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13. ABSTRACT *(Maximum 200 Words)*

Both the acute and delayed toxicities of DNA damaging agents like sulfur mustard (SM) appear to represent the outcome of a race between protective and toxic pathways triggered by DNA damage. Results obtained within the framework of our first objective – to investigate the mechanisms involved in processing SM-induced DNA modifications, have revealed that both nucleotide excision repair (NER) and base excision repair (BER) are involved in processing SM-induced DNA damage. However, in contrast to the protective effect of NER, expression of the alkyl adenine DNA glycosylase, the first enzyme in the BER repair pathway, renders cells more sensitive to SM. We believe that specific post-exposure conditions can modulate cellular response to DNA damage by facilitating protective pathways and/or diminishing toxic ones. Specifically, as our second objective, we are exploring mild hypothermia as a means of ameliorating SM toxicity. Our results show that low temperature increases survival of mammalian cells after exposure to SM and suggest that, at least in part, this may be due to post-exposure modulation of DNA repair and replication.

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INTRODUCTION

During the last several years many aspects of cellular responses to stress and mechanisms leading to the cell death have been elucidated (1-4). A better understanding of those highly regulated processes helps us also to better understand the role of DNA damage in triggering stress response and cell death. It is reasonable to assume that the overall toxic effect of sulfur mustard (SM) exposure represents the net effect of toxic and protective cellular pathways activated in response to SM-induced DNA damage. Therefore by applying specific post-exposure conditions, we should be able to modulate stress response in a way that interrupts toxic pathways and/or increases the capacity of protective ones. Our studies indicate that lowering temperature after exposure to SM may provide such conditions (5); certain features of hypothermia such as accumulation of p53 protein and effects on cell cycle progression that we discovered (6), strongly suggest that hypothermia acts by modulating the cellular response to SM-induced DNA damage.

We suggest that the initial level and type of damage to DNA and its immediate processing are the early determinants of SM toxicity. Later determinants of toxicity may involve p53-dependent and independent stress response pathways such as cell cycle arrest, repair, and apoptosis that may be modulated by hypothermia. Therefore, our main objectives are: 1) to investigate repair mechanisms involved in processing of SM-induced DNA lesion, and 2) to examine the role of hypothermia in protection against SM toxicity.

BODY

BACKGROUND

The Repair Mechanisms Involved in Processing of SM-induced DNA Lesions

DNA crosslinks are considered major toxic lesions in SM-alkylated DNA, and the repair of crosslinks is most likely a major defense against SM toxicity. At the same time, DNA monoadducts can seriously interfere with DNA functions either directly or as precursors of crosslinks, apurinic (AP) sites, or strand breaks, all of which, if unrepaired, can be deleterious. Our previous results suggest that at least two distinct DNA repair pathways, nucleotide excision repair (NER) and base excision repair (BER), are involved in processing of SM-induced DNA damages (5,7). In the present study we investigate specific features of the pathways involved.

Nucleotide Excision Repair

Increased sensitivity to SM of NER-deficient mammalian cells in culture (5) indicate that at least some cytotoxic DNA lesions induced by SM are successfully repaired by mammalian NER. It does not indicate, however, which specific lesions are substrates for NER. In order to answer the question weather NER is involved in processing of mustard-induced DNA monoadducts we used one-armed sulfur mustard, chloroethyl ethyl sulfide (CEES) which generates DNA monoadducts similar to those formed by bifunctional SM, but does not form crosslinks.

We have adapted a host cell reactivation assay using a luciferase expression plasmid to measure repair of SM- or CEES-damaged plasmid transfected into wild type or NER-deficient Chinese Hamster Ovary (CHO) cells (5, 8). The results have shown an increased repair of both, SM and CEES-alkylated DNA in wild type compared to NER-deficient cells demonstrating the role of NER in repair of mustard-induced DNA monoadducts (5, 8).

In order to examine whether monoadducts processed by NER are cytotoxic if unrepaired, we have compared survival of wild type and NER-deficient CHO cells after exposure to SM or CEES (see Results section and 8). The results demonstrate protective role of NER not only against SM but also against CEES toxicity suggesting that mustard-generated monoadducts in DNA that are substrate for NER do represent lethal lesion if unrepaired.

Base Excision Repair

We have previously reported (7) that purified bacterial AlkA glycosylase removes both monoadducts, 3HETEA and 7HETEG, from SM-alkylated DNA *in vitro*. More recent data with purified human glycosylase suggest that this enzyme recognizes at least one of the SM-modified bases in DNA (5). However, there are no studies reported so far on the biological effects of base excision repair (BER) activity in SM-exposed cells. Furthermore, very little is known about potential mechanisms that coordinate the two repair pathways, NER and BER, when they are functional in the same cell.

Base excision repair has an important role in protecting cells against the lethal and mutagenic effects of simple alkylating agents. However, there are conflicting reports on the involvement of mammalian alkyl adenine DNA glycosylase (AAG) in protecting cells against cytotoxic effects of bifunctional alkylating agents. Engelward et al. (9) reported that the loss of alkyl adenine DNA glycosylase renders mouse embryonic stem cells more sensitive not only to the methyl methane sulfonate (MMS), but also to the two crosslinking agents, 1,3-bis(2-chloroethyl)-1-nitrosourea (CNU) and mitomycin C. In contrast, Elder et al. (10) did not observe any protective effect of AAG against CNU toxicity in experiments with wild type and AAG knockout mouse embryonic fibroblasts.

Here we show that in the same experimental system where the expression of alkyl adenine DNA glycosylase clearly protects cells against MMS-induced toxic effects, it sensitizes cells to SM-induced effects. We demonstrate for the first time that, in contrast to the protective effect of NER, expression of the first enzyme in the base excision repair pathway renders bacterial as well as mammalian cells more sensitive to SM.

The Role of Hypothermia in Protection Against SM Toxicity

Cell response to hypothermia characterized by p53 accumulation and p53-dependent cell cycle arrest (6) together with the growing amount of evidence on cross-talk between the p53 protein and DNA repair processes (11-14) support our hypothesis on multiple mechanisms responsible for the beneficial effect of low temperature after SM exposure. It also encourages a more

detailed investigation of molecular and cellular mechanisms involved in response to hypothermia and its effects on DNA repair.

In the present study we measured the extent of replicative DNA synthesis in cells at 37°C and 28°C and found that the initiation of DNA synthesis is significantly delayed at 28°C (see: Results section). This delay may allow more time for the processing of damaged DNA before the onset of DNA synthesis. The incubation at 28°C following SM exposure does indeed increase survival of mouse embryonic stem cells more than ten fold above the survival at 37°C (see: the Results section).

The results on sensitizing effects of DNA glycosylase prompted us to address the question of possible modulation of glycosylase activity by low temperature. We found that mammalian cells deficient in alkyl adenine DNA glycosylase survive better than wild type cells at both temperatures and that the sensitizing effect of glycosylase activity in SM-damaged cells is much less pronounced at 28°C. It suggests that the beneficial effect of hypothermia on survival partly derives from the "weakening" of the toxic effects of glycosylase action at low temperature. This is supported by our *in vitro* studies with methylated DNA substrates showing that the cloned human AAG has lower activity at 28°C than at 37°C.

MATERIAL AND METHODS

Bis-(chloroethyl)sulfide (SM) was supplied by the US Army Institute of Chemical Defense (Aberdeen Proving Ground, MD). Methyl methanesulfonate (MMS) and chloroethyl ethyl sulfide (CEES) were purchased from Aldrich (Milwaukee, WI). ^{3}H-methyl nitrosourea (MNU) was obtained from New England Nuclear. The substrate for *in vitro* repair studies was synthesized from ^{3}H-MNU that had a specific activity (SA) of 17 Ci/mmol. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies were from Caltag Laboratories, Burlingame, CA.

The normal human diploid fibroblast cell line, AG01522, was obtained from the Aging Cell Repository, Coriell Institute for Medical Research (Camden, NJ) at a population doubling level (PDL) of 15. Chinese hamster ovary (CHO) cell lines AA8 (wild type) and UV41 (NER group 4) were obtained from the American Type Culture Collection. Mouse embryonic stem (ES) cells, wild type and null mutants for 3-alkyladenine DNA glycosylase, were obtained from Dr. Bevin Engelward (Massachusetts Institute of Technology) (9). SNL76/7 feeder cells were obtained from Dr. Allan Bradley (Baylor College of Medicine, Huston) (15).

Bacterial Cells and Plasmids

All bacterial strains used in this study were constructed by Dr. M. Volkert. Their relevant genotypes are listed in Table 1.

Table 1. Bacterial strains

Name	*Relevant Genotype*
MV 1161	*arg*E3
MV1273	*arg*E3 *uvrA*
MV1174	*arg*E3 *alk*A1
MV1302	*arg*E3 *alk*A1 *uvr*A
MV3855	*arg*E3 *alk*A1 *tag*A1 *uvr*A
MV4236	*arg*E3 *alk*A1 *tag*A1 *uvr*A / pTrc
MV4237	*arg*E3 *alk*A1 *tag*A1 *uvr*A / pTrc hAAG-1
MV4239	*arg*E3 *alk*A1 *tag*A1 *uvr*A / pTrc hAAG-2
MV4126	*arg*E3 *alk*A1 *tag*A1/pTrc hAAG-1-his_6

Cells were grown in liquid Luria-Bertani (LB) medium at 37°C with aeration. Strains containing plasmids were grown in the presence of ampicillin. Permanent stocks are maintained at –80°C in LB with 10% DMSO. Expression vectors with human alkyl adenine DNA glycosylases (hAAG) used in this study were constructed starting from the hAAG-1 gene on pBU16 plasmid obtained from Dr. Leona Samson, Harvard School of Public Health. After the deletion of pBU16 vector sequence, the hAAG gene was inserted into the pTrc99A plasmid and placed under the control of the IPTG-inducible P_{trc} promoter. The hAAG-2 isoform was constructed by PCR using the hAAG-1 gene as a template and an appropriate oligonucleotide as a PCR primer to replace exon 1a from hAAG-1 gene with the exon 1b DNA sequence from hAAG-2 gene.

Transformation

Plasmids were introduced into the bacterial cells by transformation of purified plasmid DNA into the competent cells and by selection for ampicillin resistant clones. To make them competent cells were resuspended in 0.1 M PIPES, pH 6.8 containing 2.5 M $CaCl_2$. For transformation, cells were mixed with DNA in a buffer containing 0.1 M PIPES, pH 6.8, 1 M $CaCl_2$, and 1 M $MgCl_2$, incubated on ice, heat-shocked at 37°C and cooled. After addition of growth media cells were incubated for 1 hr at 37°C to allow expression of ampicillin resistance. Cells were then plated on plates with ampicillin to select transformants.

Cell Survival and Mutation Frequency

Cells were grown in LB medium with or without ampicillin to approximately 10^8 cells per ml. In experiments with human glycosylase (hAAG), 0.1 M IPTG was added to induce hAAG expression and cells were grown for the additional 90 min at 37°C with aeration. Aliquots of cell suspension were transferred into the SterilchemGARD hood and exposed to different doses of SM or MMS for 30 min. Cell dilutions were prepared in E-salts buffer containing 4% $Na_2S_2O_3$ and plated in triplicates on ESEM media. Plates were incubated for 48 h at 37°C and surviving colonies and arg+ mutants were counted. Survival was expressed as a percent of untreated control and mutation frequency was calculated as a number of Arg+ revertants among survivors.

Isolation of Human Alkyl Adenine DNA Glycosylase

Human alkyl adenine DNA glycosylase (hAAG) was expressed as a histidine-tagged derivative of a hAAG1 isoform in MV4126 *alkA⁻ tagA⁻ E. coli* cells. Cells were grown to a Klett reading of 70 (approximately 5 x 10^8 cells/ml) in LB broth containing ampicillin (100 μg/ml), then induced by the addition of IPTG (2 mM). Fresh ampicillin (100 μg/ml) was also added at this time to insure plasmid maintenance, and cells were incubated for an additional 5 hours. After incubation, the cells were centrifuged, washed in saline buffer (10 mM Tris-Cl, pH 7.4; 1 mM EDTA; 100 mM NaCl) and lysed using a Kraft homogenizer followed by a French press. Crude cell extracts were then centrifuged at 12,000 x g and the supernatant recovered. The supernatant was applied to a Ni-agarose column (Qiagen) equilibrated with column buffer (50 mM Na_2HPO_4, pH 8.0; 300 mM NaCl; 10% glycerol). The column was washed with 200 ml of column buffer followed by 300 ml of 30 mM imidazole in column buffer, and finally elute with a 100 ml gradient of 30 mM to 500 mM imidazole in column buffer. Two ml fractions were collected and assayed for glycosylase activity with ^{3}H-MNU-modified DNA. A sharp peak of activity appeared around fraction 26.

Preparation of Methylated DNA Substrates

Substrate for glycosylase assay was prepared as described (7). Briefly, calf thymus DNA was alkylated with ^{3}H-methylnitrosourea (^{3}H-MNU) washed free of unbound radioactivity, and characterized for modified base content by acid hydrolysis and HPLC analysis.

Glycosylase Assays

Glycosylase assays followed the procedures described in reference (7). Briefly, ^{3}H-MNU substrate containing approximately 20,000 cpm of modified bases was incubated at 37°C or 28°C in pH 7.5 assay buffer for 30 to 150 min in the presence of 5 μl of glycosylase enzyme. At the end of the incubation, substrate and enzyme were precipitated with ethanol, and the mixture was centrifuged. Centrifugation leaves the bases that are released from the substrate by the glycosylase in the supernatant; the extent of the release was determined by counting an aliquot of the supernatant in a Beckman LS-6500 scintillation counter.

Mammalian Cell Culture

CHO cells were grown in monolayer culture in α-modified minimum essential medium (Eagle) without ribonucleosides or deoxyribonucleosides, supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Mouse embryonic stem (ES) cells were cultured on gelatinize plates with mitotically inactive SNL76/7 feeder cells that express leukemia inhibitory factor (LIF) to prevent ES cells differentiation (15). Feeder cells were grown on Dulbecco's modified Eagle's medium (DMEM)

supplemented with FBS (7%), glutamine and antibiotics. They were inactivated by mitomycin C (10 μg/ml, 2 h) and kept frozen until use. DMEM media for ES cells growth was supplemented with FBS (15%), glutamine, antibiotics, 2-mercaptoethanol and nonessential amino acids.

Human fibroblasts (AG01522B) were grown as a monolayer in standard minimal essential media (MEM) with a 2x concentration of amino acids and vitamins supplemented with 15% fetal bovine serum, penicillin and streptomycin.

All cell culture media were from Gibco BRL, Gaithersburg, MD or ICN Pharmaceuticals, Costa Mesa, CA. Cells were grown at either 37°C or 28°C in a humidified atmosphere of 7% or 4.6% CO_2, respectively. Incubation temperatures in these experiments were monitored with a Yellow Springs Instrument Model 4600 Digital Thermometer.

Survival Studies

Cells were plated in 12- or 6-well plates at a density of 2 x 10^4 cells/cm^2. After 24 h, the medium was replaced with fresh medium containing the indicated concentrations of SM, CEES or MMS; dilute solutions of these compounds in absolute alcohol were prepared immediately before treatment. Cells were exposed to the chemicals for 1 h at room temperature in a SterilchemGard hood and then incubated in fresh medium at 37°C or 28°C. At the indicated times, cell viability was determined by the trypan blue exclusion assay. In order to include cells that may have been detached from the monolayer, the medium above the monolayer was collected and centrifuged prior to trypsinization, and the pellet was combined with cells detached by trypsin. Analysis of duplicate determinations showed that these counts were reproducible with a standard deviation of ± 6.3%.

Cell Cycle Analysis

Replicative DNA synthesis was determined as 5-bromo-2'-deoxyuridine (BrdU) incorporation following a slightly modified procedure described by Jones *et al.* (16). Cells were labeled in 13 μM BrdU for 2 h, harvested by trypsinization, fixed with 70% ethanol and stored at -20°C until analysis. Samples were treated with 0.1 N HCl containing 0.5% Triton X-100 for 30 min at room temperature, boiled for 2 min and rapidly cooled to denature DNA. Cells were then washed twice with 0.1 M $Na_2B_4O_7$ solution (pH 8.5), incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies for 30 min (1:4 dilution; Caltag Laboratories, Burlingame, CA) and counterstained with propidium iodide. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA). At least 15,000 events were collected per sample; cell doublets and aggregates were electronically eliminated from analysis. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME).

RESULTS AND DISCUSSION

Repair Mechanisms Involved in Processing of SM-induced DNA Lesions

Nucleotide Excision Repair

In order to examine the toxicity of mustard mono-adducts and the role of NER in survival independently from the effects of crosslinks, we used single armed mustard CEES for survival studies with NER-competent and NER-deficient CHO cells.

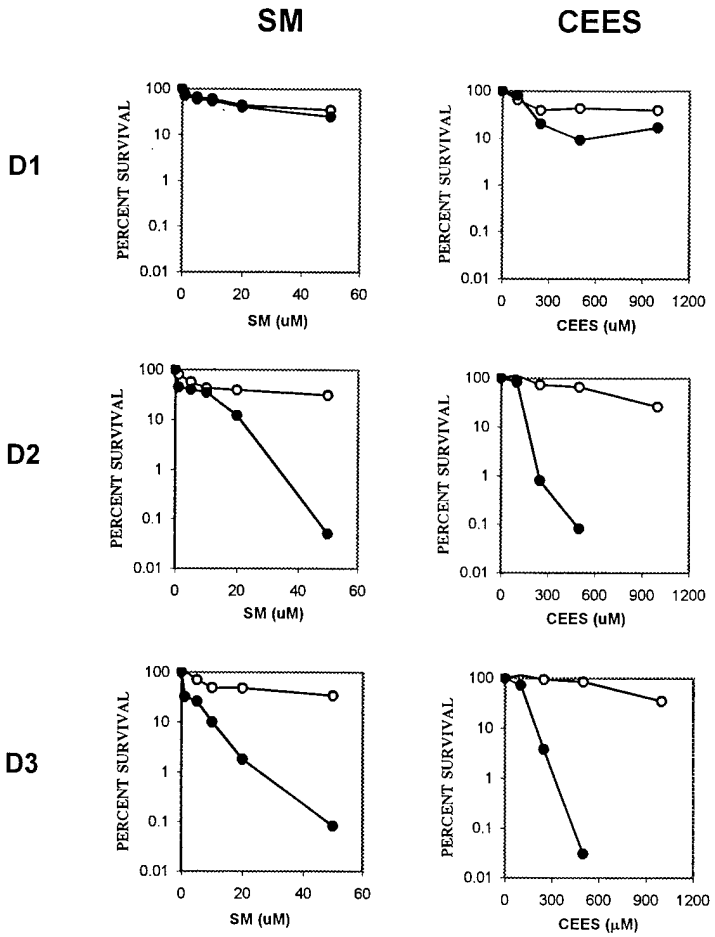

Figure 1. Survival of CHO cells after exposure to SM or CEES. Viable cell numbers were determined by trypan blue exclusion and are expressed as a percent of control on day 1 (D1), day 2 (D2), and day 3 (D3). Wild type cells (O); NER-deficient cells (●).

The results in Figure 1 demonstrate the importance of NER in protecting cells from cytotoxicity of both SM and CEES. Three days after exposure to 20 μM SM, more than 50% of wild type cells survive, while NER-deficient cells show a survival of less than 2%. A similar difference is noted after exposure to CEES except that concentrations of CEES more than ten times as great as the SM concentrations are needed to produce the same level of cytotoxicity. Three days after exposure to 300 μM CEES, survival is close to 100% for wild type cells but only about 5% for NER-deficient cells. The difference in cytotoxicity between SM and CEES is assumed to be the result of crosslink formation. In addition to demonstrating that monoadducts are substrate for NER, the results also suggest that at least one of the mustard DNA monoadducts, if unrepaired, represents a lethal lesion for mammalian cells.

Base Excision Repair

Bacterial Glycosylase

The base excision repair pathways are present in diverse organisms from bacteria to mammalian cells. In all organisms the substrate specificity of the base excision repair pathway is determined by DNA glycosylases. In order to determine whether alkyl adenine DNA glycosylase is able to process biologically relevant alkylation products in SM-exposed cells, we used *E. coli* cells that are either wild type or repair deficient, and measured their survival as a function of SM dose.

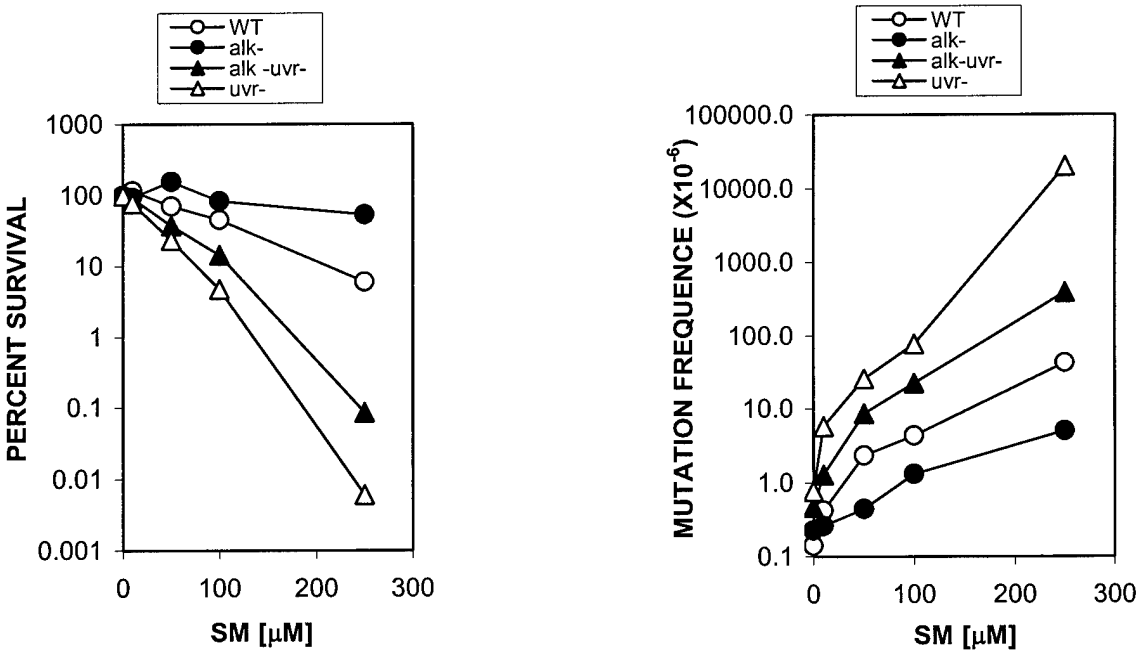

Figure 2. Role of DNA repair on survival and mutation induction in repair deficient *E. coli* cells exposed to sulfur mustard. Wild type (○), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to SM for 30 min and plated for total cell number and for Arg+ revertants. Survival (left panel) was calculated as a percent of untreated control and mutation frequency (right panel) was calculated as a number of revertants among survivors.

Surprisingly, as the results in Figure 2 (left panel) show, *E. coli* cells lacking functional AlkA glycosylase (Alk$^-$, close symbols) survive better than cells that are wild type for glycosylase (open symbols). In contrast, lack of NER (uvrA$^-$) in *E. coli* cells increases their sensitivity to SM, similar to the effects in CHO cells. This sensitizing effect of AlkA glycosylase function occurs in cells regardless of their NER status; AlkA+ cells are more sensitive to SM than AlkA$^-$ cells whether or not they have functional nucleotide excision repair.

Another potential consequence of DNA damage, induction of mutations, was tested as the reversion of Arg$^-$ into the Arg+ phenotype in cells with different repair background. The results in Figure 2 (right panel) show that, in contrast to NER which protects cells from accumulation of mutations, alkA function increases mutation frequency in *E. coli* above that seen in the AlkA-deficient strain.

Human Glycosylase Expressed in E.coli

In order to study the effects of human alkyl adenine DNA glycosylase (hAAG) in SM exposed cells, cloned isoforms of hAAG that can be expressed from an IPTG-inducible promoter were introduced into *alkA tagA uvrA E. coli* cells by transformation. Human alkyl adenine DNA glycosylase has been shown to exist in two isoforms and both are expressed in wild type mammalian cells. The two forms are produced by alternative splicing and differ with respect to exon 1 (17). Both isoforms can complement methyl methansulfonate (MMS) sensitivity of *E. coli* cells deficient in Alk and Tag glycosylases (18). In order to confirm that human glycosylase is expressed under the conditions of our experiment, we measured survival after the exposure to MMS in parallel with SM experiment. The results in Figure 3, left panel, show that hAAG is IPTG-inducible and that it protects *E. coli* cells from MMS toxicity. However, under the same experimental conditions, expression of hAAG increases sensitivity of *E. coli* cells to sulfur mustard (Figure 3, right panel). This effect is more pronounced in cells containing isoform 2 (hAAG2) than isoform 1 (hAAG1) glycosylase.

The results described so far indicate that neither bacterial nor human DNA glycosylase protect bacteria from SM toxicity. However, increased sensitivity of cells expressing glycosylase compared to the glycosylase-deficient cells indicate that human and bacterial glycosylases do act on SM lesions, but process them in a manner that increases toxicity. There are several possible explanations for this. First, glycosylases, especially human glycosylase that is expressed from multicopy plasmids, may be hyperactive and may process lesions too rapidly, thereby overwhelming subsequent steps in DNA repair.

This possibility is ruled out by the results from MMS experiments. These studies suggest that the highly expressed human glycosylase does not overwhelm the subsequent repair steps when DNA is modified by methylation. In both SM and MMS treated cells, a glycosylase-performed repair step would result in the production of abasic sites (AP sites). The AP endonucleases, DNA polymerase and ligase, required for processing of the abasic sites appear to be capable of accomplishing the repair process on MMS-modified substrate. In fact, the rate limiting step in base excision repair appears to be the glycosylase step, since the highly expressed hAAG provides a high level of protection against MMS damage.

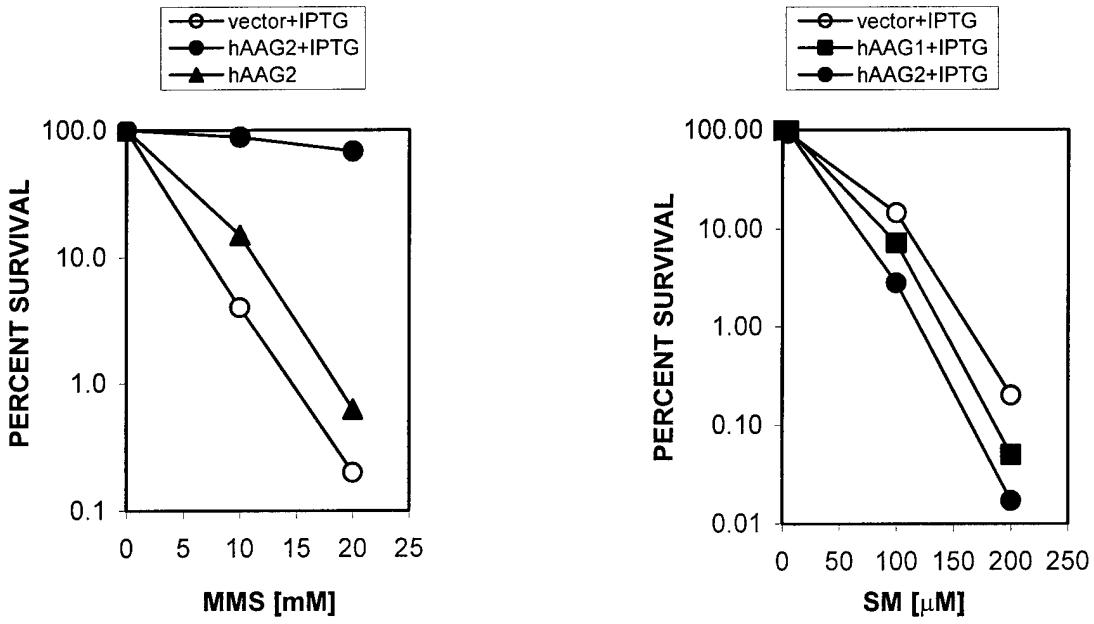

Figure 3. Effects of cloned human DNA glycosylase on survival of *E. coli* cells exposed to MMS or SM. Cloned human alkyl adenine DNA glycosylase hAAG1 (■), hAAG2 (●) or cloning vector only (○) were introduced into the *E. coli* cells deficient for bacterial glycosylase and NER (uvrA⁻ alkA⁻ tagA⁻). Left panel: hAAG provides full protection against MMS toxicity in IPTG-induced cells (●) and slightly increases resistance in un-induced cells (▲). Right panel: both hAAG1 and hAAG2 isoforms increase sensitivity of E. coli cells to SM.

Second, ineffective repair by glycosylases may interfere with the repair by other, more effective, repair processes such as NER. If sensitization derives from the interference with NER, then we should not see sensitization by glycosylase expression in NER-defective cells. The results in Figures 2 rule out such possibility, at least for the bacterial glycosylase, since both NER+ and NER⁻ cells manifest increased sensitivity to SM treatment by glycosylase expression.

Third, glycosylases may convert SM lesions to repair intermediates that are more toxic than the primary DNA lesion. The unrepaired AP sites represent highly toxic and mutagenic lesions, however, they are also formed by DNA glycosylase during the repair of MMS-modified bases suggesting that the sensitizing step and the lesion responsible for sensitization are unique to SM-modified DNA. It is possible that glycosylase initiates repair and either is unable to dissociate, or AP site processing can not take place, due to the effect of neighboring DNA crosslinks. Alternatively, glycosylase may "unhook" a certain fraction of SM-induced crosslinks generating more toxic intermediates. It is also possible that the mammalian glycosylase can repair SM lesions, but requires additional factors, not present in bacterial cells, for processing.

AAG-deficient Mouse Embryonic Stem Cells

In order to examine whether the sensitization to SM by DNA glycosylase also occurs in mammalian cells we performed survival studies with wild type and 3-alkyl adenine DNA glycosylase (AAG) null mutants of mouse embryonic stem (ES) cells. The results in Figure 4 (left panel) demonstrate protective effect of glycosylase against the methylating agent, MMS, toxicity. However, when cells are exposed to SM (right panel), the presence of glycosylase function in wild type cells (WT, open symbols) has a similar effect as it has in bacterial cells, it increases sensitivity to SM; lack of AAG in null mutant cells (AAG-/-, close symbols) make cells more resistance to SM.

Therefore, the results with ES cells indicate that the sensitizing effect of alkyl adenine DNA glycosylase on SM toxicity is not unique for bacterial cells, but also occurs at least in some undifferentiated mammalian cells. It also suggests that it may be possible to manipulate, diminish or inhibit, glycosylase activity in order to both enhance survival and reduce mutagenic effect after SM exposure.

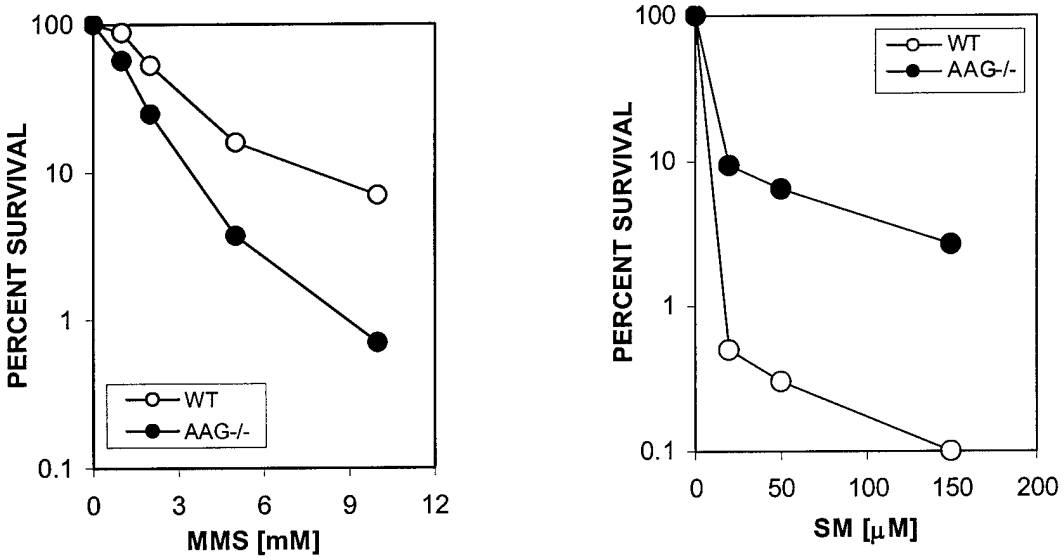

Figure 4. Effect of DNA glycosylase on survival of mouse embryonic stem cells exposed to MMS or SM. Mouse embryonic stem cells, wild type (WT, O) or DNA glycosylase null mutants (AAG-/-, ●) were exposed to MMS (left panel) or to SM (right panel) for 1h and incubated at 37°C for 2 days. Cell viability was determined by trypan blue exclusion assay. While presence of glycosylase protects cells from toxic effects of MMS, it increases their sensitivity to SM.

The Role of Hypothermia in Protection Against SM Toxicity

The Effects on Cell Cycle Progression

In contrast to the heat shock, very little is known at the molecular level about the response of mammalian cells to cold. We have demonstrated (6) that hypothermia represents a p53-inducing stress condition and that lowering temperature to 28°C causes reversible growth arrest in population of normal human fibroblasts. FACS analysis of DNA content per cell demonstrated that this growth arrest is due to the cell cycle arrest that occurs at 28°C (6).

In the present study we used the 5-bromo-2'-deoxiuridine (BrdU) incorporation assay to compare the extent of DNA synthesis in undamaged normal human fibroblasts at 37°C and 28°C. The results in Figure 5 show that the entrance into the S phase is delayed for at least two days when cells are incubated at 28°C. The effect of low temperature on cell cycle in damaged cells is likely to be dependent on a number of factors such as the cell cycle stage at the time of treatment,

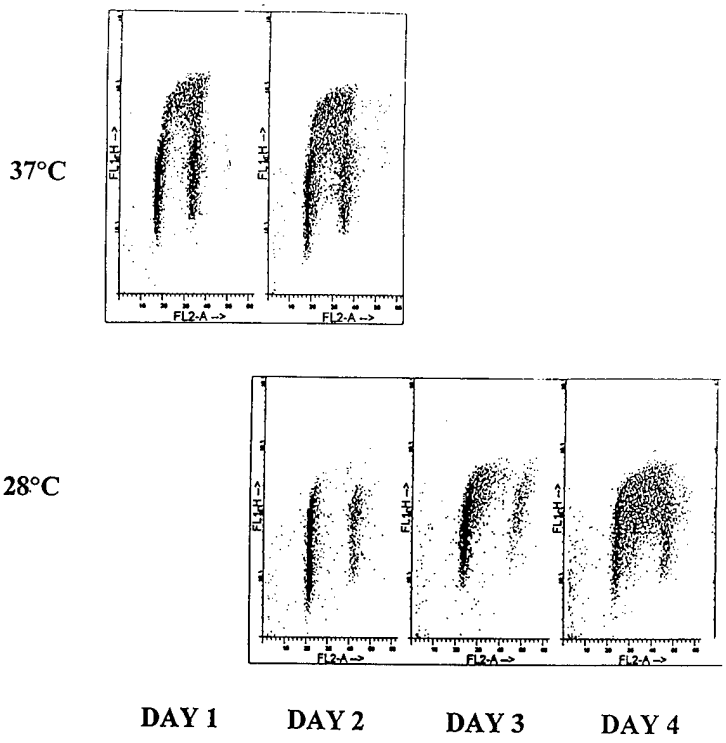

Figure 5. Effect of hypothermia on DNA synthesis in normal human fibroblasts. Cells were incubated at 37°C or 28°C and labeled with BrdU for 2 h. After staining with anti-BrdU-FITC and propidium iodide, cells were analyzed by flow cytometry. The BrdU incorporation is delayed in cells at 28°C for at least two days.

effects of treatment itself on cell cycle progression, and others. Providing low temperature does not compromise protective pathways or intensify toxic ones, the delay in progression is likely to be beneficial for survival by allowing more time for processing of damaged DNA before the onset of DNA synthesis.

The Effects on DNA repair

In order to investigate the role of DNA repair under hypothermic conditions we examined survival of mouse embryonic stem cells, wild type or DNA glycosylase null mutants that were exposed to SM and then incubated at either 37°C or 28°C. The results in Figure 6 (left panel) clearly demonstrate a protective effect of post-exposure incubation at 28°C on cells with functional DNA repair (WT). Increased survival may be the result of either increased (or prolonged) activity of protective pathway(s) or weakened activity of toxic pathway(s). Compared to the wild type, glycosylase-deficient cells (AAG-/-, right panel) survive better at both temperatures and the beneficial effect of low temperature is much less pronounced than in the wild type cells. The results strongly suggest that at least one component of protection from toxicity of SM by hypothermia is due to the modulation of repair pathway initiated by DNA glycosylase.

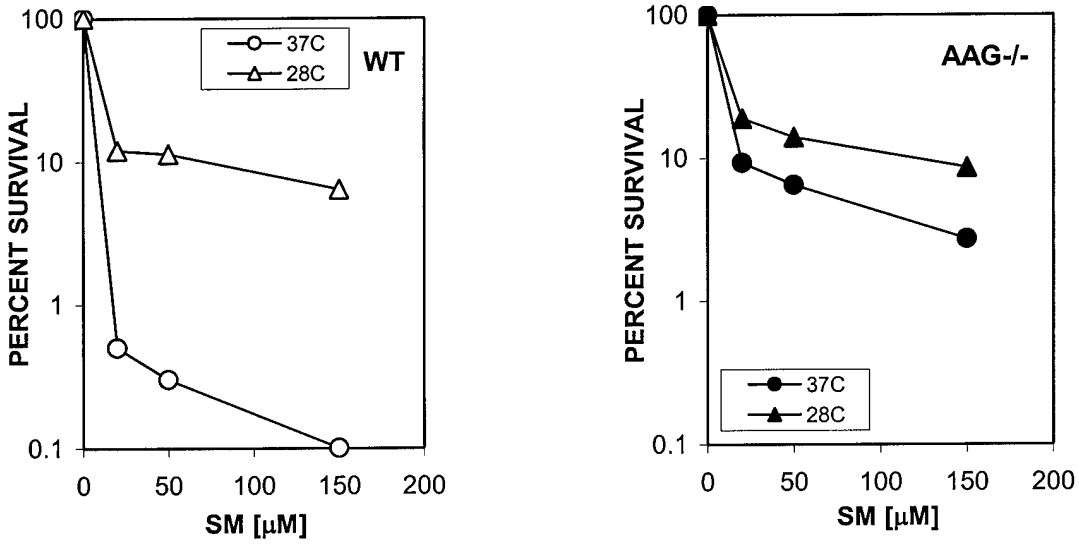

Figure 6. Hypothermia protects mammalian cells from SM toxicity by diminishing sensitizing effect of DNA glycosylase activity. Mouse embryonic stem cells, wild type (left panel, O and △) or DNA glycosylase null mutants (right panel, ● and ▲) were exposed to SM for 1h and then incubated at either 37°C (● and O) or 28°C (▲ and △) for two days. Cell viability was determined by trypan blue exclusion assay.

This conclusion is supported by the results from *in vitro* studies with methylated DNA substrate shown in Figure 7. When ^{3}H-MNU-modified DNA is incubated with cloned human alkyl adenine DNA glycosylase, the release of modified bases by glycosylase is lower at 28°C than at 37°C. It is likely that lowering temperature reduces glycosylase activity *in vivo* as well, and it is possible that it also affects the toxic component of its activity described above.

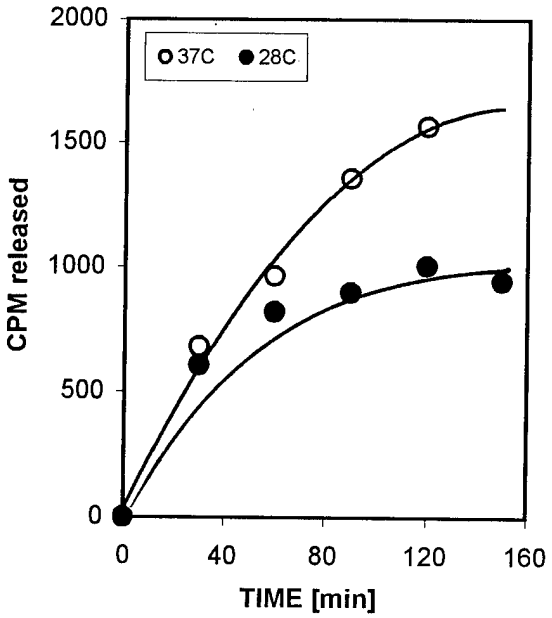

Figure 7. Effect of temperature on human alkyl adenine DNA glycosylase activity. ^{3}H-MNU-DNA was incubated with purified human DNA glycosylase for indicated periods of time at either 37°C (O) or 28°C (●); DNA was then precipitated with ethanol and released radioactivity was measured in supernatant

KEY RESEARCH ACCOMPLISHMENTS

- SM monoadducts processed by nucleotide excision repair (NER) are lethal for mammalian cells if unrepaired.
- NER protects both mammalian and bacterial cells against toxic effects and, at least bacterial cells, against the mutagenic effects of SM.
- In contrast to the protective effect of NER, bacterial alkyl adenine DNA glycosylase, the first enzyme on the base excision repair pathway, increases sensitivity to toxic and mutagenic effects of SM.

- Sensitizing effect of DNA glycosylase occurs, at least in bacterial cells, regardless of their NER status.

- While cloned human DNA glycosylase complements the sensitivity of glycosylase-deficient *E. coli* cells to methylating agent MMS it sensitizes cells to SM toxic effects.

- Sensitizing effect of DNA glycosylase activity is not limited to bacterial cells; mouse embryonic stem (ES) cells with functional DNA glycosylase are more sensitive to SM than the isogenic cells lacking glycosylase activity.

- Hypothermia (28°C) significantly delays the initiation of replicative DNA synthesis in normal human fibroblasts. This delay may provide more time for the repair of damaged DNA before the onset of DNA synthesis.

- There is a more than tenfold increase in survival of mouse embryonic stem cells when they are incubated after SM treatment at 28°C instead at 37°C.

- Protective effect of low temperature derives, at least in part, from the modulation of the toxic effect of glycosylase activity.

REPORTABLE OUTCOMES

Publications Supported by this Contract

Matijasevic, Z., Precopio, M., Snyder, J. E. and. Ludlum, D. B. 2001. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host-cell reactivation assay. Carcinogenesis, *22*: 661-664.

Matijasevic, Z., J. E. Snyder, and D.B.Ludlum. 2000. Hypothermia as a model for selective protection of normal cells from the toxicity of antitumor agents. Proc. Am. Assoc. Cancer Res. 41, 250-251.

Matijasevic, Z., M. Precopio, J. E. Snyder, and D. B. Ludlum. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host-cell reactivation assay. US Army Medical Defense Bioscience Review, 4 June – 9 June, 2000.

Bonnano, K. C., Wryzykowski, J., Chong, W., Matijasevic, Z. and Volkert M.R. Repair of alkylation damage by human alkyl adenine DNA glycosylase expressed in *Escherichia coli*. In preparation for Mutation Res.

Personnel Receiving Contract Support

Zdenka Matijasevic, Ph.D.	Principal Investigator
Michael Volkert, Ph.D.	Co-investigator
Christine Powers	Research Associate

CONCLUSIONS

The two most important conclusions from our studies reported here are that: 1) two independent repair pathways that have opposite effects on survival are involved in processing of SM-damaged DNA, and 2) hypothermia has a beneficial effect on survival which may be due to the modulation of DNA repair activity.

In cells with SM-damaged DNA nucleotide excision repair (NER) is apparently the major protective pathway repairing the most toxic SM lesions, DNA crosslinks. However, we show here that NER activity also extends to the repair of toxic SM monoadducts. Very little is known about the mechanisms of SM crosslink repair and this is a long-term goal beyond the objectives of this proposal.

Interestingly, we have found that the expression of alkyl adenine DNA glycosylase, the first enzyme of another repair pathway, base excision repair (BER), sensitizes cells to SM exposure. This effect is present in bacterial cells expressing either bacterial or cloned human glycosylase and in undifferentiated mammalian cells. Some recent reports suggest that the response to DNA damage in undifferentiated and differentiated cells may not be the same due to the modulation of protein p53 activities. We will address this question in experiments with glycosylase-deficient differentiated mammalian cells that are now available to us, and will investigate the nature of the effect.

Sensitization by DNA repair activity is a new finding that may lead to new post-exposure conditions that protect against SM toxicity. Specifically, it may be possible to manipulate, diminish or inhibit glycosylase activity in order to enhance survival after the exposure to SM.

Hypothermia is the post-exposure condition that we have found is most beneficial for survival of SM-damaged mammalian cells. We are investigating specific conditions for optimal protective effect and the mechanisms involved in this protection. The results reported here suggest that delay in initiation of replicative DNA synthesis and inhibition of the toxic effects of BER may contribute to the protection.

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APPENDICES

Abbreviations

AAG	alkyladenine glycosylase
AP	apurinic/apyrimidinic
BER	base excision repair
CEES	chloroethyl ethyl sulfide
CHO	Chinese hamster ovary
CL	crosslink
FBS	fetal bovine serum
hAPG	human alkylpurine glycosylase
HPLC	high performance liquid chromatography
3HETEA	3-hydroxyethylthioethyl adenine
7HETEG	7-hydroxyethylthioethyl guanine
IPTG	isopropyl β-D-thiogalactopyramoside
MEM,	minimal essential media
MMS	methyl methane sulfonate
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
NER	nucleotide excision repair
NHF	normal human fibroblasts
PCR	polymerase chain reaction
PI	propidium iodide
SM	sulfur mustard
TBE	trypan blue excluding assay
WT	wild type

Journal Article

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Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay

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DNA damage is thought to be the initial event that causes sulfur mustard (SM) toxicity, while the ability of cells to repair this damage is thought to provide a degree of natural protection. To investigate the repair process, we have damaged plasmids containing the firefly luciferase gene with either SM or its monofunctional analog, 2-chloroethyl ethyl sulfide (CEES). Damaged plasmids were transfected into wild-type and nucleotide excision repair (NER) deficient Chinese hamster ovary cells; these cells were also transfected with a second reporter plasmid containing *Renilla* luciferase as an internal control on the efficiency of transfection. Transfected cells were incubated at 37°C for 27 h and then both firefly and *Renilla* luciferase intensities were measured on the same samples with the dual luciferase reporter assay. Bioluminescence in lysates from cells transfected with damaged plasmid, expressed as a percentage of the bioluminescence from cells transfected with undamaged plasmid, is increased by host cell repair activity. The results show that NER-competent cells have a higher reactivation capacity than NER-deficient cells for plasmids damaged by either SM or CEES. Significantly, NER-competent cells are also more resistant to the toxic effects of SM and CEES, indicating that NER is not only proficient in repairing DNA damage caused by either agent but also in decreasing their toxicity. This host cell repair assay can now be used to determine what other cellular mechanisms protect cells from mustard toxicity and under what conditions these mechanisms are most effective.

Introduction

Sulfur mustard, bis-(2-chloroethyl) sulfide (SM), is a bifunctional alkylating agent that has cytotoxic, mutagenic and vesicant properties, and is considered carcinogenic by the IARC (1). Sulfur mustard interacts with cellular DNA to form the cross-link, di-(2-guanin-7-yl-ethyl)-sulfide, and two monoadducts, 7-(2-hydroxyethylthioethyl) guanine (HETEG) and 3-(2-hydroxyethylthioethyl) adenine (HETEA) (reviewed in ref. 2). DNA modification by SM has been shown to interfere with replication and transcription and is probably responsible for its various toxicities (3–5).

Since the resistance of *Escherichia coli* cells to the lethal effect of SM correlates with their ability to remove cross-links, it has generally been assumed that the formation of DNA cross-links is a major cause of SM toxicity (3,6). However, the toxicity and vesicating properties of monofunctional derivatives of sulfur mustard, such as 2-chloroethyl ethyl sulfide (CEES), which generate similar monoadducts but do not form cross-links (2), suggest that monoadducts also contribute to the biological effects of sulfur mustard.

Previously, the cellular repair of SM-damaged DNA has been demonstrated either by measuring the disappearance of alkyl groups from DNA (7,8) or by monitoring the occurrence of non-semiconservative DNA synthesis ('repair synthesis') in cells exposed to sulfur mustard (9). The removal of DNA cross-links specifically has been demonstrated by several investigators in both *E.coli* and mammalian cells (3,8,10–12).

However, the specific cellular repair pathways and enzymes that act on SM-induced DNA adducts have not been completely established. *In vitro* studies have shown that bacterial 3-alkyl adenine DNA glycosylase II releases both of the SM monoadducts, 7HETEG and 3HETEA, from SM-modified DNA indicating that base excision repair may play a role in repairing sulfur mustard lesions (13). The involvement of another repair pathway in eukaryotic organisms, nucleotide excision repair (NER), has been suggested by the study of Kircher *et al.* (14) who have shown that yeast mutants deficient in nucleotide excision repair are much more sensitive to sulfur mustard than wild-type cells.

Although the biochemical studies mentioned above indicate that DNA repair processes act on SM-modified DNA, they do not demonstrate whether or not the damaged DNA has been restored to a functional state. To address this issue, other investigators have used a variety of host cell reactivation assays to demonstrate functional repair of DNA after damage by antitumor agents (15–18). In this manuscript, we describe the use of a dual luciferase reporter assay to demonstrate cellular repair of mustard-induced DNA damage.

We have first established that NER-competent Chinese hamster ovary cells are more able to withstand the toxic effects of SM than are NER-deficient cells. Then, using the dual luciferase host cell reactivation assay, we have shown that NER-competent cells are able to repair SM-damaged reporter plasmid and bring luciferase expression from the plasmid to higher levels than in NER-deficient cells, thus correlating DNA repair with SM toxicity.

We have also used the host cell reactivation assay to examine cellular repair of the damage caused by the single armed mustard, CEES. As shown in Figure 1, CEES forms monoadducts that are very similar to those formed by SM, thus allowing the effects of monoadducts on survival and repair to be examined independently from the effects of cross-links. These studies have shown that the toxicity of CEES is also decreased in NER-competent cells in comparison with NER-deficient cells. Luciferase expression from plasmid damaged by CEES is enhanced in NER-competent cells compared with NER-deficient cells, indicating that the monofunctional mustard adducts formed by CEES are cytotoxic and are also substrates for NER. From these results, we conclude that this

Abbreviations: CEES, 2-chloroethyl ethyl sulfide; HETEA, 3-(2-hydroxyethylthioethyl) adenine; HETEG, 7-(2-hydroxyethylthioethyl) guanine; NER, nucleotide excision repair; SM, sulfur mustard, bis-(2-chloroethyl) sulfide.

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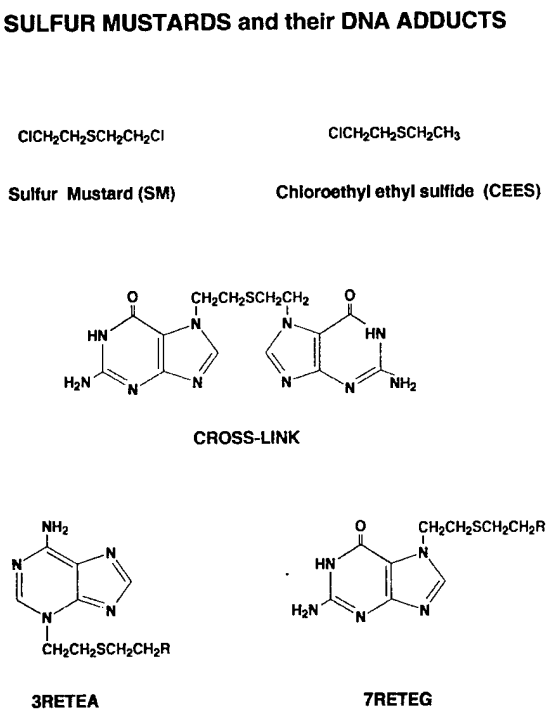

Fig. 1. Structures of SM and CEES and the adducts they form with DNA. Sulfur mustard forms the indicated cross-link and two monoadducts with R = OH; CEES forms only monoadducts with R = H.

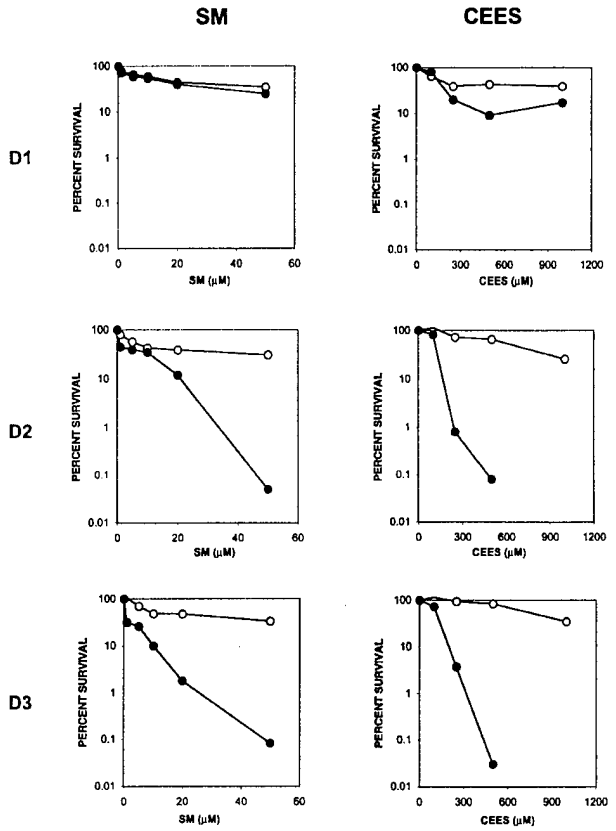

Fig 2. Survival of CHO cells after exposure to SM or CEES. Viable cell numbers were determined by trypan blue exclusion and are expressed as a percent of control on day 1 (D1), day 2 (D2) and day 3 (D3). Wild-type cells (○); NER-deficient cells (●).

relatively simple host cell reactivation assay can be used to determine what repair mechanisms restore mustard-damaged DNA to a functional state and under what conditions they are most effective.

Materials and methods

Materials

Bis-(2-chloroethyl)sulfide (SM) was supplied by the US Army Institute of Chemical Defense (Aberdeen Proving Ground, MD) and 2-chloroethyl ethyl sulfide (CEES) was purchased from Aldrich (Milwaukee, WI). The luciferase reporter vectors pGL3-Control (containing the firefly luciferase gene) and pRL-TK (containing the *Renilla* luciferase gene), the transfection reagent TransFast and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Plasmid vectors were propagated in *Escherichia coli* strain JM109 and plasmid DNAs were purified by using the EndoFree Plasmid Mega kit (Qiagen, Chatsworth, CA). Chinese hamster ovary (CHO) cell lines AA8 (wild type) and UV41 (NER group 4) were obtained from the American Type Culture Collection. These cells were grown at 37°C in monolayer culture in α-modified minimum essential medium supplemented with 10% fetal bovine serum and antibiotics.

Sulfur mustard exposure and cytotoxicity determinations

Cells were plated in 12-well plates at a density of 2×10^4 cells/cm^2. After 24 h, the medium was replaced with fresh medium containing the indicated concentrations of SM or CEES; dilute solutions of these compounds in absolute alcohol were prepared immediately before treatment. Cells were exposed to SM or CEES for 1 h at room temperature in a SterilchemGard hood and then incubated in fresh medium at 37°C. At the indicated times, cell viability was determined by the trypan blue exclusion assay.

Alkylation of plasmid DNA

Purified pGL3 DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) at a concentration of 1 μg/μl and incubated with SM or CEES in a SterilchemGard hood at room temperature for 1 h. DNA was precipitated with ethanol, dissolved in TE buffer and stored at –20°C until used for transfection. Aliquots were analyzed by gel electrophoresis on 1% agarose gel. The percent conversion of supercoiled DNA (Form I) into the nicked circular form (Form II) after exposure to SM or CEES was determined densitometrically from a UV photograph of the ethidium bromide-stained agarose gel.

Transfection conditions

For transfection experiments, cells were plated in 24-well plates at a density of 2×10^4 cells/cm^2 and incubated for 24 h at 37°C. Transfection was performed using the liposome-based transfection reagent, TransFast. Optimal conditions for transfection were established as 0.5 μg plasmid DNA per well, at a charge ratio of transfection reagent to DNA of 1:1, and a ratio of pGL3 to pRL-TK of 10:1. Twenty-four hours after plating, the growth medium was replaced with 0.2 ml serum-free medium containing the transfection mixture. After the cells were incubated for 1.5 h at 37°C, the transfection reagent was diluted by the addition of 1 ml complete medium and cells were further incubated for luciferase expression.

Preliminary experiments with undamaged pGL3 and pRL-TK plasmids have shown that the difference in genetic background between the AA8 and UV41 cells does not affect the kinetics of expression of either firefly or *Renilla* luciferase. The level of expression of both luciferases increases during the first 25 h of incubation at 37°C and then reaches a plateau. Therefore in repair experiments, cells were incubated for 27 h after transfection to allow a maximal level of luciferase expression. At that time, cells were approximately 90% confluent.

Cell lysis and assay for luciferase activity

Cells were lysed in multi-well plates with 150 μl/well passive lysis buffer provided with the Dual Luciferase Reporter Assay kit; lysates were stored frozen at –20°C until they were assayed. The assays for firefly luciferase activity and *Renilla* luciferase activity were performed sequentially in one reaction tube using 20 μl aliquots of cell lysates. The supplier's standard protocol for the dual luciferase assay was followed. The luminescent signal from the luciferase reaction was monitored by a single-sample luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA) with spectral sensitivity over the range 360–620 nm. The values shown are means ± SD from three to five separate assays.

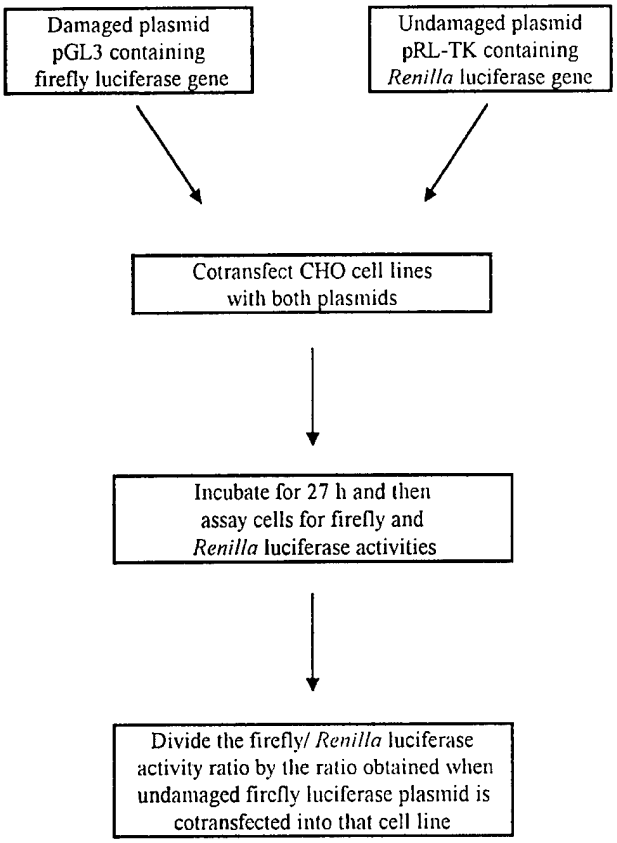

Fig 3. Scheme for monitoring DNA repair using damaged luciferase reporter gene in CHO cells. The level of firefly luciferase activity relative to *Renilla* luciferase activity is a measure of how efficiently the damaged firefly luciferase gene has been repaired.

Results

The data in Figure 2 show that CHO cells exposed to either SM or CEES are protected from cytotoxicity by the NER mechanism. Referring to data on day 3 (D3), >50% of NER-competent cells survive exposure to 20 μM SM while fewer than 2% of the NER-deficient cells survive. A similar difference is noted after exposure to CEES except that concentrations of CEES >10-fold greater than those of SM are required to produce the same level of cytotoxicity. Again at day 3, survival for wild-type cells is close to 100% after exposure to 300 μM CEES, but only ~5% for NER-deficient cells.

Based on the assumption that unrepaired DNA damage is responsible for SM cytotoxicity, we would expect that the increased survival shown in Figure 2 would be accompanied by an increase in cellular repair of DNA. This has been confirmed by measuring cellular DNA repair directly with the host cell repair assay as described below. In this assay, a plasmid that contains a gene for firefly luciferase is damaged by SM or CEES and then transfected into the cells that are to be evaluated for repair. Cells that can repair the DNA damage will express the luciferase gene at a higher level (i.e. the cells will 'reactivate' the gene). This results in an increased intensity of firefly luciferase bioluminescence that can be used to determine the extent of repair.

To compensate for variations in the efficiency of transfection and other experimental variables, luciferase reporter gene assays are usually run using dual transfection with DNA from

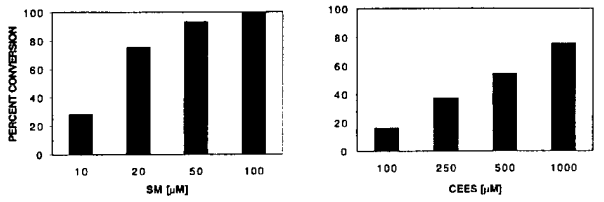

Fig 4. DNA damage caused by SM and CEES. The percent conversion of the firefly luciferase plasmid to Form II is plotted *versus* the concentration of mustard to which it was exposed.

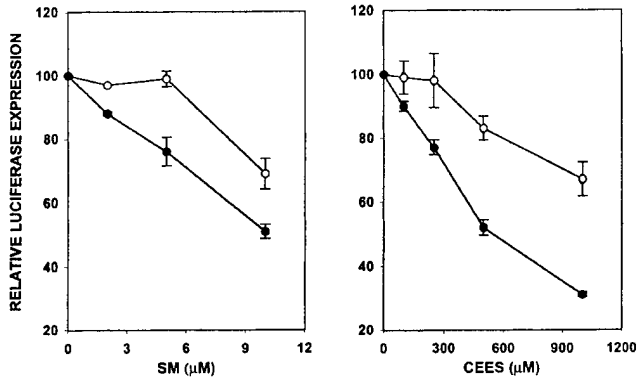

Fig 5. Relative luciferase expression in wild type (○) and NER-deficient cells (●); values are means ± SD from three to five separate assays. See text for details.

two plasmids, one containing the damaged firefly luciferase gene and the other (pRL) containing an undamaged *Renilla* luciferase gene. The intensity of firefly luciferase enzyme activity can then be compared with the level of *Renilla* luciferase activity as a control. This protocol is shown in Figure 3.

The firefly luciferase gene was damaged as described in the Materials and methods by exposing plasmid pGL3 to either SM or CEES *in vitro*. Damaged plasmid was then recovered and used in the host cell reactivation assays as shown in Figure 3.

Damage to plasmid DNA can be detected by measuring the conversion of the supercoiled form of the plasmid (Form I) to the nicked circular form (Form II). We assume that the extent of DNA damage that causes this conversion parallels the DNA damage that interferes with expression of the luciferase gene. Accordingly, we have measured conversion of the plasmid to Form II as an indication of the DNA damage caused to the firefly luciferase gene by SM and CEES. The level of conversion of Form I to Form II is shown in Figure 4. As expected, both SM and CEES convert Form I to Form II, but it takes an ~10-fold higher concentration of CEES to cause as much damage as is caused by a given concentration of SM.

The host cell reactivation data in Figure 5 show the extent to which this damage was repaired in the two different cell lines. In these experiments, plasmid containing damaged firefly luciferase gene was transfected separately into either wild-type or NER-deficient CHO cells. As shown in Figure 5, wild-type cells can return luciferase expression to normal if the plasmids are damaged with low concentrations of SM or CEES. In contrast, NER-deficient cells show much less repair, and levels of firefly luciferase expression fall off as the concentrations of SM or CEES are raised.

It is possible that some of the damage to the luciferase gene

could be repaired by mechanisms other than NER in the NER-deficient cells, but the lack of a shoulder on the curve for NER-deficient cells at low concentrations of damaging agent suggests that NER is needed to restore full expression. In any case, the data in Figure 5 indicate that the host cell reactivation assay can be used to examine repair of SM- or CEES-induced DNA damage within the cell. It is also apparent from the right hand panel of Figure 5 that NER removes the monofunctional adducts caused by CEES and, by analogy, the monofunctional adducts caused by SM as well.

Discussion

The survival curves in Figure 2 show that the difunctional agent SM is ~10-fold more cytotoxic than CEES for both cell lines, in agreement with previous observations (2). Since CEES forms monofunctional adducts that are similar to those formed by SM, this difference in cytotoxicity between SM and CEES has been assumed to be the result of cross-link formation (2). The data (Figure 2, left panel) show that NER competent cells are more resistant to the cytotoxic action of SM than are NER deficient cells suggesting that the cytotoxic cross-link is removed by the NER repair mechanism. Again, this would agree with previous observations that the NER pathway recognizes bulky adducts like DNA cross-links.

Since the data (Figure 2, right panel) show that NER competent cells are also more resistant to the cytotoxic action of CEES, we can conclude that NER repairs the monoadducts formed by CEES and, presumably, the similar monoadducts formed by SM as well. These adducts, as well as the cross-link, would probably be classified as bulky because of the size of their adducted groups.

Host cell reactivation data provide direct information on cellular repair of DNA damage, and the data in Figure 5 support the role of the NER mechanism in protecting cells from mustard toxicity since firefly luciferase gene damaged by either SM or CEES is restored to a higher level of expression in the NER-competent CHO cells. This reinforces the belief that DNA repair mechanisms protect against SM toxicity.

Thus the host cell reactivation assay described in this manuscript demonstrates that there is a direct relationship between resistance to SM and CEES toxicity and the cellular repair of DNA damage caused by these agents. This not only validates the hypothesis that DNA damage is the root cause of mustard toxicity, but provides a method of testing environmental conditions such as hypothermia that may increase the extent of DNA repair.

Acknowledgements

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