Reduced Level of DNA Cross-Links and Sister Chromatid Exchanges in 1,3-Bis(2-chloroethyl)-1-nitrosourea-resistant Rat Brain Tumor Cells


ABSTRACT

We found that 9L-2 cells, a cell line derived from the in vivo 9L rat brain tumor model, are approximately 8-fold more resistant to the cytotoxic effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) than are sensitive 9L cells. Treatment with BCNU induces sister chromatid exchanges in both lines, but to produce similar levels of exchanges, 9L-2 cells must be treated with a 14-fold higher concentration of BCNU. The extent of DNA methylation was the same in both cell lines after a 1-hr treatment with 100 \( \mu \text{M} \) methylnitrosourea. While the levels of the alkylation products N7-methylguanine and N3-methyladenine were similar in both lines, the level of O6-methylguanine was 20% lower in 9L-2 than in 9L cells, which implies that 9L-2 cells repair O6-alkylguanine derivatives more efficiently than do 9L cells. The number of DNA interstrand cross-links formed in 9L-2 cells after treatment with BCNU was approximately 50% of the number formed in 9L cells. These results suggest that the repair of O6-alkylguanine derivatives formed in BCNU-treated 9L-2 cells may be related to the reduced number of DNA interstrand cross-links formed and may have a role in the mechanism of cellular resistance of 9L-2 cells to BCNU. However, our results indicate that, in itself, the reduction in the number of DNA cross-links may not be sufficient to account entirely for the cellular resistance of 9L-2 cells to BCNU and suggest that additional mechanisms may be involved in cellular resistance of 9L-2 cells to BCNU treatment.

INTRODUCTION

Resistance of cells to the cytotoxic effects of chemotherapeutic agents appears to be one cause of the ineffectiveness of chemotherapy for human cancer (12, 30). Approximately 60% of human brain tumors are resistant to the cytotoxic effects of BCNU* (41, 42). Studies of the properties of cell lines that are resistant to chemotherapeutic agents may help define more fully the molecular mechanisms involved in cellular resistance.

Resistance to different chemotherapeutic agents is caused by many different mechanisms in a variety of cell lines. The uptake of alkaloids (20) and mustards (28) is reduced in some resistant cell lines. Bioactivation of phenylalanine mustard by enzymes such as glutathione S-transferase may cause resistance to the drug (36). Gene amplification of target enzymes mediates cellular resistance to methotrexate (1), hydroxyurea (19), 5-fluorouracil (32), and N-(phosphoacetyl-L-aspartate) (39). Deletion of or mutation in the gene coding for hypoxanthine-guanine phosphoribosyltransferase are probable causes of resistance to 6-thioguanine (8, 15). Increased repair of DNA adducts that can form DNA cross-links or repair of DNA cross-links after they are formed has been implicated as a major factor in the resistance of cells to DNA cross-linking agents (4, 5, 13, 22, 23, 44).

CENUs are chemotherapeutic agents used to treat brain tumors and a variety of other systemic tumors (41, 42). Under physiological conditions, CENUs are hydrolyzed to reactive species that alkylate cellular DNA, RNA, and proteins (25). Some monoalkylation products may form DNA interstrand cross-links in subsequent reactions (17, 38). Cross-link formation is thought to cause the cytotoxic effects of CENUs (13, 30, 44).

Although the chemical structure of the cross-links formed in cells treated with CENUs has not been determined definitively, there is evidence that the formation of O6-(2-chloroethyl)guanine is an initial important alkylaion event that leads to the subsequent formation of DNA cross-links. This hypothesis is supported by several observations: (a) cells that can remove O6-mGua from DNA are resistant to the cytotoxic effects of CENUs and have fewer DNA interstrand cross-links induced after treatment with CENUs (13, 44), and (b) O6-(2-fluoroethyl)guanine has been isolated from DNA treated with N-(2-fluoroethyl)-N'-cyclohexyl-N-nitrosourea (38).

The enzyme that repairs O6-alkylguanine in DNA is O6-mGua-DNA methyltransferase (7, 10, 26), a receptor protein that catalyzes the dealkylation of O6-alkylguanine in DNA to produce the alkylated protein and guanine (7, 10, 26). The transferase is inactivated after a single event (7, 10, 26). Human tumor cells have been classified as Mex° or Mex° based on their capability to remove O6-mGua from DNA (11, 16, 33, 43). Mex° cells have 20,000 to 60,000 transferase molecules/cell, and Mex° cells have about 1200 molecules/cell (16, 21, 40, 43). Because the maximum number of DNA interstrand cross-links are formed 6 hr after treatment with CENUs (17), in Mex° cells, there is an adequate time for repair of O6-2-chloroethylguanine adducts before DNA interstrand cross-links are formed. These results suggest that repair of O6-alkylguanine derivatives from DNA and inhibition of DNA cross-link formation may be important factors in human tumor cell resistance to CENUs.

Currently, a number of rodent-derived cell lines are used to test the effects of chemotherapeutic agents. One of these, 9L, is a well-characterized gliosarcoma cell line that has been used...
for both in vivo and in vitro cytotoxicity studies (3). CENU-resistant sublines have been isolated from the 9L cell line (30). We have begun a study of the mechanisms of resistance to CENU using one of these sublines, 9L-2 cells. Results of the initial phase of this project are reported here.

MATERIALS AND METHODS

Cell Lines. The 9L rat gliosarcoma is a well-established cell line (3). A subline resistant to CENUs was produced by the following procedure. Fisher 344 rats bearing intracerebral 9L tumors were treated with a single dose of BCNU (26.7 mg/kg). Rats were sacrificed 24 hr later, and tumors were removed and disaggregated to single cells with an enzyme cocktail using a reported method (31). Cells that survived treatment, designated 9L-2, were passaged in culture and then stored in liquid nitrogen. 9L and 9L-2 cell lines were subcultured approximately twice a week.

CFE Assay. Single-cell suspensions of 9L and 9L-2 cells were obtained by incubation of confluent cultures with trypsin/EDTA; 50 to 1 × 10⁶ cells were plated into Costar 6-well plates with heavily irradiated 9L feeder cells in minimal essential medium containing 10% fetal calf serum and gentamicin (50.0 μg/ml). After 24 hr of incubation, cells were treated with various concentrations of BCNU for 1 hr. Medium was then replaced, and cultures were incubated for an additional 10 to 14 days. After being fixed with methanol/acetic acid and stained with crystal violet, colonies consisting of 50 or more cells were counted. Cell survival was calculated as the ratio of the CFEs of treated and untreated cells.

SCF Assay. 9L and 9L-2 cells (1.5 to 2.0 × 10⁶) were seeded into 75-cm² flasks. The following day, cells were treated with various concentrations of BCNU dissolved in 100% ethanol. After 1 hr of treatment, medium was removed and replaced with medium containing 0.05 M KCl for 8 to 10 min and fixed twice with glacial acetic acid and centrifugation (1000 rpm for 5 min). The pellet was treated with 2.0 ml of 0.05 M KCl for 8 to 10 min and fixed twice with glacial acetic acid and methanol, and metaphase chromosomes were spread on glass microscope slides. The method of Perry and Wolff was used for differential staining of sister chromatids (27). For each experiment, the frequency of SCEs was determined in 25 metaphase cells.

Analysis of DNA Alkylation Products. 9L or 9L-2 cells were grown in 850-cm² roller bottles. After cells had reached confluency, they were trypsinized and collected by centrifugation at 1500 rpm for 3 min. Cells (1 to 2 × 10⁶) were suspended in cold 5 ml of cold 100% ethanol and stored at −40° until the DNA was isolated.

The DNA was isolated and purified from the cellular pellet using a published method (6). The DNA was precipitated repeatedly with sodium acetate and 100% ethanol until a constant specific activity was obtained, stored at −40° until the DNA was isolated.

The method of Perry and Wolff was used for differential staining of sister chromatids (27). For each experiment, the frequency of SCEs was determined in 25 metaphase cells.

Calculation of the number of DNA cross-links was done using the procedure of Ewrig and Kohn (14). The cross-link index was calculated using the formula:

\[
[(1 - R_0)/(1 - R_i)]^{1/2} - 1
\]

where \(R_0\) and \(R_i\) are the relative retention for untreated and BCNU-treated cells, respectively. Relative retention was defined as the fraction of the [³¹C]DNA remaining on the filter when 50% of the [³¹H]DNA remained on the filter.

RESULTS

Cell Survival. Survival plots for 9L and 9L-2 cells treated with various concentrations of BCNU are shown in Chart 1. 9L-2 cells

HPLC was performed using a Chromatotronix 3500 pump coupled to a Rheodyne 7120 sample injector valve with a 500-μl sample loop. A 5-μm C-18 reverse-phase column (Alttech 605-RP) was used for the separation. UV absorbance at 282 nm was monitored with a Perkin Elmer Model 55 detector. The mobile phase was 0.01 M NH₄H₂PO₄, pH 5.1, with 16% methanol; the flow rate was 1.2 ml/min. The average retention times were: polyguanines, 3.5 min; guanine, 5.3 min; m⁷Gua, 8.4 min; adenine, 9.6 min; m³Ade, 13.2 min; and O⁶-mGua, 16.7 min.

Fractions (0.6 ml) were collected continuously and mixed with 10 ml of Aquasol. The levels of radioactivity were determined using a Beckman LS-250 liquid scintillation counter. The counting efficiency was 40%, and the average overall recovery of the sample was 92%.

Alkaline Elution Assay. 9L or 9L-2 cells (0.5 to 1 × 10⁶) were seeded into 100-cm² dishes and grown for 2 to 3 days in medium containing either [³¹C]thymidine (0.01 μCi/ml) or [³¹H]thymidine (0.1 μCi/ml). Medium containing labeled compounds was removed and replaced with fresh medium. [³¹C]-Labeled cells were treated with various concentrations of BCNU at 1 hr after, after which medium was replaced with fresh medium, and cells were incubated for an additional 6 hr. Immediately before elution, medium was replaced with cold Hanks' balanced salt solution. The [³¹H]-labeled cells, which served as the internal control, were irradiated with 300 rads of X-rays, and the [³¹C]-labeled cells were irradiated with 600 rads of X-rays (General Electric Maxitron 300; 300 kVp, 20 ma; nominal half-value layer, 2.0 mm copper).

The procedure used for alkaline elution was a modification of that described by Kohn et al. (18). Approximately 5 × 10⁶ treated [³¹C]-labeled cells and a similar number of control [³¹H]-labeled cells were filtered onto polycryl chloride filters (diameter, 25 mm; pore size, 2 μm) (Millipore Corp., Bedford, MA) and washed twice with 5 ml of cold calcium-magnesium-free Dulbecco's phosphate-buffered saline. Cells were lysed on the filter with 5 ml of Sarkosyl-NaCl-EDTA lysis solution (pH 10.0) containing Proteinase K (0.5 mg/ml). The lysate was digested with Proteinase K for 1 hr, and then the lysis solution was allowed to flow through by gravity. The filters were then rinsed with 4 ml of 0.02 M disodium EDTA, pH 10.0. No suction was applied before or after lysis. Elution was carried out in the dark with a solution of tetrahydrofuryl-EDTA plus tetraperopylammonium hydroxide (Eastman Kodak, Rochester, NY), 2% in water, pH 12.2 at a flow rate of 2 ml/hr (0.03 ml/min). Fractions were collected at 1-hr intervals for 13 to 15 hr. Samples were mixed with 4 ml of Aquasol for scintillation counting. Radioactivity remaining on the filter was determined by treating filters with 0.4 ml of 1 N HCl at 70° for 1 hr, followed by 2.5 ml of 4 N NaOH at room temperature for 30 min, after which 6 ml of Aquasol were added. The remaining radioactivity in the column and pump tubing was recovered by passing 2.5 ml of 4 N NaOH through the funnel into the tube; the wash was left in the tubing for 1 hr and then collected. 1 N HCl (0.4 ml) was added, and the solution was mixed with 6 ml of Aquasol. Radioactivity was determined using either a Packard Tri-Carb or a Beckman LS-330 liquid scintillation spectrometer. Results were calculated as the fraction of [³¹C]DNA and [³¹H]-DNA retained on the filter at each fraction.

Calculation of the number of DNA cross-links was done using the procedure of Ewrig and Kohn (14). The cross-link index was calculated using the formula:

\[
[(1 - R_0)/(1 - R_i)]^{1/2} - 1
\]

where \(R_0\) and \(R_i\) are the relative retention for untreated and BCNU-treated cells, respectively. Relative retention was defined as the fraction of the [³¹C]DNA remaining on the filter when 50% of the [³¹H]DNA remained on the filter.

RESULTS

Cell Survival. Survival plots for 9L and 9L-2 cells treated with various concentrations of BCNU are shown in Chart 1. 9L-2 cells

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are markedly more resistant to the cytotoxic effects of BCNU than are 9L cells. Ninety % of the 9L cells cannot form colonies after a 1-hr treatment with 30 μM BCNU. In contrast, 9L-2 cells must be treated with 120 μM BCNU to achieve the same effect. Thus 9L-2 cells are at least 4-fold more resistant to the cytotoxic effects of BCNU than are 9L cells; however, when the amount of resistance is calculated as the ratio of the slopes of the survival plots (cell kill/μM of BCNU), 9L-2 cells are 8-fold more resistant to the cytotoxic effects of BCNU than are 9L cells.

SCE Induction. BCNU efficiently induces SCEs in 9L cells; the dose-response curve is linear (Chart 2A), with a slope of 13.5 SCEs/μM of BCNU. However, very few SCEs were induced in 9L-2 cells treated over the same dose range (Chart 2B). A 10-fold higher concentration of BCNU was needed to induce a similar number of SCEs in 9L-2 cells (Chart 2B). The slope of the dose-response relationship for SCE induction in 9L-2 cells was 0.93 SCEs/μM of BCNU. Therefore, as calculated by the ratio of the slopes of the dose-response plots, 9L-2 cells are 14-fold less susceptible to the induction of SCEs than are 9L cells. Data plotted in Charts 1 and 2 were obtained with successive passages of 9L-2 cells. Results indicate that resistance to BCNU is a stable property of 9L-2 cells, at least for the 6-month period during which the data plotted in Charts 1 and 2 were collected.

Because recently published data (13, 44) suggest that a relationship exists between the repair of O6-alkylguanine derivatives and resistance to CENUs in human tumor cells, we examined the possibility that similar mechanisms were responsible for the observed resistance of 9L-2 cells to SCE induction and cytotoxicity. O6-mGua is rapidly excised in Mex+ cells, but repair of O6-mGua could not be detected in Mex- cells (11, 16, 21, 33). We treated 9L and 9L-2 cells with 100 μM [3H]MNU, a dose of MNU that does not saturate the methyltransferase activity in Mex+ cells (21). The extent of methylation of DNA in 9L and 9L-2 cells after a 1-hr treatment with 100 μM [3H]MNU was very similar (Table 1).

Methylation product were separated by HPLC and quantitated by liquid scintillation spectrometry. The formation of m3Ade and m7Gua was the same in the 2 cell lines. In 9L cells, the O6-mGua/m7Gua ratio was 0.118, which is similar to values reported for other cell lines that do not repair O6-mGua (11). In contrast, the O6-mGua/m7Gua ratio in 9L-2 cells was 0.094, approximately 20% lower than in 9L. Because O6-mGua is a stable alkylation product of DNA, the lower O6-mGua/m7Gua ratio in 9L-2 cells represents repair of O6-mGua by 9L-2 cells during the 1-hr treatment period.

Differences in cellular repair of O6-mGua in 9L and 9L-2 cells are smaller than those observed by others for the repair of O6-mGua in Mex+ human tumor cell lines (11, 16). Because of the possibility that the methods used might influence the results, we treated HeLa cells classified as Mex- with the same protocol used to treat 9L and 9L-2 cells. The O6-mGua/m7Gua ratio was 0.064 in cells treated for 1 hr with 100 μM [3H]MNU (Table 1), a

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**Mechanisms of Resistance to BCNU**

**Table 1**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Total methylation (μmol methylated purine/mol of DNA)</th>
<th>m3Ade μmol methylated purine/mol of DNA</th>
<th>m7Gua μmol methylated purine/mol of DNA</th>
<th>O6-mGua/ m7Gua</th>
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<td>9L E-1</td>
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<td>1.2</td>
<td>18.3</td>
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<td>1.6</td>
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<td>21.6</td>
<td>2.5</td>
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<tr>
<td>9L-2 E-1</td>
<td>30.7</td>
<td>1.8</td>
<td>22.9</td>
<td>2.3</td>
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<tr>
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<td>27.7</td>
<td>1.3</td>
<td>21.4</td>
<td>1.9</td>
</tr>
<tr>
<td>CCL2</td>
<td>21.4</td>
<td>0.7</td>
<td>15.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Chart 1.** Survival of 9L (●) and 9L-2 (▲) cells treated for 1 hr with BCNU. Each symbol represents the mean value for a single experiment. The survival of 9L and 9L-2 cells were measured at several different passages during a 6-month period.

**Chart 2.** Induction of SCEs in 9L (●) and 9L-2 (▲) cells treated with various doses of BCNU. A, BCNU (0 to 10 μM), B, BCNU (0 to 55 μM). The frequency of BCNU-induced SCEs was calculated by subtracting the number of SCEs in cells treated with 10 μM bromodeoxyuridine alone. The dose-response curves for SCE induction were calculated by linear regression analysis using the equation y = Ax, where y is the number of SCEs induced, A is the slope of the line, and x is the BCNU dose. Each symbol represents the mean value for a single experiment.

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value approximately 47% lower than that found for 9L cells. Similar values for HeLa CCL2 cells were reported by Day et al. (11).

Compared with sensitive cells, the number of DNA interstrand cross-links formed after BCNU treatment is reduced in a number of human cell lines resistant to the cytotoxic effects of CENUs (13, 44). The relative number of DNA interstrand cross-links formed in 9L and 9L-2 cells after treatment with either 50 or 100 μM BCNU for 1 hr, followed by a 6-hr incubation period, are listed in Table 2. Compared to 9L cells, the number of cross-links in 9L-2 cells was 50% lower.

**DISCUSSION**

Our results show that 9L-2 cells are approximately 8-fold more resistant to the cytotoxic effects of BCNU treatment than are 9L cells. The 14-fold difference in BCNU concentration needed to induce the same level of SCEs in 9L-2 and 9L cells suggests that the mechanism(s) that lead to the reduced level of SCE induction and reduced sensitivity to BCNU of 9L-2 cells may be related. A correlation between induction of SCEs and cytotoxicity, measured by the CFE assay, has been reported (24, 37). Results reported here extend this correlation to resistant cell lines.

As discussed in “Introduction,” many mechanisms could account for cellular resistance to chemotherapeutic agents. However, the mechanisms that might cause resistance to BCNU in 9L-2 cells are reduced uptake of BCNU, decreased alkylation of cellular DNA, increased repair of the chloroethyl alkylation products of DNA, and a decrease in the number of DNA interstrand cross-links formed.

The extent of DNA alkylation was the same in both cell lines and was similar to levels of DNA alkylation reported for other cell lines treated with MNU (21). This result suggests that drug uptake and the level of alkylation of DNA by nitrosoureas is probably not the cause of the resistance in 9L-2 cells to BCNU observed in these studies. This conclusion is supported by the work of Connors and Hare (9), who found very little difference in total alkylation between sensitive and resistant tumor cells treated with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

The levels of m7Gua and m3Ade found after a 1-hr treatment with MNU were similar in 9L and 9L-2 cells, which indicates that there was no differential repair of these adducts. However, after a 1-hr treatment with 100 μM MNU, the ratio of O6-mGua/m7Gua was 20% lower in 9L-2 than in 9L cells; therefore, there is more repair of O6-mGua in 9L-2 than in 9L cells. This difference in the repair of O6-mGua is smaller than difference in repair found for Mex+ and Mex− human cell lines (11, 16, 33). Our finding that O6-mGua was more efficiently repaired in HeLa CCL2 cells than in 9L-2 cells suggests that resistant human cells may have a higher level of O6-mGua-DNA methyltransferase than do resistant rodent cells. However, more extensive studies of differences between resistant human and rodent cell lines must be conducted before a definitive conclusion can be drawn.

Erickson et al. (13) and Zlotogorski and Erickson (44) found the Mex+ cells have fewer DNA interstrand cross-links after BCNU treatment than do Mex− cells. Because the level of repair of O6-mGua was higher in 9L-2 cells compared to 9L cells, 9L-2 cells resemble the Mex+ phenotype. Alkaline elution profiles indicate that 9L-2 cells had about 2-fold fewer DNA cross-links than did the 9L cells, probably because O6-alkylguanine derivatives are repaired more efficiently in 9L-2 cells. This is consistent with the findings of Robins et al. (29), who reported that O6-mGua-DNA methyltransferase removes the chloroethyl alkylation product that forms DNA cross-links (29). Fewer DNA interstrand cross-links are formed in Mex+ human tumor cells (44) than in 9L-2 cells treated with BCNU, which probably reflects the different levels of repair of O6-alkylguanine between the 2 cell lines.

An important question is whether the 2-fold difference observed in cross-link formation between 9L-2 and 9L cells is sufficient to account for the 14-fold difference observed in SCE induction and the 8-fold difference in cytotoxicity. Standberg et al. (34, 35) have observed that the differences in cellular DNA interstrand cross-link formation induced by cis-platinum in sensitive and resistant L1210 cells did not account for the observed differences in cellular cytotoxicity. This suggests that, in addition to differences in the induction of DNA cross-links, other mechanisms that modify BCNU cytotoxicity could be operative in 9L-2 cells. Further work is required to clarify the role of DNA repair and other biochemical mechanisms in cellular resistance to the cytotoxicity effects of BCNU.

**ACKNOWLEDGMENTS**

We thank Rufus Day for kindly providing HeLa CCL2 cells, Ann Schlee for typing the manuscript in draft, and Neil Buckley for editorial assistance.

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Mechanisms of Resistance to BCNU

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Reduced Level of DNA Cross-Links and Sister Chromatid Exchanges in 1,3-Bis(2-chloroethyl)-1-nitrosourea-resistant Rat Brain Tumor Cells


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