[CANCER RESEARCH 60, 5187–5195, September 15, 2000]

Direct Reversal of DNA Damage by Mutant Methyltransferase Protein Protects Mice against Dose-intensified Chemotherapy and Leads to *in Vivo* Selection of Hematopoietic Stem Cells[1]

Susanne Ragg, Meng Xu-Welliver, Jeff Bailey, Maria D'Souza, Ryan Cooper, Saurabh Chandra, Roopa Seshadri, Anthony E. Pegg, and David A. Williams[2]

Howard Hughes Medical Institute, Section of Pediatric Hematology/Oncology [S. R., D. A. W.], Department of Pediatrics, Herman No. Wells Center for Pediatric Research [S. R., J. B., M. D., R. C., S. C., D. A. W.], and Division of Biostatistics, Department of Medicine [R. S.], Indiana University School of Medicine, Indianapolis, Indiana 46202, and Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033 [M. X-W., A. E. P.]

ABSTRACT

Direct reversal of O^6 adducts caused by chemotherapy agents is accomplished in mammalian cells by the protein O^6-methylguanine DNA methyltransferase (MGMT). Some tumors overexpress MGMT and are resistant to alkylator therapy. One future approach to treatment of these tumors may rely on concurrent pharmacological depletion of tumor MGMT with O^6-benzylguanine (6-BG) and protection of sensitive tissues, such as hematopoietic stem and progenitor cells, using genetic modification with 6-BG-resistant MGMT mutants. We have used retroviral-mediated gene transfer to transduce murine hematopoietic bone marrow cells with MGMT point mutants showing resistance to 6-BG depletion *in vitro*. These mutants include proline to alanine and proline to lysine substitutions at the 140 position (P140A and P140K, respectively), which show 40- and 1000-fold resistance to 6-BG compared with wild-type (WT) MGMT. Lethally irradiated mice were reconstituted with murine stem cells transduced with murine stem cell virus retrovirus expressing each mutant, WT MGMT, or mock-infected cells and then treated with a combination of 30 mg/kg 6-BG and 10 mg/kg 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or with 40 mg/kg BCNU alone. Compared with mice treated with BCNU alone, significant myeloid toxicity and death occurred in mice reconstituted with mock-infected or WT MGMT (<0.1 probability of survival) or the P140A mutant (0.13 probability of survival) MGMT cDNAs. In contrast, after an initial period of mild cytopenia, mice reconstituted with the P140K mutant (0.83 probability of survival) recovered nearly normal blood counts, even during continued treatment. Comparison of peripheral blood neutrophils after completion of 5 weekly treatments in these animals showed a direct correlation between the treatment and *in vivo* selection for progeny of transduced cells (pretreatment, ~8–12% transduced cells; no treatment, ~6% transduced cells; BCNU only, 51% transduced cells; 6-BG/BCNU, 93% transduced cells). To determine whether this selection occurred at the stem cell level, bone marrow from each treatment group was infused into secondary recipients. Whereas animals that received bone marrow from untreated animals reconstituted with 2% transduced cells, animals receiving marrow from 6-BG/BCNU-treated animals reconstituted with 94% transduced cells, demonstrating nearly complete selection for stem cells in the primary animals. Mice reconstituted with marrow from animals treated with BCNU only demonstrated 23% transduced cells, consistent with partial selection of stem cells in the primary mice. The levels of transduced cells also correlated with survival during a second round of intensive combination chemotherapy (probability of survival: 6-BG/BCNU, 1.0; BCNU alone, >0.70; no treatment, <0.1). These data demonstrate that mutant MGMT expressed in the bone marrow can protect mice from time- and dose-intensive chemotherapy and that the combination of 6-BG and BCNU leads to uniform selection of transduced stem cells *in vivo* in mice.

INTRODUCTION

The utility of gene transfer technology in the treatment of cancer and a number of genetic diseases is currently limited by gene transfer efficiencies for retroviral vectors in clinical protocols. Typically, current protocols result in modification of less than 5% of peripheral blood cells (1, 2). One strategy to compensate for this limitation in transduction efficiency is the selection of cells *in vivo* that have a growth advantage. Selection of blood cells has been observed in female carriers of certain X-linked diseases (3) or in preclinical studies with the introduction of a selectable marker such as a drug resistance gene. Retroviral vectors containing sequences encoding dihydrofolate reductase (4–6), p-glycoprotein (7, 8), or MGMT[3] (9–12) have been tested for this purpose with varying success. To date none of these systems has shown uniform selection at the level of the stem cells.

One application where a rapid selection of transduced cells would be desirable is the use of hematopoietic cells resistant to certain chemotherapeutic agents. Rapid amplification of drug-resistant cells might allow the use of more intensified chemotherapy regimens to treat tumors resistant to conventional therapy. Among the best-studied mechanisms of tumor resistance is the increased expression of MGMT that mediates resistance to CENUs and other alkylating agents (13–15). 6-BG, an effective pharmacological inhibitor of MGMT activity, has been shown to restore tumor cell sensitivity to some alkylators, in particular, CENUs, *in vitro* (16–18) and in human xenografts *in vivo* (19, 20). However, pharmacological manipulation of MGMT will likely enhance toxicity to normal tissues simultaneously. In many cases, the use of dose intensification is limited by excessive toxicity of normal cells, including blood cells. Early Phase I human trials appear to show that hematopoietic cells are particularly sensitive to the combination of 6-BG and CENU cytotoxicity (21, 22).

Several human mutant MGMT proteins have been shown to confer resistance to 6-BG while retaining the ability to remove alkylator-induced O^6 adducts (23–25). The identification of such mutants has allowed for the development of new strategies to increase the therapeutic index of alkylating agents by using 6-BG to inactivate tumor MGMT activity and gene transfer of 6-BG-resistant mutant MGMT cDNAs to protect hematopoietic cells. Although current approaches are focused on blood cells, if this approach is successful, it could have implications for use in other sensitive tissues. MGMT mutants P140A and G156A expressed and purified from *Escherichia coli* have shown a 40- and 240-fold higher resistance to 6-BG depletion compared with WT MGMT (24). Xu-Welliver *et al.* (26) have generated several additional MGMT mutant proteins, including the single mutant P140K that has been shown to be over 1000-fold more resistant to

Received 3/17/00; accepted 7/20/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grant P01 CA75426-02, a William Kennedy Research Fellowship of the National Childhood Cancer Foundation, and a Fellow Scholar Award of the American Society of Hematology.

[2] To whom requests for reprints should be addressed, at Herman B. Wells Center for Pediatric Research, Indiana University, Cancer Research Institute, 1044 West Walnut Street, #414, Indianapolis, IN 46202.

[3] The abbreviations used are: MGMT, O^6-methylguanine DNA methyltransferase; 6-BG, O^6-benzylguanine; CENU, chloroethylnitrosourea; WT, wild-type; MSCV, murine stem cell virus; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GFP, green fluorescence protein; eGFP, enhanced GFP; PE, phycoerythrin; ANC, absolute neutrophil count; IRES, internal ribosome entry site.

6-BG. All of these mutants showed a reduced rate of repair of methylated DNA substrates *in vitro* compared with human WT MGMT by a factor of 2.5 (P140A), 10 (P140K), and 25 (G156A). However, each mutant protected Chinese hamster ovary cells from BCNU-induced cytotoxicity to a level comparable with WT MGMT (27). Expression of either of P140A or G156A has been shown to protect hematopoietic cells from combination 6-BG and BCNU treatment *in vitro* (28, 29). Mice transplanted with MGMT mutant G156A-transduced bone marrow were also protected from 6-BG and BCNU hematopoietic toxicity (10, 30). However, the G156A mutant shows severely reduced DNA repair kinetics in addition to being relatively unstable in mammalian cells compared with WT MGMT and P140K (31). Taken together, all of the *in vitro* data suggest that the P140K mutant should provide superior protection to primary hematopoietic cells; however, the significance of the reduced kinetic properties of this mutant compared with WT MGMT and P140A is not clear. To date, no studies have directly compared WT MGMT and these MGMT mutants with respect to protection of susceptible tissue from combined 6-BG/BCNU treatment.

To identify the most promising MGMT mutant for hematopoietic cell protection, we used a murine model to compare the mutant P140K with the previously tested P140A mutant and WT MGMT. Our results demonstrate that the P140K mutant is superior to the P140A mutant and WT MGMT in protecting bone marrow from 6-BG/BCNU toxicity *in vivo*. The data also suggest that the combined use of 6-BG and BCNU leads to significant selection of chemoresistant primitive hematopoietic stem cells *in vivo*. To study *in vivo* selection more carefully, we generated a bicistronic retroviral construct containing the P140K mutant linked to the eGFP marker gene. The expression of GFP from this construct allowed serial determinations of the number of vector-expressing blood cells in individual mice. After several courses of 6-BG/BCNU treatment, transduced stem cells were uniformly selected *in vivo*. The resistance phenotype was amplified and transmitted to secondary and tertiary transplant recipients following bone marrow transplant confirming modification and resistance at the stem cell level. Animals receiving *in vivo* selected bone marrow were highly resistant to the cytotoxic effects of 6-BG/BCNU on subsequent exposure. These data further support the potential utility of mutant MGMT gene transfer in effecting chemoresistance and suggest a powerful selection method for hematopoietic cells in cancer patients undergoing intensive chemotherapy.

MATERIALS AND METHODS

Retroviral Vectors and Producer Cell Lines. The MSCV WT and MSCV P140A retrovirus vector has been described previously (28). A second retrovirus vector containing P140K cDNA was generated by cloning full-length P140K MGMT cDNA into the *Eco*RI-*Xho*I restriction sites 5′ of the encephalomyocarditis virus IRES (Clontech, Palo Alto, CA; Ref. 32) linked to the eGFP sequence (Clontech) in a MSCV retroviral vector (MSCV 2.1; kindly provided by R. Hawley; Sunnybrooks Research Institute, Toronto, Ontario, Canada). Retroviral producer cells were generated by transfecting plasmid DNA into GP+envAmma12 packaging cells using LipofectAMINE (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. The transient viral supernatant collected 60 h after the transfection was used to infect GP+E86 cells (33) in the presence of 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI). Producer populations were then used to harvest virus supernatant for each vector. Virus-containing supernatant was harvested after an overnight incubation at 32°C, filtered through 0.45 μm filter, and stored at −80°C before use (34). Virus supernatant was titered on NIH/3T3 cells as described previously. MGMT repair activity and 6-BG resistance for each virus vector was confirmed after infection of U937 cells with virus supernatant passaged first onto GP+envAmma 12 cells. Cell extracts of infected and selected U937 cells were used for repair activity assays as described previously (28), with or without prior treatment with 10–100 μM 6-BG. For each vector, transmission of the full-length vector genome was also confirmed by Southern blot analysis of infected and selected cells.

Bone Marrow Transduction and Transplantation. Bone marrow was harvested from femurs of 8–10-week-old C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) 48 h after treatment with 5-fluorouracil (150 mg/kg; SoloPak Laboratories, Franklin Park, IL) as described previously (35, 36). Harvested bone marrow cells were prestimulated for 48 h at 37°C in 5% CO_2 with 100 units/ml recombinant human interleukin 6 (Pepro Tech, Inc. Rock Hill, NJ) and 100 ng/ml recombinant rat stem cell factor (Amgen, Thousand Oaks, CA) in α-modified Eagle's medium (Life Technologies, Inc.) supplemented with 20% FCS (Hyclone, Logan, UT). Retroviral transduction was done on fibronectin fragment CH296 (RetroNectin-; Takara Shuzo, Biotechnology Group, Otsu, Japan) at a concentration of 8 μg/cm^2 as described previously (36, 37), with a multiplicity of infection of 0.5 in the presence of cytokines. Over a period of 48 h, the cells were incubated with viral supernatant twice for 4 h. After infection, hematopoietic cells were harvested using cell dissociation buffer (Life Technologies, Inc.), and 1.5×10^6 cells were injected via tail vein into lethally irradiated mice (^{139}Cs source, 11 Gy, split dose with a minimum of 3 h between doses; Nordion International, Kanata, Canada).

Drug Administration and Blood Analysis. Four weeks after transplantation, the mice were randomly assigned to the different treatment groups: mice were injected weekly with five doses of 40 mg/kg BCNU (Drug Synthesis and Chemistry Branch, Developmental Therapeutics program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) injected i.p. in a solution of 10% (v/v) ethanol and 90% (v/v) normal saline or injected with 30 mg/kg 6-BG (provided by R. Moschel; Frederick Cancer Research Center, Frederick, MD) in a solution of 40% (v/v) polyethylene glycol-400 and 60% (v/v) 0.05 M PBS, and BCNU (10 mg/kg) was injected 1 h after 6-BG injection (both were injected i.p.). BCNU was used within 10 min of reconstitution.

In some studies, primary mice transplanted with bone marrow cells transduced with P140K retrovirus that had been left untreated, treated with BCNU only, or treated with a combination of 6-BG and BCNU were randomly chosen as a stem cell source for secondary transplants. After irradiation of recipient mice (as described above), 4×10^6 bone marrow cells harvested from primary mice were transplanted into secondary recipients. Four weeks after infusion of bone marrow cells, all animals were treated with 4 weekly doses of 6-BG and BCNU as described above.

Blood was collected each week before chemotherapy treatment. Blood counts were run on a Cell Dyn 3500 hematology analyzer (Abbott Laboratories, South Pasadena, CA) using veterinary software (Abbott Laboratories).

Analysis GFP Expression by Flow Cytometric Analysis. For analysis of GFP expression in myeloid and lymphoid lineages, peripheral blood, bone marrow, and spleen were first depleted of RBCs using RBC lysis buffer (Gentra Systems, Minneapolis, MN) for 30 min on ice and blocked with 10% normal rat serum (Caltag Laboratories, Burlingame, CA) in PBS for 10 min on ice. Cells were then incubated with one or two antibodies for 30 min on ice, washed once in 0.2% BSA in PBS, and then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Antibodies used were: (*a*) PE-conjugated GR-1 (RB6-8C5); (*b*) PE-conjugated B220 (RA3-6B2); (*c*) PE-conjugated CD3 (145-2C11); (*d*) PE-conjugated Rat IgG_{2a}; (*e*) PE-conjugated Armenian hamster IgG group 1; and (*f*) PE-conjugated Rat IgG_{2b} [all were purchased from PharMingen (San Diego, CA) and used at the concentrations recommended by the manufacturer]. The percentage of GFP-expressing granulocytes (GR-1), B-cells (B220), and T-cells (CD3, CD4, and CD8) within peripheral blood, bone marrow, and spleen was determined by dividing the number of double-positive cells by the number of lineage-positive cells (corrected for background using isotope-specific control antibody; Ref. 34).

DNA Isolation and Analysis. Genomic DNA was isolated using the Puregene DNA Isolation kit (Gentra Systems) according to the manufacturer's instructions. Genomic DNA (10–20 μg) was digested with *Eco*RI (New England BioLabs, Beverly, MA) and separated on a 0.8% agarose gel. The DNA was transferred to a nylon membrane (Magna Graph, Micron Separation INC, Westboro, MA). The membrane was then hybridized with a ^{32}P-labeled full-length eGFP probe. Prehybridization and hybridization were completed with ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's instructions. Posthybridization washes were done in 2× SSC/0.05% SCS at room temperature and then in 0.1× SSC/0.01% SDS at 50°C. The membrane was then exposed to X-ray film at −70°C.

Table 1 *Peripheral blood counts in mice treated with BCNU*

	P140A	P140K	*P*
ANC			
Pretreatment	1402 ± 470[a]	1236 ± 517	0.902
Posttreatment	1050 ± 280	949 ± 350	0.4
Platelet count			
Pretreatment	590 ± 278[b]	633 ± 170	0.4
Posttreatment	698 ± 119	869 ± 334	0.165

[a] Cells/μl.
[b] $\times 10^3$ cells/μl.

Statistical Methods. Sample sizes in this study were sufficient to ensure adequate power. The normality assumption required for the large sample tests is frequently not satisfied by these data. Hence, nonparametric tests were used for all analyses. To compare between two independent samples, Wilcoxon test was used, and multiple sample comparisons were done using the Kruskal-Wallis test. When comparing pretreatment values with posttreatment values (paired data), the sign rank test was used. All conclusions are made using 0.05 as the level of significance. Survival rates of the groups (mutant and treatment groups) were described using Kaplan-Meier curves and compared using the log-rank test. All data are presented as the mean ± SD unless otherwise indicated.

RESULTS

Establishment of a Dose-intensive Model to Study the Combination of 6-BG/BCNU. To compare resistance of MGMT mutants to 6-BG depletion *in vivo*, we first completed a pilot study examining the resistance of mice transplanted with mock-infected *versus* WT MGMT vector-transduced cells to a dose-intensive regimen of 6-BG (30 mg/kg) combined with BCNU (10 mg/kg) given once weekly for 5 weeks. The WT MGMT vector and the method of bone marrow transduction and bone marrow transplantation have been described previously (12, 36). For comparison, some mice were treated with BCNU alone at 40 mg/kg given at the same schedule because this dose has been used previously in experiments without 6-BG administration. The dose of BCNU in combination with 6-BG was chosen based on published data demonstrating that higher doses of BCNU in combination with 6-BG were lethal, even as a single dosage (38). The dose of BCNU given alone has been shown previously by our laboratory to induce fatal pancytopenia in 75% of mice transplanted with mock-infected bone marrow (12). After transduction by WT MGMT or mock infection, mice were allowed to reconstitute and were then treated with 6-BG/BCNU or with BCNU alone. All mice treated with the combination of 6-BG/BCNU in both groups died with a median survival time of 50 days (range, 33–66 days) in the mock-infected group and 43 days (range, 33–66 days) in the WT MGMT group (nonsignificant, $P = 0.69$). In agreement with our previous reports (9, 12), all mice transplanted with bone marrow transduced with WT MGMT survived treatment with the higher dose of BCNU when given alone. Thus, in this dose-intensive model, overexpression of WT MGMT does not protect animals from combined 6-BG/BCNU treatment; therefore, in the remaining experiments, mock-infected cells were used as concurrent controls.

Comparison of P140A and P140K MGMT Mutant Function *in Vivo*. To compare resistance of MGMT mutants to 6-BG depletion *in vivo*, retroviral vectors expressing a mutant containing an amino acid substitution of alanine for proline (P140A) and a mutant containing a substitution of lysine for proline (P140K) at position 140 were constructed. The full-length cDNA of each mutant was cloned into the MSCV 2.1, allowing cDNA expression to be directed from the viral long terminal repeat. Each plasmid DNA was transfected into GP+envAmma12 packaging cells, and transient virus supernatant harvested from the transfected populations was used to infect GP+E86 producer cells. Subsequently, producer populations were used to harvest virus supernatant for each vector. Titers of recombinant virus used for infection of bone marrow cells were as follows: P140A vector, 4×10^5 infectious units/ml; and P140K vector, 5×10^5 infectious units/ml assayed on NIH/3T3. Cells infected with either virus demonstrated MGMT repair activity using a standard oligonucleotide repair assay and were resistant to BCNU in standard survival assay *in vitro* (data not shown).

To determine the relative resistance of hematopoietic cells transduced with each vector *in vivo*, murine bone marrow cells were infected with supernatant virus on FN-CH296. To simulate the low number of transduced cells characteristically seen in human clinical trials, the multiplicity of infection was kept close to 0.5 (virus:bone marrow cells). Lethally irradiated mice were reconstituted with 1.5×10^6 transduced bone marrow cells expressing either no transgene-encoded MGMT (mock-infected)-, P140A mutant-, or P140K mutant-transduced bone marrow cells. Four weeks after initial transplantation of transduced bone marrow cells, mice were randomly assigned to receive five weekly injections of the combination treatment of 6-BG (30 mg/kg) and BCNU (10 mg/kg) or BCNU (40 mg/kg) alone. The third group was left untreated. We used mice treated with BCNU at the same dose used in the pilot study as a control arm. Before treatment, both MGMT mutant groups were statistically identical with regard to ANCs and platelet count (Table 1). Mice transplanted with bone marrow cells transduced with P140K or P140A vectors uniformly survived treatment with BCNU alone. There was no difference in the ANC or platelet counts among these groups after treatment (Table 1). Thus, the resistance of each mutant to BCNU alone appeared similar both *in vitro* and *in vivo*. This is in agreement with the findings of Loktionova *et al.* (27) and Maze *et al.* (28), who have shown in cell culture experiments that no difference in cell survival after transduction with these mutants is observed, even at high BCNU doses, despite the reduced rate of DNA repair of the mutants P140A and P140K compared with WT MGMT.

We next examined the effect of combination 6-BG and BCNU on mice transplanted with mock-infected, P140A-transduced, or P140K-transduced bone marrow cells. During and after treatment with the combination 6-BG and BCNU, mice transplanted with P140K-transduced bone marrow cells demonstrated 83% survival compared with 13% survival in mice transplanted with P140A-transduced cells (Fig. 1). None of the animals transplanted with mock-infected control cells survived this treatment at 120 days ($P < 0.001$). Peripheral blood counts were measured each week before chemotherapy. Before initiation of treatment, both groups were statistically identical with regard to ANC ($P = 0.4$) and platelet counts ($P = 0.43$; Table 2). Between the third and fourth treatment, the animals transplanted with cells transduced with P140K demonstrated significantly higher ANCs

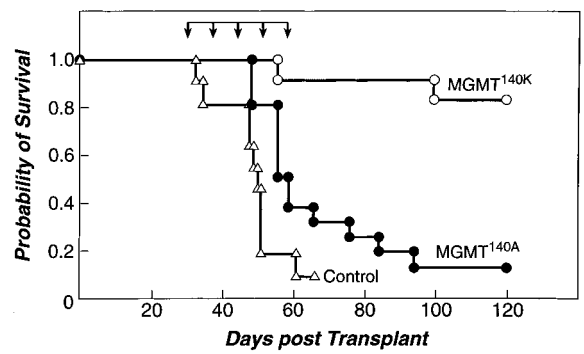

Fig. 1. Kaplan-Meier survival curve of mice transplanted with transduced bone marrow cells. Mice transplanted with bone marrow cells transduced with either P140K (○; $n = 13$), P140A (●; $n = 16$), or mock-transduced (control, △; $n = 11$) cells were treated weekly with 6-BG/BCNU (*arrows*). $P < 0.001$, log-rank test.

Table 2 *Peripheral blood counts in mice treated with 6-BG/BCNU*

	Pretreatment	Posttreatment	*P*
ANC			
P140A	1362 ± 419[a]	209 ± 320	<0.001
P140K	1161 ± 720	1469 ± 641	0.088
Platelet count			
P140A	620 ± 105[b]	97 ± 56	0.004
P140K	647 ± 91	591 ± 412	0.43

[a] Cells/μl.
[b] $\times 10^3$ cells/μl.

(P140K, 496 ± 266 cells/μl; P140A, 149 ± 75 cells/μl; $P < 0.001$) and platelet counts (P140K, 1027 ± 671 $\times 10^3$ cells/μl; P140A, 239 ± 127 $\times 10^3$ cells/μl; $P < 0.001$) compared with mice transplanted with cells transduced with P140A. In mice transplanted with P140K, peripheral blood counts reached pretreatment values for ANC and platelets after the last weekly treatment dose (Table 2 and Fig. 2). In contrast, mice transplanted with cells transduced with P140A-expressing retrovirus demonstrated significantly lower ANC and platelet counts at the end of the treatment period (Table 2 and Fig. 2).

***In Vivo* Selection of MGMT P140K-expressing Cells.** The recovery of peripheral blood counts during ongoing combination 6-BG/BCNU treatment in mice transplanted with bone marrow cells transduced with P140K retrovirus suggested that hematopoietic cells resistant to this therapy were being selected *in vivo*. Because the bicistronic P140K retroviral vector expresses P140K MGMT and eGFP from the same message, GFP expression is a useful marker for following the number of transduced cells in peripheral blood. Specific antibodies were used for dual-color analysis of neutrophils (GR-1+) and lymphocytes (B220+/CD3+). In experimental animals, the percentage of GFP-expressing (GFP+) cells in the peripheral blood before treatment was fairly uniform, ranging from 8–12% of GR-1+ and 7–9% of B220+/CD3+ cells (Table 3). Although mice randomly assigned to BCNU-only treatment showed a slightly higher number of neutrophils expressing GFP than the two other groups (Table 3; $P = 0.044$), this difference, which is probably due to differences in the kinetics of recovery from the transplant, does not alter the conclusions derived from the subsequent treatment. As seen in Fig. 3, after only three treatments, the mean percentage of GFP+ peripheral blood neutrophils increased markedly in mice treated with the combination treatment (from 8% to 80%; $P < 0.001$) but only increased from 12% to 24% ($P = 0.008$) in mice treated with BCNU alone (6-BG/BCNU group *versus* BCNU only, $P = 0.008$) and remained unchanged in the untreated group (8% *versus* 8%). After five treatments, 93% of the neutrophils in the peripheral blood expressed GFP in mice treated with 6-BG/BCNU compared with 51% of the neutrophils in the group of mice treated with BCNU alone ($P < 0.001$; Table 3 and Fig. 3).

After treatment, the percentage of GFP-expressing neutrophils remained unchanged (94%) for 7 weeks in the group of mice treated with combination therapy and in the group of mice that received no treatment (6%) but decreased significantly to 35% in the group treated with BCNU alone ($P = 0.039$). The decrease in GFP+ GR-1+ cells seen after the treatment ended in the peripheral blood was also seen in the bone marrow with only 32 ± 21% of cells being GFP+ in the group treated with BCNU only (Table 3). This may suggest that treatment with BCNU alone selects less efficiently than the combination therapy for long-lived stem cells compared with short-lived progenitor cells. The persistence of GFP+ cells at high levels in mice treated with combination therapy after completion of treatment suggested that selection of a more primitive hematopoietic progenitor cell was occurring *in vivo*.

To examine mice for evidence of stem and progenitor cell selection after BCNU treatment alone or with the combination of 6-BG/BCNU, serial transplants were performed. Theoretically, combination treatment may provide greater selective pressure because endogenous MGMT in some hematopoietic cells may protect against treatment with BCNU alone. Furthermore, if transduced stem cells were enriched with either treatment, then the drug resistance phenotype seen in peripheral blood of treated mice should be transplantable to secondary recipients. Moreover, effective elimination of nonresistant stem cells in the primary donor animals should increase the resistance

Table 3 *Analysis of peripheral blood and bone marrow of primary mice*

	Untreated	BCNU	6-BG/BCNU
Pretreatment/blood			
Gr-1	8 ± 4[a]	12 ± 3	8 ± 3
B220/CD3	7 ± 1	9 ± 2	9 ± 3
Posttreatment/blood			
Gr-1	7 ± 2[b,c]	51 ± 11[b,d]	93 ± 7[c,d]
B220/CD3	5 ± 2[b,e]	32 ± 14[b,f]	53 ± 16[e,f]
Posttreatment/bone marrow			
Gr-1	6 ± 3[g,h]	32 ± 21[g,i]	89 ± 10[h,i]
B220/CD3	5 ± 6[j]	27 ± 15	46 ± 26[j]

[a] Percentage.
[b] Untreated *versus* BCNU, $P = 0.012$.
[c] Untreated *versus* 6-BG/BCNU, $P = 0.002$.
[d] BCNU *versus* 6-BG/BCNU, $P < 0.001$.
[e] Untreated *versus* 6-BG/BCNU, $P = 0.003$.
[f] BCNU *versus* 6-BG/BCNU, $P = 0.026$.
[g] Untreated *versus* BCNU, $P = 0.045$.
[h] Untreated *versus* 6-BG/BCNU, $P = 0.012$.
[i] BCNU *versus* 6-BG/BCNU, $P < 0.001$.
[j] Untreated *versus* 6-BG/BCNU, $P = 0.048$.

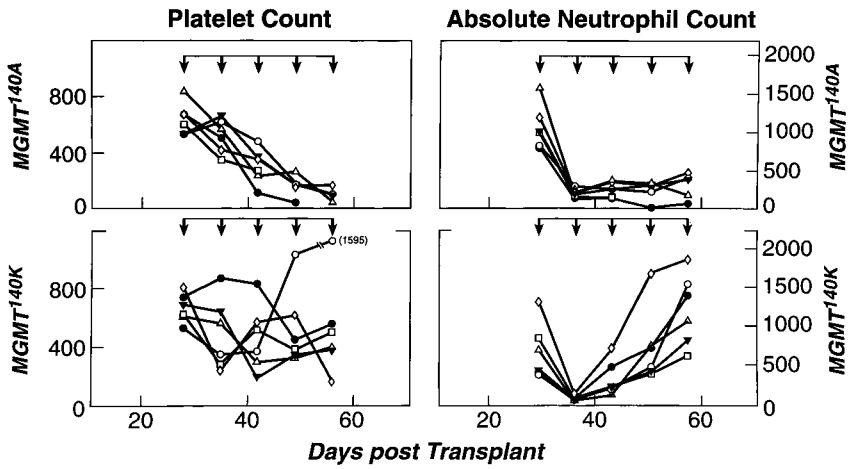

Fig. 2. Sequential analysis of neutrophil and platelet counts in transplanted mice. Mice were transplanted with P140A (*top panels*), P140K (*bottom panels*), or mock-transduced (data not shown) bone marrow cells and treated with weekly 6-BG/BCNU. Data are from one representative experiment, and each line represents serial determination of platelets (*left*) and ANCs (*right*) from the same individual mice. *Arrows*, drug treatment.

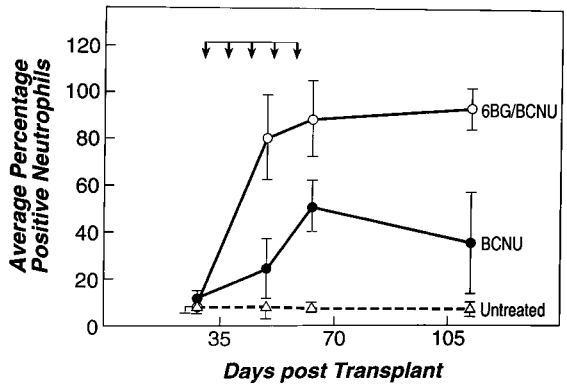

Fig. 3. Sequential flow cytometric analysis showing the mean percentage of GFP+ peripheral blood neutrophils in primary transplant recipients. The percentage of GFP+ neutrophils was determined before treatment, after three treatments, posttreatment, and before sacrifice of each animal. The percentage of GFP+ cells is shown for mice transplanted with cells transduced with the P140K retrovirus and receiving 6-BG/BCNU (○) or BCNU alone (●) or left untreated (△). *Arrows*, time of treatments. Data represent the mean ± SD for 4–14 animals/group.

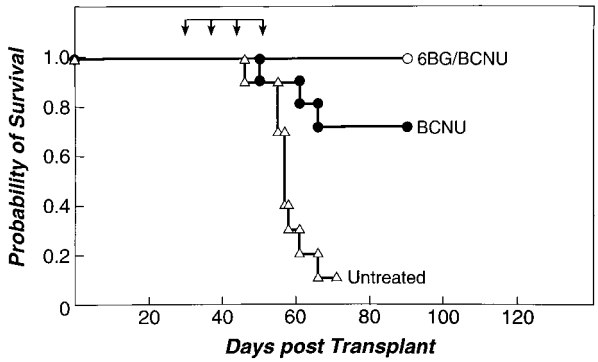

Fig. 4. Kaplan-Meier survival curve of secondary bone marrow transplant recipients. Secondary animals received bone marrow from primary animals treated with 6-BG/BCNU (○; $n = 10$) or BCNU only (●; $n = 12$) or left untreated (△; $n = 10$). Secondary animals received weekly 6-BG/BCNU at the times indicated by *arrows* ($P < 0.001$, log-rank test).

of secondary recipient mice to hematopoietic toxicity during subsequent chemotherapy treatment. Primary mice transplanted with bone marrow cells transduced with the P140K vector that were left untreated, treated with BCNU, or treated with combination 6-BG and BCNU were used as bone marrow donors in secondary transplants. Seven weeks after the fifth treatment of these primary mice, 4×10^6 bone marrow cells were harvested and transplanted into secondary irradiated recipients. Four weeks after the infusion of cells, the secondary recipients were treated with 4 weekly doses of 6-BG and BCNU.

All secondary transplant recipients of donor stem cells derived from the 6-BG/BCNU-treated mice survived an additional four weekly doses of 6-BG/BCNU, whereas only 75% of recipients of stem cells derived from BCNU-treated donors survived this treatment (Fig. 4). None of the animals transplanted with cells derived from untreated donors survived (Fig. 4; $P < 0.001$). Before treatment, the three groups were similar with regard to the ANC ($P = 0.31$) and the platelet count ($P = 0.11$; data not shown). The effects of combination therapy on peripheral blood counts varied significantly among the three groups. Mice transplanted with cells harvested from previously untreated mice or mice treated with BCNU alone demonstrated significant declines in ANC (Fig. 5*A*). In contrast, mice transplanted with bone marrow cells harvested from primary mice treated previously with combination 6-BG/BCNU showed no significant neutropenia in the face of retreatment with combination drugs (Fig. 5*A*). There was no significant drop in the platelet counts in secondary transplant recipients derived from BCNU-pretreated or 6-BG/BCNU-pretreated primary animals (Fig. 5*B*). However, secondary transplant recipients derived from untreated mice had significantly lower platelet counts after the second treatment (Fig. 5*B*). These data suggest that selection of stem cells is occurring in primary mice treated with 6-BG/BCNU to a greater extent than in mice treated with BCNU alone. In addition, the lack of significant hematopoietic toxicity in these secondary transplant recipients suggests that few nontransduced cells survive treatment in the primary animals.

Selection of transduced cells was furthered analyzed in individual primary and secondary mice by sequential flow cytometric analysis measuring the percentage of GFP+ cells in peripheral blood. Analyses of one representative mouse from each group are shown in Fig. 6. The number of GFP+ peripheral blood cells remained low (4–5%) and constant in the representative mouse from the untreated group during the entire experimental observation period and in secondary transplant recipients (Fig. 6, *A-C*). In the representative mouse treated only with BCNU, the number of GFP+ cells increased from 12% (pretreatment) to 63% after BCNU treatment and then fell to 28% after transplantation into a secondary animal (Fig. 6, *D—F*). However, subsequent treatment of this secondary animal with combination 6-BG/BCNU led to GFP expression in 99% of peripheral blood cells of this secondary animal (Fig. 6*G*). In contrast, in the representative mouse treated with 6-BG/BCNU, the number of GFP+ cells increased from 8% to 98% after combined treatment in primary mice. This high level of GFP-expressing cells was maintained in the secondary animal

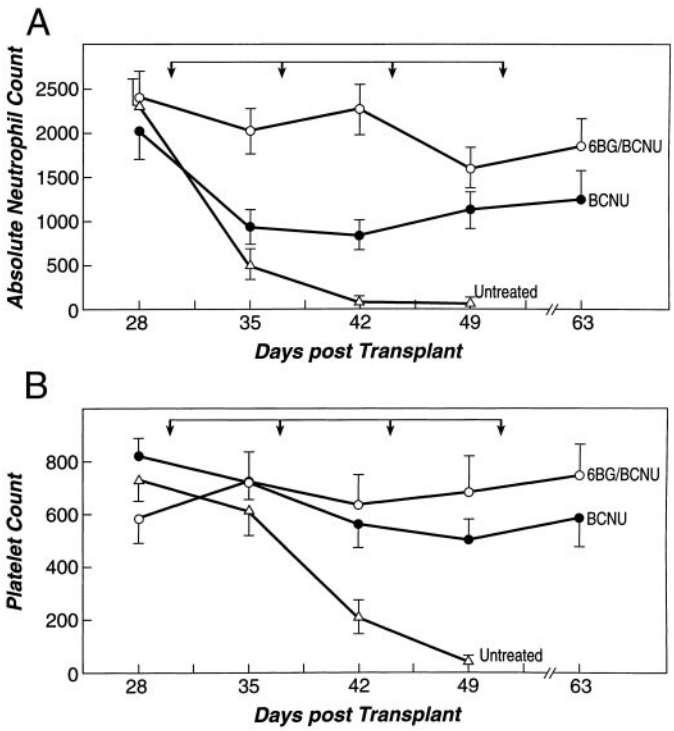

Fig. 5. Sequential analysis of mean neutrophil and platelet counts of secondary transplant recipients during 6-BG/BCNU treatment at the times indicated by the *arrows*. Peripheral blood counts were done each week before treatment. *A*, peripheral ANCs of 10–12 animals/time point. The ANC was significantly higher in 6-BG/BCNU-treated animals *versus* BCNU-treated animals at days 35 and 42 ($P < 0.001$). *B*, peripheral blood platelet counts of 10–12 animals/time point. The platelet counts were significantly lower in untreated *versus* BCNU- and 6-BG/BCNU-treated mice at days 42 and 49 ($P = 0.009$). There were no significant differences in ANC and platelet count at week 9 in 6-BG/BCNU-treated *versus* BCNU-treated mice. ○, 6-BG/BCNU-treated mice; ●, BCNU only-treated mice; △, untreated mice. Primary animals are as described in the legend to Fig. 3.

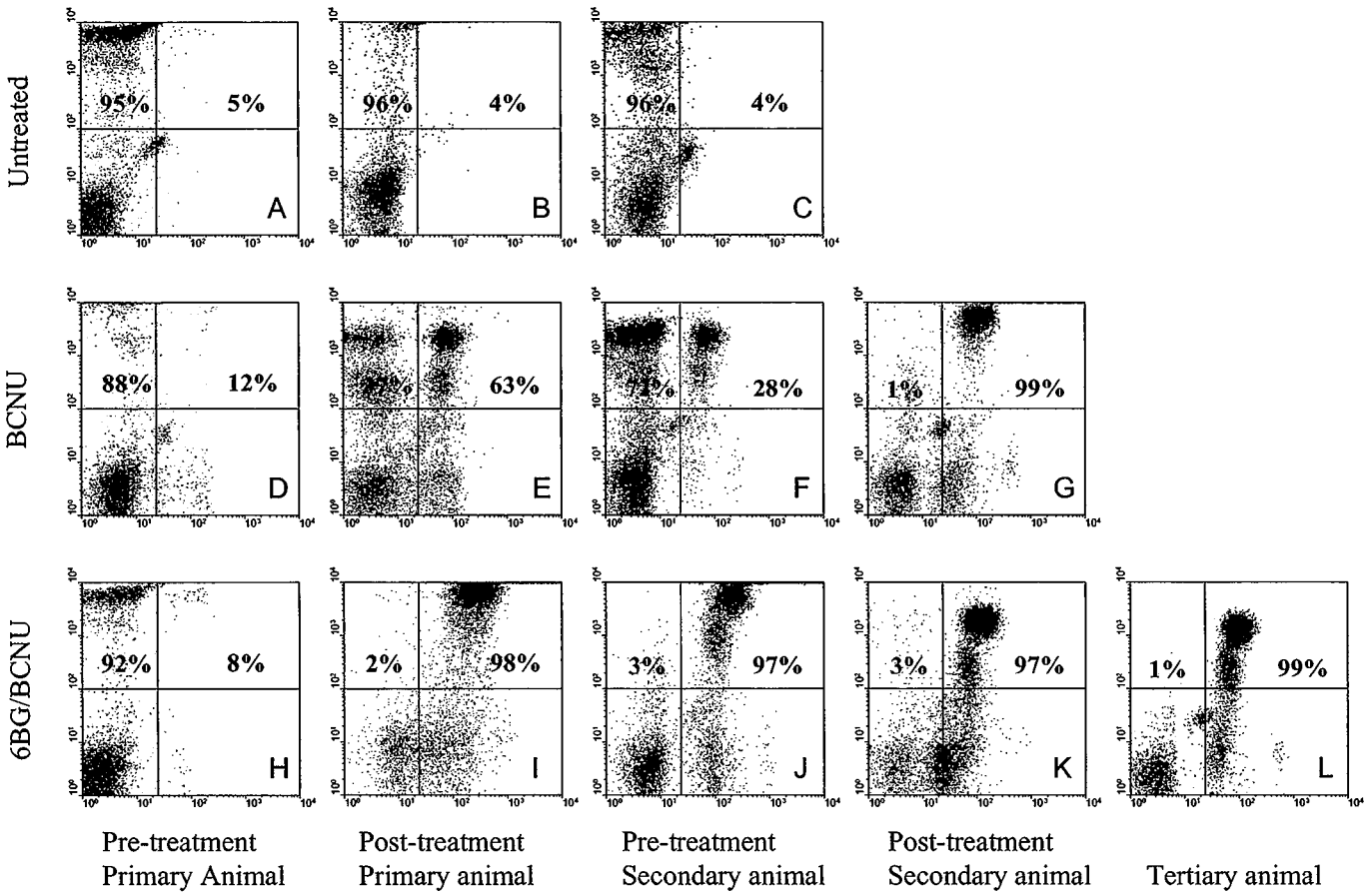

Fig. 6. Flow cytometric analysis of GFP expression in peripheral blood neutrophils of representative primary, secondary, and tertiary transplanted mice. Peripheral blood was obtained from representative individual mice transplanted with P140K transduced bone marrow cells before each treatment and after the last treatment in primary, secondary and tertiary recipient mice. Bone marrow harvested from primary mice left untreated (*A*—*C*), treated with BCNU alone (*D*—*G*), or treated with the combination of 6-BG/BCNU (*H*—*L*) was transplanted into secondary recipients (three to four secondary mice/each primary mouse). Tertiary animal was analyzed at 4 weeks after transplant and again at 12 weeks, with identical results. GFP+ (*X* axis) neutrophils were identified by both light scatter and staining with PE-conjugated GR-1 antibody (*Y* axis). The percentage of GFP+ neutrophils is indicated at the *top right* of each dot blot.

before and after treatment (Fig. 6, *H*—*K*). The high level of GFP+ cells persisted in tertiary transplant recipients of bone marrow derived from this animal (Fig. 6*L*). Of interest, the level of GFP expression (analyzed by GFP intensity) in neutrophils was identical in cohorts of mice transplanted from the same initial donor.

For all animals studied, after transplantation and before additional treatment, the proportion of GFP+ peripheral blood neutrophils was significantly higher in secondary recipients derived from donors treated with the combination therapy compared with secondary animals that were transplanted with cells from BCNU only-treated primary mice (Fig. 7 and Table 4; $P < 0.001$). After combination treatment, there was no significant difference in the number of GFP+ cells between secondary animals derived from mice treated initially with BCNU only or with combination therapy (Table 4 and Fig. 7). The increase in GFP+ neutrophils in the peripheral blood was also reflected in the bone marrow after treatment (Table 4).

These data demonstrate effective selection of stem cells in mice treated with combination 6-BG/BCNU and suggest that in contrast to treatment with BCNU alone, essentially all nontransduced myeloid progenitor cells are eliminated in primary mice receiving combination therapy.

Analysis of Lymphoid Recovery. Our laboratory has demonstrated previously that BCNU treatment of mice posttransplant is toxic to immune recovery (9). To evaluate whether lymphocytes were protected from combination therapy in mice transplanted with bone marrow cells transduced with the P140K retrovirus, the percentage of GFP+ B and T cells in peripheral blood and spleen were evaluated before and after treatment in primary and secondary animals. Thymic cell numbers could not be analyzed due to the lack of identifiable thymic tissue in mice treated with 6-BG/BCNU. Primary transplant recipients showed a marked reduction in peripheral blood lymphocyte

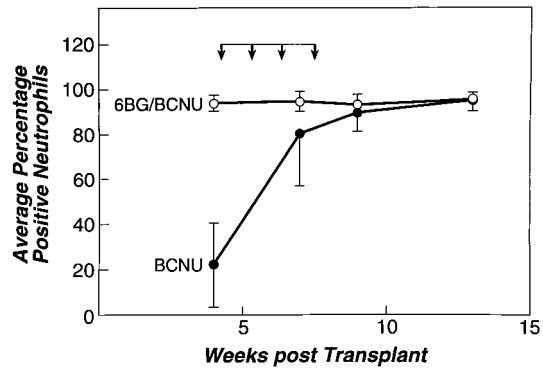

Fig. 7. Sequential flow cytometric analysis showing the mean percentage of GFP+ peripheral blood neutrophils in secondary transplant recipients. Bone marrow cells harvested from primary recipients were transplanted into lethally irradiated secondary recipients. All secondary mice received 6-BG/BCNU at the times indicated by the *arrows*. Data represent the mean ± SD of 10–13 animals/determination.

Table 4 *Analysis of peripheral blood and bone marrow in secondary mice*

	BCNU	6-BG/BCNU	*P*
Pretreatment/blood			
Gr-1	23 ± 19[a]	94 ± 4	<0.001
B220/CD3	7 ± 4	23 ± 14	<0.001
Posttreatment/blood			
Gr-1	96 ± 5	96 ± 3	0.523
B220/CD3	57 ± 15	63 ± 14	0.388
Posttreatment/bone marrow			
Gr-1	90 ± 18	97 ± 2	0.919

[a] Percentage.

count after treatment (before treatment, 4405 ± 1435 cells/μl; after treatment, 1611 ± 1014 cells/μl; $P = 0.002$), whereas secondary transplant recipients showed lower but stable peripheral blood lymphocyte counts throughout the treatment period (before treatment, 1614 ± 841 cells/μl; after treatment, 1572 ± 427 cells/μl; $P > 0.99$). In primary mice, the percentage of GFP+ B220+/CD3+ cells in the peripheral blood increased to 32% ($P = 0.008$) in BCNU only-treated mice and 53% ($P < 0.001$) in 6-BG/BCNU-treated mice compared with 5% ($P = 0.375$) in untreated mice (Table 3). In addition, all secondary animals showed a significantly higher percentage of GFP+ B220+/CD3+ cells in the peripheral blood after treatment (Table 4; 6-BG/BCNU, $P = 0.002$; BCNU, $P = 0.004$). There was no significant difference in the number of GFP+ B220+/CD3+ cells between the peripheral blood and spleen of primary and secondary transplant recipients (data not shown). Flow cytometric analysis of splenocytes showed heterogeneous GFP expression in lymphocytes of primary transplant recipients (Fig. 8*A* and *B*, *bottom panel*). These data suggest that although protection and selection of transduced lymphocytes occurs *in vivo*, the clonal nature of the selected lymphocyte population appears to be more complex than that seen in the myeloid compartment in the time course analyzed in these studies.

DNA Integration Analysis. Southern blot analysis was performed on genomic DNA isolated from nucleated bone marrow cells and splenocytes from primary, secondary, and some tertiary mice sacrificed after chemotherapy treatment at 13–16 weeks posttransplant. As noted above, flow cytometric analysis demonstrated that GFP expression patterns remained unchanged among primary, secondary, and tertiary animals treated with either BCNU or combination therapy (Fig. 7 and 8), with a discrete, narrowly confined pattern of expression suggestive of oligoclonality. To confirm identical integration patterns in primary and secondary mice at the molecular level, which would provide further evidence of selection of the stem cell compartment, DNA was digested with *Eco*RI, which cuts only once in the proviral genome, and probed with the full-length eGFP probe to determine the integration fragments of transduced cells. As seen in Fig. 8, *A* and *B*, identical hybridizing bands were seen in the DNA of bone marrow and spleen cells of the primary and all corresponding secondary and tertiary (data not shown) animals. These data confirm at the molecular level repopulation of multiple secondary animals with transduced stem cells. The limited number of integration sites seen in each animal also suggests oligoclonality of hematopoiesis. Thus, these data are consistent with engraftment and effective protection of mice by a limited number of transduced stem cells.

DISCUSSION

CENUs show moderate activity against a variety of tumors and have been used extensively in the treatment of brain tumors (39, 40). Increasing evidence suggests that tumor resistance to these agents occurs in part due to enhanced tumor repair activity associated with increased expression of MGMT (41, 42), a protein that repairs cytotoxic O^6 alkyl adducts in DNA caused by these and other alkylating agents (43, 44) in stochiometric fashion. Treatment of tumors with 6-BG, an agent that competes with mutated DNA adducts for MGMT binding, has been shown to increase tumor sensitivity to CENUs *in vitro* and deplete MGMT activity in primary tumor samples *in vivo* (16, 17). However, increased sensitivity documented in hematopoietic cell lines (45) and the enhanced myeloid toxicity seen in early human safety trials are the main limitations for the clinical treatments using the combination of 6-BG and BCNU (21, 22).

Genetic strategies to decrease sensitivity of bone marrow cells to CENUs have been explored by a number of investigators using retrovirus-mediated gene transfer of MGMT (9, 10, 12, 29, 45–48). In addition, many MGMT mutants have been generated using random *in vitro* mutagenesis or based on the primary sequence and structure of the bacterial gene product, ada, that is naturally resistant to 6-BG (24–26). The stability and resistance of these mutant proteins to 6-BG depletion and BCNU treatment varies over several logs (27, 31). The use of gene transfer to express these mutants in bone marrow cells thus provides a unique approach to increase resistance of normal blood forming cells to the combined effects of 6-BG and CENUs, while allowing sensitization of tumor cells *in vivo*. To date, limited data are available that analyze MGMT mutants in this context, particularly with respect to *in vivo* resistance to the combination of both classes of agents.

In the study reported here, we compared *in vivo* the effects of expression of the MGMT mutants P140A and P140K with WT MGMT in bone marrow cells. Our results demonstrate that the P140K mutant is superior to both the P140A mtuant and human WT MGMT in protecting bone marrow from 6-BG/BCNU toxicities *in vivo*. Mice expressing P140K showed significantly higher resistance to the combination therapy of 6-BG/BCNU, resulting in increased survival and protection of myeloid, lymphoid, and megakaryocytic lineages. In

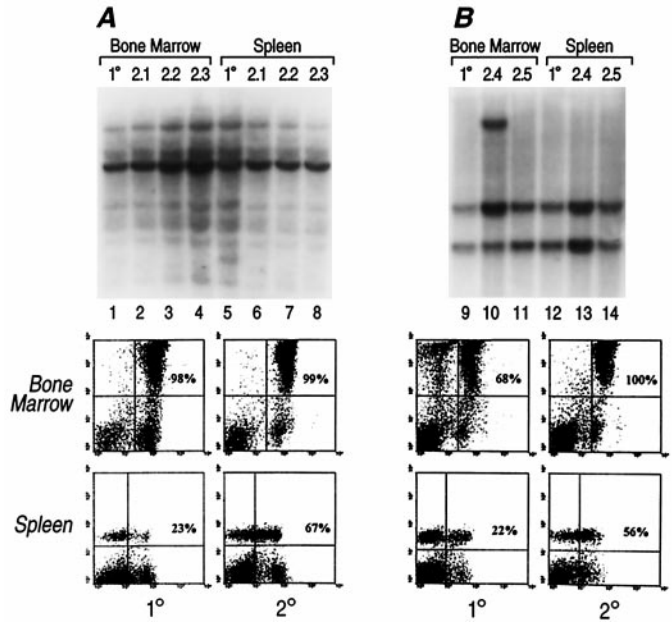

Fig. 8. Analysis of DNA integration and GFP expression in selected primary mice and derivative secondary mice. *A* and *B* show different primary mice and secondary animals transplanted with bone marrow from the primary mice. *Top panels*, integration blot using *Eco*RI restriction enzyme and ^{32}P-labeled GFP sequence as a probe. Note that the integration bands in *A* and *B* are common to 1° and 2° animals in both bone marrow and spleen cells. A sole integration band in *Lane 10* (mouse 2.4) represents a clone of cells derived from a marked stem cell in the primary animal that has become mitotically active in only this secondary animal. *Bottom panels* show GFP expression of bone marrow and spleen cells from the primary animal and representative secondary animal. Note the heterogeneity of GFP expression in the spleen that is absent in bone marrow, likely representing multiple lymphocyte clones expressing the transgene at different levels.

contrast, the dose-intensive chemotherapy leads to severe bone marrow aplasia in mice reconstituted with mock-infected bone marrow cells or with bone marrow cells transduced with WT MGMT or the P140A mutant. These data confirm and extend the results of Loktionova *et al.* (27) showing that the reduction in repair kinetics of the P140K mutant compared with WT MGMT and the P140A mutant is not as critical for determining cellular resistance to combined 6-BG/BCNU as the increase in resistance to 6-BG. This may be due to the overall rapid kinetics of this protein.

Studies examining the effect of expression of other mutants *in vivo* on the sensitivity to drug therapy have used different treatment protocols, making comparison of data derived from different mutants difficult. For example, Davis *et al.* (49) reported increased survival of mice expressing G156A in bone marrow after exposure of mice to two doses of 30 mg/kg 6-BG and 10 mg/kg BCNU given 3 weeks apart. An additional study in nonablated mice examined infusion of G156A-expressing cells during treatment with higher doses of 6-BG and BCNU. Mice expressing G156A in bone marrow demonstrated significant survival compared with mice infused with cells not expressing a MGMT transgene. Thus, in each of these studies, resistance to BCNU and moderate resistance to 6-BG depletion were noted along with protection from myelosuppression. However, Davis *et al.* (31) also reported reduced stability of the G156A mutant in mammalian cells compared with the P140K mutant, making this mutant a less optimal candidate to confer protection to hematopoietic stem cells in a dose-intensified chemotherapy trial.

The P140A/G156A double mutant has been shown to be highly resistant to 6-BG depletion (24). However, there are conflicting results with respect to the degree of protection of hematopoietic and other cells after gene transfer of this mutant. Hickson *et al.* (50) have previously demonstrated protection of fibroblasts cells using P140A/G156A expressed off a cytomegalovirus promoter, despite a 10-fold decrease in repair activity, whereas Maze *et al.* (28) demonstrated no protection in murine L1210 cells after retroviral transduction. The P140A/G156A mutant protein appears unstable and is therefore unlikely to be superior to P140K in hematopoietic cell protection. Christians *et al.* (51) and Encell *et al.* (52) have isolated several additional mutants generated by random mutagenesis that show a high degree of resistance to 6-BG as well as protection from alkylating agent cytotoxicity in bacteria. One potential problem with all MGMT mutants when used in human studies is the risk of immunogenicity, which may be increased by multiple mutations. Direct comparison of these mutants *in vivo* with P140K is warranted to determine the optimal mutant to be used in future studies.

Previously, no studies using gene transfer of MGMT have critically examined the effect of CENUs or a combination of CENUs with 6-BG on hematopoietic stem cell populations. Here we have examined the effect of a 6-BG/BCNU combination on the stem cell compartment because hematopoietic toxicity has been seen in early human safety trials. The recovery of peripheral blood counts during ongoing treatment with combination therapy along with the emergence of uniformly GFP+ cells in the peripheral blood of primary animals strongly suggested that P140K expression and combination drug treatment lead to selection of transduced hematopoietic cells *in vivo*. Data from serial transplants demonstrated that this selection is likely occurring at the level of the stem cell. Secondary transplanted mice reconstituted with bone marrow cells derived from primary animals expressing P140K and treated with combination therapy demonstrated no hematopoietic toxicity on subsequent exposure to the dose of intensive chemotherapy regimen. In contrast, mice derived from primary animals not treated previously developed severe and fatal cytopenias. The level of blood cells derived from transduced stem/progenitor cells after secondary and even tertiary transplants remained high and unchanged without any further treatment. In addition, the pattern of GFP expression in peripheral blood was nearly identical in secondary and tertiary transplant recipients compared with the initial donor. Finally, DNA analysis demonstrated common retrovirus integration bands in primary and multiple secondary mice, confirming survival and expansion of transduced and selected stem cells in these mice *in vivo*. Interestingly, selection in the lymphoid compartment appeared less uniform, suggesting that some T-cell precursors are more resistant to the combination therapy. Delayed maturation of T cells after high-dose chemotherapy is seen in humans and may also contribute to the recovery kinetics seen in the model described here. In addition, the heterogeneity in GFP expression in the lymphoid compartment may relate to peripheral expansion of transduced mature T lymphocytes as seen in human studies (53, 54).

Data derived from mice treated with BCNU alone show a decrease in the number of GFP+ cells in the peripheral blood after cessation of selective pressure. Studies by Allay *et al.* (6) also demonstrated incomplete selection of stem cells after gene transfer of mutant dihydrofolate reductase and treatment with trimetrexate and nitrobenzylmercaptopurine riboside 5′ monophosphate. In both cases, the decline in transduced cells is likely due to higher selective pressure on more differentiated progenitors compared with transduced stem cells. The contribution of these more mature progenitors to the peripheral blood declines over time due to the limited proliferative capacity of cells in this compartment. Taken together, these data suggest that stringent selection of MGMT-expressing stem cells occurs in the setting of combined 6-BG/BCNU treatment *in vivo* and that such a selection requires expression of a mutant MGMT highly resistant to 6-BG depletion in the setting of retrovirus-encoded expression. Such a selection may not be possible with other drug resistance markers, such as MDR or dihydrofolate reductase.

The clinical utility of this approach remains to be defined, and issues related to potential toxicities will need further clarification in mice, in human xenograft models, and in nonhuman primates. The data presented here and recent improvements in gene transfer technology suggest that the combined use of pharmacological depletion of tumor MGMT activity and genetic manipulation of hematopoietic cells may ultimately be useful in human chemotherapy protocols.

ACKNOWLEDGMENTS

We thank Eva Meunier and Sharon Smoot for administrative assistance. We thank members of our laboratory and Dr. Mark Kelley for many helpful discussions. We thank Takara Shuzo Co., Ltd., Biomedical Group (Otsu, Japan) for supplying CH296.

REFERENCES

1. Dunbar, C. E., Cottler-Fox, M., O'Shaughnessy, J. A., Doren, S., Carter, C., Berenson, R., Brown, S., Moen, R. C., Greenblatt, J., Stewart, F. M., Leitman, S. F., Wilson, W. H., Cowan, K., Young, N. S., and Nienhuis, A. W. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. Blood, *85:* 3048–3057, 1995.
2. Brenner, M. K., Rill, D. R., Moen, R. C., Krance, R. A., Mirro, J. J., Anderson, W. F., and Ihle, J. N. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet, *341:* 85–86, 1993.
3. Puck, J. M., and Willard, H. F. X inactivation in females with X-linked disease. N. Engl. J. Med., *338:* 325–328, 1998.
4. Williams, D. A., Hsieh, K., DeSilva, A., and Mulligan, R. C. Protection of bone marrow transplant recipients from lethal doses of methotrexate by the generation of methotrexate-resistant bone marrow. J. Exp. Med., *166:* 210–218, 1987.
5. Corey, C. A., DeSilva, A., Holland, C., and Williams, D. A. Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. Blood, *75:* 337–343, 1990.
6. Allay, J. A., Persons, D. A., Galipeau, J., Riberdy, J. M., Ashmun, R. A., Blakley, R. L., and Sorrentino, B. P. *In vivo* selection of retrovirally transduced hematopoietic stem cells. Nat. Med., *4:* 1136–1143, 1998.
7. Sorrentino, B. P., Brandt, S. J., Bodine, D., Gottesman, M., Pastan, R., Cline, A., and Nienhuis, A. W. Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. Science (Washington DC), *257:* 99–103, 1992.

8. Podda, S., Ward, M., Himelstein, A., Richardson, C., de la Flor-Weiss, E., Smith, L., Gottesman, M., Pastan, I., and Bank, A. Transfer and expression of the human multiple drug resistance gene into live mice. Proc. Natl. Acad. Sci. USA, *89:* 9676–9680, 1992.
9. Maze, R., Kapur, R., Kelley, M. R., Hansen, W. K., Oh, S. Y., and Williams, D. A. Reversal of 1,3-bis(2-chlorethyl1)-1-nitrosourea-induced severe immunodeficiency by transduction of murine long-lived hematopoietic progenitor cells using O^6-methylguanine DNA methyltransferase complementary DNA. J. Immunol. *158:* 1006–1013, 1997.
10. Davis, B. M., Reese, J. S., Koç, O. N., Lee, K., Schupp, J. E., and Gerson, S. L. Selection for G156A O^6-methylguanine DNA methyltransferase gene-transduced hematopoietic progenitors and protection from lethality in mice treated with O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res., *57:* 5093–5099, 1997.
11. Allay, J. A., Davis, B. M., and Gerson, S. L. Human alkyltransferase-transduced murine myeloid progenitors are enriched *in vivo* by BCNU treatment of transplanted mice. Exp. Hematol., *25:* 1069–1076, 1997.
12. Maze, R., Carney, J. P., Kelley, M. R., Glassner, B. J., Williams, D. A., and Samson, L. Increasing DNA repair methyltransferase levels via bone marrow stem cell transduction rescues mice from the toxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea, a chemotherapeutic alkylating agent. Proc. Natl. Acad. Sci. USA, *93:* 206–210, 1996.
13. Pegg, A. E. Mammalian O^6-alkylguanine-DNA alkyltransferases: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res., *50:* 6119–6129, 1990.
14. Pegg, A. E., and Byers, T. L. Repair of DNA containing O^6-alkylguanine. FASEB J., *6:* 2302–2310, 1992.
15. Mitra, S., and Kaina, B. Regulation of repair of alkylation damage in mammalian genomes. Prog. Nucleic Acid Res. Mol. Biol., *44:* 109–142, 1993.
16. Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C., and Pegg, A. E. Effect of O^6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. Cancer Res., *51:* 3367–3372, 1991.
17. Baer, J. C., Freeman, A. A., Newlands, E. S., Watson, A. J., Rafferty, J. A., and Margison, G. P. Depletion of O^6-alkylguanine-DNA alkyltransferase correlates with potentiation of temozolomide and CCNU toxicity in human tumor cells. Br. J. Cancer, *67:* 1299–1302, 1993.
18. Magull-Seltenreich, A., and Zeller, W. J. Sensitization of human colon tumour cell lines to carmustine by depletion of O^6-alkylguanine-DNA alkyltransferase. J. Cancer Res. Clin. Oncol., *121:* 225–229, 1995.
19. Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C., and Pegg, A. E. Modulation of mammalian O^6-alkylguanine-DNA alkyltransferase *in vivo* by O^6-benzylguanine and its effect on the sensitivity of a human glioma tumor to 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea. Cancer Commun., *2:* 371–377, 1990.
20. Mitchell, R. B., Moschel, R. C., and Dolan, M. E. Effect of O^6-benzylguanine on the sensitivity of human tumor xenografts to 1,3-bis (2-chloroethyl)-1-nitrosourea and on DNA interstrand cross-link formation. Cancer Res., *52:* 1171–1175, 1992.
21. Dolan, M. E., Roy, S. K., Fasanmade, A. A., Paras, P. R., Schilsky, R. L., and Ratain, M. J. O^6-Benzylguanine in humans: metabolic, pharmacokinetic, and pharmacodynamic findings. J. Clin. Oncol., *16:* 1803–1810, 1998.
22. Spiro, T. P., Gerson, S. L., Liu, L., Majka, S., Haaga, J., Hoppel, C. L., Ingalls, S. T., Pluda, J. M., and Willson, J. K. V. O^6-Benzylguanine: a clinical trial establishing the biochemical modulatory dose in tumor tissue for alkyltransferase-directed DNA repair. Cancer Res., *59:* 2402–2410, 1999.
23. Crone, T. M., and Pegg, A. E. A single amino acid change in human O^6-alkylguanine-DNA alkyltransferase decreasing sensitivity to inactivation by O^6-benzylguanine. Cancer Res., *53:* 4750–4753, 1993.
24. Crone, T. M., Goodtzova, K., Edara, S., and Pegg, A. E. Mutations in human O^6-alkylguanine-DNA alkyltransferase imparting resistance to O^6-benzylguanine. Cancer Res., *54:* 6221–6227, 1994.
25. Crone, T., Kanugula, S., and Pegg, A. E. Mutations in the ADA O^6-alkylguanine-DNA alkyltransferase conferring sensitivity to inactivation by O^6-benzylguanine and 2,4-diamino-6-benzyloxy-5-nitrosopyrimidine. Carcinogenesis (Lond.), *16:* 1687–1692, 1995.
26. Xu-Welliver, M., Kanugula, S., and Pegg, A. E. Isolation of human O^6-alkylguanine-DNA alkyltransferase mutants highly resistant to inactivation by O^6-benzylguanine. Cancer Res., *58:* 1936–1945, 1998.
27. Loktionova, N. A., Xu-Welliver, M., Crone, T. M., Kanugula, S., and Pegg, A. E. Protection of CHO cells by mutant forms of O^6-alkylguanine-DNA alkyltransferase from killing by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) plus O^6-benzylguanine or O^6-benzyl-8-oxoguanine. Biochem. Pharmacol., *58:* 237–244, 1999.
28. Maze, R., Kurpad, C., Pegg, A., Erickson, L., and Williams, D. Retroviral-mediated expression of the P140A, but not P140A/G156A, mutant form of O^6-methylguanine DNA methyltransferase protects hematopoietic cells against O^6-benzylguanine sensitization to chloroethylnitrosourea treatment. J. Pharmacol. Exp. Ther., *290:* 1467–1474, 1999.
29. Reese, J. S., Koç, O., Lee, K. M., Liu, L., Allay, J., Phillips, W., and Gerson, S. L. Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O^6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea. Proc. Natl. Acad. Sci. USA, *93:* 14088–14093, 1996.
30. Koç, O. N., Reese, J. S., Davis, B. M., Liu, L., Majczenko, K. J., and Gerson, S. L. ΔMGMT-transduced bone marrow infusion increases tolerance to O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea and allows intensive therapy of 1,3-bis(2-chloroethyl)-1-nitrosourea-resistant human colon cancer xenografts. Hum. Gene Ther., *10:* 1021–1030, 1999.
31. Davis, B. M., Roth, J. C., Liu, L., Xu-Welliver, M., Pegg, A. E., and Gerson, S. L. Characterization of the P140K, PVP(138–140)MLK, and G156A O^6-methylguanine-DNA methyltransferase mutants: implications for drug resistance gene therapy. Hum. Gene Ther., *10:* 2769–2778, 1999.
32. Morgan, R. A., Couture, L., Elroy-Stein, O., Ragheb, J., Moss, B., and Anderson, W. F. Retroviral vectors containing putative internal ribosome entry sites: development of a polycistronic gene transfer system and applications to human gene therapy. Nucleic Acids Res., *20:* 1293–1299, 1992.
33. Markowitz, D., Goff, S., and Bank, A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. J. Virol., *62:* 1120–1124, 1988.
34. Persons, D. A., Allay, J. A., Allay, E. R., Smeyne, R. J., Ashmun, R. A., Sorrentino, B. P., and Nienhuis, A. W. Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors *in vitro* and identification of genetically modified cells *in vivo*. Blood, *90:* 1777–1786, 1997.
35. Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A., and Bernstein, A. Introduction of a selectable gene in primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell, *42:* 71–79, 1985.
36. Hanenberg, H., Xiao, X. L., Dilloo, D., Hashino, K., Kato, I., and Williams, D. A. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat. Med., *2:* 876–882, 1996.
37. Pollok, K. E., Hanenberg, H., Noblitt, T. W., Schroeder, W. L., Kato, I., Emanuel, D., and Williams, D. A. High-efficiency gene transfer into normal and adenosine deaminase-deficient T-lymphocytes is mediated by transduction on recombinant fibronectin fragments. J. Virol., *72:* 4882–4892, 1998.
38. Dolan, M. E., Pegg, A. E., Moschel, R. C., Vishnuvajjala, B. R., Flora, K. P., Grever, M. R., and Friedman, H. S. Biodistribution of O^6-benzylguanine and its effectiveness against human brain tumor xenografts when given in polyethylene glycol or cremophor-EL. Cancer Chemother. Pharmacol., *35:* 121–126, 1994.
39. Levin, V. A., Prados, M. R., Wara, W. M., Davis, R. L., Gutin, P. H., Phillips, T. L., Lamborn, K., and Wilson, C. B. Radiation therapy and bromodeoxyuridine chemotherapy followed by procarbazine, lomustine, and vincristine for the treatment of anaplastic gliomas. Int. J. Radiat. Oncol. Biol. Phys., *32:* 75–83, 1995.
40. Fine, H. A. The basis for current treatment recommendations for malignant gliomas. J. Neurooncol., *20:* 111–120, 1994.
41. Preuss, I., Haas, S., Eichhorn, U., Eberhagen, I., Kaufmann, M., Beck, T., Eibl, R. H., Dall, P., Bauknecht, T., Hengstler, J., Wittig, B., Dippold, W., and Kaina, B. Activity of the DNA repair protein O^6-methylguanine-DNA methyltransferase in human tumor and corresponding normal tissue. Cancer Detect. Prev., *20:* 130–136, 1996.
42. Silber, J. R., Bobola, M. S., Ghatan, S., Blank, A., Kolstoe, D. D., and Berger, M. S. O^6-Methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. Cancer Res., *58:* 1068–1073, 1998.
43. Kohn, K. W. Interstrand cross-linking of DNA by 1,3-bis (2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. Cancer Res., *37:* 1450–1454, 1977.
44. Tong, W. P., Kirk, M. C., and Ludlum, D. B. Mechanism of action of the nitrosoureas. V. Formation of O^6-(2- fluoroethyl)guanine and its probable role in the crosslinking of deoxyribonucleic acid. Biochem. Pharmacol., *32:* 2011–2015, 1983.
45. Wang, G., Weiss, C., Sheng, P., and Bresnick, E. Retrovirus-mediated transfer of the human O^6-methylguanine-DNA methyltransferase gene into a murine hematopoietic stem cell line and resistance to the toxic effects of certain alkylating agents. Biochem. Pharmacol., *51:* 1221–1228, 1996.
46. Allay, J. A., Dumenco, L. L., Koç, O. N., Liu, L., and Gerson, S. L. Retroviral transduction and expression of the human alkyltransferase cDNA provides nitrosourea resistance to hematopoietic cells. Blood, *85:* 3342–3351, 1995.
47. Moritz, T., Mackay, W., Glassner, B. J., Williams, D. A., and Samson, L. Retroviral-mediated expression of a DNA repair protein in bone marrow protects hematopoietic cells from nitrosourea-induced toxicity *in vitro* and *in vivo*. Cancer Res., *55:* 2608–2614, 1995.
48. Jelinek, J., Fairbairn, L. J., Dexter, T. M., Rafferty, J. A., Stocking, C., Ostertag, W., and Margison, G. P. Long-term protection of hematopoiesis against the cytotoxic effects of multiple doses of nitrosourea by retrovirus-mediated expression of human O^6-alkylguanine-DNA-alkyltransferase. Blood, *87:* 1957–1961, 1996.
49. Davis, B. M., Koç, O. N., and Gerson, S. L. Bone marrow repopulation by limited numbers of G156A MGMT transduced hematopoietic cells in non-myeloablated mice after O^6-benzylguanine and BCNU mediated selection. Blood, *92:* 691a, 1998.
50. Hickson, I., Fairbairn, L. J., Chinnasamy, N., Lashford, L. S., Thatcher, N., Margison, G. P., Dexter, T. M., and Rafferty, J. A. Chemoprotective gene transfer. I. Transduction of human haemopoietic progenitors with O^6-benzylguanine-resistant O^6-alkylguanine-DNA alkyltransferase attenuates the toxic effects of O^6-alkylating agents *in vitro*. Gene Ther., *5:* 835–841, 1998.
51. Christians, F. C., Dawson, B. J., Coates, M. M., and Loeb, L. A. Creation of human alkyltransferases resistant to O^6-benzylguanine. Cancer Res., *57:* 2007–2012, 1997.
52. Encell, L. P., Coates, M. M., and Loeb, L. A. Engineering human DNA alkyltransferases for gene therapy using random sequence mutagenesis. Cancer Res., *58:* 1013–1020, 1998.
53. Mackall, C. L., Fleisher, T. A., Brown, M. R., Andrich, M. P., Chen, C. C., Feuerstein, I. M., Horowitz, M. E., Magrath, I. T., Shad, A. T., Steinberg, S. M., Wexler, L. H., and Gress, R. E. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N. Engl. J. Med., *332:* 143–149, 1995.
54. Mackall, C. L., and Gress, R. E. Pathways of T-cell regeneration in mice and humans: implications for bone marrow transplantation and immunotherapy. Immunol. Rev., *157:* 61–72, 1997.

Direct Reversal of DNA Damage by Mutant Methyltransferase Protein Protects Mice against Dose-intensified Chemotherapy and Leads to *in Vivo* Selection of Hematopoietic Stem Cells

Susanne Ragg, Meng Xu-Welliver, Jeff Bailey, et al.

Cancer Res 2000;60:5187-5195.