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CYTOMETRIC ANALYSIS OF DNA CHANGES
INDUCED BY SULFUR MUSTARD

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AND C.L. GROSS

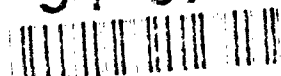
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ABSTRACT

Sulfur mustard is an alkylating agent which causes severe, potentially debilitating blisters following cutaneous exposure. Its mechanism of pathogenesis is unknown and no antidote exists to prevent its pathology. The biochemical basis of sulfur mustard's vesicating activity has been hypothesized to be a cascade of events beginning with alkylation of DNA.

Using human cells in culture, we have assessed the effects of sulfur mustard on cell cycle activity using flow cytometry with propidium iodide. Two distinct patterns emerged, a G1/S interface block at concentrations equivalent to vesicating doses ($>50\mu\text{M}$) and a G2 block at 10-fold lower concentrations. In addition, noticeable increases in amount of dye uptake were observed at 4 and 24 hours after sulfur mustard exposure. These increases are believed to be related to DNA repair activities and can be prevented by treatment of the cells with niacinamide, which inhibits DNA repair. Other drugs which provide alternate alkylating sites or inhibit cell cycle progression were shown to lower the cytotoxicity of sulfur mustard and to protect against its direct DNA damaging effects.

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INTRODUCTION

Sulfur mustard (HD) is an alkylating agent which has been shown to have mutagenic (1,2), cytotoxic(3), and vesicating (4) properties. Its use in combat situations has resulted in lethal, incapacitating, and disfiguring injuries. The principal incapacitating injuries following cutaneous exposure to HD come from its vesicating capacity, i.e., production of skin blisters.

Despite decades of medical research, the mechanism by which HD induces vesication is not known and no effective antidotes are currently available. In 1985, Papirmeister *et al.* (5) proposed a hypothesis delineating the following events for the generation of HD-induced pathology: HD alkylates DNA, DNA strand breaks occur, poly(ADP-ribose)polymerase is activated and utilizes cellular NAD⁺ as substrate, NAD⁺ is depleted, glycolysis is inhibited, hexose monophosphate shunt is activated, proteases are released, and pathology results.

Previous studies from our laboratories have established that human peripheral blood lymphocytes (PBL) and human epidermal keratinocytes (HEK) in culture could serve as useful models for biochemical investigations into the toxicity of HD. (3,6,7,8) The results of these investigations suggested that resting PBL and actively proliferating HEK demonstrated somewhat different response to HD in both cytotoxicity and depletion of cellular NAD⁺. Since the dividing basal epidermal cell in skin appears to be the major target of HD, proliferating epithelial cells were used as *in vitro* models of HD-induced skin injury to determine the direct effects of HD on cell cycle activity. The studies in this report utilize flow cytometric analyses of PBL, HeLa, and HEK for the toxicologic assessment of HD-induced DNA damage. Using cytometric analysis, levels of human cell cytotoxic and genotoxic sensitivity to HD could be estimated. These results should provide data to aid in the development of therapeutic intervention in the human pathology produced by HD.

MATERIALS AND METHODS

Reagents:

The lymphocyte studies used RPMI 1640 containing gentamicin (50 µg/ml) for cell washing and RPMI 1640 supplemented with 5% fetal bovine serum for final cell suspension and assay (RPMI-FBS). All tissue culture reagents were from Sigma, St. Louis, MO. Buoyant density isolations were performed using histopaque, d=1.077 (Sigma, St. Louis, MO).

Epithelial cell studies used HEK, Keratinocyte Growth Medium (KGM), trypsin-EDTA, and trypsin neutralizing solution from Clonetics Corporation (San Diego, CA). The HeLa cell line (American Type Culture Collection, MD) was grown in RPMI-FBS.

HD (2,2'-dichlorodiethyl sulfide) was obtained from the Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD. The following chemicals were purchased from commercial sources: RNase A from Boehringer Mannheim (Indianapolis,

IN), propidium iodide (PI), dextran T-500, N-acetyl-L-cysteine (NAC) and mimosine from Sigma (St. Louis, MO).

PI was dissolved in RPMI 1640 at 20 μ g/ml for use in viability studies and at 250 μ g/ml in 5% triton X-100 for DNA staining of PBL. RNase A (1 mg/ml) was added to the DNA staining solution when needed (9). DNA studies in epithelial cells used the trypsin-detergent method described by Vindelov *et al.* (10).

Lymphocyte Isolation:

Human PBL were isolated from whole blood obtained by venipuncture from volunteers under an approved human use protocol. One ml of 6% dextran was added per 10 ml of blood to expedite red blood cell sedimentation. After allowing sedimentation for 30 minutes at 37°C, the white cell rich supernate was collected, layered onto histopaque (d=1.077), and centrifuged at 450 X g for 20 minutes. The mononuclear cell layer was harvested, washed twice with 15 ml of RPMI, and centrifuged at 250 X g for 10 minutes. The isolated PBL were resuspended in 5 ml of RPMI-FBS for cell counting, and initial cell viability determinations were performed by trypan blue dye exclusion.

Epithelial Cell Culture System:

Both HEK and HeLa cultures were maintained in 75 cm² flasks in medium at 37°C in 5% CO₂. When the cultures became 80% to 90% confluent, cells were removed with 0.05% trypsin-0.53mM EDTA. After removal, the cells were placed in a conical tube containing trypsin-neutralizing solution, and centrifuged at 250 X g for 10 minutes. Cells were resuspended in 5 ml of medium for determination of cell number and viability.

Microplate Preparation:

PBL were resuspended in RPMI-FBS at 4 X 10⁶ cells/ml, and 50 μ l/well dispensed into 96-well microtest plates (Falcon, Oxnard, CA) to yield a final concentration of 2 X 10⁵ cells/well. RPMI-FBS and HD were added to the wells to yield a final volume of 200 μ l/well. The plates were vented in a fume hood at room temperature for 1 hour to exhaust volatile agent. They were then incubated at 37°C for 0-72 hours in 5% CO₂ after which time they were prepared for flow cytometric analysis.

HEK or HeLa (1 X 10⁵ cells/well) were placed in the wells of a 24-well plate in a total volume of 1 ml. The plates were incubated for 24 hours. The media was discarded, and fresh media containing HD was added to a final volume of 1 ml. The plates were vented and incubated for 24-48 hours.

Flow Cytometry:

PBL. Propidium iodide in RPMI was added to each well to yield a final concentration of $6 \mu\text{g}/10^5$ cells. After 3 minutes at room temperature, the plates were placed on a Multiple Sample Delivery System (MSDS, Coulter Electronic, Hialeah, FL) attached to an EPICS C flow cytometer (Coulter) for viability analysis. The flow cytometer operates with a 5 watt argon laser generating a 488 nm line at 200 mW. The MSDS permits automatic sampling of all wells of the 96-well plate.

Immediately after conclusion of the viability analysis, $50 \mu\text{l}$ of PI in triton X-100 was added to each well, and the plate was returned to the MSDS for cytometric analysis of DNA.

Data analysis was performed using the EASY 2 computer system (Coulter) with cell viability analyzed using the Intgra program (Coulter) and DNA histogram analysis by the Para1 program (Coulter).

Epithelial cells. To determine HEK viability by PI, the supernatant media containing the floating dead cells were collected in 12 x 75 mm plastic tubes. Cells were trypsinized from the wells, pooled in the respective tubes containing supernatant cells and 1 ml trypsin neutralizing solution, and centrifuged at $250 \times g$ for 10 minutes. The pellet was resuspended in 1 ml of medium and PI ($1 \mu\text{g}$) was added. After 3 minutes at room temperature, viability determinations were performed on the flow cytometer. Immediately after the viability determinations, DNA cell cycle analysis was conducted following preparation of the DNA by the trypsin-detergent PI staining procedure of Vindelov *et al.* (10). Data analysis was performed as described for PBL except that cell cycle analysis was conducted using the Multicycle program (Phoenix Flow Systems, San Diego, CA).

Therapeutic Drug Treatment:

N-acetyl-L-cysteine (NAC) was added to the cell cultures at a final concentration of 10 mM. The cultures were incubated at 37°C for 24 hours. The media containing NAC was removed and fresh media without NAC but with HD was added. Following 24 hour incubation at 37°C , the cells were prepared for flow cytometric analysis as described above.

Mimosine ($300 \mu\text{M}$) was incubated with HeLa cells for 2 hours, the cells were exposed to HD, and then incubated in mimosine containing medium for an additional 1, 3, 5, or 21 hours at which times the mimosine containing medium was replaced with fresh medium without mimosine. The cells were then incubated at 37° to complete a full 24 hours post-HD exposure. The viability of the cells was measured by PI uptake.

RESULTS

DNA analysis by flow cytometry using propidium iodide (PI) allows for graphical and statistical demonstration of cell cycle progression of the cell population under study. We have previously demonstrated (7,8) that vesicating doses of HD ($>50\mu\text{M}$) caused a blockage of cell cycle progression at the G₁/S interface in dividing PBL, HEK, and HeLa. Other reports have suggested that alkylating agents cause a G₂ blockage (reviewed in 11). A dose response over the HD concentration range of 1 to $500\mu\text{M}$ was done in HeLa cells and two distinct patterns emerged (Figure 1). As previously reported, a G₁/S interface block was seen at concentrations equivalent to vesicating doses ($100\mu\text{M}$) (Figure 1C). At lower concentrations of HD ($1-25\mu\text{M}$) the cell cycle blockage was seen at the G₂/M interface (Figure 1B). Identical results were seen using HEK (Data not shown).

Besides being an indicator of cell cycle progression, PI stained cellular DNA has been shown to exhibit changes in dye uptake following monofunctional alkylation (7). These increased amounts of dye uptake may indicate repair processes producing more PI intercalation sites. When the position of the PI stained G₁ peaks following HD exposure of human PBL were compared to that of control cells, a dose dependent increase in dye uptake was seen at 4 hours with a greater increase seen at 24 hours (Figure 2). When the amount of PI staining was tested at 48 hours after HD exposure, the staining was much less than control cells, suggesting that the DNA had been fragmented or became condensed and excluded PI intercalation. The increases in PI uptake seen at 4 and 24 hours can be prevented by treatment of cells with 1mM niacinamide, which inhibits DNA repair (Data not shown). Similar patterns of dye uptake following HD exposure were seen with both HEK and HeLa (Data not shown).

Drugs which provide alternate alkylating sites or inhibit cell cycle progression should provide a therapeutic benefit following HD exposure. Two candidate compounds, N-acetyl cysteine (NAC) and mimosine, were tested for their potential therapeutic effects. NAC, a prodrug of glutathione, has been shown to provide a level of protection against HD-induced cytotoxicity in PBL (12). Incubation of PBL in 10mM NAC solution for 24 hours before HD exposure blocked the increased dye uptake at 50 and $100\mu\text{M}$ HD (Figure 3) but had no effect against $500\mu\text{M}$.

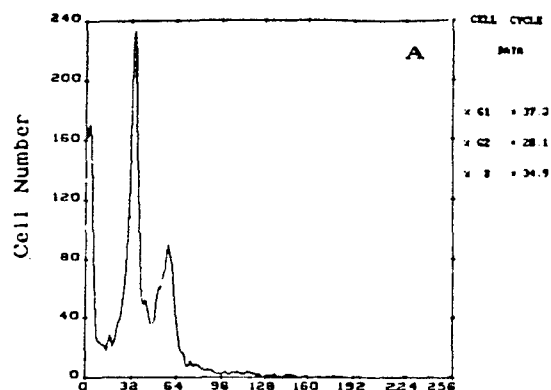
Mimosine ($300\mu\text{M}$), a reversible G₁ inhibitor of the cell cycle (13), provided a slight protection (13-17%) against $500\mu\text{M}$ HD cytotoxicity when exposed HeLa cells were held in G₁ by mimosine for 3, 5 or 21 hours after the HD exposure (Figure 4). The same level of protection was observed with mimosine treated HEK (Data not shown).

DISCUSSION

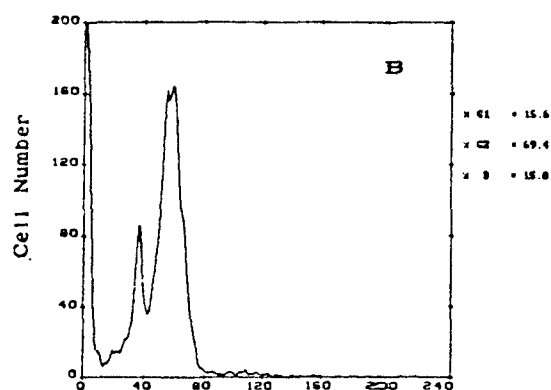
The demonstration that different concentrations of HD affect different areas of the cell cycle provides several important

Figure 1. Flow cytometric DNA histogram using propidium iodide obtained 24 hours after exposure.

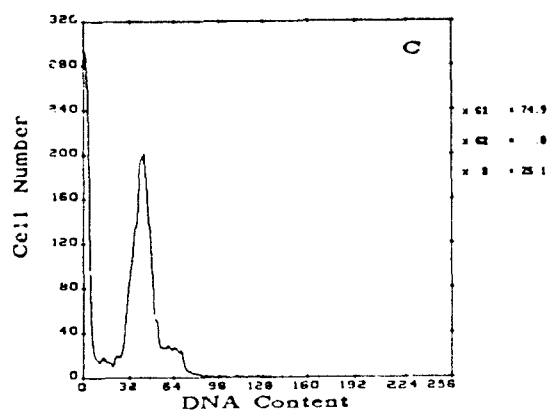
a) Control HeLa cells exhibiting G1 and G2 peaks with relatively even distribution of population throughout the cell cycle.



b). HeLa cells exposed to 10 μ M HD exhibiting a G2/M blockage and build up of cells in the G2 phase.

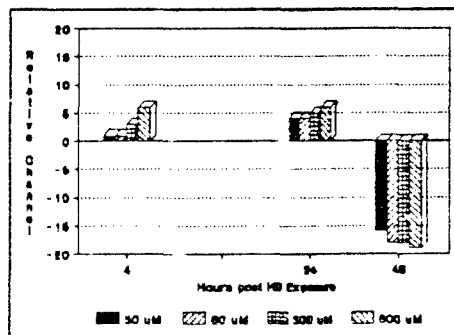


c). HeLa cells exposed to 100 μ M HD exhibiting a G1/S blockage with build up of cells in the G1 phase.



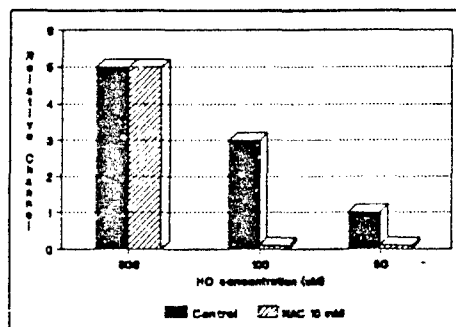
considerations for therapy of the HD injury. At vesicating equivalent doses ($100\mu\text{M}$), the blockage occurs at the G1/S interface. At 10-fold lower doses, the blockage is in the G2/M region. This difference is probably related to the level of monofunctional adducts and crosslinks present at high versus low

Figure 2. Time course of propidium iodide uptake by DNA from human PBL exposed to HD. A positive fluorescence channel shift compared to controls indicates increased dye uptake as seen at 4 and 24 hours post-HD. A negative shift indicates reduced dye uptake as seen at 48 hours. Similar results were seen with both HEK and HeLa cells (Data not shown). Results given as mean and S.E.M. for triplicate samples from a representative experiment repeated four times.



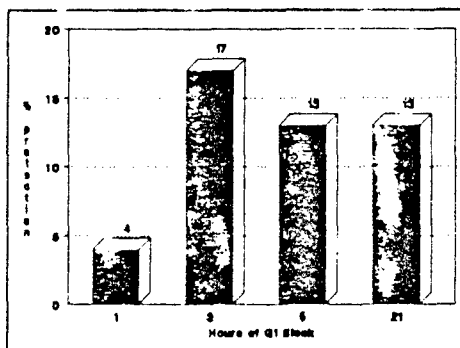
doses. With many adducts and crosslinks present in the DNA, the overall chromatin conformation would be seriously disrupted, thereby interfering with DNA polymerase activities. At lower levels of damage, the DNA could undergo synthesis but post-replication packaging of the chromatin would be inhibited (14). Preliminary studies in our laboratory suggest that the G2 blockage can be overcome, presumably by endogenous DNA repair processes, and the cells can resume normal cycling by 144 hours. Cells blocked in the G1/S phase do not recover and the DNA fragments by 72 hours. These preliminary observations are currently under study and will be the subject of a future report.

Figure 3. Propidium iodide staining of human PBL DNA following HD exposure in the presence or absence of N-acetyl cysteine (NAC, 10mM). Cells pretreated with NAC fail to show the increased dye uptake caused by 50 and $100\mu\text{M}$ HD. Results given as mean and S.E.M. of triplicate samples from a representative experiment repeated four times.



The data presented showing increased dye uptake suggest that DNA repair processes are activated within 4 hours and continue through the first 24 hours post-HD exposure. If no intervention is accomplished, the DNA is destroyed by 48 hours. This suggests that a window of therapeutic intervention that was not formerly appreciated may exist for enhancing DNA repair after alkylation. We had previously thought that once the adducts formed within the DNA, the DNA was doomed for fragmentation. We have not yet confirmed that this increased dye uptake is related to DNA repair processes, but this assumption is supported by the observation that niacinamide, which is known to inhibit poly(ADP-ribose) polymerase and subsequently DNA repair, inhibits this phenomenon.

Figure 4. Mimosine ($300\mu\text{M}$) protection of HeLa cells exposed to $500\mu\text{M}$ HD. Cells were incubated with mimosine for 2 hours prior to HD exposure then incubated with mimosine for 1, 3, 5 or 21 hours before medium was replaced with fresh medium without mimosine. At 24 hours after HD exposure the cells were harvested and viabilities determined using propidium iodide as a vital fluorescent stain. Results given as mean and S.E.M. of triplicate samples from a representative experiment repeated three times.



Both the cell cycle blockage data and the increased dye uptake data suggest that dose reduction of the HD damage does provide a therapeutic benefit. One of the likely intracellular scavengers capable of dose reducing the intracellular concentration of a potent alkylating agent such as HD would be glutathione. This principle was enunciated by Gross *et al.* (12) and they showed that N-acetyl cysteine (NAC) could provide a limited degree of protection against the cytotoxicity of HD. Our data, showing the reduction of HD-induced increases in PI uptake in cells pretreated with NAC, support the concept that the protection by NAC is due to lowering the damage at the level of the DNA.

Since the damage caused by alkylation with HD consists of the addition of an ethylthioethyl adduct to purines, it is conceivable that holding the cells in the G1 phase of the cell cycle might allow time for the endogenous repair capacity of the cells to remove the affected nucleotides and repair the damaged DNA. This concept was shown to be practical by Chen *et al.* (15) using benzopyrene. Mimosine, a compound which inhibits molecules involved in generation of the START signal responsible for moving cells from G1 to S (16), provides a limited amount of protection against the cytotoxicity of HD. While the protection was low (<20%), it is

important to note that the protection was against a very high concentration of HD (500 μ M) and was not accompanied by any other therapeutic support.

The exact mechanism of HD-induced vesication is unknown and currently there are several theories to account for potential biochemical pathways to pathogenesis (reviewed in 11). The most clearly presented has been that of Papirmeister *et al.* (5), which has DNA damage as the initial cause of vesication. This has recently been challenged by Mol and deVries-VanderRuit (17) who show that inhibition of DNA synthesis occurs at vesicating and non-vesicating doses while inhibition of protein synthesis and metabolic activities are unique features at vesicating concentrations of HD. Our data, that the extent of DNA damage may differ at vesicating and non-vesicating concentration of HD, suggest that blockage of DNA synthesis may not be as important as the nature of the blockage. Furthermore, at concentrations of HD with concomitant production of high levels of adducts and crosslinks, G1/S blockage, early and pronounced activation of poly(ADP-ribose) polymerase (18) and other DNA damage related activities may combine to set in motion biochemical cascades that culminate in blister formation.

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