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We are investigating the hypothesis that modalities which cause a reversible cell cycle arrest will decrease sulfur mustard toxicity by allowing more time for DNA repair before cell division takes place.

Both human fibroblasts and NM-1 keratinocytes have been exposed to hypothermic conditions (28°C). This temperature arrests the growth of both cell lines, but they recover rapidly on transfer back to 37°C. Similarly, exposure to 8 μM ciclopirox olamine produces a readily reversible cell cycle arrest. Experiments are currently in progress to determine the efficacy of these treatments in reducing sulfur mustard cytotoxicity.

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We have shown that both bacterial and partially purified human glycosylases release 7-hydroxyethylthioethylguanine from sulfur mustard-modified DNA. This repair enzyme is not inhibited by either hypothermic conditions or exposure to ciclopirox olamine, providing support for the hypothesis that DNA repair will continue during a reversible cell cycle arrest.

At the same time, we have developed a ^{32}P-postlabeling method which can detect one 7-hydroxyethylthioethylguanine adduct in 10^7 DNA nucleotides. This success has been achieved by performing the digestion step at 10°C, by including butyldeoxyguanosine 3'-phosphate from the beginning of the analysis as an internal standard, and by using disposable anion columns in early separation steps. The sensitivity which has been achieved is more than adequate for measuring the levels of DNA damage encountered at minimally cytotoxic concentrations of sulfur mustard and can be used to test DNA repair during cell cycle arrest.

FOREWORD

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David B Ludlum 4 Jun 94

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INTRODUCTION

Background

Sulfur mustard (SM) exposure can result in serious injury to the skin, respiratory tract, and other mucous membranes. It is generally believed that the initiating event which causes this toxicity is DNA alkylation. This damage can interfere with cellular function or, if it is extensive enough, lead to cell death (1). No effective antidote for SM toxicity is yet available.

Perhaps surprisingly, DNA damaging agents are also found in nature. An important cellular metabolite, S-adenosylmethionine, is actually a one-armed sulfur mustard which can alkylate DNA (2,3). Consequently, cells have developed some defenses against the kinds of DNA damage inflicted by SM and other alkylating agents. A major objective of our work is to enhance these natural defenses.

Overall, we are investigating the hypotheses that (a) DNA damage is the initiating event in sulfur mustard toxicity, (b) repair of this damage by cellular enzymes can reduce cytotoxicity, and (c) alteration of the cell cycle by physical or chemical means may allow increased repair and lead to decreased cytotoxicity.

In this Introduction, we review briefly what is known about DNA damage by SM and consider the repair of this damage by cellular enzymes. Methods of slowing cell division in order to allow further repair will also be considered. The bulk of this Report will summarize our progress in the three areas mentioned above.

Sulfur Mustard-induced DNA Modifications

The modified DNA bases which are produced by SM exposure have been reviewed recently by Papirmeister *et al.* (1). As reported originally by Brooks and Lawley (4), the principal product is 7-hydroxyethylthioethyl guanine (HETEG), which accounts for approximately 60% of the total DNA alkylation. This finding has been confirmed on numerous occasions, most recently by Benschop *et al.* (5) and by our laboratory (6). Smaller amounts of 3-hydroxyethylthioethyl adenine and di-(2-guanin-7-yl-ethyl)sulfide also have been found consistently.

In addition to these major products, several minor products including O^6-hydroxyethylthioethylguanine have been found in the reaction of chloroethyl ethyl sulfide with DNA (1,7,8). Similar minor products are probably formed by SM as well, and these could be more important than HETEG in causing SM toxicity since the biological significance of a particular DNA modification depends on other factors besides its prevalence. However, for the purposes of evaluating exposure, we have focused on the most abundant DNA modification.

Clearly, if the level of damage is measured accurately, methods of protecting cells or minimizing the consequences of exposure can be evaluated more precisely. The successful application of the ^{32}P-postlabeling technique to the problem of measuring low levels of DNA damage by SM is described herein.

Repair of SM-induced DNA Modifications

Since alkylating agents occur in nature, it is not surprising that cells have developed defenses against such agents. Intracellular material, particularly glutathione, can combine with alkylating agents and prevent their reactions with DNA. Once reaction with DNA has occurred, however, the cell still has the ability to repair this damage.

Papirmeister *et al.* (9) found that bacterial extracts catalyzed the release of 3-substituted adenines produced in DNA by 2-chloroethyl-2-hydroxyethyl sulfide (hemisulfur mustard) and attributed this release to the presence of a glycosylase. Glycosylase action results in the formation of apurinic sites which are subject to endonuclease action and subsequent repair. Although the extracts tested by Papirmeister *et al.* (9) were inactive in releasing the 7-substituted guanine formed by hemisulfur mustard, Habraken *et al.* (10) showed that bacterial 3-methyladenine DNA glycosylase II releases two monoadducts formed by chloroethylethyl sulfide (CEES), 3-hydroxyethylthioethyl adenine and 7-hydroxyethylthioethyl guanine. In fact, activity towards these bases was comparable to the activity towards methylated bases.

Mammalian and, in particular, human enzymes have not yet been tested towards sulfur mustard-modified DNAs. However, we have obtained the cloned human enzyme from Professor Leona Samson at Harvard University and have shown that this enzyme has activity towards DNA modifications formed by the therapeutic chloroethylnitrosoureas (unpublished results). Thus, the human enzyme appears to have the broad substrate specificity associated with 3-methyladenine DNA glycosylase II, and we anticipate that it will have activity toward sulfur mustard-induced DNA modifications. If this enzyme has all of the activities associated with Gly II, it will probably release the cross-links formed in DNA by sulfur mustard since Gly II releases similar cross-links formed by chloroethylnitrosourea (11).

As outlined below, we have made substantial progress in the purification of the human glycosylase. Rapid assay of its action towards sulfur mustard-modified DNA will be possible when radiolabeled sulfur mustard with a high specific activity becomes available.

Cytotoxicity of Sulfur Mustard

An important consideration in the laboratory investigation of SM cytotoxicity is the choice of the experimental system. Biochemical studies can be carried out most precisely in a tissue culture system, preferably using keratinocytes because skin is a major target organ for SM toxicity. However, keratinocytes have proved to be difficult to culture in the past. In this report, we will describe the use of NM-1 cells (12) which we can now grow under defined media conditions, simplifying the interpretation of data. It should be noted, however, that some characteristics of these cells including levels of enzymes associated with the cell cycle may differ from those of cells that are undergoing terminal differentiation.

At low levels of DNA modification, cells are still able to carry out some metabolic functions but may be arrested in G_2. Clearly, if damaged cells had sufficient time for DNA repair before progressing through the cell cycle, their chances of survival might be enhanced. Both chemical and physical methods are available for slowing the progression of cells through their replicative cycle and the application of these modalities may allow time for more organized tis-

sue repair even if the damaged cells ultimately do not replicate.

The agent, ciclopirox olamine (CPX), causes a G_1/S arrest which is reversible when the agent is removed (13,14). If DNA repair continues in the presence of CPX, survival might be increased after a period of recovery under CPX treatment. Preliminary data contained in this report show that CPX does not interfere with glycosylase action at concentrations that result in G_1/S arrest. Consequently, there is a theoretical reason for believing that CPX treatment could enhance DNA repair.

Although other chemical agents besides CPX might also be effective in causing G_1/S arrest, a period of hypothermia might produce a similar result. Hypothermia would probably slow all metabolic processes including repair, but repair of radiation-induced DNA damage has been shown to be increased by a period of hypothermia (15,16). Conversely, hyperthermia increases cell damage by cancer chemotherapeutic agents (17). Data presented in this report show that progression through the cell cycle is indeed prevented by mild hypothermia which does not affect the activity of glycosylase-mediated repair. Thus, we believe that it will be possible to find hypothermic conditions under which repair continues during cell cycle arrest.

MATERIALS AND METHODS

Materials

Reagents

[^{14}C]-SM (0.88 mCi/mmol), uniformly labeled in the chloroethyl group, and unlabeled SM were supplied by the Analytical Chemistry Branch, U.S. Army Medical Research Institute of Chemical Defense. 2-Chloroethyl-2-hydroxyethyl sulfide (hemisulfur mustard, HSM) was prepared by reacting 2-mercaptoethanol with 1,2-dichloroethane according to Tsou *et al* (18) as described previously (19). 6-Chloro-2-amino purine deoxyriboside was a gift of Prof. George Wright, University of Massachusetts (20). Mercaptoethanol, ciclopirox olamine (CPX), MTT (dimethylthiazolyl-diphenyltetrazolium), micrococcal nuclease, P1 nuclease, proteinase K, calf thymus DNA and all nucleosides and nucleotides were purchased from Sigma. 1-Iodobutane, triethylamine and 1,2-dichloroethane were obtained from Aldrich; spleen phosphodiesterase, from Worthington Biochemical Corporation; and T4 polynucleotide kinase, from New England Biolabs. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear. DEAE Sephadex A-25 anion exchange resin (capacity, 3.5 meq/g; particle size, 40-120μ) was obtained from Pharmacia. Serum-free media (SFM) for the growth of keratinocytes was obtained from Gibco. Chromatography-grade solvents, media ingredients, and other reagents were obtained from standard sources.

Cell Lines

Human fibroblasts (AG1522B) were obtained from Coriell Cell Repository, and human keratinocytes (NM-1 cells) were obtained from Dr. Howard P. Baden's laboratory, Massachusetts General Hospital. The plasmid containing the cloned gene for human 3-methyladenine DNA glycosylase was obtained from Prof. Leona Samson, Harvard School of Public Health. Methylated DNA substrate for assaying this enzyme was prepared by our published procedure (21).

Methods

Synthesis of HPLC Markers and [^{14}C]SM-modified DNA

7-Hydroxyethylthioethyldeoxyguanosine 3'-phosphate (HETEdGp) was synthesized as described previously (19). Briefly, HSM (30 μl, 0.21 mmol) was reacted with 2'-deoxyguanosine 3'-phosphate (dGp, 10 mg, 0.029 mmol) in 1 ml of 100 mM KH_2PO_4 buffer, pH 3.5, for 2 hr at room temperature. The pH was adjusted to 7 and the reaction mixture was separated, 0.5 ml at a time, on an A-25 anion exchange column (1 x 10 cm) eluted with 60 mM triethylammonium acetate (TEAA), pH 7.0, at a flow rate of 0.8 ml/min; purification was performed in a cold cabinet to minimize depurination. Twenty min fractions were collected and the major product appeared in fractions 4 to 6. This product had the same UV spectrum as HETEdG, and released HETEG on depurination. 7-Hydroxyethylthioethyldeoxyguanosine 5'-phosphate (HETEpdG) was produced by the corresponding reaction with pdG.

7-Butyldeoxyguanosine 3'-phosphate (BudGp) and 7-butyldeoxyguanosine 5'-phosphate (BupdG) for use in the internal standardization procedure were synthesized by reacting n-butyliodide in DMSO with the triethylammonium salts of dGp and pdG, respectively. These nucleotides, purified as for HETEdGp and HETEpdG, depurinated to 7-butylguanine; the structure of this base was established by its characteristic ultraviolet spectra in acid, neutral, and basic pH, and its molecular weight of 207 obtained by fast atom bombardment mass spectrometry.

DNA was modified with [^{14}C]SM to provide DNAs with known levels of DNA modification for calibration of the ^{32}P-postlabeling method. [^{14}C]SM (1 mg/ml in toluene) was added to 10 ml calf thymus DNA (6 mg/ml) in 50 mM of sodium cacodylate buffer, pH 7.0, to bring the final concentration of SM to approximately 200 μM. After incubation with continuous mixing for 2 hr at 37°C, 1/20th volume of 6N NaCl and two volumes of 95% ethanol were added to precipitate the DNA; the precipitate was washed free of noncovalently bound material by repeated dissolutions and reprecipitations from buffer solution. The level of HETEG in this sample was determined from the optical density and HETEG content of an aliquot to be 630 HETEG/10^6 DNA nucleotides. HETEG content was determined by depurinating the sample and measuring the radioactivity of the HETEG peak in an HPLC separation as described previously (19,22). A solution of unmodified DNA was blended with a solution of this sample to obtain DNAs ranging in HETEG content down to 0.087 HETEpdG/10^6 DNA nucleotides.

^{32}P-Postlabeling Method for Detecting SM Damage to DNA

^{32}P-Postlabeling is performed in discreet steps which are shown in Figure 1 (next page). The method is dependent on the successful digestion of DNA into its constituent deoxynucleotide 3'-phosphates. This is necessary because the presence of the 3'-phosphate is required for the labeling process to work; the labeling enzyme (T4 kinase) adds a 5'-phosphate only when a 3'-phosphate is present. A critical step in the successful application of the ^{32}P-postlabeling method to SM-modified DNA was the discovery that low temperature digestion was possible and that it preserved the unstable HETEdGp. Another critical step in obtaining accurate analyses was the inclusion of an internal standard of BudGp from the beginning of the analysis. Since this nucleotide has chromatographic and stability characteristics similar to those of HETEdGp, it can be used to correct for physical losses and decomposition of

HETEdGp. Furthermore, since amounts of HETEpdG are determined by a ratio to the internal standard, it is not necessary to know the specific activity of the [^{32}P]ATP accurately.

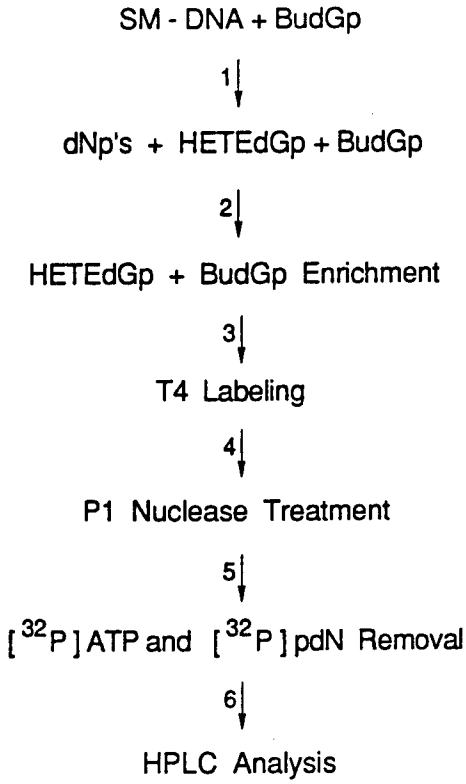

Figure 1. ^{32}P-Postlabeling Method for Detecting SM Damage to DNA. A known amount of BudGp is added as internal standard to an amount of DNA calculated from its A_{260} value, and enzymatic digestion to the 3'-deoxynucleotides is carried out at 10°C in step (1). HETEdGp and BudGp are separated from unmodified nucleotides quickly on a small column to minimize decomposition in step (2), and the solution is concentrated by lyophilization. In step (3), a ^{32}P label is added to the nucleotide 5' position by T4 kinase. 3'-Phosphates are removed by P1 nuclease in step (4), and the labeled HETEpdG and BupdG are recovered from a disposable anion column in step (5). Residual radioactivity is discarded with the column at this point, minimizing laboratory handling of ^{32}P. Finally, HETEpdG and BupdG are separated from contaminating radioactivity by HPLC in step (6), and the amount of HETEpdG is calculated from the ratio of HETEpdG to BupdG radioactivity.

Details of these steps are described in the next few paragraphs.

DNA was digested into its constituent 3'-nucleotides by two enzymes: micrococcal nuclease, an endonuclease that cleaves phosphodiester bonds to leave 3'-phosphates, and spleen phosphodiesterase, an exonuclease that releases 3'-mononucleotides. Typically, 50 μg of DNA was digested overnight at 10°C in 160 μl containing 0.3 M triethylammonium acetate buffer, pH 7.5; 5 mM $CaCl_2$; 0.3 unit spleen phosphodiesterase and 6 units micrococcal nuclease.

We have found that HETEdGp is depurinated if this hydrolysis reaction is performed at the usual temperature, 37°C. However, if the digestion is per-

formed at 10°C, approximately 50% of the derivative is released as the 3'-mononucleotide and is, therefore, capable of being phosphorylated by the T4 enzyme.

Since the normal nucleotides greatly exceed the derivative nucleotides in number, perhaps by 10^7 to 1, it is desirable to remove these before the ^{32}P-postlabeling step. N-7-substituted guanine nucleotides are separated efficiently from normal nucleotides on an anion exchange column because they bear a positive charge at the N-7 position (23,24). We have found it convenient to perform both the initial separation of the nucleotides in the enzymatic digest and the later separation of labeled N-7 adducts from [^{32}P]ATP on disposable columns. These are made by packing 1 ml plastic tuberculin syringes with 1 ml of A-25 resin pre-equilibrated in 0.2 M TEAA buffer, pH 7.0, to form columns measuring 4 x 68 mm. The columns are attached in an easily removable way to a Milton Roy pump through a plastic connector inserted into the syringe barrel, and to a UV monitor through a modified #17 needle. In the initial separation of N-7 adducts from normal nucleotides, an optical marker of BupdG was added and in the separation of labeled adducts from [^{32}P]ATP, an optical marker of HETEpdG was added. The column was eluted with 80 mM TEAA, pH 7.0, at a flow rate of 1 ml/min in both separations; in the initial separation involving unstable HETEdGp, cold buffer was used. Fractions containing N-7 guanine nucleotides, identified by the BupdG marker, were frozen immediately and lyophilized to dryness.

For levels of modification exceeding one HETEpdG/10^6DNA nucleotide, N-7 guanine nucleotides collected from 1 nmol (0.3 μg) of digested SM-DNA containing 2 fmol of BudGp were labeled in 5 μl of 0.14 M Tris-acetate buffer, pH 7.5, containing 28 mM $MgCl_2$, 28 mM dithiothreitol, 2.8 mM EDTA, and 2 mM spermidine. One μl of ATP (0.023 mM) and 5 μl of [^{32}P]ATP (50 μCi) were added, and the substrate was incubated with twenty units of T4 kinase for 90 min at 37°C. Then, 2 μl of 2 mM $ZnCl_2$ and two units of P1 nuclease were added and the incubation was continued for 1 hr at 37°C to remove 3'-phosphates. For levels below one HETEpdG/10^6 DNA nucleotides, N-7 guanine nucleotides from as much as 40 nmol of DNA digest (13 μg) were labeled.

The labeling solution was mixed with an optical marker of HETEpdG and loaded onto a disposable A-25 column eluted at 1 ml/min with 80 mM TEAA buffer, pH 7.0. About 10 ml of buffer containing the HETEpdG optical peak were collected and lyophilized to dryness. Most of the [^{32}P]ATP remained on the column and was discarded in the radioactive waste.

The final HPLC analysis was performed on a modular apparatus consisting of a Milton Roy minipump, Rheodyne 7125 injection valve, and a 5 μm Spherisorb (4.6 x 250 mm) C_{18} column from Alltech. Elution profiles were monitored at 254 nm with a Perkin-Elmer LC-55B spectrophotometric detector interfaced with a Hewlett-Packard 3396A integrator. The column was eluted at 1 ml/min with 1% acetonitrile in 70 mM TEAA, pH 7.0, for 40 min; then with a gradient of 1 to 3% acetonitrile in TEAA from 40 to 70 min, and finally with 3% acetonitrile in TEAA from 70 to 84 min. One ml fractions were collected and the Cherenkov radiation in each fraction was determined with an LS-1800 Beckman scintillation counter. Radioactivity in individual peaks was automatically totaled by a computer program which subtracts background radiation.

DNA Isolation

The ^{32}P-postlabeling method can be used to determine the extent of DNA damage in tissue culture or other cells exposed to SM. Our procedure for

isolating DNA from the cells follows the flow diagram shown in Figure 2. The composition of the TCNS buffer used in this procedure is: 10 mM Tris-Cl, pH 7.4; 2 mM $CaCl_2$; 150 mM NaCl; 0.4% SDS.

Collect cells from monolayer with trypsin

↓

Incubate in TCNS buffer for 30 min at 37°C

↓

Add RNase and continue incubation for 1.5 hr at 37°C

↓

Incubate with proteinase K for 2 hr at 37°C

↓

Chelate Ca^{++} with EGTA; 30 min at 25°C

↓

Extract with $CHCl_3$-isoamyl alcohol (x3)

↓

Precipitate with cold ethanol

Figure 2. Procedure for Isolating DNA from Keratinocytes.

DNA Repair Studies

The activity of bacterial Gly II against SM-modified DNA was tested by incubating Gly II isolated as described previously (10) with a blend of SM-modified DNA which contained 5.2 HETEpdG/10^6 nucleotides. After incubation for 1 hr at 37°C, DNA was precipitated with two volumes of cold ethanol, and the remaining HETEpdG in the substrate was analyzed by the ^{32}P-postlabeling method.

Human 3-methyladenine DNA glycosylase has been purified from a plasmid containing the cloned gene (pP5-3, ref 25) grown in *E. coli* (*alkA tag*) cells which do not contain any endogenous glycosylase activity. Cells (3.5 gm) grown to early log phase were homogenized in 60 ml of 20 mM Tris-HCl, pH 7.5, buffer containing 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 30 units/l of Aprotinin, 0.2 M NaCl, and 10% glycerol. The homogenized cells were lysed in an ice cold French press and the lysate was centrifuged in a refrigerated centrifuge at 10,000 g for 40 min. All subsequent steps were carried out in a cold cabinet.

The supernatant was pumped onto a DEAE-cellulose column (DE23, 2 x 17.5 cm) at a flow rate of 0.75 ml/min to remove DNA. The column was washed with 135

ml of 20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1 mM DTT; 10% glycerol (buffer G) containing 0.2 M NaCl and then eluted with a 50 ml gradient to 1 M NaCl in buffer G; 15 min fractions were collected. Glycosylase activity appeared in fraction 5 and gradually tapered off to fraction 25. Fractions 5 through 13 were pooled and pumped at 0.7 ml/min onto a double stranded DNA cellulose column (1.5 x 9.5 cm) previously equilibrated with buffer G containing 0.2 M NaCl. The enzyme was eluted with an 80 ml gradient to 1 M NaCl in buffer G; 15 min fractions were collected. In addition to a broad peak near the front, a sharp peak of glycosylase activity was detected at fraction 18, approximately one third of the way through the gradient.

The peak fraction from the DNA-cellulose column was diluted to 72 ml with buffer G and pumped at 0.3 ml/min onto a P11 phosphocellulose column (2 x 8 cm) equilibrated with buffer G containing 0.15 M NaCl. The column was washed with 40 ml of buffer G containing 0.15 M NaCl and then eluted with an 80 ml gradient from 0.15 M to 1 M NaCl in buffer G. Again, 15 ml fractions were collected and immediately divided in half; 2 mg of serum albumin was added to one half to stabilize the glycosylase. Assays of these fractions showed a sharp peak of activity at fraction 27. SDS-PAGE analysis of this fraction is shown in the RESULTS section.

Cell Growth

Human fibroblasts (AG01522B cells) were grown as a monolayer in standard minimum essential media (MEM) with a 2 x concentration of amino acids and vitamins supplemented with 15% fetal bovine serum (FBS), penicillin and streptomycin. Human keratinocytes (NM-1 cells) were grown: (a) in the presence of feeder layer cells (mitomycin-treated mouse 3T3 cells) on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% FBS, hydrocortisone, cholera toxin, penicillin and streptomycin; (b) in the absence of both feeder layer cells and serum on Ham's Nutrient Mixture F-12 (Gibco) supplemented with epidermal growth factor (EGF), insulin, bovine pituitary extract (BPE), ethanolamine and hydrocortisone; and (c) in Serum Free Media (SFM) for keratinocytes from Gibco, supplemented with BPE and EGF. Supplemented SFM was selected for growth of cells for cytotoxicity studies and growth on feeder layers for long term maintenance. Both cell lines were grown in a humidified atmosphere of 5% CO_2 at 37°C.

Cytotoxicity Studies

Exposure Conditions

The cytotoxicity of SM was investigated by plating cells in 6-, 12-, or 96-well plates at an initial density from 2 x 10^4 to 4 x 10^4 cells/cm^2. After 24 hr for fibroblasts or 48 hr for keratinocytes, the medium was replaced with fresh medium containing SM at the indicated concentration. Dilute solutions of mustard in absolute alcohol were prepared separately for each dose immediately before treatment. Cells were exposed to SM for 1 hr at room temperature in the SterilchemGard hood and then incubated in fresh medium at 37°C. Medium was changed every 2 days thereafter. At the indicated times, cell viability was determined. The effect of temperature on cytotoxicity was examined by incubating one set of plates for 1 or 2 days at 28°C before transferring them back to 37°C.

The effect of the cell cycle modulator, ciclopirox olamine (CPX), on cytotoxicity was examined by adding CPX dissolved in the media to the cells at the indicated times before and/or after exposure to the alkylating agent. After

the required time, CPX was removed by replacing CPX-containing media with fresh media.

Viability Assays

Trypan Blue Assay

For the trypan blue exclusion assay, cells in 6- or 12-well plates were trypsinized, washed and resuspended in 100 μl of media. After staining, trypan blue-negative cells were counted in a hemocytometer. Analysis of duplicate determinations shows that these counts are reproducible with a standard deviation of $\pm$ 6.3%.

MTT Assay

The MTT (dimethylthiazolyl diphenyl tetrazolium) assay for viability was performed as described by Sobbotka and Berger (26). Cells in 96-well plates were incubated for 2 hr at 37°C in the presence of MTT (4 mg/ml of medium). The medium was then removed and 100 μl of DMSO was added to each well. Plates were shaken for a short time to improve dissolution of formazan crystals, and the absorbance at 560 nm, representing MTT activity, was measured using a THERMOMAX microplate reader.

Determination of Cellular DNA Content

Cell growth and cytotoxicity were also monitored by measuring the DNA content of cells fluorometrically with Hoechst 33258 dye. The procedure described by Rao and Otto (27) that employes a fluorescence plate reader was modified for cells grown in 12-well plates instead of 96-well plates, and fluorescence measurements were performed in cuvettes instead of *in situ*. Cells were exposed to the alkylating agent as described and incubated at 37°C. At the indicated times, medium was removed, cells were trypsinized, transferred into the 1.5 ml-centrifuge tubes, and rinsed with PBS. The cell pellet was frozen until time for the assay. At that time, cells were solubilized by adding 0.25 ml of saline sodium citrate (SSC) buffer, pH 7, containing SDS (0.05%) and urea (8M) to each tube and incubated at 37°C for 1.5 hr. Solubilized cells were diluted with 1.75 ml of SSC buffer, and 50 μl of Hoechst 33258 in SSC (80 μg/ml) was added to 2 ml of sample. Fluorescence was measured at an emission wavelength of 460 nm, using an excitation wavelength of 355 nm.

Cell Cycle Analysis

The effect of sulfur mustard or potential modulators of cytotoxicity on cell cycle progression was examined by flow-cytometry. Cells grown in monolayers in 75 cm^2 flasks (~ 1 x 10^6 cells) were trypsinized after treatment, rinsed with PBS and resuspended in 0.1 ml of PBS. The cells were immediately fixed with 0.9 ml of ice-cold 95% ethanol and stored at +4°C for up to 1 week before analysis. Cells were stained by propidium iodide (PI) and fluorescence was measured using a FACScan flow cytometer (Becton & Dickinson, San Jose, CA).

RESULTS

Synthesis of SM-Modified Deoxynucleotides and Markers

Analysis by the ^{32}P-postlabeling method depends on the availability of well-characterized deoxynucleotide markers because DNA adducts are detected as peaks of radioactivity which must be identified by comparison with known markers. Indeed, the required separations cannot be developed efficiently unless these markers are available.

The markers required for the analysis of HETEpdG, the predominant SM-induced DNA modification, are: HETEpdG itself, HETEdGp, and the two markers used in the internal standardization procedure, BupdG and BudGp. The synthesis of these deoxynucleotides is described in the METHODS section and proceeded without difficulty.

Because the biological significance of a particular DNA modification may not parallel its abundance, we are interested in the analysis of less prevalent adducts including, particularly, O^6-substituted deoxyguanosines. The adduct expected from SM, by analogy with that found for CEES (7), is O^6-hydroxyethylthioethyldeoxyguanosine. This compound was synthesized by the reaction shown in Figure 3.

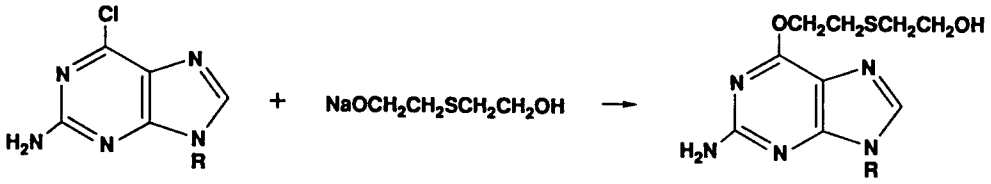

Figure 3. Synthesis of O^6-Hydroxyethylthioethyl Deoxyguanosine.

The structure of this compound was established by its uv spectra which are typical of an O^6-substituted guanine nucleoside; the nature of the substituent was confirmed by its FABMS molecular weight of 371. The uv spectra are shown in Figure 4 (next page)

With this marker available, it was possible to determine whether O^6-hydroxyethylthioethyl deoxyguanosine was formed when SM reacted with deoxyguanosine. Deoxyguanosine (2 mg) was reacted with HSM (4 μl) for 4 hr at 37°C in 50 mM sodium cacodylate buffer, pH 7. The mixture was then analyzed on a C_{18} column eluted with an acetonitrile gradient. As shown in Figure 5, a small peak eluted at 82 min which was identified as O^6-hydroxyethylthioethyl deoxyguanosine on the basis of its retention time, uv spectrum captured on a diode detector, and its intense fluorescence. Attempts to demonstrate this adduct in DNA reacted with HSM or SM were unsuccessful, however, presumably since it is such a minor product.

The low yield of the O^6-substituted product makes the direct synthesis of the substituted deoxynucleotides from dGp and pdG impractical and it will be necessary to use an enzymatic method to produce the marker required for analysis by ^{32}P-postlabeling (see DISCUSSION).

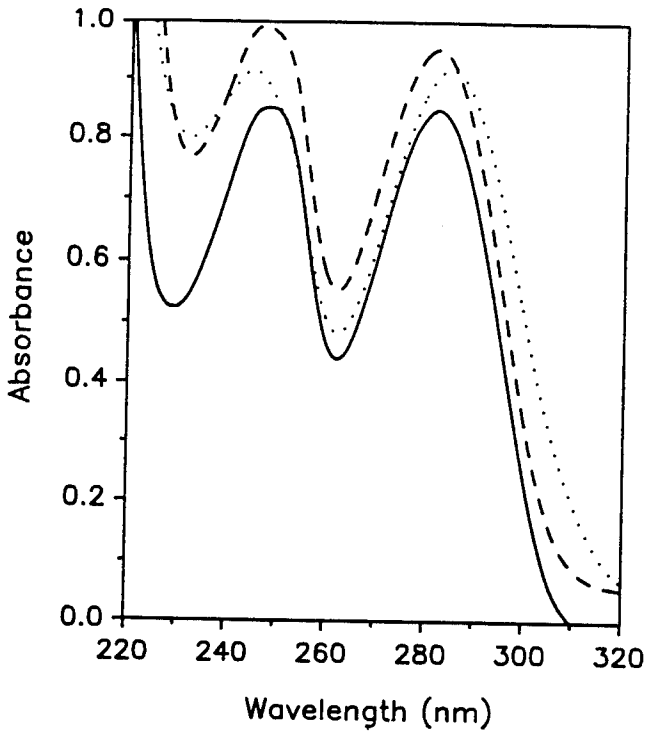

Figure 4. UV Spectra of O^6-Hydroxyethylthioethyl Deoxyguanosine. Spectra were obtained in acidic (··· pH 1), neutral (——, pH 7), and basic (---, pH 12) solution.

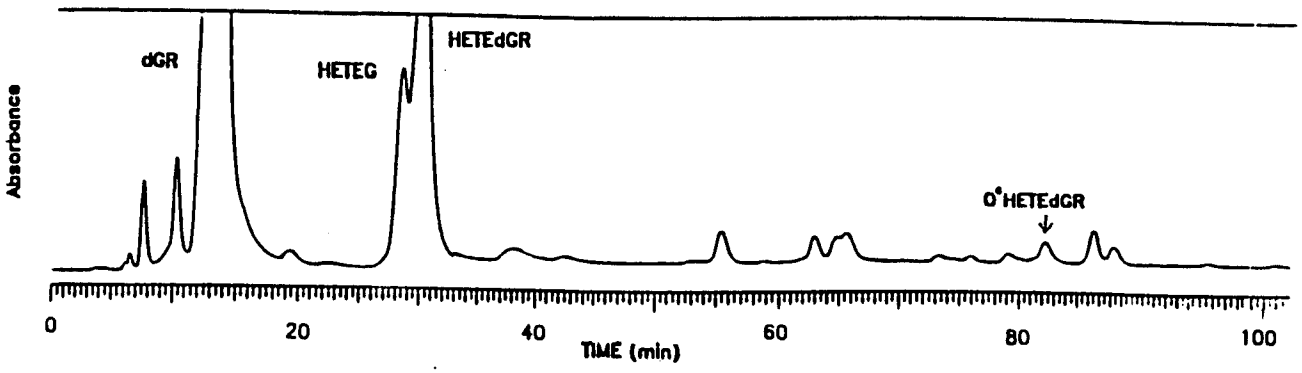

Figure 5. HPLC Profile of Sulfur Mustard-Deoxyguanosine Reaction Mixture. A small peak of O^6-hydroxyethylthioethyl deoxyguanosine appeared at 82 min.

^{32}P-Postlabeling Analysis of SM-induced DNA Modifications

Our ^{32}P-postlabeling technique has been improved substantially since our last report (19) and can now detect HETEpdG at the level of one modification in 10^7 DNA nucleotides. These improvements have been achieved by including an internal standard with HPLC and stability characteristics similar to those of HETEdGp from the beginning of the analysis, and by making more extensive use of disposable A-25 cartridges to separate HETEdGp from unmodified deoxynucleotide 3'-phosphates (step 2) as well as from [^{32}P]ATP (step 5).

As shown in Table 1, the disposable A-25 columns separate nucleotides into three groups: 7-substituted guanine nucleotides which elute between 14 and 16 min, unsubstituted nucleotides which elute between 35 and 71 min, and ATP which is retained on the column. Consequently, similar columns can be used in both the HETEdGp enrichment step 2 and the ATP removal step 5. BupdG was added as an optical marker in step 2 and HETEpdG as an optical marker in steps 5 and 6. The nature of the alkyl substituent, n-butyl, was chosen to produce a satisfactory separation during the final HPLC analysis, step 6.

Table 1. Retention Times on Disposable Sephadex A-25 Columns

Nucleotide	Retention time (min)	Nucleotide	Retention time (min)
HETEdGp	15.7	dTp	35
HETEpdG	15.3	dCp	41
BudGp	14.3	dAp	50
BupdG	14.6	dGp	71
		ATP	> 120

Figure 6 (next page) is an example of the assay. An aliquot of a digest from 1 nmol of [^{14}C]SM-DNA which contained 2 fmol of BudGp as internal standard was separated on a C_{18} column. From our computer program, HETEpdG radioactivity equaled 0.75 times BupdG radioactivity, indicating that 1.5 fmol of HETEpdG were present. Separately, however, we had determined from its [^{14}C] content that 1 nmol of this [^{14}C]SM-DNA blend originally contained 10.2 fmol of HETEpdG. Consequently, the overall recovery of HETEpdG in the analysis was 15%. However, by increasing the amount of DNA analyzed to 40 nmol (13.2 μg), it was possible to measure the HETEpdG content of [^{14}C]SM-DNA blends containing as little as one HETEpdG/10^7 DNA nucleotide accurately.

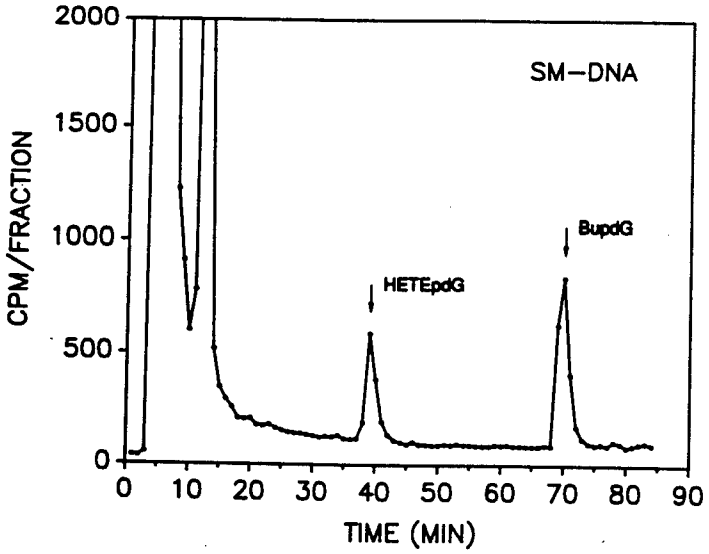

Figure 6. HPLC Profiles Showing Detection of 1.5 fmol of HETEpdG. Top panel, labeled digest from 1 nmol (0.3 μg) of [^{14}C]SM-DNA to which 2 fmol of BudGp was added as internal standard. Bottom panel, labeled digest from 1 nmol (0.3 μg) of unmodified DNA with 2 fmol of BudGp added.

To test the reproducibility of the assay, a representative blend containing 5.2 fmol HETEpdG per nmol of DNA was analyzed a total of four times by two different operators. Recoveries were 13.0%, 20.7%, 21.7%, and 25.6% for a mean of 20.2 ±5.2.

The calibration curve obtained by analyzing a range of blends containing from 8.7×10^{-8} to 3.1×10^{-5} HETEG per DNA nucleotide is shown in Figure 7 (next page). The data show a linear dependence of the log of HETEpdG recovered on the log of HETEpdG in the DNA blend analyzed. The actual relationship is given by the equation: $\log y = 2.47 + 1.61 \log x$ with a correlation coefficient of 0.99.

The ^{32}P-postlabeling method has now been applied successful to the detection of DNA modification in tissue culture cells exposed to SM. In order to do this, DNA has to be isolated and its amount has to be accurately determined. The method shown in Figure 2 of the Methods section has resulted in the recovery of good yields of DNA of satisfactory purity as judged by the A_{260}/A_{280} ratios. Typically, we obtain 20 to 50 μg of DNA from 10^6 to 10^7 cells with an A_{260}/A_{280} ratio of approximately 1.6. We have found that measuring the A_{260} of an aliquot in a 50 μl cuvette is a rapid and accurate method of obtaining the DNA concentration.

Figure 8 illustrates the analysis of DNA isolated from fibroblasts exposed to 5 μM (left panel) or 20 μM SM (right panel) for 1 hr at room temperature. Back calculating from the calibration curve shown in Figure 7, we find that these samples contain 1.8 and 3.0 HETEpdG/10^6 DNA nucleotides, respectively.

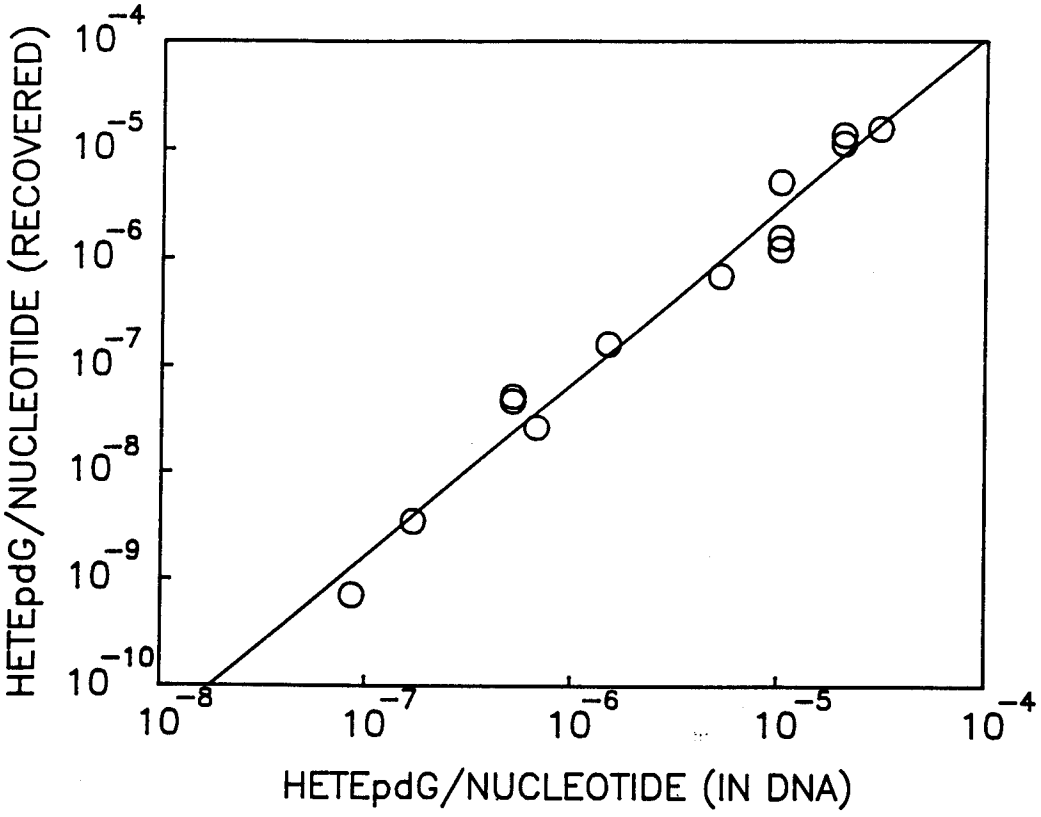

Figure 7. Calibration Curve for Detecting HETEpdG in DNA. Samples of DNA containing the indicated ratio of HETEpdG to normal nucleotides were digested and labeled in the presence of internal standard; the calculated amount of HETEpdG recovered is plotted on the y axis.

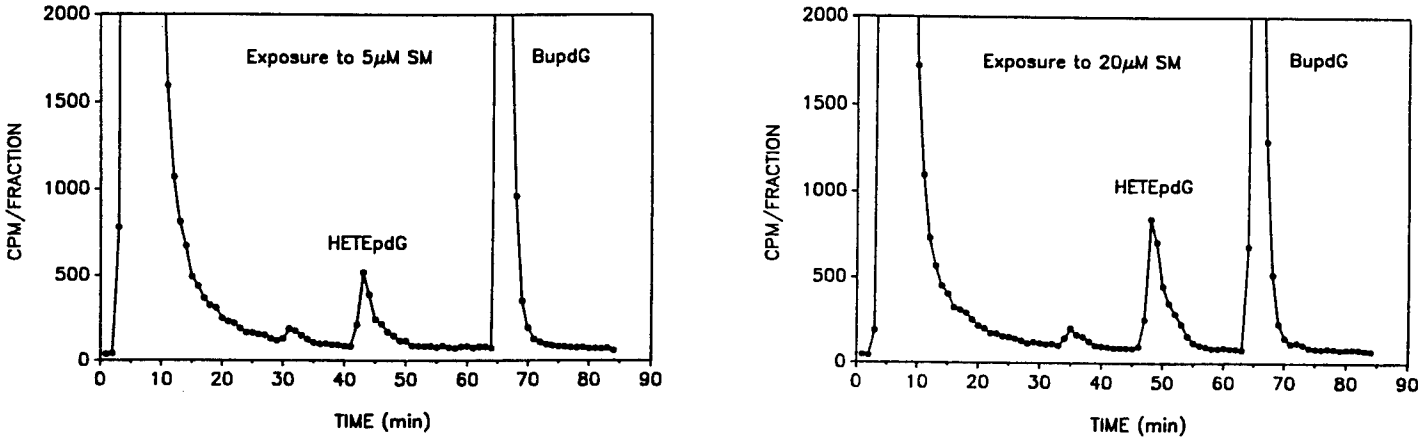

Figure 8. ^{32}P-Postlabeling Analysis of DNA from Fibroblasts Exposed to SM. DNA was isolated from fibroblasts exposed for 1 hr at room temperature to 5 μM (left panel) or 20 μM (right panel) SM. HETEpdG levels, calculated from the ratio of HETEpdG to BupdG peak sizes and the ^{32}P-postlabeling calibration curve, are 1.8 and 3.0/10^6 nucleotides, respectively.

DNA Repair Studies

Glycosylase action on SM-modified DNA will be more easily demonstrated when radiolabeled SM of high-specific activity becomes available. However, the high sensitivity of the ^{32}P-postlabeling method is required for studies involving tissue culture. Accordingly, we have piloted the study of glycosylase action on SM-modified DNA using ^{32}P-postlabeling.

Figure 9 shows the results of incubating SM-modified DNA with bacterial Gly II. After a 1 hr incubation at 37°C, the HETEpdG content of DNA incubated with glycosylase (left panel) is clearly much diminished in comparison with the control (right panel). We conclude from this that bacterial Gly II recognizes and releases HETEG from SM-modified DNA.

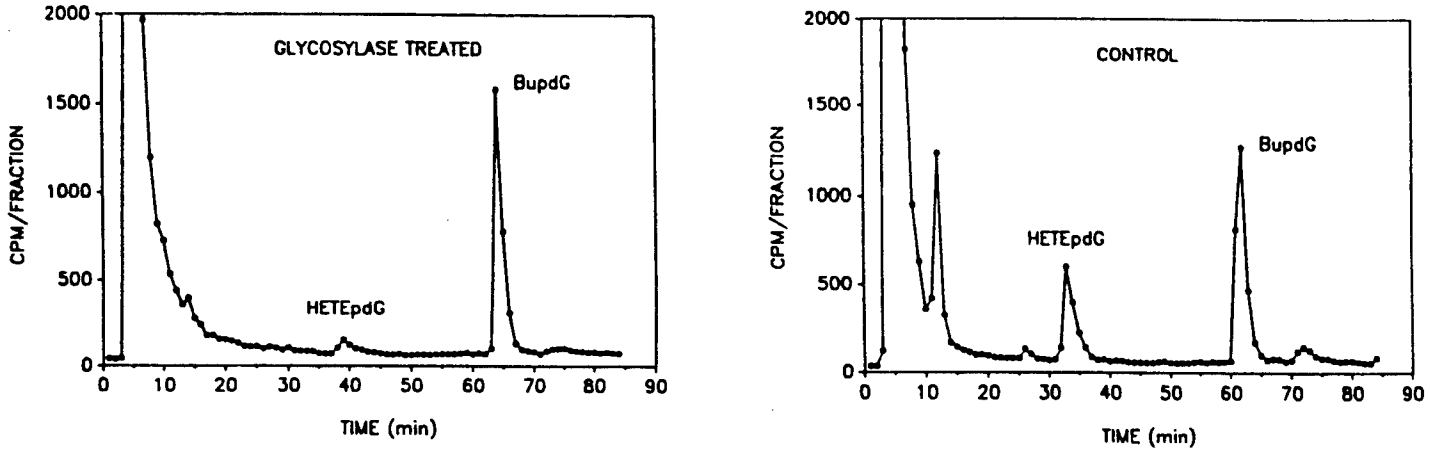

Figure 9. Release of HETEG by Bacterial Gly II. Left panel: 16 μg of DNA containing 247 fmol of HETEGpdG was incubated in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM EDTA and 3 mM DTT in the presence of 0.4 μg of Gly II for 1 hr at 37° C and then analyzed by the ^{32}P-postlabeling method. Right panel: DNA incubated in the absence of Gly II and analyzed as in the left panel. In comparison with the internal standard peak of BupdG, it is apparent that the HETEpdG peak has been decreased in the enzyme-treated sample.

Although evidence is mounting that the human enzyme resembles bacterial Gly II, it is clearly of much greater importance to study the action of the human glycosylase on this substrate. We have put a major emphasis on purifying this enzyme from the cloned gene.

As described in the METHODS section, we have made good progress in purifying this enzyme. We have followed the scheme originally used by Gallagher and Brent (28) through a phosphocellulose column, but may have been more successful in obtaining an intact protein because we started with a richer source (Gallagher, personal communication). In comparison with the recently published procedure of O'Connor (29), we use a different DNA removal step and obtain good purification on a DNA cellulose column. The next steps are to perform the purification on a larger scale and to add a final FPLC purification step. Stability of the enzyme remains a problem although it has been largely solved by adding purified albumen as a stabilizer. However, we will explore the use of higher concentrations of glycerol and/or sucrose as an alternative.

Figure 10 shows that Fraction 27 from the phosphocellulose column consists on one major band with a mobility corresponding to approximately 39 kD.

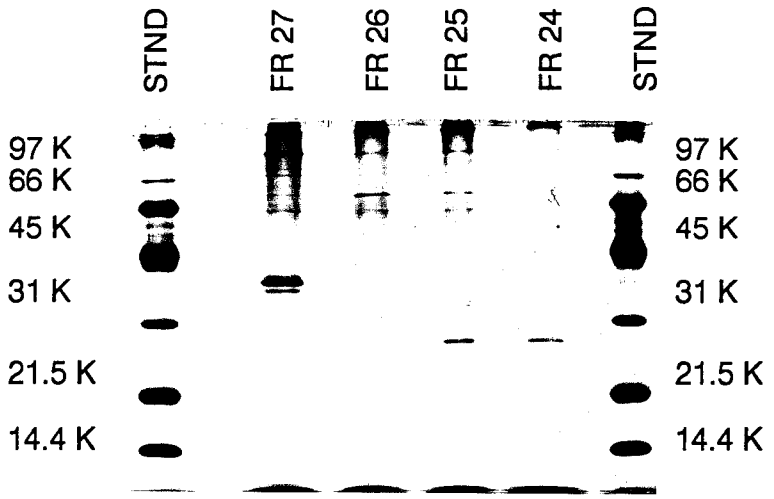

Figure 10. SDS-PAGE Analysis of Purified Human Glycosylase. The gel was stained by the silver staining technique.

We have not yet obtained sufficient quantities of purified human glycosylase to test this fraction against the SM-modified DNA substrate, but preliminary experiments with partially purified human glycosylase indicate that the human enzyme also releases HETEG from SM-modified DNA.

Cytotoxicity Studies

Studies with Fibroblasts

Effect of Sulfur Mustard on Cell Survival

Since fibroblasts divide more quickly and are easier to grow than keratinocytes, we began our studies of SM toxicity on the human fibroblast line, AG01522B. Cytotoxicity was measured by counting the number of cells which were able to exclude trypan blue. As shown in Figure 11 (next page), this number increased rapidly in the control population, but not in a population exposed to 2 μM SM for 1 hr at room temperature. The lack of increase in the viable cell number may reflect either a balance between cell death and growth, or cell arrest.

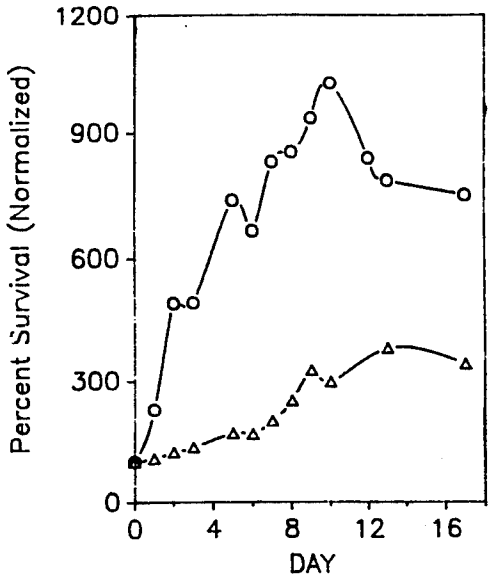

Figure 11. Cytotoxicity of SM for Human Fibroblasts as Measured by the Trypan Blue Method. Fibroblasts grown as described in METHODS and plated at 1.3 x 10^5 cells/well in 12-well plates were exposed 1 day after plating to 0 μM (O) or 2 μM (Δ) SM for 1 hr at room temperature and then incubated at 37°C. Results are expressed as a percentage of the trypan blue-excluding cells on the day of treatment.

The increase in cell DNA content is another measure of growth. As shown in Figure 12, the increase in total DNA content measured as DNA fluorescence was also markedly reduced by exposure to 2 μM SM. However, the DNA content per viable trypan blue-excluding cell shown in Table 2 (next page) is higher than in the control population, suggesting polyploidy among the SM-treated cells.

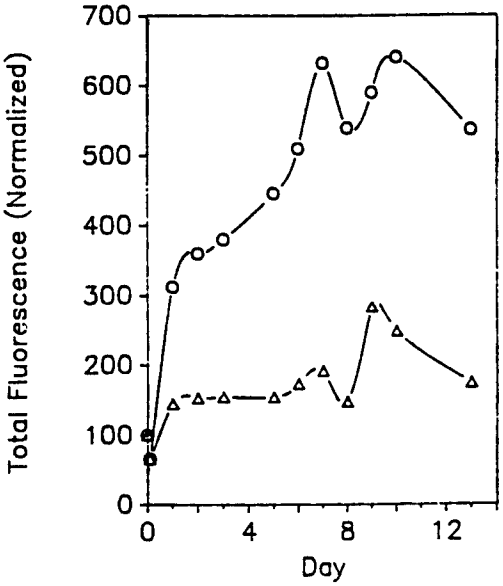

Figure 12. Cytotoxicity of SM for Human Fibroblasts as Measured by DNA Fluorescence. Fibroblasts were grown and exposed to 0 μM (O) or 2 μM (Δ) SM as in Figure 10. The results are expressed as a percentage of the fluorescence on the day of treatment.

Table 2. Effect of SM on the DNA Content per Cell

Day	Fluorescence/Cell	
	Control Cells	Cells Exposed to 2 μM SM
0	15.7	15.7
1	16.2	14.5
2	9.1	16.8
3	8.9	14.7
5	7.1	10.2
6	8.2	12.3
7	8.6	10.6
8	7.9	6.9
9	7.8	10.1
Mean	9.9 ± 3.5	12.4 ± 3.2

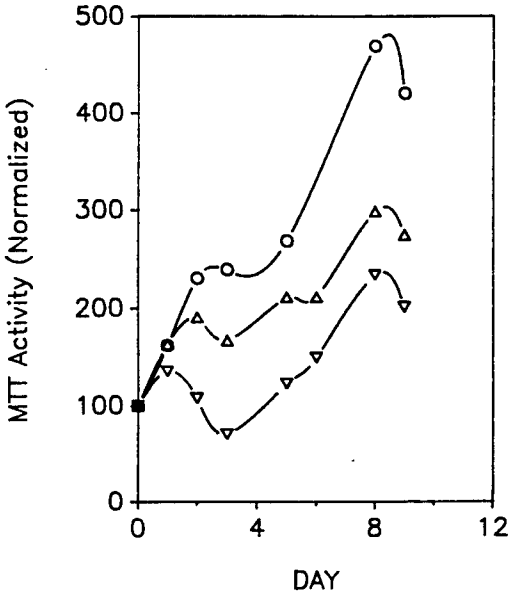

Figure 13. MTT Activity of Fibroblasts after Exposure to SM. Fibroblasts grown in 96-well plates were exposed to 0 μM (O), 5 μM (Δ) or 100 μM (∇) SM 1 day after plating, and MTT activity was determined by measuring the A_{560} as described in the METHODS section.

We have also used the MTT test to measure cytoxicity since this test is easily automated and has proved useful in the evaluation of new antitumor agents. In contrast to the viable cell number, cell metabolism expressed as MTT activity increases over time (Figure 13), even in cell populations exposed to SM doses of 5 and 100 μM. Since the trypan blue data (Figure 11) indicate that the

number of viable cells is decreased by an even lower doses of SM, these data indicate that the level of metabolic activity in damaged cells is highly elevated.

Effects on Cell Cycle Progression

The data on cytotoxicity of SM obtained by the trypan blue, MTT, and DNA assays, combined with the microscopic appearance of these cells (an increase in size of SM-treated cells) all suggest that cell cycle progression is inhibited by SM. To evaluate this effect, we performed cell cycle analyses of fibroblasts exposed to 2 μM SM for 1 hr. Figure 14 shows the cell cycle distribution in a control cell population (top row) and a SM-treated cell population (bottom row) at 6, 11, 21, and 46 hr after the treatment.

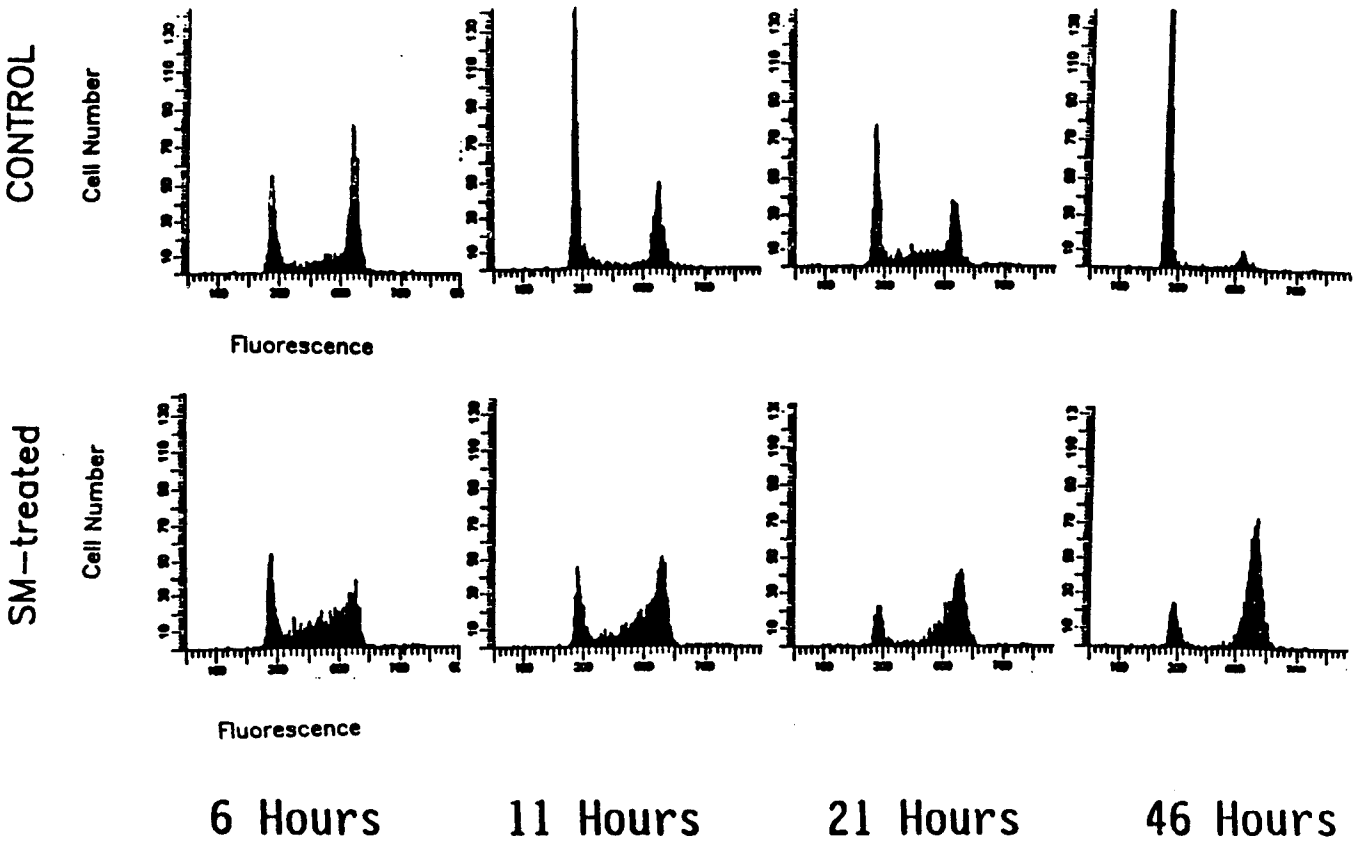

Figure 14. Cell Cycle Distribution in Control and SM-exposed Fibroblasts. Control cells (top row) and cells exposed for 1 hr to 2 μM SM (bottom row) were trypsinized 6, 11, 21, and 46 hr after exposure, stained with PI and analyzed.

Control cells proceed through the entire cell cycle while SM-exposed cells accumulate in G_2. Thus, fibroblasts exposed to as little as 2 μM SM undergo G_2 arrest and, as indicated by the results in Figures 11 and 13, do not divide, but do maintain a certain level of metabolic activity.

Effect of Temperature

The next series of experiments investigated methods of halting cell cycle progression in a nontoxic way that would permit DNA repair to continue.

Two modalities were tested: (1) hypothermia, and (2) the addition of CPX to the growth media.

As shown in Figure 15, fibroblasts held for 1 day at 28°C before being returned to 37°C show an increase in number of trypan blue-excluding cells that parallels the increase in control cells incubated at 37°C throughout. This indicates that the cells suffered no permanent damage by being held at 28°C.

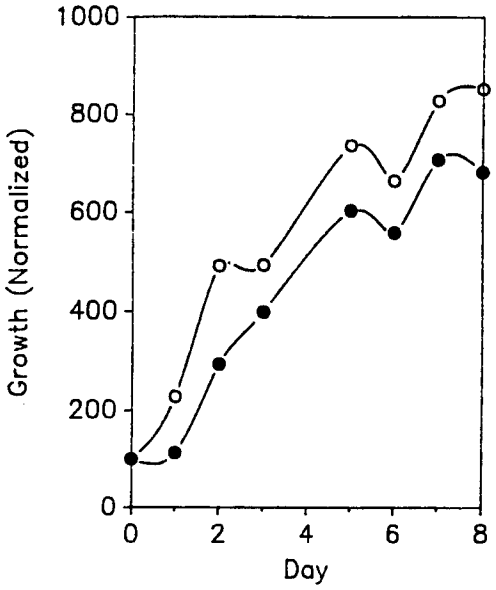

Figure 15. Effect of Temperature on Growth of Fibroblasts. Cells were grown at 37°C (O) or were held at 28°C for 1 day before being returned to 37°C (●).

Cell cycle progressions under the two temperature regimes are shown in Figure 16 (next page). Cells were grown in monolayers for 1 day at 37°C, media was changed and incubation was continued at either 37°C or 28°C. Control cells (cells at 37°C) were analyzed at 6, 11, 21, and 46 hr following the media change. Cells held at 28°C for 21 hr were analyzed before being returned to 37°C and then again 25 hr later. At 21 hr, the cells which had been held at 28°C show a distribution very much like control cells at 6 hr, but once returned to 37°C, they progressed through the cell cycle in a manner very similar to that of the control cells. We conclude from this that exposure to 28°C has, indeed, arrested cell cycle progression in a reversible way.

Experiments have begun to determine whether this cell cycle arrest enhances survival after SM exposure. Figure 17 (next page) shows the effect of post-treatment temperature on cytotoxicity of SM. Control cells or cells exposed to 2 μM SM for 1 hr at room temperature were either incubated at 37°C (left panel), or held at 28°C for 1 day before being returned to 37°C (right panel). Again, cells not exposed to SM, temporarily held at 28°C, recovered after 1 day; the number of trypan blue-excluding cells grew at approximately the same rate as it did for cells which had been at 37°C throughout. Cells exposed to 5 μM SM grew poorly at 37°C. Exposed cells held at 28°C grew at least as well, and there is a slight suggestion that toxicity was diminished by the period of hypothermia. However, it will be necessary to extend these experiments to lower temperatures and lower SM concentrations to determine whether this effect is real. Additional evidence as to the potential protective value of hypothermia will be obtained by monitoring the level of DNA damage and progression through the cell cycle.

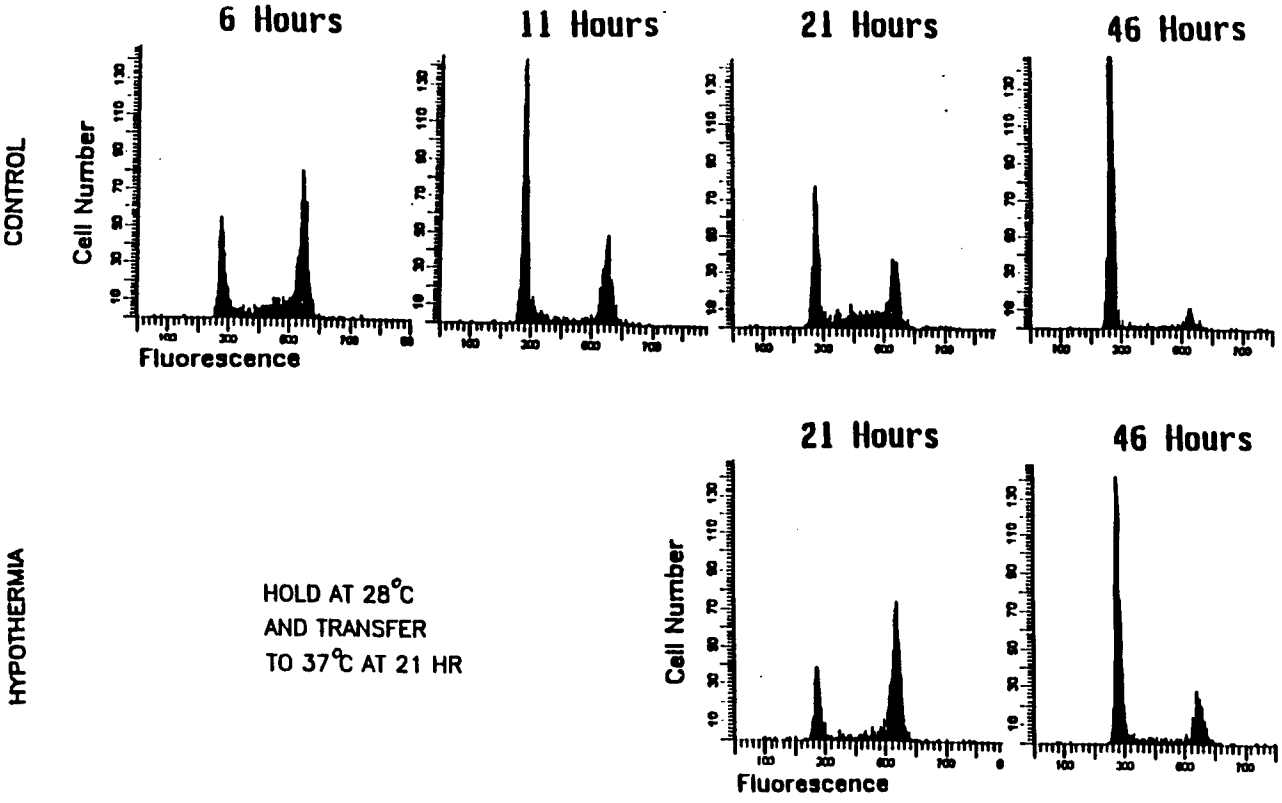

Figure 16. Effect of Temperature on Cell Cycle Distribution of Fibroblasts. Cells plated one day earlier were grown at 37°C or held at 28°C for 21 hr before being returned to 37°C; they were trypsinized 6, 11, 21, or 46 hr after the beginning of the temperature cycle, stained with PI and analyzed.

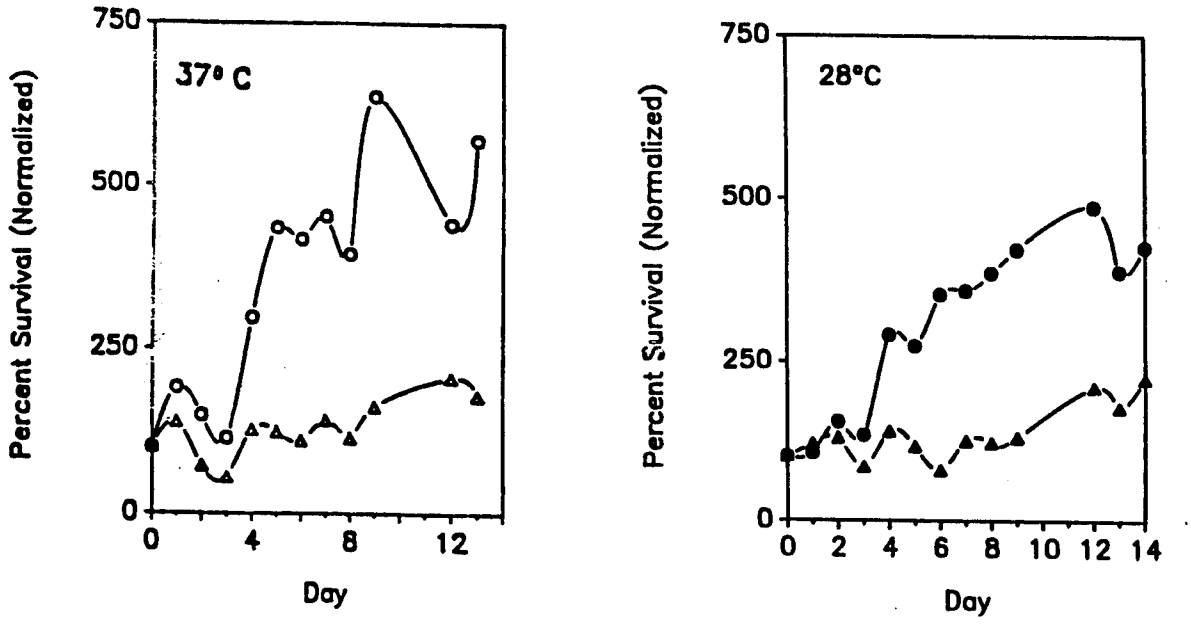

Figure 17. Effect of Temperature on Growth of Fibroblasts Exposed to SM. Cells grown in 12-well plates were exposed 1 day after plating to 0 μM (○, ●) or 5 μM (△, ▲) SM at room temperature for 1 hr. They were then grown at 37°C (left panel) or held at 28°C for 1 day before being returned to 37°C (right panel).

Effect of Ciclopirox

As mentioned in the INTRODUCTION, an alternate method of arresting the cell cycle reversibly is through the use of chemicals like ciclopirox olamine (CPX). Figure 18 compares the growth of fibroblasts held in 10 μM CPX for 9 hr with the growth of control cells. CPX clearly slows the growth of these cells as measured by the trypan blue-exclusion assay.

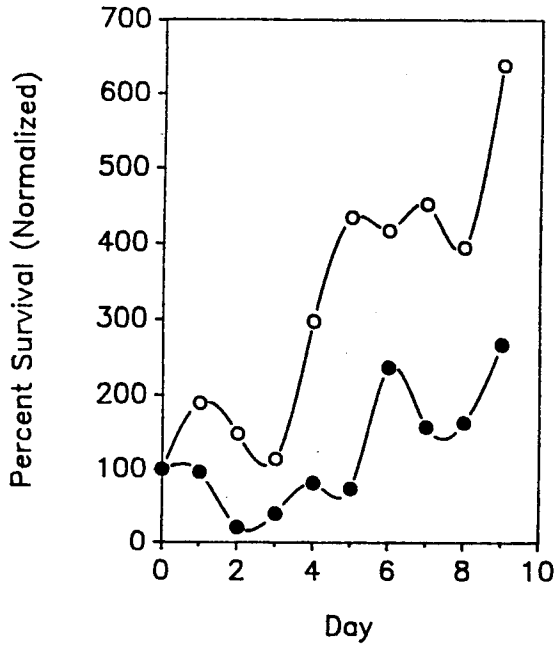

Figure 18. Effect of CPX on Growth of Human Fibroblasts. Fibroblasts plated at 2.2 x 10^5 cells/well in 12-well plates were exposed to 0 μM (○) or 10 μM (●) CPX for 9 hr and grown at 37°C. Survival was determined by the trypan blue test and expressed as a percentage of the trypan blue-excluding cells on day 0.

Figures 19 and 20 (next page) show that CPX does cause a reversible cell cycle arrest. Figure 19 compares the cell cycle distribution in a cell population held in 8 μM CPX for 6 hr with control cells at 6, 11, and 21 hr after the addition of CPX. As shown by the analyses in the bottom row, a 6-hr exposure to CPX results in a G_1/S arrest; however, 5 hr after the removal of CPX, at 11 hr from time zero, the cell cycle distribution resembles that of the control population. By 21 hr, the distribution is almost indistinguishable from that of the control. Thus, we conclude that the use of CPX is an alternate method of a reversible cell cycle arrest. Figure 20 shows that cells held for 11 hr in 8 μM CPX also recover by 21 hr after the beginning of the experiment. We are currently investigating whether or not this arrest allows time for additional DNA repair and recovery from SM exposure.

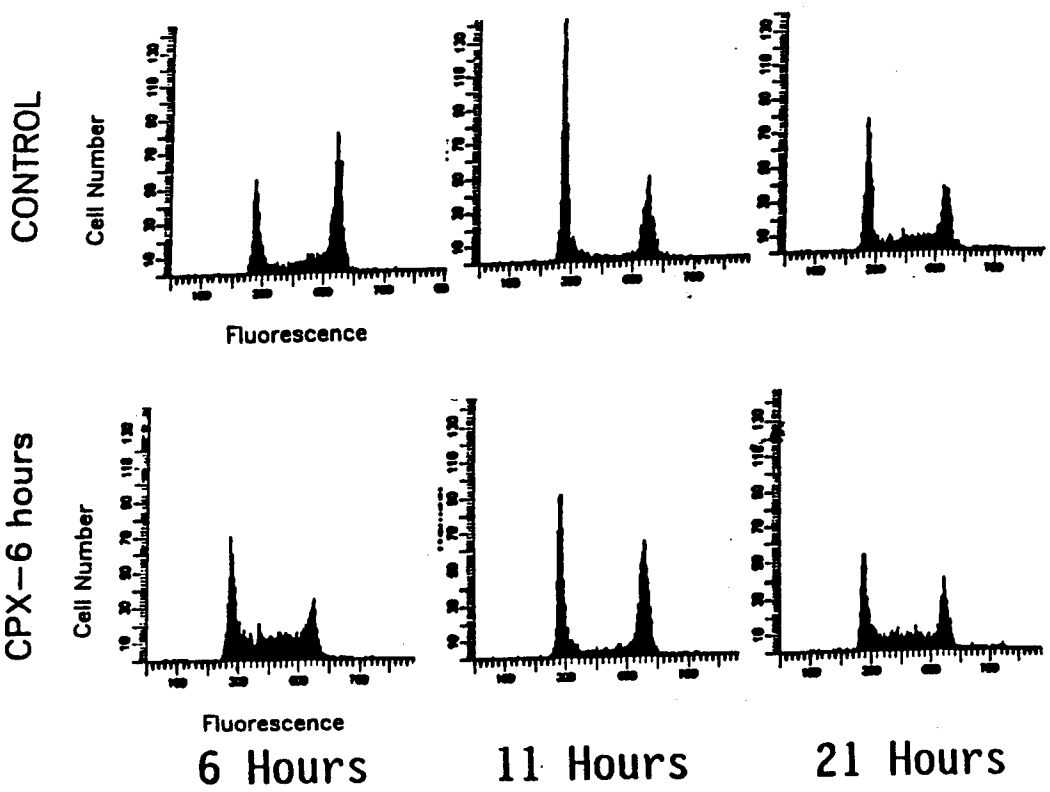

Figure 19. Effect of 6-Hour CPX Treatment on Cell Cycle Distribution. Cell cycle analysis was performed on control cells (top row) and cells held for 6 hr in 8 μM CPX (bottom row) at 6, 11, and 21 hr after the addition of CPX.

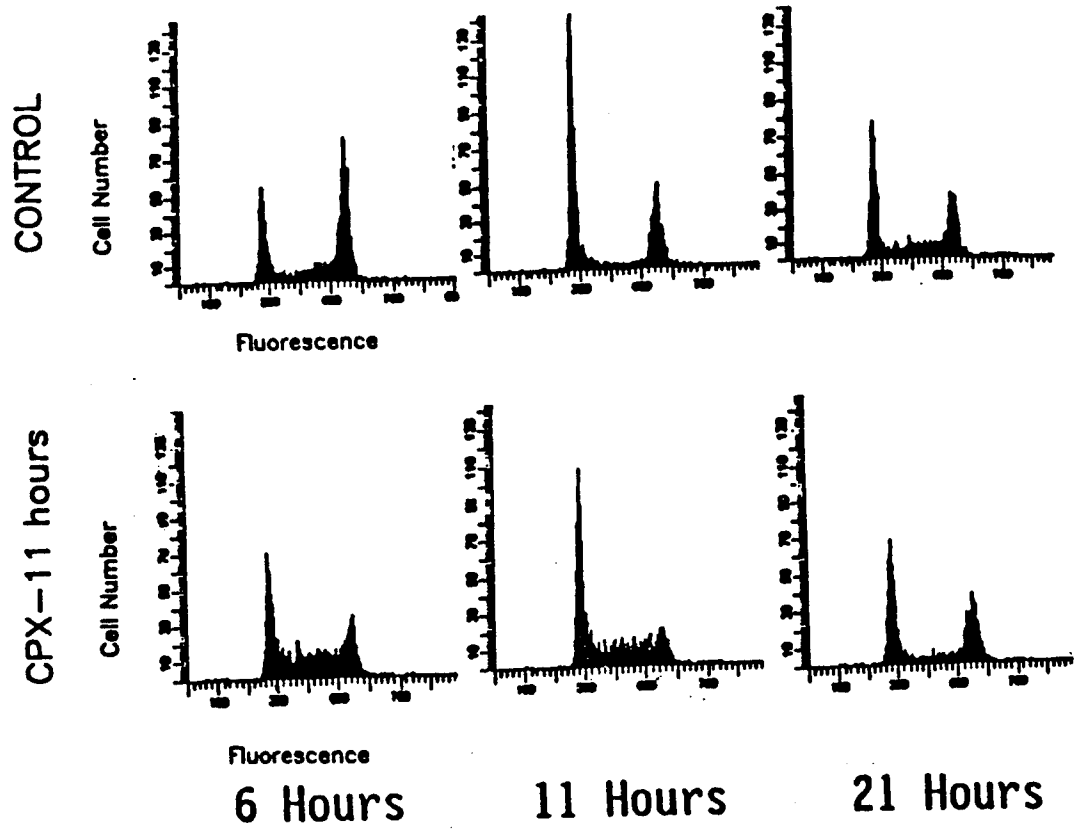

Figure 20. Effect of 11-Hour CPX Treatment on Cell Cycle Distribution. Cell cycle analysis was performed on control cells (top row) and cells held for 11 hr in 8 μM CPX (bottom row) at 6, 11, and 21 hr after the addition of CPX.

Studies with NM-1 Keratinocytes

Effect of Sulfur Mustard on Cell Survival

With our success in growing NM-1 keratinocytes under defined media conditions, similar experiments have been performed with these cells. Figure 21 shows the increase in number of trypan blue-excluding cells exposed to 1 or 5 μM SM in comparison with control cells. Cells exposed to 1 μM SM show only a short-term delay in growth, while cells exposed to 5 μM SM seem to be arrested for at least 2 weeks. However, when the total fluorescence, as a measure of DNA content, is determined as shown in Figure 22 (next page), even the cells exposed to 5 μM SM show an increase. We assume that, as is the case of fibroblasts, this represents "unbalanced" cellular growth due to the G_2 arrest, and we are investigating this phenomenon further.

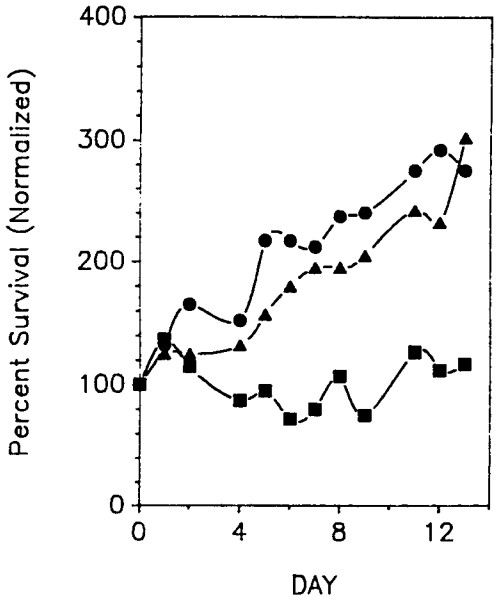

Figure 21. Cytotoxicity of Sulfur Mustard for Human NM-1 Keratinocytes as Measured by the Trypan Blue Method. Cells grown as described in METHODS were exposed 2 days after plating to 0 μM (●), 1 μM (▲), and 5 μM (■) SM for 1 hr and then incubated at 37°C. Survival was determined by the trypan blue test and expressed as a percentage of the trypan blue-excluding cells on the day of treatment.

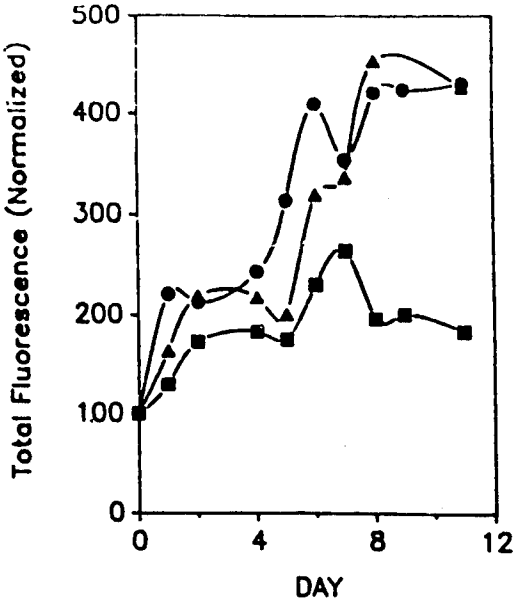

Figure 22. Cytotoxicity of SM for Human NM-1 Keratinocytes as Measured by DNA Fluorescence. Cells were grown and exposed 2 days after plating to 0 μM (●), 1 μM (▲), and 5 μM (■) SM for 1 hr and then incubated at 37°C as in Figure 20. The results are expressed as a percentage of the fluorescence on the day of treatment.

Effect of Temperature on Growth of NM-1 Keratinocytes

The effect of temperature on the cytotoxicity of mustard for keratinocytes is shown in Figure 23 (next page). Cells held for 1 day at 28°C after exposure to 5 μM CEES (right panel) again grow as well or perhaps better than cells returned immediately to the 37°C incubator (left panel). Especially in comparison with the growth of control cells held for 1 day at 28°C before being returned to the 37°C, this short period of hypothermia seems to have decreased the toxicity of CEES slightly. Since CEES is a monofunctional agent, however, these data may not be representative of SM which can form cross-links. These experiments were performed before our laboratory was certified for the use of SM, and are currently being repeated with this agent.

Reversible cell cycle arrest will result in decreased cytotoxicity if recovery occurs before cell division is re-initiated. Since we believe that glycosylase action contributes to the repair of SM-induced DNA modification, it is important to show that the activity of this enzyme is not decreased by the modalities used to produce the reversible cell cycle arrest. The data in Table 3 (next page) show that glycosylase does, indeed, retain its activity at 28°C or in the presence of as much as 100 μM CPX. Thus, we believe that glycosylase activity would be unimpaired during the reversible cell cycle arrest phase.

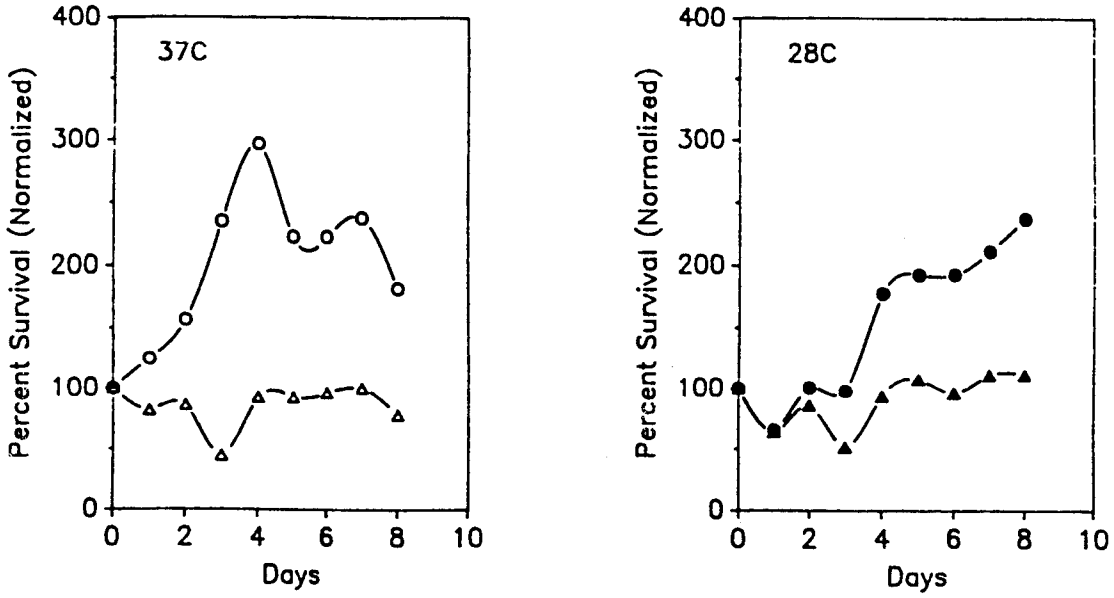

Figure 23. Effect of Temperature on Growth of NM-1 Keratinocytes Exposed to CEES. Cells grown in 12-well plates were exposed 1 day after plating to 0 μM (○, ●) or 5 μM (△, ▲) CEES at room temperature for 1 hr. They were then grown at 37°C (left panel) or held at 28°C for 1 day before being returned to 37°C (right panel).

Table 3. Effect of Temperature and Ciclopirox Olamine (CPX) on Human Glycosylase Activity

Temperature (°C)	CPX (μM)	Enzyme Activity (cpm released)
37	0	1283
28	0	1153
37	1	1253
37	10	1353
37	100	1283

DISCUSSION

The results presented above provide excellent support for the proposal that progression through the cell cycle can be reversibly arrested by relatively non-toxic modalities to provide time for DNA repair. Our data suggest that glycosylase action plays a role in this repair and that its action is not inhibited by hypothermia or CPX, two modalities that arrest cells reversibly. At the same time, the ^{32}P-postlabeling method provides a tool for determining the extent of DNA damage by SM and for monitoring the repair of this damage during cell cycle arrest.

The following sections discuss the analysis of SM-induced DNA modifications, the repair of these modifications, and the possibility of reducing cytotoxicity by allowing further time for DNA repair.

^{32}P-Postlabeling Analysis of SM-induced DNA Modifications

The ^{32}P-postlabeling method for detecting SM-induced damage to DNA has been greatly improved and is now capable of measuring a single HETEpdG in 10^7 normal nucleotides as shown in Figure 7. Critical steps include performing the enzymatic digestion at 10°C, including BudGp from the beginning of the analysis as an internal standard, and using disposable anion columns in early separation steps. The sensitivity which has been achieved is more than adequate for measuring the levels of DNA damage encountered at minimally cytotoxic concentrations of SM.

As shown in Figure 8, we detected 1.8 HETEpdG in 10^6 nucleotides in the DNA of fibroblasts exposed to 5 μM SM. This value can be compared with that obtained by van der Schans and colleagues (30) on studies of DNA modification in whole blood exposed to SM. They report 48 HETEpdG/10^6 in the DNA of white cells exposed to 10 μM SM. This difference is probably explained by differences in the biological system examined and by the fact that the tissue culture cells were exposed at room temperature whereas blood was exposed at 37°C. Further comparison will be possible as more data are accumulated by both methods. Recent cytometric data from our laboratory indicate that concentrations of SM even lower than 5 μM produce biological effects, and the ^{32}P-postlabeling method will allow damage to be monitored at these lower levels.

The ^{32}P-postlabeling method can also be used to measure glycosylase action *in vitro* without the use of radiolabeled SM. Similar analyses can be performed on DNA extracted from cells exposed to SM, and the resulting data can be used to evaluate methods of enhancing this repair.

Other DNA modifications, particularly guanine-guanine cross-links and possible O^6-substituted guanines, are probably of more biological significance than the monosubstituted adduct, HETEpdG. In order to measure these other adducts, it is necessary to synthesize the corresponding deoxyguanylic acid markers. We believe that direct reaction of SM with pdG may yield the required cross-link marker, but these were not obtained when the reaction was run in aqueous solution. We will repeat the reaction in an organic solvent using pdG solubilized by the presence of a hydrophobic cation.

As indicated in the RESULTS section, we have been successful in the synthesis of O^6-hydroxyethylthioethyldeoxyguanosine. This nucleoside will be converted to the required 5'-phosphate by the method that we used previously for the synthesis of O^6-methyldeoxyguanosine-5'-phosphate (31).

Since O^6-ethylthioethylguanine has been found in DNA as a product of CEES reaction, we expect to find the corresponding adduct, O^6-hydroxyethylthioethylguanine, in DNA exposed to SM. As shown in Figure 5, this product is obtained when SM is reacted with monomeric deoxyguanosine.

Repair of SM-induced DNA Modifications

Previous studies have shown that the CEES modification, 7-ethylthioethylguanine, is released from DNA by glycosylase action (10). As shown in Figure 9, we now have evidence that bacterial Gly II releases 7-hydroxyethylthioethylguanine from SM-modified DNA. These studies provide strong support for the hypothesis that glycosylase action plays a role in protecting cells from the cytotoxic action of SM. A similar protective role for glycosylase has been demonstrated against the cytotoxic action of the antitumor agent, chloroethylnitrosourea (32).

Although studies with bacterial enzymes can provide important clues as to what is occuring in eukaryotic systems, it is obviously important to study the human enzyme. Consequently, we have emphasized studies of the human glycosylase which has been cloned by Professor Leona Samson.

It is important to perform glycosylase studies with purified enzymes because the combination of endonuclease action and chemical instability can cause artifacts in these experiments. Our progress towards the goal of purification is shown in Figure 10 where we have obtained an excellent product; the next steps are to scale the preparation up and include a final FPLC purification step. Studies with the partially purified enzyme indicate that it does recognize SM-induced DNA modifications.

Cytotoxicity of SM

With the introduction of NM-1 keratinocytes into the laboratory, we now have a defined keratinocyte population for exploring the effects of SM. Based on trypan blue and cytometric analyses, this cell line appears to have approximately the same sensitivity towards SM as the fibroblast line (AG01522B). As shown in Figure 21, 5 μM SM results in nearly complete arrest of keratinocyte growth.

We have obtained very encouraging results on the ability of both hypothermia and CPX to produce a reversible cell cycle arrest. As shown in Figure 16, a temperature of 28°C holds fibroblasts in arrest, but they recover rapidly on transfer back to 37°C. Similarly, as shown in Figures 19 and 20, 8 μM CPX also produces a readily reversible cell cycle arrest although there is an indication that CPX itself may be cytotoxic. However, either one of these modalities would allow more time for DNA repair to occur before cell division takes place.

Importantly, the data shown in Table 3 indicate that neither of the two modalities interferes with the DNA repair activity of human glycosylase *in vitro*.

Cell cycle analyses (see Figure 14) have confirmed that low doses of SM cause a G_2 arrest (33). We believe that this type of analysis will prove to be very valuable in studying marginally damaged cells, especially those for which additional time for DNA repair might result in recovery.

In summary, we now have a sensitive analytical method that can be used to correlate DNA damage with biological effects. We have preliminary evidence that human glycosylase repairs SM-modified DNA and that this repair is not inhibited by either hypothermia or CPX, modalities which cause a reversible cell cycle arrest and may provide more time for DNA repair before cell division takes place. Cytometric analysis appears to be a highly useful tool for monitoring the biological effects for SM toxicity and can be used to monitor the biological success of interventions which may increase DNA repair.

CONCLUSIONS

The initial event that causes SM toxicity is evidently DNA alkylation. Since cells are capable of repairing DNA damage, we are investigating methods of enhancing repair as a means of decreasing toxicity. We believe that use of modalities which cause reversible cell cycle arrest may decrease toxicity by allowing more time for DNA repair before cell division takes place.

Two modalities have resulted in reversible cell cycle arrest: hypothermia and use of the antiproliferative agent, ciclopirox olamine (CPX). Neither modality interferes with the *in vitro* action of human glycosylase, an enzyme which takes part in DNA repair.

Previous data have shown that bacterial glycosylase releases the CEES adduct, 7-ethylthioethylguanine, from CEES-modified DNA; data reported here indicate that this enzyme also acts on the SM adduct, 7-hydroxyethylthioethylguanine. In order to investigate the action of the more relevant human glycosylase, we have purified the human enzyme from *E. coli* cells carrying a plasmid with the cloned human gene. A purification procedure has been developed that produces a highly purified but not quite homogeneous product. Partially purified human enzyme, like the bacterial enzyme, releases 7-hydroxyethylthioethylguanine from SM-treated DNA.

At the same time, we have developed ^{32}P-postlabeling as a highly sensitive method for detecting SM-induced damage to DNA. Use of this technique will provide a direct evaluation of protocols designed to protect cells from damage and/or enhance the subsequent repair of such damage.

We are currently investigating the protective effect of regimens which result in a reversible cell cycle arrest. We are exploiting our observations that both hypothermia and the use of CPX result in such a reversible arrest, and we will determine whether conditions can be found that significantly ameliorate toxicity. Efficacy will be evaluated by effects on cell survival as evaluated by trypan blue exclusion and by cell cycle progression. Increased DNA repair, which may correlate with decreased cytotoxicity, will be monitored by our highly sensitive ^{32}P-postlabeling technique.

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APPENDIX

List of Publications Supported by this Contract:

1. Yu, D., Niu, T.-q., Austin-Ritchie, P., and Ludlum, D. B. A ^{32}P-post-labeling method for detecting unstable N-7 substituted deoxyguanosine adducts in DNA. Proc. Natl. Acad. Sci. USA *91*, 7232-7236, 1994.

2. Ludlum, D. B., Austin-Ritchie, P., Hagopian, M., Niu, T.-q., and Yu, D. Detection of sulfur mustard-induced DNA modifications. Chem.-Biol. Interactions *91*, 39-49, 1994.

3. Yu, D., Niu, T.-q., Austin-Ritchie, P., and Ludlum, D. B. ^{32}P-Postlabeling of unstable deoxyguanosine adducts in DNA. Proc. Am. Assoc. for Cancer Res. *35*, 99, 1994.

4. Matijasevic, Z., Niu, T.-q., Austin-Ritchie, P., and Ludlum, D. B. Release of the sulfur mustard DNA adduct, 7-hydroxyethylthioethylguanine, by 3-methyladenine DNA glycosylase II. In preparation.

5. Niu, T.-q., Matijasevic, Z., Austin-Ritchie, P., and Ludlum, D. B. Application of a ^{32}P-postlabeling method for detecting unstable DNA adducts in tissue culture samples. In preparation.

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