Attenuation of Cytogenetic Damage by 2-Mercaptoethanesulfonate in Cultured Human Lymphocytes Exposed to Cyclophosphamide and Its Reactive Metabolites

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ABSTRACT

Cyclophosphamide (CP) is metabolized to the reactive intermediates, phosphoramide mustard (PAM) and acrolein (AC), which have generally different molecular binding targets. Sodium-2-mercaptopethanesulfonic acid (MESNA) has been used clinically to alleviate hemorrhagic cystitis caused by CP chemotherapy, has exhibited anticarcinogenic effects in rats exposed to CP during a long-term bioassay, and acts in the urogenital tract by reacting with 4'-OH-CP and AC. The purpose of this study was to: (a) compare the relative abilities of PAM and AC to induce cytogenetic damage and cytotoxicity in cultured human lymphocytes; (b) assess the efficacy of MESNA to attenuate the cytogenetic damage and cytotoxicity induced by CP, AC, PAM, and diethyl-4'-hydroperoxycyclophosphamide (DEHP-CP), an activated AC-generating compound; and (c) determine if concanavalin A-stimulated T-lymphocytes, which differentiate into suppressor cells upon lectin activation, exhibit any heightened cytogenetic sensitivity compared to a variety of cultured mammalian cells during exposure to PAM or AC as reported by other investigators.

Purified mononuclear leukocytes were stimulated with concanavalin A and exposed to CP (0.5–2.0 mM) without an exogenous activation system, AC (0.001–40.0 μM), PAM (0.0014–27.1 μM), or DEHP-CP (0.1–100.0 μM) in the presence or absence of MESNA (1, 5, or 10 mM). All four compounds induced significant concentration-related increases in the SCE frequency, but only PAM was clastogenic. On an induced SCE/MM basis, PAM was about 130 and 193 times more potent than were DEHP-CP and AC, respectively. MESNA protected against the cytogenetic damage and cytotoxicity induced by the four compounds, but it was particularly effective against AC and DEHP-CP by abolishing SCE induction completely. SCEs and chromosome aberrations differed considerably in their induction kinetics in lymphocytes exposed to PAM, and these disparities suggested an uncoupling of the two phenomena. Although SCE induction was not consistently associated with cytotoxicity with the four agents, chromosome aberration induction coincided with an inhibition of cell cycle kinetics in PAM-treated cells. The exceptionally high SCE frequency of up to 21 times baseline in cells exposed to PAM indicates that T-suppressor lymphocytes stimulated with concanavalin A may be particularly sensitive to the DNA-damaging effects of PAM. Finally, these data suggest that the anticarcinogenicity of MESNA correlates with its ability to attenuate cytogenetic damage and cytotoxicity induced by reactive CP metabolites.

INTRODUCTION

There are two recent developments in the treatment of malignant diseases with oxazaphosphorine chemotherapy. The first approach involves treating patients with a sulphydryl compound, especially MESNA, immediately prior to or concurrently with CP therapy to provide detoxification of activated CP metabolites in the urogenital tract (1, 2). Because hemorrhagic cystitis during CP therapy is a major side effect due to AC release in the urine or in urothelial cells (1–6), adjuvant chemotherapy with MESNA makes it possible to use even higher doses of CP for direct tumor cell killing (1, 3, 7). MESNA does not affect plasma alkylating activity or, by inference, antitumor activity of isophosphamide in humans (8). Concentrations of up to 10 mM MESNA did not induce SCEs or inhibit cell cycle kinetics in PHA-stimulated, human T-lymphocytes in vitro (9). More importantly, MESNA has been demonstrated to abolish the induction of urinary bladder cancer in rats on long-term exposure to CP (10).

The second approach to the treatment of malignant diseases takes into consideration that in many instances the host’s immune response to the tumor is suppressed by cells of T- and B-lymphocytes or macrophage origin (11, 12). Within these leucocyte populations, the pre-suppressor T-cells of the delayed-type hypersensitivity response (13), the pre-suppressor T-cells of the pokeweed mitogen-driven differentiation of B-cells (14), and B-cells (15–17) are highly sensitive to the toxicity of activated CP and its metabolites, whereas macrophages require much higher concentrations of activated CP in vitro before functional impairment occurs (18). Con A-stimulated T-lymphocytes have been used as a model system to understand the cell-specific toxicity of activated CP (14, 19), because these lectin-stimulated T-cells can suppress the pokeweed mitogen-driven production of immunoglobulin in B-lymphocytes (20). Therefore, a practical aspect of determining the differential sensitivities of lymphoid subpopulations to activated CP by immune function or cytogenetic assays may be the possibility of treating patients with much lower CP doses, either alone or in combination with tumor antigens, to augment a delayed-type hypersensitivity response.
against the tumor (21). In addition, clinical methods involving the
adoptive transfer of immune lymphocytes require the prior admin-
istration of CP to inactive suppressor T-cells (22). Thus, the
first method may result in severely immunosuppressed patients
having many cells with extensive DNA damage. The second
method relies on selective CP toxicity to pre-suppressor and
suppressor T-cell populations while leaving a functional immune
system relatively intact and possibly having enhanced antitumor
activity through release of cytotoxic lymphocytes from suppres-
sor factors.

CP is metabolized primarily in the liver to 4'-OH-CP, which
is in tautomeric equilibrium with aldoxophamid (23, 24). There
is substantial evidence that, apart from hepatic activation of CP,
cultured mammalian lymphocytes can metabolize CP to geno-
toxic intermediates, as evidenced by an increased SCE fre-
quency (17, 25-27). 4'-OH-CP and aldoxophamid are thought
to be the primary transport forms of CP to tumor cells
either as unbound metabolites or bound to plasma proteins (23,
24). There is some controversy as to whether PAM is also a
transport form (28), because it has been detected in high
concentrations (50-100 μM) in human peripheral blood during CP
therapy (29). Once inside the cells, aldoxophamid decom-
poses to two highly toxic agents, AC and PAM, which act by
killing tumor cells directly or as a delayed effect during DNA
synthesis and cell division.

Generally, these two activated CP metabolites have different
molecular binding targets. AC has a high binding affinity for
proteins and can denature critical enzymes necessary for drug
metabolism (7, 30, 31), replicative DNA synthesis (32), RNA
transcription (33), and cell membrane integrity (34). AC also
reacts rapidly and irreversibly with reduced glutathione (7), which
serves, among other functions, to protect critical macromole-
cules from oxidative and xenobiotic damage (35). The toxicity of
AC alone or through release during CP metabolism can be
eliminated by an exogenous source of reduced sulphhydrils, in-
cluding MESNA (1-7). The results of point mutation assays on
AC in prokaryotic and eukaryotic systems have generally been
equivalent, and the results of the few chromosome aberration
assays performed have been equally conflicting (36, 37). AC was
reported to be a potent clastogen based on the observations of
balanced chromosomes or "ball metaphases" in CHO cells (38).
AC has been shown also to react in situ with deoxyguanosine
by Michael addition to form 1, 2-propanodeoxyguanosine ad-
ducts (39) and can induce DNA single strand breaks in L1210
mouse leukemia cells (40).

The bifunctional alkylating agent PAM binds to guanosine and
deoxyguanosine in vitro, especially at the N-7 position (41)
leading to opened imidazole rings (42). Similar to nitrogen mus-
tard (43), PAM also forms cross-links in DNA, which has been
proposed as a mechanism for its antiproliferative action (40, 44).
PAM has been reported to be a strong mutagen in Salmonella
typhimurium TA1535 (27) and has been shown to be a potent
SCE inducer in a number of cultured rodent (27, 38, 45, 46) and
human cells (27, 45). The only study on the comparative geno-
toxic effects of PAM and AC showed that similar frequencies of
SCEs were induced at equimolar concentrations in CHO cells
exposed in exponential growth phase to a 1-h pulse treatment
(38).

The purposes of the present study were as follows: (a) to
compare the relative abilities of PAM and AC to induce cytoge-
netic damage and cytotoxicity in cultured human lymphocytes;
(b) to assess the efficacy of MESNA to attenuate the cytogenetic
damage and cytotoxicity induced by CP, PAM, AC, and DEHP-
CP (an activated AC-generating compound) as a means to
understand the nature of their respective molecular binding tar-
gets; and (c) to determine if Con A-stimulated T-lymphocytes,
which differentiate into suppressor cells upon lectin activation,
exhibit any heightened cytogenetic sensitivity compared to a
variety of cultured mammalian cells during exposure to PAM and
AC as reported by other investigators.

MATERIALS AND METHODS

Chemicals. All test chemical stock solutions were prepared from 5-
20 min prior to addition to the lymphocyte cultures. CP monohydrate
and MESNA were obtained from Sigma Chemical Co. (St. Louis, MO)
and were dissolved in RPMI 1640. AC (99%, Gold Label) was purchased
from Aldrich Chemical Co. (Milwaukee, WI) and contained 3% water
and 200 ppm hydroquinone to inhibit polymerization. AC was dissolved in
ice-cold sterile deionized water, and all of the dilutions were kept on ice
to minimize volatilization. MESNA (1, 5, or 10 mw), CP (0.5, 1, or 2 rniM), and bromodeoxyuridine
co's phosphate-buffered saline (pH 7.3). DEHP-CP (Ast 7037) was a
gift from Mr. Leonard H. Kedda (Drug Synthesis and Chemistry Branch,
National Cancer Institute, Bethesda, MD) and was prepared in Dulbec-
co's phosphate-buffered saline (pH 7.3). DEHP-CP (Ast 7037) was a
gift from Dr. Norbert Brock (Asta-Werke AG, Degussa Pharma Gruppe,
Bielefeld, Federal Republic of Germany) and was dissolved in ice-cold
deionized water. DEHP-CP undergoes spontaneous hydrolysis or enzym-
atic reduction to diethy-4'-OH-CP (47), which then can release AC and
N,N-bis(ethyl)phosphorodiamidic acid without the alkylating activity nor-
malized with the 2-chloroethyl side chains (48). Stock solutions of
CP, MESNA, PAM, and DEHP-CP were sterilized using 0.22 μm Millex-
GSI filter units (Millipore Corp., Bedford, MA). All test chemicals were
added to the cultures in 20-μ1 aliquots, and the final culture volumes were
the same.

Blood Processing, Lymphocyte Culture Technique, and Chemical
Exposure. Whole blood (~50 ml) was drawn by venipuncture into a
heparinized syringe. The same healthy adult male (34 years old) was
used as a source of the blood over the course of 9 months. MNLs were
separated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ)
density gradients and cultured as described previously (49). Erythrocytes
were removed because AC has been shown to have a high affinity for
the cytoskeletal protein spectrin (34), which could interfere with the
determination of the antagonistic effects of MESNA. The cultures were
established by inoculating 10^6 MNLs into 1.9 ml of complete medium
comprised of RPMI 1640 plus 25 mm N-2-hydroxyethylpiperezine-N'-2-
thanesulftic acid as buffer, 10% heat-inactivated fetal bovine serum,
100 units of penicillin and 100 μg of streptomycin sulfate/ml, and an
additional 292 μg of L-glutamine/ml. Two to 4 MNL cultures were used
for each treatment group. T-lymphocytes were stimulated mitogenically
with Con A (4 μg/ml). The cultures were incubated at 37° in a humidified
atmosphere containing 5% CO2 for 22 or 24 h. In the CP experiment,
MESNA (1, 5, or 10 mw), CP (0.5, 1, or 2 mw), and bromodeoxyuridine
(5 μM) were added concurrently at 24 h. In the experiments using AC,
PAM, and DEHP-CP, MESNA (1 mw) was added at 22 h after culture
initiation to allow equilibration. Bromodeoxyuridine (5 μM) was added 2
h later, and then the test compounds were added separately to the
cultures over the following concentration ranges: AC (0.001-40 μM);
PAM (excluding cyclohexylamine, 0.0014-27.1 μM); and DEHP-CP (0.1-
100 μM). The culture tubes were loosely capped during the subsequent
incubation. The cultures were harvested at 72 h following a 4-h exposure
to demecolcine (1.35 μM). The procedure for cell harvest was as de-
scribed previously (49).

Slide Preparation. The microscope slides were prepared as described
previously, coded, and stained using a modified fluorescence-plus-
Giromska technique (50, 51). Nomarski phase contrast optics were used during cytogenetic analysis. Twenty-five second division metaphases, 100 consecutive metaphases, and 1000 nuclei were scored from each culture for SCE frequency, cell cycle kinetics, and mitotic index, respectively. Only metaphases with 46 chromosomes were included in the final tabulations, and SCEs occurring at the centromere were counted unless there was obvious twisting of the chromatids. The replicative index (RI) was calculated from the cell cycle kinetic data by the formula: RI = ([1 × percentage of first division cells] + [2 × percentage of second division cells] + [3 × percentage of third division cells] + [4 × percentage of fourth division cells]) (52). Chromosome aberrations were analyzed from 100 first division metaphases per culture on the same slides using the criteria of Evans and O’Riordan (53).

Statistical Analyses. All cytogenetic data, with the exception of the chromosome aberration data, were tested for normality and then subjected to a one-way analysis of variance (54). A square root transformation of individual means was used to equalize the variances in all of the experiments (55). A one-tailed Dunnett’s multiple comparison test was used to compare the various treatment groups to the concurrent control if warranted (54, 56). Another method of statistical analysis was also used and involved the cell-to-cell variation in the SCE frequency rather than culture-to-culture variation in the mean SCE frequency. By using the pooled SCE counts from each treatment group, a potentially more stable estimate of variance could be obtained by larger degrees of freedom. The pooled SCE counts were subjected to a one-way analysis of variance, and then Dunnett’s multiple range test was used to compare the various treatment groups to the concurrent control. However, these two statistical methods gave essentially the same results for the in vitro experiments described here. A one-tailed Student’s t test was used to compare the mean SCE frequencies of cultures treated with the test chemicals in the presence or absence of MESNA to determine at which point a significant attenuation of cytogenetic damage appeared. A χ² test was used to analyze the incidence of chromosome aberrations in the first division metaphases. The level of significance was chosen as 0.05 for all of the determinations.

RESULTS

CP induced significant concentration-related increases in the SCE frequency of up to 3 times baseline at 2 μM in the absence of any exogenous metabolizing system (Table 1). However, CP did not depress mitotic activity or inhibit cell cycle progression significantly (Table 1). When MESNA was added to the lymphocyte cultures containing CP, the SCE frequency was reduced significantly by 27–45% (Table 1) for all 3 MESNA concentrations. Although MESNA alone did not induce SCE or perturb cell cycle kinetics, there was some evidence of cell cycle inhibition in cultures exposed to a combination of 2 mM CP and 1 mM MESNA (Table 1).

Because MNLs were apparently capable of metabolizing CP at a slow rate, as shown by an increase in the SCE frequency, it was important to know the relative capabilities of the activated CP metabolites to induce cytogenetic damage and cytotoxicity. AC induced significant concentration-related increases in the SCE frequency of up to 1.6-fold at 20 μM, but the induction occurred over a narrow concentration range (5–20 μM) (Table 2). AC was not clastogenic at the highest analyzable concentration (20 μM) (data not shown). Forty μM AC was lethal to MNLs, as evidenced by many pyknotic nuclei and no mitoses. Although AC did not significantly depress the mitotic index, it slowed the cell cycle kinetics significantly at 10 and 20 μM (Table 2). 1 mM MESNA protected completely against AC-induced SCEs and cell lethality (Table 2) up to 40 μM AC; however, significant cell cycle inhibition occurred at 10 and 40 μM AC.

Since the extracellular exposure of lymphocytes to AC is unlikely to occur in vivo due to rapid scavenging by nucleophilic plasma components, it was necessary to use an activated cyclophosphamide analogue, DEHP-CP, to mimic the intracellular release of AC during CP degradation to reactive intermediates. DEHP-CP was comparable to AC in its SCE-inducing capability over a similar narrow range (10–20 μM) where a 1.8-fold increase in the SCE frequency was seen at 20 μM (Table 3). DEHP-CP concentrations of 15 and 20 μM decreased the mitotic activity significantly, and the cell cycle progression was inhibited significantly at concentrations ≥0.5 μM (Table 3). No mitoses were observed at DEHP-CP concentrations ≥40 μM. One mM MESNA protected completely against SCE induction at 10 and 15 μM DEHP-CP and provided partial protection up to 100 μM DEHP-CP (Table 3). For example, the SCE frequency of the cells exposed to MESNA and 100 μM DEHP-CP was about equal to cells exposed to 20 μM DEHP-CP alone. MESNA protected completely against cytotoxicity up to 40 μM DEHP-CP (Table 3).

Although a significant depression in mitotic activity and inhibition of cell cycle kinetics was observed in cultures containing ≥80 μM DEHP-CP and MESNA, the extent of cytotoxicity was comparable to that seen at ≥15 μM DEHP-CP alone.

PAM induced significant concentration-related increases in the SCE frequency over a 500-fold range (Table 4). A significant increase in the SCE frequency was detected at 0.0068 μM PAM (1.5 ng/ml), and a 21-fold increase in the SCE frequency (185.4 ± 4.3 SCEs/cell) was observed at 3.39 μM PAM. PAM also induced a 33-fold increase above baseline in the number of damaged first division metaphases at 3.39 μM (Table 5). Whether increases in the number of damaged cells occurred at 0.399 μM PAM, a concentration which is 50 times higher than that required to see a significant elevation in SCE induction. The disparity in the kinetics of SCE and chromosome aberration induction can

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of MESNA on the SCE frequency, mitotic activity, and cell cycle kinetics in cultured human lymphocytes exposed to CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP concentration (μM)</td>
<td>MESNA concentration (μM)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>12.3 ± 0.6° (0.53)</td>
</tr>
<tr>
<td>1.0</td>
<td>17.0 ± 0.2° (0.46)</td>
</tr>
<tr>
<td>2.0</td>
<td>25.6 ± 1.4° (0.56)</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>8.9 ± 0.4° (0.42)</td>
</tr>
<tr>
<td>1.0</td>
<td>14.7 ± 0.5° (0.51)</td>
</tr>
<tr>
<td>2.0</td>
<td>20.2 ± 0.2° (0.54)</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>10.9 ± 0.5° (0.44)</td>
</tr>
<tr>
<td>1.0</td>
<td>12.2 ± 0.4° (0.45)</td>
</tr>
<tr>
<td>2.0</td>
<td>18.3 ± 0.2° (0.53)</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
</tr>
<tr>
<td>0.5</td>
<td>10.0 ± 1.1° (0.51)</td>
</tr>
<tr>
<td>1.0</td>
<td>10.3 ± 0.7° (0.57)</td>
</tr>
<tr>
<td>2.0</td>
<td>18.0 ± 1.4° (0.60)</td>
</tr>
</tbody>
</table>

a Mean ± SD of triplicate cultures.

b Numbers in parentheses, estimated standard error of the mean for pooled SCE counts.

Significantly different from the concurrent control using Dunnett's multiple comparison test (P < 0.05).

d Significant reductions in the SCE frequency at all CP concentrations compared to cultures without MESNA.
Table 2

Effect of MESNA on SCE induction, mitotic activity, and cell cycle kinetics in cultured human lymphocytes exposed to DEHP-CP

<table>
<thead>
<tr>
<th>PAM concentration (µM)</th>
<th>-1 mM MESNA</th>
<th>+1 mM MESNA</th>
<th>Mitotic index (%)</th>
<th>Replicative index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4 ± 0.77 (0.30)</td>
<td>7.3 ± 0.5 (0.26)</td>
<td>2.5 ± 0.3</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>0.001</td>
<td>8.0 ± 0.1 (0.46)</td>
<td>7.7 ± 0.7 (0.49)</td>
<td>3.9 ± 2.1</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>0.01</td>
<td>8.1 ± 0.3 (0.49)</td>
<td>8.1 ± 0.5 (0.52)</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>9.0 ± 1.4 (0.48)</td>
<td>8.4 ± 1.6 (0.48)</td>
<td>3.9 ± 0.3</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>9.5 ± 0.2 (0.58)</td>
<td>8.2 ± 0.4 (0.44)</td>
<td>4.0 ± 0.8</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>1.0</td>
<td>9.5 ± 0.3 (0.53)</td>
<td>8.0 ± 0.3 (0.47)</td>
<td>3.1 ± 0.3</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>5.0</td>
<td>10.6 ± 0.1 (0.49)</td>
<td>7.2 ± 4.5° (0.36)</td>
<td>3.2 ± 0.7</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>10.0</td>
<td>9.8 ± 0.5 (0.59)</td>
<td>8.1 ± 0.5 (0.53)</td>
<td>2.6 ± 0.2</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>15.0</td>
<td>10.8 ± 0.6° (0.65)</td>
<td>7.8 ± 0.3 (0.46)</td>
<td>1.5 ± 0.3</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>20.0</td>
<td>13.3 ± 0.7° (0.70)</td>
<td>8.5 ± 2.0° (0.46)</td>
<td>No mitoses</td>
<td>No mitoses</td>
</tr>
<tr>
<td>40.0</td>
<td>No mitoses</td>
<td>8.4 ± 0.0 (0.41)</td>
<td>No mitoses</td>
<td>No mitoses</td>
</tr>
</tbody>
</table>

Table 3

Effect of MESNA on SCE induction, mitotic activity, and cell cycle kinetics in cultured human lymphocytes exposed to DEHP-CP

<table>
<thead>
<tr>
<th>DEHP-CP concentration (µM)</th>
<th>-1 mM MESNA</th>
<th>+1 mM MESNA</th>
<th>Mitotic index (%)</th>
<th>Replicative index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.0 ± 0.8 (0.41)</td>
<td>8.4 ± 0.5 (0.38)</td>
<td>6.1 ± 1.3</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>9.7 ± 0.7 (0.56)</td>
<td>8.7 ± 0.0 (0.49)</td>
<td>6.6 ± 0.0</td>
<td>9.7 ± 3.6</td>
</tr>
<tr>
<td>0.5</td>
<td>9.8 ± 0.9 (0.54)</td>
<td>8.7 ± 1.7 (0.48)</td>
<td>5.0 ± 1.6</td>
<td>8.1 ± 1.1</td>
</tr>
<tr>
<td>1.0</td>
<td>10.7 ± 0.3 (0.66)</td>
<td>8.7 ± 0.3 (0.52)</td>
<td>6.5 ± 0.4</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>5.0</td>
<td>10.5 ± 0.6 (0.47)</td>
<td>8.8 ± 1.2 (0.57)</td>
<td>5.7 ± 1.2</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>10.0</td>
<td>12.6 ± 0.5° (0.66)</td>
<td>8.6 ± 1.4 (0.46)</td>
<td>4.0 ± 0.1</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>15.0</td>
<td>12.2 ± 1.4° (0.60)</td>
<td>10.3 ± 0.1 (0.50)</td>
<td>3.5 ± 0.1°</td>
<td>7.1 ± 0.0</td>
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<tr>
<td>20.0</td>
<td>16.4 ± 1.1° (0.66)</td>
<td>11.1 ± 0.8° (0.65)</td>
<td>1.9 ± 1.0°</td>
<td>7.2 ± 1.1</td>
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<tr>
<td>40.0</td>
<td>No mitoses</td>
<td>10.4 ± 0.2 (0.56)</td>
<td>No mitoses</td>
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<td>80.0</td>
<td>No mitoses</td>
<td>14.4 ± 2.1° (0.60)</td>
<td>No mitoses</td>
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<td>100.0</td>
<td>No mitoses</td>
<td>15.3 ± 1.1° (0.71)</td>
<td>No mitoses</td>
<td>2.0 ± 0.1°</td>
</tr>
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</table>

Table 4

Effect of MESNA on SCE induction, mitotic activity, and cell cycle kinetics in cultured human lymphocytes exposed to PAM

<table>
<thead>
<tr>
<th>PAM concentration (µM)</th>
<th>-1 mM MESNA</th>
<th>+1 mM MESNA</th>
<th>Mitotic index (%)</th>
<th>Replicative index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.3 ± 0.9° (0.33)</td>
<td>7.7 ± 0.9 (0.34)</td>
<td>5.3 ± 0.7</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>0.001</td>
<td>9.4 ± 0.1 (0.55)</td>
<td>9.3 ± 0.6 (0.61)</td>
<td>6.0 ± 2.7</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>0.0008</td>
<td>11.7 ± 0.1° (0.63)</td>
<td>9.7 ± 0.2° (0.64)</td>
<td>5.9 ± 0.8</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>0.068</td>
<td>15.7 ± 0.8° (0.53)</td>
<td>12.0 ± 0.4° (0.53)</td>
<td>6.3 ± 0.5</td>
<td>5.2 ± 1.7</td>
</tr>
<tr>
<td>0.339</td>
<td>42.4 ± 2.9° (1.06)</td>
<td>24.7 ± 0.1° (0.77)</td>
<td>3.7 ± 0.1</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>0.48</td>
<td>18.4 ± 2.2° (0.19)</td>
<td>49.2 ± 3.5° (1.15)</td>
<td>3.8 ± 0.8</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>3.39</td>
<td>185.4 ± 4.3° (4.25)</td>
<td>117.4 ± 7.5° (2.13)</td>
<td>1.1 ± 0.4°</td>
<td>2.9 ± 0.6°</td>
</tr>
<tr>
<td>≥6.8</td>
<td>No mitoses</td>
<td>No mitoses</td>
<td>No mitoses</td>
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</tr>
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</table>
DISCUSSION

These data show that human lymphocytes can activate CP to genotoxic intermediates, as shown by a 3-fold increase in the SCE frequency, and that both CP metabolites, AC and PAM, can induce cytogenetic damage and cytotoxicity. These data demonstrate also that MESNA attenuates the cytogenetic damage induced by CP, AC, DEHP-CP, and PAM and provide an insight into the molecular mechanism of action of these agents. The kinetics of SCE and chromosome aberration induction in the PAM-exposed cells provides evidence for the uncoupling of these two cytogenetic endpoints and for the closer relationship of chromosome aberration induction to cytotoxicity. The unusually high SCE and chromosome aberration frequencies induced by PAM indicate not only that PAM is one of the most potent chromosomal mutagens known but also that Con A-stimulated T-lymphocytes are highly sensitive to PAM-induced DNA damage.

The ability of MESNA to provide protection against the genotoxicity and cytotoxicity of AC and PAM indicates the nature and affinity of their respective molecular binding targets. One mM MESNA protected completely against AC-induced SCEs and cell lethality at a molar ratio of 25:1 (Table 2). When a different means of delivering AC to the lymphocytes' nuclei was used, 1 mM MESNA protected completely against DEHP-CP-induced SCEs at a molar ratio of 67:1 and provided partial protection at a ratio of 10:1 (Table 3). The cytotoxicity of DEHP-CP was inhibited completely by MESNA at a ratio of 25:1, and lower molar ratios were partially effective (Table 3). In contrast, although the decreases in SCE induction by addition of MESNA to the lymphocyte cultures exposed to PAM were dramatic, MESNA did not completely attenuate the PAM-induced SCEs, even at a molar ratio of 147,600:1. This large dichotomy in the protective effects of MESNA indicates that the affinity of DEHP-CP and AC for reduced sulphydryls is about 2200 and 5900 times, respectively, that of PAM. These results support the observations of a high reactivity of AC with proteins and a significantly lower affinity of PAM for proteins in two separate studies. Wildenauer and Oehlmann (34) showed that there was preferential binding of [14C]AC to microsomes and erythrocytes during CP metabolism, but little binding of [3H]chloroethyl side chains was detected. Gurtoo et al. (57) demonstrated that 2100 times more [14C]AC than [3H]PAM was bound to liver microsomes, whereas 22 times more [3H]PAM than [14C]AC was bound to native calf thymus DNA during metabolism of radiolabeled CP in vitro. Thus, besides the nature of their respective induced DNA lesions (39–42, 44), the ability of PAM and AC to induce cytogenetic damage might be due primarily to their divergent affinities for low molecular weight thiols and proteins compared to nucleic acids.

An important aspect of the antagonistic effects of MESNA on AC-induced toxicity was that MESNA inhibited cell lethality observed at 40 μM AC and 40–100 μM DEHP-CP. These results obtained from using lymphocytes as a model cell type support and amplify the hypothesis of Cox (58) regarding the relationship of CP-induced urinary bladder cystitis and bladder cancer. The cause of cystitis during CP chemotherapy has been shown conclusively to be mediated by AC rather than PAM (2, 4, 5). Because the urine is normally low in reduced thiols, urothelial cells represent the major target for CP metabolites. The decomposition of activated CP releases AC in the urine or in urothelial cells, which damages cell membranes (34) and cytoplasmic components (7, 30, 31) and induces SCEs (38; present study). The rapid death of urothelial cells induced by AC leads to compensatory regeneration of the underlying diploid cell populations (59, 60). Because PAM is also released simultaneously with AC, it induces DNA lesions which are expressed as SCEs and chromosome aberrations during S-phase. Thus, fixation of cytogenetic damage during induced cell turnover may be an important first step in cell transformation to a malignant phenotype in urothelium and possibly in lymphoid cells as well.

Table 5

<table>
<thead>
<tr>
<th>PAM concentration (μM)</th>
<th>±MESNA (1 mM)</th>
<th>% of damaged metaphases</th>
<th>Frequency of aberrations per 100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>0.0014</td>
<td>+</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>0.0068</td>
<td>+</td>
<td>3.5</td>
<td>30.6</td>
</tr>
<tr>
<td>0.068</td>
<td>+</td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.339</td>
<td>+</td>
<td>11.0a</td>
<td>0.5</td>
</tr>
<tr>
<td>0.68</td>
<td>+</td>
<td>10.5b</td>
<td>2.0</td>
</tr>
<tr>
<td>3.39</td>
<td>+</td>
<td>65.5c</td>
<td>1.5</td>
</tr>
<tr>
<td>≥6.8</td>
<td>+</td>
<td>No mitoses</td>
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</tr>
<tr>
<td>3.39</td>
<td>+</td>
<td>30.5d</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Footnotes:

1. Significantly different from the control using $\chi^2$ test ($P < 0.05$).
2. Significantly different from the control using $\chi^2$ test ($P < 0.05$).
3. Significantly different from the respective cultures without MESNA using the $\chi^2$ test ($P < 0.05$).
The administration of MESNA concurrently with oxazaphosphorine therapy has been shown to abolish hemorrhagic cystitis in humans (1, 2) and rodents (2, 3), as well as to attenuate the induction of urinary bladder cancer in rats during long-term exposure to CP (10). Besides binding to AC and PAM, MESNA also reacts with 4′-OH-CP to form the condensation product, 4- (2-sulfonatoethylthio)-CP, which is thought to be a temporary inactivation product in the urine (4). MESNA was shown to be the least toxic thiol compound in rats and to provide the greatest amount of regional protection to the urogenital tract among many experimental compounds tested (3). The present study has confirmed the observations of Becher et al. (9) that concentrations of up to 10 nm MESNA are not genotoxic or cytotoxic. The present study suggests that the anticarcinogenicity of MESNA correlates with its ability to attenuate the cytogenetic damage and cytotoxicity induced by reactive CP metabolites.

The results of the present study with SCE induction by CP in purified human lymphocyte cultures support the findings in a number of other studies that mammalian lymphocytes can metabolize CP to genotoxic intermediates (17, 25–27). However, the rate of CP metabolism in blood cultures is slow, as evidenced by only a 3-fold increase in the SCE frequency at 2 mm CP (Table 1). Because PAM was such a potent SCE inducer at low concentrations relative to AC and DEHP-CP, most of the genotoxicity of CP in lymphocyte cultures is probably originating from PAM. This assumption is partly supported by the fact that MESNA did not protect completely against CP-induced SCEs but reduced the SCE frequency by about the same percentage as in PAM-treated cells; that is, a 27–45% reduction in the SCE frequency by MESNA was observed in the CP-exposed cells (Table 1) and a 21–49% reduction was seen in the PAM-exposed cells (Table 4).

The findings of the present study that AC induces SCEs over a narrow concentration range (5–20 μM) (Table 2) agree well with the results of Au et al. (38). They showed that AC induced about a 2.6-fold increase in the SCE frequency in CHO cells exposed for 1 h, whereas a 1.6-fold increase was seen in the present study. However, they reported that AC induced chromosome tangling at concentrations ≥40 μM, which was taken as an indication of "potential" clastogenicity. There was no evidence of clastogenicity or tangled chromosomes at 20 μM AC in the present study. The depression in mitotic activity by 20 μM AC was much less in human lymphocytes (Table 2) compared to the complete inhibition by 10 μM AC in CHO cells exposed for 5 h (38). These results suggest that human lymphocytes may be less susceptible than CHO cells to the cytotoxic effects of AC.

The present study demonstrated that PAM is one of the most potent chromosome mutagens known and is comparable to another bifunctional alkylating agent, mitomycin C, in its SCE-inducing capability (61). On an induced SCE/μM basis, PAM is about 130 and 193 times more potent than are DEHP-CP and AC, respectively. The form of structural chromosome aberrations of the chromatid-type support the conclusions of Winckler et al. (27) that PAM is an S-phase dependent agent (Table 5). The present results contrast sharply with the findings of Au et al. (38), who reported that the SCE-inducing ability of PAM is similar to that of AC over equimolar concentrations. These large discrepancies might be explained by a species or cell-type difference, but a more likely possibility is that a 1 h pulse treatment with PAM is not a long enough exposure for a compound that is ionized at physiological pH (23) and would be taken up slowly by cells.

Although PAM proved to be a strong chromosome mutagen, the magnitude of the cytogenetic response suggested that Con A-stimulated lymphocytes are more sensitive than any mammalian cell tested to date. In the present study a significant increase in the SCE frequency was detected at 6.8 nm, whereas the other studies reported significant increases at concentrations from about 14.8–33 times higher (27, 45). Treatment of Con A-stimulated lymphocytes with 3.39 μM PAM for 48 h resulted in a 21-fold increase in the SCE frequency. Dearfield (45) observed a 9.5-fold increase in SCE frequency in human IMR-90 lung fibroblasts exposed to 4.52 μM PAM for two cell cycles. Similarly, Winckler et al. (27) saw a 11.4-fold increase in the SCE frequency in PHA-stimulated, whole blood T-lymphocytes exposed to 10 μM PAM for 54 h using a virtually identical treatment procedure to the present study. Con A is a non-specific, polyclonal activator of T-lymphocytes which differentiate into cells exhibiting suppressor phenotypes (14, 20). These suppressor T-cells and their progenitors are highly sensitive to the toxicity of activated CP (14, 19). Generally, PHA-stimulated lymphocytes are affected to a lesser extent by the toxicity of activated CP, as measured by the incorporation of [3H]thymidine into DNA (62). Deknudt (63) has shown nearly a 2-fold higher SCE response in Con-A-stimulated whole blood lymphocytes compared to PHA-stimulated lymphocytes during a 1-h exposure to S-9 activated CP at 48 h post-culture initiation. The inhibition of differentiation by activated CP can occur without the loss of mitogen responsiveness or cell viability and in the absence of detectable DNA cross-links (14) at comparable concentrations to PAM in the present study where SCEs, but not chromosome aberrations, were induced. The significantly higher SCE frequency observed in Con A-stimulated lymphocytes compared to other mammalian cells may reflect a cytogenetic basis for their greater susceptibility to inhibition of differentiation by activated CP.

The kinetics of SCE and chromosome aberration induction were different in the experiment with PAM (Tables 4 and 5). One obvious difference was that a significant increase in SCEs was induced at a concentration of PAM about 50 times less than the concentration required to induce a significant increase in chromosome aberrations. This disparity between the two endpoints has been observed many times with alkylating agents (64). The second difference was that chromosome aberrations reached a plateau phase from 0.339–0.68 μM PAM in the presence or absence of MESNA and then rose dramatically at 3.39 μM, whereas there was no evidence of a plateau phase in SCE induction. These two differences in the kinetics of induction indicate that SCEs and chromosome aberrations arise from either different mechanisms of formation or different DNA lesions, as suggested by others (65, 66). The relatively greater decline in chromosome aberrations compared to SCEs in cultures treated with MESNA suggests that MESNA reacts with one of the chloroethyl side chains of PAM and partially abolishes the cross-linking capability, while the monofunctional alkylating activity remains. These data support the hypothesis that cross-links are more likely to lead to chromosome aberration induction through the processes of misrepair and deletion (67), while monoadducts are efficient stimulators of SCE induction (66).

Although previous investigations have shown a correlation of SCE induction and cytotoxicity (68, 69), there did not appear to
be a consistent correlation of SCE to cytotoxicity by the agents used in the present study. SCE induction could occur in the absence of depressed mitotic activity and inhibition of cell cycle kinetics. SCE induction reached 2–3 times baseline, especially with CP (Table 1) and PAM (Table 4), without significant inhibition of cell cycle progression. However, the induction of chromosome aberrations coincided with a distinct inhibition of cell cycle kinetics, which was evident at 0.339 μM PAM (Tables 4 and 5). The coincident occurrence of cell cycle inhibition and chromosome aberrations reinforces the concept that chromosome aberrations generally lead to cell death (66). The results of the present study support the findings of Kaina (70) and Nishi et al. (71) that SCE induction by mono- and bifunctional alkylating agents is not related to cytotoxicity. Additionally, because AC has been shown to induce DNA single strand breaks in cultured mouse leukemia cells (40), single strand breaks might be relatively inefficient stimuli for SCE induction (72). Because nitrogen mustard, an analogue of PAM, exhibits many of the characteristics of PAM in its interactions with DNA (43) and is a poor inducer of specific-locus mutations compared to SCEs in mammalian cells (71), it is doubtful that SCE induction by PAM can be strictly equated with specific-locus mutagenesis. The results of the present study and other studies on immunotoxic effects at low PAM or activated CP concentrations suggest that SCE induction is more closely related to inhibition of differentiation in lymphoid cells and is not an innocuous event, but further experimentation will be needed to test this hypothesis.

In conclusion this study has demonstrated that SCE induction is probably not closely related to cytotoxicity or chromosome aberration formation. This study has also shown a possible cytogenetic basis for the heightened sensitivity of Con A-stimulated, suppressor T-lymphocytes to activated CP and PAM and may offer a means of understanding differential toxicity in lymphoid subpopulations using cytogenetic techniques. The attenuation of cytogenetic damage by MESNA provides insights into the molecular mechanisms of induced toxicity by CP and its reactive metabolites and lends support for the concept of regional chemoprotection during CP therapy to abrogate the induction of cytisitis and urinary bladder cancer.

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REFERENCES

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Attenuation of Cytogenetic Damage by 2-Mercaptoethanesulfonate in Cultured Human Lymphocytes Exposed to Cyclophosphamide and Its Reactive Metabolites

James L. Wilmer, Gregory L. Ereksen and Andrew D. Kligerman


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