ABSTRACT

Two human colon carcinoma cell lines which differ greatly in their content of O6-alkylguanine-DNAalkyltransferase were analyzed for their response to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 2-chloroethyl(methylsulfonyl)methanesulfonate (ClEtSoSo) before and after treatment with O6-alkylguanines. HT29 cells contained about 17 times more alkyltransferase activity than BE cells. The alkyltransferase activity of HT29 cells was reduced by 60–80% by treatment for 24 h with 0.05–0.4 mM O6-methylguanine or O6-N-butylyguanine. Such pretreatment prior to exposure to CCNU or ClEtSoSo increased the sensitivity of HT29 cells to these drugs. The exposure to O6-alkylguanines gave a greater enhancement of the toxic effects of ClEtSoSo than of CCNU. There was no significant increase in the toxicity of these agents towards the BE cells which contained much lower levels of the alkyltransferase. When added alone neither O6-methylguanine nor O6-N-butylyguanine showed any toxicity towards HT29 or BE cells at the doses used. These results provide strong evidence that the formation of adducts at the O6-position of guanine by these agents contributes significantly to their lethality and that this reaction is more critical for ClEtSoSo than CCNU. The enhancement of the activity of the alkylating agents by pretreatment with nontoxic doses of O6-alkylguanines may be clinically useful in terms of increasing their therapeutic efficacy towards cells containing high levels of alkyltransferase.

INTRODUCTION

CENUs2 are clinically useful against a variety of malignant diseases, particularly brain tumors and Hodgkin’s disease (1–3). These agents, which include CCNU, exert their biological effect through a spontaneous decomposition reaction in which the parent drug is converted to active alkylating and carbamoylating intermediates (4, 5). Their antitumor activity appears to be a result of alkylation of DNA, in particular chloroethylation at the O6-position of guanine. Evidence suggests that lethal cross-links may form between complementary strands of DNA following generation of the O6-chloroethylguanine adduct. After initial attack at the O6-position of guanine, there is a slow intramolecular rearrangement followed by reaction with cytosine in the opposite DNA strand which requires several hours (6–8). In support of this, Erickson et al. (9, 10) and Kohn et al. (11) have found that cells exhibiting a higher sensitivity to the cytotoxic activity of the CENU form interstrand cross-links to a much greater extent.

Another class of alkylating compounds, the 2-haloethyl (methylsulfonyl)methanesulfonates, was found to be highly effective against P388 leukemia in vivo (12, 13). Gibson et al. (14) determined that ClEtSoSo produced interstrand cross-links after a 6–12 h delay in a cell strain also sensitive to CENUs but not in cell strains resistant to CENUs. It was suggested that ClEtSoSo acts in much the same way by chloroethylating the O6-position of guanine in DNA and producing lethal cross-links. Recent data suggest that ClEtSoSo may react more selectively than CENUs since ClEtSoSo does not produce toxic carbamoylating side products or hydroxethylated DNA adducts, neither of which contribute to antitumor activity (15).

Mammalian cells contain a protein termed AGT which transfers an alkyl group from the O6-position of guanine in DNA onto one of its own cytosine residues. This protein repairs methyl, ethyl, hydroxethyl, propyl, and butyl adducts on guanine (16–18). There is indirect evidence that the chloroethyl adduct is also repaired by this protein which thus prevents interstrand cross-links from forming (18, 19). A unique property of this repair protein is that it is not regenerated in the reaction; therefore, the number of O6-alkylguanine lesions repaired is limited by the amount of AGT present (20, 21). Cells containing high levels of AGT (termed Mer+) are resistant to the cytotoxic effects of mono- and bifunctional alkylnitrosoureas, whereas cells with little or no AGT (termed Mer−) are sensitive to these agents (22–24).

Zlotogorski and Erickson (25, 26) have determined that pretreatment of Mer+ cells with a dose of methylating agent sufficient to saturate the AGT repair system enhances the sensitivity of cells to CENUs and allows the formation of interstrand cross-links. Although this may be a method to enhance the activity of CENUs, its usefulness may be limited because methylating agents are highly carcinogenic and mutagenic (20, 22).

Recently, we and others have found that exposure of cells to nontoxic doses of O6-alkylguanines can specifically deplete the cells of AGT (27–30). In this paper, we have described the effect of treating two human colon carcinoma cell lines, HT29 (Mer+) and BE (Mer−), with m6Gua or n-bu6Gua prior to treatment with CCNU or ClEtSoSo. The objective was to test whether the biological effects of chemotherapeutic cross-linking agents can be enhanced by depleting cells of AGT; furthermore, a comparison was made between an agent which has carbamoylating activity as well as alkylating activity with an agent possessing the latter only.

MATERIALS AND METHODS

Cell Culture. Human colon carcinoma cell lines (HT29 and BE) were obtained from Dr. L. C. Erickson, Loyola Stritch School of Medicine, Maywood, IL (10). Both cell lines were grown in Dulbecco’s minimal essential medium containing 36 mM NaHCO3 supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml), and 3% glutamine. Cells were maintained in a humidified atmosphere of 10% CO2 at 37°C and were seeded at weekly intervals with 2.5 × 104 cells/75-cm2 flask. The cells were harvested by rinsing with 0.02% (w/v) EDTA/phosphate-buffered saline prior to the addition of a 0.25% (w/v) solution of trypsin 4500

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The abbreviations used are: CENU, chloroethylnitrosourea; ClEtSoSo, 2-chloroethyl(methylsulfonyl)methanesulfonate; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; ACT, O’-alkylguanine-DNA alkyltransferase; m6Gua, O6-methylguanine; n-bu6Gua, O6-N-butylyguanine; CNU, 1-(2-chloroethyl)-1-nitrosourea; ED50, the effective dose required to inhibit cell proliferation by 50%.

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m'Gua and n-bu'Gua. Cells in exponential growth were preincubated for 24 h at a density of 2 x 10^6 cells/25-cm^2 flask in the presence or absence of 0.4 mM m'Gua or n-bu'Gua. The cells were then treated for 2 h with 20 μl of different dilutions of ClEtSoSo or CCNU dissolved in ethanol as shown in the figure legends.

Cell Proliferation and Survival. After treatment with the chloroethylating agent, cytotoxicity was determined by inhibition of cell growth and loss of ability to form colonies. For the cell proliferation studies, cells were allowed to grow for 3 or 4 days with a change to fresh medium 24 h after treatment with the alkylating agent. Cell number was determined by counting on an electronic Coulter Counter. For determination of colony forming ability, cells were replated in fresh medium 24 h after treatment with the alkylating agent. Cell number was determined by counting on an electronic Coulter Counter. For determination of colony forming ability, cells were replated in fresh medium after drug treatment at densities between 90 and 2000 cells in 25-cm^2 flasks. Colonies were counted 9–12 days after plating by rinsing with 0.9% saline solution and staining with 0.5% crystal violet in ethanol. The plating efficiencies for HT29 and BE cells were 38 and 42%, respectively.

Assay of AGT Activity. The cells were allowed to grow for 4 days, and the medium was replaced with fresh medium or fresh medium containing 0.4 mM m'Gua or n-bu'Gua. After 24 h, the cells were harvested, collected by centrifugation, and an extract was prepared as previously described (24). The AGT activity was analyzed using two different methods. One method involved a measurement of the decrease in radioactivity of a partially depurinated, H-methyalted DNA substrate after incubation with extract (27). The second method measured radioactivity in the m'Gua peak after separation by high-pressure liquid chromatography of acid-hydrolyzed DNA following incubation of DNA with extract for 30 min at 37°C (23). The purine bases were separated on a Microsil 5-μm C8 column (Micromeritics, Norcross, GA) and a precolumn of Bio-Sil ODS-SS (Bio-Rad, Richmond, CA) eluted isocratically with 0.05 M ammonium formate (pH 3.5) containing 7.5% methanol at 35°C. The flow rate was 1.0 ml/min. Protein was determined by the method of Bradford (32).

RESULTS

Two human colon carcinoma cell lines, HT29 and BE, were analyzed for alkyltransferase activity by incubating extract with DNA containing O^6-[^3H]-methyldeoxyguanosine. The amount of O^6-methyldeoxyguanosine remaining in the DNA was analyzed by high-pressure liquid chromatography (Fig. 1). HT29 cells were found to contain 0.51 unit AGT/mg protein and BE cells were found to contain about 0.03 ± 0.01 (S.D.) unit ACT/mg protein (27, 30) or Raji cells (28). There appeared to be a reduction in AGT activity upon exposure of BE cells to 0.4 mM m'Gua for 24 h, yet these values are very low and difficult to measure accurately.

The effect of depletion of AGT activity on the sensitivity of cells to the cytotoxic effects of chloroethylation agents was examined either by inhibition of cell proliferation or inhibition of colony forming ability. Exposure of HT29 or BE cells to 0.4 mM m'Gua or n-bu'Gua for up to 48 h did not affect cell proliferation or ability of cells to form colonies. HT29 cells were treated with medium alone or medium containing 0.4 mM m'Gua for 24 h prior to exposure to concentrations of CCNU up to 100 μM for 2 h. The ED50 as measured 4 days after treatment was 45 μM for cells exposed to medium alone. This value dropped to 28 μM when cells were pretreated with 0.4 mM m'Gua (Fig. 3). There was a 1.5-fold decrease in the ED50 of HT29 cells treated with m'Gua prior to CCNU treatment compared to treatment with medium alone if cell proliferation was measured after 2 days (data not shown). BE cells are more sensitive to the effects of CCNU than are HT29 cells. There was no significant enhancement of toxicity when BE cells were pretreated with m'Gua as measured by inhibition of cell growth (Fig. 3).

The differential cytotoxicity of ClEtSoSo against HT29 cells compared to BE was significantly larger than that produced by CCNU (Fig. 4). This is consistent with results obtained using IMR-90, a Mer^+ cell line, and VA-13, a Mer^- cell line (14). Pretreatment of HT29 cells with 0.4 mM m'Gua decreased the ED50 of ClEtSoSo from 560–170 μM as measured by cell growth 3 days after treatment (Fig. 4). This decrease in ED50 for ClEtSoSo is greater than that observed using CCNU as the chloroethylating agent.

These results were confirmed and extended by studies in which toxicity was measured by studying colony forming ability and are shown in Figs. 5 and 6. It was found that exposure of HT29 cells to 0.4 mM m'Gua decreased the ED50 of CCNU from 42–27 μM, which is a 1.6-fold increase in sensitivity. Exposure of HT29 cells to m'Gua resulted in a decrease in the ED50 of ClEtSoSo from a dose greater than 500–130 μM, which represents more than a 3.8-fold difference in sensitivity of cells to this drug. There was no significant difference in the sensitivity of BE cells to CCNU or ClEtSoSo by prior treatment with m'Gua (Figs. 5 and 6).

The ability of n-bu'Gua to enhance the cytotoxicity of CCNU and ClEtSoSo was also evaluated (Table 1). There was a 2-fold decrease in the percentage of HT29 cells surviving a dose of 50 μM CCNU and a 5.3-fold decrease in cells surviving a dose of 250 μM ClEtSoSo if pretreated with 0.4 mM n-bu'Gua. The
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Fig. 2. Reduction of AGT activity after incubation with O'-alkylguanines. HT29 cells were treated with O'-methylguanine (m6G) (●) or O'-n-butylguanine (n-bu6G) (●) or BE cells were treated with O'-methylguanine (●) at the concentrations indicated for 24 h. Alkyltransferase activity was determined and expressed as pmol AGT per mg protein, where 1 pmol AGT represents loss of 1 pmol of O'-methylguanine from the DNA substrate.

Fig. 3. Effect of O'-methylguanine (m6G) on proliferation of HT29 or BE cells treated with CCNU. HT29 and BE cells were exposed to fresh medium (●) or medium containing 0.4 mM O'-methylguanine (●) for 24 h prior to treatment with CCNU for 2 h at the concentrations indicated. Cell number was determined after 4 days of growth. Bars, SD of the mean of three determinations. C, control.

Fig. 4. Effect of O'-methylguanine (m6G) on proliferation of HT29 or BE cells treated with CIEtSoSo. HT29 and BE cells were exposed to fresh medium (●) or medium containing 0.4 mM O'-methylguanine (●) for 24 h prior to treatment with CIEtSoSo for 2 h at the concentrations indicated. Cell number after three days of growth was determined. Bars, SD of the mean of three determinations. C, control.

Fig. 5. Effect of O'-methylguanine (m6G) on cloning efficiency of HT29 and BE cells treated with CCNU. HT29 and BE cells were exposed to fresh medium (●) or medium containing 0.4 mM O'-methylguanine (●) for 24 h prior to treatment with CCNU for 2 h. Treatment with O'-methylguanine alone did not effect colony forming ability of HT29 or BE cells. Cells were plated at densities between 60 and 4500 cells/60-mm dish and colonies were counted 9-11 days later. Bars, SD of the mean of six determinations, each containing four dishes. C.F.E., colony forming efficiency; C, control.

**DISCUSSION**

Our results are in disagreement with those reported by Karran and Williams (28). They reduced AGT levels in a human lymphoma cell line (Raji) with m6Gua but found no enhancement in cell killing by CNU. It was concluded that adducts at the O'-position of guanine in DNA are not potentially cytotoxic lesions. This discrepancy could be due to the recovery time of AGT activity in the different cell lines studied. Raji cells recovered maximal AGT activity within 3-4 h after removal of m6Gua (28). Since cross-link formation requires 6-12 h, the regeneration of AGT within four hours would allow for repair of O'-chloroethylguanine before lethal cross-links formed. The recovery time for AGT in HeLa cells is much slower requiring 48 h to reach control levels (27). The rate of resynthesis of
AGT may have a profound effect on the enhancement of the cytotoxic activity of chloroethylyating agents by prior treatment with $O^6$-alkylguanines.

These results clearly show that the specific reduction of AGT in cultured cells can greatly enhance the cytotoxic effect of chloroethylyating agents suggesting that adducts repaired by AGT contribute significantly to cell lethality. Unlike the bacterial source, AGT isolated from mammalian sources cannot repair $O^6$-methylthymine (33, 34); thus, it is specific for $O^6$-alkylguanines lesions. We conclude that $O^6$-chloroethylyating guanine in DNA must be one of the adducts in cells that can exert toxic effects. Although there is no direct evidence that AGT repairs $O^6$-chloroethylyating guanine, it has been shown to repair long-chain alkyl derivatives such as $O^6$-hydroxyethylyating (16, 18). CCNU and CIEtSoSo are thought to exert their cytotoxic effect via attack at the $O^6$-position of guanine followed by cross-linking (6–9). CIEtSoSo is more specific in its reaction with DNA, producing chloroethylyated adducts with no generation of hydroxyethylyated products (15); thus, in the case of CIEtSoSo, unlike CCNU, there is no $O^6$-hydroxyethylyating guanine formed, which further supports the concept that AGT protects cells from toxicity due to chloroethylyating agents by repairing the initial $O^6$-chloroethylyating lesion prior to cross-link formation.

When BE cells were pretreated with m$^6$Gua, there was no significant increase in cell kill by CCNU or CIEtSoSo. Gibson et al. (35) found that administration of 1-methyl-3-nitro-1-nitrosoguanidine to deplete AGT levels in BE cells produced only a slight increase in cell kill and no change in cross-linking levels after treatment with CNU. There was a dramatic difference in both effects when HT29 cells were treated with 1-methyl-3-nitro-1-nitrosoguanidine prior to CNU. Similar observations were also found to be the case with another Mer$^+$ and Mer$^-$ pair of cell lines, IMR-90 and VA-13, respectively (26). The level of AGT is much lower in BE cells than HT29 cells. Although there was a decrease in AGT activity when BE cells were pretreated with m$^6$Gua, the values obtained were very low and therefore difficult to quantitate accurately. In BE cells, the amount of damage caused by chloroethylyating agents may far exceed the capacity of the cells to repair the damage so that a decrease in the already low levels of AGT will not affect the activity of these cross-linking compounds. Whatever the explanation is for the lack of enhancement in cell kill in Mer$^-$ cells, there appears to be a selective advantage in pretreating Mer$^+$ cells with agents that deplete AGT levels.

Although the mechanisms of action of CIEtSoSo and CCNU are similar in that both produce interstrand cross-links which are thought to be responsible for their antitumor activity, the additional side products generated from these agents are different. CCNU has the disadvantage of decomposing to a carbamylyating species which reacts with cellular protein but does not contribute to the therapeutic activity; furthermore, CENUs form hydroxyethylation products in DNA which are carcinoogenic and also contribute little if at all to antitumor activity (36, 37). The selective advantages of CIEtSoSo over CENUs as an antineoplastic agent are that CIEtSoSo does not hydroxyethylate DNA and does not generate carbamylyating species (15). Although the effective dose of CIEtSoSo required for the same cell kill is higher than CCNU, this dose can be reduced 4-fold by prior treatment with nontoxic doses of m$^6$Gua or n-bu'Gua. This effect is also observed with CCNU, but the differential toxicity by pretreatment with m$^6$Gua or n-bu'Gua is only 2-fold. This may be due to the fact that CIEtSoSo does not produce toxic side products through carbamylyation reactions. Treatment with m$^6$Gua or n-bu'Gua may be useful clinically in terms of increasing the therapeutic efficacy of chloroethylyating agents provided that the relatively high concentrations required can be maintained in appropriate clinical protocols. This is especially important in tumors resistant to alkylyating agents due to high levels of AGT.

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Effect of $O^6$-Alkylguanine Pretreatment on the Sensitivity of Human Colon Tumor Cells to the Cytotoxic Effects of Chloroethylating Agents

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