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Research Article

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O⁶Alkylguanine-DNA Alkyltransferase Activity in Human Myeloid Cells

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Abstract

The association between alkylating agent exposure and acute nonlymphocytic leukemia in humans indicates that myeloid cells may be particularly susceptible to mutagenic damage. Alkylating agent mutagenesis is frequently mediated through formation and persistence of a particular DNA base adduct, O⁶alkylguanine, which preferentially mispairs with thymine rather than cytosine, leading to point mutations. O⁶alkylguanine is repaired by O⁶alkylguanine-DNA alkyltransferase (alkyltransferase), a protein that removes the adduct, leaving an intact guanine base in DNA. We measured alkyltransferase activity in myeloid precursors and compared it with levels in other cells and tissues. In peripheral blood granulocytes, monocytes, T lymphocytes, and B lymphocytes, there was an eightfold range of activity between individuals but only a twofold range in the mean activity between cell types. Normal donors maintained stable levels of alkyltransferase activity over time. In bone marrow T lymphocytes and myeloid precursors, there was an eightfold range of alkyltransferase activity between donors. Alkyltransferase activity in the two cell types was closely correlated in individual donors, $r = 0.69$, $P < 0.005$, but was significantly higher in the T lymphocytes than the myeloid precursors, $P < 0.05$. Liver contained the highest levels of alkyltransferase of all tissues tested. By comparison, small intestine contained 34%, colon 14%, T lymphocytes 11%, brain 11%, and myeloid precursors 6.6% of the activity found in liver. Thus, human myeloid precursors have low levels of O⁶alkylguanine-DNA alkyltransferase compared with other tissues. Low levels of this DNA repair protein may increase the susceptibility of myeloid precursors to malignant transformation after exposure to certain alkylating agents.

Introduction

There is a very strong association in humans between alkylating agent exposure and subsequent development of acute nonlymphocytic leukemia (ANLL)¹ (1-3). Although initially described in patients receiving combination chemotherapy and radiation therapy for Hodgkins disease (4), ANLL has now been reported to follow the use of alkylating agents as therapy for a variety of malignant and nonmalignant conditions (5-8). The cumulative

incidence of ANLL in patients with alkylating agent exposure who survive 5 yr is as high as 17% (5, 8). While considerable efforts have been devoted to epidemiologic documentation of alkylating agent-induced ANLL, there has been little work focused on the apparent sensitivity of myeloid precursors to alkylating agent-induced mutagenesis. In this paper, we have evaluated the capacity of myeloid cells to repair DNA damage induced by alkylating agents and have compared it with the capacity present in other human tissues. Understanding the response of myeloid cells to alkylating agent mutagenic damage will help unravel the process of chemical leukemogenesis.

Alkylating agents cause damage to DNA by formation of DNA base adducts (9, 10) which, in the case of bifunctional agents, results in DNA cross-links (9-11). Secondary damage is induced by DNA protein cross-links (11, 12) and by repair processes that can result in base substitution, strand breaks, and rearrangements (13). Cytotoxicity results from sufficient alkylation of DNA and proteins to disrupt cellular metabolism and replication (14). Mutagenesis and carcinogenesis, on the other hand, occur in less severely affected cells that retain their ability to replicate but are unable to completely repair point mutations, deletions, rearrangements, or translocations (13).

14 major sites of DNA adduct formation have been described for the alkylating agents (15). Most of these adducts are not directly mutagenic (15, 16). In contrast, the O⁶alkylguanine adduct is highly mutagenic because the altered base preferentially mispairs with thymine rather than undergoing the normal base pairing of guanine with cytosine (17). In subsequent rounds of replication, this mispairing results in propagation of a point mutation in which an adenine-thymine base pair is substituted for a guanine-cytosine base pair (15, 16). Furthermore, the O⁶alkylguanine adduct is a necessary intermediate for the bifunctional chemotherapeutic chloroethylnitrosoureas that form biguaninyl DNA cross-links (11, 12). Many studies have shown a good correlation between persistent O⁶alkylguanine adducts and mutagenesis in the Ames test (18) and carcinogenesis in rat brain (19), kidney, liver (20, 21), and thymus (22, 23). In addition, this type of guanine to adenine point mutation has been found to be the single change responsible for activation of the H-ras oncogene present in the methylnitrosourea (MNU)-induced rat mammary carcinoma (24). Because O⁶methylguanine (MG) is an adduct formed by MNU (10, 15, 22), it appears that this adduct is likely to be responsible for oncogene activation in this particular rat mammary carcinoma.

DNA alkyl-adduct repair proceeds by three basic mechanisms. Some adducts, such as N⁷alkylguanine and N³alkyladenine, are removed by specific DNA-glycosylases (13, 25). The DNA strand is then cleaved at the apurinic site by the action of an apurinic endonuclease. Larger adducts are removed by nucleases that excise the abnormal and adjacent bases (13). In both instances the correct nucleotides are inserted into DNA by DNA polymerase and the strand break closed with DNA ligase (13). O⁶alkylguanine, on the other hand, is specifically repaired

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1. Abbreviations used in this paper: ANLL, acute nonlymphocytic leukemia; MG, methylguanine; MNU, methylnitrosourea.

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by a protein called O⁶alkylguanine-DNA alkyltransferase, which transfers the alkyl group from the O⁶ of guanine to a cysteine moiety in the protein, leaving an intact guanine molecule in the DNA (26, 27).

The O⁶alkylguanine-DNA alkyltransferase (alkyltransferase) has been described in both prokaryotes and eukaryotes (26–29). The protein has a molecular weight of 18,000–26,000 (26, 28). It acts in a stoichiometric fashion, in which each protein molecule is capable of transferring one alkyl group from O⁶alkylguanine to the protein with a $t_{1/2}$ of between 15 and 47 min (28, 29). Once the alkyl group is covalently transferred to a cysteine residue in the protein, the protein becomes permanently inactivated and activity is not regenerated in the absence of protein synthesis (30). Consequently, alkylating agents that cause a large number of O⁶alkylguanine adducts can saturate the cellular content of O⁶alkylguanine-DNA alkyltransferase, producing a threshold effect in the number of persistent O⁶alkylguanine lesions and point mutations (18) in cells undergoing DNA replication.

A cell's susceptibility to the mutagenic effects of alkylating agents depends, in part, on the relative amount of O⁶alkylguanine-DNA alkyltransferase. Previous studies have shown that human liver contains the highest level of activity (27), with lower levels seen in stomach (31), small intestine, colon (32, 33), and brain (34). All fresh human tissues analyzed contain alkyltransferase, whereas studies with human cell lines have identified some that lack the enzyme (35). There have been no studies on the level of the alkyltransferase in human bone marrow cells or myeloid precursors.

This study was conducted to determine the range of activity of O⁶alkylguanine-DNA alkyltransferase in myeloid precursors and to compare this range with that found in other tissues. We hypothesized that if the activity were found to be low in myeloid precursors, it could provide a rationale for the susceptibility of myeloid cells to alkylating agent-induced transformation.

Methods

Cell line. The HL-60 promyelocytic cell line (36) was obtained from the American type culture collection (Camden, NJ). The cell line was maintained in exponential growth phase by biweekly passage in culture medium consisting of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (Sterile Systems, Logan, UT), 100 U/ml penicillin-streptomycin solution (Gibco), 2 mM glutamine, and 25 mM Hepes (Sigma Chemical Co., St. Louis, MO).

Peripheral blood and bone marrow fractionation. After informed consent, 150 ml of blood was drawn from healthy donors into plastic syringes. Blood was anticoagulated with heparin or was defibrinated in the presence of 50 ml 5% dextran in order to remove platelets as previously described (37). Preparation of cells by either method gave similar results. Defibrinated blood was sedimented for 1 h at unit gravity, 37°C, and the buffy coat-rich plasma-dextran upper layer collected. The defibrinated buffy coat or heparinized whole blood was layered over Ficoll-Hypaque (specific gravity = 1.077, Sigma Chemical Co.) and separated by density gradient centrifugation at 1,000 $g \times 30$ min at 22°C as previously described (38). Granulocytes in the pellet were isolated after osmotic shock lysis of the erythrocytes by incubation at 4°C for 3 min in 4 vol of lysing buffer consisting of 0.155 M NH₄Cl, 0.015 M NH₄HCO₃, and 0.1 mM EDTA followed by dilution into phosphate-buffered saline (PBS) (37) and centrifugation at 500 $g \times 10$ min. Low density mononuclear cells were recovered at the Ficoll-Hypaque interface. Monocytes were isolated by adherence during a 90-min incubation in RPMI-1640 culture medium at 1 $\times 10^6$ cells/ml in 75-cm² tissue culture flasks (Costar, Cambridge,

MA) preincubated for 90 min with heat inactivated, pooled human A/B serum, using published methods (38, 39). After the nonadherent cells were removed, the adherent monocytes were washed twice with 10 ml PBS, exposed to 20 ml PBS-1 mM EDTA for 20 min at 4°C, and collected by sweeping the flask with a rubber policeman.

The nonadherent, mononuclear cells were further separated into T and B lymphocyte fractions using the capacity of T cells to form rosettes with neuraminidase (Sigma Chemical Co.) treated sheep erythrocytes (Wilfer Laboratories, Stillwater, MN) during a 90-min incubation at 4°C as previously described (40).

Bone marrow was obtained either from healthy donors, after informed consent, or from individuals with normal bone marrows undergoing bone marrow harvest for bone marrow transplantation. 8 ml of bone marrow were aspirated from the posterior iliac crest into a syringe containing 250 U heparin as previously described (38, 41). The bone marrow was fractionated using methods similar to that described above for peripheral blood. Briefly, granulocytes, bands, and metamyelocytes were recovered in the pellet of the Ficoll-Hypaque gradient. Adherent cells were recovered as described above and consisted of monocytes with 1–5% fibroblasts. The residual bone marrow nonadherent mononuclear cells consisted of T lymphocytes (25–35%), B lymphocytes (2–5%), and myeloid precursors (60–73%) from the hematopoietic stem cell through the myelocyte stage. T lymphocytes were isolated by rosette formation with sheep erythrocytes as described above. The myeloid precursor pool remained contaminated with 2–6% B lymphocytes. Purity of cell preparations was determined as previously described (38, 41) by morphologic analysis of cytospin preparations stained with Wright-Geimsa stain, nonspecific esterase histochemical staining of monocytes, or monoclonal antibody staining of T lymphocytes using leu 4 (Becton-Dickenson & Co., Sunnyvale, CA), with fluorescein isothiocyanate conjugated goat anti-mouse F(ab')₂ (Cappel Laboratories, Cochranville, PA) as secondary reagent (42). Fluorescent antibody staining was quantitated by a 200 cell count under a Ortholux II fluorescent microscope (Leitz E., Inc., Rockleigh, NJ) or a 10,000 cell count using a FACS analyzer (Becton-Dickenson & Co.).

Tissue samples. Tissue samples were obtained from surgical specimens after approval of the Institutional Review Board on Human Studies, University Hospitals of Cleveland. Samples were immediately placed in PBS-1 mM EDTA at 4°C and frozen at –20°C in 3 vol of cell extract buffer that consisted of 1 mM EDTA, 70 mM Hepes, 5% glycerol, and 1 mM dithiothreitol, adjusted to pH 7.8.

Cell extracts. Cells obtained by fractionation of blood or bone marrow or from cultured cell lines were washed twice in 40 ml PBS-1 mM EDTA, 4°C. They were then resuspended at 4 $\times 10^7$ cells/ml in cell extract buffer and frozen at –20°C. Cell extracts were prepared by sonication three times for 5 s at 4°C to complete cell disruption using a microsonicator equipped with a 3/32-in. diameter probe (Kontes Glass Co., Vineland, NJ) followed by centrifugation at 10,000 $g \times 2$ min to remove cellular debris (43, 44). Tissue samples were homogenized in a Ten Broeck cell disrupter (Fisher Scientific Co., Pittsburgh, PA) and then sonicated and centrifuged as described above. Cell extract supernatant was collected and the protein concentration determined by the method of Bradford (45). The DNA concentration was determined fluorometrically using Hoescht dye 33258 (Calbiochem-Behring Corp., La Jolla, CA) and calf thymus DNA (type 5, Sigma Chemical Co.) as standard (46). Enzyme activity in cell extracts was stable for 2 wk when stored at –20°C, but underwent a 20% loss in activity over a 3-mo period.

O⁶Alkylguanine-DNA alkyltransferase assay. The activity of O⁶alkylguanine-DNA alkyltransferase (alkyltransferase) in cell extracts was measured as removal of the [³H]methyl adduct from the O⁶ position of guanine in [³H]methyl DNA alkylated with [³H]MNU as previously described (27, 32, 44). The [³H]methyl DNA was prepared (25) by dissolving 4.0 mg calf thymus DNA at 1.0 mg/ml in 0.01 M ammonium buffer (Sigma Chemical Co.), pH 10, overnight at 4°C and reacting it with 2 mCi [³H]MNU (specific activity 4.7 Ci/mM [Amersham Corp., Arlington Heights, IL]), at 35°C for 15 min. The mixture was cooled to 4°C and 8 ml 95% ethanol were added to precipitate the DNA that was

collected onto a glass rod by vigorous stirring. The DNA was washed twice in ethanol, twice in ether, and 14 times in ethanol. The DNA was then dissolved in 10 ml 0.01 M Tris-HCl, pH 8.0, and stored at -80°C until use. The specific activity of the preparation was 2.7×10^6 dpm/mg DNA or 0.096 fmol $\text{O}^6\text{MG}/\text{dpm}$ of $[\text{}^3\text{H}]\text{O}^6\text{MG}$.

To assay O^6 alkylguanine-DNA alkyltransferase, a sample of cell extract containing 250 μg protein was combined with 7.2 μg $[\text{}^3\text{H}]\text{methyl DNA}$ (containing 160 fmol O^6MG) in assay buffer consisting of 70 mM Hepes, pH 7.8, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, and 25 μM spermidine in a total volume of 200 μl , using a modification of methods previously described (43, 44). The control for each assay consisted of $[\text{}^3\text{H}]\text{methyl DNA}$ mixed with 10 μg cold calf thymus DNA in assay buffer. The reaction mixture was incubated for 60 min at 37°C , stopped with 40 μl 50% TCA at 4°C for 30 min, and the precipitate collected by centrifugation at $10,000 g \times 2$ min and washed with 400 μl 95% ethanol. In preliminary experiments, both the incubation period and the amount of cell extract were varied to determine optimal conditions. The pellet was hydrolyzed with 80 μl 0.1 N HCl at 70°C for 45 min, which removed $>95\%$ of the $[\text{}^3\text{H}]\text{methyl purines}$. The hydrolysate was neutralized by addition of 400 μl 0.02 M Tris base, pH 10.6. The supernatant was collected after centrifugation at $10,000 g \times 2$ min and combined with a 200- μl distilled water wash of the residual pellet. Large molecular weight fragments ($>2,000$ mol wt) were removed from the supernatant by filtration through a low binding affinity TM membrane in a Centrifree apparatus (Amicon Corp., Lexington, MA).

The methyl purines in the sample were separated by high performance liquid chromatography (HPLC) using a 5000 liquid chromatograph equipped with a 1.0-ml loop, an internal UV-100 detector, and an MCH-5 15-cm, 5- μm silica-bonded C_{18} column (Varian Assoc., Sunnyvale, CA) coupled to a guard column packed with 40 μm Vydex reverse-phase silica bonded particles. The column was equilibrated in 0.01 M KH_2PO_4 , pH 6.2, and eluted at 1.0 ml/min with a gradient of 100% 0.01 M KH_2PO_4 to 75% 0.01 M $\text{KH}_2\text{PO}_4/25\%$ methanol (HPLC grade, Fisher Scientific Co.) run over 25 min. Elution times of the methyl purines were identified by ultraviolet absorption of internal standards of unlabeled $\text{N}^3\text{methyladenine}$, N^7MG (Vega Scientific, Tucson, AZ) and O^6MG . The O^6MG was prepared by hydrolysis of $\text{O}^6\text{methylguanosine}$ (Vega Scientific) in 0.1 N HCl at 70°C for 180 min, using a modification of published methods (47), and confirmed to be O^6MG by comparison to a sample obtained from Dr. D. Goldthwait, Casewestern Reserve University School of Medicine, Cleveland, OH (25). Eluate from the HPLC was collected as 1.7-ml fractions, mixed with 10 ml Scintiverse II (Fisher Scientific Co.), and counted in an LS7800 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

The O^6 alkylguanine-DNA alkyltransferase activity was calculated as the femtomoles of O^6MG removed per milligram protein of cell extract and reported as femtomoles O^6MG per milligrams protein. The amount of $[\text{}^3\text{H}]\text{O}^6\text{MG}$ removed was measured as the difference in magnitude of the O^6MG peak in the sample compared with the control, and was converted to femtomole O^6MG based on the specific activity of the $[\text{}^3\text{H}]\text{MNU}$. In certain experiments, alkyltransferase activity was expressed as femtomole O^6MG per microgram cellular DNA. In the reaction mixture, excess $[\text{}^3\text{H}]\text{O}^6\text{MG}$ was present so that total alkyltransferase activity could be determined.

Chromatographic analysis of *S*-methylcysteine formation. To determine whether $[\text{}^3\text{H}]\text{methyl}$ moieties lost from $[\text{}^3\text{H}]\text{MG}$ during the reaction incubation were transferred to protein, we digested the reaction mixture with proteases and performed an amino acid analysis using a modification of a previously described method (26, 30). Briefly, after collection of the TCA precipitate formed in the reaction mixture described above, the pellet was washed twice in 100% ethanol at 4°C and air dried. The pellet was redissolved in 0.2 ml of 75 mM Hepes, pH 7.8, 1 mM EDTA, and 1 mM dithiothreitol buffer and digested with 60 μg proteinase K for 12 h at 37°C and with 1 U microsomal leucine aminopeptidase (Sigma Chemical Co.) for 4 h. The remaining large molecular weight molecules were precipitated with 0.5 ml 100% ethanol at -20°C for 1 h and the supernatant collected after centrifugation at $10,000 g \times 2$ min. The

ethanol was allowed to evaporate and the remaining solution spotted onto 3-M chromatography paper (Whatman Ltd., London, England), which was subjected to descending chromatography in a humidified chamber for 20 h using a solvent of ethanol, butanol, and water (2:2:1), as previously described (26). Amino acid standards run at the same time were *S*-methylcysteine, cysteine, serine, glycine, and methionine (Sigma Chemical Co). The chromatograph was developed with ninhydrin, cut into $\frac{1}{2}$ -in. strips, solubilized overnight in 0.05 ml water and 0.5 ml Protosol tissue solubilizer (New England Nuclear, Boston, MA), dissolved in scintillation fluid (48), and the radioactivity determined by scintillation counting.

Results

Characterization of *O*⁶alkylguanine-DNA alkyltransferase in the myeloid cell line HL-60. To determine whether O^6 alkylguanine-DNA alkyltransferase existed in myeloid cells and if its characteristics were similar to those described for other cell types, we assayed for removal of $[\text{}^3\text{H}]\text{O}^6\text{MG}$ by cell extracts of the myeloid cell line, HL-60. Fig. 1 presents the time course of the removal of O^6MG residues in methyl DNA by the alkyltransferase present in HL-60 cell extract. Removal of methyl adducts from O^6MG is essentially complete in <30 min at concentrations of cell extract of both 250 and 500 μg protein. We therefore selected 60 min for subsequent reactions to ensure sufficient time for the reaction to go to completion. Using this incubation period, we found that removal of O^6MG by extracts of HL-60 is linearly dependent on the amount of cell extract added between 25 and 500 μg protein of cell extract.

We next evaluated the location of the methyl moieties lost from O^6MG during the reaction with HL-60 extract, to determine whether they became protein bound. This is the reported mechanism by which O^6 alkylguanine-DNA alkyltransferase acts in other cells (27, 28). The alkyltransferase reaction mixture was subject to protease digestion and the amino acids separated by paper chromatography. As can be seen in Fig. 2, the radioactivity has become transferred to *S*-methylcysteine residues. The amount of radioactivity present in the $[\text{}^3\text{H}]\text{methylcysteine}$ peak was, within experimental error, the same as that lost from the $[\text{}^3\text{H}]\text{O}^6\text{MG}$ peak recovered by HPLC. Thus, myeloid cells removed the methyl adduct from O^6MG in DNA by transfer of the methyl group from O^6MG onto a cysteine residue in proteins in a rapid, stoichiometric fashion, which was linearly dependent on the amount of alkyltransferase present in the reaction.

***O*⁶Alkylguanine-DNA alkyltransferase in peripheral blood cells.** To determine whether normal nucleated blood cells contained alkyltransferase and to determine the differences in alkyltransferase activity that occurred in different cell lineages and

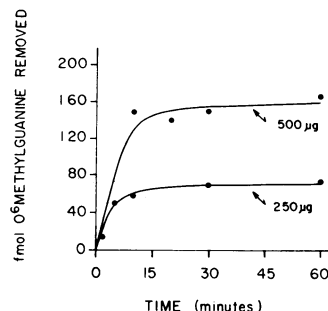


Figure 1. Time course for removal of O^6MG from methyl DNA by cell extract of HL-60. Cell extracts of HL-60 were incubated at 37°C in the presence of substrate $[\text{}^3\text{H}]\text{methyl DNA}$ that contained 160 fmol O^6MG . At the indicated times, the reactions were terminated with 50% TCA and the amount of O^6MG removed quantitated by separation of the residual O^6MG present in the sample by HPLC.

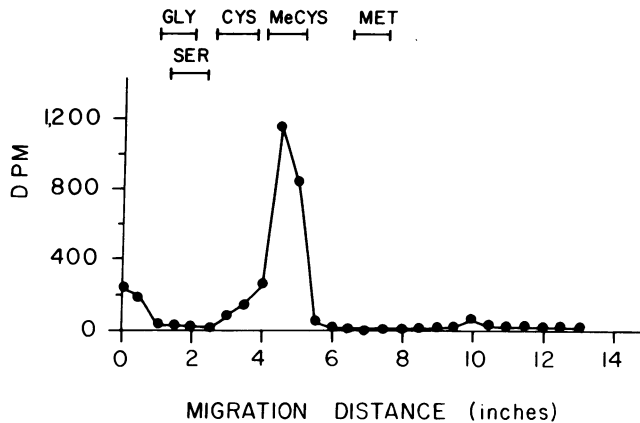


Figure 2. Transfer of [³H]methyl moieties from [³H]O⁶MG to protein by cell extract of HL-60. After incubation of 500 μg of HL-60 cell extract with [³H]methyl DNA for 60 min, the reaction product was subjected to proteolysis, and the TCA solubilized fraction was separated by descending chromatography using a mixture of ethanol, butanol, and water (2:2:1), as described in the text. GLY, glycine; CYS, cysteine; Me CYS, S-methylcysteine; SER, serine; MET, methionine.

stages of differentiation, we quantitated the alkyltransferase activity in distinct cell populations separated from peripheral blood and bone marrow. Peripheral white blood cells were separated from normal donors into the four major peripheral blood cell types. The granulocyte fraction was 92±1% pure; the monocyte fraction was 80±9% pure, with the major contaminant band forms; the T lymphocyte fraction was 89±5% pure, with the major contaminant being B lymphocytes; and the B lymphocyte fraction was 96±1% pure.

The range of O⁶alkylguanine-DNA alkyltransferase activity found in blood cells is shown in Fig. 3. There was a 4–6-fold range of activity among the 7–20 donors shown for each cell type. The mean value for alkyltransferase activity in each cell type ranged from 99±39 fmol O⁶MG/mg protein (mean±SD) in granulocytes to 189±60 fmol O⁶MG/mg protein in T lymphocytes.

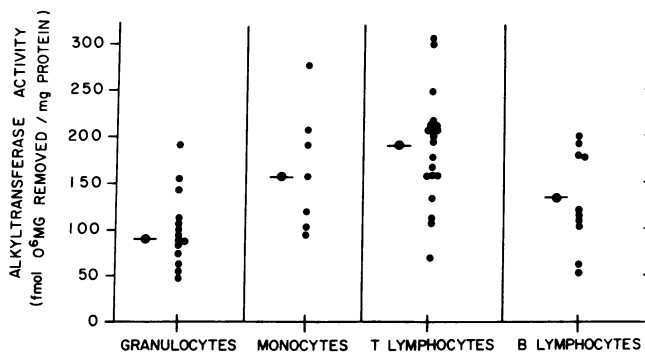


Figure 3. O⁶alkylguanine-DNA alkyltransferase activity in isolated peripheral blood nucleated cells. Nucleated white blood cells were separated into granulocytes, monocytes, and T and B lymphocytes. Extracts were prepared and assayed for alkyltransferase as described in the text. Cells were prepared from 20 donors. Samples in which insufficient cells of a particular type were obtained or in which the purity of the preparation was poor were excluded from analysis. The mean level of alkyltransferase present in each cell type is indicated by the larger circle to the left of the column of points.

phocytes. There was no correlation of alkyltransferase activity with age or sex.

O⁶Alkylguanine-DNA alkyltransferase in bone marrow cells.

Bone marrow cells were separated to yield granulocytes, monocytes, T lymphocytes, and myeloid precursors. The myeloid precursor fraction was 92±6% pure, with contaminants being either B lymphocytes (3–5%) or monocytes (0–8%). We have previously found that hematopoietic stem cells are increased in this fraction compared with unseparated bone marrow by about tenfold. The purity of the other cell fractions was similar to that obtained in the fractions from peripheral blood. The level of O⁶alkylguanine-DNA alkyltransferase activity in bone marrow-derived granulocytes was 89±40 fmol O⁶MG/mg protein (*n* = 21), and of bone marrow-derived monocytes, 105±37 fmol O⁶MG/mg protein (*n* = 7). These values are similar to those obtained in peripheral blood.

The level of O⁶alkylguanine-DNA alkyltransferase in bone marrow-derived T lymphocytes and myeloid precursors is shown in Fig. 4. Data are divided into bone marrow samples obtained from individuals younger and older than 30 yr. As can be seen, there is no difference in the mean or range of activity in the two age groups. There is an 8–9-fold range of activity between individuals. The mean alkyltransferase levels were slightly higher in T lymphocytes, 210±121 fmol O⁶MG/mg protein, than in myeloid precursors, 143±86 fmol O⁶MG/mg protein. The higher level of alkyltransferase activity found in myeloid precursors than in mature granulocytes (99±39 fmol O⁶MG/mg protein) indicates that during maturation there is loss of enzyme activity.

Given the wide range of alkyltransferase activity found between individuals, we wished to determine whether different cell lineages within the same individual had similar levels of alkyltransferase activity. To do this analysis, we compared individuals from whom we had assayed both T lymphocytes and myeloid precursors. These individuals are identified by the solid lines

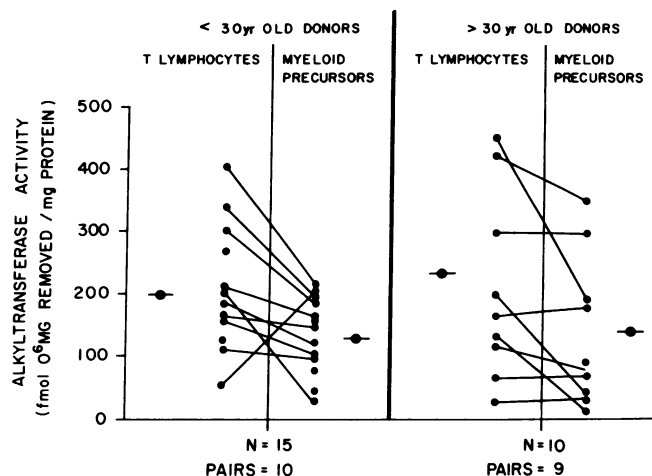


Figure 4. O⁶alkylguanine-DNA alkyltransferase present in nucleated cells isolated from the bone marrow. Nucleated cells from the bone marrow were separated into granulocytes, monocytes, T lymphocytes, and myeloid precursors, and assayed for alkyltransferase as described in the text. Each point represents the mean value from an individual donor. The mean level of alkyltransferase present in each cell type is indicated by the larger circle to the left of the column of points. Paired samples of T lymphocytes and myeloid precursors isolated from the same donor are connected by a solid line.

between values in Fig. 4. In 18 of these 19 donors, the myeloid precursors had lower or equal alkyltransferase activity than the T lymphocytes, and in 13, the myeloid precursors were actually lower ($P < 0.05$, paired t test, two sided). In only one individual was the alkyltransferase activity markedly higher in the myeloid precursors than the T lymphocytes. In addition, in these 19 donors there was a significant correlation between the alkyltransferase level found in T lymphocytes and the activity found in myeloid precursors ($r = 0.69$, $P < 0.005$). These results indicate that despite the wide range of alkyltransferase activity observed between individuals, the activity is usually higher in T lymphocytes than myeloid precursors in the same individual. In addition, because of the reasonably good correlation between T lymphocytes and myeloid precursors in alkyltransferase activity, it may be feasible to screen individuals for activity in their myeloid precursors by examining the peripheral blood T lymphocytes.

Comparison of O⁶alkylguanine-DNA alkyltransferase in myeloid precursors with activity in other tissues. To compare the capacity to repair the O⁶alkylguanine adduct in myeloid precursors with that of other tissues, we assayed fresh surgical biopsies of normal tissue for O⁶alkylguanine-DNA alkyltransferase. Most published analyses of the alkyltransferase are done on the basis of the protein content of the cell extract (32–34). However, since tissues vary widely in protein content per cell, we performed our comparisons on the basis of alkyltransferase per unit protein and per unit DNA present in the cell extract. Since DNA is the substrate both for mutagenic alkylating agent damage and for alkyltransferase repair, it seemed reasonable to compare repair capacity in different tissues on the basis of DNA content. Expressing alkyltransferase activity in this way also corrects for changes in cellular DNA content during different phases of the cell cycle.

Figs. 5 and 6 show the O⁶alkylguanine-DNA alkyltransferase activity in various tissues compared with myeloid precursors and T lymphocytes. On the basis of cell protein content (Fig. 5), only a fourfold variation in mean alkyltransferase activity is observed between tissues. However, comparing the alkyltransferase activity between tissues on the basis of DNA content (Fig. 6), there is a 15-fold range in activity. In each tissue, there is a 3–6-fold range of activity between individuals. Based on a relative mean alkyltransferase activity in human liver of 100%, the relative alkyltransferase activity in small intestine was 35%, in colon

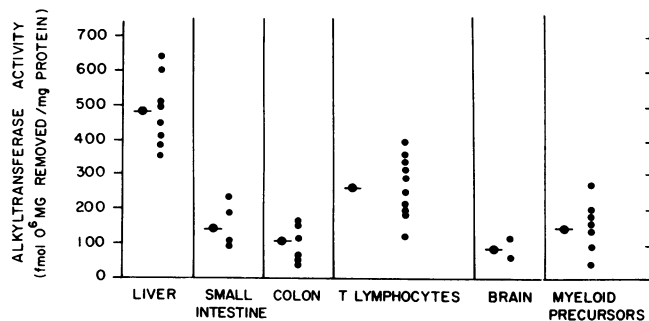


Figure 5. O⁶alkylguanine-DNA alkyltransferase in human tissues based on cellular protein content. Normal human tissues were homogenized to form cell extracts and assayed for alkyltransferase activity. Each point represents the mean value from an individual donor. Mean values for each tissue are represented by the larger circle to the side of the column of points.

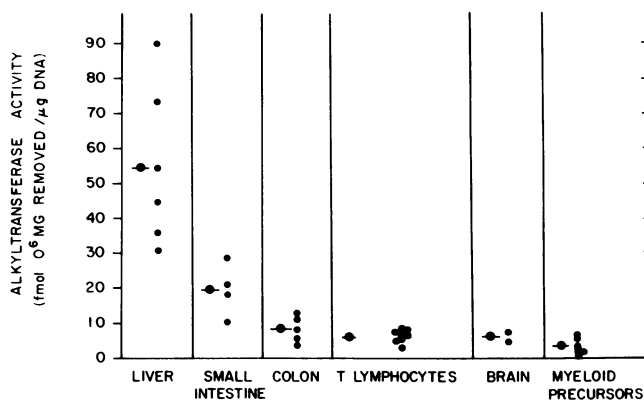


Figure 6. O⁶alkylguanine-DNA alkyltransferase in human tissues based on cellular DNA content. Normal human tissues were assayed for alkyltransferase activity as described in the text and in Fig. 5. Values shown are derived from those in Fig. 5, converted to alkyltransferase activity per microgram DNA based on the cellular DNA content.

was 14%, in T lymphocytes and brain was 11%, and in myeloid precursors only 6.6% (Table I). The highest levels observed in T lymphocytes and myeloid precursors are less than the lowest values observed with liver or small intestine. Thus, on the basis of O⁶alkylguanine-DNA alkyltransferase per microgram cellular DNA, normal myeloid precursors contain the lowest levels of activity of the tissues tested. These studies suggest that myeloid precursors are a likely target for mutagenic DNA damage by alkylating agents and that, in these cells, the level of alkyltransferase activity varies greatly between individuals.

Before it is reasonable to interpret the significance of low levels of alkyltransferase in terms of mutagenic risk, it is important to determine how stable the alkyltransferase level is over time in the same individual. Since we noted a good correlation between T lymphocytes and myeloid precursors, we tested lymphocytes from seven individuals on more than one occasion and found similar alkyltransferase levels on each occasion. In addition, two individuals were tested over a 4-wk period (Fig. 7). As can be seen, the two individuals maintained a similar level

Table I. Relative O⁶Alkylguanine-DNA Alkyltransferase Activity in Human Tissues and Cells

Tissue	No	Relative alkyltransferase activity* (fmol O ⁶ methylguanine removed)	
		per mg protein	per μg DNA
Liver	8	1.00	1.00
Small intestine	4	0.28	0.35
Colon	6	0.22	0.15
T lymphocytes	10	0.55	0.11
Brain	2	0.18	0.11
Myeloid precursors	7	0.31	0.066

No., numbers of samples.

* All assays were performed by incubating 250 μg protein of cell extract with 145 fmol O⁶MG present in [³H]methyl DNA for 60 min at 37°C. Values reported are the mean for each tissue relative to the value in human liver.

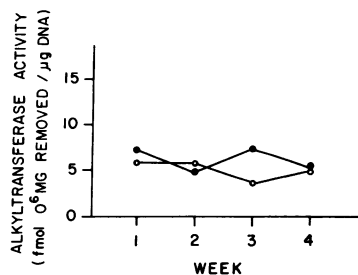


Figure 7. Stability of O⁶alkylguanine-DNA alkyltransferase in T lymphocytes from individual donors over time. T lymphocytes were isolated weekly from the peripheral blood of two donors and assayed for alkyltransferase activity as described in the text.

Data are presented as removal of O⁶MG per microgram cellular DNA. A similar stable pattern of alkyltransferase activity was obtained expressing the activity as removal of O⁶MG per milligram cellular protein.

of activity over time. Thus, individuals found to have low levels of alkyltransferase activity can be expected to maintain that level and may be at increased risk for mutagenic damage from alkylating agent exposure.

Discussion

In this study, we have measured the O⁶alkylguanine-DNA alkyltransferase activity in a variety of tissues to evaluate the possibility that low levels of this DNA repair protein may contribute to the susceptibility of myeloid precursors to alkylating agent induced DNA damage. Because ANLL is the most frequent malignancy to follow alkylating agent exposure in humans (1–8), it was our hypothesis that myeloid precursors may lack the mechanisms necessary for removal of mutagenic lesions from DNA. Such a relationship between low levels of DNA repair capacity and malignancy have been described for ultraviolet damage and xeroderma pigmentosa (49), ionizing radiation damage and ataxia telangiectasia (50), and DNA cross-linking agents and Fanconi's anemia (51). We chose to study repair of O⁶alkylguanine, a mutagenic adduct formed by some alkylating agents, and in particular, the nitrosoureas. The repair protein for this adduct is O⁶alkylguanine-DNA alkyltransferase (28). It is known that removal of this adduct can prevent two forms of mutagenic damage to the DNA, specifically point mutations due to preferential base mispairing of the O⁶alkylguanine adduct with thymine rather than cytosine during DNA replication (15, 16), and biguaninyl-DNA cross-links produced by the O⁶chloroethylguanine adduct formed by the chemotherapeutic chloroethylnitrosoureas (52). Our studies indicate that, compared with other tissues, bone marrow myeloid precursors contain low levels of the alkyltransferase, suggesting that these cells may be a target for mutagenic damage.

Three factors contribute to the mutagenicity of O⁶alkylguanine in a particular cell. The first is the extent of alkylation in the cell after alkylating agent exposure (22, 23), the second is the capacity for repair (26–28), and the third is the proliferative state of the cell after formation of the adduct (20). The first two factors control the number and persistence of adducts, while the third factor controls the rate at which the adduct is converted into a point mutation during DNA replication (15, 16, 52). The degree of alkylation experienced by various organs in vivo after drug exposure has not been measured in human tissues or bone marrow. However, animal studies indicate that the level of alkylation after intraperitoneal injection of MNU varies less than threefold in liver, intestine, kidney, spleen, and bone mar-

row (22, 23). Less variation might be expected when exposure is by the intravenous route. This suggests that during the initial passage of the compound into cells, the direct acting alkylating agents undergo nucleophilic substitution reactions with cellular DNA (9–11), producing immediate alkylation adducts. Consequently, levels of alkylation depend on the tissue distribution of drug, not on the presence of various enzymes that might activate or inactivate the agent. Since the rate of alkylation by nitrosoureas is similar in many tissues, it cannot explain the particular susceptibility of bone marrow cells to mutagenic damage by these agents.

The second factor contributing to the mutagenicity of O⁶alkylguanine, capacity for adduct repair, greatly influences the susceptibility of cells to mutagenic damage (19, 20, 22, 35). Because we found that myeloid precursors have low levels of the alkyltransferase and are more likely than other tissues to harbor persistent O⁶alkylguanine adducts after exposure to alkylating agents, these cells appear to be a target for alkylating agent-induced DNA damage. Mutagenic damage that arises from this DNA damage could be responsible for a variety of random point mutations as well as mutations in critical genes, such as transforming oncogenes (24).

This study is the first to demonstrate the presence of alkyltransferase activity in human myeloid precursors and to compare its activity with a number of other human tissues. Other investigators (27, 29–34) have found mean levels of alkyltransferase based on cellular protein content that agree quite closely with levels we observed for liver, small intestine, colon, and brain (27, 32–34). According to the published data for stomach and kidney (32, 34), both tissues appear to contain more alkyltransferase activity than we found in myeloid precursors. We base this conclusion on the published values of alkyltransferase activity per milligram cellular protein, because determinations of alkyltransferase activity per microgram cellular DNA have not been done. As mentioned above, we feel that alkyltransferase activity based on cellular DNA content is a more accurate way to express relative activity between tissues because of the variation between tissues in cellular protein content. Consequently, it remains possible that the alkyltransferase activity per microgram DNA in stomach and kidney is lower than that in brain and myeloid precursors.

Nonetheless, of the organs tested to date, those with the highest relative risk for mutagenic damage appear to be myeloid precursors, brain, and lymphocytes. In mammalian species, the brain is a target for carcinogenic damage by alkylating agents (19), but there is no association of brain tumors with alkylating agent exposure in humans. The reason for this in humans is unclear, but may have to do with the proliferative activity of glial cells in adult human brain, the existence of the blood-brain barrier, and the lag time necessary between exposure and onset of a detectable tumor. Colon epithelium, on the other hand, does undergo proliferation, and the relatively low levels of alkyltransferase activity in this tissue could contribute to the high level of colon carcinoma.

Cell proliferation is the third factor contributing to mutagenic damage by alkylating agents. In the bone marrow, alkylating agents cause a significant destruction of myeloid precursors (53), and, as a consequence, the release of stimulating factors for hematopoiesis (54). These factors induce intense cell proliferation 2–5 d after alkylating agent exposure (53), allowing mutations to be efficiently locked into the genome of myeloid cells. Re-

peated exposure to alkylating agents during this period of active DNA synthesis would be particularly mutagenic to hematopoietic stem cells recruited into cell cycle. The end result would be clonal growth of myeloid cells containing a large number of point mutations.

In contrast, lymphocytes protect themselves from alkylating agent-induced DNA damage. First, lymphocytes do not, in the absence of an antigenic stimulus, undergo a strong proliferative burst after alkylating agent exposure, so that the cells have time to repair DNA damage before replication. Second, lymphocytes increase their O⁶alkylguanine-DNA alkyltransferase levels after mitogen stimulation (44), thereby repairing adducts during DNA replication. This may explain why lymphoma or lymphoid leukemia are infrequently observed in humans after alkylating agent exposure (1-8).

The second important observation of this paper is that individuals vary greatly in the level of alkyltransferase activity found in their myeloid precursors, and that this level appears to be maintained over time. Others have found a similar wide range in interindividual alkyltransferase activity in lymphocytes (44), colon (32), liver (32, 33), intestine (32), stomach (32, 33), and brain (34). However, these earlier studies were not able to evaluate the stability of alkyltransferase activity in various organs over time. We observed that there was a stable level of activity within T lymphocytes over time and that there was a reasonably good correlation between the alkyltransferase activity in lymphocytes and that in myeloid precursors. We, as others (32-34, 44), found that the 3-9-fold range of alkyltransferase activity between individuals was not dependent on age or sex of the individual. These findings suggest that certain individuals may be particularly susceptible to mutagenic damage and malignant transformation from nitrosoureas and other alkylating agents that produce the O⁶alkylguanine adduct. Furthermore, this susceptibility may involve more than one cell lineage.

The bone marrow also appears to be susceptible to the cytotoxic and mutagenic effects of other alkylating agents, such as the nitrogen mustard derivatives. These agents are mutagenic in the Ames assay (55) but produce much lower levels of O⁶alkylguanine than the nitrosoureas. The most common adduct produced by the nitrogen mustard derivatives is N⁷alkylguanine, which itself is not mutagenic but can form the basis of intra- and interstrand cross-links (56). These cross-links may be responsible for the observed increase in sister chromatid exchanges seen with these agents (57). Furthermore, the susceptibility of the myeloid precursors to these agents in addition to the nitrosoureas (1-8) suggests that these cells may have low levels of other DNA repair enzymes, such as those involved in excision repair, in addition to the alkyltransferase.

Because animal studies indicate that the nitrosoureas are the most potent leukemogenic alkylating agents (58), and other studies indicate that O⁶alkylguanine is the critical adduct for transformation (59), it would appear that O⁶alkylguanine is an important adduct involved in the induction of leukemia. However, in other systems, it has been noted that O⁴alkylthymine is a potent mutagenic adduct that persists in those organs that are targets for malignant transformation after alkylating agent exposure (60). Like O⁶alkylguanine, this adduct is produced by the nitrosoureas, and thus may also be involved in the pathogenesis of alkylating agent-induced leukemia.

As results of cancer chemotherapy improve, more individuals enjoy a prolonged survival after alkylating agent exposure. For

this reason, it may become important to identify individuals susceptible to alkylating agents before they are treated. Based on our studies, it would be appropriate to screen patients blood cells for O⁶alkylguanine-DNA alkyltransferase activity. Individuals with low levels of alkyltransferase might be encouraged to limit their exposure to these agents. Because of the strong correlation between myeloid precursor and T lymphocyte alkyltransferase levels within the same individual, it would be possible to screen the peripheral blood cells rather than necessitating repeated bone marrow examinations.

Finally, given that a particular tissue and particular individuals may be at risk for mutagenic damage from alkylating agents, the question arises of how to increase the endogenous alkyltransferase activity in the bone marrow of individuals with low levels of activity. Other investigators have found that exposure to alkylating agents themselves may induce the alkyltransferase protein in prokaryotes and certain mammalian tissues (20, 21, 26). However, there are no reports of other, more benign inducing agents. The search for such agents remains an active area of investigation.

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