Retroviral Transfer of a Bacterial Alkyltransferase Gene into Murine Bone Marrow Protects against Chloroethylnitrosourea Cytotoxicity

Linda C. Harris,2 Upendra K. Marathi, Carol C. Edwards, Peter J. Houghton, Deo Kumar Srivastava, Elio F. Vanin, Brian P. Sorrentino, and Thomas P. Brent

Department of Molecular Pharmacology [L. C. H., U. K. M., C. C. E., P. J. H., T. P. B.], Department of Biostatistics [D. K. S.], Division of Experimental Hematology [E. F. V.], and Department of Biochemistry [B. P. S.]. St. Jude Children’s Research Hospital, Memphis, Tennessee 38105; Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, Tennessee 38163 [T. P. B.]; and Genetic Therapy, Inc., Gaithersburg, Maryland 20878 [E. F. V.]

ABSTRACT

The chloroethylnitrosoureas (CENUs) are important antineoplastic drugs for which clinical utility has been restricted by the development of severe delayed myelosuppression in most patients. To investigate the potential of DNA repair proteins to reduce bone marrow sensitivity to the CENUs, we transferred the Escherichia coli ada gene, which encodes a Mr 39,000 O'-alkylguanine-DNA alkyltransferase (ATase), into murine bone marrow cells by the use of a high-titer ecotropic retrovirus. The ada-encoded ATase is resistant to O'-benzyguanine (O'-BG), a potent inhibitor of the mammalian ATases, thus affording the bone marrow an additional level of protection against CENUs. In methylcellulose cultures, ada-infected hematopoietic progenitor cells were twice as resistant as uninfected cells to the toxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) following treatment with O'-BG. Although showing no obvious protective effects against leukopenia, overexpression of the bacterial ATase activity reduced the severity of anemia and thrombocytopenia in mice treated with O'-BG and BCNU. These effects, which were maximal at a BCNU dose of 12.5 mg/kg, were associated with improved survival when BCNU was given at this dose. At lower BCNU doses cytotoxicity was limited in both transduced and control mice, and at higher doses the protective effect was saturated due to cytotoxicity. These results suggest that ada gene therapy may be a feasible approach to amelioration of delayed myelosuppression following O'-BG plus CENU combination chemotherapy.

INTRODUCTION

O'-alkylguanine-DNA ATases3 are conserved DNA repair proteins found in all living organisms studied to date (1, 2). They are responsible for the repair of O'-alkylguanine and O'-alkylthymine, which are toxic, mutagenic DNA adducts generated by the alkylnitrosoureas MNU and N-methyl-N'-nitro-N-nitrosoguanidine (1, 2). ATases also repair O'-chloroethylguanine lesions, the DNA cross-link precursors produced by the CENUs, such as BCNU and 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea (3). Because DNA cross-links are extremely cytotoxic, the CENUs possess low mutagenic potential (4), and, therefore, these drugs are useful antineoplastic agents (5). The ATase repair reaction is stoichiometric and autoinactivating, so that the degree of cytotoxicity produced by a CENU depends on the amount of repair protein within the cell (6). Consequently, human tumor xenografts expressing high levels of this repair enzyme are resistant to CENU therapy (6), and tissues expressing very low levels (e.g., bone marrow) are highly sensitive (7, 8). These observations are especially pertinent to BCNU, because of its central role in primary therapy for brain tumors (9); as for all CENUs, its dose-limiting toxicity is delayed myelosuppression (5, 9).

An attractive strategy to overcome bone marrow sensitivity to the CENUs would rely on overexpression of an ATase gene and incorporating a mechanism by which endogenous tumor ATase activity may be selectively inhibited. Several groups have introduced drug resistance genes into murine bone marrow with retroviral vectors [e.g., the multidrug resistance 1 gene (MDR1; Ref. 10) and mutant dihydrofolate reductase cDNAs (11–13)] demonstrating gene expression by selection of taxol-resistant marrow in vivo or improvement in the survival of methotrexate-treated mice.

In the study reported here, we tested the feasibility of this approach using the ada ATase gene of Escherichia coli (14), which encodes a Mr 39,000 protein consisting of two subunits that repair either O'-alkylguanine and O'-alkylthymine or alkylphosphotriester adducts (14, 15). Previously, its expression in mammalian cells in vitro has been shown to protect against nitrosourea cytotoxicity (16, 20). The ada protein is similar to other ATases in that it is autoinactivated following reaction with its substrate, although in contrast to mammalian ATases, it has a very low affinity for O'-BG (21, 22), which has been used to

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2 To whom requests for reprints should be addressed, at Department of Molecular Pharmacology, St. Jude Children’s Research Hospital, P.O. Box 318, 332 North Lauderdale, Memphis, TN 38105. Phone: (901) 495-3440; Fax: (901) 521-1668.
3 The abbreviations used are: ATase, alkyltransferase; CENU, chloroethylnitrosourea; MNU, N-methyl-N-nitrosourea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MDR1, multidrug resistance 1; O'-BG, O'-benzyguanine; PGK, phosphoglycerate kinase; IL, interleukin; LTR, long terminal repeat; RT, reverse transcription; PEG, polyethylene glycol.

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sensitize resistant human tumor xenografts to CENU chemotherapy by depleting their ATase (23–26).

Theoretically, O6-BG treatment should sensitize tumors to the cytotoxic effect of the CENUs, whereas overexpression of the O6-BG-resistant ATase increases the resistance of normal bone marrow. We describe successful transfer of the bacterial ada gene into the hematopoietic cells of CB/CAJ mice, and protection against O6-BG plus BCNU-induced myelosuppression and death.

MATERIALS AND METHODS

The plasmid containing the ada gene p062C (27) was generously provided by Dr. G. P. Margison (Paterson Laboratories, Manchester, England), and the retroviral plasmid pG1Na (28) was provided by Genetic Therapy, Inc. (Gaithersburg, MD). The pUC007pgk plasmid contained the PGK promoter (29), and the N2 control retroviral producer clone (30) expressed the neo gene. NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% newborn calf serum (Sigma Chemical Co., St. Louis, MO), as were GP+E86 cells (31). PA317 cells (32) were grown in DMEM containing 10% FCS (Sigma). CBA/CaJ female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in isolation cages in a negative pressure cubical with access to food and water ad libitum. Human IL-6 was a gift from Amgen (Thousand Oaks, CA), whereas murine IL-3 was obtained from GIBCO-BRL. O6-BG was obtained from Bristol-Myers Squibb Co. (Princeton, NJ). Moschel (National Cancer Institute, Frederick, MD), and BCNU (carmustine) was obtained from GIBCO-BRL. O6-BG was dissolved in 100% ethanol. For in vitro studies, O6-BG was dissolved in DMSO, and BCNU was dissolved in 100% ethanol. For in vivo studies, the former compound was dissolved in 40% PEG 400 in saline, with the latter dissolved in 10% ethanol. All radiolabeled isotopes were from Amersham (Arlington Heights, IL); other reagents were from Sigma unless otherwise stated.

Generation of Ecotropic Retroviral Producer Clones by Transinfection. Plasmid DNA was initially transfected into the amphotropic packaging cell line PA317 by a modified calcium phosphate coprecipitation method (Stratagene, La Jolla CA; Ref. 33). Forty-eight h later, the medium--containing virus was removed and diluted 1:5, 1:10, 1:20, 1:40, and 1:80, and then centrifuged for 5 mm at 14,000 rpm at 4°C. The pellets were resuspended in 0.2 ml VTR [0.5 ml Vanadyl Ribonucleoside Complex (GIBCO-BRL), 10 mg/ml trNA, and 5 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)], after which 0.2 ml X-lys buffer (1% SDS, 0.6 mM NaCl, 20 mM EDTA, and 20 mM Tris, pH 7.4) was added. The mixed solution was extracted once with an equal volume of TE-saturated phenol and then with an equal volume of chloroform:isoamyl alcohol (24:1). The RNA was then precipitated with the addition of 0.9 ml ethanol and by holding on dry ice for 15 min. After centrifugation at 14,000 rpm for 15 min at 4°C, the pelleted RNA was dissolved in 250 μl 15% formaldehyde solution (2.1 ml 37.5% formaldehyde, 2.9 ml H2O); 250 μl 20X SSC were added; and the samples were heated at 50°C for 15 min. They were then held on ice before being applied to the slot blot apparatus (Minifold II; Schleicher & Schuell, Keene, NH), which contained a GeneScreen plus membrane (New England Nuclear DuPont, Wilmington, DE) prewetted in 10X SSC. The membrane was hybridized with a virus-specific probe using standard procedures (34).

Clones that generated the strongest signals, hence the highest levels of viral RNA, were subjected to biological titering by G418 selection of infected NIH 3T3 cells and counting resistant colonies.

ATase Assays and Fluorography. The O6-A Tase assay 1, used to quantitate the activity in the producer clones, relied on [3H]MNU-treated calf thymus DNA substrate as previously described (35). Assay 2, originally described by Wu et al. (36) and modified by Marathi et al. (37), relies on PvuII digestion of a double-stranded oligonucleotide once the O6-methylguanine adduct within its cleavage recognition sequence has been repaired by ATase. The second assay has greater sensitivity than the first and therefore allowed qualitative assessment of ATase activity in WBCs isolated from mouse peripheral blood. Blood (0.7–1.5 ml) was obtained by cardiac puncture from anesthetized mice, which were subsequently killed by cervical dislocation. RBCs were lysed in 10 ml RBC lysis buffer (150 mM NaCl, 10 mM KHCO3, and 0.1 mM EDTA) and centrifuged for 12 min at 2000 rpm to pellet the WBCs, from which extracts were prepared as described for assay 1 (35).

Fluorography was performed by incubating producer cell extracts with [3H]MNU-treated substrate DNA at 37°C for 30 min before PAGE and electroblotting of proteins to Immobilon-P membranes (Millipore, Bedford MA). Because the ATase proteins covalently bind [3H]methyl groups following their repair, radiolabeled protein was visualized by the addition of scintillation fluid (Scint-A XF, Meriden, CT) to the membrane and autoradiography (14).

Genomic DNA Isolation, Labeling of DNA Probes, and Southern Blot Analysis. Genomic DNA was isolated from producer cells using a standard protocol, essentially as described by Sambrook et al. (34), which involves SDS lysis, incubation in proteinase K (GIBCO-BRL), phenol-chloroform extraction, and ethanol precipitation. Southern blot analysis was performed as described by Sambrook et al. (34). 32P labeling of DNA probes was carried out with a Random Prime Labeling kit (Boehringer Mannheim, Indianapolis, IN).

Genomic DNA was isolated from mouse blood (200 μl) by lysing RBCs in 10 ml RBC lysis buffer (see above). WBCs were...
blood was subjected to PCR analysis with either ada and 3-globin-specific primers. The sequences of these oligonucleotides were: ada, 5'-GCTGATGCAATGCGGC-3' and 5'-GATAGAAGGCGAT-GCGCTGGGAATCG-3', which generated a 404-bp fragment, and β-globin, 5'-GAAGTTGGGAGAGCTTTGAGAC-3' and 5'-GAGAAGCTTCCCAGAAATCG-3', which generated a 401-bp fragment. Each PCR reaction contained 200 ng DNA, 1× Promega PCR buffer (Promega, Madison, WI), 1 mM MgCl₂, 0.2 mM of each of the four nucleotides, 2 μCi [³²P]dCTP, 50 ng of each oligonucleotide primer, and 2.5 units Taq polymerase (Promega). Amplifications were performed in a minicycler (M. J. Research, Watertown, MA) for 25 cycles at 94°C for 45 s, 56°C for 1 min 30 s, and 72°C for 2 min. Amplified products were separated by nondenaturing PAGE (6%) and visualized by autoradiography of the dried gel.

**RT-PCR.** RNA was prepared from hematopoietic colonies with RNAzol (Tel-Test, Friendswood, TX), as described by the manufacturer; RT was performed with a cDNA cycle kit (Invitrogen, San Diego, CA). The conditions of RT-PCR were essentially the same as those described for DNA, except that reactions were extended to 30 cycles.

**Hematological Analysis.** Blood (20 μl) was diluted with 9980 μl hematological diluent (0.1 mM NaCl, 16 mM boric acid, 0.1 mM sodium-tetaborate, and 0.5 mM EDTA) and analyzed using a Sysmex automated hematology analyzer.

**Statistical Analysis.** The measurements for the variables hemoglobin, hematocrit, RBCs, platelets, and WBCs were recorded on days 0, 3, 9, 14, 21, 28, 42, 45, 51, 56, 63, and 70 for each mouse on various days are correlated, the data were analyzed using ANOVA for repeated measures (40). For the two groups (ada and neo) of mice. Because the observations for each mouse on various days are correlated, the data were analyzed using ANOVA for repeated measures (40). For the analysis, it was assumed that the underlying covariance structure had compound symmetry, and the observations were missing at random; i.e., information missing because of death or for any other reason did not provide any information about the process. The analysis was performed using the program 5V in the BMDP statistical software package (41). The significance of the parameters in the model was tested using a Wald-type χ² statistic. For the two groups (ada and neo), Kaplan-Meier plots (42) were obtained and compared using the exact Wilcoxon-Gehan test as implemented in StatXact statistical software (43).

**Retroviral Infection of Murine Bone Marrow Cells.** CBA/CaJ mice were thymectomized at 8 weeks of age so that future studies may use human tumor xenografts, and, at 10 weeks, received i.v. injections of 150 mg/kg 5-fluorouracil (Solopak Laboratories, Inc., Elk Grove Village, IL). Forty-eight h later, they were sacrificed for isolation of bone marrow from their tibias and femurs (38). Marrow was flushed from the bones with a needle and syringe containing PBS and 2% FCS (HyClone FCS, 2× glutamine, IL-3 (3 ng/ml), and IL-6 (100 ng/ml) (39) at a concentration of 10⁶ cells/100-mm suspension dish (Corning Glass Works, Corning, NY). After another 48 h, the marrow cells were harvested and cocultured with retroviral producer cells at the same density and in the same media as above with the addition of polybrene (6 μg/ml). Retrovirally infected bone marrow cells were harvested and used for either transplantation of irradiated recipients or in vitro methylcellulose colony-forming assays.

**Assay for Clonogenic Hematopoietic Progenitor Cells.** Methylcellulose (Stem Cell Technologies, Vancouver, British Columbia, Canada) was divided into 4-ml aliquots for each drug dose to be tested. Retrovirally infected bone marrow cells were then added at a concentration of 1 × 10⁵/ml followed by the addition of 10 μM O²-BG. After 2 h, BCNU was added, and 1-ml aliquots were plated into 35-mm dishes and incubated for 11 days. The number of colonies was counted by light microscopy on day 4. Colonies for RT-PCR analysis were isolated on day 11.

**PCR Analysis.** DNA isolated from peripheral mouse blood was subjected to PCR analysis with either ada and β-globin or neo- and β-globin-specific primers. The sequences of these oligonucleotides were: ada, 5'-GCTGATGCAATGCGGC-3' and 5'-GATAGAAGGCGAT-GCGCTGGGAATCG-3', which generated a 319-bp fragment; neo, 5'-TCCACTATG-GCTGATGCAATGCGGC-3' and 5'-GATAGAAGGCGAT-GCGCTGGGAATCG-3', which generated a 401-bp fragment; and 3-globin, 5'-GAAGTTGGGAGAGCTTTGAGAC-3' and 5'-GAGAAGCTTCCCAGAAATCG-3', which generated a 401-bp fragment. Each PCR reaction contained 200 ng DNA, 1× Promega PCR buffer (Promega, Madison, WI), 1 mM MgCl₂, 0.2 mM of each of the four nucleotides, 2 μCi [³²P]dCTP, 50 ng of each oligonucleotide primer, and 2.5 units Taq polymerase (Promega). Amplifications were performed in a minicycler (M. J. Research, Watertown, MA) for 25 cycles at 94°C for 45 s, 56°C for 1 min 30 s, and 72°C for 2 min. Amplified products were separated by nondenaturing PAGE (6%) and visualized by autoradiography of the dried gel.

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**RESULTS**

**Generation of an ada Ecotropic Retrovirus.** The Moloney leukemia virus-based vector pG1Nada was generated from the pG1Na plasmid by inserting the PGK promoter (29) and the ada gene (Fig. 1; Ref. 27). The resulting plasmid has the potential to express the ada gene in mammalian cells under control of the PGK promoter and the neo gene under control of the viral promoter contained within the LTR. pG1Nada was then used to generate retroviral producer clones, as described in "Materials and Methods." The clone with the highest titer, clone 18 (approximately 2 x 10^6 virus particles/ml,) was used to generate retroviral producer clones, as described in all subsequent experiments described in this article. It was shown to be free of the helper virus, because supernatants collected from infected NIH 3T3 cells failed to infect a secondary culture as determined by a marker rescue assay (32).

**Characterization of Producer Clones.** The O"-ATase activity of clone 18 (1.5 pmol/mg protein) was 5-fold greater than the endogenous activity of parental GP+E86 cells (0.3 pmol/mg). Moreover, clone 18 ATase was not inhibited by a 1-h exposure to 10 μM O"-BG (data not shown). Incubation of whole-cell extract with [3H]MNU-treated substrate DNA, followed by PAGE and fluorography, enabled us to visualize the sizes of radiolabeled ATases, namely, the M, 39,000 ada and M, 22,000 mouse proteins (Fig. 2). Although clone 18 expresses both the neo and ada genes, it was designated the ada-expressing virus to distinguish it from N2, which expresses only the ada gene.

Genomic DNA isolated from several producer clones was digested with the NheI restriction endonuclease (which cuts the viral DNA once within each of its two LTRs) and subjected to Southern blot analysis. After hybridization with a neo probe, a single 4-kb band was observed in each of the lanes (Fig. 3), demonstrating that full-length proviral DNA had been uniformly inserted into the host cell genome.

**ada Gene Transfer to Murine Hematopoietic Commited Progenitor Cells in Vitro.** CB/CAJ bone marrow cells were infected with either the clone 18 or N2 retrovirus and exposed to BCNU, added alone or 1 h after treatment with O"-BG. After 4 days of growth in methylcellulose, the total number of hematopoietic progenitor cell colonies was counted. The survival responses to BCNU alone of cells infected with either ada- or neo-expressing viruses were similar; however, with preexposure to O"-BG the neo-transfected cells became sensitized to BCNU, whereas those bearing the ada gene did not (Fig. 4). The D50 for neo-transfected cells was 10 μM BCNU compared with 20 μM for the the ada-positive cells, representing a 2-fold increase in sensitivity following preincubation with O"-BG.

The presence of ada mRNA was demonstrated by RT-PCR (Fig. 5) within individual colonies, indicative of ada gene expression. Only two of the eight colonies exposed to O"-BG but not to BCNU were positive for ada, compared with eight of eight exposed to both O"-BG and BCNU (30 μM; Fig. 5).

**Reconstitution of Lethally Irradiated Mice with Bone Marrow Infected ex Vitro with the ada or neo Gene.** Thirty-seven female CB/CAJ donor mice were sacrificed 48 h after i.v.

**Fig. 2** Fluorography of cell extracts showing sizes of active ATase proteins. Clones 18 and 29 are two ada-expressing retroviral producer clones, GP+E86 is the parental murine line from which they were derived, and CEM is a human T-lymphoblast cell line that served as a control. Arrows, ada, human, and murine ATase proteins.

**Fig. 3** Southern blot analysis of DNA extracted from six retroviral producer cell lines probed with the neo gene. The DNA was digested with NheI, which cuts once within each viral LTR to generate a 4063-bp fragment.

**Fig. 4** Methylcellulose assay for clonogenic survival of hematopoietic progenitor cells among ada- and neo-infected bone marrow cells treated in vitro with either BCNU alone or BCNU in combination with O"-BG. Points, mean colony number relative to the number in untreated plates; bars, SDs.
administration of 150 mg/kg 5-fluorouracil (38), and bone marrow cells were harvested as described in "Materials and Methods." Fifty percent of the cells were infected with the clone 18 virus containing the ada gene, and the remainder were infected with the N2 virus containing neo. Cells (4 × 10⁷) from either manipulation were then injected into tail veins of each of 73 CB/CaJ female recipient mice that had been thymectomized and lethally irradiated (850 cGy). All mice became reconstituted with donor marrow cells, as determined by 100% survival beyond 2 weeks after transplantation. After 3 weeks, DNA was isolated from 200 µl blood isolated from the retroorbital venous plexus of each mouse. PCR analysis performed on blood samples from 28 of 38 ada and 12 of 35 neo recipient mice revealed the presence of the respective gene in every case examined (Fig. 6). Thus, all mice were likely positive for the virally inserted genes. At 12 weeks after transplantation, three ada-positive mice were sacrificed, and their marrow was used to reconstitute 24 additional mice, all of which became PCR positive for the gene. This finding, together with the observation that three ada mice remained PCR positive when tested 8 months after transplantation, indicates that long-lived primitive hematopoietic progenitors had been infected with the retrovirus ex vivo.

**O³-BG and BCNU Treatment of Mice Transplanted with Retrovirally Infected Bone Marrow Cells.** Five weeks after transplantation, 35 ada- and 35 neo-bearing mice, produced as described above, were divided into five treatment groups each comprising 7 ada and 7 neo mice. They were treated as follows: (group 1) vehicle alone; (group 2) 30 mg/kg O³-BG and 7.5 mg/kg BCNU; (group 3) 30 mg/kg O³-BG and 10 mg/kg BCNU; (group 4) 30 mg/kg O³-BG and 12.5 mg/kg BCNU; and (group 5) 30 mg/kg O³-BG and 15 mg/kg BCNU. The drugs were injected i.p., with O³-BG administered 2 h before BCNU. The mice in group 1 received both vehicles, 40% PEG in saline and 10% ethanol.

Blood samples (20 µl each) were taken from the tail vein of each mouse on the day of drug treatment (day 0) and subsequently on days 3, 9, 14, 21, and 28. On day 42, surviving mice received a second dose of O³-BG and BCNU that was identical to the first treatment, with additional blood collected on days 42, 45, 51, 56, 63, and 70. Measurements of hemoglobin, hematocrit, erythrocytes, WBCs, and platelets were obtained for all doses at each time point and subjected to statistical analysis. The two phases of the experiment from day 0 through 28 and from day 42 through 70 were analyzed either separately and also after combining them together. The most significant differences in the blood parameters for the two groups were observed in group 4 following treatment with O³-BG and 12.5 mg/kg BCNU. Fig. 7 shows the hematopoietic data for this dose. For group 4, the combined statistical analysis showed there was a significant difference (at a level of significance α = 0.01) between the two groups (ada and neo) for the variables hemoglobin (P < 0.0001), hematocrit (P = 0.0001), and RBCs (P =
0.0002). The P value corresponding to the platelet counts for the two groups was 0.014, which indicates that the two groups differ significantly at the 0.05 level of significance but not at the 0.01 level of significance. However, there was no difference in the mean of WBC levels in the two groups (P = 0.136). A similar trend was also noted when the data in the two phases were analyzed separately, with the only exception that the difference between ada and neo in platelet counts was significant at α = 0.01 in the second phase (P = 0.004). There was no difference in any of the variables in the two groups (ada and neo) when treated with vehicle alone.

The vast majority of mice survived the first drug treatment; however, death rates increased substantially after the second dose (Fig. 8). A clear survival advantage was conferred by the ada protein in mice treated with BCNU at 12.5 mg/kg (P = 0.021) consistent with the protection of hematological parameters shown in Fig. 7. There was no significant difference in the survival of the two groups for all other dose levels. Necropsies performed on four mice within 1 h of death revealed severe myeloid hypoplasia and anemia due to BCNU-induced deficits in blood elements.

**ATase Activity Assay of WBCs Isolated from Transduced Mouse Blood.** ATase assay 2 was performed on WBC extracts isolated from 12 ada mice and 2 neo mice 21 weeks after reconstitution with transduced marrow (Fig. 9). Extracts (50 µg) were pretreated for 30 min at 37°C with 50 µM O6-BG to inhibit endogenous murine ATase prior to incubation with 0.05 pmol oligonucleotide substrate. A positive control (Fig. 9, C3) consisted of 10 µg ada producer cell extract expressing 3 pmol/mg ada ATase. As indicated in Fig. 9, O6-BG-resistant activity was evident in at least three of the mice analyzed, demonstrating that ada ATase was being expressed, albeit at...
low levels, in WBCs after transduction and transplantation of virally infected marrow cells. ATase cannot be measured in any other hematopoietic cell compartment.

DISCUSSION

We have generated a high-titer retroviral producer clone that expresses high levels of the bacterial ATase gene, designated ada. This replication-defective retrovirus infects long-lived murine hematopoietic progenitor cells, as demonstrated by the persistence of ada gene sequences in peripheral blood and by the generation of ada-positive secondary bone marrow recipient mice. The functionally active, full-length M,

$M = 39,000$ ada protein expressed by producer clone 18 was easily distinguished from the smaller $M = 22,000$ endogenous murine ATase expressed by GP+E86 cells (Fig. 2). The identity of the ada protein was confirmed by its resistance to the mammalian ATase inhibitor O^6-BG (21). Southern blot analysis of genomic DNA isolated from clone 18 and other producer clones (Fig. 3) showed that the correct full-length proviral DNA had been inserted into all clones analyzed, without gross rearrangements or deletions.

The in vitro methylcellulose assay demonstrated that the clone 18 retrovirus was able to infect hematopoietic progenitor cells (Fig. 4). Functional expression of the ATase was indicated by the 2-fold greater resistance of clone 18 versus N2-infected cells to BCNU-induced cytotoxicity. Because these cells were only partially transduced (~25%), it is likely that the resistance of the ada transduced component was greater than 2-fold. It was surprising that after exposure to BCNU alone, the ada ATase provided no additional protection to cells beyond that provided by endogenous ATase levels (7, 8). It is possible that the levels of ada activity were so low that the total increase in DNA repair capacity was too small to be significant. However, once the murine enzyme was inhibited with 10 μM O^6-BG, the neo-expressing cells became sensitized to BCNU, whereas the ada-expressing cells retained their resistance, indicating that the bacterial protein was functionally active in murine hematopoietic cells. The 2-fold increase in resistance generated by clone 18 may have been greater if a higher proportion of cells expressed the gene. ada mRNA was detected in only two of eight colonies exposed to the virus in vitro, suggesting that a low proportion (~25%) of cells expressed the ada gene (Fig. 5). However, this proportion increased to 100% following treatment with 10 μM O^6-BG and 30 μM BCNU, presumably due to selection for ada-expressing cells containing functionally active ATase encoded by the bacterial gene.
The ability of the ada ATase protein to protect hematopoietic progenitor cells against BCNU in vitro prompted us to use clone 18 to infect CB/CaJ mouse bone marrow. The modified marrow was then injected into marrow-depleted recipients for the study of ada protective effects in vivo. We chose thymectomized CB/CaJ mice for our experiments because they have been successfully used at this center in therapeutic studies with human tumor xenografts (6). Our in vivo experiments revealed that transduction of the ada gene into hematopoietic cells can protect against nitrosourea-induced myelosuppression. After the first treatment of O'-BG and BCNU, all measured blood elements (platelets, erythrocytes, hematocrit, hemoglobin, and WBCs) were significantly suppressed (Fig. 7), but there were few deaths (Fig. 8). The rationale for giving a second treatment after the blood values had returned to normal was that ada-expressing cells might be selected during the first treatment, so that marrow would become enriched with such cells and be more resistant when challenged with a second dose. However, validity of this approach could not be determined because of the large number of animal deaths that occurred shortly after the second treatment. Although some blood components were protected after both 12.5-mg/kg doses of BCNU in ada-positive compared with neo-positive mice, only one of the seven animals given the latter gene was alive on day 70, precluding a meaningful statistical test of differences in blood profiles. Nonetheless, the fact that six times more ada-positive than neo-positive mice survived this treatment clearly indicates a protective effect from the bacterial enzyme. The low frequency of deaths in both treatment groups at doses below 12.5 mg/kg and the equally high frequency at 15 mg/kg suggest a very narrow BCNU dose range, over which the therapeutic benefits of ATase gene transfer could be realized. This is consistent with the known steep dose response for BCNU (44).

One might be able to enhance the protective effects of ada gene transfer by increasing the level of gene expression. Assay of ATase activity in transduced mouse peripheral blood leukocytes demonstrated a relatively low level of ada expression (Fig. 9). The pattern of ada expression in these cells is probably different from that in the rapidly proliferating stem cells from which they were derived and that are the presumed target for BCNU. The present data suggest that the erythroid progenitor cells from which RBCs and platelets are derived express a higher level of ada ATase, because it was in these lineages that protection was evident. Because the blood was taken for ATase assay 21 weeks after transplantation, it is also possible that the measured levels of ada expressed underrepresent the level of expression at the time of drug treatment at weeks 5 and 11 after reconstitution.

The question also arises as to whether the bacterial ada ATase, with no nuclear localization signal, is able to accumulate in the cell nucleus (19). Its ability to protect mammalian cells in vitro against DNA damage (16–20) suggests that at least some of the protein reaches the nucleus, but it is unknown how much ATase activity measured in these cells is available for DNA repair in the nucleus. The human ATase also lacks a recognizable nuclear localization signal, yet it is localized there (45–47), possibly as a result of its DNA binding properties. To address these issues in future studies, we plan to use a mutant human ATase that resists inhibition by O'-BG (48).

We have demonstrated that ada gene transfer to murine hematopoietic cells confers protection against the toxic effects of O'-BG plus BCNU, both in vitro and in vivo. Such selective protection of the bone marrow while retaining the capacity for sensitizing tumor cells to BCNU with O'-BG provides a strategy for increasing the therapeutic index for BCNU.
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