Transduction of NIH 3T3 Cells with a Retrovirus Carrying Both Human MDR1 and Glutathione S-Transferase TT Produces Broad-Range Multidrug Resistance

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ABSTRACT

In these experiments, we examined the ability of a retroviral vector, pHaMSV, to encode two potential chemoprotective genes on separate transcription units. We previously described the pHaMSV vector, which includes the human MDR1 gene as a selectable marker and chemoprotective gene, plus an internal SV40 promoter for expressing a second heterologous gene along with MDR1 [M. E. Metz, D. M. Best, and S. E. Kane. Virology, 208: 634–643, 1995]. To test the ability of this vector to deliver two therapeutic genes simultaneously, the cDNA for human glutathione S-transferase TT (GSTTT, the most abundant member of the glutathione S-transferase family in human tumors) was inserted into pHaMSV, and this plasmid was transduced into ecotropic packaging cells. The resulting pHaMSV.GSTTT retrovirus, which was produced at a titer of \( 2 \times 10^6 \) colony-forming units/ml, was used to transduce NIH 3T3 cells. After initial selection in 60 ng/ml colchicine, a population of transduced cells was exposed to stepwise increasing colchicine concentrations to select for amplified expression of MDR1. As MDR1 expression increased, the expression of GSTTT increased in concert, as demonstrated by Northern analysis, Western analysis, and measurements of glutathione S-transferase activity. Transduced cells growing in 1280 ng/ml colchicine had about 3-fold higher total glutathione S-transferase activity than nontransduced cells and 2.5-fold higher activity than transduced cells growing in 60 ng/ml colchicine. Northern hybridizations demonstrated a 3–5-fold increase in both the full-length retroviral message encoding MDR1 and the subgenomic mRNA encoding GSTTT after amplification of resistance from 60 to 1280 ng/ml colchicine. The cytotoxic effects of several xenobiotics were evaluated in NIH 3T3 cells transected with MDR1 (3T3.MDR) or transduced with the MDR1-GSTTT retrovirus (3T3.GSTTT60 or 3T3.GSTTT1280) to evaluate the ability of our vector to produce a spectrum of drug resistances specific for the genes expressed. 3T3.MDR and 3T3.GSTTT1280 cells expressing equivalent levels of MDR1 had identical levels of resistance to doxorubicin or colchicine. These results suggest that GSTTT expression did not contribute to doxorubicin resistance in this model system. However, 3T3.GSTTT640 cells were about 4-fold resistant to etacrynate acid and 1-chloro-2,4-dinitrobenzene compared to cells expressing MDR1 alone, consistent with the ability of GSTTT to conjugate both of these cytotoxins. Increases in drug resistance paralleled increases in gene-specific mRNA and recombinant protein levels in all cases. Thus, our studies suggest that the amplifiable coexpression of MDR1 plus a second potentially therapeutic gene of interest is a feasible strategy for the delivery of multiple drug resistance genes to normal cells for protection against the toxic side effects of combination chemotherapy.

INTRODUCTION

Despite the availability of hematopoietic growth factors, hematological toxicities associated with combination chemotherapy remain a significant therapeutic challenge to the application of dose-intensive treatment regimens (1, 2), including those that utilize autologous bone marrow or peripheral blood progenitor cell support (3). Considering the importance of dose in the therapeutic outcome of most chemotherapy programs (4, 5), clinically applicable strategies to diminish the toxicity of cancer chemotherapy for the bone marrow beyond those possible by transplantation or treatment with bone marrow cytokines could substantially enhance the therapeutic index of currently available cytotoxic agents.

Several recent studies have focused on using the MDR1 gene to protect hematopoietic stem cells (6, 7). The human MDR1 gene, which encodes the P-glycoprotein multidrug transporter, confers resistance to a wide variety of structurally dissimilar chemotherapeutic agents including taxol, etoposide, and the anthracycline antibiotics (8). Whereas recent experiments have demonstrated low levels of P-glycoprotein in some populations of early bone marrow progenitors (9), this degree of expression does not persist in more highly committed cells and is insufficient to protect most hematopoietic precursors from drug-induced toxicity. Studies with MDR1-transgenic mice (that constitutively express high levels of MDR1 in bone marrow elements) and mice reconstituted with MDR1-transduced bone marrow support the notion that MDR1 expression can protect mice from the myelosuppressive side effects of drugs participating in the multidrug resistance phenotype (7, 10, 11). A similar approach has proved successful in studies of the dihydrofolate reductase gene, which has been shown to confer resistance to methotrexate after retroviral transduction into murine bone marrow (12, 13).

Murine bone marrow reconstitution experiments suggest further that it is possible to select for MDR1 in vivo to enrich for a minor population of stem cells transduced with MDR1 within a background of nontransduced cells (10, 11). These findings are consistent with the activity of MDR1 in vivo as a selectable and amplifiable marker in gene transfection experiments (14, 15), and suggest that MDR1 might also be useful as a selectable marker in the clinic for the expression of genes with potential therapeutic use that are not themselves selectable. Because there are many classes of chemotherapeutic agents that are not substrates for the P-glycoprotein efflux pump (including the alkylating agents and the antimitotoblasts), an important expansion of studies with MDR1 in the bone marrow would be the combination of one or more drug resistance genes with MDR1 for the protection of hematopoietic precursors against the toxic effects of several families of anticancer drugs.

One such class of drug resistance genes is the glutathione transferase family (16). Members of this family of enzymes (including the \( \pi \), \( \alpha \), and \( \mu \) isoenzymes) are found ubiquitously in human tumor cells (17) and have been shown to contribute to the resistance of these cells to a variety of xenobiotics as well as commonly used chemotherapeutic agents (18, 19). Thus, in the experiments presented here, we chose to evaluate the role of GST\( \pi \), the most abundant glutathione transferase in human tumors, in concert with MDR1 in producing xenobiotic resistance.

Whether for chemoprotection with multiple drug resistance genes or for the coexpression of selectable and nonselectable therapeutic genes, the abbreviations used are: GST\( \pi \), glutathione S-transferase \( \pi \); MDR, multidrug resistance; \( \pi \), nucleotide; LTR, long terminal repeat; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid.
it is necessary to package *MDR1* with other gene(s) of interest into a single delivery vehicle suitable for gene therapy. In early experiments with *MDR1*, this was accomplished by creating a retrovirus encoding a fusion gene between *MDR1* and the *ADA* gene (20). Cells transduced in culture with the MDR-ADA virus express a chimeric gene product that is localized to the plasma membrane, confers multidrug resistance, and has *ADA* activity. This retrovirus has the potential for selection in vivo (by virtue of *MDR1* expression) as well as the therapeutic potential to correct the metabolic defect caused by *ADA* deficiency. However, the use of a fusion gene including *MDR1* prevents independent regulation of the two genes and might dictate the colocalization of the fused gene products to the plasma membrane, potentially limiting the function of the non-*MDR1* gene product. We recently reported a modified HaMSV-based retroviral vector, pHaMSV, which encodes *MDR1* plus a second, heterologous gene regulated by an internal SV40 promoter (21). Using the human *MDR1* and GST\(\pi\) genes inserted into pHaMSV, we now demonstrate the simultaneous transfer, expression, and activity of two drug resistance genes in NIH 3T3 cells. These studies suggest that the coexpression of *MDR1* with a second therapeutic gene of interest is a feasible strategy for a variety of approaches to the gene therapy of cancer in the clinic.

**MATERIALS AND METHODS**

**Cell Lines.** NIH 3T3 cells were maintained in DMEM containing 10% bovine serum (Irvine Scientific, Irvine, CA), 5 mm glutamine, 50 units/ml of penicillin, and 50 \(\mu\)g/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The 3T3.MDR cell line, which has been described previously (15), was developed by transfection of NIH 3T3 cells with the *MDR1* gene; these cells were maintained in tissue culture medium containing either 640 or 1280 ng/ml of colchicine (Sigma Chemical Co., St. Louis, MO).

**Construction of pHaMSV.GST\(\pi\).** Vector pHaMSV.GST\(\pi\) was derived from pHaMSV, the construction of which is described elsewhere (21). Cloning of the human GST\(\pi\) cDNA has been described previously (22). To obtain an appropriate fragment of GST\(\pi\) for cloning into pHaMSV, PCR was performed on the GST\(\pi\) template using manufacturer’s recommended reaction conditions for Taq polymerase (Perkin Elmer Cetus), 200 \(\mu\)M of each dNTP. 

**PCR:**

1. **Amplification:** Five nanograms of GST\(\pi\) cDNA was used as the template for PCR, and the reaction was carried out using the following cycling parameters: 30 cycles at 94°C (1 min), 42°C (1 min), and 72°C (30 sec) for each cycle. The upstream PCR primer spanned nt 6 - 6, and the downstream primer was complementary to nt 673 - 684 just upstream of the GST\(\pi\) polycladenylation signal (numbering according to Kano et al. (22)). SalI restriction sites were included at the 5' ends of each of these primers. The GST\(\pi\) PCR product was digested with SalI, purified, and ligated into the SalI site of pHaMSV. The resulting plasmid, designated pHaMSV.GST\(\pi\), is shown in Fig. 1.

**Retrovirus Production and Transduction of NIH 3T3 Cells.** Plasmid pHaMSV.GST\(\pi\) was transfected into GP + E86 ecotropic packaging cells that were obtained from Dr. A. Bank (23). Cells were selected in the presence of 20 ng/ml colchicine; drug-resistant cells were pooled and grown to 80% confluence. Fresh medium lacking colchicine was added to the cells, and virus was collected after 20-24 h. Virus titer, assayed on NIH 3T3 cells, was determined to be 2 \(\times\) 10\(^6\) colony-forming units/ml. For transduction of NIH 3T3 cells, 5 \(\times\) 10\(^5\) cells were plated in 35-mm dishes on day 0. On day 1, 1 ml of virus was added to the cells in the presence of 1 \(\mu\)g of polybrene. On day 3, 60 ng/ml colchicine was added to select for transduced cells. Colchicine-resistant cells were pooled, and drug resistance was amplified by growing cells in increasing concentrations of colchicine as described previously (14, 15). Amplification was continued until cells were growing in 640 or 1280 ng/ml of colchicine.

**Northern Analysis.** For Northern analysis, total RNA was extracted from cell lines with RNAzol B (Cinna/Biotexc Laboratories International) according to manufacturer’s instructions. Ten \(\mu\)g of each RNA were loaded onto duplicate lanes of a 1% agarose-formaldehyde gel; separated RNAs were transferred to a Hybond N+ nylon membrane (Amersham). Filters were hybridized with either an *MDR1*-specific probe spanning nt 191-2188 (where +1 is at the initiating ATO) or the full-length GST\(\pi\)-specific SalI fragment from pHaMSV.GST\(\pi\). Hybridizations were performed in Rapid-hyb buffer (Amersham) at 65°C overnight using 2 \(\times\) 10\(^5\) cpm/ml of random primer-labeled probe. Filters were washed according to a standard protocol with the following washes at 0.1 \(\times\) SSC plus 0.1% SDS (1 \(\times\) SSC is 150 mm NaCl-15 mm sodium citrate, pH 7.0) at 65°C for 20 min. Dried filters were exposed to XAR-5 film at -80°C with an intensifying screen. For quantification, filters were exposed to a Molecular Dynamics PhosphorImager screen for 72 h and analyzed with the Image Quant software. Band intensities were normalized to the intensities of the endogenous GST\(\pi\) mRNAs in each cell line to control for different amounts of RNA loaded in the gel lanes.

**Flow Cytometry.** Flow cytometry was performed on live cells as described elsewhere (21). Primary antibody was mAb 4E3 (Signet Laboratories, Inc.; Ref. 24), which detects an external epitope of the *MDR1* gene product. Secondary antibody was fluorescein-conjugated goat anti-mouse IgG (H + L) F(ab')\(_2\) (Jackson ImmunoResearch).

**Western Analysis.** Cells were lysed in 50 mm Tris (pH 8.0)-5 mm EDTA-150 mm NaCl-0.5% NP40-1 mm phenylmethylsulfonyl fluoride by sonication on ice. Protein concentrations were determined using the BioRad DC protein assay. One hundred \(\mu\)g of protein from each lysate were run on 12% SDS-PAGE then electrobotted onto Immobilon P polyvinylidene difluoride membrane (Millipore). The filter was blocked overnight with 10% Carnation dried milk in PBS containing 0.1% Tween 20 and then incubated for 1 h with a GST\(\pi\)-specific polyclonal antibody (BioGenex) diluted 1:100 in PBS, 0.1% Tween 20, and 1% BSA. Hybridized antibody was visualized using protein A-peroxidase (Boehringer Mannheim) and the enhanced chemiluminescence Western detection system from Amersham. As a loading control, filters were stripped and subsequently reacted with mAb directed against actin (data not shown). Hybridization signals were quantified using a GS-250 Molecular Imager (BioRad) and PhosphorAnalyst/PC software.

**Enzyme Activities in Transduced Cells.** Enzyme activities were determined from cytosolic preparations of NIH 3T3 cells, 3T3.MDR cells, and a population of NIH 3T3 cells transduced with retroviral supernatants, selected for colchicine resistance, and grown to 1280 ng/ml colchicine resistance. Glutathione and glutathione reductase were purchased from Sigma; bovine liver catalase (devoid of superoxide dismutase activity) was obtained from Boehringer Mannheim (Indianapolis, IN). To determine enzymatic activities, cells in logarithmic phase growth were harvested with trypsin-EDTA typically 4 days after plating. Approximately 6 \(\times\) 10\(^5\) cells were harvested for each experiment. The cells were washed twice in PBS (pH 7.4), resuspended in 1 ml of 0.25 m sucrose buffer containing 5 mm HEPES (pH 7.4), and then disrupted at 4°C with 20–25 bursts of 1–2 s each with a Branson ultrasonicator. The cytosolic fraction of the cell was separated by centrifugation of the sonicates for 20 min at 100,000 \(\times\) g at 4°C. The resulting supernatants were assayed for enzymatic activity. GST, glutathione peroxidase (with hydrogen peroxide as substrate), glutathione reductase, glucose-6-phosphate dehydrogenase, and catalase activities were determined by established methods as described previously (25). Glutathione levels were determined as total nonprotein thiols using \(\sim\)1 \(\times\) 10\(^6\) cells as reported previously (26, 27). Protein concentrations of the cytosols were determined by the method of Lowry et al. (28).

**Cytotoxicity Assays.** Clonogenic cytotoxicity assays were performed using logarhythmically growing cells that had been taken out of colchicine for at least one passage; the drug of interest was added to tissue culture flasks for 1 h. Colchicine, CDNB, and EA were obtained from Sigma. Doxorubicin was purchased from Adria Laboratories (Columbus, OH), cisplatinum was from Bristol-Myers Squibb Co. (Princeton, NJ), and chlorambucil was obtained from Burroughs Wellcome Co. (Research Triangle Park, NC). Clinical formulations were used for all cancer chemotherapeutic agents. Cells were harvested with trypsin-EDTA, washed with PBS, and 2500 cells were plated in 60 \(\times\) 15-mm dishes, in triplicate. Colonies of >50 cells were counted after incubation for 8–10 days at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The percent clonogenic survival has been expressed as the number of colonies produced by drug-treated cells divided by the number of colonies produced by untreated control cells \(\times\) 100. Assays were performed on nontransduced NIH 3T3 cells, on 3T3.MDR cells carrying only the *MDR1* gene, and on 3T3.GST640 or 3T3.GST1280 cells carrying transduced *MDR1* plus GST\(\pi\) genes. 3T3.GST1280 cells expressed about 45% more functional *MDR1* mRNA than 3T3.GST640 cells, and the two cell lines had similar GST\(\pi\) mRNA and protein levels. The doubling times and plating efficiencies (15–25%) of these cell lines did not differ significantly.

**Statistical Analyses.** Cytotoxicity assays were analyzed using Student’s two-tailed \(t\) test for independent means; significance required \(P < 0.05\) (29).
RESULTS

Retroviral Transduction of NIH 3T3 Cells. A map of the retroviral vector used in this study is shown in Fig. 1. Briefly, the vector contains LTRs and a viral packaging signal derived from Harvey murine sarcoma virus (30); the human MDRI cDNA is under the control of the upstream LTR, and the SV40 early promoter and enhancer drive the expression of the human GSTπ cDNA. The plasmid is designated pHaMASV.GSTπ. The pHaMASV.GSTπ plasmid was used to produce ecotropic retrovirus as described in “Materials and Methods.” Virus was used to transduce NIH 3T3 cells at a multiplicity of infection of 1, and transduced cells were selected with 60 ng/ml of colchicine to select for cells expressing the MDRI gene. A pooled population of drug resistant cells was grown in stepwise increasing concentrations of colchicine, up to 640 or 1280 ng/ml, to select for amplified MDRI expression (14, 15).

To measure the extent of amplified MDRI expression in transduced cells, we used flow cytometry to detect P-glycoprotein on the cell surface of those cells. Results are shown in Fig. 2. P-glycoprotein expression increased with increasing selective pressure up to 640 ng/ml of colchicine, with an additional slight increase at 1280 ng/ml. Furthermore, the level of P-glycoprotein on 3T3.GST1280 cells was comparable to that on control 3T3.MDR cells that had been transfected previously with just the MDRI gene and grown in 1280 ng/ml colchicine (15).

Enzyme Activities and Gene Expression in Transduced Cells. To determine whether the GSTπ gene was transduced along with MDRI, we measured total GST activity in control and transduced NIH 3T3 cells as well as in the 3T3.MDR line. The results are shown in Fig. 3 and Table 1. The population of MDRI-GSTπ-transduced cells had about 2.5-fold higher total GST activity (assayed with CDNB) than either nontransduced NIH 3T3 cells or cells transfected with MDRI and independently selected for resistance to 1280 ng/ml of colchicine (P < 0.02). Furthermore, the level of GST increased progressively with selection in higher concentrations of colchicine (Fig. 3). Whereas the total GST activity increased in concert with the degree of colchicine resistance, the activities of other glutathione-dependent enzymes and of glutathione itself were unchanged in response to selection in colchicine, even at the highest level of colchicine resistance (Table 1). The level of glucose-6-phosphate dehydrogenase, however, was lower in cells transfected with MDRI than in control NIH 3T3 cells or in transduced cells. The explanation for this result is not clear.

To demonstrate that the elevated GST activity was derived from the transduced GSTπ gene, we performed Northern analysis on total RNA extracted from these cell lines. As illustrated in Fig. 4, with an MDRI-specific probe (see “Materials and Methods” and Fig. 1), we detected the expected full-length mRNA of about 7000 nt, transcribed from the upstream LTR and terminating in the downstream LTR (as diagrammed in Fig. 1). This message was present in all cell lines transduced with pHaMASV.GSTπ but not in nontransduced NIH 3T3 cells. With a GSTπ-specific probe and pHaMASV.GSTπ-transduced cells, we detected both the full-length message, which includes GSTπ sequences, and a subgenomic mRNA of about 1500 nt that was transcribed from the internal SV40 promoter and terminated in the downstream LTR. Endogenous GSTπ mRNA was detected as an approximately 800-nt band in nontransduced and transduced cell lines. PhosphorImager quantification of the Northern hybridization indicated that there was a 5-fold increase in the full-length message and a nearly 3-fold increase in the subgenomic message after amplification from 60 to 1280 ng/ml colchicine resistance. There was nearly 4-fold more of the transduced, subgenomic GSTπ mRNA than of endogenous GSTπ mRNA in the cell line growing in 1280 ng/ml colchicine.

Table 1 Enzyme activities in nontransduced NIH 3T3 cells and in 3T3.MDR and 3T3.GST1280 cells selected for resistance to 1280 ng/ml colchicine

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>NIH 3T3</th>
<th>3T3.MDR</th>
<th>3T3.GST1280</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase (nmol/min/mg)</td>
<td>45 ± 7</td>
<td>51 ± 9</td>
<td>115 ± 11a</td>
</tr>
<tr>
<td>Catalase (μmol/min/mg)</td>
<td>2.58 ± 0.12</td>
<td>2.34 ± 0.19</td>
<td>2.51 ± 0.23</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (μmol/min/mg)</td>
<td>53.9</td>
<td>30.7 ± 1.3</td>
<td>54.8 ± 2.9a</td>
</tr>
<tr>
<td>Glutathione peroxidase (nmol/min/mg)</td>
<td>3.8 ± 1.6</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Glutathione reductase (nmol/min/mg)</td>
<td>9.5 ± 0.5</td>
<td>12.9 ± 3.3</td>
<td>12.2 ± 2.3</td>
</tr>
<tr>
<td>Glutathione (nmol/10^6 cells)</td>
<td>7.4 ± 0.6</td>
<td>8.6 ± 0.6</td>
<td>10.9 ± 1.7</td>
</tr>
</tbody>
</table>

a P < 0.02 versus 3T3.MDR and NIH 3T3.
b P < 0.02 versus 3T3.MDR.

Table 2 Enzyme activities and gene expression in transduced NIH 3T3 cells

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>NIH 3T3</th>
<th>GST640</th>
<th>GST60</th>
<th>GST1280</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase (nmol/min/mg)</td>
<td>45 ± 7</td>
<td>51 ± 9</td>
<td>68 ± 10</td>
<td>64 ± 10</td>
</tr>
<tr>
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</tr>
</tbody>
</table>

a P < 0.02 versus 3T3.MDR and NIH 3T3.
b P < 0.02 versus 3T3.MDR.

c P < 0.02 versus NIH 3T3.
Arntws. full-length retroviral message (MDRI) and subgenomic message expressed from concept, we compared the toxicity of a variety of xenobiotics in our coexpression of two drug resistance genes produced cell lines. The goal of these experiments was to determine whether transduction and consistent with the Northern analysis, transduced cells growing at 60, 640, or 1280 ng/ml concentrations of colchicine (GST-60, GST-640, and GST-1280).

Fig. 4. Northern analysis. Northern hybridizations were performed on total RNA as described in “Materials and Methods.” Cell lines are nontransduced NIH 3T3 cells (3T3) or cells transduced with pHaM VS.GSTG virus and grown in 60, 640, or 1280 ng/ml colchicine, as labeled. Membranes were hybridized with MDRI or GST-specific probes, as indicated. Probes are described in “Materials and Methods” and shown in Fig. 1. Arrows, full-length retroviral message (MDRI) and subgenomic message expressed from the internal SV40 promoter (GST) as illustrated in Fig. 1. * position of endogenous mouse GST mRNA expressed by all four cell lines analyzed.

Relative Resistance of Transduced Cells to Xenobiotics. The goal of these experiments was to determine whether transduction and coexpression of two drug resistance genes produced cell lines that were resistant to a broad range of cytotoxic agents. To test this concept, we compared the toxicity of a variety of xenobiotics in our MDRI-GST-7 transduced cell lines (3T3.GST640 or 3T3.GST1280) to that in nontransduced NIH 3T3 cells or in 3T3.MDR cells expressing MDRI alone. When adapted to growth in 1280 ng/ml colchicine, 3T3.MDR cells and the MDRI-GST-7 transduced line expressed comparable amounts of P-glycoprotein on their cell surface (Fig. 2) and had equivalent plating efficiencies and growth rates (data not shown). As illustrated in Fig. 6 A and B, both these cell lines were ~100-fold resistant to doxorubicin and about 10-fold resistant to colchicine by comparison of IC50 versus nontransduced NIH 3T3 cells, consistent with the expression of the MDRI gene product in both lines. There was no apparent difference in the toxicity of colchicine or doxorubicin between the transfected and transduced cells (Fig. 6 A and B). Furthermore, there was no effect of enhanced GST levels on the cytotoxicity of doxorubicin or cisplatinum (Fig. 6C). These results are of interest in light of previous studies suggesting that GST overexpression contributes to doxorubicin or cisplatinum resistance in tumor cell lines selected in vitro (31–34).

On the other hand, the MDRI-GST-7 transduced line (compared to NIH 3T3 and 3T3.MDR cells) was ~4–5-fold resistant to EA or CDNB (Fig. 7 A and B). These findings are compatible with the relative levels of GST activity and GST-7 protein in these two cell lines and with the level of transduced GST mRNA compared to the endogenous message in the transduced cell lines. As shown in Fig. 7C, enhanced expression of GST-7 had no effect on the toxicity of chlorambucil.

DISCUSSION

Gene therapy with MDRI has been proposed as a means of protecting patients against myelosuppression produced by dose-intensive chemotherapy (10, 11). The feasibility of this concept has been demonstrated in transgenic mice (8) and in mice reconstituted with MDRI-transduced bone marrow cells (10, 11). Other drug resistance genes are being explored for this same purpose. In particular, a modified version of the DHFR gene product has been shown to protect transplanted mice from lethal doses of methotrexate (12, 13). These approaches aim to exploit the potential of drug resistance genes to provide genetic selection for transduced hematopoietic cells in vivo, allowing them to survive under conditions which normally destroy their nontransduced counterparts. An extension of this application would be to deliver two or more drug resistance genes simultaneously so as to protect transduced cells from a wide variety of toxic agents. In addition, it should be possible to use the in vivo selectability of MDRI to deliver a second therapeutic, but nonselectable, gene to appropriate target cells and sustain the expression of the therapeutic gene by maintaining selective pressure on transduced cells via the MDRI gene.

**MDRI Retroviruses.** Previous work with MDRI has used the pHaMDRA vector for retrovirus production (10, 11, 35). A similar vector has proven capable in DNA-mediated transfections of cultured cell lines of mediating expression and amplification of MDRI under the selective pressure of high cytotoxic drug concentrations (14, 15). MDRI retrovirus encoded by pHaMDRA has also been used in mouse bone marrow transduction and reconstitution experiments, during which MDRI-transduced marrow is protected from the myelosuppressive effects of taxol (10, 11). In addition, the vector was used to create a fusion gene encoding a chimeric, bifunctional MDRI-ADA gene product (20). Finally, we have recently described a modified, reduced-size version of pHaMDRA that is capable of encoding MDRI plus a second gene under the independent control of an internal SV40 promoter (21). In the current work, we created

![Fig. 5. Western analysis. One hundred μg of protein from whole cell lysates of NIH 3T3 cells (3T3), transfected 3T3.MDR cells growing at 1280 ng/ml colchicine (3T3.MDR), and transduced cells growing at 60, 640, or 1280 ng/ml colchicine (GST60, GST640, and GST1280, respectively) were run on 12% SDS-PAGE. Western hybridization was performed with polyclonal antibody specific for the GST-7 isozyme and visualized as described in “Materials and Methods.”](image-url)
pHaMASV.GSTp to demonstrate efficient retrovirus-mediated transduction and expression of two potentially therapeutic genes in NIH 3T3 cells.

**MDR1 and GSTp Coselection in Transduced Cells.** NIH 3T3 cells transduced with the pHaMASV.GSTp retrovirus were efficiently selected with colchicine, and drug resistance was readily amplified by exposing cells to increasing concentrations of this drug. As drug resistance increased, expression of both MDR1 and the transduced GSTp increased, as demonstrated by Northern analysis, protein (flow cytometry and Western) analysis, and enzyme activity measurements. The ability to obtain high GSTp expression by selecting for high colchicine drug resistance mimics the previously reported role of MDR1 as a selectable and amplifiable marker (14, 15). These results suggest that if MDR1 is selectable and amplifiable in vivo, it will also serve as a means for achieving elevated, long-term expression of therapeutic sequences in gene therapy.

The levels of total GST activity and GSTp expression in our transduced cells are comparable to or exceed those reported in studies using GSTp transfections to investigate mechanisms of drug resistance (18, 36), suggesting that expression from the pHaMASV.GSTp vector is good. Nevertheless, the levels of GSTp obtained are relatively modest compared with heterologous gene expression achieved in previous work with the MDR1-based transfection, selection, and amplification system (14, 15). It is possible, though speculative, that very high levels of GST activity do not carry a survival advantage and that high expressers do not survive the colchicine amplification process. It is also possible that amplification of transduced copies of MDR1 is not as efficient as amplification of transfected, tandemly integrated copies of MDR1. This issue has not yet been explored in the literature, but it could have an impact on how gene therapy with in vivo selectable markers will proceed in the future.

**Transduction of Multiple Drug Resistance Genes.** With the advent of gene therapy, it has become theoretically possible to deliver drug resistance genes to normal cells for protection against the toxic side effects of chemotherapy. The MDR1 gene can confer resistance to a wide variety of chemotherapeutic agents including taxol and etoposide, which have dose-limiting bone marrow toxicities. However, there are a number of common drugs, in particular the alkylating agents and antimitobolites, that are not substrates for transport by P-glycoprotein. Additional gene products will be needed if marrow cells are to be made resistant to a broad range of potential chemotherapeutic agents. The advantage of this approach to gene therapy is that it is not necessary for the therapeutic drug resistance genes to be routinely responsible for multidrug resistance in tumors. It is sufficient that these genes are able to act as dominant genetic factors capable of conferring resistance to the chemotherapeutic drugs of interest in normal host tissues.

We have used the human MDR1 and GSTp genes as a model for this approach and have obtained transduced cells which are simultaneously resistant to both MDR1- and GSTp-related drugs. For the MDR1-related drugs doxorubicin and colchicine, our transduced cells were ~100-fold and about 10-fold resistant, respectively, relative to nontransduced NIH 3T3 cells. Although some laboratories have found low levels of resistance to doxorubicin after transfection of GSTp into suitable host cells (19, 37), our experiments are consistent with those of Fairchild et al. (36), who failed to demonstrate alterations in doxorubicin cytotoxicity for MCF-7 human breast cancer cells overexpressing GSTp. Although GSTp levels are frequently elevated in tumor tissue, the absence of convincing data demonstrating that doxorubicin is a substrate for GSTp-mediated conjugation reactions probably explains the results presented in Fig. 64.

On the other hand, the toxicity of EA and CDNB for NIH 3T3 cells was modulated by GSTp expression (Fig. 7 A and B). Because EA and CDNB are efficiently conjugated by GSTp (38, 39), and because tumor cells selected for resistance to EA demonstrate increased GSTp mRNA at a level consistent with the degree of resistance (40), it is not surprising that our 3T3.GST640 cells were 4–5-fold resistant to EA and CDNB. Because GSTp is quite inefficient at utilizing chlorambucil as a substrate (19), the similar
cytotoxicity profile for this agent in NIH 3T3, 3T3.MDR and 3T3.GST640 cells was also expected.

Other drug resistance or drug-metabolizing genes are potential candidates for use in gene therapy. These include the peroxide and alkylating agent detoxifying genes glutathione peroxidase and GST α; antimitotilate target genes such as thymidylate synthase and DHFR; and a variety of DNA repair genes. In concert with MDR1, the selective coexpression of several drug resistance-enhancing genes in the bone marrow or other normal tissues has the potential to decrease or eliminate much of the host toxicity associated with combination chemotherapy. The experiments outlined herein provide a first step toward the attainment of that goal.

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Transduction of NIH 3T3 Cells with a Retrovirus Carrying Both Human MDR1 and Glutathione S-Transferase π Produces Broad-Range Multidrug Resistance


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