Modulation of Nitrosourea Resistance in Myeloid Leukemias

By Stanton L. Gerson and Joan E. Trey

Drug resistance in myeloid leukemias may be mediated by an increased capacity to repair chemotherapy-induced DNA damage. Some tumor cell lines that are resistant to nitrosoureas contain the DNA repair protein O6-alkylguanine-DNA alkyltransferase (alkyltransferase). This protects cells by removing cytotoxic, nitrosourea-induced O6-alkylguanine adducts. We measured the level of alkyltransferase activity in myeloid leukemic cells freshly obtained from patients to determine whether the alkyltransferase was an important factor in nitrosourea resistance in these cells and whether inactivation of this protein could sensitize leukemic cells to nitrosoureas. Myeloid leukemic cells from patients with acute nonlymphocytic leukemia and chronic myelogenous leukemia had higher levels of alkyltransferase than did myelid precursors from normal donors (P < .01). This difference did not appear to be due to the state of differentiation of the leukemia or normal cells. To show that this repair protein mediated nitrosourea resistance in leukemic cells, cells were treated with the modified base O4-methylguanine to selectively and irreversibly inactivate the alkyltransferase and then exposed to 1.3-bis (2-chloroethyl)-1-nitrosourea (BCNU). An 18-hour incubation in 0.5 mmol/L O4-methylguanine caused an 87% ± 3.6% decrease in alkyltransferase activity in leukemic cells and a 73% ± 8.6% decrease in normal myeloid precursors. After treatment with O4-methylguanine, clonogenic leukemic cells from ten different donors became much more sensitive to BCNU, with a decrease in the dose needed to reduce colony survival by 50% (LD50) of 6.3 ± 1.4-fold. A lesser effect was seen on CFU-GM, BFU-E, and CFU-GEM where the LD50 decreased two- to threefold. These studies show that nitrosourea resistance in myeloid leukemic cells can be abrogated by inactivation of the DNA repair protein O4-alkylguanine-DNA alkyltransferase. This method of biochemical modulation of DNA repair will sensitize leukemic cells to nitrosoureas in vitro and has the potential of increasing the therapeutic index of nitrosoureas in this disease.

Although the majority of acute nonlymphocytic leukemias (ANLL) can be effectively treated, a proportion of these leukemias remain resistant to even the newer chemotherapeutic agents.12 One group of agents, the nitrosoureas, has not been useful in the treatment of ANLL14 but is effective in many lymphoid malignancies and gliomas.5,6 This suggests that there may be a common mechanism of nitrosourea resistance in myeloid leukemias that is absent in nitrosourea-sensitive cells.

One of the best-studied mechanisms of nitrosourea resistance is the DNA repair protein O6-alkylguanine-DNA alkyltransferase (alkyltransferase).7 This particular protein repairs the O6-alkylguanine adduct, an alkylation product formed when nitrosoureas react with double-stranded DNA.8 The adduct and its repair are important for a number of reasons. First, O6-alkylguanine adducts are largely responsible for DNA cross-linking and resulting cytotoxicity of clinically used chloroethylnitrosoureas.9,10 Second, the protein acts by covalent transfer of the alkyl group from the modified base into a cysteine moiety within the active site of the protein, which results in irreversible inactivation of the protein.11 Consequently, it has been hypothesized that cells with high levels of this protein are protected from the cytotoxic effect of nitrosoureas because they repair the lesions before cross-links are formed, whereas cells with low levels of activity are sensitive to nitrosoureas.7

Early data to support the hypothesis that alkyltransferase was important in nitrosourea resistance was suggested by studies that showed that cells were sensitized to nitrosoureas when the alkyltransferase was selectively inactivated by methylating agents that were themselves cytotoxic.12 More recently, it has been found that the modified base O4-methylguanine (O4mG) was an effective, nontoxic compound that could also inactivate the alkyltransferase.13,14 O4mG reacts with the alkyltransferase in the same way as the normal substrate. By using O4mG, it was possible to show that cell lines that contain high levels of alkyltransferase activity became sensitized to nitrosoureas when pretreated with O4mG.14,15 Thus, alkyltransferase appears to be a significant factor in the nitrosourea resistance of a number of cell lines.7,10,14

In earlier studies, we measured the levels of alkyltransferase activity in human myeloid precursors and compared them with the promyelocytic cell line HL60.16 Normal human bone marrow was separated to yield granulocytes, monocytes, T lymphocytes, and myeloid precursors. The myeloid precursor fraction consisted of myeloblasts, promyelocytes, and myelocytes and were contaminated by less than 8% B lymphocytes and monocytes. Bone marrow-derived myeloid precursors contained significantly lower levels of alkyltransferase activity (3.1 ± 1.6 fmol O4mG removed/µg cellular DNA) than did HL60 cells (11.6 ± 0.3 fmol O4mG).

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1487
fmol O6mG/µg DNA) and lower levels than normal human liver, lung, colon, lymphocytes, or spleen. This suggested that the susceptibility of normal myeloid precursors treated with nitrosoureas may arise from their lower levels of alkyltransferase activity compared with other cell types. Our results also suggested a possible explanation for the relative resistance to nitrosoureas observed with human myeloid leukemias and a mechanism to overcome this resistance. Human myeloid leukemias may be resistant to nitrosoureas if, like HL60, they have high levels of alkyltransferase activity. Furthermore, through the use of O6mG to inactivate the alkyltransferase, it may be possible to sensitize myeloid leukemia cells to nitrosoureas, possibly by increasing the therapeutic index when compared with the relative toxicity observed in normal human bone marrow cells treated in a similar manner.

This possibility was first evaluated by showing that the promyelocytic cell line HL-60, which contains high levels of alkyltransferase activity, was resistant to nitrosoureas. When these cells were pretreated with O6mG, there was a decrease in the dose needed to decrease survival by 50% (LD50) of between 2.5- and 5.1-fold for five different nitrosoureas. We also observed that the K562 chronic myelogenous leukemia (CML) cell line had very low levels of alkyltransferase activity and could not be further sensitized to nitrosoureas by O6mG. These results indicated that alkyltransferase activity may vary considerably between myeloid leukemia cell lines and indicated the need to test the relationship between alkyltransferase and nitrosourea resistance directly in patient-derived leukemic cell samples.

In this study, we provide information as to whether the alkyltransferase was an important mechanism of nitrosourea resistance in myeloid leukemic cells taken directly from patients. We also evaluate the role of O6mG as a sensitizer to nitrosoureas in these cells. We report that freshly obtained myeloid leukemic cells contain high levels of alkyltransferase and that O6mG is able to significantly increase the sensitivity of clonal myeloid leukemic cells to the cytotoxic effects of the nitrosourea 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Normal hematopoietic stem cells are also sensitized to nitrosoureas by preincubation with O6mG, but the therapeutic index of these agents is increased in vitro due to the ability of O6mG to markedly increase the sensitivity of leukemic cells to nitrosoureas compared with the effect seen in normal cells.

**METHODS**

**Reagents.** RPMI 1640 medium and tissue culture supplements were obtained from KC Biologicals Lenexa, KS. Fetal calf serum (FCS, Hyclone Laboratories, Logan, UT) was screened for optimal growth of normal and malignant myeloid colony formation. Phytohemagglutin in (PHA) (HA-15) was obtained from Burroughs Wellcome (Dartford, England) and reconstituted as directed by the company. BCNU was obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutics Program, National Cancer Institute, and was reconstituted in 0.5 mL 100% ethanol at 22°C and diluted to 10 mmol/L in sterile phosphate-buffered saline (PBS) immediately before use. [3H] methylisourea (MNU) (Amersham Corp, Arlington Heights, IL; specific activity, 4.7 Ci/mmol) was used to synthesize [3H] methylated DNA substrate. Dimethyl sulfoxide (DMSO), 12-0-tetradecanoylphorbol-13-acetate (TPA) and all trans-retinoic acid were obtained from Sigma Chemical Co, St. Louis. TPA was resuspended at 1.6 x 10−4 mol/L in acetone and diluted further in PBS. Trans-retinoic acid was resuspended at 10−3 mol/L in 95% ethanol in the dark and diluted further directly into culture medium. O6mG was synthesized by Dr P. Howard, Department of Environmental Health Sciences, Case Western Reserve University, from 6-chloroguanine and anhydrous sodium methoxide (Sigma) by using a modification of the method described by Demple et al. The preparation contained 98.8% O6mG by high-performance liquid chromatography (HPLC) analysis, with the major contaminant (0.5%) being 6-chloroguanine. At 5 µmol/L, 6-chloroguanine had no effect on leukemic cell growth, cellular sensitivity to BCNU, or alkyltransferase activity. Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF), the gift of Dr K. Kaushansky, Department of Medicine, University of Washington Medical School, was received as the supernatant from transfected Cos cells and had an activity of 2.5 x 105 U/mL. It was diluted to 1,000 U/mL in tissue culture medium before use and stored at −20°C for up to 4 months without a loss of activity. An alternative source of colony-stimulating activity was 10% (vol/vol) conditioned medium from PHA-stimulated peripheral blood leukocytes (PHA-LCM) prepared and stored as described. Erythropoietin (rh) was obtained from Amgen Corp, Thousand Oaks, CA.

**Cell preparation.** Bone marrow from normal donors or patients in first remission of ANLL and blood or bone marrow from patients with newly diagnosed ANLL, CML in blast crisis, or untreated CML was obtained after informed consent by using procedures approved by University Hospitals of Cleveland Institutional Review Board for Human Experimentation as previously described. One sample was obtained from a discarded leukapheresis pack from an 18-month-old child with ANLL who presented with a white blood count of >150,000 cells/µL. All samples from patients with acute leukemia that were chosen for study had >68% blasts confirmed by Wright-Giemsa stain of the unprocessed sample. Low-density mononuclear cells were collected at the interface of an isopaque Ficoll-Hypaque gradient as previously described, and adherent cells were removed after incubation in serum-coated flasks. T lymphocytes were removed by incubating the cell suspension with neuraminidase-treated sheep RBCs for two hours at 4°C to allow E rosette formation; this was followed by separation of the rosetting T lymphocytes by Ficoll-Hypaque gradient centrifugation as previously described. In all samples from the bone marrow of normal donors the resulting nonadherent T lymphocyte–depleted mononuclear cells consisted of 85% ± 6.6% myeloid precursors defined as myeloblasts, promyelocytes, myelocytes, and metamyelocytes as determined by morphology and confirmed by monoclonal antibody analysis as previously described.

**Drug exposure and hematopoietic stem cell assay.** The low-density nonadherent mononuclear cells were suspended at 5 x 104 cells/mL in RPMI 1640 medium supplemented with 15% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (tissue culture medium) and incubated for 18 hours at 37°C, 5% CO2. One half of the cells were incubated in 0.5 mmol/L O6mG for 18 hours, which was previously shown to be sufficient to inactivate the alkyltransferase in myeloid cell lines. Cell cultures were readjusted to 5 x 104 cells/mL in culture medium and exposed to 0 to 50 µmol/L BCNU for two hours at 37°C. The cell suspensions were centrifuged at 500 g for ten minutes and resuspended in fresh culture medium. To measure the clonal survival of leukemic cells, the cells were then immediately suspended at 5 x 105 cells/mL in 0.3% agar in Dulbecco's modified Eagle's medium with 20% FCS, diethylaminoethyl dextran, 1% penicillin, streptomycin solution, and 10 µg/mL asparagine in triplicate 0.5-mL wells (Costar, Cambridge, MA) and incubated at 37°C at 5% CO2 in a fully humidified atmosphere as
previously described.29 Either GM-CSF or PHA-LCM was added to agar plates containing leukemic cells, and both gave similar clonal growth of leukemic cells. Leukemic colonies (L-CFU) were defined as aggregates of >20 cells and were read after ten days of culture under an inverted microscope.

Growth of normal human hematopoietic stem cells was performed as previously described.17,22 Briefly, cells were suspended at 1 x 10^7 cells/mL in 0.3% agar in 1-mL, 35-mm plates in Iscove's modified Dulbecco's medium supplemented with 1% deionized bovine serum albumin, 0.1 mmol/L α-thioglycerol, 0.2 mmol/L hemin, and 30% FCS. Erythropoietin, 1 U/mL, was added to stimulate BFU-E growth, and 100 units of rh GM-CSF or PHA-LCM (10% [vol/vol]) was added to stimulate the CFU-GM or CFU-GEM growth of normal precursors. Normal hematopoietic colony types were defined as stated previously21 and were scored on day 12 of growth. BFU-E consisted of aggregates of three or more erythroid cell clusters, each greater than ten cells, or a single large erythroid colony of >200 cells. CFU-GM were defined as large (>200 cells) mixed colonies containing granulocyte, monocyte, erythroid, and occasionally megakaryocyte cells. CFU-GM were defined as colonies >50 cells of either granulocyte, macrophage, or mixed origin. To confirm the morphology of the colonies, the entire agar gels were fixed, transferred to glass slides, and stained for myeloid and erythroid elements as previously described.20,21 No lymphoid colonies were detected when using either PHA-LCM or rh GM-CSF.

The potentiation of BCNU toxicity by O6mG was determined by measuring two parameters from the survival curves. The ability of O6mG to reduce the LD50 of BCNU was termed the O6mG dose potentiation factor. This factor measures the ability of O6mG to decrease the LD50 of BCNU and was calculated as the ratio of the LD50 observed with BCNU alone to the LD50 observed with O6mG pretreatment followed by BCNU. To measure the ability of O6mG to increase the cytotoxicity of BCNU, the O6mG cytotoxicity potentiation factor was used. This factor was determined by comparing colony survival in the presence or absence of O6mG and using the BCNU concentration that caused 50% colony survival in the absence of O6mG. This factor was calculated by dividing 0.50 (the fractional survival [by definition] of cells treated with the LD50 of BCNU alone) by the fractional survival of O6mG-pretreated cells exposed to the same dose of BCNU.

**Alkyltransferase assay.** Cells to be assayed for alkyltransferase activity were centrifuged at 500 g for ten minutes at 4°C, resuspended in PBS and 1 mmol/L EDTA, washed twice in this buffer by centrifugation, and stored at −20°C. Cell extracts were prepared as previously described18 by using a sonic cell disrupter, and the protein and DNA content was determined as described.18 The substrate for the alkyltransferase present in the cell extracts was [3H]methyl DNA prepared by reacting [3H]MNU with calf thymus DNA followed by purification of the DNA as previously described.16 The major alkylation sites in this substrate are N7-methylguanine (72%) and O6mG (8.6%). The assay of alkyltransferase activity was performed as previously described.18,22 Briefly, alkyltransferase activity was measured as the removal of the [3H]methyl adduct from O6mG by methylguanine in the substrate DNA.16,22 The reaction mixture was incubated for 60 minutes at 37°C, the DNA was precipitated with 7.5% trichloroacetic acid at 4°C, and the methyl purines were liberated by acid hydrolysis and separated by HPLC.18 One unit of alkyltransferase activity was defined as the removal of 1 fmol of O6mG/μg cellular DNA. Each cell extract was assayed in duplicate.

**Induction of differentiation in HL-60.** HL-60 promyelocytic cells (American Type Culture Collection, Camden, NJ) were maintained in exponential growth in RPMI tissue culture medium in 5% CO₂, 37°C. The cells were resuspended at 2 x 10^7 cells/mL and incubated in either culture medium alone or 2 culture medium supplemented with 1.6 x 10⁻⁷ mol/L TPA, 1% (vol/vol) DMSO, or 2 x 10⁻⁴ mol/L all trans-retinoic acid for 72 hours at 37°C in tissue culture flasks. Cells were harvested by the removal of nonadherent cells and treatment of residual adherent cells with PBS and 1% EDTA at 4°C for 15 minutes followed by detachment using a cell scraper. The cells were analyzed for their state of differentiation by Wright-Giemsa stain, chloroacetate esterase stain, and nitroblue tetrazolium (NBT) reduction by using established techniques.16,20,23 Cells were also harvested and assayed for alkyltransferase activity as described earlier.

**RESULTS**

**Alkyltransferase activity in myeloid leukemias.** To determine whether the alkyltransferase was likely to contribute to the nitrosourea resistance observed with myeloid leukemias, we measured the levels of alkyltransferase activity in freshly obtained, low-density, nonadherent mononuclear leukemic cells from patients with ANLL, CML, and CML in blast crisis. For comparative purposes, the activity in these leukemic cells was compared with that obtained in normal bone marrow cells enriched for myeloid precursors by depletion of high-density, adherent, and E rosette-positive cells.16 The results shown in Fig 1 indicate the the alkyltransferase activity in ANLL cells (9.2 ± 4.4 units, n = 10), CML cells (7.6 ± 2.7 units, n = 8), and CML in blast crisis (8.0 ± 4.1 units, n = 5) was higher than that observed in myeloid bone marrow cells obtained from normal donors or patients with ANLL in remission (2.5 ± 0.3 units, n = 23, P < .01). The levels of alkyltransferase activity in many of the freshly obtained leukemic cells are similar to those observed in the HL-60 cell line (see later), which is known to have high levels of alkyltransferase activity and to be resistant to nitrosoureas.16,17 Leukemic cells from patients with CML were all greater in alkyltransferase activity than that observed in the K562 cell line. Of interest, there appeared to be no correlation between the French-American-British (FAB) classification of ANLL and the alkyltransferase activity, although the
numbers for each type of leukemia were small. Thus, the alkyltransferase activity in myeloid leukemic cells is sufficiently high to suggest that this repair protein may contribute to nitrosourea resistance in these leukemic cells.

Role of myeloid cell differentiation in alkyltransferase activity. One possible explanation for the difference in alkyltransferase activity between leukemic cells and normal bone marrow myeloid precursors is the state of differentiation of the cells tested. The enrichment procedures used to isolate normal bone marrow myeloid precursors removed granulocytes, band forms, nucleated RBCs, monocytes, and T lymphocytes. These procedures, however, did not produce cells that were identical in morphology to the malignant cells. Specifically, the normal cells assayed for alkyltransferase activity had a differential of 4.9% ± 3.6% (mean ± SD) myeloblasts, 8.8% ± 5.6% promyelocytes, 48.7% ± 12.2% myelocytes, 23.0% ± 7.7% metamyelocytes, 5.4% ± 4.9% bands, 2.2% ± 2.9% polymorphonuclear granulocytes, 5.0% ± 4.1% monocytes, and 1.5% ± 1.8% lymphocytes. In contrast, leukemic cell samples contained a minimum of 69% leukemic cells on the basis of morphology, with the specific cell type determined by the type of leukemia and the FAB classification (Table 1).

Figure 2 indicates that there was no correlation between alkyltransferase activity and the percentages of blast and promyelocytes or myelocytes in individual cell preparations from normal bone marrow. This coupled with our earlier data showing a similar level of alkyltransferase activity in myeloid precursors, peripheral blood monocytes, and granulocytes suggests that early myeloid precursors in fact have low levels of alkyltransferase activity. To further investigate this question, we examined the effect of differentiating agents on the alkyltransferase activity present in the cells. Cumulatively, there is little evidence to suggest that the state of differentiation is a major factor in the level of alkyltransferase activity in myeloid cells. Rather, some other factor such as the state of proliferation or gene deregulation may explain the difference between the alkyltransferase activity observed in leukemic compared with normal myeloid precursors.

Inactivation of the alkyltransferase by O6mG. Ten additional samples from patients with either ANLL or CML in blast crisis were analyzed in greater detail. The diagnostic criteria and treatment received before the date the sample was obtained are shown in Table 1. None had prior exposure to nitrosoureas. The ability of O6mG to inactivate the alkyltransferase in myeloid leukemia cells was measured by incubating cells for 18 hours in the presence or absence of 0.5 mmol/L O6mG, washing the cells to remove the culture

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**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Diagnosis*</th>
<th>Chromosome Abnormalities</th>
<th>Blasts† (%)</th>
<th>Prior Treatment</th>
</tr>
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<tr>
<td>1</td>
<td>45</td>
<td>CML, blast crisis</td>
<td>46 X; (t9:22; t9:22; t9:19)</td>
<td>69 (Bl)</td>
<td>Busulfan</td>
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<tr>
<td>2</td>
<td>24</td>
<td>ANLL, M2</td>
<td>nml</td>
<td>78 (Blm)</td>
<td>Ara-C, DNR, 6-TG, mitoxantrone, VP-16</td>
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<td>3</td>
<td>56</td>
<td>ANLL, M4</td>
<td>nml</td>
<td>70 (Bl)</td>
<td>Ara-C, DNR, 6-TG, m-AMSA</td>
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<td>4</td>
<td>35</td>
<td>ANLL, M1</td>
<td>46 X; (t8:21; -4; 5p+; 8q-; 21)</td>
<td>72 (Blm)</td>
<td>Ara-C, DNR, 6-TG</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>CML, blast crisis</td>
<td>(t9:22)</td>
<td>78 (Blm)</td>
<td>Busulfan</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>ANLL, M1</td>
<td>nml</td>
<td>72 (Blm)</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>64</td>
<td>ANLL, M2</td>
<td>nml</td>
<td>74 (Blm)</td>
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<td>nml</td>
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<tr>
<td>10</td>
<td>54</td>
<td>ANLL, M2</td>
<td>nml</td>
<td>74 (Blm)</td>
<td>None</td>
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</table>

Abbreviations: Bl, blood; Blm, bone marrow; Ara-C, cytosine arabinoside; DNR, daunorubicin; 6-TG, 6-thioguanine; nml, normal.

†Percentage of blasts indicates the proportion of leukemic cells in the sample as indicated by their immature morphology and includes myeloblasts, promyelocytes, monoblasts, and myelocytes, depending on the leukemic subtype.
medium containing O6mG, and assaying the cell extract for residual alkyltransferase activity. As shown in Table 3, O6mG inactivated the alkyltransferase in the seven leukemic cell samples tested from a mean of 5.9 ± 0.8 units to 0.8 ± 0.3 units, a decrease of 87% ± 3.6%. The effect of O6mG on the alkyltransferase was not measured in three leukemic cell samples because the amount of cell extract was insufficient for the alkyltransferase assay. In bone marrow myeloid precursors from six normal donors, O6mG also decreased the alkyltransferase activity from a mean of 2.4 ± 0.2 units to 0.6 ± 0.2 units, a decrease of 73% ± 8.6%. Thus, the effect of O6mG was to reduce the alkyltransferase to similar low but measurable levels in leukemic and normal myeloid precursors. Of note, however, the percent decrease in the leukemic samples was greater than that in the normal bone marrow samples.

Effect of O6mG on nitrosourea sensitivity in normal hematopoietic stem cells. To determine the extent to which pretreatment with O6mG would increase the sensitivity of normal hematopoietic cells to nitrosoureas, the dose-dependent cytotoxicity of BCNU with or without O6mG preincubation was measured. As shown in Fig 3, an 18-hour preincubation in 0.5 mmol/L O6mG increased the sensitivity of CFU-GM, BFU-E, and CFU-GEM from five normal donors to BCNU to a similar extent. The effect was to decrease the LD50 of BCNU by 3.0-fold in BFU-E, and 2.0-fold in CFU-GEM. There was a decrease in the cloning efficiency of BFU-E and CFU-GEM observed with O6mG pretreatment alone, but this was not statistically significant. A similar mild decrease in cloning efficiency was previously observed with HL-60 and K562 cells.17

Sensitization of leukemic cells to nitrosoureas by O6mG. Next, the degree to which inactivation of the alkyltransferase by O6mG could sensitize myeloid leukemic cells to nitrosoureas was analyzed. Two representative survival curves are shown in Fig 4, and the results of all ten donors are shown in Table 4. In each case, preincubation in the presence of O6mG increased the sensitivity of leukemic cells to nitrosoureas. O6mG preincubation itself had no significant cytotoxic effect. The ability of O6mG to decrease the LD50 of BCNU or the O6mG dose potentiation factor was 6.3 ± 1.4 (range, 2.3 to 14.0). By similar analysis, the ability of O6mG to increase the cytotoxicity of BCNU at a constant exposure dose, or the O6mG cytotoxicity potentiation factor, was 28.8 ± 9.2 and >50 in samples from three patients (Table 4). Of interest, the patient with the smallest degree of O6mG-induced potentiation of BCNU cytotoxicity, patient 3, also had the highest level of residual alkyltransferase activity after exposure to O6mG. Unfortunately, this patient had refractory leukemia and died during reinduction therapy, so it was not possible to determine why O6mG did not completely inactivate the alkyltransferase in these leukemic cells.

Table 2. Effect of Differentiation on Alkyltransferase Activity in HL-60 Cells

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. Experiments</th>
<th>Protein (μg)</th>
<th>DNA (μg)</th>
<th>Alkyltransferase Activity</th>
<th>Differentiation Parameter (Percentage of Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>460 ± 36</td>
<td>12.3 ± 0.6</td>
<td>0.8 ± 0.2</td>
<td>Granulocytes 60 ± 80, Macrophages 6 ± 5, Chloroacetate 5 ± 3, Esterase 4 ± 2, NBT Reduction 26 ± 3.6</td>
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<tr>
<td>TPA (1.6 × 10⁻⁷ mol/L)</td>
<td>6</td>
<td>445 ± 99</td>
<td>12.3 ± 0.6</td>
<td>4 ± 0.5</td>
<td>40 ± 6, 6, 30, 36, 42</td>
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<tr>
<td>DMSO (1%)</td>
<td>3</td>
<td>467 ± 69</td>
<td>14.0 ± 1.7</td>
<td>42 ± 0</td>
<td>36 ± 42</td>
</tr>
<tr>
<td>Retinoic acid (2 × 10⁻⁶ mol/L)</td>
<td>5</td>
<td>353 ± 14</td>
<td>12.0 ± 0.5</td>
<td>55 ± 0</td>
<td>32 ± 62</td>
</tr>
</tbody>
</table>

*Cells were resuspended at 2 × 10⁹ cells/mL in tissue culture medium and treated with the agent shown for three days at 37°C except that cells were incubated for five days in cis-retinoic acid. Control cells had similar levels of alkyltransferase activity after both three and five days. Cells were collected as described in Methods, assayed for alkyltransferase activity, and analyzed by cytochemical stain for evidence of differentiation.

†Alkyltransferase activity is expressed both in terms of activity per milligram cellular protein and per microgram cellular DNA because the differentiation process may affect the relative ratio of protein to DNA in the cell. Data shown are means ± SE.

Table 3. Inactivation of Alkyltransferase Activity by O6mG

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>−O6mG</th>
<th>+O6mG</th>
<th>Inactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemic cells†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.7</td>
<td>0.1</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
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<td>2.4</td>
<td>68</td>
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<td>4</td>
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<td>0.8</td>
<td>90</td>
</tr>
<tr>
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<td>4.1</td>
<td>0.5</td>
<td>88</td>
</tr>
<tr>
<td>8</td>
<td>6.8</td>
<td>0.6</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>3.8</td>
<td>0.2</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>7.3</td>
<td>1.0</td>
<td>84</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>5.9 ± 0.8</td>
<td>0.8 ± 0.3</td>
<td>87 ± 3.6</td>
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</tbody>
</table>

Normal myeloid precursors

| n = 6 | 2.4 ± 0.2 | 0.6 ± 0.2 | 73 ± 8.6 |

*Activity is expressed as femtomoles O6mG removed per microgram cellular DNA.
†Cells were incubated in tissue culture medium with or without 0.5 mmol/L O6mG for 18 hours and then harvested and assayed for alkyltransferase activity as described in Methods.

Fig 3. O6mG potentiation of BCNU cytotoxicity of normal marrow stem cells. Normal bone marrow low-density, nonadherent mononuclear cells were incubated with or without 0.5 mmol/L O6mG in culture medium and then exposed to BCNU for two hours before being assayed for stem cells as described in Methods. CFU-GM, BFU-E, and CFU-GEM were scored on day 12 of culture. Data represent means ± SEM from five normal donors.
One possible factor in the development of drug resistance in malignant cells is prior exposure to chemotherapeutic agents, which may select for cells with resistance to a variety of drugs.\textsuperscript{22} It is of interest to note that patients 1 to 5 were previously treated (two patients with CML in blast crisis, three with relapsing ANLL, Table 1). Although the mean level of alkyltransferase activity in the treated leukemias was no different than de novo ANLL (5.9 ± 1.2 units vs 6.0 ± 1.1 units, Table 3), the toxic effect of O\textsuperscript{6}mG was less in these cases than de novo ANLL, with a mean O\textsuperscript{6}mG dose potentiation factor of 3.5 ± 0.7 vs 4.2 ± 2.1 and a mean O\textsuperscript{6}mG cytotoxicity potentiation factor of 9.0 ± 3.6 vs >49 for the treated and de novo leukemias, respectively (Table 4).

DISCUSSION

There are many possible causes of drug resistance in leukemic cells. These include decreased drug uptake,\textsuperscript{24,25} increased drug metabolism to inactive products,\textsuperscript{26} altered cell cycle progression,\textsuperscript{27} and altered DNA repair capacity.\textsuperscript{28} Resistance to alkylating agents and, in particular, nitrosoureas is not observed in the pleotropic drug resistance phenotype,\textsuperscript{29} and cross-resistance between the nitrosoureas and nitrogen mustards is not common.\textsuperscript{30}

In this study we evaluated the role of a DNA repair protein, O\textsuperscript{6}-alkylguanine–DNA alkyltransferase in the observed nitrosourea resistance of myeloid leukemias. This alkyltransferase has been shown to be critical to nitrosourea resistance in many normal, transformed, and malignant cell lines.\textsuperscript{16,17} We document that the nitrosourea resistance of patient-derived leukemic cells can be overcome by selective inactivation of the alkyltransferase. Although inactivation of the alkyltransferase also had effects on normal hematopoietic precursors, the therapeutic index of BCNU was increased.

Previously we have shown that HL-60 cells contain high levels of alkyltransferase and are much more resistant to both methyl and chloroethyl nitrosoureas than is the K562 CML cell line, which has very low levels of alkyltransferase activity.\textsuperscript{16,17} The nitrosourea resistance of HL-60 cells was eliminated by inactivation of the alkyltransferase by the modified base O\textsuperscript{6}mG.\textsuperscript{17} We were concerned that the results of this earlier work in leukemic cell lines might not mimic those found in freshly obtained leukemic cells for the following reasons. First, there is evidence that the alkyltransferase may not be the only factor contributing to nitrosourea resistance. Some cell lines in which O\textsuperscript{6}mG is able to inactivate the alkyltransferase cannot be sensitized to the nitrosoureas with O\textsuperscript{6}mG,\textsuperscript{13} and other cell lines that lack the alkyltransferase are not sensitive to methylating agents.\textsuperscript{28} Second, there is a great variation in alkyltransferase activity levels found in the same tissue between donors\textsuperscript{6} and between normal and malignant tissues as well as between normal and transformed cells from the same tissue.\textsuperscript{31,32} Thus, although it was possible that previous results with HL-60 would be similar to those observed with freshly obtained leukemic samples, this was by no means ensured.

In data presented here, the mean level of alkyltransferase in myeloid leukemic cells freshly harvested from patients, like that for HL-60 cells, was significantly higher than that

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|}
\hline
Patient No. & L-CFU/10\(^6\) Cells & \text{BCNU Cytotoxicity} & \text{O\textsuperscript{6}mG Dose Potentiation Factor*} & \text{O\textsuperscript{6}mG Cytotoxicity Potentiation}\tabularnewline
\hline
1 & 78 ± 6 & 8.0 & 3.0 & 2.6 & 3.1\tabularnewline
2 & 42 ± 2 & 23.0 & 7.5 & 3.3 & 10.6\tabularnewline
3 & 134 ± 13 & 8.1 & 3.7 & 2.3 & 2.4\tabularnewline
4 & 224 ± 14 & 5.6 & 1.5 & 3.7 & 8.3\tabularnewline
5 & 204 ± 16 & 12.2 & 2.2 & 5.6 & 20.8\tabularnewline
6 & 54 ± 2 & 18.5 & 1.3 & 14.0 & >50\tabularnewline
7 & 52 ± 4 & 24.5 & 3.0 & 8.1 & >50\tabularnewline
8 & 453 ± 19 & 5.0 & 1.3 & 3.8 & 9.3\tabularnewline
9 & 546 ± 12 & 13.2 & 1.8 & 7.2 & 83.3\tabularnewline
10 & 172 ± 6 & 15.0 & 1.2 & 12.5 & >50\tabularnewline
\hline
\text{Mean ± SEM} & 195 ± 58 & 13.3 ± 2.3 & 2.7 ± 0.6 & 6.3 ± 1.4 & 28.8 ± 9.2\tabularnewline
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\end{tabular}
\caption{Effect of O\textsuperscript{6}mG on BCNU Cytotoxicity of Human Myeloid Leukemias}
\end{table}

*The ratio of the LD\textsubscript{50} observed with BCNU alone to the LD\textsubscript{50} observed with O\textsuperscript{6}mG pretreatment followed by BCNU.
†The ratio of 0.5 (the fractional survival [by definition] of cells treated with the LD\textsubscript{50} of BCNU alone) to the fractional survival of cells pretreated with O\textsuperscript{6}mG and then exposed to the same dose of BCNU, i.e., the LD\textsubscript{50} of BCNU alone. Values reported as greater than could not be calculated exactly because no L-CFU were present in cultures treated with O\textsuperscript{6}mG at the BCNU dose used to calculate the ratio.
observed in normal bone marrow cells enriched for myeloid precursors. This suggested that both leukemic cell lines and fresh leukemic cells use high levels of alkyltransferase activity as a common mechanism of nitrosourea resistance.

On the basis of the cell enrichment methods used, it is not possible to be certain that the differences in alkyltransferase activity observed between myeloid cells of normal donors (predominantly myelocytes) and leukemic cells (myeloblasts through myelocytes) from patients with ANLL or CML in blast crisis are due to differences in the enzyme activity of the clonally proliferating progenitor or simply reflect a decrease in enzyme activity that occurs during maturation of the cell. There was no correlation, however, between alkyltransferase activity and the percentage of blasts, promyelocytes, or myelocytes in normal bone marrow, and a decrease in enzyme activity did not occur during experimentally induced differentiation of the HL-60 promyelocytic cell line after exposure to TPA, DMSO, or all-trans-retinoic acid. Two more likely explanations for the difference in alkyltransferase activity between normal and leukemic myeloid cells are (a) the state of proliferation of the cells at the time the enzyme is assayed and (b) alterations in gene expression that are a result of the leukemic process. Both of these possibilities have not been studied at the present time. It has been shown that proliferation induces alkyltransferase activity in normal human lymphocytes, tissue culture fibroblasts, and regenerating rat liver, which raises the possibility that the transition from resting to proliferation will increase the alkyltransferase activity in normal myeloid precursors. We are currently evaluating this possibility by using interleukin-3 and GM-CSF to induce the proliferation of normal myeloid precursors. We are also examining whether leukemic cells synthesize the alkyltransferase protein at a higher rate than do normal myeloid cells.

Although we are unable to examine the alkyltransferase activity in a homogeneous population of normal hematopoietic stem cells as was possible when using leukemic cells, it was possible to gauge the impact of the alkyltransferase by measuring the ability of the alkyltransferase protein to protect both normal and leukemic stem cells from the cytotoxic effect of BCNU. To measure the protection, the alkyltransferase was inactivated with O-demethylation, and the degree to which the cells were sensitized to nitrosoureas was measured. O-demethylation was an effective inhibitor of alkyltransferase activity in leukemic cells, and the addition of it resulted in a mean decrease in activity of 87% ± 3.6%. A lesser degree of inactivation of alkyltransferase occurred in normal bone marrow cells, 73% ± 8.6%, and both leukemic and bone marrow cells retained a low but measurable level of alkyltransferase activity. Because the decrease in alkyltransferase activity was greater in leukemic cells than in normal cells, it was not surprising to find a greater potentiation of nitrosourea cytotoxicity in leukemic than in normal hematopoietic colony-forming cells.

The O-demethylation dose potentiation factor for BCNU in freshly obtained leukemic cells was, in fact, quite high, a factor of 6.3 ± 1.4, compared with 2.0- to 3.4-fold observed during similar treatment of normal bone marrow cells and the HL-60, HELA, and HT-29 cell lines. This marked potentiation of BCNU cytotoxicity by O-demethylation was observed in leukemic cell samples compared with the malignant cell lines cannot be explained by differences in the degree of alkyltransferase inactivation induced by O-demethylation, which was similar in leukemic cells to that reported in malignant cell lines. More likely, it indicates that the freshly obtained leukemic cells rely on the alkyltransferase for nitrosourea resistance and that other potential mechanisms of nitrosourea resistance that have been reported to exist in malignant cell lines are less important. Of interest, other drug resistance mechanisms may be responsible for the lesser degree of cytotoxicity potentiation observed in leukemic cells harvested from relapsing compared with de novo patients.

When examining the role of compounds that potentiate cytotoxicity, it is important to consider the therapeutic index achieved by the combination. Our data show that O-demethylation has a slight effect on the cloning efficiency of CFU-GEM and BFU-E but that leukemic cells were more sensitive to the combination of O-demethylation and BCNU than are normal hematopoietic stem cells. From these in vitro data, it appears that the therapeutic index of nitrosoureas may be increased two- to threefold by the use of O-demethylation to modulate the level of alkyltransferase. Generally of course, treatment of acute leukemia is designed to result in bone marrow aplasia, and the issue of therapeutic index is not usually addressed in such treatments. Furthermore, the in vivo therapeutic index cannot be gauged by in vitro studies because of the unknown role of the proliferative status of normal hematopoietic stem cells, the protective role of the marrow microenvironment, and the importance of drug metabolism and excretion. Consequently, although these in vitro observations provide a biochemical basis to the observed nitrosourea resistance of myeloid leukemias and indicate that inactivation of the alkyltransferase may be an effective strategy for using nitrosoureas in the treatment of these leukemias, the ultimate utility of this combination will depend on the results of pharmacokinetic and animal tumor models.

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Modulation of nitrosourea resistance in myeloid leukemias

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