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13. ABSTRACT (Maximum 200 Words)

The mechanism of toxicity of sulfur mustard was investigated by examining the biochemical effects of the analog 2-chloroethylethyl sulfide (CEES) in both human Jurkat cells as well as normal human lymphocytes. Exposure of both cells to CEES resulted in a marked decrease in the intracellular concentration of the reduced form of glutathione (GSH), and CEES-induced cell death was potentiated by L-buthionine sulfoximine, an inhibitor of GSH synthesis. CEES increased the endogenous production of reactive oxygen species (ROS) in Jurkat cells, and CEES-induced cell death was potentiated by hydrogen peroxide. CEES induced various hallmarks of apoptosis, including collapse of the mitochondrial membrane potential, proteolytic processing and activation of procaspase-3, and cleavage of poly (ADP-ribose) polymerase. The effects of CEES on the accumulation of ROS, the intracellular concentration of GSH, the mitochondrial membrane potential, and caspase-3 activity were all inhibited by pretreatment of cells with the GSH precursor *N*-acetyl cysteine or with GSH-ethyl ester. Furthermore, CEES-induced cell death was also prevented by these antioxidants CEES toxicity appears to be mediated, at least in part, by the generation of ROS and consequent depletion of GSH. Given that sulfur mustard is still a potential biohazard, the protective effects of antioxidants against CEES toxicity demonstrated in Jurkat cells and normal human lymphocytes may provide the basis for development of a therapeutic strategy to counteract exposure to this chemical weapon.

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(Research from May 1, 2002 – April 30, 2004)

Introduction:

I have made excellent progress on setting up a new cell systems (Jurkat cells, Normal Human Lymphocytes, Ha-Cat keratinocytes) and have identified a profitable research direction for investigating at the protective role of oxidant related cell injury by mustards. While this research does not account for all the original SOW objectives, due to earlier reported staffing problems, and additionally cut back in time and funding, it clearly meets the critical ones aimed at defining the mechanisms of cellular injury by sulfur mustard. Several of the 10 original objectives of the amended Contract were, in fact, related to ATP/NAD levels in cells, and stresses placed on mitochondria membrane potentials, We thus began a detailed study on Protective roles of anti-oxidants in Jurkat T-cells on sulfur half mustard (CEES). A manuscript on the first year's work on this topic was recently published in *The British Journal of Pharmacology*. The report from this paper constitutes the bulk of the results for this FINAL REPORT. Responsibilities on other projects and grants. Based upon my long-term association with USAMRAA and having fully met my contractual terms since 1990, I report now in the FINAL REPORT on the new and exciting direction of ant-oxidant protection of SM damage.

STATEMENT OF WORK

SOW 1.3.Aterationa due to SM on Mitochondrial Properties and Associated activities Related to Apoptosis:

Description Of Work Accomplished:
[Reference citations and Methods will be placed in the Appendix.]
2-chloroethylethyl sulfide (CEES), the sulfur mustard derivative, is considered a valid biochemical analog of sulfur mustard since it causes, albeit, limited vesication but alkylates a wide range of biological molecules. SM is absorbed into the general circulation at sites of direct contact, such as the skin, eyes and airways, and those organs are the most commonly effected. Leucopoenia and bone marrow depletion are observed in humans or rats after being poisoned by sulfur mustards, indicating that leukocytes are also targets of sulfur mustards. Recent studies on CEES have shown that exposure of a Jurkat T cell line to CEES induces biochemical and morphological changes that are characteristic of apoptotic cell death (Zhang et al., 2002). This is one reason why we chose Jurkat T cells for out initial studies during the initial 12 months of this 18 (research) month contract. The effects shown in T cells included chromatin condensation, degradation of genomic DNA into both high molecular weight and oligonucleosomal fragments, up-regulation of caspases 3, 6, 8 and 9 (Many of these topics have been included in aims that were intentionally not pursued in the first year protocols of my SOW, since they had already been done by Zhang and co-workers were thus available to the current work we have performed during the Contract. The above workers showed that CEES exposure Jurkat cells causes down regulation of the transcription of Akt, protein kinase B, which normally inhibits apoptosis.

A major factor that contributes to the commitment of cells to apoptotic death is the alteration of mitochondrial function and the consequent loss of mitochondrial membrane potential

($\Delta\Psi_{mito}$) (Mancini *et al.*, 1997; Salvioli *et al.*, 1997). Our lab in the past had recently performed a number experiments measuring the effects of toxic agents other than mustards on mitochondrial membrane potentials and also effects of antioxidants and protection. The perturbation of mitochondrial function during earlier apoptosis increases generation of reactive oxygen species (ROS) (Jabs 1999), which results in an oxidative environment that contributes to the apoptotic death process, in part, by depleting cellular antioxidants.

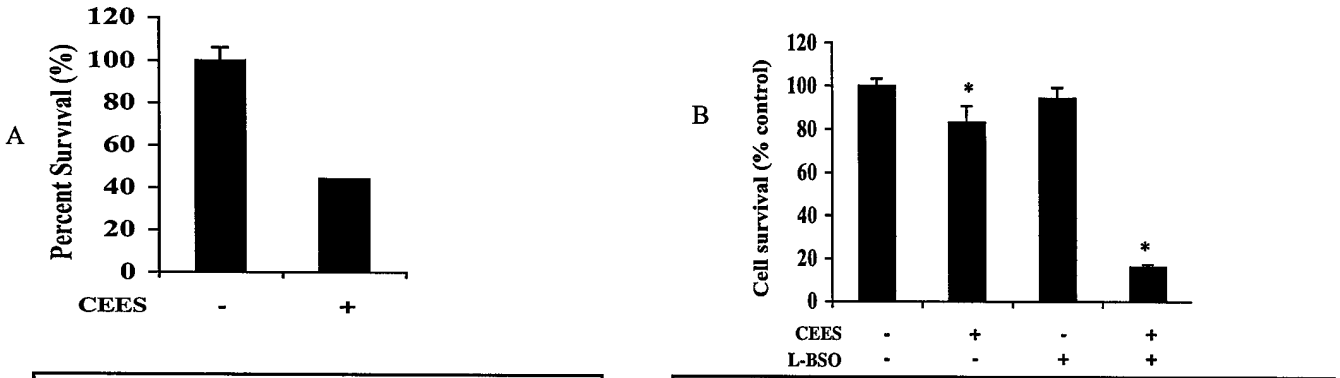
Based on these past studies with other toxic agents, we were fascinated by the possibility that thiol anti-oxidants would be highly effective in rescuing cells from SM toxic cell death. Given that the toxicity of SM and concomitant generation of ROS has been proposed to result from electrophilic or oxidative stress. During the last 18 months our results have been quite encouraging in demonstrating that oxidative stress of CEES toxicity in Jurkat T cells as well as the protective properties of GSH and NAC against CEES were quite pronounced and constitute possibly a beneficial effect for military personnel. However, some differing results were observed when we turned to skin cells, in the latter period of the contract (i.e. see below).

The detailed methods for how the experiments to be described below were performed is given in the appendix, included with the application (*Appended publication*).

GSH depletion affects CEES–induced death in Jurkat T cells

As noted, a recent study had appeared after we began this SOW, which confirmed that exposure of Jurkat T cells to CEES induces apoptotic cell death (Zhang et al. 2002). In our experiments incubation of Jurkat T cells for 18 h with CEES caused an approximate 60% loss of cell viability, as assessed by calcein-AM staining (Figure 1A).

Figure 1. The influence of the depletion GSH on CEES-induced death in Jurkat T cells



Jurkat T cells were incubated for 18 h in the absence (control) or presence of a 600 uM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence.

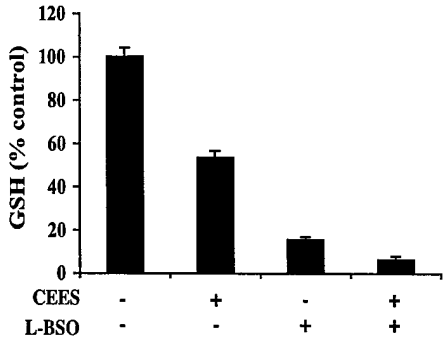
Jurkat T cells were incubated initially for 20 h in the presence or absence of 200 mM L-BSO. Subsequent incubation for 6 h with or without CEES in the continued presence or absence of L-BSO was accomplished.

The intracellular concentration of GSH has shown to be altered by a number of toxic agents (Hathway 2000). Accordingly, an investigation of whether the concentration of GSH is of consequence to CEES toxicity in Jurkat T cells was investigated. In order to deplete cells of GSH,

Jurkat T cells were incubated for 20 h with 200 μM L-BSO, an agent which is well established to selectively inhibit γ-glutamylcysteine synthestase, which represents the rate-limiting enzyme in GSH biosynthesis (Chiba *et al.*, 1996; Oda et al. 1999). Significantly, L-BSO reduced Jurkat T cell GSH levels to 83% of untreated cells. The cells were then treated with CEES for 6 h and then cell viability was measured by calcein-AM staining. The inhibition of GSH synthesis by L-BSO significantly potentiated CEES–induced cell death (Figure. 1B). While CEES alone resulted in an ~20% lowering of cell viability, the combination of CEES and the GSH inhibitor, L-BSO, resulted in >80% cell death. In the case of Jurkat cells, L-BSO alone induced a 20% decrease in cell viability in the absence of the sulfur mustard derivative (Figure 1B). The above experiments provided evidence that the intracellular concentration of GSH is of importance in the CEES toxicity observed in Jurkat T cells. The direct effect of CEES exposure was subsequently examined with respect to the intracellular levels of GSH by measurement of this metabolite in Jurkat T cells after 6 h exposure to the sulfur mustard derivative.

Figure 1. The influence of the depletion GSH on CEES-induced death in Jurkat T cells

C



Intracellular concentrations of GSH in Jurkat T cells upon exposure to CEES and L-BSO. Jurkat T cells were incubated first for 20 h in the presence or absence of 200 mM L-BSO, and then for 3 h with or without CEES in the continued presence or absence of L-BSO. Cell extracts were then assayed for GSH.

CEES markedly reduced the level of GSH (~50%) during this 6 h time exposure (Figure 1C). L-BSO (an inhibitor of GSH synthesis) potentiated the lowering of GSH by CEES to 10% of the control. The data emphasizes the important role of GSH level perturbation by an inhibitor such as L-BSO in the overall cytoxicity caused by CEES.

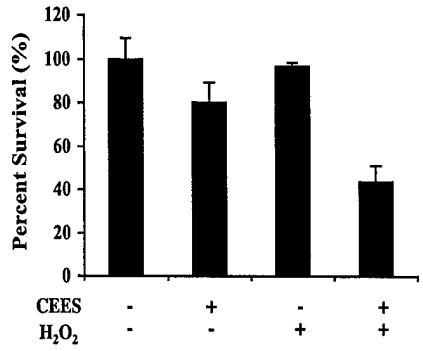
Influence of oxidative stress on CEES–induced cell death

We reasoned that the generation of an oxidative intracellular environment presumably resulted from the increased levels of ROS as occasioned by the chemical reactivity of the sulfur mustard derivative were directly related to the decrease in intracellular GSH levels. Accordingly we tested whether several thiol anti-oxidants such as GSH and its precursor NAC resulted in a protective effect against CEES-induced cell toxicity and death. To test this hypothesis, we first examined the effect of a low concentration of H_2O_2 on CEES–induced death in Jurkat cells. The cells were pretreated with 25 μM H_2O_2 for 1 h, subsequently, incubated with CEES for 6 h in the continued presence of H_2O_2, and then assayed for viability by calcein-AM staining. It was of significance that treatment of the cells with H_2O_2 alone had no significant effect on cell viability

(Figure 2A), however, while CEES alone induced approximately 20% loss of cell viability, **the combination of CEES and H_2O_2 resulted in greater than 50% cell death.**

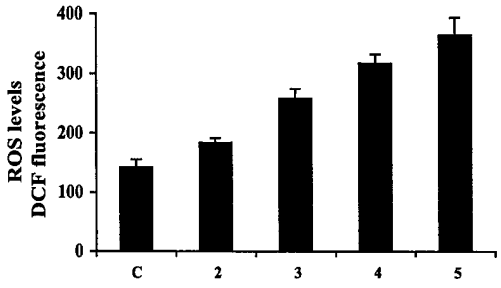
Figure. 2 CEES-*induced cell death: effect of oxidative stress*

A



Jurkat T cells were incubated first in the absence or presence of 25 μM H2O2 for 1 h and then with or without 600 uM CEES for 6 h (in the continued presence or absence of H2O2), after which cell viability was assessed by measurement of calcein-AM fluorescence.

B

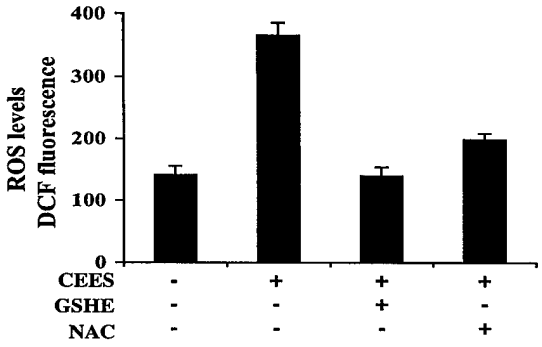


CEES-treated cells at the indicated times demonstrate progressively increasing ROS generation as compared with the controls.

CEES generated heightened ROS in cells As Assessed by Direct Fluorometric Measurement

The data shown above (Figure. 1C, 2B) indicated a synergistic effect of H_2O_2 on CEES killing further suggested that the sulfur mustard analog was presumably acting by a direct generation of active oxygen species in cells. The latter was directly measured by oxidation of H2DCF to the fluorescent DCF moiety, which is measured flourometrically and is a valid indicator of the presence of ROS synthesis in cells. The data in Figure 2B above showed that CEES alone dramatically increased the levels of ROS in Jurkat cells by 2 fold at 5 h time course over the control cells. However preincubation of the cells with GSH and NAC significantly reduced the induced ROS generation. These results corroborate that the observed increase in ROS formation is indeed due to a pro-oxidant cellular activity of CEES (see Figure. 2C below).

Figure. 2 C



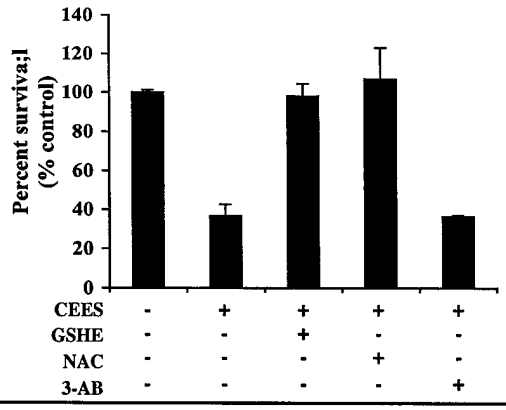
Jurkat cells were first loaded for 1 h with 1 mM GSH-ethyl ester (GSHE) or 5mM NAC and then incubated in the presence or absence of CEES for 5 h, after which cells were incubated with H2DCF probe and then the fluorescence, was measured immediately. In all data above are expressed are means ± S.D. of three independent experiments. *Significant difference in DCF fluorescence was assessed using Student's *t* test ($p < 0.05$).

Protective effect of thiol antioxidants on apoptotic death in Jurkat cells as occasioned by CEES

The data shown above indicated that CEES lowered intracellular GSH levels and that significant increase in ROS levels also resulted by this treatment to Jurkat T cells. Accordingly, it was logical to test whether the co-incubation of selective anti-oxidants would negate the above effects, which were contributing to cell death in Jurkat cells. We thus determined whether thiol anti-oxidants such as GSH and NAC exert a protective effect against cell death induced by CEES.

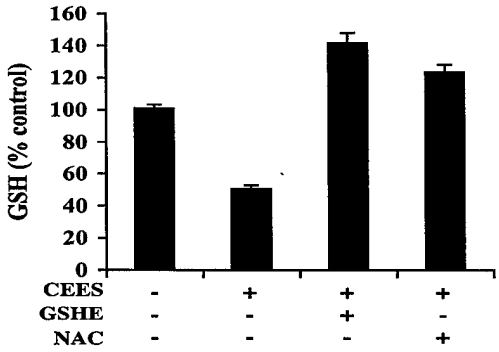
Figure 3. ***Influence of thiol antioxidants (GSH and NAC) on CEES-induced apoptosis.***

A



Jurkat T cells were incubated first for 1 h in the presence or absence of 5 mM NAC, 1 mM GSH-ethyl ester or 5 mM 3-AB, and then for 18 h with or without 600 uM CEES in the continued presence or absence of test agent. Cell viability was then assessed by measurement of calcein-AM fluorescence.

B



Jurkat T cells were initially incubated for 1 h in the presence or absence of 5 mM NAC or 1 mM GSH-ethyl ester and then for 3 h with or without CEES in the continued absence or presence of test agent. Cell extracts were then assayed for GSH. Data are means ± SD of triplicates from a representative experiment. *Significant difference from control on values normalized to the control mean for each treatment ($n = 4$; $p < 0.05$).

In the experiment shown in Figure 3A above, Jurkat cells were incubated in the presence or absence of 5mM NAC for 1 h and subsequently exposed to sulfur mustard half derivative for 18 h (in the continued presence or absence of NAC). It was highly significant that while treatment of Jurkat cells with CEES alone induced >60% cell death, **the presence of NAC totally protected the cells against cell death**. These data as well as similar data using the GSH-ethyl ester rather than NAC indicate that these agents might be useful in the human as a protective and non-toxic measure to overcome potential inflammatory and vesicating activities of sulfur mustard during human exposure. *The use of the Jurkat Cell model, for its experimental convenience will be translated in the second year to keratinocytes and fiberglass as well as mouse skin to test these hypotheses. Nevertheless, T-cells have been shown to be affected in individuals who have inhaled large quantities of SM, validating use of Jurkat cells in these preliminary experiments.* The data below will indicate that part of the protective effects of both these anti-oxidants was involved in the protection against the very early stages of apoptotic activation such as caspase-3 and PARP cleavage. It also should be noted that caspase-3 activity and PARP cleavage assays have been included in the original SOW in sections 1 and 2 of the amended application as well as in other aspects of the various 10 SOW's that were included in the original grant.

The Effects of inhibition of PARP on the Protection of cells to the Toxicity of sulfur mustard ----

COMPARISON TO ANTIOXIDANTS

Using either mouse fibroblasts, stably transfected with PARP antisense cDNA, or fibroblasts derived from PARP knockout mice, we earlier observed that in order to initiate the apoptotic pathway utilizing anti-Fas and cycloheximide as inducers, a transient early "burst" of poly ADP-ribosylation was essential (Simbulan-Rosenthal, et al., 1998). Accordingly, we examined the effect of the PARP inhibitor 3-AB on CEES–induced cell death (Figure 3A, 3B shown above). In Jurkat cells exposed to CEES we did not find a major effect of PARP inhibition by 3-AB for protection against CEES toxicity as was noted above due to the thiol anti-oxidants, GSH-ethyl ester and NAC.

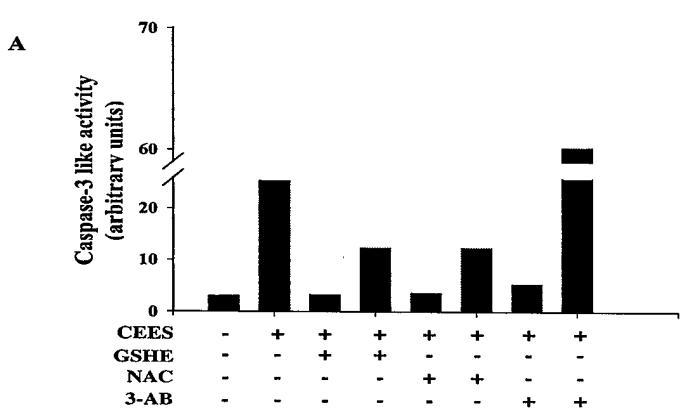
We then wished to determine whether NAC and GSH-mediated protection against CEES cytotoxicity is a result of prevention of a decrease in intracellular GSH levels. Jurkat T cells were therefore incubated in the presence or absence of 5 mM NAC or 1 mM GSH-ethyl ester for 1 h and then exposed to CEES for an additional 6 h (in the continued absence or presence of NAC or GSH-ethyl ester). Treatments with NAC and GSH-ethyl ester completely blocked CEES-induced decrease in GSH levels as shown above (Figure 3B). This data further supports the hypothesis that CEES cytotoxicity is mediated by an increase in ROS and a consequent decrease in intracellular GSH levels.

The Effects of CEES–induced activation of caspase-3 by both thiol antioxidants and 3-AB

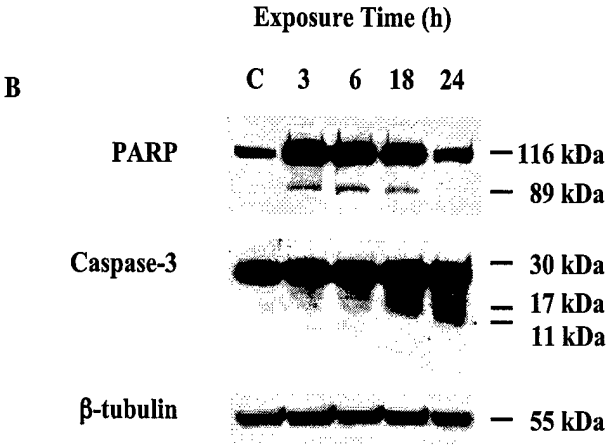
Caspase-3 is one of the early executioner proteases, which commits cells towards the apoptotic death cascade. Among other proteins, it has been characterized by its ability to cleave PARP at aspartic acid 218, which is located adjacent to the end terminus DNA binding domain of PARP; thus separating PARP into an 89 kDA fragment which contains a modification and activation domain and the 24 kDA DNA binding domain. As noted, PARP is only active catalytically

when bound to DNA strand breaks. Since it appeared that CEES was in part causing cell death due to generation of ROS and general oxidative stress on cells causing DNA strand breaks, we determined whether the protective effect of the thiol antioxidants studies above against CEES toxicity in Jurkat T cells was associated with inhibition of caspase-3 activation from its precursor status. Accordingly, we measured caspase-3–like activity in extracts prepared from cells that had been treated with CEES either alone or in combination with NAC or GSH-ethyl ester. NAC and GSH-ethyl ester each greatly reduced the extent of the increase in caspase-3–like activity induced by CEES as shown below (Figure 4A), a result which is also consistent with the ability of thiol antioxidants to inhibit apoptosis in other cellular systems (Hour et al. 1999; Li et al. 2000).

Figure 4. Influence of thiol antioxidants on activation of caspase-3 by CEES incubation.



Jurkat T cells were incubated first for 1 h in the presence or absence of 5 mM NAC, 1 mM GSH-ethyl ester, or 5 mM 3-AB, and then for 12 h in the continued presence or absence of test agent. Cell extracts were then prepared and assayed for caspase-3–like activity. Data are expressed in arbitrary units and are from a representative experiment.



Time course study on CEES-induced proteolytic cleavage of PARP and caspase-3 by Western blot analysis. β-tubulin was used as a sample loading control.

Consistent with the earlier results shown above in Figure 3A, inhibition of PARP activity did not affect the induction of caspase-3 activation.

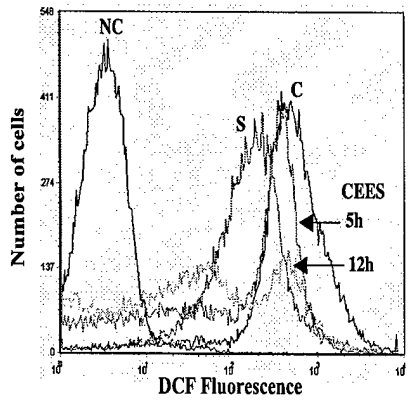
A direct examination by immunoblotting of CEES (600 uM) effect on activity of caspase-3 is shown by the experiment in Figure 4B above during a 24 h incubation period of Jurkat cells with CEES. As early as 3 hours, some PARP can be detected to be cleaved to the 89 kDa fragment that continued progressively between 17 and 24 h. There was approximately 50% of PARP totally cleaved by caspase-3 as caused by CEES incubation and presumably its ROS generating activities. Additionally, CEES also induces the cleavage of procaspase-3 into its catalytically active p17/p20 form as shown by the Middle Western Blot of figure 4B.

Mitochondrial membrane potential ($\Delta\Psi_{mito}$) in vivo----- Direct influence of CEES incubation of Jurkat cells

Since the major thrust of part 3 of the aims in The Contract concerned mitochondrial changes t, the following experiments were performed (and published) to directly address this aspect of the project. The loss of Ψ_{mito} and hence mitochondrial dysfunction has been observed to accompany apoptotic cell death in various cells (Mancini *et al.*, 1997; Salvioli *et al.*, 1997). The opening of mitochondria permeability transition pores during loss of $\Delta\Psi_{mito}$ has been established to lead to the release of several pro-apoptotic factors, including cytochrome c. This in turn contributes to the activation of caspase-9, which then with a number of other factors converts pre-caspase-3 to caspase-3, and the irreversible apoptotic cascade is established (Gottlieb, 2000). Accordingly, we examined whether the sulfur mustard half ester- induced cell death is associated with a loss of $\Delta\Psi_{mito}$. We were also interested in whether the thiol antioxidants, utilized above, prevented this mitochondrial transition. Jurkat T cells were first thus exposed to CEES for 5 h and then $\Delta\Psi_{mito}$ was examined with the specific probe JC-1 by flow cytometry.

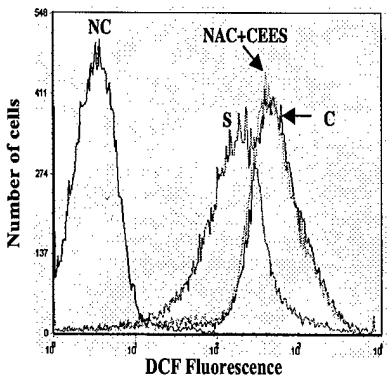
Figure 5. Influence of thiol antioxidants on CEES-induced loss of mitochondrial.

A



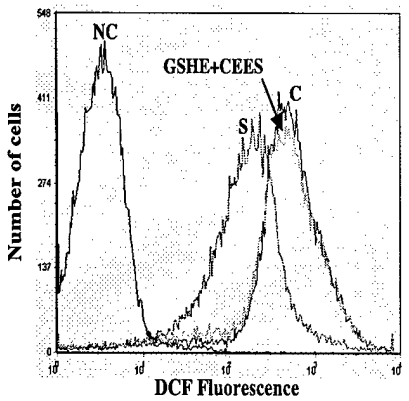
Jurkat T cells were incubated for 5 and 12 h in the continued presence or absence of test agent. NC, cells without any treatment and JC-1; S, cells treated with 1 μM of staurosporine; C, control cells. Cells were pre-incubated first for 1 h in the presence or absence of 5 mM NAC.

B



1 mM GSH-ethyl ester

C



For 5 h with or without 600uM CEES. Ten minutes before termination of the incubation, the cells were exposed to JC-1after, which $\Delta\Psi_{mito}$ was assessed by flow cytometry.

Figure 5A above shows that exposure of cells to CEES indeed resulted in a slow decrease in $\Delta\Psi_{mito}$. Because the cells exhibited more than one peak at 5 h treatment with CEES, we subsequently increased the exposure time to 12 h, and also a control used with 1 μM of staurosporine, an established inducer of apoptosis, membrane maintenance and lowering of $\Delta\Psi_{mito}$, for 5 h, to exclude that the two peaks were due to a mixture of a possible mixture of viable and some dead cells. At longer exposure with CEES, we observed a marked breakdown of $\Delta\Psi_{mito}$ that is reflected in the increased JC-1 green fluorescence, however the pattern characterized differed from that shown for cells treated with staurosporine. The effects of NAC

and GSH-ethyl ester treatment on the loss of $\Delta\Psi_{mito}$ in Jurkat cells was subsequently examined by incubating the cells in the presence or absence of 5 mM NAC or 1 mM GSH-ethyl ester for 1 h followed by exposure to CEES for 5 h (in the continued presence or absence of NAC or GSH-ethyl ester). The data in Figure 5B demonstrate that **the loss of $\Delta\Psi_{mito}$ by CEES was prevented by the presence of NAC and GSH-ethyl ester (Figure 5C).** *These data support the theory that part of cell toxicity due to sulfur mustard and its derivatives is via increased oxidative stress to cells and that the mitochondria per se is a major target of sulfur mustards.* The data clearly show that the sulfur mustard analog, CEES, has a major effect on $\Delta\Psi_{mito}$, which clearly is associated with the ability of CEES and presumably sulfur mustard *per se* to cause cell death via apoptosis in a number of cells including Jurkat. Thus, we favor the view that the loss of mitochondria membrane potential might be the results in both ROS generation and depletion of intracellular GSH that induce an oxidative modification of the mitochondrial membrane, leading to an osmotic imbalance that can favor apoptosis induction.

Employing a variety of techniques, including a caspase-3 resistant mutant of PARP, our lab previously showed that the proteolytic cleavage of PARP-1 plays a key role in the normal progression of apoptosis in both human osteosarcoma cells and PARP mouse knockout cells (Boulares, et al., 1999, Herceg and Wang, 1999). Blocking this cleavage increases the rate of apoptotic cell death, based upon an excessive depletion of the PARP substrate NAD. It was additionally demonstrated (Boulares, et al., 1999, Herceg and Wang, 1999) that expression of a caspase-3 resistant PARP mutant alters cell death induced by TNFα to both an increase rate of apoptosis as well as necrosis. By reducing excessive depletion of energy reserves such as ATP and NAD and a switch to necrosis, cells exposed to inducers of apoptosis cleave PARP rapidly into inactive peptides and inactivate the enzymatic activity and recombinant depletion of NAD and ATP. In the current experiments however, inhibition of PARP by 3-AB did not protect CEES cells against toxicity nor did it prevent the loss of $\Delta\Psi_{mito}$ induced by this sulfur mustard analog. Nevertheless, our data strongly implicates mitochondrial stress in CEES toxicity, presumably by generation of high levels of ROS, and furthermore demonstrates that CEES-induced loss of $\Delta\Psi_{mito}$ is prevented by treatment of cells with thiol antioxidants. These observations, thus, may provide the development of rational new strategies to protect and potentially prevent the toxic effects of sulfur mustard on both cells and especially tissues such as normal lymphocytes as well as keratinocytes. Accordingly, in the final experiments performed on this contract, prior to submitting this FINAL REPORT, the latter cells have been explored. Some data (normal human lymphocytes) has been subsequently published. In contrast, the keratinocytes experiments are still preliminary.

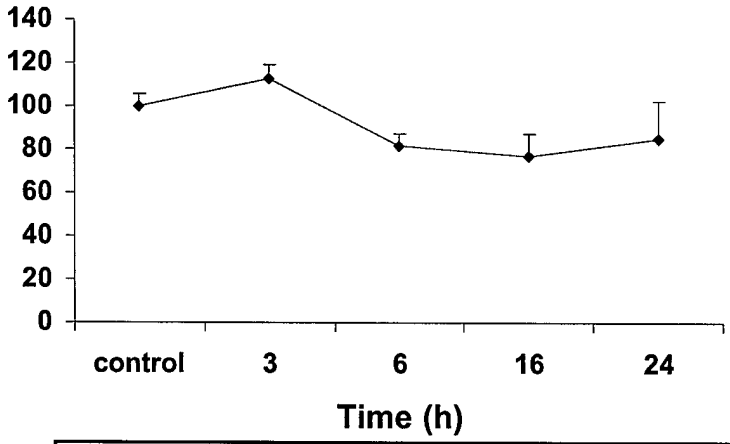
STUDIES ON NORMAL LYMPHOCYTES (CONTRACT YEAR TWO)

Comparison of intracellular GSH in CEES-induced death of Malignant versus Normal Human Lymphocytes

In the study below, calcein-AM staining revealed that incubation of normal lymphocytes and Jurkat cells for 24 h with 600 μM CEES resulted in an approximately 60% and 80% loss of viability respectively (Figure 6A). Previous data suggest that the intracellular concentration of GSH is a determinant of CEES cytotoxicity. Therefore, in order to evaluate the kinetics of GSH reduction in normal lymphocytes and Jurkat cells exposed to CEES, we measured the levels of

GSH for several time intervals from 0 to 24 h. The results (Figure 6B) indicate that CEES induces depletion of GSH content in both cells types in a time-dependent manner as observed in the cell survival data (Figure 6A).

Figure 6 CEES-induced death in normal lung fibroblast cells.



Normal lung fibroblast cells (IMR-90) were incubated for the indicated times with 600 μM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence.

Protective effect of thiol antioxidants against CEES-induced cell death in Normal Human Lymphocytes

We next determined (Fig 7, below) whether thiol antioxidants such as GSH and NAC are able to protect normal lymphocytes against CEES toxicity. Pretreatment of normal lymphocytes with NAC or GSH-ethyl ester for 1 h before exposure to the SM derivative for 18 h (in the continued presence of antioxidant) significantly inhibited cell death in normal lymphocytes than cells exposed to CEES alone as what we found in Jurkat cells (Figure 4). These data thus provided further support for the notion that CEES cytotoxicity is mediated by an increase in ROS generation and a consequent decrease in the intracellular concentration of GSH or vice versa.

With the use of both mouse fibroblasts stably transfected with PARP antisense cDNA and fibroblasts derived from PARP knockout mice, we have previously shown that a transient early phase of poly (ADP-ribosyl)ation is required for apoptosis triggered by antibodies to Fas or by cycloheximide (Simbulan-Rosenthal et al., 1998). We therefore examined the effect of the PARP inhibitor 3-aminobenzamide (3-AB) (5 mM) on CEES-induced death of normal lymphocytes. The PARP inhibitor did not substantially affect CEES cytotoxicity (Figure 7) in normal lymphocytes also, confirming that PARP is not required for cell death induced by the SM derivative.

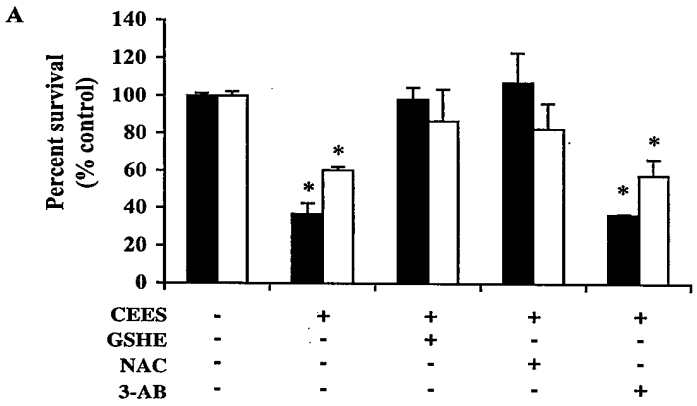


Figure 7. Role of intracellular GSH in CEES-induced death in lymphocytes. (**A**) Jurkat cells (closed circles) and normal lymphocytes (open circles) were incubated for the indicated times with 600 μM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence. (B) Jurkat cells (closed bars) and lymphocytes (open bars) were treated with 600 μM CEES at the indicated times, after which GSH content was analyzed. All data are means ± s.d. of triplicates from an experiment that was repeated a total of three times with similar results. (*)$P < 0.05$ versus control value for untreated cells.

CEES-induced activation of caspase-3 in Normal Human Lymphocytes

Caspase-3 is an executioner protease whose activation commits cells to apoptotic death (Nicholson *et al.*, 1995). In addition to its other substrates, caspase-3 cleaves PARP into a 24-kDa fragment that contains the DNA binding domain and an 89-kDa fragment that contains the automodification and catalytic domains (Tewari *et al.*, 1995). This cleavage results in inactivation of PARP, given that its activity requires its binding to the ends of DNA strand breaks (Smulson *et al.*, 1994). We had already observed caspases-3 activation **in malignant lymphocytes** (Fig 4 above) , accordingly we now examined the effect of CEES on caspase-3–like activity **in normal human lymphocytes** (Fig 8).
Immunoblot analysis confirmed that CEES induced the cleavage of procaspase-3 to the active form of the enzyme in a time-dependent manner (Figure 7). Furthermore, the CEES-induced cleavage of pro-caspase-3 in normal human lymphocytes as shown earlier with Jurkat cells was accompanied by the cleavage of PARP, which was detected as early as 3 h after exposure to the SM derivative.

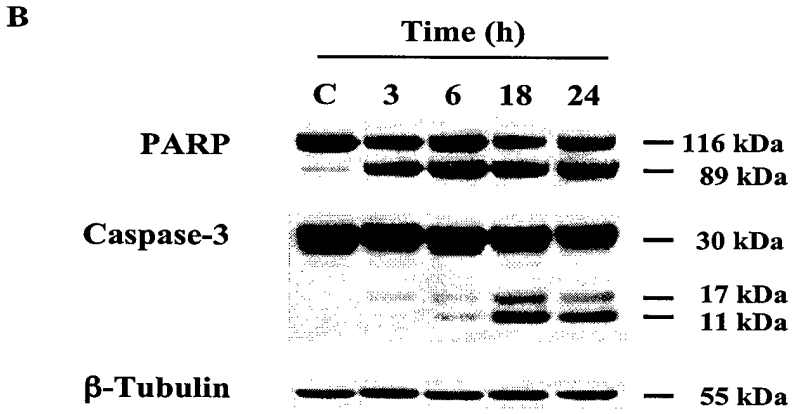


Figure 8. CEES induces PARP cleavage and activation of procaspase-3 in normal lymphocytes. Normal lymphocytes were incubated for the indicated times with CEES, after which cell lysates were subjected to immunoblot analysis with antibodies to PARP, to caspase-3, or to β-tubulin (control).

Furthermore, the CEES-induced cleavage of pro-caspase-3 in normal human lymphocytes and Jurkat cells was accompanied by the cleavage of PARP, which was detected as early as 3 h after exposure to the SM derivative.

CEES-induced death in normal lung fibroblast cells

The data above has shown that incubation of normal lymphocytes and Jurkat cells for 24h with 600 μM CEES resulted in an approximately 60% and 80% loss of viability respectively. However when exponentially growing IMR-90 cells (Fig 9) were treated with the same concentrations of CEES, there was less than 20% loss of viability induced, and this appeared to be Necrotic , which indicates lung fibroblast cells are more apoptotic resistant to CEES than that of lymphocytes.

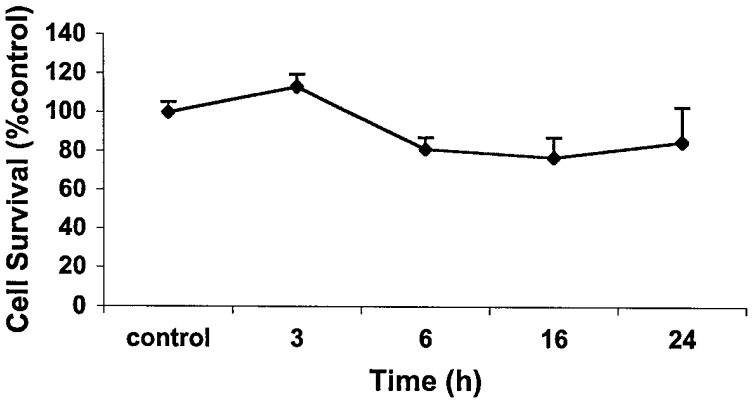


Figure 9. CEES-induced death in normal lung fibroblast cells. Normal lung fibroblast cells (IMR-90) were incubated for the indicated times with 600 μM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence.

When the Jurkat cells, however are treated with the PARP inhibitor 3-AB, the CEES-induced increase in Caspase-3 activation was not affected. Immunoblot analysis confirmed that CEES induced the cleavage of procaspase-3 to the active form of the enzyme in a time-dependent manner starting at 3h. Furthermore, the CEES-induced cleavage of pro-Caspase-3 in normal human lymphocytes and Jurkat cells was accompanied by the cleavage of PARP, which was detected as early as 3 h after exposure to the SM derivative. When IMR-90 cells were treated with 600 μM CEES (Fig 10), however, there were no cleavage of pro-Caspase-3 or the cleavage of PARP even 24h after exposure to CEES. **These results indicate that apoptosis may not play the major role in CEES induced death of lung fibroblast çells.**

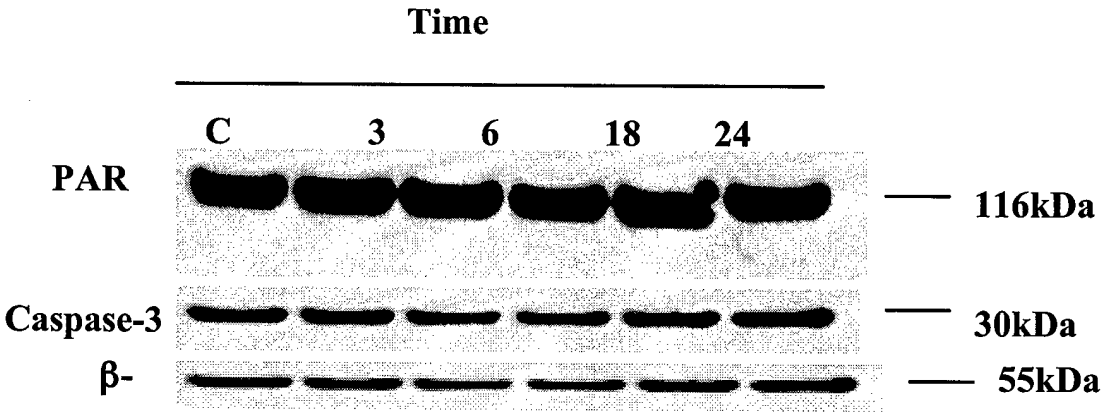


Figure 10. CEES induces PARP cleavage and activation of procaspase-3 in normal lung fibroblast cells. IMP-90 were incubated for the indicated times with CEES, after which cell lysates were subjected to immunoblot analysis with antibodies to PARP, to caspase-3.

Previous data have shown, whereas exposure of Jurkat cells to H_2O_2 alone had no effect on viability, when the cells were exposed to CEES alone induced an ~20% loss of viability, the combination of these agents resulted in >50% cell death. Nevertheless, when IMR-90 cells (Fig. 11) were treated with 600 μM CEES alone induced ~20% loss of viability, the combination of H_2O_2 and CEES did not increase the loss of viability.

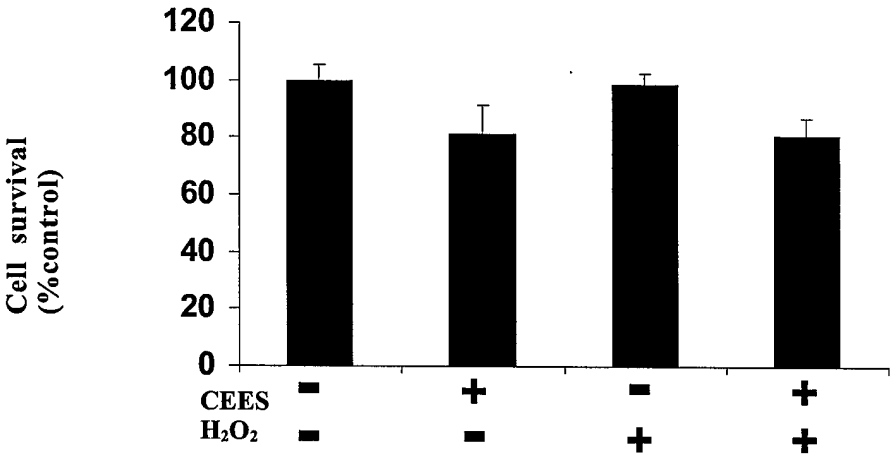


Fig. 11. Role of ROS in CEES-induced death of normal lung fibroblast cells. IMR-90 cells were incubated first for 1 h with or without 25 μM H_2O_2 and then for 6 h in the additional absence or presence of 600 μM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence.

Differing Effects of SM on Cell Death in Human Skin Fibroblasts-HaCat Cells

Previous data have shown, whereas exposure of Jurkat cells to H_2O_2 alone had no effect on viability, when the cells were exposed to CEES alone induced an ~20% loss of viability, the combination of these agents resulted in >50% cell death. However, HaCat cells were treated with 600 μM CEES alone(Fig.11) induced ~5% loss of viability, the combination of H_2O_2 and CEES resulted in an ~30% loss of viability. Which indicates that HaCat cells are more tolerable to CEES than that of Jurkat and normal lymphocytes, however, their vulnerability was dramatically increased when they were under ROS stress.

HaCat Skin Cells and Nitrogen Mustard Treatment

Pretreatment of normal lymphocytes and Jurkat cells with NAC or GSH-ethyl ester for 1 h before exposure to the SM derivative for 18 h (in the continued presence of antioxidant) significantly inhibited cell death in both types of cells exposed to CEES alone. The PARP inhibitor3-AB did not substantially affect CEES cytotoxicity, suggesting that PARP is not required for cell death induced by the SM derivative. We lastly examined the effects of thiol antioxidants in HaCat cells treated with another mustard derivative nitrogen mustard (Fig. 12). While HACat cells treated with 10 μM NM alone resulted in an ~95% loss of cell viability, pretreatment with NAC or GSH-ethyl ester for 1h significantly increased cell survival to a 55% and 45% respectively. The PARP inhibitor 3-AB did not substantially affect NM cytotoxicity, suggesting that PARP is not required for cell death induced by nitrogen mustard also.

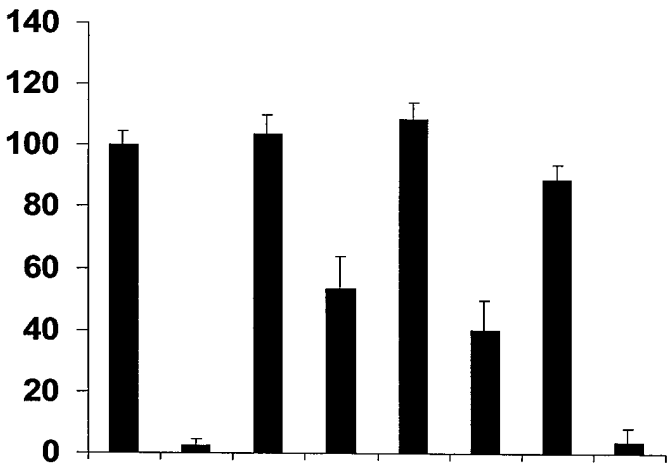


Fig. 12 Inhibitory effects of thiol antioxidants in HaCat cells.
HaCat Cells were incubated first for 1 h with or without 5 mM NAC, 1 mM GSH-ethyl ester, or 5 mM 3-AB and then for 12 h in the additional absence or presence of 10μM nitrogen mustard. after which cell viability was assessed by measurement of calcein-AM fluorescence.

KEY RESEARCH ACCOMPLISHMENTS

- Apoptotic characteristic biochemical changes and subsequent mechanism of CEES toxicity in Jurkat T cells has been further investigated in order to associate and identify rational, therapeutic, protective mechanisms against this toxicity.
- Intracellular concentrations of GSH were observed in Jurkat T cells almost immediately after exposure to CEES. This effect was accompanied by increased intracellular ROS generation and by loss of mitochondrial membrane potential that declined slowly at 5 h and more markedly by 12 h. Additionally, L-BSO, an inhibitor of an early step of GSH synthesis, increased CEES induced cell death.
- Additionally, CEES exposure in Jurkat cells induces biochemical markers of apoptotic cell death such as: PARP cleavage, caspase-3 processing and activity.
- CEES' pro-oxidant effect of was inhibited by either exogenously added thiol-containing antioxidant NAC or GSH-ethyl ester. Accordingly, CEES toxicity appears to be mediated, in part, by generation of ROS and depletion of GSH.
- intracellular GSH levels were significantly(60-80%) reduced in CEES-induced death of Malignant as well as Normal Human Lymphocytes.
- Thiol antioxidants such as GSH and NAC are able to also protect normal lymphocytes against CEES toxicity which further supports the notion that CEES cytotoxicity is mediated by an increase in ROS generation and a consequent decrease in the intracellular concentration of GSH or vice versa.
- CEES additionally was shown to induce activation of caspase-3 in Normal Human Lymphocytes.
- When exponentially growing IMR-90 cells (normal lung fibroblast cells) were treated with the same concentrations of CEES, there was less than 20% loss of viability induced, and this appeared to be Necrotic , which suggests that lung fibroblast cells are more apoptotic resistant to CEES than that of lymphocytes. When IMR-90 cells were treated with 600 μM CEES (there were no cleavage of pro-Caspase-3 or the cleavage of PARP even 24h after exposure to CEES. **These results indicate that apoptosis may not play the major role in CEES induced death of lung fibroblast cells.**
- HaCat cells(normal human keratinocytes) were treated with 600 μM CEES alone e induced only ~5% loss of viability, the combination of H_2O_2 and CEES resulted in an ~30% loss of viability. Which indicates that **HaCat cells are more tolerable to CEES than** that of Jurkat and normal lymphocytes.
- **We lastly examined the effects of thiol antioxidants in HaCat cells treated with nitrogen mustard. HACat cells treated with 10** μM NM alone resulted in an ~95% loss of cell viability, pretreatment with NAC or GSH-ethyl ester significantly increased cell survival.

REPORTABLE OUTCOMES

(Research From May 1, 2002- April 30,2004)

CHAPTERS, ABSTRACTS, PRESENTATIONS, AND MANUSCRIPTS

Han S, Espinoza LA, Boulares AH, and **Smulson ME.** Protection by Antioxidants Against Toxicity and Apoptosis Induced by the Sulfur Mustard Analog CEES (2-chloroethylethyl sulfide) in Jurkat T cells *British Jour. Pharmacol.* 141(5): 795-802, 2004

Espinoza LA, and **Smulson ME**. Macroarray Analysis of the Effects of JP-8 Jet Fuel on Gene Expression in Jurkat Cells. *Toxicology*. 189(3): 181-90, 2003.

Simbulan-Rosenthal CM, Rosenthal DS, Luo R, Samara R, Espinoza LA, Hassa PO, Hottiger MO, and **Smulson ME**. PARP-1 Binds E2F-1 Independently of its DNA Binding and Catalytic Domains, and Acts as a Novel Co Activator of E2F-1-Mediated Transcription During Reentry of Quiescent Cells into S-phase, *Oncogene* 22: 8460-71, 2003.

Boulares, A.H., Zoltoski, A.J., Sherif, Z.A., Jolly, P., Massaro, D., **Smulson, M.E.** Gene knockout or pharmacological inhibition of poly(ADP-ribose) polymerase-1 prevents lung inflammation in a murine model of asthma. *Am J Respir Cell Mol Biol.* 2003 Mar;28(3):322-9.

Trofimova, I., Dimtchev, A., Jung, M., Rosenthal, D., Smulson, M., Dritschilo, A., Soldatenkov, V. Gene therapy for prostate cancer by targeting poly(ADP-ribose) polymerase. *Cancer Res.* 2002 Dec 1;62(23):6879-83.

Mandir, A.S., Simbulan-Rosenthal, C.M., Poitras, M.F., Lumpkin, J.R., Dawson, V.L., **Smulson, M.E.,** Dawson, T.M. A novel in vivo post-translational modification of p53 by PARP-1 in MPTP-induced parkinsonism. *J Neurochem.* 2002 Oct;83(1):186-92.

Boulares, A.H., Zoltoski, A.J., **Smulson, M.E.** Acetaminophen induces acaspase-dependent and Bcl-xL sensitive apoptosis in human hepatoma cells and lymphocytes. *Pharmacol. Toxicol.* 90, 38-50 (2002)

Boulares,A H., Contreras, F., Espinoza, L., and **Smulson, M**. Roles of glutathione and oxidative stress in JP-8 jet fuel-induced apoptosis in rat lung epithelial cells. (2002). *Toxicol. Appl. Pharmacol.* **180:** 92-99.

Boulares,A.H. ,Zoltoski, A.J.,Zaki, A. S, Yakovlev, A., and **Smulson, M. E.** Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase 1 in sensitization of fibroblasts to tumor necrosis factor-induced apoptosis *Biochem. Biophys. Res. Com.,* 290:796-801(2002)

Boulares, A.H., Zoltoski, A.J., Contreras, F.J., Yakovlev, A.G., Yoshihara, K., **Smulson, M.E.** Regulation of DNAS1L3 Endonuclease Activity by Poly(ADP-ribosyl)ation during Etoposide-induced Apoptosis. *J. Biol. Chem.*, 277(1):372-378 (2002)

Soldatenkov, VA., Chasovskikh, S., Potaman, V.N., Trofimova, I., **Smulson, M.E.**, Dritschilo, A. Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J. Biol. Chem.* ;277(1):665-670. (2002).

Rosenthal, D.S., Simbulan-Rosenthal, C.M., Valena, A., Anderson, D., Benton, B., Wang, Z.Q., Smith, B., Ray, R., & **Smulson, M.E.** PARP determines the mode of cell death in skin fibroblasts but not in keratinocytes exposed to sulfur mustard. *J.Invest.Dermatol.* 117(6): 1566-1573 (2001)

REFERENCES

BOULARES, A.H., YAKOVLEV, A.G., IVANOVA, V., STOICA, B.A., WANG, G., IYER, S. & SMULSON, M. (1999). Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *J. Biol. Chem.*, **274,** 22932–22940.

CHIBA, T., TAKAHASHI, S., SATO, N., ISHII, S. & KIKUCHI, K. (1996). Fas-mediated apoptosis is modulated by intracellular glutathione in human T cells. *Eur. J. Immunol.*, **26,** 1164–1169.

GOTTLIEB, R.A. (2000). Mitochondria: execution central. *FEBS Lett.*, **482,** 6–12.

HATHWAY, D.E. (2000). Toxic action/toxicity. *Biol. Rev. Camb. Philos. Soc.* **75,** 95127.

HERCEG, Z. & WANG, Z.Q. (1999). Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. *Mol. Cell. Biol.*, **19,** 5124–5133.

HOUR, T.C., SHIAU, S.Y. & LIN, J.K. (1999). Suppression of N-methyl-N'-nitro-N-nitrosoguanidine- and S-nitrosoglutathione-induced apoptosis by Bcl-2 through inhibiting glutathione-S-transferase pi in NIH3T3 cells. *Toxicol. Lett.*, **110,** 191–202.

JABS, T. (1999). Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol.*, **57,** 231–245.

LI, J., HUANG, C.Y., ZHENG, R.L., CUI, K.R. & LI, J.F. (2000). Hydrogen peroxide induces apoptosis in human hepatoma cells and alters cell redox status. *Cell. Biol. Int.* **24,** 9–23.

MANCINI, M., ANDERSON, B.O., CALDWELL, E., SEDGHINASAB, M., PATY, P.B. & HOCKENBERY, D.M. (1997). Mitochondrial proliferation and paradoxicalmembrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. *J. Cell Biol.*, **138,** 449–469.

NICHOLSON, D.W., ALI, A., THORNBERRY, N.A., VAILLANCOURT, J.P., DING, C.K., GALLANT, M., GAREAU, Y., GRIFFIN, P.R., LABELLE, M., LAZEBNIK, Y.A., MUNDAY, N.A. RAJU, S.M., SMULSON, M.E., YAMIN, T.T., YU, V.L. & MILLER, D.K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376,** 37-43.

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.

SALVIOLI, S., ARDIZZONI, A., FRANCESCHI, C. & COSSARIZZA, A. (1997). JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess DY mito changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett.*, **411,** 77–82.

SIMBULAN-ROSENTHAL, C.M., ROSENTHAL, D.S., DING, R., BHATIA, K. & SMULSON, M.E. (1998). Prolongation of the p53 response to DNA strand breaks in cells depleted of PARP by antisense RNA expression. *Biochem. Biophys. Res. Commun.*, **253,** 864–868.

SMULSON, M,, ISTOCKM N,, DING, R. & CHERNEY, B. (1994). Deletion mutants of poly(ADP-ribose) polymerase support a model of cyclic association and dissociation of enzyme from DNA ends during DNA repair. *Biochemistry* **33,** 6186-6191.

TEWARI, M., QUAN, L.T., O'ROURKE, K., DESNOYERS, S., ZENG, Z., BEIDLER, D.R., POIRIER, G.G., SALVESEN, G.S. & DIXIT, V.M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substratepoly(ADP-ribose) polymerase. *Cell* **81,** 801-809.

ZHANG, P., NG, P., CARIDHA, D., LEACH, R.A., ASHER, L.V., NOVAK, M.J., SMITH, W.J., ZEICHNER, S.L. & CHIANG, P.K. (2002). Gene expressions in Jurkat cells poisoned by a sulphur mustard vesicant and the induction of apoptosis. *Br. J. Pharmacol.*, **137,** 245–252.

APPENDICES

Experimental Procedures

Materials Fetal bovine serum was obtained from Quality Biological, INC. (Gaithersburg, MD, U.S.A.). RPMI 1640 was obtained from Invitrogen (Carlsbad, CA, U.S.A.). GSH-ethyl ester, NAC, L-BSO, hydrogen peroxide and anti-β-tubulin were purchased from Sigma Chemical Company (St, Louis, MO, U.S.A.). CEES was obtained from Aldrich (Milwaukee, WI, U.S.A.). Calcein-AM, JC-1 and H2DCF were obtained from Molecular probes (Eugene, OR, U.S.A.) and anti-PARP from BD Transduction Laboratories (San Diego, CA, U.S.A.). Anti-mouse IgG peroxidase-conjugated secondary antibody was purchased from Amersham (Piscataway, NJ, U.S.A.) and anti-caspase-3 from Santa Cruz (Santa Cruz, CA, U.S.A.). Ac-DEVD-AMC was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). Cell 100 mg/ml). A 14% (v/v) CEES solution was prepared in absolute ethanol and was further diluted 1:2000 with complete growth medium. A 0.05% solution of ethanol in culture medium (vehicle) had no significant effect on cell viability (data not shown). The density of CEES is 1.07 g/ml; a 1/14,000 dilution therefore corresponds to a CEES concentration of 600 μM.

Cell viability assay

For determination of cell viability, cells were seeded in 24-well plates. After treatment with 600 μM CEES, cells were exposed to calcein-AM (Molecular Probes, Eugene, OR) to a final concentration of 2.5 μM and then the plate was incubated for 30 min at 37°C. Fluorescence resulting from the deesterification of calcein-AM was monitored with a CytoFluor 4000 fluorometer (PerSeptive Biosystems, Framingham, MA) at excitation and emission settings of 488 and 520 nm, respectively.

Assay for intracellular GSH

Cells were incubated with CEES at previously indicated concentrations and immediately washed twice with PBS. The cell pellets were then lysed in 10 mM HCl by freezing and thawing three times followed of deproteinization with 10% 5-sulfosalicylic acid and centrifuged at 1000 x g for 5 min. The supernatant solution was assayed for nonprotein sulfhydryls by quantifying, using a spectrophotometer, the reduction of DNTB (5,5'-dithio-bis-[2-nitrobenzoic acid]) through its conversion to 5-thio-2-nitrobenzoic acid at 412 nm. Standard curves were run in all experiments with known amounts of GSH.

Caspase-3 activity measurement

Caspase-3 activity was assessed using z-Asp-Glu-Val-Asp (DEVD) as a substrate in the established fluorescent assay. Cell extracts (30 μg of protein) were incubated with 40 mM DEVD-AMC peptide substrate in a total volume of 200 μl. The free aminomethylcoumarin (AMC) fluorescence, which is produced by the cleavage of the aspartate–AMC bond, was measured continuously utilizing a CytoFluor 4000 fluorometer at excitation and emission wavelengths of 360 and 460 nm, respectively for 30 min. The fluorescent emission from each well was plotted versus time, and linear regression analysis of the initial velocity for each curve yielded the activity.

Measurement of ROS levels

The assay for ROS measure was monitored using the cell-permeate probe H2DCF. Cells growing on 96-well culture plates were pretreated with CEES for 5 h. Then cells were loaded with H2DCF by addition of the diacetate form of this compound (Molecular Probes) to the medium at a final concentration of 10 μM in the dark for 15 min. In the presence of ROS, the nonfluorescent dichlorodi-hydrofluorescein is oxidized to the highly fluorescent 2,7-dichlorofluorescein. Fluorimetric analysis was measured with an excitation of 485 nm and emission of 530 nm.

Mitochondrial Membrane Potential Measurement ($\Delta\Psi_{mito}$)

After treatment with CEES, cells were assayed for mitochondrial membrane potential with 5,5', 6,6', -tetrachloro-1, 1'3, 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes), a cell-permeable dye that becomes concentrated in the mitochondria and generates red fluorescence in mitochondria with a high $\Delta\Psi_{mito}$. After CEES exposure for 5 h, cells were incubated for 10 min at 37°C with 15 μg/ml of JC-1, and then analyzed for loss of JC-1 fluoresce as $\Delta\Psi_{mito}$ using a Becton Dickinson FACS flow cytometer (Boulares *et al.*, 2001).

Immunoblot Analysis

Cells were harvested, washed with ice–cold phosphate-buffered saline. Cells were lysed in ice-cold lysis buffer containing (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulphonylfluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 20 μg/ml leupeptin. Thirty micrograms of protein per lane was subjected to SDS-PAGE on 4-20% gradient gels, followed by transfer to nitrocellulose membrane and incubation with the first antibodies overnight. Proteins were visualized with horseradish peroxidase-conjugated anti-mouse followed by use of ECL chemiluminescence's kit (Pierce, Rockgold, IL).

Statistical analysis

All statistical analyses to determine the significances of differences between experimental conditions were performed using the Student's *t* test. A difference of $P < 0.05$ was considered significant.