

# Generation of Dual Resistance to 4-Hydroperoxycyclophosphamide and Methotrexate by Retroviral Transfer of the Human Aldehyde Dehydrogenase Class 1 Gene and a Mutated Dihydrofolate Reductase Gene

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The genetic transfer of drug resistance to hematopoietic cells is an attractive approach to overcoming myelosuppression caused by high-dose chemotherapy. Because cyclophosphamide (CTX) and methotrexate (MTX) are commonly used non-cross-resistant drugs, generation of dual drug resistance in hematopoietic cells that allows dose intensification may increase anti-tumor effects and circumvent the emergence of drug-resistant tumors. We constructed a retroviral vector containing both a human cytosolic ALDH-1 cDNA and a human doubly mutated DHFR cDNA (Phe22/Ser31; termed F/S in the description of constructs) to generate increased resistance to both CTX and MTX. Infection of NIH3T3 cells resulted in increased resistance to both 4-hydroperoxycyclophosphamide (4HC) ( $1.9 \pm 0.1$ -fold) and MTX ( $73 \pm 2.8$ -fold). Transduced human CD34<sup>+</sup> enriched hematopoietic progenitor cells were also resistant to both 4HC and MTX by CFU-GM readout. Lethally irradiated mice transplanted with SFG-ALDH-IRES-F/S or mock-transduced bone marrow cells were treated with high-dose pulse CTX or high-dose CTX/MTX. Animals receiving marrow not transduced with ALDH-1 or mutated DHFR cDNA died from CTX or CTX/MTX toxicity, whereas mice transduced with ALDH-1 and mutated DHFR cDNA-containing marrow were able to tolerate the same doses of CTX or CTX/MTX treatment posttransplant. These data taken together indicate that ALDH-1 overexpression and mutant DHFR increased both 4HC and MTX resistance *in vitro* and in the *in vivo* mouse model. This construct may be useful for protecting patients from high-dose CTX- and MTX-induced myelosuppression.

## INTRODUCTION

Transfer of drug-resistance genes into bone marrow progenitor cells may protect patients from dose-limiting drug-induced myelosuppression (1–7), thus permitting safer use of chemotherapy and even administration of higher doses of chemotherapy which may improve cure rates (8, 9). Cyclophosphamide (CTX) and methotrexate

(MTX) are commonly used chemotherapy agents, e.g., in breast cancer and lymphoma patients. CTX, an alkylating agent, is known to demonstrate a steep dose-response relationship and modest increases in dose may result in improved anti-tumor effects. Mutated DHFR enzymes with inhibition weakened by MTX and trimetrexate (TMTX) confer resistance to these folate analogs in mouse marrow transplantation models and cell line studies (11–15), as well as in CD34<sup>+</sup> selected human hematopoietic cells (16).

Cytosolic aldehyde dehydrogenase (ALDH-1), a member of the aldehyde dehydrogenase family of enzymes, has been shown to be associated with CTX resistance in a

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L1210 leukemia cell line (17). Overexpression of the human ALDH-1 gene by retroviral gene transfer in human hematopoietic cells conferred resistance to maphosphamide (an active analog of CTX) (18). ALDH-1 is the only known isozyme of ALDH that is expressed in early marrow progenitor cells and its level decreases with hematopoietic differentiation (19). This fact suggests that ALDH-1 protects very early progenitor cells from CTX toxicity, but not more committed progenitors, an observation that is supported clinically with the known hematologic effects of this drug. Therefore, ALDH-1 transduction may preferentially protect more committed progenitor cells, i.e., CFU-GM, from CTX myelotoxicity.

The purpose of this study was to generate increased resistance of human CD34<sup>+</sup> cells to both CTX and MTX with the ultimate goal of allowing dose-intensive treatment with these drugs with less myelosuppression in patients. In this paper, we report on the construction of a SFG-based dicistronic vector containing human ALDH-1 cDNA and human doubly mutated DHFR Phe22/Ser31 (F/S) cDNA. In this study we show that coexpression of ALDH-1 and F/S DHFR in transduced NIH3T3 cells and human CD34<sup>+</sup> cells conferred resistance to both 4-hydroperoxycyclophosphamide (4HC) and MTX. Mice transplanted with bone marrow transduced with the F/S DHFR and ALDH cDNA tolerated doses of either CTX or CTX/MTX lethal to mock-transduced animals.

## METHODS

**Cells.** NIH3T3 cells were cultured in Dulbecco's modified Eagle-high glucose (4.5 g/L) (DME-HG) medium supplemented with 10% fetal bovine serum (FBS). The viral packaging cell line GP-envAM12 line, an amphotropic line, was cultured in DME-HG as described previously (20).

**Construction of retrovirus vectors containing human ALDH-1 cDNA.** The human liver cytosolic ALDH-1 cDNA in the PT7-7 vector (21) (provided by Dr. Henry Weiner, Purdue University, West Lafayette, IN) was digested with *NcoI* (876 bp downstream of the ATG start codon) and *BamHI* (multiple cloning site in PT7-7) and this 3' partial fragment was subcloned into the *NcoI/BamHI* sites of a modified SFG vector. This SFG vector contains the *NheI/SacI* myeloproliferative sarcoma virus (MPSV) LTR sequence which was subcloned into the corresponding sites of the Moloney murine leukemia virus 3' LTR of the SFG vector (14). The expression of the gene of interest is driven by the MPSV LTR promoter. To subclone the rest of the ALDH-1 cDNA containing the 5' part, a *NcoI* site at the ALDH-1 cDNA 5' end was created by using a primer that changes the second amino acid serine (TCA) to alanine (GCA) in the ALDH-1 cDNA. This primer was used to amplify the first 900 bp of sequence of ALDH-1 cDNA: ALDH *NcoI*, 5'-GACGCAGGCAATGCCATGGCATCCTCAGGCACGCCAGAC. The 3' primer had the following sequence: ALDH 898, 5'-CGATCAGTTATCCACCCTCTTCAGATTGCTTTCCCGGC.

Polymerase chain reaction (PCR) conditions were 94°C for 5 min followed by 94°C for 30 s, 60°C for 30 s, and then 72°C for 30 s, for 35 cycles using the Thermocycler 9600 (Perkin-Elmer Cetus, Thousand Oaks, CA). After amplification by PCR, the product was electrophoresed on an agarose gel to confirm the size of the product. The product was then digested with *NcoI* to obtain the fragment with *NcoI* at both ends. This fragment was subcloned into the SFG vector containing the 3' partial sequence of ALDH-1 cDNA to construct the SFG-ALDH vector.

SFG-ALDH-IRES-Neo was constructed by excising the internal ribosomal entry site (IRES) and neomycin phosphotransferase (Neo) cDNA from a SFG-F/S-IRES-Neo vector (14) with *BamHI* digestion. The *BamHI* fragment was then inserted into the *BamHI* site of the SFG-ALDH vector (Fig. 1a). SFG-ALDH-IRES-F/S was constructed by the following procedure: the IRES and double-mutant DHFR cDNA (F/S) was subcloned into pBluescript SKII

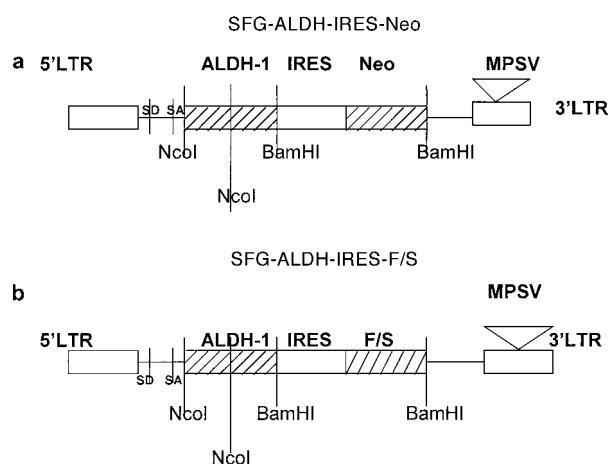


FIG. 1. (a and b) The structure of the SFG-ALDH-IRES-Neo and SFG-ALDH-IRES-F/S retroviral vectors. The chimeric 3' long terminal repeat (LTR) contains the myeloproliferative sarcoma virus (MPSV) promoter/enhancer. Spliced donor (SD) and splice acceptor (SA) sites are located between the 5' LTR and the *NcoI* site. The unique restriction sites used for cloning are shown.

(Stratagene, La Jolla, CA), as pBlue-IRES-F/S. The IRES-F/S fragment was released by digestion with *BamHI* and then cloned into the *BamHI* site of SFG-ALDH vector to generate SFG-ALDH-IRES-F/S (Fig. 1b).

**Generation of high-titer amphotropic retrovirus producer cells.** Plasmid vectors containing human ALDH-1 cDNA and Neo or mutated DHFR (F/S) were transfected into GP-envAM12 cells using the DOTAP reagent (Boehringer Mannheim, Indianapolis, IN). The vector containing human ALDH-1 cDNA and mutated DHFR (F/S) was cotransfected with the pSV2 Neo plasmid at a molar ratio of 10 to 1 (22). Transfected cells were selected with G418 (750  $\mu\text{g/ml}$ ) for 14 days. The selection of the best producer clones containing retroviral vector SFG-ALDH-IRES-F/S was carried out by measuring the viral titer using NIH3T3 cells as targets and selection with MTX (150 nM) in dialyzed FBS for 14 days, while the SFG-ALDH-IRES-Neo titer was measured using G418 selection (750  $\mu\text{g/ml}$ ) for 14 days. Dialyzed FBS was used to measure viral titer with MTX to minimize thymidine salvage. The virus-containing supernatant used in this experiment was harvested as follows: the day before the harvest when the producer cells became 80% confluent (in a 100-mm culture dish), 10 ml fresh medium was added. The viral supernatant was harvested 24 h later and centrifuged at 1300g for 20 min to spin down the cell debris, and the supernatant was carefully aspirated. The supernatant was immediately stored at  $-70^\circ\text{C}$  until use. Titters of frozen supernatant stocks were approximately  $1-2 \times 10^6$  colony-forming units/ml for each vector. These producer cell lines were free of replication-competent retrovirus as demonstrated by the failure of MTX or G418 resistance to be transferred from virally transduced NIH3T3 cells to nontransduced NIH3T3 cells as described by Persons *et al.* (23). In brief, 10 ml of supernatant from producer cells was used to transduce  $1 \times 10^5$  NIH3T3 which were passaged for 3 weeks without selection. Ten milliliters of conditioned medium from these cells was then used to transduce  $1 \times 10^5$  nontransduced NIH3T3 cells, and 48 h later selection was applied with 150 nM MTX or 750  $\mu\text{g/ml}$  G418 in DME-HG with 10% FBS. No MTX- or G418-resistant colonies were observed.

**Infection of NIH3T3 cells.** NIH3T3 cells were exposed to virus-containing supernatant for 6 h in the presence of 8  $\mu\text{g/ml}$  Polybrene. After 6 h, fresh medium was added to dilute the supernatant, then at 24 h, the medium was changed, and cells were selected with G418 (750  $\mu\text{g/ml}$ ) for 7 days or TMTX (20 nM) for 3 days, for the vectors containing Neo gene and mutated DHFR (F/S) gene, respectively. This concentration of TMTX is 100% lethal to uninfected 3T3 cells but is not inhibitory to infected cells. Moreover, this 3-day selection does not change the  $\text{IC}_{50}$  of infected cells (24). After selection using either G418 or TMTX, the infected NIH3T3 cells were harvested and used for sodium 3'-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cytotoxicity assays, molecular analysis, and enzyme analysis.

**Protection of cells infected with retroviral vectors containing human ALDH-1 and mutated DHFR (F/S) from 4HC and MTX toxicity.** 4HC was stored in powder form at  $-70^{\circ}\text{C}$ . A 1 mg/ml solution was prepared with distilled water immediately before use. MTX was prepared from a stock solution ( $10^{-2}$  M) stored at  $-70^{\circ}\text{C}$ . 4HC and MTX cytotoxicity was measured by a colorimetric assay using XTT, as described previously (25). The cells infected with viral supernatant and selected as described (vide supra) were plated in 96-well plates, 400 cells per well, and then exposed to increasing doses of 4HC the following day. Dialyzed FBS was used to test MTX cytotoxicity. After 5 days of exposure to drug (either MTX or 4HC), 50  $\mu\text{l}$  of XTT at a concentration of 1 mg/ml was added together with phenazine methosulfate. After a 4-h incubation at  $37^{\circ}\text{C}$ , the OD at 450 nm was measured using a 96-well plate reader. The absorbance was plotted with wells without drugs (with cells) as 100% and wells without cells as 0%.

**RNA analysis.** Total cellular RNA was prepared using the guanidinium thiocyanate method using the reagent Ultraspec RNA (Biotex, Houston, TX) (26). cDNA was synthesized from 5  $\mu\text{g}$  of RNA per sample by the random primer method, using a cDNA Cycle kit for RT-PCR (Invitrogen, San Diego, CA), as per the manufacturer's instructions. The DNA was aliquoted into 5 or 10  $\mu\text{l}$  per sample and amplified by PCR for detection of human ALDH-1 cDNA or 36B4 acidic ribosomal phosphoprotein cDNA (27) as an internal control.  $^{32}\text{P}$ -labeled dCTP was added to each reaction. PCR primers were as follows: ALDH866, 5'-GCCTAGCTAGCGAAGCTCGCGGAAAAGCAATCTGAAG; ALDH BamHI, 5'-GCGGTCTGTCAAGGATCCTTTATGAGTTCCTCTGAGAGAT; 36B4 A, 5'-GGCCGAATTCTGCTGTGGAGACGGATTACACC; 36B4 B, 5'-GGCCGATCCGACTCTCTTGGCTTCAACCTTAG.

PCR conditions were  $94^{\circ}\text{C}$  for 5 min in the denaturing period, then  $94^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min for 40 cycles. After PCR, the products were run on an agarose gel with the molecular markers to confirm the size of the product. Separate aliquots of the same products were also electrophoresed on a polyacrylamide gel and the bands visualized by autoradiography.

For Northern blot analysis, 10  $\mu\text{g}/\text{ml}$  RNA was electrophoresed in an agarose gel with Mops buffer and then transferred to a nitrocellulose filter, dried, UV crosslinked, and hybridized to  $^{32}\text{P}$ -labeled human ALDH-1 cDNA or 36B4 acidic ribosomal phosphoprotein cDNA probes and exposed to X-ray films (28). 36B4 normalized content of mRNA level in each lane was compared to the control cell line using the Bio-Rad Gel Doc 1000 (Bio-Rad, Hercules, CA).

**Western blotting.** Protein lysates were prepared from cells transduced by the SFG-ALDH-IRES-F/S vector and mock-transduced cells by lysing cells in the lysis buffer described previously (29). After 20 min incubation on ice and centrifugation at 15,000g for 20 min, the supernatant fluid was recovered. Protein concentration was determined by the Bio-Rad protein determination reagent according to the manufacturer's instructions using BSA as a standard. Lysate containing 100  $\mu\text{g}$  of protein was used for electrophoresis on 12.5% SDS-PAGE. After transfer to a nitrocellulose membrane, the membrane was incubated for 2 h with a rabbit polyclonal antiserum raised against recombinantly expressed human DHFR protein or polyclonal anti-human ALDH-1 rabbit antibody followed by goat anti-rabbit secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). The protein bands were visualized on X-ray film using the enhanced chemiluminescence reagent from Amersham (Arlington Heights, IL).

**Aldehyde dehydrogenase enzyme assay.** Wild-type or transduced NIH3T3 cells ( $5-10 \times 10^7$ ) were collected and centrifuged at 350g for 5 min, resuspended in 0.5 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 0.02% bovine serum albumin, and sonicated on ice for 5 s with a 30-s interval three times. Supernatant containing cytosolic protein was collected after centrifugation at 32,500g for 20 min. ALDH enzyme activity was measured as described previously (30) using acetoaldehyde as a substrate. In brief,  $\beta$ -nicotinamide adenine dinucleotide, acetoaldehyde, potassium chloride solution, and 2-mercaptoethanol were mixed in Tris-HCl buffer (pH 8.0) and equilibrated to  $25^{\circ}\text{C}$ . The absorbance at 340 nm was monitored until constant, then enzyme was added to the test sample or buffer with BSA was added to the blank sample, and the increase in the absorbance at 340 nm was recorded for 5 min. Protein concentrations were determined by the Bio-Rad protein determination reagent (Bio-Rad) per the manufacturer's instructions using BSA as a standard.

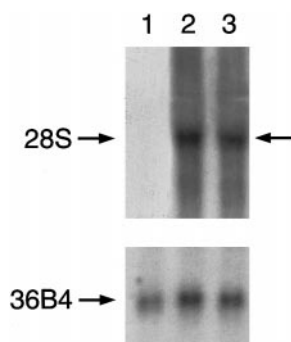
**Human CD34-positive enriched peripheral blood progenitor cell transduction and CFU-GM assay.** A CD34-positive enriched population of cells (PBPC) from donors undergoing G-CSF mobilized leukapheresis was obtained using the Ceptrate SC stem cell concentrator (CellPro, Bothell, WA) (31). CD34<sup>+</sup> enriched cells were stimulated into cycle with a cocktail containing recombinant human stem cell factor (rhSCF) 20 ng/ml (Kirin Pharmaceuticals, Tokyo, Japan), recombinant human thrombopoietin 100 ng/ml (Kirin), recombinant human FLT3-L 100 ng/ml (Immunex, Seattle, WA), recombinant human interleukin 6 (rhIL-6) 100 U/ml (Sigma, St. Louis, MO), and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) 10 ng/ml (Sigma) at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  incubator. After 2 days of stimulation at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  incubator, cells were cocultured with AM12 producer cell lines either transduced by SFG-ALDH-IRES F/S or mock transduced (32). AM12 producer cells were plated onto T25 flasks ( $1 \times 10^5$  cells/flask) the day before the transduction procedure and irradiated at 1500 cGy the following day. Prestimulated hematopoietic cells were collected, added onto each flask ( $1 \times 10^5$  cells/flask), and incubated for another 3 days in the presence of cytokines described above. CD34<sup>+</sup> enriched transduced cells were then harvested, counted, and plated on  $10 \times 35$ -mm culture dishes containing 2 ml of semisolid medium that contained 1% methylcellulose, 30% thymidine phosphorylase-treated FBS, 20 ng/ml rhSCF, 20 ng/ml rhIL-6, 50 ng/ml rhIL-3, 10 ng/ml rhG-CSF, 100 ng/ml rhGM-CSF, 1  $\mu\text{M}$   $\beta$ -mercaptoethanol, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 1% L-glutamine. The cells were incubated with increasing doses of 4HC alone, MTX alone, or 4HC and MTX. The plates were kept at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  incubator, and colonies of more than 50 cells (HPP-CFU-C) were scored after 14 days. The experiment was repeated more than three times.

**CFU-GM colony PCR.** To determine the viral transduction efficiency of human CD34<sup>+</sup> enriched cells, day 14 CFU-GM colonies were analyzed by PCR done on individual colonies. Ten large colonies were harvested from SFG-ALDH-IRES-F/S-transduced colonies without drug exposure, transferred to 0.5-ml Eppendorf tubes (one tube for each colony), resuspended in PBS, and washed twice. The pellet was resuspended with Higuchi's buffer (33) containing proteinase K and incubated at  $55^{\circ}\text{C}$  for 3 h, followed by inactivation of proteinase K at  $95^{\circ}\text{C}$  for 10 min. PCR amplification was done using ALDH primers (forward, ALDHNcoI, and reverse, ALDH898, sequence described above) for transgene detection. PCR conditions were  $95^{\circ}\text{C}$  for 1 min,  $59^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 2 min for 40 cycles. Five microliters of genomic DNA was used in a total volume of 50  $\mu\text{l}$  per reaction. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Infection of mouse bone marrow cells, bone marrow transplantation, and treatment of recipients.** Bone marrow cells from BD2F1 (7- to 11-week-old male) donor mice were treated with 5-FU (150 mg/kg). After 4 days, bone marrow cells from the mice were harvested in IMDM medium, and a mononuclear cell suspension was prepared. The recovered mononuclear cells were then cocultured with the amphotropic virus-producing cell line that had been irradiated with 1500 cGy 2 h previously. Coculture was carried out as described previously (34). Nonadherent cells ( $2 \times 10^6$ ) were injected via the tail vein into lethally irradiated mice (900 cGy whole body, 24 h previously). Control mice received bone marrow cells treated in the identical fashion but incubated with the non-virus-producing AM12 packaging cell line.

Two separate experiments were performed. The drug administration schedule for the first experiment was as follows: the mock-transduced group consisting of eight animals and the ALDH-IRES-F/S vector-transduced group consisting of eight animals were treated with CTX 200 mg/kg daily for three doses (day 29, 30, and 31). The second experiment was conducted as follows: 4 weeks after transplantation, the ALDH-IRES-F/S vector-transduced (six animals) or mock-transduced (six animals) groups were treated with MTX 300 mg/kg and CTX 150 mg/kg weekly for two doses (4th and 5th weeks).

**Secondary bone marrow transplantation.** One animal from the six surviving animals that received ALDH-IRES-F/S vector-transduced marrow and had survived MTX and CTX treatment was used as donor for a secondary bone marrow transplant (BMT) into three recipients. A total of  $2 \times 10^6$  cells (per recipient) were transplanted into irradiated recipients who were treated with MTX (300 mg/kg) and CTX (150 mg/kg) days 1 and 4 after transplantation (twice a week). These animals were then sacrificed on day 11



**FIG. 2.** Northern blot analysis of transduced cell lines using a human ALDH-1 cDNA probe. RNA from nontransduced NIH3T3 cells (lane 1) and 3T3 cells transduced with SFG-ALDH-IRES-F/S (lanes 2 and 3) was probed with ALDH-1 cDNA as described under Methods. A loading control probed with 36B4 is also shown. The arrow shows a 4.6K nucleotide mRNA, consistent with the size expected for human ALDH-1 mRNA in SFG-ALDH-IRES-F/S-transduced 3T3 cells. Lanes 2 and 3 have at least a threefold increased level of mRNA compared to the control (lane 1).

posttransplant and CFU-S colonies were analyzed for the presence of the human ALDH-1 cDNA fragment by PCR as described below. Bone marrow cells from these animals were also analyzed for the presence of the human ALDH cDNA fragment by PCR.

**Genomic DNA isolation from CFU-S and bone marrow.** CFU-S colonies and bone marrow cells were incubated overnight in digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) and DNA was isolated using phenol-chloroform extraction and alcohol precipitation.

**Measurement of hematologic parameters and demonstration of proviral DNA in bone marrow cells and in CFU-S by PCR.** White blood cells (WBC), platelets, reticulocytes, weights, and survival were monitored in all groups of animals. Integration of the human ALDH-1 cDNA in bone marrow cells and CFU-S from secondary BMT recipients was evaluated by PCR amplification of a 900-bp fragment from genomic DNA using the ALDH *Nco*I and ALDH898 primers described as above. PCR analysis was carried out for 40 cycles in 50- $\mu$ l reaction mixtures containing genomic DNA prepared from mouse tissues, 1.25 mmol/L of each dNTP, 1 $\times$  PCR buffer (Perkin-Elmer Cetus), 1  $\mu$ l of each primer (300 ng/ $\mu$ l), and 0.5  $\mu$ l of *Taq* polymerase (Perkin-Elmer Cetus). PCR conditions were 95°C for 1 min, 59°C for 1 min, and 72°C for 2 min for 40 cycles. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

## RESULTS

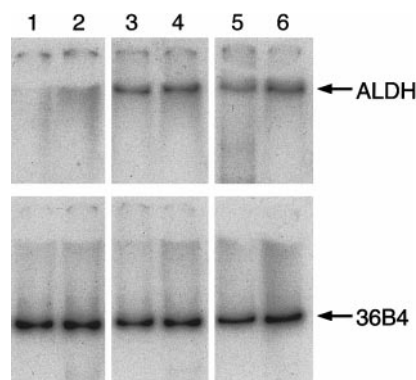
**ALDH-1 and mutated DHFR cDNA transduction and expression in NIH3T3 cells.** NIH3T3 cells were utilized prior to testing bone marrow progenitor cells because the antibodies to human ALDH-1 and DHFR preferentially recognize the human over the mouse proteins; in addition, this cell line is relatively sensitive to 4HC and MTX, and thus an increase in resistance to these drugs may be readily observed. NIH3T3 cells were transduced with the supernatants of amphotropic retrovirus carrying human ALDH-1 cDNA, and the cells were harvested and expanded. Northern blot analysis using a <sup>32</sup>P-labeled ALDH-1 probe demonstrated increased expression of the expected 4.6K nucleotide mRNA in transduced cells infected by various producer cell lines (Fig. 2). Compared to the nontransduced cell line, the ALDH-1 mRNA/36B4 ratio of the transduced cell lines was more than threefold increased

over control (lanes 2 and 3). RT-PCR analysis using human ALDH-1 cDNA coding sequence primers showed that these primers did not detect murine endogenous ALDH-1 mRNA in the mock-transduced clones, whereas the expected product of 880 bp was detected in cell lines transduced with the ALDH-IRES-Neo and ALDH-IRES-F/S constructs (Fig. 3).

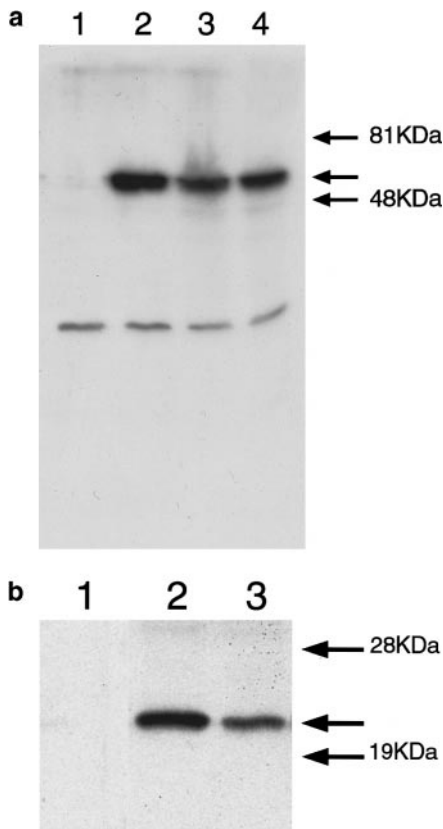
In order to demonstrate expression at the protein level, cell lysates from ALDH-IRES-Neo-, ALDH-IRES-F/S-, and mock-transduced clones were analyzed by Western blot using antibodies to human DHFR and human ALDH-1 (Figs. 4A and 4B). No endogenous DHFR and ALDH-1 protein was detected in mock-transduced NIH3T3 cells. A 55-kDa band, corresponding to the size of human ALDH-1 protein, was detected in cell lines transduced with the ALDH-1 retroviral construct. Furthermore, a 21-kDa band, corresponding to the size of human DHFR protein, was detected in cell lines transduced with the mutated DHFR (F/S)-containing construct.

**Assay of ALDH-1 activity.** In order to show that ALDH-1 protein detected by Western blot was enzymatically active, a spectrophotometric assay was used to measure ALDH activity. The ALDH activity in NIH3T3 cells transduced with the SFG-ALDH-IRES-Neo vector, SFG-ALDH-IRES-F/S vector, and SFG-ALDH-IRES-F/S vector with 10<sup>-6</sup> M MTX selection for 3 weeks increased by 3.1  $\pm$  0.8-fold, 1.8  $\pm$  0.2-fold, and 3.0  $\pm$  0.4-fold, respectively, compared to base line activity (nontransduced cells) (Table 1).

**4HC and MTX cytotoxicity assays.** To test whether increased expression of both human ALDH-1 and mutated DHFR led to both 4HC and MTX resistance, SFG-ALDH-IRES-F/S and mock-transduced 3T3 cells were exposed to increasing doses of 4HC or MTX. The vector-transduced cell line was treated with 20 nM TMTX for 3 days after infection with viral supernatant to eliminate nontransduced cells (see Methods). TMTX-treated cells were har-



**FIG. 3.** RT-PCR using ALDH primers. RT-PCR using ALDH primers was used to confirm the presence of ALDH-1 mRNA (see Methods). Lanes 1 and 2, NIH3T3 mock-transduced cells; lanes 3 and 4, NIH3T3 cells transduced with SFG-ALDH-IRES-Neo; and lanes 5 and 6, NIH3T3 cells transduced with SFG-ALDH-IRES-F/S. Lanes 1, 3, and 5, 5  $\mu$ l of cDNA was amplified by PCR; lanes 2, 4, and 6, 10  $\mu$ l of cDNA was amplified by PCR, using ALDH primers. The bottom lanes are the loading controls, amplified by PCR using the same amount of cDNA as above, using 36B4 primers.



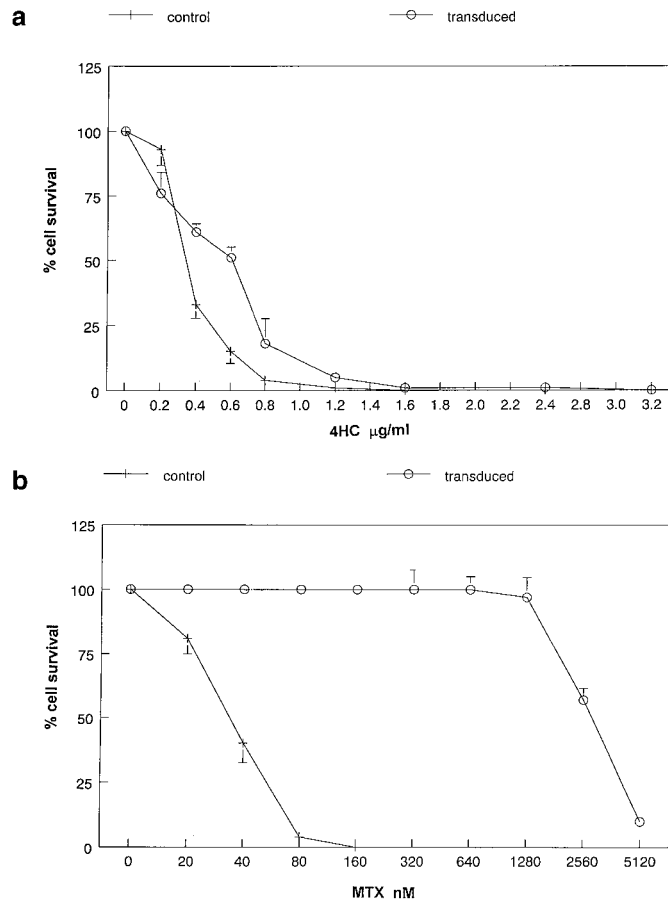
**FIG. 4.** (a) Western blot analysis for the detection of human ALDH using cytosolic extracts from NIH3T3 cells transduced with SFG-ALDH-IRES-F/S, SFG-ALDH-IRES-Neo, or mock. 100  $\mu$ g of protein was loaded onto each lane. Lane 1, mock-transduced NIH3T3; lane 2, NIH3T3 cells transduced with SFG-ALDH-IRES-Neo; lanes 3 and 4, NIH3T3 cells transduced with SFG-ALDH-IRES-F/S. The expected size of the ALDH protein was 55 kDa (see Methods for details). (b) Western blot analysis for the detection of human DHFR using cytosolic extracts from NIH3T3 cells transduced with SFG-ALDH-IRES-F/S and mock. 100  $\mu$ g of protein was loaded onto each lane. Lane 1, mock-transduced NIH3T3; lanes 2 and 3, NIH3T3 cells transduced with SFG-ALDH-IRES-F/S. The expected size of the DHFR protein was 21 kDa.

vested in bulk and expanded for the cytotoxicity studies. The transduced cell line had one gene copy/cell on average as tested by dot blot analysis using control cell lines with known DHFR gene copy number (data not shown).

**TABLE 1**  
ALDH-1 Enzyme Activity

Cell line	ALDH-1 activity (nmol/min/mg)	Ratio
3T3	0.24 $\pm$ 0.07	1
ALDH-IRES-Neo	0.75 $\pm$ 0.24	3.1 $\pm$ 0.8
ALDH-IRES-F/S	0.44 $\pm$ 0.20	1.8 $\pm$ 0.2
ALDH-IRES-F/S (10 <sup>-6</sup> M MTX)	0.73 $\pm$ 0.19	3.0 $\pm$ 0.4

*Note.* ALDH-1 enzyme activity was measured by a spectrophotometric assay (see Methods). Experiments were repeated three times. Results are shown as means  $\pm$  standard deviations.



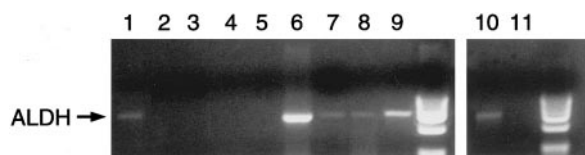
**FIG. 5.** (a) XTT colorimetric assay of the cytotoxicity of 4HC of NIH3T3 cells (control) and NIH3T3 cells transduced with SFG-ALDH-IRES-F/S (transduced). Cells were transfected and XTT assay was performed as described under Methods. The experiment was repeated three times, in triplicate each time. Results are shown with standard deviations. (b) XTT colorimetric assay of the cytotoxicity of MTX of NIH3T3 cells (control) and NIH3T3 cells transduced with SFG-ALDH-IRES-F/S (transduced). Cells were transfected and after incubation with various concentrations of MTX, the XTT assay was performed (see Methods). Dialyzed serum was used in these experiments to decrease the levels of exogenous thymidine (see Methods). Results of these experiments done in triplicate with standard deviations are shown.

**TABLE 2**  
4HC and MTX Resistance in Virally Transduced Human CD34<sup>+</sup> Cells

	Number of colonies (%)			
	No drugs	4HC [1 $\mu$ g/ml]	MTX [2 $\times$ 10 <sup>-8</sup> M]	4HC + MTX
AM12	323 (100)	0	6 (2 $\pm$ 0.5)	0 (0)
AIF/S <sup>a</sup>	313 (100)	82 (26 $\pm$ 1.5)	68 (22 $\pm$ 1.5)	65 (21 $\pm$ 1.5)

*Note.* Human CD34<sup>+</sup> enriched cells were infected by coculture with amphotropic viral producer cells (see Methods). CFU-GM colonies obtained with and without 4HC or MTX are indicated. The values are shown as means (%)  $\pm$  SD from three separate experiments.

<sup>a</sup> AIF/S represents the ALDH-IRES-F/S construct-transduced cells.

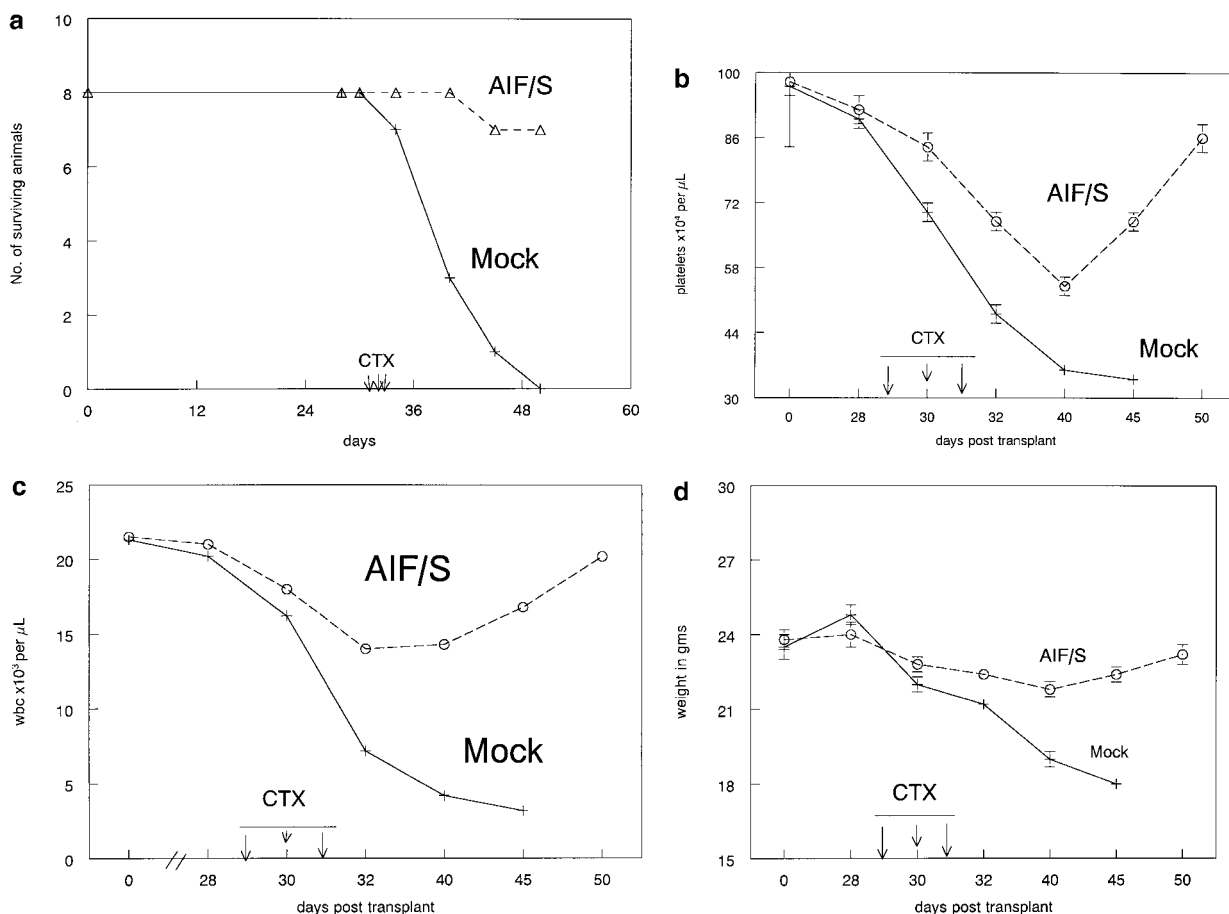


**FIG. 6.** Detection of proviral integration into human progenitor cells by PCR amplifications. Genomic DNA from day 14 CFU-GM colonies was prepared (see Methods), and detection of human ALDH-1 sequences utilized primers as described under Methods. Lanes 1 to 10 were from PCR reactions using DNA from ALDH-IRES-F/S-transduced CFU-GM colonies and the lane 11 was amplified using DNA from a mock-transduced CFU-GM colony. Samples were run with a molecular weight marker,  $\phi$ X174, as shown. Lanes 1 and 6–10 (6 of 10 colonies) showed the expected-size human ALDH cDNA products, whereas no products were seen from mock-transduced CFU-GM (10 negatives were not shown in this picture, instead the PCR from only 1 colony was shown as lane 11).

The XTT assay was used for determination of  $IC_{50}$ . In transduced cells, the  $IC_{50}$  for 4HC was  $0.6 \mu\text{g/ml}$ , a  $1.9 \pm 0.1$ -fold increase over mock-transduced cells (Fig. 5a). The  $IC_{50}$  for MTX was increased  $73 \pm 2.8$ -fold compared to mock-transduced cells (Fig. 5b). Therefore the cells

transduced by SFG-ALDH-IRES-F/S were more resistant to both 4HC and MTX than the nontransduced ones.

*Transduction of human CD34-positive enriched hematopoietic progenitor cells.* Encouraged by the results obtained in NIH3T3 cells, we infected human CD34<sup>+</sup> enriched PBPCs to determine if the SFG-ALDH-IRES-F/S retroviral construct could confer resistance to progenitors as measured by CFU-GM colony assays. SFG-ALDH-IRES-F/S transduced human CD34<sup>+</sup> cells exposed to 4HC at  $1 \mu\text{g/ml}$  for 14 days showed  $26 \pm 1.5\%$  colony survival, compared to no surviving colonies from mock-transduced CD34<sup>+</sup> cells. SFG-ALDH-IRES-F/S and mock-transduced CD34<sup>+</sup> cells incubated with  $2 \times 10^{-8}$  M MTX showed  $22 \pm 1.5$  and  $2 \pm 0.5\%$  colony survival, respectively. SFG-ALDH-IRES-F/S and mock-transduced CD34<sup>+</sup> cells exposed to both 4HC  $1 \mu\text{g/ml}$  and MTX  $2 \times 10^{-8}$  M simultaneously showed  $21 \pm 1.5$  and  $0\%$  colony survival, respectively. Thus, the SFG-ALDH-IRES-F/S-transduced CD34<sup>+</sup> cells showed increased resistance to 4HC alone or MTX alone, as well as resistance to 4HC/MTX together (Table 2).



**FIG. 7.** Mouse bone marrow transplantation and drug treatment from experiment 1. Eight animals from each group were transplanted with mouse bone marrow cells transduced with ALDH-IRES-F/S or with mock. (a) The survival of mice after BMT followed by CTX (200 mg/kg) daily on days 29, 30, and 31. All mice receiving mock-transduced marrow died of toxicity (8 of 8), whereas 7 of 8 experimental group mice survived. (b) The effect of drug treatment on the platelet count from the same animals and (c) the effect of drug treatment on the WBC count. (d) The animal weight. All the values shown are the means  $\pm$  SD.

### Detection of proviral integration in CD34<sup>+</sup> enriched PBPCs.

Evidence for integration of the retrovirus into human CD34<sup>+</sup> PBPC DNA was detected by PCR amplification of the ALDH-1 cDNA fragment in genomic DNA isolated from 14-day CFU-GM colonies (see Methods). Ten individual colonies transduced with ALDH-IRES-F/S and 10 mock-transduced colonies were harvested and used for genomic DNA isolation. Since genes encoding the human ALDH-1 are very large and contain many introns (21), PCR primers used in this experiment were designed to amplify a portion of the ALDH-1 cDNA of 900 bp size. Detection of the 900-bp marker ALDH-1 cDNA was observed in 6 of 10 colonies from SFG-ALDH-IRES-FS-transduced cells but not in colonies from mock-transduced cells (Fig. 6), giving a transduction efficiency of approximately 60%.

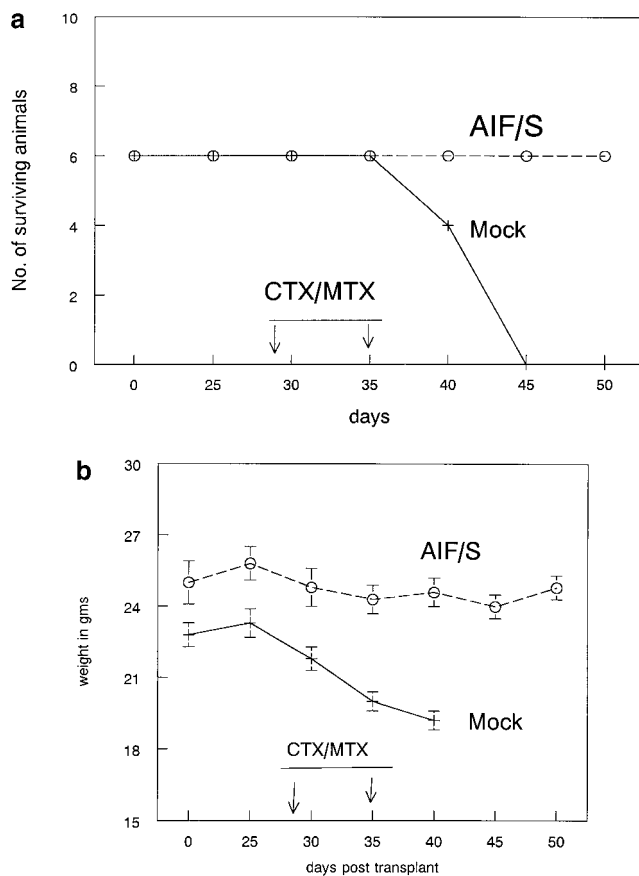
### Mouse bone marrow transplantation and drug treatment.

Two experiments were performed in mice transplanted with SFG-ALDH-IRES-F/S-transduced marrow cells. In the first experiment (Fig. 7a), in which CTX treatment was administered on days 29, 30, and 31 (200 mg/kg), seven of eight animals in the experimental group survived (followed until day 50), whereas eight of eight animals in the control group died from chemotherapy toxicity.

In experiment 2 (Fig. 8a), in which both CTX and MTX were administered (see figure legend) six of six animals in the experimental group (transfected with the SFG-ALDH-IRES-F/S vector as described under Methods) survived (followed until day 50 posttransplant), whereas all six animals in the control group (mock transduced) died.

Mice that received bone marrow cells transduced with ALDH-IRES-F/S showed similar recovery of platelets and WBC after high-dose CTX treatment (Figs. 7b and 7c) or MTX/CTX treatment (not shown), whereas the mice transplanted with mock-transduced cells did not show such recovery and succumbed due to drug-related toxicities. The body weight of animals transplanted with bone marrow cells transduced with ALDH-IRES-F/S in both experiments did not change during the drug treatment while a steady decline of body weight was observed in the animals transplanted with the mock-transduced cells (Figs. 7d and 8b).

**Secondary BMT.** Evidence for integration of the retrovirus into early hematopoietic progenitors was obtained by transplantation of marrow from mice into secondary recipients. One of the animals in the experimental group that survived was sacrificed on day 52 and the bone marrow cells obtained were used for transplantation into three lethally irradiated recipients. Following the treatment with CTX and MTX (see Methods) the secondary recipients were sacrificed on day 11 posttransplant, and genomic DNA was isolated from spleen colonies (CFU-S) and bone marrow cells. Successful amplification of the diagnostic 900-bp human ALDH fragment (11 of 18 colonies positive) indicated proviral integration in the bone marrow cells of the primary recipient (Fig. 9). DNA from the bone marrow cells from the two recipients also showed amplification of the diagnostic 900-bp human ALDH frag-



**FIG. 8.** Mouse bone marrow transplantation and drug treatment from experiment 2. Six animals from each group were transplanted with mouse bone marrow cells transduced with ALDH-IRES-F/S (○) or with mock (+). (a) Survival of mice after BMT followed by CTX (150 mg/kg) and MTX (300 mg/kg) at weeks 4 and 5. All mice receiving mock-transduced marrow died of toxicity (6 of 6), whereas mice in the experimental group survived (6 of 6). (b) The animal weight from the same animals. All the values shown are the means  $\pm$  SD. Surviving mice were sacrificed on day 50 for further analysis.

ment (Fig. 10, lanes 1 and 2 are from recipients, lane 3 is from mock-transduced bone marrow).

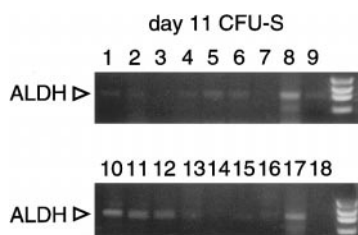
## DISCUSSION

We have shown previously that expression of the double-mutant human DHFR in mouse cells and in CD34<sup>+</sup> enriched cells protects them from MTX cytotoxicity (14). In this study we now demonstrate that expression of both human ALDH-1 and human mutated DHFR in murine fibroblast cells, and in a human CD34<sup>+</sup> enriched population of cells, protects these cells from both 4HC and MTX toxicity. Further, transduction of the retroviral construct containing both human mutated DHFR and ALDH-1 cDNA into mouse bone marrow cells protected animals from high-dose MTX and CTX toxicity. Transduction of ALDH-1 in murine and human hematopoietic progenitor cells was reported in one study to result in increased resistance to maphosphamide, an analog of CTX (18). However, ALDH-1 mRNA expression or ALDH-1 protein

expression in cell lines transduced by a retroviral vector containing human ALDH-1 cDNA could not be demonstrated by other investigators (32, 33) and was attributed to unstable ALDH-1 mRNA (34). ALDH-1 expression in K562 human leukemia cells was detected only after treatment of transduced cells with high-dose pulses of 4HC which resulted in an increase in the copy number of transduced genes (35). Our results using a SFG-based vector containing ALDH-IRES-F/S clearly demonstrated increased expression of ALDH-1 and mutant DHFR at both the mRNA and the protein level, correlating with increased resistance to both 4HC and MTX. Probably the major reason for the different results between our studies and those of other investigators is the use of different retroviral vectors. The SFG vector used in our experiment contains the MPSV promoter/enhancer in the 3' LTR, which may provide stronger expression of transgenes than the other vectors used in previous experiments. This vector contains an IRES sequence which allowed dual expression of both human ALDH-1 and mutated DHFR.

The advantages of using a retroviral vector containing both ALDH-1 and mutated DHFR rather than a vector containing only ALDH-1 or DHFR cDNA for future clinical use are that the two drugs, CTX and MTX, are used in combination frequently for treatment of breast cancer and lymphoma. Mutant forms of DHFR may also find use as *in vivo* selectable markers allowing enrichment of the transduced cells by MTX or TMTX treatment. Recent evidence of *in vivo* enrichment of mutant-DHFR-transduced marrow cells in mice due to antifolate treatment together with an inhibitor of nucleoside transport (37) further supports the use of this strategy *in vivo*.

Previous studies from this laboratory and elsewhere have demonstrated that transduction of mouse bone marrow cells with human DHFR variants protected lethally irradiated mice transplanted with these cells from high-dose MTX toxicity (11–13, 15, 32, 36). Moreover, mice bearing a murine mammary adenocarcinoma that received bone marrow transplantation after transduction with mutated-DHFR-transduced cells were able to tolerate



**FIG. 9.** Detection of proviral integration of human ALDH in CFU-S. Mice from experiment 1 that received ALDH-IRES-F/S vector-transduced marrow and survived CTX/MTX treatment (day 50) were used as donors to transplant secondary recipients. On day 11, the secondary recipients were sacrificed and DNA was isolated from CFU-S and analyzed by PCR using primers to detect human ALDH sequences (see Methods). DNA from 11 of 18 colonies showed a positive signal (indicated by arrow) for ALDH cDNA (lanes 1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 17), while lanes 3, 7, 13–16, and 18 do not show any signal. DNA size markers ( $\phi$ X174 *Hae*III digest) are in the far right lanes.



**FIG. 10.** Detection of the human ALDH cDNA by PCR amplification from bone marrow of secondary recipients. DNA from secondary-recipient bone marrow (2 animals) was examined for presence of human ALDH-1 cDNA by PCR using primers (see Methods) (lanes 1 and 2). Lane 3, negative control using mock-transduced bone marrow cells as template DNA. DNA size markers ( $\phi$ X174 *Hae*III digest) are in the far right lane. Signal is weaker in lane 1 compared to lane 2.

high doses of MTX and as a consequence showed increased survival over control tumor-bearing mice (32).

Unlike the F/S variant DHFR, which confers a high level of resistance to MTX in transduced cells, the modest level of increased expression of wt ALDH-1, a catalytic enzyme, only conferred a ca. two- to threefold resistance to 4HC in cell lines. Since CTX has a steep dose-response curve, even a twofold increase in expression of ALDH-1 resulted in generation of significant protection from CTX toxicity *in vitro* and *in vivo*. Taken together, these results with the SFG-ALDH-IRES-F/S construct point to the potential usefulness of this construct to protect patients with malignancies from the hematologic toxicity of MTX and CTX combinations.

#### ACKNOWLEDGMENTS

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#### REFERENCES

- Sorrentino, B. P., et al. (1992). Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 257: 99–103.
- Deisseroth, A., Holmes, F., Hortobagyi, G., and Champlin, R. (1996). Use of safety modified retroviruses to introduce chemotherapy resistance sequences into normal hematopoietic cells for chemoprotection during the therapy of breast cancer: A pilot trial. *Hum. Gene Ther.* 10: 401–406.
- O'Shaughnessy, J., Cowan, K., and Nienhuis, A. (1994). Retroviral mediated transfer of the human multidrug resistance gene (MDR-1) into hematopoietic stem cells during autologous transplantation after intensive chemotherapy for metastatic breast cancer. *Hum. Gene Ther.* 5: 891–911.
- Hesdorffer, C., et al. (1998). A phase I clinical trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. *J. Clin. Oncol.* 16: 165–172.
- Rahman, Z., et al. (1998). Chemotherapy immediately following autologous stem-cell transplantation in patients with advanced breast cancer. *Clin. Cancer Res.* 11: 2717–2721.
- Mompalmer, R. L., Ellipoulos, N., Bovenzi, V., Letourneau, S., Greenbaum, M., and Cournoyer, D. (1996). Resistance to cytosine arabinoside by retrovirally mediated gene transfer of human cytidine deaminase into murine fibroblast and hematopoietic cells. *Cancer Gene Ther.* 3: 331–338.
- Jelinek, J., et al. (1996). Long-term protection of hematopoiesis against the cytotoxic effects of multiple doses of nitrosourea by retrovirus-mediated expression of human O6-alkylguanine DNA-alkyltransferase. *Blood* 87: 1957–1961.
- Vahdat, L., and Antman, K. H. (1995). Dose-intensive therapy in breast cancer. In *High Dose Cancer Therapy: Pharmacology, Hematopoietins, Stem Cells* (J. O. Armitage and K. H. Antman, Eds.), 2nd ed., p. 802. Williams & Wilkins, Baltimore.
- Moskowitz, C. H., et al. (1999). Ifosfamide, carboplatin, and etoposide: A highly effective cytoreduction and peripheral-blood progenitor-cell mobilization regimen for transplant-eligible patients with non-Hodgkin's lymphoma. *J. Clin. Oncol.* 17: 3776–3785.
- Bunting, K. D., Gallipeau, J., Topham, D., Beaim, E., and Sorrentino, B. P. (1998). Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. *Blood* 92: 2269–2279.
- Corey, C. A., DeSilva, A. D., Holland, C. A., and Williams, D. A. (1990). Serial trans-

plantation of methotrexate resistant bone marrow: Protection of murine recipients from drug toxicity by progeny of transduced stem cells. *Blood* **76**: 337–343.

<sup>12</sup> Li, M. X., et al. (1994). Development of a retroviral construct containing a human mutated dihydrofolate reductase cDNA for hematopoietic stem cell transduction. *Blood* **83**: 3403–3408.

<sup>13</sup> May, C., Gunther, R., and Mclvor, R. S. (1995). Protection of mice from lethal doses of methotrexate by transplantation with transgenic marrow expressing drug-resistant dihydrofolate reductase activity. *Blood* **86**: 2439–2448.

<sup>14</sup> Ercikan-Abali, E. A., et al. (1996). Active site-directed double mutants of dihydrofolate reductase. *Cancer Res.* **56**: 4142–4145.

<sup>15</sup> Allay, J. A., et al. (1997). Sensitization of hematopoietic stem and progenitor cells to trimetrexate using nucleoside transport inhibitors. *Blood* **90**: 3546–3554.

<sup>16</sup> Flasshove, M., Banerjee, D., Mineishi, S., Li, M. X., Bertino, J. R., and Moore, M. A. S. (1995). Ex vivo expansion and selection of human CD34<sup>+</sup> peripheral blood progenitor cells after introduction of a mutated dihydrofolate reductase cDNA via retroviral gene transfer. *Blood* **85**: 566–574.

<sup>17</sup> Hilton, J. (1984). Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res.* **44**: 5156–5160.

<sup>18</sup> Magni, M., Shammah, S., Schiro, R., Mellado, W., Dalla-Favera, R., and Gianni, A. M. (1996). Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. *Blood* **87**: 1097–1103.

<sup>19</sup> Kastan, M. B., Schaffer, E., Russo, J. E., Colvin, O. M., Civin, C. L., and Hilton, J. (1990). Direct demonstration of elevated aldehyde-dehydrogenase in human hematopoietic progenitor cells. *Blood* **75**: 1947–1950.

<sup>20</sup> Markowitz, D., Goff, S., and Bank, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167**: 400–406.

<sup>21</sup> Zheng, C. F., Wang, T. T. Y., and Weiner, H. (1993). Cloning and expression of the full-length cDNAs encoding human liver class 1 and class 2 aldehyde dehydrogenase. *Alcohol Clin. Exp. Res.* **17**: 828–831.

<sup>22</sup> Riviere, I., and Sadelain, M. (1997). Methods for the construction of retroviral vectors and the generation of high-titer producers. In *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, Ed.), p. 59. Humana Press, Totowa, NJ.

<sup>23</sup> Persons, D. A., et al. (1997). 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.

<sup>24</sup> Mineishi, S., Nakahara, S., Takebe, N., Banerjee, D., Zhao, S. C., and Bertino, J. R. (1997). Co-expression of the herpes simplex virus thymidine kinase gene potentiates methotrexate resistance conferred by transfer of a mutated dihydrofolate reductase gene. *Gene Ther.* **4**: 570–576.

<sup>25</sup> Rodgers, G. H., Hatfield, S. M., and Glasbrook, A. L. (1991). An improved colorimetric assay for cell protection and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods* **142**: 257–265.

<sup>26</sup> Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162**: 156–159.

<sup>27</sup> Laborda, J. (1991). 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucleic Acid Res.* **19**: 3998.

<sup>28</sup> Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. USA* **7**: 5350–5354.

<sup>29</sup> Banerjee, D., Schnieders, B., Fu, J. Z., Adikri, D., Zhao, S. C., and Bertino, J. R. (1998). Role of E2F-1 in chemosensitivity. *Cancer Res.* **58**: 4292–4296.

<sup>30</sup> Manthey, C. L., Landkamer, G. J., and Sladek, N. E. (1990). Identification of the mouse aldehyde dehydrogenases important in aldophosphamide detoxification. *Cancer Res.* **50**: 4991–5002.

<sup>31</sup> von Kalle, C., et al. (1994). Increased gene transfer into human hematopoietic progenitor cells by extended *in vitro* exposure to a pseudotyped retroviral vector. *Blood* **84**: 2890–2897.

<sup>32</sup> Zhao, S. C., Banerjee, D., Mineishi, S., and Bertino, J. R. (1997). Post-transplant methotrexate administration leads to improved curability of mice bearing a mammary tumor transplanted with marrow transduced with a mutant human dihydrofolate reductase cDNA. *Hum. Gene Ther.* **8**: 903–909.

<sup>33</sup> Xu, L. C., Kluepfel-Stahl, S., Blanco, M., Schiffmann, R., Dunbar, C., and Karlsson, S. (1995). Growth factors and stromal support generates very efficient retroviral transduction of peripheral blood CD34<sup>+</sup> cells from a Gaucher patient. *Blood* **86**: 141–146.

<sup>34</sup> Bunting, D. K., et al. (1997). Coding region-specific destabilization of mRNA transcripts attenuates expression from retroviral vectors containing class I aldehyde dehydrogenase cDNAs. *Hum. Gene Ther.* **8**: 1531–1543.

<sup>35</sup> Moreb, J. S., Schweder, M., Gray, B., Zucali, J., and Zori, R. (1998). *In vitro* selection for K562 cells with higher retrovirally mediated copy number of aldehyde dehydrogenase class-I and higher resistance to 4-hydroperoxycyclophosphamide. *Hum. Gene Ther.* **9**: 611–619.

<sup>36</sup> Zhao, S. C., Li, M. X., Banerjee, D., Mineishi, S., Gilboa, E., and Bertino, J. R. (1994). Long term protection of recipient mice from lethal doses of methotrexate by marrow infected with a double copy vector retrovirus containing a mutant dihydrofolate reductase. *Cancer Gene Ther.* **1**: 27–33.

<sup>37</sup> Allay, J. A., et al. (1998). *In vivo* selection of retrovirally transduced hematopoietic stem cells. *Nat. Med.* **4**: 1136–1143.