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# Cross-Linking Interferes with Assessing Sulfur Mustard-Induced DNA Damage in Human Peripheral Blood Lymphocytes Using the Comet Assay

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Sulfur mustard (SM) is a blistering agent that produces DNA strand breaks. To detect SM-induced DNA single strand breaks in human peripheral blood lymphocytes (PBL), cells were exposed to various concentrations of SM (10 to 1000  $\mu\text{M}$ ), and the comet assay (single-cell gel electrophoresis) was performed. We observed a SM concentration- and time-dependent increase in detectable DNA damage. To test whether SM-induced DNA cross-linking inhibits DNA migration in the comet assay, PBL were exposed to a) SM alone (10 to 1000  $\mu\text{M}$ ), b)  $\text{H}_2\text{O}_2$  (0.001%), which produces DNA single strand breaks with no cross-links, or c) SM followed at 2, 4, or 6 h by  $\text{H}_2\text{O}_2$ . With  $\text{H}_2\text{O}_2$  alone, a large amount of strand breakage was detected. With  $\text{H}_2\text{O}_2$  plus SM, detectable  $\text{H}_2\text{O}_2$ -induced strand breaks decreased as SM concentration increased up to 30  $\mu\text{M}$ ; at 30  $\mu\text{M}$  and above, the response with  $\text{H}_2\text{O}_2$  plus SM was similar to that with SM alone. Interference with the detection of  $\text{H}_2\text{O}_2$ -induced DNA strand breaks appears to be SM concentration-dependent up to 30  $\mu\text{M}$ , and independent of SM concentration at  $\geq 30 \mu\text{M}$ . This is presumably due to SM-induced cross-linking. It follows that cross-linking in DNA of SM-exposed PBL also interferes with DNA migration and detection of DNA strand breaks when cells are exposed to SM alone.

**Keywords** Comet Assay, DNA Cross-Linking, DNA Damage, Lymphocytes, Sulfur Mustard

Sulfur mustard (SM) is a cytotoxic vesicant chemical warfare agent, or “blister agent,” that produces severe lesions on exposed epithelial tissues. There is, to date, no effective antidote for SM. The most sensitive target of SM is cellular DNA. SM preferentially alkylates at the N-7 position of guanine or the

N-3 position of adenine, and alkylation leads to depurination of DNA strands. Subsequent breakage of phosphodiester bonds at the apurinic sites produces DNA single-strand breaks (reviewed in Papirmeister et al. 1991). To repair DNA breaks, the resulting gap in the DNA chain is filled with appropriate bases (DNA polymerization), and these added bases are then ligated to complete the DNA strand repair.

Interstrand or intrastrand DNA cross-links can form when a guanine-alkyl-guanine adduct is produced by the alkylation of 2 guanines on opposite strands or 2 guanines on the same strand, respectively (Reid and Walker 1969; Walker 1971). In DNA isolated from SM-exposed cells, approximately 75% of these cross-links are intrastrand (Walker 1971). The excision rate of SM-induced DNA cross-links is logarithmic with a half-life of approximately 2 h; a rapid exponential reduction in cross-links occurs between 1 and 6 h after SM exposure (Reid and Walker 1969; Walker and Reid 1971).

We used the comet assay, also known as single-cell gel electrophoresis, to detect DNA single-strand breaks in SM-exposed human peripheral blood lymphocytes (PBL). The comet assay is a fast, efficient, and very sensitive method for detecting DNA single-strand breaks in individual cells (Singh et al. 1988; de With et al. 1994; Slamenová et al. 1997). This assay can be used to monitor the rate of DNA repair by measuring the disappearance of DNA single-strand breaks over time (Fairbairn et al. 1995).

To perform the comet assay, nucleated cells are embedded in agarose gel on microscope slides and lysed by detergents at high salt concentrations. The cellular DNA is treated with a strong alkaline buffer to produce single-stranded DNA, electrophoresed under alkaline conditions, and stained with a fluorescent dye for visualization. Individual cells are analyzed with a fluorescence microscope and image analysis system. DNA of an undamaged cell appears as a spherical mass occupying the cavity formed by the lysed cell. When DNA is damaged, the negatively charged pieces of DNA are free to migrate in the electric field toward the anode and are seen streaming from the spherical mass (the

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comet head) in the shape of a comet tail (Kent et al. 1995). Single-stranded DNA migrate according to molecular length (Freeman et al. 1986), with shorter DNA (that with more damage) migrating further from the nucleus than longer DNA (that with less damage). The comet moment (product of comet tail length and intensity of the tail) describes the relative amount of DNA strand breakage in a cell, and it has been correlated quantitatively with the frequency of DNA strand breaks (Olive and Banath 1993). The sensitivity of the comet assay is 0.1 break per  $10^9$  daltons, or 6.6 breaks per average chromosome, in UV-C damaged cells (Gedik et al. 1992).

Cross-linking agents can reduce or entirely inhibit detection of DNA damage in the comet assay by inhibiting DNA migration through the agarose gel. Known cross-linking agents such as nitrogen mustard (a compound closely related to sulfur mustard), cisplatin, and mitomycin C have been reported to reduce, in a concentration-dependent manner, DNA migration in cells containing DNA single-stranded breaks (Olive et al. 1992; Olive and Banath 1995; Pfuhrer and Wolf 1996).

In this study, we found that the comet assay did not detect DNA damage in PBL at the low SM concentrations or the early time points following exposure as reported using alternative methods (2 h after exposure to 300  $\mu$ M SM or 24 h after exposure to 6  $\mu$ M SM; Meier and Millard 1998). We hypothesized that SM-induced DNA cross-linking inhibits DNA migration in the comet assay, resulting in decreased detection of DNA strand breaks. We tested our hypothesis by determining whether SM interferes with the migration of DNA from PBL exposed to  $H_2O_2$ , an oxidizing agent that produces a random distribution of DNA single-strand breaks with no cross-links (Szmigiero and Studszian 1988).

## MATERIALS AND METHODS

### PBL Isolation

Human peripheral blood lymphocytes were acquired from normal adult volunteers under an approved human use protocol. PBL were isolated from whole blood ( $\leq 200$  ml) by differential centrifugation on a Percoll gradient (Pharmacia Biotech, Piscataway, NJ, USA; density = 1.080 g/ml at 20°C) (Meier et al. 1987). Cells were washed twice with calcium-free and magnesium-free Tyrode's buffer and once with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 0.05 mg gentamicin/ml. Between each wash, the cells were centrifuged at  $900 \times g$  for 20 min at 20°C. The number of PBL obtained was determined by counting the cells on a hemocytometer. Following the final centrifugation, the isolated PBL were resuspended at  $2 \times 10^7$  cells/ml in RPMI 1640 medium. The cells were then dispensed into 24-well tissue culture plates to yield a final concentration of  $2-3 \times 10^6$  cells/well.

### SM and $H_2O_2$ Treatment of PBL

Sulfur mustard with a purity of  $\geq 98\%$  was obtained from the Edgewood Chemical and Biological Center (Aberdeen Proving

Ground, MD, USA). Stock solutions of SM were prepared as 4 mM solutions in RPMI 1640 medium.

PBL were exposed to seven SM concentrations ranging from 10 to 1000  $\mu$ M by diluting stock SM in RPMI 1640 medium and immediately adding aliquots to PBL cultures (Meier and Johnson 1992). The PBL were exposed to SM directly after plating into 24-well plates. Final volume was 1 ml/well.

To allow venting of volatile SM and permit its complete hydrolysis, control and exposed cells were incubated at room temperature (20 to 22°C) for 1 h after exposure in a fume hood. Following room temperature incubation, the lymphocytes were transferred to a 37°C, 5%  $CO_2$ -95% air incubator until harvested.

PBL were exposed to a) SM alone, as described, b)  $H_2O_2$  (0.001%) for 5 min at 2, 4, or 6 h after PBL isolation, or c) SM followed at 2, 4, or 6 h by a 5-min exposure to 0.001%  $H_2O_2$ . Unexposed cells served as the negative control for DNA damage.

### Harvesting PBL

PBL exposed to SM alone were harvested at 2, 4, or 6 h after exposure. PBL exposed to  $H_2O_2$  or to SM plus  $H_2O_2$  were harvested at 5 min after  $H_2O_2$  exposure. After triturating several times, the PBL were suspended in RPMI 1640 medium and transferred from each well of the 24-well plate into individual 1.5-ml microcentrifuge tubes. The cells were centrifuged at  $3500 \times g$  for 5 min at 4°C and resuspended in 600  $\mu$ l RPMI 1640 medium.

### Viability Determination

After harvesting the cells, a 200- $\mu$ l aliquot from each PBL sample was dispensed into a 12  $\times$  75 mm test tube and diluted by the addition of 200  $\mu$ l of RPMI 1640 medium to each tube. Then 50  $\mu$ l of propidium iodide (Sigma Chemical Company, St. Louis, MO, USA; dissolved in RPMI 1640 medium at 0.3 mg/ml) was added to each tube containing PBL. After a 2- to 5-min room temperature incubation in the dark, propidium iodide incorporation into the cells was assessed using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with the Lysis II computer software program (Becton Dickinson), and viability results were expressed as the percent of the total number of cells that excluded propidium iodide (Smith et al. 1991; Clayson et al. 1993).

### Comet Assay

The comet assay was performed as described (Singh et al. 1988) with some modifications. Fully frosted microscope slides (Fisher Scientific, Pittsburgh, PA, USA) were covered with a base layer of 1% normal melting agarose (Type I; Sigma Chemical Company, St. Louis, MO, USA) in phosphate buffered saline (PBS; 0.15 M NaCl, 3 mM  $Na_2HPO_4$ , both from Sigma; pH 7.4), and the slides were completely air dried at room temperature. A sample of the PBL suspension harvested from one well of the 24-well plate was diluted 1:10 in 1% low melting agarose

(Sea Plaque, FMC Bioproducts, Rockland, ME, USA) in PBS. Then 75  $\mu$ l of the cell-agarose mixture was added as a second layer onto the microscope slide and covered with a No. 1 coverslip (Corning, Inc., Horseheads, NY, USA; 24  $\times$  60 mm). The agarose was allowed to solidify on the slides at room temperature for approximately 15 min. After removing the coverslip, a third layer of agarose was formed on each slide by the addition of 100  $\mu$ l of 1% low melting agarose. The coverslip was immediately replaced, and the agarose was allowed to solidify at 4°C for at least 5 min.

To lyse the cells, the coverslips were removed, and the slides were submerged in cold (4°C) lysis solution (2.5 M NaCl, 100 mM EDTA, 1% N-lauroylsarcosine, 10 mM Tris base, pH 10, and 1% Triton X-100 added fresh, all from Sigma) and incubated at 4°C for a minimum of 1 h and a maximum of 3 days. The lysis solution was then poured from the slides. To denature the DNA, the slides were submerged in cold (4°C) electrophoresis solution (300 mM NaOH, 1 mM EDTA, both from Sigma; pH  $\geq$  13) and incubated at 4°C for 40 min. The slides were then placed randomly side by side in a horizontal electrophoresis chamber (Fisher Scientific, Fisher Biotech Recirculating Large Horizontal System #FB-SBR-2025). The chamber was filled with cold (4°C) electrophoresis solution, and electrophoresis proceeded for 40 min at 25 V and 300 mA (Electrophoresis Power Supply, EPS 600; Pharmacia Biotech, Piscataway, NJ, USA). Up to this point, all steps of the comet assay were performed under dim light to minimize artificial induction of DNA single strand breaks.

Following electrophoresis, the slides were rinsed four times with neutralization solution (0.4 M Tris, pH 7.5). If necessary, the slides were stored in neutralization solution in sealed containers at 4°C for up to 1 week. After draining the neutralization solution from the slides, 100  $\mu$ l of 4  $\mu$ g/ml ethidium bromide (Sigma) was pipetted onto each slide to stain the DNA, and a new coverslip was applied. After 5 min, the coverslips were removed, each slide was rinsed with a small amount of deionized distilled water, 2 additional drops of 4  $\mu$ g/ml ethidium bromide were pipetted onto each slide, and new coverslips applied. If necessary, the slides were stored in air-tight containers for up to 3 days before analysis.

The ethidium bromide-stained DNA was visualized using a fluorescence microscope equipped with a 515–560 nm excitation filter and a 590 nm barrier filter, connected through a gated CCD camera to a computer-aided image analyzer. Komet 3.1 imaging software (Kinetic Imaging Ltd., Liverpool, UK) was used for image analysis. Results were expressed as the comet moment (product of comet tail length and intensity of the tail).

### Data Analysis

Data are summarized as mean  $\pm$  SEM of comet moment from 5 separate experiments that were conducted independently. In each experiment and for each PBL sample, 2 slides were prepared, and 25 cells/slide were selected at random for analysis using the comet assay.

Statistical analyses were conducted using the SigmaStat, version 2.0 computer program (Jandel Scientific Corporation, San Rafael, CA, USA). First, a three-way analysis of variance (ANOVA) was used on all the data to determine whether there were significant differences in comet moment a) between treatments (cells exposed to SM alone, H<sub>2</sub>O<sub>2</sub> alone, or SM plus H<sub>2</sub>O<sub>2</sub>), b) between time points following SM exposure, and c) between cells exposed to different concentrations of SM. Significant two-way interactions were observed, and a two-way ANOVA was therefore performed on each treatment group, comparing the comet moment at different times following SM exposure and the comet moment of cells exposed to different SM concentrations. Significant two-way interactions in each treatment group were observed, and a one-way ANOVA was subsequently used to assess differences in comet moment between SM concentrations at each time point for each treatment group. A one-way ANOVA was again used to determine whether there were differences in comet moment between different time points following SM exposure at each SM concentration for each treatment group. A one-way ANOVA was also used to assess differences in comet moment between treatments at each SM concentration and each time point. When a significant difference was found, Newman-Keuls multiple comparison method was used to identify pairwise differences. Differences were considered significant at  $p < 0.05$ .

For viability determinations, each PBL sample was assayed in triplicate.

## RESULTS

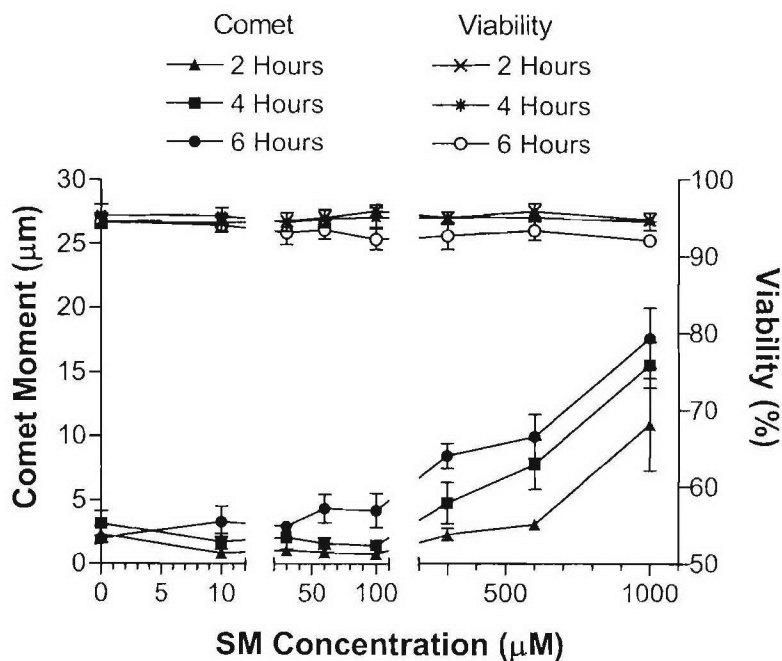
### DNA Damage Detected in PBL Exposed to SM Alone

PBL exposed to SM demonstrated a SM concentration- and time-dependent increase in detectable DNA damage (Fig. 1). Compared with control cells, a significant increase in DNA damage was detected at SM concentrations  $\geq 600$   $\mu$ M at 2 h,  $\geq 300$   $\mu$ M at 4 h, and  $\geq 60$   $\mu$ M at 6 h.

The amount of DNA single strand breaks observed in control cells was minimal (Figs. 1 and 3) and did not significantly change over the 6 h of incubation. Although not statistically significant, a consistent pattern was observed throughout all of the 2 and 4 h experiments in which DNA single strand breakage measured in control cells was higher than that measured in cells exposed to SM concentrations  $\leq 100$   $\mu$ M. At 6 h, DNA single strand breakage detected at  $\leq 100$   $\mu$ M SM was equivalent to that of control cells.

Detectable DNA damage did not differ significantly between the 2- and 4-h time points in cells exposed to  $\leq 100$   $\mu$ M SM; at 6 h, detectable DNA damage was significantly higher than that measured at 2 or 4 h after exposure to  $\geq 60$   $\mu$ M SM. However, there was no significant difference between the 4- and 6-h time points in strand breakage detected following exposure to 600 or 1000  $\mu$ M SM.

Over 6 h following SM exposure, viability remained above 90%.



**FIG. 1.** Effect of sulfur mustard (SM) concentration on detectable DNA damage and on viability over time following SM exposure. Peripheral blood lymphocytes (PBL) were exposed to buffer (control) or to buffer plus the indicated SM concentrations, harvested at 2, 4, or 6 h after exposure, and the comet assay then performed. For each PBL sample, slides were prepared in duplicate, and 25 cells/slide were analyzed. Data are mean  $\pm$  SEM of comet moment of 5 separate experiments (250 comets analyzed for each data point). There was a SM concentration- and time-dependent increase in the detectable amount of DNA single strand breakage in PBL exposed to  $\geq 600 \mu\text{M}$  SM at 2 h,  $\geq 300 \mu\text{M}$  SM at 4 h, and  $\geq 60 \mu\text{M}$  SM at 6 h. Viability was  $>90\%$  at all SM concentrations and time points tested.

#### DNA Damage Detected in PBL Exposed to $\text{H}_2\text{O}_2$ Alone

DNA damage in  $\text{H}_2\text{O}_2$ -exposed cells was approximately 11 times larger than that in control cells (Fig. 2A–C;  $0 \mu\text{M}$  SM). There was no significant difference in DNA damage measured in cells exposed to  $\text{H}_2\text{O}_2$  at 2, 4, or 6 h after PBL isolation (Fig. 2A–C).

The amount of DNA single strand breaks observed in cells exposed to  $\text{H}_2\text{O}_2$  alone was significantly larger than that observed in SM- or SM/ $\text{H}_2\text{O}_2$ -exposed cells at all time points and all SM concentrations tested (Fig. 2A–C). At 4 and 6 h after SM exposure, detectable DNA damage averaged 1.7 times larger for cells exposed to  $\text{H}_2\text{O}_2$  alone than for cells exposed to  $1000 \mu\text{M}$  SM, with or without  $\text{H}_2\text{O}_2$ ; it was 2.6 times larger at 2 h after exposure.

Viability of cells exposed to  $\text{H}_2\text{O}_2$  alone at 2, 4, or 6 h after PBL isolation remained above 90% and averaged 94% at each time point.

#### DNA Damage Detected in PBL Exposed to SM Followed by $\text{H}_2\text{O}_2$

As SM concentration increased, detectable DNA damage in  $\text{H}_2\text{O}_2$ -exposed cells decreased up to  $30 \mu\text{M}$  SM at each time point (Fig. 2). At SM concentrations  $\geq 30 \mu\text{M}$ , strand breakage measured in cells exposed to both SM and  $\text{H}_2\text{O}_2$  was not significantly different from that in cells exposed to SM alone. The amount of measurable DNA damage in cells exposed to  $\text{H}_2\text{O}_2$  increased over time following SM exposure and with increasing SM concentrations  $\geq 300 \mu\text{M}$ .

In photomicrographs, control cells appeared to show the least amount of DNA damage, followed by cells exposed to SM alone and cells exposed to both  $\text{H}_2\text{O}_2$  and SM (Fig. 3). The most damage appeared in those cells exposed to  $\text{H}_2\text{O}_2$  alone.

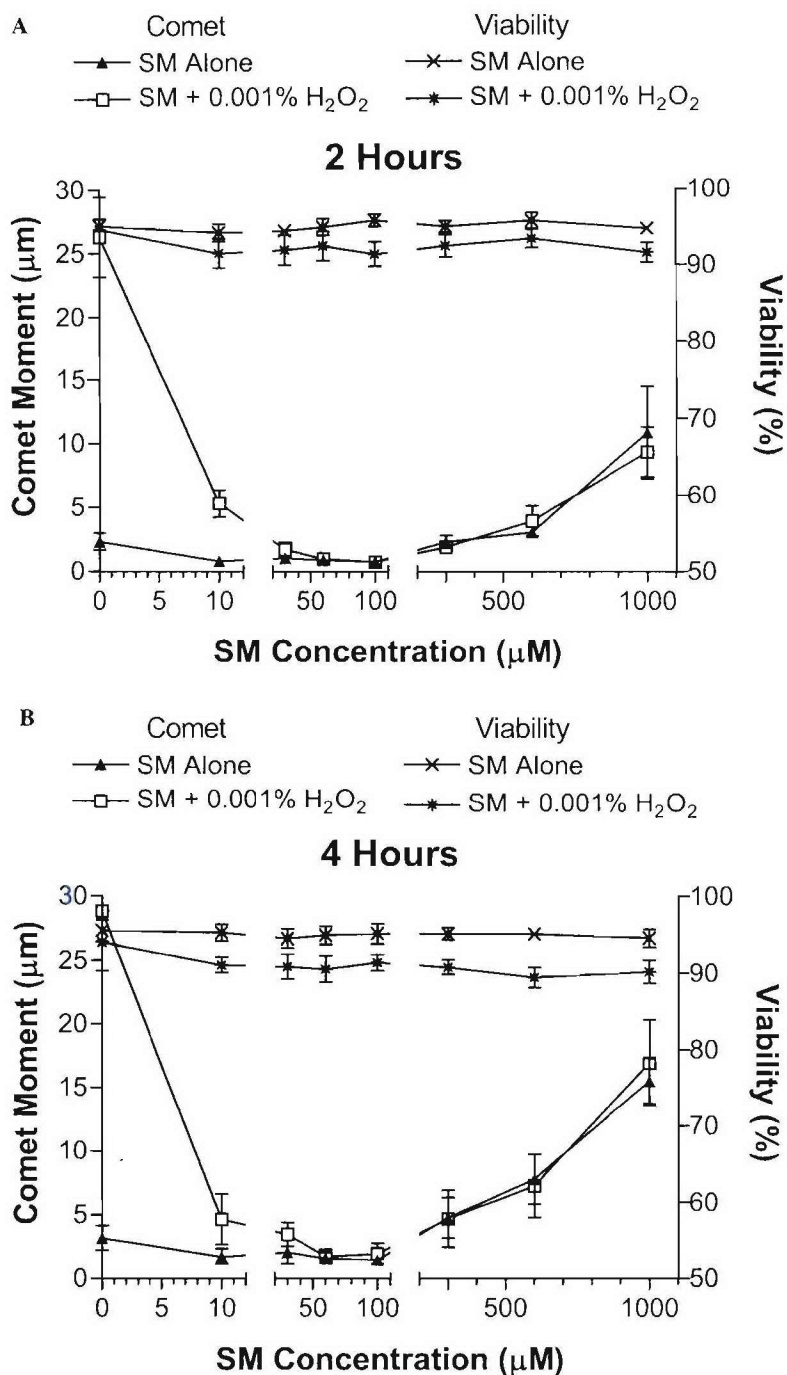
The viability of cells exposed to both SM and  $\text{H}_2\text{O}_2$  remained above 85% at all time points and SM concentrations tested.

#### DISCUSSION

This study shows that, in the comet assay, SM interferes with DNA migration and detection of DNA single strand breaks. This is presumably due to the formation of SM-induced DNA cross-links.

It appears that DNA cross-linking is SM concentration-dependent up to  $30 \mu\text{M}$ , with maximum DNA cross-linking occurring with an exposure between 10 and  $30 \mu\text{M}$  SM. This is suggested by the fact that, up to  $30 \mu\text{M}$  SM, there is a SM concentration-dependent decrease in the detection of  $\text{H}_2\text{O}_2$ -induced DNA single strand breaks at all time points tested. Similar results were obtained in an earlier study in which cross-linking was detected in the DNA of SM-exposed rat cutaneous epidermal keratinocytes using an alkaline DNA unwinding assay (Sorscher and Conolly 1989). These investigators found that DNA cross-linking did not increase at SM concentrations higher than  $5 \mu\text{M}$ . The lowest SM concentration at which maximum DNA cross-linking occurs may depend upon cell type.

At SM concentrations  $\geq 30 \mu\text{M}$ , detection of DNA single strand breaks produced by SM plus  $\text{H}_2\text{O}_2$  is no different from that



**FIG. 2.** Effect of sulfur mustard (SM) concentration on detectable DNA damage and on viability in H<sub>2</sub>O<sub>2</sub>-exposed cells. Peripheral blood lymphocytes (PBL) were exposed to either buffer (control); buffer plus a 5-min exposure to 0.001% H<sub>2</sub>O<sub>2</sub> at 2, 4, or 6 h after PBL isolation; buffer plus the indicated SM concentrations; or buffer plus the indicated SM concentrations followed by a 5-min exposure to 0.001% H<sub>2</sub>O<sub>2</sub> at 2, 4, or 6 h after SM exposure. PBL exposed to SM alone were harvested at 2, 4, or 6 h after exposure. PBL exposed to H<sub>2</sub>O<sub>2</sub> alone or SM plus H<sub>2</sub>O<sub>2</sub> were harvested immediately after exposure to H<sub>2</sub>O<sub>2</sub>. Cells were analyzed with the comet assay. For each PBL sample, slides were prepared in duplicate, and 25 cells/slide were analyzed. Data are mean  $\pm$  SEM of comet moment of 5 separate experiments (250 comets analyzed for each data point). At all time points tested, the amount of DNA damage measured was significantly larger in cells exposed to H<sub>2</sub>O<sub>2</sub> alone than in SM- or SM/H<sub>2</sub>O<sub>2</sub>-exposed cells. In cells exposed to H<sub>2</sub>O<sub>2</sub> plus SM, there was a SM concentration-dependent decrease in the detectable amount of single strand breaks up to 30  $\mu$ M SM. At 30  $\mu$ M SM and above, the amount of DNA damage measured in cells exposed to H<sub>2</sub>O<sub>2</sub> plus SM was similar to that in cells exposed to SM alone. Viability of cells exposed to H<sub>2</sub>O<sub>2</sub> alone was  $>90\%$  at each time point. For cells exposed to H<sub>2</sub>O<sub>2</sub> plus SM, viability was  $>85\%$  at each time point and SM concentration tested. (Continued)

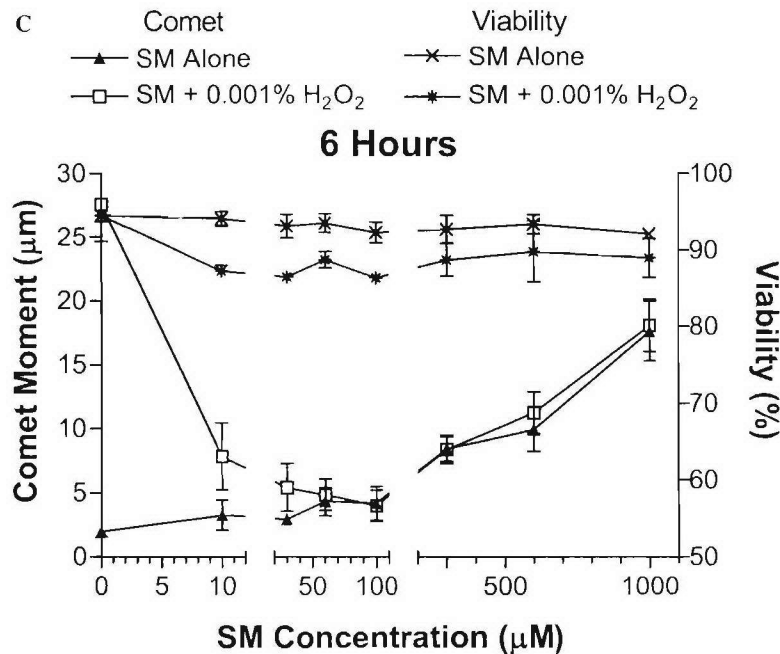


FIG. 2. Continued.

produced by SM alone. This indicates that detection of H<sub>2</sub>O<sub>2</sub>-damaged DNA is completely masked by SM-induced cross-links and that the concentration-dependent increase in detectable DNA damage observed at SM concentrations  $\geq 100$   $\mu$ M SM reflects only the damage produced by SM. At high concentrations of SM, the amount of DNA single strand breakage is apparently so great that the effect of cross-linking does not completely prevent detection of strand breaks. Therefore, although DNA damage produced by 1000  $\mu$ M SM appears lower than that produced by 0.001% H<sub>2</sub>O<sub>2</sub> alone, the amount of DNA damage produced by 1000  $\mu$ M SM may actually be close to the same or higher than that produced by 0.001% H<sub>2</sub>O<sub>2</sub>, the true amount of damage being concealed by the presence of SM-induced cross-links.

The migration of DNA from cells exposed to lower concentrations of SM in the absence of H<sub>2</sub>O<sub>2</sub> appears to be completely inhibited by DNA cross-linking. Compared with control PBL in which a very small amount of DNA single-strand breakage is expected (Jostes et al. 1989) and which is, in fact, detected with the comet assay, the DNA of cells exposed to lower concentrations of SM consistently demonstrates either less than or the same amount of DNA damage as control cells.

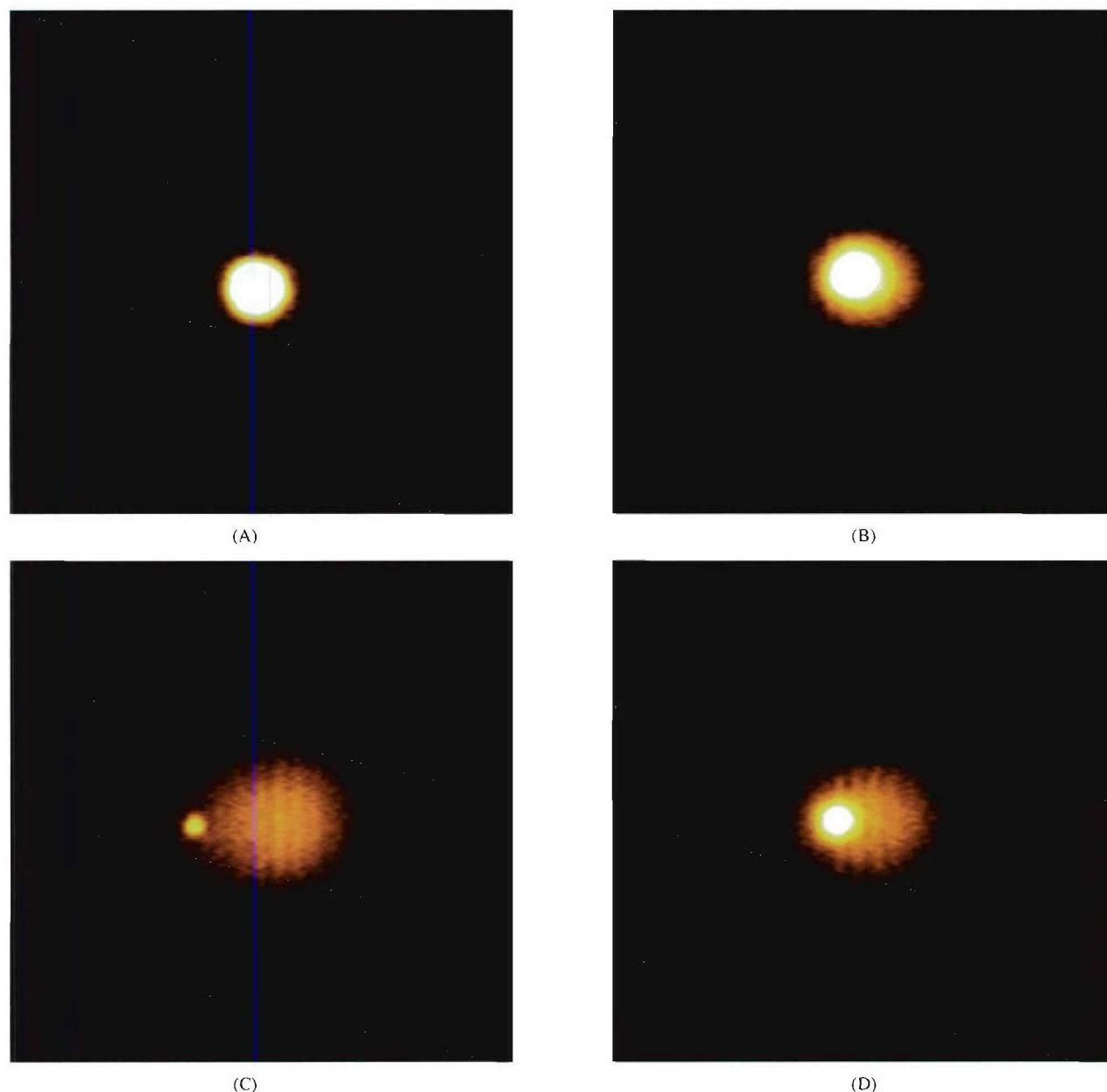
The time-dependent increase in detection of DNA single strand breaks following SM exposure may be due to an increase in the actual number of DNA single strand breaks produced by SM and/or an increase in the detectable number of DNA single strand breaks as a result of DNA cross-link repair. It is assumed that repair of both DNA single strand breaks and DNA cross-links occurs over time, which would either decrease or increase, respectively, the amount of DNA damage reflected in the comet assay. Since there is an overall increase in the amount of DNA damage detected over time, cross-link repair appears to take

place sooner and/or at a higher rate than strand break repair. Previous studies with rat cutaneous keratinocytes suggest that at low SM concentrations, cells are capable of completely repairing SM-induced DNA damage within 22 h following exposure, but at high concentrations the damage is so extensive that the cell loses its ability to completely repair damaged DNA (Ribeiro et al. 1991). Up to at least 6 h following SM exposure, the rate of DNA single strand break formation may be higher than the rate of DNA single strand break repair, which would also contribute to the overall effect.

At 6 h following SM exposure, detection of H<sub>2</sub>O<sub>2</sub>-induced DNA damage is prevented to a large extent by SM. This indicates that a significant number of DNA cross-links are still present at 6 h after SM exposure. Indeed, in studies using SM-treated human epidermal keratinocytes (Mol et al. 1993) and various types of mammalian cells treated with nitrogen mustard (Ewig and Kohn 1977; Ross et al. 1978; Murnane and Byfield 1981), it was observed that most SM- or nitrogen mustard-induced cross-links are repaired within 24 h after exposure; however, some cross-links are persistent and remain at 24 h, independent of the concentration of the agent. Evidence indicates that the ability to repair DNA cross-links may depend upon the phase in the cell cycle during which cells are exposed (Lin et al. 1996).

SM and H<sub>2</sub>O<sub>2</sub> concentrations employed in these experiments are not cytotoxic within the times to which the PBL were exposed. As previously reported (Henderson et al. 1998), to avoid false positive responses, the viability of cells used in the comet assay should exceed 75% since cytotoxins induce an increase in DNA migration when cell viability is  $\leq 75\%$ .

In the comet assay, SM-induced DNA cross-linking inhibits the migration of H<sub>2</sub>O<sub>2</sub>-damaged DNA at concentrations of SM



**FIG. 3.** Photomicrographs of comets from cells exposed to sulfur mustard (SM) and/or  $\text{H}_2\text{O}_2$ . Peripheral blood lymphocytes (PBL) were exposed to A) buffer (control), B) buffer plus  $1000 \mu\text{M}$  SM, C) buffer plus a 5-min exposure to  $0.001\%$   $\text{H}_2\text{O}_2$  at 2 h after PBL isolation or D) buffer plus  $1000 \mu\text{M}$  SM followed 2 h later by a 5-min exposure to  $0.001\%$   $\text{H}_2\text{O}_2$ . Cells exposed to SM alone were harvested at 2 h after exposure. Cells exposed to  $\text{H}_2\text{O}_2$  alone or SM plus  $\text{H}_2\text{O}_2$  were harvested immediately after  $\text{H}_2\text{O}_2$  exposure. The comet assay was then performed. For visualization of DNA damage, observations were made of ethidium bromide-stained DNA using a fluorescent microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Cells are magnified  $1000\times$ . Length and fluorescence intensity of the comet tail denote the relative amount of DNA single strand breaks (longer length and higher intensity indicate more DNA damage). The anode is to the right. There appeared to be less DNA damage in cells exposed to  $\text{H}_2\text{O}_2$  plus SM (D) than in those exposed to  $\text{H}_2\text{O}_2$  alone (C). Apart from the control (A), cells exposed to SM alone (B) showed the least amount of DNA damage.

at which there is no detectable DNA damage due to SM alone. This implies that even when no DNA migration is apparent in the comet assay, there is still significant SM-induced damage. This is in agreement with the findings of others who evaluated by electrophoresis total genomic DNA extracted from SM-exposed PBL (Meier and Millard 1998); DNA fragmentation

was detected as early as 2 h after exposure to  $300 \mu\text{M}$  SM. In another study, using the nucleoid sedimentation assay, it was demonstrated that within 1 h of exposure to as little as  $0.1 \mu\text{M}$  SM, the structural integrity of DNA from rat cutaneous keratinocytes was compromised, assumed to be the result of DNA single-strand breakage (Ribeiro et al. 1991).



Due to DNA cross-linking, the comet assay as performed in this study does not reflect the full extent of DNA damage induced by SM and is therefore limited in its ability to detect DNA single strand breaks in SM-exposed cells. To directly assess the relative amount of DNA single strand breaks in SM-exposed cells, assay modification to eliminate cross-links or to correct for the presence of cross-links is indicated.

The ability of SM to impede the migration of H<sub>2</sub>O<sub>2</sub>-damaged DNA is an indication of the amount of SM-induced DNA cross-linking. Thus, with the utilization of H<sub>2</sub>O<sub>2</sub> or other compounds that create DNA single-stranded breaks, the comet assay may potentially be used to provide information about the induction of DNA cross-links and their repair in cells exposed to SM.

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