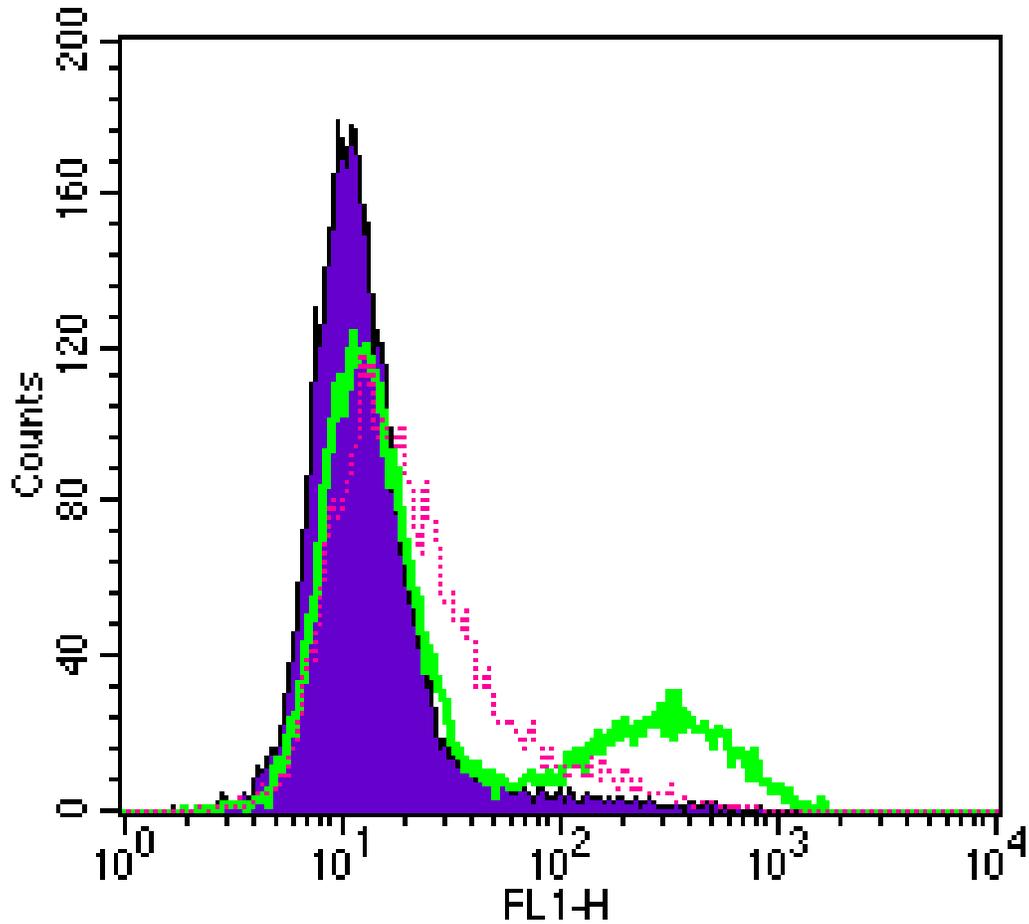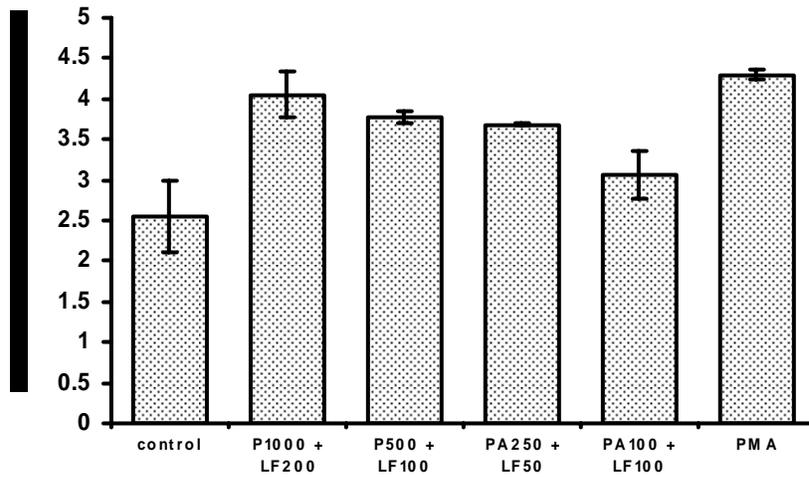
Figure 3. Reactive oxidative species (ROS) produced by RAW264.7 cells after challenged with LeTx for 120 mins (control-shaded purple; lethal toxin-green line; PMA-dotted red line). PMA treatment serves as positive control. The highly fluorescent DCH was measured by flow cytometer.
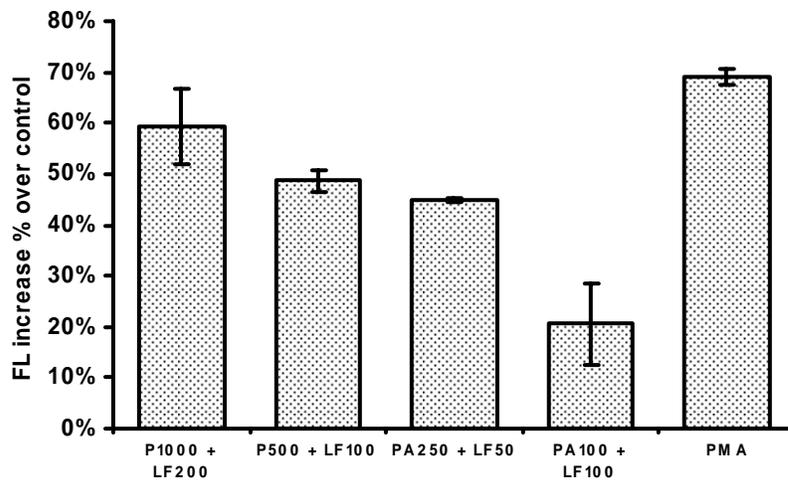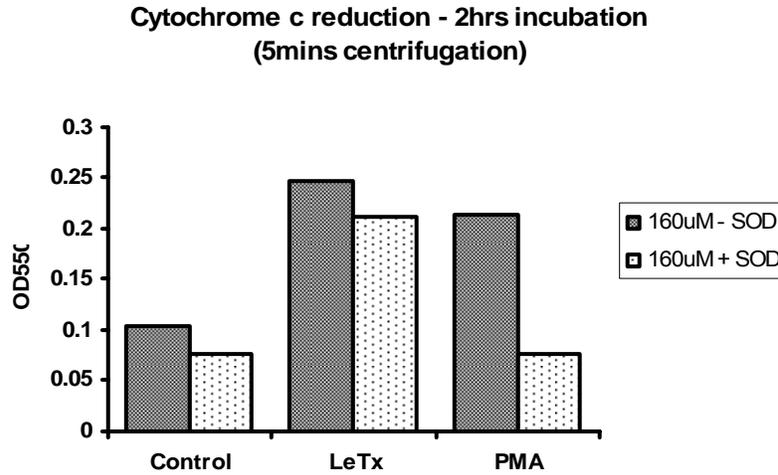
A.

B.

Figure 4. Reactive oxidative species (ROS) produced by RAW264.7 cells after challenged with LeTx for 3 hours. PMA treatment serves as positive control. The highly fluorescent DCH was measured by FluorosKan plate reader.

A.

**Cytochrome c reduction - 2hrs incubation
(5mins centrifugation)**

B.

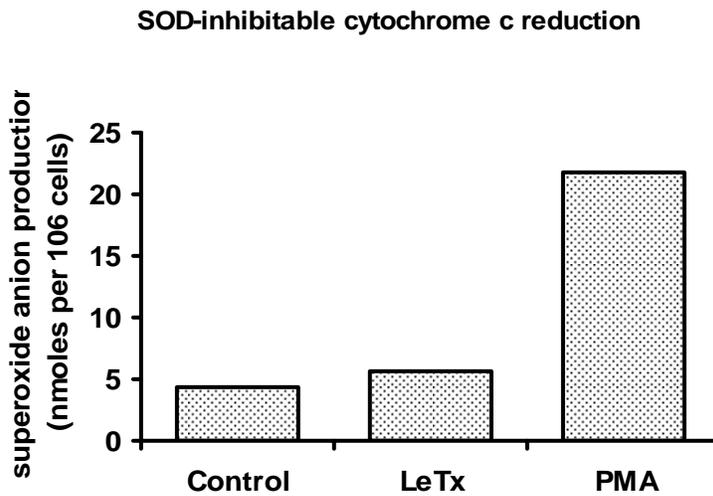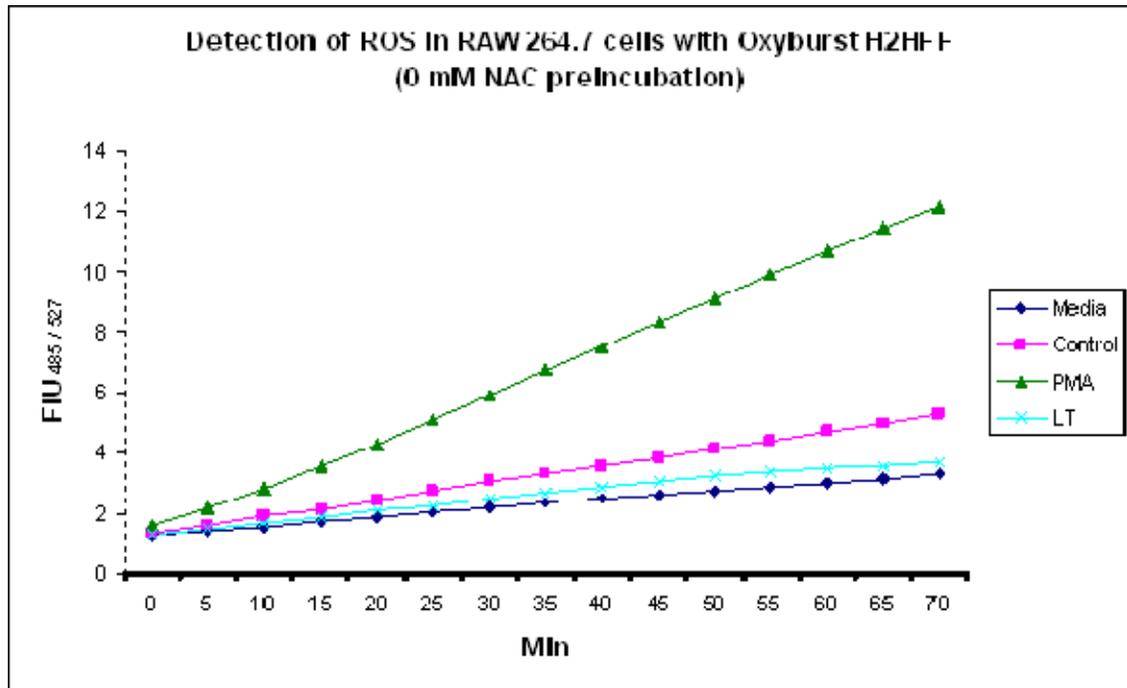**SOD-inhibitable cytochrome c reduction**

Figure 5. Measurement of superoxide (O_2^-) generation produced by RAW264.7 after challenged with LeTx as determined by superoxide dismutase (SOD) inhibitable reduction of cytochrome-c. PMA treatment serves as positive control.

A.

B.
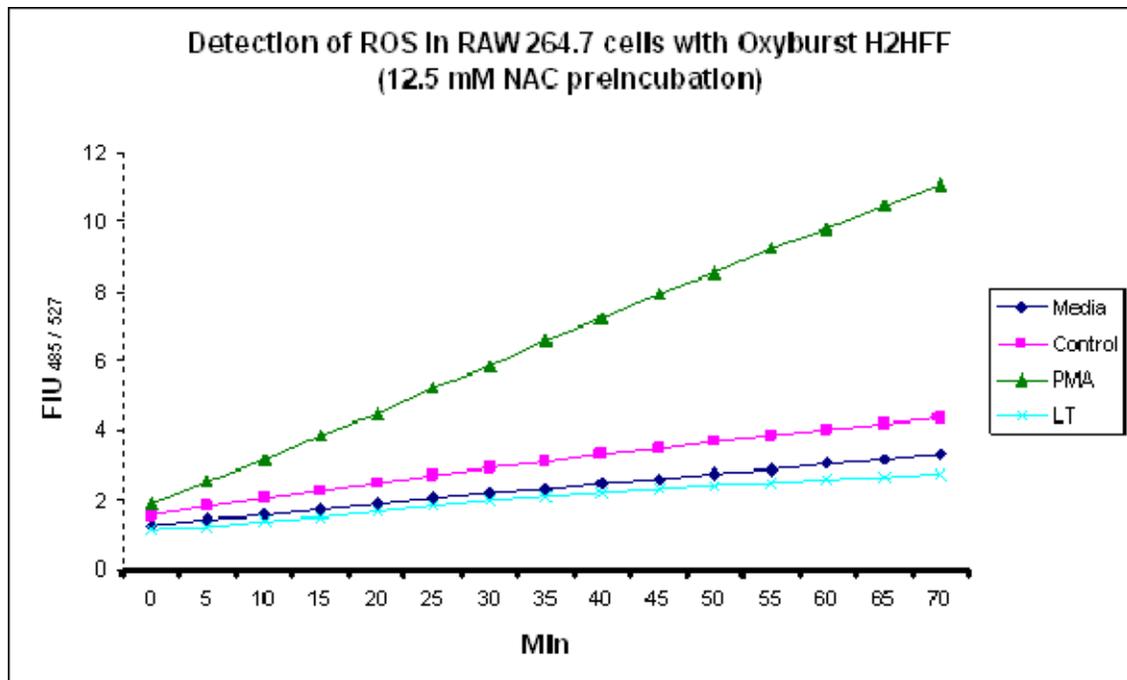
C.
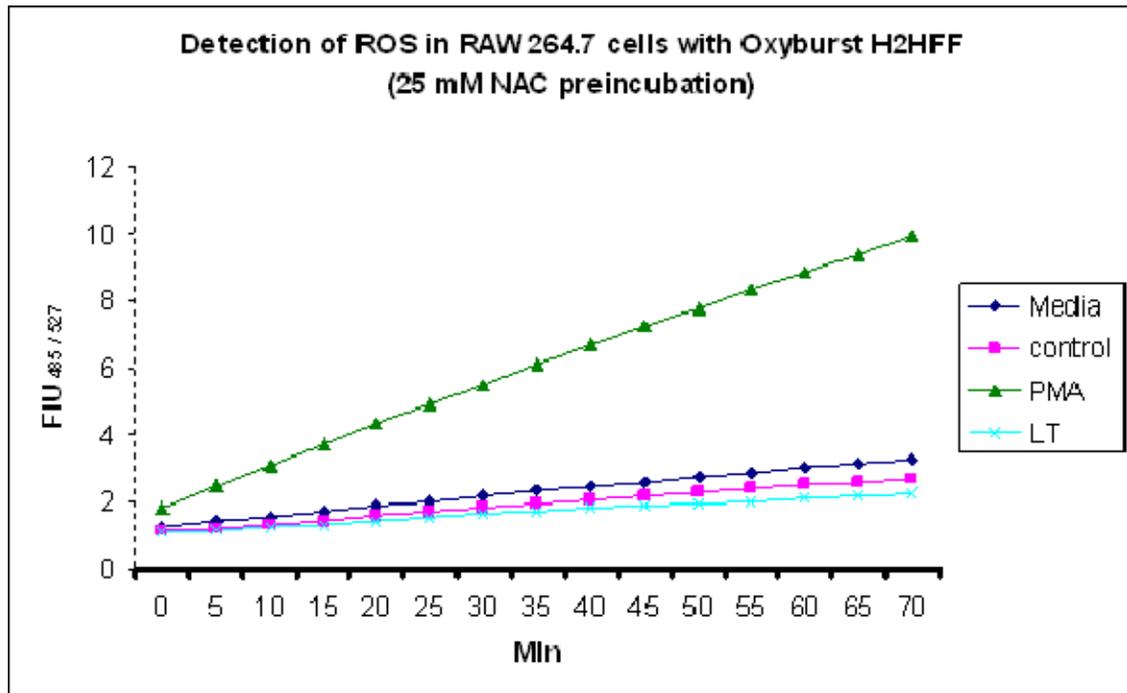
Figure 6. Reactive oxidative species (ROS) produced by murine macrophage RAW264.7 cells that were preincubated with (A) 0mM NAC, (B) 12.5mM NAC or (C) 25mM NAC for 16 hours and challenged with LeTx. PMA treatment serves as positive control. The detection of ROS in RAW264.7 was measured with Oxyburst green H2HFF.
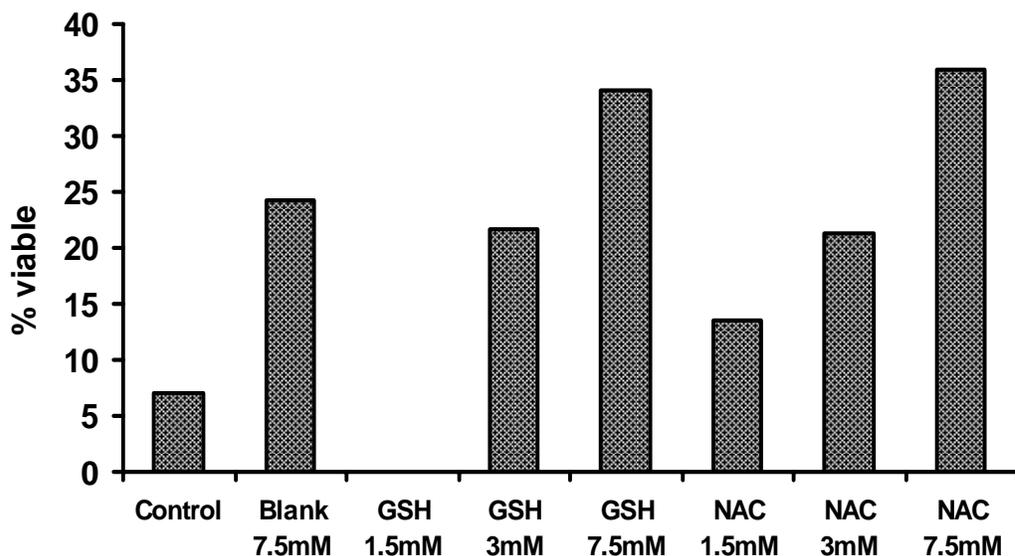
Figure 7. RAW 264.7 cells were treated with LeTx and indicated concentrations of antioxidant liposome in DMEM supplemented with 5% FBS at 37^0C. Three hours after treatment, percent cell cytotoxicity was determined by CytoTox 96 Assay by measuring released lactate dehydrogenase (LDH) levels upon cell lysis.
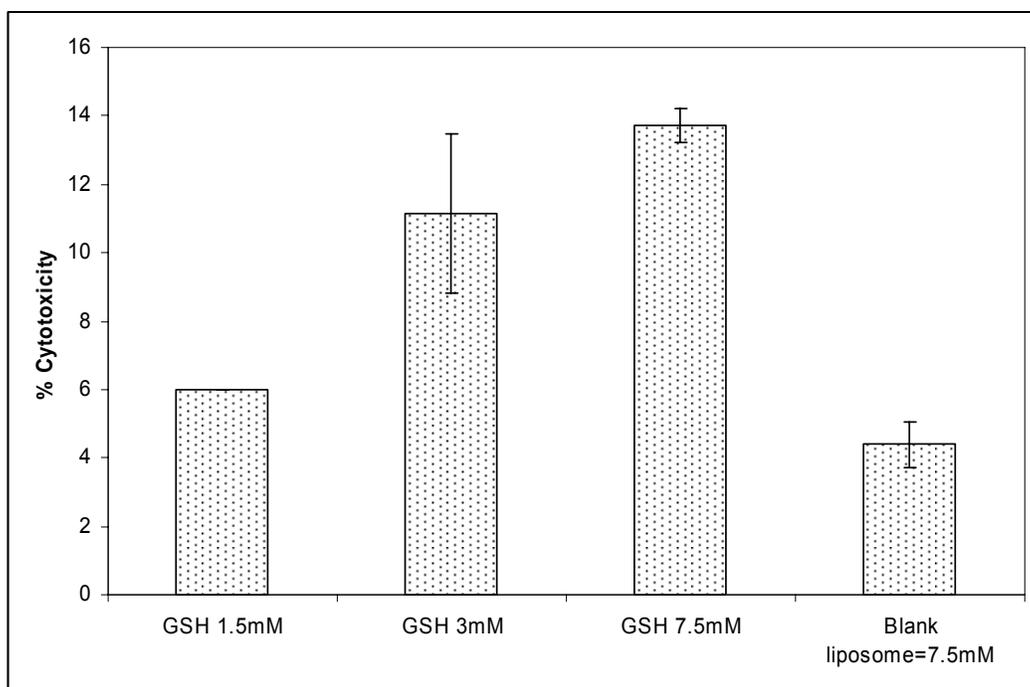
Figure 8. RAW 264.7 cells were treated with various concentration of GSH-liposome in DMEM supplemented with 5% FBS at 37^0C. Three hours after treatment, percent cell cytotoxicity was determined by CytoTox 96 Assay.

Figure 9. Cytokine level in human PBMC after LeTx treatment. LPS at 100ng/ml was used as a positive control. Human PBMC challenged with LeTx and the cytokine response was measured after 2 hours or 3 hours post challenge using BD CBA kit.

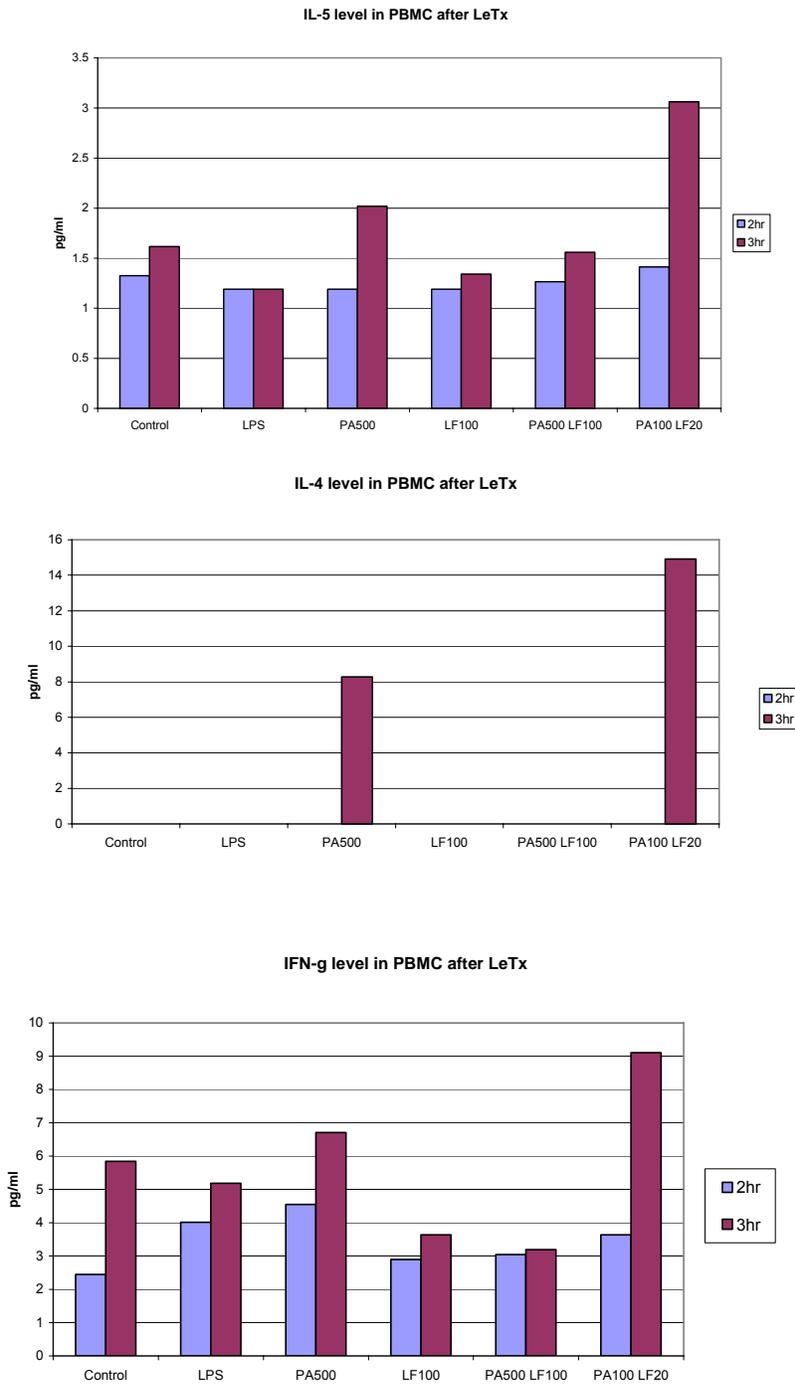| Sample	A 405nM	A405nM	A405nM	Average	Std dev	Hemoglobin conc (mg/dL)
Blank	0.036	0.034	0.037	**0.036**	0.002	
Calibrator	0.578	0.575	0.591	**0.581**	0.009	
PBS	0.208	0.189	0.223	**0.207**	0.017	31.338
PA 500ng/ml	0.165	0.191	0.21	**0.189**	0.023	28.039
PA 100ng/ml	0.207	0.22	0.223	**0.217**	0.009	33.170
LF 100ng/ml	0.191	0.23	0.218	**0.213**	0.020	32.498
LF 10ng/ml	0.214	0.22	0.206	**0.213**	0.007	32.560
PA 500ng/ml:LF100ng/ml	0.218	0.237	0.205	**0.220**	0.016	33.781
PA 100ng/ml:LF10ng/ml	0.164	0.198	0.206	**0.189**	0.022	28.161
Triton-X 1:10	1.434	1.338		**1.386**	0.068	2474.649

Table 1. Hemoglobin concentration (mg/dL) in plasma and supernatants. Controls were treated with PBS (as negative control) and Triton X (as positive control). Quantichrom hemoglobin assay was performed accordance with the manufacturer's instruction. Absorbency at 405nm was measured, and the hemoglobin concentration was calculated as (OD sample – OD blank)/(OD calibrator – OD Blank) x 100

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Harvard Medical School
Brigham & Womens Hospitals

Development of the APC Biosensor as a diagnostic tool for
infectious disease.

W81XWH-06-2-0044

Report is Pending at this time

Salil K. Das, Ph.D.
Meharry Institution

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Statement of Work

We have established in our laboratory that intratracheal exposure of 2-chloroethyl ethyl sulfide (CEES), a mustard gas analog to guinea pigs causes reactive oxygen species (ROS) mediated lung injury by stimulation of inflammatory cytokines, such as TNF-α. The purpose of the present investigation is to determine whether CEES-induced activation of inflammatory cytokines is followed by the activation of several key transcription factors (nuclear factor-κB [NF-κB], activation protein-1 [AP-1], serum accelerator factor [SAF], and CCAAT/enhancer binding protein [C/EBP]). These transcription factors induce expression of many target genes including, alpha 1-antitrypsin [α1-AT], matrix metalloproteinases [MMP], and tissue inhibitors of metalloproteinases [TIMPs]. Increased expression of these genes will lead to altered expression of proteins that may exacerbate the injury in lung or lung cells.

We intend to achieve this goal by conducting the following specific aims:

1. Analysis of the transcripts of target genes (alpha 1-antitrypsin [α1-AT], matrix metalloproteinase [MMP], and tissue inhibitors of metalloproteinases [TIMPs] in the lung by Northern blot or RT-PCR.
2. Analysis of the target proteins in the lung by immunohistochemistry.
3. Analysis of SAF-1, AP-1, NF-κB and C/EBP transcription factor in the lung by immunohistochemistry. Co-localization analysis of transcription factor and target genes.
4. Functional analysis of transcription factor activity in the tracheal epithelial cells by assessing promoter-binding activity.
5. Analysis of the signaling mechanisms (protein kinase A [PKA], protein kinase C [PKC], and mitogen activated protein kinase [MAPK]) involved in the activation of SAF-1 in the lung cells following CEES exposure.
6. Analysis of the effectiveness of the treatment by (a) delivery of a liposome containing an antioxidant, such as N-acetyl cysteine (NAC) either intravenously or subcutaneously, and (b) in combination therapy with a specific inhibitor or inhibitors of transcription factors in abolishing the induction of the transcription factors involved in lung injury.

Introduction

For several years we have been studying in a guinea pig model the molecular mechanisms by which CEES causes lung injury associated with ARDS. Our earlier publication indicated that CEES causes lung injury and significantly decreases expression and activity.of cholinephosphotransferase (CPT), the terminal enzyme in CDP-choline pathway for phosphatidylcholine synthesis (1). 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, NF-κB and ceramides in the lung (2). However, NF-κB disappeared after 2 hours indicating an intricate interplay of pro- and anti- apoptotic inflammatory cytokines. In subsequent studies, we utilized a state of the art cytokine array technology to identify other cytokines affected by CEES exposure (3). 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. We also showed that antioxidant liposomes were protective against acute lung injury induced by exposure to CEES (4-6). We have also observed that CEES exposure causes progressive lung fibrosis (5-7).

Body

1. Report on studies involving cytokines induced by mustard gas analog (2-chloroethyl ethyl sulfide, CEES)

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. 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 upregulation (>1.5x) of several growth factors (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.

2. Activation of Inflammatory cytokines induced by mustard gas analog (2-chloroethyl ethyl sulfide, CEES)

We reported earlier that guinea pigs exposed to 2-chloroethyl ethyl sulfide (CEES) as a mustard gas analog, accumulate inflammatory cytokines, including TNF-$\tilde{\alpha}$. Since angiogenesis may play a role, in inflammation and pathophysiology of tissue remodeling, we evaluated the early induction profile of angiogenic cytokines and selected target genes using cytokine antibody arrays. VEGF and bFGF levels showed 1.5-fold increase within 1 hr. of CEES exposure. However, while VEGF remained high after one day, bFGF level was decreased. Increase in VEGF receptors Flk-1 and Flt-1and MMP-2 were also detected by Western blot analysis in 1 day. Our results strongly suggest that angiogenic cytokines may play an essential role in inducing chronic inflammation following lung injury.

3. Effects of CEES exposure on the protein levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases [TIMPs]

In order to accomplish this goal, we induced lung injury in the guinea pig model in a dose and time dependent manner. Lungs were initially analyzed for changes in the protein levels of the above biochemical parameters by protein antibody array and Western blot analysis. Initial

results on Western blot analysis indicated an increase in the protein levels of two metalloproteinases: 1) MMP-2 [72 kd target protein (gelatinase A) for IFN-γinvolved in angiogenesis] and 2) MMP-9 [92 kd protein (gelatinase B)], in a dose (0.5 mg – 4 mg/kg of CEES) and time dependent manner (1 h – 14 d). Protein antibody array analysis indicated that while a short-term exposure (1 h) of CEES (0.5 mg/kg) causes an up-regulation of TIMP-2 (an anti-angiogenic cytokine), a longer exposure (24 h) causes its down-regulation. These studies are being continued for statistical validity.

4. Activation of mitogen activated protein kinases (MAPKs) signaling pathway in guinea pig lung in mustard gas induced lung

Adult guinea pigs were intratracheally injected single doses (0.5 mg/kg body weight) of 2-chloroethyl ethyl sulfide (CEES) in ethanol. Control animals were injected with ethanol in the same way. The animals were sacrificed at two time points, after 1 h and 30 days of CEES exposure and lungs were removed after perfusion with physiological saline. CEES exposure caused lung injury with evidence of fibrosis. The expression of members of the AP-1 family of transcription factors was investigated by Western blot analysis. The activation/phosphorylation of p38 protein was increased in both time points. This in turn increased the expression of c-fos, Fos-B and Fra-2 as well as Jun-D proteins. Since c-fos and c-jun dimerization is necessary to activate AP-1 transcription factors, it is expected that CEES-exposure will cause activation of AP-1 transcription factor. Thus, the overall pathway by which CEES-induced lung injury causes activation of AP-1 is as follows: Generation of reactive oxygen species - activation of TNF-alpha – activation of MAPKs - activation of AP-1.

5. Report on studies involving protection of CEES-induced lung injury by antioxidant liposomes

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. Those kinds of treatments, however, are not possible for lung injury and no prophylactic treatment has been available for pulmonary injury by mustards. Because of this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. The objective of this study was to develop antioxidant liposomes as antidotes for mustard gas induced lung injury. In vitro work with our cell models strongly supports the notion that antioxidant liposomes are effective in preventing cytotoxicity of 2-chloroethyl ethyl sulfide (CEES), a mustard gas analog. In a study with rats, we observed protection of CEES-induced lung injury by liposomes containing catalase, SOD or reducing agents, NAC, GSH or resveratrol. Recently, we obtained data on the attenuation of CEES-induced lung injury in guinea pigs by intratracheal delivery of antioxidant liposomes containing NAC, GSH and Vitamin E.

Five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5) were prepared differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), N-acetylcysteine (NAC) and GSH. A single dose (200 μl per animal) of each liposome was administered intratracheally after 5 min and 60 minutes of CEES exposure to guinea pigs and the animals were sacrificed after 2 h of CEES exposure. Lung injury was monitored by studying the leakage of ^{125}I-BSA into the lung after CEES exposure.

These antioxidant liposomes offered 9 to 75% protection against lung injury as evidenced by leakage of BSA from blood into the lung. The maximum protection was achieved with two liposomes, LIP-2 (71.5%) and LIP-4 (75.4%), when administered after 5 minutes of CEES

exposure. Delaying the administration of the liposomes after 1 h of CEES exposure decreased the efficacy. Both liposomes contained 55 mM phospholipid, 22 mM cholesterol, 0.6 mM phosphatidic acid, 11 mM α-tocopherol, 11 mM γ-tocopherol and 75 mM NAC. The only difference was that LIP-2 contained additionally 5 mM-δ-tocopherol. Our study clearly suggests that NAC can be used in combination with tocopherol as a liposome for effective antidote against CEES–induced lung injury.

These results were presented at the 5th SISPAT Symposium in Singapore, Nov 27-Dec 1, 2006 (7).

6. <u>Report on studies involving superoxide dismutase gene in lung injury induced by mustard gas analog (2-chloroethyl ethyl sulfide, CEES)</u>

Mustard gas exposure causes inflammatory lung diseases. Many inflammatory lung diseases are associated with oxidative stress. Reactive oxygen species (ROS) are involved in the maintenance of physiological functions. In tissues, it is therefore essential to maintain a steady-state level of antioxidant activity to allow both for the physiological functions of ROS to proceed and at the same time preventing tissue damage. We have recently reported that mustard gas exposure decreases the overall activity of superoxide dismutase (SOD). In the present study, we investigated the effects of mustard gas on each of the three isozymes [SOD-1 (Cu/Zn), SOD-2 (Mn) and SOD-3 (extracellular)]. Adult guinea pigs were intratracheally injected single doses of CEES (2mg/kg body weight) in ethanol. Control animals were injected with vehicle in the same way. The animals were sacrificed after 7 days and lungs were removed after perfusion with physiological saline. Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue.

Mustard gas exposure caused a significant increase in the activity of SOD-1 (35%). However, the SOD-3 activity which is the predominant type in lung was significantly decreased (62%), whereas, no change was observed in SOD-2 activity. Thus the decrease in the total activity of SOD was primarily due to the SOD-3 isozyme. Northern blot analysis indicated 3.5-fold increased expression of SOD-1 in mustard gas-exposed lung, but no significant change in the expression of SOD-2 and SOD-3 was observed. Mustard gas exposure did not cause mutation in the coding region of SOD-1 gene while causing modulation in expression levels. The protein levels of SOD-1, SOD-2 and SOD-3 were not altered significantly in the mustard gas exposed lung.

Our results indicate that the overall decrease in the activity of SOD by mustard gas exposure is probably mediated by direct inactivation of the SOD-3 gene or the enzyme itself. This decrease in the activity of SOD-3 may be due to the cleavage of active form of the protein to an inactive form. Existence of active and inactive forms of SOD-3 as a result of shifts in Cys-Cys disulfide bonding has been described in human, recently. Studies are underway in our lab to investigate whether mustard gas-induced inactivation of SOD-3 in lung is similarly mediated by a change in Cys-Cys disulfide bonding.

These results have been recently published (8).

7. <u>Report on studies involving pulmonary fibrosis induced by mustard gas analog (2-chloroethyl ethyl sulfide, CEES)</u>

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 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 within 7 days (5). Pulmonary fibrosis has also been demonstrated in rats by our collaborative studies with Dr. Peter Ward (6).

Key Research Accomplishment

See items 1-7, above and reprints 1- , included in the Appendix.

Reportable Outcomes
Presentations:
1   Das, S.K., Stone, W.L., Smith, M.G. and Mukherjee, S. Prophylactic Protection by Antioxidant Liposomes Against Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog, 5th International Symposium on Protection Against Toxic Substances, Nov 27-Dec 1, 2006, Singapore.
2   Invited Speaker for the Guinea Pig Genome Meeting, Broad Institute, MIT, December 13, 2006. Guinea Pig as a Model of Lung Injury.
3   Mukherjee S, Stone WL, Yang H, Smith M, and Das SK. Attenuation of Half Sulfur Mustard Gas-Induced Acute Lung Injury in Guinea Pigs by Antioxidant Liposomes., FASEB Meeting, April 28 – May 2, 2007, Washington, DC (FASEB J. 21: A261, 2007)
4   Advanced Medical Countermeasures Consortium Meeting, June 28, 2007, Crystal City, VA: Protection of Mustard Gas-Induced Lung Injury by Antioxidant Liposomes

Manuscripts (published during the last year of funding)

1.   McClintock, S.D., Hoesel L.M., Das, S. K., Till, G.O., Neff, T., Kunkel R.G., Smith M.G. and Ward, P.A. Attenuation of Half Sulfur Mustard Gas-Induced Acute Lung Injury in Rats. J. Applied Toxicology. 26: Issue 2, 126-131, 2006.
2.   Das SK, Stone WL, Smith M and Mukherjee S. Protection of Mustard Gas-Induced Lung Injury by Antioxidant Liposomes. Proc 5th International SISPAT Symposium on Toxic Substances, Singapore, Nov 27-Dec 2, p 10-15, 2006.
3.   Mukhopadhyay S, Rajaratnam V., Mukherjee S., Smith M and Das, S.K. Modulation of the Expression of Superoxide Dismutase Gene in Lung Injury by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog. J. Biochem Mol. Toxicol. 20: 142-149, 2006.
4.   Smith M.G., Stone W., Ren-Feng G., Ward, P. A., Suntress Z., Mukherjee, S. and Das, S. K. Vesicants and Oxidative Stress, In: "Chemical Welfare Agents Chemistry Pharmacology Toxicology, ed: J.A. Romano", Taylor & Francis Publisher, In Press.

Conclusion

Our over-all goal is to elucidate the molecular mechanisms by which mustard gas exposure causes inflammatory lung diseases. The on-going studies reveal that lung injury induced by

CEES, a mustard gas analog involves a signal transduction pathway including (a) generation of reactive oxygen species, (b) activation of inflammatory cytokines, such as TNF-α, (c) activation of MAPKs, and (d) activation of AP-1 transcription factors. Elucidation of the molecular mechanisms will help us in developing therapeutic strategies to counteract the mustard gas-induced lung injury.

<u>**References for Text**</u>

1. Sinha Roy, S; Mukherjee, S; Kabir, M; Rajaratnam, V; Smith, M and Das, S.K. Inhibition of Cholinephosphotransferase Activity in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog, J. Biochem. Molecular Toxicol. 19: 289-297, 2005.
2. Chatterjee, D; Mukherjee, S; Smith M, and Das, S.K. Signal Transduction Events in Lung Injury Induced by 2-Choloroethyl Ethyl Sulfide, a Mustard Analog. J. Biochem. Molecular Toxicol. 17: 114-121, 2003.
3. 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.
4. Das, S. K., Mukherjee, S., Smith, M and Chatterjee, D. Prophylactic Protection by N-Acetylcysteine Against the Pulmonary Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analogue, J. Biochem. Molecular Toxicol. 17: 177-184, 2003
5. 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.
6. McClintock, S.D., Hoesel L.M., Das, S. K., Till, G.O., Neff, T., Kunkel R.G., Smith M.G. and Ward, P.A. Attenuation of Half Sulfur Mustard Gas-Induced Acute Lung Injury in Rats. J. Applied Toxicology. 26: Issue 2, 126-131, 2006.
7. Das SK, Stone WL, Smith M and Mukherjee S. Protection of Mustard Gas-Induced Lung Injury by Antioxidant Liposomes. Proc 5th International SISPAT Symposium on Toxic Substances, Singapore, Nov 27-Dec 2, p 10-15, 2006.
8. Mukhopadhyay S, Rajaratnam V., Mukherjee S., Smith M and Das, S.K. Modulation of the Expression of Superoxide Dismutase Gene in Lung Injury by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog. J. Biochem Mol. Toxicol. 20: 142-149, 2006.

<u>**Appendices (available in PDF files)**</u>

9. Stone, Mukherjee, Smith and Das, Methods in Molecular Biology, Vol 199: Liposome Methods and Protocols (Eds: Basu and Basu), Humana Press, 145-161, 2002.
10. Chatterjee, Mukherjee, Smith and Das, J. Biochem. Molecular Toxicol. 17: 114-121, 2003.
11. Das, Mukherjee, Smith and Chatterjee, J. Biochem. Molecular Toxicol. 17: 177-184, 2003
12. Chatterjee, Mukherjee, Smith and Das, LIPIDS: Sphingolipid Metabolizing Enzymes (Eds: Haldar and Das), Research Signpost, 117-139, 2004.
13. Chatterjee, Mukherjee, Smith and Das, J. Biochem. Molecular Toxicol. 18: 150-153, 2004.

14. Sinha Roy, Mukherjee, Kabir, Rajaratnam, Smith and Das, J. Biochem. Molecular Toxicol. 19: 289-297, 2005.
15. Das, Chatterjee, Mukherjee, Grimes, Shen, Smith and Ghosh, Biochem. Biophys. Res. Commun. 339: 55-58, 2006
16. Mukhopadhyay, Rajaratnam, Mukherjee, Smith and Das, J. Biochem. Molecular Toxicol. 20: 142-149, 2006.
17. McClintock, Hosesel, Das, Till, Neff, Kunkel, Smith and Ward, J. Appl. Toxicol. 26: 126-131, 2006
18. Das, Stone, Milton and Mukherjee, Proc. 5th International SISPAT Symposium on Toxic Substances, Session 4, Medical Countermeasures, Singapore, 10-15, 2006.

PROTECTION OF MUSTARD GAS-INDUCED LUNG INJURY BY ANTIOXIDANT LIPOSOMES

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ABSTRACT

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. Those kinds of treatments, however, are not possible for lung injury and no prophylactic treatment has been available for pulmonary injury by mustards. Because of this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. The objective of this study was to develop antioxidant liposomes as antidotes for mustard gas induced lung injury. In vitro work with our cell models strongly supports the notion that antioxidant liposomes are effective in preventing cytotoxicity of 2-chloroethyl ethyl sulfide (CEES), a mustard gas analog. In a study with rats, we observed protection of CEES-induced lung injury by liposomes containing catalase, SOD or reducing agents, NAC, GSH or resveratrol. Recently, we obtained data on the attenuation of CEES-induced lung injury in guinea pigs by intratracheal delivery of antioxidant liposomes containing NAC, GSH and Vitamin E.

Five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5) were prepared differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), N-acetylcysteine (NAC) and GSH. A single dose (200 μl per animal) of each liposome was administered intratracheally after 5 min and 60 minutes of CEES exposure to guinea pigs and the animals were sacrificed after 2 h of CEES exposure. Lung injury was monitored by studying the leakage of ^{125}I-BSA into the lung after CEES exposure.

These antioxidant liposomes offered 9 to 75% protection against lung injury as evidenced by leakage of BSA from blood into the lung. The maximum protection was achieved with two liposomes, LIP-2 (71.5%) and LIP-4 (75.4%), when administered after 5 minutes of CEES exposure. Delaying the administration of the liposomes after 1 h of CEES exposure decreased the efficacy. Both liposomes contained 55 mM phospholipid, 22 mM cholesterol, 0.6 mM phosphatidic acid, 11 mM α-tocopherol, 11 mM γ-tocopherol and 75 mM NAC. The only difference was that LIP-2 contained additionally 5 mM-δ-tocopherol.

Our study clearly suggests that NAC can be used in combination with tocopherol as a liposome for effective antidote against CEES–induced lung injury. Work is under progress to develop devices to deliver this drug directly into the lungs even immediately after CEES exposure.

Key Words: Mustard gas, lung injury, liposomes, NAC, tocopherol

INTRODUCTION

Mustard gas, an alkylating agent, is an extensively used chemical warfare agent. Upon exposure, it is known to exert local actions on eyes, skin, and respiratory tissue followed by impairment of nervous, cardiac, and digestive systems in humans and laboratory animals [1-3]. Upper and lower respiratory tracts may be damaged acutely, because of hemorrhagic inflammation after inhalation of mustard gas. Subsequently, a variety of chronic pulmonary complications may develop, including acute respiratory distress syndrome (ARDS), chronic bronchitis, and pulmonary fibrosis [4-8].

Although the exact mechanism is not well understood, it is not unreasonable to postulate a causal role of oxidative stress in the pathology that follows exposure to mustard gas. Many inflammatory lung diseases, including ARDS, are associated with oxidative stress [9]. For example, inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), provoke the generation of reactive oxygen species (ROS), mediators of oxidative stress [10].

Recently, we reported that intratracheal exposure of a sulfur mustard analog, 2-chloroethyl ethyl sulfide (CEES), to guinea pigs causes accumulation of high levels of TNF-α in the lung [11]. TNF-α elevations result in activation of acid and neutral sphingomyelinases and production of excessive ceramides, a second messenger involved in programmed cell death (apoptosis) [11]. In addition, intratracheal exposure to CEES leads to an immediate but transient activation of NF-κB, which is regarded as an anti-apoptotic signaling molecule. The abrupt disappearance of NF-κB, however, resulted in activation of several caspases, leading to apoptosis [11].

Ultrastructural assessment of the lungs from guinea pigs exposed to a single low dose of CEES (0.5 mg/kg b.wt.) resulted in detection of neutrophilic alveolitis and varying degrees of interstitial fibrosis [8, 12]. The lung injury was characteristic of that caused by oxidative stress secondary to the inhibition of defense enzymes against oxygen injury, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase [11].

Recently we reported delivery of an antioxidant N-acetylcysteine (NAC) through drinking water to offer *prophylactic* protection against CEES-induced lung injury [12]. This protection is associated with (a) inhibition of CEES-induced activation of TNF-α, NF-κB, sphingomyelinases and caspases, (b) inhibition of CEES-induced accumulation of ceramides, and (c) protection of the oxygen defense system by counteracting CEES-induced inhibition of SOD, GSH-Px, and catalase activities [12]. We also observed protection against CEES-induced lung injury in rats by intratracheal administration of liposomes containing catalase, SOD or the reducing agents, NAC, GSH, or resveratrol [13].

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. Those kinds of treatments, however, are not possible for lung injury and no prophylactic treatment has been available for

pulmonary injury by mustards. Because of this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. Therefore, it is important that we develop a suitable antioxidant liposome therapy for the protection of injury induced by mustard gas exposure. The present study reports data on the attenuation of CEES-induced acute lung injury in guinea pigs by five antioxidant liposomes differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), N-acetylcysteine (NAC) and GSH.

CONCLUSION

Male guinea pigs (Hartley strain, 5-6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley Inc (Indianapolis, Indiana). Animals were infused intratracheally with single doses of CEES (2 mg/kg body weight) in ethanol (infusion volume was 100 μl/animal). Control animals were infused with 100 μl of ethanol in the same way. Liposomes were injected intratracheally in a volume of 200 μl per animal through the same catheter setup used for CEES instillation. Liposomes were given after 5 and 60 minutes of CEES exposure and sacrificed after 2 h.

Liposomes	Liposome Content (mM)							
	Phospholipid (PL90H)	Cholesterol	Phosphatidic Acid (PA)	Tocopherol (Vitamin E)			NAC	GSH
				α	γ	δ		
LIP-1 (Blank)	71	28	0.67	-	-	-	-	-
LIP- 2	55	22	0.6	11	11	5	75	-
LIP- 3	62	25	0.6	6	6	-	75	-
LIP- 4	55	22	0.6	11	11	-	75	-
LIP- 5	55	22	0.6	11	11	-	-	75

Antioxidant liposomes were prepared using a M-110L Laboratory Microfluidizer Processor (Microfluidics Headquarters, MA). Altogether, five different antioxidant liposome preparations were tested, as outlined in Table 1 which shows the composition and concentrations of the stock liposomal formulations.

TABLE 1. **Composition of Antioxidant Liposomes**

Lung injury was monitored by studying the leakage of ^{125}I-BSA into lung after CEES exposure [12]. Guinea pigs were injected into ear vein slowly with the ^{125}I-BSA solution (8 μCi/animal). CEES (2 mg/kg body weight) was infused into the animals intratracheally 3 h after ^{125}I-BSA injection. Control animals were infused intratracheally with 100 μl of solvent only. After 5 h of the injection of ^{125}I-BSA (i.e. 2 h after CEES infusion), the animals were sacrificed and 1 ml of blood was collected. The chest cavity was opened and lung was perfused with buffer to get rid of any residual blood from the animals individually. The perfused lung was taken out and after removal of heart and trachea; the radioactivity content of the lung was monitored in a gamma counter. Lung injury was expressed as permeability index that was obtained by dividing total radioactive counts in lung by counts in 1 ml of blood from the same animal.

The data presented in Table 2 indicate that all liposomes offered prophylactic protection; however the efficiency varied (9.2–75.4%) depending on their composition. Two liposomes offered maximum protection (LIP 2 – 71.5%, LIP-4 – 75.4%) when given within 5 minutes of CEES exposure. Liposome

2 contained 11 mM α-Vit E, 11 mM γ-Vit E, 5 mM δ-Vit E and 75 mM NAC. The only difference between LIP 2 and LIP 4 is that LIP 4 did not have any δ-VIT E. The protection was diminished if the liposome delivery was delayed for 1 h. Maximum protection was 43% if the liposome was given after

Treatment	Permeability Index	
	Liposomes injected 5 min after CEES Exposure	Liposomes injected 60 min after CEES Exposure
Control (No CEES)	0.20 ± 0.04	
Control (CEES only)	1.30 ± 0.22	
CEES + LIP-1	1.18 ± 0.03	1.11 ± 0.21
CEES + LIP-2	0.37 ± 0.03	0.74 ± 0.04
CEES + LIP-3	0.60 ± 0.02	0.80 ± 0.06
CEES + LIP-4	0.32 ± 0.01	0.76 ± 0.03
CEES + LIP-5	0.64 ± 0.05	0.83 ± 0.04

1 h of CEES exposure.

TABLE 2. Effects of Liposome Treatment on CEES-Induced Lung Injury in Guinea Pigs (Leakage of ^{125}I-BSA from Blood into the Lung).

CEES was infused (2 mg/kg body weight) intratracheally into guinea pigs with or without treatment with five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5). Liposomes were infused intratracheally either 5 or 60 minutes after CEES exposure. The lung injury was measured after 2h of CEES exposure and expressed as permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. ^{125}I-BSA was injected into the ear veins 3h prior CEES exposure. Each group had 3 animals. Values are mean ± SE.

The extensive leakage of albumin into the lung after intratracheal infusion of CEES indicates that the blood-gas barrier has been seriously compromised and that causes a functional impairment or destruction of both vascular endothelial and alveolar epithelial cells resulting in hypoxia. In the present study, we report data on the attenuation of CEES-induced acute lung injury in guinea pigs by five antioxidant liposomes differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), NAC and GSH. Empty liposome (LIP-1) infusion did not change the permeability index. LIP-2 and LIP 4 offered substantial attenuation of the massive leak of blood albumin into the lung (Table 2). Furthermore, a matter of considerable interest is that delayed delivery (for as long as 60 minutes) of liposomes into the lung after CEES infusion still provides substantial attenuation of lung injury. It is well known that liposomes delivered into the airways are phagocytized by macrophages and internalized [14]. Thus it can be implied from the current studies that liposomal delivery selectively enhances reducing environment in lung macrophages and thereby compromises CEES toxicity in these cells. This data indicates that addition of tocopherols causes more protection than NAC or GSH only.

Vitamin E is believed to be involved in a variety of physiological and biochemical functions. The molecular mechanism of these functions is believed to be mediated by either the antioxidant action of the vitamin or by its action as a membrane stabilizer and most potent lipid-soluble antioxidants in blood, breaking free-radical chain reactions of lipid peroxidation (15) Furthermore, the antioxidative roles of the different tocopherol isoforms are highly interdependent and may be complementary in function. There is some evidence suggesting that isoforms concentrations relative to each other may be important in preventing specific types of oxidative damage, with γ-tocopherol possibly being more important than α-tocopherol in removing nitrogen oxides and other electrophilic mutagens, whereas δ-tocopherol has stronger antiproliferative effect than α- and γ - tocopherols (16).

ACNOWLEDGMENT

REFERENCES

1. Papirmeister B., Fenster AJ, Robinson SI and Ford RD. Sulfur mustard injury: description of lesions and resulting incapacitations. In: Medical Defense Against Mustard Gas. Toxic Mechanisms and Pharmacological Implications, edited by A.J. Fenster. Boca Raton, FL; CRC, p.13-42, 1991
2. Dacre JC and Goldman M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard, Pharmacol. Rev., 48, 289-326, 1996.
3. Smith MG, Stone W, Crawford K, Ward P, Till GO and Das SK. A promising new treatment for mustard gas with the potential to substantially reduce the threat posed by chemical, biological and radiological agents. Jane's Chem-Bio Web, 1-5, 2003.
4. Worwser U. Toxicology of mustard gas. Trends Pharmacol Sci 12: 164-167, 1991.
5. Momeni AZ, Enshaelh S., Meghdadi SM and Amindjavaheri M. Skin manifestation of mustard gas. Clinical study of 535 patients exposed to mustard gas. Archs Dermatol 128: 775-780, 1992.
6. Calvet JH, Jarrean PH, . Levame M, D'ortho MP, Lorino H, Harf A and Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 76: 681-688, 1994.
7. Emad A and Rezaian GR. The diversity of the effects of sulfur mustard gas inhalation on respiratory system 10 years after a single, heavy exposure: Analysis of 197 cases. Chest 112: 734-738, 1997.
8. Mukherjee S. and Das SK. Pulmonary fibrosis in guinea pig by 2-chloroethyl ethyl sulfide. FASEB J. 19: A280, 2005.
9. Leff JA, Parsons PE, Day CE, Taniguchi N, Jochum M, Fritz H, Moore FA, Moore EE, McCord JM and Repine JE. Serum antioxidants as predictors of adult respiratory distress syndrome in patients with sepsis. Lancet 1003; 341: 777-780, 1993.
10. Lloyd SS, Cheng AK, Taylor FB and McCay JEG. Free radicals and septic shock in primates: the role of tumor necrosis factor. Free Rad Biol & Medicine 14: 233-242, 1993.
11. Chatterjee D, Mukherjee S, Smith MG and Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide (CEES), a mustard analog. J Biochem Mol Toxicol 17: 1-8, 2003.

12. Das SK, Mukherjee S, Smith MG and. Chatterjee D. Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. J Biochem Mol Toxicol 17: 177-184, 2003.

13. McClintock SD, Hoesel LM, Das SK, Till GO, Neff T, Kunkel RG, Smith MG and Ward PA. Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 26: Issue 2, 126-131, 2006.

14. Gonzalez-Rothi RJ, Straub L, Cacace JL and Schreier H Liposomes and pulmonary alveolar macrophages: functional and morphologic interactions. Exp Lung Res 17, 687-705, 1991.

15. Wang X and Quinn PJ Vitamin E and its function in membranes Prog Lipid Res 38:309-336 1999. Mayes PA Structure and function of the lipid-soluble vitamins. In: Murray RK, Granner DK, Mayes PA et al. Eds) Harper's Biochemistry. Stamford, CT: Appleton & Lange, 1996, 614-624.

16. Christen S, Woodall AA, Shigenaga MK et al. Gamma-tocopherol traps mutagenic electrophiles such as NO_2 and complements alpha-tocopherol: physiological implications. Proc Natl Acad Sci USA 94, 3217-3222, 1997.

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Modulation of the Expression of Superoxide Dismutase Gene in Lung Injury by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog

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ABSTRACT: Mustard gas exposure causes inflammatory lung diseases. Many inflammatory lung diseases are associated with oxidative stress. Reactive oxygen species (ROS) are involved in the maintenance of physiological functions. In tissues, it is therefore essential to maintain a steady-state level of antioxidant activity to allow both for the physiological functions of ROS to proceed and at the same time preventing tissue damage. We have recently reported that mustard gas exposure decreases the overall activity of superoxide dismutase (SOD). In the present study, we investigated the effects of mustard gas on each of the three isozymes: SOD-1 (Cu/Zn), SOD-2 (Mn), and SOD-3 (extracellular). Adult guinea pigs were intratracheally injected single doses of 2-chloroethyl ethyl sulfide (CEES) (2 mg/kg body weight) in ethanol. Control animals were injected with vehicle in the same way. The animals were sacrificed after 7 days, and lungs were removed after perfusion with physiological saline. Lung injury was established by measuring the leakage of iodinated-BSA into lung tissue. Mustard gas exposure caused a significant increase in the activity of SOD-1 (35%). However, the SOD-3 activity which is the predominant type in lung was significantly decreased (62%), whereas no change was observed in SOD-2 activity. Thus the decrease in the total activity of SOD was primarily due to the SOD-3 isozyme. Northern blot analysis indicated 3.5-fold increased expression of SOD-1 in mustard gas exposed lung, but no significant change in the expression of SOD-2 and SOD-3 was observed. Mustard gas exposure did not cause mutation in the coding region of SOD-1 gene while causing modulation in expression levels. The protein levels of SOD-1, SOD-2, and SOD-3 were not altered significantly in the mustard gas exposed lung. Our results indicate that the overall decrease in the activity of SOD by mustard gas exposure is probably mediated by direct inactivation of the SOD-3 gene or the enzyme itself. This decrease in the activity of SOD-3 may be due to the cleavage of active form of the protein to an inactive form. The existence of active and inactive forms of SOD-3 as a result of shifts in Cys–Cys disulfide bonding has been described in human, recently. Studies are underway in our laboratory to investigate whether mustard gas induced inactivation of SOD-3 in lung is similarly mediated by a change in Cys–Cys disulfide bonding. © 2006 Wiley Periodicals, Inc. J Biochem Mol Toxicol 20:1–, 2006; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20128

INTRODUCTION

Mustard gas is the most widely used chemical warfare vesicant [1–3]. It is a poisonous chemical agent that exerts a local action on eyes, skin, and respiratory tissue, followed by impairment of nervous, cardiac, and digestive systems in humans and laboratory animals [4–7]. Inhalation of mustard gas causes hemorrhagic inflammation to the tracheobronchial tree, with severe pulmonary complications, including adult respiratory distress syndrome (ARDS) [8]. 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 [9].

The basis for the tissue injuries caused by mustard gas remains unclear. Mustard gas belongs to a group

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of bifunctional alkylating agents and has electrophilic properties. It affects DNA synthesis and intracellular and membrane proteins, thus exerting its pathophysiological effects [10,11]. Several studies in rats and mice have shown that the mechanism of mustard gas action on lung, skin, or other organ includes DNA alkylation; cross-linking of DNA [12]; activation of proteases, resulting in proteolysis of several vital intracellular enzymes and structural proteins [13]; and production of free radicals and free radical-mediated oxidative stress [14,15]. Studies on thermal trauma in animal models and accumulation of clinical data implicate oxygen radicals as a causative agent in local response, shock and distant organ damage. Oxygen radicals may be produced by activated neutrophils after thermal trauma or as a result of tissue ATP loss [16]. It has been demonstrated that ATP degradation and the production of oxygen radicals are part of the initial response to thermal injuries [16,17]. In experimental animal models, protection from acute lung injury following skin burns has been achieved by depleting neutrophils, complement or by systemic treatment with a combination of superoxide dismutase (SOD) and catalase [18]. The protective effect of SOD and catalase provides strong evidence that oxygen free radicals released from activated neutrophils and macrophages are important mediators of lung injury, secondary to skin burns [17–21].

We have earlier reported that exposure to mustard gas significantly inhibits the activity of SOD, glutathione peroxidase, and catalase [22]. We postulated that oxygen-free radicals might be implicated as causing at least part of the mustard gas toxicity to internal organs and to the local tissue secondary injury. At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described. Two isoforms of SOD have Cu and Zn in their catalytic center and are localized to either intracellular cytoplasmic compartments (Cu-Zn-SOD or SOD-1) or to extracellular elements (EC-SOD or SOD-3) [23,24]. A third isoform of SODs has manganese (Mn) as a cofactor and has been localized to mitochondria of aerobic cells (Mn-SOD or SOD-2) [25]. On the basis of this information, we investigated in this study the effects of mustard gas on three isoforms of SOD in both molecular and functional levels.

MATERIALS AND METHODS

Chemicals

2-Chloroethyl ethyl sulfide (CEES, Cl-CH$_2$CH$_2$-S-CH$_2$CH$_3$), dimethylsulfoxide (DMSO), and cytochrome C were obtained from Sigma Chemicals (St. Louis, MO).

RNA isolation kit was obtained from Qiagen. One-step RT-PCR kit was obtained from Invitrogen, MD.

Animals and CEES Treatment

Male guinea pigs (Hartley strain, 5 to 6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals were infused intratracheally with single doses of CEES (2 mg/kg body weight) in ethanol (infusion volume was 100 μL/animal). Control animals were infused with 100 μL of ethanol in the same way. The animals were sacrificed after 7 days of CEES infusion, and lung was taken out for the experiment.

Subcellular Fractionation

After sacrifice, the lung was quickly excised, washed in ice-cold saline (0.9% NaCl), blotted dry, and weighed. The excised lung was then minced with scissors, and homogenized in four volumes of 0.25 M sucrose/1 mM EDTA (pH 7.4), in a Potter–Elvehjem homogenizer. One aliquot of whole homogenate was kept for SOD assay. The nuclear fraction was removed by centrifuging the whole homogenate at $600 \times g$ for 10 min in a refrigerated Sorvall RC-5 centrifuge using an SS-34 rotor. The supernatant was centrifuged at $10,000 \times g$ for 10 min to obtain the mitochondrial fraction. The supernatant was centrifuged in a Beckman L8-M ultracentrifuge at $105,000 \times g$ for 60 min using a 70.1 Ti rotor to obtain the cytosolic fractions.

Assay of SOD Activity

SOD activity was measured using alkaline dimethylsulfoxide as superoxide anion generating system as follows: Samples to be assayed (200 μL) were added to 1 mL of 0.20 M potassium phosphate buffer (pH 8.6) containing 10^{-4} M EDTA and 2×10^{-5} M cytochrome C. Tubes were kept in an ice bath for 20 min. Then, 0.5 mL alkaline DMSO (DMSO containing 1% water and 5 mM NaOH) was added with stirring. After the addition of alkaline DMSO, the final pH of the solution was usually between 9.5 and 9.6. Absorbance of reduced cytochrome C was determined at 550 nm against samples prepared under similar conditions except that DMSO did not contain NaOH. A unit of enzymatic activity is the amount of enzyme, which causes 50% inhibition of alkaline DMSO-mediated cytochrome C reduction [26]. Whole homogenate was used for total SOD activity, whereas mitochondria and cytosol were used for measurement of activity of SOD-2 and SOD-1,

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respectively. Data on SOD 3 activity were obtained by subtracting the values of combined SOD-1 and SOD-2 from total SOD activity.

Isolation of RNA

RNA was isolated from 100 mg tissue using Qiagen method (Qiagen, MD). Tissues were suspended in 2 mL of homogenization buffer (RLT) along with 20 μL of mercaptoethanol and homogenized for 1 min, and centrifuged for 10 min at 5000 rpm. Then equal volume of 70% ethanol was added to the supernatant and the mixture was vigorously shaken. The samples were applied to a midi column and centrifuged at 4500 rpm for 5 min and discarded the flow through. Then 4 mL of wash buffer (RWI) was added to the column, incubated for 5 min and then centrifuged, and the flow through was discarded. RNA was eluted in 100 μL of RNase free water. The concentration and the purity of the RNA were analyzed using a UV spectrophotometer.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR of SOD-1, SOD-2, SOD-3, and GAPDH was performed using 5 μg total RNA from each sample, using the one-step RT-PCR kit (Invitrogen, MD). The primers were synthesized to amplify cDNA fragments, based on the sequences from the GenBank. The primer sequences were as follows:

SOD1 (accession # X02317): sense, 5′ATGGCGAC GAAGGCCGTGTGCGTG-3′, antisense, 5′-AGGG AATGTTTATTGGGCGATCCC-3′
SOD2 (accession # M36693): sense,5′-ATGTTG (T/A)G(T/C)CGGGC(G/A)G(C/T)GTGC-3′, antisense, 5′-ACATTCTCCCAGTTGATTACATTC-3′
SOD3 (accession # NM_003102): sense, 5′-CATGCT (N)GCGCTACT(N)TGTT-3′, antisense, 5′-(N)GCG GC(Y)TTGCACTCGCTCT-3′
GAPDH (accession # U51572): sense, 5′GGTA TCGTGGAAGGACTCATGACC-3′, antisense, 5′ TCGCTGTTGAAGTCACAGGACACA-3′.

RT-PCR was performed in a thermal cycler (Biometra, Tgradient) as follows: 1 cycle for 30 min at 45°C, 1 cycle at 94°C for 2 min, 39 cycles for 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and 1 cycle for 10 min at 72°C. The RT-PCR products were electrophoresed on a 1% agarose gel, purified using QIAquick PCR purification kit (Qiagen, CA), and sequenced using BidDye-terminators kit (Applied Biosystems, CA). The sequences were analyzed using applied biosystems automated sequencer (ABI 3700 model, Meharry Molecular Core Facility).

Northern Blot Hybridization

Total RNA (30 μg) was loaded on a 1.6% agarose gel containing 20% formaldehyde and 1× MOPS. After 4–6 h of electrophoresis at 70 V, RNA was transferred overnight onto a Bright Star (Ambion) nylon membrane in 20× sodium chloride–sodium citrate (SSC) and UV cross-linked. Total RNA was DNAse treated to remove genomic DNA before reverse transcribed into cDNA for PCR amplification of the SOD (1,2,3) and GAPDH probes. The cDNA probes for each isoenzymes were prepared according to the method described previously and labeled with [^{32}P] ATP (3000 Ci/mmol) using DNA polymerase Nick translation kit (Invitrogen). Following a 3-h prehybridization at 42°C, the filters were hybridized overnight at 42°C with the ^{32}P-labeled SOD probe added in ultrahybridization buffer (Ambion). Blots were washed twice in 2× SSC/0.1% SDS for 30 min, once with 1× SSC/0.1%SDS at 42°C for 30 min, 0.5× SSC/0.1% SDS at 50°C for 30 min, and finally with 0.1× SSC/0.1% SDS at 55°C for 30 min, and exposed at −80°C to Kodak OMAT Xs film for 1 day to a week. Following autoradiography, blots were stripped and reprobed with ^{32}P-labeled guinea pig GAPDH cDNA probe to enable standardization between samples. Band intensities on films were analyzed using the alpha imager (Inotech).

Western Blot Analysis

Total lung homogenates were prepared according to the method described previously. Protein concentration of tissue homogenates was determined using the Lowry method [27], and 50 μg protein was resolved by 12% SDS-PAGE under reducing conditions. Proteins were transferred onto PVDF membranes (Millipore). Following incubation with SOD-1, SOD-2 (SantaCruz Biotechnology Inc., CA), and SOD-3 (Stressgen Biotechnologies, Canada), primary antibodies blots were washed and incubated with HRP-conjugated secondary antibody. Binding of antibodies to the blots was detected with an ECL-detection system (Perkin Elmer) following manufacturers' instructions. Stripped blots were reprobed with β-actin specific polyclonal antibodies to enable standardization of signals between samples. Band intensities were analyzed by densitometric scan for standardization and semiquantitation.

Statistical Analysis

Differences between control and mustard gas treated samples were assessed by student t-test, and the significance level was set for $p \leq 0.05$.
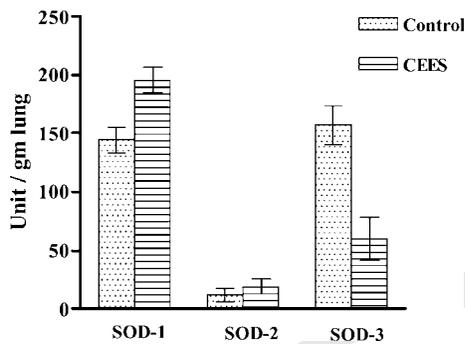
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FIGURE 1. Effects of CEES on superoxide dismutase activity of guinea pig lung. Animals were exposed to 2 mg/kg body weight CEES for 7 days. SOD activity was expressed as unit/gm lung, $p \leq 0.05$, $N = 5$.

RESULTS

Effects of CEES Exposure on Lung SOD Activity

Figure 1 shows the effect of CEES at a single dose (2 mg/kg body weight) on the activity of the three isoforms of SOD. According to this figure, activity of SOD-1 was increased by 35% ($p \leq 0.05$) in CEES-treated lung compared to the control. No significant changes in the activity of SOD-2 were observed in lung (Figure 1). A significant decrease (62%) in SOD-3 activity ($p \leq 0.05$) was observed after 7 days of CEES infusion (2 mg/kg body weight).

Effect of CEES Exposure on Lung SOD Gene Expression

RT-PCR from both control and CEES-treated lung yielded a product of 0.47 Kb cDNA of SOD-1, 0.6 Kb cDNA of SOD-2, and 0.4 Kb cDNA of SOD-3 (figure not shown). No mutation was observed in the nucleotide sequence of these cDNA fragments of SOD-1, SOD-2, and SOD-3 gene as a result of the CEES exposure. However, an upregulation (3.5-fold) in SOD-1 gene expression was evident from the northern blot analysis as shown in Figure 2. No significant change was observed for the SOD-2 and SOD-3 gene expression (Figures 3 and 4).
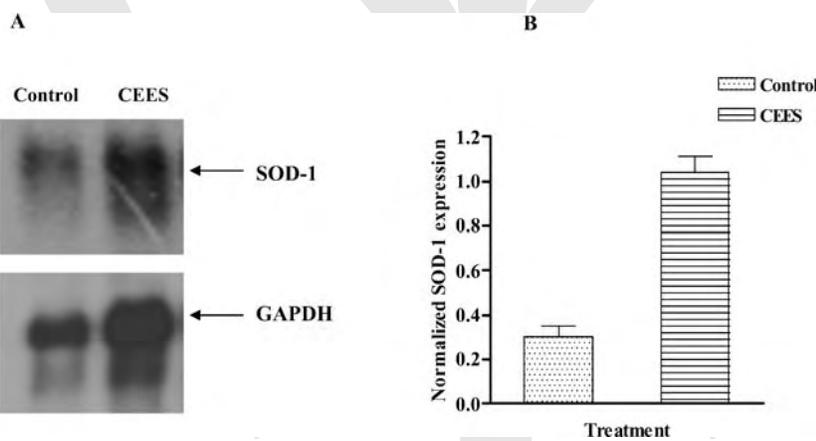

FIGURE 2. Effects of CEES on expression of SOD-1 gene in guinea pig lung. (A) One representative blot for SOD-1 mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (B) Histograms summarizing the northern blot analysis data for SOD-1, $p \leq 0.05$, $N = 3$.
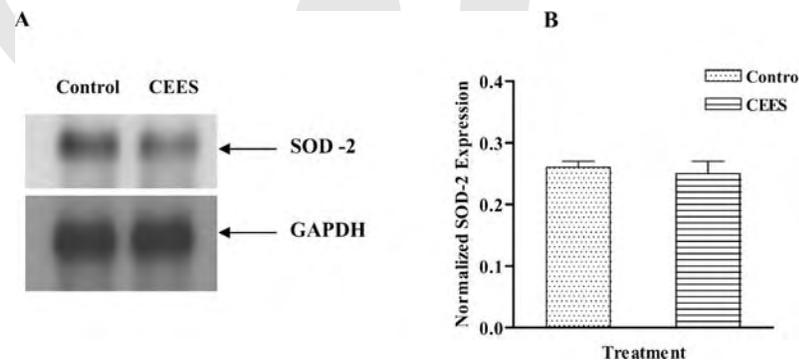

FIGURE 3. Effects of CEES on expression of SOD-2 gene in guinea pig lung. (A) One representative blot for SOD-2 mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (B) Histograms summarizing northern blot analysis data for SOD-2, $N = 3$.

FIGURE 4. Effects of CEES on expression of SOD-3 gene in guinea pig lung. (A) One representative blot for SOD-3 mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (B) Histograms summarizing northern blot analysis data for SOD-3, $N = 3$.
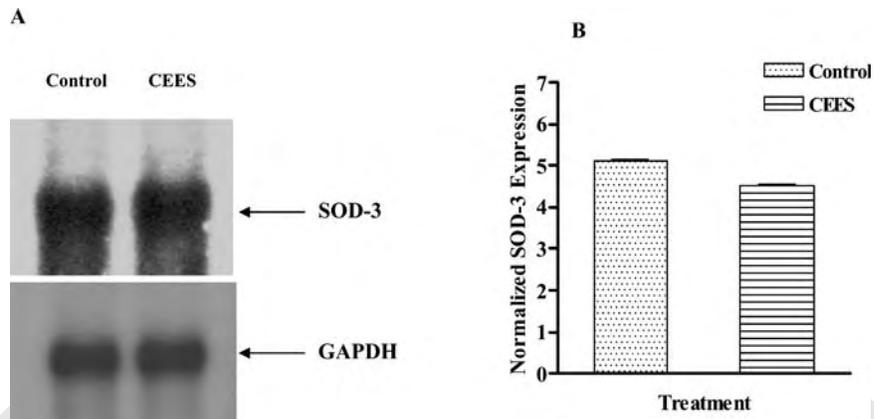
Effect of CEES Exposure on Lung SOD Protein Expression

To check the protein level, we determined the level of all the three isoforms of SOD by the western blot analysis. We found no significant difference in the level of SOD-1 and SOD-2 when treated with CEES (Figures 5 and 6). In the case of SOD-3, we got three bands (Figure 7A). In this case, SOD-3 was predominantly identified as a tetrameric 135 kDa band along with a 64-kDa dimer on the western blot. The blot also displayed a single monomeric band in the range of 30–35 kDa. No significant difference was observed in all the three bands when the lung was exposed

FIGURE 5. Effects of CEES on SOD-1 protein expression in guinea pig lung. (A) Western blot analysis of SOD-1 in control and CEES-induced guinea pig lung. (B) Histograms summarizing western blot analysis data for SOD-1, $N = 3$.
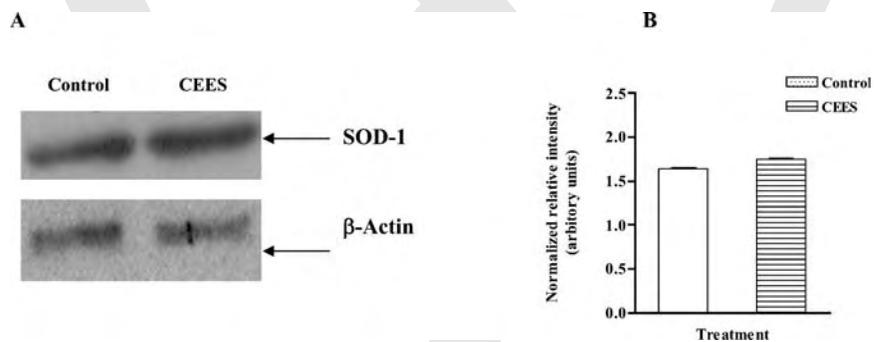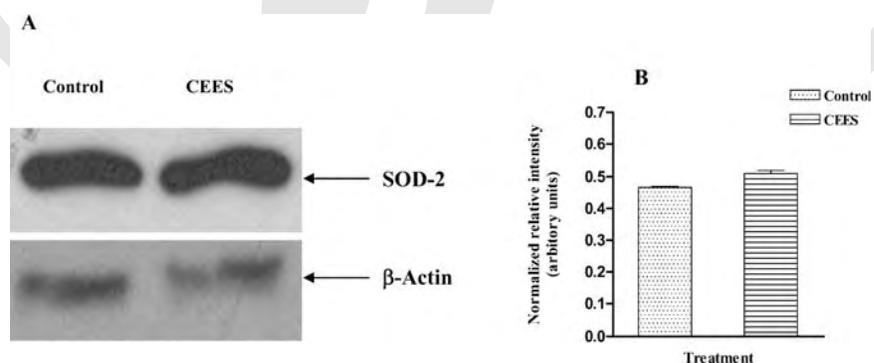

FIGURE 6. Effects of CEES on SOD-2 protein expression in guinea pig lung. (A) Western blot analysis of SOD-2 in control and CEES-induced guinea pig lung. (B) Histograms summarizing western blot analysis data for SOD-2, $N = 3$.
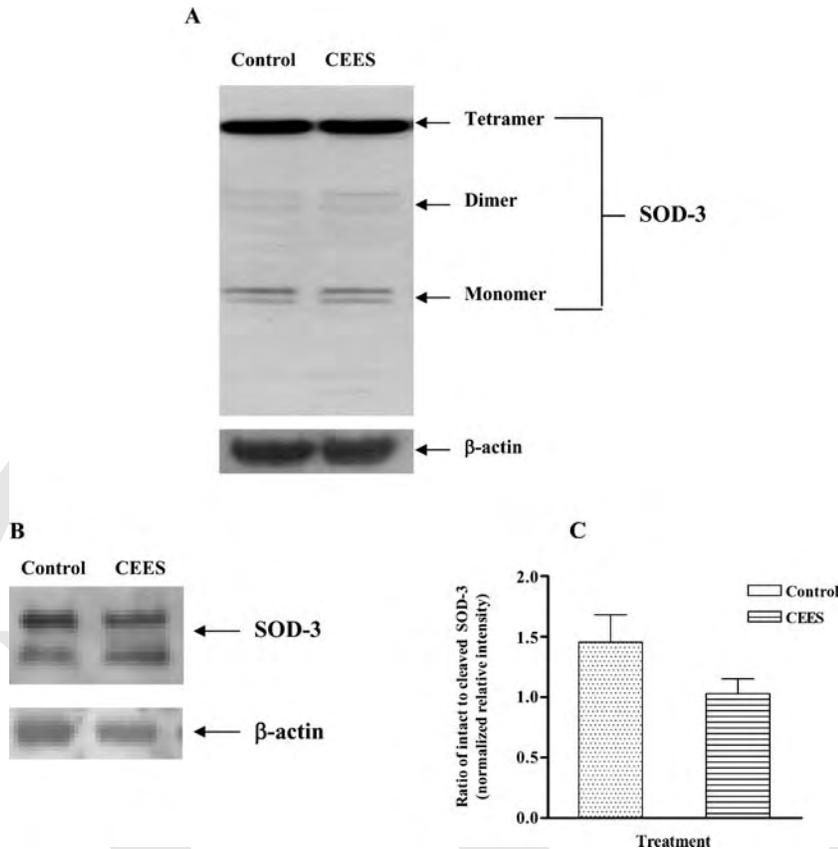
FIGURE 7. Effects of CEES on SOD-3 protein expression in guinea pig lung. (A) Tissue homogenates were electrophoresed on a 12% SDS-polyacrylamide gel electrophoresis following exposure (95°C for 5 min) to reducing agents 5% β-mercaptoethanol (BME) or (B) 100 mM dithiothreitol (DTT). (C) Histograms summarizing western blot analysis data for SOD-2, $p \leq 0.05$, $N = 3$. 17.

to CEES; however, when we treated the sample with dithiothreitol (dTT), we found a prominent monomeric doublet bands in the range of 30–35 kDa (Figure 7B). The doublet bands were separated; however, and the ratio of the top to bottom subunit band was significantly less in CEES-treated lung than control lung (Figures 7B and 7C), suggesting an increase in the intensity of the lower band and a decrease in the intensity of the upper band.

DISCUSSION

Reactive oxygen intermediates are thought to be primary mediators of neutrophils and macrophage-induced tissue injury, and they have been implicated in the pathogenesis of xenobiotic-induced lung toxicity [21]. Thus, following inhalation of irritants, pulmonary levels of reactive oxygen intermediates increase in the lung [27,28]. Moreover, antioxidants have been shown to abrogate tissue injury in several of these models. The present study focuses on analyzing the role of antiox-

idant enzyme in the mustard gas induced lung injury. In accordance with other studies [29,30], we found that SOD-3 isoform is predominant in the lung. The relatively high SOD-3 levels in lung likely relate to the dense airway and vascular network in the lung and the higher potential of extracellular inflammatory events resulting from direct exposure of the lung to the external environment. In contrast, SOD-1 activity is extensively higher in liver followed by SOD-2 and SOD-3 (data not shown).

We observed that exposure of mustard gas resulted in significant lung injury, as evidenced by a decrease in the activity of the predominant SOD (SOD-3) in the lung. But the mechanism of the damage caused by mustard gas at the cellular and molecular level is still not completely understood [11]. It is possible that reactive oxygen species generated by CEES exposure may cause the lung damage [31].

Though we found that the overall activity of SOD is decreased in the mustard gas exposed lung (Figure 1), we could not find any decrease in the gene or protein expression levels of any of the SOD isoforms (Figures 2–6). The decrease in the activity of total SOD

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by CEES is thus not regulated through gene or protein expression of any of the isoforms of SOD. The reason for such discrepancy between enzymatic activity and protein level is not clear, but one explanation may be that some of the SOD-3 protein which is localized to intracellular compartments may be inactive by cleavage [32].

SOD-3 is the only extracellular scavenger of the superoxide radical. SOD-3 is a tetramer of 135 kDa [22]. It contains two dimers with disulfide bonds linking the heparin-binding domain together [33,34]. The central region of SOD-3 (His 96 to Gly-193) is homologous to human Cu/Zn-SOD and contains all of the ligands essential for the coordination of the active site [35,36]. The N-terminal region of SOD-3 is important for the formation of tetramers (37–39), and the C-terminal region (Val-194 to Ala-222) encompasses a heparin-binding region, which is responsible for the immobilization of SOD-3 in extracellular matrix [40,41]. Recently, Peterson et al. [42] have shown that human SOD-3 subunit exists in two forms, each with a distinct disulfide bridge pattern. One form is enzymatically active, while the other displays no SOD activity. The active form contains a disulfide bridge homologous to Cu/Zn-SOD, thus supporting enzymatic activity [42,43]. By intracellular proteolytic processing and generation of active and inactive molecules, SOD-3 represents a flexible protein with the capacity to finetune the tissue localization and the antioxidant level in the extracellular space [44]. Our results suggest that the decrease in SOD-3 activity in response to CEES exposure is most likely through an inactivation mechanism. The inactivation may be due to conformational change in the SOD-3 isoform in direct response to CEES exposure in lung.

In an experimental animal model of thermal injury, it was shown that acute lung injury could be prevented by treatment with scavengers of hydroxyl radical such as DMSO [18]. In the same way, we hypothesize that administration of SOD (especially SOD3) before mustard gas exposure may have protective effects on lung injury.

REFERENCES

1. Warthin AS, Weller CV. The medical aspects of mustard gas poisoning. London: Henry Kimpton, 1919.
2. Willems JL. Clinical management of mustard gas casualties. Ann Med Mil Belg 1989;3:1–61.
3. Mellor SG, Rice P, Cooper GC. Vesicant burns. Br J Plast Surg 1991;44:434–437.
4. Momeni AZ, Enshaeih S, Meghadadi M, Amindjavaheri M. Skin manifestations of mustard gas: A clinical study of 535 patients exposed to mustard gas. Arch Dermatol 1992;128:775–780.
5. Debrowska MI, Becks LL, Lelli JL, Levee MG, Hinshaw DB. Sulfur mustard induces apoptosis and necrosis in endothelial cells. Toxicol Appl Pharmacol 1996;141:568–583.
6. Dacre JC, Goldman M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. Pharmacol Rev 1996;48:289–326.
7. Elsayed NM, Ta PN, Korte DW. Biochemical alterations in mouse liver induced by nitrogen mustard. Toxicologist 1989;9:26–34.
8. Elsayed NM, Omaye ST, Klain GJ, Inase JL, Dahlberg ET, Wheeler CR, Korte DW. Response of mouse brain to a single subcutaneous injection of the monofunctional sulfur mustard, butyl-2-chloroethyl sulfide (BCS). Toxocology 1989;58:11–20.
9. Calvet JH, Jarrean PH, Levame M, D'ortho MP, Lorino H, Harf A, Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 1994;76:681–688.
10. Yamakido M, Ishinka S, Hiyama K, Maeda A. Former poison gas workers and cancer: Incidence and inhibition of tumor formation by treatment with biological response modifier N-CWS. Environ Health Perspect 1996;104:485–488.
11. Gross CL, Meier HL, Papirmeister B, Brinkley FB, Johnson JB. Sulfur mustard lowers nicotinamide adenine dinucleotide concentrations in human skin grafted to nude mice. Toxicol Appl Pharmacol 1985;81:85–90.
12. Elsayed NM, Omaye ST, Klain GJ, Korte DW. Free radical mediated lung response to the monofunctional sulfur mustard, butyl-2-chloroethyl sulfide after subcutaneous injection. Toxicology 1992;72:153–165.
13. Cowman FM, Broomfield CA. Putative roles of inflammation in the dermatopathology of sulfur mustard. Cell Biol Toxicol 1993;9:201–213.
14. Husain K, Dube SN, Sugendran K, Singh R, DasGupta S, Somani SM. Effect of topically applied sulphur mustard on antioxidant enzymes in blood cells and body tissues of rats. J Appl Toxicol 1996;16:245–248.
15. Kopff M, Zakrzewska I, Strzelczyk M, Klem J, Dubiecki W. Superoxide dismutase and catalase activity in psoriatic patients treated topically with ointment containing 2-chloroethyl-3-chloropropyl sulfide. Pol J Pharmacol 1994;46:439–444.
16. Wooliscroft JO, Prasad JK, Thomson PD. Metabolic alterations in burn patients: Detection of adenosine triphosphate degradation products and lipid peroxides. Burns 1990;16:92–96.
17. Johnson KJ, Ward PA. Role of oxygen metabolites in immune complex injury of lung. J Immunol 1981;126:2365–2371.
18. Till GO, Beauchamp C, Menapace D, Tourtellotte W, Kunkel R, Johnson KJ, Ward PA. Oxygen radical-dependent lung injury following thermal injury of rat skin. Trauma 1983;23:269–277.
19. Till GO, Hatherill JR, Tourtellotte AW. Lipid peroxidation and acute lung injury after thermal trauma to skin. Am J Pathol 1985;119:376–384.
20. McCormick JR, Harkin MM, Johnson KJ. Suppression by superoxide dismutase of immune-complex induced pulmonary alveolitis and dermal inflammation. Am J Pathol 1981;102:55–64.
21. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell. Mol Physiol 2002;283:L246–L255.

Author Proof

22. Das SK, Mukherjee S, Smith MG, Chatterjee D. Prophylactic protection by *N*-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. J Biochem Mol Toxicol 2003;17: 177–184.

23. Marklund SL. Human copper-containing superoxide dismutase of high molecular weight, Proc Natl Acad Sci USA 1982;79:7634–7638.

24. Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. Proc Natl Acad Sci USA 1992;89:10405–10409.

25. Weisiger RA, Fridovich I. Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localisation. J Biol Chem 1973;248:4793–4796.

26. Hyland K, Voisin E, Banoun H, Auclair C. Superoxide dismutase assay using alkaline dimethylsulfoxide as superoxide anion-generating system. Anal Biochem 1983;135:280–287.

27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:1772–1775.

28. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol 1995;35:655–677.

29. Marklund SL. Extracellular superoxide dismutase in human tissues and human cell lines. J Clin Invest 1984;74:1398–1403.

30. Nakao C, Ookawara T, Kizaki T, Ishi S, Miyazaki H, Haga S, Sato Y, Ji LL, Ohno H. Effects of swimming training on three superoxide dismutase isoenzymes in mouse tissues. J Appl Physiol 2000;88:649–654.

31. Kinnula VL, Crapo JD, Raivio KO. Generation and disposal of reactive oxygen metabolites in the lung. Lab Invest 1995;19:3–19.

32. Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 2002;33:337–349.

33. Enghild JJ, Thogersen IB, Oury TD, Valnickova Z, Hojrup P, Crapo JD. The heparin-binding domain of extracellular superoxide dismutase is proteolytically processed intracellularly during biosynthesis. J Biol Chem 1999;274:14818–14822.

34. Oury TD, Crapo JD, Valnickova Z, Enghild JJ. Human extracellular superoxide dismutase is a tetramer composed of two disulphide-linked dimmers: A simplified, high-yield purification of extracellular superoxide dismutase. Biochem J 1996;317:51–57.

35. Tainer JA, Getzoff ED, Beem KM, Richardson JS, Richardson DC. Determination and analysis of the 2 A-structure of copper, zinc superoxide dismutase. J Mol Biol 1982;160:181–217.

36. Hjalmarsson K, Marklund SL, Engstrom A, Edlund T. Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. Proc Natl Acad Sci USA 1987;84:6340–6344.

37. Carlsson LM, Marklund SL, Edlund T. The rat extracellular superoxide dismutase dimer is converted to a tetramer by the exchange of a single amino acid. Proc Natl Acad Sci USA 1996;93:5219–5222.

38. Tibell LA, Skarfstad E, Jonsson BH. Determination of the structural role of the N-terminal domain of human extracellular superoxide dismutase by use of protein fusions. Biochim Biophys Acta 1996;1292:47–52.

39. Stenlund P, Andersson D, Tibell LA. Subunit interaction in extracellular superoxide dismutase: Effects of mutations in the N-terminal domain. Protein Sci 1997;6:2350–2358.

40. Adachi T, Kodera T, Ohta H, Hayashi K, Hirano K. The heparin binding site of human extracellular-superoxide dismutase. Arch Biochem Biophys 1992;297:155–161.

41. Sandstrom J, Carlsson L, Marklund SL, Edlund T. The heparin-binding domain of extracellular superoxide dismutase C and formation of variants with reduced heparin affinity. J Biol Chem 1992;267:18205–18209.

42. Petersen SV, Oury TD, Valnickova Z, Thogersen IB, Hojrup P, Crapo JD, Enghild JJ. The dual nature of human extracellular superoxide dismutase: One sequence and two structures. Proc Natl Acad Sci USA 2003;100:13875–13880.

43. Petersen SV, Due AV, Valnickova Z, Oury TD, Crapo JD, Enghild JJ. The structure of rabbit extracellular superoxide dismutase differs from the human protein. Biochemistry 2004;43:14275–14281.

44. Petersen SV, Enghild JJ. Extracellular superoxide dismutase: Structural and functional considerations of a protein shaped by two different disulfide bridge patterns. Biomed Pharmacother 2005;59:175–182.

Decrease in brain POMC mRNA expression and onset of obesity in guinea pigs exposed to 2-chloroethyl ethyl sulfide, a mustard analogue

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Abstract

The full spectrum of physiological effects resulting from exposure to sulfur mustard and its analogs is currently unknown. In a guinea pig model, initially selected to study the role of an inflammatory cytokine cascade in mustard gas induced lung injury, we observed significant body weight gain in guinea pigs exposed to an intratracheally injected single dose of 2-chloroethyl ethyl sulfide, a mustard analog. The body weight gain was not associated with any apparent change in appetite. To further elucidate a molecular basis for the observed weight gain, we evaluated candidate genes for the obese phenotype by quantitative RT-PCR. We observed a time- and dose-dependent decrease in guinea pig pro-opiomelanocortin (POMC) message following treatment with mustard gas. This reduction in POMC message is consistent with the onset of obesity in the animals. We hypothesize that the POMC melanocortin pathway provides a mechanistic basis for the observed effects of sulfur mustard on body weight.
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Keywords: 2-Chloroethyl ethyl sulfide; Obesity; POMC; Guinea pig

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 to 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 remain 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]; and inflammation [15]. We have recently developed the guinea pig model to study the role of inflammatory cytokine cascade in mustard gas induced

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lung injury [16,17]. In these studies, we have observed activation of NF-κB and tumor necrosis factor (TNF-α), and accumulation of ceramides, a second messenger involved in cell apoptosis. Surprisingly, during the course of these investigations, we observed significant body weight gain in guinea pigs exposed to intratracheally injected single dose of CEES. This was interesting in view of the fact that there was no difference in food intake between control and experimental animals. To understand the phenotype better, we decided to monitor the status of some of the obesity-associated genes in guinea pig tissues in response to mustard gas exposure. To our knowledge, only three obesity genes [pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and lipoprotein lipase (LPL)] have been characterized in guinea pigs, whereas at least 25 genes have been characterized in humans. Deficiency of POMC and peripheral melanocortins has been etiologically implicated in the initiation of obesity in mouse [18,19]. The objective of the present study was to determine whether the mustard gas-induced obesity in guinea pigs is associated with down-regulation of POMC in brain. Insights from these experiments will increase our understanding of the physiological effects of sulfur mustards and might unravel new mechanisms for the induction of obesity.

Materials and methods

Chemicals. 2-Chloroethyl ethyl sulfide [Cl-CH$_2$CH$_2$-S-CH$_2$CH$_3$, CEES] was obtained from Sigma Chemicals (St. Louis, MO).

Animals and CEES treatment. Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight) were obtained from Harlan Sprague–Dawley (Indianapolis, Indiana). Animals were injected intratracheally single doses of CEES (0.5–6.0 mg/kg body weight) in ethanol (total injection volume was 100 μl per animal). Control animals were injected with 100 μl ethanol in the same way intratracheally. The animals were sacrificed at different time intervals (1, 4, and 6 h, 1 day, 7 day, 14 day, and 21 day) after CEES injection. Excepting for timepoints up to 6 h, the animals were sacrificed at the same time each day to offset gene changes induced by diurnal variations. Body weight gain was recorded every week. The tissues were flash-frozen in liquid N$_2$ and kept at −70 °C for future use.

Preparation of RNA. Total RNA was isolated from frozen brain slices of male guinea pigs treated with varying doses of CEES (0.5–6.0 mg/kg body weight) for 7, 14 or 21 days. Untreated male animals were used as controls. Total RNA was isolated from guinea pig brain using TRI-Reagent supplemented with 1 μl/ml polyacryl carrier, according to the manufacturer's recommended protocol (Molecular Research Center, Cincinnati, OH, USA). Briefly, 50 mg tissue/ml of TRI-Reagent was homogenized for 45 s and then incubated at room temperature for 5 min. Following elution of RNA, the A_{260}/A_{280} ratio of the samples was determined by spectrophotometry. RNA integrity was confirmed by electrophoretic traces on a BioAnalyzer instrument (Agilent Technologies, Wilmington DE).

Preparation of cDNA templates. All RNA samples were DNased using the DNA-free kit (Ambion, Austin TX) according to protocol. The samples were then quantitated by RiboGreen (Molecular Probes). Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) gene expression was analyzed using real-time PCR [PE Applied Biosystems (ABI), Foster City, CA, USA] in the absence of reverse transcriptase to ensure the samples were free of genomic DNA. The samples were then converted to cDNA using the High Capacity cDNA Archive Kit according to manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Samples were diluted to a final concentration of 5 ng/μl of cDNA.

Analysis of mRNA gene expression. Guinea pig oligonucleotides were designed for use in real-time PCR using a modified version of Primer 3 (MIT, Cambridge, MA) and synthesized according to the protocols of Keystone labs (BioSource International, Camarillo, CA) (forward: 5′-CCTTGCTGCTTCAGATCTCCA-3′; reverse: 5′-AGGCACTCCAG-CAGGTGTCT-3′, encompassing nucleotides 44–140 of guinea pig POMC mRNA, GenBank Accession No. S78260). The synthesized oligonucleotides were diluted in 10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0 buffer at a mix stock concentration of 3 μM each of forward and reverse primers. Pro-opiomelanocortin gene was assayed using real-time PCR, the SYBR Green 1 Double-stranded Binding Dye assay, and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Ten microliter of a mix containing final concentrations of 1× PCR SYBR master mix, 300 nM primer mix and water, was added to a well of a 384-well optical plate containing 12.5 ng of template (5 ng/μl). The PCR cycling conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Dissociation curve analysis was performed for all experimental samples and the resulting melting temperature was generated to confirm the specificity of the product.

Statistical analysis. Data for biochemical and physiological measurements were analyzed by ANOVA and the significance level was set for $p < 0.05$. Gene expression results are normalized to expression of GAPDH gene in each sample. A non-parametric, Wilcoxon rank-sum test was used to evaluate the changes in POMC gene expression in control and CEES treated brains.

Results

Effects of CEES exposure on body weight gain

There was a gradual weight gain in guinea pigs exposed to varying doses of mustard gas. As shown in Fig. 1, body weight gain was significantly higher ($p < 0.05$) in the CEES exposed group at every time period tested (7, 14, and 21 days post-treatment at 0.5 mg/kg dose), as assessed by a two-tailed t test. No significant difference in feeding behav-
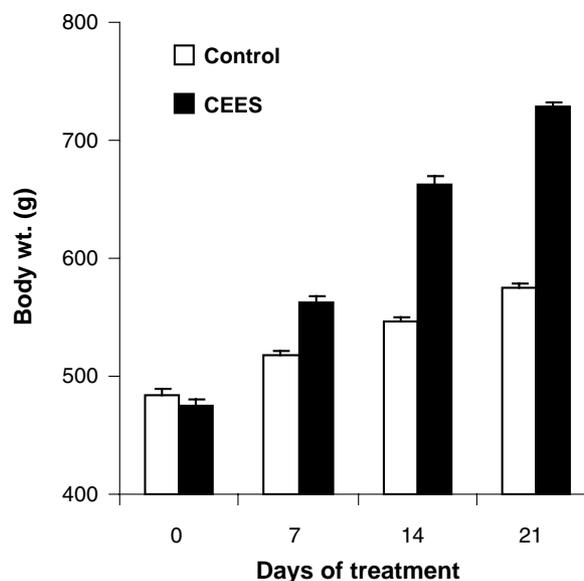

Fig. 1. Effect of CEES exposure on body weight gain in guinea pigs. CEES was injected at a dose of 0.5 mg/kg body weight intratracheally. Values reported are means ± SEM of six animals. Body weight gain was significantly higher ($p < 0.05$) in experimental group at each time periods (7, 14, and 21 days post-treatment), assessed by a two-tailed t test.

149 ior was noted among the control and treated groups, sug-
150 gesting that the weight gain was not related to food intake.

151 *Effects of CEES exposure on POMC gene expression*

152   Using real-time PCR, we investigated the levels of
153 POMC gene expression in the brains of guinea pigs that
154 had been exposed to mustard gas at various concentrations
155 for various periods of time. The results from three indepen-
156 dent studies are summarized in Figs. 2A–B. As shown in
157 Fig. 2A, exposure of guinea pigs for 24 h with 1 mg/kg
158 CEES resulted in a statistically significant reduction in
159 POMC RNA levels in the brain (POMC message was
160 unchanged in lung and liver tissues; also message levels
161 for neuropeptide Y and lipoprotein lipase genes were

162 unchanged in brain, lung, and liver tissues, data not shown)
163 as estimated by Wilcoxon rank-sum test ($p < 0.03$) or two-
164 tailed t test ($p < 0.06$). The reduction in POMC message did
165 not occur until after 6 h of exposure to CEES and near
166 maximal levels of reduction were achieved by 24 h of treat-
167 ment when compared to 7 and 14 days of CEES exposure
168 (Fig. 2B). Similar reduction in POMC gene expression was
169 also observed for higher doses of CEES (data not shown).

Discussion   170

171   Pro-opiomelanocortin (POMC) is a prohormone yield-
172 ing bioactive peptides that are generated in the hypotha-
173 lamic neurons and act as endogenous ligands for the
174 melanocortin-4 receptor, a key molecule involved in appe-
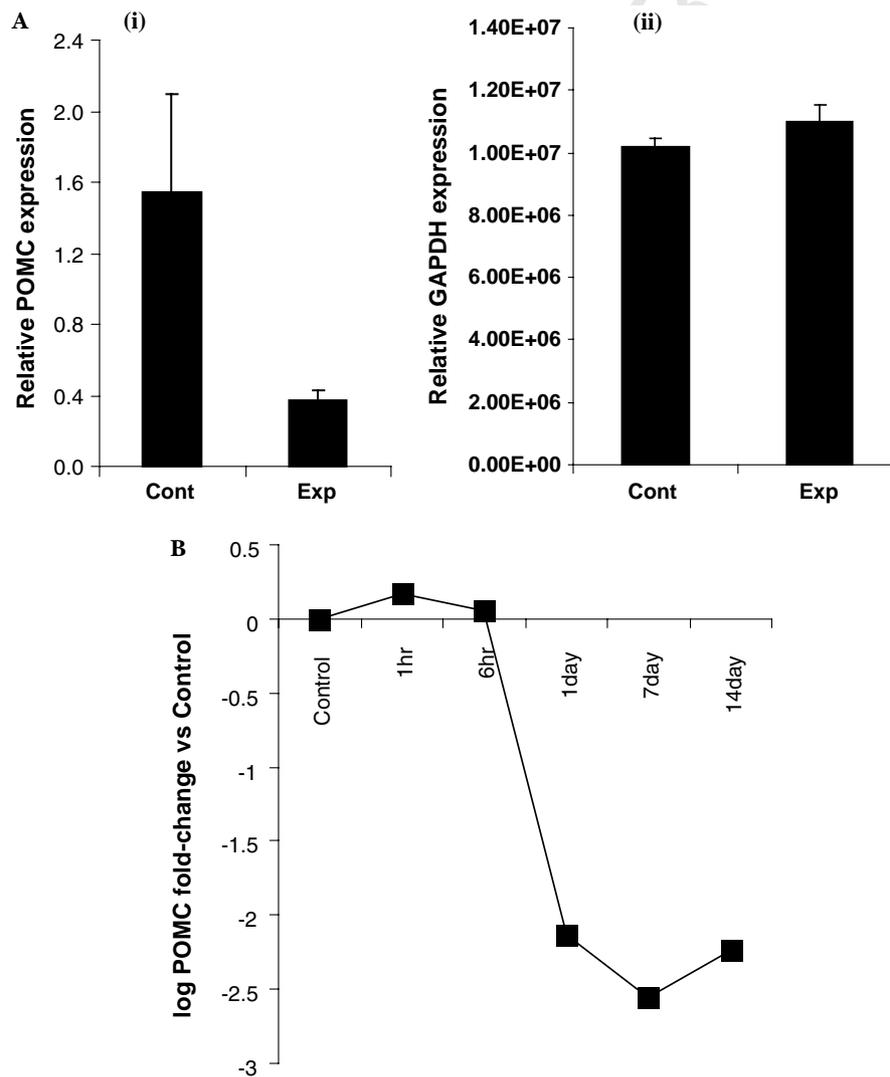
Fig. 2. Effect of CEES exposure on POMC RNA levels measured by quantitative RT-PCR. (A) Relative expression of (i) POMC and (ii) GAPDH in control and CEES treated guinea pig brains (1 mg/kg CEES for 1 day). POMC message levels were normalized to the expression of GAPDH in the same sample. Since POMC message levels were very low compared to GAPDH message levels, an arbitrary multiplier of 10,000 was applied to the normalized POMC values to arrive at the 'Relative POMC Expression' plotted on the y-axis. Mean expression values for the two groups are plotted, and the SEM for each group is shown ($n = 4$ in each group). (B) Time course of POMC expression in guinea pigs treated with 0.5 mg/kg CEES. POMC expression values were obtained by the method described in (A). The fold-change in POMC message levels compared to control are plotted for every time point (values expressed in the $\log_{10}$ scale).

tite control and energy homeostasis. Several lines of evidence establish a role of POMC in the development of obesity. Although rare, inactivating mutations in POMC in severely obese patients have been found [20] and the phenotype displayed by POMC null mice is remarkably similar to that seen in human mutant POMC subjects [21]. POMC also serves as a quantitative trait loci in obesity and correlates, in part, with variation in serum leptin levels [22]. POMC messenger RNA levels are decreased in leptin deficient (ob/ob) and leptin receptor deficient (db/db) mice compared with controls [23]. Thus, the inactivation or reduction in POMC levels appears to be positively associated with the development of obesity. In this study, we report the unexpected finding relating a gradual weight gain in guinea pigs exposed to mustard gas. The biochemical mechanism for the observed weight gain appears to involve POMC since POMC levels decrease as the animals gain weight. Thus, the sulfur mustard, CEES, appears to act as a signaling trigger for the reduction in POMC. The exact mechanism of how CEES induces a decline in POMC message presently is not clear. One could hypothesize that CEES acts as a mutagen and reproducibly induces inactivating mutations in the POMC gene; alternatively, CEES may act as a cell signaling trigger that results in a decrease in POMC transcription in the guinea pig model. The magnitude of the decrease in POMC message varied considerably (approximately 3- to 100-fold over control) but consistently among independent experiments. It is also interesting to note that although POMC is traditionally related to feeding behavior, we observed no noticeable change in feeding in the animals despite decrease in their POMC transcripts. This is suggestive of additional functions of POMC in the control of body weight.

Acknowledgments

References

[1] M.I. Debrowska, L.L. Becks, J.L. Lelli, M.G. Levee, D.B. Hinshaw, Sulfur mustard induces apoptosis and necrosis in endothelial cells, Toxicol. Appl. Pharmacol. 141 (1996) 568–583.

[2] J.C. Dacre, M. Goldman, Toxicology and pharmacology of the chemical warfare agent sulfur mustard, Pharmacol. Rev. 48 (1996) 289–326.

[3] N.M. Elsayed, P.N. Ta, D.W. Korte, Biochemical alterations in mouse liver induced by nitrogen mustard, Toxicologist 9 (1989) 26–34.

[4] N.M. Elsayed, S.T. Omaye, G.J. Klain, J.L. Inase, E.T. Dahlberg, C.R. Wheeler, D.W. Korte, Response of mouse brain to a single subcutaneous injection of the monofunctional sulfur mustard, butyl-2-chloroethyl sulfide (BCS), Toxicology 58 (1989) 11–20.

[5] J.H. Calvet, P.H. Jarrean, M. Levame, M.P. D'ortho, H. Lorino, A. Harf, I.M. Mavier, Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs, J. Appl. Physiol. 76 (1994) 681–688.

[6] A.Z. Momeni, S. Enshaelh, M. Meghdadi, M. Amindjavaheri, Skin manifestation of mustard gas. Clinical study of 535 patients exposed to mustard gas, Arch. Dermatol. 128 (1992) 775–780.

[7] M. Dixon, D.M. Needham, Biochemical research on chemical warfare agents, Nature 158 (1946) 432–433.

[8] M. Yamakido, S. Ishinka, K. Hiyama, A. Maeda, Former poison gas workers and cancer: incidence and inhibition of tumor formation by treatment with biological response modifier N-CWS, Environ. Health Perspect. 104 (Suppl.) (1996) 485–488.

[9] S. Adachi, K. Takemoto, Occupational lung cancer. A comparison between humans and experimental animals, Sangyo Igaku 29 (1987) 345–357.

[10] L.B. Sasser, J.A. Cushing, J.C. Dacre, Two-generation reproduction study of sulfur mustard in rats, Reprod. Toxicol. 10 (1996) 311–319.

[11] N.M. Elsayed, S.T. Omaye, G.J. Klain, D.W. Korte, Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection, Toxicology 72 (1992) 153–165.

[12] F.M. Cowan, C.A. Broomfield, Putative roles of inflammation in the dermato-pathology of sulfur mustard, Cell Biol. Toxicol. 9 (1993) 201–213.

[13] K. Husain, S.N. Dube, K. Sugendran, R. Singh, S. Das Gupta, S.M. Somani, Effect of topically applied sulphur mustard on antioxidant enzymes in blood cells and body tissues of rats, J. Appl. Toxicol. 16 (1996) 245–248.

[14] M. Kopff, I. Zakrzewska, M. Strzelczyk, J. Klem, W. Dubiecki, Superoxide dismutase and catalase activity in psoriatic patients treated topically with ointment containing 2-chloroethyl-3-chloro-propyl sulfide, Pol. J. Pharmacol. 46 (1994) 439–444.

[15] D.B. Kuhns, E. Decarlo, D.M. Hawk, J.I. Gallin, Dynamics of the cellular and humoral components of the inflammatory response elicited in skin blisters in humans, J. Clin. Invest. 89 (1992) 1734–1740.

[16] D. Chatterjee, S. Mukherjee, M.G. Smith, S.K. Das, Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog, J. Biochem. Mol. Toxicol. 17 (2003) 114–121.

[17] S.K. Das, S. Mukherjee, M.G. Smith, D. Chatterjee, Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue, J. Biochem. Mol. Toxicol. 17 (2003) 177–184.

[18] M.B. Zemel et al., Pro-opiomelanocortin (POMC) deficiency and peripheral melanocortins in obesity, Nutr. Rev. 58 (6) (2000) 177–180.

[19] S. Lin, L.H. Storlien, X.F. Huang, Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain, Brain Res. 875 (1–2) (2000) 89–95.

[20] H. Krude, H. Biebermann, W. Luck, R. Horn, G. Brabant, A. Gruters, Sever early-onset obesity, adrenal insufficiency, and red hair pigmentation caused by POMC mutations in humans, Nat. Genet. 20 (1998) 111–112.

[21] L. Yaswen, N. Diehl, M.B. Brennan, U. Hochgeschwender, Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin, Nat. Med. 5 (1999) 1066–1070.

[22] J.E. Hixson, L. Almasy, S. Cole, S. Birnbaum, B.D. Mitchell, M.C. Mahaney, M.P. Stern, J.W. MacCluer, J. Blangero, A.G. Comuzzie, Normal variation in leptin levels associated with polymorphisms in the proopiomelanocortin gene, POMC J. Clin. Endocrinol. Metab. 84 (1999) 3187–3191.

[23] T.M. Mizuno, S.P. Iliopoulos, H.T. Bergen, J.L. Roberts, C.A. Priest, C.V. Mobbs, Hypthalamic pro-opiomelanocortin Mrna is reduced by fasting and in ob/ob and db/db mice, but is stimulated by leptin, Diabetes 47 (1998) 294–297.

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 the lung. Morphologically, early changes in the 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 the 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 1 h 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. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: 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 extreme 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 and Rezaian, 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 the 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 and Shek, 1996). In the 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. The current studies demonstrated that liposomes containing anti-oxidants or anti-oxidant enzymes cause a reduction in acute lung injury in rats following airway delivery of CEES. Furthermore, delivery of such liposomes, when delayed 1 h after CEES administration, still provides significant attenuation of acute lung injury.

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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^{-1} body weight) (Fort Dodge Animal Health, Fort Dodge, IA) was used for anesthesia and intraperitoneal xylazine (13 mg kg^{-1} 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 per 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 so as to facilitate a unilateral, left-lung injury. A small volume of CEES (2 µl per rat; about 6 mg kg^{-1}) 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, 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 killed 4 h 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 killing as described elsewhere (McClintock *et al.*, 2002). ^{125}I-BSA present in the 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 the loss of the vascular and epithelial barriers (Johnson and Ward, 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 and Cochrane, 1969). 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 (Mayer, 1961). Experiments were performed 24 h after the second CVF injection.

Liposome Preparation

Dipalmitoylphosphatidylcholine (DPPC, Avanti Polar Lipids) was dissolved 20 mg ml^{-1} 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 1 h 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 100 nm 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

In order morphologically to 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 the deposition of fibrin and collagen (Luna, 1968).

Statistical Analysis

The 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 0, 6, 12 and 24 h 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 Fig. 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 h, increased evidence of trichrome stained deposition in alveolar walls was likely related to fibrin deposition (frame C). By 24 h after lung instillation of CEES, dense interstitial and intra-alveolar accumulations of 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 h (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 the lung, together with a collapse of alveolar

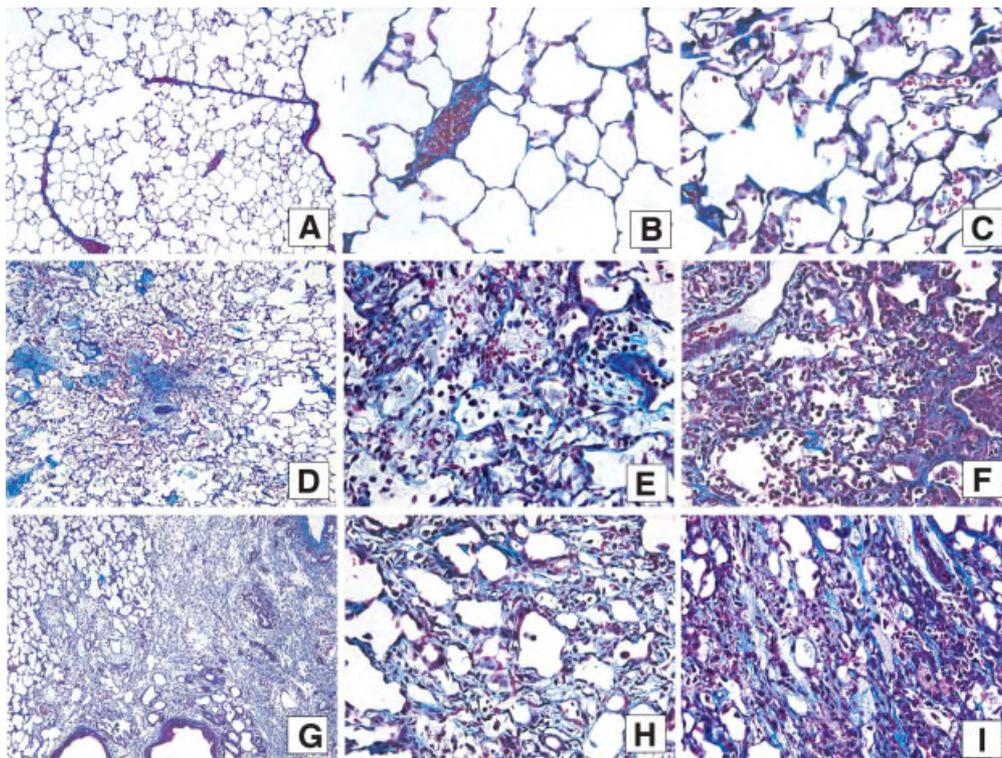**Figure 1.** Tissue sections of lungs with trichrome stain. Lungs were obtained after airway instillation of CEES at time 0 (A, B, 10× and 40×); 6 h (C, 40×); 24 h (D, E, 10× and 40×); 3 days (F, 10×), 6 days (G, 10× and H, 40×); and 3 weeks (I, 40×). All tissue sections were reacted with trichrome stain
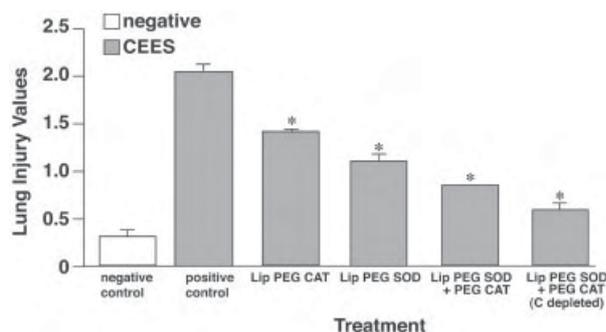
Figure 2. Attenuative effects of liposomes loaded with anti-oxidant enzymes. Rats received either saline followed by unloaded liposomes (negative control), CEES followed by airway delivery of unloaded liposomes (positive control), or CEES with liposomes containing either pegylated (PEG)-CAT or PEG-SOD, individually 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 h after airway delivery of CEES (see text). * Represents *P* values of <0.05 when compared with the positive control group

structures and the appearance of honeycombing (frames G, H). By week 3, 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 Fig. 2, the airway instillation of CEES together with unloaded liposomes resulted 4 h later in approximately a 10-fold increase in lung injury, as defined by the leakage of ^{125}I-albumin from blood into the lung. When instilled into the 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
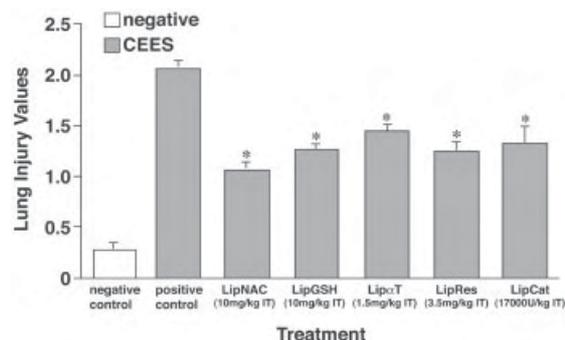

Figure 3. Attenuative effects of liposomes loaded with reducing agents in CEES lung injury. The positive and negative controls are similar to those described in Fig. 1. When used, liposomes were injected intra-tracheally immediately after the airway instillation of CEES. Lung injury was determined by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

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 Fig. 3), there was approximately a 10-fold increase in leakage of albumin from the circulation into the lungs of animals receiving airway instillation of CEES 4 h 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 Fig. 4, approximately a 10-fold increase in the leakage of ^{125}I-albumin into the lungs occurred following instillation of CEES together with unloaded liposomes. When the animals were complement (C) depleted, the instillation
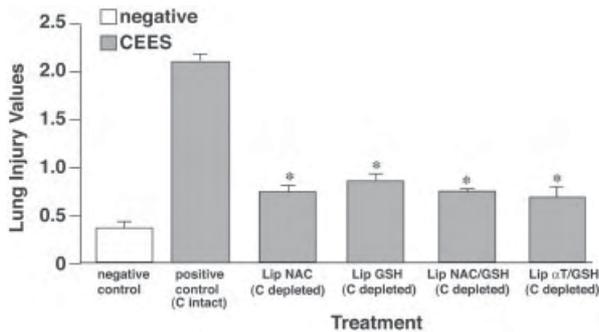
Figure 4. Enhanced effects of anti-oxidant containing liposomes in complement depleted rats. The negative and positive control groups are similar to those described in Fig. 1. Complement depletion was induced in four groups of animals by the prior intraperitoneal injection of purified cobra venom factor (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 Figs 1–3

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 significantly to attenuate CEES-induced acute lung injury in an additive manner.

Effects of Delayed Lung Instillation of Anti-Oxidant Liposomes

As shown in Fig. 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 Fig. 5, 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 the 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.
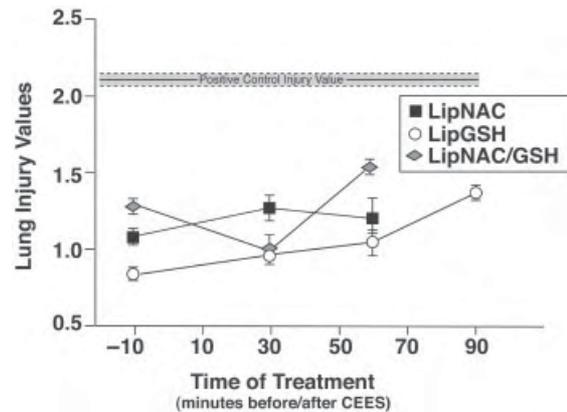
Discussion

The data described in this report indicate that CEES instillation into the lung produces acute lung injury in a manner that seems related to the loss of the redox balance in the lung, although this has not been demonstrated directly. 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 the 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 the alveolar spaces. Deposition of fibrin reflects a nonspecific reaction to tissue damage. It remains to be shown

that CEES-exposure causes rapid development of interstitial fibrosis, as confirmed biochemically by collagen accumulation. It is hypothesized 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 the lung may be associated with the release of mediators (such as TGF$_\beta$) 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 a 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 with the use of either type of liposomes given to complement-intact rats (Figs 3 and 4) suggests that it may be both the loss of reducing potential in the lung as well as an 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 it was shown 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 the 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 the lung. It should be noted that there is not much increased albumin leak into the lung in the first 60 min after administration of CEES (McClintock *et al.*, 2002). In fact, compared with values at 1 h, the permeability index at 2, 4 and 6 h after instillation of CEES rose 2 fold, 2.9 fold, 7.7 fold and 16.2 fold, respectively, when compared with uninjured lung values. Accordingly, the 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 the 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.

References

Angelov A, Belchen L, Angelov G. 1996. Experimental sulfur mustard gas poisoning and protective effect of different medicines in rats and rabbits. *Indian Vet. J.* **73**: 546–551.

Ballow M, Cochrane CG. 1969. Two anticomplementary factors in cobra venom: hemolysis of guinea pig erythrocytes by one of them. *J. Immunol.* **103**: 944–952.

Dube SN, Husain K, Sugendran K, Vijayaraghavan R, Somani SM. 1998. Dose response of sulphur mustard: behavioral and toxic signs in rats. *Indian J. Physiol. Pharmacol.* **42**: 389–394.

Eisenmenger W, Drasch G, von Clarmann M, Kretschmer E, Roider G. 1991. Clinical and morphological findings on mustard gas [bis(2-chloroethyl)sulfide] poisoning. *J. Forensic Sci.* **36**: 1688–1698.

Emad A, Rezaian GR. 1999. Immunoglobulins and cellular constituents of the BAL fluid of patients with sulfur mustard gas-induced pulmonary fibrosis. *Chest* **115**: 1346–1351.

Fan J, Shek PN, Suntres ZE, Li YH, Oreopoulos GD, Rotstein OD. 2000. Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome. *Surgery* **128**: 332–338.

Freeman BA, Turrens JF, Mirza Z, Crapo JD, Young SL. 1985. Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase. *Fed. Proc.* **44**: 2591–2595.

Gonzalez-Rothi RJ, Straub L, Cacace JL, Schreier H. 1991. Liposomes and pulmonary alveolar macrophages: functional and morphologic interactions. *Exp. Lung Res.* **17**: 687–705.

Johnson KJ, Ward PA. 1974. Acute immunologic pulmonary alveolitis. *J. Clin. Invest.* **54**: 349–357.

Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. 2003. Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. *J. Occup. Environ. Med.* **45**: 1136–1143.

Lakshmana Rao PV, Vijayaraghavan R, Bhaskar AS. 1999. Sulphur mustard induced DNA damage in mice after dermal and inhalation exposure. *Toxicology* **139**: 39–51.

Lentsch AB, Czermak BJ, Bless NM, Van Rooijen N, Ward PA. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-kappaB. *Am. J. Respir. Cell Mol. Biol.* **20**: 692–698.

Luna L. 1968. *Manual of Histologic Staining Methods of the AFIP*, 3rd edn. McGraw-Hill: New York.

Mayer MM. 1961. Complement and complement fixation. In *Experimental Immunochemistry*, Kabat EA, Mayer MM (eds). Springfield: Thomas, 133–240.

McClintock SD, Till GO, Smith MG, Ward PA. 2002. Protection from half-mustard-gas-induced acute lung injury in the rat. *J. Appl. Toxicol.* **22**: 257–262.

Shephard EG, Joubert JR, Finkelstein MC, Kuhn SH. 1981. Phagocytosis of liposomes by human alveolar macrophages. *Life Sci.* **29**: 2691–2698.

Sone S, Poste G, Fidler IJ. 1980. Rat alveolar macrophages are susceptible to activation by free and liposome-encapsulated lymphokines. *J. Immunol.* **124**: 2197–2202.

Suntres ZE, Shek PN. 1996. Treatment of LPS-induced tissue injury: role of liposomal antioxidants. *Shock* **6** (Suppl 1): S57–S64.

PROTECTION OF MUSTARD GAS-INDUCED LUNG INJURY BY ANTIOXIDANT LIPOSOMES

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ABSTRACT

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. Those kinds of treatments, however, are not possible for lung injury and no prophylactic treatment has been available for pulmonary injury by mustards. Because of this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. The objective of this study was to develop antioxidant liposomes as antidotes for mustard gas induced lung injury. In vitro work with our cell models strongly supports the notion that antioxidant liposomes are effective in preventing cytotoxicity of 2-chloroethyl ethyl sulfide (CEES), a mustard gas analog. In a study with rats, we observed protection of CEES-induced lung injury by liposomes containing catalase, SOD or reducing agents, NAC, GSH or resveratrol. Recently, we obtained data on the attenuation of CEES-induced lung injury in guinea pigs by intratracheal delivery of antioxidant liposomes containing NAC, GSH and Vitamin E.

Five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5) were prepared differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), N-acetylcysteine (NAC) and GSH. A single dose (200 μl per animal) of each liposome was administered intratracheally after 5 min and 60 minutes of CEES exposure to guinea pigs and the animals were sacrificed after 2 h of CEES exposure. Lung injury was monitored by studying the leakage of ^{125}I-BSA into the lung after CEES exposure.

These antioxidant liposomes offered 9 to 75% protection against lung injury as evidenced by leakage of BSA from blood into the lung. The maximum protection was achieved with two liposomes, LIP-2 (71.5%) and LIP-4 (75.4%), when administered after 5 minutes of CEES exposure. Delaying the administration of the liposomes after 1 h of CEES exposure decreased the efficacy. Both liposomes contained 55 mM phospholipid, 22 mM cholesterol, 0.6 mM phosphatidic acid, 11 mM α-tocopherol, 11 mM γ-tocopherol and 75 mM NAC. The only difference was that LIP-2 contained additionally 5 mM-δ-tocopherol.

Our study clearly suggests that NAC can be used in combination with tocopherol as a liposome for effective antidote against CEES–induced lung injury. Work is under progress to develop devices to deliver this drug directly into the lungs even immediately after CEES exposure.

Key Words: Mustard gas, lung injury, liposomes, NAC, tocopherol

INTRODUCTION

Mustard gas, an alkylating agent, is an extensively used chemical warfare agent. Upon exposure, it is known to exert local actions on eyes, skin, and respiratory tissue followed by impairment of nervous, cardiac, and digestive systems in humans and laboratory animals [1-3]. Upper and lower respiratory tracts may be damaged acutely, because of hemorrhagic inflammation after inhalation of mustard gas. Subsequently, a variety of chronic pulmonary complications may develop, including acute respiratory distress syndrome (ARDS), chronic bronchitis, and pulmonary fibrosis [4-8].

Although the exact mechanism is not well understood, it is not unreasonable to postulate a causal role of oxidative stress in the pathology that follows exposure to mustard gas. Many inflammatory lung diseases, including ARDS, are associated with oxidative stress [9]. For example, inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), provoke the generation of reactive oxygen species (ROS), mediators of oxidative stress [10].

Recently, we reported that intratracheal exposure of a sulfur mustard analog, 2-chloroethyl ethyl sulfide (CEES), to guinea pigs causes accumulation of high levels of TNF-α in the lung [11]. TNF-α elevations result in activation of acid and neutral sphingomyelinases and production of excessive ceramides, a second messenger involved in programmed cell death (apoptosis) [11]. In addition, intratracheal exposure to CEES leads to an immediate but transient activation of NF-κB, which is regarded as an anti-apoptotic signaling molecule. The abrupt disappearance of NF-κB, however, resulted in activation of several caspases, leading to apoptosis [11].

Ultrastructural assessment of the lungs from guinea pigs exposed to a single low dose of CEES (0.5 mg/kg b.wt.) resulted in detection of neutrophilic alveolitis and varying degrees of interstitial fibrosis [8, 12]. The lung injury was characteristic of that caused by oxidative stress secondary to the inhibition of defense enzymes against oxygen injury, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase [11].

Recently we reported delivery of an antioxidant N-acetylcysteine (NAC) through drinking water to offer *prophylactic* protection against CEES-induced lung injury [12]. This protection is associated with (a) inhibition of CEES-induced activation of TNF-α, NF-κB, sphingomyelinases and caspases, (b) inhibition of CEES-induced accumulation of ceramides, and (c) protection of the oxygen defense system by counteracting CEES-induced inhibition of SOD, GSH-Px, and catalase activities [12]. We also observed protection against CEES-induced lung injury in rats by intratracheal administration of liposomes containing catalase, SOD or the reducing agents, NAC, GSH, or resveratrol [13].

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. Those kinds of treatments, however, are not possible for lung injury and no prophylactic treatment has been available for

pulmonary injury by mustards. Because of this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury from mustards. Therefore, it is important that we develop a suitable antioxidant liposome therapy for the protection of injury induced by mustard gas exposure. The present study reports data on the attenuation of CEES-induced acute lung injury in guinea pigs by five antioxidant liposomes differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), N-acetylcysteine (NAC) and GSH.

CONCLUSION

Male guinea pigs (Hartley strain, 5-6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley Inc (Indianapolis, Indiana). Animals were infused intratracheally with single doses of CEES (2 mg/kg body weight) in ethanol (infusion volume was 100 μl/animal). Control animals were infused with 100 μl of ethanol in the same way. Liposomes were injected intratracheally in a volume of 200 μl per animal through the same catheter setup used for CEES instillation. Liposomes were given after 5 and 60 minutes of CEES exposure and sacrificed after 2 h.

Liposomes	Liposome Content (mM)							
	Phospholipid (PL90H)	Cholesterol	Phosphatidic Acid (PA)	Tocopherol (Vitamin E)			NAC	GSH
				α	γ	δ		
LIP-1 (Blank)	71	28	0.67	-	-	-	-	-
LIP- 2	55	22	0.6	11	11	5	75	-
LIP- 3	62	25	0.6	6	6	-	75	-
LIP- 4	55	22	0.6	11	11	-	75	-
LIP- 5	55	22	0.6	11	11	-	-	75

Antioxidant liposomes were prepared using a M-110L Laboratory Microfluidizer Processor (Microfluidics Headquarters, MA). Altogether, five different antioxidant liposome preparations were tested, as outlined in Table 1 which shows the composition and concentrations of the stock liposomal formulations.

TABLE 1. **Composition of Antioxidant Liposomes**

Lung injury was monitored by studying the leakage of ^{125}I-BSA into lung after CEES exposure [12]. Guinea pigs were injected into ear vein slowly with the ^{125}I-BSA solution (8 μCi/animal). CEES (2 mg/kg body weight) was infused into the animals intratracheally 3 h after ^{125}I-BSA injection. Control animals were infused intratracheally with 100 μl of solvent only. After 5 h of the injection of ^{125}I-BSA (i.e. 2 h after CEES infusion), the animals were sacrificed and 1 ml of blood was collected. The chest cavity was opened and lung was perfused with buffer to get rid of any residual blood from the animals individually. The perfused lung was taken out and after removal of heart and trachea; the radioactivity content of the lung was monitored in a gamma counter. Lung injury was expressed as permeability index that was obtained by dividing total radioactive counts in lung by counts in 1 ml of blood from the same animal.

The data presented in Table 2 indicate that all liposomes offered prophylactic protection; however the efficiency varied (9.2–75.4%) depending on their composition. Two liposomes offered maximum protection (LIP 2 – 71.5%, LIP-4 – 75.4%) when given within 5 minutes of CEES exposure. Liposome

2 contained 11 mM α-Vit E, 11 mM γ-Vit E, 5 mM δ-Vit E and 75 mM NAC. The only difference between LIP 2 and LIP 4 is that LIP 4 did not have any δ-VIT E. The protection was diminished if the liposome delivery was delayed for 1 h. Maximum protection was 43% if the liposome was given after

Treatment	Permeability Index	
	Liposomes injected 5 min after CEES Exposure	Liposomes injected 60 min after CEES Exposure
Control (No CEES)	0.20 ± 0.04	
Control (CEES only)	1.30 ± 0.22	
CEES + LIP-1	1.18 ± 0.03	1.11 ± 0.21
CEES + LIP-2	0.37 ± 0.03	0.74 ± 0.04
CEES + LIP-3	0.60 ± 0.02	0.80 ± 0.06
CEES + LIP-4	0.32 ± 0.01	0.76 ± 0.03
CEES + LIP-5	0.64 ± 0.05	0.83 ± 0.04

1 h of CEES exposure.

TABLE 2. **Effects of Liposome Treatment on CEES-Induced Lung Injury in Guinea Pigs (Leakage of ^{125}I-BSA from Blood into the Lung).**

CEES was infused (2 mg/kg body weight) intratracheally into guinea pigs with or without treatment with five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5). Liposomes were infused intratracheally either 5 or 60 minutes after CEES exposure. The lung injury was measured after 2h of CEES exposure and expressed as permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. ^{125}I-BSA was injected into the ear veins 3h prior CEES exposure. Each group had 3 animals. Values are mean ± SE.

The extensive leakage of albumin into the lung after intratracheal infusion of CEES indicates that the blood-gas barrier has been seriously compromised and that causes a functional impairment or destruction of both vascular endothelial and alveolar epithelial cells resulting in hypoxia. In the present study, we report data on the attenuation of CEES-induced acute lung injury in guinea pigs by five antioxidant liposomes differing in the levels of phospholipid, cholesterol, phosphatidic acid, tocopherol (α, γ, δ), NAC and GSH. Empty liposome (LIP-1) infusion did not change the permeability index. LIP-2 and LIP 4 offered substantial attenuation of the massive leak of blood albumin into the lung (Table 2). Furthermore, a matter of considerable interest is that delayed delivery (for as long as 60 minutes) of liposomes into the lung after CEES infusion still provides substantial attenuation of lung injury. It is well known that liposomes delivered into the airways are phagocytized by macrophages and internalized [14]. Thus it can be implied from the current studies that liposomal delivery selectively enhances reducing environment in lung macrophages and thereby compromises CEES toxicity in these cells. This data indicates that addition of tocopherols causes more protection than NAC or GSH only.

Vitamin E is believed to be involved in a variety of physiological and biochemical functions. The molecular mechanism of these functions is believed to be mediated by either the antioxidant action of the vitamin or by its action as a membrane stabilizer and most potent lipid-soluble antioxidants in blood, breaking free-radical chain reactions of lipid peroxidation (15) Furthermore, the antioxidative roles of the different tocopherol isoforms are highly interdependent and may be complementary in function. There is some evidence suggesting that isoforms concentrations relative to each other may be important in preventing specific types of oxidative damage, with γ-tocopherol possibly being more important than α-tocopherol in removing nitrogen oxides and other electrophilic mutagens, whereas δ-tocopherol has stronger antiproliferative effect than α- and γ - tocopherols (16).

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REFERENCES

1. Papirmeister B., Fenster AJ, Robinson SI and Ford RD. Sulfur mustard injury: description of lesions and resulting incapacitations. In: Medical Defense Against Mustard Gas. Toxic Mechanisms and Pharmacological Implications, edited by A.J. Fenster. Boca Raton, FL; CRC, p.13-42, 1991
2. Dacre JC and Goldman M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard, Pharmacol. Rev., 48, 289-326, 1996.
3. Smith MG, Stone W, Crawford K, Ward P, Till GO and Das SK. A promising new treatment for mustard gas with the potential to substantially reduce the threat posed by chemical, biological and radiological agents. Jane's Chem-Bio Web, 1-5, 2003.
4. Worwser U. Toxicology of mustard gas. Trends Pharmacol Sci 12: 164-167, 1991.
5. Momeni AZ, Enshaelh S., Meghdadi SM and Amindjavaheri M. Skin manifestation of mustard gas. Clinical study of 535 patients exposed to mustard gas. Archs Dermatol 128: 775-780, 1992.
6. Calvet JH, Jarrean PH, . Levame M, D'ortho MP, Lorino H, Harf A and Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 76: 681-688, 1994.
7. Emad A and   Rezaian GR. The diversity of the effects of sulfur mustard gas inhalation on respiratory system 10 years after a single, heavy exposure: Analysis of 197 cases. Chest 112: 734-738, 1997.
8. Mukherjee S. and Das SK. Pulmonary fibrosis in guinea pig by 2-chloroethyl ethyl sulfide. FASEB J. 19: A280, 2005.
9. Leff JA,   Parsons PE, Day CE, Taniguchi N, Jochum M,   Fritz H, Moore FA,   Moore EE, McCord JM and Repine JE. Serum antioxidants as predictors of adult respiratory distress syndrome in patients with sepsis. Lancet 1003; 341: 777-780, 1993.
10. Lloyd SS, Cheng AK, Taylor FB and McCay JEG. Free radicals and septic shock in primates: the role of tumor necrosis factor. Free Rad Biol & Medicine 14: 233-242, 1993.
11. Chatterjee D, Mukherjee S, Smith MG and Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide (CEES), a mustard analog. J Biochem Mol Toxicol 17: 1-8, 2003.

12. Das SK, Mukherjee S, Smith MG and. Chatterjee D. Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. J Biochem Mol Toxicol 17: 177-184, 2003.

13. McClintock SD, Hoesel LM, Das SK, Till GO, Neff T, Kunkel RG, Smith MG and Ward PA. Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J Appl Toxicol. 26: Issue 2, 126-131, 2006.

14. Gonzalez-Rothi RJ, Straub L, Cacace JL and Schreier H Liposomes and pulmonary alveolar macrophages: functional and morphologic interactions. Exp Lung Res 17, 687-705, 1991.

15. Wang X and Quinn PJ Vitamin E and its function in membranes Prog Lipid Res 38:309-336 1999. Mayes PA Structure and function of the lipid-soluble vitamins. In: Murray RK, Granner DK, Mayes PA et al. Eds) Harper's Biochemistry. Stamford, CT: Appleton & Lange, 1996, 614-624.

16. Christen S, Woodall AA, Shigenaga MK et al. Gamma-tocopherol traps mutagenic electrophiles such as NO_2 and complements alpha-tocopherol: physiological implications. Proc Natl Acad Sci USA 94, 3217-3222, 1997.

9

Therapeutic Uses of Antioxidant Liposomes

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1. Introduction

This chapter 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 *(1–3)*. 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, often highly reactive and damaging to biological systems. The biological membranes of subcellular organelles are a major site of free radical damage but proteins and DNA are also significant targets. Moreover, free radicals can alter cellular signal transduction pathways and stimulate the synthesis of inflammatory cytokines. Oxygen radicals and other reactive oxygen species (ROS) arise from the single electron reductions of oxygen.

$$O_2 + e^- \rightarrow O_2^{\bullet -} \tag{1}$$

$$O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2 \tag{2}$$

$$H_2O_2 + e^- + H^+ \rightarrow H_2O + OH^{\bullet} \tag{3}$$

$$OH^{\bullet} + e^- + H^+ \rightarrow H_2O \tag{4}$$

$$O_2^{\bullet -} + NO \rightarrow ONOO^- \tag{5}$$

In addition, the superoxide radical $(O_2^{\bullet-})$ can react rapidly with nitric oxide to yield peroxynitrite $(ONOO^-)$ as shown in **Eq. 5**. Peroxynitrite is a reactive nitrogen oxide species (RNOS) that can also cause damage to DNA, proteins, and membranes. 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 potential clinical use of such bifunctional liposomes has been extremely limited (4).

A primary use of antioxidant liposomes has been to define the molecular mechanism of action for various antioxidants (5–13). Antioxidants such as butylated hydroxytoluene (BHT) and α-tocopherol have also been used to prevent the oxidation of unsaturated fatty acid moieties in the phospholipids of liposomes during storage (14) or sonication (15). This chapter, however, focuses on the potential therapeutic uses of antioxidant liposomes. This is a rapidly evolving area of medical research not 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 (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 (1). In the following subheadings, 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 (tocopherols and tocotrienols) (16), ubiquinones (17), retinoids (18–20), carotenoids (21,22), lipid-soluble flavonoids (e.g., quercetin, hesperetin, naringenin) (23), tamoxifen (24,25), as well as synthetic lipid-soluble antioxidants such as BHT, tertiary butylhydroquinone (TBHQ), and probucol. Tocopherols can readily be incorporated into both monolayers of unilamellar liposomes in a monomeric form (16). Furthermore, tocopherol in liposomes can undergo spontaneous intermembrane transfer to an acceptor membrane without the fusion of the tocopherol liposome (16). This intermembrane transfer is more pronounced when the tocopherol liposome contains

polyunsaturated fatty acids (16). R,R,R-α-tocopherol and R,R,R-α-tocotrienol are forms of vitamin E that have the same aromatic chromanol head group but differ in the structure of their hydrocarbon tails. R,R,R-α-tocotrienol is, however, a better peroxyl radical scavenger than R,R,R-α-tocopherol in phosphatidylcholine liposomes (26).

β-Carotene (a carotinoid) can be incorporated into liposomes to a maximum of about 0.5 mol% (based on phospholipid) whereas tocopherol 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-tocopherol (27). β-Carotene at 0.45 mol% (of phospholipid) is, however, a more powerful inhibitor of singlet oxygen mediated lipid peroxidation than α-tocopherol at 0.45 mol% (28). α-Tocopherol at 4.5 mol% is, however, also effective at inhibiting both free radical lipid peroxidation as well as singlet oxygen mediated lipid peroxidation (28). Singlet oxygen can be generated by photosensitization and this reactive oxygen species may contribute to light-induced skin toxicity as well as the aging of skin.

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 (29,30). Liposomes constructed with ethanolamine plasmalogen inhibit both iron- and copper-dependent peroxidation in the presence of preformed lipid hydroperoxides (31). Koga et al. have synthesized a novel phospholipid containing a chromanol structure as its polar head group (12,32). This phosphatidyl derivative of vitamin E is almost as effective an antioxidant as α-tocopherol 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. This is particularly important in the case of liposomes with both tocopherol (TOH) and ascorbate (Asc), as it has been demonstrated that ascorbate can regenerate tocopherol from the tocopheroxyl radical $(TO^{\bullet})$ (33).

$$TO^{\bullet} + Asc \rightarrow TOH + Asc^{\bullet} \tag{6}$$

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 (34). 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 (6) and can also function as a substrate for glutathione peroxidase, a key (selenium containing) antioxidant enzyme that converts lipid hydroperoxides (LOOH) 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 (35). Recently, we have observed 50–60% protection by N-acetylcysteine in the generation of free radicals in lungs by mustard gas induced lung injury in guinea pigs (Das and coworkers unpublished observations).

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 (36). 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 (37). The various procedures for preparing liposomes with entrapped antioxidant enzymes have been evaluated by Aoki et al. (38). This group and others (39) 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. (40) 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. (41) found that endothelial cells treated with liposomes containing entrapped superoxide dismutase 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 a major site 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, 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 (42). Topical administration of antioxidant liposomes could also be useful in situations where individuals were exposed to toxic substances (e.g., chemical warfare agents) causing skin damage by free radical mechanisms. 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 (4).

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-tocopherol are about 10 times higher than the levels of γ-tocopherol despite the fact that dietary levels of γ-tocopherol are at least two times that of α-tocopherol. Nevertheless, γ-tocopherol has a unique chemical ability to detoxify peroxynitrite that is not shared with α-tocopherol (43). 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-tocopherol. The poor bioavailability of orally administered γ-tocopherol makes this very difficult to accomplish. This limitation could, however, be overcome by the intravenous administration of liposomes containing γ-tocopherol.

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 upon by intestinal esterases to liberate the unesterified tocopherol. It is interesting, therefore, that α-tocopheryl succinate but not α-tocopherol has been found to inhibit the activation of nuclear factor κB (NFκB) in cultured macrophages (44). 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

(45). 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 *(46,47).* Moreover, the intravenous administration of Cu,ZnSOD liposomes to rats can reduce cerebral infarction caused by ischemia *(47)* and also inhibit learning dysfunction caused by a low dose of total body irradiation *(48).* 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 *(49).*

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. *(45)* found that intravenous administration of liposomes containing vitamin E (TOH liposomes) was very effective in decreasing mortality in mice given a lethal dose of CCl4. 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 well 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–PE). Recently, the preparation of pH-sensitive stealth liposomes has been described *(50).* 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.

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 *(51),* and cancer *(52,53).* Schwartz et al. *(54)* 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 *(54).* 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 *(55,56).* Several investigators have documented dramatic improvement in patients with acute promyelocytic leukemia after treatment with all-*trans*-retinoic acid *(57–59).* 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 antitumor properties, that is, growth inhibition and differentiation induction of all-*trans*-retinoic acid are maintained or even enhanced *(60,61).* 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 *(62).* In general, the use of retinoids is safe and induces complete remission in 80–90% 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 *(51,63–66),* respiratory distress syndrome *(67),* trauma *(3),* irradiation *(48),* cold injury *(68),* and certain types of infectious diseases such as influenza and human immunodeficiency virus (HIV) infection. Evidence suggests that trauma to the brain results in the overproduction of superoxide radicals that may contribute to edema *(69,70).* Antioxidant liposomes containing SOD have been used effectively to treat posttraumatic brain edema *(69,70)* and neurological dysfunctions in rats *(71).*

Retinopathy of prematurity is a leading cause of blindness in premature and low birth weight infants who are often treated with high levels of oxygen due to surfactant deficiency. Considerable evidence *(72)* indicates that oxidative stress is a major contributor to this disease. In an animal model, Niesman et al. *(73)* found that intraperitoneal administration of SOD encapsulated in PEG-modified 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 *(74).* Surfactant is

necessary to maintain proper expansion of the small air sacs in the lung. 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 (75,76). 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. (77) 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 (78) demonstrated that intratracheally administered liposomes (with phosphatidyl-choline, cholesterol, and stearylamine) have a long retention time (> 5 d) in the mouse lung. Liposomes with entrapped Cu,Zn superoxide dismutase and catalase (Cu,ZnSOD+CAT liposomes) were intratracheally instilled in rabbits and the alveolar distribution of the antioxidants measured after 4 and 24 hours (79). The results indicate that Cu,ZnSOD+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. (80) 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 (81).

Archer et al. (82) 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 catalase liposomes inhibited decreases in pulmonary vascular reactivity. Superoxide dismutase administered free in solution or combined with catalase in liposomes, increased the normoxic pulmonary arterial pressure, and enhanced vascular reactivity to angiotensin II and hypoxia (82).

In a rat model, Freeman et al. (83) 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 non-entrapped (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 (84).

Barnard et al. (85) 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 catalase conferred protection against the cytotoxic effects of 50% and 95% oxygen (86,87) and also protect against cell death (88).

Briscoe et al. (89) 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 the culture cells were incubated with the pH-sensitive SOD liposomes (89). 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 (89,90). The presence of SP-A in the SOD liposomes facilitates their uptake by pulmonary epithelial cells (89,90).

Considerable evidence suggests that oxidative injury to lung tissues can be mediated by neutrophils (91). 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-tocopherol are able to counteract some PMA-induced lung injury in a rat model (91). In contrast, rats pretreated with blank liposomes (no α-tocopherol) showed no protection from PMA-induced lung injury (91).

Paraquat has also been used to induce oxidative lung injuries in animal models *(4)*. Suntres and Shek *(4)* have compared the ability of α-tocopherol liposomes (TOH liposome) or liposomes with both α-tocopherol 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 *(4)*. Interestingly, neither antioxidant liposome was effective in preventing lung edema *(4)*.

Liposomes encapsulated with catalase (CAT liposomes) have also been found to be efficacious in preventing chronic pulmonary oxygen toxicity in young rats *(92)*. 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 superoxide dismutase (SOD liposomes) or with lower levels of CAT (50 U 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 *(93)*.

Acknowledgments

References

1. Papas, A. M. (1999) *Contemporary Food Science. Antioxidant Status, Diet, Nutrition and Health* (Clydesdale, F. M., ed.), CRC Press, Boca Raton, FL.
2. Krinsky, N. I. (1998) The antioxidant and biological properties of the carotenoids. *Ann. NY Acad. Sci.* **854**, 443–447.
3. Oldham, K. M. and Bowen, P. E. (1998) Oxidative stress in critical care: Is antioxidant supplementation beneficial? *J. Am. Diet. Assoc.* **98**, 1001–1008.
4. Suntres, Z. and Shek, P. (1996) Alleviation of paraquat-induced lung injury by pretreatment with bifunctional liposomes containing alpha-tocopherol and glutathione. *Biochem. Pharmacol.* **52**, 1515–1520.
5. Barclay, L., Bailey, A., and Kong, D. (1985) The antioxidant activity of alpha-tocopherol-bovine serum albumin complex in micellar and liposome autoxidations. *J. Biol. Chem.* **260**, 15809–15814.
6. Barclay, L. (1988) The cooperative antioxidant role of glutathione with a lipid-soluble and a water-soluble antioxidant during peroxidation of liposomes initiated in the aqueous phase and in the lipid phase. *J. Biol. Chem.* **263**, 16138–16142.
7. Barclay, L. and Vinqvist, M. (1994) Membrane peroxidation: inhibiting effects of water-soluble antioxidants on phospholipids of different charge types. *Free Radic. Biol. Med.* **16**, 779–788.
8. Barclay, L., Antunes, F., Egawa, Y., McAllister, K., Mukai, K., Nishi, T., and Vinqvist, M. (1997) The efficiency of antioxidants delivered by liposomal transfer. *Biochim. Biophys. Acta* **1328**, 1–12.
9. Di Giulio, A., Saletti, A., Oratore, A., and Bozzi, A. (1996) Monitoring by *cis*-parinaric fluorescence of free radical induced lipid peroxidation in aqueous liposome suspensions. *J. Microencapsul.* **13**, 435–445.
10. Doba, T., Burton, G., and Ingold, K. (1985) Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water-soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim. Biophys. Acta* **835**, 298–303.
11. Hayashi, K., Noguchi, N., and Niki, E. (1995) Action of nitric oxide as an antioxidant against oxidation of soybean phosphatidylcholine liposomal membranes. *FEBS Lett.* **370**, 37–40.
12. Koga, T. and Terao, J. (1996) Antioxidant behaviors of vitamin E analogues in unilamellar vesicles. *Biosci. Biotechnol. Biochem.* **60**, 1043–1045.
13. Takahashi, M., Tsuchiya, J., Niki, E., and Urano, S. (1988) Action of vitamin E as antioxidant in phospholipid liposomal membranes as studied by spin label technique. *J. Nutr. Sci. Vitaminol. (Tokyo)* **34**, 25–34.
14. Chow, C. and Heath, T. (1995) Rapid diffusion of the lipid phosphorus of phosphatidylglycerol liposomes through polycarbonate membranes is caused by the oxidation of the unsaturated fatty acids. *Biochim. Biophys. Acta* **1239**, 168–176.
15. Gabrielska, J., Sarapuk, J., and Przestalski, S. (1995) Antioxidant protection of egg lecithin liposomes during sonication. *Z. Naturforsch. [C]* **50**, 561–564.
16. Kagan, V., Bakalova, R., Zhelev, Z., Rangelova, D., Serbinova, E., Tyurin, V., et al. (1990) Intermembrane transfer and antioxidant action of alpha-tocopherol in liposomes. *Arch. Biochem. Biophys.* **280**, 147–152.
17. Yamamoto, Y., Komuro, E., and Niki, E. (1990) Antioxidant activity of ubiquinol in solution and phosphatidylcholine liposome. *J. Nutr. Sci. Vitaminol. (Tokyo)* **36**, 505–511.
18. Tesoriere, L., Bongiorno, A., Pintaudi, A., DqAnna, R., DqArpa, D., and Livrea, M. (1996) Synergistic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Arch. Biochem. Biophys.* **326**, 57–63.
19. Tesoriere, L., Ciaccio, M., Bongiorno, A., Riccio, A., Pintaudi, A., and Livrea, M. (1993) Antioxidant activity of all-*trans*-retinol in homogeneous solution and in phosphatidylcholine liposomes. *Arch. Biochem. Biophys.* **307**, 217–223.

20. Tesoriere, L., DqArpa, D., Re, R., and Livrea, M. (1997) Antioxidant reactions of all-*trans* retinol in phospholipid bilayers: effect of oxygen partial pressure, radical fluxes, and retinol concentration. *Arch. Biochem. Biophys.* **343,** 13–18.

21. Stahl, W., Junghans, A., de Boer, B., Driomina, E., Briviba, K., and Sies, H. (1998) Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. *FEBS Lett.* **427,** 305–308.

22. Woodall, A., Britton, G., and Jackson, M. (1995) Antioxidant activity of carotenoids in phosphatidylcholine vesicles: chemical and structural considerations. *Biochem. Soc. Trans.* **23,** 133S.

23. Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., and Castelli, F. (1995) Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radic. Biol. Med.* **19,** 481–486.

24. Wiseman, H., Laughton, M., Arnstein, H., Cannon, M., and Halliwell, B. (1990) The antioxidant action of tamoxifen and its metabolites. Inhibition of lipid peroxidation. *FEBS Lett.* **263,** 192–194.

25. Wiseman, H. (1994) Tamoxifen and estrogens as membrane antioxidants: comparison with cholesterol. *Methods Enzymol.* **234,** 590–602.

26. Suzuki, Y., Tsuchiya, M., Wassall, S., Choo, Y., Govil, G., Kagan, V., and Packer, L. (1993) Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: implication to the molecular mechanism of their antioxidant potency. *Biochemistry* **32,** 10692–10699.

27. Liebler, D., Stratton, S., and Kaysen, K. (1997) Antioxidant actions of beta-carotene in liposomal and microsomal membranes: role of carotenoid-membrane incorporation and alpha-tocopherol. *Arch. Biochem. Biophys.* **338,** 244–250.

28. Stratton, S. P. and Liebler, D. C. (1997) Determination of singlet oxygen-specific versus radical-mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of beta-carotene and alpha-tocopherol. *Biochemistry* **36,** 12911–12920.

29. Engelmann, B., Bräutigam, C., and Thiery, J. (1994) Plasmalogen phospholipids as potential protectors against lipid peroxidation of low density lipoproteins. *Biochem. Biophys. Res. Commun.* **204,** 1235–1242.

30. Vance, J. E. (1990) Lipoproteins secreted by cultured rat hepatocytes contain the antioxidant 1-alk-1-enyl-2-acylglycerophosphoethanolamine. *Biochim. Biophys. Acta* **1045,** 128–134.

31. Zommara, M., Tachibana, N., Mitsui, K., Nakatani, N., Sakono, M., Ikeda, I., and Imaizumi, K. (1995) Inhibitory effect of ethanolamine plasmalogen on iron- and copper-dependent lipid peroxidation. *Free Radic. Biol. Med.* **18,** 599–602.

32. Koga, T., Nagao, A., Terao, J., Sawada, K., and Mukai, K. (1994) Synthesis of a phosphatidyl derivative of vitamin E and its antioxidant activity in phospholipid bilayers. *Lipids* **29,** 83–89.

33. Thomas, C., McLean, L., Parker, R., and Ohlweiler, D. (1992) Ascorbate and phenolic antioxidant interactions in prevention of liposomal oxidation. *Lipids* **27,** 543–550.

34. Kagan, V., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell, R., and Packer, L. (1992) Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxyl, ascorbyl and chromanoxyl radicals. *Biochem. Pharmacol.* **44,** 1637–1649.

35. Ma, Y. S., Stone, W. L., and LeClair, I. O. (1994) The effects of vitamin C and urate on the oxidation kinetics of human low-density lipoprotein. *Proc. Soc. Exp. Biol. Med.* **206,** 53–59.

36. Turrens, J. (1991) The potential of antioxidant enzymes as pharmacological agents in vivo. *Xenobiotica* **21,** 1033–1040.

37. White, C., Jackson, J., Abuchowski, A., Kazo, G., Mimmack, R., Berger, E., et al. (1989) Polyethylene glycol-attached antioxidant enzymes decrease pulmonary oxygen toxicity in rats. *J. Appl. Physiol.* **66,** 584–590.

38. Aoki, H., Fujita, M., Sun, C., Fuji, K., and Miyajima, K. (1997) High-efficiency entrapment of superoxide dismutase into cationic liposomes containing synthetic aminoglycolipid. *Chem. Pharmaceut. Bull. (Tokyo)* **45,** 1327–1331.

39. Miyajima, K., Komatsu, H., Sun, C., Aoki, H., Handa, T., Xu, H., et al. (1993) Effects of cholesterol on the miscibility of synthetic glucosamine diesters in lipid bilayers and the entrapment of superoxide dismutase into the positively charged liposomes. *Chem. Pharmaceut. Bull. (Tokyo)* **41,** 1889–1894.

40. Freeman, B. A., Young, S. L., and Crapo, J. D. (1983) Liposome-mediated augmentation of superoxide dismutase in endothelial cells prevents oxygen injury. *J. Biol. Chem.* **258,** 12534–12542.

41. Beckman, J., Minor, R. L, Jr., and Freeman, B. (1986) Augmentation of antioxidant enzymes in vascular endothelium. *J. Free Radic. Biol. Med.* **2,** 359–365.

42. Natsuki, R., Morita, Y., Osawa, S., and Takeda, Y. (1996) Effects of liposome size on penetration of dl-tocopherol acetate into skin. *Biol. Pharmaceut. Bull.* **19,** 758–761.

43. Christen, S., Woodall, A., Shigenaga, M., Southwell-Keely, P., Duncan, M., and Ames, B. (1997) Gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. *Proc. Natl. Acad. Sci. USA* **94,** 3217–3222.

44. Nakamura, T., Goto, M., Matsumoto, A., and Tanaka, I. (1998) Inhibition of NF-kappa B transcriptional activity by alpha-tocopheryl succinate. *Biofactors* **7,** 21–30.

45. Yao, T., Degli Esposti, S., Huang, L., Arnon, R., Spangenberger, A., and Zern, M. (1994) Inhibition of carbon tetrachloride-induced liver injury by liposomes containing vitamin E. *Am. J. Physiol.* **267,** G476–484

46. Imaizumi, S., Woolworth, V., Kinouchi, H., Chen, S. F., Fishman, R. A., and Chan, P. H. (1990) Liposome-entrapped superoxide dismutase ameliorates infarct volume in focal cerebral ischaemia. *Acta Neurochir. Suppl. (Wien)* **51,** 236–238.

47. Imaizumi, S., Woolworth, V., Fishman, R. A., and Chan, P. H. (1990) Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats. *Stroke* **21,** 1312–1317.

48. Lamproglou, I., Magdelenat, H., Boisserie, G., Baillet, F., Mayo, W., Fessi, H., et al. J. Y. (1998) An experimental model of acute encephalopathy after total body irradiation in the rat: effect of liposome-entrapped Cu/Zn superoxide dismutase. *Int. J. Radiat. Oncol. Biol. Phys.* **42,** 179–184.

49. Stanimirovic, D. B., Markovic, M., Micic, D. V., Spatz, M., and Mrsulja, B. B. (1994) Liposome-entrapped superoxide dismutase reduces ischemia/reperfusion 'oxidative stress' in gerbil brain. *Neurochem. Res.* **19,** 1473–1478.

50. Slepushkin, V. A., Simões, S., Dazin, P., Newman, M. S., Guo, L. S., Pedroso de Lima, M. C., and Duzgunes, N. (1997) Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. *J. Biol. Chem.* **272,** 2382–2388.

51. Tang, C. S., Su, J. Y., Li, Z. P., Zhang, L. Z., Yang, J., Qi, M., et al. (1993) Possibility of targeting treatment for ischemic heart disease with liposome (II). *Sci. China B.* **36,** 809–816.

52. Demopoulos, H., Pietronigro, D., Flamm, E., and Seligman, M. (1980) The possible role of free radical reactions in carcinogenesis. *J. Environ. Pathol. Toxicol.* **3,** 273–303.

53. Stone, W. L. and Papas, A. M. (1997) Tocopherols and the etiology of colon cancer. *J. Natl. Cancer Inst.* **89,** 1006–1014.

54. Schwartz, J., Shklar, G., Flynn, E., and Trickler, D. (1990) The administration of beta carotene to prevent and regress oral carcinoma in the hamster cheek pouch and the associated enhancement of the immune response. *Adv. Exp. Med. Biol.* **262,** 77–93.

55. Lippman, S. M., Kessler, J. F., and Meyskens, F. L. J. (1987) Retinoids as preventive and therapeutic anticancer agents (Part II). *Cancer Treat. Rep.* **71,** 493–515.

56. Smith, M. A., Parkinson, D. R., Cheson, B. D., and Friedman, M. A. (1992) Retinoids in cancer therapy. *J. Clin. Oncol.* **10,** 839–864.

57. Chomienne, C., Ballerini, P., Balitrand, N., Huang, M. E., Krawice, I., Castaigne, S., et al., (1990) The retinoic acid receptor alpha gene is rearranged in retinoic acid-sensitive promyelocytic leukemias. *Leukemia* **4,** 802–807.

58. Castaigne, S., Chomienne, C., Daniel, M. T., Berger, R., Miclea, J. M., Ballerini, P., and Degos, L. (1990) Retinoic acids in the treatment of acute promyelocytic leukemia. *Nouv. Rev. Fr. Hematol.* **32,** 36–38.

59. Chomienne, C., Ballerini, P., Balitrand, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. (1990) All-trans retinoic acid in acute promyelocytic leukemias. II. In vitro studies: structure–function relationship. *Blood* **76,** 1710–1717.

60. Sacks, P. G., Oke, V., and Mehta, K. (1992) Antiproliferative effects of free and liposome-encapsulated retinoic acid in a squamous carcinoma model: monolayer cells and multicellular tumor spheroids. *J. Cancer Res. Clin. Oncol.* **118,** 490–496.

61. Parthasarathy, R., Sacks, P. G., Harris, D., Brock, H., and Mehta, K. (1994) Interaction of liposome-associated all-*trans*-retinoic acid with squamous carcinoma cells. *Cancer Chemother. Pharmacol.* **34,** 527–534.

62. Fiorentini, D., Cabrini, L., and Landi, L. (1993) Ubiquinol-3 and ubiquinol-7 exhibit similar antioxidant activity in model membranes. *Free Radic. Res. Commun.* **18,** 201–209.

63. Bilenko, M., Morgunov, A., Churakova, T., Bulgakov, V., and Komarov, P. (1989) Disorders of cardiac contractile function in ischemic shock; the protective effect of antioxidants and liposomes made from egg phospholipids. *Bull. Eksp. Biol. Med.* **108,** 660–663.

64. Ferrari, R., Agnoletti, L., Comini, L., Gaia, G., Bachetti, T., Cargnoni, A., et al. (1998) Oxidative stress during myocardial ischaemia and heart failure. *Eur. Heart J.* **19** (Suppl B), B2–11.

65. Janero, D. and Burghardt, B. (1989) Oxidative injury to myocardial membrane: direct modulation by endogenous alpha-tocopherol. *J. Mol. Cell Cardiol.* **21,** 1111–1124.

66. Sjogren, K., Hjalmarson, A., and Ek, B. (1992) Antioxidants protect against reoxygenation-induced cell damage in ventricular myocytes. *Biochem. Soc. Trans.* **20,** 233S.

67. Gupta, A., Majumdar, S., and Sanyal, S. (1996) Effect of lung surfactant liposomes on the rabbit fetal lung type II cell antioxidant enzymes following prenatal dexamethasone treatment. *Res. Exp. Med. (Berl.)* **196,** 67–76.

68. Das, D. K., Russell, J. C., and Jones, R. M. (1991) Reduction of cold injury by superoxide dismutase and catalase. *Free Radic. Res. Commun.* **12–13** (Pt 2), 653–662.

69. Chan, P. H., Longar, S., and Fishman, R. A. (1987) Protective effects of liposome-entrapped superoxide dismutase on posttraumatic brain edema. *Ann. Neurol.* **21,** 540–547.

70. Chan, P. H. (1992) Antioxidant-dependent amelioration of brain injury: role of CuZn-superoxide dismutase. *J. Neurotrauma* **9** (Suppl 2), 417–423.

71. Michelson, A. M., Jadot, G., and Puget, K. (1988) Treatment of brain trauma with liposomal superoxide dismutase. *Free Radic. Res. Commun.* **4,** 209–224.

72. Papp, A., Németh, I., Pelle, Z., and Tekulics, P. (1997) Prospective biochemical study of the antioxidant defense capacity in retinopathy of prematurity. *Orv. Hetil.* **138,** 201–205.

73. Niesman, M., Johnson, K., and Penn, J. (1997) Therapeutic effect of liposomal superoxide dismutase in an animal model of retinopathy of prematurity. *Neurochem. Res.* **22,** 597–605.

74. Stone, W. L. (1999). Oxidative stress and antioxidants in premature infants, in *Antioxidant Status, Diet, Nutrition, and Health* (Papas, A. M., ed.), CRC Press, Boca Raton, FL, pp. 277–297.

75. Bernard, G. R. (1991) N-Acetylcysteine in experimental and clinical acute lung injury. *Am. J. Med.* **91,** 54S.

76. Bernard, G. R., Wheeler, A. P., Arons, M. M., Morris, P. E., Paz, H. L., Russell, J. A., and Wright, P. E. (1997) A trial of antioxidants N-acetylcysteine and procysteine in ARDS. The Antioxidant in ARDS Study Group. *Chest,* **112,** 164–172.

77. Shek, P., Suntres, Z., and Brooks, J. (1994) Liposomes in pulmonary applications: physicochemical considerations, pulmonary distribution and antioxidant delivery. *J. Drug Target* **2**, 431–442.

78. Smith, L. and Anderson, J. (1993) Lung retention of phosphatidylcholine and cholesterol from liposomes: effects of oxygen exposure and fasting. *J. Appl. Physiol.* **74**, 1899–1904.

79. Baker, R. R., Czopf, L., Jilling, T., Freeman, B. A., Kirk, K. L., and Matalon, S. (1992) Quantitation of alveolar distribution of liposome-entrapped antioxidant enzymes. *Am. J. Physiol.* **263**, 585–594.

80. Walther, F. J., David-Cu, R., and Lopez, S. L. (1995) Antioxidant-surfactant liposomes mitigate hyperoxic lung injury in premature rabbits. *Am. J. Physiol.* **269**, 613–617.

81. Walther, F., Mehta, E., and Padbury, J. (1996) Lung CuZn-superoxide dismutase and catalase gene expression in premature rabbits treated intratracheally with antioxidant-surfactant liposomes. *Biochem. Mol. Med.* **59**, 169–173.

82. Archer, S. L., Peterson, D., Nelson, D. P., DeMaster, E. G., Kelly, B., Eaton, J. W., and Weir, E. K. (1989) Oxygen radicals and antioxidant enzymes alter pulmonary vascular reactivity in the rat lung. *J. Appl. Physiol.* **66**, 102–111.

83. Freeman, B. A., Turrens, J. F., Mirza, Z., Crapo, J. D., and Young, S. L. (1985) Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase. *Fed. Proc.* **44**, 2591–2595.

84. Padmanabhan, R. V., Gudapaty, R., Liener, I. E., Schwartz, B. A., and Hoidal, J. R. (1985) Protection against pulmonary oxygen toxicity in rats by the intratracheal administration of liposome-encapsulated superoxide dismutase or catalase. *Am. Rev. Respir. Dis.* **132**, 164–167.

85. Barnard, M. L., Baker, R. R., and Matalon, S. (1993) Mitigation of oxidant injury to lung microvasculature by intratracheal instillation of antioxidant enzymes. *Am. J. Physiol.* **265**, L340–L345.

86. Tanswell, A. K. and Freeman, B. A. (1987) Liposome-entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat. *J. Appl. Physiol.* **63**, 347–352.

87. Tanswell, A. K., Olson, D. M., and Freeman, B. A. (1990) Liposome-mediated augmentation of antioxidant defenses in fetal rat pneumocytes. *Am. J. Physiol.* **258**, L165–L172.

88. Tanswell, A. K., Olson, D. M., and Freeman, B. A. (1990) Response of fetal rat lung fibroblasts to elevated oxygen concentrations after liposome-mediated augmentation of antioxidant enzymes. *Biochim. Biophys. Acta* **1044**, 269–274.

89. Briscoe, P., Caniggia, I., Graves, A., Benson, B., Huang, L., Tanswell, A. K., and Freeman, B. A. (1995) Delivery of superoxide dismutase to pulmonary epithelium via pH-sensitive liposomes. *Am. J. Physiol.* **268**, 374–380.

90. Walther, F., David-Cu, R., Supnet, M., Longo, M., Fan, B., and Bruni, R. (1993) Uptake of antioxidants in surfactant liposomes by cultured alveolar type II cells is enhanced by SP-A. *Am. J. Physiol.* **265**, L330–339.

91. Suntres, Z. and Shek, P. (1995) Prevention of phorbol myristate acetate-induced acute lung injury by alpha-tocopherol liposomes. *J. Drug Target* **3**, 201–208.

92. Thibeault, D., Rezaiekhaligh, M., Mabry, S., and Beringer, T. (1991) Prevention of chronic pulmonary oxygen toxicity in young rats with liposome-encapsulated catalase administered intratracheally. *Pediatr. Pulmonol.* **11**, 318–327.

93. Ledwozyw, A. (1991) Protective effect of liposome-entrapped superoxide dismutase and catalase on bleomycin-induced lung injury in rats. I. Antioxidant enzyme activities and lipid peroxidation. *Acta Vet. Hung.* **39**, 215–224.

Prophylactic Protection by *N*-Acetylcysteine Against the Pulmonary Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analogue

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ABSTRACT: Mustard gas exposure causes adult respiratory distress syndrome associated with lung injury. The purpose of this study was to investigate whether an antioxidant, such as *N*-acetylcysteine (NAC), has any protective effect. Guinea pigs were given single exposure (0.5–6 mg/kg body weight) of 2-chloroethyl ethyl sulfide (CEES) as a mustard analogue intratracheally and maintained for various lengths of time (1 h to 21 days). Within 1 h of CEES infusion at 4 mg/kg, high levels of tumor necrosis factor α (TNF-α), ceramides, and nuclear factor κB accumulated in lung and alveolar macrophages. Both acid and neutral sphingomyelinases were activated within 4 h. These signal transduction events were associated with alteration in the oxygen defense system. Within 1 h of exposure to CEES (6 mg/kg body weight), there was 10-fold increase in the ^{125}I-BSA leakage into lung tissue, indicating severe lung injury. Although low level of CEES exposure (0.5 mg/kg body weight) produced symptoms of chemical burn in lung as early as 1 h after exposure, the severity of edema, congestion, hemorrhage, and inflammation increased progressively with time (1 h to 21 days). Feeding of single dose of NAC (0.5 g) by gavage just before the CEES infusion was ineffective to counteract these effects. However, consumption of the antioxidant in drinking water for 3 or 30 days prior to CEES exposure significantly inhibited the induction of TNF-α, activation of neutral and acid sphingomyelinases, production of ceramides, activation of caspases, leakage of ^{125}I-bovine serum albumin (^{125}I-BSA) into lung tissue, and histological alterations in lung. Pretreatment with NAC for 3 and 30 days protected against 69–76% of the acute lung injury. Therefore, NAC may be an antidote for CEES-induced lung injury.

INTRODUCTION

Mustard gas functions as a highly cytotoxic blisterogen in both humans and animals [1]. The primary targets of mustard gas are eyes, skin, and lung. Most mustard gas induced cytotoxicity has been studied in skin or skin-derived cell lines in vitro [2–5]. Previously, the cytotoxic effects of mustard gas were reported to be mainly due to DNA alkylation, leading to vascular leakage, leukocyte infiltration, slow death of basal epidermal cells [6], generation of free radicals [7,8], and activation of protein degradation enzymes [9–11]. On the basis of this information, several groups investigated the beneficial effects of agents that scavenge free radicals and other oxidant species. They monitored the beneficial effects of those agents mainly by showing the recovery from damaged skin [12] or by showing inhibition of the induction of proteolytic enzymes [13]. Favorable response of systemic cortisone and local hydrocortisones on rabbit skin lesions, produced after sulfur mustard application, was obtained by Vogt et al. [6]. In vitro, the lethal effects of sulfur mustard on L-cells were reduced by thiol reagents, viz., dithiothretol and *N*-acetylcysteine (NAC) [14,15]. Protective effects of various antioxidants, viz. vitamin E, glutathione, etc., have been studied and found to be effective to some extent to prevent mustard gas induced tissue damage [16]. Skin damage by sulfur mustard has been reported to be treated successfully with iodine-containing ointments [17,18].

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Recent studies indicated that within 30 min of the mustard gas exposure, induction of tumor necrosis factor α (TNF-α) occurs that initiates a series of signaling events leading to apoptosis [19–21]. Inhibition of the initiation of signaling events has not been previously demonstrated. An effective prophylactic strategy would be to decrease the signal transduction that starts the inflammatory cascade. We have tested different agents to block mustard gas induced signal transduction events in guinea pig lung, including dimethylthiourea (DMT), which others have used to block free-radical-induced lung injury in chronic hypoxia [22]. As NAC was found most effective among the agents we have tested, we have studied in detail the beneficial effects of NAC prophylactic treatment on the chloroethyl ethyl sulfide induced signal transduction events, which lead to lung injury in guinea pigs.

MATERIALS AND METHODS

Chemicals

2-Chloroethyl ethyl sulfide (CEES, Cl—CH_2CH_2—S—CH_2CH_3) NAC, and DMT were obtained from Sigma Chemicals (St. Louis, MO). Ondrox was obtained from LSI America Corp., USA. TNF-α ELISA kit was obtained from BioSource International (Camarillo, CA). ^{14}C-Sphingomyelin (50 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Diacyglycerol kinase (DAG kinase) kit and ^{32}P-γ-ATP were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Oligonucleotides and antibodies for nuclear factor κB (NF-κB) assay were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals and CEES Treatment

Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals were infused intratracheally with single doses of CEES (0.5–6 mg/kg body weight) in ethanol (infusion volume was 100 μL/animal). Control animals were infused with 100 μL of ethanol in the same way. The animals were sacrificed at different time intervals after CEES infusion and lung was lavaged as described [23]. The tissues were stored at −70°C for future use.

Treatment of Guinea Pigs with NAC and Other Drugs

NAC was given to the guinea pigs in three different ways. For single-dose treatment, 5 mg of NAC (in 0.5 ml phosphate buffered saline, pH 7.4 per animal)

was given directly into stomach 10 min before CEES infusion. In another set of experiments (short-term pretreatment), guinea pigs were given NAC in drinking water (0.5 g/(day animal) in 10 mM phosphate buffer, pH 7.4) for 3 days prior to CEES infusion. For long-term pretreatment, each guinea pig received 0.5 g of NAC each day in drinking water containing 10 mM phosphate buffer, pH 7.4, for 30 days before exposing to CEES. Control guinea pigs were given 10 mM phosphate buffer, pH 7.4, for drinking.

Ondrox, a multi-antioxidant complex, was suspended in distilled water and given directly into guinea pig's stomach (34 mg/kg body weight) 10 min prior to CEES infusion (single dose). For short-term pretreatment, ondrox was given to the guinea pigs in the similar way for 3 consecutive days prior to CEES exposure. For some studies, we injected L-glutathione (GSH) into ear veins of guinea pigs (18 μmol/kg body weight in 60 μL of normal saline) 30 min before the CEES infusion. DMT (500 mg/kg body weight) was injected into guinea pigs intraperitoneally for 3 days prior to CEES infusion.

Lung Injury Study

Lung injury was monitored by studying the leakage of ^{125}I-bovine serum albumin (^{125}I-BSA) from lung after CEES exposure [24]. Ear veins of guinea pigs (body weight 350–450 g) were injected slowly with the ^{125}I-BSA solution (8 μCi/animal). CEES (6 mg/kg body weight) was infused into the animals intratracheally 3 h after ^{125}I-BSA injection. Control animals were infused intratracheally with 100 μL of solvent only. After 4 h of the injection of ^{125}I-BSA (i.e. 1 h after CEES infusion), the animals were sacrificed and 1 ml of blood was collected. The chest cavity was opened and lung was perfused with buffer to get rid of any residual blood from the animals individually. The perfused lung was taken out after removal of heart and trachea; the radioactivity content of the lung was monitored in a gamma counter. Lung injury was expressed as permeability index, which was obtained by dividing total radioactive counts in lung by counts in 1 ml of blood from the same animal.

Assay of TNF-α

Lungs were sonicated in Tris buffered saline, pH 7.4, centrifuged at 10,000 rpm for 10 min. TNF-α was measured in the supernatant using TNF-α ELISA kit. Supernatants were added to TNF-α antibody coated micro wells along with biotin conjugated second antibody and incubated for 1 h at room temperature. After washing, the wells were incubated

with streptovadin–horseradish peroxidase complex. The wells were washed and color reactions were carried out using horseradish peroxidase substrate. The color developments were measured using ELISA reader after addition of stop solution. For this study, we sacrificed animals 1 h postexposure to CEES (4 mg/kg body weight), since our previous study indicated that maximum activation of TNF-α in lung occurs 1 h after the CEES infusion [21].

Assay of Acid and Neutral Sphingomyelinases

Both acid and neutral sphingomyelinases were assayed using ^{14}C-sphingomyelin as described by Gulbins and Kolesnick [25]. Briefly, tissue homogenates in neutral (pH 7.4) or acidic (pH 5.0) buffer were centrifuged at 1000 rpm for 5 min to remove nuclear fraction. The supernatants were incubated with ^{14}C-sphingomyelin in acidic (for acid sphingomyelinase assay) or neutral (for neutral sphingomyelinase assay) buffer for 30 min. Following enzyme assay the products were separated by chloroform/methanol/water (2:1:1) extraction. Radioactivity in the upper aqueous layer gave a measure of the sphingomyelinase activity. For this study, we sacrificed animals 4 h after CEES infusion (4 mg/kg body weight), since optimum activation occurs 4 h postexposure to CEES [21].

Assay of Ceramides

Ceramides were assayed in total lipids isolated from lung according to Cao et al. [26] using DAG kinase kit (Amersham Pharmacia Biotech, Piscataway, NJ). Total lipids were extracted from lung tissue by chloroform/methanol (2:1) extraction and dried under nitrogen. Dried lipids were dissolved in imidazole buffer containing n-octyl β-glucopyranoside and DAG kinase. The reaction was initiated by addition of ^{32}P-γ-ATP. Following reaction, the products were separated by thin layer chromatography (TLC) and autoradiographed to visualize ceramide level. Ceramide standard was run side by side to identify ceramides on TLC. The ceramide-containing portions of the TLC were scraped out and counted in scintillation counter after addition of scintillation fluid to estimate ceramide content quantitatively. For this study, we sacrificed animals 1 h postexposure to CEES (4 mg/kg body weight), since optimum activation occurs within 1–2 h of CEES infusion [21].

Mobility Shift Assay for NF-κB Activation

Activation of NF-κB was measured in the nuclear extract from control and CEES exposed lung tissues according to Mackay et al. [27] by gel shift assay. Nuclear fractions were isolated by low-speed centrifugation and extracted with high-salt buffer. The nuclear extracts were incubated with ^{32}P-γ-ATP-labeled oligonucleotide probe for NF-κB (NF-κB in its activated form binds specifically to this oligonucleotide probe). The reaction mixtures were separated by native polyacrylamide gel electrophoresis and autoradiographed to visualize the activation of NF-κB. For this study, we sacrificed animals 1 h after the CEES infusion (4 mg/kg body weight), since optimum activation occurs within 1 h of CEES infusion [2].

Biochemical Assays

Superoxide dismutase (SOD) activity was determined as described by Hyalnd et al. [28]. Glutathione peroxidase (GSH-Px) activity was measured by the method of Flohe and Gunzler [29]. Catalase activity was estimated as described earlier [30]. For this study, we sacrificed animals 4 h postexposure to CEES (4 mg/kg body weight).

Light Microscopy Studies

Histological examinations were carried out as described before [31]. Briefly, a portion of lung tissue from all animals was fixed overnight in neutral buffered formalin, pH 7.5, and embedded in paraffin. Five microns sections were stained with hematoxylin and eosin stain for light microscopy studies. For this study, we sacrificed animals 1 h, 2 h, 6 h, 1 day, 7 days, 14 days, and 21 days postexposure to CEES (0.5 mg/kg). However, for NAC effects, we have studied so far for only two periods postexposure to CEES (1 h for short-term and 21 days for long-term).

Statistical Analysis

Results are presented as mean $\pm$ SEM in the text and figures. Groups ($n = 6$) were subjected to statistical analysis using the Student's t-test. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of NAC Treatment on ^{125}I-BSA Leakage in CEES-Induced Lung Injury

CEES-induced lung injury was expressed as permeability index, which is a measure of ^{125}I-BSA leakage from injured lung. The data presented in Table 1 indicated that about 69% of the lung injury was eliminated

TABLE 1. Effects of NAC Pretreatment on CEES-Induced Lung Injury in Guinea Pigs

Treatment	Permeability Index
Control	0.20 ± 0.04
Control + NAC (3 days)	0.08 ± 0.01
CEES	2.01 ± 0.16
CEES + NAC (single dose)	2.07 ± 0.11
CEES + NAC (3 days)	$0.62 \pm 0.09*$
CEES + NAC (30 days)	$0.48 \pm 0.06*$
CEES + Ondrox (single dose)	2.04 ± 0.03
CEES + Ondrox (30 days)	2.00 ± 0.08
CEES + GSH (single dose)	1.98 ± 0.10

CEES was infused (6 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/(day/animal), for either 3 days or 30 days prior to CEES infusion. Information on treatment with ondrox and GSH is given in text. The lung injury was measured after 1 h of CEES exposure and expressed by permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. Each group had 6 animals. Values are mean $\pm$ SE ($n = 6$). *NAC supplementation in drinking water blocked the CEES-induced lung injury significantly ($p < 0.05$).

by 3 days pretreatment of NAC. A single dose of NAC could not block the CEES-induced lung injury at all. On the other hand, about 76% of lung injury could be blocked by long-term pretreatment (30 days) with NAC. There was no significant protection of lung injury by pretreatment with either ondrox (single-dose or 3 days pretreatment) or GSH (single dose).

Effects of NAC Treatment on CEES-Induced TNF-α Accumulation

Data on the effects of antioxidants on the induction of TNF-α in lung 1 h after CEES exposure are presented in Table 2. Single-dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 62% of TNF-α induction was inhibited by

TABLE 2. Inhibition of CEES-Induced TNF-α Accumulation in Guinea Pig Lung by NAC Treatment

Treatment	Level of TNF-α (pg/mg protein)
Control	20 ± 7
CEES	708 ± 38
CEES + NAC (single dose)	705 ± 24
CEES + NAC (3 days)	$270 \pm 40*$
CEES + NAC (30 days)	$190 \pm 64*$
CEES + Ondrox (single dose)	716 ± 22
CEES + Ondrox (3 days)	610 ± 54
CEES + DMT (3 days)	648 ± 62
CEES + GSH (single dose)	720 ± 41

CEES was injected (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before injection of CEES or with drinking water (0.5 g/(day/animal), for either 3 days or 30 days prior to CEES injection). Information on treatment with ondrox, DMT, and GSH is given in the text. TNF-α was measured after 1 h exposure of CEES. Values are mean $\pm$ SE ($n = 6$). *NAC supplementation in drinking water blocked the CEES-induced accumulation of TNF-α significantly ($p < 0.05$).

3 days pretreatment with NAC. Long-term treatment with NAC gave more protection (inhibition of 73% of TNF-α induction). There was no protection for TNF-α accumulation by single dose of either ondrox or GSH. Three days pretreatment with ondrox and DMT could only block 13 and 8% of the TNF-α induction.

Effects of NAC Treatment on CEES-Induced Sphingomyelinase Activation

Data on the activation of neutral and acid sphingomyelinases are shown in Figures 1A and 1B. The activation of both neutral and acid sphingomyelinases, which was observed 4 h after exposure to CEES (4 mg/kg body weight), was blocked significantly (42 and 46%, respectively) by only 3 days pretreatment with NAC. Thirty days of pretreatment with NAC blocked 49 and 61% of the activation of neutral and acid sphingomyelinases. Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase.

Effects of NAC Treatment on CEES-Induced Ceramide Accumulation

Data on the accumulation of ceramide 1 h after CEES exposure at 4 mg/kg body weight are shown in Table 3. Short-term (3 days) and long-term (30 days) pretreatment with NAC decreased the levels of ceramide by 71 and 77%. However, a single dose of NAC was ineffective in blocking the ceramide accumulation. There was no significant protection from ceramide accumulation by pretreatment (single dose or 3 days) with either ondrox or GSH (data not shown).

Effects of NAC Treatment on CEES-Induced NF-κB Activation

Both short-term (3 days) and long-term (30 days) treatment of NAC significantly ($p < 0.05$) blocked the

TABLE 3. Inhibition of CEES-Induced Ceramide Accumulation in Guinea Pig Lung by NAC Treatment

Treatment	Levels of Ceramide (μg/mg protein)
Control	9 ± 2
CEES	148 ± 14
CEES + NAC (single dose)	146 ± 10
CEES + NAC (3 days)	$39 \pm 8*$
CEES + NAC (30 days)	$28 \pm 12*$

CEES was infused (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/(day/animal), for either 3 days or 30 days prior to CEES infusion). Ceramide accumulations were assayed after 1 h of infusion of CEES. Values are mean $\pm$ SE ($n = 6$). *NAC supplementation in drinking water blocked the CEES-induced accumulation of ceramide significantly ($p < 0.05$).
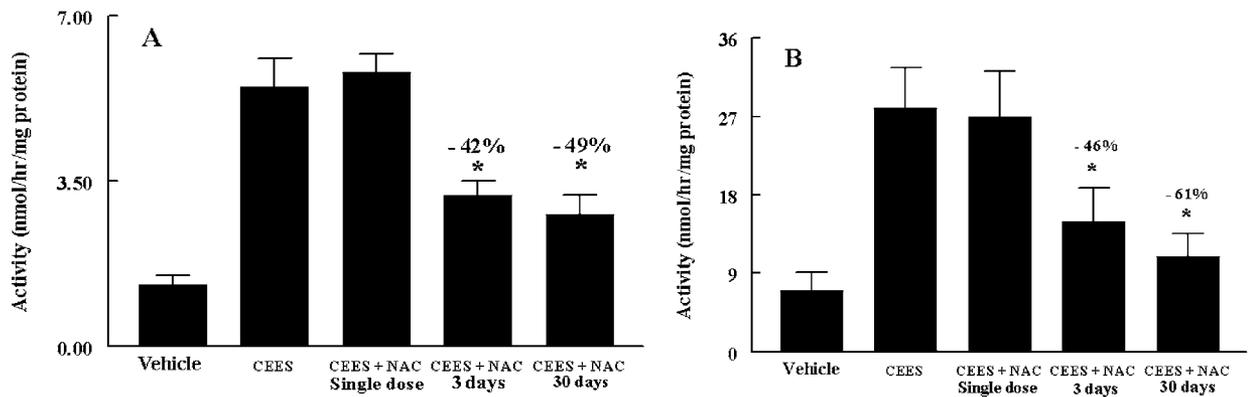
FIGURE 1. Inhibition of CEES-induced activation of neutral and acid sphingomyelinases in guinea pig lung by NAC treatment. Accumulation of neutral (A) and acid (B) sphingomyelinases at 4 h after exposure to CEES (4 mg/kg body weight) and their inhibition by 3 and 30 days pretreatment with NAC. Values are mean ± SE ($n = 6$). Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase. *Pretreatment with NAC for 3 and 30 days prior to CEES exposure caused a significant inhibition of both neutral and acid sphingomyelinases ($p < 0.05$).

CEES-induced activation of NF-κB that was observed 1 h after CEES exposure (Figure 2).

Effects of NAC Treatment on CEES-Induced Caspase Activities

Different caspases (caspase 2, caspase 3, caspase 8, and caspase 9) were activated in lung tissue within 4 h of CEES infusion (Figure 3). Only 3 days pretreatment

with NAC could block the activation of caspase 2, caspase 3, caspase 8, and caspase 9 by 41, 44, 55, and 51%.

Effects of NAC Treatment on the CEES-Induced Changes in the Free-Radical Metabolizing Enzymes

Exposure to CEES significantly inhibited ($p < 0.05$) the activity of SOD (31%), GSH-Px (67%), and catalase (25%) (Figure 4). Pretreatment of guinea pigs for 3 days with NAC before CEES infusion significantly ($p < 0.05$) decreased CEES-induced inhibition of SOD (from −31% to +4.1%), GSH-Px (from −67% to −50%), and catalase (from −25% to −9%). Pretreatment of guinea pigs for 30 days with NAC provided additional
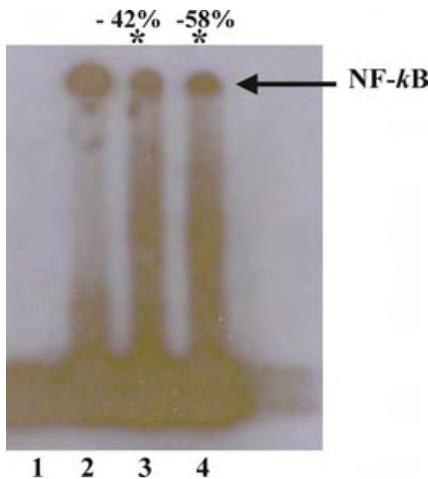

FIGURE 2. Effects of NAC pretreatment on CEES-mediated activation of NF-κB in guinea pig lung. Activation of NF-κB after 1 h of intratracheal infusion of CEES (4 mg/kg body weight) was monitored by mobility shift assay. The guinea pigs were treated with NAC either for 3 days or for 30 days prior to CEES exposure. Panels 1, 2, 3, and 4 represent vehicle, CEES, CEES + NAC (3 days), and CEES + NAC (30 days), respectively. *NAC treatment caused a significant inhibition (46 and 58% for 3 and 30 days, respectively, $p < 0.05$) in the accumulation of NF-κB.
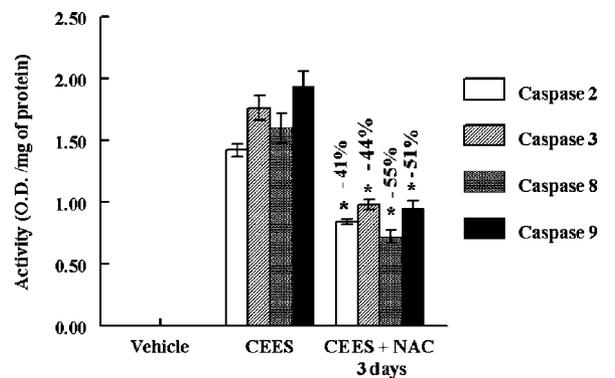

FIGURE 3. Inhibition of the activation of different caspases in guinea pig lung after CEES exposure by pretreatment with NAC. Accumulation of caspases (caspase 2, caspase 3, caspase 8, and caspase 9) at 4 h after exposure to CEES (4 mg/kg body weight) and the prevention of this caspases activation by short-term pretreatment (3 days) with NAC. Values are mean ± SE ($n = 6$). *NAC treatment inhibited the activation of all caspases significantly ($p < 0.05$).
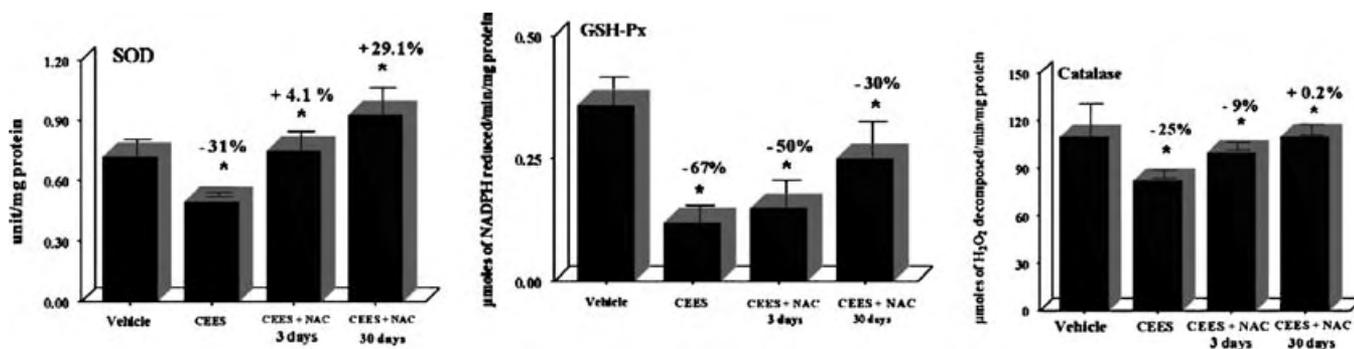
FIGURE 4. Effects of NAC pretreatment on the CEES-induced alterations in the free-radical metabolizing enzymes. Guinea pigs were infused with CEES (4 mg/kg body weight) intratracheally and the free-radical metabolizing enzymes (SOD, GSH-Px, catalase) were assayed in perfused lung after 4 h of CEES exposure. The samples were taken from guinea pigs with or without pretreatment with NAC for 3 days. *CEES exposure caused a significant decrease in the activity of SOD, GSH-Px, and catalase ($p < 0.05$). NAC treatment blocked the CEES-induced changes significantly ($p < 0.05$) for all enzymes.

resistance. For example, the activity of SOD was increased by 29% over the basal value, the activity of GSH-Px was decreased by only 30%, and the activity of catalase was brought back to the basal level.

Effects of NAC Treatment on CEES-Induced Lung Morphology

We studied the morphology of the lung at different time points (1 h, 2 h, 6 h, 1 day, 7 days, 14 days, and 21 days) after exposure to CEES (0.5 mg/kg body weight). Figures 5A and 5B show the effects of NAC after 1 h and after 21 days. For both Figures 5A and 5B, the upper panel represents bronchial region and the lower panel represents the alveoli. Animals exposed to CEES showed symptoms of chemical burn within 1 h; however, the severity of damage progressively increased with time. Severe bronchial constriction with occasional apoptotic nucleus and accumulation of viscid secretion of mucin were observed in CEES-treated animals. Furthermore, both polymorphonucleus and eosinophilic leukocytes migration were observed in both alveoli and bronchi. However, pretreatment with NAC protected lung from all these changes remarkably except mucin secretion.

DISCUSSION

Having examined the signal transduction events that cause lung injury by mustard gas exposure in earlier studies, we wanted to develop antidotes by monitoring the effects of different chemical prophylactics on the mustard gas mediated cell signaling in lung. In these studies, we have used guinea pigs to study mustard gas induced lung injury because guinea pigs are closer to humans than other rodents in so far as

structure and function of lungs are concerned. Furthermore, we have used CEES here to induce lung injury because it is a mustard stimulant commonly used as a surrogate compound in investigations of the mechanism of sulfur mustard activity.

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. Owing 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 detail as that was found to be most effective. 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. [32]. 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
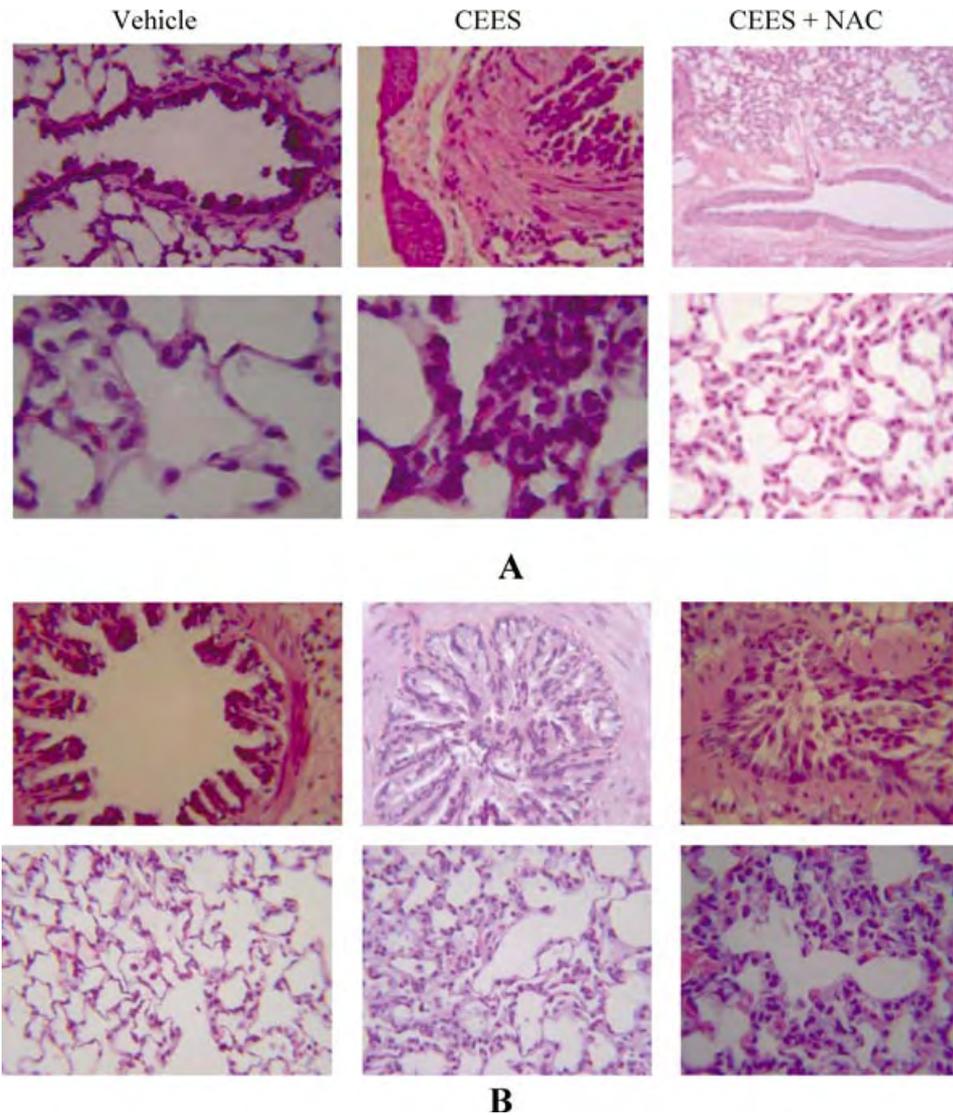
FIGURE 5. Histological analysis showing recovery from CEES-induced lung damage by pretreatment of NAC. Guinea pig lungs were examined under light microscope after 1 h (A) and after 21 days (B) of exposure to CEES (0.5 mg/kg body weight). Upper panel represents morphology of bronchi and lower panel represents morphology of the alveoli. Magnifications: ×400.

was not elucidated in the studies by McClintock et al. [32]. In our study, we have demonstrated that NAC inhibits the production of NF-κB. The protection of lung injury thus may be etiologically related to the inhibition of oxidative activation of the transcription factor NF-κB, which is usually upregulated by stress signals. In fact, Atkins et al. [33] 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-κB.

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 3 days or more 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, nonaddicting, safe, and readily obtainable. It is important to note here that there is no other known example of the downregulation of the activity of NF-κB 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.

REFERENCES

1. Papirmeister B, Gross CL, Meier HL, Petrali JP, Johnson JB. Molecular basis for mustard gas induced vesication. Fundam Appl Toxicol 1985;5:S134–S149.

2. Papirmeister B, Gross CL, Petrali JP, Meier HL. Pathology produced by sulfur mustard in human skin grafts on athymic nude mice: II. Ultrastructural changes. J Toxicol Cutan Ocular Toxicol 1984;3:395–410.

3. Bos JD, Zonneveld I, Das PK, Krieg SR, Van der Loos KM, Kapsenberg ML. The skin immune system: Distribution and immunophenotype of lymphocytes subpopulation in normal human skin. J Invest Dermatol 1987;88:569–573.

4. Eldad A, Ben Mein P, Breiterman S, Chaouat M, Shafran A, Ben-Bassat H. Superoxide dismutase for mustard gas burns. Burns 1998;24:114–119.

5. Graham JS, Bryant MA, Braue EH. Effects of sulfur mustard on mast cells in hairless guinea pig skin. J Toxicol Cutan Ocular Toxicol 1993;10:210–217.

6. Vogt RF, Dannenberg AL, Schofiels B, Hynes N, Papirmeister B. Pathogenesis of skin caused by sulfur mustard. Fundam Appl Toxicol 1984;4:S71–S83.

7. Husain K, Dube SN, Sugendran K, Singh R, Dasgupta S, Somani SM. Effects of topically applied sulfur mustard on antioxidant enzymes in blood cells and body tissue of rats. J Appl Toxicol 1992;16:245–248.

8. Elsayed NM, Omaye ST, Klain GJ, Korte DW. Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection. Toxicology 1992;72:153–165.

9. Cowan FM, Broomfield CA, Smith WJ. Effects of sulfur mustard on protease activity in human peripheral blood lymphocytes. Cell Biol Toxicol 1991;7:239–248.

10. Cowan FM, Yourick JJ, Hurst CG, Broomfield CA, Smith WJ. Sulfur mustard increased proteolysis following in vitro and in vivo exposures. Cell Biol Toxicol 1993;9:269–277.

11. Cowan FM, Anderson DR, Broomfield CA, Byers SL, Smith WJ. Biochemical alterations in rat lung lavage fluid following acute sulfur mustard inhalation: II. Increases in proteolytic activity. Inhal Toxicol 1997;9:53–61.

12. Cowan FM, Broomfield CA. Putative roles of inflammation in the dermato-pathology of sulfur mustard. Cell Biol Toxicol 1993;9:201–213.

13. Cowan FM, Broomfield CA, Smith WJ. Inhibition of sulfur mustard-increased protease activity by niacinamide, N-acetyl cysteine or dexamethasone. Cell Biol Toxicol 1992;8:129–138.

14. Walker IG. Protection of animal cells against mustard gas by pretreatment with dithiothreitol. In: Proc 5th Intern Cong Chemotherapy, Vienna, 1967, p 1063.

15. Walker IG, Smith WJ. Protection of L-cells by thiols against the toxicity of sulfur mustard. Can J Physiol Pharmacol 1969;47:143–151.

16. Kumar O, Sugendran K, Vijayaraghavan R. Protective effects of various antioxidants on the toxicity of sulfur mustard administration by inhalation or percutaneous routes. Chem Biol Interact 2001;134:1–12.

17. Wormser U, Sintov A, Brodsky B, Nyska A. Topical iodine preparation as therapy against sulfur mustard induced skin lesions. Toxicol Appl Pharmacol 2000;15:33–39.

18. Wormser U, Brodsky B, Green BS, Arad-Yellin R, Nyska A. Protective effect of povidone-iodine ointment against skin lesions induced by sulfur mustards and by non-mustard vesicants. Arch Toxicol 1997;71:165–170.

19. Arroyo CM, Von Tersch RL, Broomfield CA. Activation of alpha-human tumor necrosis factor (TNF-alpha) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulfide (HMG). Hum Exp Toxicol 1995;14:547–553.

20. Tsuruta J, Sugisaki K, Dannenberg AM, Yoshmura T, Abe Y, Mounts P. The cytokines NAP-1 (IL-8), MCP-1, IL-1 beta and *gro* in rabbit inflammatory skin lesion produced by the chemical irritant sulfur mustard. Inflammation 1996;20:293–318.

21. Chatterjee D, Mukherjee S, Smith MG, Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide (CEES), a mustard analog. J Biochem Mol Toxicol 2003;17:1–8.

22. Langleben D, Fox RB, Jones RC, Reid LM. Effects of dimethylthiourea on chronic hypoxia-induced pulmonary arterial remodeling and ventricular hypertrophy in rats. Clin Invest Med 1989;12:235–240.

23. Khan AQ, Sikpi MO, Das SK. Phospholipid composition of guinea pig lung lavage. Lipids 1985;20:7–10.

24. Ward PA, Till GO, Kunel R, Beaucham C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J Clin Invest 1983;72:789–801.

25. Gulbins E, Kolesnick R. Measurement of sphingomyelinase activity. Methods Enzymol 2000;322:382–388.

26. Cao LC, Honeyman T, Jonassen J, Scheid C. Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells. Kidney International 2000;57:2403–2411.

27. Mackay F, Majeau GR, Hochman PS, Browning JL. Lymphotoxin receptor triggering induces activation of the nuclear factor κB transcription factor in some cell type. J Biol Chem 1996;271:24934–24938.

28. Hyalnd K, Voisin E, Banoun H, Auclair C. Superoxide dismutase assay using alkaline dimethylsulfoxide as superoxide anion-generating system. Anal Biochem 1983;135:280–287.

29. Flohe L, Gunzler WA. Assay of glutathione peroxidase. Methods Enzymol 1984;105:114–121.

30. Das SK, Nair CR. Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation of normal and sickled erythrocytes. Br J Haematol 1980;44:87–92.

31. Das SK, Mukherjee S, Desai U. Development of pancellular toxicity in guinea pig lung by ingestion of oleylanilide. J Biochem Toxicol 1994;9:41–49.

32. McClintock SD, Till GO, Smith MG, Ward PA. Protection from half-mustard-gas-induced acute lung injury in the rat. J Appl Toxicol 2002;22:257–262.

33. Atkins KB, Lodhi IJ, Hurley LL, Hinshaw DB. N-Acetylcysteine and endothelial cell injury by sulfur mustard. Appl Toxicol 2000;20:S125–S128.

Signal Transduction Events in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, A Mustard Analog

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ABSTRACT: Sulfur mustard has been used as a vesicant chemical warfare agent. To understand the mechanism by which mustard gas exposure causes respiratory damage, we have used 2-chloroethyl ethyl sulfide (CEES) as a mustard analog. Our initial studies have shown that guinea pigs exposed to CEES intratracheally accumulate high levels of TNF-α. Accumulation of TNF-α leads to activation of both acid and neutral sphingomyelinases, resulting in high accumulation of ceramides, a second messenger involved in cell apoptosis. In addition, NF-κB was activated for a short period (1–2 h after exposure) as determined by mobility shift assay. Supershift assays indicated that both p50 and p65 of NF-κB were activated due to CEES exposure. However, NF-κB rapidly disappeared after 2 h. It is possible that the initial activation of NF-κB was an adaptive response to protect the cells from damage since NF-κB is known to inhibit TNF-α/ceramide-induced cell apoptosis. Since NF-κB disappeared after 2 h, the cells continued being damaged owing to accumulation of ceramides and activation of several caspases, leading to apoptosis. © 2003 Wiley Periodicals, Inc. J Biochem Mol Toxicol 17:114–121, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10068

KEYWORDS: 2-Chloroethyl Ethyl Sulfide (CEES); Lung Injury; TNF-α; Sphingomyelinase; Ceramides; NF-κB; Caspases; Apoptosis

INTRODUCTION

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 systems 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 remain 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. Hence, the objective of this study is to understand the downstream signal transduction events in lung following mustard gas exposure.

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 used guinea pigs to understand the mechanisms of

mustard gas mediated lung injury. Insights from these experiments will help us to design drugs to prevent mustard gas induced pulmonary dysfunction.

MATERIALS AND METHODS

Chemicals

2-Chloroethyl ethyl sulfide (Cl—CH$_2$CH$_2$—S—CH$_2$CH$_3$, CEES) was obtained from Sigma Chemicals (St. Louis, MO). TNF-α ELISA kit was obtained from BioSource International (Camarillo, CA). ^{14}C-sphingomyelin (50 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Diacylglycerol kinase kit and ^{32}P-γ-ATP were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Probes and antibodies for NF-κB activation assay were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals and CEES Treatment

Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were intratracheally injected single doses of CEES (0.5 mg/kg body weight) in ethanol (total injection volume was 100 μL per animal). Control animals were injected with 100 μL of ethanol in the same way (i.e. intratracheally). The animals were sacrificed at different time intervals after CEES injection and lungs were lavaged as described before [19]. Alveolar macrophages were obtained from lavaged fluid by centrifugation at 600 × g for 10 min. The cells, tissue, and lavage fluid were flash-frozen in liquid N$_2$, and kept at −70°C for future use.

Assay of TNF-α

Cells or tissue were sonicated in Tris buffered saline (0.05 M Tris-HCl, 0.15 M sodium chloride, pH 7.4), centrifuged at 10,000 rpm for 10 min. TNF-α was measured in the supernatant using TNF-α ELISA kit. Supernatants were added to TNF-α antibody-coated micro wells (supplied with the kit) along with biotin-conjugated second antibody in a buffer containing blocking agent to block nonspecific binding (supplied with the kit) and incubated for 1 h at room temperature. After washing, the wells were incubated with streptovadin–horseradish peroxidase complex. The wells were washed and color reactions were carried out using horseradish peroxidase substrate. The color developments were measured using ELISA reader after addition of stop solution.

Assay of Acid and Neutral Sphingomyelinase

Both acid and neutral sphingomyelinases were assayed using ^{14}C-sphingomyelin as described by Gulbins and Kolesnick [20]. Briefly, tissue homogenates in neutral (100 mM Tris-HCl, pH 7.4) or acidic (100 mM sodium acetate, pH 5.0) buffer were centrifuged at 1000 rpm for 5 min to remove nuclear fraction. The supernatants were incubated with ^{14}C-sphingomyelin in acidic (for acid sphingomyelinase assay) or neutral buffer (for neutral sphingomyelinase assay) for 30 min. Following enzyme assay, the products were separated by chloroform/methanol/water (2:1:1) extraction. Radioactivity of the upper aqueous layer gave a measure of the sphingomyelinase activity.

Assay of Ceramides

Ceramides were assayed in total lipid from lung tissue according to Cao et al. [21] using diacylglycerol kinase (DAG kinase) kit (Amersham Pharmacia Biotech, Piscataway, NJ). Total lipids were extracted from lung tissue by chloroform/methanol (2:1) extraction and dried under nitrogen. Dried lipids were dissolved in imidazole buffer (100 mM imidazole/HCl, pH 6.6) containing 1 mM n-octyl-β-glucopyranoside and DAG kinase (supplied with the kit). The reaction was initiated by addition of ^{32}P-γ-ATP. Following reaction, the products were separated by TLC and autoradiographed to visualize ceramide level. A ceramide standard was run side by side to identify ceramides on TLC. The ceramide-containing portions of the TLC were scraped out and counted in scintillation counter after addition of scintillation fluid to estimate ceramide content quantitatively.

Mobility Shift Assay for NF-κB Activation

Activation of NF-κB was measured in the nuclear extract from control and CEES-exposed lung tissues by gel shift assay, according to Mackay et al. [22]. Nuclear fractions were isolated by low speed centrifugation and extracted with high salt buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1 mM PMSF). The nuclear extracts were incubated with ^{32}P-γ-ATP-labeled oligo nucleotide probe for NF-κB (NF-κB in its activated form binds specifically to this oligo nucleotide probe). The reaction mixtures were separated by native polyacrylamide gel electrophoresis and autoradiographed to visualize the activation of NF-κB. The super shift assay was also carried out using p50 and p65 specific antibodies against NF-κB subunits, as described by Mackay et al. [22].

Assay of Caspases

Caspases were assayed chemically using caspase 2, caspase 3, caspase 8, and caspase 9 assay kits of Bio Source International (CA, USA).

Statistical Analysis

Data were treated statistically using the Student's t test [23]. The variability of data is presented as a mean ± standard error.

RESULTS

Effects of CEES Exposure on TNF-α Levels in Lungs and Lung Macrophages

We have given single intratracheal injection (0.5 mg/kg body weight) of CEES to guinea pigs. At different time points, the guinea pigs were sacrificed and lungs were removed after perfusion. The lung was lavaged and TNF-α concentrations were measured in lung lavage fluid, lung lavage macrophages, and in lung tissue. The level of TNF-α in lavage fluid was very low, whereas a high level of TNF-α accumulated in lung as well as in lung macrophages within 1 h of CEES exposure. The level of TNF-α decreased rapidly after 1 h and came to the normal level within 24 h of CEES exposure (Figure 1A). Further studies revealed that the induction of TNF-α by CEES is dose-dependent: optimal TNF-α accumulation was observed at 2 mg/kg dose of CEES exposure (Figure 1B).

Activation of Sphingomyelinase Activities After CEES Exposure

Both the neutral and acid sphingomyelinase activities increased 4- to 5-fold after CEES treatment. As the control level of acid sphingomyelinase activity (Figures 2B and 2D) was much higher than the control level of neutral sphingomyelinase (Figures 2A and 2C), we could see much higher activity of acid sphingomyelinase compared to neutral sphingomyelinase after CEES treatment. Both neutral and acid sphingomyelinase activities started to increase along with the increase of TNF-α; these activities reached a maximum peak between 4 and 6 h in lung and between 3 and 4 h in macrophages. It is not known at this time whether this difference between lung and macrophages is functionally significant or not. The difference may occur because macrophages were exposed to CEES more quickly than the whole lung tissue, where CEES must diffuse throughout the tissue to give the maximum effect. The sphingomyelinase activities (both neutral and acid) were found to be higher in lavage macrophages than in the lung tissue. It is possible that macrophages are more sensitive to CEES than other cell types in the lung. The level of sphingomyelinase decreased rapidly and returned to near normal level within 24 h. In this case also we found that 2 mg/kg dose of CEES is sufficient to reach the maximum level of both neutral and acid sphingomyelinase activity (Figures 2C and 2D).

Accumulation of Ceramide in Lungs After CEES Exposure

The ceramides accumulation after CEES exposure demonstrated a biphasic pattern. Within 1 h of CEES
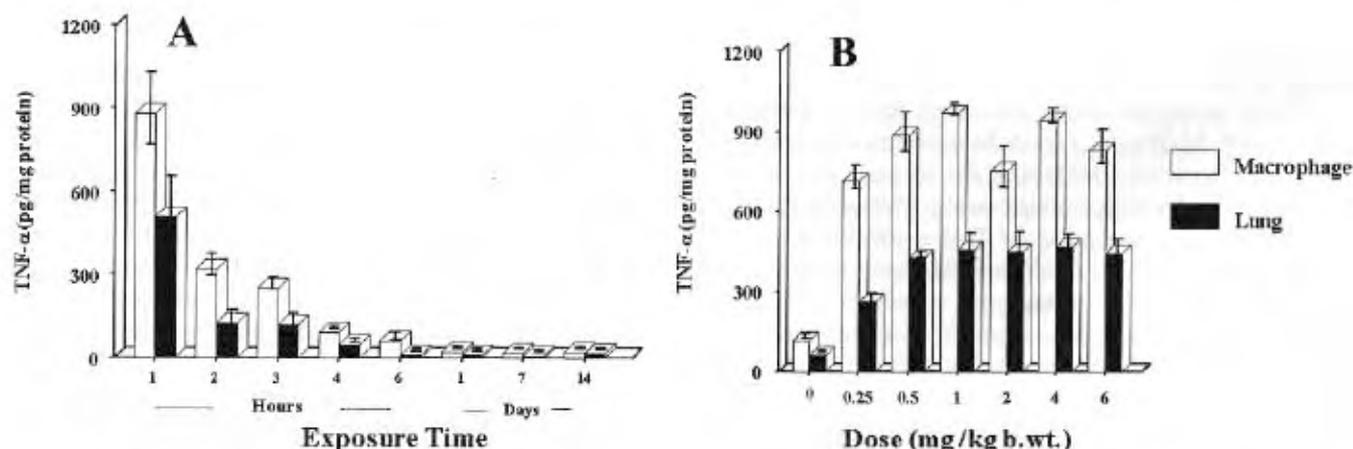

FIGURE 1. Accumulation of TNF-α in guinea pig lung and macrophages after exposure to CEES. A. Time-dependent induction of TNF-α after intratracheal injection of CEES (0.5 mg/kg body wt.). B. Accumulation of TNF-α 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.).
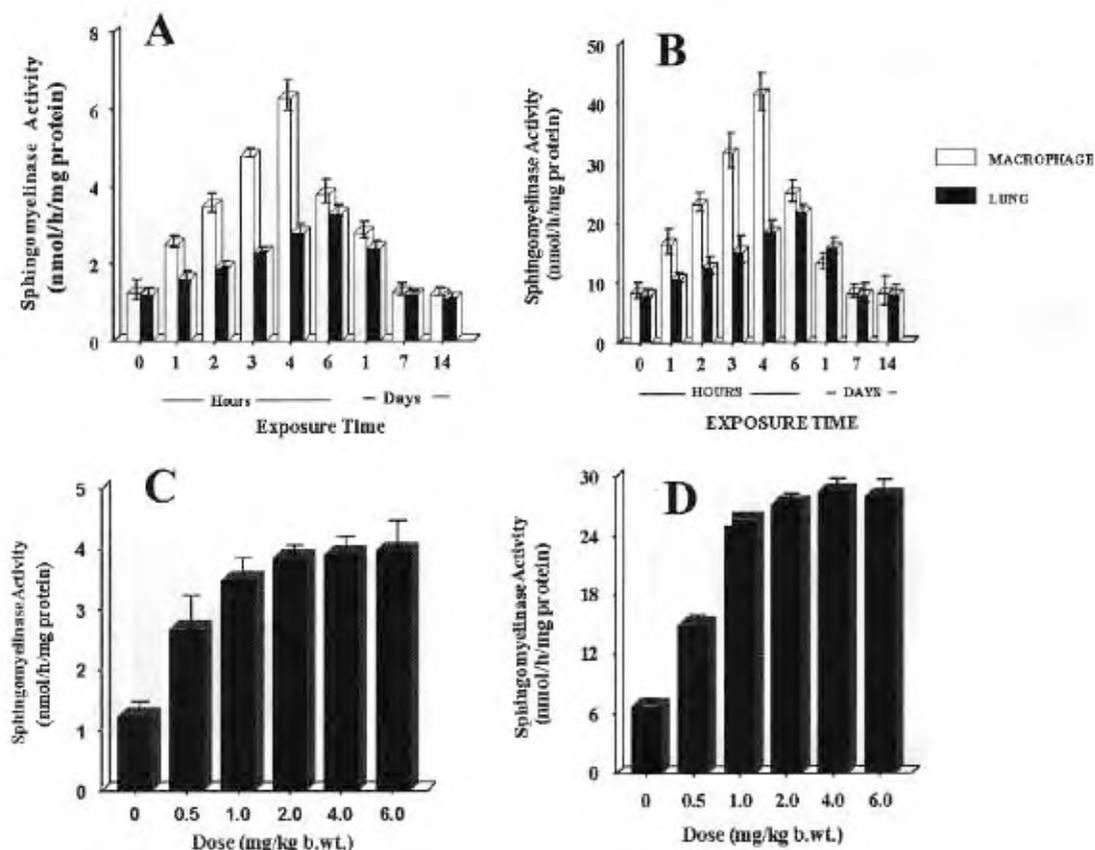
FIGURE 2. Activation of acid and neutral sphingomyelinase in guinea pig lung and macrophages following CEES exposure. Time course of induction of neutral (A) and acid (B) sphingomyelinase after intratracheal injection of CEES (0.5 mg/kg body wt.). Accumulation of neutral (C) and acid (D) sphingomyelinase 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.).

exposure, ceramide levels became very high and reached a peak accumulation within 2 h (Figure 3A). After 2 h, there was some decrease in the ceramide levels but then they increased to a very high level and remained almost at a steady state, even up to 14 days (Figure 3A). CEES-induced ceramide accumulation was found to be saturated at 4 mg/kg dose of CEES. At 2 mg/kg dose of CEES, about 90% induction of ceramide was achieved (Figure 3B).

Activation of NF-κB in Lungs After CEES Exposure

The activation of NF-κB was measured in the nuclear extracts of lungs after exposure to CEES. NF-κB, which is well known to inhibit TNF-α-mediated apoptosis, showed activation only up to 1–2 h after CEES exposure (Figure 4A). This may explain the biphasic effect of CEES on lung. After initial lung damage by TNF-α within 2 h, there was some recovery due to activation of NF-κB. After 2 h, NF-κB level went down, ceramide level increased, and secondary lung damages

were observed. Dose-dependent studies revealed that 4 mg/kg CEES was needed for the optimum activation of NF-κB (Figure 4B). Supershift assay using specific antibodies to p50 and p65 revealed that both the p50 and p65 subunits were activated in lung owing to CEES exposure (Figure 4C).

Activation of Different Caspases in Lungs After CEES Exposure

Figure 5 demonstrates the activation of different caspases in guinea pig lung after CEES exposure. Within 1 h of exposure some activation was observed for all the four caspases, but the activity returned to basal levels within 2 h of exposure (Figure 5A). The activity of caspase 2, caspase 3, caspase 8, as well as caspase 9 increased significantly in between 4 to 6 h of exposure and then went down again. No activity for any of the caspases were observed at 24 h and thereafter (Figure 5A). Here also, the activation of caspases were found to be optimum at 2 mg/kg body weight of CEES (Figure 5B).
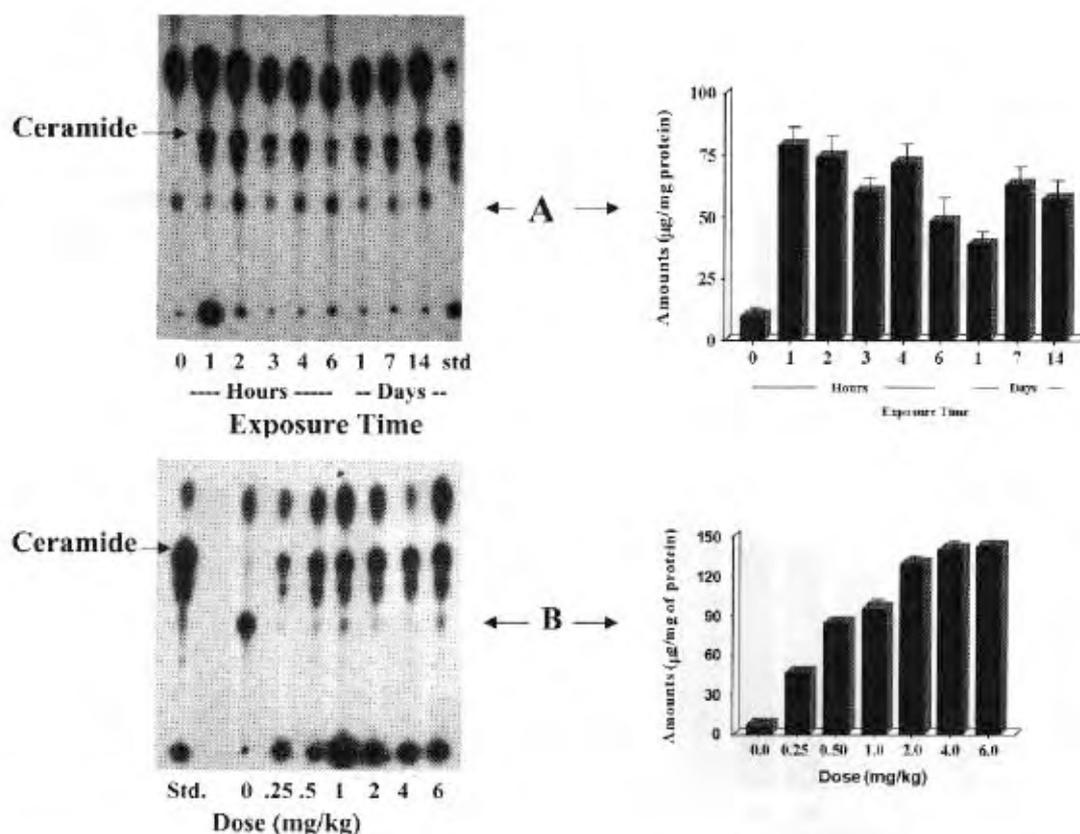
FIGURE 3. Accumulation of ceramide in guinea pig lung after CEES exposure. (A) Time course of induction of ceramide after intratracheal injection of CEES (0.5 mg/kg body wt.). (B) Accumulation of ceramide 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.). In both cases (A and B), left panel is the autoradiograph showing the accumulated ceramides, and the right panel represents the quantitative analysis of accumulated ceramide as determined by ^{32}P incorporation.

DISCUSSION

In these studies, we have used guinea pigs to study mustard gas induced lung injury. As guinea pigs are closer to humans than other primates in so far as structure and function of lungs are concerned, a guinea pig model will be ideal for studying the mechanism of lung injury by mustard gas.

Apoptosis is a genetically programmed, morphologically distinct form of cell death that can be triggered by a variety of physiological and pathological stimuli, leading to dysfunction of the organ [24–27]. Intracellular changes accompanying apoptosis include cleavage of a specific subset of cellular polypeptides, which is accomplished by a specialized family of cysteine-dependent aspartate-directed proteases termed caspases [28]. Exogenous stimuli initiate activation of the cascade of caspases. It is the activation of caspase 3 that ultimately cleaves several important cellular proteins and leads to cell death [29].

The activation of ceramides, sphingomyelin-derived lipid metabolites, in the apoptotic process is

well documented [30–32]. The results presented here 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 1 h of exposure. TNF-α level started declining after 1 h and returned to basal levels within 24 h. After the accumulation of TNF-α, both acid and neutral sphingomyelinase activities were stimulated, and both peaked within 4–6 h after CEES exposure. Although 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 1 h of CEES exposure. However, there was a slight fall in the ceramide level between 3 and 6 h; it increased again to a high level even up to 14 days
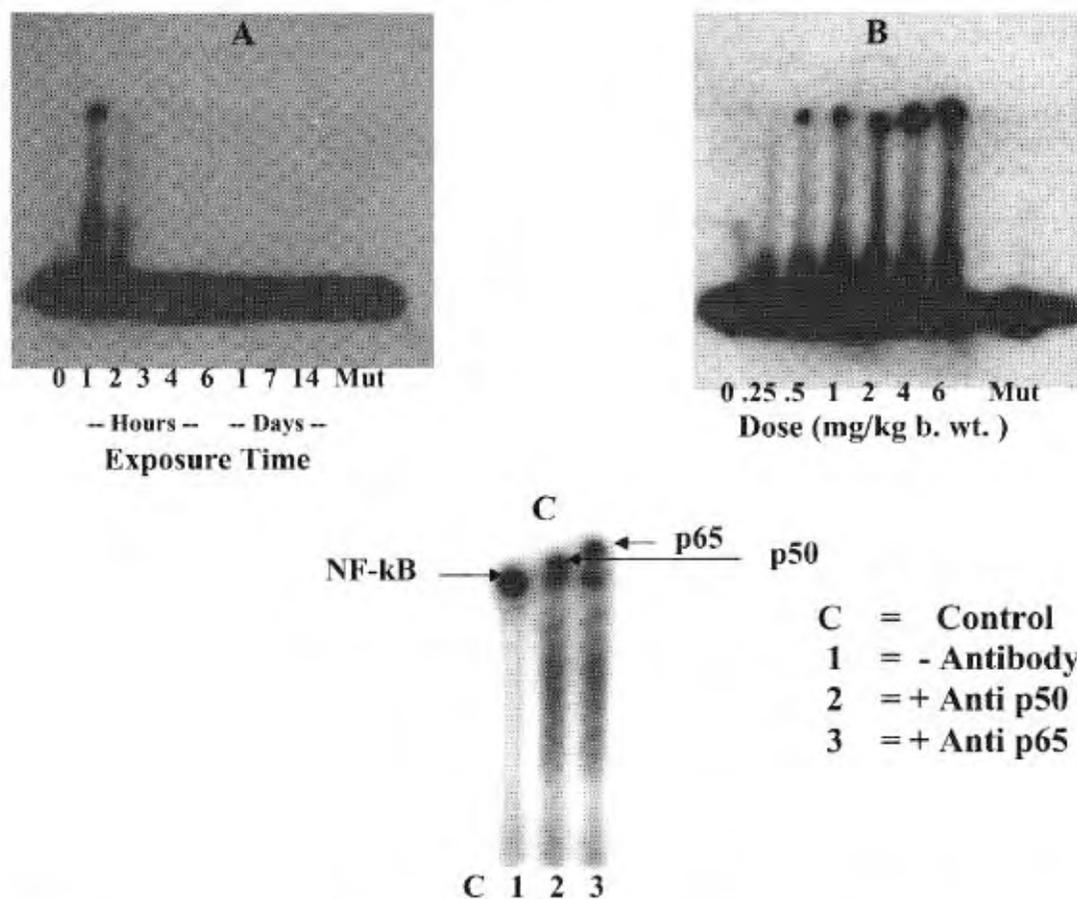
FIGURE 4. Activation of NF-κB in guinea pig lung following CEES exposure. (A) Time-dependent activation of NF-κB after intratracheal injection of CEES (0.5 mg/kg body wt.) as observed by mobility shift assay. (B) Mobility shift assay showing the accumulation of NF-κB after 1 h of CEES exposure at different doses (ranging from 0.5 to 6.0 mg/kg body wt.). (C) Supershift assay, using subunit specific antibodies to identify the subunits (p50 and p65) of NF-κB activated due to CEES exposure in guinea pig lung.

after CEES exposure. It is unknown at this time what is the physiological significance of this 14-day elevation of ceramides.

The slight drop in the ceramide level between 3 and 6 h might have been due to the activation of NF-κB, which showed a sharp transient activation at 1–2 h after CEES exposure. The activation of NF-κB coincided with the increase of TNF-α in lung tissue. It is well known that TNF-α is proapoptotic [33] and NF-κB acts as antiapoptotic by opposing the TNF-α-induced apoptosis [34–36]. Here, we observed a biphasic effect of CEES on lung. After the initial damage by TNF-α, there was some recovery owing to activation of NF-κB within 2 h. 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 h of CEES exposure. This activation of caspases declined thereafter and reappeared in between 4 and 6 h, initiating cell apoptosis in lung as observed by light as well as electron microscopy (photographs not shown). This second phase of caspase activation disappeared

within 24 h 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 [37], where an initial phase of injury after 1 h is followed by a delayed phase, which becomes evident after 8 h 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 [38–40].

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
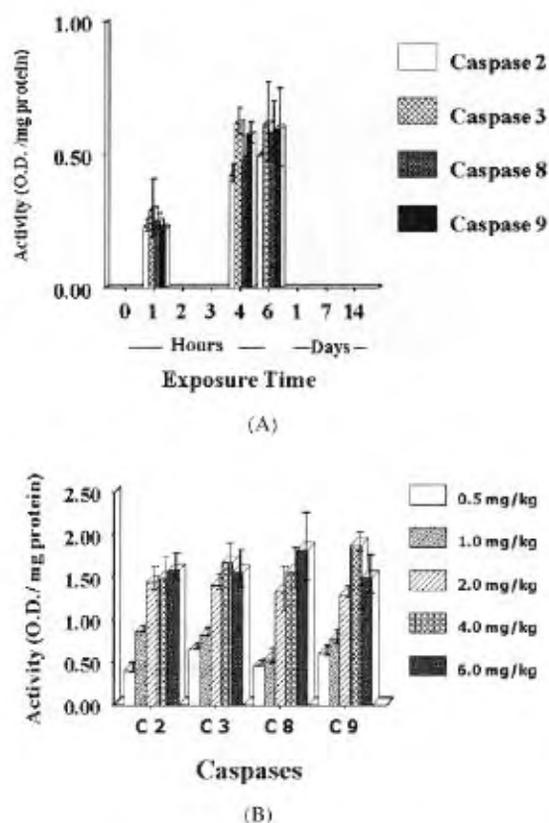
FIGURE 5. Activation of different caspases in guinea pig lung after CEES exposure. (A) Time course of activation of different caspases after intratracheal injection of CEES (0.5 mg/kg body wt.). (B) Accumulation of different caspases 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.).

of mustard gas mediated proapoptotic 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 that 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 that 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.

REFERENCES

1. Debrowska MI, Becks LL, Lelli JL, Levee MG, Hinshaw DB. Sulfur mustard induces apoptosis and necrosis in endothelial cells. Toxicol Appl Pharmacol 1996;141:568–583.

2. Dacre JC, Goldman M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. Pharmacol Rev 1996;48:289–326.

3. Elsayed NM, Ta PN, Korte DW. Biochemical alterations in mouse liver induced by nitrogen mustard. Toxicologist 1989;9:26–34.

4. Elsayed NM, Omaye ST, Klain GJ, Inase JL, Dahlberg ET, Wheeler CR, Korte DW. Response of mouse brain to a single subcutaneous injection of the monofunctional sulfur mustard, butyl-2-chloroethyl sulfide (BCS). Toxicology 1989;58:11–20.

5. Calvet JH, Jarrean PH, Levame M, D'ortho MP, Lorino H, Harf A, Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 1994;76:681–688.

6. Momeni AZ, Enshaelh S, Meghdadi SM, Amindjavaheri M. Skin manifestation of mustard gas. Clinical study of 535 patients exposed to mustard gas. Arch Dermatol 1992;128:775–780.

7. Dixon M, Needham DM. Biochemical research on chemical warfare agents. Nature 1946;158:432–433.

8. Yamakido M, Ishinka S, Hiyama K, Maeda A. Former poison gas workers and cancer: Incidence and inhibition of tumor formation by treatment with biological response modifier N-CWS. Environ Health Perspect 1996;104(Suppl.):485–488.

9. Adachi S, Takemoto K. Occupational lung cancer. A comparison between humans and experimental animals. Sangyo Igaku 1987;29:345–357.

10. Sasser LB, Cushing JA, Dacre JC. Two-generation reproduction study of sulfur mustard in rats. Reprod Toxicol 1996;10:311–319.

11. Elsayed NM, Omaye ST, Klain GJ, Korte DW. Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection. Toxicology 1992;72:153–165.

12. Cowan FM, Broomfield CA. Putative roles of inflammation in the dermatopathology of sulfur mustard. Cell Biol Toxicol 1993;9:201–213.

13. Husain K, Dube SN, Sugendran K, Singh R, Das Gupta S, Somani SM. Effect of topically applied sulphur mustard on antioxidant enzymes in blood cells and body tissues of rats. J Appl Toxicol 1996;16:245–248.

14. Kopff M, Zakrzewska I, Strzelczyk M, Klem J, Dubiecki W. Superoxide dismutase and catalase activity in psoriatic patients treated topically with ointment containing 2-chloroethyl-3-chloropropyl sulfide. Pol J Pharmacol 1994;46:439–444.

15. Kuhns DB, Decarlo E, Hawk DM, Gallin JI. Dynamics of the cellular and humoral components of the inflammatory response elicited in skin blisters in humans. J Clin Invest 1992;89:1734–1740.

16. Arroyo CM, Von Tersch RL, Broomfield CA. Activation of alpha-human tumor necrosis factor (TNF-alpha) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulfide (H-MG). Hum Exp Toxicol 1995;14:547–553.

17. Ricketts KM, Santai CT, France JA, Graziosi AM, Doyel TD, Gazaway MY, Casillas RP. Inflammatory cytokine response in sulfur mustard-exposed mouse skin. J Appl Toxicol 2000;20(Suppl. 1):S73–S76.

18. Stith IE, Das SK. Development of cholinephosphotransferase in guinea pig lung mitochondria and microsomes. Biochem Biophys Acta 1982;714:250–256.

19. Khan AQ, Sikpi MO, Das SK. Phospholipid composition of guinea pig lung lavage. Lipids 1985;20:7–10.

20. Gulbins E, Kolesnick R. Measurement of sphingomyelinase activity. Methods Enzymol 2000;322:382–388.

21. Cao LC, Honeyman T, Jonassen J, Scheid C. Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells. Kidney Int 2000;57:2403–2411.

22. Mackay F, Majeau GR, Hochman PS, Browning JL. Lymphotoxin receptor triggering induces activation of the nuclear factor kB transcription factor in some cell type. J Biol Chem 1996;271:24934–24938.

23. Steel RGD, Torrie JH. In: Principles and Procedures of Statistics: A Biometric Approach, 2nd edition, New York: McGraw-Hill Book Co.; 1980. pp 86–121.

24. Orlov SN, Tremblay J, Deblois D, Hamet P. Genetics of programmed cell death and proliferation. Semin Nephrol 2002;22:161–171.

25. Liversidge J, Dick A, Gordon S. Nitric oxide mediates apoptosis through formation of peroxynitrite and fas/fas-ligand interactions in experimental autoimmune uveitis. Am J Pathol 2002;160:905–916.

26. Puig B, Ferrer I. Caspase-3-associated apoptotic cell death in excitotoxic necrosis of the entorhinal cortex following intraperitoneal injection of kainic acid in the rat. Neurosci Lett 2001;321:182–186.

27. Helal MA, Mehmet H, Thomas NS, Cox PM, Ralph DJ, Bajoria R, Chatterjee R. Ontogeny of human fetal testicular apoptosis during first, second, and third trimesters of pregnancy. J Clin Endocrinol Metab 2002;87:1189–1193.

28. Earnshaw WC, Martin LM, Kaufmann SH. Mammalian caspases: Structure, activation, substrates and functions during apoptosis. Annu Rev Biochem 1999;68:383–424.

29. Nagata S. Apoptosis by death factor. Cell 1997;88:355–365.

30. Kolesnick RN, Kronke M. Regulation of ceramide production and apoptosis. Annu Rev Physiol 1998;60:643–665.

31. Chmura SJ, Nodzenski E, Beckett MA, Kufe DW, Quintans J, Weichselbaum RR. Loss of ceramide production confers resistance to rediation induced apoptosis. Cancer Res 1997;57:1270–1275.

32. Hannun YA, Bell RM. Functions of sphingolipids and sphingolipid degradation break down product in cellular regulation. Science 1976;243:500–507.

33. Shang J, Eberle J, Geilen CC, Hossini AM, Fecker LF, Orfanos CE, Tebbe B. The role of nuclear factor-kappa B and melanogenesis in tumor necrosis factor-alpha-induced apoptosis of normal human melanocytes. Skin Pharmacol Appl Skin Physiol 2002;15:321–329.

34. Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α induced cell death. Science 1996;274:782–787.

35. Xu Y, Bialik S, Jones BE, Iimuro Y, Kitsis RN, Srinivasan A, Brenner DA, Czaja MJ. NF-κB inactivation converts a hepatocyte cell line TNF-α response from proliferation to apoptosis. Am J Physiol 1998;275:1058–1066.

36. Plumpe J, Malek NP, Bock CT, Rakemann T, Manns MP, Trautwein C. NF-κB determines between apoptosis and proliferation in hepatocytes during liver regeneration. Am J Physiol 2000;278:173–183.

37. Vogt RF, Dannenberg AM, Schofield BH, Hynes NA, Papirmeister B. Pathogenesis of skin lesions caused by sulfur mustard. Fundam Appl Toxicol 1984;4:S71–S83.

38. Alphonse G, Aloy MT, Broquet P, Gerard JP, Louisot P, Rousson R, Rodriguez-Lafrasse C. Ceramide induces activation of the mitochondrial/caspases pathway in Jurkat and SCC61 cells sensitive to gamma-radiation but activation of this sequence is defective in radioresistant SQ20B cells. Int J Radiat Biol 2002;78:821–835.

39. Hetz CA, Hunn M, Rojas P, Torres V, Leyton L, Quest AF. Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: Onset of necrosis is associated with delayed ceramide increase. J Cell Sci 2002;115:4671–4683.

40. Hearps AC, Burrows J, Connor CE, Woods GM, Lowenthal RM, Ragg SJ. Mitochondrial cytochrome c release precedes transmembrane depolarization and caspase-3 activation during ceramide-induced apoptosis in Jurkat T cells. Apoptosis 2002;7:387–394.

Evidence of Hair Loss After Subacute Exposure to 2-Chloroethyl Ethyl Sulfide, A Mustard Analog, and Beneficial Effects of *N*-Acetyl Cysteine

Short Communication

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ABSTRACT: Mustard gas has been used as a vesicant chemical warfare agent. However, a suitable biomarker for monitoring mustard gas exposure is not known. We observed that the hairs of the guinea pigs exposed intratracheally to subacute doses of 2-chloroethyl ethyl sulfide (CEES), a mustard analog, came out very easily though there was no sign of skin lesions or skin damage. Also the hairs looked rough and dry and lost the shiny glaze. There was no recovery from this hair loss, though the animals never became hairless, following CEES exposure. Hairs were observed in this study both visually and with light microscopy. Treatment with *N*-acetylcysteine (NAC) prior to CEES exposure could prevent the hair loss completely. Hence, sudden hair loss might be a good biomarker for subacute exposure of mustard gas to subjects at risks when the victims might have no other visible symptom of toxicity. © 2004 Wiley Periodicals, Inc. J Biochem Mol Toxicol 18:150–153, 2004; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20020

KEYWORDS: 2-Chloroethyl Ethyl Sulfide (CEES); Skin; Hair Loss; Biomarker; Guinea Pig

INTRODUCTION

Sulfur mustard is a blistering agent, which has been used as poisonous vesicant chemical warfare agent [1–4]. Exposure to mustard gas at high doses causes immediate hemorrhagic inflammation to the eye, lung, and skin [5,6]. Skin exposed to mustard gas develops edema, vesicle and blister formation, ulceration, necrosis, and desquamation [6–12]. However, there is no proper sign of subacute mustard gas toxicity. Slow poisoning due to low mustard gas exposure has been reported to cause ultimately lung or skin cancer as observed in cases of workers in mustard gas factories [13,14]. Hence, it is essential to search for a good biomarker for subacute mustard gas toxicity. This will help us to determine whether military personnel, who are returning from the battlefield, are exposed to mustard gas, and if so whether they are taking proper medication to prevent further damage due to mustard gas exposure. We have recently reported that *N*-acetylcysteine (NAC), a well-known antioxidant, offers prophylactic protection against the lung injury induced by mustard gas in guinea pigs [15]. This protection may be etiologically related to the inhibition of oxidative activation of the transcription factor NF-κB, which is upregulated by stress signals [15].

The objective of this study was to demonstrate in a guinea pig model that sudden hair loss can be a very good indication for subacute mustard gas toxicity.

MATERIALS AND METHODS

Chemicals

2-Chloroethyl ethyl sulfide (Cl-CH$_2$CH$_2$-S-CH$_2$CH$_3$, CEES), a mustard analog, was obtained from Sigma Chemicals (St. Louis, MO).

Treatment of the Animals with NAC

NAC was given to the animals in drinking water (0.5 g/day/animal in 10 mM phosphate buffer, pH 7.4) for 30 days starting from 3 days prior to CEES injection. Control animals were given 10 mM phosphate buffer, pH 7.4, for drinking.

Animals and CEES Treatment

Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). Animals were intratracheally injected with single doses of CEES (0.5, 1, 2, 4, and 6 mg/kg body weight) in ethanol (total injection volume was 100 μL per animal). Control animals were injected with 100 μL of ethanol in the same way (i.e. intratracheally). Animals were sacrificed after 27 days of CEES exposure.

Light Microscopy Studies

Morphological examinations of skins from experimental and control animals were carried out as described before [16]. Briefly, portions of skin from control and CEES exposed animals (with or without NAC treatment) were fixed overnight in neutral buffered formalin, pH 7.5, and embedded in paraffin. Five micron sections were stained with hematoxylin and eosin stain for light microscopy studies. For this study, we sacrificed animals 27 days post exposure to CEES (0.5 mg/kg).

RESULTS

Hair Loss Due to Subacute Doses of CEES Exposure

Guinea pig hairs are normally bright milky white and silky. When we injected CEES at a dose of either 4 or 6 mg/kg body weight intratracheally, we observed hemorrhagic skin lesions within 48 h and the animals became very sick. However, if the animals received 0.5–2 mg/kg body weight doses, they were not sick and no skin lesions were observed visually. For the subacute exposure, we observed changes only in the hair of the animals. The hairs became rough, lost their silky
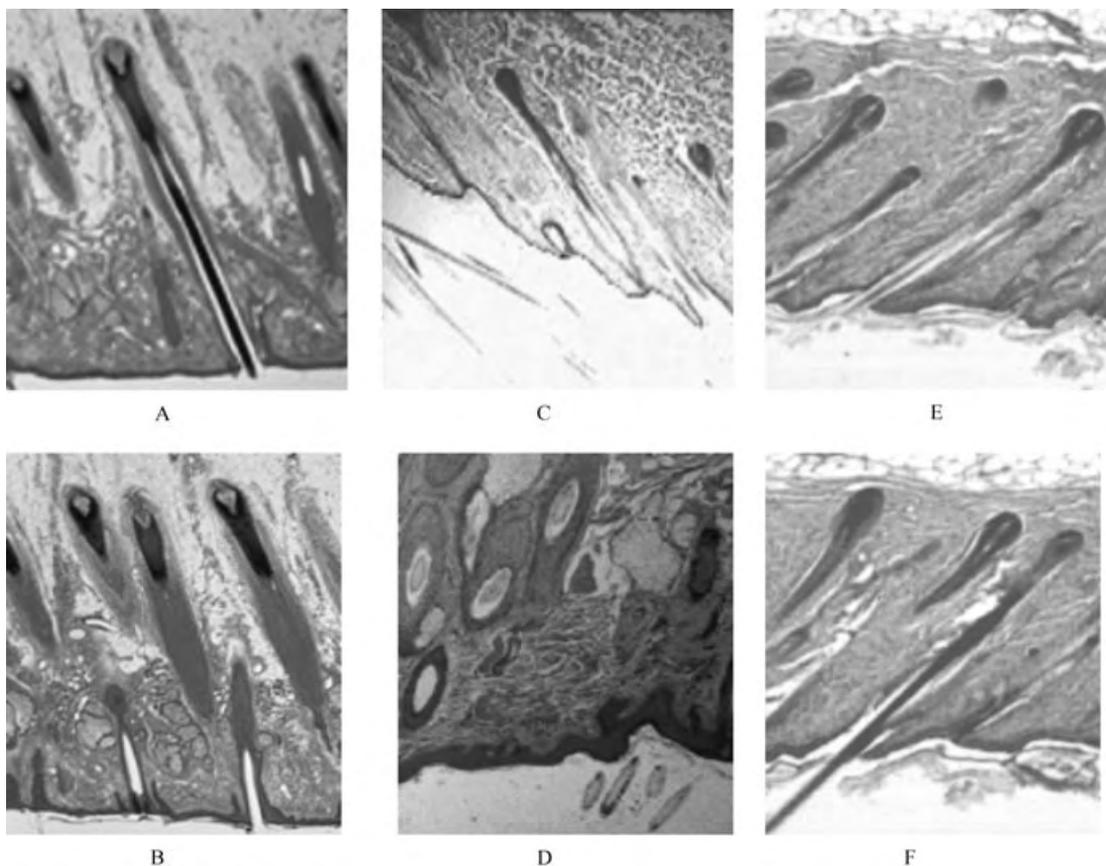
FIGURE 1. Light micrographs of skin sections of the control animals, and those exposed to CEES with or without the NAC treatment. NAC was given to the animals in drinking water (0.5 g/day/animal in 10 mM phosphate buffer, pH 7.4) for 30 days starting from 3 days prior to CEES injection. Control animals were given 10 mM phosphate buffer, pH 7.4, for drinking. Light microscopic studies were done at 27 days post exposure to CEES. A and B: skin from control animals showing healthy and growing hair shafts; C and D: skin from CEES exposed animals showing shedding of hair shaft; E and F: skin sections of the animals exposed to CEES and treated with NAC, showing healthy growing hair shaft as observed for control animals.

appearance, and appeared to be pale yellow colored. The hairs were so loose that lots of hairs came out easily even during very gentle handling of the animals. This type of hair loss is mostly observed in chronic telogen effluvium [17]. Treatment with NAC completely prevented this hair loss.

Light Microscopy Studies of CEES Exposed Skin

Light microscopy studies (Figure 1) showed no evidence of epidermal necrosis or ulceration but dramatic effects were observed in the hair follicles after subacute dose of CEES exposure (0.5 mg/kg body weight). Compared to the healthy mature and growing hair follicles, as seen in the control animals (Figure 1A, 1B), we observed hair follicle regression (catagen) in the CEES exposed animals (Figure 1C, 1D). Furthermore, there was dramatic reduction of proliferation and differentiation of hair matrix keratinocytes with cessation of hair shaft production. This was accompanied with massive apoptosis in the proximal hair follicle epithelium and shortening of the outer root sheath and shortening of hair follicle length. Some of the skin area showed intense degenerative effects of the hair follicle, such as detachment of the inner root sheath, cystic dilation of the hair shaft, and presence of epithelial cells within the lumen (Figure 1D). When the animals were treated with NAC, the skin and the hair shaft looked healthy similar to that observed in the skin of control animals (Figure 1E, 1F).

DISCUSSION

Skin of animals exposed to mustard gas at high doses shows epidermal necrosis and ulceration associated with dermal necrosis and acute inflammation [6]. Hence, this type of acute exposure can be easily identified and the patient can be given proper care immediately. The data presented in our study demonstrate that in case of subacute exposure of mustard gas, there is no visible sign of skin damage. However, the only visible sign of subacute mustard gas intoxication is hair loss, which is associated with shedding of hair shaft and development of hair follicle regression (catagen) as observed for chronic effluvium and alopecia. Such type of hair loss without hair follicle regression (catagen) has been reported in guinea pigs after external application of selenium disulfide [18]. As there is no other visible sign of subacute mustard gas exposure, sudden excessive hair loss might be a very good marker for slow poisoning by mustard gas. Furthermore, our results indicate that subacute mustard gas toxicity can be treated with NAC. Thus, hair loss is a potential biomarker for

subacute exposure of military personnel and civilians to mustard gas exposure. However, further studies are needed to elucidate the mechanisms by which mustard gas exposure induces effluvium and/or alopecia as observed for variety of insults, including stress [19].

REFERENCES

1. Papirmeister B, Gross CL, Meier HL, Petrali JP, Johnson JB. Molecular basis for mustard gas induced vesication. Fundam Appl Toxicol 1985;5:S134–S149.
2. Eisenmenger W, Drasch G, Von Clarmann M, Kretschmer E, Roider G. Clinical and morphological findings on mustard gas [bis(2-chloroethyl)sulfide] poisoning. J Forensic Sci 1991;36:1688–1698.
3. Hochmeister M, Vycudilik W. Morpho-toxicologic findings following war gas effect (S-Lost). Beirt Gerichtl Med 1989;47:533–538.
4. Mohamed-Ali H. Late lesion due to poison gas in survivors of the Iraqi poison warfare against the Kurdish people. Wien Med Wochenschr 1992;142:8–15.
5. Calvet JH, Jarrean PH, Levame M, D'ortho MP, Lorino H, Harf A, Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 1994;76:681–688.
6. Wormser U, Sintov A, Brodsky B, Nyska A. Topical iodine preparation as therapy against sulfur mustard induced skin lesions. Toxicol Appl Pharmacol 2000;15:33–39.
7. Wormser U, Brodsky B, Green BS, Arad-Yellin R, Nyska A. Protective effect of povidone-iodine oinment against skin lesions induced by sulfur mustards and by non-mustard vesicants. Arch Toxicol 1997;71:165–170.
8. Tsuruta J, Sugisaki K, Dannenberg AM, Yoshmura T, Abe Y, Mounts P. The cytokines NAP-1 (IL-8), MCP-1, IL-1 beta and gro in rabbit inflammatory skin lesion produced by the chemical irritant sulfur mustard. Inflammation 1996;20:293–318.
9. Eldad A, Ben Mein P, Breiterman S, Chaouat M, Shafran A, Ben-Bassat H. Superoxide dismutase for mustard gas burns. Burns 1998;24:114–119.
10. Vogt RF, Dannenberg AL, Schofiels B, Hynes N, Papirmeister B. Pathogenesis of skin caused by sulfur mustard. Fundam Appl Toxicol 1984;4:S71–S83.
11. Papirmeister B, Gross CL, Petrali JP, Meier HL. Pathology produced by sulfur mustard in human skin grafts on athymic nude mice. II. Ultrastructural changes. J Toxicol Cutan Ocular Toxicol 1984;3:395–410.
12. Cowan FM, Broomfield CA. Putative roles of inflammation in the dermato-pathology of sulfur mustard. Cell Biol & Toxicol 1993;9:201–213.
13. Yamakido M, Ishinka S, Hiyama K, Maeda A. Former poison gas workers and cancer: Incidence and inhibition of tumor formation by treatment with biological response modifier N-CWS. Environ Health Perspect 1996;104(Suppl.):485–488.
14. Adachi S, Takemoto K. Occupational lung cancer. A comparison between humans and experimental animals. Sangyo Igaku 1987;29:345–357.
15. Das SK, Mukherjee S, Smith MG, Chatterjee D. Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. J Biochem Mol Toxicol 2003;17:177–184.

16. Das SK, Mukherjee S, Desai U. Development of pancellular toxicity in guinea pig lung by ingestion of oleylanilide. J Biochem Toxicol 1994;9:41–49.

17. Whiting DA. Chronic telogen effluvium. Dermatol Clin 1996;14:723–731.

18. Wirth H, Dunsing W, Gloor M. Telogen effluvium following application of selenium disulfide in the guinea pig. Hautarzt 1980;31:502–504.

19. Arck PC, Handjiski B, Peters EM, Peter AS, Hagen E, Fischer A, Klapp BF, Paus R. Stress inhibits hair growth in mice by induction of premature catagen development and deleterious perifollicular inflammatory events via neuropeptide substance P-dependent pathways. Am J Pathol 2003;162:803–814.

Inhibition of Cholinephosphotransferase Activity in Lung Injury Induced by 2-Chloroethyl Ethyl Sulfide, a Mustard Analog

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ABSTRACT: 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 4 h and then decreased at 6 h and stabilized at 7 days in both mitochondria and microsomes. To determine the dose-dependent effect of CEES on CPT activity, we varied the doses of CEES (0.5–6.0 mg/kg b.wt.) and sacrificed the animals at 1 h and 4 h. CPT activity showed a dose-dependent increase of 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. 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 determined by measuring the leakage of iodinated-bovine serum albumin into lung tissue and expressed as the permeability index. CEES exposure (2.0 mg/kg b.wt. for 7 days) caused a significant decrease of both CPT gene expression ($\sim$1.7-fold) and activity ($\sim$1.5-fold) in the lung. This decrease in CPT activity was not associated with any mutation of the CPT gene. 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. 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. © 2005 Wiley Periodicals, Inc. J Biochem Mol Toxicol 19:289–297, 2005; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20092

INTRODUCTION

Mustard gas is a well-known chemical warfare agent and was extensively used in World War I. It exerts local action on the eyes, skin, and respiratory tissues and then impairs the nervous, cardiac, and digestive systems in humans and laboratory animals [1–4]. Previous studies with rats and mice have shown that mustard gas action on the lungs, skin, and other organs involves DNA alkylation; cross-linking of DNA [5]; activation of proteases, resulting in proteolysis of several important enzymes and structural proteins [6];

production of free radicals and induction of free radical-mediated oxidative stress [7,8]; inflammation [9]; and activation of TNF-α, part of the inflammatory cytokine cascade [10,11]. Recently, we demonstrated that the downstream signal transduction events in lung following chloroethyl ethyl sulfide (CEES) exposure involve the induction of TNF-α, which in turn activates both acid and neutral sphingomyelinases, resulting in the subsequent accumulation of ceramides in the lung [12].

Mustard gas exposure also causes inflammatory lung diseases, including acute respiratory distress syndrome (ARDS) [13]. A defective secretion of surfactant by alveolar type II cells has been implicated as one of the causative factors for the development of ARDS [14]. A major component of lung surfactant is dipalmitoyl phosphatidylcholine (DPPC) [15]. The precursor of DPPC is normally 1-palmitoyl-2-oleolyl PC. DPPC is produced by deacylation and subsequent reacylation with palmitic acid at 2-position of glycerol moiety of the unsaturated phospholipid.

The cytidine disphosphocholine (CDP-choline) pathway is the major pathway for the synthesis of PC in the lung and cholinephosphotransferase (CPT) is a terminal enzyme in this pathway. Regulation of PC metabolism is one of the vital aspects of the cell cycle, with implications in the control of cell proliferation as well as in apoptosis [16,17]. It is not known whether mustard gas induces ARDS by exposure is mediated by acting directly on some of the enzymes in the CDP-choline pathway.

Ceramides are intracellular signaling molecules implicated in the induction of cellular apoptosis [18,19] and are known to induce several protein kinases and phosphatases [20–22]. Ceramide analogs have been shown to inhibit PC synthesis [23–26]. Ceramides may directly affect the biosynthesis of PC and phosphatidylethanolamine by inhibiting the enzymes of the CDP-choline and CDP-ethanolamine pathways [23,27,28]. In the present study, our objective was to investigate whether lung injury associated with CEES exposure causes a decrease in the activity of CPT and whether this decrease of CPT activity is mediated by accumulation of ceramides.

MATERIALS AND METHODS

Animals and CEES Treatment

Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight (b.wt.)) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). To determine the optimum treatment time, a single dose (0.5 mg/kg b.wt.) of 2-CEES (Cl-CH$_2$CH$_2$-S-CH$_2$CH$_3$, Sigma, MO) in 100% ethanol (infusion volume was 100 μL/animal) was given intratracheally to the animals. The animals

were then sacrificed after intervals of 1, 2, 4, and 6 h, and 1, 7, 14, and 21 days. For dose-dependent studies, animals were intratracheally infused with single injection of different doses (0.5, 1, 2, 4, and 6 mg/kg b.wt.) of CEES in ethanol. Control animals were infused with 100 μL of vehicle (100% ethanol). The animals were sacrificed after 1 h and 4 h of CEES infusion. Lungs were perfused with 2.6 mM phosphate buffered saline containing 5.6 mM glucose, removed from the chest cavity and immediately flash frozen in liquid nitrogen and stored at $-80°C$ for further analysis.

Since the aim of the present study was to look for the long-term effects of CEES which might lead to the development of ARDS, we performed further experiments after 7 days incubation with 2 mg/kg dose, because higher doses caused irreparable lung damage (burns) and cannot be incubated for a longer time period.

Lung Injury Study

Lung injury was monitored by studying the leakage of ^{125}I-bovine serum albumin (^{125}I-BSA) from lung after CEES exposure as described previously by us [29]. Ear veins of guinea pigs were injected slowly with 4 μL (8 μCi/animal) of ^{125}I-BSA (Amersham, NJ) solution. CEES (2 mg/kg b.wt.) was infused into the animals intratracheally 3 h after ^{125}I-BSA injection. Control animals were infused intratracheally with 100 μL of solvent only. After 4 h of injection of ^{125}I-BSA (i.e., 1 h after CEES infusion), the animals were sacrificed and 1 mL of blood was collected. The chest cavity was opened and lung was perfused with buffer. The perfused lung was taken out; the radioactivity content of the lung was monitored using a gamma counter. Lung injury was expressed as a permeability index, which was obtained by dividing total radioactive counts in the lung by counts in 1 mL of blood from the same animal.

Subcellular Fractionation

Lung was homogenized in four volumes of 0.25 M sucrose/1 mM EDTA (pH 7.4), in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at $600 \times g$ for 10 min in a refrigerated Sorvall RC-5 centrifuge using an SS-34 rotor to remove the nuclear fraction, then at $10,000 \times g$ for 10 min to obtain the mitochondrial fraction. The supernatant obtained was then centrifuged at $105,000 \times g$ for 60 min in a Beckman L8-M ultracentrifuge using a 70.1 Ti rotor to obtain the microsomal fraction as described by us previously [30].

Assay of CPT Activity

CPT activity was measured in the mitochondrial and microsomal fractions by monitoring the

incorporation of [methyl-^{14}C] CDP-choline into PC, according to our previously published method [31]. The final reaction mixture contained: 10 mM MgCl$_2$, 5 mM reduced glutathione, 50 mM Tris-HCl (pH 8.5), 80 μM [methyl-^{14}C] CDP-choline (American Radioligand, St. Louis, MO, specific activity 52.5 Ci/mol), 6 mM of 1,2-dioleolyl-glycerol, and the protein in the total volume of 100 μL. The reaction was started by adding 10 μL of samples (20 μg protein) and incubated at 37°C for 2 min. The reaction was stopped by adding 550 μL of *n*-butanol. Lipids were extracted by adding 500 μL of butanol-saturated water. The mixture was allowed to equilibrate for 10 min and centrifuged at 800 × *g* for 10 min. A measure of 350-μL butanol layer was removed and carefully placed in counting vial. The radioactivity was determined after adding 5 mL Universal Cocktail (ICN Radiochemicals, Irvine, CA) and counted in a Beckman LS-355 scintillation counter.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from lung using a Qiagen RNAEASY kit, and treated with DNAse to remove genomic DNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using 5 μg of RNA from all the samples, using one-step RT-PCR kit (Invitrogen, MD). The primers were synthesized based on the sequences from the GenBank Accession No. NM_020244. Two degenerate primers were used. The primer sequences were 5′-GCNCANATGTGGGAYTAYAC-3′ (forward, Y = C, T; N = A, T, C, G) and 5′-CTCTTCARTCCATRTTRTT-YTGRTG-3′(reverse, R = A, G; Y = C, T) with expected product size of 600 bp. RT-PCR products were electrophoresed on a 1% agarose gel and were purified (QIAquick PCR purification kit, Qiagen, CA) and sequenced using BidDye-terminators kit (Applied Biosystems, CA). The sequences were analyzed using Applied Biosystems Automated sequencer (ABI 3700 model). An alpha imager (Alpha Innotech Corporation, CA) quantitation of the band intensities was also obtained.

Northern Blot Analysis

Total RNA (30 μg) was loaded on a 1.6% agarose gel containing 20% formaldehyde and 1× MOPS [3-(N-morpholino) propane sulfonic acid]. Electrophoresis was allowed to proceed for 3–4 h at 100 V, after which the RNA was transferred overnight onto a BrightStar membrane (Ambion, TX) in 20 × sodium chloride–sodium citrate and UV cross-linked. cDNA obtained by RT-PCR was ^{32}P-labeled (Amersham, NJ)

with the DNA polymerase Nick Translation kit (Invitrogen) and used as probe. Following prehybridization, the filter was hybridized overnight at 42°C with the ^{32}P-labeled CPT probe in ultrahyb buffer (Ambion, TX). Following stringent washes, the blots were exposed to X-ray film and the quantitation of band intensities was done using an alpha imager (Alpha Innotech Corporation, CA). Blots were then stripped and rehybridized with ^{32}P-labeled guinea pig-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. CPT band intensities were normalized with GAPDH band intensities for all the samples.

Treatment of Microsomal Fraction with Ceramides

To determine the effect of ceramides on CPT activity, *N*-acetyl-sphingosine (C$_2$ ceramide, Calbiochem, CA) at different concentrations (25, 50, 100, and 200 μg in 10 μL volume) was added directly into CPT assay mixture (for 0 min incubation) or preincubated with the microsomal fraction at different time points (0.5, 1, and 6 h) at 37°C.

Statistical Analysis

Differences between control and CEES-treated samples were assessed by using Student's *t*-test, and the significance level was $p \leq 0.05$.

RESULTS

Effects of CEES Exposure on Mitochondrial and Microsomal CPT Activity in Lung

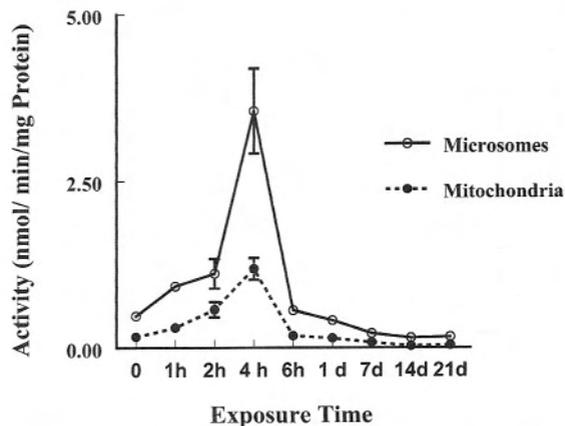Figure 1 shows the effects of CEES at a single dose (0.5 mg/kg b.wt.) on both mitochondrial and

FIGURE 1. Time-dependent effect of 0.5 mg/kg b.wt. CEES treatment on mitochondrial and microsomal CPT activity. $N = 3$.

microsomal CPT activity over time. These results indicate that CPT activity gradually increases in a time-dependent manner for exposure up to 4 h, decreases sharply at 6 h, and then continually decreases at a slow rate up to 21 days post-treatment. Thus, it is evident that there are two distinct phases in the effects of CEES. In the acute phase (up to 4 h), CPT activity increases, whereas in the chronic phase of post-treatment (6 h to 21 days), the CPT activity decreases. The effect is more pronounced in the microsomal fraction than in the mitochondrial fraction.

Figures 2A and B show the dose-dependent effects of CEES (0, 0.5, 1, 2, 4, and 6 mg/kg b.wt.) on mitochondrial and microsomal CPT activity after 1 h (Figure 2A) and 4 h (Figure 2B). At both time periods, CPT activity increased in a dose-dependent manner up to 2 mg/kg b.wt. and decreased at higher doses (4 and 6 mg/kg b.wt.). However, the effect was more pronounced in the microsomal fraction than in the mitochondrial fraction. Thus CEES has a biphasic effect on both mitochondrial and microsomal CPT activity in lung.
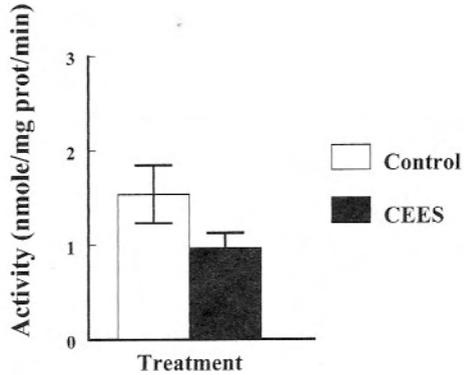

FIGURE 3. CPT enzyme activity in the lung microsomal fraction of 2 mg/kg b.wt. CEES (for 7 days) treated lung as compared to the control (only vehicle) showing significant decrease in activity. $p \leq 0.05$, $N = 5$.

A significant decrease in microsomal CPT activity ($p \leq 0.05$) was observed after 7 days of 2 mg/kg CEES infusion (Figure 3).

Effects of CEES Exposure on Lung Injury

A fivefold increase in lung permeability was observed 1 h after exposure to CEES (2 mg/kg b.wt.), indicating a high degree of immediate lung injury (Figure 4). A similar result was previously observed by us using 0.5 mg/kg b.wt. CEES treatment [32]. The electron micrographs indicate that CEES causes irregular expansion of alveolar spaces, structural changes in the alveolar type II cells, and accumulation of lamellar bodies as a result of disruption of surfactant secretion (Figure 5).

Effects of CEES Exposure on Lung CPT Expression

RT-PCR from both control and CEES-treated lung yielded a product of 0.6 Kb cDNA (Figure 6). No

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. $N = 3$.
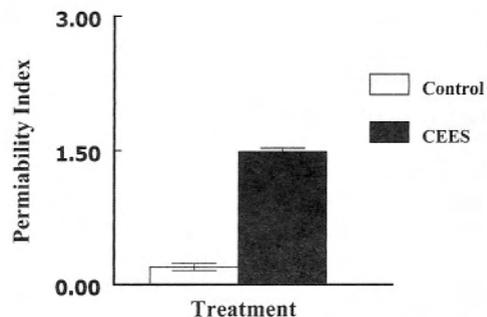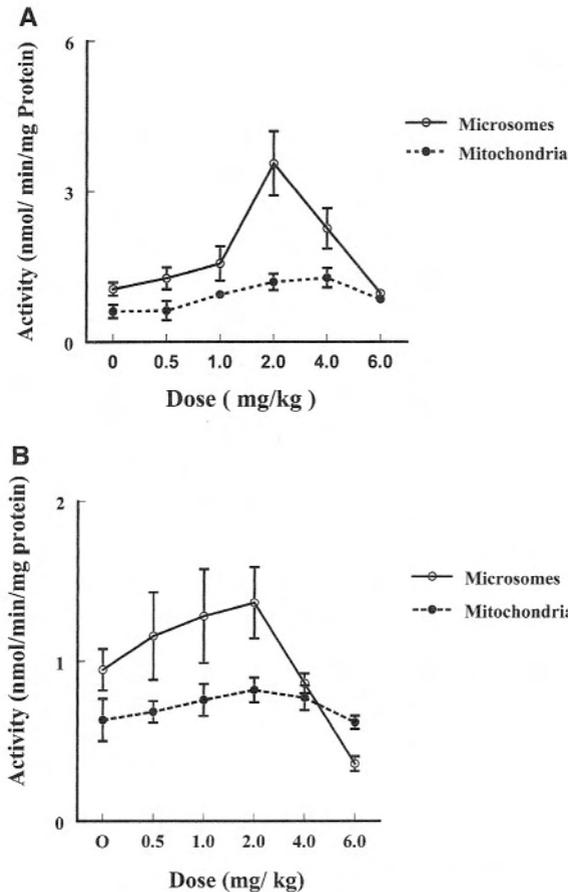

FIGURE 4. Lung injury measured by leakage of ^{125}I from lung. Animals were exposed to 2 mg/kg b.wt. CEES for 1 h. $p \leq 0.05$, $N = 3$.
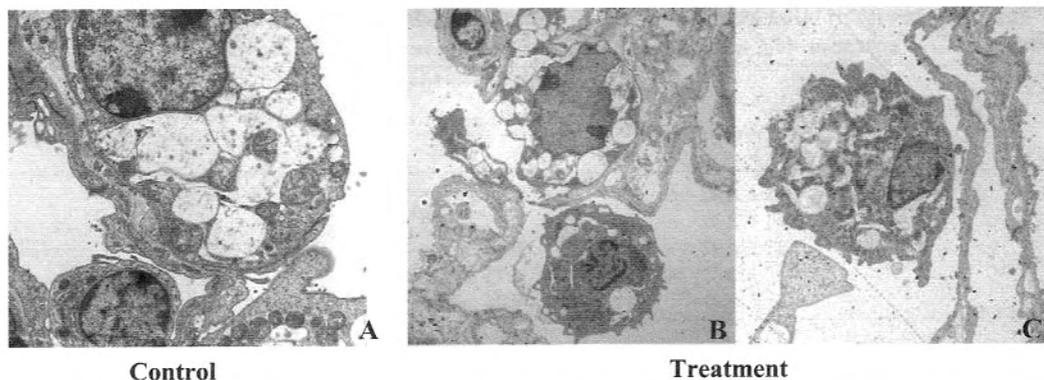
FIGURE 5. Electron micrograph of the lung. The control (A) lung (4400×) shows smooth surfactant layer and uninjured alveolar type II cells and mustard gas-treated lung, (B) (3400×) and (C) (5600×) showing irregular expansion of alveolar spaces, structural changes in the alveolar type II cells, and accumulation of lamellar bodies as a result of disruption of the surfactant secretion.
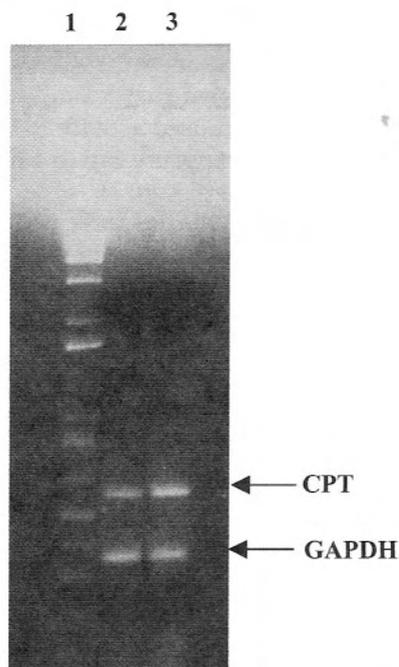
FIGURE 6. Reverse transcriptase-polymerase chain reaction. A representative gel showing 0.6 Kb product of CPT and 0.3 Kb product for GAPDH. Lane 1: 100 Kb plus marker; lane 2: CEES treated; lane 3: Control. $N = 5$.

FIGURE 7. Northern blot analysis for the expression of the CPT gene. (A) One representative blot for CPT mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (B) Graph showing down-regulation of CPT expression as the result of mustard gas treatment normalized with GAPDH. $N = 3$.

mutation was observed in the nucleotide sequence of this 0.6 Kb cDNA fragment of CPT gene as a result of the CEES treatment. However, a down-regulation in CPT gene expression is evident from the Northern blot analysis as shown in Figures 7A and 7B. Figure 7A depicts the blot of ~0.6 Kb CPT mRNA and Figure 7B shows the values after normalizing with the values of GAPDH in both control and CEES-treated samples ($N = 3$).

Effects of Ceramide Treatment on Lung Microsomal CPT Activity

When the lung microsomal fraction from control animals was incubated with C_2 ceramide at different concentrations (50, 100, and 200 μM) and time periods (0, 0.5, 1, and 6 h) prior to the assay for CPT activity, CPT activity decreased significantly in a time- and

TABLE 1. CPT Enzyme Activity in Lung Microsomal Fraction in the Presence of Different Concentration of C_2 Ceramide. Table Showing Effect of Both Concentration of Ceramide and Incubation Time on the CPT Enzyme

	Specific Activity (nmol/mg protein/min) Incubation Time (h)			
C_2 Ceramide Conc. (μM)	0	0.5	1	6
0	0.36 ± 0.02	0.36 ± 0.03	0.27 ± 0.01	0.17 ± 0.04
25	0.40 ± 0.02	0.28 ± 0.02	0.26 ± 0.01	0.16 ± 0.02
50	0.27 ± 0.02	0.26 ± 0.04	0.19 ± 0.01	0.10 ± 0.02
100	0.27 ± 0.02	0.23 ± 0.02	0.18 ± 0.01	0.08 ± 0.02
200	0.28 ± 0.01	0.24 ± 0.01	0.16 ± 0.02	0.10 ± 0.01
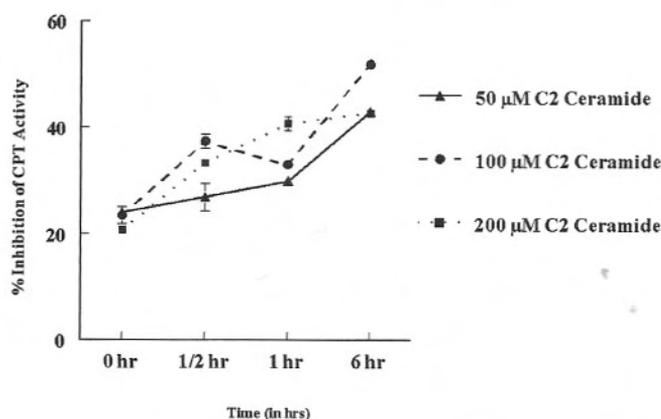
FIGURE 8. Percent inhibition of the CPT enzyme activity as the result of C_2 ceramide treatment of the lung microsomal fraction. $N = 3$.

dose-dependent manner (Table 1, Figure 8). However, the effect was more pronounced when the microsomal fraction was preincubated with the ceramide prior to assay of the CPT activity. The degree of inhibition was increased with the increase in incubation time (0.5, 1, and 6 h). Highest inhibition (50%) was achieved after 6 h of incubation. However, only 20% inhibition was observed when ceramide was directly added into the assay mixture.

DISCUSSION

In the present study, we have used a guinea pig model to study CPT enzyme activity in lung injury induced by a mustard gas analog, CEES. Guinea pigs are close to humans in their lung development and function [33], and therefore are an ideal animal model for studying lung injury induced by mustard gas and its subsequent long-term effects on lung function.

PC is the most abundant phospholipid in mammalian cells and it is synthesized via the CDP-choline pathway [34]. CPT is the terminal enzyme of this pathway and plays a direct role in the final production of PC in lung. This pathway is important for both cell proliferation and cell death [35,36], and selective inhibition of this pathway has been shown to induce cellular apop-

tosis [37]. Any modulation in the expression and/or activity of this enzyme is expected to result in abnormal functioning of the cells. In the present study, an initial experiment was done to study the time-dependent effects of CEES treatment at a low dose (0.5 mg/kg b.wt.). This study showed a biphasic effect on CPT activity in both mitochondria and microsomes. The time-dependent studies indicated that a single infusion of CEES (0.5 mg/kg b.wt.) caused activity to increase for a short time after CEES exposure (up to 4 h), and then to decrease (6 h onwards) (Figure 1).

The dose-dependent studies indicated that CEES treatment caused an initial increase in the CPT activity at low doses (0–2 mg/kg b.wt.) followed by a decrease at higher doses (4 and 6 mg/kg b.wt.) at incubation times of 1 h and 4 h. This decrease was more acute in microsomes than that in mitochondria (Figure 2). We have previously demonstrated that in addition to its predominant localization in the microsomes, CPT exists also in mitochondria [38,39]. Thus, it is possible that during early stage of lung injury as observed in this study, cells try to repair the membrane damage by stimulating PC synthesis and therefore increased CPT activity, but with time lung cells lose their ability to repair membrane damage as evident from decreased CPT activity in both mitochondria and microsomes isolated from lung of CEES-treated animals (Figures 1 and 2). Hence, CEES has both short-term (stimulation) and long-term (inhibition) effects on lung CPT activity. Since CPT activity is crucial to synthesis of surfactant, these effects may cause ARDS after long term of CEES exposure due to lack of surfactant synthesis.

In our previous study [12], we reported that CEES exposure causes induction of the TNF-α cascade associated with accumulation of ceramides in the lung. TNF-α is not only involved in various cellular functions including proliferation, differentiation, and programmed cell death; it also alters pulmonary surfactant lipid metabolism and modifies surfactant biophysical properties [40,41]. The biphasic modulation of CPT activity, as observed in the present study, is similar to the effects of CEES on ceramide accumulation

in lung reported earlier by us [12]. Thus, the initial increase in the CPT enzyme activity can be termed as the acute effect of CEES exposure, which also corresponds to the induction of various cytokines like TNF-α and NF-κB as observed by us previously [12]. But, if we look at the long-term effects of CEES, which may be considered as the chronic effect, CPT activity is significantly decreased. CPT activity showed a significant ($p \leq 0.05$) decrease in the lung microsomal fraction at 2 mg/kg b.wt. dose of CEES after 7 days. Thus, we can predict that as its chronic effect, CEES is effecting the lung surfactant production by modulating the CPT enzyme.

Similar to our earlier finding [32], we observed in this study an immediate and severe injury to lung epithelium by CEES exposure. This was further evident from the electron micrographs of the lung, which showed clear morphological changes in the type II alveolar cells and disruption in their secretory function due to accumulation of lamellar bodies (Figure 5). These cells are responsible for the secretion of lung surfactant, and any injury to these cells is expected to cause a modulation in surfactant secretion.

In our present study, we investigated whether the decrease in CPT enzyme activity in the CEES exposed group results from mutation of the CPT gene. We obtained a ~600 bp CPT cDNA from lung of both control and CEES-treated animals (Figure 6). Nucleotide sequencing of this 0.6 kb fragment showed no random point mutations ($N = 5$) as a result of the CEES treatment. However, Northern blot analysis, using this 0.6 kb fragment as probe from both control and CEES-exposed lung, showed a 40% down-regulation in the CPT gene expression as the result of the CEES treatment (Figure 7). These observations suggest that there may be a genetic control operating, which results in the reduction of the CPT gene expression in the lungs of the CEES-treated animals.

It has been reported that cells treated with ceramides may undergo programmed cell death, become growth arrested, or in rare cases, become stimulated to proliferate. The diversity of biological responses of cells to ceramides reflects the complexity of the role of these sphingolipids as second signal molecules [42]. Furthermore, ceramide treatment of lung cancer-derived A-549 cells promotes apoptosis in a caspase-dependent process [43]. Our previous study [12] showed dose-and time-dependent accumulation of ceramides and activation of several caspases as the result of CEES treatment. The level of ceramide was found to be ~60 μg/mg protein after 7 days (with 0.5 mg/kg b.wt. of CEES) and ~130 μg/mg protein for 2 mg/kg b.wt. of CEES (after 1 h). Therefore, in the present study, to determine whether ceramides have any direct effect on CPT activity, we measured microsomal CPT activity in the presence and absence of different concentrations of C_2 ceramide (25, 50, 100, and 200 μM concentrations of the C_2 ceramide).

Other laboratories have also shown that enzymes of the CDP-choline pathway for the production of PC in the cells, including CPT, show reduced activity when cells are incubated with cell-permeable C_2/C_6 ceramides [23,27], and it has been predicted that this inhibition may be due to the competitive inhibition by ceramides owing to the similarity in the structure to one of the substrates for CPT, diacylglycerol (DAG).

Bladergroen et al. [23] have also shown that a 30% inhibition could be obtained in CPT activity when ceramides were directly added to the assay mixture at 50 μM concentration. Since this inhibition of 30% was less than 64% obtained when cells were incubated directly, they suggested that the competitive inhibition was not the only mechanism. In the present work, we found similar results with lung microsome fraction, that is, with increase in the incubation time with ceramides, the inhibition of the enzyme activity increases. Therefore, we support the observations by Bladergroen et al. [23] that ceramide inhibition of CPT activity may be only partially through direct competitive inhibition with DAG; ceramide may act through interaction with other CPT enzyme inhibitors present in the microsomal fraction.

It is known that the short-chain ceramides often do not mimic the endogenous long-chain ceramides produced as a result of sphingomyelinase activity. However, the lipophilic nature of both the short- and long-chained ceramides makes these molecules the likely candidates to alter biological processes as components of the lipid bilayer [44]. We can therefore predict that ceramides accumulated in lung due to exposure of CEES can alter the activity of the membrane-bound enzymes like CPT and can also act as a membrane perturbant. This ceramide-induced membrane perturbation can result in mitochondrial release of cytochrome C and subsequent release of different caspases (as shown by us previously, Ref. 12) as a part of the apoptotic pathway.

Thus, from the present study, we can conclude that inhibition of the CPT activity as a chronic effect of CEES treatment may be directly responsible for reduction in the lung surfactant production, resulting in subsequent development of ARDS and pulmonary fibrosis. This inhibition of CPT activity is not only controlled at the transcriptional level in lungs; our study also suggests that CEES-induced ceramide production may play a direct role in the reduction of CPT activity, in turn reducing lung surfactant production. Thus, we suggest the following model for the action of CEES on guinea pig lung and development of ARDS (Figure 9).
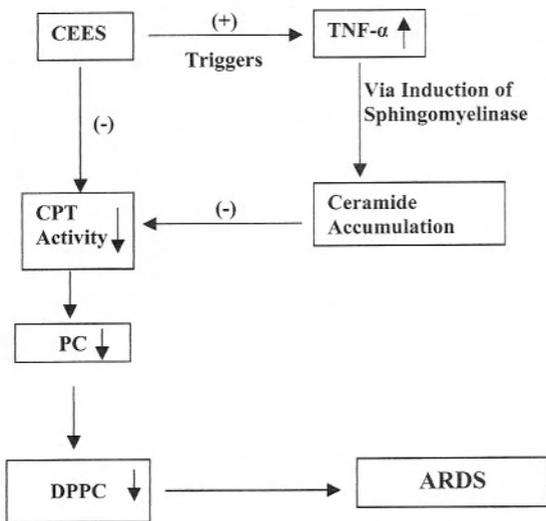
**FIGURE 9.** Proposed pathway for induction of ARDS by CEES.

ACKNOWLEDGMENTS

REFERENCES

1. Debrowska MI, Becks LL, Lelli JL, Levee MG, Hinshaw DB. Sulfur mustard induces apoptosis and necrosis in endothelial cells. Toxicol Appl Pharmacol 1996;141:568–583.

2. Dacre JC, Goldman M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. Pharmacol Rev 1996;48:289–326.

3. Elsayed NM, Ta PN, Korte DW. Biochemical alterations in mouse liver induced by sulfur mustard. Toxicologist 1989;9:26–34.

4. Elsayed NM, Omaye ST, Klain GJ, Inase JL, Dahlberg ET, Wheeler CR, Korte DW. Response of mouse brain to a single subcutaneous injection of the monofunctional sulfur mustard, butyl-2-chloroethyl sulfide (BCS). Toxicology 1989;58:11–20.

5. Elsayed NM, Omaye ST, Klain GJ, Korte DW. Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection. Toxicology 1992;72:153–165.

6. Cowan FM, Broomfield CA. Putative roles of inflammation in the dermatopathology of sulfur mustard. Cell Biol Toxicol 1993;9:201–213.

7. Husain K, Dube SN, Sugendran K, Singh R, Das Gupta S, Somani SM. Effect of topically applied sulphur mustard on antioxidant enzymes in blood cells and body tissues of rats. J Appl Toxicol 1996;16:245–248.

8. Kopff M, Zakrzewska I, Strzelczyk M, Klem J, Dubiecki W. Superoxide dismutase and catalase activity in psoriatic patients treated topically with oinment containing 2-chloroethyl-3-chloropropyl sulphide. Pol J Pharmacol 1994;46:439–444.

9. Kuhns DB, Decarlo E, Hawk DM, Gallin JI. Dynamics of the cellular and humoral components of the inflammatory response elicited in skin blisters in humans. J. Clin. Invest. 1992;89:1734–1740.

10. Arroyo CM, Von Tersch RL, Broomfield CA. Activation of alpha-human necrosis factor (TNFα) by human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulfide (H-MG). Hum Exp Toxicol 1995;14:547–553.

11. Ricketts KM, Santai CT, France JA, Graziosi AM, Doyel TD, Gazaway MY, Casillas RP. Inflammatory cytokine response in sulfur mustard-exposed mouse skin. J Appl Toxicol 2000;20(Suppl 1):S73–S76.

12. Chatterjee D, Mukherjee S, Smith MG, Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog. J Biochem Mol Toxicol 2003;17:114–121.

13. Calvet JH, Jarrean PH, Levame M, D'ortho MP, Lorino H, Harf A, Mavier IM. Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pigs. J Appl Physiol 1994;76:681–688.

14. Ansceschi MM. The alveolar type II cells as a model for investigating the metabolism of surfactant phospholipids. Eur Respir J Suppl 1989;3:105–125.

15. Stith IE, Das SK. Pulmonary surfactant lipids: Studies on cholinephosphotransferase in developing guinea pig lung. Indian Biologist 1981;13:120–128.

16. Cui Z, Houweling M, Chen MH, Record M, Chap H, Vance DE, Tercé F. A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in chinese hamster ovary cells. J Biol Chem 1996;271:14668–14671.

17. Baburina I, Jackowski S. Apoptosis triggered by 1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphocholine is prevented by increased expression of CTP:Phosphocholine Cytidylyltransferase. J Biol Chem 1998; 273: 2169–2173.

18. Hannun YA, Luberto C. Ceramide in the eukaryotic stress response. Trends Cell Biol 2000;10:73–80.

19. Kolesnick RN, Kronke M. Regulation of ceramide production and apoptosis. Annu Rev Physiol 1998;60:643–665.

20. Vietor I, Schwenger P, Li W, Schlessinger J, Vilcek J. Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. J Biol Chem 1993;268:18994–18999.

21. Mathias S, Dressler KA, Kolesnick RN. Characterization of a ceramide-activated protein kinase: Stimulation by tumor necrosis factor α. Proc Natl Acad Sci USA 1991;88:10009–10013.

22. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. J Biol Chem 1993;268:15523–15530.

23. Bladergroen BA, Bussiere M, Klein W, Geelen MJH, Van Golde LMG, Houweling M. Inhibition of phosphatidylcholine and phosphatidylethanolamine biosynthesis in rat-2 fibroblasts by cell-permiable ceramides. Eur J Biochem 1999;264:152–160.

24. Allan D. Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis. Biochem J 2000;345:603–610.

25. Ramos B, Salido GM, Campo ML, Claro E. Inhibition of phosphatidylcholine synthesis precedes apoptosis induced by C2-ceramide: Protection by exogenous phosphatidylcholine. Neuroreport 2000;11:3103–3108.

26. Vivekananda J, Smith D, King RJ. Sphingomyelin metabolites inhibit sphingomyelin synthase and CTP: Phosphocholine cytidylyltransferase. Am J Physiol 2001;281:L98–L107.

27. Ramos B, Mouedden ML, Claro E, Jackowski S. Inhibition of CTP:Phosphocholine cytidyltransferase by C_2-ceramide and its relationship to apoptosis. Mol Pharmacol 2002;62:1068–1075.

28. Awasthi S, Vivekananda J, Awasthi V, Smith D, King RJ. CTP: Phosphocholine cytidylyltransferase inhibition by ceramide via PKC-α, p38 MAPK, cPLA$_2$ and 5-lipoxygenase. Am J Physiol Lung Cell Mol Physiol 2001;281:L108–L118.

29. Ward PA, Till GO, Kunel R, Beauchamp C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J Clin Invest 1983;72:789–801.

30. Mukherjee S, Das SK. Subcellular distribution of "peripheral type" binding sites for [^{3}H] Ro5-4864 in guinea pig lung. J Biol Chem 1989;264:16713–16718.

31. Sinha Roy S, Mukherjee S, Mukhopadhay S, Das SK. Differential effect of cadmium on cholinephosphotransferase activity in normal and cancerous human mammary epithelial cell lines. Mol Cancer Ther 2004;3:199–204.

32. Das SK, Mukherjee S, Smith MG, Chatterjee D. Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. J Biochem Mol Toxicol 2003;17:177–184.

33. Mukherjee S, Chatterjee D, Das SK. Cholinephosphotransferase. In: Halder D, Das SK, editors. Lipids: Glycerolipid metabolizing enzymes; Research Signpost, Trivandrum, India; 2002. pp 86–107.

34. Kent C. Eukaryotic phospholipid biosynthesis. Annu Rev Biochem 1995;64:315–343.

35. Ryan AJ, McCoy DM, McGowan SE, Salome RG, Mallampalli RK. Alveolar sphingolipids generated in response to TNF-α modifies surfactant biophysical activity. J Appl Physiol 2003;94:253–258.

36. Ghosh A, Akech J, Mukherjee S, Das SK. Differential expression of cholinephosphotransferase in normal and cancerous human mammary epithelial cells. Biochem Biophy Res Commun 2002;297:1043–1048.

37. Miquel K, Pradines A, Tercé F, Selmi S, Favre G. Competitive inhibition of choline phosphotransferase by geranylgeraniol and farnesol inhibits phosphatidylcholine synthesis and induces apoptosis in human lung adenocarcinoma A549 cells. J Biol Chem 1998;273:26179–26186.

38. Stith IE, Das SK. Development of cholinephosphotransferase in guinea pig lung mitochondria and microsomes. Biochim Biophys Acta 1982;714: 250–256.

39. Sikpi MO, Das SK. The localization of cholinephosphotransferase in the outer membrane of guinea-pig lung mitochondria. Biochim Biophy Acta 1987;899:35–43.

40. Carroll JL, McCoy DM, McGowan SE, Salome RG, Ryan AJ, Mallampalli RK. Pulmonary-specific expression of tumor necrosis factor-α alters surfactant lipid metabolism. Am J Physiol Lung Cell Mol Physiol 2002;282:L735–L742.

41. Wright MM, McMaster CR. Phospholipid synthesis, diacylglycerol compartmentation, and apoptosis. Biol Res 2002;35:223–229.

42. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J. Ceramide: Second messenger or modulator of membrane structure and dynamics? Biochem J 2003;369:199–211.

43. Kurinna SM, Tsao CC, Nica AF, Jiffar T, Ruvolo PP. Ceramide promotes apoptosis in lung cancer-derived A549 cells by a mechanism involving c-Jun NH2-terminal kinase. Cancer Res 2004;64:7852–7856.

44. Gidwani A, Brown HA, Holowka D, Baird B. Disruption of lipid order by short-chain ceramides correlates with inhibition of phospholipase D and down stream signaling by FcεRI. J Cell Sci 2003;116:3177–3187.
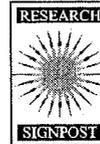
8
Role of sphingomyelinase in the environmental toxin induced apoptosis of pulmonary cells

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Abstract

Sphingomyelinase is an enzyme that plays a very important role in cell apoptosis. Ceramide, the breakdown product of sphingomyelin by sphingomyelinase, is one of the major intermediates for the signal transduction that leads to cell apoptosis by various agents or factors. Environmental pollution is known to cause severe lung dysfunctions. The mechanisms of actions of most of the environmental toxins were not clearly understood till now. It will be useful to understand the mechanisms by which these toxins cause lung cell damage to develop specific antidotes or prophylactic drugs for prevention of environmental toxin induced lung injury. We have

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recently studied the signal transduction events in lung after exposure to one of the analogs of sulfur mustard gas, 2-chloroethyl ethyl sulfide (CEES). Mustard gas has been used as a vesicant chemical warfare agent and well known to cause ARDS associated with lung injury. Mustard gas induced acute lung injury can be demonstrated by ^{125}I-BSA leakage into lung and microscopic studies. The initial signal transduction event after CEES exposure is the rapid, transient induction of TNF-α followed by activation of both acid and neutral sphingomyelinases, resulting in high accumulation of ceramides, a second messenger involved in cell apoptosis. At the same period of time there was a transient activation of NF-κB due to CEES exposure. It is predicted that the initial activation of NF-κB was due to an adaptive response to protect the cells from damage since NF-κB is known to be an inhibitor of TNF-α/ sphingomyelinases/ceramide induced cell apoptosis. Since NF-κB disappeared after 2 hours, the cells continued being damaged due to accumulation of ceramides and activation of several caspases leading to apoptosis. These signal transduction events were associated with alteration in oxygen defense system. Hence, we further investigated whether an antioxidant, such as N-acetylcysteine (NAC) has any protective effect. It was observed that feeding of single dose of NAC (0.5 g) by gavage just before the CEES infusion was ineffective to counteract any of the CEES induced signal transduction events that lead to lung damage. However, consumption of the antioxidant (0.5 g/day) via drinking water for 3 or 30 days prior to CEES exposure caused a significant inhibition in the induction of TNF-α, activation of neutral and acid sphingomyelinases, production of ceramides, activation of caspases, leakage of ^{125}I-BSA into lung tissue, and histological alterations in lung. Therefore, NAC may be a potential antidote for CEES induced lung injury.

Introduction

Sphingomyelinase (SM-ase) is the enzyme that breaks down sphingomyelin (SM) into ceramide and plays important roles in cell apoptosis. Recent evidence shows that, besides their structural role, sphingolipids play many important roles in cellular responses such as differentiation, death, proliferation and signal transduction [1,2]. Among this class of lipids is ceramide (N-acylsphingosine), which also serves as a precursor for sphingolipid biosynthesis [2-4]. Hydrolysis of SM is one of the major sources of ceramide production in response to a variety of stimulus. However, it can also be derived from the stepwise degradation of all sphingolipids through the action of specific hydrolases, the best documented of which are located in lysosomes. A wide array of biological responses to extracellular stimuli has been described to be mediated by the intracellular generation of ceramides [5-11]. Ligands of various members of the tumor necrosis factor (TNF) and CD95 (Fas/APO1) receptor family, ionizing and UV radiation, anticancer drugs, and heat shock oxidants, are known to

induce cellular stress [6, 12-16]. Stress-induced SM hydrolysis is one of the early phases of apoptosis [17]. The rate and degree of SM hydrolysis depends on the stimulus and cell type. Many apoptosis-inducing stimuli increase ceramide and/or decrease sphingomyelin [18-22]. Ceramide generated by hydrolysis of SM by SM-ase, is a crucial intermediate of stress responses and the intracellular level of ceramide plays a key role in determining the fate of cells [7].

The hallmarks of cytokine biology are pleiotropism and redundancy. Although a number of signaling pathways have been implicated in mediating the cellular effects of inflammatory cytokines, there is still considerable uncertainty about the relative importance of each of these pathways during inflammatory processes in vivo, including asthma, inflammatory allergic reactions and endotoxic shock [23-25]. One class of signaling pathways that has received considerable attention involves the action of cytokine-stimulated SM-ase [26]. Ceramide generated from SM-ase activation plays critical roles in cytokine-mediated apoptosis, cellular differentiation and senescence, each of which may be important in the inflammatory response.

Ceramide can be generated through several different pathways such as synthesis within the cell, hydrolysis of SM by SM-ase and breakdown of glycosphingolipids. Then, in the lysosomal compartment, acid ceramidase degrades ceramide to liberate sphingosine and a free fatty acid [3,5]. Ceramide can also be increased by the inhibition of ceramide breakdown by ceramidase, inhibition of sphingomyelin synthase. SM-ase has been proposed as key enzymes involved in stress-induced ceramide formation [26]. Multiple pathways may be regulated which in turn ultimately determine the levels of ceramide.

At least five different types of SM-ase have been reported thus far but among these, acid SM-ase and neutral membrane-bound N-SM-ase have been investigated in response to cell stimulation. Whereas different SM-ases having distinct subcellular locations may implement different biological effects of ceramide [8,10], an apoptotic function has been attributed by some investigators to the ceramide produced in acidic organelles through the action of an acid SM-ase. Acid SM-ase, a lysosomal enzyme is involved in the intracellular signal transduction pathway of TNF-α leading to activation of NF-κB [27]. Most N-SM-ase activity is located in the cellular membrane fraction. The activity of N-SM-ase and the subsequent SM hydrolysis/ceramide release can be modulated either positively or negatively by several cellular molecules [28-30], including lipids [31,32], protein kinases [33-36], proteases [37], and other signaling molecules [38-42].

Several recent literatures describe roles for ROS in pulmonary diseases such as acute respiratory distress syndrome, chronic obstructive pulmonary disease, including asthma and interstitial pulmonary fibrosis [43]. Lavrentiadou and colleagues have demonstrated a direct link between two important aspects of

mammalian stress responses: the generation of ROS and activation of the sphingomyelin/ceramide cycle leading to apoptosis [44]. In the lung, epithelial cells of the airway and the alveolar compartments are constantly exposed to airborne environmental stresses. Their ability to adapt to injury from these insults is essential in maintaining lung function. One response to cellular injury is to give up the fight and die to permit neighboring cells to replicate and replace the injured cell. Apoptosis is also an essential mechanism by which the lung purges itself of pathogen-invaded cells [45]. SM-ase involves the production of reactive oxygen species (ROS) with ceramide generation in the process of cell apoptosis [46-49]. Both neutral and acidic SM-ase are involved in these processes. Furthermore, ceramide has also been associated with many of the effects of the cytokine TNF-α [50-52].

TNF-α involves the expression of specific genes via activation of the NF-kB/Rel family of transcription factors [53-55]. The mammalian transcription factor NF-kB regulates a wide variety of genes, including those involved in the immune response, cell growth control, and apoptosis [56-58]. The five members of the NF-kB family, p65/RelA, c-Rel, Rel B, p50 (Nf-kB1), and p52 (NF-kB), are able to form various homo- and heterodimeric complexes with a range of transactivation potentials in regulating transcription [53-58]. The interaction of dimmers containing the p 65 or c-rel NF-kb subunits with IkBα or IkBβ blocks the nuclear localization signal of NF-kB, leading to the preferential accumulation of these dimmers in the cytoplasm, thus inhibiting their DNA binding potential [57,58]. Upon stimulation with TNFα, the inhibitory IkB molecules are rapidly phosphorylated on N-terminal serines i.e. serines 32 and 36 for IkBα, leading to subsequent ubiquitination and degradation by proteasome-mediated mechanisms, allowing NF-kB to accumulate in the nucleus where it activates gene expression [58]. It has also been demonstrated that the unactivated NF-kB/IkB complex shuttles between the cytoplasm and nucleus, with the primary, uninduced localization being the cytoplasm [58-60]. The two catalytic subunits of the complex, IKK and IKKα, interact with IKKβ to comprise the core IKKγ activity [61,62]. Additionally, the inducible expression of a number of NF-kB-dependent genes in response to TNF treatment requires IKKα in controling this process is unclear. Furthermore, it has also been found that IKKα controls histone H3 phosphorylation at NF-kB-dependent promoters, presumably contributing to the control of positive gene expression [63,64]. Some studies suggest that ceramide plays an essential role in NF-kB activation [65,66]. Diacylglycerol (DAG) generated by phosphatidylcholine-specific phospholipase C causes accumulation of ceramide via activation of acid sphingomyelinase [67]. Glutathione (GSH) has a key role in the regulation of N-SM-ase activity. GSH inhibits N-SM-ase activity and depletion of GSH increases intracellular ceramide [41,68]. TNF-α mediates cell

apoptosis by decreasing the intracellular GSH level and facilitating the hydrolysis of SM to generate ceramide [68]. Hence, N-SM-ase acts as a sensor of oxidative stress, thereby coupling intracellular oxidation and the apoptotic cascade through ceramide formation. However, relatively little is known regarding the specific mechanism(s) that activates SM-ase.

Environmental toxicity leads to different types of pulmonary complication including bronchitis, lung congestions, lung cancer, asthma, chronic obstructive pulmonary disease (COPD) and Adult Respiratory Distress Syndrome (ARDS) [69-74]. The mechanisms of action of several environmental pollutants are unknown. But one of the most reproducible inducers of apoptosis is mild oxidative stress. H_2O_2 is a ubiquitous molecule, freely miscible and able to cross cell membranes readily. It is present in several air pollutants, including the vapor phase of cigarette smoke. It is also detected in exhaled air of humans [75], although it is not clearly known where this H_2O_2 originates. Amounts of exhaled H_2O_2 appear greater in subjects with lung inflammation [76-78], and in cigarette smokers [79]. Importantly, several agonists increase generation of H_2O_2 by epithelial cells. These include cytokines (TNF-α, IL-1 and Fas ligand), cytotxic agents, ionizing radiation, infections and environmental pollutants. Understanding the mechanisms by which those agents harm the lung will be helpful in finding ways to develop remedies for those lung disorders. We have shown the involvement of SM-ase/ceramide pathways in lung injury induced by mustard gas, a toxic chemical that has been used in several warfares, and tested different antagonists for SM-ase/ceramide system to recover the mustard gas induced lung injury to develop antidotes for that toxin [80,81]. For our study, we have used a mustard gas analog, 2-Chloroethyl ethyl sulfide (CEES), and guinea pigs as the model system.

A. Signal transduction events during mustard gas induced lung injury

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 [82], activation of proteases resulting proteolysis of several vital intracellular enzymes and structural proteins [83], free radicals production and free radical-mediated oxidative stress [84,85], inflammations [86] and activation of tumor necrosis factor (TNF-α), a part of the inflammatory cytokine cascade [87,88]. Hence, our first objective was to understand the down-stream signal transduction events in lung following mustard gas exposure.

Elevation of TNF-α levels after CEES exposure

After single intratracheal injection (0.5 mg/kg body weight) of CEES to guinea pigs, guinea pigs were sacrificed at different time points and lung was removed after perfusion. The lung was lavaged and TNF-α concentrations were

measured in lung lavage fluid, lung lavage macrophages and in lung tissue. The TNF-α level in lavage fluid was very low whereas a high level of TNF-α accumulated in lung as well as in lung macrophages within 1 hour of CEES exposure. The level of TNF-α decreased rapidly after 1 hour and came to the normal level within 24 hours of CEES exposure (Fig 1A). Furthermore, the induction of TNF-α by CEES is dose dependent – optimal TNF-α accumulation was observed at 2mg/kg dose of CEES exposure (Fig. 1B).
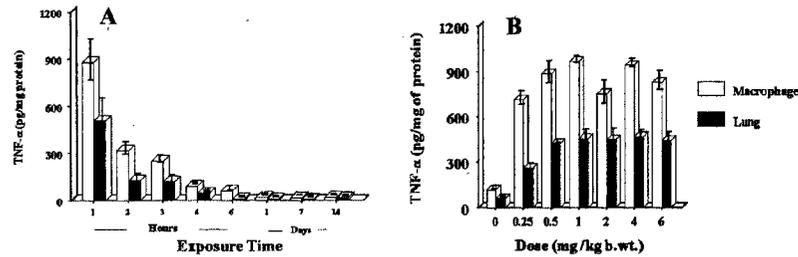
Figure 1. Accumulation of TNF-α in guinea pig lung and macrophages after exposure to CEES. **A.** Time dependent induction of TNF-α after intratracheal injection of CEES (0.5 mg/kg body wt.). **B.** Accumulation of TNF-α 1 hr after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.).

Activation of sphingomyelinase activities after CEES exposure

Both the neutral and acid sphingomyelinase activities were increased 4-5 fold after CEES treatment. As the control level of acidic sphingomyelinase activity (Fig. 2B, Fig. 2D) was much higher than the control level of neutral sphingomyelinase (Fig. 2A, Fig. 2C), we could see much higher activity of acid sphingomyelinase compared to neutral sphingomyelinase after CEES treatment. Both neutral and acid sphingomyelinase activities started to increase along with the increase of TNF-α and give a maximum peak between 4 and 6 hours in lung and between 3 and 4 hours in macrophages. It is not clear whether this difference between lung and macrophages is functionally significant or not. It might be due to the fact that macrophages were exposed to CEES quicker than the whole lung tissue where CEES needs to be diffused within the tissue to give the maximum effect. The sphingomyelinase activities (both neutral and acidic) were found to be higher in lavage macrophages compared to the lung tissue. It is possible that in comparison to other cell types in lung, macrophages are more sensitive to CEES. The level of sphingomyelinase decreased rapidly to come back to normal level within 24 hours. In this case we also found that a 2mg/kg dose of CEES is sufficient to reach the maximum level of both neutral and acid sphingomyelinase activity (Fig. 2C and 2D).
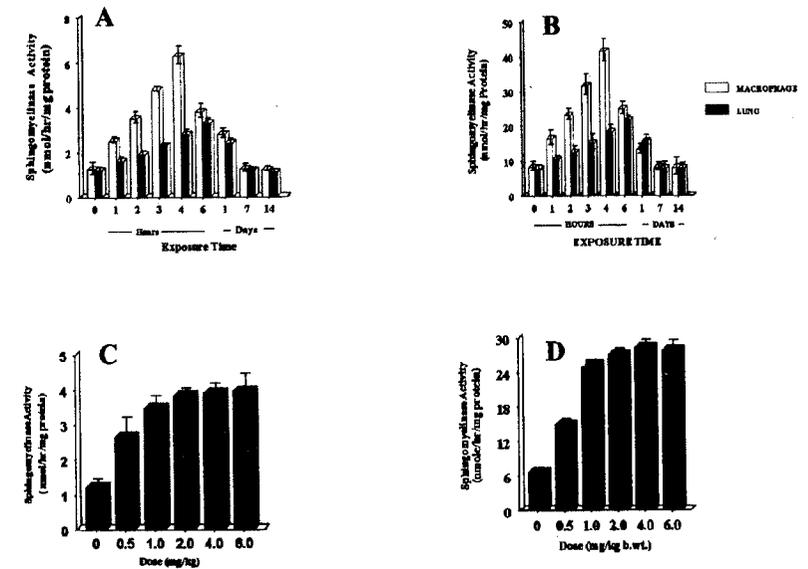
Figure 2. Activation of acid and neutral sphingomyelinase in guinea pig lung and macrophages following CEES exposure. Time course of induction of neutral (**A**) and acid (**B**) sphingomyelinase after intratracheal injection of CEES (0.5 mg/kg body wt.). Accumulation of neutral (**C**) and acid (**D**) sphingomyelinase at 4 hr after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.).

Ceramide accumulation after CEES exposure

The accumulation of ceramides after CEES exposure showed a biphasic pattern. Ceramide levels became very high within 1 hour of CEES exposure and gives a peak accumulation within 2 hours (Fig. 3A). After 2 hours, there was some decrease in the ceramide levels but again the level increased to a very high level and remained almost steady even up to 14 days (Fig. 3A). CEES induced ceramide accumulation was found to be saturated at 4 mg/kg dose of CEES. At 2 mg/kg dose of CEES about 90% induction of ceramide was achieved (Fig. 3B).

NF-κB activation after CEES exposure

The NF-κB activation was measured in the nuclear extracts of lung tissue after exposure to CEES. NF-κB showed activation only up to 1-2 hours after CEES exposure (Fig. 4A). After initial lung damage by TNF-α, within 2 hours there was some recovery due to activation of NF-kB. After 2 hours NF-κB levels
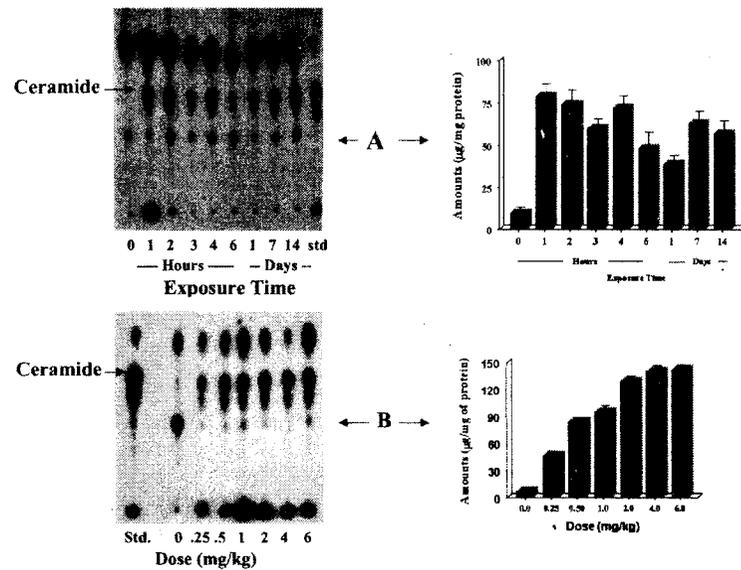
Figure 3. Accumulation of ceramide in guinea pig lung after CEES exposure. **(A)** Time course of induction of ceramide after intratracheal injection of CEES (0.5 mg/kg body wt.). **(B)** Accumulation of ceramide at 1 hr after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body wt.). In both cases (A and B), left panel is the autoradiograph showing the accumulated ceramides, and the right panel represents the quantitative analysis of accumulated ceramide as determined by ^{32}P incorporation.

went down, ceramide level increased and secondary lung damages were observed. Dose dependent studies revealed that 4mg/kg CEES was needed for the optimum activation of NF-κB (Fig. 4B). Supershift assay using specific antibodies to p50 and p65 revealed that both the p50 and p65 subunits were activated in lung due to CEES exposure (Fig. 4C).

Activation of caspases after CEES exposure

Activation of different caspases in guinea pig lung after the CEES exposure is shown in Fig. 5. Within 1 hour of exposure, some activation was observed for all the four caspases but the activity went to basal level within 2 hours of exposure. The activity of caspase 2, caspase 3, caspase 8 as well as caspase 9 increased significantly between 4 to 6 hours of exposure and then went down again. No activity for any of the caspases was observed at 24 h and thereafter. Here also, the activation of caspases was found to be optimum at 2 mg/kg body weight of CEES.
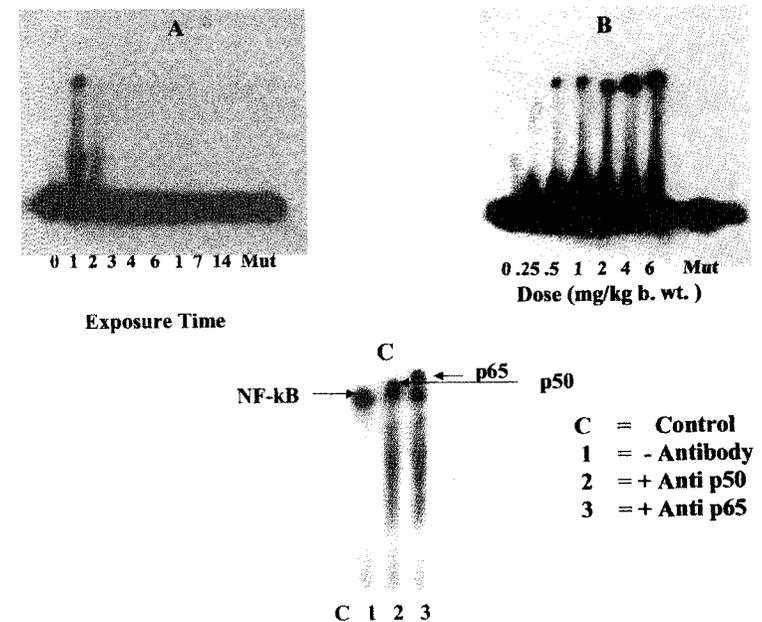
Figure 4. Activation of NF-kB in guinea pig lung following CEES exposure. **(A)** Time dependent activation of NF-kB after intratracheal injection of CEES (0.5 mg/kg body wt.) as observed by mobility shift assay. **(B)** Mobility shift assay showing the accumulation of NF-kB after 1 hr of CEES exposure at different doses (ranging from 0.5 to 6.0 mg/kg body wt.). **(C)** Supershift assay, using subunit specific antibodies, to identify the subunits (p50 and p65) of NF-kB activated due to CEES exposure in guinea pig lung.
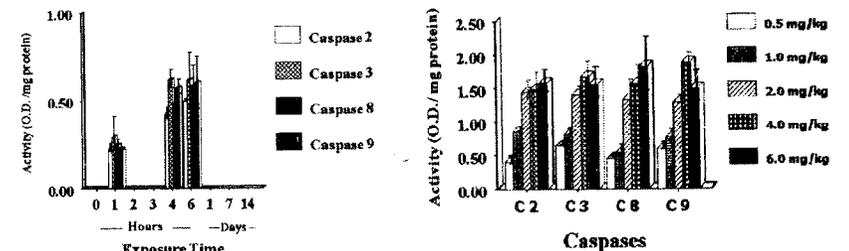
Figure 5. Activation of different caspases in guinea pig lung after CEES exposure. **A.** Time course of activation of different caspases after intratracheal injection of CEES (0.5 mg/kg body wt.). **(B)** Accumulation of different caspases at 4 hr after exposure to CEES at different doses (ranging from 0.5-to 6.0 mg/kg body wt.).

B. Recovery from mustard gas induced lung injury by antioxidants

Protective effects of various antioxidants, viz. vitamin E, glutathione etc. have been studied and found to be effective to some extent to prevent mustard gas induced tissue damage [89] but inhibition of the initiation of signaling events has not been previously demonstrated. An effective means of prophylaxis would accomplish a significant decrease in signal transduction that is associated with the initiation of the inflammatory cascade. We have tested different agents to block mustard gas induced signal transduction events in guinea pig lung including dimethylthiourea (DMT), which was used to block free radical induced lung injury in chronic hypoxia [90]. As NAC was found most effective among the agents we have tested, we have studied in detail the beneficial effects of NAC prophylactic treatment on mustard gas induced signal transduction events, which lead to lung injury in guinea pigs.

NAC treatment and ^{125}I-BSA leakage in CEES injured lung

Lung injury followed by CEES exposure was expressed as permeability index, which is a measure of ^{125}I-BSA leakage from injured lung. The results (Table 1) indicated that about 69% of the lung injury was protected by 3 days pretreatment of NAC. Single dose of NAC could not block the CEES induced lung injury at all. On the other hand, about 76% of lung injury could be blocked by long term pretreatment (30 days) with NAC. No significant protection of lung injury was observed by pretreatment with either ondrox (single dose or 3 days pretreatment) or GSH (single dose).

NAC treatment and CEES induced TNF-α accumulation

Effects of antioxidants on the induction of TNF-α in lung 1 h after CEES exposure are presented in Table 2. Single dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 3 days pretreatment with NAC inhibit about 62% of TNF-α induction. Long-term treatment with NAC gave more protection (inhibition of 73 % of TNF-α induction). There was no protection for TNF-α accumulation by single dose of either ondrox or GSH. Three days pretreatment with ondrox and DMT could only block 13 and 8 % of the TNF-α induction, respectively.

NAC treatment and CEES induced sphingomyelinases activation

The activation of both neutral and acid sphingomyelinases that was observed 4 h post exposure to CEES (4 mg/ kg body weight) was blocked significantly (42% and 46%, respectively) by only 3 days pretreatment with NAC. 30 days of pretreatment with NAC blocked 49% and 61% of the activation of neutral and acid sphingomyelinases, respectively (Fig. 6).

Table I. Effects of N-acetyl cysteine (NAC) pretreatment on CEES induced lung injury in guinea pigs.

Treatment	Permeability Index
Control	0.20 ± 0.04
Control + NAC (3 days)	0.08 ± 0.01
CEES	2.01 ± 0.16
CEES + NAC (single dose)	2.07 ± 0.11
CEES + NAC (3 days)	0.62 ± 0.09*
CEES + NAC (30 days)	0.48 ± 0.06*
CEES + Ondrox (single dose)	2.04 ± 0.03
CEES + Ondrox (30 days)	2.00 ± 0.08
CEES + GSH (single dose)	1.98 ± 0.10

CEES was infused (6 mg/kg body wt.) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0. 5 g/day/animal, for either 3 days or 30 days prior to CEES infusion). Information on treatment with ondox and GSH is given in text. The lung injury was measured after 1 h of CEES exposure and expressed by permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. Each group had 6 animals. Values are mean ± SE (n=6). *NAC supplementation in drinking water blocked the CEES-induced lung injury significantly (p< 0.05).

Table 2. Inhibition of CEES induced TNF-α accumulation in guinea pig lung by NAC treatment

Treatment	Level of TNF-α (pg/mg protein)
Control	20 ± 7
CEES	708 ± 38
CEES + NAC (single dose)	705 ± 24
CEES + NAC (3 days)	270 + 40*
CEES + NAC (30 days)	190 ± 64*
CEES + Ondox (single dose)	716 ± 22
CEES + Ondrox (3 days)	610 ± 54
CEES + DMT (3 days)	648 ± 62
CEES + GSH (single dose)	720 ± 41

CEES was injected (4 mg/ kg body wt.) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before injection of CEES or with drinking water (0. 5 g/day/animal, for either 3 days or 30 days prior to CEES injection). Information on treatment with ondox, DMT and GSH is given in the text. TNF-α was measured after 1 h exposure of CEES. Values are mean ± SE (n = 6). *NAC supplementation in drinking water blocked the CEES-induced accumulation of TNF-α significantly (P< 0.05).
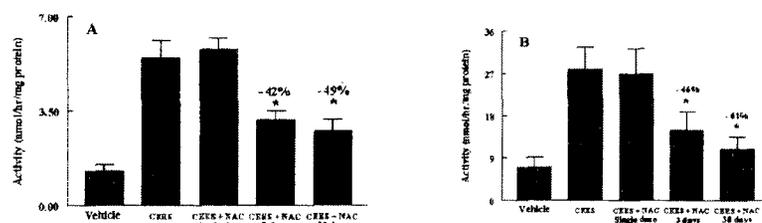
Figure 6. Inhibition of CEES induced activation of neutral and acid sphingomyelinases in guinea pig lung by NAC treatment. Accumulation of neutral (**A**) and acid (**B**) sphingomyelinases at 4 h after exposure to CEES (4 mg/kg body weight) and their inhibition by 3 days and 30 days pretreatment with NAC. Values are mean ± SE (n = 6). Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase. *Pretreatment with NAC for 3 and 30 days prior to CEES exposure caused a significant inhibition of both neutral and acid sphingomyelinases (p< 0.05%).

Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase.

NAC treatment and CEES induced ceramide accumulation

Ceramide accumulation after 1 h of CEES exposure at 4 mg/kg body weight are shown in Table 3. Short term (3 days) and long term (30 days) pretreatment with NAC caused 71% and 77% decrease in the levels of ceramide, respectively, but a single dose of NAC was ineffective in blocking the ceramide accumulation.

Table 3. Inhibition of CEES induced ceramide accumulation in guinea pig lung after NAC treatment

Treatment	Levels of Ceramide (μg/mg protein)
Control	9 ± 2
CEES	148 ± 14
CEES + NAC (single dose)	146 ± 10
CEES + NAC (3 days)	39 ± 8*
CEES + NAC (30days)	28 ± 12*

CEES was infused (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0. 5 g/day/animal, for either 3 days or 30 days prior to CEES infusion. Ceramide accumulations were assayed after 1 h of infusion of CEES. Values are mean ± SE (n = 6). *NAC supplementation in drinking water blocked the CEES-induced accumulation of ceramide significantly (p < 0.05).

There was no significant protection from ceramide accumulation by pretreatment (single dose or 3 days) with either ondrox or GSH (data not shown).

NAC treatment and CEES induced NF-κB and caspases activation

CEES induced activation of NF-κB, observed 1 h after CEES exposure, was significantly (p < 0.05) blocked both by short term (3 days) and long term (30 days) pretreatment of NAC (Fig. 7). Different caspases (caspase 2, caspase 3, caspase 8 and caspase 9) were activated in lung tissue within 4 h of CEES infusion (Fig. 8). Only 3 days pretreatment with NAC blocked 41, 44, 55 and 51% of the activation of caspase 2, caspase 3, caspase 8 and caspase 9, respectively.
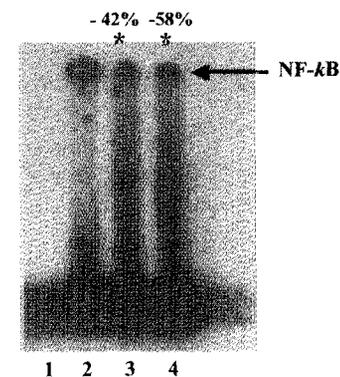
Figure 7. Effects of NAC pretreatment on CEES mediated activation of NF-κB in guinea pig lung. Activation of NF-κB after 1 hr of intratracheal infusion of CEES (4 mg/kg body weight) was monitored by mobility shift assay. The guinea pigs were treated with NAC either for 3 days or for 30 days prior to CEES exposure. Panels 1, 2, 3 and 4 represent vehicle, CEES, CEES + NAC (3 days) and CEES + NAC (30days), respectively. *NAC treatment caused a significant inhibition (46% and 58% for 3 and 30 days, respectively, p < 0.05) in the accumulation of NF-κB.

NAC treatment and CEES induced changes in the free radical metabolizing enzymes

Exposure to CEES caused a significant (p < 0.05) inhibition in the activity of SOD (31%), GSH-Px (67%) and catalase (25%) (Fig. 9). Pretreatment of guinea pigs for 3 days with NAC before CEES infusion significantly (p < 0.05) decreased CEES induced inhibition of SOD (from 31% decrease to 4.1% increase), GSH-Px (from 67% decrease to 50% decrease), and catalase (from 25% decrease to 9% decrease). Pretreatment of guinea pigs for 30 days with
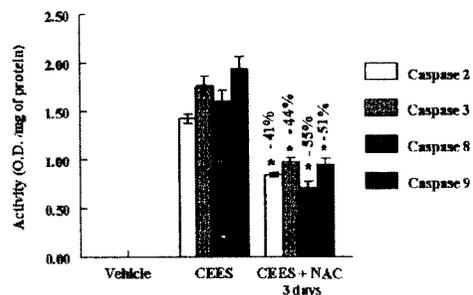
Figure 8. Inhibition of the activation of different caspases in guinea pig lung after CEES exposure by pretreatment with NAC. Accumulation of caspases (caspase 2, caspase 3, caspase 8, caspase 9) at 4 h after exposure to CEES (4 mg/kg body weight) and the prevention of this caspases activation by short term pretreatment (3 days) with NAC. Values are mean $\pm$ SE (n = 6). *NAC treatment inhibited the activation of all caspases significantly (p < 0.05).
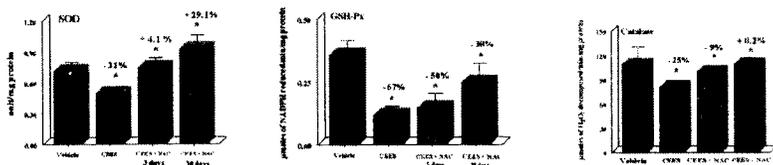
Figure 9. Effects of NAC pretreatment on the CEES induced alterations in the free radical metabolizing enzymes. Guinea pigs were infused with CEES (4 mg/kg body weight) intratracheally and the free radical metabolizing enzymes (SOD, GSH-Px, catalase) were assayed in perfused lung after 4 h of CEES exposure. The samples were taken from guinea pigs with or without pretreatment with NAC for 3 days. *CEES exposure caused a significant decrease in the activity of SOD, GSH-Px and catalase (p < 0.05). NAC treatment blocked the CEES-induced changes significantly (p < 0.05) for all enzymes.

NAC provided additional resistance. For example, the activity of SOD was increased by 29% over the basal value, the activity of GSH-Px was decreased by only 30%, and the activity of catalase was brought back to the basal level.

NAC treatment and CEES induced lung morphology changes

Morphology of the lung was examined at different time points (1 h, 2 h, 6 h, 1 day, 7 days, 14 days, and 21 days) post exposure to CEES (0.5 mg/kg body weight). However, we have shown here the photographs for only two periods post exposure to CEES (Fig. 10A for 1 h and Fig. 10B for 21 days), since the

effects of NAC were studied only for these two periods. For both Fig. 10A and Fig. 10B, the top panel represents the bronchial region and the bottom panel represents the alveoli. Animals exposed to CEES showed symptoms of chemical burn within 1 h; however the severity of damage progressively increased with time. Severe bronchial constriction with occasional apoptotic nucleus, and accumulation of viscid secretion of mucin was observed in CEES treated animals. Furthermore, both polymorphonucleus (PMNs) and eosinophilic leukocytes migration were observed in both alveoli and bronchi. However, pretreatment with NAC protected lung from all these changes remarkably except mucin secretion.
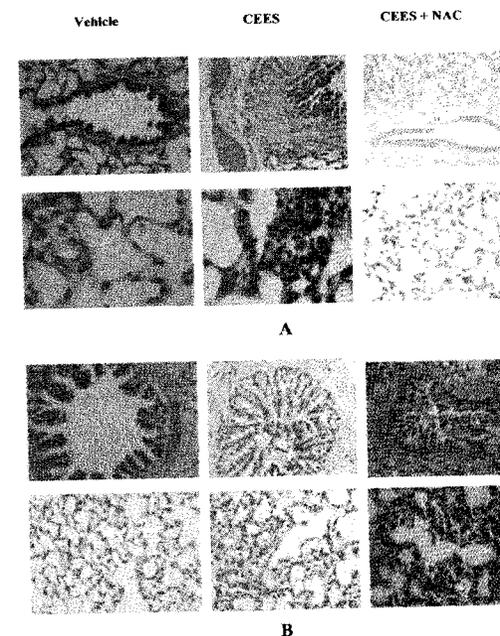
Figure 10. Histological analysis showing recovery from CEES induced lung damage by pretreatment of NAC. Guinea pig lungs were examined under light microscope after 1 h (**A**) and after 21 days (**B**) of exposure to CEES (0.5 mg/kg body weight). Upper panel represents morphology of bronchi and lower panel represents morphology of the alveoli. Magnifications: X 400.

Discussion

We have used the guinea pigs for studying mustard gas induced lung injury. As guinea pigs are closer to humans than other primates as far as structure and

function of lungs are concerned, a guinea pig model will be ideal for studying the mechanism of lung injury by mustard gas. The results clearly demonstrated the involvement of a SM-ase/ceramide signal transduction pathway in the mustard gas mediated lung injury. After intratracheal injection of CEES to guinea pigs, the TNF-α level increased sharply within one hour of exposure. TNF-α level started declining after one hour and came back to basal level within 24 hours. Followed by the accumulation of TNF-α, both the acid and neutral sphingomyelinase activities were stimulated giving a peak 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. The higher levels of TNF-α as well as both the acid and neutral sphingomyelinase activities in lung macrophages compared to those in lung tissue were expected because lung tissue consists of several types of cells all of which were not responsive to CEES. Ryan et al. (91) have recently been reported that intracheal instillation of TNF-α in adult rats led to a twofold increase in the amount of surfactant-associated ceramides and tended to decrease levels of sphingomyelin without significantly altering sphingosine or sphiganine content. Furthermore, the results of this study also indicated that the secretory alveolar sphingomyelinase, that is TNF-α responsive, mediates effects of cytokine on alveolar sphingolipid metabolism.

As the sphingomyelinase activity increased, the accumulation of ceramides started. Ceramide level increased within one hour of CEES exposure and after a slight fall between 3-6 hour, it increased again and remained at high level even upto 14 days after CEES exposure.

Slight drop in the ceramide level in between 3-6 hours might be due to the activation of NF-κB, which showed a sharp transitional activation at 1 to 2 hours after CEES exposure. The activation of NF-κB coincided with the increase of TNF-α in lung tissue. It is well known that TNF-α is proapoptotic [92,93] and NF-κB acts as antiapoptotic by opposing the TNF-α induced apoptosis [94-96]. Here, we observed a biphasic effect of CEES on lung. After 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 the cell apoptosis in lung as observed by light as well as electron microscopy (unpublished observation). This second phase of caspase activation was disappeared within 24 hours and we could not observe any further activation of any of the above caspases. This type of biphasic action was observed in mustard gas induced skin lesions also [97] where initial phase of injury by 1 hr was followed by a delayed phase, which became evident after 8 hr of exposure.

One explanation for this biphasic action of mustard gas is that NF-kB is activated by TNF-α through a phosphatidylcholine-specific phopholipase C/diacyglycerol/protein kinase C or phosphatidylcholine-specific phopholipase C/diacyglycerol/acid SM-ase/ ceramide model. It has been reported that ξ isoform of protein kinase C is a specific activator of NF-kB [98]. Another explanation is that TNF-α can stimulate phosphorylation of inactive dimers of NF-kB subunits by stimulating N-SM-ase /cermide pathway. Marchesini et al. (99) have recently reported that GW4869 can significantly inhibit activity of N-SM-ase activity but not acid SM-ase activity. Subsequent activation of both pathways causes increased cellular concentration of ceramide, which is known to inhibit NF-kB at high concentration [99]. Sphingomyelin synthetase can use ceramide as a precursor for the synthesis of sphingomyelin and release diacylglycerol, converting a sphingolipid bioeffector into a glycolipid messenger, thus resulting in the activation of NF-kB. Luberto et al (68) have reported that effects of ceramide on NF-kB signalling differ due to the presence and absence of sphingomyelin synthetase.

Our result is the first report that provided the series of events leading to cell death after mustard gas exposure. Our 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 the damage and prevent from further damage. It was the balance between these two opposite effects, which will determine the ultimate damage of the tissue. Different possible pathways by which mustard gas might induce lung injury have been schematically presented in Fig. 11.
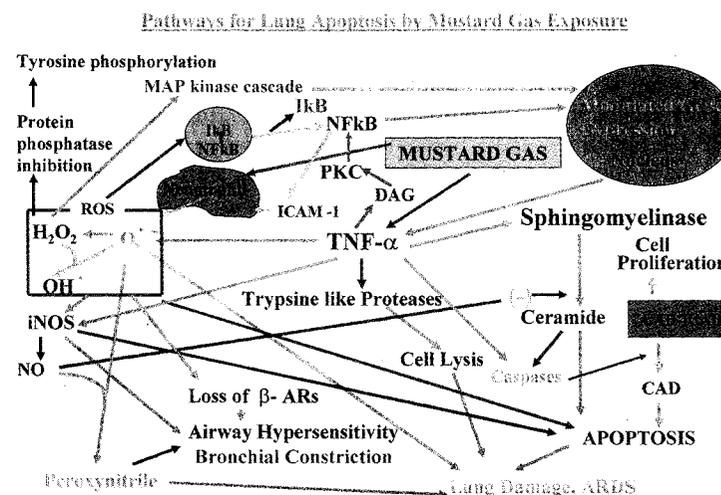

Figure 11.

There has been no prophylactic treatment previously available for pulmonary injury by mustards. Due to this lack of a prophylactic treatment, the battlefield soldier is at risk for pulmonary injury due to the exposure to mustards. After understanding the signal transduction events which cause lung injury by mustard gas exposure, we wanted to develop antidotes by monitoring the effects of different chemical agents on the mustard gas mediated cell signaling in lung.

We have tested several antioxidants and decided to study NAC in details as that was found to be most effective. We observed that only 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 only 3 days prior to CEES exposure was found to be 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 100% 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.* [100]. 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.* [100]. In our study, we have demonstrated that NAC inhibits the production of NF-kB. It can be suggested that the protection of lung injury may be etiologically related to the inhibition of oxidative activation of the transcription factor NF-kB, which is usually upregulated by stress signals. In fact, Atkins *et al.* [101] 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-kB.

In summary, our study clearly suggests that NAC, a well-known antioxidant, can be used as an effective antidote against CEES–induced lung injury that involves SM-ase/ceramide pathways of lung cell apoptosis. 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 known 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. This important

finding might be extended for treatment of other environmental pollutants those take the same signaling pathway to damage lung tissue.

Acknowledgement

References

1.   Hannun,Y.A. (1994) J. Biol. Chem 269: 3125-3128.
2.   Merrill, A.H., Jr., and Jones, D.D. (1990) Biochim. Biophys. Acta 1044: 1-12.
3.   Kolesnick, R.N. (1991) Prog. Lipid Res. 30: 1-38.
4.   Sandhoff, K., and Kolter, T. (1996) Trends Cell Biol. 6: 98-103.
5.   Ballou, L.R., Laulederkind, S.J.F., Rosloniec, E.F., and Raghow, L.R. (1996) Biochim. Biophys. Acta 1301: 273-287.
6.   Spiegel, S., Foster, D., and Kolesnick, R.N. (1996) Current Opin Cell Biol 8: 159-167.
7.   Hannun, Y.A. (1996) Science 274: 1855-1859.
8.   Testi, R. (1996) Trends Biol. Sci. 21: 468-471.
9.   Riboni, L., Viani, P., Bassi, R., Prinetti, A., and Tettamanti, G. (1997) Prog. Lipid Res. 36: 153-195.
10. Kolesnick, R.N., and Kronke, M. (1998) Annual Rev. Physiol. 60: 643-665.
11. Levade, T., and Jaffrezou, J.P. (1999) Biochim. Biophys. Acta 1438: 1-17.
12. Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993) Science 259: 1769-1771.
13. Cifone, M.G., De Maria, R., Roncaioli, P., Rippo, M.R., Azuma, M., Lanier, L.L., Santoni, A., and Testi, R. (1994) J. Exp. Med. 180: 1547-1552.
14. Haimovitz-Friedman, A., Kan, C.C., Ehleiter, D., Persaud, R.S., McLoughlin, M., Fuks, Z., and Kolesnick, R.N. (1994) J. Exp. Med. 180: 525-535.
15. Jaffrezou, J.P., Levade, T., Bettaieb, A., Andrieu, N., Bezombes, C., Maestre, N., Vermeersch, S., Rousse, A., and Laurent, G. (1996) EMBO J. 15: 2417-2424.
16. Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, J.M. (1996) Nature (London) 380: 75-79.
17. Segui, B., Benzombes, C., Uro-Coste, E., Medin, J.A., Andrieu-Abadie, N., Auge, N., Brouchet, A., Laurent, G., Salvayre, R., Jaffrezou, J.-P., and Levade, T. (2000) FASEB J. 14: 36-47.
18. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., and Kolesnick, R. (1995) Cell 82: 405–414.
19. Venable, M.E., Bielawska, A., and Obeid, L.M. (1996) J. Biol. Chem. 271: 24800-24805.
20. Strum, J.C., Small, G.W., Pauig, S.B., and Daniel, L.W. (1994) J. Biol. Chem. 269: 15493-15497.
21. Cifone, M.G., De Maria, R., Roncaioli, P., Rippo, M.R., Azuma, M., Lanier, L.L., Santoni, A., and Testi, R. (1994) J. Exp. Med. 177: 1547-1552.
22. Jayadev, S., Linardic, C.M., and Hannun, Y.A. (1994) J. Biol. Chem. 269: 5757-5763.

23. Schuh, J.M., Blease, K., Kunkel, S.L., and Hogaboam, C.M. (2003) Cytokine and Growth Factor Reviews 14: (2003) 503-510.
24. Oliveira, S.H.P. and Lukacs, N.W. (2003) Braz J Med Biol Res 36: 1455-1463.
25. Holberg, C.J., and Halonen, M. (2003) Lancet 362: 1166-1167.
26. Hannun, Y.A., and Obeid, L.M. (1997) Adv Exp Med Biol 407: 145-149.
27. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) Cell 71: 765-776.
28. Levade, T., and Jaffrézou, J.P. (1999) Biochim. Biophys. Acta 1438: 1-17.
29. Hannun, Y.A., Luberto, C., and Argraves, K.M. (2001) Biochemistry 40: 4893-4903.
30. Jaffrézou, J.P., Laurent, G., and Levade, T., In: P.J. Quinn and Kagan, V.E. (2002) Editors, Subcellular Biochemistry, "Phospholipid Metabolism in Apoptosis" vol. 36, Kluwer Academic/Plenum Publishers, London, 269-284.
31. Robinson, B.S., Hii, C.S., Poulos, A., and Ferrante A. (1997) Immunology 91: 274-280.
32. Jayadev, S., Linardic, C.M., and Hannun Y.A. (1994) J. Biol. Chem. 269: 5757-5763.
33. Chmura, S.J., Nodzenski, E., Weichselbaum, R.R., and Quintans, J. (1996) Cancer Res. 56: 2711-2714.
34. S.J. Chmura, E. Nodzenski, M.A. Beckett, D.W. Kufe, J. Quintans and Weichselbaum, R.R. (1997) Cancer Res. 57: 1270-1275.
35. Mansat, V., Laurent, G., Levade, T., Bettaieb, A., and Jaffrézou, J.P. (1997) Cancer Res. 57: 5300-5304.
36. Dawson, G., Goswami, R., Kilkus, J., Wiesner D., and Dawson S. (1998) Acta Biochim. Pol. 45: 287-292.
37. Mansat, V., Bettaieb, A., Levade, T., Laurent, G., and Jaffrézou, J.P. (1997) FASEB J. 11: 695-702.
38. Sawada, M., Nakashima, S., Kiyono, T., Nakagawa, M., Yamada, J. Yamakawa, H., Banno, Y., Shinoda, J., Nishimura, Y., Nozawa, Y., and Sakai N. (2001) Oncogene, 20:1368-1378.
39. Sawada, M., Nakashima, S., Banno, Y., Yamakawa, H., Takenaka, K., Shinoda, J., Nishimura, Y., Sakai, N., and Nozawa Y. (2000) Oncogene 19: 3508-3520.
40. Liu, B., and Hannun, Y.A. (1997) J. Biol. Chem. 272: 16281-16287.
41. Yoshimura, S., Banno, Y., Nakashima, S., Hayashi, K., Yamakawa, H., Sawada, M., Sakai, N., and Nozawa, Y. (1999) J Neurochem 73: 675-683.
42. Marchesini, N., Luberto, C., and Hannun, Y.A. (2003) J Biol Chem 278: 13775-13783.
43. Hoidal, J.H.(2001) Am J Respair Cell Mol Biol 25: 661-663.
44. Lavrentiadou, S.N., Chan, C., Kaweak, T.N., Ravid, T., Tsaba, A., van der Vliet, A., Rasooly, R., and Goldkorn, T. (2001) Am J Respir Cell Mol Biol 25: 676-684.
45. Grassme, H., Kirschnek, S., Riethmueller, J., Riehle, A., von Kurthy, G., Lang, F., Weller, M., and Gulbins, E. (2000) Science 290: 527-530.
46. Bezombes, C., Plo, I., Mansat-De Mas, V., Quillet-Mary, A., Nègre-Salvayre, A., Laurent, G., and Jaffrézou, J.P. (2001) FASEB J. 15: 1583-1585.
47. Takeda, Y., Tashima, M., Takahashi, A., Uchiyama, T., and Okazaki, T. (1999) J. Biol. Chem 274: 10654-10660.

48. Chan, C., and Goldkorn, T. (2000) Am. J. Respir. Cell Mol. Biol. 22: 460-468.
49. Fernandez-Ayala, D.J., Martin, S.F., Barroso, M.P., Gomez-Diaz, C., Villalba, J.M., Rodriguez-Aguilera, J.C., Lopez-Lluch, G., and Navas, P. (2000) Antioxid. Redox Signal. 2: 263-275.
50. Dressler, K.A., Mathias, S., and Kolesnick, RN. (1992) Science 255: 1715-1718.
51. Dbaibo, G. S., Obeid, L. M., and Hannun, Y. A. (1993) J. Biol. Chem. 268: 17762-17766.
52. Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y. A., and Seldin, M. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92: 8443-8447.
53. Osborn, L., Kunkel, W., and Nabel, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86: 2336-2340.
54. Lowenthal, J. W., Ballard, D. W., Bogerd, H., Böhnlein, E., and Greene, W. C. (1989) J. Immunol. 142: 3121-3128.
55. Ghosh, S., May, M., and Kopp E. (1998) Ann. Rev. Immunol. 16: 225-260.
56. Baldwin, A. (2201) J. Clin. Invest. 17: 241-246.
57. Ghosh, S, and Karin, M. Cell 109: S81-S96.
58. Van Antwerp, D. J., Martin, S. J., Verma, I. M., and Green, D. R. (1998) Trends Cell Biol. 8: 107-111.
59. Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S. (1993) Mol. Cell. Biol. 13: 3301-3310.
60. Delhase, M., and Karin, M., (2002) Cold Spring Harbor Symposia on Quantitative Biology 64: 491-503.
61. Li, X, Massa, P., Hanidu, G., Peet, P., Aro, A, Savitt, S., Mische, J., Li, J., and Marcu, K. (2002) J. Biol. Chem. 277: 45129-45140.
62. Anest, V., Hanson, J., Cogswell, P. Steinbrecher, K., Strahl, B., and Baldwin, A., (2003) Nature 423: 659-663.
63. Yamamoto, Y., Verma, U., Prajapati, S., Kwak, Y., and Gaynor (2003) Nature 423: 655-659.
64. Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., and Baeuerle, P. (1993) Nature 365: 182-185.
65. Johns, L. D., Sarr, T., and Ranges, G. E. (1994) J. Immunol. 152, 5877-5882.
66. Schütze, S., Potthof, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992) Cell 71: 765-776.
67. Luberto, C., Yoo , D. S., Suidan, H. S., Bartoli, G.M., and. Hannun Y. A.(2000) J Biol Chem, 275: 14760-14766.
68. Liu, B., Andrieu-Abadie, N., Levade, T., Zhang, P., Obeid, L.M., and Hannun, Y.A. (1998) J. Biol. Chem. 273: 11313-11320.
69. Calvet, J.H., Jarrean, P. H., Levame, M., D'ortho, M.P., Lorino, H., Harf, A., and Mavier, I. M. (1994) J. Appl. Physiol 76: 681-688.
70. Bijani, K.H., and Moghadamnia, A.A. (2002) Ecotoxicol. Environ. Saf. 53: 422-424.
71. Emad, A., and Rezaian, G.R. (1997) Chest 112: 734-738.
72. Emad, A., and Rezaian, G.R. (1999) Am. J. Med.106: 625-628.
73. Mukherjee S, Ghosh S, Rodgers, L Nayyar T Desai U and Das S K (1994) Toxic effects of fatty acid Anilides on the oxygen defense systems of guinea pig lungs and erythrocytes J Biochem Toxicol 9: 1-7.

74. Mukherjee S, Woods L, Weston Z, Williams AB and Das SK (1993) The effect of mainstream and sidestream cigarette smoke exposure on oxygen defense mechanisms of guinea pig erythrocytes. J. Biochem. Toxicology 8: 119-125.

75. Williams, M.D., and Chance, B. (1983) J. Biol. Chem 258: 3628-3631.

76. Sznajder, J.I., Fraiman, A., Hall, J.B., Sanders, W., Schmidt, G., Crawford, G. Nahum, A., Factor, P., and Wood, L.D. (1989) Chest 96: 606-612.

77. Loukides, S., Horvath, I., Wodehouse, T., Cole, P.J., and Barnes, P.J. (1998) Am. J. Respir. Crit. Care Med. 158: 991-994.

78. Dekhuijzen, P.N., Aben, K.K., Dekker, I., Aarts, L.P., Wielders, P.L., van Herwaarden, C.L., and Bast, A. (1996) Am. J. Respir. Crit. Care Med. 154: 813-816.

79. Nowak, D., Antczak, A., Krol, M., Pietras, T., Shariati, B., Bialasiewicz, P., Jeczkowski, K., and Kula, P. (1996) Eur. Respir. J. 9: 652-657.

80. Chatterjee, D., Mukherjee, S., Smith, M.G., Das, S.K. (2003), J. Biochem. Mol. Toxicol. 17: 114-121.

81. Das, S.K., Mukherjee, S., Smith, M.G., and Chatterjee, D. (2003) J. Biochem. Mol. Toxicol. 17: 177-184.

82. Elsayed, N. M., Omaye, S. T., Klain, G.J., and Korte, D.W. (1992) Toxicology, 72:153-165.

83. Cowan, F.M., and Broomfield, C.A. (1993) Cell Biol. Toxicol. 9: 201-213.

84. Husain, K., Dube, S.N., Sugendran, K., Singh, R., Das Gupta, S., and Somani, S. M. (1996) J. Appl. Toxicol. 16: 245-248.

85. Kopff, M., Zakrzewska, I., Strzelczyk, M., Klem, J., and Dubiecki, W. (1994) Pol. J. Pharmacol. 46: 439-444.

86. Kuhns, D.B., Decarlo, E., Hawk, D.M., and Gallin, J.I. (1992) J. Clin. Invest. 89: 1734-1740.

87. Arroyo, C.M., Von Tersch, R.L., and Broomfield, C.A. (1995) Human and Experimental Toxicology 14: 547-553.

88. Ricketts, K.M., Santai, C.T., France, J.A., Graziosi, A.M., Doyel, T.D., Gazaway, M.Y., and Casillas, R.P. (2000) J Appl Toxicol 20 (Suppl. 1): S73-S76.

89. Kumar, O., Sugendran, K., and Vijayaraghavan, R. (2001) Chemico Biological Interactions 134: 1-12.

90. Papirmeister, B., Gross, C.L., Petrali, J.P., and Meier, H.L. (1984) J. Toxicol. Cutan Ocular Toxicol. 3: 395-410.

91. Ryan, A.J., McCoy, D.M., McGowan, S.E., Salom, R.G., and Mallampall, R.K. (2003) J Appl Physiol 94: 253-258.

92. Shang, J., Eberle, J., Geilen, C.C., Hossini, A. M., Fecker, L. F., Orfanos, C. E., and Tebbe, B. (2002) Pharmacol. Appl. Skin Physiol. 15: 321-329.

93. Fujiki, H., Suganuma, M., Okabe S., Kurusu, M., Imai, K., and Nakachi, K. (2002) Mech. Ageing Dev. 123: 1655-1663.

94. Beg, A.A., and Baltimore, D. (1996) Science 274: 782-787.

95. Xu, Y., Bialik, S., Jones, B.E., Iimuro, Y., Kitsis, R.N. Srinivasan, A., Brenner, D.A., and Czaja, M.J. (1998) Am. J. Physiol. 275: 1058-1066.

96. Plumpe, J., Malek, N.P., Bock, C.T., Rakemann, T., Manns, M.P., and Trautwein, C. (2000) Am. J. Physiol. 278: 173-183.

97. Vogt, R.F., Dannenberg, A.M., Schofield, B.H., Hynes, N.A., and Papirmeister, B. (1984) Fundam. Appl. Toxicol. 4: S71-S83.

98. Signorelli, P., Luberto, C., and Hannun, Y.A. (2001) FASEB J. 15: 2401-2414.

99. Marchesini, N. Luberto, C., and Hannun, Y.A. (2003) J. Biol. Chem. 278: 13775-13783.

100. McClintock, S.D., Till, G.O., Smith, M.G., and Ward, P.A. (2002) J. Appl. Toxicol. 2: 257-262.

101. Atkins, K.B., Lodhi, I.J., Hurley, L.L., and Hinshaw, D.B. (2000) Appl Toxicol 20: S125-S128.

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TITLE: Effect of Antioxidants on Bacillus anthracis – Macrophage Interactions

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Table of Contents

INTRODUCTION SUBJECT: Oxidative stress, including the production of reduced oxygen products during the interaction of macrophages with pathogens, alters the host response, often exacerbating the host-parasite interaction, leading to more severe inflammation or disease. Inhibiting or dampening the oxidative response can help ameliorate inflammation and reduce disease manifestations. PURPOSE and SCOPE: With this in mind, we hypothesized that anti-inflammatory agents, particularly liposome-encapsulated reducing agents such as N-acetylcysteine, glutathione, and tocopherols, would help human macrophages limit growth of *Bacillus anthracis* (BA), in an *in vitro* system where BA spores and macrophages are incubated together in small culture dishes. We found that reducing agents have a dramatic effect on helping macrophages control germination and replication of BA.

BODY This section of the report summarizes what has been presented in each quarterly report. The data presented in the quarterly reports will not be repeated, and no additional data have been added that isn't already in the quarterly reports.

Liposomes for all of our studies were from the laboratory of Dr. William Stone, East Tennessee State University, a member of the consortium.

Task 1. Determine if antioxidant-loaded liposomes alter the cellular functions of human monocyte derived macrophages and human macrophage-like cell lines treated in vitro with sublethal concentrations of anthrolysin O (ALO) and anthrax lethal toxin (LT), alone or in combination (months 1-12).

   a) *Measure macrophage viability, apoptosis, chemotaxis, phagocytosis, phagosome-lysosome fusion, phagolysosome pH, phagocytic killing and upregulation of surface markers.*
   b) *Determine the effects of empty or antioxidant-loaded liposomes on the functions measured in 1.a).*

In dose and time experiments, we measured the effects of free NAC and glutathione and NAC-loaded liposomes on the viability of human monocyte-derived macrophages (mdm) and on macrophage cell lines. Concentrations of antioxidants and/or liposomes did not cause cell death.

NAC completely reverses the ability of the anthrax toxin ALO to inhibit phagocyte chemotaxis. We investigated the effects of NAC and NAC liposomes on macrophage and neutrophil chemotaxis, in the absence or presence of Anthrolysin O (ALO), a newly described anthrax toxin (Shannon JG, Ross CL, Koehler TM, Rest RF, 2003 Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin. Infect Immun. 71:3183; Park JM, Ng VH, Maeda S, Rest RF, Karin M, 2004 Anthrolysin O and other gram-positive cytolysins are toll-like receptor 4 agonists. J Exp Med. 200(12):1647; Mosser EM, Rest RF, 2006 The *Bacillus anthracis* cholesterol-dependent cytolysin, Anthrolysin O, kills human neutrophils, monocytes and macrophages. BMC Microbiol. 6:56). Chemotaxis was measured in a NeuroProbe disposable micro well chemotaxis chamber. He had previously observed that LPS-free recombinant ALO inhibits neutrophil chemotaxis by >90%. Upon pre-addition or simultaneous addition of 125 uM NAC (a sublethal concentration), chemotaxis was restored to control levels. Thus, inhibition of phagocyte chemotaxis by ALO is redox sensitive, and is completely reversed by NAC.

In the first quarter, we showed that inhibition of phagocyte chemotaxis by the *B. anthracis* pore-forming toxin anthrolysin O (ALO) is redox sensitive, and is completely reversed by NAC. One possible mechanism to explain this observation is that NAC affects activation, i.e., phosphorylation of mitogen activated protein kinases (MAPK) including p38 MAPK. To investigate this possibility, we incubated hMDM +/- NAC liposomes for 30 min, and then added 100 ng/ml ALO for 10 min. Cells were then washed, disrupted, and analyzed via Western analysis for activated, phospho-p38 (bottom panel, below) or for native, un-phosphorylated p38, (top panel, below). Native p38 was quantified to determine total cellular p38, and to measure similar loading of sample in different lanes. As expected, ALO alone induces p38 phosphorylation. The data indicate that NAC liposomes have little or no effect on the ability of ALO to stimulate p38 phosphorylation. NAC liposomes alone do not activate p38 above background. *Thus, the ability of NAC to relieve inhibition of macrophage chemotaxis by ALO is not mediated through p38 MAPK, even though p38 MPK is involved in ALO inhibition of macrophage chemotaxis (see below).*

In an effort to determine the mechanism of action of ALO on macrophage function, and, eventually, to get a better handle on how NAC affects macrophage function, we are studying the contribution of calcium and p38 MAPK on such effects. *We noted that chelation of extracellular calcium, and to some extent the presence of p38 MAPK inhibitors, inhibits ALO-mediated differentiated human THP-1 cytotoxicity (data not shown).*

Interestingly, neutrophils, which are more sensitive to lethal ALO-mediated lysis (due to large pore formation), are also more sensitive to sublethal ALO-mediated p38 MAPK phosphorylation. From this we hypothesized that some of the ALO-induced p38 MAPK phosphorylation we observe is a result of small sublethal pore formation and calcium fluxes. To test this hypothesis and better determine the mechanism of ALO's ability to stimulate p38 MAPK, we pre-incubated PMA-differentiated THP-1s with 5 mM EGTA 30 min before stimulation with rALO. Macrophages in which the extracellular calcium was chelated had a decreased amount of p38 MAPK stimulation as compared to controls without EGTA (Western analysis to the left, --Suramin, +EGTA). EGTA alone did not effect p38 MAPK stimulation. THP-1s remained viable under all conditions. *These results show that ALO-mediated p38 MAPK phosphorylation is due, at least in part, to the presence of extracellular calcium, and probably flow of calcium into the cell.*

In the first quarter, we noted that chemotaxis of macrophages towards IL-8 and fMLP is inhibited > 90% by as little as 10 ng/of rALO (p<.05, n=3). To examine the role of p38 MAPK in the observed rALO-mediated inhibition, we incubated the p38 MAPK inhibitor, SKF86002, with phagocytes 30 min prior to stimulation with rALO. The p38 MAPK inhibitor completely prevented rALO-mediated inhibition of chemotaxis (data not shown). *Thus, p38 MAPK is involved in rALO inhibition of chemotaxis.*

Task 2. Determine if antioxidant-loaded liposomes alter the release of pro-inflammatory cytokines from human monocyte-derived macrophages and human macrophage-like cell lines incubated with sublethal concentrations of ALO and LT, alone or in combination (months 3-12).

a) Measure IL-1α, IL-1β, IL-6 and TNFα mRNA and cytokine release. Other cytokines will be measured as necessary and for controls.

b) Determine the effects of empty or antioxidant-loaded liposomes on the functions measured in 2.a).

We did not perform studies related to this Task.

Task 3. Determine if antioxidant-loaded liposomes alter the interactions and outcomes of viable, virulent Bacillus anthracis spores with human macrophages (months 5-12).

a) Use wild type B. anthracis and mutants – lethal factor, protective antigen, edema factor, capsule biosynthesis, and anthrolysin O – to probe B. anthracis-macrophage interactions. Observe spore germination, B. anthracis viability and replication, and macrophage responses, including viability, apoptosis, upregulation of surface markers, and cytokine release.

b) Determine the effects of empty or antioxidant-loaded liposomes on the functions measured in 3.a).

NAC decreases BA growth in association with macrophages. We incubated BA spores with monolayers of mdms (data shown immediately below), THP-1 cells (a human monocyte/macrophage-like cell line; data not shown) or RAW264.7 cells (a mouse macrophage-like cell line; data not shown), with or without antioxidant. NAC and NAC-liposomes inhibited the growth or germination of BA in association with macrophages, in a dose and time dependent manner (n=4). In the presence of untreated macrophages (i.e., with no antioxidant), BA spores germinated and then the vegetative cells multiplied, appearing in the supernatant about 5 hours after infection. The same time frame was seen with NAC treatment; however, at 8 hours there were significantly fewer vegetative BA both intracellularly and extracellularly compared to controls without NAC.

NAC inhibits germination of anthrax spores. The observation that NAC dramatically decreased the number of viable BA in the presence of macrophages in culture (Fig 2 above), prompted us to look at the effects of NAC on BA germination in the absence of macrophages. We have begun trying to determine whether NAC is affecting the ability of BA to germinate or whether it is increasing the ability of macrophages to kill BA (or both). We mixed NAC and fresh BA spores together at 37C in cell culture medium plus 10% serum, and at 10 minute intervals diluted and plated samples without or with heat treatment (65C, 20 min). NAC liposomes (and soluble NAC) dramatically inhibited germination of BA spores in a time-dependent (Fig 3) and dose dependent (data not shown) manner.

In the first quarter we showed that NAC significantly decreases *B. anthracis* growth in association with macrophages. In studies performed this quarter, and exemplified by the experiment below, we incubated *B. anthracis* spores with monolayers of differentiated THP-1 human monocyte cultures, with or without NAC liposomes. NAC-liposomes inhibited the germination of macrophage-associated *B. anthracis* spores, in a dose and time dependent manner (n=4). In the presence of untreated macrophages, *B. anthracis* spores began to germinate at about 5 hours after infection, whereas in the presence of NAC-treated macrophages, spore germination was >80% complete by 3 hours. Paradoxically, in the last quarter we also showed that NAC (in a dose and

time dependent manner) **inhibits** germination of anthrax spores in the absence of macrophages. Thus, there is a complex relationship between macrophages, *B. anthracis* spores, and NAC.

In the third quarter, we used the light microscope and camera purchased with funds from this contract. We show with phase contrast and Nomarski Differential Interference Contrast (DIC) optics that in the presence of macrophages (without NAC), *B. anthracis* spores germinate and grow into dense 'ropes' or 'mats' of vegetative bacteria at ~4 hours after infection (and are very obvious at 6 hours post infection), whereas with 5 mM NAC obvious outgrowth into vegetative cells is significantly delayed (figure 1, below). In addition, there is less macrophage death with NAC than without; at 8 hrs, in the absence of NAC, there are no viable macrophages remaining in culture. Since our data from last quarter indicate that germination itself is not inhibited by NAC in the presence of macrophages, it appears that germination and outgrowth into actively growing, tightly packed vegetative cells are two quite distinct functions.

In the fourth quarter we repeated earlier experiments and we were excited that we confirmed what we have seen up to this point. For instance, we took publication quality Nomarski DIC photomicrographs, as shown below. It is clear that NAC allows human macrophages to limit BA infection *in vitro*. The photos (taken at 0, 4 and 8 hours) show the interaction of BA Sterne strain 7702 spores with THP-1 cells in culture with macrophages that were or were not treated with 10 mM NAC. At the 8 hour time point WITHOUT NAC, notice the large number of vegetative BA and the low numbers of macrophages, compared to the low number of vegetative BA and the larger number of healthy macrophages WITH NAC. Very striking!

We are in the process of writing 3 manuscripts, (i) one on the effects of reducing agents on *B. anthracis* germination, (ii) one on the effects of reducing agents with or without liposomes on the ability of human macrophages to limit BA infection *in vitro*, and (iii) one on the effects of antioxidants on ALO-treated human macrophages.

KEY RESEARCH ACCOMPLISHMENTS

> ➢ NAC accelerates BA sporulation (in the absence of macrophages).
> ➢ NAC inhibits BA germination (in the absence of macrophages).
> ➢ NAC **reverses** the inhibition of human macrophage and neutrophil chemotaxis caused by the anthrax toxin Anthrolysin O.
> ➢ NAC **prevents** the inhibition of human macrophage and neutrophil chemotaxis caused by the anthrax toxin Anthrolysin O.
> ➢ NAC prevents degranulation of human neutrophils caused by the anthrax toxin Anthrolysin O.
> ➢ NAC, in a dose and time-dependent manner, stimulates the ability of human macrophages to kill intracellular BA.
> ➢ NAC is not directly toxic to vegetative BA or to BA spores, or to macrophages.

REPORTABLE OUTCOMES Two abstracts were submitted and accepted:

1) BARDA (Biomedical Advanced Research Development Authority) *2007 Industry Day* Conference, **August 3, 2007**, Washington, DC.

Abstract entitled "STIMAL (Liposome-Encapsulated N-Acetylcysteine) Increases the Ability of Human Macrophages to Kill *Bacillus anthracis*"

2) *2007 Biodefense Research Conference – Bridging the Gap: Biodefense and Beyond*, **Nov 5-7, 2007,** Philadelphia, PA (sponsored by the Joint Science and Technology Office for Chemical and Biological Defense (JSTO-CBD) / Defense Threat Reduction Agency (DTRA))

Abstract entitled "STIMAL (Liposome-Encapsulated N-Acetylcysteine) Increases the Ability of Human Macrophages to Kill *Bacillus anthracis*"

CONCLUSIONS In this first year of funding, we have made very significant progress towards our goals, and have made several surprising, dramatic and relevant observations vis-à-vis mechanisms of disease and treatment of anthrax. We observed that NAC (and other reducing agents) has dramatic effects on several aspects of the interaction of *Bacillus anthracis* with human macrophages and neutrophils. NAC completely reverses or inhibits the negative effects of the anthrax toxin Anthrolysin O on human phagocytes, as measured by chemotaxis and degranulation. NAC helps human macrophages limit the growth of BA in vitro. It also directly inhibits (in the absence of macrophages) the ability of BA spores to germinate. If BA spores don't germinate, there is no disease. Alternatively, if NAC delays germination, host defenses would have time to mount a better attack on germinated bacteria. In case of an anthrax attack, NAC could be used as an immediate therapeutic aid to help prevent the initial intra-alveolar events of BA-macrophage interactions in humans, and thus diminish or eliminate the disease potential of the infecting BA spores. STIMAL, and its active component NAC, which is approved for human use, could be an ancillary therapeutic agent, as the first multithread treatment countermeasure, in conjunction with vaccines and antimicrobials.

REFERENCES - NONE

APPENDICES   (Two abstracts)

BARDA INDUSTRY DAY (August 3, 2007)

STIMAL (Liposome-Encapsulated N-Acetylcysteine) Increases the Ability of Human Macrophages to Kill *Bacillus anthracis*

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Category: B. Therapeutics
TRL1 / TRL2

Background STIMAL is a non-toxic anti-oxidant and anti-inflammatory therapeutic based on liposome-encapsulated NAC. We have previously shown that STIMAL ameliorates the pathophysiology associated with *in vivo* and *in vitro* administration of CEES, a mustard gas analogue. During inhalation anthrax, *B. anthracis* (BA) interacts directly with alveolar macrophages, and infection and disease is initiated following this critical interaction. In the present studies, we investigated whether STIMAL might help human macrophages limit the *in vitro* growth of BA, and thus limit disease progression in the host.

Methods We treated BA spores or vegetative cells with various concentrations of NAC *in vitro* in the presence or absence of human macrophage monolayers, and quantified BA and macrophage viability and functions. Proper negative and positive controls were included.

Results (i) NAC accelerates BA sporulation. (ii) NAC completely delays / inhibits BA germination (in the absence of macrophages). (iii) NAC, in a dose and time-dependent manner, stimulates the ability of macrophages to kill intracellular BA. (iv) NAC is not directly toxic to vegetative BA or to BA spores.

Conclusions NAC has dramatic protective activities regarding macrophage killing of BA. In case of an anthrax attack, NAC could be used as an immediate therapeutic aid to help prevent the initial intra-alveolar events of BA-macrophage interactions in humans. STIMAL, and its active component NAC, which is approved for human use, could be an ancillary therapeutic agent, as the first multithread treatment countermeasure, in conjunction with vaccines and antimicrobials.

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2007 Biodefense Research Conference (November 5-7, 2007)

STIMAL (Liposome-Encapsulated N-Acetylcysteine) Increases the Ability of Human Macrophages to Kill *Bacillus anthracis*

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Anthrax is caused *by Bacillus anthracis* (BA), a spore-forming, facultative aerobic, Gram positive bacillus. Anthrax in humans and many animals is initiated by introduction of BA spores into the body by penetration of the epidermis, the gut, or the respiratory epithelium. *In vivo* experiments have demonstrated that once inhaled, anthrax spores can reach the bronchioles and alveoli of the lung, and most spores rapidly and efficiently associate with alveolar macrophages and dendritic cells. The germination of spores and emergence of vegetative bacilli, in an environment allowing rapid out-growth in the body, are essential in the early stages of infection in order to establish disease. After germination, the bacilli multiply and spread to the regional lymph nodes and lymph, and from there to the blood stream where they can reach $>10^8$/ml.

In vitro, there are contradicting studies about how successful macrophages are at clearing anthrax spores. Most agree spore uptake is inefficient, yet there is extensive adherence of spores and vegetative cells to the macrophage surface. Four to six hours post infection, there is extensive germination and vegetative cell outgrowth, with chains of bacteria extending across several macrophages. The degree of BA clearance varies depending on the type of macrophage cell (or cell line) and the methodology used in each study.

In studies presented here, we use (i) human monocyte-derived macrophages (hMDMs), (ii) the RAW 264.7 murine macrophage cell line, or (iii) the differentiated human THP-1 cell line, infected with BA Sterne strain 7702 spores, to study the effect of individual or liposome encased antioxidants (STIMAL) on the interaction between BA and macrophages. Liposomes are cholesterol and fatty-acid rich carriers that can readily fuse with cell membranes and successfully deliver their contents inside the cell. STIMAL is a non-toxic, anti-inflammatory therapeutic based on liposome-encapsulated N-acetylcysteine (NAC). STIMAL ameliorates the pathophysiology associated with *in vivo* and *in vitro* administration of CEES, a mustard gas analogue (J Appl Toxicol. 2002, 22:257). The antioxidants we focused on in the present studies are NAC and glutathione, and the fat soluble tocopherols. NAC is a thiol, a mucolytic agent, a precursor of intracellular L-cysteine and reduced glutathione, and a source of sulfhydryl groups in cells and scavenger of free radicals as it interacts with reactive oxygen species such as OH• and H_2O_2. NAC's interaction with bacterial pathogens like BA and its effect upon BA infection have yet to be elucidated. At the initiation of these studies, we hypothesized that NAC would affect the interplay between BA and macrophages, providing the latter with means to better clear the infection.

We incubated various macrophages or macrophage cell lines in 24 well plates with or without 5 mM NAC and subsequently infected them with BA. At 2 hrs post-infection, untreated and treated macrophages had similar numbers of cell-associated spores. However, starting at 4 hrs, spores germinated 2 hrs earlier in NAC treated hMDMs, compared to untreated cells, and the germinated BA were rapidly killed. In addition, and logically following the intracellular killing of germinated spores, there was a >3-fold decrease in the number of extracellular vegetative bacteria 6 hrs post-infection in NAC treated hMDMs, compared to untreated controls. A similar phenomenon occurred with RAW 264.7 or differentiated THP-1 macrophages. Via *in vitro* viability assays and via light microscopy, a significantly greater percentage of NAC pretreated macrophages were viable at the end of assays (8 hours) compared to untreated macrophages. Thus, macrophages pre-treated with 5 mM NAC kill germinating BA spores more effectively than do untreated macrophages, strictly limit total BA growth, and remain viable. In the absence of macrophages, 5 mM NAC reduced germination of BA spores >95% in a 90 min study. Importantly, NAC was not toxic to spores or vegetative bacilli at the times or concentrations used in our studies. Therefore, the interplay between the effect of NAC on macrophages and its direct effect on BA spores may lead to successful clearance of BA infection. We suggest that STIMAL could be used in conjunction with antibiotics and vaccination in the event of an anthrax attack.

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EDUCATIONAL HISTORY

1970-1974, Ph.D., Microbiology, University of Kansas, Lawrence
        Advisor: Donald C. Robertson, Ph.D.
1966-1970, B.S., Microbiology, University of Massachusetts, Amherst
        Advisor: Martin S. Wilder, Ph.D.

PROFESSIONAL EXPERIENCE & POSITIONS

(Drexel Univ College of Medicine was formerly MCP Hahnemann Univ Sch of Medicine was formerly Hahnemann Univ)
2005-present, Director, Biomed Grad and Postgrad Professional Development, Drexel University College of Medicine
1999-2001, Associate Dean, Biomedical Graduate Studies, MCP Hahnemann University School of Medicine
1994-present, Professor, Dept. of Pathology and Laboratory Medicine, Drexel Univ College of Medicine
1990-present, Professor, Dept. of Microbiology & Immunology, Drexel Univ College of Medicine
1983-1990, Associate Professor, Dept. of Microbiology & Immunology, Hahnemann University School of Medicine
1983, Associate Professor (with tenure), University of Arizona Health Sciences Center
1977-1983, Assistant Professor, Dept. Molecular & Medical Microbiology
        University of Arizona Health Sciences Center, Tucson
1974-1977, Post-doctoral Fellow, Department of Bacteriology and Immunology
        University of North Carolina, Chapel Hill
        Advisor: John K. Spitznagel, M.D.

RESEARCH INTERESTS
        I am interested in how *Neisseria gonorrhoeae, N. meningitidis* and *Bacillus anthracis* cause disease. My laboratory personnel have investigated two neisseria virulence factors: Opa proteins and sialyltransferase. (1) Opa proteins, a family of outer membrane proteins, are pluripotent virulence factors, allowing gonococci and meningococci to adhere to, invade and survive within human phagocytes, epithelial cells and endothelial cells. Using the yeast two hybrid system, we showed that intracellular Opa+ gonococci bind at least two human epithelial cell cytosolic proteins, including pyruvate kinase (PK) and thyroid hormone receptor interacting protein 6 (TRIP6). Gonococci bind PK and appear to require pyruvate for optimal intracellular survival and growth. We hypothesize that gonococci bind TRIP6, which is a cytoskeletal-associated protein and a nuclear messenger & transcriptional activator, to signal their presence within the cell, and alter host responses. (2) We have shown sialyltransferase (stase) to be an outer membrane, surface-exposed glycosyltransferase that transfers sialic acid (N-acetylneuraminic acid, Neu5Ac) from CMP-Neu5Ac to terminal galactoses of neisseria lipooligosaccharide (LOS). Sialylation of LOS can render gonococci and meningococci resistant to the bactericidal action of human serum, and modulate how these pathogens interact with host cells. We study regulation of expression of stase by environmental cues including contact with serum and human cells. (3) More recently we have studied the effect of antioxidants, including N-acetylcysteine (NAC), on interactions of *B. anthracis* with human macrophages and neutrophils, focusing on spores and the role of Anthrolysin O (ALO), a pore-forming, cholesterol-dependent cytolysin. ALO has rapid lethal effects and potent sublethal effects on host cells, due at least in part to signaling through TLR4 (Toll like Receptor 4). Most recently, we are characterizing a new global transcription regulator, unique to the *B. cereus* group.

ACTIVITIES, COMMITTEES, POSITIONS AND AWARDS

National and International
Research Career Development Award, NIH, 80-85
Ad hoc member, various NIH study sections, 82 – present
Member, Bacteriology and Mycology 2 (BM2) Study Section, NIH, 84-88
Editorial board: Infection and Immunity, 88-present
Editorial Advisory Board, The Journal of Infectious Diseases, 06 – present
Member, NIH Reviewer's Reserve, 89-94
Ad hoc grant proposal review: American Cancer Society, NIH, NSF, US - Israel Cooperative Program, Veterans
      Administration, The Wellcome Trust (England), US Civilian Research & Development Foundation (CRDF)
Invited referee: Amer J Respiratory Cell & Molecular Biology, Antimicrobial Agents and Chemotherapy; Cellular
      Microbiol, Clin Immunol & Immunopath, FEMS Immuno & Med Micro, Gene, Infect & Immun, Int'l J Exptl
      Pathology, J Bact, J Clin Immunol, J Clin Invest, J Immunol, J Infect Dis, J Leukocyte Biol, J Virology,
      Microbial Pathogenesis, Microbiology, Molec Microbiol, Nature Medicine, PNAS, Scanning Electron
      Microscopy, Vaccine
Sabbatical leave with Drs. Barry Eisenstein and Laurence Boxer, Univ Mich Med Sch, 8/89-8/90
Member, national ASM Biotechnology Conference Committee, 90-92
Member, national ASM Public and Scientific Affairs Board, 91-97
Co-organizer and co-chair, *Microbial Pathogenesis and Immune Response I & II*; N.Y. Academy of Sciences,
      sponsor; 9/93, Orlando, FL; 10/95, New York, NY.
Sabbatical leave with Dr. E. Richard Moxon, Oxford Univ, England, 8/96-8/97
Outside Reviewer, FDA Laboratory of Enterics and Sexually Transmitted Diseases, 8/97
Member, USMLE Step 1 Microbiology Test Material Development Committee, 03 – 05
Chair, Microbiology and Immunology Test Committee, US Podiatric Medical Licensing Exam, 06 - present

Local and Regional
President Elect/President, Arizona Branch American Society for Microbiology (ASM), 81-83
Chair, Program Committee, Eastern Pennsylvania Branch ASM, 91-93
Chair and organizer, *92, 93, 94, 96, 02, 03, 04 Phila Infection & Immunity Forum* (Eastern PA Branch ASM)
Volunteer teacher, middle and high school students; STDs and their prevention, 92-96
1994 Teaching Faculty Award (best teacher), from the Hahnemann University Graduate Student Society
President Elect/President, Eastern Pennsylvania Branch ASM, 95-97/97-99
Nominated by MCPHU class of 2000 for the Golden Apple Award, best medical school teacher, 1998
Nominated by MCPHU class of 2004 for the Golden Apple Award, best medical school teacher, 2001
Nominated by DUCOM class of 2008 for the Golden Apple Award, best medical school teacher, 2005
Board of Directors, Families with Children from China – Delaware Valley (FCC-DV), 00-04
Vice President, Families with Children from China – Delaware Valley (FCC-DV), 01-04
Board of Directors, Radnor Elementary School PTO, 01-03
President, Ding Hao Chinese School PTO, 01-03
Board of Directors, Chinese Culture Association of Greater Philadelphia, 02-present
Board of Directors, Ding Hao Chinese School, 02-present

University & Medical School Committees and Positions
University Safety Comm 87-89, 93-95
Comm on Academic Quality, Graduate School 87-89, 93-95, 97-99
Admissions Comm, COM Graduate School 90-91
Admissions Comm, COM doctoral subcommittee, Graduate School 90-91
University Research Comm, University, 90-99, ex officio 99-01
Medical School Research Comm, University, co-chair, 91-96; ad hoc 97-01
MD/PhD Advisory Comm, University, 92 (inception)-95
University Biosafety Comm / Institutional Biosafety Committee, Chair, 93-95
Microbiology & Immunology Dept Graduate Program coordinator, 92-97; co-director 97-99; 01-04
University Council, 96
Department of Microbiology & Immunology Graduate Program course coordinator, 96-03
Committee to Investigate Endowments (appointed by University President) 98

Research Support Services Task Force, chair (appointed by COM Dean) 98-99
University Library Committee 99-00
Associate Dean for Biomedical Graduate Studies 99-01 (appointed by COM Dean)
University Research Finance Committee, co-chair (appointed by University Provost), 01-03
The National Bioterrorism Civilian Medical Response Center, steering committee, 01-present
Medical Student Summer Research Program, Director, 00-02; review committee chair, 03–07
Research Strategic Planning at COM, Collaborations subcommittee chair (appointed by COM Dean), 02
Strategic Planning Committee, COM, co-chair (appointed by COM Dean), 04-present
Capital Committee, COM, member (appointed by University Provost), 04-present
Institute of Basic and Applied Protein Science (IBAPS), steering comm & member, 03 (inception) - 05
Committee on future of IBAPS (appointed by University Provost), 05
Committee on University Institutes, Centers and Programs (appointed by University Provost), 05-present
Biomed Grad and Postdoc Professional Development, Director (appointed by COM Dean), 05-present
Committee on Drexel Faculty Diversity (appointed by University Provost), 06 - present
DUCOM Core Curriculum sub-committee, Biomedical Graduate Studies, 06 - present

University Activities

Organized and wrote grant proposals for Hahnemann, through NIH Shared Instrumentation Program, for: DuPont
ultracentrifuge, 88; ABI peptide synthesizer, 91; BioRad molecular imaging system, 93; ABI DNA
sequencer, 98
Advise Hahnemann medical and graduate students who volunteer teach middle and high school students about
STDs and their prevention, 92-96
Research mentor for:
high school students in HUMRAP (HU Minority Research Apprentice Program): Rosemary Ali, 89 & 90;
Jenny Solis, 91 & 92; Nya Lewis 95
>MCPHU/Drexel medical students: Nicolle Lee, 86-87; Christopher Bowden, 87; Bo Hong Yoo, 88-89; Greg
Ahearn, 92; Sarah Arnold, 92; Sarah Chang, 01; Karlyn Powell, 02; John Galiote, 03; Steven Boyle, 04
>MCPHU Medical Sciences Track Students: Michael DiMarino, 94; John Merlino,94-95; Yetunde Olesande,
99-00
>Minority high school teachers in HU Summer Research Program: Michael Bailey, 93
>Bucknell Univ. "Jan Plan" work-study program: John Van Kirk, 92; Duane Godshall, 93
>Summer Undergraduate Research Fellowship (SURF): Michael Quayle, 01; Alissa Romano, 03; Amit
Prasad, 04
>Other undergraduates: Denise Ireland, Drexel, 01-02; Alexandra Bortnick, Univ Maryland, 02; Krista
Kolbach, Penn State Univ, 02; Cassie Gunnis, Bradford Univ (UK), 03
Member, Microbiology and Immunology Graduate Program, 83 (inception) -present; Molecular Biology Graduate
Program, 88 (inception)-present; Radiation Sciences Graduate Program, 99-present; Molecular
Pathobiology graduate program, 88 (inception)-present

TEACHING EXPERIENCE

University of Arizona
1977-78: *Pathogenic Microbiology* (MICRO 220); 12 lectures, 75 undergraduates.
1977-83: *Pathogenic Mechanisms*, literature seminar (MMIC 596g), 6-8 graduate students.
1978-79: *Medical Microbiology* (MMIC 801), Initiated and re-designed 10 laboratory exercises; 12 lectures, 88
medical students. MICRO 220; 12 lectures, 40 undergraduate students. *Molecular Mechanisms of
Microbial Pathogenesis* (MMIC 550); 20 lectures of 1½ hour each, 8 graduate students.
1979-81: MMIC 801; Course Coordinator, 15 lectures.
1981-83: MMIC 801; Course Coordinator, 12 lectures. MMIC 550; 20 lectures of 1½ hours each, 6-12 graduate
students.

Drexel University College of Medicine (formerly MCP and Hahnemann School of Medicine)
1984-85: *Medical Microbiology*; 3-4 hrs/wk laboratory for semester (15 weeks), and four 50 min lectures to 175
medical students.
85-89: As 84-85; plus *Molecular Mechanisms of Microbial Pathogenesis*; ten 2-3 hr lectures, to 3-6 graduate
students, annually. 88-89; chaired committee to reorganize the Microbiology Department's M.S. and Ph.D.
curricula.
89-90: ***On sabbatical leave***

90-93: 8-12 lectures and 5-10 laboratory sessions in *Medical Microbiology,* annually. 12 2-3 hr lectures in two graduate courses, *Introduction to Infection and Immunity: molecular pathogenesis* and *Advanced Infection and Immunity: molecular pathogenesis*, annually or semi-annually.

93-96: As 90-93, plus, organized, direct and team teach a graduate course, entitled *Microbial Physiology and Genetics*, for 1st/2nd year graduate students, annually or semi-annually.

96-97: ***On sabbatical leave***

97-present: Similar to 90-96. Plus scattered lectures in our graduate core courses, including Molecular Biology & Genetics, and Cell Biology.

2001-2003: Course director, graduate Biomedical Ethics (Research Integrity) core course taken by all Biomed Grad students.

03-04: Course director and lecturer, Biomedical Ethics (Research Integrity), graduate core course; Course director and lecturer, Molecular Biology graduate core course; Course co-director and lecturer; Molecular Pathogenesis, a new 1 year course taken by first year Microbiology & Immunology graduate students; Lecturer, Medical Microbiology taken by ~200 medical students

04-05: Course director and lecturer, Biomedical Ethics graduate core course; Course co-director and lecturer, Molecular Biology and Genetics graduate core course; Course director and lecturer, Microbial and Molecular Pathogenesis I & II, graduate courses; Lecturer, Medical Microbiology, medical student course

05-06: Similar to 04-05.

06-08: Course Director and lecturer, Biomedical Ethics, a biomedical graduate core course; Course Director and lecturer, Molecular Biology and Genetics, a biomedical graduate core course; Course Director and lecturer, Microbial and Molecular Pathogenesis I & II, a core course for Microbiology and Immunology graduate students; Lecturer, Medical Microbiology, a medical student course.

STUDENT EXAMINATION COMMITTEES (not including my own students)
Mary Pully, MS Nursing/Psychology, 1987; Susan Jordan, PhD Biochemistry, 1989; Brian Finnegan, MS Microbiology and Immunology (M&I), 1989; Michael Autieri, PhD Pathology, 1992; Katie Zaifert, MS M&I, 1992; Peter Kima, PhD Molecular Biology and Biotechnology (MB&B), 1992; Marguerite Dalton, PhD Biochemistry, 1993; Bruce Kuo, PhD MB&B, 1994; Richard Tomko, PhD M&I, 1995; Patrick Farley, PhD MB&B, 1995; Barbara Timblin, PhD M&I, 1996; Dalei Shao, PhD MB&B, 1995; Feng Zhu, MS Radiation Sciences 1996; Thomas Nesspor, MS MB&B, 1996; Terry Rowland, MS Radiation Sciences, 1996; Luan Tao, PhD MB&B 1997; Sumita Bhaduri, PhD MB&B 1997; Norman Waters, MS M&I 1997; Paul Calvo, MS MB&B 1997; Yvette Murley, PhD M&I 1997; Ana Gabrea, PhD M&I 1998; Connie Kang, PhD M&I 1998; Rocio Mulero, PhD M&I 1998; Christopher Hand, PhD Radiation Sciences 2000; Elizabeth Javazon, MS Molecular and Human Genetics; Angela MacIntyre, PhD Molecular Pathobiology; Thomas Caltagirone, PhD Neurosciences 2003; Donghui Zhang, MS Molec Biol 2003; Devrim Eren, PhD Molec Human Genetics 2003; Ajaya Kumar Devabhaktuni, Radiation Sciences 2003; Xin Fan, PhD M&I, Univ of Penn School of Medicine 2004; Jason Stumhofer, PhD M&I 2004; Daniel Woods, Ph.D. M&I, Temple University School of Medicine 2005; Shannon Morgan, Ph.D. M&I, Temple Univ School of Medicine 2006. ***Present*** Jean-Paul McGovern, PhD, Biomedical Engineering, Drexel; Swati Thorat, PhD, M&I.

POST-DOCTORAL FELLOWS
Linda J. Eaton, Ph.D., 1980-83. Biochemical analysis of human neutrophil phagolysosomes: bactericidal activities and oxidative metabolism. Present position: Director of Research & Development, List Biological Labs, Campbell, CA.

Barbara Belisle, Ph.D., 1986-87. The role of outer membrane protein PII in gonococcal adherence to human cervical and endometrial epithelial cells.

Susan T. Hingley, Ph.D., 1988 - 1991. Interactions of *N. gonorrhoeae with* human epithelial cells: the roles of pili, PII (Opa) proteins and lipooligosaccharide (LOS). Present position: Associate Professor of Biology, Pennsylvania College of Osteopathic Medicine, Philadelphia.

Daniel J. J. Simon, Ph.D., 1991 - 1994. Molecular studies on the interactions of Opa proteins with their neutrophil and epithelial cell receptors. Present position: Senior scientist, Glaxo-Wellcome, Verona, Italy.

Janice (Dobrowolski) Bennett, Ph.D. 1996-1999. Molecular structure-function studies on the interactions of Neisserial Opa proteins with cytosolic epithelial cell proteins - pyruvate kinase (PK-M2) and thyroid hormone interacting protein 6 (TRIP6).

Shi V. Liu, M.D., Ph.D. 1997-2000. Environmental regulation of Neisseria sialyltransferase gene expression, and genomic analysis of complex repeats in the genomes of pathogenic Neisseria. Present position: Scientist, USDA, Research Triangle Park, NC.

Vida Irani, Ph.D.. 2000 – 2001. Environmental regulation of sialyltransferase gene expression. Current position, postdoc, Department of Pathology, University of Pennsylvania.

Dawn (Bell) Shell, Ph.D. 2000–2004. Molecular and functional analysis of sialyltransferase gene expression; human immune response to sialyltransferase. Current position: Assistant Professor of Biology, Pennsylvania College of Osteopathic Medicine, Philadelphia.

Vincent Ng, Ph.D. 2004 – 2005. Interaction of *Bacillus anthracis* with human macrophages and neutrophils; biological role of Anthrolysin O.

GRADUATE STUDENTS

Paul Buck, 1978-80, M.S. Effects of lysosomal enzymes on gonococcal metabolism.

Rosalie A. Fowler, 1978-83, M.S. Biochemical analysis of phagosome-lysosome fusion in neutrophils. Present position, Director, Clinical Microbiology, St. Christopher's Hospital, Tucson, AZ.

Elizabeth E. Vela, 1980-83, M.S. (Research advisor). Biochemical analysis of macrophage maturation.

Joseph R. Mezzatesta, M.S., 1979-83, Ph.D. Phagocytosis and killing of gonococci by human mononuclear phagocytes. Present position: Senior Scientist, Research and Development, Wyeth-Lederle Laboratories, Pearl River, NY.

John P. Rock, 1980-86, Ph.D. Effects of human neutrophil lysosomal enzymes on gonococcal metabolism and physiology. Present position: Manager, Strategic Project Management, AgrEvo Environmental Health, Research Triangle Park, NC.

Steven H. Fischer, 1981-88, MD/PhD. Role of gonococcal outer membrane protein PII (Opa) in phagocytosis by human PMN. Present position: Private practice, Florida.

Christopher Elkins M.S., 1983-88, Ph.D. Role of gonococcal outer membrane PII (Opa) in neutrophil stimulation and adherence. Present position: Research Associate Professor, Dept of Microbiology & Immunology, UNC, Chapel Hill.

Catherine Farrell, 1984-1990, Ph.D. A human neutrophil receptor for gonococcal outer membrane protein PII (Opa). Present position: Senior scientific writer, Sanofi Ltd., Philadelphia, PA.

Fredrick Naids, 1984-1991, Ph.D. Molecular and cellular studies on the role of PII in gonococcal stimulation of human neutrophils. Present position: Vice President for Marketing and Sales, Cerup Corp, US/France.

Joseph V. Frangipane, 1988-1993, Ph.D. Interaction of gonococci with human neutrophils and serum: role of oxygen and CMP-NANA (cytidinemonophosphate N-acetyl-neuraminic acid). Present position: Applications specialist, Life Technologies Corp, Gaithersburg, MD.

David McGee, 1992-1997, Ph.D. Molecular analysis of the regulation of expression of gonococcal and meningococcal sialyltransferase gene(s). Present position: Assistant Professor, Department of Microbiology & Immunology, University of South Alabama.

Janine Ferguson, 1993-1997, M.S. Regulation of gonococcal sialyltransferase activity by *in vivo* signals and substrates. Present position: Nurse Practitioner, New Jersey.

John M. Williams, 1993-1999, M.D./Ph.D. Use of the yeast two hybrid system to identify gonococcal Opa receptors in the cytoplasm of human epithelial cells. Present position: Resident, Harvard Univ.

Dawn (Bell) Shell, 1992-1999, Ph.D. Physical and immunologic characterization of neisserial sialyltransferases; the role of PIII (Rmp) in gonococcal adhesion and invasion. Present position: Assistant Professor of Biology, Pennsylvania College of Osteopathic Medicine, Philadelphia.

Jeff Shannon, 1999-2003, Ph.D. The role of Opa proteins in the intracellular lifestyle of *Neisseria gonorrhoeae* and *Neisseria meningitidis*; Characterization of *Bacillus anthracis* Anthralysin O. Present position: postdoctoral fellow, Rocky Mountain National Laboratories, Montana.

Mathanraj Packiam, 2001-2006, Ph.D. Regulation of sialyltransferase expression by *N. gonorrhoeae* and *N. meningitidis*. Present position: postdoc, USUHS.

Yognandan Pandya, 2003 – 2005, M.S. Biomedical Engineering. The role of Anthrolysin O in the interaction of *Bacillus anthracis* with human macrophages and neutrophils. Present position: biopharma, NJ.

Elise (Bifano) Mosser, 2003-2007, Ph.D. Transcriptional regulation and mechanism of action of Anthrolysin O, a *Bacillus anthracis* virulence factor.

Mariana Bernui, 2005 – present, Ph.D. candidate. The effect of antioxidants on the interaction of *Bacillus anthracis* and its toxins with human macrophages and neutrophils.

Mitali Purohit, M.S. 2006 – present, Ph.D., candidate. The effects of antioxidants on the interaction of *Bacillus anthracis* and its toxins with human macrophages and neutrophils.

Judy Rieger, 2006 – present, Ph.D. candidate. The effects of antioxidants on the interaction of Anthrolysin O with human macrophages and neutrophils.

Meghan Wynosky, 2006 – present, Ph.D. candidate. The role of Arp1 and Arp2 in transcriptional regulation of *B. anthracis* virulence factors.

RESEARCH SUPPORT (@ = active)

Bactericidal action of cationic proteins from human PMN. Postdoctoral Fellowship, 1 F32 AI01533, NIH ($28,500 total), 6/75-5/77.

Wellcome Research Travel Grant from the Burroughs Wellcome Fund, to study in the laboratory of H. Smith, Ph.D., Head, Department of Microbiology, University of Birmingham, England ($3,450), 5/21/79-7/13/79.

Macrophage maturation and lysosomal adaptations. Co-investigator for bacterial studies, 5% effort, Program No. 821 from the Veterans Administration, 10/78-9/81. S. Axline, M.D., PI.

> *Effects of phagolysosomal contents on Neisseria gonorrhoeae*, R01 AI15881, NIH ($200,000 total direct costs), PI, 4/79-3/82.

Mechanisms of intracellular pathogenesis, K04 AI00594, NIH ($175,000, total direct costs), Research Career Development Award, PI, 7/80-6/85.

Gonococcal surface components in pathogenesis, F32 AI07874, NIH NRSA post-doctoral fellowship to S. T. Hingley, Ph.D., PI and applicant; R. Rest, Sponsor, 4/89-3/91.

Wellcome Research Travel Grant, Burroughs Wellcome Fund, to study with E. R. Moxon, M.D., Dept of Paediatrics, Univ of Oxford, England ($10,400), 8/96 - 8/97.

Gonococcal Opa proteins and intracellular survival, F32 AI10046, NIH NRSA post-doctoral fellowship to Janice (Dobrowolski) Bennett, Ph.D., postdoc; R.F. Rest, Sponsor; 12/97-12/99.

Intracellular fate of Neisseria gonorrhoeae, R01 AI20897, NIH ($175,000 direct, 6/00-5/01). PI at 25% effort, 9/78 - 5/01. To be renewed.

Development of a biodetector for bioterrorism agents. Drexel University Synergy Award ($10,000). Co-investigator, 7/02 – 6/03.

Carbide-derived carbon filters for biohazardous agents. Drexel University Synergy Award ($20,000). Co-investigator, 7/02-6/03.

Active Filtration System for Building Defense Against Chemical/Biological Attacks. N00175-03-C-0024, Collaboration on a DARPA contract with PlasmaSol Corp, Hoboken, NJ ($45,000 to Rest). Co-investigator at 10% effort, 9/03-3/04.

Sialylation of the pathogenic Neisseria, R01 AI33505 supplement, NIH ($50,000 direct annually). This is a minority postdoctoral supplement for Dr. Dawn Shell, 9/01-7/04.

BSL3 Laboratory Renovations. Pennsylvania Tobacco Settlement Act 77 Formula Funds, PI, 1/03-12/04. This grant provided funds to renovate one of my BSL2 labs to a BSL3 lab; PI, $65,000.

Interaction of Bacillus anthracis with Macrophages. Pennsylvania Tobacco Settlement Act 77 Formula Funds, PI, 1/03-6/04, $50,000.

Interaction of Anthrolysin O with other Anthrax Toxins, U54 AI57168, NIH, Mid-Atlantic Regional Center of Excellence in Bioterrorism and Emerging Infectious Diseases, Myron Levine M.D., PI, Univ of Maryland Center for Vaccine Development. Rest, co-I, 10% effort, developmental sub-proposal, 09/03 – 03/05, $150,000 to Rest.

Molecular and Structural Mechanisms, Detection and Antagonism of Anthrax Toxins. Pennsylvania Tobacco Settlement Act 77 Formula Funds, PI, 5/04-4/05, co-PI, 0%, $37,500.

Microbiologic Support for Biosensor Development. Ben Franklin Technology Partners, Nanotechnology Institute, Kambiz Pourrezaei, PI, Drexel University. Rest, co-I, 5%, 8/03 – 12/04, $46,000 to Rest.

Sialylation of the pathogenic Neisseria, R01 AI33505, NIH ($200,000 direct annually). PI at 35% effort, 6/94-6/05.

@ *Piezoelectric Biosensor for Anthrax Detection.* Contract number: PA 26-0017-00, recipient ID: 6346, Transportation Security Administration, US Department of Transportation, Department of Homeland Security, Banu Onaral, PI, Drexel University. Rest, co-I, 10%, 10/04 – 3/08, $3,250,000 total, ~$300,000 direct to Rest.

@ *The Advanced Medical Countermeasures Consortium: Use of Free and Liposome-encapsulated Antioxidants as a Countermeasure to Mustards and Anthrax.* DOD contract no. W81XWH-06-2-0044, PI, Peter A. Ward, University of Michigan. Rest is co-I at 25% effort, 5/06 – 9/07, $6,000,000 total, $375,000 direct to Rest, "Effects of Antioxidant Liposomes on *Bacillus anthracis* – Macrophage Interactions."

@ *The Advanced Medical Countermeasures Consortium: Use of Free and Liposome-encapsulated Antioxidants as a Countermeasure to Anthrax.* DOD contract no. to be determined, PI, Peter A. Ward, University of Michigan. Rest is co-I at 25% effort, 10/07 – 8/08, $2,200,000 total, $225,000 direct to Rest, "Effects of Antioxidant Liposomes on *Bacillus anthracis* – Macrophage Interactions."

@ *Arp: a Bacillus anthracis transcription regulator*. U54 AI57168, NIH, Mid-Atlantic Regional Center of Excellence in Biodefense and Emerging Infectious Diseases (MARCE), Myron Levine M.D., PI. Rest co-I, supplementary funds, at 5% effort. 2007-2008, $35,000.

@ *PDAG: a naturally occurring regulator of inflammation.* TherimuneX Pharmaceuticals, Inc – Drexel University College of Medicine Collaborative. Rest, PI at 25% effort. 10/07 – 9/08, $400,000 direct.

Anthrolysin O and Phagocytes. 1 R21 AI073827-01A1, NIH, Rest PI at 15% effort, submitted

Arp: a Bacillus anthracis transcription regulator. 1 R01 AI074999-01A1, NIH. Daniel Simon, PI; Rest co-I at 10% effort, submitted

PUBLICATIONS

Primary, Peer-reviewed

Rest, R.F. and D.C. Robertson. 1974. Glucose transport in *Brucella abortus.* J. Bacteriol. 118:250-258.

Rest, R.F. and D.C. Robertson. 1975. Characterization of the electron transport system in *Brucella abortus.* J. Bacteriol. 122:139-144.

Rest, R.F. and J.K. Spitznagel. 1977. Subcellular distribution of superoxide dismutases in human neutrophils. Influence of myeloperoxidase on the measurement of superoxide dismutase activity. Bioch. J. 166:145-153.

Rest, R.F., M.H. Cooney, and J.K. Spitznagel. 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. Infect. Immun. 16:145-151.

Rest, R.F. and J.K. Spitznagel. 1978. The myeloperoxidase/Cl-/H2O2 bactericidal system. Effect of bacterial membrane structure and growth conditions. Infect. Immun. 19:1110-1112.

Rest, R.F., M.H. Cooney, and J.K. Spitznagel. 1978. Bactericidal activity of specific and azurophil granule contents from human neutrophils. Studies with outer membrane mutants of *Salmonella typhimurium* LT-2. Infect. Immun. 19:131-137.

Rest, R.F., M.H. Cooney, and J.K. Spitznagel. 1978. Subcellular distribution of various glycosidases in human polymorphonuclear leukocytes. Bioch. J. 174:53-59.

Rest, R.F. 1979. Killing of *Neisseria gonorrhoeae* by human polymorphonuclear neutrophil granule extracts. Infect. Immun. 25:574-579.

Buck, P. and **R.F. Rest**. 1981. Effects of human neutrophil granule extracts on macromolecular synthesis in *Neisseria gonorrhoeae*. Infect. Immun. 33:426-433.

Rest, R.F. and E. Pretzer. 1981. Degradation of outer membrane proteins of *Neisseria gonorrhoeae* by human neutrophil lysosomal enzymes. Infect. Immun. 34:62-68.

Rest, R.F., S.H. Fischer, Z.Z. Ingham, and J.F. Jones. 1982. Interactions of *Neisseria gonorrhoeae* with human neutrophils: Effects of serum and gonococcal opacity on killing and chemiluminescence. Infect. Immun. 36:737-744.

Mezzatesta, J.R., and **R.F. Rest.** 1983. Interactions of *Neisseria gonorrhoeae* with human mononuclear phagocytes. Infect. Immun. 42:99-105.

Eaton, L.J., and **R.F. Rest**. 1983. Degradation of gonococcal outer membrane proteins within phagolysosomes of phagocytizing human neutrophils. Infect. Immun. 42:1034-1040.

Speer, C.P., M.J. Pabst, H.B. Hedegaard, **R.F. Rest**, and R.B. Johnston, Jr. 1984. Enhanced release of oxygen metabolites by monocyte-derived macrophages exposed to proteolytic enzymes: Activity of neutrophil elastase and cathepsin G. J. Immun. 133: 2151-2156.

Rest, R.F., N. Lee, and C. Bowden. 1985. Stimulation of human leukocytes by P.II+ gonococci is mediated by lectin-like gonococcal components. Infect. Immun. 50:116-122.

Striker, R., M.E. Kline, R.A. Haak, **R.F. Rest**, and R. S. Rosenthal. 1987. Degradation of gonococcal peptidoglycan by granule extract from human neutrophils: demonstration of N-acetyl-glucosaminidase activity that utilizes peptidoglycan substrates. Infect. Immun. 55:2579-84.

Rest, R.F., C. Farrell, and F. Naids. 1988. Mannose inhibits the human polymorphonuclear neutrophil oxidative burst. J. Leukocyte Biol. 43:158-164.

Rock, J., and **R. F. Rest.** 1988. Rapid damage to membranes of *Neisseria gonorrhoeae* caused by human neutrophil granule extracts. J. Gen. Microbiol. 134:509-519.

Fischer, S.H., and **R. F. Rest**. 1988. Gonococci possessing only certain P.II outer membrane proteins interact with human neutrophils. Infect. Immun. 56:1574-1579.

Elkins, C., and **R. F. Rest**. 1990. Monoclonal antibodies to outer membrane protein PII block interactions of *Neisseria gonorrhoeae* with human neutrophils. Infect. Immun. 58:1078-1084.

Farrell, C., and **R. F. Rest**. 1990. Up-regulation of human neutrophil receptors for *Neisseria gonorrhoeae* expressing PII outer membrane proteins. Infect. Immun. 58:2777-2784.

Blomfield, I. C., V. Vaughn, **R. F. Rest**, and B. I. Eisenstein. 1991. Allelic exchange in *Escherichia coli* using *Bacillus subtilis* sac B gene and a temperature-sensitive pSC101 replicon. Molec. Microbiol. 5:1447-1458.

Naids, F. and **R. F. Rest**. 1991. Stimulation of human neutrophil oxidative metabolism by nonopsonized *Neisseria gonorrhoeae*. Infect. Immun. 59:4383-4390.

Naids, F., B. Belisle, N. Lee and **R. F. Rest**. 1991. Interactions of *Neisseria gonorrhoeae* with human neutrophils. Studies with purified PII (Opa) outer membrane proteins and synthetic Opa peptides. Infect. Immun. 59:4628-4635.

R. F. Rest and J. Frangipane. 1992. Growth of *Neisseria gonorrhoeae* in cytidine-3'-monophosphate-N-acetyl neuraminic acid (CMP-NANA) inhibits their nonopsonic (Opa-mediated) association with human neutrophils. Infect. Immun. 60:989-997.

Frangipane, J.V. and **R. F. Rest**. 1992. Anaerobic growth does not affect Opa-mediated (nonopsonic) association of *Neisseria gonorrhoeae* with human neutrophils. Infect. Immun. 60:1793-1799.

Simon, D. and **R. F. Rest**. 1992. *Escherichia coli* expressing a *Neisseria gonorrhoeae* opacity associated (Opa) outer membrane protein invade human cervical and endometrial epithelial cell lines. P.N.A.S. (USA) 89:55112-55116.

Frangipane, J. and **R. F. Rest**. 1993. Anaerobiosis and cytidine-3'-monophosphate-N-acetyl neuraminic acid (CMP-NANA) act synergistically to induce high level resistance to normal human serum by *Neisseria gonorrhoeae*. Infect. Immun. 61:1657-1666.

McGee, D., and **R. F. Rest**. 1996. Regulation of gonococcal sialyltransferase, lipooligosaccharide and serum resistance by glucose, pyruvate and lactate. Infect. Immun. 64:4630-4637.

McGee, D., and **R. F. Rest**. 1996. Expression of sialyltransferase is not required for interaction of *Neisseria gonorrhoeae* with human epithelial cells and human neutrophils. Infect. Immun. 64:4129-4136.

Williams, J.M., L. Zhu, G.-C. Chen and **R. F. Rest**. 1998. Using the yeast two-hybrid system to identify human epithelial cell proteins that bind gonococcal Opa proteins: Intracellular gonococci bind pyruvate kinase via their Opa proteins and require host pyruvate for growth. Molec Microbiol, 27:171-186.

Hood, D. W., K. Makepeace, M. E. Deadman, **R. F. Rest**, P. Thibault, A. Martin, J. C. Richards, & E. R. Moxon. 1999. Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization. Molec. Microbiol. 33:679-692.

Liu, S. V., N. J. Saunders, A. Jeffries and **R. F. Rest**. 2002. Genome analysis and strain comparison of Correia repeats and Correia Repeat-Enclosed Elements (CREE) in pathogenic neisseria. J. Bacteriology, 184:6163-6173.

Shell, D., D. Chiles, R. Judd, S. Seal and **R. F. Rest**. 2002. Outer membrane localization of sialyltransferase in the pathogenic *Neisseria*. Infect Immun 70:3744-3751.

J. G. Shannon, C. L. Ross, T. M. Koehler and **R. F. Rest**. 2003. Characterization of Anthrolysin O, the *Bacillus anthracis* Cholesterol-Dependent Cytolysin. Infect. Immun. 71:3183-3189.

Park, J. M., V. H. Ng, **R. F. Rest**, S. Maeda and M. Karin. 2004. Anthrolysin O and other Gram-positive cytolysins are Toll-like receptor 4 agonists. J. Exp Med 200:1647-1655.

Packiam, M., D. Shell, D., S. V. Liu, Y.B. Liu, D. J. McGee, R. Srivastava, S. Seal and **R. F. Rest**. 2006. Differential expression and transcriptional analysis of the α-2,3-sialyltransferase gene in pathogenic *Neisseria*. Infect. Immun. 74:2637-2650.

Cocklin, S., M. Jost, N. M. Robertson, S. D. Weeks, H.-W. Weber, E. Young, S. Seal, M. Zhang, P. J. Loll, A. J. Saunders, **R. F Rest,** and I. M. Chaiken. 2006. Real-time monitoring of the membrane-binding and insertion properties of the cholesterol-dependent cytolysin Anthrolysin O from *Bacillus anthracis*. J Molec Recognition 19: 354-362.

Mosser, E. M. and **R. F. Rest**. 2006. The *Bacillus anthracis* cholesterol-dependent cytolysin, Anthrolysin O, kills human neutrophils, monocytes and macrophages. BMC Microbiology, 6:56.

Gogotsi, Y, R. K. Dash, G. Yushin, B. E. Carroll, S. R. Altork, S. Sassi-Gaha and **R. F. Rest**. 2007. Bactericidal activity of chlorine-loaded carbide-derived carbon against *Escherichia coli* and *Bacillus anthracis*. J Biomed Materials Res: Part A, in press

SUBMITTED

Viator, R. J., **R. F. Rest** and D. J. McGee. 2007. *Bacillus anthracis* arginase: Characterization and development of a whole cell assay. J. Bact. In revision

W. Wang, G. Jung, T. Nguyen, P. Ruchala, Y. OLuyang, E. J. Gillespie, K. A. Bradley, A. J. Ratner, **R. F. Rest**, W. Lu, and R. I. Lehrer. 2007. Inactivation of cholesterol-dependent cytolysins by human β-defensins. In revision

M. Purohit, S. Sassi-Gaha and **R.F. Rest**. 2007. Rapid sporulation of *Bacillus anthracis*. Submitted

IN PREPARATION

Shannon, J. F, S. R. Altork, J. Dobrowolski, R. Srivastava, **R. F. Rest**. Characterization of binding of gonococcal Opa proteins to TRIP6 within human cells.

Mosser, E. M., Y. Pandya, **R. F. Rest**. Effects of the *Bacillus anthracis* toxin Anthrolysin O (ALO) on human neutrophil and macrophage intracellular signaling.

Mosser, E. M., **R. F. Rest**, O. Crasta, S. Mohapatra, C. Evans, B. Sobral, D. J. Simon. 2007. Anthrolysin Regulatory Protein 1 (Arp1): a unique, global transcription regulator in the *Bacillus cereus* group.

Books, Book Chapters, Proceedings, Reviews, Etc.

Rest, R.F. 1974. Dissertation: Metabolite transport and the electron transport system in *Brucella abortus*.

Buck, P. and **R.F. Rest**. 1980. Differential inhibition of colony formation and macromolecular synthesis in *Neisseria gonorrhoeae* by human neutrophil lysosomal contents. In: Proceedings of the EMBO Workshop on Genetics and Immunology of Pathogenic Neisseria, pp. 247-250, Hemavan, Sweden (S. Normark and D. Danielsson, eds).

Johnston, R.B., Jr., M.J. Pabst, C.P. Speer, H.B. Hedegaard, **R.F. Rest** and S.M. Bryant. 1984. In: Mononuclear Phagocytes in Inflammation. R. van Furth, editor. Martinus Nijhof, The Hague.

Rest, R.F., N. Lee, and C. Bowden. 1985. Stimulation of human leukocyte oxidative metabolism by P.II+ gonococci is mediated by lectin like gonococcal surface components, p. 495-501. In G. Schoolnick, (ed), The Pathogenic Neisseria: Proceedings of the Fourth International Symposium. American Society for Microbiology, Washington.

Rock, J.P., and **R.F. Rest**. 1985. Effect of human neutrophil lysosomal contents on peptidoglycan turnover and membrane structure in *Neisseria gonorrhoeae*, p. 344-351. In G. Schoolnick, (ed.), The Pathogenic Neisseria: Proceedings of the Fourth Inter-national Symposium. American Society for Microbiology, Washington.

Elkins, C., C. Wilde, C. Farrell, and **R.F. Rest**. 1988. The role of P.II outer membrane proteins in gonococcus - host cell interactions, pp699-702. In J. T. Poolman et al (Eds), Gonococci and Meningococci. Kluwar Academic Publishers, Dordrecht, Netherlands.

E.L. Thomas, R.I. Lehrer, and **R.F. Rest**. 1988. Human neutrophil antimicrobial activity. Proceedings of a meeting entitled "Microbial Surfaces: Determinants of Virulence and Host Responsiveness". Rev. Infect. Dis. 10:S450-S456.

Rest, R.F. 1988. Chapter 28, Human neutrophil and mast cell proteases implicated in inflammation. IN Meth. in Enzym., Volume 163, Chemotaxis and Inflammation, Part M, G. DiSabato, ed, Academic Press, New York.

Rest, R.F. and W.M. Shafer. 1989. Interaction of *Neisseria gonorrhoeae* with human neutrophils. Clin. Microbiol. Rev. 2:S83-S91.

Shafer, W.M. and **R.F. Rest**. 1989. Interaction of gonococci with phagocytic cells. Annual Rev. Microbiol. 43:121-145.

Farrell, C.F., F.L. Naids and **R.F. Rest**. 1991. Identification of a human neutrophil receptor for gonococcal outer membrane protein PII, pp 579-584. IN "Neisseriae 1990," eds, M Achtman, et al, Walter de Gruyter, publishers, Berlin.

Cassell, G.H., L.A. Miller, and **R.F. Rest**. 1992. Biological warfare: role of biological societies. IN "The Microbiologist and Biological Defense Research: Ethics, Politics and International Security," ed, R. A. Zilinskas. Ann. N. Y. Acad. Sci. 666:230-238.

I. Ofek, **R.F. Rest** and N. Sharon. 1992. Nonopsonic phagocytosis of microorganisms. Phagocytes use several molecular mechanisms to recognize, bind, and eventually kill microorganisms. ASM News 58:429-435.

Rest. R.F. and D. P. Speert. 1994. Chapter 8, Measurement of Nonopsonic Phagocytic Killing by Human and Mouse Phagocytes. IN Meth. Enzym., Volume 236, *Bacterial Pathogenesis,* Part B, Interaction of Pathogenic Bacteria with Host Cells, V.L. Clark and P.M. Bavoil, eds, Academic Press, New York.

Rest, R.F. 1994. Chapter 10, Measurement of the Human Neutrophil Respiratory Burst during Phagocytosis and Killing of Bacteria. IN Meth. Enzym., Volume 236, *Bacterial Pathogenesis*, Part B, Interaction of Pathogenic Bacteria with Host Cells, V. L. Clark and P. M. Bavoil, eds, Academic Press, New York.

Rest, R.F., J. Liu, R. Talukdar, J. V. Frangipane and D. Simon. 1994. "Interaction of Pathogenic *Neisseria* with host defenses: what happens *in vivo*?" IN Microbial Pathogenesis and Immune Response, eds., E. Ades, S. Morse & **R. Rest**, Ann. N. Y. Acad. Sci. 730:182-196.

G. Gorby, D. Simon and **R.F. Rest**. 1994. *Escherichia coli* that express *Neisseria gonorrhoeae* Opacity-associated proteins attach to and invade human fallopian tube epithelium, IN Microbial Pathogenesis and Immune Response , eds., E. Ades, S. Morse & **R. Rest**, Ann. N. Y. Acad. Sci. 730: 286-289.

E.W. Ades, **R.F. Rest**, and S.A. Morse, eds. 1994. Microbial Pathogenesis and Immune Response. Ann. N.Y.A.S. vol. 730.

J.V. Frangipane and **R.F. Rest**. 1994. Anaerobic growth induces increased gonococcal sialyltransferase activity and acts synergistically with CMP-NANA to increase gonococcal serum resistance, IN Pathobiology and Immunobiology of *Neisseriaceae*, Proceedings of the VIIIth International Pathogenic *Neisseria* conference. C.J. Conde-Glez, S.A. Morse, P. Rice, P.F. Sparling and E. Calderon, eds. INSP, Mexico.

Simon, D.J.J., S.T. Hingley and **R.F. Rest**. 1994. Interaction with human epithelial cell lines and neutrophils of *Escherichia coli* expressing gonococcal Opa proteins, IN Pathobiology and Immunobiology of *Neisseriaceae*, Proceedings of the VIIIth International Pathogenic *Neisseria* conference. C.J. Conde-Glez, S.A. Morse, P. Rice, P.F. Sparling and E. Calderon, eds. INSP, Mexico.

Rest, R.F. 1995. Chapter 2. Association of Bacteria with Phagocytic Cells. IN Methods in Enzymology, Volume 253, *Adhesion of Microbial Pathogens*, R. J. Doyle and I. Ofek, eds, Academic Press, New York.

Rest, R.F. and R.E. Mandrell. 1995. Neisseria sialyltransferases and their role in pathogenesis. Microbial Pathogenesis, 19:379-390.

Ades, E.W., **R.F. Rest**, and S.A. Morse, eds. 1996. Microbial Pathogenesis and Immune Response II. Ann. N.Y.A.S. vol. 797.

Simon, D., J. T. Liu, M. S. Blake, C. R. Blake and **R. F. Rest**. 1996. Structure-function studies with *Neisseria gonorrhoeae* Opa outer membrane proteins expressed in *Escherichia coli*. Annals N. Y. Acad. Sci 797:253-254.

Williams, J. M. and **R. F. Rest.** 1996. Cytoplasmic proteins involved in thyroid hormone response also bind *Neisseria gonorrhoeae* Opa outer membrane proteins. Annals N. Y. Acad. Sci. 797:288-289.

Jerse, A., and **R.F. Rest**. 1997. Adhesion and invasion by the pathogenic neisseria. Trends in Microbiol 5:217-221.

Rest. R.F. and D. P. Speert. 1997. Measurement of Nonopsonic Phagocytic Killing by Human and Mouse Phagocytes. IN *Bacterial Pathogenesis: selected Methods in Enzymology*, V.L. Clark and P.M. Bavoil, eds, Academic Press, New York.

Rest, R.F. 1997. Measurement of the Human Neutrophil Respiratory Burst during Phagocytosis and Killing of Bacteria. IN *Bacterial Pathogenesis: selected Methods in Enzymology*, V. L. Clark and P. M. Bavoil, eds, Academic Press, New York.

Rest, R.F. 2008. Regulation of gene expression leading to resistance to immune response by the pathogenic neisseria. In preparation

ABSTRACTS AND INVITED TALKS

I attend three or four international meetings annually at which I, or more often one of my laboratory personnel, present data. I present invited talks at international meetings once or twice a year. I do not keep formal records of such activities.

PATENT APPLICATIONS

1. Compositions and Methods for Activating Toll-like Receptor 4, 2004
2. Compositions and Methods for Preventing and Treating Anthrax Disease, 2004
3. Chlorine-Loaded Carbide-Derived Carbon with Bactericidal Properties, 2005
4. Regulation of Expression of Anthrolysin O: The Bacillus anthracis Cholesterol Dependent Cytolysin, 2006
5. Regulation of Gene Expression by Bacillus anthracis Arp, 2007

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East Tennessee State University

W81XWH-06-2-0044

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Introduction

Statement of Work

Sulfur mustard or (bis-2-(chloroethyl) sulfide (military code HD) is a dangerous vesicant and a chemical warfare agent. Inflammation and oxidative stress play key roles in the toxicity of HD and its monofunctional analog 2-chloroethyl-ethyl sulfide (CEES) [1]. This suggests that antioxidant liposomes could provide a unique therapeutic strategy for mustard gas exposure. Immunostimulators like lipopolysaccaride (LPS) induce intracellular generation of NO and reactive oxygen species (ROS) and greatly enhance CEES toxicity in RAW 264.7 macrophages [2]. We also found that CEES inhibits NO generation in these cells via downregulation of inducible NO synthase (iNOS) protein expression [3]. Nitric oxide is known to inhibit mast cell degranulation in various species [4, 5]. Therefore, it is likely that CEES might have an effect on mast cell degranulation. Our first set of goals are to: (1) investigate the effect of CEES on the degranulation of mast cells; (2) determine if CEES inhibits the production of nitric oxide (NO) in mast cells; (3) determine if antioxidant liposomes inhibit mast cell degranulation induced by CEES.

A second series of goals focus on utilizing redox proteomics to determine how CEES (or HD/pathogen) treatment influences changes in the levels and distribution of oxidized proteins using either mast cell lines or animal tissue samples obtained from other members of the Consortium. We will also determine the identities and functional significance of the oxidized proteins. The effect of antioxidant liposomes on protein expression and oxidation will be studied in mast cells and animal models exposed to CEES or HD.

Our **Specific Goals** are:

Task 1: To determine if mast cell degranulation is induced or inhibited by CEES treatment; and if antioxidant liposomes influence this effect. More specifically we will: (1) investigate the effect of CEES on degranulation/cytokine release of rat basophilic leukemia RBL-2H3 and human HMC-1 mast cell lines; (2) determine if CEES inhibits the production of nitric oxide (NO) in the mast cell lines by use of the cell permeable fluorescent dyes; (3) determine if antioxidant liposomes reduce the effect of CEES on mast cell degranulation.

Task 2: Proteomics of various biological samples exposed to CEES or pathogens will be analyzed by 2-dimensional gel electrophoresis (2D-GE). Oxidized proteins from the 2D-GE gels will be identified using tandem mass spectrometry. Utilizing this proteomics approach we will analyze samples from: (1) the *in vivo* animal models, and (2) *in vitro* cellular studies (mast cell lines) exposed and not exposed to CEES/HD; (3) antigen presenting cell (APC) samples (from Dr. Crawford's laboratory) exposed and not exposed to various pathogens. The tissue samples (snap frozen in liquid nitrogen) from the animal models will be obtained from other members of the mustard consortium. In addition, the

influence of antioxidant-liposomes on the levels and distribution of oxidized proteins from RBL-2H3 and HMC-1 mast cells (both exposed and not exposed to CEES/HD) will be evaluated by proteomics.

Task 3: My laboratory will continue to supply well characterized various antioxidant-liposomes of various formulations to all other members of the Research Consortium and provide the analytical methodology and services to measure antioxidant (vitamin E and glutathione) levels in tissues collected from the various animal models used by other members of the mustard consortium. We are continuously improving the stability and effectiveness of the antioxidant liposomes.

Body

Specific Tasks 1:

Mast cell degranulation induced by 2-chloroethyl ethyl sulfide (CEES)

We have set up and validated a colorimetric assay to measure the release (via degranulation) of β-hexosaminidase, one of the major components of mast cell granules. We also examined various chemical stimulators in order to choose the most efficient degranulating agent (Ca-ionophore A23187) to be used as a positive control for the experiments with CEES.

We have now completed a series of experiments, in which we: (1) explored the effect of CEES toxicity in RBL-2H3 cells; (2) test the ability of CEES to stimulate degranulation; (3) explored the effect of various levels of CEES (0.01 to 5 mM) on the degranulation stimulated by Ca-ionophore A23187.
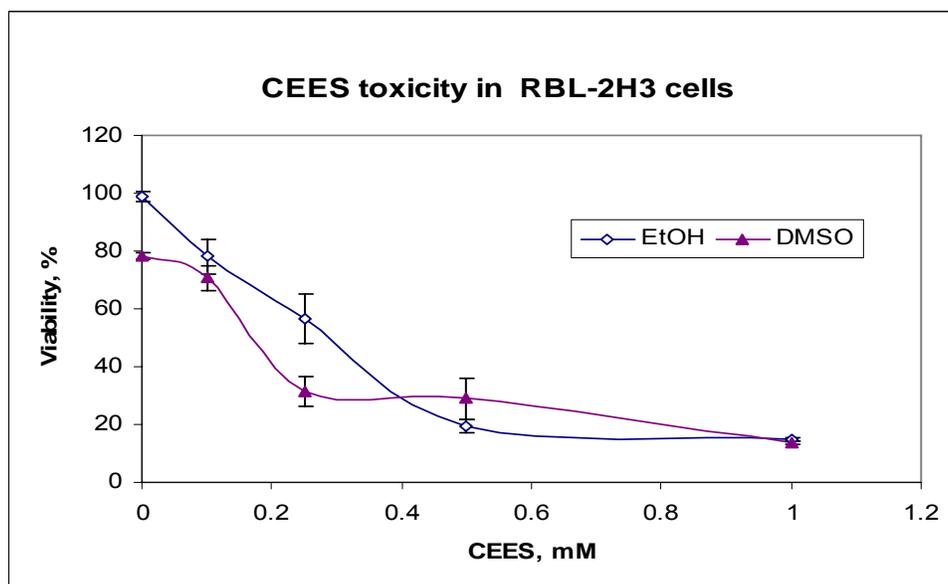
Fig.1 RBL-2H3 cells were exposed to various levels of **CEES** for 24 hours. Stock solutions of CEES were prepared in EtOH or DMSO. Final concentrations of the vehicles were 1% (vol/vol). Cell viability was measured by the MTT assay.

Figure 1 shows the dose-dependent effect of CEES on rat mast cell viability. RBL-2H3 cells were placed in a 24-well plates (~10^5 cells/well). CEES was applied as a stock solution in ethanol (EtOH) or DMSO with a final concentration of each vehicle at 1% (vol/vol). Cell viability was measured after 24 hour of incubation by the standard MTT assay. Under these conditions DMSO was somewhat toxic to the cells with 80% viable cells (in the absence of CEES) and markedly increased the toxic effect of CEES at low doses, i.e., less than 0.5 mM. Ethanol at 1% did not show any toxic effect in the absence of CEES. We, therefore, chose ethanol as a vehicle for our next experiments.

We next tried to induce degranulation in the rat mast cells using three different doses of CEES (Figure 2). Since the doses of CEES used in this experiment were toxic in 24 hour incubations (Figure 1), we measured cell viability within the first 2 hours in a series of separate MTT assays (data not shown) and normalized the percentage of degranulation (after 1.5 hour β-hexosaminidase assays) to the number of viable cells. The effect of CEES was compared with that of 200 nM Ca-ionophore A23187 (positive control). In this experiment CEES, at all the chosen doses, failed to induce degranulation in RBL-2H3 cells whereas the Ca-ionophore A23187 induced 64% degranulation.

Degranulation of RBL-2H3 cells stimulated with CEES
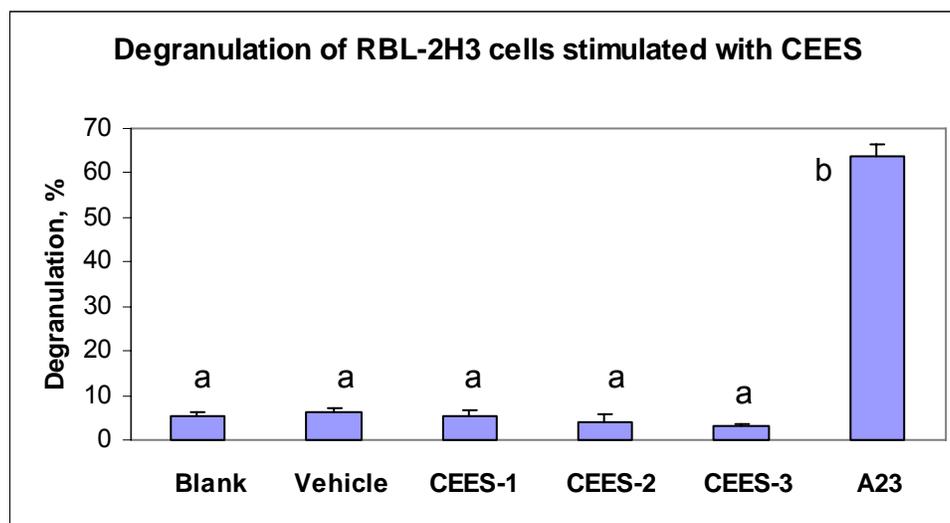
Fig.2 RBL-2H3 cells were stimulated for 1.5 hour with various levels of CEES: **CEES-1**, 0.5 mM; **CEES-2**, 1 mM; **CEES-3**, 5 mM; or with 200 nM Ca-ionophore A23187 (**A23**) as positive control; **Veh.**, 1%EtOH; **Blank**, no additives. Mast cell degranulation was measured as ß-hexosaminidase release and normalized to the percentage of viable cells (measured in a separate experiment by the standard MTT assay). Means not sharing a common letter are significantly different ($P < 0.05$).

Degranulation of RBL-2H3 cells pretreated with CEES
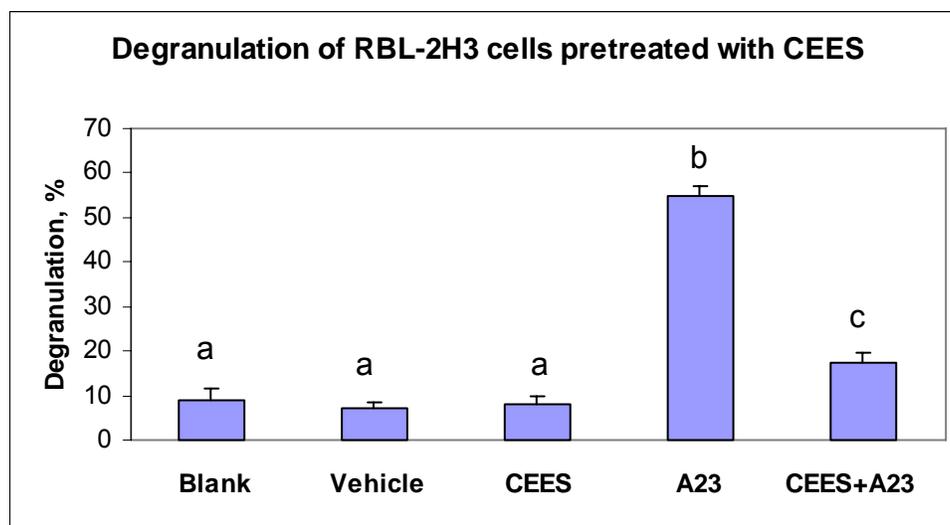
Fig.3 RBL-2H3 cells were incubated with 1 mM **CEES** or 1%EtOH (**Vehicle**) for 3.5 hours. **Blank**, no additives. **A23**, cells were incubated with the vehicle for 2 hours, and then stimulated by additional treatment with 200 nM Ca-ionophore A23187 for 1.5 hour; **CEES+A23**, cells were pretreated with 1 mM CEES for 2 hours, and then stimulated by additional incubation with 200 nM Ca-ionophore A23187 for 1.5 hour. Mast cell degranulation was measured as ß-hexosaminidase release and normalized to the percentage of viable cells (measured in a separate MTT assay). Means not sharing a common letter are significantly different ($P < 0.05$).

We next sought to determine if CEES might be able to affect mast cell degranulation induced by other stimulators, such as the Ca-ionophore A23187. We, therefore, performed an experiment in which we treated RBL-2H3 cells with both CEES and A23187 simultaneously. Similar to the previous experiments, we normalized the percentage of degranulation measured after 1.5 hour to the number of viable cells. Simultaneous application of CEES and A23187 for 1.5 hour reduced degranulation of the rat mast cells if compared to the A23187 alone (data not shown).

We also performed a similar experiment to find out if the pretreatment of RBL-2H3 cells with CEES also reduces degranulation induced by A23187. Figure 3 show that pretreatment of the cells with 1 mM CEES for 2 hours before the A23187 stimulation drastically reduced the percentage of degranulated cells if compared with A23187 stimulated cells pretreated with the vehicle only. The result was very similar to the previous experiment (see above), in which CEES and A23187 were added to the cells simultaneously.

Based on the above described experiments we concluded that toxic doses of CEES impair degranulation in RBL-2H3 rat mast cells. Initially, we proposed that CEES might stimulate mast cell degranulation since it inhibits the generation of nitric oxide (NO) in RAW 264.7 macrophages [3]. Nitric oxide is known to inhibit mast cell degranulation in various species [4, 5]. However, a recent study has shown that endogenous nitric oxide does not modulate degranulation of mesenteric or skin mast cells in rats [6]. Interestingly, another recent study revealed that a number of compounds capable of nitric oxide inhibition in RAW 264.7 macrophages also exhibited inhibition of degranulation in RBL-2H3 cells [7]. Our initial hypotheses may, therefore, have to be reconsidered. It is clear, nevertheless, that CEES indeed is capable of modulation of mast cell degranulation. The molecular mechanisms for this effect and its relation to oxidative stress will be elucidated in our future experiments.

As a next step we further examined the effect of CEES on A23187-induced degranulation. We initiated a series of experiments with rat mast cells in order to confirm the effect of CEES on A23187-induced degranulation when the two agents are applied simultaneously. However, experiments in which RBL-2H3 cells were treated with 1 mM CEES and 200 nM A23187 simultaneously did not produce any stable degranulation results due to a marked drop in cell viability (data not shown). We reasoned that if both A23187 and CEES were applied simultaneously CEES there could be an enhanced cytotoxicity. Using the standard MTT assay we, therefore, monitored cell viability after 24 hour incubations with CEES (20-200 µM) (Fig. 4, upper panel) or Ca-ionophore A23187 (20-500 nM) (Fig. 4, lower panel). We found that CEES cytotoxicity alone was detectable only at 100 µM level (viability 87.3%); lower doses were not significantly toxic. Ca-ionophore A23187 had no cytotoxic effect on RBL-2H3 cells at concentrations of 200 nM and lower. We next sought to determine the combined toxicity of low levels of CEES and Ca-ionophore A23187 when applied simultaneously to RBL-3H3 cells. Figure 5 shows the effect of Ca-ionophore A23187 on CEES cytotoxicity at low concentrations. Although 20 nM A23187 had no effect on the CEES cytotoxicity, 50 nM and higher concentrations of A23187 drastically reduced cell viability for all CEES level examined.

Notably, 50 nM A23187 (a dose much lower than highest non-toxic concentration of A23187) if combined with 20 µM CEES (again, a dose much lower than highest non-toxic concentration of CEES) did reduce cell viability to 63.1%.

Based upon the above described experiments we concluded that very low doses of Ca-ionophore A23187 (even levels much lower than highest non-toxic concentration) are still able to induce toxic effect in mast cells if combined with low-toxic doses of CEES. It is likely that this effect could be tightly interconnected with the NO generation.
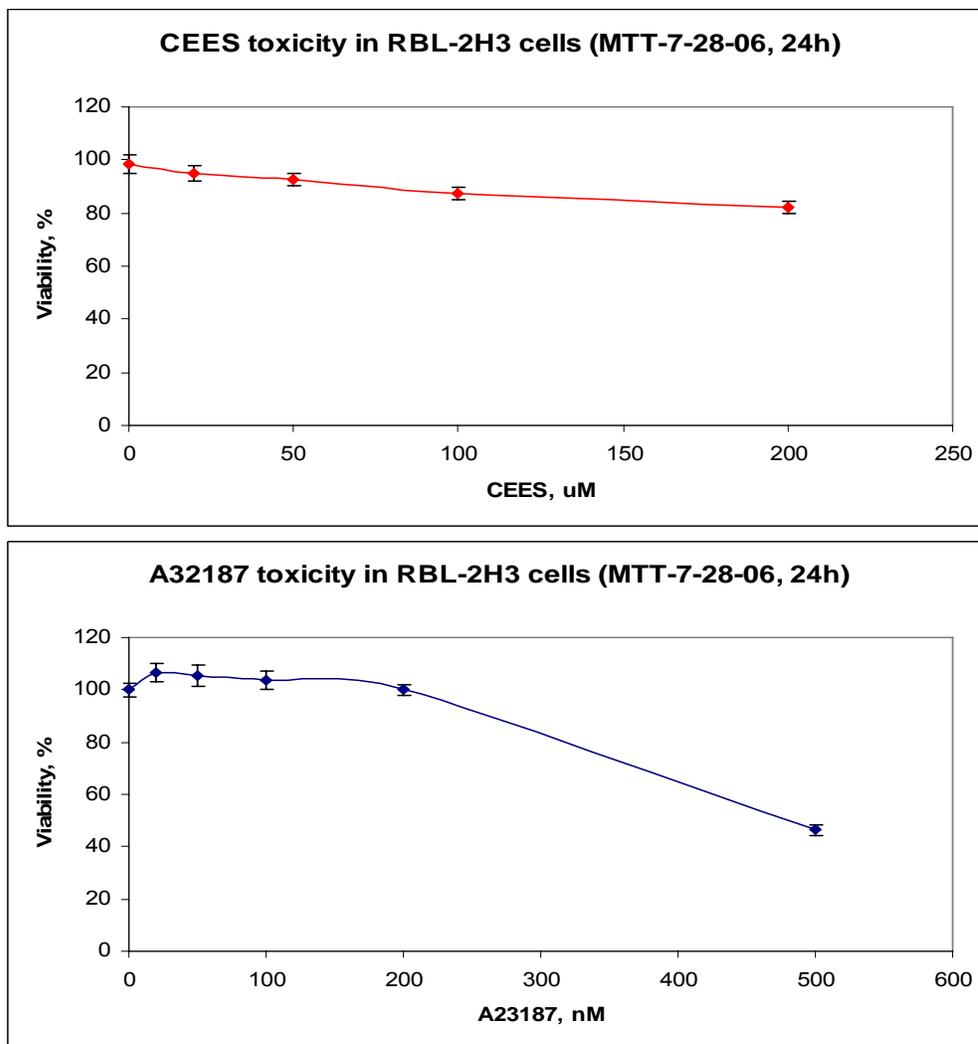
Fig.4 RBL-2H3 cells were placed in 24-well plates (~10^5 cells/well) and exposed to low levels of CEES (upper panel) or Ca-ionophore A23187 for 24 hours. Stock solutions of CEES were prepared in EtOH; final concentrations of the vehicles were 1% (vol/vol). Cell viability was measured by the standard MTT assay.
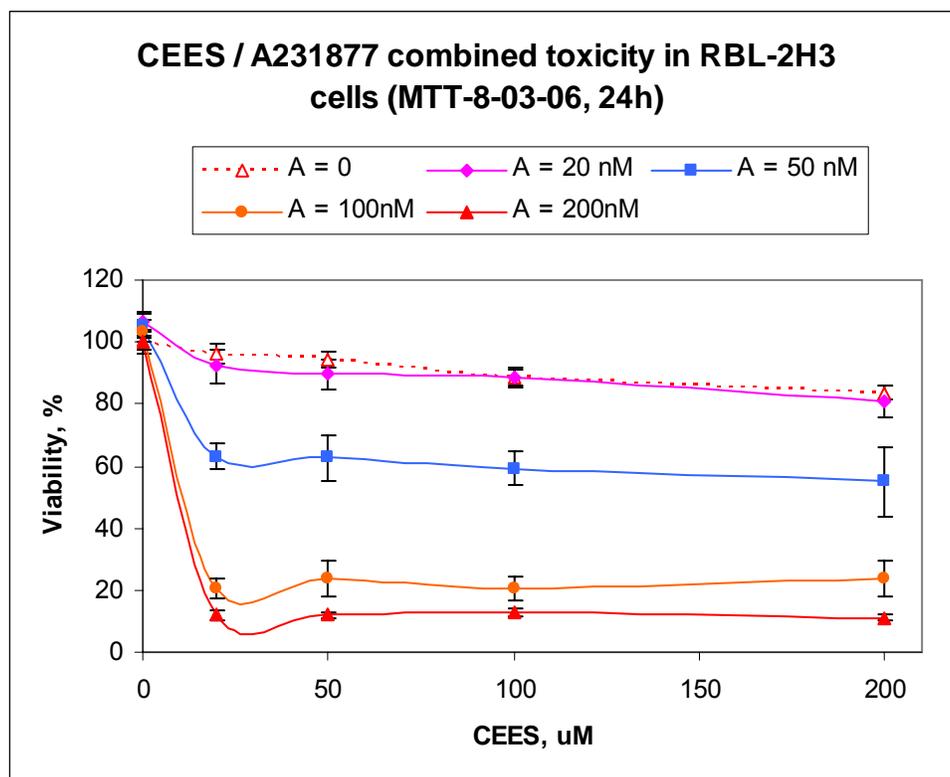
Fig.5 RBL-2H3 cells were treated with various low levels of CEES and Ca-ionophore A23187 (as indicated) for 24 hours. Both agents were applied simultaneously. Stock solutions of CEES were prepared in EtOH; final concentrations of the vehicles were 1% (vol/vol). Cell viability was measured by the standard MTT assay.

 We next sought to determine how low (≤ 0.1 mM) levels of CEES would affect A23187-induced degranulation in RBL-2H3 cells. We performed a series of experiments monitoring both mast cell degranulation after 1 h (via β-hexosaminidase assays) and viability after 24 h (via MTT assays). A23187 concentrations were 20 nM, 50 nM, 100 nM, and 200 nM (nontoxic in the absence of CEES); CEES concentrations were 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM (viability not less than 80% in the absence of A23187). We found that low levels of CEES did not affect A23187-induced mast cell degranulation (data not shown). We plan to repeat all of the above experiments before publishing our data.

Specific Tasks 2:

Oxidized proteins (protein carbonyls) will be analyzed by 2-dimensional gel electrophoresis (2D-GE) and identified by mass spectrometry

We have initiated a series of experiments utilizing proteomics approach in order to evaluate modulations in protein expression induced by CEES/HD. We have obtained protein samples from (1) the *in vivo* animal models (tissue samples provided by USAMRICD) exposed and not exposed to CEES or HD and; (2) *in vitro* cellular studies

on rat mast cells exposed and not exposed to CEES. We have initiated two dimensional gel electrophoresis (2D-GE) analyses of these samples utilizing an XCell6™ Multi Gel Unit (Bio-Rad), which allows us to run, simultaneously up to six gradient electrophoresis gels (second dimension) after isoelectric focusing with the ZOOM® IPGRunner™ System (Bio-Rad).

Figure 7 shows trial separation of cellular proteins obtained from RBL-2H3 cells (~10^6cells/sample) treated with 0.5 mM CEES or vehicle (1% EtOH) for 24 hours. After the incubation cells were washed twice with cold PBS and destroyed with 0.5 mL of Protein Solublizer 1 (Bio-Rad) containing protease inhibitor cocktail and 20 mM DTT. Cell debris was removed by centrifugation, and supernatant aliquots containing 20 µg of total protein were analyzed by 2D-GE and stained by SilverSNAP Stain Kit (Bio-Rad). Differences in expression of various proteins will be evaluated by comparison of these two images.
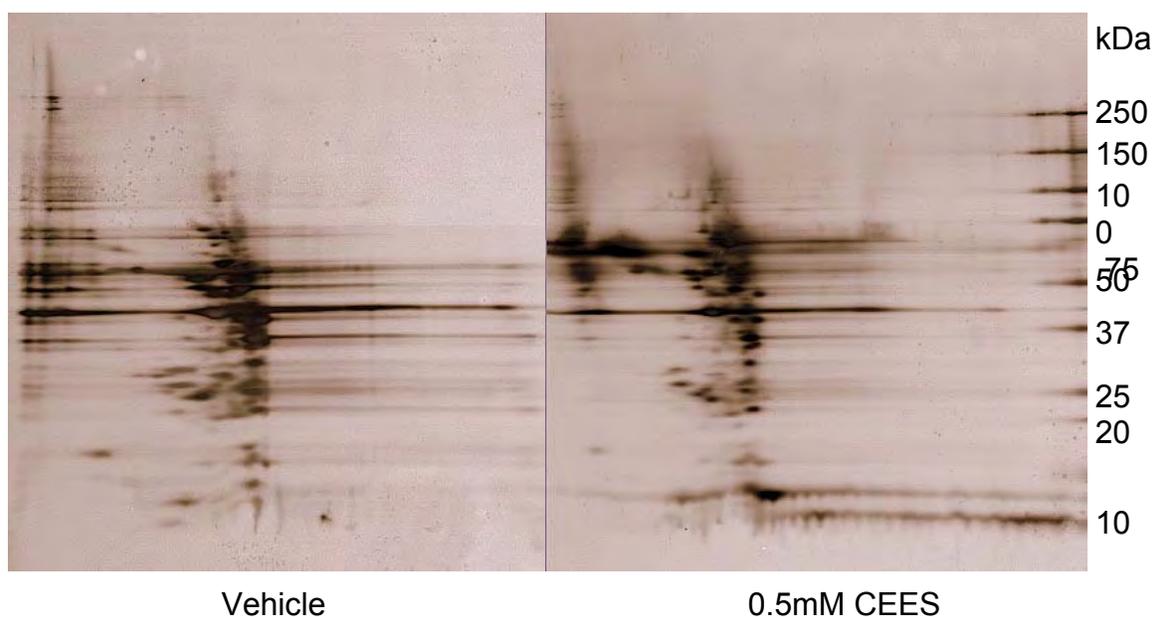
Vehicle   0.5mM CEES

Fig.7 RBL-2H3 cells were treated with 0.5mM CEES or vehicle (1% EtOH) for 24 hours. Cells were washed twice with cold PBS and lysates were prepared. Aliquots containing 20 µg of total protein were separated by 2D-GE and sliver stained. Isoelectric focusing (pH 3-10) – horizontal separation; SDS gel gradient (4-20%) electrophoresis – vertical separation. Molecular weights of protein standards are shown on the right.

We currently optimizing 2D-GE separation conditions for rat mast cell samples using narrow range IEF strips (pH 4-7) instead of broad range strips (pH 3-10). Figure 8 shows the difference in overall protein separation of a non-treated sample of RBL-2H3 mast cell lyzate using different IEF strips. We also optimizing 2D-GE separation conditions for a number of animal tissue samples treated and not treated with HD (data not shown).

kDa

	250
	150
	10
	0
	75
	50
	37
	25
	20
	15
	10

pH 3-10   pH 4-7
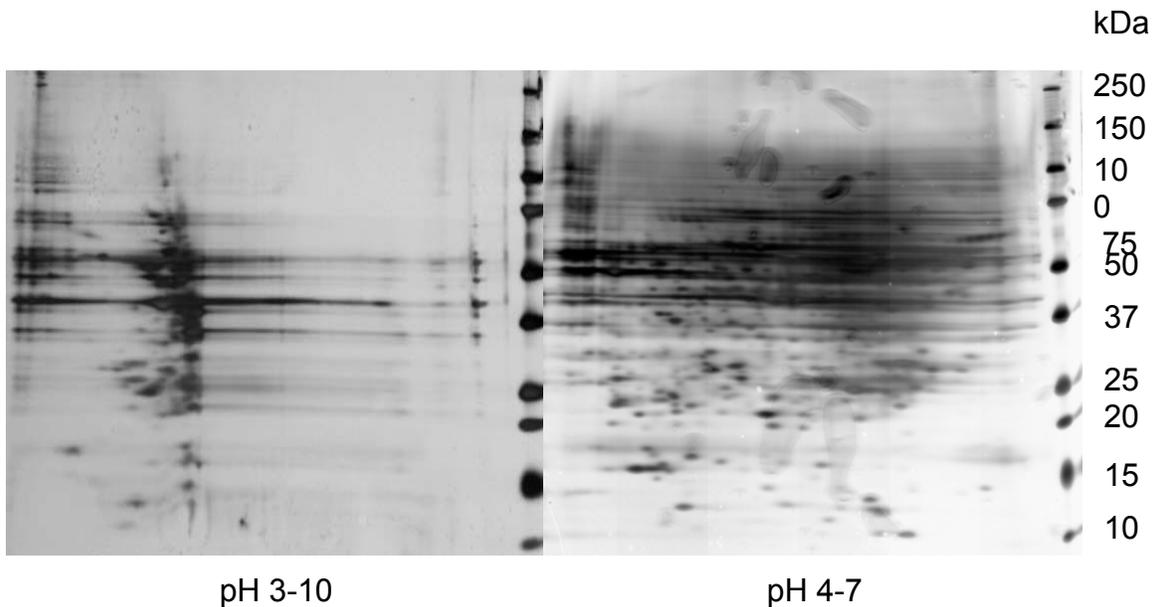
Fig.8 RBL-2H3 cell lyzate aliquots containing 20 µg of total protein were separated by 2D-GE and sliver stained. Isoelectric focusing (as indicated) – horizontal separation; SDS polyacrylamide gradient (4-20%) gel electrophoresis – vertical separation. Molecular weights of protein standards are shown on the right.

Next, we will analyze the differences in protein "finger prints" on the gels quantitivly using Dymension software (SynGene). The most affected proteins will be extracted from gel, trypsinized, and resulting peptide mixtures analyzed using nano-spray technique on our LTQ Linear Ion Trap Mass Spectrometer (ThermoFisher). By performing such analyses we will be able to identify oxidized proteins (protein carbonyls) as well as proteins affected (down-regulated or up-regulated) by CEES or HD.

Specific Tasks 3:

Preparation of well characterized antioxidant-liposomes

Guinea Pig Model

Dr. Hongsong Yang has been preparing and characterizing a number of antioxidant liposomes and shipping them to members of the Consortium. Dr. Salil Das at Meharry Medical College has found that some of these liposomal formulations are very effective at reducing CEES pulmonary toxicity in a guinea pig model [8, 9]. Dr. Das has been invited to present this work at the Singapore International Symposium on Protection against Toxic Substances.

Five antioxidant liposomes (LIP-1, LIP-2, LIP-3, LIP-4 and LIP-5) were sent to Dr. Das (see Table below) with differing levels of phospholipids, cholesterol, phosphatidic acid, tocopherols, N-acetylcysteine (NAC) and GSH as shown in the Table below.

Maximum lung protection was achieved with two liposomes, LIP-2 (71.5%) and LIP-4 (75.4%), when administered after 5 minutes of CEES exposure. Delaying the administration of the liposomes after 1 h of CEES exposure decreased the efficacy. This work clearly suggests that antioxidant liposomes can be used as an effective antidote against CEES-induced lung injury in this animal model. We continue to modify the antioxidant liposomes and hope that we can further enhance their therapeutic potential.

Rat Model

Liposomes	Liposome Content (mM)							
	Phospholipid (PL90H)	Cholesterol	Phosphatidic Acid (PA)	Tocopherol (Vitamin E)			NAC	GSH
				α	γ	δ		
LIP1 (Blank)	71	28	0.67	-	-	-	-	-
LIP 2	55	22	0.6	11	11	5	75	-
LIP 3	62	25	0.6	6	6	-	75	-
LIP 4	55	22	0.6	11	11	-	75	-
LIP 5	55	22	0.6	11	11	-	-	75

Dr. Yang has also prepared and shipped antioxidant liposomes to Dr. Peter Wards's laboratory. The Ward Group recently demonstrated that acute oxidant-related lung injury (ALI) in rats following instillation of chloroethyl ethyl sulfide (CEES) was attenuated by the airway instillation of anti-oxidant agents. This group hypothesized that anti-oxidant containing liposomes would attenuate acute as well as long-term (fibrotic) effects of CEES-induced lung injury. In a recently submitted publication (see below) this group found that in the acute injury model (at 4 hr), N-acetylcysteine (NAC)-containing liposomes were protective and reduced to baseline levels both the lung permeability index and the appearance of pro-inflammatory mediators in bronchoalveolar lavage fluids from CEES exposed lungs. Similar results were obtained when rat alveolar macrophages were incubated in vitro with either CEES or lipopolysaccharide (LPS) in the presence or of NAC-liposomes. When lung fibrosis three weeks after CEES was quantitated using hydroxyproline content, liposomes containing NAC or NAC + glutathione (GSH) had no effects, but liposomes containing E/α,γ-tocopherol alone or with NAC significantly suppressed the increase in lung hydroxyproline. These findings were confirmed by histopathology evaluation of lung sections. These data demonstrate that delivery of antioxidants via liposomes to CEES injured lungs are protective against ALI, prevent the appearance of pro-inflammatory mediators, and suppress progressive lung injury

(fibrosis). Accordingly, the Ward Group has concluded that "liposomal strategy may be therapeutically useful in oxidant mediated lung injury in humans."

Key Research Accomplishments

1. The effect of various levels of CEES (10 µM – 5 mM) on mast cell degranulation was studied in RBL-2H3 cells
2. An inhibition of mast cell degranulation by CEES was found in RBL-2H3 cells
3. An enhancement of CEES toxicity by non-toxic doses of Ca-ionophore A23187 was documented in RBL-2H3 cells
4. Rat mast cells and various animal tissue samples treated/untreated with CEES or HD were analyzed by 2D-GE separation
5. Second generation antioxidant liposomes were formulated, prepared and distributed to members of the Consortium. These antioxidant liposomes were found to be particularly effective in preventing chronic lung damage.

Reportable Outcomes

Presentations:

1. Advanced Medical Countermeasures Consortium Meeting, June 28, 2007, Crystal City, VA: "Protective effects of anti-oxidant liposomes in acute and progressive lung injury after CEES."
2. BARDA 2007 Industry Day Conference, August 3, 2007, Washington, DC: "Protective effects of anti-oxidant liposomes."
3. Biotech 2007 Poster (Richard Rest, Mariana Bernui, Milton Smith and William Stone). STIMAL (Liposome-Encapsulated N-Acetylcysteine) increases the ability of human macrophages to kill *Bacillus anthracis*.

Manuscripts (during the past year of funding):

1. Qui M, Paromov VM, Yang H, Smith M, **Stone WL:** Inhibition of inducible Nitric Oxide Synthase by a mustard gas analog in murine macrophages**. *BMC Cell Biology* 2006,** 7:39.

2. Viktor Paromov, Min Qui, Hongsong Yang, Milton Smith, and **William L. Stone**: The influence of N-acetyl-L-cysteine and polymyxin B on oxidative stress and nitric oxide synthesis in stimulated macrophages treated with a mustard gas analog. *BMC Cell Biology* (submitted)

3. Viktor Paromov, Zacharias Suntres, Milton Smith, **William L. Stone**: Sulfur Mustard Toxicity in Human Skin: Role of Oxidative Stress, and Antioxidant Therapy in: *Journal of Burns and Wounds* (in press)

4. Milton G. Smith, **William L. Stone**, Ren-Feng Guo, Peter A. Ward, Zacharias Suntres, Shyamali Mukherjee and Salil K. Das, Chapter 12 Vesicants and Oxidative Stress, pp 249-294 in Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology and Therapeutics. Romano editor (in press).

Conclusion

It is now clear that CEES-induced cell injury is associated with oxidative stress. The acute phase of injury is an oxidant-mediated process that is associated with intensive inflammatory responses. Liposomes containing antioxidants (GSH, NAC, pyruvates, tocopherols and tocotrienols) are expected to be highly protective in this setting. We are currently analyzing overall intracellular changes due to the oxidative stress in CEES/HD treated samples utilizing a systems biology approach. We will continue to analyze the differences in protein "finger prints" on the 2D-gels quantitivly using Dymension software (SynGene). The most affected proteins will be extracted from gels, and identified by nano-spray technique on our LTQ Linear Ion Trap Mass Spectrometer (ThermoFisher). By performing such analyses we will be able to identify oxidized proteins (protein carbonyls) and proteins whose expression was affected (down-regulated or up-regulated) by CEES or HD. The effect of antioxidant liposomes on these proteins also will be determined.

We found that toxic doses of CEES impair degranulation in RBL-2H3 rat mast cells. Initially, we proposed that CEES might stimulate mast cell degranulation since we found that CEES inhibits the generation of NO in RAW 264.7 macrophages [3]. Nitric oxide is known to inhibit mast cell degranulation in various species [4, 5]. However, recent study has shown that endogenous nitric oxide does not modulate degranulation of mesenteric or skin mast cells in rats [6]. It is clear, nevertheless that CEES is capable of modulating mast cell degranulation. The molecular mechanisms of this effect and its relation to the oxidative stress will be elucidated in our future experiments.

References

1. Victor Paromov ZS, Milton Smith, William L. Stone: **Sulfur Mustard Toxicity Following Dermal Exposure: Role of Oxidative Stress, and Antioxidant Therapy**. *Journal of burns and wounds* 2007, **in press**.

2. Stone WL, Qui M, Smith M: **Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide**. *BMC cell biology* 2003, **4**(1):1.

3. Qui M, Paromov VM, Yang H, Smith M, Stone WL: **Inhibition of inducible Nitric Oxide Synthase by a mustard gas analog in murine macrophages**. *BMC cell biology* 2006, **7**:39.

4. Eastmond NC, Banks EM, Coleman JW: **Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis**. *J Immunol* 1997, **159**(3):1444-1450.

5. Iikura M, Takaishi T, Hirai K, Yamada H, Iida M, Koshino T, Morita Y: **Exogenous nitric oxide regulates the degranulation of human basophils and rat peritoneal mast cells**. *International archives of allergy and immunology* 1998, **115**(2):129-136.

6. Kwasniewski FH, Tavares de Lima W, Bakhle YS, Jancar S: **Endogenous nitric oxide does not modulate mesenteric mast cell degranulation in rats**. *Biochemical pharmacology* 2003, **65**(12):2073-2080.

7. Seol IW, Kuo NY, Kim KM: **Effects of dopaminergic drugs on the mast cell degranulation and nitric oxide generation in RAW 264.7 cells**. *Archives of pharmacal research* 2004, **27**(1):94-98.

8. Das SK, Mukherjee S, Smith MG, Chatterjee D: **Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue**. *Journal of biochemical and molecular toxicology* 2003, **17**(3):177-184.

9. McClintock SD, Hoesel LM, Das SK, Till GO, Neff T, Kunkel RG, Smith MG, Ward PA: **Attenuation of half sulfur mustard gas-induced acute lung injury in rats**. *J Appl Toxicol* 2006, **26**(2):126-131.

Appendices (available as PDF files)

1. *BMC Cell Biology* Paper 2006
2. *Journal of Burns and Wounds* Review 2007
3. *BMC Cell Biology* Paper 2007

BMC Cell Biology

Research article

Open Access

Inhibition of inducible Nitric Oxide Synthase by a mustard gas analog in murine macrophages

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Abstract

Background: 2-Chloroethyl ethyl sulphide (CEES) is a sulphur vesicating agent and an analogue of the chemical warfare agent 2,2'-dichlorodiethyl sulphide, or sulphur mustard gas (HD). Both CEES and HD are alkylating agents that influence cellular thiols and are highly toxic. In a previous publication, we reported that lipopolysaccharide (LPS) enhances the cytotoxicity of CEES in murine RAW264.7 macrophages. In the present investigation, we studied the influence of CEES on nitric oxide (NO) production in LPS stimulated RAW264.7 cells since NO signalling affects inflammation, cell death, and wound healing. Murine macrophages stimulated with LPS produce NO almost exclusively via inducible nitric oxide synthase (iNOS) activity. We suggest that the influence of CEES or HD on the cellular production of NO could play an important role in the pathophysiological responses of tissues to these toxicants. In particular, it is known that macrophage generated NO synthesised by iNOS plays a critical role in wound healing.

Results: We initially confirmed that in LPS stimulated RAW264.7 macrophages NO is exclusively generated by the iNOS form of nitric oxide synthase. CEES treatment inhibited the synthesis of NO (after 24 hours) in viable LPS-stimulated RAW264.7 macrophages as measured by either nitrite secretion into the culture medium or the intracellular conversion of 4,5-diaminofluorescein diacetate (DAF-2DA) or dichlorofluorescin diacetate (DCFH-DA). Western blots showed that CEES transiently decreased the expression of iNOS protein; however, treatment of active iNOS with CEES *in vitro* did not inhibit its enzymatic activity

Conclusion: CEES inhibits NO production in LPS stimulated macrophages by decreasing iNOS protein expression. Decreased iNOS expression is likely the result of CEES induced alteration in the nuclear factor kappa B (NF-κB) signalling pathway. Since NO can act as an antioxidant, the CEES induced down-regulation of iNOS in LPS-stimulated macrophages could elevate oxidative stress. Since macrophage generated NO is known to play a key role in cutaneous wound healing, it is possible that this work has physiological relevance with respect to the healing of HD induced skin blisters.

Background

HD is a chemical weapon that can produce casualties in military situations and has been used with devastating results against civilian populations [1]. Extensive and slow healing lesions following exposure to HD can place a heavy burden on the medical services of military and public health organizations. The design of effective countermeasures to HD depends upon a detailed understanding of the molecular mechanisms for its toxicity. Important mechanisms of HD induced skin injury are alkylation of DNA and other macromolecules, accompanied by enhanced reactive oxygen species (ROS) generation and depletion of intracellular glutathione (GSH) [2-5]. Depletion of GSH by HD and its metabolites is known to shift the intracellular redox milieu toward a more oxidized state with a subsequent loss of protection against oxidative free radicals and an activation of inflammatory responses[6,7].

It has been shown that HD induces a vast "spectrum" of inflammatory cytokines released from keratinocytes [8,9]. It is likely that CEES cause similar changes in macrophages and leukocytes. We previously found that LPS, as well as inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin one-beta (IL-1β), significantly amplify the toxicity of CEES in RAW264.7 macrophages [10]. In macrophages, stimulation by LPS, as well as by pro-inflammatory cytokines, leads to the activation and nuclear translocation of NF-κB [11]. One of the major consequences of such activation in macrophages is an induction of iNOS expression with subsequent elevation of intracellular NO [12]. The effect of CEES on NO generation and on the NF-κB pathway is potentially significant since NO signalling plays an important role in inflammation, the mechanisms of cell death NF-κB [13,14], and wound healing [15,16]. The present work describes the inhibition of NO production and iNOS expression in LPS stimulated macrophages treated with CEES.

Results

CEES transiently suppresses NO production and iNOS expression in LPS stimulated cells

In Figure 1a, we examined nitrite secretion into the cell culture medium by RAW 264.7 murine macrophages after 24 hours of treatment with CEES and various levels of LPS. Nitrite level in the cell culture medium, as measured by the Griess reagent, is a reliable indicator of nitric oxide secretion. These data show that CEES (100–500 µM) inhibited the secretion of NO into the cell medium by LPS stimulated macrophages in a dose-dependent manner. Low levels of CEES (≤ 100 µM) only partially inhibited NO production, whereas levels higher than 300 µM completely inhibited NO production. Although CEES does decrease the viability of LPS stimulated macrophages [10],

the decreased generation of NO cannot be accounted simply for the loss of viable cells. Figure 1b shows that in case nitrite levels in the culture medium (as measured by OD at 532 nm) are normalized to the amount of viable cells (OD at 580 nm, MTT assay, measured separately) there is still a significant CEES dose dependent inhibition of NO formation.

In order to determine if CEES influenced cellular levels of iNOS, we performed Western blot analyses (Figure 1c) of the cell lysates using highly selective anti-iNOS antibodies with equal amounts of total protein applied to each lane. Control RAW 264.7 macrophages had no detectable iNOS protein, CEES treatment alone did not induce any iNOS protein but LPS (10 ng/ml for 24 hours) produced a marked induction of iNOS protein. When simultaneously treated with LPS (10 ng/ml) and CEES (300 µM) there was a marked reduction in the LPS induction of iNOS protein.

We then examined the influence of 300 µM CEES on the time course of NO production in macrophage stimulated with 10 ng/ml LPS. Figure 2a shows that CEES delays, but does not prevent, the production of NO (as measured by nitrite formation) in LPS-stimulated macrophages. In fact, after 12 hours the rate of NO production is about the same in cells treated with LPS alone compared with cells treated with both LPS and CEES. Western blot data (Figure 2b) from the cells used in Figure 2a show a similar pattern: LPS alone induces robust iNOS protein expression which is completely inhibited by CEES for up to 6 hours. After 12 hours, however, the cells incubated with both CEES and LPS show a rebound in the expression of iNOS and after 24 hours the iNOS protein level in cells treated with both CEES and LPS is very similar to that observed in cells treated with LPS alone. These data show that the influence of CEES on both nitric oxide synthesis and iNOS expression is transient.

CEES does not inhibit iNOS enzymatic activity *in vitro*

In order to evaluate the possible direct inhibitory effect of CEES on iNOS activity *in vitro*, we measured the intracellular rates of 4,5-diaminofluorescein (DAF-2) or dichlorofluorescin (DCFH) oxidation in intact macrophages. Dichlorofluorescin diacetate (DCFH-DA) is permeable to the cell plasma membrane and intracellular esterases convert it into a membrane impermeable (DCFH) form which is can be oxidized to highly fluorescent dichlorofluorescein (DCF) by free radicals. In macrophages, the oxidation of DCFH has been shown to be a sensitive and relatively selective probe for monitoring intracellular NO formation by iNOS [17].

Using DCFH-DA and DAF-2DA, we were able to continuously monitor NO formation in intact macrophages under a variety of conditions. Previously, we [18] and oth-
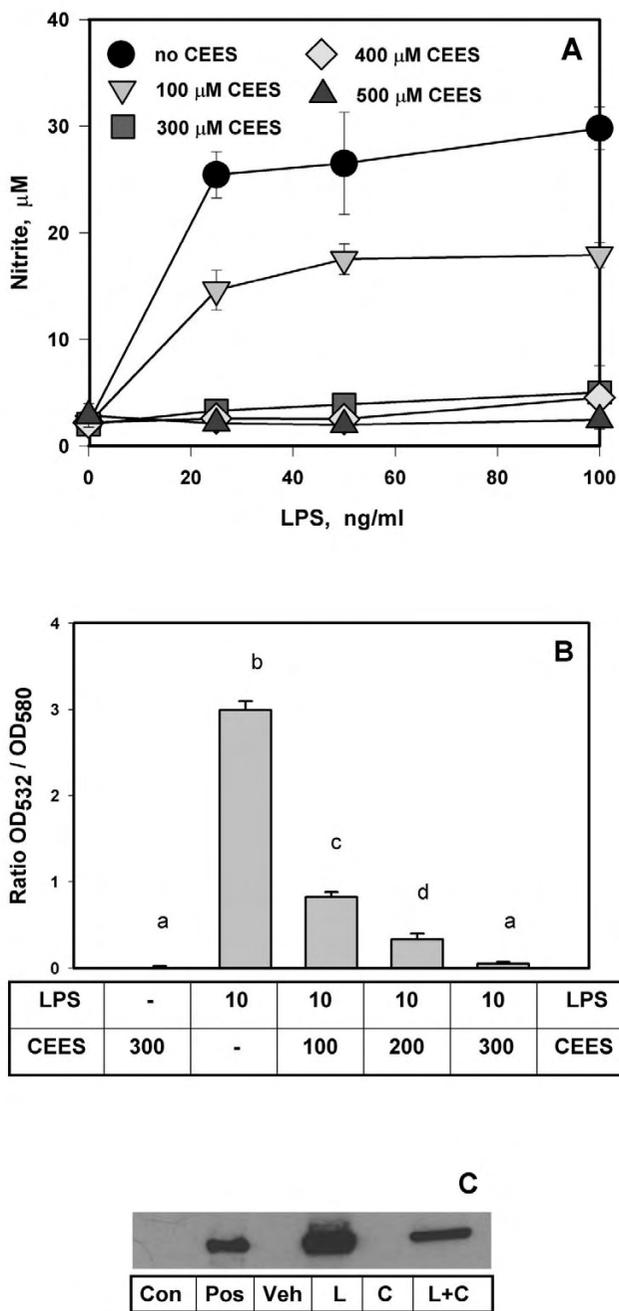
Figure 1
CEES inhibits NO production and iNOS expression in LPS stimulated RAW264.7 macrophages. *Panel A*: Macrophages were simultaneously treated with various levels of CEES (as indicated) and low doses of LPS (as indicated). NO production was monitored as the concentration of nitrite in the culture medium after 24 h. *Panel B*: Cells were treated similarly as for *Panel A*; LPS, 10 ng/ml; CEES, 100, 200, or 300 μM (as indicated). Means not sharing a common letter are significantly different (p < 0.05). Nitrite levels in the culture medium (OD at 532 nm) were normalized to the amount of viable cells (OD of the MTT product at 580 nm). *Panel C*: Western blot analysis of iNOS protein from cells simultaneously incubated with 300 μM CEES and/or 10 ng/ml LPS for 24 h; cell lysates were prepared as described in Materials and Methods: Con, control cells; Pos, iNOS protein for positive control; Veh, vehicle; L, 10 ng/ml LPS stimulated cells; C, 300 μM CEES treated cells; L+C, LPS/CEES treated cells.
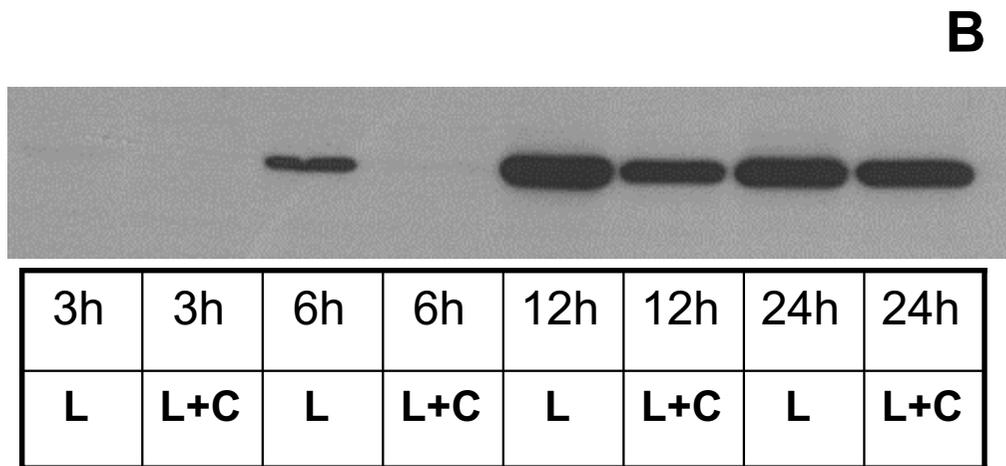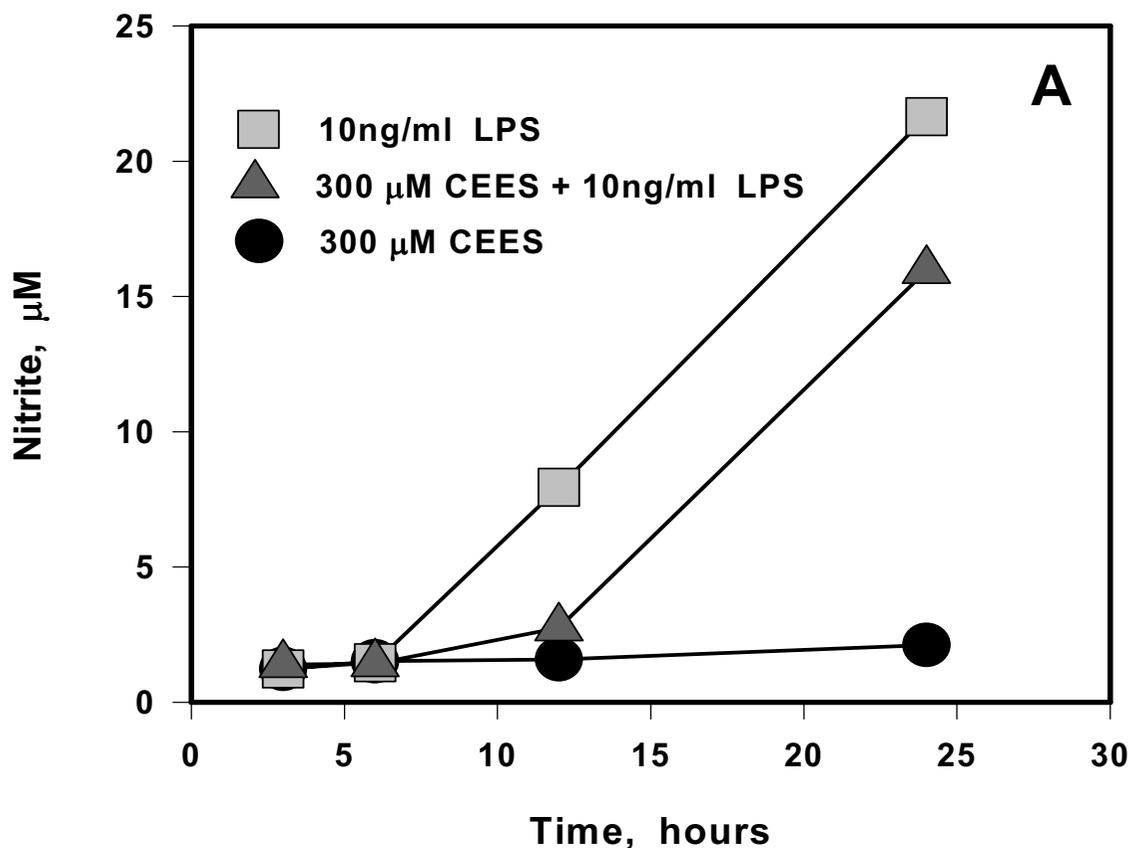
Figure 2
Time course of NO production and iNOS expression in LPS stimulated RAW264.7 macrophages incubated with CEES. *Panel A*: Macrophages were incubated with 10 ng/ml LPS alone, 300 µM CEES alone or simultaneously with both 300 µM CEES 10 ng/ml LPS for various time intervals (as indicated). NO production measured as concentration of nitrite in culture medium. *Panel B*: Western blot analysis of iNOS protein from the cells incubated with 300 µM CEES with or without 10 ng/ml LPS; cell lysates were prepared after 3, 6, 12, or 24 hour incubation (as indicated) as described in Materials and Methods; L, LPS; C, CEES.

ers [19] have shown that LPS exclusively induces the iNOS form of nitric oxide synthase in murine macrophages. Figure 3a shows DCFH oxidation in RAW 264.7 cells stimulated with different levels of LPS for 24 hours. In the absence of LPS, the rate of DCFH oxidation was extremely low but increased with increasing exposure to LPS; however, this effect was nearly saturated at LPS levels above 15 ng/ml.

We then measured the rates of DAF-2 oxidation in RAW 264.7 macrophages stimulated with 20 ng/ml LPS in the presence or absence of 500 μM CEES during 24 hour incubations (Figure 3b). In the absence of LPS or CEES, minimal DAF-2 oxidation was observed. As expected, LPS alone induced a marked increase in DAF-2 oxidation. Next, macrophages incubated with LPS for 24 hours were then exposed (post-treatment) to 500 μM CEES and the rate of DAF-2 oxidation immediately measured. As shown in Figure 3b, there was no change in rate of DAF-2 oxidation compared to cells treated with LPS alone. These data strongly support the notion that CEES does not directly inhibit iNOS enzymatic activity. Similar results were obtained with DCFH-DA staining (data not shown). As expected, macrophages simultaneously treated with both LPS and CEES for 24 hours show a marked decrease in either DAF-2 or DCFH oxidation.

To further confirm that DCFH oxidation is overwhelmingly due to iNOS, we incubated LPS-stimulated macrophages with ebselen (see Figure 3c). Ebselen is a selenoorganic compound that can inhibit both the activity of iNOS [20] and its induction by LPS [21]. Ebselen (25 μM) almost completely inhibited the DCFH oxidation in RAW 264.7 cells treated with 10 ng/ml or 20 ng/ml LPS. Ebselen was not cytotoxic at the levels used in Figure 3 (data not shown).

Discussion
Overall, the experiments detailed in this work show that CEES treatment in LPS-stimulated RAW264.7 murine macrophages transiently inhibits intracellular NO generation by interfering with iNOS expression rather than by direct inhibition of iNOS enzymatic activity. CEES (as well as HD) undergo rapid hydrolysis in aqueous solutions and this may account, in part, for the transitory nature of its inhibiting effect on iNOS induction [22]. LPS is a major component of the cell wall of gram-negative bacteria and is known to trigger a variety of inflammatory reactions in macrophages and other cells expressing CD14 receptors [23,24]. LPS is ubiquitous and is present in serum, tap water, and dust. Military and civilian personnel would, indeed, always have some degree of exposure to environmental LPS.

LPS stimulation of macrophages is known to involve the activation of protein phosphorylation by kinases as well as the activation of nuclear transcription factors such as NF-κB [25-28]. An important consequence of NF-κB activation in macrophages is the induction of iNOS expression followed with highly elevated NO production [12]. Nitric oxide has been demonstrated to have an important role in promoting cell death; however, the precise nature of this role varies with cell type and the dose. Low levels of nitric oxide protect RAW 264.7 macrophages from hydrogen peroxide induced apoptosis [29], however, nitric oxide has also been reported to induce apoptosis in J774 macrophages [14]. Nitric oxide can induce cell death through energy depletion-induced necrosis and oxidant-induced apoptosis.

We are currently exploring the potential molecular mechanism(s) whereby CEES interferes with iNOS expression in LPS stimulated macrophages. It is possible that GSH depletion caused by CEES determines iNOS expression. There are strong evidences suggesting that thiol depletion and iNOS expression are interrelated [30-32]. For example, LPS stimulated macrophages depleted of GSH exhibit a decreased level of iNOS protein and nitrite production [32]. Similarly, both *in vitro* [30] and *in vivo* [31] studies show that hepatocytes depleted of GSH have a diminished production of nitric oxide which is primarily due to a decreased level of iNOS mRNA. Vos et al. [31] have also presented evidence showing that GSH modulation of iNOS expression in hepatocytes is correlated with NF-kB activation, i.e., GSH depletion is associated with a lack of NF-kB activation. The influence of GSH depletion is not, however, consistent in all cell types. Glucose induced reduction of GSH in intestinal epithelial cells is associated with NF-kB activation and upregulation of iNOS gene expression [33].

It is also possible that CEES decreases iNOS expression by interfering with the LPS-induced activation of transcription factor NF-κB and/or signal transducer and activator of transcription-1α (STAT-1α). It is interesting, therefore, that Gray [34] has found that both CEES and HD inhibit the *in vitro* binding of transcription factor activating protein-2 (AP-2) via alkylating the AP-2 DNA consensus binding sequence rather than by direct damage to the AP-2 protein. Furthermore, it is significant that neither CESS nor its hydrolysis products were found to damage the AP-2 transcription factoring in a manner that prevented its DNA binding [35]. Similar experiments have yet to be done with NF-κB. Chen et al. [36] have also found that nitrogen mustard (bis(2-chloroethul) methylamine) similarly inhibits the binding of AP-2 to its consensus sequence. Nitrogen mustard also was shown to inhibit the binding of NF-κB to the GC-rich consensus sequence due to the interactions with DNA [37]. It is possible, therefore,
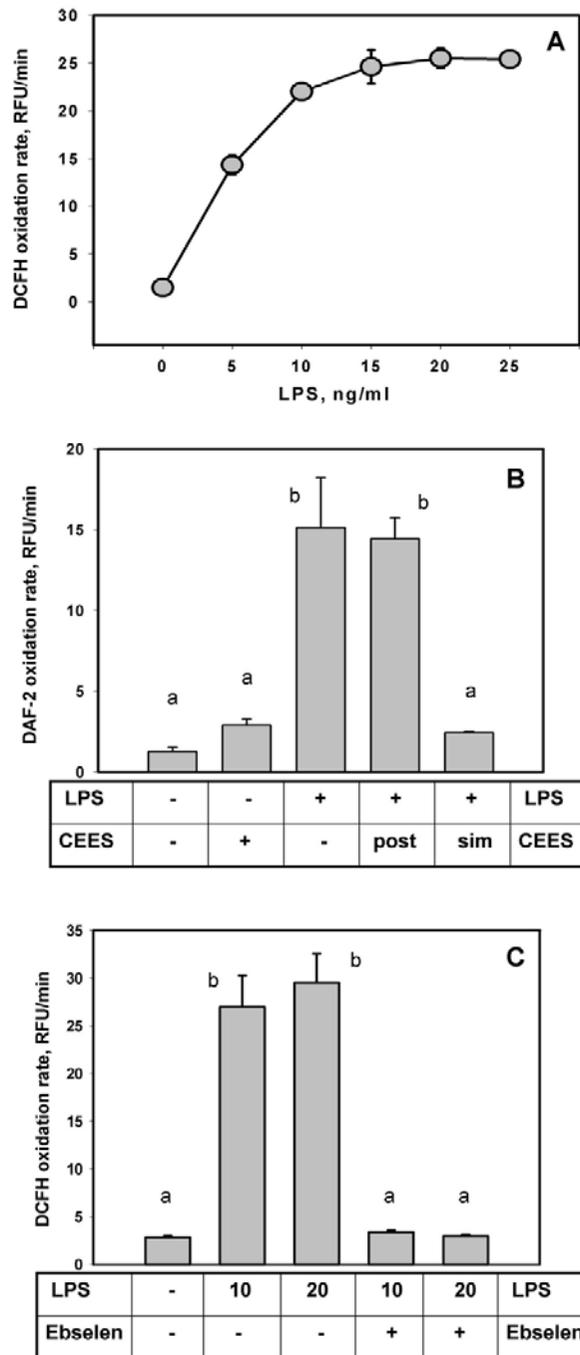
Figure 3
CEES reduces intracellular NO in LPS stimulated RAW264.7 macrophages. *Panel A*: Intracellular DCFH (20 μM) oxidation in LPS stimulated macrophages (as indicated) incubated for 2 h. Fluorescence (excitation 485 nm, emission 520 nm) was measured in Relative Fluorescence Units (RFU); the oxidation rate was expressed as RFU/min. *Panel B*: Macrophages stimulated with 20 ng/ml LPS, were incubated in the presence or absence of 500 μM CEES (as indicated) for 24 h. Post, CEES was applied after the 24 hours of LPS stimulation; Sim, CEES was applied simultaneously with LPS. *Panel C*: LPS stimulated cells were incubated in the presence or absence of 25 μM ebselen, a selective iNOS inhibitor (as indicated). 10, 10 ng/ml LPS; 20, 20 ng/ml LPS. Mean values not sharing a common letter are significantly different ($p < 0.05$).

that CEES also alkylates the NF-κB consensus sequence thereby preventing the binding of the NF-κB to the iNOS promoter. LPS and/or cytokine-inducible NF-κB binding elements of the murine iNOS promoter have been identified [38], and they are rich of guanine, which is the major alkylation site for HD or CEES. The possible effect of CEES on iNOS promoter regulation is currently being explored.

Although the activation of NF-κB due to mustard or CEES exposure have been shown in various cell lines [7,37,39], the detailed mechanism of this event is still unclear. Recent report [39] showed that NF-κB-driven gene expression has maximum at 9 hours in HD treated keratinocytes. In contrast, in a guinea pig model, Chatterjee et al. [40] have shown that NF-κB activation in lung tissues occurs shortly after CEES expose (1 hour), then disappears within 2 hours completely. However, in our experiments we did not observe any short term stimulating effect of CEES on NO production or iNOS expression (data not shown). Notably, the electrophoretic mobility shift assays used by Chatterjee et al. to measure NF-κB activation show only the state of NF-κB protein complex and provide no information regarding its binding to the DNA consensus sequences.

The physiological significance of potentially decreased iNOS expression by exposure to CEES or HD is not known. Considerable evidence, however, supports the view that nitric oxide production via iNOS plays a key role in wound healing [41-43]. Animal studies [16] have shown that the iNOS knockout mice have impaired wound healing that is reversed by iNOS gene transfer. Soneja et al. [44] have suggested that wound healing could be accelerated under circumstances where oxidative stress is minimized and nitric oxide production enhanced. We have initiated work to explore the role of antioxidants in preventing HD induced pathology in skin.

Conclusion

Our results show that CEES transiently inhibits NO production in LPS stimulated macrophages by inhibiting the expression of iNOS protein and not by modulating the enzymatic activity of iNOS. The decreased iNOS expression induced by CEES suggests that this alkylating agent inhibits the LPS stimulated activation of NF-κB and/or STAT-1α transcription factors, and this possibility is being investigated. We cannot directly address the physiological significance of our *in vitro* results, however, both decreased expression of iNOS and decreased production of nitric oxide are associated with impaired wound healing [16,41,43,44]. It is likely that the CEES or HD toxicity is modulated by a complex balance between nitric oxide production, thiol depletion and oxidative stress.

Methods

Materials

RPMI-1640 medium without phenol red and fetal bovine serum with a low endotoxin level were purchased from Life Technologies (Gaithersburg, MD). Rabbit anti-mouse iNOS antibody was obtained from Transduction Laboratory (Lexington, KY). Horseradish peroxidase conjugated anti-rabbit polyclonal antibodies, Escherichia coli lipopolysaccharide serotype 0111:B4, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and 2-chloroethyl ethyl sulphide were obtained from Sigma Chemical Company (St. Louis, MO).

Cell culture and treatments

RAW264.7 murine macrophage-like cells (American Type Culture Collection, Rockville, MD) were cultured at 37°C in a humidified incubator with 5% CO_2 in RPMI-1640 medium with 10% fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin (GiBcoBRL Grand Island, NY). CEES was used as a fresh (2 week old or less) 50 mM stock solution in dried ethanol. LPS was prepared as a 1 mg/ml stock solution in PBS and stored at -20°C for up to 3 months.

MTT assay

The MTT (3-(4,5-dimethylthiazool-2yl)-2,5-diphenyltetrazolium bromide) assay was performed by a slight modification of the method described by Wasserman et al. [45,46]. Briefly, at the end of each experiment, cultured cells in 96 well plates (with 200 μl of medium per well) were incubated with MTT (20 μl of 5 μg/ml per well) at 37°C for 4 hours. The formazan product was solubilized by addition of 100 μl of dimethyl sulfoxide (DMSO) and 100 μl of 10% SDS in 0.01 M HCl and the OD measured at 575 nm (Molecular Devices SPECTRAmax Plus microplate reader).

Western blot analysis

Cellular protein lysates were prepared as described in the protocol from Transduction Laboratory (Lexington, KY). Briefly, about 10^6 adherent cells were rinsed once with cold PBS and solublized by boiling in 0.1 ml of SDS-PAGE sample buffer for 5 min. Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). A 30 μg aliquot of protein was separated via 8% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Western blotting was performed with a rabbit polyclonal antiserum against the C-terminal (961 to 1144 amino acids) sequence of mouse iNOS (Transduction Lab, Lexington, KY). The protein was detected using an enhanced chemiluminescence kit from Amersham Life Science (Arlington Heights, IL). Murine iNOS (Calbiochem, CA) was used as a positive control.

Determination of NO production

The production of NO, reflecting cellular NO synthase activity, was estimated from the accumulation of nitrite (NO_2^-), a stable breakdown product of NO, in the medium. Nitrite was measured using the Griess reagent according to the method of Green et al. [47]. Briefly, an aliquot of cell culture medium was mixed with an equal volume of Greiss reagent which reacts with nitrite to form an azo-product. Absorbance of the reaction product was determined at 532 nm using a microplate reader (Molecular Devices Microplate Reader). Sodium nitrite was used as a standard to calculate nitrite concentrations.

Intracellular NO measurement

Assays were performed using 96-well tissue culture plates as described by Imrich and Kobzik [17]. The cell density was adjusted to 2×10^5/ml, and a 100 µl aliquot of the cell suspension in media was placed put in each well. CEES and LPS solutions to achieve desired concentrations were added and the plate incubated for 24 h at 37°C in 5% CO_2. Following the removal of media, serum free 1640 RPMI supplemented with 10 mM HEPES containing 20 µM DCFH-DA or 10 µM DAF-2DA (final concentration) was added, and the plates incubated for 2 h at 37°C. Fluorescence intensity (relative fluorescence unit, RFU) was continuously monitored using 485 nm for excitation and 520 nm emission in a florescence microplate reader (FluoStar Microplate Reader, BMG).

Statistical analyses

Data were analyzed by followed with the Scheffe test for significance with $p < 0.05$. Results were expressed as the mean ± SD. In all the Figures, mean values not sharing a common letter are significantly different ($p < 0.05$). Mean values sharing a common letter are not significantly different. The mean values and standard deviations of at least three independent experiments are provided in all the Figures.

Abbreviations

HD, sulphur mustard gas

CEES, 2-chloroethyl ethyl sulphide

LPS, lipopolysaccharide

NO, nitric oxide

iNOS, inducible nitric oxide synthase

NF-κB, nuclear factor kappa B

STAT-1α, signal transducer and activator of transcription-1α

DCF, dichlorofluorescein

DCFH, dichlorofluorescin

DCFH-DA, dichlorofluorescin diacetate

TNF-α, tumor necrosis factor-alpha

IL-1β, interleukin-1 beta

AP2, activating protein 2

MTT, 3-(4,5-dimethylthiazool-2yl)-2,5-diphenyltetrazolium bromide

DMSO, dimethyl sulfoxide

DEM, diethylmaleate

BSO, buthionine sulfoximine

DAF-2DA, 4,5-diaminofluorescein diacetate

Authors' contributions

WLS supervised the overall conduct of the research, which was performed in his laboratory. MQ and HY carried out all of the experimental work in this study and performed the statistical analyses. WLS and VP analyzed the data and drafted the manuscript. MS (along with WLS) conceived of the study, participated in the study design, and provided continuous evaluation of the experimental data. All authors read and approved the final manuscript.

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References

1. Smith KJ, Skelton H: **Chemical warfare agents: their past and continuing threat and evolving therapies. Part I of II.** *Skinmed* 2003, **2(4):**215-221.
2. Yourick JJ, Dawson JS, Benton CD, Craig ME, Mitcheltree LW: **Pathogenesis of 2,2'-dichlorodiethyl sulfide in hairless guinea pigs.** *Toxicology* 1993, **84(1-3):**185-197.
3. Elsayed NM, Omaye ST, Klain GJ, Korte DW Jr.: **Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection.** *Toxicology* 1992, **72(2):**153-165.
4. Elsayed NM, Omaye ST: **Biochemical changes in mouse lung after subcutaneous injection of the sulfur mustard 2-chloroethyl 4-chlorobutyl sulfide.** *Toxicology* 2004, **199(2-3):**195-206.
5. Kadar T, Turetz J, Fishbine E, Sahar R, Chapman S, Amir A: **Characterization of acute and delayed ocular lesions induced by sulfur mustard in rabbits.** *Curr Eye Res* 2001, **22(1):**42-53.
6. Gross CL, Innace JK, Hovatter RC, Meier HL, Smith WJ: **Biochemical manipulation of intracellular glutathione levels influences**

cytotoxicity to isolated human lymphocytes by sulfur mustard. *Cell Biol Toxicol* 1993, **9(3):**259-267.

7. Atkins KB, Lodhi IJ, Hurley LL, Hinshaw DB: **N-acetylcysteine and endothelial cell injury by sulfur mustard.** *J Appl Toxicol* 2000, **20 Suppl 1:**S125-8.

8. Arroyo CM, Schafer RJ, Kurt EM, Broomfield CA, Carmichael AJ: **Response of normal human keratinocytes to sulfur mustard (HD): cytokine release using a non-enzymatic detachment procedure.** In *Hum Exp Toxicol Volume 18.* Issue 1 ENGLAND ; 1999:1-11.

9. Sabourin CL, Petrali JP, Casillas RP: **Alterations in inflammatory cytokine gene expression in sulfur mustard-exposed mouse skin.** *J Biochem Mol Toxicol* 2000, **14(6):**291-302.

10. Stone WL, Qui M, Smith M: **Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide.** *BMC Cell Biol* 2003, **4(1):**1.

11. Li YH, Yan ZQ, Brauner A, Tullus K: **Activation of macrophage nuclear factor-kappa B and induction of inducible nitric oxide synthase by LPS.** *Respiratory research* 2002, **3:**23.

12. Kleinert H, Pautz A, Linker K, Schwarz PM: **Regulation of the expression of inducible nitric oxide synthase.** *European journal of pharmacology* 2004, **500(1-3):**255-266.

13. Borutaite V, Brown G: **What else has to happen for nitric oxide to induce cell death?** *Biochem Soc Trans* 2005, **33(Pt 6):**1394-1396.

14. Borutaite V, Brown GC: **Nitric oxide induces apoptosis via hydrogen peroxide, but necrosis via energy and thiol depletion.** *Free Radic Biol Med* 2003, **35(11):**1457-1468.

15. Nakai K, Kubota Y, Kosaka H: **Inhibition of nuclear factor kappa B activation and inducible nitric oxide synthase transcription by prolonged exposure to high glucose in the human keratinocyte cell line HaCaT.** *The British journal of dermatology* 2004, **150(4):**640-646.

16. Yamasaki K, Edington HD, McClosky C, Tzeng E, Lizonova A, Kovesdi I, Steed DL, Billiar TR: **Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer.** *J Clin Invest* 1998, **101(5):**967-971.

17. Imrich A, Kobzik L: **Fluorescence-based measurement of nitric oxide synthase activity in activated rat macrophages using dichlorofluorescin.** *Nitric Oxide* 1997, **1(4):**359-369.

18. Huang A, Li C, Kao RL, Stone WL: **Lipid hydroperoxides inhibit nitric oxide production in RAW264.7 macrophages.** *Free Radic Biol Med* 1999, **26:**526-537.

19. Schmidt HH, Warner TD, Nakane M, Forstermann U, Murad F: **Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages.** *Molecular pharmacology* 1992, **41(4):**615-624.

20. Hattori R, Inoue R, Sase K, Eizawa H, Kosuga K, Aoyama T, Masayasu H, Kawai C, Sasayama S, Yui Y: **Preferential inhibition of inducible nitric oxide synthase by ebselen.** *Eur J Pharmacol* 1994, **267(2):**R1-2.

21. Zhang N, Weber A, Li B, Lyons R, Contag PR, Purchio AF, West DB: **An inducible nitric oxide synthase-luciferase reporter system for in vivo testing of anti-inflammatory compounds in transgenic mice.** In *J Immunol Volume 170.* Issue 12 United States ; 2003:6307-6319.

22. Gray PJ: **Sulphur mustards inhibit binding of transcription factor AP2 in vitro.** In *Nucleic Acids Res Volume 23.* Issue 21 ENGLAND ; 1995:4378-4382.

23. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC: **CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein.** *Science* 1990, **249(4975):**1431-1433.

24. Downey JS, Han J: **Cellular activation mechanisms in septic shock.** *Front Biosci* 1998, **3:**d468-76.

25. Chen CC, Wang JK, Lin SB: **Antisense oligonucleotides targeting protein kinase C-alpha, -beta I, or -delta but not -eta inhibit lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages: involvement of a nuclear factor kappa B-dependent mechanism.** In *J Immunol Volume 161.* Issue 11 UNITED STATES ; 1998:6206-6214.

26. Fujihara M, Connolly N, Ito N, Suzuki T: **Properties of protein kinase C isoforms (beta II, epsilon, and zeta) in a macrophage cell line (J774) and their roles in LPS-induced nitric oxide production.** In *J Immunol Volume 152.* Issue 4 UNITED STATES ; 1994:1898-1906.

27. Shapira L, Takashiba S, Champagne C, Amar S, Van Dyke TE: **Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-alpha and IL-1 beta production by human monocytes.** *J Immunol* 1994, **153(4):**1818-1824.

28. Shapira L, Sylvia VL, Halabi A, Soskolne WA, Van Dyke TE, Dean DD, Boyan BD, Schwartz Z: **Bacterial lipopolysaccharide induces early and late activation of protein kinase C in inflammatory macrophages by selective activation of PKC-epsilon.** *Biochem Biophys Res Commun* 1997, **240(3):**629-634.

29. Yoshioka Y, Kitao T, Kishino T, Yamamuro A, Maeda S: **Nitric oxide protects macrophages from hydrogen peroxide-induced apoptosis by inducing the formation of catalase.** *J Immunol* 2006, **176(8):**4675-4681.

30. Harbrecht BG, Di Silvio M, Chough V, Kim YM, Simmons RL, Billiar TR: **Glutathione regulates nitric oxide synthase in cultured hepatocytes.** *Ann Surg* 1997, **225(1):**76-87.

31. Vos TA, Van Goor H, Tuyt L, De Jager-Krikken A, Leuvenink R, Kuipers F, Jansen PL, Moshage H: **Expression of inducible nitric oxide synthase in endotoxemic rat hepatocytes is dependent on the cellular glutathione status.** *Hepatology* 1999, **29(2):**421-426.

32. Buchmuller-Rouiller Y, Corrandin SB, Smith J, Schneider P, Ransijn A, Jongeneel CV, Mauel J: **Role of glutathione in macrophage activation: effect of cellular glutathione depletion on nitrite production and leishmanicidal activity.** *Cell Immunol* 1995, **164(1):**73-80.

33. Powell LA, Warpeha KM, Xu W, Walker B, Trimble ER: **High glucose decreases intracellular glutathione concentrations and upregulates inducible nitric oxide synthase gene expression in intestinal epithelial cells.** *J Mol Endocrinol* 2004, **33(3):**797-803.

34. Gray PJ: **Sulphur mustards inhibit binding of transcription factor AP2.** *Nucleic Acids Research* 1995, **23(Vol. 23):**4378-4382.

35. Gray PJ: **Sulphur mustards inhibit binding of transcription factor AP2 in vitro.** *Nucleic Acids Res* 1995, **23(21):**4378-4382.

36. Chen XM, Gray PJ, Cullinane C, Phillips DR: **Differential sensitivity of transcription factors to mustard-damaged DNA.** *Chemico-biological interactions* 1999, **118(1):**51-67.

37. Fabbri S, Prontera C, Broggini M, D'Incalci M: **Differential inhibition of the DNA binding of transcription factors NF kappa B and OTF-1 by nitrogen mustard and quinacrine mustard: transcriptional implications.** *Carcinogenesis* 1993, **14(9):**1963-1967.

38. Spink J, Cohen J, Evans TJ: **The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor-kappa B.** *The Journal of biological chemistry* 1995, **270(49):**29541-29547.

39. Minsavage GD, Dillman 3d JF: **Protective role of CAPE on bifunctional alkylating agent-induced toxicity in keratinocytes via modulation of NF-kappaB, p53 and ARE/EpRE signaling .** In *Bioscience 2006 Medical Defense Review* ; 2006:94-94.

40. Chatterjee D, Mukherjee S, Smith MG, Das SK: **Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog.** *J Biochem Mol Toxicol* 2003, **17(2):**114-121.

41. Schwentker A, Billiar TR: **Nitric oxide and wound repair.** *Surg Clin North Am* 2003, **83(3):**521-530.

42. Weller R: **Nitric oxide: a key mediator in cutaneous physiology.** *Clin Exp Dermatol* 2003, **28(5):**511-514.

43. Witte MB, Barbul A: **Role of nitric oxide in wound repair.** *Am J Surg* 2002, **183(4):**406-412.

44. Soneja A, Drews M, Malinski T: **Role of nitric oxide, nitroxidative and oxidative stress in wound healing.** *Pharmacol Rep* 2005, **57 Suppl:**108-119.

45. Wasserman TH, Twentyman P: **Use of a colorimetric microtiter (MTT) assay in determining the radiosensitivity of cells from murine solid tumors.** *Int J Radiat Oncol Biol Phys* 1988, **15(3):**699-702.

46. Twentyman PR, Luscombe M: **A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity.** *Br J Cancer* 1987, **56(3):**279-285.

47. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: **Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids.** In *Anal Biochem Volume 126.* Issue 1 UNITED STATES ; 1982:131-138.

Sulfur Mustard Toxicity Following Dermal Exposure

Role of Oxidative Stress, and Antioxidant Therapy

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Objective: Sulfur mustard (bis-2-(chloroethyl) sulfide) is a chemical warfare agent (military code: HD) causing extensive skin injury. The mechanisms underlying HD-induced skin damage are not fully elucidated. This review will critically evaluate the evidence showing that oxidative stress is an important factor in HD skin toxicity. Oxidative stress results when the production of reactive oxygen (ROS) and/or reactive nitrogen oxide species (RNOS) exceeds the capacity of antioxidant defense mechanisms. **Methods:** This review will discuss the role of oxidative stress in the pathophysiology of HD skin toxicity in both in vivo and in vitro model systems with emphasis on the limitations of the various model systems. Evidence supporting the therapeutic potential of antioxidants and antioxidant liposomes will be evaluated. Antioxidant liposomes are effective vehicles for delivering both lipophilic (incorporated into the lipid bilayers) and water-soluble (encapsulated in the aqueous inner-spaces) antioxidants to skin. The molecular mechanisms interconnecting oxidative stress to HD skin toxicity are also detailed. **Results:** DNA repair and inflammation, in association with oxidative stress, induce intracellular events leading to apoptosis or to a programmable form of necrosis. The free radical, nitric oxide (NO), is of considerable interest with respect to the mechanisms of HD toxicity. NO signaling pathways are important in modulating inflammation, cell death, and wound healing in skin cells. **Conclusions:** Potential future directions are summarized with emphasis on a systems biology approach to studying sulfur mustard toxicity to skin as well as the newly emerging area of redox proteomics.

SULFUR MUSTARD: A CENTURY OF THREAT

Sulfur mustard (SM) or mustard gas (bis-2-(chloroethyl) sulfide, military code: HD) is a chemical warfare agent classified as a weapon of mass destruction. Mustard gas was one of the first chemical weapons deployed against troops on a battlefield during World War I, almost hundred years ago. Since then, the military use of mustard gas has been documented in a number of situations. In 1988, HD was used with devastating results

Figure 1. Distribution and accumulation of HD via circulation after dermal/inhalation exposure.

by Saddam Hussein's military forces against civilian targets in Halabja and later during the Iran-Iraq war. Mustard gas produces casualties in the battlefield and forces opposing troops to wear full protective equipment thus slowing the tempo of military operations. It is highly probable that mustard gas could be used by terrorists since it is a simple chemical compound readily synthesized without elaborate technology. Moreover, as a "persistent agent" (US Army classification) aerosolized mustard gas presents a threat for up to 1 week under dry and warm weather conditions because it remains in the environment until fully hydrolyzed. Along with nerve agents, mustard gas presents a major threat as a potential and effective chemical weapon. The possibility of low technology production, easy stockpiling, and difficulty in verifying its storage makes mustard gas a continuing worldwide threat. Presently, there is no antidote or effective treatment for mustard gas intoxication.

PATHOPHYSIOLOGY OF SULFUR MUSTARD ON SKIN

Clinical and physiological characteristics

Mustard gas is lethal in high doses and causes severe damage to the interface organs, that is, skin, lungs, respiratory tract, and eyes. The most prominent toxic effects of HD are on skin where it produces severe damage including extremely slow healing lesions and blisters which can ulcerate, vesicate, and promote secondary infections. Because of its hydrophobic nature, mustard gas easily penetrates and accumulates in the lipid component of exposed tissues. Upon contact with the skin, about 80% of HD evaporates and only about 20% is absorbed by the skin. Skin not only accumulates but also distributes HD to other tissues. Only about 10%–12% of the initially absorbed HD is retained in the skin, whereas up to 90% of HD enters circulation as indicated in Figure 1[1] Extractable skin reservoirs of HD

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can be found in the dermis and epidermis even 24 to 48 hours postexposure.[2] In the case of a lethal poisoning, HD concentration in skin blisters remains very high even 7 days after exposure.[3] Consequently, even after the initial exposure skin reservoirs continue to distribute HD via circulation to the body tissues thereby increasing damage to several organs. Figure 1 schematically shows the distribution pathway of HD toxicity throughout the human body. We would like to point that, although skin is the initial accumulator of HD, its toxic effect is also prominent in distal organs. Therefore, the effect of HD after dermal exposure is not limited only to skin tissues

While the epidermis contains no blood vessels, both the dermis and the subcutaneous regions are rich in blood vessels. Adipose cells in the subcutaneous skin layer are likely to be a depository for HD due to their high lipid content (as indicated in Figure 1). Moreover, HD soublized in adipose cells would be out of contact with water and thereby resistant to hydrolysis. After acute skin exposure, HD would be systemically delivered to various tissues in the body via lipid rich blood cell membranes and plasma lipoproteins and accumulate in lipid rich tissues (adipose tissues, brain, and skin). Chemical analyses following acute HD exposure show a high accumulation in thigh fat, brain, abdominal skin, kidney, and muscle tissues, in decreasing order.[3] In addition, HD can be found in the spleen, liver, and bone marrow.[4] The organs acquiring the most damage after dermal and/or respiratory exposure are indicated in Figure 1.

Skin damage caused by aerosolized HD appears after a latent period of up to 24 hours. First symptoms, such as itching, burning, and erythema, are followed by hyperpigmentation, tissue necrosis, and blister formation in warm moist areas of the body. When a large skin area is exposed to HD, medical conditions can be complicated by fluid imbalance, general inflammation, systemic intoxication, and secondary infection. At high doses, HD can also produce systemic effects with gastrointestinal symptoms (nausea and vomiting), respiratory distress due to the bronchospasm, temporary blindness as well as corneal damage. In most lethal cases, massive skin burns and wounds, as well as lung damage, are the primary causes of death. Since it damages DNA, mustard gas promotes mutagenesis and carcinogenesis.[1,5–7] Acute and severe exposures to HD have been shown to produce skin cancers.[8,9]

A few limited cases of HD exposure in humans provide some evidence for oxidative stress. HD metabolites derived from hydrolysis (thiodiglycol, thiodiglycol sulphoxide), as well as HD metabolites from glutathione (GSH) conjugates by the beta-lyase pathway, can be found in human urine after HD exposure.[10,11] Both thiodiglycol sulphoxide and beta-lyase metabolites can be detected indicating GSH conjugation. Thiodiglycol has also been detected in urine samples from individuals not exposed to HD and is therefore not useful as a definitive marker for HD exposure.[11] In contrast, HD metabolites from the glutathione (GSH)/beta-lyase pathway are specific for HD exposure.[10,11] These observations suggest that GSH depletion occurs in humans, and that GSH-HD/beta-lyase pathway metabolites provide a specific and useful biomarker for diagnosing HD exposure. GSH is a key intracellular antioxidant and its depletion by HD would be expected to increase oxidative stress.

The effects of HD in humans are very complicated and not fully elucidated. Figure 2 summarizes some of the key potential molecular mechanisms for HD toxicity in skin cells (as discussed in more detail below). Macromolecular damage and thiol depletion are primary and presumably the most dangerous intracellular events following HD exposure to skin
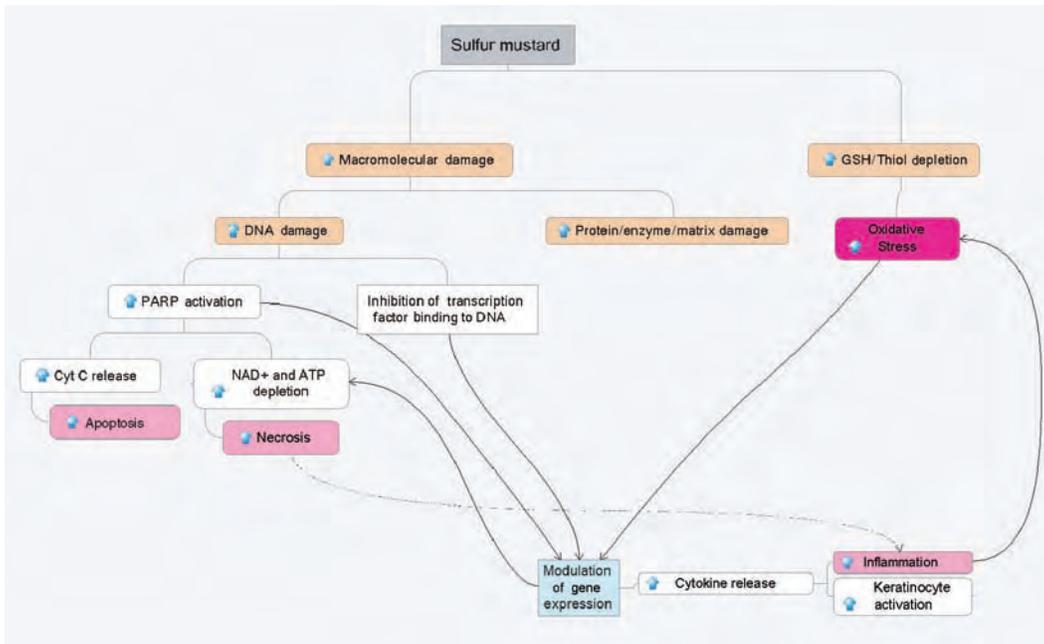
Figure 2. Schematic representation of the hypothesized molecular mechanisms of HD toxicity in skin cells.

cells. Macromolecular damage includes DNA damage as well as covalent modification of proteins and inactivation of enzymes. HD can affect cellular proteins both directly or indirectly by influencing expression and thereby altering the function of various enzymes, causing fragmentation of the extracellular matrix and cell detachment. GSH and total cellular thiol depletion is considered to be the major source of the oxidative stress. These primary damaging events modulate gene expression and induce inflammation and oxidative stress, which finally leads to apoptosis and/or necrosis (see Fig 2).

GENERAL COUNTERMEASURES

Presently, elimination of contact, decontamination, and supportive therapies are the only primary treatments for the vesicant exposure. Respirators and protective masks are effective in preventing inhalation, and special protective clothing can be used to eliminate skin exposure. Various decontaminating agents can eliminate or effectively reduce the toxic effect of HD if used immediately after the exposure. Ambergard XE-555 Resin reactive powder, hypochlorite neutralizing solutions, reactive skin lotions, and absorbent powders can be used to remove HD from human skin. Substantial HD reservoirs can be found in human skin even 24 hours after exposure.[12] These reservoirs can account for up to 35% of the total dose, and it is important, therefore, to develop decontaminating techniques capable of the removing such reservoirs thereby reducing further skin and systemic damage. Graham et al[13] have provided an excellent review of the strategies and current therapies for treating

cutaneous HD toxicity and promoting wound healing. An optimal therapeutic approach is, however, still lacking.

In this review, we will focus on the potential role of antioxidant therapy; we will review the data from in vivo and in vitro models, suggesting that oxidative stress is an important molecular mechanism underlying HD toxicity and that antioxidants can be therapeutically useful. The strengths and limitations of the in vivo and in vitro models will be detailed.

MUSTARD GAS/ANALOG-INDUCED OXIDATIVE STRESS IN ANIMAL MODELS AND THE EFFECTS OF ANTIOXIDANTS

Detailed information about HD toxicity to human skin, especially at the molecular level, is very limited. Animal models are, therefore, the major source of information about the pharmacokinetics and the molecular mechanisms of HD skin toxicity. Unfortunately, there is no animal model that exactly mimics the development of HD injury in human skin. Young swine and miniature swine skin are, however, considered to be the best models since they have a similar skin structure (epidermis, dermis, and subcutaneous tissue) and barrier function. Furred animals are poor models probably because their skin is not as well keratinized as human skin, thereby permitting more rapid penetration of drugs or toxins.

Despite limitations, the mouse ear model, the rabbit, the hairless guinea pig, the nude mouse, and the weanling swine have all been useful for studying the (1) pathophysiology, (2) molecular mechanism of action, and (3) efficacy of countermeasures for HD injury. Studies on the Yucatan mini-pig have demonstrated that laminin in the dermo-epithelial junction is a target for partial protease degradation following HD exposure. The protease cleavage of laminin networks may account for the blistering effect of HD.[14] The logistics of dealing with even miniature swine has, however, limited their use in HD studies.

In 2002, Naghii[15] reviewed much of the existing literature connecting HD toxicity and oxidative stress and suggested that further studies in animal models were well justified. Direct evidence for free radical formation in rat lung lavage following inhalation of HD vapor has been obtained by using electron paramagnetic resonance (EPR) and spin trapping techniques.[16] These studies show a rapid formation of ascorbyl radicals followed by the formation of carbon-centered radicals.[16]

Elsayed et al[17,18] have demonstrated that subcutaneous injections of either a butyl 2-chloroethyl sulfide (a monofunctional mustard analog) or 2-chloroethyl 4-chlorobutyl sulfide (a bifunctional mustard gas analog) in animal models caused an elevation in lung tissue lipid peroxidation as assayed by the thiobarbituric acid (TBA) assay. The TBA assay is, however, not very specific: rather then directly measuring levels of lipid hydroperoxide, this assay is generally considered a measure of total "thiobarbituric acid reactive substances" (TBARS). Total (GSH+GSSG) and oxidized (GSSG) glutathione contents in lung tissue were found to increase 1 hour and 24 hours after subcutaneous injection of butyl 2-chloroethyl sulfide.[18] Subcutaneous injection of 2-chloroethyl 4-chlorobutyl sulfide was associated with increased GSSG and decreased GSH at 1 hour postexposure. The increased formation of GSSG and TBARS in lung tissues following subcutaneous injection of mustard analogs is consistent with oxidative stress and suggests that dermal exposure can impact distal organs. This notion is supported by the work of Vijayaraghavan et al,[19] who found that dermally applied HD induces hepatic lipid peroxidation and GSH depletion in mice. In

this study, the generation of malondialdehyde (MDA) was used as an indirect measure of lipid peroxidation Vitamin E or flavonoids, while not influencing hepatic GSH depletion, did reduce MDA levels, suggesting a therapeutic potential.[19]

The effects of topically applied HD on key antioxidant enzymes has been measured but with conflicting results. For example, Husain et al[20] found that HD decreased the levels of glutathione peroxidase in white blood cells, spleen, and liver compared to control. Elsayed et al,[18] however, found an increased level of glutathione peroxidase compared to controls. Elsayed[17] interpreted the increased level of glutathione peroxidase (and other antioxidant enzymes) as an upregulation in response to oxidative stress, whereas Husain et al[20] interpreted the decreased levels of glutathione peroxidase as a potential cause of oxidative stress. Careful in vitro work with purified enzymes may help clarify these issues.

Despite the importance of skin itself as a primary target for HD toxicity, this organ has not been extensively studied with respect to oxidative stress. Yourick et al,[21] using the hairless guinea pig model, analyzed the skin NAD+ and NADP+ content as a function of time after HD exposure. Skin NAD+ content was found to decrease to a minimum after 16 hours (20% of control) whereas NADP+ levels increased (260%) between 1 and 2 hours and returned to control levels at 4 hours. This marked increase in NADP+ levels was thought to be an early marker of oxidative stress and a contributory factor for HD toxicity.[21] Increased NADP+ levels are a result of increased NADPH consumption: NADPH is a major source of reducing equivalents for key antioxidant enzymes such as glutathione reductase/peroxidase and thioredoxine reductase/peroxidase and lack of NADPH would be a source of oxidative stress.

The data present above support the view that diminished antioxidant protective mechanisms are a consequence of HD exposure. It is less clear, however, whether or not the resulting oxidative stress is a direct contributing factor to mustard toxicity or a secondary effect due to inflammation. In any event, the ability of exogenous antioxidants (as discussed below) to decrease HD toxicity supports the hypothesis that decreasing oxidative stress and/or inflammation is a viable therapeutic strategy.

Antioxidant protection in animal models

As early as 1985, work by Vojvodic et al[22] demonstrated that vitamin E was very effective in extending the survival time of rats acutely poisoned by HD. Vitamin E is, however, a generic term referring to at least 4 different tocopherols (alpha-, beta-, gamma-, and delta-) and 4 tocotrienols (alpha-, beta-, gamma-, and delta-). The particular form of vitamin E used in the Vojvodic et al experiments was not specified.[22] Vitamin E is generally considered to be the primary lipid soluble antioxidant but it is now recognized that vitamin E has important "nonantioxidant" roles in modulating various signal transduction and gene regulation pathways.[23–25] Moreover, the different chemical forms of vitamin E are now known to have distinct chemical and biological properties.[26,27] It is important, therefore, to specify the particular chemical and stereochemical form of vitamin E used in a given experiment.

Superoxide dismutase (SOD, EC 1.15.1.1) is a key antioxidant enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. Eldad et al[28] studied the therapeutic role of both Cu-Zn-SOD (cytosolic form) and Mn-SOD (mitochondrial form) in HD skin damage, using the Hartley guinea pig model. Pretreatment of the animals

by intraperitoneal injection with either form of SOD resulted in a dramatically reduced skin lesion area induced by HD.[28] Treatment with SOD was, however, not effective when given 1 hour after HD poisoning.[28] These data strongly suggest that superoxide radicals play a key role in HD-induced skin toxicity. Superoxide radicals alone are not a particularly damaging form of free radicals but they rapidly react with nitric oxide radicals to form peroxynitrite, which is a potent oxidant capable of causing tissue damage.[29-31]

HD and its analogs are alkylating agents that chemically react with and deplete biological thiols such as GSH, which is a key intracellular antioxidant. By promoting ROS generation and lipid peroxidation (as discussed above), HD will also promote the consumption of GSH and a reduced level of NADPH (see above) will inhibit the regeneration of GSH from GSSG. It is reasonable, therefore, that exogenous GSH or N-acetyl-L-cysteine (NAC) would help minimize oxidative stress induced by HD or its analogs. Kumar et al[32] tested the potential protective effect of GSH given to Swiss albino female mice following acute exposure to HD by either inhalation or percutaneous routes. GSH was administered by intraperitoneal injection and the dose was 400 mg/kg of body weight, which translates into about a 20 mM concentration in blood. Survival time following inhalation exposure to HD was increased by GSH administration as well as 2 other antioxidants: trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is a water-soluble derivative of alpha-tocopherol, and quercetin, which a flavonoid.[32] Inhalation exposure to HD depleted hepatic GSH levels, and increased hepatic and lung lipid peroxidation (as indirectly measured by MDA levels), and exogenous GSH was able to reduce lung and hepatic lipid peroxidation as well as prevent GSH depletion in these tissues.[32] None of the 3 antioxidants tested were able to significantly increase survival time following percutaneous exposure to HD but exogenous GSH was effective in preventing GSH depletion in blood and liver. Surprisingly, lung levels of GSH were not altered by percutaneous HD exposure.[32] The data present in work by Kumar et al[32] show that the potential effectiveness of antioxidant therapy is dependent on the route of HD exposure.

The role of GSH and NAC (and other antioxidants) in attenuating acute lung injury by 2-chloroethyl ethyl sulfide (CEES) has recently been studied in a rat model in which lung damage was quantitatively measured by the extravasation of.[125]-I-bovine serum albumin into the extravascular compartment.[33,34] CEES is a monofunctional analog of HD that has proven very useful in mimicking HD exposure. When the experimental animals were depleted of either complement or neutrophils prior to CEES exposure (by intrapulmonary injection) lung damage was significantly decreased.[34] Neutrophil depletion was accomplished by IP injection of rabbit anti-serum to rat polymorphonuclear neutrophils and complement depletion by IP injections cobra venom factor.[34] Antioxidants such as catalase, dimethyl sulfoxide, dimethyl urea, resveratrol, and NAC all provided significant protection in this animal model.[34] NAC (an acetylated form of L-cysteine) can directly function as free radical scavenger and its metabolites are capable of stimulating GSH synthesis.[35] NAC was found to be the most effective antioxidant among those tested[33,34] and was effective even when given up to 90 minutes after lung exposure to CEES.

In the work of McClintock et al,[33] NAC was superior to GSH. In vitro by Gross et al[36] found that pretreatment of human peripheral blood lymphocytes (PBL) with 10 mM NAC elevated GSH level to 122% of untreated control but caused only a partial protective effect on HD-induced cytotoxicity. These researches also noted work by Meister and **[AQ1]** Anderson, suggesting[37] that exogenously added GSH does not appear to enter the cell very

effectively. This may help explain why NAC is superior to GSH in the work by McClintock et al.[33]

Bhat et al[38] have studied the potential therapeutic use of lipoic acid to decrease oxidative stress and mustard gas toxicity in a rat model. Lipoic acid is a disulphide derivative of octanoic acid, and it is known to be a crucial prosthetic group for various cellular enzymatic complexes. Lipoic acid has been identified as a potent antioxidant and a potential therapeutic agent for the prevention or treatment of pathological conditions mediated via oxidative stress, as in the case of ischemia-reperfusion injury, diabetes, radiation injury, and oxidative damage of the central nervous system.[39-43] Lipoic acid is taken up and reduced by cells to dihydrolipoate, a more powerful antioxidant than the parent compound, which is also exported to the extracellular medium; hence, protection is affected in both extracellular and intracellular environments. Both lipoic acid and dihydrolipoate, in addition to their direct antioxidant properties, have been shown to regenerate, through redox cycling, other antioxidants such as vitamin C and vitamin E, and to raise intracellular glutathione levels.[44,45] Bhat et al[38] found that lipoic acid pretreatment decreased the levels of lipid peroxidation (measured as MDA) in lung, skin, and eyes in HD treated rats but was not effective posttreatment.

Antioxidant liposomes as a potential countermeasure

Antioxidant liposomes may represent an optimal means of treating HD-induced skin lesions. The authors' laboratory is currently testing this hypothesis. 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. Antioxidant liposomes hold great promise in the treatment of many diseases and conditions in which oxidative stress plays a prominent role.[46,47] The relative ease of incorporating hydrophilic and lipophilic therapeutic agents into liposomes; the possibility of directly delivering liposomes to an accessible body site; and the relative nonimmunogenicity and low toxicity of liposomes have rendered this system highly attractive for drug delivery. Moreover, several studies have clearly indicated that the liposomal antioxidant formulations, compared to that of the free nonencapsulated antioxidants, exert a far superior protective effect against oxidative stress-induced tissue injuries.[48]

Experimental studies have shown that liposomes and their constituents effectively penetrate skin.[49,50] Topical application of antioxidant-liposomes is likely, therefore, to be particularly effective in enhancing the antioxidant status of skin. Work by Kirjavainen et al[49] suggests that liposomes containing dioleylphosphatidyl ethanolamine (DOPE) are better able to penetrate into the stratum corneum than liposomes without DOPE. Similarly, ultradeformable liposomes, lipid vesicles with special membrane flexibility due to incorporation of an edge activator such as sodium cholate, have been shown to be superior in comparison to ordinary phosphatidylcholine liposomes (see http://www.skin-forum.org.uk/abstracts/ebtassam-essa.php).

At present there are no data on the potential use of antioxidant liposomes in treating HD-induced skin lesions but McClintock et al[33] have found that liposomes containing pegylated (PEG) catalase, PEG-SOD, or the combination were very effective in reducing

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CEES-induced lung injury in a rat model. Similarly, liposomes containing NAC, GSH, or resveratrol also were effective according to this study.

In vitro studies using human skin models

Keratinocyte cell lines

In vivo models are essential for testing countermeasures to HD or its analogs, however, in vitro models are also critical for rapid screening of potential therapeutic agents and for detailed studies at the molecular level. Skin is the largest organ of the human body with a complicated multilayer multicell type structure. As mentioned above, there is no model system perfectly mimicking human skin. Normal or immortalized human keratinocytes cultured on plastic as a monolayer represent the simplest and least inexpensive model and are suitable for an initial approach for HD toxicity studies. Normal human epidermal keratinocytes (NHEK) isolated from adult or infant fetal skin tissue are available commercially. These cells are easy to handle, can be frozen for long-term storage but require special medium containing a mixture of growth factors.[51] Even then, NHEK cells spontaneously transform after 3–5 passages as they continuously undergo terminal differentiation.

Nevertheless, NHEK remains the only commercially available normal cell line possessing all of the structural and functional features of normal skin keratinocytes and is being used by many investigators to study mustard gas toxicity.[52] However, the requirement of special growth medium and a short lifespan make this model more expensive than immortalized human keratinocytes such as human pappiloma virus (HPV)–immortalized cell lines or spontaneously immortalized HaCaT cells. There are also a number of commercially available human keratinocyte cell lines immortalized via transfection with DNA coding E6 and/or E7 viral oncoproteins. All of these cell lines still require special medium with growth factors and, like NHEK, have a limited lifespan since they spontaneously transform after 10 to 15 passages.[53]

The HaCaT cell line, originating in Germany, has recently become commercially available; it represents spontaneously immortalized adult human keratinocytes.[54] HaCaT cells are extremely easy to handle and do not require special medium. Theoretically, HaCaT cells have an unlimited lifespan but they do show morphological changes after 10 to 20 passages. Despite the altered growth potential, HaCaT cells still express differentiation-specific markers[54] and unlike HPV-immortalized cell lines, HaCaT cells are not tumorigenic when transplanted into nude mice.[54]

It is well known that HD, like UV radiation, affects mostly proliferating keratinocytes within the lower dermis and basement membrane. Differentiating keratinocytes of the epidermis are much less susceptible to toxicity since they do not undergo apoptosis and respond weakly to inflammatory stimuli. Normal keratinocytes undergo terminal differentiation (so-called "cornification") in response to a high (1 mM) exogenous Ca^{++} concentration.[51] Normal keratinocytes in vivo start to differentiate when they detach from the basement membrane and migrate to the suprabasal layers.[55] Thus, NHEK and HPV-immortalized keratinocytes, unlike HaCaT cells, spontaneously differentiate when subcultured in response to the cell detachment. Therefore, only the first passages of NHEK cells are truly proliferating, whereas every passage of HaCaT culture consists of proliferating cells. On the other hand, HaCaT cells show impaired production and release of IL-1[56] which is crucial for normal

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keratinocyte proliferation and also plays an important role in keratinocyte activation and keratinocyte/fibroblast crosstalk in normal skin.[57]

As previously acknowledged, HD-induced depletion of intracellular glutathione (GSH) is a triggering event for oxidative stress in skin. Smith et al[58] have shown that pretreatment of the human keratinocyte cell line, SVK-14, with GSH markedly increases the resistance to HD-induced cytotoxicity. Conversely, pretreatment with buthionine sulfoximine (BSO) increases the sensitivity of G361, SVK14, HaCaT, and NCTC 2544 human keratinocytes to HD toxicity.[59] BSO lowers intracellular GSH by irreversibly inhibiting the rate-limiting GSH synthesis enzyme γ-glutamylcysteine synthetase. Surprisingly, there is no reported direct evidence to date for the enhanced generation of ROS and/or RNOS in HD-treated keratinocytes.

As pointed out earlier, HD and its chemical analogs cause massive leukocyte infiltration in animal skin and lungs[60,61] It is likely that lymphocytes and macrophages, attracted to the burned area by cytokines released from keratinocytes/fibroblasts, could be a major source of oxidative stress to skin cells. It has been demonstrated that HD-exposed NHEK cells express chemoattractants such as TNF-α, IL-1β, IL-8, and GM-CSF.[62-64] Moreover, an enhanced ability of NHEK cells to attract lymphocytes in vitro was demonstrated in an experiment in which the media from HD-treated keratinocytes was tested for chemoattractant activity to polymorphonuclear leukocytes purified from human blood.[65]

Multilayer keratinocyte tissues

Multilayer skin tissues (so-called "3D skin models") are a more realistic model for toxicological studies. The simplest models of this class consist only of keratinocytes such as the commercially available Epiderm, which is a few millimeters thick structure of human NHEK cells grown on top of a wet membrane. Epiderm provides the possibility of applying HD (or other gaseous agents) in vapor or aerosol form which closely simulates a real HD attack. However, this model represents differentiating keratinocytes on a collagen matrix and practically all of the cells within the tissue start to cornify at the moment they are fully grown. Blaha et al[66-68] have characterized the ultrastructural, histological, and molecular response of the Epiderm model to CEES. The Epiderm system not only has great potential for identifying and developing sulfur mustard therapeutic agents but also has limitations. In vivo, skin damage would be accompanied by the rapid leakage of serum, leukocyte infiltration, and perhaps mast cell degranulation (see below) but these events will not occur in any of the available in vitro skin models.

More advanced tissue models, like EpidermFT full thickness skin tissue model, consist of 2 cell types: a bottom layer of human fibroblasts imbedded in gelatin and an upper multilayer of human keratinocytes. This particular model is particularly valuable for studies involving paracrine signaling (keratinocyte/fibroblast interactions). HaCaT cells, with normal human or mouse fibroblasts, have also been used to construct 3D models of human skin. However, the impaired IL-1 production in these cells presents some technical difficulties that can be overcome with the addition of human growth factors.[56] These multilayer skin models morphologically mimic the dermis and epidermis of human skin including the cuboidal appearance of the basal cell layer, the presence of the stratum spinosum and stratum granulosum with typical stellate-shaped keratohyalin granules, and the presence of numerous lamellar bodies that are extruded at the stratum granulosum–stratum corneum interface.

In a key experiment, Blaha et al[67] compared the effects of CEES on the secretion of key inflammatory mediators using 2 model human skin systems, the Epiderm system (from MatTek Corporation) and the Skin2 system (a 3D skin model) from Advanced Tissue Sciences, which consists of differentiating keratinocytes on a fibroblast-collagen matrix. In the Skin2 system, the proinflammatory cytokine IL-1alpha increased in response to CEES but the proinflammatory cytokine IL-6 decreased: the Epiderm showed undetectable levels of IL-6 and the levels of IL-1alpha did not change in response to CEES.[67] These data show that the presence of fibroblasts in the Skin2 model dramatically changes the cytokine secretion response to CEES.

More recently, Hayden et al[69] evaluated the effects of HD on the EpiDermFT skin model which has a 3D, highly differentiated human skin-like structure with an epidermis and a dermis. This in vitro model permits the study of dermal phenomena in which fibroblast-keratinocyte cell interactions are important as appears to be the case for CEES-induced skin injury (see above). Hayden et al[69] treated the EpiDerm-FT model with HD for 8 minutes and evaluated the structural effects at 6 and 12 hours postexposure. Histological analyses showed typical HD targeting of basal keratinocytes (cytopathology, condensed chromatin, pyknotic nuclei, and increased eosinophilia) and epidermal cleavage at the dermal/epidermal junction. Transmission electron microscopy showed that lamina densa of the basement membrane to be largely intact. The EpiDerm-FT model represents a major advance in the development of human skin models and its use in studying the molecular mechanisms/proteomics for HD/CEES toxicity is just beginning to be exploited.

Human skin allografts in immunodeficient mice

A third class of human skin model is provided by the use of human skin allografts in immunodeficient mice. Human skin cells, either genetically modified[70] or normal,[71] were grafted onto nude mice and successfully used to examine HD-induced biochemical alterations in skin. In 1995, Rosenthal et al[70] described an engineered human skin model, in which human keratinocyte clones, with some genetic modifications, were grafted onto nude mice, where they formed histologically normal human skin. Later, the same group reported an advanced model developed in immunodeficient nude mice, where a pellet of cells containing human keratinocytes and fibroblasts were placed on top of the muscular layer at the graft site and grown for 1 week.[71] Glass bulbs filled with HD can be directly applied to the sections of mouse skin containing the human skin allograft. Although these in vivo models are expensive and complicated, they possess a number of advantages over any of the in vitro cultured skin models. Grafted human skin models make it possible to obtain a detailed picture of HD-induced morphological, ultrastructural, and inflammatory alterations in various layers of skin cells possessing the realistic complexity of multiple cell–cell interactions. Recently, 3D human skin allografts in mice have allowed investigators to identify distinctive pre-vesication and postvesication phases and to monitor both dermal-epidermal separation and basal membrane alterations in response to HD exposure.[72,73] However, a limitation of this model is the lack of a functional immune response in the recipient mice.

In spite of the ever higher degrees of physiological complexity, there is not a single model that reflects all the features of human skin. The choice of a particular model may, therefore, be dictated by the particular experimental design and goals. Wound healing studies, for example, would require an in vivo system with an intact immune system since

immune cells are known to contribute to skin regeneration. Moreover, immune cells are thought to play important roles both in HD-induced skin inflammation and in postexposure wound healing through the expression of proinflammatory cytokines (such as Il-1β, TNF-α, IL-6, and GM-CSF) within the first hour after exposure and proceeding through vesication and blister formation.[64] Leukocyte infiltration always starts shortly after HD treatment in mice, rabbits, or guinea pigs. In wound healing, leukocytes and macrophages provide many of the molecular signals regulating fibroblast and keratinocyte proliferation.

The effects of HD on all of the various cell types that come in contact with skin, including macrophages and mast cells, are also important in understanding the overall effects of HD on skin in vivo. Rikimaru et al[74] have used full-thickness human skin explants to study inflammatory mediators in response to topically applied HD. These investigators found that culture fluids from the HD-treated skin contained increased levels of histamine, plasminogen-activating activity, and prostaglandin E2 compared to control explants. It was concluded that both mast cells and epidermal cells were apparently involved in early mediation of the inflammatory response to HD.[74] In contrast, Inoue et al[75] found that the inflammatory response of the mouse ear to HD did not differ in mast cell deficient mice compared to normal mice. At present, there is no obvious explanation for the differences observed between the work of Rikimaru et al[74] and that of Inoue et al[75] It may well be that the mouse ear is not an optimal model for human skin. It is critically important to determine whether HD, or other toxic vesicants, degranulate mast cells since this process could be a major source of inflammatory mediators and, therefore, a major factor in modulating the immune response to HD. In particular, mast cell degranulation would release large amounts of TNF-alpha, which is an inflammatory cytokine.

We have previously reported that lipopolysaccharide (LPS) as well as other inflammatory factors such as TNF-alpha and IL-1-beta amplify the toxicity of CEES[76] and that CEES is a potent inhibitor of nitric oxide production from inducible nitric oxide synthase (iNOS).[77] LPS is a major component of the cell wall of gram-negative bacteria and is known to trigger a variety of inflammatory reactions in macrophages and other cells having CD14 receptors.[78,79] In particular, LPS is known to stimulate the macrophage secretion of nitric oxide[80] and inflammatory cytokines such as tumor TNF-alpha and IL-1-beta[81] Figure 3 shows that RAW 264.7 macrophages stimulated with LPS at 100 ng/mL are markedly more susceptible ($P < .05$) to CEES cytotoxicity (24 hours with 500 μM) than resting macrophages as indicated by a dramatic drop in dehydrogenase activity as measured by the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In the absence of LPS, CEES at a level of 500 μM did not significantly affect cell viability.[76] Figure 4 shows that CEES (100–500 μM for 24 hours) inhibits the secretion of nitric oxide into the cell medium by LPS stimulated macrophages in a dose-dependent manner[77] In these experiments, nitrite secretion into the cell culture medium was used as a measure of nitric oxide synthesis. Macrophages (and mast cells) are both present in dermal tissues.

IgE-mediated mast cell degranulation is known to be inhibited by nitric oxide production.[82] NO is a powerful antioxidant[83] and increased intracellular levels of NO are known to inhibit mast cell degranulation.[84] Significantly, mast cell degranulation and histamine release are stimulated by membrane lipid peroxidation and inhibited by antioxidants such as alpha-tocopherol.[85] Collectively, the information presented above suggests that HD/CEES could induce mast cell degranulation by increasing oxidative stress and/or
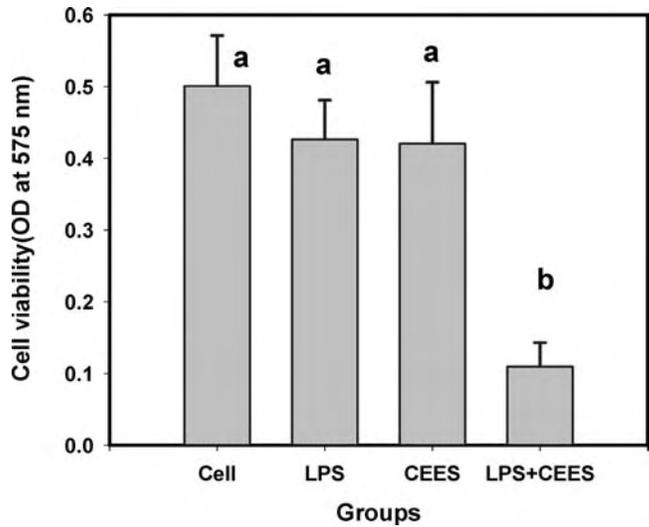
Figure 3. LPS (100 ng/mL) enhances the cytotoxicity of CEES (500 μM). Means not sharing a common letter are significantly different ($P < .05$). Cytotoxicity was measured after 24 hours by the MTT assay.
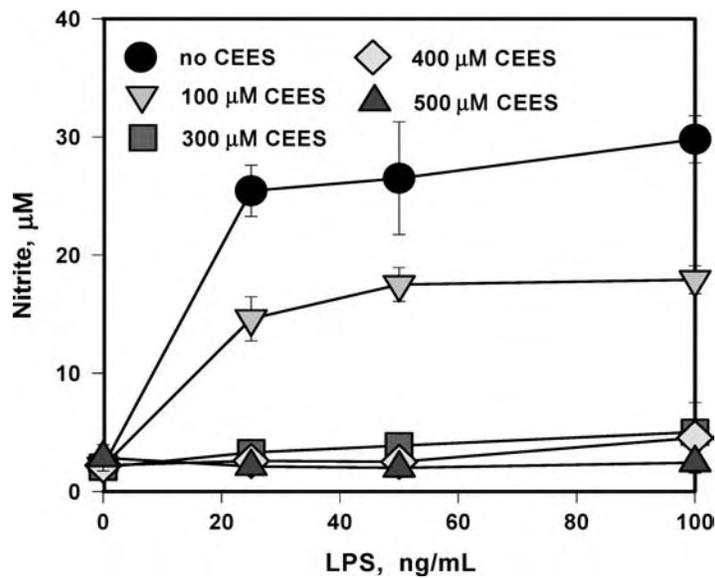
Figure 4. CEES inhibits NO production in LPS stimulated RAW 264.7 macrophages. Cells were simultaneously treated with various levels of CEES (as indicated) and low doses of LPS (as indicated). NO production was monitored as the concentration of nitrite in the culture media after 24 hours.

decreasing nitric oxide production. The subsequent release of TNF-alpha could enhance the cellular toxicity of HD/CEES.

NO generation, mediated by iNOS, is also crucial for the rapid healing of human skin wounds. Although, keratinocytes are known to express iNOS and generate NO in wound healing, it is likely that macrophages, known for their ability to express iNOS and generate high levels of NO, also contribute to the healing stimuli. Thus, it is tempting to suggest that the development of a 3-cell-type (macrophages/fibroblasts/keratinocytes) model would provide a unique and optimal model for studying skin vesication, blistering, and wound healing under very reproducible experimental conditions.

MOLECULAR MECHANISMS FOR MUSTARD TOXICITY

The molecular mechanisms of HD skin toxicity are complex and not yet fully understood. We will focus on 3 major types of interrelated events: primary macromolecule damage, oxidative stress, and inflammation. All of these processes are tightly interconnected and play central roles in HD toxicity. The hypothesized mechanisms of HD toxic effect in skin cells are summarized in Figure 2. In addition, we will discuss the importance of NO signaling modulation in HD toxicity since it is likely to be important for the postexposure wound healing process in skin.

Primary macromolecule damage

HD easily penetrates both cellular and nuclear membranes due to its hydrophobic nature. In the cytosol, it reacts with water forming a highly electrophilic ethylene episulfonium derivative that is the ultimate alkylating agent. DNA alkylation and crosslinking are well-documented primary intracellular damaging reactions of HD. Extensive DNA damage, due to alkylating agents, activates and overloads the DNA repair machinery. In particular, DNA damage induces expression of poly (ADP-ribose) polymerase (PARP), the key regulatory enzyme involved in DNA repair and hypothesized to regulate cell fate by modulating death and survival transcriptional programs.[86] HD induces PARP expression in normal human keratinocytes and the possible involvement of this nuclear enzyme in the regulation of HD cell death mechanisms has been extensively studied.[87–91]

PARP-1 is the most abundant member of the of PARP protein family. PARP-1 binds to DNA structures that have single- and double-strand breaks, crossovers, cruciforms, or supercoils[92]; it signals DNA rupture and facilitates base-excision repair.[93,94] Normally, the intracellular level of PARP-1 is very low, and this enzyme can be detected in the cytosol only under stressful conditions. Upon binding to the damaged DNA sites, PARP-1 metabolizes β-nicotinamide adenine dinucleotide (NAD^+) into branched polymers of ADP-ribose that are transferred to a set of nuclear proteins. This process also results in a very large decrease in the pyridine nucleotide pool. Poly(ADP-ribosyl)ation is thought to be beneficial for genome repair since modifications of proteins proximal to the DNA breaks facilitate multiple local openings of the condensed chromatin structure allowing the binding of the repair protein complex.[93,95]

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Despite the beneficial effect, PARP can induce apoptosis or necrosis in skin cells treated with HD[91] or other alkylating agents.[86,96] Thus, limited expression of PARP proteins helps in DNA repair and promotes cell survival but its overexpression (as in case of massive DNA damage) can induce cell death.[97] PARP overproduction, especially in cells utilizing aerobic glycolysis, can lead to the depletion of cellular NAD^+ and ATP (see Fig 2) which rapidly promotes general intracellular bioenergetic collapse and oxidative stress resulting in a regulated form of necrosis.[86,96,98–100] HD is cytotoxic to both dermal fibroblasts and epidermal keratinocytes. It has been suggested that PARP determines the mode of HD-induced cell death in skin fibroblast but not in keratinocytes.[91] In mouse skin fibroblast the absence of PARP shifts the mode of HD-induced cell death shifts from necrosis to apoptosis,[91] whereas keratinocytes, with or without PARP, primarily express an apoptotic form of cell death.[91] HD-treated human keratinocytes show a PARP activation, an upregulation of proapoptotic p53 accompanied by a downregulation of antiapoptotic Bcl-2, and, finally, to caspase activation and apoptosis.[87,90] This pathway was found to be Ca^{++} and calmodulin dependent.[90]

Necrosis due to PARP-induced depletion of NAD^+ and ATP exhaustion during aerobic glycolysis is thought to be the main mechanism of cell death induced by DNA damaging agents, especially in proliferating cells.[96] However, these observations may vary with the dose of alkylating agent, with cell type and perhaps the particular composition of the culture medium (see below) in the case of in vitro studies. HD promotes apoptosis in HeLa cells (10–100 μM),[101] peripheral blood lymphocytes (6–300 μM),[102] keratinocytes (50–300 μM),[53,87] and endothelial cells (<250 μM).[103] A time-dependent shift to necrosis was observed in HD-treated lymphocytes.[102] but a shift toward necrosis is observed at higher levels of HD in endothelial cells (>500 μM)[103] and HeLa (1 mM)[101] Interestingly, human fibroblasts undergo necrosis even at lower concentrations of HD (100–500 μM).[91] In most human cell types, apoptosis predominates within 6–12 hours of postexposure time, whereas necrotic events markedly increase after 12–24 hours.

Countermeasures capable of preventing rapid ATP depletion and mitochondrial dysfunction could be protective against HD-induced necrosis. Unfortunately, this approach would not eliminate cell death completely since apoptosis would likely proceed. However, a shift from necrosis to the less inflammatory apoptotic pathway could possibly be beneficial by helping eliminate secondary infections and improving wound healing. PARP activation causes NAD^+ depletion and NAD+ is required for glycolysis and pyruvate synthesis.[104] In the absence of pyruvate, mitochondrial respiration fails causing bioenergetic collapse and cell death via necrosis.[86] Therefore, the addition of a mitochondria substrate, such as pyruvate, glutamate, or glutamine, to the cell medium could be protective against necrosis.[104] A protective effect of pyruvate treatment has, indeed, been documented in genotoxic stress caused by nitrogen mustard or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a chemical analog of HD used as anticancer drugs.[96,100,104]

Alkyl pyruvates, such as methyl pyruvate and ethyl pyruvate, are excellent alternative mitochondrial substrates since they (unlike pyruvate) are stable in solution. In aqueous solutions, pyruvate spontaneously undergoes a series of chemical reactions yielding 2, 4-dihydroxy-2-methylglutarate, which is a mitochondrial poison.[105] In addition, alkyl pyruvates also function as effective and potent scavengers of free radicals. Pyruvates are capable of scavenging hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH&rad;$^-$).[106,107] Administration of pyruvates was shown to protect against various types of oxidant-mediated

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cellular and organ injuries in numerous in vitro and in vivo studies.[108,109] These data further suggest that HD activation of PARP and the subsequent depletion of pyruvate is also a contributing factor for HD-induced oxidative stress.

In preliminary results, the authors' laboratory has observed that methyl pyruvate provides protection to human keratinocytes (HaCaT cell line) treated with CEES and a similar effect was observed with ethyl pyruvate (unpublished data). It is worth noting that commercially available serum-free media, formulated to culture NHEK cells, contains 0.5 mM sodium pyruvate. Keratinocyte media from Gibco, Sigma, and Cambrex all contain 0.5 mM pyruvate. It is possible, therefore, that necrosis has not been observed in HD-treated NHEK cells due to the protective effect of sodium pyruvate in the culture media.

In our experiments (unpublished data), however, we used immortalized HaCaT keratinocytes, which proliferate continuously but do not differentiate. Actively proliferating cells utilizes aerobic glycolysis and are more susceptible to mitochondrial dysfunction and necrosis.[86,96,100] After limited number of passages NHEK cells, unlike HaCaT cells, undergo terminal differentiation which is a form of cell death different from either apoptosis or necrosis.[55] This difference between HaCaT and NHEK cells theoretically could cause discrepancies in the cell death pathway caused by HD. Parallel experiments are now being done with NHEK and HaCaT cells to further characterize the protective effect of pyruvate to HD/CEES.

Inflammation

HD-treated normal human keratinocytes release proinflammatory TNF-α, IL-6, IL-1β in a dose-dependent manner[62] but the particular cytokine profiles observed differ depending on the skin model used and the dose of HD.[63,64,110] Cytokine production and responses are known to be regulated by the activation of nuclear transcription factor-kappaB (NF-kappaB) and this activation also plays a key role in determining the fate of a damaged cell. There are numerous activators of NF-kappaB such as bacterial and viral infections, chemical damage, radiation, and oxidative stress. In response to these stimuli, an active NF-kappaB protein complex is liberated in the cytoplasm and it subsequently translocates to the nucleus and triggers selective gene expression. Among the genes regulated by NF-kappaB are adhesion molecules, pro-inflammatory mediators (IL-1 beta, TNF-alpha, interleukin 6), chemokines, IL-8, iNOS, E-selectin, vascular cell adhesion molecule 1 (ICAM-1), and granulocyte-macrophage colony stimulating factor (GM-CSF).[34,111–114] In general, NF-kappaB activation also triggers antiapoptotic genes and promotes cell survival.

Although the precise mechanism(s) of HD-induced gene expression has not yet been fully described in skin cells, it is very likely connected to the DNA damaging effect of HD (see Fig 2) and could, therefore, be PARP-dependent. PARP-1 is known to be a coactivator of NF-kappaB.[115]; however, this pathway has not been fully explored in HD-treated keratinocytes or fibroblasts. It is also possible that HD modulates NF-kappaB and other nuclear factors by covalently modifying DNA binding sequences for transcription factors. Grey et al[116] have shown that HD inhibits the in vitro binding of transcription factor activating protein-2 (AP-2) via alkylating the AP-2 DNA consensus binding sequence rather than by direct damage to the AP-2 protein.

In addition, it is highly possible that HD-induced oxidative stress also can stimulate inflammatory responses via transcription factors. Many of the activators of NF-kappaB can

be blocked with the use of antioxidants.[117,118] Transcription factors AP-1,[119,120] MAF and NRL,[121,122] and NF-IL6[123,124] are regulated by oxygen-dependent mechanisms, and sensitive to ROS. Interestingly, chemical compounds indirectly disrupting NF-kappaB activation induce apoptosis in cancer cells,[125–130] whereas inhibitors of NF-kappaB activation protect HD-treated human keratinocytes.[131,132]

It is also unclear how exactly the proinflammatory cytokines, such as IL-6, contribute to the HD-induced skin damage. It is known, however, that inflammatory processes contribute to the skin damage. In animal models, both skin and lung exposure to HD or CEES causes massive leukocyte infiltration, which starts shortly after the exposure and builds up continuously.[60,61] The fact that skin burns and blistering have a latent period also suggests that secondary responses in skin cells/immune cells also contribute to the mechanisms of HD toxicity. HD treatment is known to induce NF-kappaB activation and release of inflammatory cytokines in both keratinocytes and macrophages.[63,110,133–135] TNF-alpha, in general, induces apoptosis in keratinocytes and treatment with anti-TNF-alpha antibodies is protective against UV-induced skin lesions.[136] However, the effect of TNF-alpha in HD-treated skin is complex and an attempt to reduce cell death in normal human keratinocytes by blocking TNFR1, the major cell receptor for TNH-alphas was not successful.[71]

It is likely that keratinocyte activation (see Fig 2) plays an important role in HD toxicity. Activation of keratinocytes is a multistep pathway induced in response to skin injury. Activated keratinocytes are hyperproliferative and able to migrate to the site of injury in order to form layers of fresh cells in the dermis and epidermis. Activation is regulated by various cell signaling pathways including TNF-alpha.[57,137] Since HD-treated keratinocytes release high levels of TNF-alpha in the medium,[133] we suggest that HD promotes keratinocyte activation, subsequent hyperproliferation, and an enhanced susceptibility to the PARP-mediated bioenergetic collapse. This series of molecular events would cause a shift from apoptosis to necrosis. Although a time- or concentration-dependent shift from apoptosis to necrosis has been well documented for HD-treated lymphocytes,[102] endothelial cells,[103] and HeLa cells,[101] such changes have not been noted for human keratinocytes. As discussed above, this may be due to the fact that NHEK cells are always protected from necrosis by pyruvate-containing media. The recently documented protective effect of NF-kappaB inhibitors in NHEK and HaCaT cells treated with HD[131,132] also supports our speculation since these inhibitors downregulate TNF-alpha which would impair keratinocyte activation.

WOUND HEALING

NO signaling plays a key role in the inflammation and wound healing.[138–140] Animal studies[141] have shown that in iNOS knockout mice, wound healing is impaired but restored by iNOS gene transfer. Lack of NO and impaired expression of iNOS after the HD exposure are thought to be important events promoting skin burns and blistering. We have shown that HD treatment inhibits iNOS expression and NO synthesis (see Fig 4) in LPS-stimulated murine macrophages.[77] Suppression of iNOS expression and several protein activators of wound healing have also been found in human keratinocytes treated with HD.[142]

In keratinocytes, the beginning stage of the wound healing process is determined by the activation process. Under conditions of physical injury, the keratinocyte cell cycle is activated and the cells become hyperproliferative and migrate to the site of injury in response to chemokines.[57,137] Activation of keratinocytes, as well as their return to the healthy basal phenotype, is controlled by cytokines and growth factors produced by various cutaneous cell types, including keratinocytes and lymphocytes infiltrated at the wound site. Various intracellular signaling pathways are involved at the different stages of activation. Interestingly, NF-kappaB activation and consequent autocrine TNF-alpha production occur at the initial stages of the activation and allow keratinocytes to become hyperproliferative and migratory.[57,137] Activation is terminated when lymphocytes, present at the wound site, release interferon-gamma (IFN-gamma), which induces the activation of STAT-1 and makes keratinocytes contract newly deposit fibronectin-rich basement membrane. Finally, transforming growth factor-beta (TGF-beta) secreted by fibroblasts induces the expression of K5 and K14, fully reverting the keratinocytes to a healthy basal phenotype and making them responsive to differentiation stimuli.[57]

In human cells, expression of iNOS, which is the main NO-generating protein in keratinocytes,[143] is regulated synergistically by 2 major pathways: NF-kappaB and STAT-1.[144] As pointed above, both HD and its chemical analog CEES downregulate iNOS expression in murine macrophages[77] and human keratinocytes.[142] Since NF-kappaB activation is well documented in NHEK cells,[131–133] it is possible that the impaired expression of iNOS in HD-treated cells could be attributed to a STAT-1-dependent mechanisms. In addition, the possible STAT-1 inhibition by HD could disrupt the IFN-gamma signaling pathway resulting in keratinocytes unable to terminate their wound healing state.[57] Thus, simultaneous HD-induced NF-kappaB activation and STAT-1 inhibition could alter necrosis, inhibit NO generation, prevent wound healing, and possibly affect vesication and blistering.

The molecular mechanisms whereby HD alters transcription factors activation are not fully elucidated. However, it is highly possible that the ability of mustards to alkylate DNA is involved. As pointed above, HD is capable of chemically modifying both proteins (via crosslinking of Cys residues) and DNA (via alkylation of guanine rich sequences, and crosslinking). It seems likely that HD would more effectively damage "more exposed" regions of DNA with accessibility to transcription factors. Interestingly, Gray[116] had shown that HD inhibits the in vitro binding of transcription factor AP-2 via alkylation of the guanine-rich consensus DNA sequences but not by directly damaging the AP-2 protein. It is tempting to assume that other transcription factor functions could be affected by HD in a similar manner. However, the effects of HD on NF-kappaB and STAT-1α have not been elucidated and AP-2 remains the only transcription factor studied in relation to HD toxicity.

Since some transcription factors are sensitive to ROS and to the redox state of the cell in general, it is likely that oxidative stress, inflammation, and NO signaling are tightly interconnected in skin and its dynamic responses to toxic agents. Soneja et al[145] have suggested that wound healing could be accelerated under the circumstances in which oxidative stress is minimized but NO production remains elevated. On the other hand, under conditions elevating oxidative stress, the toxicity of mustards can be greatly enhanced. For instance, the HD analog CEES shows much higher toxicity in cells stimulated by LPS, TNF-alpha, or IL-1beta, which enhance inflammation and oxidative stress.[76]

FUTURE DIRECTIONS

A systems biology approach to mustard toxicity

As discussed above, HD toxicity in skin results from a multistep complex mechanism involving a number of signaling cascades and various cell types. It is extremely difficult to follow each step in this mechanism even in a simple in vitro model. A systems biology approach would view HD toxicity as time-dependent disruption of an integrated and interacting network of genes, proteins, and biochemical reactions. This approach would emphasize integrating data obtained from transcriptomics, metabolomics, and proteomics with the purpose of constructing and validating a comprehensive model of HD toxicity. The computational tools for this task would include network mapping as well as correlation, logical, and kinetic modeling.[146] This comprehensive model would be the best way to address the question: "how relevant to the HD-induced cell death pathways are the direct chemical alterations caused by HD to various cellular proteins (oxidation, crosslinking, and fragmentation) and the indirect chemical protein alterations caused by ROS and RNOS?"

A transcriptomic approach to studying HD toxicity is already yielding useful results. In NHEK cells, DNA array techniques have been applied to studying HD-altered gene expression,[147,148] and mRNA differential display has been used to examine HD-induced transcriptional modulations in human epidermal keratinocytes.[149] Microarray analyses of gene expression in CEES- or HD-exposed mouse skin in vivo have also been accomplished.[147,150] These studies are providing a deeper insight into the mechanism of HD toxicity since they have identified a number of genes upregulated at the early (0.5–4 hours) and intermediate (24 hours) stages of postexposure. DNA array analyses are capable of providing crucial information regarding the changes in transcriptional activity in the cell and are useful in the search for "the key" signaling pathways involved in HD toxicity. These studies will help in the design of evermore effective countermeasures and help identify key biomarkers for therapeutic efficacy. Proteomic data on HD toxicity are currently very limited but this approach would complement the previously accumulated microarray data by helping identify all the key proteins involved in HD toxicity at different stages.

Collectively, the literature reviewed here supports the notion that oxidative stress, free radical damage to biomolecules, and alterations in redox sensitive signaling pathways are key factors in understanding vesicant toxicology. It is likely, therefore, that the newly emerging area of redox proteomics would be particularly useful in understanding HD damage to skin. Redox proteomics is focused on characterizing (1) the chemical modifications of specific proteins induced by ROS and RNOS; (2) alterations in specific proteins induced by changes in redox sensitive transcription factors; and (3) alterations in the function/structure of specific proteins caused by redox sensitive posttranslational modifications.[151–153] In this regard, small thiols, like GSH, are no longer viewed just as protective antioxidants but as redox regulators of proteins via glutathionylation or by oxidation of protein cysteine residue.[152] Redox proteomics is rapidly emerging as a very powerful tool for characterizing and identifying proteins based on their redox state.[153] This approach has recently been used to specifically identify oxidized proteins in Alzheimer's disease and this information has proven useful in identifying new therapeutic targets and in providing new molecular insights into disease etiology.[154]

Multicomponent antioxidant liposomes

HD, due to its hydrophobic nature, effectively penetrates deep into the skin and affects mostly proliferating cells within basement membrane, that is, the lowest layer of proliferating keratinocytes. These growing cells would be highly susceptible to the PARP-mediated bioenergetic collapse since they actively utilize aerobic glycolysis. HD is likely, therefore, to induce necrosis rather than apoptosis in these cells, which would subsequently promote severe inflammation, skin blistering, and vesication. Thus, it is critically important to provide fast and efficient delivery of the desired drugs to the deeper skin layers and liposomes hold promise in this regard. By encapsulating a lipid soluble thiol, antioxidant liposomes could also effectively diminish (by direct covalent reaction) the stores of HD in skin lipid depots. Although this review has emphasized antioxidants, there is practically no limit to the possible encapsulated agents that can be incorporated into liposomes and delivered to the skin cells. These agents could include PARP inhibitors, protease inhibitors, anti-inflammatory agents, and chemical or enzymatic antioxidants. Currently, we are testing novel formulations of multiagent antioxidant liposomes containing both antiapoptotic (NAC) and antinecrotic (ethyl pyruvate) agents. Liposomes can also be formulated with agents designed to accelerate wound healing such as epidermal growth factor, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor, keratinocyte growth factor, and fibroblast growth factor.

REFERENCES

1. Somani SM, Babu SR. Toxicodynamics of sulfur mustard. *Int J Clin Pharmacol Ther Toxicol*. 1989;27:419–435.
2. Sinclair DC. Disability produced by exposure of skin to mustard-gas vapour. *Br Med J*. 1950;1:346–349.
3. Drasch G, Kretschmer E, Kauert G, von Meyer L. Concentrations of mustard gas [bis(2-chloroethyl)sulfide] in the tissues of a victim of a vesicant exposure. *J Forensic Sci*. 1987;32:1788–1793.
4. Vijayaraghavan R, Kulkarni A, Pant SC, et al. Differential toxicity of sulfur mustard administered through percutaneous, subcutaneous, and oral routes. *Toxicol Appl Pharmacol*. 2005;202:180–188.
5. Papirmeister B, Gross CL, Meier HL, Petrali JP, Johnson JB. Molecular basis for mustard-induced vesication. *Fundam Appl Toxicol*. 1985;5:S134–S149.
6. Langenberg JP, van der Schans GP, Spruit HE, et al. Toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig. *Drug Chem Toxicol*. 1998;21(suppl 1):131–147.
7. Wormser U. Toxicology of mustard gas. *Trends Pharmacol Sci*. 1991;12:164–167.
8. Hay A. Effects on health of mustard gas. *Nature*. 1993;366:398.
9. Pearson GS. Veterans at risk: the health effects of mustard gas and lewisite. *Nature*. 1993;365:218.
10. Black RM, Read RW. Biological fate of sulphur mustard, 1,1′-thiobis(2-chloroethane): identification of beta-lyase metabolites and hydrolysis products in human urine. *Xenobiotica*. 1995;25:167–73.
11. Boyer AE, Ash D, Barr DB, et al. Quantitation of the sulfur mustard metabolites 1,1′-sulfonylbis[2-(methylthio)ethane] and thiodiglycol in urine using isotope-dilution Gas chromatography-tandem mass spectrometry. *J Anal Toxicol*. 2004;28:327–332.
12. Chilcott RP, Jenner J, Carrick W, Hotchkiss SA, Rice P. Human skin absorption of bis-2-(chloroethyl)sulphide (sulphur mustard) in vitro. *J Appl Toxicol*. 2000;20:349–355.
13. Graham JS, Chilcott RP, Rice P, Milner SM, Hurst CG, Maliner BI. Wound healing of cutaneous sulfur mustard injuries: strategies for the development of improved therapies. *J Burns Wounds*. 2005;4:e1.
14. Lindsay CD, Rice P. Changes in connective tissue macromolecular components of Yucatan mini-pig skin following application of sulphur mustard vapour. *Hum Exp Toxicol*. 1995;14:341–348.
15. Naghii MR. Sulfur mustard intoxication, oxidative stress, and antioxidants. *Mil Med*. 2002;167:573–575.

16. Anderson DR, Yourick JJ, Arroyo CM, Young GD, Harris LW. Use of EPR spin-trapping techniques to detect radicals from rat lung lavage fluid following sulfur mustard vapor exposure. *Med Defense Biosci Rev Proc*. 1993;1:113–121.

17. Elsayed NM, Omaye ST. Biochemical changes in mouse lung after subcutaneous injection of the sulfur mustard 2-chloroethyl 4-chlorobutyl sulfide. *Toxicol*. 2004;199:195–206.

18. Elsayed NM, Omaye ST, Klain GJ, Korte DW Jr. Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection. *Toxicol*. 1992;72:153–165.

19. Vijayaraghavan R, Sugendran K, Pant SC, Husain K, Malhotra RC. Dermal intoxication of mice with bis(2-chloroethyl)sulphide and the protective effect of flavonoids. *Toxicol*. 1991;69:35–42.

20. Husain K, Dube SN, Sugendran K, Singh R, Das Gupta S, Somani SM. Effect of topically applied sulphur mustard on antioxidant enzymes in blood cells and body tissues of rats. *J Appl Toxicol*. 1996;16:245–248.

21. Yourick JJ, Dawson JS, Benton CD, Craig ME, Mitcheltree LW. Pathogenesis of 2,2′-dichlorodiethyl sulfide in hairless guinea pigs. *Toxicol*. 1993;84:185–197.

22. Vojvodic V, Milosavljevic Z, Boskovic B, Bojanic N. The protective effect of different drugs in rats poisoned by sulfur and nitrogen mustards. *Fundam Appl Toxicol*. 1985;5:160–168.

23. Azzi A, Gysin R, Kempna P, et al. Vitamin E mediates cell signaling and regulation of gene expression. *Ann N Y Acad Sci*. 2004;1031:86–95.

24. Ricciarelli R, Zingg JM, Azzi A. Vitamin E: protective role of a Janus molecule. *Faseb J*. 2001;15:2314–2325.

25. Zingg JM, Azzi A. Non-antioxidant activities of vitamin E. *Curr Med Chem*. 2004;11:1113–1133.

26. Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. *Proc Natl Acad Sci U S A*. 1993;90:1771–1775.

27. Samandari E, Visarius T, Zingg JM, Azzi A. The effect of gamma-tocopherol on proliferation, integrin expression, adhesion, and migration of human glioma cells. *Biochem Biophys Res Commun*. 2006;342:1329–1333.

28. Eldad A, Ben Meir P, Breiterman S, Chaouat M, Shafran A, Ben-Bassat H. Superoxide dismutase (SOD) for mustard gas burns. *Burns*. 1998;24:114–119.

29. Alvarez MN, Trujillo M, Radi R. Peroxynitrite formation from biochemical and cellular fluxes of nitric oxide and superoxide. *Methods Enzymol*. 2002;359:353–366.

30. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. 1996;271:1424–1437.

31. Fukuyama N, Nakazawa H. [Superoxide, nitric oxide and peroxynitrite]. *Nippon Yakurigaku Zasshi*. 1998;112:169–176.

32. Kumar O, Sugendran K, Vijayaraghavan R. Protective effect of various antioxidants on the toxicity of sulphur mustard administered to mice by inhalation or percutaneous routes. *Chem Biol Interact*. 2001;134:1–12.

33. McClintock SD, Hoesel LM, Das SK, et al. Attenuation of half sulfur mustard gas-induced acute lung injury in rats. *J Appl Toxicol*. 2006;26:126–131.

34. McClintock SD, Till GO, Smith MG, Ward PA. Protection from half-mustard-gas-induced acute lung injury in the rat. *J Appl Toxicol*. 2002;22:257–262.

35. Kelly GS. Clinical applications of *N*-acetylcysteine. *Altern Med Rev*. 1998;3:114–127.

36. Gross CL, Innace JK, Hovatter RC, Meier HL, Smith WJ. Biochemical manipulation of intracellular glutathione levels influences cytotoxicity to isolated human lymphocytes by sulfur mustard. *Cell Biol Toxicol*. 1993;9:259–267.

37. Meister A, Anderson ME. Glutathione. *Annu Rev Biochem*. 1983;52:711–760.

38. Bhat S, Gulati S, Husain K, Milner SM. Lipoic acid decreases oxidative stress in sulphur mustard toxicity. Proceedings of American Burn Association, 38th Annual Meeting, S139. Las Vegas, Nevada: *J Burn Care Res*, 27(2, suppl):S139, 2006.

39. Bast A, Haenen GR. Lipoic acid: a multifunctional antioxidant. *Biofactors*. 2003;17:207–213.

40. Dincer Y, Telci A, Kayali R, Yilmaz IA, Cakatay U, Akcay T. Effect of alpha-lipoic acid on lipid peroxidation and anti-oxidant enzyme activities in diabetic rats. *Clin Exp Pharmacol Physiol*. 2002;29:281–284.

41. Ernst A, Stolzing A, Sandig G, Grune T. Antioxidants effectively prevent oxidation-induced protein damage in OLN 93 cells. *Arch Biochem Biophys*. 2004;421:54–60.

42. Maritim AC, Sanders RA, Watkins JB, III. Effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. *J Nutr Biochem*. 2003;14:288–294.

43. Wollin SD, Jones PJ. Alpha-lipoic acid and cardiovascular disease. *J Nutr*. 2003;133:3327–3330.

44. Busse E, Zimmer G, Schopohl B, Kornhuber B. Influence of alpha-lipoic acid on intracellular glutathione in vitro and in vivo. *Arzneimittelforschung*. 1992;42:829–831.

45. Han D, Handelman G, Marcocci L, et al. Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. *Biofactors*. 1997;6:321–338.

46. Stone WL, Mukherjee S, Smith M, Das SK. Therapeutic uses of antioxidant liposomes. *Methods Mol Biol*. 2002;199:145–161.

47. Stone WL, Smith M. Therapeutic uses of antioxidant liposomes. *Mol Biotechnol*. 2004;27:217–230.

48. Fan J, Shek PN, Suntres ZE, Li YH, Oreopoulos GD, Rotstein OD. Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome. *Surg*. 2000;128:332–338.

49. Kirjavainen M, Urtti A, Jaaskelainen I, et al. Interaction of liposomes with human skin in vitro—the influence of lipid composition and structure. *Biochim Biophys Acta*. 1996;1304:179–189.

50. Kirjavainen M, Urtti A, Valjakka-Koskela R, Kiesvaara J, Monkkonen J. Liposome-skin interactions and their effects on the skin permeation of drugs. *Eur J Pharm Sci*. 1999;7:279–286.

51. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol*. 1983;81:33s–40s.

52. Smith WJ, Gross CL, Chan P, Meier HL. The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. *Cell Biol Toxicol*. 1990;6:285–291.

53. Stoppler H, Stoppler MC, Johnson E, et al. The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. *Oncogene*. 1998;17:1207–1214.

54. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol*. 1988;106:761–771.

55. Lippens S, Denecker G, Ovaere P, Vandenabeele P, Declercq W. Death penalty for keratinocytes: apoptosis versus cornification. *Cell Death Differ*. 2005;12(suppl 2):1497–1508.

56. Maas-Szabowski N, Starker A, Fusenig NE. Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-alpha. *J Cell Sci*. 2003;116:2937–2948.

57. Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the keratinocyte activation cycle. *J Invest Dermatol*. 2001;116:633–640.

58. Smith CN, Lindsay CD, Upshall DG. Presence of methenamine/glutathione mixtures reduces the cytotoxic effect of sulphur mustard on cultured SVK-14 human keratinocytes in vitro. *Hum Exp Toxicol*. 1997;16:247–253.

59. Simpson R, Lindsay CD. Effect of sulphur mustard on human skin cell lines with differential agent sensitivity. *J Appl Toxicol*. 2005;25:115–128.

60. Wormser U, Sintov A, Brodsky B, Nyska A. Topical iodine preparation as therapy against sulfur mustard-induced skin lesions. *Toxicol Appl Pharmacol*. 2000;169:33–39.

61. Das SK, Mukherjee S, Smith MG, Chatterjee D. Prophylactic protection by *N*-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. *J Biochem Mol Toxicol*. 2003;17:177–184.

62. Arroyo CM, Schafer RJ, Kurt EM, Broomfield CA, Carmichael AJ. Response of normal human keratinocytes to sulfur mustard: cytokine release. *J Appl Toxicol. Jat*. 2000;20(suppl 1):S63–S72.

63. Ricketts KM, Santai CT, France JA, et al. Inflammatory cytokine response in sulfur mustard-exposed mouse skin. *J Appl Toxicol. Jat*. 2000;20(suppl 1):73–76.

64. Sabourin CL, Petrali JP, Casillas RP. Alterations in inflammatory cytokine gene expression in sulfur mustard-exposed mouse skin. *J Biochem Mol Toxicol*. 2000;14:291–302.

65. Vavra AK, Laurent CJ, Ngo V, Sweeney JF, Levitt JM. Sulfur mustard primes phagocytosis and degranulation in human polymorphonuclear leukocytes. *Int Immunopharmacol*. 2004;4:437–445.

66. Blaha M, Bowers W, Kohl J, DuBose D, Walker J. Il-1-related cytokine responses of nonimmune skin cells subjected to CEES exposure with and without potential vesicant antagonists. 2000;13:99–111.

67. Blaha M, Bowers W, Kohl J, et al. Effects of CEES on inflammatory mediators, heat shock protein 70A, histology and ultrastructure in two skin models. *J Appl Toxicol. Jat*. 2000;20(suppl 1):S101–S108.

VICTOR PAROMOV ET AL

68. Blaha M, Kohl J, DuBose D, Bowers W Jr, Walker J. Ultrastructural and histological effects of exposure to CEES or heat in a human epidermal model. *In Vitr Mol Toxicol*. 2001;14:15–23.

69. Hayden PJ, Petrali JP, Hamilton TA, Kubilus J, Smith WJ, Klausner M. Development of a full thickness in vitro human skin equivalent (EpiDerm-FT) for sulfur mustard research, SID Abstract #174. 2005 Society for Investigative Dermatology Annual Meeting, 2005.

70. Rosenthal DS, Shima TB, Celli G, De Luca LM, Smulson ME. Engineered human skin model using poly(ADP-ribose) polymerase antisense expression shows a reduced response to DNA damage. *J Invest Dermatol*. 1995;105:38–43.

71. Rosenthal DS, Velena A, Chou FP, et al. Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard. *J Biol Chem*. 2003;278:8531–8540.

72. Greenberg S, Margulis A, Garlick JA. In vivo transplantation of engineered human skin. *Methods Mol Biol*. 2005;289:425–430.

73. Greenberg S, Kamath P, Petrali J, Hamilton T, Garfield J, Garlick JA. Characterization of the initial response of engineered human skin to sulfur mustard. *Toxicol Sci*. 2006;90:549–557.

74. Rikimaru T, Nakamura M, Yano T, et al. Mediators, initiating the inflammatory response, released in organ culture by full-thickness human skin explants exposed to the irritant, sulfur mustard. *J Invest Dermatol*. 1991;96:888–897.

75. Inoue H, Asaka T, Nagata N, Koshihara Y. Mechanism of mustard oil-induced skin inflammation in mice. *Eur J Pharmacol*. 1997;333:231–240.

76. Stone WL, Qui M, Smith M. Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide. *BMC Cell Biol*. 2003;4:1.

77. Qui M, Paromov VM, Yang H, Smith M, Stone WL. Inhibition of inducible nitric oxide synthase by a mustard gas analog in murine macrophages. *BMC Cell Biol*. 2006;7:39.

78. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Sci*. 1990;249:1431–1433.

79. Downey JS, Han J. Cellular activation mechanisms in septic shock. *Front Biosci*. 1998;3:d468–d476.

80. Tepperman BL, Chang Q, Soper BD. Protein kinase C mediates lipopolysaccharide- and phorbol-induced nitric-oxide synthase activity and cellular injury in the rat colon. *J Pharmacol Exp Ther*. 2000;295:1249–1257.

81. Shapira L, Takashiba S, Champagne C, Amar S, Van Dyke TE. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-alpha and IL-1 beta production by human monocytes. *J Immunol*. 1994;153:1818–1824.

82. Eastmond NC, Banks EM, Coleman JW. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J Immunol*. 1997;159:1444–1450.

83. Wink DA, Miranda KM, Espey MG, et al. Mechanisms of the antioxidant effects of nitric oxide. *Antioxid Redox Signal*. 2001;3:203–213.

84. McCauley SD, Gilchrist M, Befus AD. Nitric oxide: a major determinant of mast cell phenotype and function. *Mem Inst Oswaldo Cruz*. 2005;100(suppl 1):11–14.

85. Masini E, Palmerani B, Gambassi F, et al. Histamine release from rat mast cells induced by metabolic activation of polyunsaturated fatty acids into free radicals. *Biochem Pharmacol*. 1990;39:879–889.

86. Chiarugi A. Poly(ADP-ribose) polymerase: killer or conspirator? The "suicide hypothesis" revisited. *Trends Pharmacol Sci*. 2002;23:122–129.

87. Rosenthal DS, Simbulan-Rosenthal CM, Iyer S, et al. Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and caspase-dependent pathway. *J Invest Dermatol*. 1998;111:64–71.

88. Hinshaw DB, Lodhi IJ, Hurley LL, Atkins KB, Dabrowska MI. Activation of poly [ADP-Ribose] polymerase in endothelial cells and keratinocytes: role in an in vitro model of sulfur mustard-mediated vesication. *Toxicol Appl Pharmacol*. 1999;156:17–29.

89. Bhat KR, Benton BJ, Rosenthal DS, Smulson ME, Ray R. Role of poly(ADP-ribose) polymerase (PARP) in DNA repair in sulfur mustard-exposed normal human epidermal keratinocytes (NHEK). *J Appl Toxicol*. 2000;20(suppl 1):S13–S17.

90. Rosenthal DS, Simbulan-Rosenthal CM, Iyer S, Smith WJ, Ray R, Smulson ME. Calmodulin, poly(ADP-ribose)polymerase and p53 are targets for modulating the effects of sulfur mustard. *J Appl Toxicol*. 2000;20(suppl 1):S43–S49.

91. Rosenthal DS, Simbulan-Rosenthal CM, Liu WF, et al. PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. *J Invest Dermatol*. 2001;117:1566–1573.

92. Kim MY, Zhang T, Kraus WL. Poly(ADP-ribosyl)ation by PARP-1: "PAR-laying" NAD+ into a nuclear signal. *Genes Dev*. 2005;19:1951–1967.

93. D'Amours D, Desnoyers S, D'Silva I, Poirier GG. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J*. 1999;342(Pt 2):249–268.

94. Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res*. 2001;477:97–110.

95. Shall S, de Murcia G. Poly(ADP-ribose) polymerase–1: what have we learned from the deficient mouse model? *Mutat Res*. 2000;460:1–15.

96. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev*. 2004;18:1272–1282.

97. Burkle A. PARP-1: a regulator of genomic stability linked with mammalian longevity. *Chembiochem*. 2001;2:725–728.

98. Bouchard VJ, Rouleau M, Poirier GG. PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol*. 2003;31:446–454.

99. Decker P, Muller S. Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Curr Pharm Biotechnol*. 2002;3:275–283.

100. Ying W, Alano CC, Garnier P, Swanson RA. NAD+ as a metabolic link between DNA damage and cell death. *J Neurosci Res*. 2005;79:216–223.

101. Sun J, Wang YX, Sun MJ. Apoptosis and necrosis induced by sulfur mustard in Hela cells. *Zhongguo Yao Li Xue Bao*. 1999;20:445–448.

102. Meier HL, Millard CB. Alterations in human lymphocyte DNA caused by sulfur mustard can be mitigated by selective inhibitors of poly(ADP-ribose) polymerase. *Biochim Biophys Acta*. 1998;1404:367–376.

103. Dabrowska MI, Becks LL, Lelli JL Jr, Levee MG, Hinshaw DB. Sulfur mustard induces apoptosis and necrosis in endothelial cells. *Toxicol Appl Pharmacol*. 1996;141:568–583.

104. Ying W, Chen Y, Alano CC, Swanson RA. Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *J Cereb Blood Flow Metab*. 2002;22:774–779.

105. Willems JL, de Kort AF, Vree TB, Trijbels JM, Veerkamp JH, Monnens LA. Non-enzymic conversion of pyruvate in aqueous solution to 2,4-dihydroxy-2-methylglutaric acid. *FEBS Lett*. 1978;86:42–44.

106. O'Donnell-Tormey J, Nathan CF, Lanks K, DeBoer CJ, de la Harpe J. Secretion of pyruvate. An antioxidant defense of mammalian cells. *J Exp Med*. 1987;165:500–514.

107. Dobsak P, Courderot-Masuyer C, Zeller M, et al. Antioxidative properties of pyruvate and protection of the ischemic rat heart during cardioplegia. *J Cardiovasc Pharmacol*. 1999;34:651–659.

108. Das UN. Pyruvate is an endogenous anti-inflammatory and anti-oxidant molecule. *Med Sci Monit*. 2006;12:RA79–RA84.

109. Mallet RT, Sun J, Knott EM, Sharma AB, Olivencia-Yurvati AH. Metabolic cardioprotection by pyruvate: recent progress. *Exp Biol Med (Maywood)*. 2005;230:435–443.

110. Sabourin CL, Danne MM, Buxton KL, Casillas RP, Schlager JJ. Cytokine, chemokine, and matrix metallo-proteinase response after sulfur mustard injury to weanling pig skin. *J Biochem Mol Toxicol*. 2002;16:263–272.

111. Akira S, Kishimoto T. NF-IL6 and NF-kappa B in cytokine gene regulation. *Adv Immunol*. 1997;65:1–46.

112. Brennan P, O'Neill LA. Effects of oxidants and antioxidants on nuclear factor kappa B activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochim Biophys Acta*. 1995;1260:167–175.

113. Barnes PJ, Adcock IM. Transcription factors and asthma. *Eur Respir J*. 1998;12:221–234.

114. Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax*. 1998;53:601–612.

115. Martin-Oliva D, O'Valle F, Munoz-Gamez JA, et al. Crosstalk between PARP-1 and NF-kappaB modulates the promotion of skin neoplasia. *Oncog*. 2004;23:5275–5283.

116. Gray PJ. Sulphur mustards inhibit binding of transcription factor AP2 in vitro. *Nucleic Acids Res*. 1995;23:4378–4382.

117. Schulze-Osthoff K, Bauer MK, Vogt M, Wesselborg S. Oxidative stress and signal transduction. *Int J Vitam Nutr Res*. 1997;67:336–342.

118. Schwager J, Schulze J. Influence of ascorbic acid on the response to mitogens and interleukin production of porcine lymphocytes. *Int J Vitam Nutr Res*. 1997;67:10–16.

119. Xanthoudakis S, Miao GG, Curran T. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc Natl Acad Sci U S A*. 1994;91:23–27.

120. Yao KS, Xanthoudakis S, Curran T, O'Dwyer PJ. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. *Mol Cell Biol*. 1994;14:5997–6003.

121. Kerppola TK, Curran T. A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. *Oncogene*. 1994;9:3149–3158.

122. Kerppola TK, Curran T. Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene*. 1994;9:675–684.

123. Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S. NF-IL6 and AP-1 cooperatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. *Mol Cell Biol*. 1994;14:6561–6569.

124. Hsu W, Kerppola TK, Chen PL, Curran T, Chen-Kiang S. Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. *Mol Cell Biol*. 1994;14:268–276.

125. Crispen PL, Uzzo RG, Golovine K, et al. Vitamin E succinate inhibits NF-kappaB and prevents the development of a metastatic phenotype in prostate cancer cells: implications for chemoprevention. *Prostate*. 2007. **[AQ2]**

126. Campbell SE, Stone WL, Lee S, et al. Comparative effects of RRR-alpha- and RRR-gamma-tocopherol on proliferation and apoptosis in human colon cancer cell lines. *BMC Cancer*. 2006;6:13.

127. Smirnov AS, Ruzov AS, Budanov AV, Prokhortchouk AV, Ivanov AV, Prokhortchouk EB. High constitutive level of NF-kappaB is crucial for viability of adenocarcinoma cells. *Cell Death Differ*. 2001;8:621–630.

128. Dhanalakshmi S, Singh RP, Agarwal C, Agarwal R. Silibinin inhibits constitutive and TNFalpha-induced activation of NF-kappaB and sensitizes human prostate carcinoma DU145 cells to TNFalpha-induced apoptosis. *Oncogene*. 2002;21:1759–1767.

129. Ma Z, Otsuyama K, Liu S, et al. Baicalein, a component of Scutellaria radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. *Blood*. 2005;105:3312–3318.

130. Taguchi T, Takao T, Iwasaki Y, Nishiyama M, Asaba K, Hashimoto K. Suppressive effects of dehydroepiandrosterone and the nuclear factor-kappaB inhibitor parthenolide on corticotroph tumor cell growth and function in vitro and in vivo. *J Endocrinol*. 2006;188:321–331.

131. Minsavage GD, Dillman 3d JF. Protective role of CAPE on bi-functional alkylating agent-induced toxicity in keratinocytes via modulation of NF-kappaB, p53 and ARE/EpRE signaling. *Biosci 2006 Med Defense Rev*, 2006;94–94. **[AQ3]**

132. Minsavage GD, Dillman Iii JF. Bifunctional alkylating agent-induced p53 and nonclassical nuclear factor-kappa b (nf-{kappa}b) responses and cell death are altered by caffeic acid phenethyl ester (cape): A potential role for antioxidant/electrophilic response element (ARE/EpRE) signaling. *J Pharmacol Exp Ther*. 2007. **[AQ4]**

133. Arroyo CM, Burman DL, Kahler DW, et al. TNF-alpha expression patterns as potential molecular biomarker for human skin cells exposed to vesicant chemical warfare agents: sulfur mustard (HD) and Lewisite (L). *Cell Biol Toxicol*. 2004;20:345–359.

134. Atkins KB, Lodhi IJ, Hurley LL, Hinshaw DB. *N*-acetylcysteine and endothelial cell injury by sulfur mustard. *J Appl Toxicol*. 2000;20(suppl 1):S125–S128.

135. Chatterjee D, Mukherjee S, Smith MG, Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog. *J Biochem Mol Toxicol*. 2003;17:114–121.

136. Duthie MS, Kimber I, Dearman RJ, Norval M. Differential effects of UVA1 and UVB radiation on Langerhans cell migration in mice. *J Photochem Photobiol B*. 2000;57:123–131.

137. Steffensen B, Hakkinen L, Larjava H. Proteolytic events of wound-healing–coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. *Crit Rev Oral Biol Med*. 2001;12:373–398.

138. Schwentker A, Billiar TR. Nitric oxide and wound repair. *Surg Clin North Am*. 2003;83:521–530.

139. Weller R. Nitric oxide: a key mediator in cutaneous physiology. *Clin Exp Dermatol*. 2003;28:511–514.

140. Witte MB, Barbul A. Role of nitric oxide in wound repair. *Am J Surg*. 2002;183:406–412.

141. Yamasaki K, Edington HD, McClosky C, et al. Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J Clin invest*. 1998;101:967–971.

142. Ishida H, Ray R, Cao Y, Ray P. Role of nitric oxide (NO) in wound healing. *Biosci 2006 Med Defense Rev.* 2006;1:112.

143. Arany I, Brysk MM, Brysk H, Tyring SK. Regulation of inducible nitric oxide s ynthase mRNA levels by differentiation and cytokines in human keratinocytes. *Biochem Biophys Res Commun*. 1996;220:618–622.

144. Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem*. 2003;384:1343–1364.

145. Soneja A, Drews M, Malinski T. Role of nitric oxide, nitroxidative and oxidative stress in wound healing. *Pharmacol Rep*. 2005;57(suppl):108–119.

146. Perkel JM. What can systems biology do for you? Four computational modeling strategies and the data that build them. *Scientist*. 2007;21:68.

147. Rogers JV, Choi YW, Kiser RC, et al. Microarray analysis of gene expression in murine skin exposed to sulfur mustard. *J Biochem Mol Toxicol*. 2004;18:289–299.

148. Sabourin CL, Rogers JV, Choi YW, et al. Time- and dose-dependent analysis of gene expression using microarrays in sulfur mustard-exposed mice. *J Biochem Mol Toxicol*. 2004;18:300–312.

149. Platteborze PL. Effects of sulfur mustard on transcription in human epidermal keratinocytes: analysis by mRNA differential display. *J Appl Toxicol*. 2003;23:249–254.

150. Dillman JF, III, Hege AI, Phillips CS, et al. Microarray analysis of mouse ear tissue exposed to bis-(2-chloroethyl) sulfide: gene expression profiles correlate with treatment efficacy and an established clinical endpoint. *J Pharmacol Exp Ther*. 2006;317:76–87.

[AQ5]

151. Sultana R, Boyd-Kimball D, Poon HF, et al. Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD. *Neurobiol Aging*. 2005.

152. Ghezzi P, Bonetto V, Fratelli M. Thiol-disulfide balance: from the concept of oxidative stress to that of redox regulation. *Antioxid Redox Signal*. 2005;7:964–972.

153. Ghezzi P, Bonetto V. Redox proteomics: identification of oxidatively modified proteins. *Proteomics*. 2003;3:1145–1153.

154. Butterfield DA. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res*. 2004;1000:1–7.

Author Queries

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The influence of N-acetyl-L-cysteine and polymyxin B on oxidative stress and nitric oxide synthesis in stimulated macrophages treated with a mustard gas analog

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Abstract

Background

Sulphur mustard gas, 2, 2'-dichlorodiethyl sulphide (HD), is a chemical warfare agent. Both mustard gas and its monofunctional analogue, 2-chloroethyl ethyl sulphide (CEES), are alkylating agents that influence cellular thiols and are highly toxic. Previously, we reported that lipopolysaccharide (LPS) significantly enhances the cytotoxicity of CEES in murine RAW 264.7 macrophages and that CEES transiently inhibits nitric oxide (NO) production via suppression of inducible NO synthase (iNOS) protein expression. NO generation is an important factor in wound healing. In this paper, we explored the hypotheses that LPS increases CEES toxicity by increasing oxidative stress and that treatment with N-acetyl-L-cysteine (NAC) would block LPS induced oxidative stress and protect against loss of NO production. NAC stimulates glutathione (GSH) synthesis and also acts directly as a free radical scavenger. The therapeutic use of the antibiotic, polymyxin B, was also evaluated since it binds to LPS and could thereby block the enhancement of CEES toxicity by LPS and also inhibit the secondary infections characteristic of HD/CEES wounds.

Results

We found that 10 mM NAC, when administered simultaneously or prior to treatment with 500 μM CEES, increased the viability of LPS stimulated macrophages. Surprisingly, NAC failed to protect LPS stimulated macrophages from CEES induced loss of NO production. Macrophages treated with both LPS and CEES show increased oxidative stress parameters (cellular thiol depletion and increased protein carbonyl levels). NAC effectively protected CEES+LPS treated cells from GSH loss and oxidative stress. Polymyxin B was found to partially block nitric oxide production and diminish CEES toxicity in LPS-treated macrophages.

Conclusion

The present study shows that oxidative stress is an important mechanism contributing to CEES toxicity in LPS stimulated macrophages and supports the notion that antioxidants could play a therapeutic role in preventing mustard gas toxicity. Although NAC reduced oxidative stress in CEES+LPS stimulated macrophages, it did not reverse CEES induced loss of NO production. NAC and polymyxin B were found to help prevent CEES toxicity in LPS-treated macrophages.

Background

Mustard gas (HD) is a chemical weapon that can easily and inexpensively be produced and used against military or civilian populations with both acute and devastating long-term effects {Balali-Mood, 2005 #262}. It produces rapid damage to eyes, skin and pulmonary tissues as well as subsequent damage to many internal organ systems {Dacre, 1996 #269; Paromov, 2007 #513}. Despite its long history of use, starting in World War I, the molecular mechanisms for HD toxicity are not fully understood and there is continuing research on the design of optimal countermeasures. Mustard gas acts as

alkylating agent covalently modifying DNA, proteins and other macromolecules. There is increasing evidence that HD or CEES toxicity is due, in part, to an enhanced production of inflammatory cytokines {Arroyo, 2004 #253; Arroyo, 1999 #92; Arroyo, 2000 #93; Arroyo, 1995 #275; Das, 2003 #270}, increased oxidative stress {Mukhopadhyay, 2006 #276} and the generation of damaging reactive oxygen species (ROS) {Das, 2003 #277; Arroyo, 1995 #278; Elsayed, 1992 #285}. HD and CEES have been shown to shift the intracellular redox milieu toward a more oxidized state by reacting with and depleting the intracellular antioxidant GSH with a subsequent loss of protection against ROS and an activation of inflammatory responses {Elsayed, 2004 #186; Han, 2004 #280; Naghii, 2002 #188}.

In a previous publication, we showed that the cytotoxicity of CEES towards RAW 264.7 macrophages was markedly enhanced by the presence of low levels of LPS (25 ng/ml), or pro-inflammatory cytokines (50 ng/ml IL-1ß; or 50 ng/ml TNF-α) {Stone, 2003 #116}. LPS is part of the cell wall of gram negative bacteria: it is ubiquitous and is found in serum, tap water and dust. Both civilian and military personnel would always have some degree of exposure to environmental LPS. HD induced skin lesions often have secondary infections which could markedly increase LPS levels. In macrophages, stimulation by LPS, as well as by pro-inflammatory cytokines, leads to the activation and nuclear translocation of transcription factor NF-κB (nuclear factor-kappa B). One of the major consequences of such activation in macrophages is an induction of iNOS expression with subsequent elevation of intracellular NO {Ganster, 2001 #515; Gao, 1997 #334}. In addition to NF-κB activation, the binding of transcription factor Stat1α (STAT stands for signal transducer and activator of transcription) to the iNOS promoter is required for optimal induction of the iNOS gene by LPS {Gao, 1997 #334}.

In a recent publication, we found that CEES transiently inhibits nitric oxide (NO) production by suppressing inducible nitric oxide synthase (iNOS) protein expression in LPS stimulated macrophages {Qui, 2006 #1200}. NO production is an important factor in promoting wound healing {Schwentker, 2003 #239; Witte, 2002 #240} and iNOS deficiency impairs wound healing in animal models {Yamasaki, 1998 #251}. RAW 264.7 macrophages have undetectable levels of iNOS or NO production in the absence of LPS and in the presence of LPS they show a marked induction of iNOS and NO production {Qui, 2006 #212}.

In the present study, we tested the hypothesis that the synergistic cytotoxic effect of CEES with LPS is due to increased oxidative stress with a subsequent depletion of intracellular GSH levels and an increase in protein carbonyls. In some cell types, GSH has also been found to regulate NO generation with decreased GSH levels associated with decreased NO production {Duval, 1995 #1197; Harbrecht, 1997 #1196; Tirmenstein, 2000 #1195}. Vos et al. {Vos, 1999 #1198} found that GSH depletion in hepatocytes prevented iNOS induction by cytokines but this effect could be reversed by the addition of NAC. We, therefore, also hypothesized that the addition of NAC to stimulated macrophages would reverse the loss of NO production caused by CEES. We also reasoned that polymyxin B, by binding to LPS, would diminish CEES toxicity in LPS treated macrophages.

Results

The influence of NAC on cell viability and NO production in CEES/LPS treated macrophages

Figure 1a shows the effect of NAC treatment on RAW 264.7 macrophages treated with LPS and/or 500 µM CEES for 24 hours. In this experiment, NAC was added simultaneously with LPS and CEES. In the absence of NAC, LPS, at either 50 ng/ml or 100 ng/ml level, markedly decreased cell viability in CEES treated cells compared to cells treated with LPS or CEES alone. This is similar to our previous observations {Stone, 2003 #116}. The addition of 10 mM NAC increased the viability of macrophages treated with both CEES and LPS (50 ng/ml or 100 ng/ml) to the same level observed for control cells (treated with vehicle alone). Figure 1b shows NO release, measured as the nitrite levels in the cell culture medium, for the same cells/treatments used in Figure 1a. As expected, LPS treatment alone resulted in a marked increase of NO generation, and LPS-stimulated macrophages treated with CEES showed a marked reduction in NO production. Surprisingly, NAC treatment did not prevent the decrease in NO production caused by CEES. In cells treated with LPS alone, NAC treatment actually resulted in a decreased production of NO (up to 40% reduction).

In order to further evaluate NAC as a potential protective agent for CEES toxicity in stimulated macrophages, we did two additional experiments in which NAC was added to macrophages 5 hours prior to CEES application or 5 hours after CEES application. These additional experiments provide a measure of the potential "window" during which NAC could be therapeutically useful. Similar to the previous experiment, LPS and CEES were added simultaneously (as indicated). As shown in Figure 2a, NAC had a substantial protective effect on cell viability when added 5 hours before CEES/LPS; however NAC did not protect against loss of NO production in CEES/LPS-treated cells (Figure 2b). When added 5 hours after CEES treatment (Figure 3a), NAC was much less effective in protecting the macrophages but still resulted in at least a doubling of the cell viability compared to the cells not treated with NAC. As shown in Figure 3b, NAC added 5 hours after CEES/LPS, also failed to restore NO production.

The influence of NAC on oxidative stress and NO production, intracellular GSH and thiols in CEES/LPS treated macrophages by fluorescence microscopy

The influence of NAC on macrophages treated with CEES/LPS was also examined by fluorescent microscopy using three fluorescent probes: a) carboxy-dichlorofluorescin diacetate (carDCFH-DA), a sensor for combined ROS and reactive nitrogen oxide species (RNOS) generation { Myhre, 2003 #495; Imrich, 1997 #62; LeBel, 1992 #494}; b) 7-amino-4-chloromethylcoumarin (CMAC), an indicator of intracellular GSH {Sebastia, 2003 #496}, and; c) 5-chloromethylfluorescein diacetate (CMF-DA), a probe for total non-protein cellular thiol levels that lacks specificity for GSH {Poot, 1991 #498; Sebastia, 2003 #496}.

Figure 4a shows the results using the lipid soluble carDCFH-DA probe. This probe enters cells and is trapped after being converted to a nonfluorescent polar derivative by cellular esterases. CarDCFH can then be oxidized by either ROS {LeBel, 1992 #494; Myhre, 2003 #495} or reactive nitrogen oxide species (RNOS) {Imrich, 1997 #62; Myhre, 2003 #495} to the fluorescent product carboxydichlorofluorescein (car-DCF) and thereby provide a qualitative index of oxidation stress. As expected, treatment with LPS[A2] alone (50 ng/ml) induced a marked generation of ROS plus RNOS in macrophages. We and others have shown that car-DCF fluorescence in activated macrophages is almost entirely from NO generation rather than ROS generation {Imrich, 1997 #62; Qui, 2006 #363}. Figure 4a also shows that treatment with CEES alone (500 μM) or CEES+LPS induces a higher level of car-DCF fluorescence than observed in control cells treated with vehicle alone. We previously reported that CEES markedly reduces NO generation in LPS stimulated cells by reducing the expression of inducible iNOS {Qui, 2006 #363}. The car-DCF fluorescence observed in CEES treated cells or CEES+LPS cells is likely, therefore, to be due to an enhanced generation of ROS alone with a minimal contribution from RNOS.

Simultaneous treatment with 10 mM NAC reduced the car-DCF fluorescence observed in LPS stimulated cells, as well as in CEES or CEES+LPS treated RAW 264.7 macrophages (Figure 4a, compare top row to bottom row). These data qualitatively suggest that CEES and CEES+LPS treatments induce oxidative stress in RAW 264.7 macrophages that can be diminished by NAC treatment.

As a next step we examined intracellular levels of GSH using the CMAC probe (Figure 4b, top row) and levels of total intracellular thiols using the CMF-DA probe (Figure 4c, top row). Both the CMAC and CMF probes revealed similar qualitative patterns: CEES or CEES+LPS treatment caused cellular GSH and thiol depletion but treatment with LPS alone did not. These data reinforce the notion that treatment with either CEES alone or treatment with CEES+LPS induces sufficient oxidative stress to reduce intracellular GSH and thiol levels. LPS alone, however, did not induce GSH or thiol depletion. NAC application was found to inhibit the loss of GSH and thiol levels caused by CEES or CEES+LPS treatment (see Figures 4b and 4c, bottom rows).

Quantitative effects of CEES on GSH status and protein carbonyl levels in LPS-stimulated RAW 264.7 macrophages

Since the fluorescence microscopy data presented above are primarily qualitative, we wanted to confirm our results by a more quantitative approach. We, therefore, determined the effect of CEES on the GSH/GSSG status of RAW 264.7 macrophage treated or untreated with LPS. Total GSH (GSH+GSSG) and GSSG concentrations were measured in cell lysates using a quantitative GSH assay kit and the values normalized to total protein content of the lysate (see Materials and Methods). Figure 5 shows that both total GSH and GSSG levels in macrophages treated with either vehicle alone or LPS were not significantly different (this is similar to our fluorescent microscopy data). However, cells treated with CEES alone showed a depletion in total GSH as well as an increase in

GSSG levels; cells treated with both CEES and LPS were further depleted in total GSH and the percentage of GSSG in these cells was the highest (40%). These result show that LPS alone does not induce a significant oxidative stress, CEES alone induced a moderate oxidative stress but the combination of both CEES and LPS induced the highest observed level of oxidative stress.

In addition, we measured the protein carbonyl levels in control cells, cells treated with CEES (500 µM or cells treated with ═S+CEES. Protein carbonyls are stable protein oxidation products. As shown in Figure 6, the combination of LPS+CEES produced a significant increase in protein carbonyl levels but cells treated with CEES alone were not significantly different from control cells treated with vehicle alone. Cells treated with LPS alone were not assayed in this experiment since both our qualitative (Figure 4b and 4c) and quantitative data (Figure 5) showed no evidence of oxidative stress with this treatment.

The inability of NAC to reverse NO loss in CEES/LPS treated cells is not GSH dependent

The data in Figure 1b show that NAC has almost no ability to restore NO production in LPS-stimulated macrophages treated with CEES. An inability of NAC to prevent the depletion of GSH in LPS-stimulated cells treated with CEES could possibly explain these results. In order to explore this possibility, we examined the ability of 5 mM NAC to prevent GSH depletion in LPS (50 ng/ml) stimulated and CEES treated (500 µM for 24 hours) RAW 264.7 cells. Figure 7 shows that CEES treatment alone decreased intracellular GSH by only about 10% compared to LPS stimulated cells in the absence NAC. As expected, the decrease in GSH levels was quite large in cells treated with both LPS+CEES (in the absence of NAC) but treatment with 5 mM NAC was effective in preventing this loss. The data shown in Figure 7 were obtained by HPLC analyses of the cell lysates but similar results were obtained by using a fluorometric assay for GSH {Kamencic, 2000 #123} (data not shown).   Despite the fact that NAC can increase the GSH level by three fold in CEES+LPS treated cells it does almost nothing to increase NO production (see Figure 1). These data suggest that the loss of NO production in CEES treated stimulated macrophages is not GSH dependent as has been observed in some other cell lines {Tirmenstein, 2000 #1195; Harbrecht, 1997 #1196; Duval, 1995 #1197; Vos, 1999 #1198}.

Polymyxin B diminishes CEES toxicity in LPS-treated macrophages and partially blocks LPS induced NO production.

Polymyxin B is an antibiotic drug, which selectively binds and neutralizes LPS. Since LPS enhances CEES toxicity, we tested the ability of polymyxin B to reduce CEES toxicity (500 µM) and decrease NO generation in LPS (50 ng/ml) stimulated macrophages. Figure 8a shows that polymyxin B (═µg/ml) had no cytotoxic effect on RAW 264.7 macrophages but partially reduced the cytotoxicity of CEES+LPS. Nevertheless, polymyxin B produced at least a six fold increase in cell viability compared to cell treated with both LPS and CEES.   Polymyxin B effectively blocked the

production of NO (measured as nitrite levels) in LPS (50 ng/ml) treated macrophages as would be expected if it bound and blocked the action of LPS.

Discussion

The cytotoxic effect of HD, and its analogue CEES, is believed to involve an increased generation of damaging free radicals and ROS {Arroyo, 1995 #4; Elsayed, 2004 #186; Elsayed, 1989 #196; Elsayed, 1992 #171; Han, 2004 #280}. The data presented here show that LPS in combination with CEES induces intracellular GSH and thiol depletion as well as increased levels of protein carbonyls. The measurement of protein carbonyls is one of the best indices for oxidative stress due to the stability of protein carbonyls and sensitivity of the measurement {Berlett, 1997 #1204}. Cellular thiols are important markers the redox state of the cell. In particular, GSH is one of the major components of the intracellular redox system and a key intracellular antioxidant that functions as a substrate for glutathione peroxidase which detoxifies both hydrogen peroxide and lipid hydroperoxides {Stone, 2004 #201; Droge, 2002 #318}. Depletion of intracellular stores of GSH plays an important role in the development of oxidative stress {Kadar, 2001 #190; Elsayed, 2004 #186; Han, 2004 #280}. Recent work also suggests that the anti-apoptotic protein Bcl-2 directly interacts with GSH to regulate an important mitochondrial GSH pool that influences mitochondrial oxidative stress and subsequent apoptosis {Zimmermann, 2007 #514}.

Taken together, our data strongly suggest that CEES induces oxidative stress in stimulated macrophages. Moreover, the pattern of oxidative stress parallels the pattern observed for CEES cytotoxicity, i.e., cytotoxicity and oxidative stress are amplified in cells treated with both CEES and LPS. The addition of �ю mM NAC, a well characterized water-soluble antioxidant, was found to be very effective in minimizing CEES toxicity in stimulated macrophages and in preventing GSH depletion. Our data suggests that NAC can be added even five hours before or five hours after CEES and still expert a cytoprotective effect.   Das et al. {Das, 2003 #151} recently found that NAC in drinking water was effective in reducing CEES-induced lung toxicity to Guinea pigs. Fan et al. {Fan, 2000 #10} have shown that liposomal encapsulated NAC delivered intratracheally was more effective than free NAC against acute respiratory distress syndrome in a rat model. It is interesting, therefore, that McClintock et al. {McClintock, 2006 #1203} have shown that reducing agents (NAC or GSH), as well as some anti-oxidant enzymes, delivered via liposomes, can substantially diminish CEES-induced injury in rat lungs. We are currently formulating an optimal antioxidant liposome preparation for treating either lung or skin induced CEES/HD injury.

We previously reported that CEES induces a transient loss of iNOS protein expression in LPS stimulated RAW 264.7 macrophages but does not inhibit the enzymatic activity of iNOS. Based of the work of others {Duval, 1995 #1197; Harbrecht, 1997 #1196; Tirmenstein, 2000 #1195;Vos, 1999 #1198}, we hypothesized that NAC treatment would not only protective against CEES toxicity but would also reverse the loss of NO production in CEES+LPS treated macrophages. Our results indicate, however, that this was not the case. Our data did, however, show that NAC effectively increases cell

viability, increases GSH levels and reduces oxidative stress in CEES+LPS treated macrophages.

CEES could inhibit iNOS protein synthesis by a number of possible molecular mechanisms which we are currently exploring {Qui, 2006 #363}. It is generally accepted that both the transcription factor NF-κB and signal transducer and STAT-1 play central roles in the LPS induction of iNOS {Liu, 1997 #1199; Gao, 1997 #1202; Kleinert, 2004 #249}. It is possible CEES/HD could inhibit the NF-κB and/or the STAT-1 pathways in RAW 264.7 macrophages and consequently block iNOS gene expression. For instance, CEES could alkylate the NF-κB consensus nucleotide binding sequences thereby preventing the binding of activated NF-κB to the iNOS promoter and block the subsequent production of iNOS mRNA and protein expression. Previous studies *in vitro* have shown that DNA alkylation by CEES {Chen, 1999 #322; Gray, 1995 #323} or by nitrogen mustard {Fabbri, 1993 #356} can inhibit the DNA binding of transcription factor AP2 or NF-κB.

Alternatively, the DNA binding ability of the NF-κB and/or STAT-1 transcription factors could be reduced by direct covalent modification by CEES or as an indirect result of GSH depletion, i.e., redox regulation. Nishi et al. {Nishi, 2002 #1201} have found, for example, that the cysteine-62 (Cys-62) residue of the p50 NF-κB protein subunit is oxidized in the cytoplasm but reduced in the nucleus, and that the reduced form is essential for NF-κB DNA binding. It is possible that CEES could rapidly react with Cys-62 of the p50 NF-κB subunit and prevent its DNA binding. However, since NAC was found to restore GSH levels without restoring iNOS activity (see Figures 1 and 7), it is unlikely that GSH redox modulation of the p50 Cys-62 is a likely molecular mechanism for CEES induced loss of iNOS protein in LPS-stimulated macrophages.

Moreover, there is evidence suggesting that CEES does not inhibit but rather promotes NF-κB activation. It is known that CEES or HD treated cells release elevated levels of TNF-α and also show NF-κB activation both *in vitro* and *in vivo* as measured by electrophoretic mobility shift assays (EMSAs){Arroyo, 2000 #93; Atkins, 2000 #189; Chatterjee, 2003 #143}. Minsavage and Dillman recently demonstrated that NF-κB is activated by HD treatment in human cell lines as evidenced by loss of the inhibitory subunit IκBα as well as by a NF-κB reporter gene {Minsavage, 2007 #375}. Collectively, these data suggest the inhibition of iNOS expression by CEES or HD could be due to a downregulation of the STAT-1 pathway. We are currently exploring these various molecular mechanisms.

In this regard, we tested the ability of polymyxin B to block the effect of LPS. Polymyxin B binds to the lipid A domain of LPS and neutralizes its activity. Our data show that polymyxin B effectively inhibits CEES toxicity in LPS stimulated cells. *In vivo*, LPS could directly enhance CEES/HD toxicity in cells with functional CD14 receptors or by triggering the release of pro-inflammatory cytokines, such as TNF-a and IL-1ß, by

immune cells. We have previously demonstrated that inflammatory cytokines also enhance CEES cytotoxicity {Stone, 2003 #116}.

Conclusions

Our *in vitro* work presents novel evidences supporting the view that oxidative stress is an important component of CEES/HD toxicity and that antioxidants have therapeutic potential. We anticipated that NAC would prevent GSH depletion and restore the loss of iNOS activity in CEES treated macrophages stimulated with LPS. Although NAC was effective in preventing both CEES toxicity and GSH depletion, it failed, however, to restore iNOS expression. Our results to date indicate that CEES causes a transient decrease in iNOS protein syntheses rather than an inhibition of iNOS activity due to its direct covalent modification by CEES. We are currently investigating the molecular mechanism(s) for the down regulation of iNOS expression by CEES.

Inhibition of iNOS and NO production could be an important element in the slow wound healing observed by exposure to CEES/HD. Considerable evidence suggests that iNOS is an important component of wound healing {Schwentker, 2003 #239; Witte, 2002 #240; Soneja, 2005 #258}. Although NAC maybe effective at reducing CEES/HD toxicity it is not effective at elevating NO production due to iNOS inhibition (by CEES/HD).

The fact that LPS was found to enhance CEES toxicity highlights the potential importance of the secondary infection prevention in the treatment of HD toxicity. LPS is a component of gram negative bacteria and a ubiquitous environmental contaminant. Its presence at very low levels (ng/ml) amplifies the toxicity of CEES. Polymyxin B, a typically applied antibiotic that binds LPS, was shown to block the iNOS inducing ability of LPS and to reduce CEES toxicity in LPS stimulated cells. Polymyxin B could, therefore, be useful as a supportive treatment in order to prevent secondary infections and to reduce HD toxicity, since it both neutralizes LPS and prevents the growth of gram-negative bacteria in healing wounds. The path to an optimal countermeasure to CEES/HD exposure may lie in a poly-drug formulation that minimizes oxidative stress, prevents inflammation and secondary infections, and, also, protects iNOS activity. Antioxidant liposomes are currently being investigated since they have the ability to deliver water-soluble and lipid soluble antioxidants {Stone, 2002 #117} and can also encapsulate polymyxin B (personal communications, Dr. Zach Suntres) as well as anti-inflammatory agents.

Methods

Materials

RPMI-1640 medium without phenol red and fetal bovine serum with a low endotoxin level were purchased from Life Technologies (Gaithersburg, MD). Escherichia coli lipopolysaccharide serotype 0111:B4, 3-(4,5-dimethylthiazolyl-2)-2,5-

diphenyltetrazolium bromide (MTT), CEES, NAC, Greiss reagent, GSH, BHT, EDTA, and all organic solvents used were obtained from Sigma Chemical Company (St. Louis, MO). Fluorescent dyes carDCFH-DA, CMAC, and CMF-DA were purchased from Molecular Probes (Invitrogen Corp., Carlsbad, CA).

Cell Culture and Treatments

RAW264.7 murine macrophage-like cells (American Type Culture Collection, Rockville, MD) were cultured at 37°C in a humidified incubator with 5% CO_2 in RPMI-1640 medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (GiBcoBRL Grand Island, NY). Adherent cells were subcultured over night in 96 well Costar tissue culture plates in the medium and treated with CEES and/or LPS in the presence or absence of various concentrations of NAC as indicated in the Figure legends. CEES was used only as a fresh 50 mM stock solution in anhydrous ethanol. LPS was prepared as a 0.5 µg/ml stock solution in PBS, filter-sterilized and stored at -20^{0} C for up to 6 months. NAC was prepared as a 0.5 M stock solution in PBS (pH adjusted to 7.4), filter-sterilized and stored at 4^{0} C for up to four weeks.

MTT Assay

MTT assay was performed by a slight modification of the method described by Wasserman et al. {Wasserman, 1988 #110; Twentyman, 1987 #111}. Briefly, at the end of each experiment, cultured cells in 96 well plates (with 200 µl of medium per well) were incubated with MTT (20 µl of 5 µg/ml per well) at 37°C for 4 hours. The formazan product was solubilized by addition of 100 µl of dimethyl sulfoxide (DMSO) and the OD measured at 575 nm with a Spectramax Plus 384 microplate reader (Molecular Devices Corp, Sunnyvale, CA)

Determination of NO production

The production of NO, reflecting cellular NO synthase activity, was estimated from the accumulation of nitrite (NO_2^-), a stable breakdown product of NO, in the medium. NO_2^- was assayed by the method of Green et al. {Green, 1982 #81}. Briefly, an aliquot of cell culture medium was mixed with an equal volume of Greiss reagent which reacts with NO_2^- to form an azo-product. Absorbance of the reaction product was determined at 532 nm using a Spectramax Plus 384 microplate reader (Molecular Devices Corp, Sunnyvale, CA). Sodium nitrite was used as a standard to calculate NO_2^- concentrations.

Quantitative GSH analyses

RAW264.7 macrophages incubated in 96-well plate (~10^6 adherent cells/well) and treated with LPS/CEES/NAC as indicated in the Figure legends was assayed for total GSH (GSH plus GSSG) using a kit (GSH assay kit, World Precision Instruments, Sarasota, FL) according to the company's protocol. This assay uses the Tietze's enzymatic recycling method {Tietze, 1969 #516}. In order to measure just GSSG, 2-vinylpyridine was first used to derivatize GSH alone {Griffith, 1980 #517}. Total GSH and GSSG levels were normalized to the total protein (as determined by the standard

BCA assay). Alternatively, GSH analyses of the cell lysates were analyzed by isocratic HPLC with electrochemical detector composed of Coulochem II model 5200A and a Coulochem 5011 analytical cell (ESA Inc, Chelmsford, MA) as described by {Houze, 2001 #518}. Since the cell lysates contained no measurable levels of homocysteine, this aminothiol was used as an internal standard.

Protein Carbonyl Levels

Protein carbonyl levels were measured by an enzyme immunoassay kit from Cell Biolabs (San Diego, CA) as per the manufacture's instructions. In this assay, the protein samples are derivatized by making use of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls to form a DNP hydrazone which is assayed using an anti-DNP antibody and a HRP conjugated secondary antibody. A standard curve from oxidized BSA standards was run with each microplate. This kit assay is essentially a modification of the method described by Buss et al. {Buss, 1997 #520; Buss, 2002 #519}.

orescent microscopic analyses

The cell density was adjusted to 2×10^5/ml, and a 100 µl aliquot of the cell suspension in media was placed in each well of an 8-well Lab-Tek chamber glass slide (Nunc, Rochester, NY). CEES (?) and/or LPS solutions (?) were added to achieve desired concentrations and the plate incubated for 12 h at 37°C in 5% CO_2. At the end of the treatment a stock solution of desired fluorescent probe in DMSO was added and the slides incubated for an additional 30 min at 37°C. The cells were washed with fresh PBS twice, observed and digitally photographed using a MOTIC inverted phase contrast fluorescence microscope equipped with a Nikon Coolpix E4300 4-megapixel camera (Martin Microscope, Easley, SC). A 20 µM carDCFH-DA and a standard FITC filter were used to monitor combined ROS and RNOS generation; a 20 µM CMAC and a standard DAPI filter were used to monitor intracellular GSH; a 20 µM CMF-DA and a standard FITC filter were used to monitor cellular thiol levels. All the optical filters were obtained from Chroma Technology Corp (Rockingham, VT).

Statistical Analyses

Data were analyzed ANOVA followed with the Scheffe test for significance with $p < 0.05$ using SPSS 14.0 for Windows (Chicago, IL). Results were expressed as the mean ± SD. In all the Figures, mean values not sharing a common letter are significantly different ($p<0.05$). Mean values sharing a common letter are not significantly different. The mean values and standard deviations of at least three independent experiments are provided in all the Figures.

Abbreviations

AP2, activating protein 2

CEES, 2-chloroethyl ethyl sulphide
carDCFH-DA, carboxy-dichlorofluorescin diacetate
CMAC, 7-amino-4-chloromethylcoumarin
CMF-DA, 5-chloromethylfluorescein diacetate
DNPH, 2,4-dinitrophenylhydrazine
GSH, reduced glutathione
GSSG, oxidized glutathione
HD, sulphur mustard gas
IL-1ß, interleukin-1 beta
LPS, lipopolysaccharide
MTT, 3-(4,5-dimethylthiazool-2yl)-2,5-diphenyltetrazolium bromide
NAC, N-acetyl-L-cysteine
NO, nitric oxide
iNOS, inducible nitric oxide synthase
NF-κB, nuclear factor kappa B
STAT-1, signal transducer and activator of transcription-1
TNF-α, tumor necrosis factor-alpha

Authors' contributions

WLS and VP analyzed the data and drafted the manuscript. WLS supervised the overall conduct of the research, which was performed in his laboratory. MQ, HY and VP carried out the experimental work in this study and performed the statistical analyses. MS (along with WLS) conceived of the study, participated in the study design, and provided continuous evaluation of the experimental data. All authors read and approved the final manuscript.

Acknowledgements

Figure legends

Figure 1 – NAC effect on viability and NO production in CEES/LPS treated RAW264.7 cells (simultaneous NAC/CEES/LPS application). *Panel A:* Macrophages incubated with 50 or 100 ng/ml of LPS or/and 500 µM CEES were simultaneously treated with or without 10 mM NAC (as indicated) for 24 hours. Cell viability was measured using the MTT assay (see Materials and Methods) and expressed as OD at 575 nm. *Panel B:* Macrophages were incubated as described above and NO production measured as the concentration of nitrite in the culture media as described in Materials and Methods.

Figure 2 – NAC effect on viability and NO production in CEES/LPS incubated RAW264.7 cells (NAC pre-treatment). *Panel A:* Macrophages were pre-treated with or without 10 mM NAC for 5 hours and then incubated with 50 or 100 ng/ml of LPS or/and 500 µM CEES (as indicated) for 24 hours. Cell viability was measured using the MTT assay (see Materials and Methods) and expressed as OD at 575 nm. *Panel B:* Macrophages were incubated as described above and NO production measured as concentration of nitrite in the culture media as described in Materials and Methods.

Figure 3 – NAC effect on viability and NO production in CEES/LPS treated RAW264.7 cells (NAC post-treatment). *Panel A:* Macrophages were incubated with 50 or 100 ng/ml of LPS or/and 500 µM CEES (as indicated) for 24 hours and 10 mM NAC was added to the cell culture medium after 5 hours after the CEES/LPS application. Cell viability was measured using the MTT assay (see Materials and Methods) and expressed as OD at 575 nm. *Panel B:* Macrophages were incubated as described above and NO production measured as concentration of nitrite in the culture media as described in Materials and Methods.

Figure 4- Fluorescent Microscopy Probes for Oxidative Stress, GSH and thiols in RAW 264.7 Cells. *Panel A*: Combined generation of ROS and RNOS were monitored using 20 µM carDCFH-DA ; *Panel B*: Intracellular GSH levels were examined using 20 µM CMAC; *Panel C*: Levels of non-protein cellular thiols were monitored using 20 µM CMF-DA under a fluorescent microscope. Macrophages were treated with CEES (500 µM) and/or LPS (x ng/ml) and incubated in the absence (top row in each panel) or NAC or in the presence of NAC (xx).

Figure 5 – Glutathione status in RAW264.7 cells incubated with CEES/LPS. Macrophages were incubated with 50 ng/ml LPS or/and 500 µM CEES . Total GSH (GSH+GSSG) and GSSG levels were measured using a GSH assay kit (see Materials and Methods section) in cell lysates and normalized to total protein. Numbers show the percentage of GSSG in total GSH.

Figure 6- Protein Carbonyl Levels in RAW264.7 cells incubated with CEES/LPS.

Figure 7– NAC restores intracellular GSH in RAW264.7 cells incubated with CEES/LPS. Macrophages incubated with 50 ng/ml LPS or/and 500 µM CEES were simultaneously treated with or without 5 mM NAC (as indicated). Reduced GSH was measured after 4 hour incubation in the cell lysates via HPLC technique as described in Materials and Methods. The GSH levels were normalized to an internal homocysteine standard.

Figure 8 – Polmyxin B partially protects RAW 264.7 Macrophages from CEES+LPS toxicity and blocks NO production. *Panel A*: Macrophages incubated with 50 ng/ml LPS or/and 500 µM CEES were simultaneously treated with or without 10 µg/ml polymyxin B (as indicated) for 18 hours. Cell viability measured in MTT assay (see Materials and Methods) and expressed as OD at 575 nm. *Panel B:* Macrophages were incubated as described above. NO production WAS measured as nitrite concentration in the culture media as described in Materials and Methods.

Ricin-Induced Toxicity: The Role of Oxidative Stress

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ABSTRACT

Ricin belongs to the type II ribosome inactivating proteins which are heterodimeric glycoproteins that contain a toxophoric A-chain and a lectin B-chain joined together by a disulfide bond. Worldwide, one million tons of castor beans (*Ricinus communis*) are processed annually in the production of castor oil; the waste mash from this process is five percent ricin by weight. Ricin has a history of use as a weapon of war, terror, and assassination, it can be prepared as liquid, crystals, or dry powder and it can be disseminated as an aerosol, injected into a victim, or used to contaminate food or water on a small scale. The toxicity of ricin varies according to the route of administration, but the clinical symptoms frequently are related to a severe inflammatory response and multiorgan failure. The cytotoxicity of ricin is commonly attributed to the inhibition of protein synthesis consequent to ribosomal damage. Several studies have also shown that administration of ricin to animals or exposure of cellular systems to ricin result in biochemical, cellular, and functional disturbances consistent with the occurrence of oxidative stress. The ability of antioxidants to ameliorate ricin-induced biochemical and cellular alterations also implicates oxidative stress as a possible contributing mechanism in ricin toxicity. In this review, the role of oxidative stress in ricin-induced cytotoxicity will be discussed.

INTRODUCTION

Ricin belongs to the type II ribosome inactivating proteins which are heterodimeric glycoproteins that contain a toxophoric A-chain and a lectin B-chain joined together by a disulfide bond (Endo, 1987a, b; Olsnes, 2004; Stirpe, 2004). The A-chain is transferred across the cell membrane by the B-chain via endocytotic vesicles into cells (Lord, 1998; Sandvig, 2002; Hartley, 2004; Roberts, 2004). The B chain binds to cell surface carbohydrates containing galactose or *N*-acetylgalactosamine residue which then allows the A-chain to enter the cell. Once inside the cell, the A-chain undergoes retrograde transport by receptor-mediated endocytosis, resulting in the toxin being transported through the Golgi complex into the cytosol after the reduction of the disulphide bond. Once in the cytosol, the A chain exhibits RNA N-glycosidase activity and inactivates ribosomes by enzymatically removing a specific adenine residue from the 28S RNA of the 60S ribosomal subunit. The adenine residue removed by ricin action is crucial for the binding of elongation factors. A consequence of the ricin-induced depurination is the cessation of protein synthesis (Olsnes, 1972; Endo, 1987a, b; Lord, 1998; Sandvig, 2002; Hartley, 2004; Roberts, 2004). The clinical latency between exposure and symptoms, ranging between 8 to 24 hours, has been attributed to the transport of the ricin to the interior of the cell.

The exact cause of ricin-induced cell death is unknown but results from several studies have shown that, regardless of the route of administration, ricin A-chain cause organ and tissue lesions that might be the result of vascular disturbances induced by the toxin rather than a direct effect of the toxin itself (Franz, 1997; Howat, 1988). Clinical trials have shown that administration of ricin A-chain immunotoxin caused vascular syndrome characterized by hypoalbuminemia and edema (Soler-Rodriquez, 1993; Schnell, 2003). In humans, the estimated lethal dose of ricin is 1 to 10 µg per kg body weight following inhalation or injection (Smallshaw, 2002). In mice, rats, and primates, high doses of ricin via inhalation produces severe enough pulmonary damage to cause death probably due to hypoxemia resulting from massive pulmonary edema and alveolar flooding (Wilhelmsen, 1996; DaSilva, 2003; Roy, 2003). In rats, inhaled ricin has an LD_{50} of 3.7 µg/kg and is associated with pulmonary edema, acute destructive alveolitis and necrosis/apoptosis of the lower respiratory tract epithelium (Gareth, 1995). Rats treated with ricin at a dose of 10 µg/kg body weight (intravenous administration- iv) showed no alterations in hepatic protein synthesis but treatment adversely affected the sinusoidal cells in the liver consequently resulting in hepatocyte necrosis (Derenzini, 1976). Intravenous administration of ricin to mice (120 µg/kg body weight) resulted in hemolytic uremic syndrome, including thrombotic microangiopathy, hemolytic anemia, and acute renal failure (Korcheva, 2005; Fu, 2004). In vitro studies with human umbilical vein endothelial cells showed that ricin damages endothelial cells (Baluna, 2000). The cytotoxicity of ricin and other ribosome-inactivating proteins are commonly attributed to the inhibition of protein synthesis consequent to ribosomal damage. A lesser known, but nevertheless important pathophysiologic event in ricin-induced toxicity appears to be oxidative stress (Schulze-Osthoff, 1992; 1993). Oxidative stress can be defined as a disturbance in the prooxidant-antioxidant balance in favor of the prooxidant, leading to damage.

Oxidative stress can be caused by an elevation in the steady-state concentration of reactive oxygen species (ROS), reactive nitrosative species (RNS) and reactive chlorine species (RCS). A large number of drugs and other xenobiotics can stimulate the generation of reactive oxygen species by redox cycling (Sies, 1987; Comporti, 1989; Stohs, 1995; Suntres, 2002). Cellular enzymatic processes, either in the normal metabolic function (electron transport in the mitochondria, microsomes) or disease states (xanthine oxidase in ischemia-reperfusion, cytochrome P-450-dependent activation of xenobiotics) are also responsible for the generation of reactive oxygen species (Sies, 1987; Fang, 2002; Djordjevic, 2004). Release of reactive oxygen species have also been demonstrated in the respiratory burst of neutrophils and macrophages (Forman and Torres, 2001; Di Virgilio, 2004). ROS, RNS and RCS indiscriminantly react with any cellular structure (Sies, 1987; Toyokuni, 1999; Forman, 2001; Fang, 2002; Di Virgilio, 2004; Djordjevic, 2004) and regulate a broad array of signal transduction pathways (Forman, 2002; Virgilio, 2004; Djordjevic, 2004). Several transcription factors such as AP-1, NFkB, MAF, NRL and NF-IL6 are regulated by the intracellular redox state of cells, a status that determines the magnitude of oxidative stress (Kamata, 1999; Hsu, 1994; Xanthoudakis, 1994; Kerrpola, 1994; Jang, 2004; Tas, 2005).

Oxidative stress can also trigger apoptosis or programmed cell death (Forman, 2002; Djordjevic, 2004; Goldenthal, 2004; Kadenbach, 2004). Activated neutrophils responding to inflammatory stimulation produce reactive oxygen species which are known to attack endothelial cells lining the vascular wall and trigger apoptosis. Endothelial cells also produce reactive oxygen species inside the cell that can contribute to oxidative stress and apoptosis, such as during ischemia/reperfusion injury (Djordjevic, 2004; Kadenbach, 2004). Oxidants can also induce apoptosis by changing cellular redox potentials by altering the reduced glutathione to oxidized glutathione (GSH/ GSSH) as well as by decreasing reducing equivalents, such as NADH and NADPH in the mitochondria (Zoratti, 1995; Bernardi, 1996; Bernardi and Petronilli, 1996). A change in the redox state, an effect also observed following administration of several toxins, facilitates the formation of permeability transition pores (PT pores), leading to the subsequent release of cytochrome c (Dalton, 1999). Mitochondrial release of cytochrome c serves as the key regulator of apoptosis because once it is released from the intermembrane space the cell is irreversibly committed to death (Orrenius, 2004).

Maintenance of cell integrity depends on the balance between cellular activation and antioxidant defense systems. Imbalances may occur due to: i) increased generation of reactive oxygen species overwhelms the defense system; ii) the defense system is severely compromised and incapable of detoxifying the normal flux or reactive metabolites; and iii) a combination of increased production and decreased detoxication occurs. The antioxidant defense system consists of enzymatic or non-enzymatic antioxidants.

The major antioxidant enzymes found in cells are superoxide dismutase, catalase and glutathione peroxidase. Three different types of SOD have been isolated and characterized: a copper and zinc containing form (Cu-Zn-SOD) that is localized in the

cytosol; a manganese-containing SOD (MnSOD) localized in the mitochondria; and, an extracellular form (EC-SOD) in the extracellular matrix. Catalase, which catalyzes the detoxication of hydrogen peroxide to water and oxygen, is localized mainly in peroxisomes, although some activity has been detected in mitochondria and cell cytoplasm. Glutathione peroxidase, plays a major role in the detoxication of hydrogen peroxide and other hydroperoxides and lipid peroxides via the glutathione redox cycle (Sies, 1987; Gutteridge, 2000).

The non-enzymatic antioxidants include vitamin C (ascorbic acid), urate, vitamin E, beta-carotene and other micronutrients such as carotenoids, polyphenols and selenium. Vitamin E, the principal lipid-soluble antioxidant in the body, is composed of four tocopherols and four tocotrienols and due to its lipophilicity it is present in all cellular membranes (Chow, 2004). Vitamin E neutralizes the highly reactive singlet oxygen molecules and protects polyunsaturated fatty acids in cell membranes from peroxidation (Buettner, 1993). Ascorbic acid, a water-soluble vitamin, is effective in scavenging free radicals, including hydroxyl radical, aqueous peroxyl radicals, and superoxide anion and is considered to be one of the most important antioxidants in extra cellular fluids (Halliwell, 1996; Carr, 1999; Evans, 2001). Glutathione (GSH) is the most abundant non-protein thiol in living organisms and plays a crucial role in intracellular protection against toxic compounds, such as reactive oxygen species and other free radicals (Anderson, 1997; Anderson, 1998). It can function as a nucleophile to form conjugates with many xenobiotic compounds and/or their metabolites and can also serve as a reductant in the metabolism of hydrogen peroxide, as well as other organic hydroperoxides. Also, glutathione is important as a redox buffer (GSH/GSSH) by playing a critical role in the regulation of redox regulated transcription factors (e.g. NFkB) (Schafer, 2001).

RICIN-INDUCED TISSUE TOXICITY.

Ricin is quite stable and extremely toxic to the cells of different organs such as the liver, kidney, lung, pancreas, intestine, and thyroid (Sadani, 1997; Franz, 1997; Greenfield, 2002; DaSilva, 2003). This toxicity is largely dependent on the route of ricin exposure, an effect most likely attributed to the lectin properties of ricin. As indicated previously, the chain B of ricin contains lectin which recognizes and binds to galactosides of cell-surface carbohydrates (Sandvig, 2002; Hartley, 2004; Roberts, 2004). Lectins are being investigated as carrier molecules to target drugs specifically to different cells and tissues including the gastrointestinal tract, lungs, liver, nasal mucosa, buccal cavity and the eye (Bies, 2004). Inhalation of ricin results in pathologic changes within 8 hours. Severe respiratory distress and other symptoms are followed by acute hypoxic respiratory failure in 36-72 hours. Ingestion of ricin results in gastrointestinal hemorrhage, necrosis of the liver, spleen and kidneys; severe localized muscle pain; regional lymph node necrosis, and moderate involvement of visceral organs. Intravenously administered ricin is found in the spleen followed by kidneys, heart, liver, and thymus (Fodstad, 1976, 1979; Ramsden, 1989; Franz, 1997; Stirpe, 2004; Bismuth, 2004).

THE ROLE OF OXIDATIVE STRESS IN RICIN TOXICITY.

Studies have demonstrated that following administration of ricin to animals or exposure of cellular systems to ricin there are biochemical, cellular, and functional disturbances consistent with the occurrence of oxidative stress. Challenge of mice with ricin has resulted in hepatic lipid peroxidation, glutathione depletion, DNA single strand breaks and increased urinary excretion of carbonyl compounds (Muldoon et al., 1992, 1994; Kumar, 2003). Increases in kidney lipid peroxidation with concomitant decreases in GSH also occur. In addition decreases in plasma SOD and increases in catalase activities following exposure of animals to ricin also suggest a significant oxidative stress component (Kumar, 2003). In another study, it was demonstrated that the enzyme xanthine oxidoreductase was converted from the dehydrogenase to the oxidase form in the liver of ricin-challenged animals (Battelli, 1996). Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid leading to the production of both superoxide radicals (O_2^-) and H_2O_2. Also, it has been shown that ricin administration resulted in the destruction of thyroid follicles and a reduction in circulating thyroid hormones. The effect on the thyroid follicles and circulating hormone are attributed to the production of reactive oxygen species (Sadani, 1997). Exposure of human cervical cancer cells to ricin resulted in time-dependent increases in intracellular reactive oxygen species production with concomitant reductions in cellular GSH levels and cell viability (Rao, 2005). Muldoon *et al.,* (Muldoon, 1996) reported that challenge of mice with ricin resulted in dose-dependent increases in macrophage, microsomal, and mitochondrial superoxide anion production.

The ability of antioxidants to ameliorate ricin-induced biochemical and cellular alterations also implicates oxidative stress as a possible contributing mechanism in ricin toxicity. In a lethality study based on percentage survival and time to death following a ricin LD_{100} of 25 µg/kg (ip), vitamin E succinate and butylated hydroxyanisole extended the survival time in response to a lethal dose of ricin (Muldoon and Stohs, 1994). Challenge of U937 human myeloid leukemia cells with ricin resulted in significant reductions in cellular glutathione levels and the antioxidant N-acetylcysteine strongly inhibited ricin-induced apoptotic cell death in U937 cells, as judged by cytotoxicity, nuclear morphological change, and DNA fragmentation (Oda, 1999). Abrin, a toxin that belongs to the type II ribosome inactivating proteins and, is similar in structure and properties to ricin, induces cells to generate reactive oxygen species and the antioxidant N-acetylcysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl can delay abrin-induced cell death (Shih, 2001).

Sadani and Nadkarni (Sadani, 1994) demonstrated that injection of animals with low doses of ricin (1.5 µg/kg, ip) is specifically toxic to Kupffer cells without affecting the parenchymal cells. The resistance of the parenchymal cells to ricin was attributed to an elevation in superoxide dismutase, catalase, and glutathione peroxidase levels in the parenchymal cells and the elevation of lipid peroxidation products in the hepatic homogenates originated mainly from damaged kupffer cells. The observation that Kupfer cells are the first target of ricin, occurring within 4 h after ricin administration, has also been confirmed by other investigators (Skilleter, 1981; Bingen, 1987; Derenzini, 1987;

Zenilman, 1988; 1989; Magnusson, 1993). Activation of macrophages is associated with increased cytokine production, an enhanced oxidative metabolism, and an increased phagocytotic activity. The high sensitivity of Kupffer cells and other macrophages to ricin has been ascribed to the ability of ricin to bind effectively to the mannose receptors present in these cells via its high-mannose oligosaccharide side chain (Skilleter, 1981; Magnusson, 1993). It is known that mannose receptors are expressed on the membranes of macrophagic type cells, including mouse peritoneal macrophages, rat Kupffer cells, mouse microglial cells, and rat bone marrow macrophages (Stahl, 1982; Simmons, 1986; Stein, 1992; Magnusson, 1993; Battelli, 2001).

Exposure of macrophages to ricin resulted in a time- and concentration-dependent production of superoxide anion and nitric oxide (Hassoun, 1999), known to form peroxynitrite and nitration of tyrosine residues (Ischiropoulos, 1998). The role of nitric oxide in the pathogenesis of glomerular thrombotic microangiography has been explored in a ricin-treated rat model (Williams, 2000). Ricin has shown to cause mucosal inflammation, epithelial damage and increased myoelectric activity (Sjogren, 1994; Stojadinovic, 1997). Korcheva et al. (Korcheva, 2005) showed that ricin induces a severe inflammatory response via stimulation of numerous stress-activated protein kinases and increases in mRNA that encodes for TNF-α and IL-1.

Pulmonary toxicity was correlated to ricin's effect on the alveolar macrophages. This effect was shown to be associated with the transcriptional induction of a number of cell surface inflammatory markers and chemotactic factors (i.e., CD14, ICAM-1, MCP3, and MIP1β) which participate in the recruitment of neutrophils or activation of tissue macrophages; collectively, these disturbances lead to acute lung injury mediated by formation of oxidants and release of proteases (Xu, 1995; Lukacs, 1996; Menten, 2001; DaSilva, 2003). This is also consistent with findings from other studies where pulmonary exposure to toxins resulted in infiltration and activation of phagocytes in the lung leading to pulmonary injury via the generation of reactive oxygen species. Antioxidants antagonize the infiltration and activation of activated phagocytes acting in response to the toxins (Suntres, 2002; Suntres, 1996; Suntres, 1998; Cadenas, 2002; Bhattacharyya, 2004).

In general, macrophages and other phagocytes produce reactive oxygen species during phagocytosis or stimulation with a wide variety of agents through activation of the membrane-associated nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase. Once activated, phagocytes infiltrate a site and cause additional damage through the release of neutral proteinase or the generation of superoxide anions (O_2^-) via NADPH-oxidase during the respiratory burst. Superoxide gives rise to hydrogen peroxide spontaneously or via superoxide dismutase. Production of hypochlorous acid (HOCl) can occur via the interaction with myeloperoxidase. On stimulation and phagocytosis, HOCl production by the neutrophil accounts for up to 70% of the oxygen consumed, making it the major oxidative product of neutrophils (Klebanoff, 1991). HOCl reacts readily with thiols and thioethers, Fe-S centers, and nucleotides (Alison, 1976; Winterbourn , 1985; Folkes, 1995), with amines to form reactive chloramines (Thomas, 1983), and with unsaturated fatty acids to form chlorohydrins (Winterbourn, 1992).

Excessive production of reactive oxygen species, in addition to their direct toxic actions, will also exacerbate the inflammatory process, cause further tissue damage, and drive redox-regulated gene expression.

One of the mechanisms by which ricin may initiate oxidative stress-induced cellular injury is linked to the production of tumor necrosis factor-alpha (TNF-α), which is coded for by the transcription factor NFkB. TNF-α is produced mainly by activated macrophages and in smaller amounts by other cell types (Vandenabeele, 1995; Bradham, 1998). Kupffer cells, the resident hepatic macrophages, are a rich source of cytokines in the liver (Boulton, 1997; Gregory, 1998) and as discussed previously, numerous studies have shown that Kupffer cells are the first target of ricin, occurring within 4 h after ricin administration (Skilleter, 1981; Magnuson, 1993). Furthermore, pretreatment of mice with anti-TNF-α antibody 2 h prior to ricin administration significantly reduced the urinary excretion of by-products of oxidative stress such as malondialdehyde, formaldehyde, and acetone and decreased hepatic lipid peroxidation and DNA single strand breaks (Muldoon et al., 1994). Results from other studies have also confirmed the role of TNF-α in ricin toxicity as evidenced by the findings where exposure of the macrophage J744A.1 cells to ricin resulted in TNF-α release and TNF-α antibody antagonized the cytotoxic effects of ricin (Hassoun, 2000). Ricin induced the release of TNF-α and interleukin-1 beta by human peripheral-blood mononuclear cells in a dose- and time-dependent manner (Licastro, 1993). More recently, it was reported that ricin induced the expression of mRNAs for TNF-α in the Caco-2 cell line, which is derived from a human colon carcinoma and has characteristics that closely resemble intestinal epithelial cells (Yamasaki, 2004). Exposure of RAW 264.7 cells to ricin strongly induced the activation of the JNK and p38 MAPK and produced a dose-dependent increase in TNF-α mRNA and TNF-α (Korcheva, 2005). Among the other protein synthesis inhibitors examined in another study, modeccin and anisomyxcin, known to trigger a ribotoxic stress response similar to ricin, also induced the release of TNF-α (Higuchi, 2003; Yamasaki, 2004).

It is well established that TNF-α regulates the inflammatory processes through the induction of genes that code for several immune mediators including interleukin (IL)-1, IL-6, IL-8, macrophage inflammatory protein (MIP)-2, granulocyte-macrophage colony-stimulating factor, intracellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1. These mediators are known to enhance vascular permeability, stimulate the expression of adhesion molecules on endothelial cells, and serve as leukocyte chemoattractants (chemokines). The expression of mRNAs for IL-8, monocyte chemoattractant protein, macrophage inflammatory protein (MIP)-1α and MIP-1β was also observed in the ricin-challenged Caco-2 cell line (Yamasaki, 2004). Similarly, the enhanced transcriptions of macrophage colony stimulating factor-1, CD14, monocyte chemoattractant protein-3, macrophage inflammatory protein-1β and intercellular adhesion molecule-1 were also observed in the lungs of ricin-exposed animals (DaSilva, 2003). In the same study, it was also demonstrated that ricin activated the nuclear factor (NF)-kappa B/rel family of transcription factors (nuclear factor of kappa light chain protein enhancer in B-cells inhibitor alpha (NFKBIA), I-kappa B, tumor necrosis factor alpha-induced protein (tnfaip)-3) as well as a number of inflammatory mediators,

including IL-6, serine protease inhibitor 2.2, and interferon regulatory factor-1. The findings from these studies possibly suggest that the sustained phagocytic activity and transendothelial trafficking of immune cells into the damaged lungs of ricin-challenged animals are mediated through TNF-α-regulated processes.

Various effects of TNF-α are mediated by the induction of a cellular state consistent with oxidative stress. Reactive oxygen species have been implicated in the signaling pathways initiated by TNF-α and these ROS may also function as second messengers for TNF-α mediated signaling (Hennet, 1993; Schulze-Osthoff, 1998). Exogenously added reactive oxygen species to cells reproduces many of the toxic actions of TNF-α. Cells treated with certain antioxidants or kept under anaerobic conditions were less susceptible to TNF-α-induced cytotoxicity (Schulze-Osthoff, 1992). Furthermore, ROS are critical in the regulation of transcription factors in the AP-1 and NF-κB families which have crucial functions in proliferation, differentiation, and morphogenesis and have been shown to be activated also in the absence of protein synthesis (Buscher, 1988; Devary, 1993).

Other studies have demonstrated that the mitochondrial electron transport system plays a key role in inducing TNF-α cytotoxicity, presumably by the formation of reactive oxygen species (Hennet, 1993; Schulze-Osthoff, 1998; Moreno-Manzano, 2000). Antimycin A, a mitochondrial inhibitor that increases the generation of reactive oxygen species, potentiates the TNF-α-triggered NF-κB activation while depletion of the mitochondrial oxidative metabolism resulted in resistance towards TNF-α cytotoxicity, as well as in inhibition of NF-κB activation by TNF-α (Schulze-Osthoff, 1993). It has been proposed that the apoptosis-inducing activity of abrin, a type II ribosomal inhibiting protein, could be due to a decrease in the levels of anti-apoptotic factors like Bcl-2 and oxidative stress due to mitochondrial damage leading to increased reactive oxygen production (Narayanan, 2004).

The mitochondrial pathway of apoptosis due to depletion of the intracellular NAD^+ pool is known to be a result of PARP [poly(ADP-ribose) polymerase] activation. Moderate activation of PARP facilitates the efficient repair of DNA damage induced by several stimuli, such as reactive oxygen species or ionizing radiation. Severe genotoxic stress leads to rapid energy consumption and subsequently cell death. It has been shown that ricin is capable of inducing depletion of intracellular NAD^+ and ATP levels, following PARP activation in U937 cells (Komatsu, 2000) resulting in apoptotic cell death. Another mechanism by which ricin may induce apoptotic cell death has centered on its ability to increase caspase -3-, caspase-6- and caspase-9- activities in human leukemia U937 cells (Villa, 1997; Komatsu, 1998; Tamura, 2002). Caspases are cysteinyl aspartate-specific proteases that play a crucial role in the apoptotic signal pathway, they are sensitive to the redox status of the cell, and their activity is blocked by excessive oxidative stress (Hampton, 1998). Komatsu et al., (Komatsu, 1998) have reported that a broad inhibitor of the caspase family proteases (Z-Asp-Ch2-DCB) and the specific serine protease inhibitor, dichloroisocoumarin, blocked ricin-induced apoptosis in human leukemia U937 cells. Furthermore, it was demonstrated that ricin-induced apoptosis of U937 cells (Oda, 1999) or human cervical cancer cells (Rao, 2005) was

associated with glutathione depletion. Although the exact role of glutathione depletion in apoptosis is unclear, selective depletion of reduced glutathione in mitochondria increase reactive oxygen cell accumulation and sensitizes cells to the apoptotic effects of TNF-α (Fernandez-Checa, 1998; Pierce, 2000).

Presently, the underlying mechanism(s) responsible for the release of TNF-α by ricin are not well understood. Higuchi et al., (Higuchi, 2004) have shown that treatment of RAW264.7 cells with ricin resulted in the induction of TNF-α , with the maximum levels of TNF-α secretion being attained at a ricin concentration that caused only partial inhibition of protein synthesis. Results from a limited number of studies examining the effect of different protein synthesis inhibitors on the induction of TNF-α release, suggested that ricin and anisomycin, which commonly act on 28rRNA and resulting in a ribotoxic stress response, may trigger the multiple signal transduction pathways through the activation of the stress-activated protein kinases (SAPKs), N-terminal-c-Jun-kinases (JNK), and p38 MAP kinase (Korcheva, 2005; Laskin, 2002; Iordanov, 1997). A major question remains as to the precise linkage between ribosomal RNA damage and induction of MAP kinase signaling cascades (Iordanov, 1997; Iordanov, 1998; Laskin, 2002).   It is becoming clear that the ribotoxic stress pathway is a highly specific event and ribotoxic stressors appear to be restricted to those toxicants that interact with or damage the R/S loop near the 3-end of the 28S rRNA including ricin, anisomycin, palytoxin, and ultraviolet light radiation (Iordanov, 1997; 1998; Laskin, 2002). More recently, it was shown that the IL-8 inducing activity in ricin-challenged Caco-2 cells correlates with the RNA N-glycosidase activity and not with general protein synthesis and among the RNA N-glycosidases tested , the members of the Shiga toxin are the strongest inducers of IL-8 production in Caco-2 cells (Yamasaki, 2004).

MANAGEMENT OF RICIN-INTOXICATION.

Management of ricin-intoxicated patients depends on the route of exposure. Patients with pulmonary intoxication are managed by supportive treatment for pulmonary edema. Gastrointestinal intoxication is best managed by vigorous gastric decontamination with superactivated charcoal, followed by use of cathartics such as magnesium citrate. Volume replacement of GI fluid losses is important. In percutaneous exposures, treatment would be primarily supportive (Franz, 1997).

The cell responds to ricin toxicity by activating its defensive mechanisms such as evoking inflammatory processes, altering protein trafficking, cell differentiation and turning on apoptotic signals (Day, 2001; Elson, 2001; Hu, 2001). Very little is known of the role that these or any other gene regulatory paths may play in the intoxication process initiated by ricin. However, the knowledge of such complex paths is critical for the development of effective prophylactic and therapeutic countermeasures to ricin. So far, it has been shown that the ricin induced expression of TNF-α protein in RAW 264.7 macrophages was reduced significantly following the specific inhibition of JNJ, p38 MAPK, and ERK1/2 kinases known to regulate the expression of mRNAs that encode inflammatory cytokines and chemokines.   In another study, the ricin-induced cytotoxicity in macrophage J744A.1 cells was antagonized by pretreatment with a TNF-α

-antibody (Hassoun, 2000). The unregulated release of TNF-α into the circulation results in circulatory dysfunction, increased endothelial permeability and inflammation of different organs.

Antioxidant supplementation may prove to be beneficial in decreasing the oxidative stress in animals and cellular systems exposed to ricin. Several studies have shown that TNF-α induces O_2^{--} formation in mitochondria which dismutates to H_2O_2 by the superoxide dismutase (Mn SOD). H_2O_2 can easily cross the mitochondrial membrane and be released into the cytosol where it could induce stimulate mitogen-activated protein kinases, such as ERK1/2, p38 and JNK, and the transcription factor, nuclear factor-kappaB, known to induce the expression of mRNA of a variety of pro-inflammatory mediators including TNF-α, interleukins, adhesion molecules and enzymes, such as cyclooxygenase (COX-2) and iNOS. These pro-inflammatory mediators are implicated in the pathogenesis of several acute inflammatory disorders. Also, TNF-α released during inflammation transiently activates neutrophils and macrophages, thereby causing them to release O_2^{-} as a consequence of the activation of membrane-associated NADPH oxidase (Klebanoff, 1986). Besides NADPH oxidase, another superoxide-generating enzyme thought to be induced after ricin exposure is xanthine oxidase (XO).

It is evident that antioxidants can protect both extracellularly by scavenging toxic ROS and intracellularly by interrupting lipid peroxidation within the membrane. Also, they can interfere early in inflammatory responses by blocking or modifying the signal transduction of inflammatory cytokines and ricin, thereby modulating cellular activation. Administration of vitamin E succinate and butylated hydroxyanisole extended the survival time in rats challenged with a lethal dose of ricin (Muldoon, 1994) The antioxidant N-acetylcysteine (NAC) strongly inhibited the ricin-induced apoptotic cell death in U937 cells (Oda, 1999). Vitamin E and NAC as well as other antioxidants are known to inhibit mitogen-activated protein kinases, such as ERK1/2, p38 and JNK, and the transcription factor NF-κB (Kyaw, 2004; Zafarullah, 2003; Cadenas, 2002). Antioxidants do not exert an indiscriminate stimulating effect on the immune cell function, are effective and safe in a large range of concentrations.

SUMMARY.

The cytotoxicity of ricin is commonly attributed to the inhibition of protein synthesis in ribosomes. To-date there are no effective prophylactic and therapeutic countermeasures to ricin and management of ricin intoxication remains supportive. More recent studies provide evidence that ricin can also mediate its toxic effects via oxidative stress-mediated mechanisms resulting to apoptosis and cell death. Ricin has been shown to selectively activate phagocytes, known to generate excessive amounts of reactive oxygen species, which in addition to their direct toxic actions, also exacerbate the inflammatory process and cause further tissue damage. The reduction of inflammation has been suggested as a viable drug target for the toxic pulmonary effects of ricin.

Presently, inflammatory mediators and other gene regulatory circuits are being investigated in order to assess the role they may play in the intoxication process initiated

by ricin. The knowledge of such complex paths and their manipulation is critical for the development of effective prophylactic and therapeutic countermeasures to ricin. Certainly, antioxidant supplementation may prove to be beneficial in decreasing the oxidative stress in animals and cellular systems exposed to ricin.

REFERENCES.

Allison, W. S. (1976) Formation and reactions of sulfenic acids in proteins. Acc. Chem. Res. 9, 293-299.

Anderson, M.E. (1997) Glutathione and glutathione delivery compounds. Adv. Pharmacol. 38, 65-78.

Anderson, M.E.; Luo, J.L. (1998) Glutathione therapy: from prodrugs to genes. Semin. Liver Dis. 18, 415-424.

Baluna, R.; Coleman, E.; Jones, C.; Ghetie, V.; Vitetta, E.S. (2000) The effect of a monoclonal antibody coupled to ricin A chain-derived peptides on endothelial cells in vitro: insights into toxin-mediated vascular damage. Exp Cell Res. 258, 417-424.

Battelli, M.G.; Buonamici, L.; Polito, L.; Bolognesi, A.; Stirpe, F. (1996) Hepatotoxicity of ricin, saporin or a saporin immunotoxin: xanthine oxidase activity in rat liver and blood serum. Virchows Arch 427, 529-535.

Battelli, M.G.; Muisiani, S.; Monti, B.; Buonamici, L.; Sparapani, M.; Contestabile, A.; Stirpe, F. (2001). Ricin toxicity to microglial and monocytic cells. Neurochem International 39, 83-93.

Bauer V.; Bauer F. (1999) Reactive oxygen species as mediators of tissue protection and injury. Gen Physiol Biophys.18, 7-14.

Bernardi, P. (1996) The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. Biochem. Biophys. Acta 1275, 5-9.

Bernardi, P.; Petronilli, V. (1996) The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J. Bioenerg Biomembr. 28, 129-136.

Bhattacharyya, J.; Biswas, S.; Datta, A.G. (2004) Mode of action of endotoxin: role of free radicals and antioxidants. Curr Med Chem. 11, 359-368.

Bies, C.; Lehr, C-M.; Woodley, J.F. (2004) Lectin-mediated drug targeting: history and applications. Adv Drug Deliv Rev 56:425-435.

Bingen, A.; Creppy, E.E.; Gut, J.P.; Dirheimer, G.; Kirn, A. (1987) The Kupffer cell is the first target in ricin-induced hepatitis. J Submicrosc Cytol. 19, 247-256.

Bismuth, C.; Borron, S.W.; Baud, F.J.; Barriot, P. (2004) Chemical weapons: documented use and compounds on the horizon. Toxicol. Lett. 149, 11-18.

Boulton,R.; Woodman,A.; Calnan,D.; Selden,C.; Tam,F.; Hodgson,H. (1997) Nonparenchymal cells from regenerating rat liver generate interleukin-1 alpha and -1 beta: a mechanism of negative regulation of hepatocyte proliferation. Hepatology, 26, 49–58.

Bradham,C.A.; Plümpe,J.; Manns,M.P.; Brenner,D.A.; Trautwein,C. (1998) Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am. J. Phys., 275, G387–G392.

Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys. 300, 535-543.

Buscher, M.; Rahmsdorf, H.J.; Litfin, M.; Karin, M.; Herrlich, P. (1988) Activation of the *c-fos* gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. Oncogene 3, 301-311.

Cadenas, S.; Cadenas, A.M. (2002) Fighting the stranger-antioxidant protection against endotoxin toxicity. Toxicol. 180, 45-63.

Carr, A.; Frei, B. (1999) Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J. 13, 1007-1024.

Chow, C.K. (2004) Biological functions and metabolic fate of vitamin E revisited. J Biomed Sci. 11, 295-302.

Dalton, T. P.; Shertzer, H.G.; Puga, A (1999). Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol, 39, 67-101.

DaSilva, L.; Cote, D.; Roy, C.; Martinez, M.; Duniho, S.; Pitt, M.L.; Downey, T.; Dertzbaugh, M. (2003) Pulmonary gene expression profiling of inhaled ricin. Toxicon. 41, 813-822.

Day, P.J.; Owens, S.R.; Wesche, J.; Olsnes, S.; Roberts, L.M.; Lord, J.M., (2001). An interaction between ricin and calreticulin that may have implications for toxin trafficking. J. Biol. Chem. 276, 7202–7208.

Devary, Y.; Rosette, C.; DiDonato, J.A.; Karin, A. (1993) NF-kappa B activation by ultraviolet light not dependent on a nuclear signal. Science 261, 1442-1445.

Di Virgilio, F. (2004) New pathways for reactive oxygen species generation in inflammation and potential novel pharmacological targets. Curr Pharm Des.10, 1647-1652.

Djordjevic, V.B. (2004) Free radicals in cell biology. Int Rev Cytol. 237:57-89.

Elson, D.A.; Thurston, G.; Huang, L.E.; Ginzinger, D.G.; McDonald, D.M.; Johnson, R.S.; Arbeit, J.M., (2001). Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1 alpha. Genes Dev. 15, 2520–2532.

Endo, Y.; Tsurugi, K. (1987a). RNA *N*-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J. Biol. Chem. 262, 8128–8130.

Endo, Y.; Mitsui, K.; Motizuki, M.; Tsurugi, K. (1987b). The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. J. Biol. Chem. 262, 5908–5912.

Evans, P.; Halliwell, B. (2001) Micronutrients: oxidant/antioxidant status. Br. J. Nutr. 85, S67-S74

Fang, Y.Z.; Yang, S.; Wu, G. (2002) Free radicals, antioxidants, and nutrition. Nutrition. 18, 872-879.

Folkes, L. K; Candeias, L.P.; Wardman, P. (1995) Kinetics and mechanisms of hypochlorous acid reactions. Arch. Biochem. Biophys. 323, 120-126.

Fu, X.J.; Iijima, K.; Nozu, K.; Hamahira, K.; Tanaka, R.; Oda, T.; Yoshikawa, N.; Matsuo, M. (2004) Role of p38 MAP kinase pathway in a toxin-induced model of hemolytic uremic syndrome. Pediatr. Nephrol. 19, 844-852.

Gareth, D.; Griffiths, G.D.; Rice, P.; Allenby, A.C.; Bailey, S.C.; Upshall, D.G., (1995). Inhalation toxicology and histopathology of ricin and abrin toxins. *Inhal. Toxicol.* 7, 269–288.

Halliwell, B. (1996) Vitamin C: antioxidant or pro-oxidant *in vivo*? Free Rad. Res. 25, 439-454.

Hartley, M.R.; Lord, J.M. (2004) Cytotoxic ribosome-inactivating lectins from plants. Biochem. Biophys. Acta 1701, 1-14.

Hennet, T.; Richter, C.; Peterhans, E. (1993) Tumour necrosis factor-alpha induces superoxide anion generation in mitochondria of L929 cells. Biochem J. 289, 587-592.

Fernandez-Checa, J.C.; Kaplowitz, N.; Garcia-Ruiz, C.; Colell, A. (1998) Mitochondrial glutathione:importance and transport. Semin Liver Dis. 18, 389-401.

Franz, D.R.; Jaax, N.K. (1997) Ricin Toxin. In: Medical Aspects of Chemical and Biological Warfare. Borden Institute, Walter Reed Army Medical Center, Washington, DC, Chapter 32, pp. 631-642.

Fodstad, O.; Olsnes, S.; Pihl, A. (1976) Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice. Br J Cancer 34, 418-425.

Fodstad, O.; Johannessen J.V.; Schjerven, L.; Pihl, A. (1979) Toxicity of abrin and ricin in mice and dogs. J Toxicol Environm Health 5, 1073-1084.

Forman, H.J.; Torres, M. (2001) Redox signaling in macrophages. Mol Aspects Med.22, 189-216.

Forman, H.J.; Torres, M. (2002). Reactive oxygen species and cell signaling. Respiratory burst in macrophage signaling. Am J Respir Crit Care Med 166, S4-S7.

Goldenthal, M.J.; Marin-Garcia, J. (2004) Mitochondrial signaling pathways: a receiver/integrator organelle. Mol Cell Biochem. 262, 1-16.

Greenfield, R.A.; Brown, B.R.; Hutchins, J.B.; Iandolo, J.J.; Jackson, R.; Slater, L.N.; Bronze, M.S. (2002). Microbiological, biological, and chemical weapons of warfare and terrorism. Am J Med Sci 323, 326-340.

Gregory,S.; Wing,E.; Danowski,K.; van Rooijen,N.; Dyer,K.; Tweardy,D. (1998) IL-6 produced by Kupffer cells induces STAT protein activation in hepatocytes early during the course of systemic listerial infections. J. Immunol. 160, 6056–6061.

Gutteridge, J.M.; Halliwell, B. (2000) Free radicals and antioxidants in the year 2000. A historical look to the future. Ann N Y Acad Sci. 899:136-147.

Hampton, M. B.; Orrenius S. (1998). "Redox regulation of apoptotic cell death in the immune system." Toxicol Lett 102-103: 355-358.

Hassoun, E.A.; Wang, X. (1999) Time-and concentration-dependent production of superoxide anion, nitric oxide, DNA damage and cellular death by ricin in the J774A.1 macrophage cells. J Biochem Mol Toxicol 13, 179-185.

Hassoun, E.; Wang, X. (2000) Ricin-induced toxicity in the macrophage J744A.1 cells: the role of TNF-α and the modulation effects of TNF-α polyclonal antibody. J Biochem MolToxicol 14, 95-101.

Higuchi, S.; Tamura, T.; Oda, T. (2003) Cross-talk between the pathways leading to the induction of apoptosis and the secretion of tumor necrosis factor-alpha in ricin-treated RAW 264.7 cells. J Biochem (Tokyo) 134, 927-933.

Howat, A.J. (1988) The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissues and intestine. J Pathol 154, 29-33.

Hsu, W.; Kerppola, T.K.; Chen, P.L.; Curran, T.; Chen-Kiang, S. 1994) Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. Mol Cell Biol 14: 268-276.

Hu, R.; Zhai, Q.; Liu, W.; Liu, X. (2001). An insight into the mechanisms of cytotoxicity of ricin to hepatoma cell: roles of bcl-2 family proteins, caspases, $Ca^{(2+)-}$dependent proteases and protein kinase C. J. Cell. Biochem. 81, 583–593.

Iordanov, M.S.; Pribnow, D.; Magun, J.L.; Dinh, T.H.; Pearson, J.A.; Chen, S.L.; Magun, B.E. (1997) Ribotoxic stress response: Activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the α-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol 17, 3373-3381.

Iordanov, M.S.; Magun, B.E. (1999) Different mechanisms of c-Jun NH(2)-terminal kinase-1 (JNK1) activation by ultraviolet-B radiation and by oxidativestressors. J Biol Chem 274, 25801-25806.

Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356:1-11.

Jang, J. H.; Surh, Y.J. (2004). "Bcl-2 attenuation of oxidative cell death is associated with up-regulation of gamma-glutamylcysteine ligase via constitutive NF-kappaB activation." J Biol Chem 279: 38779-38786.

Kadenbach, B.; Arnold, S.; Lee, I.; Huttemann, M. The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochem Biophys Acta 1655:400-408.

Kamata, R.; Hirata, H. (1999) Redox regulation of cellular signalling. Cell Signal. 11:1-14.

Kerppola, T.K.; Curran, T. (1994). A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. Oncogene 9: 3149-318.

Klebanoff, S.J.; Vadas, M.A.; Harlan, J.M.; Sparks, L.H.; Gamble, J.R.; Agosti, J.M.; Waltersdorph, A.M. (1986) Stimulation of neutrophils by tumor necrosis factor. J. Immunol. 136, 4220–4225.

Klebanoff, S. J. (1991) Myeloperoxidase: occurrence and biological function. In: J. Everse, K. E. Everse, and M. B. Grisham (eds) Peroxidases in Chemistry and Biology, Boca Raton, FL: CRC, p. 1-35.

Komatsu, N.; Oda, T.; Muramatsu, T. (1998) Involvement of both caspase-like proteases and serine proteases in apoptotic cell death induced by ricin, modeccin, diphtheria toxin, and Pseudomonas toxin. J. Biochem 124, 1038-1044.

Korcheva, V.; Wong, J.; Corless, C.; Iordanov, M.; Magun, B. (2005) Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and p38 MAPK and provides a mouse model of hemolytic uremic syndrome. Am. J. Pathol. 166, 323-339.

Kroemer, G. P.; Petit, N.; Zamzami, J.; Vayssiere, L.; Mignotte, B. (1995) The biochemistry of programmed cell death. FASEB J. 9, 1277-1287.

Kumar, O.; Sugendran, K.; Vijayaraghavan, R. (2003) Oxidative stress associated hepatic and renal toxicity induced by ricin in mice. Toxicon. 41, 333-338.

Kyaw,M.; Yoshizumi, M.; Tsuchiya, K.; Izawa, Y.; Kanematsu, Y.; Tamaki, T. (2004) Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases. Acta Pharmacol Sin. 25, 977-985.

Laskin, J.D.; Heck, D.E.; Laskin, D.L. (2002) The ribotoxic stress response as a potential mechanism for MAP kinase activation in xenobiotic toxicity. Toxicol Sci. 69, 289-291.

Licastro, F.; Morini, M.C.; Bolognesi, A.; Stirpe, F. (1993) Ricin induces the production of tumour necrosis factor-alpha and interleukin-1 beta by human peripheral-blood mononuclear cells. Biochem J. 294 (Pt 2), 517-520.

Lord, J.M.; Roberts, L.M. (1998). Toxin entry: retrograde transport through the secretory pathway. J Cell Biol 140, 733-736.

Lukacs, N.W.; Ward, P.A. (1996). Inflammatory mediators, cytokines, and adhesion molecules in pulmonary inflammatory and injury. Adv. Immunol. 62, 257–305.

Magnusson, S.; Berg, T. (1993) Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells. Biochem J. 291, 749-755.

McKeon, T.A.; Chen, G.Q.; Lin, J.-T. (2000) Biochemical aspects of castor oil biosynthesis. Biochem. Society Transact. 28, 972-974.

Menten, P.; Wuyts, A.; Van Damme, J. (2001). Monocyte chemoattractant protein-3 Eur. Cytokine Netw. 12, 554–560.

Moreno-Manzano, V.; Ishikawa, Y.; Lucio-Cazana, J.; Kitamura, M. (2000) Selective Involvement of Superoxide Anion, but Not Downstream Compounds Hydrogen Peroxide

and Peroxynitrite, in Tumor Necrosis Factor-α-induced Apoptosis of Rat Mesangial Cells. J Biol Chem, 275, 12684-12691.

Muldoon, D.F.; Hassoun, E.A.; Stohs, S.J. (1992) Ricin-induced hepatic lipid peroxidation, glutathione depletion, and DNA single-strand breaks in mice. Toxicon. 30, 977-984.

Muldoon, D.F.; Bagchi, D.; Hassoun, E.A.; Stohs, S.J. (1994) The modulating effects of tumor necrosis factor alpha antibody on ricin-induced oxidative stress in mice. J Biochem Toxicol. 9, 311-318.

Muldoon, D.F.; Stohs, S.J. (1994) Modulation of ricin toxicity in mice by biologically active substances. J Appl Toxicol 14, 81-86.

Narayanan, S.; Surolia, A.; Karande, A.A. (2004) Ribosome-inactivating protein and apoptosis: abrin causes cell death via mitochondrial pathway in Jurkat cells. Biochem. J. 377, 233-240.

Oda, T.; Iwaoka, J.; Komatsu, N.; Muramatsu, T. (1999) Involvement of N-acetylcysteine-sensitive pathways in ricin-induced apoptotic cell death in U937 cells. Biosci. Biotechnol. Biochem. 63, 341-348.

Olsnes, S; Pihl, A. (1972) Ricin-a potent inhibitor of protein synthesis. FEBS Lett. 20, 327-329.

Olsnes, S. (2004) The history of ricin, abrin and related toxins. Toxicon 44, 361-370.

Orrenius, S. (2004) Mitochondrial regulation of apoptotic cell death. Toxicol Lett. 149:19-23.

Pierce, R.H.; Cambnell, J.S.; Stephenson, A.B.; Franklin, C.C.; Chaisson, M.; Poot, M.; Kavanagh, T.J.; Rabinovitch, P.S.; Fausto, N. (2000) Disruption of redox homeostatsis in tumour necrosis-factor-induced apoptosis in a murine hepatocyte cell line. Am. J. Pathol. 157:221-236.

Ramsden, C.S.; Drayson, M.T.; Bell, E.B. (1989) The toxicity, distribution and excretion of ricin holotoxin in rats. Toxicol. 55, 161-171.

Rao, L.P.V.; Jayaraj, R.; Bhaskar, A.S.B.; Kumar, O.; Bhattacharya, R.; Saxena, P.; Dash, P.K.; Vijayaraghavan. (2005) Mechanism of ricin-induced apoptosis in human cervical cancer cells. Biochem. Pharmacol. 69, 855-865.

Roberts, L.M.; Smith D.C. (2004) Ricin: the endoplasmic reticulum connection. Toxicon 44, 469-472.

Roy, C.J.; Hale, M.; Hartings, J.M.; Pitt, L.; Duniho, S. (2003) Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. Inhal Toxicol. 5, 619-638.

Sadani, G.; Nadkarni, G.D. (1994) Role of antioxidant enzyme defence in sparing rat hepatocytes from toxicity of ricin at low dose. Indian J Exp Biol 32, 354-355.

Sadani, G.R.; Soman, C.S.; Deodhar, K.K.; Nadkarni, G.D. (1997) Reactive oxygen species involvement in ricin-induced thyroid toxicity in rat. Hum Exp Toxicol 16, 254-256.

Saini, K. S.; N. I. Walker. (1998) Biochemical and molecular mechanisms regulating apoptosis. Mol. Cell. Biochem. 178, 9-25.

Sandvig, K.; Van Deurs, B. (2002) Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. FEBS Lett. 529, 49-53.

Schafer, F.Q.; Buettner, G.R. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 30, 1191-1212.

Schnell, R.; Borchmann, P.; Staak, J.O.; Schindler, J.; Ghetie, V.; Vitetta, E.S.; Engert, A. (2003) Clinical evaluation of ricin A-chain immunotoxins in patients with Hodgkin's lymphoma. Ann Oncol. 14, 729-736.

Schulze-Osthoff, K.; Bakker, A. C.; Vanhaesebroeck, B.; Beyaert, R.; Jacob, W. A.; Fiers, W. (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J. Biol. Chem. 267, 5317-5323.

Schulze-Osthoff, K.; Beyaert, R.; Vandevoorde, V.; Haegeman, G.; Fiers, W. (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J. 12, 3095-3104.

Schulze-Osthoff, K.; Ferrari, D.; Los, M.; Wesselborg, S.; Peter, M.E. (1998). Apoptosis signaling by death receptors. Eur. J Biochem 254, 439-459.

Schulze-Osthoff, K.; Bakker, A.C.; Vanhaesebroeck, B.; Beyaert, R.; Jacob, W.A.; Fiers, W. (1992) Cytotoxic activity of tumour necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267, 5317-5323.

Shih, S.F.; Wu, Y.H.; Hung, C.H.; Yang, H.Y.; Lin, J.Y. (2001) Abrin triggers cell death by inactivating a thiol-specific antioxidant protein. J Biol Chem. 276, 21870-21877.

Sjogren, R.W.; Colleton, C.; Shea-Donohue, T. (1994) Intestinal myoelectric response in two different models of acute enteric inflammation. Am J Physiol. 267, G329-37.

Sies, H. (1987). Antioxidant activity in cells and organs. Am. Rev. Respir. Dis. 136, 478-480.

Simmons, B.M.; Stahl, P.D.; Russell, J.H. (1986) Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. Multiple intracellular pathways for A chain translocation. J Biol chem. 261, 7912-7920.

Skilleter, D.N.; Paine, A.J.; Stirpe, F. (1981) A comparison of the accumulation of ricin by hepatic parenchymal and non-parenchymal cells and its inhibition of protein synthesis. Biochim Biophys Acta. 677, 495-500.

Smallshaw, J.E.; Firan, A.; Fulmer, J.R.; Riback, S.L.; Ghetie, V.; Vitetta, E.S. (2002) A novel recombinant vaccine which protects mice against ricin intoxication. Vaccine 20, 3422-3427.

Soler-Rodriquez, A.; Ghetie, M.; Oppenheimer-Marks N.; Uhr, J.; Vitetta E. (1993) Ricin-A chain and ricin-A-chain immunotoxins rapidly damage human endothelial cells: Implications for vascular leak syndrome. Exp Cell Res 206, 227-234.

Stahl, P.; Gordon, S. (1982) Expression of a mannosyl-fucosylreceptor for endocytosis on cultured primary macrophages and their hybrids. J Cell Biol 93, 49-56.

Stein, M.; Keshav, S.; Harris, N.; Gordon, S. (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of altyernative immunologic macrophage activation. J Exp Med 176, 287-292.

Stohs, S. J.; Bagchi, D. (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med. 18, 321-336.

Stojadinovic, A.; Kiang, J.; Goldhill, J.; Matin, D.; Smallridg,e R.; Galloway, R.; Shea-Donohue, T. (1997) Induction of the heat shock response prevents tissue injury during acute inflammation of the rat ileum.Crit Care Med. 25, 309-317.

Stirpe, F. (2004) Ribosome-inactivating proteins. Toxicon 44, 371-383.

Suntres, Z.E. (2002) Role of Antioxidants in Paraquat Toxicity. Toxicol. 180, 65-77.

Suntres, Z.E.; Shek, P.N. (1996) The pulmonary uptake of intravenously administered liposomal α-tocopherol liposomes is augmented in acute lung injury. J. Drug Target. 4, 151-159.

Suntres, Z.E.; Shek, P.N. (1998) Prophylaxis of LPS-induced lung injury by α-tocopherol liposomes. Crit Care Med, 26, 723-729.

Tas, S.W.; Remans, P.H.; Reedquist, K.A.; Tak, P.P. (2005) Signal transduction pathways and transcription factors as therapeutic targets in inflammatory disease: towards innovative antirheumatic therap Curr Pharm Des.11:581-611..

Tamura, T.; Sadakata, N.; Oda, T.; Muramatsu, T. (2002). Role of zinc ions in ricin-induced apoptosis in U937 cells. Toxicol. Lett. 132, 141-151.

Thomas, E. L.; Grisham, M.B.; Jefferson, M.M. (1983) Myeloperoxidase-dependent effect of amines on functions of isolated neutrophils. J. Clin. Invest. 72, 441-454.

Toyokuni, S. (1999) Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int. 49, 91-102.

Vandenabeele,P.; Declercq,W.; Beyaert,R.; Fiers,W. (1995) Two tumour necrosis factor receptors: structure and function. Trends Cell Biol. 5, 392–399.

Villa, P.; Kaufmann, S.H.; Earnshaw, W.C. (1997) Caspases and caspase inhibitors. TIBS Rev. 22, 388-393.

Winterbourn, C. C. (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. Biochim. Biophys. Acta 840, 204-210.

Winterbourn, C. C; van den Berg, J.J.M.; Roitman, E.; Kuypers, F.A. (1992) Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid. Arch. Biochem. Biophys. 296, 547-555.

Wilhelmsen, C.L.; Pitt, M.L. (1996) Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys. Vet. Pathol. 33:296-302.

Williams, J.M.; Lote, C.J.; Thewles, A.; Wood, J.A.; Howie, A.J.; Williams, M.C.; Adu, D.A.; Taylor, C.M. (2000) Role of nitric oxide in a toxin-induced model of haemolytic uraemic syndrome. Pediatr Nephrol. 14, 1066-1070.

Xanthoudakis, S.; Miao, G.G.; Curran, T. (1994) The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. Proc Natl Acad Sci U S A 91: 23-27.

Xu, L.L.; McVicar, D.W.; Ben-Baruch, A.; Kunhs, D.B.; Johnston, J.; Oppenheim, J.J.; Wang, J.M. (1995) Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils. Eur. J. Immunol. 25, 2612–2617.

Yamasaki, C.; Nishikawa, K.; Zeng, X.T.; Katayama, Y.; Natori, Y.; Komatsu, N.; Oda, T.; Natori, Y (2004). Induction of cytokines by toxins that have an identical RNA N-

glycosidase activity: Shiga toxin, ricin, and modeccin. Biochim Biophys Acta. 1671,44-50.

Zafarullah, M.; Li, W.Q.; Sylvester, J.; Ahmad M., (2003) Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci. 60, 6-20.

Zenilman, M.E.; Fiani, M.; Stahl, P.D.; Brunt, E.M.; Flye, M.W. (1988) Use of ricin A-chain to selectively deplete Kupffer cells. J Surg Res. 45, 82-89.

Zenilman, M.E.; Fiani, M.; Stahl, P.D.; Brunt, E.M.; Flye, M.W. (1989) Selective depletion of Kupffer cells in mice by intact ricin. Transplantation. 47, 200-203.

Zoratti, M.; Szabo, I. (1995) The mitochondrial permeability transition. Biochem. Biophys. Acta. 124, 139-176.

12 Vesicants and Oxidative Stress

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CONTENTS

250   Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology, and Therapeutics

I. BACKGROUND

The primary treatment of vesicant exposure is decontamination and supportive therapies; there is only one vesicant that has an antidote. The paucity of the treatment options continues to confer their tactical advantage. The development of an ameliorative or antidote would accomplish, at a minimum, two goals: (1) effective treatment, if needed, and (2) decreased tactical advantage.

They are considered to be of low technology and are relatively simple to manufacture. Barrier protection gear, otherwise known as Mission Oriented Protective Posture (MOPP), provides significant protection if donned before exposure. During battlefield conditions, errors would be expected in the use of the MOPP gear because of stressful situations, tears in the suit, false positive or negative alarms, etc.

The vesicants share some common properties that are noteworthy in attempts to achieve a better understanding of their pathogenesis. One characteristic is that they all induce an acute inflammatory reaction (Sidell et al., 1997; Sciuto, 1998; Ricketts et al., 2000; Naghii, 2002; Segal and Lang, 2005), of which a subcomponent is oxidative stress (OS) (Bartsch and Nair, 2006). A consequence of OS is the oxidation of thiol groups, which is seen in all of the vesicant exposures (Vissers and Winterbourn, 1995; Pant et al., 2000; Carr et al., 2001).

The oxidation of thiol groups disrupts redox balance (defined later in this chapter), which can set into motion a cascade of events, such as apoptosis, oxidant production, and increased activity of redox-regulated transcription factors (e.g., nuclear factor kappa beta [NF-κB]). The occurrence of OS is not isolated to the vesicant class of weapons of mass destruction (WMD). Radiation (Kang et al., 2006), bacterial infections (e.g., anthrax) (Hanna et al., 1994; Kuhn et al., 2006), viral infections (e.g., influenza) (Ghezzi and Ungheri, 2004), and ricin (Kumar et al., 2003; Suntres et al., 2005) exposures also induce OS as part of the host pathogen response, which is acute inflammation.

Sublethal OS induces varying degrees of damage in cellular organelles. Lethal OS occurs when antioxidant defenses completely fail, resulting in necrosis (Virag, 2005) or apoptosis (Haddad, 2004). In this chapter, OS will be discussed as a concept, along with its occurrence in the organ systems that are most notably affected by the vesicants. Antioxidant defenses that are critical to the maintenance of redox balance are also discussed.

Achieving a deeper understanding of OS is important for the elucidation of all acute inflammatory disorders. The OS component of vesicant exposure has not been a focal point of research, by
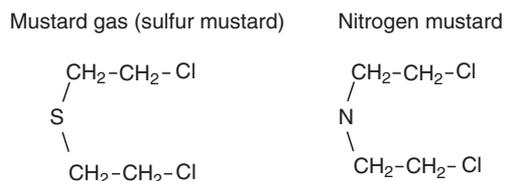
Mustard gas (sulfur mustard) Nitrogen mustard

CH$_2$–CH$_2$– Cl   CH$_2$–CH$_2$–Cl
/   /
S   N
\   \
CH$_2$–CH$_2$–Cl   CH$_2$–CH$_2$– Cl

FIGURE 12.1 Chemical structure of sulfur and nitrogen mustards.

and large for vesicants or other WMD. It is hoped that this chapter will help to spur future research in the area.

II. INTRODUCTION

There are four primary vesicating agents–mustard, phosgene, chlorine, and Lewisite. Strategically, they are poor weapons because they are subject to being redirected by the wind. Sulfur mustards (SM) are considered to be one of the vesicants that cause the most concern. There are two types of mustards, sulfur- and the nitrogen-based compounds (see Figure 12.1). The nitrogen mustards have been found to be unsuitable for warfare; therefore, there will be no further mention of them. Any further references to mustards will be to SM. Strategically, Lewisite is the least important since there is a proven antidote. British anti-Lewisite (BAL), the antidote for Lewisite, has been known for decades (Goldman and Dacre, 1989).   AQ1

A significant component of acute inflammation is OS (Nonas et al., 2006). One aspect of OS is the production of oxidants and proinflammatory cytokines. It is defined as an imbalance of antioxidant/oxidant ratio, which has a consequent effect on gene expression. The maintainence of the antioxidant/oxidant ratio in the nonstressed cell would be termed redox homeostasis. Thiol groups that are part of intracellular proteins and glutathione (GSH) are critical for the maintenance of redox homeostasis. The vesicants as a class are either oxidants themselves, or they indirectly produce oxidants. Oxidants can be water soluble and fat soluble; therefore, they can arise in any compartment of the cell. They are able to attack any cellular structure, rendering them partially or completely dysfunctional.

The chemical WMD classified as vesicants cause blistering of the skin, which is why they are referred to as blistering agents. They incapacitate more so than kill the exposed person because burns occur on any tissue that it contacts. They all have prolonged systemic effects. The reader is referred to the individual sections of this book and other excellent reviews on the agents for a more detailed classic description of their effects, usage, and history (Goldman and Dacre, 1989; Naghii, 2002; Sciuto and Hurt, 2004; Segal and Lang, 2005). The agents themselves will be reviewed with the features of OS highlighted where information was available.

III. MUSTARD

Vesicants were first deployed against troops during World War I (WWI). They were used as recently as 1988 by Saddam Hussein against the Kurds in Halabja. SM (2-bis-chloroethyl ethyl chloride) is a straw-colored liquid that disseminates with a garlic-like odor on evaporation (Duke-Elder and MacFaul, 1972; Dahl et al., 1985). SM is composed of small, oily droplets with volatility   AQ2
significantly higher in warmer climates (e.g., the Middle East). At a temperature of 38°C, it can be present in the environment for 7 h, whereas at 10°C, it persists for 100 h. The density of mustards is 5.4-fold greater than air and tends to be found 6–12 inches above the ground. This ground-hugging characteristic causes it to sink into trenches and gullies. In WWI, soldiers frequently removed their masks in the morning, assuming that the mustard threat had subsided, and were unaware that the

mustard persisted in the environment. As the ambient temperature increased at sunrise, there was an increase in evaporation of the mustard at the ground-level atmosphere, and the soldiers would unknowingly inhale the newly evaporated gas. Mustard has a high freezing temperature (57°F). This freezing temperature can be reduced by mixing it with other agents, such as chlorobenzene or carbon tetrachloride (Borak and Sidell, 1992; Sidell et al., 1997). Similar reductions in freezing point can be accomplished by mixing it with Lewisite.

SM is commonly referred to as an alkylating agent, but recent evidence has shown it to be a potent inducer of oxidants (Levitt et al., 2003; McClintock et al., 2006). Mustard gas has a strong, irritating effect on living tissue and induces long-lasting, toxic effects (Safarinejad et al., 2001). Additionally, its destructive effects are not localized to the site of application; remote cells and tissues are also affected. SM damages DNA mainly by alkylating and cross-linking the purine bases (Fox and Scott, 1980). Cysteine groups in proteins make them sensitive to SM, resulting in covalently cross-linked dimers on exposure (Byrne et al., 1996). This cross-linking causes conformational change or dysfunction of enzymes. The potent alkylating activity of SM is due to the formation of highly electrophilic ethylene episulphonium derivative (Lundlum et al., 1984; Papirmeister et al., 1991). A portion of the inhalation pathogenesis of SM is due its cholinergic properties (mediating the action of acetylcholine) that stimulates both muscarinic and nicotinic receptors (Anslow, 1946).   AQ3

On contact with human skin, 80% of the liquid evaporates and 20% penetrates (half remains on the skin and the other half is absorbed systemically). Systemic absorption results in partitioning to organs, such as the spleen, liver, and bone marrow (Langenberg et al., 1998). It is mutagenic and carcinogenic (Papirmeister et al., 1985; Somani and Babu, 1989; Wormser, 1991; Langenberg et al., 1998). The lethal dose for humans is 200 mg if ingested and 3 g with cutaneous exposure (Javadi et al., 2005). A property that has received little attention, but should be kept in mind, is that SM is also a radiomimetic, a property that is shared with other radiation-emitting agents. Additional properties that both radiation and mustards share are the inducement of apoptosis, burns, and OS.

A. POLY ADP-RIBOSE POLYMERASE

Alkylation of DNA by SM leads to the activation of poly ADP-ribose polymerase (PARP), which reduces the availability of oxidized nicotinamide adenine dinucleotide (NAD^+) and ATP in the cell (Fox and Scott, 1980). The consequent change in cellular bioenergetics leads to the inhibition of glycolysis, activation of hexosemonophosphate shunt, induction of plasminogen activator, and ultimately, production of skin lesions (Mol et al., 1989). Other alterations in cellular metabolism due to the loss of NAD^+ are microfilament architecture and function in keratinocytes (Papirmeister et al., 1985). PARP uses NAD^+ for a two-electron donor in an oxidation step to catalyze the polymerization of ADP-ribose units on target proteins to attach PARP. PARP has a multiplicity of functions, such as DNA damage sensor and repair chromatin modification, transcription, cell death pathways, insulator function, and mitotic apparatus function. These processes are important to several physiological and pathophysiological processes that involve genome maintenance, carcinogenesis, aging, inflammation, and neuronal function. Poly ADP-ribose polymerase-1 (PARP-1) is the most abundantly expressed member of the family of PARP proteins; it binds to DNA structures that have single and double strand breaks, crossovers, cruciforms, and supercoils (Kim et al., 2005). During normal cellular metabolism, the level of PARP-1 is very low. The enzymatic activity is greatly increased, as much as 500-fold, with allosteric activators, such as damaged and undamaged DNA occurrence (D'Amours et al., 1998, 1999; Kun and Bauer, 2001; Oei and Shi, 2001).

The attachment of PARP or PARrylation, in response to oxidation, alkylation, or ionizing radiation, is dramatic and immediate. At minimal levels of DNA damage, PARP-1 acts as a survival factor involved in DNA damage detections and repair. At high levels of DNA damage, PARP-1 promotes cell death (Burkle, 2001). Elevated levels of PARP in response to DNA damage canpromote necrosis secondary to exhaustion of cellular NAD^+ and ATP (cellular energy failure) (Decker and Muller, 2002; Bouchard et al., 2003). PARP-1 even facilitates apoptosis by a caspase-independent

apoptotic cell death via apoptosis inducing factor (AIF). AIF, a potent trigger of apoptosis, is a flavoprotein, which resides in the mitochondrial intermembrane (similar to cytochrome c). However, it is unclear what the exact trigger of AIF release is, but it could be the result of the depletion of NAD^+ due to excessive PARP synthesis and activity. PARP preferentially depletes cytosolic NAD^+ (Zong et al., 2004), which would be expected to affect the overall cellular bioenergetics.

B. METABOLITES OF SULFUR MUSTARD

The metabolites of SM are noteworthy because of their utilization of thiol compounds. These metabolites, in addition to OS, diminish the thiol cellular pool. Some metabolites occur by direct hydrolysis, but the majority are conjugates of GSH (Black et al., 1992; Black and Read, 1995); others are metabolites are N-acetyl-l-cysteine (NAC) conjugates or methylthio/methylsulfhinyl derivatives. Enzymatic conjugation of alkylating agents utilizing GSH can occur through gluta-thione-S-transferase (GST); but alkylating agents can also combine directly with thiols (Moore and Ray 1983; Colvin et al., 1993). Spontaneous or enzymatic conjugation of alkylating agents occurs through an aziridium intermediate (Colvin et al., 1993).

C. SIGNALING

There are a multitude of activators of NF-κB, such as viral infections, bacterial infections, radiation, interleukin (IL)-1, and tumor necrosis factor (TNF). Most of the activators of NF-κB can be blocked with the use of antioxidants (Schulze et al., 1997). Other transcription factors, such as AP-1 (Xanthoudakis et al., 1994), MAF, and NRL (Kerppola and Curran, 1994), and NF-IL6 (Hsu et al., 1994) are regulated by oxygen-dependent mechanisms that cause redox cycling of cysteinyl residues.

NF-κB regulates many genes involved in inflammation, such as inducible nitric oxide synthase (iNOS), proinflammatory cytokines, IL-1, tumor necrosis factor-alpha (TNF-α), IL-6, chemokine, IL-8, E-selectin, vascular cell adhesion molecule 1 (ICAM-1), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Brennan et al., 1995; Akira and Kishimoto, 1997; Barnes and Adcock, 1998; Rahman and MacNee, 1998; McClintock et al., 2002). Arroyo et al. (2000) found a dose-dependent increase in TNF-α, IL-6, and interleukin-1-beta (IL-1-beta) in SM-treated human keratinocyte cells.

Although inflammatory cytokine profiles differ depending on the skin models used (Ricketts et al., 2000; Sabourin et al., 2002), they were shown to be elevated in response to SM. In contrast, in guinea pig lungs exposed to 2-chloroethyl ethyl sulfide (CEES) (a mustards analog), the inflammatory cytokine TNF-α was found to be markedly elevated (Das et al., 2003). The inflammatory cytokines exacerbate the effects of CEES (Stone et al., 2003), which would imply that there is an amplification of the initial injury by the mustards. Lipopolysaccharide (LPS), a ubiquitous entity in our environ-ment, also upwardly modulates the damage that CEES inherently causes to cells (Stone et al., 2003).

SM activates phospholipase A2 and liberates arachidonic acid by acting on linolenic acid found in cellular membranes. Activated neutrophils show an amplification of their respiratory burst in the presence of arachidonic acid (Bostan et al., 2003). Metabolites of arachidonic acid produce oxidants, which would be additional contributors to OS. Arachidonic acid itself and several of its metabolites, such as 12 HETE and 15 HETE, induce FOS and JUN expression (Haliday et al., 1991; Sellmayer et al., 1991; Rao et al., 1996).

One class of signaling pathways that has received considerable attention involves the action of cytokine-stimulated sphingomyelinase (SHM-ase) (Hannun and Obeid, 1997). Ceramide generated from SHM-ase activation plays a critical role in cytokine-mediated apoptosis, cellular differenti-ation, and senescence, each of which may be important in the inflammatory response.

Ceramide can be generated through several different pathways, such as synthesis within the cell, hydrolysis of SHM by SHM-ase, and breakdown of glycosphingolipids. The degradation of ceramide by acid ceramidase liberates sphingosine, a free fatty acid in the lysosomal compartment

(Alphonse et al., 2002). Ceramide can also be increased by the inhibition of ceramide breakdown by ceramidase and the inhibition of sphingomyelin synthase.

SHM-ase has been proposed as a key enzyme involved in stress-induced ceramide formation (Hannun and Obeid, 1997). Multiple pathways may be regulated, which in turn ultimately determine the levels of ceramide.

D.   TUMOR NECROSIS FACTOR-ALPHA INCREASES WITH CEES EXPOSURE

A single, intratracheal injection (0.5 mg/kg body weight) of CEES in guinea pigs was done at different time points. The guinea pigs were dissected and the lung was removed after perfusion. The lung was lavaged and TNF-α concentrations were measured in lung lavage fluid, lung lavage macrophages, and in lung tissue. The level of TNF-α in lavage fluid was very low, whereas high levels accumulated in lung as well as in lung macrophages within 1 h of CEES exposure. The level of TNF-α decreased rapidly after 1 h and returned to normal levels within 24 h of CEES exposure (Figure 12.2a). Further studies revealed that the induction of TNF-α by CEES is dose dependent, and optimal TNF-α accumulation was observed at 2 mg/kg dose of CEES exposure (Figure 12.2b).
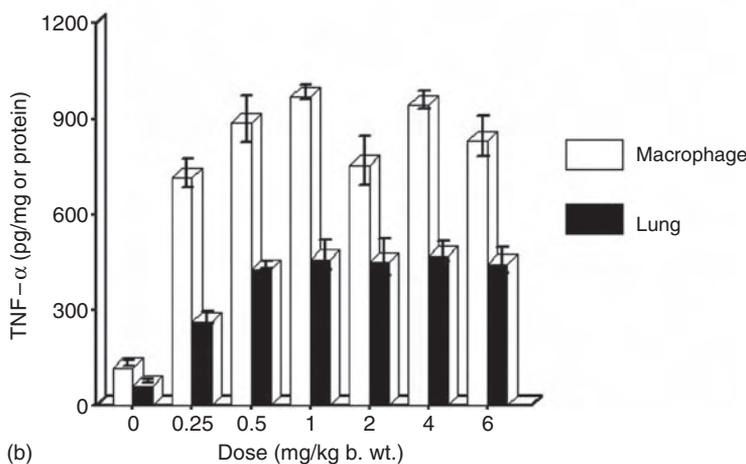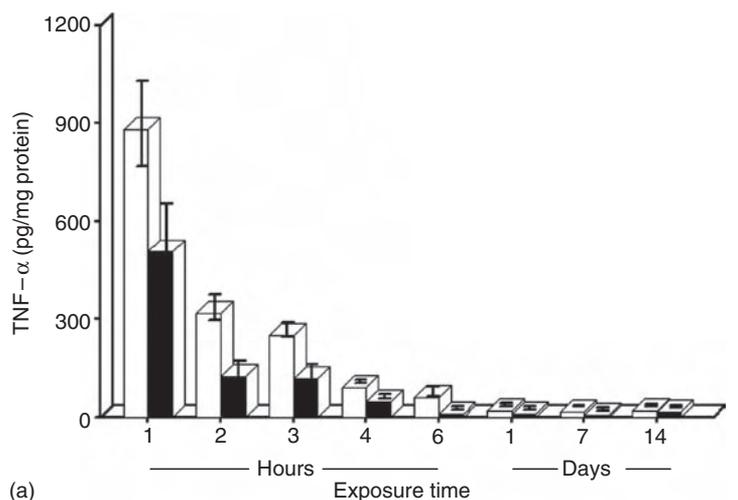

FIGURE 12.2 Accumulation of TNF-α in guinea pig lung and macrophages after exposure to CEES. (a) Time-dependent induction of TNF-α after intratracheal injection of CEES (0.5 mg/kg body weight). (B) Accumulation of TNF-α 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).

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E. ACTIVATION OF SPHINGOMYELINASE ACTIVITIES AFTER CEES EXPOSURE

Both the neutral and acid SHM-ase activities showed fourfold to fivefold increases after CEES treatment. The basal level of acidic SHM-ase activity (Figure 12.3b and D) was much higher than the basal level of neutral SHM-ase (Figure 12.3a and c).

Both neutral and acid SHM-ase activities started to increase, along with the increase of TNF-α; these activities reached a maximum peak between 4 and 6 h in the lung and between 3 and 4 h in macrophages. It is not known at this time whether this difference between lung and macrophages is functionally significant or not. The SHM-ase activities (both neutral and acidic) were found to be higher in lavage macrophages than those in the lung tissue. It is possible that macrophages are more sensitive to CEES than other cell types in the lung. The level of SHM-ase decreased rapidly and returned to near normal level within 24 h. It was also found that a 2 mg/kg dose of CEES is sufficient to reach the maximum level of both neutral and acid SHM-ase activity (Figure 12.3c and d).

F. ACCUMULATION OF CERAMIDE IN LUNGS AFTER CEES EXPOSURE

The ceramide accumulation after CEES exposure demonstrated a biphasic pattern. Within 1 h of CEES exposure, ceramide levels became very high and reached a peak accumulation within 2 h (Figure 12.4a). After 2 h, there was some decrease in the ceramide level, but then the level increased very high and remained almost to a steady state, even up to 14 days later (Figure 12.4a). CEES-induced ceramide accumulation was found to be saturated at 4 mg/kg dose of CEES. At 2 mg/kg dose of CEES, about 90% induction of ceramide was achieved (Figure 12.4b).

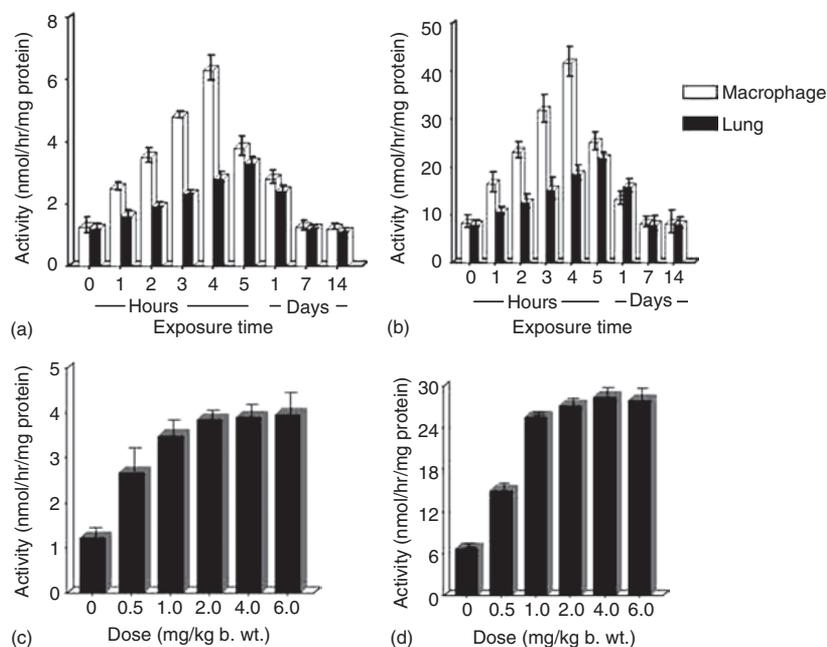**FIGURE 12.3** Activation of acid and neutral sphingomyelinase in guinea pig lung and macrophages following CEES exposure. Time course of induction of neutral (a) and acid (b) sphingomyelinase after intratracheal injection of CEES (0.5 mg/kg body weight). Accumulation of neutral (c) and acid (d) sphingomyelinase at 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).
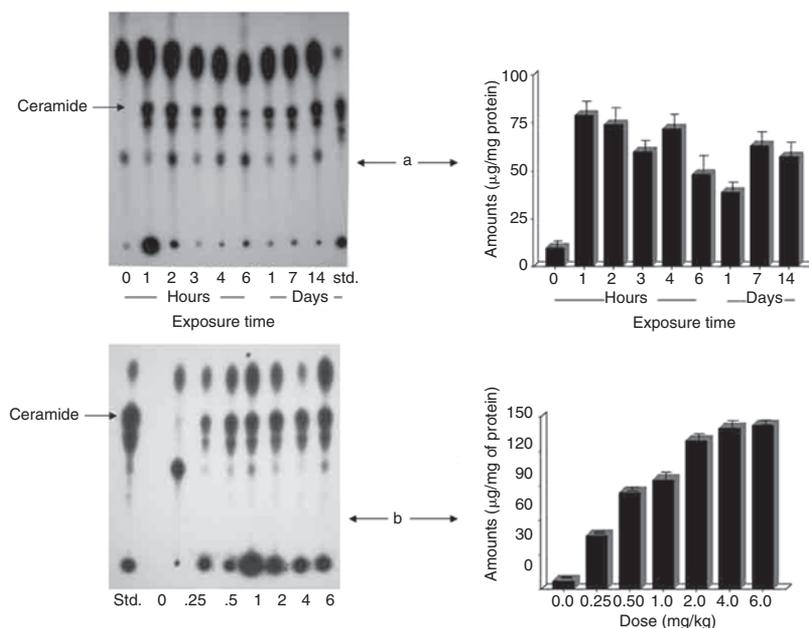
FIGURE 12.4 Accumulation of ceramide in guinea pig lung after CEES exposure. (a) Time course of induction of ceramide after intratracheal injection of CEES (0.5 mg/kg body weight). (b) Accumulation of ceramide at 1 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight). In both cases (a and b), the left panel is the autoradiograph showing the accumulated ceramides, and the right panel represents the quantitative analysis of accumulated ceramide as determined by ^{32}P incorporation.

G. ACTIVATION OF NUCLEAR FACTOR KAPPA IN LUNGS AFTER CEES EXPOSURE

The activation of NF-κB was measured in the nuclear extracts of lungs after exposure to CEES. NF-κB, which is well known to inhibit TNF-α-mediated apoptosis, showed activation only up to 1–2 h after CEES exposure (Figure 12.5a). This may explain the biphasic effect of CEES on the lung.

After initial lung damage by TNF-α, within 2 h there was some recovery due to activation of NF-κB. After 2 h, the NF-κB level went down, ceramide level increased, and secondary lung damages were observed. Dose-dependent studies revealed that 4 mg/kg CEES was needed for the optimum activation of NF-κB (Figure 12.5b). Super shift assay using specific antibodies to p50 and p65 revealed that both the p50 and p65 subunits were activated in lung because of CEES exposure (Figure 12.5c).

H. ACTIVATION OF CASPASES AFTER CEES EXPOSURE

Activation of different caspases in guinea pig lung after the CEES exposure is shown in Figure 12.6. Within 1 h of exposure, some activation was observed for all four caspases, but the activity came back to the basal level within 2 h of exposure. The activity of caspase 2, caspase 3, caspase 8, as well as caspase 9 increased significantly between 4 and 6 h of exposure and then declined again. No activity for any of the caspases was observed at 24 h and thereafter. Here also, the activation of caspases was found to be optimum at 2 mg/kg body weight of CEES.

Our study clearly demonstrated the involvement of an SHM-ase/ceramide signal transduction pathway in the mustard gas-mediated lung injury. After intratracheal injection of CEES to guinea pigs, the TNF-α level increased sharply within 1 h of exposure. The TNF-α level started, declined after 1 h, and returned to basal level within 24 h.
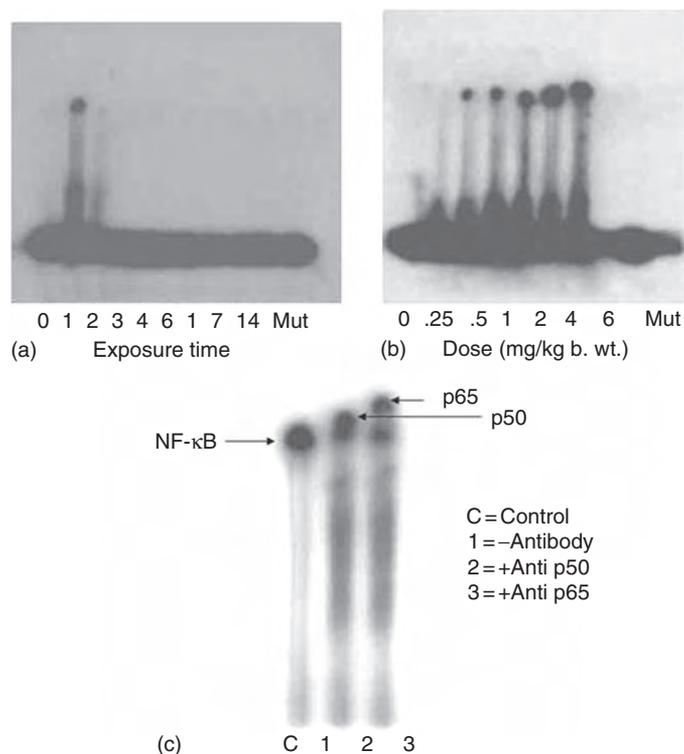
FIGURE 12.5 Activation of NF-κB in guinea pig lung following CEES exposure. (a) Time-dependent activation of NF-κB after intratracheal injection of CEES (0.5 mg/kg body weight), as observed by mobility shift assay. (b) Mobility shift assay showing the accumulation of NF-κB after 1 h of CEES exposure at different doses (ranging from 0.5 to 6.0 mg/kg body weight). (c) Supershift assay, using subunit specific antibodies, to identify the subunits (p50 and p65) of NF-κB activated due to CEES exposure in guinea pig lung.

Followed by the accumulation of TNF-α, both the acid and neutral SHM-ase activities were stimulated, peaking within 4–6 h after CEES exposure. Though both the acid and neutral SHM-ase activities were stimulated, the level of acid SHM-ase was found to be much higher after CEES exposure.

The higher levels of TNF-α, as well as both the acid and neutral SHM-ase activities in lung macrophages compared with those in lung tissue were expected because lung tissue consists of several types of cells, all of which were not responsive to CEES. We observed a biphasic effect of CEES on lung. After initial damage by TNF-α, there was some recovery due to activation of NF-κB within 2 h. This biphasic pattern was also observed in caspases activation.

Significant but small activation of caspase 2, caspase 3, caspase 8, and caspase 9 was observed within 1 h of CEES exposure. This activation of caspases declined thereafter and reappeared within 4–6 h, initiating the cell apoptosis in lung as observed by light as well as electron microscopy (unpublished observation). One explanation for this biphasic action of mustard gas is that NF-κB is activated by TNF-α through a phosphatidylcholine-specific phospholipase C/diacylglycerol (DAG)/protein kinase C (PKC) or phosphatidylcholine-specific phospholipase C/DAG/acid SHM-ase/ceramide model (Chatterjee et al., 2004).

These studies indicate that CEES exposure causes accumulation of TNF-α-activated SHM-ases, resulting in the production of ceramides and simultaneous activation of caspases and finally apoptosis. Ceramides are known to cause apoptosis via the activation of caspases (Alphonse et al., 2002; Hearps et al., 2002; Hetz et al., 2002).
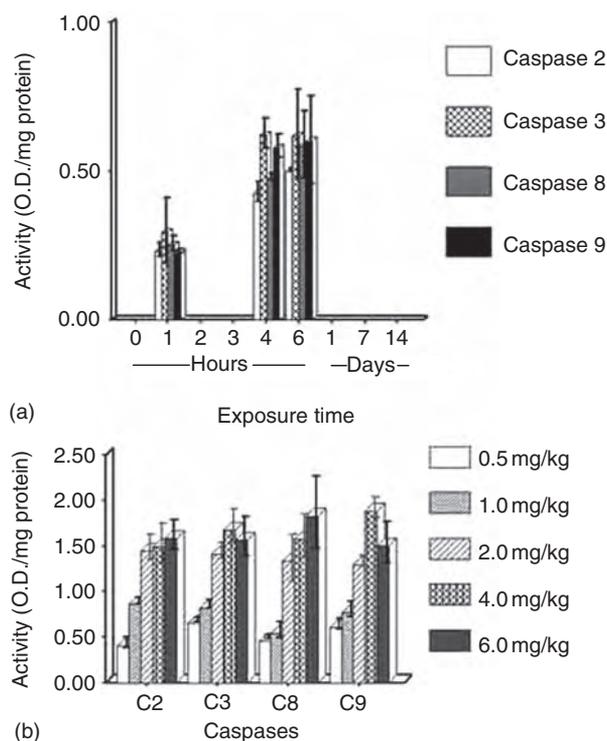
FIGURE 12.6 Activation of different caspases in guinea pig lung after CEES exposure. (a) Time course of activation of different caspases after intratracheal injection of CEES (0.5 mg/kg body weight). (b) Accumulation of different caspases at 4 h after exposure to CEES at different doses (ranging from 0.5 to 6.0 mg/kg body weight).

Our study also revealed that there was some initial damage of the lung tissue when exposed to CEES, but self-defense mechanisms of the lung played a significant role in the recovery from the damage and the prevention of any further damage. Furthermore, ~~the present~~ investigation enhances our understanding of mustard gas-mediated proapoptotic signaling pathways and characterizes the events of mustard gas-induced lung dysfunction.

Mustard gas exposure also causes inflammatory lung diseases, including acute respiratory distress syndrome (ARDS) (Calvet et al., 1994). A defective secretion of surfactant by alveolar type II cells has been implicated as one of the causative factors for the development of ARDS (Ansceschi, 1989). A major component of lung surfactant is DPPC (Stith and Das, 1982). The precursor of DPPC is normally 1-palmitoyl-2-oleolyl PC. DPPC is produced by deacylation and subsequent reacylation with palmitic acid at 2-position of glycerol moiety of the unsaturated phospholipid.

The CDP-choline pathway is the major pathway for the synthesis of PC in the lung, and cholinephosphotransferase (CPT) is a terminal enzyme in this pathway. Regulation of PC metabolism is one of the vital aspects of the cell cycle with implications in the control of cell proliferation as well as in apoptosis (Cui et al., 1996; Baburina and Jackowski, 1998).

PC is the most abundant phospholipid in mammalian cells, and it is synthesized via the CDP-choline pathway (Kent, 1995). CPT is the terminal enzyme of this pathway and plays a direct role in the final production of PC in the lung. This pathway is important for both cell proliferation and cell death (Ghosh et al., 2002; Ryan et al., 2003), and selective inhibition of this pathway has been shown to induce cellular apoptosis (Miquel et al., 1998). Any modulation in the expression and activity of this enzyme is expected to result in abnormal functioning of the cells.
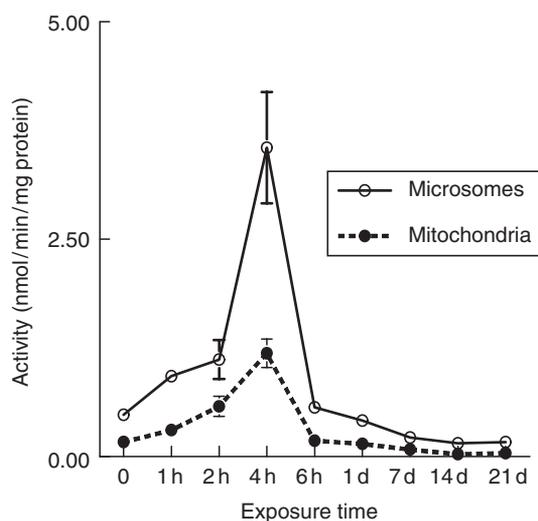
FIGURE 12.7 Time-dependent effects of 0.5 mg/kg body weight. CEES treatment on the mitochondrial and microsomal CPT activity. $N = 3$.

The time-dependent effects of CEES treatment showed a biphasic effect on CPT activity in both mitochondria and microsomes. The time-dependent studies indicated that a single infusion of CEES (0.5 mg/kg body weight) caused an increase in the activity for a short time after CEES exposure (up to 4 h), followed by a decrease (6 h onward) (Figure 12.7). The dose-dependent studies indicated that CEES treatment caused an initial increase in the CPT activity at low doses (0–2 mg/kg body weight), followed by a decrease at higher doses (4 and 6 mg/kg body weight) at incubation times of 1 and 4 h. This decrease was more acute in microsomes than in the mitochondria (Figure 12.8).

We have previously demonstrated that in addition to its predominant localization in the microsomes, CPT also exists in the mitochondria (Stith and Das, 1982; Sikpi and Das, 1987). Thus, it is possible that during the early stage of lung injury as observed in this study, cells try to repair the membrane damage by stimulating PC synthesis. Therefore, with increased CPT activity
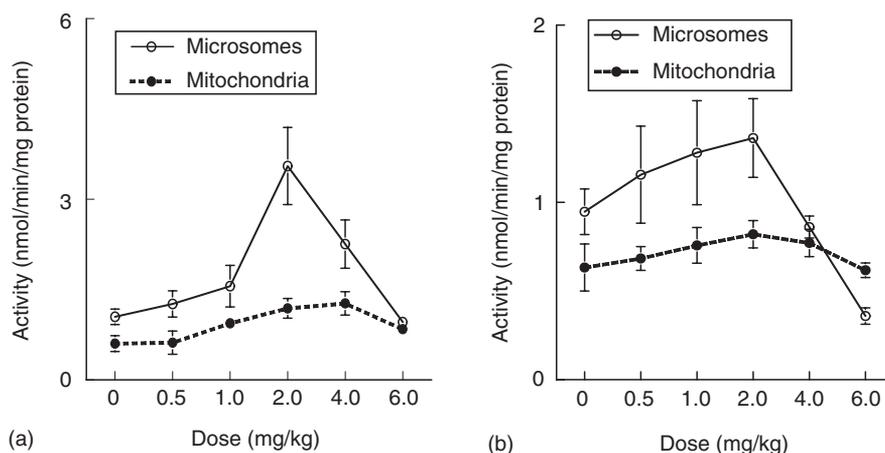
FIGURE 12.8 Dose-dependent effects of CEES on cholinephosphotransferase activity in mitochondria and microsomes. (a) After 1 h CEES treatment and (b) after 4 h CEES treatment. $N = 3$.

over time, lung cells lose their ability to repair membrane damage, as evident from decreased CPT activity in both mitochondria and microsomes isolated from lung of CEES-treated animals (Figures 12.7 and 12.8). Hence, CEES has both short-term (stimulation) and long-term (inhibition) effects on lung CPT activity. Since CPT activity is crucial to the synthesis of surfactant, these effects may cause ARDS with long-term CEES exposure because of lack of surfactant synthesis.

I. Effects of Ceramide Treatment on Lung Microsomal CPT Activity

Ceramides are intracellular signaling molecules implicated in the induction of cellular apoptosis (Kolesnick and Krönke, 1998; Hannun and Luberto, 2000), and are known to induce several protein kinases and phosphatases (Mathias et al., 1991; Dobrowsky et al., 1993; Vietor et al., 1993). Ceramide analogs have been shown to inhibit PC synthesis (Bladergroen et al., 1999; Allan, 2000; Ramos et al., 2000; Vivekananda et al., 2001). Ceramides may directly affect the biosynthesis of PC and phosphatidylethanolamine (PE) by inhibiting the enzymes of the CDP-choline and CDP-ethanolamine pathways (Bladergroen et al., 1999; Awasthi et al., 2001; Ramos et al., 2002).

It has been reported that cells treated with ceramides may undergo programmed cell death, become growth arrested, or in rare cases, become stimulated to proliferate. The diversity of biological responses of cells to ceramides reflects the complexity of the role of these sphingolipids as second signal molecules (van Blitterswijk et al., 2003). Furthermore, ceramide treatment of lung cancer-derived A-549 cells promotes apoptosis in a caspase-dependent process (Kurinna et al., 2004).

Other laboratories have also shown that enzymes of the CDP-choline pathway for the production of PC in the cells, including CPT, show reduced activity when cells are incubated with cell-permeable C_2/C_6 ceramides (Bladergroen et al., 1999; Ramos et al., 2002), and it has been predicted that this inhibition may be due to the competitive inhibition by ceramides owing to the similarity in the structure to one of the substrates for CPT, DAG.

The downstream signal transduction events in lung following CEES exposure involve the induction of TNF-α, which in turn activates both acid and neutral SHM-ases, resulting in the subsequent accumulation of ceramides in the lung (Chatterjee et al., 2003, 2004). The level of ceramide was found to be ~60 μg/mg protein after 7 days (with 0.5 mg/kg body weight of CEES) and ~130 μg/mg protein for 2 mg/kg body weight of CEES (after 1 h; Figure 12.4).

When the lung microsomal fraction from control animals was incubated with C_2 ceramide at different concentrations (50, 100, and 200 μM) and time periods (0, 0.5, 1, and 6 h) before the assay for CPT activity, CPT activity decreased significantly in a time- and dose-dependent manner (Figure 12.9).
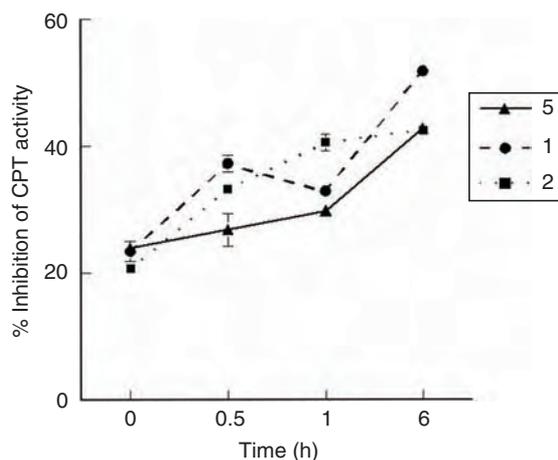
FIGURE 12.9 Effect of C_2 ceramide treatment on lung microsomal CPT activity. $N = 3$.

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However, the effect was more pronounced when the microsomal fraction was preincubated with the ceramide before assay of the CPT activity. The degree of inhibition was increased with the increase in incubation time (0.5, 1, and 6 h). The highest inhibition (50%) was achieved after 6 h of incubation. However, only 20% inhibition was observed when ceramide was directly added into the assay mixture.

It has been shown that a 30% inhibition could be obtained in CPT activity when ceramides were directly added to the assay mixture at 50 μM concentrations (Bladergroen et al., 1999). Since this inhibition of 30% was less than the 64% obtained when cells were incubated directly, it would indicate competitive inhibition was not the only mechanism. In the present work, we found similar results with lung microsomal fraction, that is, with an increase in the incubation time with ceramides, the inhibition of the enzyme activity increases. Therefore, we support the observations by Bladergroen et al. (1999) that ceramide inhibition of CPT activity may be only partially through direct competitive inhibition with DAG; ceramide may act through interaction with other CPT enzyme inhibitors present in the microsomal fraction.

It is known that the short-chain ceramides often do not mimic the endogenous long-chain ceramides produced as a result of SHM-ase activity. However, the lipophilic nature of the both short- and long-chained ceramides makes these molecules likely candidates for altering biological processes as components of the lipid bilayer (Gidwani et al., 2003). We can therefore predict that ceramides accumulated in the lung because of exposure of CEES can alter the activity of the membrane-bound enzymes like CPT and can also act as a membrane perturbant.

This ceramide-induced membrane perturbation can result in mitochondrial release of cytochrome c and subsequent release of different caspases (Figure 12.6) (Gidwani et al., 2003; Chatterjee et al., 2004) as a part of the apoptotic pathway. Furthermore, CEES exposure (2.0 mg/kg body weight for 7 days) caused a significant decrease of both CPT activity (~1.5-fold; Figure 12.10) and gene expression (~1.7-fold; Figure 12.11) in the lung (Gidwani et al., 2003; Sinha Roy et al., 2005). This decrease in CPT activity was not associated with any mutation of the CPT gene. Thus, the inhibition of CPT activity as a chronic effect of CEES exposure may be directly responsible for the
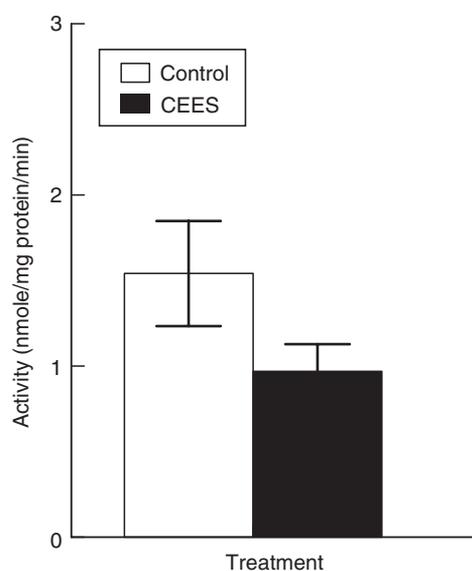
FIGURE 12.10 CPT enzyme activity in the lung microsomal fraction of 2 mg/kg body weight CEES (for 7 days) treated lung as compared with the control (only vehicle) showing significant decrease in activity. $p \leq 0.05$, $N = 3$.
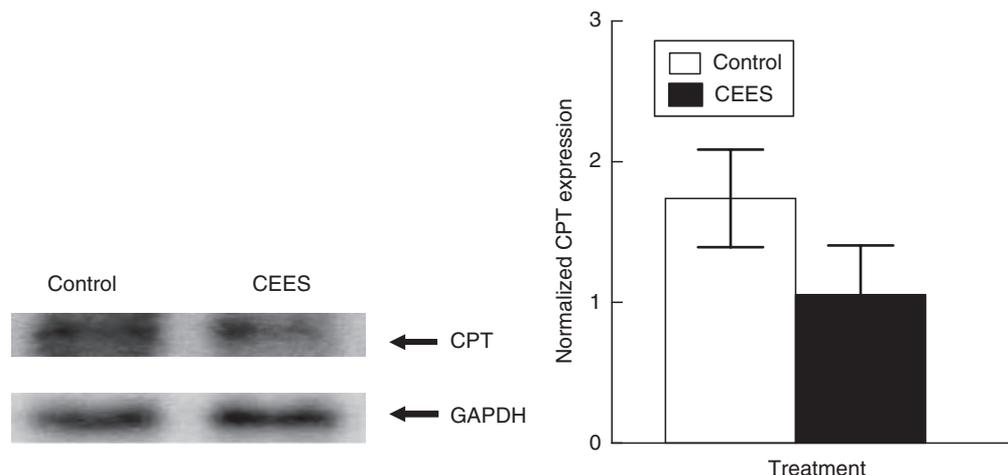
FIGURE 12.11 Northern blot analysis for the expression of the CPT gene. (a) One representative blot for CPT mRNA from control and CEES-treated guinea pig lung and same blot reprobed for GAPDH expression. (b) Graph showing downregulation of CPT expression as the result of mustards gas treatment normalized with GAPDH. $N = 3$.

reduction in the lung surfactant production, resulting in subsequent development of ARDS and pulmonary fibrosis, and is probably mediated by accumulation of ceramides.

J. CEES INDUCES OXIDATIVE STRESS

Stone et al. (2003) have found that OS and inflammatory agents play a key role in the toxicity of CEES. Both HD and CEES are known to provoke acute inflammatory responses in the skin (Blaha et al., 2000a, 2000b; Sabourin et al., 2002). It is interesting that RAW 264.7 macrophages stimulated with LPS or inflammatory cytokines are more susceptible to the cytotoxic effect of CEES than unstimulated macrophages. LPS (bacterial endotoxin) is a well-characterized inflammatory factor found in the cell wall of Gram-negative bacteria and is a ubiquitous natural agent found in the environment. LPS is present in serum, tap water, and dust. Military and civilian personnel would always have some degree of exposure to environmental LPS. Very low levels of LPS (20 ng/mL) were found to dramatically enhance the toxicity of CEES at concentrations greater than 400 μM (Stone et al., 2003). CEES alone is not toxic to RAW 264.7 macrophages at levels lower than about 500 μM (Stone et al., 2003).

LPS is known to trigger a variety of inflammatory reactions in macrophages and other cells having CD14 receptors (Wright et al., 1990; Downey and Han, 1998). In particular, LPS is known to stimulate the macrophage secretion of inflammatory cytokines such as TNF-α and IL-1-beta (Shapira et al., 1994). It is interesting, therefore, that both TNF-α and IL-1-beta were also found to enhance the cytotoxic effects of CEES but to a lesser extent than LPS (Stone et al., 2003). LPS stimulation of macrophages is known to involve the activation of protein phosphorylation by kinases as well as the activation of nuclear transcription factors such as NF-κB (Fujihara et al., 1994; Shapira et al., 1994, 1997; Chen et al., 1998;). The activation of PKC by DAG is also a key event in LPS macrophage activation (Downey and Han, 1998). In vitro experiments have shown that the secretion of TNF-α and IL-1-beta by LPS-stimulated monocytes is dependent on PKC activation (Shapira et al., 1997; Coffey et al., 2000). Stone et al. (2003) also determined that phorbol myristate acetate (PMA) activation of PKC also enhanced CEES toxicity. These data suggest that the activation of PKC may play a key role in the molecular mechanism whereby LPS and inflammatory cytokines enhance the cytotoxicity of CEES (and potentially other vesicant weapons

as well). Evidence suggests that LPS (deRojas et al., 1995; Fu et al., 2001) as well as TNF-α (Ye et al., 1999) also stimulate the production of free radicals by macrophages. Collectively, this information supports the view that inflammatory factors and OS are key factors in understanding vesicant toxicity, and are important factors in designing effective countermeasures (Veness-Meehan et al., 1991; Yourick et al., 1991; Elsayed et al., 1992; Pant et al., 2000; Schlager and Hart, 2000; Kadar et al., 2001; Naghii, 2002; Levitt et al., 2003).

IV. CHLORINE

Chlorine is a pulmonary irritant that affects both the upper and the lower respiratory tracts. It is similar to SM in that its density is greater than that of air. Therefore, it characteristically hugs the ground, as does SM when deployed. It is only slightly water soluble; on contact with water or moisture, it forms hypocholorous acid (HClO) and hydrochloric acid (HCl). HClO is unstable and readily decomposes to oxygen-centered radicals. In animal models, 2000 ppm exposure will induce respiratory arrest. Subacute exposures of 9 ppm and acute exposures of 50 ppm can cause chemical pneumonitis (an inflammatory process) and bronchiolitis olbiterans (Segal and Lang, 2005).

The LD50 is in the range of 800–1000 ppm. HOCl, whether exogenously obtained from Cl_2 gas, or from an oxidative burst from neutrophils, the product reacts with a number of functional thiol groups present in enzymes (Winterbourn, 1985; Pullar et al., 2002). HOCl reactivity with thiol groups could inactivate such enzymes as GSH peroxidase and catalase (Aruoma and Halliwell, 1987). Inactivation of enzymes involved in antioxidant defense system renders the cell vulnerable to OS (a disruption of redox homeostasis).

Glutamylcysteine synthetase, cysteine, or methionine was 100 times more reactive to hypochlorous acid in comparison with amino acids that did not contain thiol groups (Folkes et al., 1995). Sublethal exposures to HOCl decreased GSH levels in several cell types (Vissers and Winterbourn, 1995; Pullar et al., 1999). In a study by Pullar (1999) using human umbilical vein endothelial cells, doses of 25 nmol of HOCl and less were sublethal; when the exposure was done over 10 min, there was a concentration-dependent loss of intracellular GSH. Tissue exposure to HOCl resulted in a reduction of GSH. The metabolite of the HOCl interaction with GSH was an unexpected cyclic sulfonamide that was exported from the cell. The expected metabolites of glutathione disulfide (GSSH) and GSH sulfonic acid were actually minimal (Pullar et al., 2001). Inactivation of acetylcholinesterase by HOCl could be a contributory cause of airway hyperreactivity (den Hartog et al., 2002). AQ5

V. PHOSGENE

Phosgene ($COCl_2$), also referred to as carbonic dichloride, is extensively used as an industrial chemical. The gas dissolves slowly in water and is hydrolyzed to CO_2 and HCl. It produces little damage to the upper airway, although the lower airways sustain the bulk of the necrosis and inflammation. There is typically a delayed onset of symptoms in the pulmonary system occurring 1–24 h after the initial exposure. The respiratory symptoms of hypoxia (shortness of breath), and in extreme cases, respiratory arrest (discontinuation of spontaneous breathing), are apparently due to leaky alveolar capillaries and the resulting pulmonary edema (Noltkamper and Burgher, 2004); also contributory to the clinical symptoms are arachidonic acid mediators and lipid peroxides (Sciuto and Hurt, 2004).

In rodent models, phosgene elicits decreases in total GSH in lung tissue 48% within 45–60 min after exposure (Sciuto, 1998). Jaskot et al. (1991) confirmed similar results 2 years after the Sciuto publication,. The concentration of phosgene exposure inversely affected the GSH levels (Jaskot et al., 1991). In gene expression studies in the inhalation mouse model, GSH regulation and redox regulation, in particular, were affected (Sciuto et al., 2005). A gene expression response can be seen as early as 30 min, wherein glutamate cysteine ligase increases and continues to increase at 8 h. Glutamate cysteine ligase continued to be elevated, approaching control levels in the 24–72 h time period. There was an upregulation of GST α-2, GSH peroxidase-2, glutamate, and γ-glutamyl

cysteine synthetase. At 4–12 h, GSH peroxidase-2, GSH reductase was elevated. GSH synthetase increased in the 4–24 h time frame. Further evidence of the crippling of the antioxidant defense system was the decreased SOD3 gene expression and enzyme activity. Similar results were found in independent experiments by Qin et al. (2004), as were reported by Sciuto in his examination of the antioxidant enzyme defense system. In the exposed rats, the antioxidant defense enzymes GST, superoxide dismutase (SOD), catalase, GSH peroxidase, and nitric oxide synthase in serum, blood, or liver were all increased. In contrast, the nitric oxide content was decreased. Similar changes in enzymes were also noted for other gaseous toxins, such as O_3 and NO_2 (Jaskot et al., 1991).

VI. LEWISITE

The Germans developed several arsenical-based warfare chemical agents circa 1917 (Goldman and Dacre, 1989). The allies, on the other hand, developed Lewisite (2-chlorovinydichloroarsine), adamsite (diphenylaminechloroarsine), methyldichloroarsine, and arsine. Lewisite is soluble in organic solvents; it is readily absorbed by rubber, paint, varnish, and porous materials. There are labile chlorine atoms, trivalent arsenic, carbons, and multiple bonds that make it quite reactive. Some of its reactions are due to nucleophilic substitution by water, hydrogen sulfide, thiols, and acid salts.

The reactions with thiol groups (e.g., those that are found in proteins) form an alkylarsine sulfide. Mustard gas, at the same LCt50, induces vesication, whereas Lewisite does not. Apparently, Lewisite is more irritating initially to the pulmonary systems than mustard. The hydrolysis products are more persistent in soil in comparison with mustard. In ambient air, Lewisite is about 10 times more volatile than mustard.

The clinical sequelae after Lewisite absorption referred to as ''Lewisite Shock'' (L shock) are similar to that of severe burns. In dog models at high dosages, exposure resulted in retching, vomiting, extreme salivation, labored breathing, inflammation of the entire respiratory system, and pulmonary edema; respiratory distress was also common, and 80% died. Inhalation of 0.05 mg/L for 15 min results in intoxication and incapacitation for several weeks, whereas 0.5 mg/L for 5 min is lethal. The exact pathophysiology of the pulmonary symptoms seen is unknown, but is likely due to the dilatation of the capillaries. Apparently, the innate immune system is also compromised because of the occurrence of bronchopneumonia. In L shock, a hemolytic anemia can occur, however, it is unclear if this is due to an autoimmune reaction or OS (McMillan et al., 2004; Sato et al., 2006). Other complications of exposure are edema and bleeding that occur in the liver and kidneys (Wardell, 1941).   AQ6

It is the arsenic in Lewisite that reacts with thiol groups to form alkylarsine sulfides, which is the basic reaction with thiol groups in tissues (Goldman and Dacre, 1989). The degree of enzyme dysfunction is dependent on the affinity of the enzyme to arsine. For example, it binds to the alpha and gamma thiol groups of lipoic acid, a component of pyruvate oxidase, which forms a stable six-membered ring (Stocken, a1949; Johnstone, 1963). Several other known enzymes are   AQ7
also inhibited–alcohol dehydrogenase, succinic oxidase, hexokinase, and succinate dehydrogenase (Heyninen, 1941; Barron, 1947; Peters, 1946). Lewisite contact with skin results in the immediate   AQ8
elicitation of pain, in contrast to mustard, which would take 4–5 h (Wardell, 1941); it penetrates skin more rapidly than does mustard.

VII. ANTIDOTES OR AMELIORATIVE AGENTS

A. LEWISITE

Partial protection was afforded by monothiols, such as cysteine or GSH, when tested against pyruvate oxidase (Goldman and Dacre, 1989), which was thought to be one of the prime targets of Lewisite. BAL or 2,3-dimercaptopropanol preferentially binds with arsenicals over the thiol rings in proteins to form a nontoxic five-membered stable ring, which reverses the lesion induced by

Lewisite. BAL is also effective against other heavy metals, such as gold, excessive copper deposition seen in Wilson's disease (El-Youssef, 2003), and mercury (Goldman and Dacre, 1989). Newer analogs of BAL have been synthesized to improve efficacy, such as DMSA, 2,3-dimercapto-1-propane sulfonic acid, and N-(2,3 dimercaptopropyl-phthalamidic acid) (DMPA).

B. Mustard

Elsayed et al. (1992) performed subcutaneous injections of CEES that resulted in increased GST activity, increased lipid peroxides, and depleted, reduced GSH in lung tissue (Elsayed et al., 1992). Similar findings occurred in the in vitro mouse model, a neuroblastoma–rat glioma hybrid cell line, in that there was a reduction in GSH as a function of time after exposure (Moore and Ray, 1983). Within the last 5 years, there has been a greater emphasis on the inhalation effects of SM.

Peter Ward and his group developed a rat instillation CEES model (McClintock et al., 2002). Leakage of radiolabeled albumin ($[^{125}I]$ BSA) was used as a measure of lung damage, and the rats were sacrificed after 4 h after the installation of CEES into the rat lung with CEES. Neutrophil and complement depletion was found to significantly reduce the injury. Both enzymatic and nonenzymatic protocols were used to examine their ameliorative effect on the model. Dimethyl sulfoxide (DMSO) and dimethyl thiourea (DMTU) resulted in a 51% and 35% reduction in injury, respectively. The enzymatic antioxidants catalase and SOD exhibited 47% and 23% protection, respectively. In contrast, the antioxidant found in red wine, resveratrol (a phytoalexin), showed protection at 61%. Interestingly, the iron chelators 2,3 dihydroxybenzoic acid (DHBA) and deferoxamine mesylate (desferal) did not have any protective effect. NAC was administered 10 min before instillation of CEES. A dose–response curve was generated for 5, 10, 20, 30, and 40 mg/kg^{-1} body weight. All of the dosages of NAC were found to be protective. The dosage of NAC at 20 mg/kg^{-1} conferred the best protection out of all antioxidants at 70% protection. In vitro, the lethal effects of SM on L-cells were reduced by thiol reagents, namely, dithiothreitol and NAC (Walker, 1967; Walker and Smith, 1969). Several observations support the concept of a pathogenic role for toxic oxygen species in CEES-induced acute lung injury.

Other groups investigated the beneficial effects of agents that scavenge free radicals and other oxidant species (Wormser et al., 2000). They monitored the beneficial effects of those agents mainly by showing the recovery from damaged skin or by showing inhibition of the induction of proteolytic enzymes (Cowan et al., 1992; Cowan and Broomfield, 1993). Systemic and topical steroids administered revealed ameliorative properties in the rabbit SM skin model (Vogt et al., 1984).

The Das Group investigated the effects of orally administered antioxidants on signal transduction in the guinea pig model. A single dose of NAC could not block the CEES-induced lung injury at all. On the other hand, about 76% of lung injury could be blocked by long-term pretreatment (30 days) with NAC. No significant protection of lung injury was observed by pretreatment with either Ondrox (an over-the-counter antioxidant supplement as single-dose or 3 days pretreatment) or GSH (single; Table 12.1). Similarly, a single-dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 76% of lung injury could be blocked by long-term pretreatment (30 days) with NAC. There was no significant protection of lung injury by pretreatment with either Ondrox (single-dose or 3 days pretreatment) or GSH (single dose).

A single-dose treatment of NAC just before CEES exposure was found to be ineffective, whereas 62% of TNF-α induction was inhibited by 3 days pretreatment with NAC. Long-term treatment with NAC gave more protection (inhibition of 73% of TNF-α induction). There was no protection for TNF-α accumulation by single dose of either Ondrox or GSH. Three days pretreatment with Ondrox and DMT could only block 13% and 8% of the TNF-α induction (Table 12.2). Additional support for an involvement of oxidants in the pathogenesis of CEES-induced acute lung injury was provided by the protective effects seen after treatment of CEES-exposed rat with NAC (McClintock et al., 2002). The protective effect of NAC was confirmed in the guinea pig lung model, as well as by others (Wormser et al., 1997).

TABLE 12.1

Effects of *N*-acetyl-ʟ-Cysteine Pretreatment on CEES-Induced Lung Injury in Guinea Pigs

Treatment	Permeability Index
Control	0.20 ± 0.04
Control + NAC (3 days)	0.08 ± 0.01
CEES	2.01 ± 0.16
CEES + NAC (single dose)	2.07 ± 0.11
CEES + NAC (3 days)	0.62 ± 0.09[a]
CEES + NAC (30 days	0.48 ± 0.06[a]
CEES + Ondrox (single dose)	2.04 ± 0.03
CEES + Ondrox (30 days)	2.00 ± 0.08
CEES + GSH (single dose)	1.98 ± 0.10

Note: CEES was infused (6 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES infusion). Information on treatment with ondrox and GSH is given in text. The lung injury was measured after 1 h of CEES exposure and expressed by permeability index, which is a measure of ^{125}I-BSA leakage from damaged blood vessels into lung tissue. Each group had 6 animals. Values are mean $\pm$ SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced lung injury significantly ($p < 0.05$).

TABLE 12.2

Inhibition of CEES-Induced TNF-α Accumulation in Guinea Pig Lung by NAC Treatment

Treatment	Level of TNF-α (pg/mg protein)
Control	20 ± 7
CEES	708 ± 38
CEES + NAC (single dose)	705 ± 24
CEES + NAC (3 days)	$270 + 40$[a]
CEES + NAC (30 days)	190 ± 64[a]
CEES + Ondrox (single dose)	716 ± 22
CEES + Ondrox (3 days)	610 ± 54
CEES + DMT (3 days)	648 ± 62
CEES + GSH (single dose)	720 ± 41

Note: CEES was injected (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before injection of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES injection). Information on treatment with ondrox, DMT and GSH is given in the text. TNF-α was measured after 1 h exposure of CEES. Values are mean TNF-$\alpha \pm$ SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced accumulation significantly (p of TNF-$\alpha < 0.05$).

1. Effect of NAC on Signal Transduction

Short-term (3 days) and long-term (30 days) pretreatment with NAC blocked significantly the activation of acid (4% and 49%, respectively, Figure 12.12) and neutral (46% and 61%, respectively, Figure 12.12) SHM-ases and decreased the levels of ceramide by 71% and 77% (Table 12.3). However, pretreatment with a single dose of NAC did not inhibit the activity of either neutral or acid SHM-ase and accumulation of ceramide.

Exposure to CEES significantly inhibited ($p < 0.05$) the activity of SOD (31%), GSH-peroxidase (67%), and catalase (25%) (Figure 12.13). Pretreatment of guinea pigs for 3 days with NAC before CEES infusion significantly ($p < 0.05$) decreased CEES-induced inhibition of SOD (from
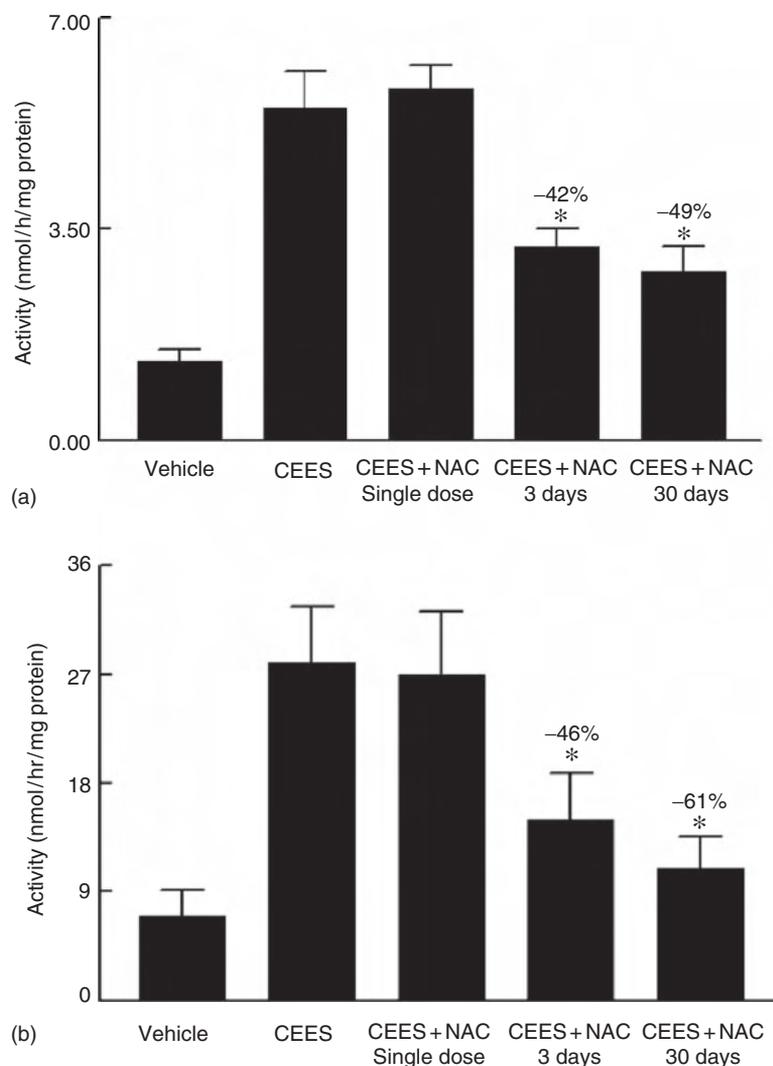
FIGURE 12.12 Inhibition of CEES-induced activation of neutral and acid sphingomyelinases in guinea pig lung by NAC treatment: Accumulation of neutral (a) and acid (b) sphingomyelinases at 4 h after exposure to CEES (4 mg/kg body weight) and their inhibition by 3 and 30 days pretreatment with NAC. Values are mean ± SE ($n = 6$). Pretreatment with single dose of NAC did not inhibit the activity of either neutral or acid sphingomyelinase. *Pretreatment with NAC for 3 and 30 days before CEES exposure caused a significant inhibition of both neutral and acid sphingomyelinases ($p < 0.05\%$).

TABLE 12.3
Inhibition of CEES-Induced Ceramide Accumulation
in Guinea Pig Lung After NAC Treatment

Treatment	Levels of Ceramide (μg/mg protein)
Control	9 ± 2
CEES	148 ± 14
CEES + NAC (single dose)	146 ± 10
CEES + NAC (3 days	39 ± 8^a
CEES + NAC (30 days)	28 ± 12^a

Note: CEES was infused (4 mg/kg body weight) intratracheally into guinea pigs with or without pretreatment with NAC. NAC was given either in single dose (5 mg) by gavage directly into stomach 10 min before infusion of CEES or with drinking water (0.5 g/day/animal, for either 3 days or 30 days before CEES infusion. Ceramide accumulations were assayed after 1 h of infusion of CEES. Values are mean $\pm$ SE ($n = 6$).

[a] NAC supplementation in drinking water blocked the CEES-induced accumulation of ceramide significantly ($p < 0.05$).
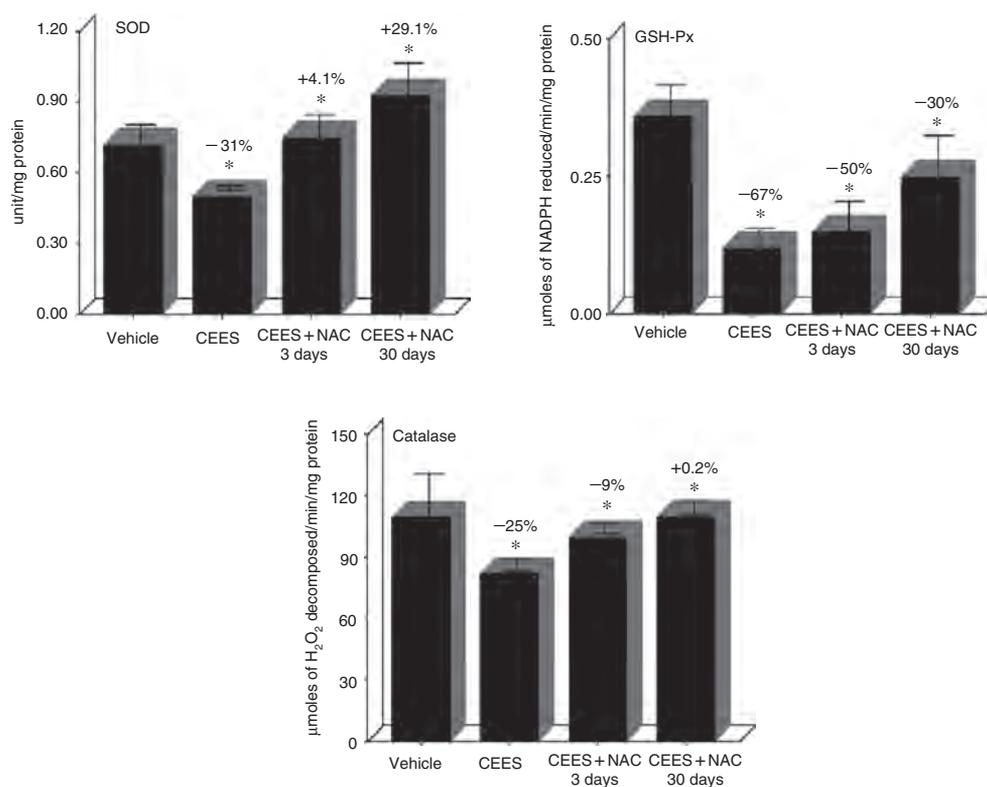
FIGURE 12.13 Effects of NAC pretreatment on the CEES-induced alterations in the free radical-metabolizing enzymes: Guinea pigs were infused with CEES (4 mg/kg body weight) intratracheally and the free radical-metabolizing enzymes (SOD, GSH pretreatment, catalase) were assayed in perfused lung after 4 h of CEES exposure. The samples were taken from guinea pigs with or without pretreatment with NAC for 3 days. *CEES exposure caused a significant decrease in the activity of SOD, GSH pretreatment, and catalase ($p < 0.05$). NAC treatment blocked the CEES-induced changes significantly ($p < 0.05$) for all enzymes.

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31% decrease to 4.1% increase), GSH-pretreatment (from 67% decrease to 50% decrease), and catalase (from 25% decrease to 9% decrease). Pretreatment of guinea pigs for 30 days with NAC provided additional resistance. For example, the activity of SOD activity was increased by 29% over the basal value, the activity of GSH pretreatment was decreased by only 30%, and the activity of catalase was brought back to the basal level.

Both short-term (3 days) and long-term (30 days) treatments significantly ($p < 0.05$) blocked the CEES-induced activation of NF-κB that was after observed 1 h after CEES infusion (Figure 12.14a). Furthermore, pretreatment with NAC for 3 days also blocked the activation of caspase 2, caspase 3, caspase 8, and caspase 9 by 41%, 44%, 55%, and 51% (Figure 12.14b).

Protection by NAC from half-mustard, gas-induced, acute lung injury has also been demonstrated recently in rats by McClintock et al. (2006). However, in these studies, NAC was administered by liposome encapsulation directly into the lung as a method of treatment for acute exposure to mustard gas. The co-instillation with CEES of liposomes containing the pegylated (PEG)-catalase, PEG-superoxide, NAC, GSH, resvertrol, or combination greatly attenuated development of rat lung injury (McClintock et al., 2006). Thus, we suggest the following model for the action of NAC on CEES-induced guinea pig lung injury (Figure 12.15).

Morphological analysis indicates that animals exposed to CEES showed symptoms of a chemical burn within 1 h; however, the severity of damage progressively increased with time. At 21 days postexposure, severe bronchial constriction with occasional apoptotic nucleus and accumulation of viscid secretion of mucins were observed in CEES-treated animals. Furthermore, both polymorphonucleus (PMNs) and eosinophilic leukocytes migration were observed in both alveoli and bronchi. However, pretreatment with NAC protected the lung from all these changes remarkably, except mucin secretion (Figure 12.16). How CEES functions as a powerful oxidant and what lung cells are targets of CEES is unclear.   AQ9

C. ANTIOXIDANT LIPOSOMES

Another method of increasing antioxidant tissue levels is facilitated by using liposomes. Liposomal drug delivery is advantageous to using the oral route in that there is a multiplicity of possible administration routes. They can be used topically, orally (using the gastrointestinal tract), or delivered by aerosol to the lung.

Intact skin allows the passage of small lipophilic substances, but in most cases, efficiently retards the diffusion of water-soluble molecules. Lipid-insoluble drugs generally penetrate the skin slowly in comparison with their rates of absorption through other body membranes. Absorption of drugs through the skin may be enhanced by iontophoresis if the compound is ionized. Certain solvents (e.g., DMSO) may facilitate the penetration of drugs through the skin, but their use for therapeutic applications is controversial. In the formulation of topical dosage forms, attempts are being made to utilize drug carriers to ensure adequate localization or penetration of the drug within or through the skin in order to enhance the local and minimize the systemic effect. For dermato-pharmacotherapy, there is a need for a drug delivery system that enhances the penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption. Antioxidant liposomes may prove very useful in this regard.

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces, and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes, in addition to their use as artificial membrane systems, are used for the selective delivery of antioxidants and other therapeutic drugs to different tissues in sufficient concentrations to be effective in ameliorating tissue injuries (Stone et al., 2002). Antioxidant liposomes containing combinations of water- and lipid-soluble antioxidants may provide a unique therapeutic strategy for mustard gas exposure because: (1) the antioxidants are nontoxic and could, therefore, be used at the earliest stages of exposure; (2) the liposomes themselves are composed of nontoxic, biodegradable, and reusable phospholipids;
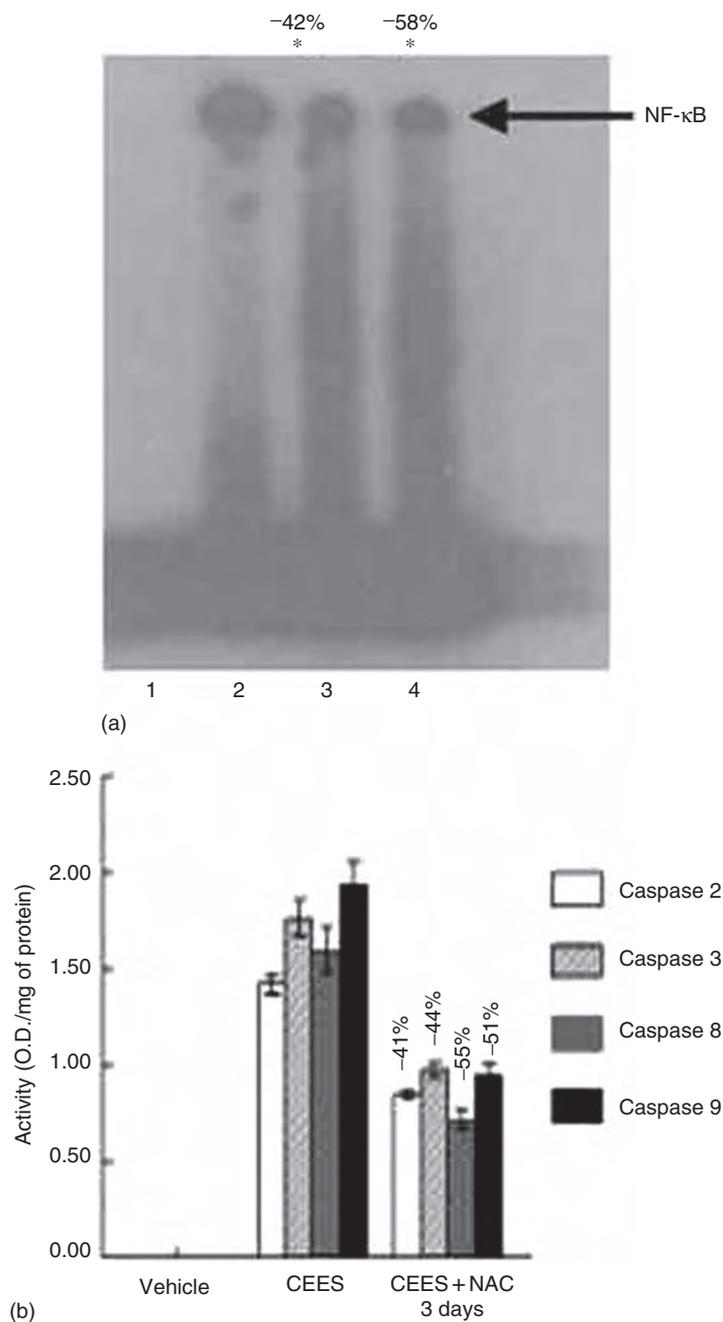
FIGURE 12.14 Inhibition of the activation of NF-κB (a) and different caspases (b) in guinea pig lung after CEES exposure by pretreatment with NAC. (a) Activation of NF-κB after 1 h of intratracheal infusion of CEES (4 mg/kg body weight) was monitored by mobility shift assay. The guinea pigs were treated with NAC either for 3 or for 30 days before CEES exposure. Panels 1, 2, 3, and 4 represent vehicle, CEES, CEES + NAC (3 days), and CEES + NAC (30 days), respectively. *NAC treatment caused a significant inhibition (46% and 58% for 3 and 30 days, respectively, $p < 0.05$) in the accumulation of NF-κB. (b) Accumulation of caspases 2, 3, 8, and 9 at 4 h after exposure to CEES (4 mg/kg body weight) and the prevention of these caspases activation by short-term pretreatment (3 days) with NAC. Values are mean ± SE ($n = 6$). *NAC treatment inhibited the activation of all caspases significantly ($p < 0.05$).
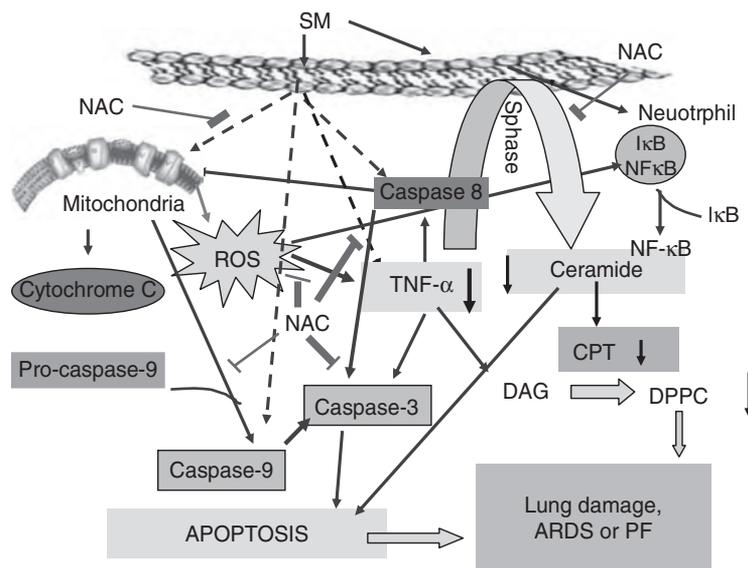
FIGURE 12.15 Proposed mechanism of action of NAC on CEES-induced lung injury.

(3) liposomes are preferentially taken up by the reticuloendothelial system which is an early target of mustards gas toxicity; (4) chemical antioxidants are relatively inexpensive and a wide range of commercial antioxidants are available; and (5) liposomes have low immunogenicity. Furthermore, results from several studies have clearly indicated that the liposomal antioxidant formulations exert a far superior protective effect compared with that of the free (unencapsulated) antioxidants, against OS-induced tissue injuries (Fan et al., 2000).

As shown in Figure 12.17, our preliminary data show that liposomes encapsulated with 5 mM GSH, a major intracellular chemical antioxidant, is effective at preventing CEES cytotoxicity to LPS-stimulated macrophages. We have also found that liposomes encapsulated with either 1 mM NAC (water-soluble antioxidant) or with 13.5 μM α-tocopherol are also effective in preventing CEES toxicity to stimulated macrophages (data not shown). Preliminary results from our ongoing collaborative research efforts have demonstrated that delivery of antioxidants as liposomal formulations were effective in protecting against CEES-induced cellular injury in an animal model.

D. CHLORINE

Similar to phosgene, chlorine causes a delayed pulmonary edema. There is no specific antidote for chlorine gas inhalational exposure. Treatment for skin or eye contact is irrigation of the site. Inhalation of the gas can be treated supportively by the use of nebulized sodium bicarbonate and bronchodilators (Aslan et al., 2006). Modest improvement in animal models was obtained with the inhalation therapy using corticosteroids (Gunnarsson et al., 2000). Thiol-containing compounds are able to scavenge HOCl, which is suspected to be a significant metabolite of chlorine gas exposure (McKenzie et al., 1999). Enhancement of systemic levels of thiols by the use of orally administered NAC may be a consideration in life-threatening exposures to chlorine gas.

E. PHOSGENE

In experiments using agents as a prophylaxis, there is promise. Rats were exposed to phosgene in a whole-body chamber after 23 days of supplementation of vitamin E (α-tocopherol) or N-propyl gallate (nPG) (Sciuto and Moran, 2001). The vitamin E-fed rats did not show any survival

Vehicle	CEES	CEES + NAC

(a)

(b)
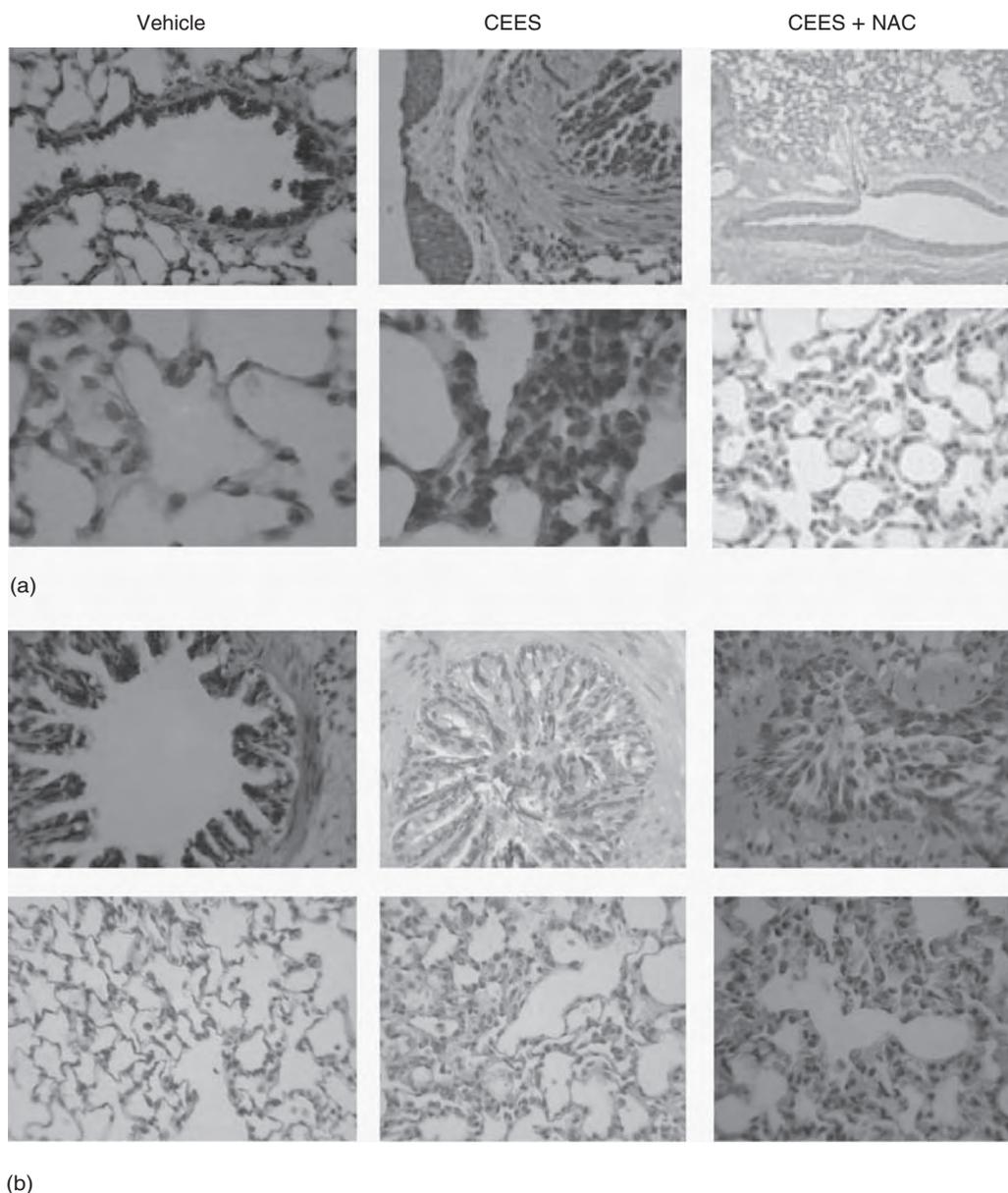
FIGURE 12.16 Histological analysis showing recovery from CEES-induced lung damage by pretreatment of NAC. Guinea pig lungs were examined under light microscope after 1 h (a) and after 21 days (b) of exposure to CEES (0.5 mg/kg body weight). Upper panel represents morphology of bronchi and lower panel represents morphology of the alveoli. Magnifications: 400×.

enhancement. The nPG-fed rats fed the lower doses of nPG (0.75%) showed the greatest increase in survival, and the higher dose of nPG (1.5%) was ineffective. In the lower dose of nPG, there was an obvious decrease in lipid peroxidation and increased lung tissue-reduced GSH. Gamma-tocopherol, in comparison with α-tocopherol, is a more potent anti-inflammatory agent and may have produced a much different result under the same experimental conditions that Sciuto and Moran (2001) performed (Jiang and Ames, 2003).
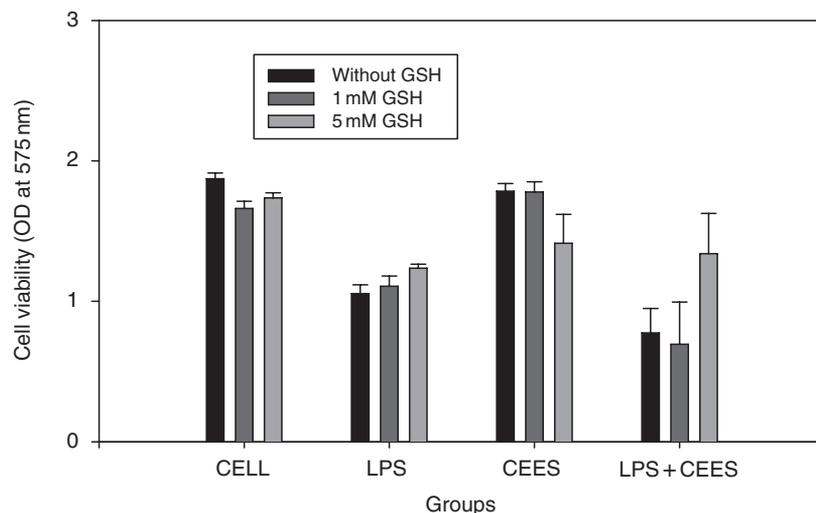
FIGURE 12.17 GSH-liposomes (5 mM) protect simulated macrophages from CEES toxicity.

Postexposure drugs used as a countermeasure to phosgene are more reflective of a clinical application or military theater exposures. The arachidonic acid analog 5-, 8-, 11-, 14-eicosatetraynoic acid (ETYA) inhibits the release of arachidonic acid metabolites, which produce both leukotrienes and prostaglandins (Farrukh et al., 1988). ETYA used as a postexposure treatment in an in situ lung model resulted in less lung edema, as well as increased gluathione levels and lower lipid peroxidation levels. It is questionable as to whether ETYA possesses any antioxidant properties (Sciuto, 2000).

VIII. OXIDATIVE STRESS IN DIFFERENT ORGAN SYSTEMS

A. LUNG

SM inhalation can produce hoarseness, laryngitis, acute airway obstruction, bronchopneumonia, pulmonary edema, and hemorrhage (Calvet et al., 1994). Bronchiolitis and dyspnea are common clinical findings. Examination of the pulmonary tree reveals a mild fibrosis in the parenchyma. Exaggerated fibroblast proliferation and increased collagen synthesis represent two critical events in the pathogenesis of this type of pulmonary fibrosis. The main initial changes are extreme hyperemia, exudation of inflammatory cells, cellular infiltration of the submucosa and detachment, necrosis, and cellular death of the respiratory epithelial lining (Chevillard et al., 1992).

Acute, heavy exposure to SM causes loss of the columnar cells of the upper respiratory tract, peribronchial edema, hyperemia of the blood vessels, cellular infiltrations in the submucosa, and intense vacuolization and disorganization of the cytoplasmic and nuclear structures (Emad and Rezaian, 1997, 1999). Pulmonary hemorrhage, pulmonary edema, and respiratory failure similar to ARDS may also occur. These cytotoxic effects are associated with acute thermal injury sustained by the airway mucosa and lead to scarring and development of stenosis of the tracheobronchial tree as was observed in 9.64% of the SM-exposed patients.

Cross-sectional study on the late pulmonary sequelae of SM-exposed veterans showed airway narrowing or stricture, asthma (10.65%), chronic bronchitis (58.88%), bronchiectasis (8.62%), and pulmonary fibrosis (PF; 12.18%) after 10 years. Crystal et al. (1984) recommended that SM be added to the various causes of interstitial lung diseases and PF (Crystal et al., 1984; DePaso and Winterbauer, 1991; Emad and Rezaian, 1997). The cellular constituents of bronchoalveolar (BAL)

fluid in patients with SM-induced PF are very similar to the cellular constituents seen in patients with idiopathic PF, and this finding indicates the presence of an ongoing active alveolitis in PF (Emad and Rezaian, 1997).

Under normal physiological conditions, airway lining fluids and extracellular spaces are maintained in a highly reduced state. Typically, the levels of antioxidants and oxidants in lung are balanced in favor of a reducing state (redox homeostasis). Decreases in antioxidants or increases in oxidants can disrupt this equilibrium and can cause OS. An imbalance in oxidant–antioxidant system has been recognized as one of the first changes that ultimately lead to inflammatory reactions (Crapo, 2003a).

An increased oxidizing environment can facilitate antigen-presenting cell (APC) maturation and T-cell activation, leading to the activation of the adaptive immune responses and, potentially, a hyperresponsive innate immune system. Thus, OS appears to play an important role in the pathogenesis of the asthmatic reaction. It is not surprising that OS occurs in many forms of lung disorders, such as pneumonia, adult respiratory distress syndrome, idiopathic pulmonary fibrosis, lung transplantation, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis ische- AQ10
mia-reperfusion, and lung cancer (Bowler and Crapo, 2002; Crapo, 2003b; Rahman and Kelly, 2003; Rahman et al., 2004).

An oxidizing state in cells can initiate intracellular signaling cascades that lead to the production of inflammatory mediators. Stress kinases (JNK, ERK, p38) and transcription factors, such as NF-κB and AP-1, are known to be redox sensitive. On their activation, these signaling pathways lead to production of TNF-α, IL-1-beta, IL-6, IL-8, IL-12, adhesion molecules (VCAM-1, ICAM-1), and GM-CSF (Mastruzzo et al., 2002). In addition to protein expression, OS favors uncoiling of DNA, thereby increasing accessibility to transcription factor binding (Rahman et al., 2004). The presence of OS can also damage alveolar epithelial cells by the induction of apoptosis. The angiotensin-converting enzyme system is also known to be redox sensitive. OS facilitates the conversion of angiotensinogen into angiotensin II, which is a bioactive peptide with a broad range of activities, including the induction of apoptosis in epithelial cells and the activation of fibroblasts. Angiotensin II can also increase the lung levels of transforming growth factor (TGF)-α, which is a crucial factor in the development of fibrosis (Mastruzzo et al., 2002).

The maintenance of a reducing environment in lung is considered to be crucial in the lung function. A balance between intracellular and extracellular oxidants and antioxidants is a prerequisite for normal lung homeostasis. The lung has highly specialized and compartmentalized antioxidant defenses to protect against reactive oxygen species (ROS) and RNS. These include the following: (1) small molecular weight antioxidants (e.g., GSH, vitamins, uric acid); (2) mucins; (3) metal-binding proteins (transferrin, lactoferrin, metallothionein, etc.); (4) SODs (e.g., mitochondrial manganese SOD [MnSOD], intracellular copper zinc SOD [CuZnSOD], and extracellular SOD [ECSOD]); (5) a group of enzymes that decomposes hydrogen peroxide (H_2O_2) (numerous GSH-associated enzymes and catalase); (6) detoxification enzyme systems (e.g., GST); and (7) other redox-regulatory thiol proteins (e.g., thioredoxin–peroxiredoxin system and glutaredoxins) (Powis et al., 2000; Fattman et al., 2003; Kinnula and Crapo, 2003).

Reduced GSH appears to be one of the most important antioxidant defense systems. In the extravascular lung fluid that coats the alveolar epithelial surfaces, GSH is present in millimolar quantities. A decrease in the ratio between GSH and oxidized glutathione (GSSG) occurring during OS leads to the activation of a variety of cellular redox-sensitive signaling pathways. Antioxidant enzymes such as SOD and catalase also play an important role in the clearance of oxidative radicals in the lung. The primary oxidant-generating enzyme is nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase in phagocytes such as alveolar macrophages and neutrophils. On activation, phagocytes generate superoxide anions, which lead to the production of H_2O_2 for host defense.

It is clear that a better understanding of the oxidative state in the lung is important for the diagnosis and treatment of lung diseases. There are many methods developed for the detection of free radicals from oxygen, ROS, and their by-products to assess the presence of OS. The techniques

include established standard protocols and advanced methodologies using HPLC, mass spectrometry, and electron paramagnetic resonance (EPR). The following sections describe the most frequently used methods to measure lung oxidative status.

1. Monitoring Oxidative Stress in Live Cells

ROS in live cells can be detected by using fluorogenic marker for ROS and observed under fluorescence microscopy. One of frequently used makers is carboxy-H_2DCFDA, a permeable fluorogenic marker, which is oxidized during OS in live cells and emits bright green fluorescence (Wan et al., 2005).

2. Hydrogen Peroxide and Superoxide Radical Generation in Bronchoalveolar Fluids

H_2O_2 fluids in BAL can be measured by the simple assay for the detection of the presence of peroxides in both aqueous and lipid environments. The basis of these assays is the complexing of ferrous ion (Fe^{+2}) by H_2O_2 in the presence of xylenol orange. Peroxides oxidize Fe^{+2} to Fe^{+3}, and Fe^{+3} forms a colored complex with xylenol orange that can be read at 560 nm (Jones et al., 1995). Superoxide radical generation can be estimated by nitroblue-tetrazolium reduction assay (Libon et al., 1993).

3. Antioxidant Status in Lung

The antioxidant status in the lungs can be evaluated by lung levels of SOD and catalase and their activities. SOD activity can be assessed by the OxyScan SOD-525 assay, which measures the activity of all forms of SOD. The method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Catalase activity can be determined by a two-step reaction scheme (Catalase-520 assay). First, catalase reacts with a known quantity of H_2O_2 to generate H_2O and O_2. In the presence of horseradish peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-amino-phenazone to form a chromophore with a color intensity. Lipid peroxidation levels in the lung can be measured by thiobarbutiric acid reactive substances assay (Erdincler et al., 1997). The GSH/GSSG ratio, a useful measure of OS, can also be determined by a colorimetric method by using Bioxytech GSH/GSSG-412.

4. Hydroxydeoxyguanosine, an Indicator of DNA Damage

The most common type of damage caused by ROS in the body is oxidative damage to DNA. Hydroxydeoxyguanosine (8-OHdG), a product of this type of DNA damage, is used as a biomarker for OS. It can be measured by the immunohistochemical procedure and a high-performance liquid chromatography system equipped with an electrochemical detector (HPLC-ECD) (Mei et al., 2003). More recently, it has been reported that capillary electrophoresis-mass spectrometry (CE/MS) can also be used for the analysis of 8-OHdG to study OS (Weiss and Lunte, 2000).

5. Direct Measurements of Oxygen Free Radicals

It is very difficult to directly measure oxygen-free radicals (OFRs) due to their short half-lives. To study these OFRs, radical spin-trapping agents have to be employed to facilitate the formation of stable radical adducts with the OFRs, for detection by EPR spectroscopy. Trapping agents are generally nitrone or nitroso-containing molecules, such as 5,5-dimethyl-1-pyrroline-*n*-oxide (DMPO), which react with OFRs to form stable nitroxide free radicals. These radical adducts can be analyzed by using EPR (Olea-Azar et al., 2003).

6. Exhaled Breath Condensate

Exhaled breath condensate collected by cooling or freezing the exhaled air is a totally noninvasive procedure. H_2O_2, leukotrienes, isoprostanes, and 3-nitrotyrosine are good candidates for OS assessments (Paredi et al., 2002). These products have been shown to elevate lung inflammation.

7. Analysis of Expired Air for Oxidation Products

Studies have shown that expired NO and CO can serve as biomarkers for OS, and ethane can serve as a marker of lipid peroxidation (Paredi et al., 2002). CO can be detected electrochemically, and it can also be measured by laser spectrophotometer and near-infrared CO analyzers. The levels of exhaled NO can be assessed by chemiluminescence. Ethane content can be detected using gas chromatography.

B. SKIN

Skin is one of the major sites of damage after exposure to vesicant chemical weapons. In the case of SM, there is a latent period after exposure, followed by an erythematous rash within 4–8 h, and then blistering some 2–18 h later. Elsayed et al. (1992) found that a nonlethal, s.c. dose of the mustard analog chloroethyl 4-chlorobutyl sulfide (CECBS) in mice caused damage to distal tissues, such as the lung, that was consistent with free radical, mediated OS. These authors suggest that ''antioxidants could potentially modulate the response and reduce the damage'' (Elsayed et al., 1992). Although useful, animal models do not exactly mimic the development of SM injury to human skin. Nevertheless, the mouse ear model, the rabbit, the hairless guinea pig, the nude mouse, and the weanling swine have all been useful for studying the: (1) pathophysiology, (2) molecular mechanism of action, and (3) efficacy of countermeasures for SM injury. Similarly, in vitro models have major limitations but have the advantage of being cost-effective and potentially very reproducible.

A synthetic human skin model, EpiDerm, showed considerable promise as an in vitro model. EpiDerm possessed all the main characteristic features of the native skin tissue, including the cuboidal appearance of the basal cell layer, the presence of the stratum spinosum and stratum granulosum with typical stellate-shaped keratohyalin granules, and the presence of numerous lamellar bodies that are extruded at the stratum granulosum–stratum corneum interface. The EpiDerm system has the potential for identifying and developing SM therapeutic agents but does have its limitations as well. In vivo, skin damage can be accompanied by the rapid leakage of serum and leukocyte infiltration but this cannot occur in the in vitro skin models.

The U.S. Army Research Institute of Environmental Medicine has used the EpiDerm model to study CEES-induced skin toxicity (Blaha et al., 2000a, 2000b, 2001). Blaha et al. (2000a), for example, studied the potential role of inflammatory cytokines on CEES-induced toxicity. Since CEES or SM is known to provoke an acute inflammatory response in skin, it is reasonable to assume that inflammatory cytokines are involved in this process and that EpiDerm would mimic the in vivo responses. Blaha et al. (2000a) found, however, that CEES depressed the levels of IL-1α and related cytokines. These authors concluded that the inflammatory responses seen in vivo are promoted by factors from sources other than keratinocytes (Blaha et al., 2000a). Stone et al. (2003, 2004) found that inflammatory cytokines dramatically enhance the toxicity of CEES in a macrophage model. It would be important, therefore, to determine if inflammatory cytokines increase CEES toxicity in the EpiDerm model. EpiDerm tissues in the presence of inflammatory cytokines may prove to be an excellent model to test the efficacy of countermeasures. Blaha et al. (2001) also demonstrated that CEES induces apoptosis in the EpiDerm model. This is a valuable observation since potential countermeasures could easily be tested for their antiapoptotic effects in this model.

1. Role of Skin Mast Cells in Vesicant Toxicity

Rikimaru et al. (1991) have used full-thickness human skin explants to study inflammatory mediators in response to topically applied SM. These investigators found that culture fluids from

the SM-treated skin contained increased levels of histamine, plasminogen-activating activity, and prostaglandin E2 compared with control explants. It was concluded that both mast cells and epidermal cells were apparently involved in early mediation of the inflammatory response to SM (Rikimaru et al., 1991). In contrast, Inoue et al. (1997) found that the inflammatory response of the mouse ear to SM did not differ in mast cell-deficient mice compared with normal mice. At present, there is no obvious explanation for the differences observed between the work of Rikimaru et al. (1991) and that of Inoue et al. (1997). It may well be that the mouse ear is not an optimal model for human skin. It is, however, critically important to determine if SM, or other toxic vesicants, degranulate mast cells, since this process could be a major source of inflammatory mediators.

C. EYES

The eye is a complex sensory organ, which receives visual information from the environment. It encodes optical information into complex electrical signals, which are transmitted to the cortex for visual imagery through the optical nerve. The visual efficiency primarily depends on the optical clarity of the eye (e.g., cornea, crystalline lens, and intraocular media) and the neural integrity of the visual pathway (e.g., retina, optic nerve, and visual cortex).

The pathogenesis of most age-related eye disorders remain poorly understood. Significant evidence points to oxidative damage as a major factor in the initiation and progression of numerous age-related diseases (Kowluru and Kennedy, 2001; Algvere and Seregard, 2002; Hogg and Chakravarthy, 2004; Shichi, 2004; Ohia et al., 2005; Truscott, 2005). Generally, OS occurs when the level of ROS exceeds the ability of the cell to respond through antioxidant defenses leading to the modification and degradation of carbohydrates, membrane lipids, proteins, and nucleic acids (Gamaley and Klyubin, 1999; Kimura et al., 2005). H_2O_2, a relatively stable oxidant, is present at low concentrations in the normal eye and is found at elevated concentrations in some patients with maturity-onset cataract (Beatty et al., 2001; Ohia et al., 2005; Truscott, 2005). Oxidative damage has also been hypothesized to play a role in the pathogenesis of glaucoma, as the trabecular meshwork is exposed to high levels of OS arising from aerobic metabolism, high aqueous concentrations of H_2O_2, and photochemical reactions in the anterior segment (Shichi, 2004; Ohia et al., 2005). The retina is particularly susceptible to OS because of its high consumption of oxygen, its high proportion of polyunsaturated fatty acids (PUFAs), its abundance of photosensitizers and its exposure to visible light (Sickel, 1972; Beatty et al., 2001). In general, it is well known that the greater the oxygen content of tissues, the more susceptible they are to oxidative and photooxidative damage. The retina is supplied with oxygen by the blood with generally high oxygen content in different portions of the retinal tissues. The lipids present in the membranes of rods contain a high percentage of PUFAs, particularly docosahexanoic acid (DHA) (22:6ω-3), known to be the most highly PUFA in nature, making the retina inherently susceptible to lipid peroxidation (Sickel, 1972; Bazan, 1989; Beatty et al., 2001).

Although the eye is continuously exposed to OS, cells have numerous protective mechanisms to reduce the incidence of severe oxidative damage. Damage to the eye by increases in ROS is typically avoided because of a very efficient antioxidant system. It is further protected by pigments such as the kynurenines and melanin (Roberts, 2001). The major water-soluble antioxidants are   AQ11
GSH and ascorbic acid (Reddy and Giblin, 1984; Reddy, 1990; Delamere, 1996; Rose et al., 1998; Lou, 2003; Rahman and Kelly, 2003). Ascorbic acid, the most effective aqueous-phase antioxidant in human blood, is present in high concentrations in the lens, cornea, retinal pigment epithelium, and aqueous humor of humans (Delamere, 1996; Rose et al., 1998). GSH is a naturally occurring tripeptide and is found mostly concentrated in the lens epithelium; its concentration in the lens is as high as that seen in the liver. GSH acts as a reductant of peroxides either by a nonenzymatic reaction or by a reaction catalyzed by GSH peroxidase. GSH may be especially important in protecting the thiol groups of crystallins, preventing them from aggregating to form opaque clusters. Aging lenses or lenses under OS show an extensively diminished size of GSH pool, with some protein thiols

being S-thiolated by oxidized nonprotein thiols to form protein thiol-mixed disulfides, as protein-S-S-glutathione (PSSG), protein-S-S-cysteine (PSSC), or protein-S-S-gamma-glutamylcysteine (Megaw, 1984; Reddy and Giblin, 1984; Reddy, 1990; Rose et al., 1998; Beatty et al., 2000; Ganea and Harding, 2006).

AQ12

The principal lipid-soluble antioxidants are vitamin E and the carotenoids (Hunt et al., 1984; Snodderly, 1995; Khachik et al., 1997; Beatty et al., 2001). Vitamin E is the major chain-breaking, lipid-soluble antioxidant in membranes, and is thus expected to play the most important role in minimizing effects of oxidation of PUFAs. Both vitamin E and the carotenoids scavenge free radicals, particularly hydroxyl radical and singlet oxygen. Vitamin E is recycled by redox coupling with vitamin C. The retina contains high quantities of α-tocopherol (outer segments of rod) and rod and retinal pigmented epithelium (RPE), and the concentrations within these tissues are very sensitive to dietary intake of the vitamin E (Hunt et al., 1984; Beatty et al., 2002). Vitamin E deficiency has been shown to result in retinal degeneration, excessive RPE lipofuscin levels, and a decrease in the PUFA content of rod outer segments and the RPE, suggesting that vitamin E protects against retinal oxidative damage (Hayes, 1974; Beatty et al., 2001). The antioxidant properties of the carotenoids are now well established, and they possess the ability to quench singlet oxygen and triplet sensitizers, interact with free radicals, and prevent lipid peroxidation. Of the many carotenoids circulating in human sera, only lutein and zeaxanthin are accumulated throughout the tissues of the eye, where they reach their highest concentration in the central retina (macula lutea). Lutein and zeaxanthin are more commonly referred to as macular pigments (Snodderly et al., 1984; Khachik et al., 1997).

AQ13

AQ14

SOD, catalase, and GSH peroxidase are antioxidant enzymes that play a significant role in protecting the retina from oxidative damage. GSH peroxidase is found in the retina and uses GSH as an electron donor to reduce fatty acid hydroperoxides, phospholipid hydroperoxides, cholesterol hydroperoxides, and H_2O_2 (Beatty et al., 2002; Ganea and Harding, 2006). SOD catalyzes the quenching of the superoxide anion to produce H_2O_2 and oxygen (Beatty et al., 2002; Lin et al., 2005). Catalase is an iron (Fe)-dependent enzyme that scavenges H_2O_2 either catalytically or peroxidatively. Catalase has been demonstrated in human neurosensory retina and RPE (Roberts, 2001; Beatty et al., 2002; Ohia et al., 2005).

AQ15

1.   Vesicant-Induced Ocular Injury and Oxidative Stress

The eyes are the organs most sensitive to vesicants, which cause cellular changes within minutes of contact; however, the onset of signs and symptoms to vesicant exposure may become evident several hours later. The time course of symptom development after exposure between SM and the nitrogen analogs is nearly the same. The initial contact of mustard gas with the eye for the most part does not cause pain and discomfort. Mild (12–70 mg/m^3/min) to moderate (100–200 mg/m^3/min) exposures might result in irritation, pain, swelling, and tearing that may occur within 3–12 h postexposure. Similar symptoms might appear 1–2 h after severe exposure (>200 mg/m^3/min) but the symptomatology might also include light sensitivity, severe pain, or temporary blindness. Physical findings include blepharospasm, periorbital edema, conjunctival injection, and inflammation of the anterior chamber (Solberg et al., 1997; Safarinejad et al., 2001; Banin et al., 2003; Javadi et al., 2005).

AQ16

In the Iran–Iraq conflict, SM was heavily used and even now, about 30,000 victims still suffer from late effects of the agent, for example, chronic obstructive lung disease, lung fibrosis, recurrent corneal ulcer disease, chronic conjunctivitis, abnormal pigmentation of the skin, and several forms of cancer (Kehe and Szinicz, 2005). Evaluation of Iranian survivors with chronic or delayed-onset mustard gas keratitis revealed that mustard gas caused chronic and delayed destructive lesions in the ocular surface and cornea, leading to progressive visual deterioration and ocular irritation. Excised conjunctival and corneal specimens revealed a mixed inflammatory response without any specific features. Light microscopy of conjunctival specimens showed decreased goblet cell density, thickened epithelium, scarring in the substantia propria associated with plasmacytic and lymphocytic

AQ17

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infiltration, and dilated lymphatic vessels. Excised corneal buttons disclosed the absence of epithe-lium and Bowman's layer, stromal scarring, and vascularization. The pathophysiologic features of these changes are not clearly identified. Based on the clinical appearance of the lesions and the histopathologic findings, an immune-mediated component seems possible (Javadi et al., 2005).

In addition to their alkylating properties, mustards are now being recognized to mediate their toxic actions, at least in part, via the formation and action of ROS (Banin et al., 2003). A dramatic increase in copper levels and a decrease in ascorbic acid within the anterior chamber after ocular exposure to mustard compounds are evidences to implicate the role of OS in mustard-induced eye injuries (McGahan and Bito, 1982; Kadar et al., 2001; Banin et al., 2003). Mustards are also known to rapidly inactivate sulfhydryl-containing proteins and peptides, such as GSH. These sulfhydryl compounds are critical in maintaining the appropriate oxidation–reduction state of cellular com-ponents, and GSH is also thought to be critical in reducing ROS in the cell and preventing peroxidation and loss of membrane integrity (Stadtman, 2001). Furthermore, the amelioration of mustard-induced ocular injuries by antioxidants is also evidence to implicate OS as a potential mechanism of injury (Banin et al., 2003; Morad et al., 2005). AQ18 AQ19

Recognizing the fact that ROS play a role in the pathogenesis of mustard-induced ocular injuries, compounds that inhibit the formation of ROS or prevent their toxic effects would be beneficial in the treatment of mustard-induced ocular injuries. The topical application of low concentrations of Zn/DFO or Ga/DFO after corneal exposure to nitrogen mustards markedly reduced conjunctival, corneal, iris, and anterior chamber injury. In the cornea, the healing of epithelial erosions was faster, the long-term opacification was reduced, and the levels of neovascularization were lowered. In the anterior chamber, decreased inflammation and better maintenance of intraocular pressure were achieved. Cataractous changes were also notably milder (Banin et al., 2003).

A combination of topically applied Zn/DFO and dexamethasone, by virtue of their additive inhibitory effects on free radical formation and inflammation, reduced nitrogen mustard-induced injury to ocular anterior segment structures. Furthermore, the combination treatment of Zn/DFO and dexamethasone resulted in a speedier corneal reepithelization, less-severe corneal neovasculariza-tion, and the intraocular pressure was not as severely elevated as in the saline or the Zn/DFO- or dexamethasone-alone groups (Morad et al., 2005).

2. Redox Proteomics

OS induces free radical damage to biomolecules and alterations in redox-sensitive signaling path-ways, both of which are key factors in understanding vesicant toxicology. In particular, small thiols, like GSH, are no longer viewed just as protective antioxidants but as redox regulators of proteins via glutathionylation or by oxidation of protein cysteine residue (Ghezzi and Ungheri, 2004). Redox proteomics is rapidly emerging as a very powerful tool for characterizing and identifying proteins based on their redox state (Ghezzi and Ungheri, 2004). This approach has not yet been applied to the study of vesicant toxicity, but studies should soon begin on exploring this area.

IX. OXIDATIVE STRESS: THE CONCEPT AND THE EFFECT ON GENE EXPRESSION

A. DEFINITION OF OXIDATIVE STRESS

Redox potential is defined by the half cell reduction potential that is created by redox couples that are primarily due to GSH, NAD^+ and nicotinamide dinucleotide phosphate. These couples are in ratios of the oxidized to reduced form of the molecules (NAD^+/NAD, $NADP^+/NADPH$, and $GSSG/2GSH$). The redox couples can be independent, as well linked to each other to form related couples. The redox environment is a reflection of these couples. These ratios can be measured by the Nernst equation, similar to a voltaic cell.

The Nernst equation is

$$E_h = E_0 + (RT/nF) \ln\{[\text{acceptor}]/[\text{donar}]\}.$$

E_h is the electromotive force at a particular pH, its units are in volts or millivolts relative to a standard hydrogen electrode (1 atom H_2, 1M H^+). The electromotive force is a quantitative measurement of a redox-active molecule that donates or accepts electrons.

R is the gas constant, T is the absolute temperature, F is the Faraday's constant, and n is the number of electrons transferred. The steady state of E_h for a redox-active component depends on the kinetic of the transfer of the reduction and oxidation reactions. Under normal homeostatic conditions (absence of OS), the E_h for 2GSH/GSSG relatively reduces because of the NADPH-coupled GSSG reductase. During periods of OS, the E_h becomes more oxidized.

Under normal cellular conditions, the redox potential is reductive. In diseased conditions, wherein OS occurs, the redox potential becomes more oxidative. The redox potential is similar in different cell types, but varies according to the cellular processes: proliferation $E = -240$ mV, differentiation $E = -200$ mV, and apoptosis $E = -170$ mV (Kirlin, 1999).   AQ20

An illustrative experimental example of a cell exposed to an oxidant was carried out by Kirlin et al. (1999). When they used HT29 cells (colon adenocarcinoma) and exposed them to sodium butyrate, it was found that there was an oxidant reaction that caused a drop from -260 to -200 mV in E_h. The 60 mV decrease resulted in a 100-fold change in protein dithiols: disulfide ratio. A correlation was noted between E_h, GST, and NADPH:quinone reductase. Kirlin further indicated that the E_h provides two additional pieces of information: (1) in reactions that use GSH as a reductant to maintain protein thiol/disulfides in their reduced form, it is an indicator of the reducing power ~~quantitatively~~ and (2) if the redox state is controlled by a GSH redox couple, it is an indication of the functional state of the protein (Kirlin et al., 1999).

B. MOLECULES THAT INFLUENCE THE REDOX POTENTIAL

GSH ~~synthase~~, a tripeptide (glutamylcysteinylglycine), is not only a water-soluble antioxidant, but is also part of a redox buffer (Smith et al., 1996). It is found in all cells and is used for a multiplicity of cellular functions, such as protein and prostaglandin synthesis, detoxification, etc. Cytosolic concentrations of GSH range from 1 to 11 mm (Smith et al., 1996) and are 100–1000 times greater than the extracellular levels. Many proteins contain sulfhydryl groups because of their cysteine content. The content of thiols in proteins is greater than that of the pool of GSH (Torchinsky, 1981).

The intracellular compartment exchanges GSH with the cytosol (Griffith and Meister, 1985; Schnellmann et al., 1988; Fernandez-Checa et al., 1998). GSH concentrations within the nucleus are critical for maintaining the redox state of protein sulfhydryls that are necessary for DNA repair and expression (Arrigo, 1999). The endoplasmic reticulum has a more oxidizing environment than the cytosol or nucleus. GSH/GSSH in the endoplasmic reticulum is in the range of 1:1 to 3:1, in comparison with the cytosol (Hwang et al., 1992). The ratio of reduced GSH to GSSG influences a variety of cellular signaling processes, such as activation and phosphorylation of stress kinases (JNK, p38, PI-3K) via sensitive cysteine-rich domains, activation of SHM-ase ceramide pathway, and activation of AP-1 and NF-κB, with subsequent gene transcription (Singh et al., 1998; Arrigo, 1999; Mercurio and Manning, 1999a, 1999b).

1. Nicotinamide Adenosine Dinucleotide Phosphate

NADPH is usually involved in reductive (biosynthetic) reactions and serves as a source of electrons. In contrast, NAD is involved in oxidative reactions and serves as a sink for electrons. In cells and tissues, the ratio of NADPH/$NADP^+$ tends to be 1:10 and 1:1000. NADPH is considered the primary source of reducing equivalents for GSH.

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2. Thioredoxin

Thioredoxin (TRX) is a pleiotropic polypeptide also known as T-cell leukemia-derived factor (Tagaya et al., 1989; Yodoi and Uchiyama, 1992). It has two redox-reactive cysteine residues in the reactive center (Cys-32 and Cys-35) (Holmgren, 1972, 1985, 1989; Buchanan et al., 1994). Reductases are used to donate electrons from NADPH to facilitate the reduction oxidation reaction between the dithiol or disulfide forms of TRX (Luthman and Holmgren, 1982; Watson et al., 2004).

TRX is responsible for the reduction of cysteine moieties in the DNA-binding sites of several transcription factors (Mathews, 1992; Okomoto, 1992), facilitates the refolding of disulfide-contain- AQ21 ing proteins, and regulates the DNA-binding activity of some transcription factors (e.g., NF-κB and Ref-1-dependent AP-1) (Sen, 1998; Arner and Holmgren, 2000). TRX facilitates gene expression in that it enables protein–nucleic acid interactions (Holmgren, 1985); it does so by reducing cysteine in the DNA-binding loop of several transcription factors (Matthews et al., 1992; Okamoto et al., 1992; Xanthoudakis et al., 1992, 1994). The redox state of TRX varies independently to that of GSH/GSSG. GSH forms intermolecular disulfides, whereas TRX is a protein that usually forms intramolecular disulfides. TRX assists in the control of apoptosis signal, regulating kinase-1 (ASK-1) (Saitoh et al., 1998).

C. APOPTOSIS

Apoptosis is induced, when tissue is exposed to SM by a calmodulin- and caspase-dependent pathway (Rosenthal et al., 1998; Sciuto and Hurt, 2004). Zhang et al. (2001) using Jurkat cells exposed to CEES, analyzed gene expression for death and survival pathways. The Akt (PKB) is an important kinase, which can block apoptosis and promote cell survival. It was downregulated in a dose-dependent manner. The antiapoptotic genes, Bcl family, were decreased: Bcl-2, 90%; bax, 80%; bcl-X_L, 67%; Bak and Mcl-1, 70%; and Bik, 57%. Caspases 3, 4, 6, 8, and 9 were upregulated. Crawford et al. (unpublished data) found that CEES exposed human dendritic cells released cytochrome *c* before detectable levels of ROS generation. NAC was found to inhibit the release of cytochrome *c* and decrease apoptosis. The release of cytochrome *c* was likely due to the opening of the mitochondrial transport permeability transition pores, which is regulated by two redox couples, NADP and GSH (Dalton et al., 1999). It is well known that a portion of the toxicity of mustard is the depletion of NAD (Mol et al., 1989; Byers et al., 2000).

A depletion of GSH alone can act as an early activation of apoptotic signaling (Coffey et al., 2000; Coppola and Ghibelli, 2000; Armstrong and Jones, 2002). Conversion from the procaspase to an active enzyme requires a reduction of the cysteine residue (Hampton et al., 1998). OS can trigger caspase activity, but can also suppress it (Hampton et al., 1998).

Studies conducted by Celli et al. (1998) used BSO (buthionine sulfoximine) to deplete cells of GSH. There was a significant decrease in the Bcl levels and associated time-dependent increase in the number of cells undergoing apoptosis. Maintenance of GSH levels with GSH ethyl ester in the presence of BSO decreased apoptosis and prevented a decrease in Bcl-2 protein. The Celli study, if contrasted to Zhang et al. (2001), one begins to suspect that there may be a strong correlation between the loss of GSH and gene expression in CEES-exposed tissues.

The suppression of Bcl-2 expression induces the relocalization of GSH to the cytosol, whereas the overexpression of Bcl-2 induces a relocalization of GSH to the nucleus. There was a direct correlation between GSH levels and the Bcl-2 nuclear protein levels. It was concluded by Voehringer et al. (1998) that one of the functions of Bcl-2 is to promote the sequestration of GSH into the nucleus.

D. GENE EXPRESSION CONTROLLED BY REDOX-STATE TRANSCRIPTION FACTORS

Redox homeostasis regulates activation and binding by transcription factors at the 5′ end of the target gene. In the transcription factors, cysteine residues are frequently located in their protein

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sequence localized in their DNA-binding domain. The cysteine molecules are often essential for recognition of the binding site due to electrostatic interactions with specific DNA bases. Inhibition of the binding factor occurs if the cysteine is oxidized, which may be a consequence of an alteration of the tridimensional structure of the transcription factors. The function of several transcription factors could be impaired or inhibited because of oxidation of cysteine groups that result in inter- or intramolecular disulfide bonds (Arrigo, 1999). Several of the transcription factors undergo redox modification posttranslationally. NF-κB binding with DNA is augmented by TRX in vitro. Ref-1 is also modulated by redox compounds (e.g., TRX) (Hirota et al., 1997).

1. NF-κB

NF-κB regulates many genes, particularly those involved in immune and inflammatory responses (Baeuerle and Baltimore, 1996). Antioxidants, such as NAC, GSH, and cysteine, inhibit the activation of NF-κB (Mihm et al., 1995). TRX regulates the binding of NF-κB to DNA (Matthews et al., 1992).

2. Ref-1

In an oxidizing environment, Ref-1 facilitates the binding transcription factors to DNA by reduction of cysteine residues within the binding domains of these proteins (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992; Walker et al., 1993). Under OS, cysteine 65 forms a disulfur bond with cysteine 93, which ceases the stimulatory activity of Rdf-1. Active Ref-1 is recycled by the contribution of hydrogen from TRX (Hirota et al., 1997).

3. Activator Protein-1

Activator protein-1 (ATP-1) binds to an enhancer element (Angel et al., 1987; Lee et al., 1987), and is modified by redox factor 1 (Ref-1), a DNA repair enzyme (Angel et al., 1987; Lee et al., 1987; Arrigo, 1999). It is present in the promoter region of several genes, which are implicated in cell proliferation and tumor promotion. Several growth factors, PKC activators, and intracellular redox activate it (Angel and Karin, 1991). It consists of c-Jun and c-Fos proteins, which are products of the c-jun and c-fos proto-oncogene. Agents that promote intracellular oxidants such as UV irradiation, H_2O_2, and mitogens induce c-fos and c-jun genes (Datta et al., 1992; Lo and Cruz, 1995). Antioxidants that have a phenolic group (e.g., d-α-tocopherol or butylated hydroxyltoluene) induce expression of c-fos and c-jun (Choi and Moore, 1993; Stauble et al., 1994). ATP-1 acts as a secondary antioxidant-responsive transcription factor (Meyer et al., 1993).

4. Heat Shock Transcription Factor

Heat shock transcription factor (HSF) is part of the family of heat shock factors that are activated and bind to DNA induced by OS. Several chemicals, heat shock, or conditions that generate abnormally folded proteins activate HSF1 conversion from a monomer to a trimer state (Liu et al., 1996). Iodoacetamide (IDAM), an alkylating agent, activates the transcription of Hsp 70 gene (Liu et al. 1996). The depletion of GSH induces oxidation of protein thiols, denaturation, and aggregation of proteins (Freeman et al., 1997). Alkylating agents that deplete GSH increase HSF1 (Liu et al., 1996) and induce trimerization of HSF1.

X. SUMMARY

Chlorine, phosgene, Lewisite, and SM all react with thiol groups as well as produce oxidants. The arsenic group in Lewisite has a high affinity to the alpha and gamma thiol groups of lipoic acid found in enzymes (e.g., pyruvate oxidase). Oxidants occur as part of the normal metabolism of cells (redox homeostasis). In the diseased state (e.g., exposure to a chemical agent), there is an acute

inflammatory response that is also inclusive of OS (redox imbalance). During significant OS, the oxidant burden exhausts the redox buffer of the cell (e.g., GSH/GSSH, NADP/NADPH, etc.) that consequently alters redox couples. The mitochondrial permeability transition pores (MPTPs) are directly regulated by the redox state. They are a cyclosporine A-sensitive, Ca^{2+}-dependent, and voltage-gated channel. There are two types. One is dependent on the GSH:GSSH ratio (Petronilli et al., 1994; Costantini et al., 1995); the other is a voltage-sensitive gate, regulated by the ratio of pyridine nucleotides (NAD^+NADP):($NADH^+NADPH$) and is independent of the reduced GSH (Reed and Savage, 1995; Costantini et al., 1996). MPTPs appear to be involved in the toxicity of several chemicals (Dalton et al., 1999). Opening of the pores results in energy uncoupling by a Ca^{2+}-dependent decrease of mitochondrial inner-membrane potential (Petronilli et al., 1994). The collapse of the membrane potential and the inhibition of oxidative phosphorylation result in diminished intracellular ATP. Consequently, there may be the release of inner-membrane cytochrome c to the cytosol, which may signal the initiation of apoptosis (Krippner et al., 1996).

In a comparison of SM to CEES done using a histological grading system, SM was shown to be about six times more potent than CEES (Dana Anderson, unpublished results). The doses that were used by our group in the in vivo models were found to be equivalent to those used at the U.S. Army Medical Research Institute of Chemical Defense. CEES increases the activity of the transcription factor NF-κB, and consequently, there is an increase in proinflammatory cytokine production (e.g., TNF-α). PARP activity increases dramatically in both CEES- (Crawford, unpublished results) and SM-exposed tissues (Bhat et al., 2006). Increased PARP activity affects the energy levels of the cell by oxidizing NADPH, which causes a redox imbalance ($NADP^+$/NADPH). The transcription factor NF-κB and caspases are increased in CEES- and SM-exposed tissues. In both CEES and SM exposures, there is a loss of GSH, likely due to direct interaction with thiols and OS. The combined loss of GSH, NADH, and NADPH has far-reaching ramifications on multiple cellular systems, particularly redox-regulated pathways.

A. ANTIDOTES

Lewisite is the only vesicant with a proven antidote—BAL (2,3-dimercaptopropanol). Increasing antioxidant levels have been found to be protective against the mustards analog, CEES. NAC, which we have used in our studies with CEES, is immediately clinically available. It is most commonly used for acetaminophen overdose. NAC has a long history of several gram quantities administered in several doses and has minimal adverse reactions. In the case of acetaminophen overdose, it is administered via the oral-gastric route, which increases hepatic GSH levels, and in turn, suppresses inflammatory cytokines (Dambach et al., 2006). Liposome encapsulation of both water- and fat-soluble antioxidants was proven to be more effective in the suppression of OS than the free molecule of NAC.

Antioxidants that are liposome encapsulated are advantageous in that they enhance delivery to sites at which inflammation occurs. In light of the common effect that the vesicants have on redox-regulated pathways and OS, it becomes a compelling reason for additional research. The down-regulation of OS may be a very significant step forward in developing treatment countermeasures against several vesicants and other WMD.

REFERENCES

Akira, S. and Kishimoto, T. (1997). NF-IL6 and NF-κB in cytokine gene regulation. *Adv Immunol* 65, 1–46.

Algvere, P.V. and Seregard, S. (2002). Age-related maculopathy: pathogenetic features and new treatment modalities. *Acta Ophthalmol Scand* 80, 136–43.

Allan, D. (2000). Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis. *Biochem J* 345 (Pt 3), 603–10.

Alphonse, G., Aloy, M.T., Broquet, P., Gerard, J.P., Louisot, P., Rousson, R., and Rodriguez-Lafrasse, C. (2002). Ceramide induces activation of the mitochondrial/caspases pathway in Jurkat and SCC61 cells

sensitive to gamma-radiation but activation of this sequence is defective in radioresistant SQ20B cells. *Int J Radiat Biol* 78, 821–35.

Angel, P. and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1072, 129–57.

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H.J., and Herrlich, P. (1987). 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. *Mol Cell Biol* 7, 2256–66.

Ansceschi, M. (1989). The alveolar type II cells as a model for investigating the metabolism of surfactant phospholipids. *Eur Respir J* Suppl 3 10s–12.

Armstrong, J.S. and Jones, D.P. (2002). Glutathione depletion enforces the mitochondrial permeability transition and causes cell death in Bcl-2 overexpressing HL60 cells. *FASEB J* 16, 1263–5.

Arner, E.S. and Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267, 6102–9.

Arrigo, A.P. (1999). Gene expression and the thiol redox state. *Free Radic Biol Med* 27, 936–44.

Arroyo, C.M., Schafer, R.J., Kurt, E.M., Broomfield, C.A., and Carmichael, A.J. (2000). Response of normal human keratinocytes to sulfur mustards: cytokine release. *J Appl Toxicol* 20 Suppl 1, S63–72.

Aruoma, O.I. and Halliwell, B. (1987). Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochem J* 248, 973–6.

Aslan, S., Kandis, H., Akgun, M., Cakir, Z., Inandi, T., and Gorguner, M. (2006). The effect of nebulized NaHCO3 treatment on "RADS" due to chlorine gas inhalation. *Inhal Toxicol* 18, 895–900.

Awasthi, S., Vivekananda, J., Awasthi, V., Smith, D., and King, R.J. (2001). CTP: phosphocholine cytidylyl-transferase inhibition by ceramide via PKC-α, p38 MAPK, cPLA2, and 5-lipoxygenase. *Am J Physiol Lung Cell Mol Physiol* 281, L108–18.

Baburina, I. and Jackowski, S. (1998). Apoptosis triggered by 1-O-octadecyl-2-O-methyl-rac-glycero-3-phos-phocholine is prevented by increased expression of CTP: phosphocholine cytidylyltransferase. *J Biol Chem* 273, 2169–73.

Baeuerle, P.A. and Baltimore, D. (1996). NF-κB: ten years after. *Cell* 87, 13–20.

Barnes, P.J. and Adcock, I.M. (1998). Transcription factors and asthma. *Eur Respir J* 12, 221–34.

Bartsch, H. and Nair, J. (2006). Chronic inflammation and OS in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch Surg*.   AQ22

Bazan, N.G. (1989). The metabolism of omega-3 polyunsaturated fatty acids in the eye: the possible role of docosahexaenoic acid and docosanoids in retinal physiology and ocular pathology. *Prog Clin Biol Res* 312, 95–112.

Beatty, S., Murray, I.J., Henson, D.B., Carden, D., Koh, H., and Boulton, M.E. (2001). Macular pigment and risk for age-related macular degeneration in subjects from a Northern European population. *Invest Ophthalmol Vis Sci* 42, 439–46.

Bhat, K.R., Benton, B.J., and Ray, R. (2006). Poly (ADP-ribose) polymerase (PARP) is essential for sulfur mustards-induced DNA damage repair, but has no role in DNA ligase activation. *J Appl Toxicol* 26, 452–7.

Black, R.M. and Read, R.W. (1995). Biological fate of sulfur mustards, 1,1′-thiobis(2-chloroethane): identification of beta-lyase metabolites and hydrolysis products in human urine. *Xenobiotica* 25, 167–73.

Black, R.M., Brewster, K., Clarke, R.J., Hambrook, J.L., Harrison, J.M., and Howells, D.J. (1992). Biological fate of sulfur mustards, 1,1′-thiobis(2-chloroethane): isolation and identification of urinary metabolites following intraperitoneal administration to rat. *Xenobiotica* 22, 405–18.

Bladergroen, B.A., Bussiere, M., Klein, W., Geelen, M.J., Van Golde, L.M., and Houweling, M. (1999). Inhibition of phosphatidylcholine and phosphatidylethanolamine biosynthesis in rat-2 fibroblasts by cell-permeable ceramides. *Eur J Biochem* 264, 152–60.

Blaha, M., Bowers, W., Kohl, J., DuBose, D., and Walker, J. (2000a). Il-1-related cytokine responses of nonimmune skin cells subjected to CEES exposure with and without potential vesicant antagonists. 13, 99–111.   AQ23

Blaha, M., Bowers, W., Kohl, J., DuBose, D., Walker, J., Alkhyyat, A., and Wong, G. (2000b). Effects of CEES on inflammatory mediators, heat shock protein 70A, histology and ultrastructure in two skin models. *J Appl Toxicol* 20 Suppl 1, S101–8.

Blaha, M., Kohl, J., DuBose, D., Bowers, W., Jr., and Walker, J. (2001). Ultrastructural and histological effects of exposure to CEES or heat in a human epidermal model. *In Vitro Mol Toxicol* 14, 15–23.

Borak, J. and Sidell, F.R. (1992). Agents of chemical warfare: sulfur mustards. *Ann Emerg Med* 21, 303–8.

Bostan, M., Galatiuc, C., Hirt, M., Constantin, M.C., Brasoveanu, L.I., and Iordachescu, D. (2003). Phospholipase A2 modulates respiratory burst developed by neutrophils in patients with rheumatoid arthritis. *J Cell Mol Med* 7, 57–66.

Bouchard, V.J., Rouleau, M., and Poirier, G.G. (2003). PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol* 31, 446–54.

Bowler, R.P. and Crapo, J.D. (2002). Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 110, 349–56.

Brennan, F.M., Maini, R.N., and Feldmann, M. (1995). Cytokine expression in chronic inflammatory disease. *Br Med Bull* 51, 368–84.

Buchanan, B.B., Schurmann, P., Decottignies, P., and Lozano, R.M. (1994). Thioredoxin: a multifunctional regulatory protein with a bright future in technology and medicine. *Arch Biochem Biophys* 314, 257–60.

Burkle, A. (2001). PARP-1: a regulator of genomic stability linked with mammalian longevity. *Chembiochem* 2, 725–8.

Byers, S., Anderson, D., Brobst, D., and Cowan, F. (2000). Automated assay for nicotinamide adenine dinucleotide (NAD$^+$). *J Appl Toxicol* 20 Suppl 1, S19–22.

Byrne, M.P., Broomfield, C.A., and Stites, W.E. (1996). Mustards gas crosslinking of proteins through preferential alkylation of cysteines. *J Protein Chem* 15, 131–6.

Calvet, J.H., Jarreau, P.H., Levame M., D'Ortho, M.P., Lorino, H., Harf, A., and Macquin-Mavier, M.I. (1994). Acute and chronic effects of sulfur mustards intoxication in the guinea pig. *J Appl Physiol* 76, 681–8.

Carr, A.C., Hawkins, C.L., Thomas, S.R., Stocker, R., and Frei, B. (2001). Relative reactivities of N-chloramines and hypochlorous acid with human plasma constituents. *Free Radic Biol Med* 30, 526–36.

Celli, A., Que, F.G., Gores, G.J., and LaRusso, N.F. (1998). Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. *Am J Physiol* 275, G749–57.

Chatterjee, D., Mukherjee, S., Smith, M.G., and Das, S.K. (2004). Role of sphinomyelinase in the environmental toxin induced apoptosis of pulmonary cells. In: *In Lipids: Sphingolipid Metabolizing Enzymes*, eds. Dipak, Haldar, and Salil K Das, Research Signpost, pp. 117–139, Trivandum, Kerala, India.

Chatterjee, D., Mukherjee, S., Smith, M.G., and Das, S.K. (2003). Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustards analog. *J Biochem Mol Toxicol* 17, 114–21.

Chen, C.C., Wang, J.K., and Lin, S.B. (1998). Antisense oligonucleotides targeting protein kinase C-alpha, -beta I, or -delta but not -eta inhibit lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages: involvement of a nuclear factor kappa B-dependent mechanism. *J Immunol* 161, 6206–14.

Chevillard, M., Lainee, P., Robineau, P., and Puchelle, E. (1992). Toxic effects of sulfur mustards on respiratory epithelial cells in culture. *Cell Biol Toxicol* 8, 171–81.

Choi, H.S. and Moore, D.D. (1993). Induction of c-fos and c-jun gene expression by phenolic antioxidants. *Mol Endocrinol* 7, 1596–602.

Coffey, R.N., Watson, R.W., Hegarty, N.J., O'Neill, A., Gibbons, N., Brady, H.R., and Fitzpatrick, J.M. (2000). Thiol-mediated apoptosis in prostate carcinoma cells. *Cancer* 88, 2092–104.

Colvin, O.M., Friedman, H.S., Gamcsik, M.P., Fenselau, C., and Hilton, J. (1993). Role of glutathione in cellular resistance to alkylating agents. *Adv Enzyme Regul* 33, 19–26.

Coppola, S. and Ghibelli, L. (2000). GSH extrusion and the mitochondrial pathway of apoptotic signalling. *Biochem Soc Trans* 28, 56–61.

Costantini, P., Petronilli, V., Colonna, R., and Bernardi, P. (1995). On the effects of paraquat on isolated mitochondria. Evidence that paraquat causes opening of the cyclosporin A-sensitive permeability transition pore synergistically with nitric oxide. *Toxicology* 99, 77–88.

Costantini, P., Chernyak, B.V., Petronilli, V., and Bernardi, P. (1996). Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J Biol Chem* 271, 6746–51.

Cowan, F.M. and Broomfield, C.A. (1993). Putative roles of inflammation in the dermato-pathology of sulfur mustards. *Cell Biol and Toxicol* 9, 201–213.

Cowan, F., Broomfield, C.A., and Smith, W.J. (1992). Inhibition of sulfur mustards-increased protease activity by niacinamide, N-acetyl cysteine or dexamethasone. *Cell Biol Toxicol* 8, 129–138.

Crapo, J.D. (2003a). Oxidative stress as an initiator of cytokine release and cell damage. *Eur Respir J Suppl* 44, 4s–6.

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Crapo, J.D. (2003b). Redox active agents in inflammatory lung injury. *Am J Respir Crit Care Med* 168, 1027–8.

Crystal, R.G., Bitterman, P.B., Rennard, S.I., Hance, A.J., and Keogh, B.A. (1984). Interstitial lung diseases of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract. *N Engl J Med* 310, 235–44.

Cui, Z., Houweling, M., Chen, M.H., Record, M., Chap, H., Vance, D.E., and Terce, F. (1996). A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in Chinese hamster ovary cells. *J Biol Chem* 271, 14668–71.

D'Amours, D., Desnoyers, S., D'Silva, I., and Poirier, G.G. (1999). Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* 342 (Pt 2), 249–68.

D'Amours, D., Germain, M., Orth, K., Dixit, V.M., and Poirier, G.G. (1998). Proteolysis of poly(ADP-ribose) polymerase by caspase 3: kinetics of cleavage of mono(ADP-ribosyl)ated and DNA-bound substrates. *Radiat Res* 150, 3–10.

Dahl, H., Gluud, B., Vangsted, P., and Norn, M. (1985). Eye lesions induced by mustards gas. *Acta Ophthamol Suppl* 173, 330–35.

Dalton, T.P., Shertzer, H.G., and Puga, A. (1999). Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol* 39, 67–101.

Dambach, D.M., Durham, S.K., Laskin, J.D., and Laskin, D.L. (2006). Distinct roles of NF-κB p50 in the regulation of acetaminophen-induced inflammatory mediator production and hepatotoxicity. *Toxicol Appl Pharmacol* 211, 157–65.

Das, S.K., Mukherjee, S., Smith, M.G., and Chatterjee, D. (2003). Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustards analogue. *J Biochem Mol Toxicol* 17, 177–84.

Datta, R., Hallahan, D.E., Kharbanda, S.M., Rubin, E., Sherman, M.L., Huberman, E., Weichselbaum, R.R., and Kufe, D.W. (1992). Involvement of reactive oxygen intermediates in the induction of c-jun gene transcription by ionizing radiation. *Biochemistry* 31, 8300–6.

Decker, P. and Muller, S. (2002). Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Curr Pharm Biotechnol* 3, 275–83.

Delamere, N.A. (1996). Ascorbic acid and the eye. *Subcell Biochem* 25, 313–29.

den Hartog, G.J., Vegt, E., van der Vijgh, W.J., Haenen, G.R., and Bast, A. (2002). Hypochlorous acid is a potent inhibitor of acetylcholinesterase. *Toxicol Appl Pharmacol* 181, 228–32.

DePaso, W.J. and Winterbauer, R.H. (1991). Interstitial lung disease. *Dis Mon* 37, 61–133.

deRojas Walker, T., Tamir, S., Ji, H., Wishnok, J.S., and Tannenbaum, S.R. (1995). Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem Res Toxicol* 8, 473–7.

Dobrowsky, R.T., Kamibayashi, C., Mumby, M.C., and Hannun, Y.A. (1993). Ceramide activates heterotrimeric protein phosphatase 2A. *J Biol Chem* 268, 15523–30.

Downey, J.S. and Han, J. (1998). Cellular activation mechanisms in septic shock. *Front Biosci* 3, d468–76.

Duke-Elder, S. and MacFaul, P.A. (1972). *Injuries, Part 2 Non-mechanical Injuries*, Henry Kimpton, London.

El-Youssef, M. (2003). Wilson disease. *Mayo Clin Proc* 78, 1126–36.

Elsayed, N.M., Omaye, S.T., Klain, G.J., and Korte, D.W., Jr. (1992). Free radical-mediated lung response to the monofunctional sulfur mustards butyl 2-chloroethyl sulfide after subcutaneous injection. *Toxicology* 72, 153–65.

Emad, A., and Rezaian, G.R. (1997). The diversity of the effects of sulfur mustards gas inhalation on respiratory system 10 years after a single, heavy exposure: analysis of 197 cases. *Chest* 112, 734–8.

Emad, A. and Rezaian, G.R. (1999). Immunoglobulins and cellular constituents of the BAL fluid of patients with sulfur mustards gas-induced pulmonary fibrosis. *Chest* 115, 1346–51.

Erdincler, D.S., Seven, A., Inci, F., Beger, T., and Candan, G. (1997). Lipid peroxidation and antioxidant status in experimental animals: effects of aging and hypercholesterolemic diet. *Clin Chim Acta* 265, 77–84.

Fan, J., Shek, P.N., Suntres, Z.E., Li, Y.H., Oreopoulos, G.D., and Rotstein, O.D. (2000). Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome. *Surgery* 128, 332–8.

Farrukh, I.S., Michael, J.R., Peters, S.P., Sciuto, A.M., Adkinson, N.F., Jr., Freeland, H.S., Paky, A., Spannhake, E.W., Summer, W.R., and Gurtner, G.H. (1988). The role of cyclooxygenase and lipoxygenase mediators in oxidant-induced lung injury. *Am Rev Respir Dis* 137, 1343–9.

Fattman, C.L., Schaefer, L.M., and Oury, T.D. (2003). Extracellular superoxide dismutase in biology and medicine. *Free Radic Biol Med* 35, 236–56.

Fernandez-Checa, J.C., Kaplowitz, N., Garcia-Ruiz, C., and Colell, A. (1998). Mitochondrial glutathione: importance and transport. *Semin Liver Dis* 18, 389–401.

Folkes, L.K., Candeias, L.P., and Wardman, P. (1995). Kinetics and mechanisms of hypochlorous acid reactions. *Arch Biochem Biophys* 323, 120–6.

Fox, M. and Scott, D. (1980). The genetic toxicology of nitrogen and sulfur mustards. *Mut Res* 75, 131–68.

Freeman, M.L., Huntley, S.A., Meredith, M.J., Senisterra, G.A., and Lepock, J. (1997). Destabilization and denaturation of cellular protein by glutathione depletion. *Cell Stress Chaperones* 2, 191–8.

Fu, Y., McCormick, C.C., Roneker, C., and Lei, X.G. (2001). Lipopolysaccharide and interferon-gamma-induced nitric oxide production and protein oxidation in mouse peritoneal macrophages are affected by glutathione peroxidase-1 gene knockout. *Free Radic Biol Med* 31, 450–9.

Fujihara, M., Connolly, N., Ito, N., and Suzuki, T. (1994). Properties of protein kinase C isoforms (beta II, epsilon, and zeta) in a macrophage cell line (J774) and their roles in LPS-induced nitric oxide production. *J Immunol* 152, 1898–906.

Gamaley, I.A. and Klyubin, I.V. (1999). Roles of reactive oxygen species: signaling and regulation of cellular functions. *Int Rev Cytol* 188, 203–55.

Ganea, E. and Harding, J.J. (2006). Glutathione-related enzymes and the eye. *Curr Eye Res* 31, 1–11.

Ghezzi, P. and Ungheri, D. (2004). Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. *Int J Immunopathol Pharmacol* 17, 99–102.

Ghosh, A., Akech, J., Mukherjee, S., and Das, S.K. (2002). Differential expression of cholinephosphotransferase in normal and cancerous human mammary epithelial cells. *Biochem Biophys Res Commun* 297, 1043–8.

Gidwani, A., Brown, H.A., Holowka, D., and Baird, B. (2003). Disruption of lipid order by short-chain ceramides correlates with inhibition of phospholipase D and downstream signaling by FcepsilonRI. *J Cell Sci* 116, 3177–87.

Goldman, M. and Dacre, J.C. (1989). Lewisite: its chemistry, toxicology, and biological effects. *Rev Environ Contam Toxicol* 110, 75–115.

Griffith, O.W. and Meister, A. (1985). Origin and turnover of mitochondrial glutathione. *Proc Natl Acad Sci U S A* 82, 4668–72.

Gunnarsson, M., Walther, S.M., Seidal, T., and Lennquist, S. (2000). Effects of inhalation of corticosteroids immediately after experimental chlorine gas lung injury. *J Trauma* 48, 101–7.

Haddad, J.J. (2004). Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno-pharmaco-redox conception of oxidative siege versus cell death commitment. *Int Immunopharmacol* 4, 475–93.

Haliday, E.M., Ramesha, C.S., and Ringold, G. (1991). TNF induces c-fos via a novel pathway requiring conversion of arachidonic acid to a lipoxygenase metabolite. *EMBO J* 10, 109–15.

Hampton, M.B., Fadeel, B., and Orrenius, S. (1998). Redox regulation of the caspases during apoptosis. *Ann N Y Acad Sci* 854, 328–35.

Hanna, P.C., Kruskal, B.A., Ezekowitz, R.A., Bloom, B.R., and Collier, R.J. (1994). Role of macrophage oxidative burst in the action of anthrax lethal toxin. *Mol Med* 1, 7–18.

Hannun, Y.A. and Obeid, L.M. (1997). Mechanisms of ceramide-mediated apoptosis. *Adv Exp Med Biol* 407, 145–9.

Hannun, Y.A. and Luberto, C. (2000). Ceramide in the eukaryotic stress response. *Trends Cell Biol* 10, 73–80.

Hayes, K.C. (1974). Retinal degeneration in monkeys induced by deficiencies of vitamin E or A. *Invest Ophthalmol* 13, 499–510.

Hearps, A.C., Burrows, J., Connor, C.E., Woods, G.M., Lowenthal, R.M., and Ragg, S.J. (2002). Mitochondrial cytochrome c release precedes transmembrane depolarisation and caspase-3 activation during ceramide-induced apoptosis of Jurkat T cells. *Apoptosis* 7, 387–94.

Hetz, C.A., Hunn, M., Rojas, P., Torres, V., Leyton, L., and Quest, A.F. (2002). Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: onset of necrosis is associated with delayed ceramide increase. *J Cell Sci* 115, 4671–83.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A* 94, 3633–8.

Hogg, R. and Chakravarthy, U. (2004). AMD and micronutrient antioxidants. *Curr Eye Res* 29, 387–401.

Holmgren, A. (1972). Tryptophan fluorescence study of conformational transitions of the oxidized and reduced form of thioredoxin. *J Biol Chem* 247, 1992–8.

Holmgren, A. (1985). Thioredoxin. *Annu Rev Biochem* 54, 237–71.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J Biol Chem* 264, 13963–6.

Hsu, W., Kerppola, T.K., Chen, P.L., Curran, T., and Chen Kiang, S. (1994). Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. *Mol Cell Biol* 14, 268–76.

Hunt, D.F., Organisciak, D.T., Wang, H.M., and Wu, R.L. (1984). Alpha-tocopherol in the developing rat retina: a high pressure liquid chromatographic analysis. *Curr Eye Res* 3, 1281–8.

Hwang, C., Sinskey, A.J., and Lodish, H.F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 257, 1496–502.

Inoue, H., Asaka, T., Nagata, N., and Koshihara, Y. (1997). Mechanism of mustards oil-induced skin inflammation in mice. *Eur J Pharmacol* 333, 231–40.

Jaskot, R.H., Grose, E.C., Richards, J.H., and Doerfler, D.L. (1991). Effects of inhaled phosgene on rat lung antioxidant systems. *Fundam Appl Toxicol* 17, 666–74.

Javadi, M.A., Yazdani, S., Sajjadi, H., Jadidi, K., Karimian, F., Einollahi, B., Ja'farinasab, M.R., and Zare, M. (2005). Chronic and delayed-onset mustards gas keratitis: report of 48 patients and review of literature. *Ophthalmology* 112, 617–25.

Jiang, Q. and Ames, B.N. (2003). Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. *FASEB J* 17, 816–822.

Jones, D.P., Maellaro, E., Jiang, S., Slater, A.F., and Orrenius, S. (1995). Effects of N-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol Lett* 45, 205–9.

Kadar, T., Turetz, J., Fishbine, E., Sahar, R., Chapman, S., and Amir, A. (2001). Characterization of acute and delayed ocular lesions induced by sulfur mustards in rabbits. *Curr Eye Res* 22, 42–53.

Kang, K.A., Zhang, R., Lee, K.H., Chae, S., Kim, B.J., Kwak, Y.S., Park, J.W., Lee, N.H., and Hyun, J.W. (2006). Protective effect of triphlorethol-A from Ecklonia cava against ionizing radiation in vitro. *J Radiat Res (Tokyo)* 47, 61–8.

Kent, C. (1995). Eukaryotic phospholipid biosynthesis. *Annu Rev Biochem* 64, 315–43.

Kerppola, T.K. and Curran, T. (1994). Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene* 9, 675–84.

Khachik, F., Bernstein, P.S., and Garland, D.L. (1997). Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Invest Ophthalmol Vis Sci* 38, 1802–11.

Kim, M.Y., Zhang, T., and Kraus, W.L. (2005). Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD^+ into a nuclear signal. *Genes Dev* 19, 1951–67.

Kimura, H., Sawada, T., Oshima, S., Kozawa, K., Ishioka, T., and Kato, M. (2005). Toxicity and roles of reactive oxygen species. *Curr Drug Targets Inflamm Allergy* 4, 489–95.

Kinnula, V.L. and Crapo, J.D. (2003). Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med* 167, 1600–19.

Kirlin, W.G., Cai, J., Thompson, S.A., Diaz, D., Kavanagh, T.J., and Jones, D.P. (1999). Glutathione redox potential in response to differentiation and enzyme inducers. *Free Radic Biol Med* 27, 1208–18.

Kolesnick, R.N. and Krönke, M. (1998). Regulation of ceramide production and apoptosis. *Annu Rev Physiol* 60, 643–665.

Kowluru, R.A. and Kennedy, A. (2001). Therapeutic potential of anti-oxidants and diabetic retinopathy. *Expert Opin Investig Drugs* 10, 1665–76.

Krippner, A., Matsuno-Yagi, A., Gottlieb, R.A., and Babior, B.M. (1996). Loss of function of cytochrome c in Jurkat cells undergoing fas-mediated apoptosis. *J Biol Chem* 271, 21629–36.

Kuhn, J.F., Hoerth, P., Hoehn, S.T., Preckel, T., and Tomer, K.B. (2006). Proteomics study of anthrax lethal toxin-treated murine macrophages. *Electrophoresis* 27, 1584–97.

Kumar, O., Sugendran, K., and Vijayaraghavan, R. (2003). Oxidative stress associated hepatic and renal toxicity induced by ricin in mice. *Toxicon* 41, 333–8.

Kun, E. and Bauer, P.I. (2001). Cell biological functions of PARP I: an overview. *Ital J Biochem* 50, 15–8.

Kurinna, S.M., Tsao, C.C., Nica, A.F., Jiffar, T., and Ruvolo, P.P. (2004). Ceramide promotes apoptosis in lung cancer-derived A549 cells by a mechanism involving c-Jun NH2-terminal kinase. *Cancer Res* 64, 7852–6.

Langenberg, J.P., van der Schans, G.P., Spruit, H.E., Kuijpers, W.C., Mars-Groenendijk, R.H., van Dijk Knijnenburg, H.C., Trap, H.C., van Helden, H.P., and Benschop, H.P. (1998). Toxicokinetics of sulfur mustards and its DNA-adducts in the hairless guinea pig. *Drug Chem Toxicol* 21 Suppl 1, 131–47.

Vesicants and Oxidative Stress **289**

Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49, 741–52.

Levitt, J.M., Lodhi, I.J., Nguyen, P.K., Ngo, V., Clift, R., Hinshaw, D.B., and Sweeney, J.F. (2003). Low-dose sulfur mustards primes oxidative function and induces apoptosis in human polymorphonuclear leukocytes. *Int Immunopharmacol* 3, 747–56.

Libon, C., Forestier, F., Cotte-Laffitte, J., Labarre, C., and Quero, A.M. (1993). Effect of acute oral administration of alcohol on superoxide anion production from mouse alveolar macrophages. *J Leukoc Biol* 53, 93–8.

Liu, H., Lightfoot, R., and Stevens, J.L. (1996). Activation of heat shock factor by alkylating agents is triggered by glutathione depletion and oxidation of protein thiols. *J Biol Chem* 271, 4805–12.

Lo, Y.Y. and Cruz, T.F. (1995). Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem* 270, 11727–30.

Lou, M.F. (2003). Redox regulation in the lens. *Prog Retin Eye Res* 22, 657–82.

Lundberg, G. (2004). Mustard gas poisoning of hundreds of Americans in the United States in 1996. *MedGenMed* 21, 39. AQ24

Lundlum, D.B., Tong, P.A., Mehata, J.R., Kirk, M.C., and Papirmeister, B. (1984). Formation of ethylthioethyl deoxy guanosine from the reaction of chloroethyl ethyl sulphide with deoxyguanosine. *Cancer Res*, 44. AQ25

Luthman, M. and Holmgren, A. (1982). Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* 21, 6628–33.

Lyriboz, Y. (2004). A recent exposure to mustards gas in the United States: clinical findings of a cohort (n = 247) 6 years after exposure. *MedGenMed* 22, 4. AQ26

Mastruzzo, C., Crimi, N., and Vancheri, C. (2002). Role of oxidative stress in pulmonary fibrosis. *Monaldi Arch Chest Dis* 57, 173–6.

Mathias, S., Dressler, K.A., and Kolesnick, R.N. (1991). Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor alpha. *Proc Natl Acad Sci U S A* 88, 10009–13.

Matthews, J.R., Wakasugi, N., Virelizier, J.L., Yodoi, J., and Hay, R.T. (1992). Thioredoxin regulates the DNA binding activity of NF-κB by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res* 20, 3821–30.

McClintock, S.D., Till, G.O., Smith, M.G., and Ward, P.A. (2002). Protection from half-mustards-gas-induced acute lung injury in the rat. *J Appl Toxicol* 22, 257–62.

McClintock, S.D., Hoesel, L.M., Das, S.K., Till, G.O., Neff, T., Kunkel, R.G., Smith, M.G., and Ward, P.A. (2006). Attenuation of half sulfur mustards gas-induced acute lung injury in rats. *J Appl Toxicol* 26, 126–31.

McKenzie, S.M., Doe, W.F., and Buffinton, G.D. (1999). 5-aminosalicylic acid prevents oxidant mediated damage of glyceraldehyde-3-phosphate dehydrogenase in colon epithelial cells. *Gut* 44, 180–5.

McMillan, D.C., Sarvate, S.D., Oatis, J.E., Jr., and Jollow, D.J. (2004). Role of oxidant stress in lawsone-induced hemolytic anemia. *Toxicol Sci* 82, 647–55.

Megaw, J.M. (1984). Glutathione and ocular photobiology. *Curr Eye Res* 3, 83–7.

Mei, N., Tamae, K., Kunugita, N., Hirano, T., and Kasai, H. (2003). Analysis of 8-hydroxydeoxyguanosine 5′-monophosphate (8-OH-dGMP) as a reliable marker of cellular oxidative DNA damage after gamma-irradiation. *Environ Mol Mutagen* 41, 332–8.

Mercurio, F. and Manning, A.M. (1999a). Multiple signals converging on NF-κB. *Curr Opin Cell Biol* 11, 226–32.

Mercurio, F. and Manning, A.M. (1999b). NF-κB as a primary regulator of the stress response. *Oncogene* 18, 6163–71.

Meyer, M., Schreck, R., and Baeuerle, P.A. (1993). H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J* 12, 2005–15.

Mihm, S., Galter, D., and Droge, W. (1995). Modulation of transcription factor NF kappa B activity by intracellular glutathione levels and by variations of the extracellular cysteine supply. *FASEB J* 9, 246–52.

Miquel, K., Pradines, A., Terce, F., Selmi, S., and Favre, G. (1998). Competitive inhibition of choline phosphotransferase by geranylgeraniol and farnesol inhibits phosphatidylcholine synthesis and induces apoptosis in human lung adenocarcinoma A549 cells. *J Biol Chem* 273, 26179–86.

Mol, M.A., van de Ruit, A.M., and Kluivers, A.W. (1989). NAD^+ levels and glucose uptake of cultured human epidermal cells exposed to sulfur mustards. *Toxicol Appl Pharmacol* 98, 159–65.

Moore, L., and Ray, P. (1983). Enhanced inhibition of hepatic microsomal calcium pump activity by CCl4 treatment of isopropanol-pretreated rats. *Toxicol Appl Pharmacol* 71, 54–8.

Naghii, M.R. (2002). Sulfur mustards intoxication, oxidative stress, and antioxidants. *Mil Med* 167, 573–5.

Noltkamper, D. and Burgher, S.W. (2004). Toxicity, phosgene. *eMedicine*.

Nonas, S., Miller, I., Kawkitinarong, K., Chatchavalvanich, S., Gorshkova, I., Bochkov, V.N., Leitinger, N., Natarajan, V., Garcia, J.G., and Birukov, K.G. (2006). Oxidized phospholipids reduce vascular leak and inflammation in rat model of acute lung injury. *Am J Respir Crit Care Med* 173, 1130–8.

Oei, S.L. and Shi, Y. (2001). Poly(ADP-ribosyl)ation of transcription factor Yin Yang 1 under conditions of DNA damage. *Biochem Biophys Res Commun* 285, 27–31.

Ohia, S.E., Opere, C.A., and Leday, A.M. (2005). Pharmacological consequences of oxidative stress in ocular tissues. *Mutat Res* 579, 22–36.

Okamoto, T., Ogiwara, H., Hayashi, T., Mitsui, A., Kawabe, T., and Yodoi, J. (1992). Human thioredox-in/adult T cell leukemia-derived factor activates the enhancer binding protein of human immunodeficiency virus type 1 by thiol redox control mechanism. *Int Immunol* 4, 811–9.

Olea-Azar, C., Rigol, C., Mendizabal, F., Morello, A., Maya, J.D., Moncada, C., Cabrera, E., Di Maio, R., Gonzalez, M., and Cerecetto, H. (2003). ESR spin trapping studies of free radicals generated from nitrofuran derivative analogues of nifurtimox by electrochemical and Trypanosoma cruzi reduction. *Free Radic Res* 37, 993–1001.

Pant, S.C., Vijayaraghavan, R., Kannan, G.M., and Ganesan, K. (2000). Sulfur mustards induced oxidative stress and its prevention by sodium 2,3-dimercapto propane sulphonic acid (DMPS) in mice. *Biomed Environ Sci* 13, 225–32.

Papirmeister, B., Gross, C.L., Meier, H.L., Petrali, J.P., and Johnson, J.B. (1985). Molecular basis for mustards-induced vesication. *Fundam Appl Toxicol* 5, S134–49.

Papirmeister, B., Feister, A.J., Robinson, S.I., and Ford, R.D. (1991). *Mustards Gas. Toxic Mechanisms and Pharmacological Implications*, CRC Press, Boca Raton, FL.

Paredi, P., Kharitonov, S.A., and Barnes, P.J. (2002). Analysis of expired air for oxidation products. *Am J Respir Crit Care Med* 166, S31–7.

Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S., and Bernardi, P. (1994). The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. *J Biol Chem* 269, 16638–42.

Powis, G., Mustacich, D., and Coon, A. (2000). The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic Biol Med* 29, 312–22.

Pullar, J.M., Winterbourn, C.C., and Vissers, M.C.M. (1999). Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid. *Am J Physiol Heart Circ Physiol* 277, H1505–12.

Pullar, J.M., Vissers, M.C., and Winterbourn, C.C. (2001). Glutathione oxidation by hypochlorous acid in endothelial cells produces glutathione sulfonamide as a major product but not glutathione disulfide. *J Biol Chem* 276, 22120–5.

Pullar, J.M., Winterbourn, C.C., and Vissers, M.C. (2002). The effect of hypochlorous acid on the expression of adhesion molecules and activation of NF-κB in cultured human endothelial cells. *Antioxid Redox Signal* 4, 5–15.

Qin, X.J., Hai, C.X., Liang, X., Wang, P., Chen, H.L., and Liu, R. (2004). [Effect of acute phosgene inhalation on antioxidant enzymes, nitric oxide and nitric-oxide synthase in rats]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 22, 200–2.

Rahman, I. and Kelly, F. (2003). Biomarkers in breath condensate: a promising new non-invasive technique in free radical research. *Free Radic Res* 37, 1253–66.

Rahman, I. and MacNee, W. (1998). Role of transcription factors in inflammatory lung diseases. *Thorax* 53, 601–12.

Rahman, I., Marwick, J., and Kirkham, P. (2004). Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-κB and pro-inflammatory gene expression. *Biochem Pharmacol* 68, 1255–67.

Ramos, B., Salido, G.M., Campo, M.L., and Claro, E. (2000). Inhibition of phosphatidylcholine synthesis precedes apoptosis induced by C2-ceramide: protection by exogenous phosphatidylcholine. *Neuroreport* 11, 3103–8.

Vesicants and Oxidative Stress **291**

Ramos, B., El Mouedden, M., Claro, E., and Jackowski, S. (2002). Inhibition of CTP: phosphocholine cytidylyltransferase by C(2)-ceramide and its relationship to apoptosis. *Mol Pharmacol* 62, 1068–75.

Rao, G.N., Glasgow, W.C., Eling, T.E., and Runge, M.S. (1996). Role of hydroperoxyeicosatetraenoic acids in oxidative stress-induced activating protein 1 (AP-1) activity. *J Biol Chem* 271, 27760–4.

Reddy, V.N. (1990). Glutathione and its function in the lens–an overview. *Exp Eye Res* 50, 771–8.

Reddy, V.N. and Giblin, F.J. (1984). Metabolism and function of glutathione in the lens, *Ciba Found Symp* 106, 65–87.

Reed, D.J. and Savage, M.K. (1995). Influence of metabolic inhibitors on mitochondrial permeability transition and glutathione status. *Biochim Biophys Acta* 1271, 43–50.

Ricketts, K.M., Santai, C.T., France, J.A., Graziosi, A.M., Doyel, T.D., Gazaway, M.Y., and Casillas, R.P. (2000). Inflammatory cytokine response in sulfur mustards-exposed mouse skin. *J Appl Toxicol* 20 Suppl 1, S73–6.

Rikimaru, T., Nakamura, M., Yano, T., Beck, G., Habicht, G.S., Rennie, L.L., Widra, M., Hirshman, C.A., Boulay, M.G., Spannhake, E.W., and et al. (1991). Mediators, initiating the inflammatory response, released in organ culture by full-thickness human skin explants exposed to the irritant, sulfur mustards. *J Invest Dermatol* 96, 888–97.

Rose, R.C., Richer, S.P., and Bode, A.M. (1998). Ocular oxidants and antioxidant protection. *Proc Soc Exp Biol Med* 217, 397–407.

Rosenthal, D.S., Simbulan Rosenthal, C.M., Iyer, S., Spoonde, A., Smith, W., Ray, R., and Smulson, M.E. (1998). Sulfur mustards induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and caspase-dependent pathway. *J Invest Dermatol* 111, 64–71.

Ryan, A.J., McCoy, D.M., McGowan, S.E., Salome, R.G., and Mallampalli, R.K. (2003). Alveolar sphingo-lipids generated in response to TNF-alpha modifies surfactant biophysical activity. *J Appl Physiol* 94, 253–8.

Sabourin, C.L., Danne, M.M., Buxton, K.L., Casillas, R.P., and Schlager, J.J. (2002). Cytokine, chemokine, and matrix metalloproteinase response after sulfur mustards injury to weanling pig skin. *J Biochem Mol Toxicol* 16, 263–72.

Safarinejad, M.R., Moosavi, S.A., and Montazeri, B. (2001). Ocular injuries caused by mustards gas: diagnosis, treatment and medical defense. *Mil Med* 166, 67–70.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17, 2596–606.

Sato, S., Furuta, K., Miyake, T., Mishiro, T., Kohge, N., Akagi, S., Uchida, Y., Rumi, M.A., Ishihara, S., Adachi, K., and Kinoshita, Y. (2006). Hemolytic anemia during 24 weeks of ribavirin and interferon-alpha2b combination therapy does not influence the cardiac function of patients with viral hepatitis C. *J Clin Gastroenterol* 40, 88–9.

Schlager, J.J. and Hart, B.W. (2000). Stress gene activity in HepG2 cells after sulfur mustards exposure. *J Appl Toxicol* 20, 395–405.

Schnellmann, R.G., Gilchrist, S.M., and Mandel, L.J. (1988). Intracellular distribution and depletion of glutathione in rabbit renal proximal tubules. *Kidney Int* 34, 229–33.

Schulze-Osthoff, K., Bauer, M.K., Vogt, M., and Wesselborg, S. (1997). Oxidative stress and signal transduc-tion. *Int J Vitam Nutr Res* 67, 336–42.

Sciuto, A.M. (1998). Assessment of early acute lung injury in rodents exposed to phosgene. *Arch Toxicol* 72, 283–8.

Sciuto, A.M. (2000). Posttreatment with ETYA protects against phosgene-induced lung injury by amplifying the glutathione to lipid peroxidation ratio. *Inhal Toxicol* 12, 347–56.

Sciuto, A.M. and Moran, T.S. (2001). Effect of dietary treatment with n-propyl gallate or vitamin E on the survival of mice exposed to phosgene. *J Appl Toxicol* 21, 33–9.

Sciuto, A.M., and Hurt, H.H. (2004). Therapeutic treatments of phosgene-induced lung injury. *Inhal Toxicol* 16, 565–80.

Sciuto, A.M., Phillips, C.S., Orzolek, L.D., Hege, A.I., Moran, T.S., and Dillman, J.F., III. (2005). Genomic analysis of murine pulmonary tissue following carbonyl chloride inhalation. *Chem Res Toxicol* 18, 1654–60.

Segal, E. and Lang, E. (2005). Toxicity, chlorine gas. *eMedicine*.

Sellmayer, A., Uedelhoven, W.M., Weber, P.C., and Bonventre, J.V. (1991). Endogenous non-cyclooxygenase metabolites of arachidonic acid modulate growth and mRNA levels of immediate-early response genes in rat mesangial cells. *J Biol Chem* 266, 3800–7.

Sen, C.K. (1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 55, 1747–58.

Shapira, L., Sylvia, V.L., Halabi, A., Soskolne, W.A., Van Dyke, T.E., Dean, D.D., Boyan, B.D., and Schwartz, Z. (1997). Bacterial lipopolysaccharide induces early and late activation of protein kinase C in inflammatory macrophages by selective activation of PKC-epsilon. *Biochem Biophys Res Commun* 240, 629–34.

Shapira, L., Takashiba, S., Champagne, C., Amar, S., and Van Dyke, T.E. (1994). Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-alpha and IL-1 beta production by human monocytes. *J Immunol* 153, 1818–24.

Shichi, H. (2004). Cataract formation and prevention. *Expert Opin Investig Drugs* 13, 691–701.

Sickel, W. (1972). *Retinal Metabolism in Dark and Light*, Springer-Verlag, Berlin.

Sidell, F., Urbanetti, J.S., Smith, W.J., and Hurst, C.G. (1997). *Chapter 7 – Vesicants*.   AQ27

Sikpi, M. and Das, S. (1987). Development of cholinephosphotransferase in guinea pig lung mitochondria and microsomes. *Biochim Biophys Acta* 899, 35–43.

Singh, I., Pahan, K., Khan, M., and Singh, A.K. (1998). Cytokine-mediated induction of ceramide production is redox-sensitive. Implications to proinflammatory cytokine-mediated apoptosis in demyelinating diseases. *J Biol Chem* 273, 20354–62.

Sinha Roy, S., Mukherjee, S., Kabir, S., Rajaratnam, V., Smith, M., and Das, S.K. (2005). Inhibition of cholinephosphotransferase activity in lung injury induced by 2-chloroethyl ethyl sulfide, a mustards analog. *J Biochem Mol Toxicol* 19, 289–97.

Smith, C.V., Jones, D.P., Guenthner, T.M., Lash, L.H., and Lauterburg, B.H. (1996). Compartmentation of glutathione: implications for the study of toxicity and disease. *Toxicol Appl Pharmacol* 140, 1–12.

Snodderly, D.M. (1995). Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am J Clin Nutr* 62, 1448S-61.

Sohrabpour, H. (1984). Clinical manifestations of chemical agents on Iranian combatants during Iran-Iraq   AQ28
conflict. *Arch Belg* Suppl, 291–7.

Somani, S.M. and Babu, S.R. (1989). Toxicodynamics of sulfur mustards. *Int J Clin Pharmacol Ther Toxicol* 27, 419–35.

Stauble, B., Boscoboinik, D., Tasinato, A., and Azzi, A. (1994). Modulation of activator protein-1 (AP-1) transcription factor and protein kinase C by hydrogen peroxide and D-alpha-tocopherol in vascular smooth muscle cells. *Eur J Biochem* 226, 393–402.

Stith, I.E. and Das, S.K. (1981). Pulmonary surfactant lipids: studies on cholinephosphotransferase in developing guinea pig lung. *Indian Biologist* 13, 120–8.

Stith, I.E. and Das, S.K. (1982). Development of cholinephosphotransferase in guinea pig lung and mitochondria and microsomes. *Biochem Biophys Acta* 714, 250–6.

Stone, W.L., Mukherjee, S., Smith, M., and Das, S.K. (2002). Therapeutic uses of antioxidant liposomes. *Methods Mol Biol* 199, 145–61.

Stone, W.L., Qui, M., and Smith, M. (2003). Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide. *BMC Cell Biol* 4, 1.

Stone, W.L., Qui, M., Yang, H., and Smith, M. (2004). Lipopolysaccharide enhances the cytotoxicity of 2-chloroethyl ethyl sulfide. *Bioscience Proceedings* Chapter 236, 1–9.

Suntres, Z., Stone, W., and Smith, M.G. (2005). Ricin-induced tissue toxicity: the role of oxidative stress.   AQ29
J Med CBR Def 3.

Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K., Yokota, T., Wakasugi, H., and et al. (1989). ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. *EMBO J* 8, 757–64.

Torchinsky, Y.M. (1981). *Sulfur in Proteins*, Pergamon, Oxford, UK.

Truscott, R.J. (2005). Age-related nuclear cataract-oxidation is the key. *Exp Eye Res* 80, 709–25.

van Blitterswijk, W.J., van der Luit, A.H., Veldman, R.J., Verheij, M., and Borst, J. (2003). Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem J* 369, 199–211.

Veness-Meehan, K.A., Cheng, E.R., Mercier, C.E., Blixt, S.L., Johnston, C.J., Watkins, R.H., and Horowitz, S. (1991). Cell-specific alterations in expression of hyperoxia-induced mRNAs of lung. *Am J Respir Cell Mol Biol* 5, 516–21.

Vietor, I., Schwenger, P., Li, W., Schlessinger, J., and Vilcek, J. (1993). Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. *J Biol Chem* 268, 18994–9.

Vesicants and Oxidative Stress   **293**

Virag, L. (2005). Structure and function of poly(ADP-ribose) polymerase-1: role in oxidative stress-related pathologies. *Curr Vasc Pharmacol* 3, 209–14.

Vissers, M.C. and Winterbourn, C.C. (1995). Oxidation of intracellular glutathione after exposure of human red blood cells to hypochlorous acid. *Biochem J* 307 (Pt 1), 57–62.

Vivekananda, J., Smith, D., and King, R.J. (2001). Sphingomyelin metabolites inhibit sphingomyelin synthase and CTP: phosphocholine cytidylyltransferase. *Am J Physiol Lung Cell Mol Physiol* 281, L98–107.

Voehringer, D.W., McConkey, D.J., McDonnell, T.J., Brisbay, S., and Meyn, R.E. (1998). Bcl-2 expression causes redistribution of glutathione to the nucleus. *Proc Natl Acad Sci U S A* 95, 2956–60.

Vogt, R.F., Dannenberg, A.L., Schofiels, B., Hynes, N., and Papirmeister, B. (1984). Pathogenesis of skin caused by sulfur mustards. *Fundam Appl Toxicol* 4.   AQ30

Walker, I. (1967). *Protection of Animal Cells Against Mustards Gas by Pretreatment with Dithiothreitol.* Proceeding of the 5th International Congress Chemotherapy, Vienna.

Walker, I.G. and Smith, W. (1969). Protection of L-cells by thiols against the toxicity of sulfur mustards. *Can J Physiol Pharmacol* 47, 143–51.

Walker, L.J., Robson, C.N., Black, E., Gillespie, D., and Hickson, I.D. (1993). Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. *Mol Cell Biol* 13, 5370–6.

Wan, X.S., Zhou, Z., Ware, J.H., and Kennedy, A.R. (2005). Standardization of a fluorometric assay for measuring oxidative stress in irradiated cells. *Radiat Res* 163, 232–40.

Watson, W.H., Yang, X., Choi, Y.E., Jones, D.P., and Kehrer, J.P. (2004). Thioredoxin and its role in toxicology. *Toxicol Sci* 78, 3–14.

Weiss, D.J. and Lunte, C.E. (2000). Detection of a urinary biomaker for oxidative DNA damage 8-hydroxy-deoxyguanosine by capillary electrophoresis with electrochemical detection. *Electrophoresis* 21, 2080–5.

Winterbourn, C.C. (1985). Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim Biophys Acta* 840, 204–10.

Wormser, U. (1991). Toxicology of mustards gas. *Trends in Pharmacological Sciences* 12, 164–7.

Wormser, U., Brodsky, B., Green, B.S., Arad-Yellin, R., and Nyska, A. (1997). Protective effect of povidone-iodine ointment against skin lesions induced by sulfur mustards and by non-mustards vesicants. *Arch Toxicol* 71, 165–70.

Wormser, U., Brodsky, B., Green, B.S., Arad-Yellin, R., and Nyska, A. (2000). Protective effect of povidone iodine ointment against skin lesions induced by chemical and thermal stimuli. *J Appl Toxicol* 20 Suppl 1, S183–5.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249, 1431–3.

Xanthoudakis, S. and Curran, T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J* 11, 653–65.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.C., and Curran, T. (1992). Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* 11, 3323–35.

Xanthoudakis, S., Miao, G.G., and Curran, T. (1994). The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc Natl Acad Sci U S A* 91, 23–7.

Ye, J., Wang, S., Leonard, S.S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castranova, V., and Shi, X. (1999). Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis. *J Biol Chem* 274, 34974–80.

Yodoi, J. and Uchiyama, T. (1992). Diseases associated with HTLV-I: virus, IL-2 receptor dysregulation and redox regulation. *Immunol Today* 13, 405–11.

Yourick, J.J., Clark, C.R., and Mitcheltree, L.W. (1991). Niacinamide pretreatment reduces microvesicle formation in hairless guinea pigs cutaneously exposed to sulfur mustards. *Fundam Appl Toxicol* 17, 533–42.

Zhang, J., Johnston, G., Stebler, B., and Keller, E.T. (2001). Hydrogen peroxide activates NFkappaB and the interleukin-6 promoter through NFkappaB-inducing kinase. 3, 493–504.   AQ31

Zong, W.X., Ditsworth, D., Bauer, D.E., Wang, Z.Q., and Thompson, C.B. (2004). Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 18, 1272–82.

AUTHOR QUERIES

[AQ1] The abbreviation BAL has been used for both bronchoalveolar and British anti-Lewsite. Please check.

[AQ2] The spelling of the author ''MacPaul'' has been changed to ''MacFaul'' in the reference citation ''Duke-Elder and MacFaul, 1972'' as per the reference list. Please check.

[AQ3] The reference ''Anslow (1946)'' is cited in the text but not listed. Please provide the full details of the reference in the list.

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[AQ9] The four paragraphs starting ''Short-term (3 days). . . .'' to '' . . . pig lung injury (Figure 12.15)'' have been repeated here and hence have been deleted.

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Introduction

The Advanced Medical Countermeasures Consortium (AMCC) is investigating the role of liposomal antioxidant formulations (STIMAL) as an effective antidote/therapeutic product to protect from exposure to chemical warfare agents, such as mustard gas. Mustard gas-induced organ and tissue injuries are mediated through mechanisms that also involve the activation of the inflammatory response and the inducement of oxidative stress (1,2). Recent studies have focused on the use of antioxidants as a treatment modality for ameliorating the injurious effects of mustard gas. So far, many natural antioxidants exhibit poor bioavailability and cannot easily cross biological barriers such as membranes. Lipophilic compounds are insoluble in water and are located almost exclusively within membranes. Hydrophilic compounds cannot penetrate many biological compartments enclosed by membranes through passive diffusion and largely remain in extracellular compartments. We have demonstrated that the protective effects of antioxidants against several experimental models of oxidant-induced tissue and organ injuries were significantly enhanced following their delivery as liposomal formulations (3-7). Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules (ie. N-acetylcysteine [NAC]) can be encapsulated in the aqueous spaces and lipophilic molecules (α-Tocopherol, γ-Tocopherol) can be incorporated into the lipid bilayers. The relative ease in incorporating hydrophilic and lipophilic antioxidants in liposomes; the possibility of directly delivering liposomes to an accessible body site; and the relative non-immunogenicity and low toxicity of liposomes have rendered the liposomal system highly attractive for drug delivery (3, 7). The overall objective of this proposal is to evaluate the safety of a liposomal formulation containing combinations of the antioxidants α-tocopherol, γ-tocopherol, and/or N-acetylcysteine. The preclinical safety studies cover the establishment of the basic pharmacology, pharmacokinetics and toxicological profile of the liposomal antioxidants, using animals (acute and subchronic toxicity studies, toxicokinetics) and *in vitro* systems (mutagenicity, genotoxicity, irritation). At the end of the preclinical safety evaluation, it will be possible to identify an initial safe dose and subsequent dose escalation schemes for human use, recognize possible target organs for toxicity, and, distinguish safety parameters for clinical monitoring.

Body

Task 1: We will perform *in vitro* studies in order to determine whether or not liposomal antioxidants exert any mutagenicity/genotoxicity or severe irritancy effects. Furthermore, in recognition that studies with cultured human cells and tissues can play an important role in studies of toxicity, other improved cell culture technologies will be utilized to assess the safety of the liposomal antioxidant formulation by predicting human metabolism, selecting appropriate animal models, and identifying potential drug-drug interactions.

1. **Report on studies involving the characterization of liposomal N-acetylcysteine (NAC) formulation.**

Experiments were carried out to identify the best possible liposomal antioxidant formulation to be used in the preclinical safety studies. Results from studies described by the consortium collaborators suggested that liposomes containing N-acetylcysteine (NAC) or glutathione (GSH) and/or alpha/gamma-tocopherol are effective against CEES and other oxidant-induced organ injuries.   We have validated the preparation and characterization of liposomes containing different antioxidants.

1.a. Stability of liposomal NAC in PBS buffer, plasma, and bronchoalveolar lavage (BAL).

In order for liposomes to be effective in the treatment of oxidant-induced tissue injuries, liposomes have to overcome structure destabilization as a result of interaction with certain serum components present in blood (following iv injection) and bronchoalveolar lavage (following inhalation). Thus, the stability of liposomal NAC formulation in biological fluids such as plasma and bronchoalveolar lavage, as well as PBS buffer as a control, was examined and the results are shown in figures 1 and 2. Liposomal NAC with alpha- or gamma-tocopherols showed better stability in PBS buffer than plasma, but the differences were not great. Inclusion of alpha- or gamma-tocopherol in liposomes improved the retention of NAC under all conditions examined.
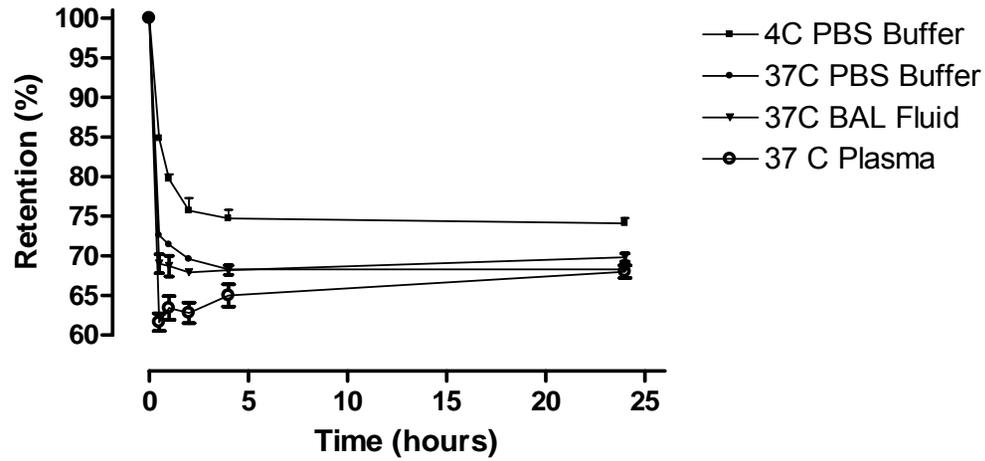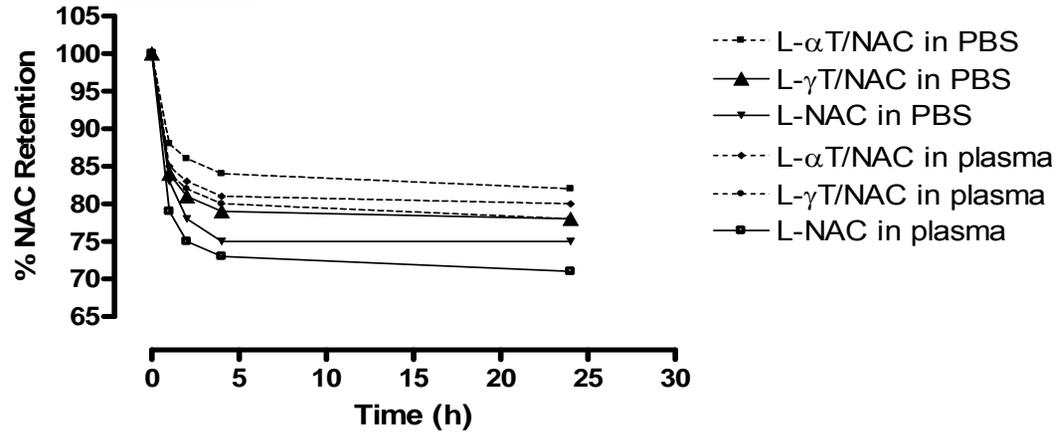
Figure 1

Figure 2

1.b. Improvement of liposomal stability by lyophilization for prolonged storage or shipment purposes.

Storage of lyophilized liposomes

We have found that the stability of the liposomal formulations can be improved significantly when prepared in the powder form following lyophilization. Lyophilization refers to the process whereby a substance is prepared in dry form by freezing and dehydration. Briefly, preformed liposomal formulations can be dehydrated by freeze-drying using a lyophilizator at -45ºC (time of freeze-drying depends on volume of sample). The vials containing the liposomal formulations can be stored at -20°C for months or shipped in dry ice.

Reconstitution Procedure for lyophilized liposomes

In the reconstitution procedure, the vials containing the liposomal formulations can be rehydrated with sterile Phosphate Buffer Saline (PBS) at pH 6.5 as follows (for a 1 ml final solution): An initial volume of 150 μL of PBS is added to the vial until all the liposomal powder is dissolved (vortexing is highly recommended if liposomes are aggregated); the vial is placed in a pre-heated water bath set at 45°C (this temperature should be maintained throughout the rehydration process) for 15 min. Then, an additional volume of 700 μL of PBS is added to the vial, vortexed well, and placed to the water bath at 45C for an additional 30 min. After heating in the water bath, the mixture is vortexed once more to dissolve any remaining aggregated liposomes. Finally, the sample is centrifuged at 4°C (28000x*g* or higher) for 30 minutes in order to separate lipids that did not form into liposomes, free drug dispersed in supernatant, and any foam produced during the vortex process. After centrifugation, the supernatant is discarded and the pellet formed (liposomes) can be reconstituted with the required buffer of choice (ex. PBS, 0.9% saline) and diluted to the concentration of preference. The liposomes are ready for use.

1.c. Mutagenicity Testing

The Ames test for mutagenicity is a mandatory test required by the FDA and Organization for Economic Cooperation and Development (OECD). The STIMAL formulations (liposomal NAC, liposomal alpha-tocopherol/NAC and liposomal gamma-tocopherol/NAC) had no mutagenic properties. We utilized the Muta-ChromoPlate kit, a sensitive approach used for the detection of mutagenic activity of chemicals, food components, cosmetics and biological fluids. The Muta-ChromoPlate kit is based on the most generally used and validated bacterial reverse-mutation test, known as the 'Ames Test'. The test employs a mutant strain, or several strains, of *Salmonella typhimurium*, carrying mutation(s) in the operon coding for histidine biosynthesis. When these bacteria are exposed to mutagenic agents, under certain conditions reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs. The system includes the Ames

Salmonella typhimurium strains TA100 for identifying base pair substitutions and TA98 for frameshift mutations.

Briefly, a mutant strain of Salmonella typhimurium (TA100/TA 98), carrying a mutation in the operon coding for histidine biosynthesis, was exposed to 2 concentrations of the STIMAL formulations. After an incubation period of 5 days at 37 C, the number of positive wells for each condition was recorded and scored against the results of the background (negative control). Sodium azide (5 mg/ml), a direct-acting mutagen, was used as a positive control.

1.d. Development of Ultrapressure Liquid Chromatography (UPLC) method for the measurement of N-acetylcysteine and other thiols.

We have developed a simple, sensitive, and rapid method that will be used to determine the levels of NAC/GSH in the different organs and tissues of animals (Task 5: Toxicokinetic Study). The method uses the Ellman's reagent derivatization procedure. The mobile phase consists of a mixture of methanol and ammonium formate (20:80 v/v) with a C18 reverse phase column as the stationary phase. Ellman's reagent can react stoichiometrically with thiols giving rise to mixed disulphides which have different retention times. The method was found to yield a quantitative recovery of GSH and NAC of more than 96%, to be sensitive (20 pmole/10 ul injection) and rapid (less than 3 min).

1.e Antioxidant effectiveness of liposomal NAC *in vitro*.

To assess the effectiveness of lipid-encapsulated and free antioxidants, the uptake and antioxidant properties of liposomal NAC and free NAC were examined in an *in vitro* model consisting of cells (hepG2 cell culture) exposed to the oxidant hydrogen peroxide (H_2O_2). The uptake of NAC in cells was assessed by measuring the cellular non-protein thiols (NPSH). The toxicity of liposomal NAC and free NAC as well as the protective effects of the antioxidant formulations against H_2O_2-induced cell injury was assessed by measuring the leakage of lactate dehydrogenase (LDH), an indicator of loss in cell permeability.

Pre-treatment of cells with either NAC or L-NAC (2 or 4 mM NAC for up to 24 h) did not produce any LDH leakage from Hep G2 cells suggesting that both formulations, at the concentrations used, are not toxic.

Exposure of hep G2 cells to free NAC was not effective in increasing the cellular thiol levels. On the other hand, cellular thiol levels were significantly increased, in a concentration-dependent and time-dependent manner, when cells were treated with liposomal NAC. Pretreatment of cells with L-NAC conferred protection against H_2O_2-induced cell membrane injury, a treatment effect not seen in cells pretreated with L-NAC. These data suggest that delivery of NAC as a liposomal formulation increases the intracellular thiol content and decreases cellular susceptibility to oxidative stresses.

Effect of NAC or L-NAC on cellular Non-protein thiols (NPSH)
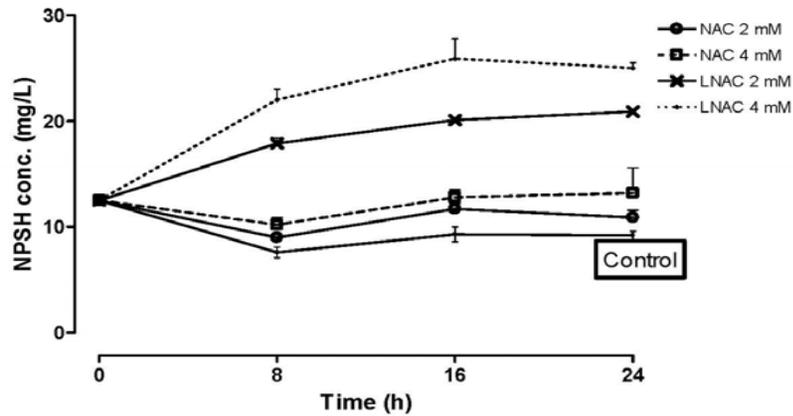
Effect of H2O2 on cells pretreated with NAC or L-NAC
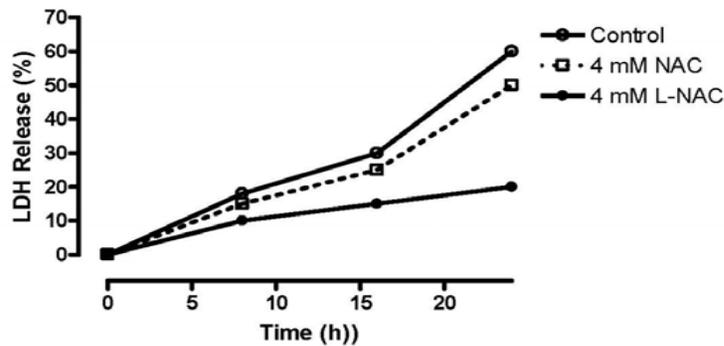
Task 2: We will determine the dosage ranges of the liposomal antioxidant formulation that will be used in the acute and subchronic safety evaluation studies. The end point in these range-finding tests is to simply determine the maximum dosage that can be given without killing an animal.

Maximum Tolerated Dose study for four liposomal antioxidant formulations in rodents

The up-and-down procedure was used in order to determine the maximum tolerated dose in both, male and female Sprague-Dawley rats. The liposomal formulations tested were: Liposomes consisting of dipalmitoylphosphatidylcholine (DPPC); Liposomal NAC; Liposomal alpha-tocopherol/NAC; and, Liposomal gamma-tocopherol/NAC. The treatments were tested as outlined in the following chart:

Range Finding Chart

Dose Sequence	Dose Level (mg/kg)		Number of Animals and Sex (male / female)
	Severe toxic effect or mortality seen at preceding dose	No effect seen at preceding dose	
1st	-	a	1 male / 1 female
2nd	a : m	a x m	1 male / 1 female
3rd	b : m	b x m	1 male / 1 female
4th	c : m	c x m	1 male / 1 female
5th	d : m	d x m	1 male / 1 female

a = initial dose
m = multiplier (1.5 – 3)

If the first group of animals survives and does not show severe toxic effect, the second group of animals will receive a higher dose. If any animals in the first group die or appear moribund, then the second group of animals will receive a lower dose. The next dose level will be chosen based on the same criteria. Based on the reaction of the previous group, the following group will be dosed at 24 to 72 hour intervals.

Findings.
The dosage range finding study used to determine the maximum tolerated doses (MTD) in male and female Sprague-Dawley rats showed that that the highest dose by a single intravenous administration of STIMAL used (consisting of: 1980 mg DPPC, 600 mg NAC, 248 mg α-T, and 248 mg γ-T) failed to produce any adverse reactions. Gross necropsy findings were unremarkable.

Due to the high viscosity of the dosing formulations the dose level of 600 mg/kg (NAC) (doses for all formulations are based on NAC, the antioxidant encapsulated in liposomes at the highest levels; IV administration to humans is approximately 150 mg/kg NAC) was considered to be the maximum feasible dose (MFD) that can be injected safely to rats by an intravenous route of administration.

Task 3: We will carry out acute systemic toxicity studies in order to assess, in more comprehensive terms, the safety of the liposomal antioxidant formulation. In these studies, both rodent and non-rodent animals will be administered different doses of the liposomal antioxidant formulations and within 24-h to 1 week, animals will be given complete physical examinations (palpations, behavioral checks, spinal reflex checks, papillary light reflexes, respiration rate, ECG recording, and rectal temperature measurement). Blood samples will also be collected following each dose to determine standard clinical chemistry, biochemistry and/or hematological profiles. The results from these studies will satisfy regulatory requirements and provide a more thorough early characterization or prediction of toxicity.

 Single dose acute intravenous toxicity study for four preparations in rodents (Appendix A)

For the single dose acute intravenous toxicity study, male and female Sprague-Dawley rats were challenged intravenously with the following liposomal formulations:

1. Liposomes (1980 mg/kg DPPC) (control)
2. Liposomal NAC (1980 mg DPPC/600 mg NAC/kg)
3. Liposomal alpha-tocopherol/NAC (1980 mg/248 mg alpha-tocopherol/600 mg NAC/kg)
4. Liposomal gamma-tocopherol/NAC (1980 mg/248 mg gamma-tocopherol/600 mg NAC/kg).

Animals were monitored on a daily basis within the 14-day experimental period. This study examined a safe dose range of three liposomal antioxidant formulations with plain liposomes being determined as a control group (liposomes/ NAC; liposomes/α-tocopherol and NAC; and liposomes/γ-tocopherol and NAC) administered by a single intravenous dose in Sprague-Dawley rats. Endpoints included daily clinical observations, weekly general physical examinations, body weights, food consumption, clinical pathology (Appendix A), gross necropsy and histopathology.

Injections were well-tolerated by animals (for all four formulations), and there were no clinical origins of toxicity associated with the treatment regiments either during injections, after injections or during the 14-day post treatment period.

Rats in all groups gained body weight during the two week observation period, and statistical analysis (ANOVA, p=0.05) showed that there was no difference in body weights and body weight gain between the control group (empty liposomes) and the three antioxidant formulations, or amongst antioxidant formulations groups.

Hematology results indicated that RBC counts, reticulocytes, hemoglobin and RBC indices (MCV, MCH and MCHC) were all within the normal ranges for both genders for all test groups. The exception was hematocrit (Hct), which was slightly below the lower limit of the normal historical ranges in males and females, for three test groups and control group which received empty liposomes. These slight decreases in Hct were not considered to be significant as they were just marginally below the normal historical ranges. These differences were most likely due to physiological variations since the control animals appeared to be similarly affected as the test animals.

Platelet counts were within normal ranges for both genders and all groups.

WBC counts and differential counts were within the normal physiological range in groups which received empty liposomes, liposome/NAC and α-tocopherol, for both genders. WBC counts, in male rats which were dosed with liposome/NAC and γ-tocopherol were slightly below the lower limit of the normal range, and were also slightly lower than WBC counts in the remaining groups. Neutrophils, lymphocytes and monocytes were within the normal range, but were slightly lower than the counts for these parameters in other groups. This is consistent with the anti-inflammatory properties of γ-tocopherol. However, it cannot be excluded that this finding is not treatment related but because of its low magnitude, this observation is of limited toxicological significance.

Prothrombin time, APTT and fibrinogen were all within the normal ranges for all groups and both genders, with the exception that APTT for female rats treated with liposomes NAC and γ-tocopherol was approximately one second below the low end of normal range. This finding is not clinically significant.

Mean total protein, albumin and globulin were within the normal ranges in all groups and both genders. A/G ratio was marginally increased in female rats treated with empty liposomes and γ-tocopherol.

Cholesterol, triglycerides and glucose levels were all within the normal ranges for all groups and both genders.

Electrolytes (Na^+, Cl^- and K^+), calcium and phosphorus values were essentially within the normal ranges for all groups and both genders. There was however a slight "increase" in Cl^- levels in some groups. This finding is mostly likely an artifact known as a "great

chloride shift", and it is not considered treatment related.

Hepatocellular / hepatobiliary panel showed that ALP, Bil(T) and ALT were within normal ranges for all groups and both genders. AST was slightly increased in males which received liposome/NAC and γ-tocopherol. AST is tissue non-specific, and most likely originated from erythrocytes.

BUN and creatinine were within the normal ranges for all groups and both genders.

LDH and CK were increased in some animals in all groups. There were substantial inter-group and intra-group variabilities in the levels of lactate dehydrogenase (LDH) and creatine kinase (CK), including the control group. These differences are nevertheless known variabilities with these enzymes and are related to their release during regular handling of rats, grasping, dosing, etc. (Yerroum *et al*, 1999), blood collection procedures (Friedel *et al*, 1974), or their release from cellular elements during clotting (Friedel *et al*, 1970).

Gross pathology findings were unremarkable.

Histopathology: pending

Task 4: We will investigate the suchronic systemic toxicity of the liposomal antioxidant formulation in order to define the safety of repeated doses of liposomal antioxidants in an animal model and to provide support for the initiation of clinical trials in humans. In these studies, both rodent and non-rodent animals will be administered with different doses of the liposomal formulation, once daily for 1 week or two weeks, and animals will be sacrificed either one or two weeks after the last dosing. Animals will be observed for clinical signs throughout the study and blood samples will be used determine standard clinical chemistry, biochemistry and/or hematological profiles. At necropsy, organs will be processed for histopathological evaluation.

Experiments have not been initiated yet.

Task 5. We will investigate the toxicokinetics of the liposomal antioxidant formulation in order to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study.

Experiments will begin in September 2007.

Key Research Accomplishments:

• Characterization of liposomal antioxidant formulations (liposomal NAC, liposomal alpha-tocopherol/NAC, liposomal gamma-tocopherol/NAC) with regards to their size, entrapment efficiencies, and stability in buffers and body fluids (plasma, bronchoalveolar lavage) at 4Cor 37C.

- Methodology for improving the stability of the liposomal formulation by lyophilization for prolonged storage or shipment purposes.
- Validation of the non-mutagenic properties of the liposomal antioxidant formulations.
- Determination of maximum tolerated dose of the liposomal antioxidant formulations in rodents.
- Determination of acute toxicity of liposomal antioxidant formulations in rodents.
- Determination of the uptake and antioxidant properties of liposomal NAC and free NAC in an *in vitro* model consisting of cells (hepG2 cell culture) exposed to oxidants.
- Development of Ultrapressure Liquid Chromatography (UPLC) method for the measurement of N-acetylcysteine and other thiols.

Reportable Outcomes.

1. Paromov, V., **Suntres, Z.,** Stone, W., Smith, M. Sulfur Mustard Toxicity if Human Skin: Role of Oxidative Stress and Antioxidant Therapy. *Journal of Burns and Wounds*, 2007 (in press).
2. Alipour, M., Omri, A.**,** Smith, M., Stone, W., Pucaj, K., and **Suntres, Z**. Pre-clinical safety evaluation of liposomal antioxidant formulation**s (STIMAL).** 2007 Biodefence Research Conference, Philadelphia, PA (Appendix B).
3. Advanced Medical Countermeasures Consortial Meeting, June 28, 2007, Crystal City, VA. "Safety of liposomal antioxidants (STIMAL).
4. Training of two graduate students.

Conclusion.

Liposomal formulations containing NAC with alpha- or gamma-tocopherols are stable in the presence body fluids such as plasma and bronchoalveolar lavage suggesting that the liposomal antioxidant formulations can be effective in the treatment of oxidant-induced tissue injuries because they can overcome structure destabilization as a result of interaction with certain serum components present in blood (following iv injection) and bronchoalveolar lavage (following inhalation). The liposomal antioxidant formulations were non mutagenic. The dosage range finding study used to determine the maximum tolerated doses (MTD) in male and female Sprague-Dawley rats, as well as the acute toxicity studies, showed that that the highest dose by a single intravenous administration of STIMAL used (consisting of: 1980 mg DPPC, 600 mg NAC, 248 mg α-T, and 248 mg γ-T) failed to produce any adverse reactions. The dose of NAC administered to rats as a liposomal formulation was approximately 3-fold higher than the dose used in humans in its free form(150 mg/kg). So far, these data suggest that our liposomal antioxidant formulations might prove to be a safe for the treatment of injuries associated with oxidative stress.

References

1.   Naghii MR. Sulfur mustard intoxication, oxidative stress, and antioxidants. Mil. Med. 2002; 167:573-5.

2.   Paromov V, Suntres Z, Stone W, Smith M. Sulfur Mustard Toxicity if Human Skin: Role of Oxidative Stress   and Antioxidant Therapy. Journal of Burns and Wounds, 2007 (in press).

3.   Stone WL, Smith MG, Therapeutic uses of antioxidant liposomes. Mol. Biotechnol 2004; 27:217-30.

4.   Suntres ZE, Hepworth SR, Shek PN, Protective effecct of liposome-associated α-tocopherol against paraquat-induced acute lung toxicity. Biochem. Pharmacol. 1992; 44:1811-18.

5.   Suntres ZE, Shek PN, Prevention of phorbol myristate acetate-induced lung injury by α-tocopherol liposomes. J. Drug Targeting 1995; 3:201-8.

6.   Suntres ZE, Shek PN, Alleviation of paraquat-induced lung injury by pretreatment with bifunctional liposomes containing α-tocopherol and glutathione. Biochem. Pharmacol. 1996; 52: 1515-20.

7.   Suntres ZE, Role of Antioxidants in Paraquat Toxicity. Toxicology 2002; 180: 65-77.

Appendix A: **Single dose acute intravenous toxicity study for four preparations in rodents**

Haematology Group Summaries – End of Treatment Period – Males

Parameters	Unit	Group Means ± S.D. (n = 5)				
		Group 1 Control (Empty Liposome)	Group 2 Liposome/NAC	Group 3 Liposome/α-toxopherol/NAC	Group 4 Liposome/γ-toxopherol/NAC	Normal Ranges
RBC	$x10^{12}$ / L	6.88 ± 0.47	7.30 ± 0.20	7.11 ± 0.38	6.93 ± 0.28	4.9 – 9.8
Hb	g / L	143 ± 5	145 ± 6	145 ± 7	141 ± 2	131 – 184
Hct	%	39.6 ± 1.5	40.5 ± 1.7	40.5 ± 2.0	39.1 ± 0.4	42 – 52
MCV	fL	57.7 ± 2.7	55.5 ± 1.0	57.0 ± 1.4	56.5 ± 1.9	50 – 67
MCH	pg	20.9 ± 1.0	19.8 ± 0.4	20.3 ± 0.4	20.4 ± 0.7	17 – 23
MCHC	g / L	361 ± 5	357 ± 2	357 ± 2	362 ± 3	320 – 366
Platelets	$x10^{9}$ / L	1104 ± 38	1332 ± 93	1134 ± 64	1094 ± 189	474 – 1535
WBC	$x10^{9}$ / L	7.96 ± 1.33	5.58 ± 2.36	9.13 ± 2.76	4.17 ± 0.94	5.0 – 14.0
Neutrophils	$x10^{9}$ / L	0.96 ± 0.19	0.93 ± 0.33	1.31 ± 0.78	0.61 ± 0.10	0.3 – 2.1
Lymphocytes	$x10^{9}$ / L	6.73 ± 1.16	4.44 ± 2.05	7.57 ± 2.51	3.41 ± 0.82	2.5 – 12.8
Monocytes	$x10^{9}$ / L	0.16 ± 0.03	0.11 ± 0.05	0.15 ± 0.03	0.08 ± 0.02	0.1 – 0.4
Eosinophils	$x10^{9}$ / L	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.03 ± 0.02	0 – 0.2
Basophils	$x10^{9}$ / L	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0 – 0.1
LUC	$x10^{9}$ / L	0.05 ± 0.04	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0 – 0.2
Reticulocytes	$x10^{9}$ / L	365.0 ± 26.7	309.9 ± 27.5	338.3 ± 42.4	361.0 ± 69.8	Up to 400

Haematology Group Summaries – End of Treatment Period – Females

Parameters	Unit	Group 1 Control (Empty Liposome)	Group 2 Liposome/NAC (	Group 3 Liposome/α-toxopherol/NAC	Group 4 Liposome/γ-toxopherol/NAC	Normal Ranges
		Group Means ± S.D. (n = 5)				
RBC	$x10^{12}$ / L	7.31 ± 0.23	7.30 ± 0.40	7.25 ± 0.05	7.26 ± 0.40	6.6 – 8.7
Hb	g / L	146 ± 5	145 ± 5	146 ± 2	143 ± 4	133 – 157
Hct	%	39.6 ± 1.5	40.1 ± 1.9	39.7 ± 0.6	39.4 ± 0.9	40 – 50
MCV	fL	54.1 ± 2.0	54.9 ± 1.1	54.8 ± 0.5	54.3 ± 2.3	53 – 63
MCH	pg	20.0 ± 0.7	19.9 ± 0.5	20.2 ± 0.3	19.7 ± 0.7	18 – 22
MCHC	g / L	369 ± 5	362 ± 9	369 ± 6	363 ± 3	329 – 374
Platelets	$x10^{9}$ / L	1238 ± 93	1133 ± 75	882 ± 257	1020 ± 164	651 – 1567
WBC	$x10^{9}$ / L	5.23 ± 1.33	5.92 ± 2.05	5.54 ± 1.05	5.59 ± 3.19	4.3 – 15.0
Neutrophils	$x10^{9}$ / L	0.72 ± 0.53	0.72 ± 0.38	0.95 ± 0.36	1.25 ± 0.96	0.1 – 1.6
Lymphocytes	$x10^{9}$ / L	4.27 ± 1.03	5.02 ± 1.66	4.31 ± 1.10	4.06 ± 2.09	2.4 – 10.7
Monocytes	$x10^{9}$ / L	0.12 ± 0.07	0.08 ± 0.06	0.16 ± 0.04	0.16 ± 0.11	0 – 0.3
Eosinophils	$x10^{9}$ / L	0.08 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.07 ± 0.04	0 – 0.2
Basophils	$x10^{9}$ / L	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0 – 0.1
LUC	$x10^{9}$ / L	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.01	0.04 ± 0.02	0 – 0.4
Reticulocytes	$x10^{9}$ / L	245.7 ± 20.8	206.7 ± 31.4	240.9 ± 34.6	263.7 ± 32.9	Up to 400

__Coagulation Group Summaries – End of Treatment Period__

Group	Parameter – Mean ± S.D. (n=5)		
	Prothrombin Time (sec.)	APTT (sec.)	Fibrinogen (g/L)
Group 1 Control (Empty Liposome) Male	17.9 ± 0.6	13.1 ± 2.0	2.92 ± 0.39
Group 2 Liposome/NAC Male	17.5 ± 0.5	15.4 ± 2.6	2.76 ± 0.08
Group 3 Liposome/α-toxopherol/NAC Male	17.9 ± 0.8	14.3 ± 1.9	2.88 ± 0.18
Group 4 Liposome/γ-toxopherol/NAC Male	17.4 ± 0.5	12.4 ± 0.8	2.51 ± 0.91
Group 1 Control (Empty Liposome) Female	16.3 ± 0.9	13.2 ± 2.8	2.49 ± 0.25
Group 2 Liposome/NAC Female	17.1 ± 1.6	13.4 ± 1.0	2.08 ± 0.76
Group 3 Liposome/α-toxopherol/NAC Female	17.2 ± 0.7	13.2 ± 2.7	2.08 ± 0.52
Group 4 Liposome/γ-toxopherol/NAC Female	16.2 ± 0.7	11.4 ± 1.9	2.50 ± 0.65
Normal Ranges	14.8 - 22.8	12.9 – 29.3	1.5 - 3.9

Serum Chemistry Group Summaries – Males – End of Treatment Period

Parameters	Unit	Group Means ± S.D. (n=5)				
		Group 1 Control (Empty Liposome)	Group 2 Liposome/NAC	Group 3 Liposome/α-toxopherol/NAC	Group 4 Liposome/γ-toxopherol/NAC	Normal Ranges
A/G	-	1.2 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.9 – 1.3
ALB	g / L	28 ± 1	29 ± 1	29 ± 1	29 ± 1	24 – 48
GLOB	g / L	23 ± 1	24 ± 1	23 ± 1	23 ± 1	22 – 39
ALP	u / L	191 ± 45	206 ± 42	207 ± 45	239 ± 35	75 – 435
Bil(T)	μmol / L	3.2 ± 1.6	2.1 ± 0.6	2.0 ± 0.4	2.8 ± 2.2	0.9 – 7.0
BUN	mmol / L	4.6 ± 0.9	4.1 ± 0.6	4.6 ± 0.4	4.4 ± 0.6	2.8 – 8.8
Ca	mmol / L	2.50 ± 0.05	2.49 ± 0.07	2.41 ± 0.05	2.52 ± 0.03	2.5 – 3.1
Cl	mmol / L	113 ± 1	112 ± 2	106 ± 1	105 ± 1	97 – 109
Creatinine	μmol / L	40 ± 3	35 ± 2	36 ± 3	34 ± 4	30 – 65
Glucose	mmol / L	5.8 ± 0.7	5.5 ± 0.6	6.7 ± 0.8	6.7 ± 0.4	1.0 – 8.6
LDH	u / L	3660 ± 1576	5621 ± 1555	4505 ± 574	3334 ± 2041	2127 - 6401
P	mmol / L	2.70 ± 0.15	2.79 ± 0.12	2.54 ± 0.08	2.95 ± 0.07	1.5 – 4.4
K	mmol / L	4.5 ± 0.2	4.7 ± 0.2	4.5 ± 0.1	4.6 ± 0.3	3.8 – 7.4
Protein (T)	g / L	51 ± 2	52 ± 2	52 ± 2	52 ± 2	52 – 80
AST	u / L	104 ± 16	128 ± 15	108 ± 9	125 ± 44	53 – 113
ALT	u / L	51 ± 4	50 ± 3	53 ± 3	55 ± 5	19 – 60
Na	mmol / L	142 ± 1	142 ± 1	141 ± 1	143 ± 1	140 – 153
Triglycerides	mmol / L	0.51 ± 0.20	0.38 ± 0.12	0.36 ± 0.21	0.35 ± 0.26	0.2 – 2.0
CK	u / L	534 ± 165	713 ± 152	594 ± 90	446 ± 290	228 - 529
Cholesterol	mmol / L	1.26 ± 0.10	1.41 ± 0.07	1.41 ± 0.23	1.28 ± 0.27	1.3 – 3.2

Serum Chemistry Group Summaries – Females – End of Treatment Period

Parameters	Unit	Group Means ± S.D. (n=5)				
		Group 1 Control (Empty Liposome)	Group 2 Liposome/NAC	Group 3 Liposome/α-toxopherol/NAC	Group 4 Liposome/γ-toxopherol/NAC	Normal Ranges
A/G	-	1.5 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	1.3 ± 0.2	0.9 – 1.3
ALB	g / L	35 ± 1	34 ± 4	34 ± 2	35 ± 3	26 – 55
GLOB	g / L	24 ± 1	25 ± 1	24 ± 1	26 ± 1	23 – 36
ALP	u / L	132 ± 34	152 ± 31	116 ± 15	140 ± 51	74 – 435
Bil(T)	μmol / L	3.7 ± 1.0	4.8 ± 3.2	3.8 ± 2.4	5.0 ± 3.3	0.9 – 7.0
BUN	mmol / L	4.7 ± 0.4	4.3 ± 0.5	4.2 ± 0.2	5.5 ± 0.8	2.9 – 8.8
Ca	mmol / L	2.54 ± 0.05	2.55 ± 0.05	2.51 ± 0.07	2.59 ± 0.03	2.5 – 3.1
Cl	mmol / L	115 ± 2	114 ± 2	108 ± 3	106 ± 1	99 – 110
Creatinine	μmol / L	35 ± 4	33 ± 4	32 ± 3	34 ± 7	31 – 68
Glucose	mmol / L	7.1 ± 0.7	8.9 ± 2.1	6.6 ± 1.0	7.2 ± 2.1	1.0 – 8.1
LDH	u / L	3038 ± 1247	3321 ± 541	3039 ± 1245	3812 ± 1932	1455 - 3081
P	mmol / L	2.43 ± 0.09	2.73 ± 0.17	2.20 ± 0.09	2.41 ± 0.09	1.5 – 3.6
K	mmol / L	4.3 ± 0.2	4.7 ± 0.5	4.3 ± 0.3	4.7 ± 0.5	4.0 – 6.7
Protein (T)	g / L	59 ± 1	59 ± 3	58 ± 2	62 ± 3	54 – 85
AST	u / L	90 ± 14	103 ± 16	101 ± 31	119 ± 37	30 - 140
ALT	u / L	43 ± 4	33 ± 17	45 ± 3	46 ± 6	19 – 60
Na	mmol / L	142 ± 1	143 ± 1	143 ± 1	142 ± 1	140 – 150
Triglycerides	mmol / L	0.14 ± 0.05	0.10 ± 0.00	0.11 ± 0.03	0.10 ± 0.00	0.2 – 2.2
CK	u / L	528 ± 151	751 ± 455	553 ± 482	665 ± 653	158 - 556
Cholesterol	mmol / L	1.46 ± 0.25	1.64 ± 0.27	1.54 ± 0.21	1.64 ± 0.43	1.2 – 3.8

Appendix B
Pre-clinical safety evaluation of liposomal antioxidant formulations (STIMAL).

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The Advanced Medical Countermeasures Consortium (AMCC) is investigating the role of liposomal antioxidant formulations (STIMAL) as an effective antidote/therapeutic product to protect from exposure to chemical warfare agents, such as mustard gas. Mustard gas-induced organ and tissue injuries are mediated through mechanisms that also involve the activation of the inflammatory response and the inducement of oxidative stress. Recent studies have focused on the use of antioxidants as a treatment modality for ameliorating the injurious effects of mustard gas. So far, many natural antioxidants exhibit poor bioavailability and cannot easily cross biological barriers such as membranes. Lipophilic compounds are insoluble in water and are located almost exclusively within membranes. Hydrophilic compounds cannot penetrate many biological compartments enclosed by membranes through passive diffusion and largely remain in extracellular compartments. We have demonstrated that the protective effects of antioxidants against several experimental models of oxidant-induced tissue and organ injuries were significantly enhanced following their delivery as liposomal formulations. Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules (ie. N-acetylcysteine [NAC]) can be encapsulated in the aqueous spaces and lipophilic molecules (α-Tocopherol, γ-Tocopherol) can be incorporated into the lipid bilayers. The relative ease in incorporating hydrophilic and lipophilic antioxidants in liposomes; the possibility of directly delivering liposomes to an accessible body site; and the relative non-immunogenicity and low toxicity of liposomes have rendered the liposomal system highly attractive for drug delivery. The present study was carried out to evaluate the safety of liposomal formulations containing combinations of the antioxidants α-tocopherol (α-T), γ-tocopherol (γ-T), and N-acetylcysteine (NAC). So far, studies have demonstrated that the STIMAL formulations (liposomal NAC, liposomal alpha-tocopherol/NAC and liposomal gamma-tocopherol/NAC) are stable in several biological fluids (plasma, bronchoalveolar lavage) without any mutagenic properties. Furthermore, the dosage range finding study used to determine the maximum tolerated doses (MTD) in rats revealed that the highest dose by a single intravenous administration of STIMAL used (consisting of: 1980 mg DPPC, 600 mg NAC, 248 mg α-T, and 248 mg γ-T) failed to produce any adverse reactions. The data so far indicate that STIMAL might prove to be a safe treatment to suppress the injurious effects associated with oxidative stress.