The Role of O^6^-Alkylguanine DNA Alkyltransferase in Limiting Nitrosourea-induced Sister Chromatid Exchanges in Proliferating Human Lymphocytes

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ABSTRACT

Although induction of sister chromatid exchanges (SCEs) following nitrosourea exposure may be greater during cell proliferation, the increase could be offset by the action of the DNA repair protein O^6^-alkylguanine DNA alkyltransferase (alkyltransferase). To evaluate these factors in resting and proliferating (phytohemagglutinin stimulated) human lymphocytes, we studied the effect of changes in alkyltransferase activity on 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-induced SCEs. Phytotohemagglutinin stimulation resulted in induction of alkyltransferase activity (5.9 ± 0.3 units, resting, versus 9.2 ± 0.2 units, proliferating). In both resting and proliferating lymphocytes the alkyltransferase activity was inactivated by 85–88% after an 18-h exposure to 0.5 mM of the modified base O^-methylguanine (O^-mGua). However, 48 h after removal of O^-mGua, proliferating lymphocytes recovered alkyltransferase activity while resting cells did not. In the absence of O^-mGua, both resting and proliferating lymphocytes were equally sensitive to BCNU-induced SCEs. Following inactivation of the alkyltransferase by O^-mGua, BCNU-induced SCEs were markedly increased, but the increase was much greater in resting than proliferating cells, 4-fold vs. 2.6-fold at each dose of BCNU (P < 0.001). The factors providing partial protection against BCNU-induced SCEs in proliferating lymphocytes appear to include the proliferation-dependent increase in alkyltransferase activity and the ability of proliferating lymphocytes to rapidly recover alkyltransferase activity after its inactivation. Thus, the alkyltransferase appears to provide an important mechanism of resistance to SCE induction in human lymphocytes.

INTRODUCTION

The ultimate biological effect of DNA-damaging chemicals is the result of a dynamic interaction between the creation of DNA damage and the ability of the cell to repair such damage. The capacity to induce SCEs has been shown to be a sensitive and quantitative method to study DNA damage (1). Although the precise mechanisms by which chemicals produce SCEs remain elusive, it is likely that SCE formation is not the result of a specific DNA lesion but rather reflects the repair of various types of DNA damage. This versatility has made SCE induction a powerful tool to study the question of whether an SCE represents the result of the interaction of DNA damage and the corresponding specific DNA repair mechanism.

Nitrosoureas are potent inducers of SCEs (1). The extent of SCE induction by nitrosoureas can be modulated by the ability of the cell to repair alkylation at the O^6^ position of guanine (15–17). This is consistent with the early suggestion that O^6^-alkylguanine lesions are important in the generation of SCEs (18, 19). O^-Alkylguanine lesions are specifically repaired by the DNA repair protein, O^-alkylguanine DNA alkyltransferase (alkyltransferase) (20, 21), which catalyzes the covalent transfer of the alkyl group from guanine to a cysteine residue in the protein. This restores guanine and results in irreversible inactivation of the alkyltransferase (21, 22). Because of the stoichiometric nature of this reaction, exposure to alkylating agents can deplete cellular stores of the alkyltransferase (21) which are renewed only by protein synthesis (21, 22). O^-Alkylguanine adducts can then persist and go on to form crosslinks or be converted to point mutations at the time of DNA synthesis. It is the presence of the persistent adduct or crosslink which is thought to give rise to an SCE (23).

The level of alkyltransferase and thus potential protection from nitrosourea-induced SCEs is not uniform among human tissues. Liver has the highest levels of alkyltransferase activity while bone marrow myeloid precursors have the lowest (24). The alkyltransferase in human lymphocytes is approximately twice that in myeloid cells, but significantly less than that of liver (24, 25). Alkyltransferase activity can be induced in human lymphocytes during in vitro mitogen stimulation (7, 8). Because lymphocyte alkyltransferase is intermediate relative to other tissues and because it increases during proliferation the lymphocyte is a useful target cell to study SCE induction by nitrosoureas. Nitrosourea-induced SCEs in lymphocytes thus serve as a sensitive marker of damage to other human tissues as well as a model to gauge the modulating impact of proliferation induced changes in alkyltransferase activity.

The role of the alkyltransferase in SCE formation in lymphocytes has previously been studied by exposing cells to MNU which forms O^-methylguanine adducts thus depleting alkyltransferase activity during the repair reaction (8). Under these conditions prior exposure to MNU results in potentiation of BCNU-induced SCEs in both resting and proliferating lymphocytes suggesting that alkyltransferase depletion sensitizes the cells to BCNU (26). After MNU exposure alkyltransferase

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Abbreviations used are: SCE, sister chromatid exchange; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BrdUrd, 5-bromo-2'-deoxyuridine; MNU, N-nitroso-N'-methylene; O^-mGua, O^-methylguanine; PHA, phytohemagglutinin; PBS, phosphate buffered saline.

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activity recovers within 48 h in proliferating lymphocytes but
does not recover in resting cells (8). Thus, residual MNU
adducts may remain unrepaired and contribute to SCE formation.
It has recently been shown that the modified base O’mGua
will inactivate the alkyltransferase specifically without directly
interacting with DNA (27-29). This provided us with a simple
experimental system to study the role of alkyltransferase in
regulating nitrosourea induced SCEs in resting and proliferat-
ing human lymphocytes.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma (St. Louis, MO)
unless otherwise specified. Lyophilized PHA (HA-15, Burroughs-Well-
come, Dartford, England) was reconstituted in sterile PBS as described
by the manufacturer and was stored at –20°C. BCNU (obtained from
the Drug Synthesis Branch, Drug Therapeutics Branch, National Can-
cer Institute) was stored at –20°C, and reconstituted in 95% ethanol
diluted to 0.01 m in PBS. O’mGua was synthesized by Dr. Paul
Howard, Department of Environmental Health Sciences, CWRU
School of Medicine, using the method of Dempie et al. (30). This
preparation was contaminated by less that 0.5% 6-chloroguanine as
determined by high-performance liquid chromatography, a concentra-
tion that has no effect on alkyltransferase activity or cell growth (27).
O’mGua was dissolved in 0.01 n HCl, and neutralized to pH 7 with NaOH.
A final concentration of 0.01 m Colcemid was obtained from
GIBCO (Grand Island, New York), BrdUrd (Calbiochem, San Diego,
CA) was stored at 1 mg/ml in PBS at –20°C. Hoescht 33258 Dye was
reconstituted to 0.01 m in H2O, and stored at 4°C in the dark. Gurr
buffer (pH 6.8) and Giemsa were obtained from Biomedical Specialties
(Los Angeles, CA). [3H]Thymidine (Du Pont-New England Nuclear,
Boston, MA) (2.0 Ci/mmol) was stored at 4°C. Tissue culture reagents
were obtained from Hyclone Labs (Logan, UT).

Isolation and Drug Exposure of Lymphocytes for Alkyltransferase
Determination. Lymphocytes were isolated from normal donors over
folic acid hypaque gradients as previously described (25), and resuspended
at 5 × 10^5 cells/ml in RPMI 1640 tissue culture medium supplemented
with 15% fetal bovine serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine-
ethane sulfonic acid, 100 units/ml penicillin, and 100 μg/ml strepto-
cyclid hypaque gradients as previously described (25), and resuspended
at 5 × 10^5 cell/ml in RPMI 1640 tissue culture medium supplemented
with 15% fetal bovine serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine-
ethanesulfonic acid, 100 units/ml penicillin, and 100 μg/ml strepto-
cin (tissue culture medium) (25). Lymphocytes were induced toproliferate by a 24-72-h culture in the presence of 1% PHA at 37°C,
5% CO2. Resting lymphocytes were isolated from the same donor on
the day of drug exposure. Cells were counted by passage through a
Coulter Counter (Model 2M) after a 1 to 20 dilution into Centrimide
(0.082 m hexadecltrimethyl ammonium bromide (Eastman Kodak Co.,
Rochester, NY), 0.16 m NaCl, 2 mM EDTA, pH 5) to disperse cell
aggregates.

To determine the effect of O’-methylguanine on alkyltransferase
activity, both resting and proliferating lymphocyte populations from
the same donor were suspended at 5 × 10^5 cells/ml in tissue culture
medium and exposed to O’mGua at concentrations of 0.01–1 mM for
24 h. To determine the time course of inactivation and recovery of
alkyltransferase activity, resting and PHA-stimulated lymphocytes were
exposed to 0.5 mM O’mGua for 24 h, washed in 50 mM PBS, and
resuspended in fresh tissue culture media without O’mGua and recul-
tured for 72 h. At appropriate times aliquots of 20 × 10^5 cells were
removed for alkyltransferase determination.

Alkyltransferase Activity. Cell extracts were prepared and samples
were taken for determination of protein and DNA content using pre-
viously described methods (24). O’-Alkylguanine DNA alkyltransferase
activity in cell extracts was measured as removal of the methyl-^H
adduct from O’-[methyl-^H]guanine in methyl-^H DNA alkylated with
N’-[methyl-^H]nitrosourea as previously described (24, 25). One unit of
alkyltransferase was defined as removal of 1 fmol of O’-methylguanine
per microgram cellular DNA. This expression adjusts for changes in
DNA content of the cell that occur during the cell cycle and during
DNA synthesis (24).

RESULTS

Inactivation of the Alkyltransferase by O’mGua. To evaluate
the role of alkyltransferase activity in modulating DNA damage
in resting and proliferating cells we used the modified base
O’mGua to inactivate the alkyltransferase. To determine the
optimal dose of O’mGua needed to inactivate the alkyltransfer-
ase, resting and proliferating lymphocytes from normal donors
were incubated for 24 h with increasing concentrations of
O’mGua from 0.01–1 mM and the residual alkyltransferase
activity was determined (Fig. 1). The alkyltransferase activity
was significantly higher in the proliferating cells (9.2 ± 0.2

Fig. 1. Inactivation of alkyltransferase activity by O’-mGua in lymphocytes.
Lymphocytes were cultured in the presence of 1% PHA for 72 h or freshly
harvested prior to culture in tissue culture media at 5 × 10^5/ml. O’mGua was
added for 24 h at concentrations from 0.01 to 1 mM prior to alkyltransferase
assay. Data represents mean ± SEM of four donors per point. Symbols used are:
Ο, PHA-stimulated lymphocytes; Φ, resting lymphocytes.

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significance was determined using a paired t test comparing control and drug-treated populations. For replication index as described in "Materials and Methods." The data shown represent the mean ± SD of the individual experimental means for N Z 3. Statistical and the determination of SCE as described in "Materials and Methods." For each individual experiment 25 metaphases were scored for SCEs and 100 metaphases cultured in 1% PHA after removal of O'mGua (data not shown). In these cultures, regeneration of alkyltransferase activity was observed if resting cells were in alkyltransferase activity was observed if resting cells were in resting and proliferating cells, maximal inactivation occurred after 18 h of exposure to 0.5 mM O'mGua. In the PHA-stimulated cells recovery of alkyltransferase activity began 6–18 h after removal of O'mGua reaching 5.8 units at 24 h and almost baseline at 48 h. In contrast the alkyltransferase activity in the resting cells recovered slowly and remained only 38% of control 48 h after removal of O'mGua. A similar slow recovery in alkyltransferase activity was observed if resting cells were cultured in 1% PHA after removal of O'mGua (data not shown). In these cultures, regeneration of alkyltransferase activity occurred at 48 h but not at 24 h which is consistent with the time course of induction of the alkyltransferase activity by PHA (8).

Effect of O'mGua on SCE Formation and Cell Cycle Progression. To examine whether inactivation of the alkyltransferase would alter spontaneous SCE formation in normal lymphocytes, we exposed resting and proliferating lymphocytes to 0.5 mM O'mGua for 24 h prior to the addition of PHA and BrdUrd to visualize SCEs. Results of multiple experiments of three normal donors are shown in Table 1. There was a small but statistically significant increase in SCEs/metaphase in proliferating lymphocytes exposed to O'mGua (P < 0.0005). In resting lymphocytes O'mGua did not increase the number of SCEs.

We have previously shown that continuous culture in the presence of O'mGua decreases the growth rate of a number of cell lines (27). A similar effect on lymphocyte proliferation kinetics was observed in these experiments by calculating changes in the replication index under different culture conditions. A change in the replication index indicates an effect on cell cycle time. The effect of a 24-h exposure to 0.5 mM O'mGua on the replication index in our cultures is shown in Table 1. The replication index in the proliferating cells exposed to O'mGua was decreased over the control proliferating cells (ratio of exposed/control = 0.91) (P < 0.0005) suggesting that there may be slowing of cell cycle progression by O'mGua. The decrease in the replication index in cells that were resting during O'mGua exposure was not statistically significant.

The effect of O'mGua on cell cycle progression in PHA-stimulated lymphocytes was confirmed by measuring the incorporation of [3H]thymidine in cells cultured with or without 0.5 mM O'mGua using a previously described method (8). A slight decrease in [3H]thymidine incorporation was noted that rebounded to normal stimulated values as soon as O'mGua was removed (data not shown). In subsequent experiments, lymphocytes were exposed to O'mGua for only 18–24 h which was sufficient to maximally inactivate the alkyltransferase activity but minimally affect cell cycle progression and proliferation.

Role of the Alkyltransferase in BCNU-induced SCEs. BCNU caused a dose-dependent increase in SCEs expressed as a percentage of control values. The induction of SCEs was linear over BCNU doses of 0–15 μM allowing the calculation of SCEs/metaphase/μM BCNU. At these low doses

<table>
<thead>
<tr>
<th>Donor (no. of experiments)</th>
<th>Condition</th>
<th>SCE (x ± SD)</th>
<th>R/I (x ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting lymphocytes</td>
<td>control</td>
<td>7.48 ± 2.29</td>
<td>2.26 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>8.14 ± 1.02</td>
<td>1.98 ± 0.49</td>
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<tr>
<td></td>
<td>control</td>
<td>8.08</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>9.52</td>
<td>2.00</td>
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<tr>
<td>X</td>
<td>control</td>
<td>7.72 ± 1.67</td>
<td>2.19 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>8.69 ± 1.13</td>
<td>1.99 ± 0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.1 &gt; P &gt; 0.05)</td>
<td>(0.1 &gt; P &gt; 0.05)</td>
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Proliferating Lymphocytes

<table>
<thead>
<tr>
<th>Donor (no. of experiments)</th>
<th>Condition</th>
<th>SCE (x ± SD)</th>
<th>R/I (x ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(7)</td>
<td>control</td>
<td>7.53 ± 1.04</td>
<td>2.47 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>9.03 ± 1.86</td>
<td>2.05 ± 0.54</td>
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<tr>
<td></td>
<td>control</td>
<td>9.80</td>
<td>2.15</td>
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<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>11.68</td>
<td>1.56</td>
</tr>
<tr>
<td>2(1)</td>
<td>control</td>
<td>6.81 ± 0.16</td>
<td>2.74 ± 0.25</td>
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<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>7.88 ± 0.84</td>
<td>1.87 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>7.82 ± 1.34</td>
<td>2.51 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>8.96 ± 1.82</td>
<td>1.95 ± 0.45</td>
</tr>
<tr>
<td>X</td>
<td>control</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM O'mGua</td>
<td>P &lt; 0.0005</td>
<td>P &lt; 0.0005</td>
</tr>
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</table>
of BCNU, resting and proliferating lymphocytes were equally sensitive to BCNU-induced SCEs (0.76 SCEs/metaphase/μM BCNU, resting versus 0.79 SCEs/metaphase/μM BCNU, proliferating). Inactivation of the alkyltransferase by O-mGua significantly increased the level of BCNU-induced SCEs in both resting and proliferating cells (Fig. 3). In proliferating lymphocytes, O-mGua pretreatment resulted in a 2.6-fold enhancement of SCE formation by BCNU (2.02 SCEs/metaphase/μM BCNU with O-mGua versus 0.79 SCEs/metaphase/μM BCNU without O-mGua, P < 0.002). An identical effect was observed in lymphocytes incubated with PHA for 24, 48, or 72 h prior to O-mGua exposure (data not shown).

The effect of O-mGua in resting lymphocytes was even greater than that seen in proliferating cells. In these cells, O-mGua pretreatment resulted in a 4-fold increase in SCE induction by BCNU (3.08 SCEs/metaphase/μM BCNU with O-mGua versus 0.76 SCEs/metaphase/μM BCNU without O-mGua, P < 0.001). Thus, following inactivation of the alkyltransferase by O-mGua, both resting and proliferating lymphocytes became much more sensitive to SCE induction by BCNU. However, this effect was significantly more pronounced in resting than proliferating cells (P < 0.001). A likely explanation for this is that proliferating lymphocytes recover alkyltransferase activity within the first 6–18 h following O-mGua exposure whereas resting cells do not (Fig. 2).

DISCUSSION

We have evaluated the role of the DNA repair protein, O-alkylguanine DNA alkyltransferase, in the induction of SCEs by BCNU in resting and proliferating human lymphocytes. Mitogen stimulation of lymphocytes alters both the baseline alkyltransferase levels and the kinetics of recovery of alkyltransferase after its depletion. These alterations in alkyltransferase activity are a prime determinant of the ability of the nitrosourea BCNU to induce SCEs in lymphocytes.

We have previously observed that the alkyltransferase activity in PHA-stimulated lymphocytes increases to 1.5 times control levels 48 h after mitogen stimulation (8). Despite higher alkyltransferase activity in the proliferating cells we show here that resting and proliferating lymphocytes are equally sensitive to SCE induction by low doses of BCNU. This suggests that there is a threshold level of alkyltransferase activity necessary to prevent those BCNU-induced SCEs mediated by O-alkylgau-

Fig. 3. Effect of alkyltransferase inactivation on BCNU-induced SCEs in resting and proliferating human lymphocytes. Lymphocytes were cultured for 24 or 72 h in tissue culture medium with or without the addition of 1% PHA and 0.5 mM O-mGua prior to exposure to BCNU and determination of SCE. Each data point represents the mean ± SEM of two to seven experiments (50–175 metaphases) using lymphocytes from three different donors.

Our data do not allow for precise determination of the critical threshold value of alkyltransferase activity but do illustrate that the ability to regenerate alkyltransferase activity may be important in protecting cells against BCNU induced SCEs. Should biochemical modulation of the alkyltransferase become an important target for cancer chemotherapy, these data indicate that O-mGua has differential effects on resting and proliferating cells. This difference could contribute both to the therapeutic efficacy and toxicity observed with the combination of O-mGua given to inactivate the alkyltransferase, and BCNU, given as the therapeutic agent.

Alkyltransferase activity is known to be an important mechanism of resistance to SCE formation induced by agents which produce alkylation at the O6 position of guanine, especially O6-chloroethyl-alkylation which can lead to interstrand cross-links (17). Cells deficient in the alkyltransferase are more sensitive to nitrosourea induced SCEs than cells with higher alkyltransferase activity (15–17). Inactivation of the alkyltransferase with MNU prior to exposure of human lymphocytes to BCNU results in potentiation of SCE formation (26). Although MNU and BCNU both cause alkylation at O6-guanine, BCNU is a more potent inducer of SCEs because it causes interstrand cross-links (23).

Brent et al. have recently elicited an additional mechanism by which cross-link precursors are repaired by the alkyltransferase (35). In his scheme, the initial O6-chloroethylguanine DNA adduct can undergo an internal rearrangement to O6-ethanoguanine both of which can either react with the alkyltransferase or go on to a cross-link reaction. In the latter case, the reaction yields an alkyltransferase DNA cross-link (35). Although this DNA-protein cross-link is likely to be less cytotoxic and less prone to the induction of SCEs than the DNA interstrand cross-link, repair of either adduct is important in determining cytotoxicity and SCE formation. Data from Brent
DNA REPAIR AND NITROSOUREA INDUCED SCEs IN LYMPHOCYTES

(35) and Kohn (36) indicate that a period of 8–12 h is required for this intermediate and the subsequent cross-link to form after BCNU exposure. Thus, changes in the level of alkyltransferase activity during this period would critically influence the number of SCEs produced especially following inactivation of the alkyltransferase with O\(\alpha\)-mGua. Since proliferating cells as opposed to resting cells begin to regenerate the alkyltransferase during this period, alkyltransferase mediated repair of these cross-link intermediates may be an important mechanism of BCNU resistance.

The alkyltransferase is not the only DNA repair enzyme which is induced in proliferating lymphocytes. The excision repair system (9), polymerase \(\beta\) (10, 11), DNA ligase (12), uracil-DNA glycosylase (13), and poly(ADP ribose) polymerase (14) are all altered by mitogen stimulation. These repair systems are likely to be important in modulating the extent of SCE formation by other DNA-damaging agents, and may explain the variable effects of mitogen stimulation on SCE induction by different chemical carcinogens. We have shown that changes in the sensitivity to SCE induction after exposure to chemicals such as BCNU which produce O\(\alpha\)-alkylguanine can be explained by corresponding changes in the levels of the alkyltransferase. As we have done with the alkyltransferase, it is possible to gauge the impact of a specific DNA repair system during the transition from the resting to the proliferating state by specifically inhibiting that system and then observing the response to DNA damage.

Monitoring human exposure to mutagenic, carcinogenic, and chemotherapeutic agents is frequently performed through the analysis of SCE induction in human lymphocytes (1). Our studies indicate that a systematic analysis of the DNA repair capacity corresponding to the type of DNA damage anticipated as well as analysis of the state of proliferation of the cells are essential to properly interpret the relationship between toxin exposure and SCE formation.

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