Low O⁶-Alkylguanine DNA Alkyltransferase Activity in the Peripheral Blood Lymphocytes of Patients with Therapy-related Acute Nonlymphocytic Leukemia

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ABSTRACT

Chemotherapeutic agents such as procarbazine, which produce methylated bases in DNA, are used to treat many Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) patients. A small proportion of such patients develop secondary malignancy. We examined the possibility that those patients who develop secondary malignancy have low endogenous levels of O⁶-alkylguanine DNA alkyltransferase (AGT) activity and are therefore more sensitive to the mutagenic and carcinogenic effects of their treatment. We assayed AGT activity in peripheral blood lymphocytes from patients with HD, NHL, acute nonlymphocytic leukemia (ANLL) de novo, and therapy-related ANLL, as well as a group of normal control subjects. Studies in normal controls showed that at least over a short term of 1 week, individuals have characteristic AGT levels, although some individuals sampled repeatedly over several months showed high variation. Mean AGT activities ± SE for the various groups are: normal control group, 7.05 ± 0.36; HD and NHL patients (prior to treatment), 4.97 ± 0.42; HD-NHL patients receiving procarbazine, 3.88 ± 0.44; ANLL de novo, 7.78 ± 1.72; and therapy-related ANLL, 4.30 ± 0.58. AGT activity decreased in the peripheral blood lymphocytes of some individuals taking procarbazine. The mean AGT activity in the procarbazine-treated patients was low, as was the activity for the therapy-related ANLL patients.

INTRODUCTION

The use of alkylating drugs in chemotherapy has been implicated in the occurrence of secondary cancers in patients treated for a primary malignancy (1, 2). In Hodgkin's disease, about 8% of the patients who have been treated with cytotoxic drugs and are in remission develop a secondary t-ANLL (3). The median time from the start of treatment to the diagnosis of the secondary malignancy is approximately 5 years (4), an interval likely to be necessary for progression from damage to the DNA into a permanently fixed genetic alteration, then to proliferation of a tumor. O⁶-MeG is thought to be an important mutagenic and carcinogenic lesion (5). Of the different drugs that are part of the treatment regimens of HD and NHL, two are suspects in producing O⁶-MeG in significant amounts: procarbazine in MOPP and the related compound dacarbazine in ABVD. Procarbazine is known to be metabolized to yield a diazonium ion (6), which is the active methylating species shown to produce O⁶-MeG in a relatively high proportion of its alklylation products. Indeed, animal studies demonstrate the existence of labeled O⁶-methylguanine in the DNA after injection of radioactive procarbazine into rats (7). If the O⁶-MeG is not repaired, guanine is likely to mispair with thymine instead of cytosine, and after a round of replication the mutation will be fixed (8). It is therefore logical to assume that cells deficient in O⁶-MeG repair will be more susceptible to mutagenesis and carcinogenesis.

The specific protein in the cell that repairs O⁶-MeG in the DNA, AGT, does so by transferring the methyl group onto one of its cysteines, restoring the intact guanine, and inactivating one of its own molecules in the process (9, 10). In Escherichia coli, this protein is induced by treatment of cells with alkylating agents. Transfer of two alkyl groups, one from the O⁶ position of guanine and one from a phosphotriester formed during the alkylation acts as a positive inducer to AGT formation (11). Although an adaptive-type response has been suggested in mammalian cells (12), the preponderance of evidence is that such processes are restricted to particular cell types, that human cells have a fixed amount of AGT, and that regeneration takes considerable periods (13, 14). It is likely that cells will be depleted of their transferase content by extensive alkylation of their DNA. We therefore decided to measure the level of AGT in lymphocytes isolated from patients with secondary ANLL as well as patients on chemotherapy and at risk of developing ANLL to test if lower levels of AGT are associated with higher risk of secondary malignancy.

The questions that we had to address were as follows, (a) Do individuals have characteristic levels of AGT, and do they vary with time? (b) Is AGT activity lower in t-ANLL patients than in other patient groups and normal subjects? (c) Is there a depletion of the AGT activity in the lymphocytes of patients being treated with procarbazine?

We assayed AGT activity in PBLs. It has been shown that AGT activity in PBLs varies from individual to individual (15) and that the activity in PBLs is higher but proportional to that of the myeloid precursors in bone marrow, the stem cells which presumably suffer the mutagenic damage (16). We therefore assume that lower activities in the lymphocytes will indicate even lower values in the stem cells. The general availability and the simple procedure for the isolation of PBLs provide a source of normal lymphoid cells that can be assayed even when the bone marrow in patients with ANLL is heavily infiltrated by malignant myeloid cells. If our hypothesis that secondary malignancy is associated with low AGT activity were correct, patients with t-ANLL will express lower AGT activity in all of their hematopoietic cell types than those with ANLL as a primary malignancy or those who received alkylating agents for HD but did not develop t-ANLL. The hypothesis is based on the assumption that the low endogenous activity persists and was present when the individual was initially treated for primary malignancy.

Our results show that: (a) normal control subjects have a characteristic AGT level which differs from individual to indi-
vidual; (b) patients with t-ANLL have lower ACT activity than those with ANLL de novo; and (c) even though patients treated with procarbazine do have low ACT activity as compared to normal subjects, a clear procarbazine-related decline in ACT activity is observed in only some patients throughout a course of treatment.

MATERIALS AND METHODS

A total of 181 individuals were studied for ACT activity in their PBLs. They were grouped as follows: group 1, ANLL de novo or a primary myelodysplastic syndrome prior to treatment (group 1A) or in remission (group 1B); group 2, t-ANLL (secondary ANLL occurring in patients previously treated with cytotoxic drugs or radiation for a different primary malignancy) prior to treatment (group 2A) or in remission (group 2B); group 3, HD and NHL patients just diagnosed and prior to any treatment; group 4, HD and NHL patients during treatment; either group 4P, procarbazine- or dacarbazine-treated, group 4R, radiation-treated, or group 4Q, other chemotherapy without procarbazine or dacarbazine; group 5, previously treated HD or NHL patients in complete remission (patients who have been free of disease for at least 3 months and return for checkups); group 6, healthy controls. All subjects gave informed consent for venipuncture and controls. All subjects gave informed consent for venipuncture and complete detailed questionnaires covering past medical history, family history, and current smoking, drinking, and medication practices. Blood specimens were obtained at the time of diagnosis and on multiple occasions thereafter during chemo/radiotherapy and the follow-up period. Patients with Hodgkin’s disease were treated with extended field megavoltage and radiotherapy or with standard MOPP/ABVD chemotherapy regimens. Patients with NHL received combination chemotherapy on a variety of treatment protocols using alkylating agents plus other drugs, and patients with ANLL received cytarabine with or without an anthracycline. When used, procarbazine was given daily at 150 mg/m² p.o. for 10–14 days. Sampling was within 6 h of the last dose. Dacarbazine was given at 750 mg/m² i.v. twice per month and blood was drawn just prior to the administration of the drug, i.e., 14 days past the last dacarbazine treatment. Some patients progressed through the different groups during the study and were sampled several times: e.g., before treatment (group 3); during treatment (group 4); and in remission (group 5). Normal volunteers were recruited from graduate students and employees of the University of Chicago.

Coding of Samples. Tubes of heparinized blood were brought to a central core laboratory for initial processing. They were coded using a four-digit specimen number before being sent on to a second laboratory where the AGT assays were performed. In this way the individual performing the AGT assay was kept blinded as to the group identity of the subject from whom the blood was drawn.

Separation of Lymphocytes (Mononuclear Cell Fraction) from Peripheral Blood. This procedure was performed in the core laboratory by density gradient centrifugation over Ficoll-Hypaque according to the method of Beyam (17). Cells were then suspended in serum free RPMI 1640. No attempt was made to remove adherent cells. Studies with normal subjects show that this mononuclear fraction contains about 75% T-lymphocytes, 10% B lymphocytes, and 15% monocytes or null cells (18).

Preparation of Extracts. Isolated lymphocytes were washed in phosphate-buffered saline and resuspended in lymphocyte buffer (70 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.8-5mM dithiothreitol-1 mM EDTA-5% glycerol) at a concentration of 3.5 × 10⁶ cells/ml (minimal volume used was 300 μl). The cell suspension was then sonicated twice for 5 s in a Branson sonifier model 200 at a power output of 60 W. Aliquots were removed for DNA determination and the sonicate was centrifuged at 15,000 × g for 10 min. The supernatant (extract) was stored in liquid N₂.

AGT Assay. This assay was carried out by a slight modification of the method of Myrnes et al. (19). Cell extracts (DNA equivalent of 15–50 μg) were incubated for 60 min at 37°C with ~45,000 dpm of [³²P]-methyl-N-nitrosourea-alkylated DNA substrate (2000–4000 dpm/μg DNA, 10% of which was O⁶MeG). Several aliquots of the same extract corresponding to 15–50 μg cellular DNA were used to obtain results in the linear range. The reaction mixture included lymphocyte buffer in a total volume of 200 μl. After 60 min at 37°C, samples were acidified to 5% trichloroacetic acid and 100 μg bovine serum albumin carrier were added. The mixture was heated at 80°C for 30 min to completely hydrolyze the modified bases of the DNA. After the mixture was cooled on ice, the protein-bound radioactivity was collected by filtration through GF/C discs, washed with 50 ml of cold 5% trichloroacetic acid and 15 ml of 95% ethanol, air dried, and solubilized with 0.3 ml Soluene tissue solubilizer overnight at room temperature. Radioactivity was measured in scintillation liquid 3a70B (Packard) after the addition of formic acid to neutralize the Soluene. The background counts, subtracted from all values, were the median of 3 no-extract controls per experiment averaged over the last 10 experiments (“running background”) and ranged from 200 to 300 dpm. After subtraction of background, radioactivity for a typical sample (7 fmol/μg DNA) was 600 dpm for 15 μg DNA and 2000 dpm for 50 μg DNA. AGT activity is expressed as fmol C3H₃ transferred to protein per μg of cellular DNA. Many investigators have reported activity in fmol/mg protein. Gerson et al. (20) report for human T-lymphocytes a value of 359 ± 72 (SE) fmol/mg protein corresponding to 7.4 ± 2.8 fmol/μg DNA. The assay is stable and repeatable; aliquots of the same sample analyzed at different times gave similar values with an intraassay coefficient of variation of about 10% (Fig. 1).

Preparation of [³²P]MNU-alkylated DNA. Preparation was according to the method of Karran et al. (21). Micrococcus luteus DNA (2.5 mg/ml) in 0.2 M sodium cacodylate, pH 7.2, was incubated with 0.1 mM [³²P]me-thyl-N-nitrosourea (2.5 Ci/mmol; Amersham) in the dark for 2 h at 37°C. The DNA was then ethanol precipitated, resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.5-1 mM EDTA and dialyzed for 20 h against the same mixture with 4 changes of buffer. The DNA was then stored in aliquots at −70°C.

Statistical Methods. Nested analysis of variance (22) was performed to assess the components of variability (i.e., variability between subjects and within subjects over time and over replicate assays) associated with AGT determinations. For all further analyses, AGT levels from repeated blood drawings on a particular patient in a given period (e.g., pretreatment or during treatment) were first averaged to obtain a representative value for that patient during the given period. Repeated samples in the controls were likewise averaged. Means from independent groups (groups 1A, 2A, 3, 6; 1C, 2C, 5, 6; 4P, 4R, 4O, and 6) were then compared by analysis of variance with the Tukey-Kramer allowance for multiple comparisons (23). In addition to comparison of group means, the effects of age, sex, alcohol use, and smoking were examined by analysis of covariance.

RESULTS

Reliability of the Assay. In order to assess the significance of AGT values obtained for any patient group it was necessary to

Fig. 1. Variability of the alkylguanine transferase assay performed on frozen extracts thawed and assayed on different days. The open square represents one outlying assay which was not linear. This value has been excluded from calculation of the correlation coefficient and coefficient of variation.
determine the reliability of the assay and the variability of the activity (a) among individuals and (b) within the same individual on repeated samplings. We sampled a group of normal donors (group 6) several times with 2–3-month intervals between samples. The results for some of the individuals are illustrated in Fig. 2. Each column represents a single sample; there are three columns for each individual representing successive sampling. It is obvious that while some individuals retain relatively constant values, others vary considerably between measurements. Analysis of variance calculations were performed with these data giving the values of $\sigma^2 = 2.4$ (36%) for interindividual and $\sigma^2 = 4.2$ (64%) for intranidividual variation. Thus the component of variability among the repeated samples of the same individual was greater than that between individuals. The results were different, however, when samples were drawn several times within a period of 1 week (Table 1). In this experiment, blood from five individuals was drawn on 3 different days, each sample was split and coded, and the total of 30 samples was assayed together. There is little variability in AGT activities from the same individual (14% contribution to the total variation) whereas differences between individuals persist (73% contribution to the total variation). Variation between split samples accounted for the remaining 13% of the total variation.

AGT Activity in the Patient and Control Groups. We analyzed PBLs from 181 individuals, in some cases several times, from groups 1 to 6 (Table 2; Fig. 3). The mean AGT activity in each

![Graph](image)

**Fig. 2. Alkylguanine transferase activities in individuals sampled at different times. Samples were taken about 3 months apart. Each column represents a separate sampling.**

<table>
<thead>
<tr>
<th>Donor</th>
<th>July 31</th>
<th>Aug. 3</th>
<th>Aug. 6</th>
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<td>5.0</td>
<td>5.5</td>
<td>4.9</td>
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<tr>
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<td>4.9</td>
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<td>7.9</td>
<td>7.0</td>
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<td>6.2</td>
</tr>
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<tr>
<td></td>
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**Table 1 Control experiment**

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<tr>
<td>T. T.</td>
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<tr>
<td>D. S.</td>
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**Table 2 Alkylguanine DNA alkyltransferase in PBLs**

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<tr>
<th>Group*</th>
<th>N</th>
<th>Mean ± SE</th>
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<tr>
<td>1. ANLL de novo</td>
<td>6</td>
<td>7.78 ± 1.72</td>
</tr>
<tr>
<td>ANLL in remission</td>
<td>12*</td>
<td>6.90 ± 1.07</td>
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<tr>
<td>2. A. t-ANLL</td>
<td>11</td>
<td>4.30 ± 0.58</td>
</tr>
<tr>
<td>B. t-ANLL in remission</td>
<td>2</td>
<td>4.35 ± 2.45</td>
</tr>
<tr>
<td>3. HD/NHL untreated</td>
<td>25</td>
<td>4.97 ± 0.42</td>
</tr>
<tr>
<td>HD untreated</td>
<td>14</td>
<td>5.59 ± 0.33</td>
</tr>
<tr>
<td>NHL untreated</td>
<td>11</td>
<td>4.19 ± 0.61</td>
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<tr>
<td>4. HD/NHL-P</td>
<td>17</td>
<td>3.88 ± 0.44</td>
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<tr>
<td>HD-P</td>
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<tr>
<td>NHL-P</td>
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</tr>
<tr>
<td>HD-R</td>
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<td>4.40 ± 1.07</td>
</tr>
<tr>
<td>5. HD/NHL-O</td>
<td>26</td>
<td>5.65 ± 0.72</td>
</tr>
<tr>
<td>HD-O</td>
<td>11</td>
<td>6.09 ± 1.08</td>
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<tr>
<td>NHL-O</td>
<td>15</td>
<td>5.33 ± 0.99</td>
</tr>
<tr>
<td>6. Controls</td>
<td>34</td>
<td>7.05 ± 0.36</td>
</tr>
</tbody>
</table>

* Group 1, ANLL de novo, with lymphocytes from patients with less than 10% blasts in the circulating blood; group 2, therapy-related ANLL; group 3, previously untreated Hodgkin's patients, previously untreated non-Hodgkin's lymphoma; group 4, HD, NHL patients receiving therapy (P, receiving MOPP and/or ABVD; R, receiving radiotherapy; O, receiving other chemotherapy); group 5, HD-NHL patients in remission, >3 months off therapy; group 6, normal controls. Values for each individual were first averaged over all samples taken. N is the number of individuals whose average value was used to calculate the group mean. One outlying value of 29.9 excluded (see Fig. 3). If this value is included, the mean and SE increase to 8.67 ± 2.02.

of the HD-NHL patient groups 3, 4P, and 5 is significantly lower ($P < 0.05$) than that of normal individuals. Although the means in groups 4O and 4R are more than 2 SE below the mean of the controls (based upon the pooled estimate of variability from the analysis of variance), these differences were not statistically significant by the Tukey-Kramer criterion. Patients treated with regimens including procarbazine or dacarbazine had the lowest activity, although the mean value is not significantly different from that measured for HD-NHL patients treated with other agents. If the hypothesis as to the role of AGT were correct (see above), individuals with t-ANLL would have AGT activity lower than that of the controls and of ANLL de novo patients. The data in Table 2 support this hypothesis. The mean AGT value for t-ANLL patients prior to treatment (group 2A) is significantly lower ($P < 0.05$) than the control mean and the mean of ANLL de novo patients sampled prior to treatment (group 1A). The mean in group 2B is also low but based on only two patients. If groups 2A and 2B are combined ($n = 12$; AGT value from one patient sampled prior to treatment
and while in remission were averaged) and compared with combined groups 1A and 1B (n = 18), the difference is again statistically significant: 4.38 ± 0.53 versus 7.19 ± 0.89, P = 0.012 by two-sample t test. The means for the ANLL de novo patients are not significantly different from the control mean. (These analyses have all omitted the single outlying value from group 1B. If this value is included in the analysis, the conclusions are not altered.) These observations suggest that the low AGT activity is not a general effect of ill health or a consequence of having a nonlymphocytic leukemia. What is not readily explicable is the finding of similarly low activity in all categories of HD-NHL patients. It has been reported that B- and T-lymphocytes differ slightly in activity (16); therefore it might be supposed that the observed differences between HD and NHL patients and normal controls are related to some difference in their distribution of B- and T-cells. However, there is a sizable literature (e.g., Ref. 24) indicating that the proportion of B- and T-cells remains similar in HD, NHL, and controls. We have also investigated and eliminated age, sex, alcohol use, and smoking differences as an explanation. Although there is some tendency for AGT activities to diminish with age (Fig. 4), the correlation (R) is low for both control and patient groups. Correction of the data for the slight age effect still leaves a significant difference in AGT values between the control and the patient groups. Similar covariate adjustments also do not alter the conclusions concerning differences between groups 2A, 4P, 5, and 6 or of differences between groups 1 and 2.

Procarbazine Treatment. If the original hypothesis was correct, treatment with procarbazine should result in a considerable reduction in cellular AGT levels. In the MOPP regimen, procarbazine is administered at a daily dose of 100 mg/m² of body surface area daily for 14 days followed by 2 weeks free of treatment. Following this rest period a cycle of treatment with ABVD is normally begun with dacarbazine administered in single doses on the 1st and 15th day. The cycles of MOPP and ABVD are repeated as long as necessary to induce remission. If the daily dose of procarbazine were uniformly distributed and completely metabolized without excretion (clearly an unrealistic assumption), it would produce a 10 μM concentration of methylating ion. Treatment of human lymphoma cells in culture medium with 2 μM MNNG decreases their AGT activity by 80% (13). Since procarbazine is excreted and not all metabolized to active diazonium ion (25), the active concentration is probably much lower than 10 μM. In fact, our examination of a group of patients throughout several cycles of treatment failed to demonstrate a drastic effect on AGT levels either by analysis of the group means (Table 2) or by sequential examination of PBLs from individual patients. In some patients we see a pattern of random increases and declines in AGT activity or a slow decline over the entire treatment period not unlike the changes seen in some of our normal subjects assayed over a comparable period (Fig. 5). In other patients (Fig. 6) we do see a more dramatic decline. Therefore, although procarbazine may result in low AGT activity, there is no evidence that the drug itself, when used in chemotherapy, significantly reduces the ability of individuals to repair O6-alkyl damage.

DISCUSSION

We assayed the activity of the DNA repair protein O6-alkylguanine DNA alkyltransferase in peripheral blood lymphocytes of normal individuals, patients with Hodgkin's disease, non-Hodgkin's lymphoma, and ANLL. Patients were
studied before treatment, during treatment, and in remission. We found that the lowest AGT values were associated with HD and NHL patients treated with procarbazine. Patients in the t-ANLL group also had low AGT values. Our original hypothesis supposed that: (a) there is a range of characteristic AGT activities in individuals; (b) chemotherapy with alkylating agents which produce $O^6$-guanine adducts would lower the existing AGT values; (c) patients with low AGT values at the time of treatment would be at risk of secondary malignancy because the mutagenic lesion produced by treatment would not be removed before cell division and fixation of the mutation. We supposed that individuals with t-ANLL come mainly from the low AGT group and that procarbazine or other alkylating drugs were the critical etiological agents.

It does seem that individuals have characteristic AGT levels (Table 1) and t-ANLL patients have lower AGT levels compared to controls and ANLL de novo patients. HD-NHL patients also have low AGT activities. No drastic lowering of AGT activity was detected as a result of procarbazine administration. The failure to detect a major effect on AGT activity in the PBLs could be the result of the pharmacokinetics of drug metabolism and excretion. If the drug were not metabolized fast enough before excretion, there would not be a high enough concentration of methylidionium ion to produce a detectable effect on the PBLs. Data on the extent of DNA methylation are available in rats following a single dose of procarbazine (7). Depending on the tissue examined, 8–13 $\mu$mol $O^6$-methylguanine/mol guanine were found. This corresponds to about 25,000 methylated $O^6$ residues per cell. The summed therapeutic dose of procarbazine administered to patients over a 14-day period is about one-fourth of that used in the experiments carried out by Wiestler et al. (7). Such a dose should produce about 5,000 residues/cell, enough to deplete about 25% of the average AGT content of PBLs, assuming no regeneration during the period. It may therefore be that the slight, but still significant, lowering of the mean activity in the lymphocytes is what should be expected from the actual concentration of the active agent. The dose of procarbazine used is sufficient to induce myelosuppression, suggesting that bone marrow cells are more susceptible to its toxic effects than PBLs.

To date, we have not observed very low AGT activities in PBLs from control subjects. Lymphocytes with low AGT activity have been found in patients with autoimmune disease (26).

It might be argued that the regeneration of AGT following treatments with procarbazine which lower the activity only slightly is so rapid as to mask any effect. Such quick regeneration would also make any long term mutational effect unlikely. Support for this argument is to be found in the work of Domoradzki et al. (27) who showed that AGT activity of fibroblasts was lowered in the presence of $O^6$-methylguanine but regenerated within 24 h following removal of the methylated purine. Experiments in which $O^6$-methylguanine and MNNG were used (28) show that use of alkylating agents requires a far longer time for regeneration than methylated purine treatment. There is therefore some fundamental difference between the two types of treatment. More relevant are the results of Trey and Gerson (29). In their studies with resting lymphocytes no regeneration was observed even 48 h after depletion of AGT with the free base. In addition, we have not seen any regeneration of AGT in nonstimulated lymphocytes treated with MNNG and maintained for up to 96 h. Under these circumstances, depletion of AGT activity in a nondividing cell might allow small quantities of the $O^6$ lesion to remain in DNA and then to result in mutation at the start of replication.

AGT measurements vary between different laboratories. Some of this variability is due to the expression of activity per unit of protein. We find, in agreement with Gerson et al. (20), that expression of activity per unit of DNA is not only theoretically justified by relating the activity to the number of cells but also practical in canceling many of the fluctuations in value obtained for the same cell type. By removing this source of variability in the assay, our methodology exposes the real variability between individuals. We still do not understand the reasons for the variability in activity in some individuals over a long term as compared to the short term constancy. It may be that we are assaying a heterogeneous lymphocyte population which changes over the long term. Notwithstanding this source of variability, which may turn out to be interesting in itself, the data indicate a significantly lower AGT activity in lymphocytes from individuals with t-ANLL as well as from those treated with regimens including procarbazine or dacarbazine.

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