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THE USE OF RAT GLUTATHIONE *S*-TRANSFERASE A3 FOR HEMATOPOIETIC CHEMOPROTECTION FROM NITROGEN MUSTARDS IN CANCER THERAPY

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SUMMARY

The effectiveness of anti-cancer chemotherapy is limited by acute dose limiting toxicities, principally myelosuppression. The introduction of drug resistance genes into hematopoietic cells may increase the bone marrow (BM) tolerance to chemotherapy and may permit safer dose escalation, and thus increase clinical efficacy. Conferring chemoprotection to nitrogen mustards would be clinically relevant because of their broad spectrum of anti-tumor activity and their predominant, dose-limiting hematotoxicity. The glutathione *S*-transferase (GST) alpha isoenzymes, particularly the rat GSTA3, have been implicated in resistance to nitrogen mustards. To determine if retrovirus-mediated gene transfer of the rat GSTA3 (previously called GST-Yc) could be used to confer resistance to nitrogen mustards, we studied the expression of rat GSTA3 and the sensitivity to nitrogen mustards in mouse NIH 3T3 fibroblasts following either transfection or transduction of GSTA3 with a Moloney-based retrovirus vector (N2Yc). Populations of GSTA3-transduced cells and single cell-derived clones demonstrated increased glutathione (GSH) peroxidase activity (associated with the A3 subunit) and moderate *in vitro* resistance to chlorambucil and mechlorethamine. To address the feasibility of using rat GSTA3 gene transfer to confer chemoprotection to the hematopoietic system, we then transduced human leukemia K-562 cells and primary murine hematopoietic progenitor cells with the N2Yc retrovirus vector. Similarly to N2Yc-expressing fibroblasts, K-562 cells and clonogenic primary murine hematopoietic cells transduced with the N2Yc retrovirus vector demonstrated increased GSH peroxidase activity and moderate *in vitro* resistance to melphalan, chlorambucil and mechlorethamine. We next explored the possibility of conferring chemoprotection against nitrogen mustards *in vivo* following transplantation of mice with GSTA3-transduced BM cells. Unfortunately, we did not observed chemoprotection from chlorambucil in mice transplanted with N2Yc-transduced

BM, possibly because of the moderate growth advantage conferred by GSTA3 expression from our vector. To circumvent this problem, we constructed a bicistronic retrovirus vector (pMFG-GIC) combining the expression of the rat GSTA3 and the expression of a gene conferring an apparently stronger growth advantage, the human cytidine deaminase (CD). Murine fibroblasts transduced with MFG/GIC displayed resistance to nitrogen mustards and cytosine arabinoside (Ara-C) administered separately or in combination. In addition, selection of transduced cells with Ara-C or melphalan augmented their level of resistance to both classes of drugs, separately and in combination. These results clearly demonstrate the ability of retrovirus-mediated gene transfer of the rat GSTA3 to confer *in vitro* resistance to nitrogen mustards in murine fibroblasts and primary clonogenic hematopoietic cells, as well as in human leukemia cells. Moreover, these results show that retroviral transfer of MFG/GIC confers *in vitro* resistance to nitrogen mustards and cytosine nucleoside analogs, and that both Ara-C and melphalan selection can be used to increase the *in vitro* expression of a bicistronic GSTA3-CD vector.

RÉSUMÉ

L'efficacité de la chimiothérapie anti-cancéreuse est réduite par ses toxicités aiguës, principalement la myelosuppression, qui limitent les doses de médicaments pouvant être administrées aux patients. L'introduction de gènes de résistance dans les cellules hématopoïétiques permettrait potentiellement d'augmenter les doses de façon sécuritaire, et ainsi d'accroître l'efficacité de la chimiothérapie. Conférer de la chimioprotection contre les moutardes azotées seraient cliniquement pertinent en raison de leur spectre étendu d'activité anti-tumorale ainsi que de la prédominance de toxicités hématologiques. Les iso-enzymes de glutathione *S*-transférases (GST) de classe alpha, en particulier la GSTA3 du rat, ont été impliqués dans la résistance aux moutardes azotées. Afin de déterminer si le transfer de gène de la GSTA3 du rat (antérieurement nommée GST-Yc) effectué par rétrovirus pouvait être utilisé pour conférer de la résistance aux moutardes azotées, nous avons étudié l'expression de la GSTA3 du rat ainsi que la sensibilité aux moutardes azotées dans des cellules NIH 3T3 (fibroblastes de souris) après transfection ou transduction de la GSTA3 avec un vecteur rétroviral de type Moloney (N2Yc). Des populations de cellules transduites avec la GSTA3, ainsi que des clones provenant de cellules isolées, ont démontré une activité de peroxidase de glutathione (GSH) accrue (associée avec la sous-unité A3), et un niveau modéré de résistance *in vitro* au chlorambucil et au mechlorethamine. Afin d'étudier la faisabilité d'utiliser le transfer du gène de la GSTA3 du rat pour conférer de la chimioprotection au système hématopoïétique, nous avons par la suite transduit des cellules de leucémie humaine K-562 ainsi que des cellules progénitrices hématopoïétiques primaires de souris avec le vecteur rétroviral N2Yc. Tout comme les fibroblastes exprimant le vecteur N2Yc, les cellules K-562 ainsi que les cellules hématopoïétiques clonogéniques murines transduites avec le vecteur rétroviral N2Yc ont démontré une activité de peroxidase de GSH accrue et

un niveau modéré de résistance *in vitro* au melphalan, au chlorambucil et au mechlorethamine. Nous avons ensuite exploré la possibilité de conférer de la chimioprotection contre les moutardes azotées *in vivo* dans des souris transplantées avec de la moëlle osseuse transduite avec le vecteur N2Yc. Malheureusement, nous n'avons pu mettre en évidence de la chimioprotection contre le chlorambucil dans les souris transplantées avec de la moëlle transduite avec le vecteur N2Yc, possiblement à cause de l'avantage de croissance modéré conféré par l'expression de la GSTA3 avec ce vecteur. Pour contourner ce problème, nous avons construit un vecteur rétroviral bicistronique (pMFG-GIC) combinant l'expression de la GSTA3 du rat avec l'expression d'un gène conférant un avantage de croissance apparemment supérieur, la cytidine déaminase (CD) humaine. Des fibroblastes murins transduits avec le vecteur MFG-GIC démontrèrent une résistance accrue aux moutardes azotées ainsi qu'à la cytosine arabinoside (Ara-C) administrées séparément ou ensemble. De plus, la sélection des cellules transduites avec l'Ara-C ou avec le melphalan augmenta le niveau de résistance aux deux classes de médicaments, administrés séparément ou ensemble. Ces résultats démontrent clairement la capacité du transfer de la GSTA3 du rat par rétrovirus à conférer de la résistance aux moutardes azotées *in vitro* dans les fibroblastes et les cellules clonogéniques hématopoïétiques primaires de souris, ainsi que dans les cellules humaines de leucémie. D'autre part, ces résultats démontrent que le transfer rétroviral du vecteur MFG-GIC confère de la résistance aux moutardes azotées ainsi qu'aux analogues de la cytosine nucléoside *in vitro*, et que la sélection avec l'Ara-C ou avec le melphalan permet d'accroître l'expression du vecteur bicistronique GSTA3-CD *in vitro*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AFB_1	Aflatoxin B_1
AGT	O^6-alkylguanine DNA alkyltransferase
ALDH	Aldehyde dehydrogenase
Ara-C	Cytosine arabinoside
ASCT	Autologous stem cell transplant
ATP	Adenosine triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BG	O^6-benzylguanine
BM	Bone marrow
BP	Base pair
BSO	Buthionine sulphoximide
CD	Cytidine deaminase
cDNA	Complementary DNA
dCTP	Deoxycytidine triphosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EA	Ethacrynic acid
FL	Flt-3 ligand
5-FU	5-fluorouracil
G-CSF	Granulocyte colony-stimulating factor
γ-GCS	γ-glutamylcysteine synthetase
GM-CSF	Granulocyte monocyte colony-stimulating factor
GSH	Glutathione

GST	Glutathione *S*-transferase
4-HC	4-hydroperoxycyclophosphamide
HDC	High-dose chemotherapy
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IL	Interleukin
IRES	Internal ribosome entry site
kb	Kilobase
kDa	Kilodalton
LTC_4	leukotriene C_4
LTC-IC	Long-term culture-initiating cell
MDR	Multidrug resistant
MGMT	Methylguanine DNA methyltransferase
MLV	Murine leukemia virus
MRP	Multidrug resistance protein
MTX	Methotrexate
NBMPR-P	Nitrobenzylmercaptopurine riboside phosphate
NSCLC	Non-small-cell lung cancer
Pgp	P-glycoprotein
RNA	Ribonucleic acid
SCF	Stem cell factor
TMTX	Trimetrexate
TPO	Thrombopoietin
WBC	White blood cell

CONTRIBUTIONS OF AUTHORS

The published and submitted manuscripts of this thesis are as follows:

Chapter 2 Greenbaum M, Létourneau S, Assar H, Schecter RL, Batist G, Cournoyer D.
Retrovirus-mediated gene transfer of rat glutathione S-transferase Yc confers alkylating drug resistance in NIH 3T3 mouse fibroblasts.
Cancer Res. 54: 4442-4447, 1994.

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Retrovirus-mediated gene transfer of rat glutathione S-transferase Yc confers *in vitro* resistance to alkylating agents in human leukemia cells and in clonogenic mouse hematopoietic progenitor cells.
Hum. Gene Ther. 7: 831-840, 1996.

Chapter 4 Létourneau S, Palerme J-S, Delisle J-S, Beauséjour CM, Momparler RL, Cournoyer D.
Coexpression of rat glutathione S-transferase A3 and human cytidine deaminase by a bicistronic retroviral vector confers *in vitro* resistance to nitrogen mustards and cytosine arabinoside in murine fibroblasts.
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In Chapter 2, the candidate was responsible for the drug sensitivity assays and the statistical analyses. The candidate also contributed to the writing of the manuscript. M.

Greenbaum performed the construction of the plasmids, the generation of the virus-producing cell lines, the determination of the virus titers, the Southern, Northern and Western Blot analyses and the enzyme assays. H. Assar performed the RNase protection assay. Dr. RL. Schecter and Dr. G. Batist provided helpful comments and suggestions. Dr. D. Cournoyer was responsible for the writing of the manuscript.

In Chapter 3, the candidate was responsible for the transduction of cells, the Southern and Northern Blot analyses, the drug sensitivity studies, the statistical analyses and the writing of part of the manuscript. M. Greenbaum performed the Western Blot analysis, the enzyme assays, the PCR assays and participated in the writing of the manuscript. Dr. D. Cournoyer helped in the revision of the manuscript.

In Chapter 4, the candidate was responsible for all experiments and the preparation of the manuscript. J-S Palerme performed the peroxidase assays on cells selected with melphalan. J-S Delisle performed the virus titer determination. CM Beauséjour cloned the IRES-CD fragment. Dr. RL Momparler provided helpful comments and suggestions. Dr. D. Cournoyer helped in the revision of the manuscript.

In Appendix 1, the candidate was responsible for all experiments. M. Greenbaum provided technical assistance for the transduction of BM cells and for the transplantation of recipient mice.

In Appendix 2, the candidate was responsible for the construction of the plasmids, the generation of the virus-producing cell lines, the transduction of cells and the selection of transduced cells with TMTX. J-S Palerme performed the peroxidase assays, the drug

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FOREWORD

Medical oncology has had a great impact on the practice of medicine in the past two decades, as curative treatments have been identified for a number of previously fatal malignancies such as testicular cancer, lymphomas, and leukemia. New drugs have entered clinical use for diseases that were previously untreatable or only amenable to local treatment with surgery or irradiation. Today, adjuvant chemotherapy routinely follows local treatment of breast cancer, colon cancer, and rectal cancer, and high-dose chemotherapy (HDC) often is employed in patients with soft-tissue sarcomas, relapsed breast cancer and lymphoma. Drugs are now routinely used earlier in the course of the patient's management, often in conjunction with radiation or surgery, to treat malignancy when it is most curable and when the patient is best able to tolerate treatment. Despite these significant advances however, cancer remains a major medical problem. In North America, it is second only to cardiovascular disease as a cause of mortality. One in three individuals will develop cancer during his life, and one in four will die of the disease.

The effectiveness of chemotherapy is limited by the presence of drug resistance in the neoplastic cells and by the inherent toxicity of the drugs. The principal dose-limiting toxicity for many of the currently available drugs is myelosuppression. Therefore, an attractive approach to circumvent these limitations would be to use somatic gene transfer of drug-resistance genes to confer chemoprotection to the hematopoietic system. Hematopoietic chemoprotection could possibly reduce the toxicities and morbidity of chemotherapy and allow safe dose intensification that might result in improved responses.

Conferring chemoprotection to nitrogen mustards would be clinically significant. Nitrogen mustards have a broad spectrum of antineoplastic activity and maintain a dose-related effect through multiple logs of tumor cell kill. Their effectiveness, however, is reduced by their dose-limiting myelosuppression. The rat GSTA3 gene may be a good

candidate to confer chemoprotection against the hematotoxicity produced by nitrogen mustards. Overexpression of the GST alpha isoenzymes has been implicated in resistance to nitrogen mustards and the highest level of resistance demonstrated to these drugs has been obtained following gene transfer of the rat GSTA3 isoform. This thesis explores the feasibility of using retrovirus-mediated gene transfer of the rat GSTA3 to confer hematopoietic chemoprotection from nitrogen mustards.

The thesis is divided into four parts: the introduction, the manuscripts, the general discussion and the appendices. In the introduction, the antineoplastic drugs pertaining to the manuscripts are reviewed, along with GSH and the GSTs. The rationale for hematopoietic chemoprotection and HDC with autologous stem-cell support are also reviewed. The general discussion summarizes the conclusions and contains suggestions for further research and the claims to originality. The references for the introduction and the general discussion are located after the latter. Additional material pertinent to the present investigation is presented in Appendices 1 to 5.

Dedicated to my late father,

for his integrity and his meticulousness;

and to my mother,

for her unconditional love and support.

CHAPTER 1

INTRODUCTION

ANTINEOPLASTIC AGENTS

NITROGEN MUSTARDS

The prototype of the nitrogen mustards, mechlorethamine, was developed in the Second World War as part of a program to develop improved chemical warfare agents. From 1942, mechlorethamine was used in clinical trials in the treatment of leukemia, and the results were published in 1946 (Gilman and Philips 1946). Nitrogen mustards are alkylating agents, which are highly reactive chemically, that dissociate into positively charged carbonium ion intermediates. These electrophilic alkyl groups then react with electron-rich nucleophiles present in the cell to form a covalently bound monoadduct, an aziridinium ion (Hopkins, et al. 1991). The aziridinium ion, being a strong electrophile, can then undergo one of three further reactions: reaction with an external nucleophile such as solvent or protein, reaction with a base on the same DNA strand to give an intrastrand crosslink, or reaction with a base in the opposite strand to give an interstrand crosslink. In contrast to many other antineoplastic agents, the effects of the nitrogen mustards, although dependent on proliferation, are not cell-cycle-specific, and the drugs may act on cells at any stage of the cycle. However, the toxicity is usually expressed when the cell enters the S phase and progression through the cycle is blocked (Calabresi and Chabner 1990).

There is considerable evidence linking alkylation of DNA to the cytotoxic, carcinogenic, and mutagenic effects of nitrogen mustards (Ludlum 1975). Common sites of alkylation on DNA include the N-7 position of guanine, the N-1, N-3, and N-7 positions of adenine, the N-3 position of cytosine, and the O-4 position of thymidine (Ludlum 1977). Formation of DNA-interstrand crosslinks has a direct inhibitory effect on DNA replication, repair, and transcription (Lawley and Brookes 1965), and correlates

very closely with the cytotoxicity of nitrogen mustards (Garcia, et al. 1988). Formation of DNA adducts may cause a variety of structural alterations, such as ring openings, base deletions, and strand breaks (Bohr, et al. 1987). Nitrogen mustards produce a wide array of mutations, including base substitutions at both G•C and A•T base pairs, intragenic as well as multilocus deletions, and chromosomal rearrangements (Povirk and Shuker 1994). Several biochemical changes have been implicated in the development of resistance to nitrogen mustards, including decreased permeation of actively transported drugs, increased production of nucleophilic substances, such as GSH, and increased GST activity (Calabresi and Chabner 1990).

Of the chemotherapeutic alkylating agents known to be carcinogenic in humans, the nitrogen mustards are amongst the most potent (Kaldor, et al. 1988). In patients with ovarian carcinoma treated with melphalan or chlorambucil, there are a number of different studies that have implicated these drugs as a cause of secondary acute leukemia (Reimer, et al. 1977). The latency period to develop leukemia after the nitrogen mustards is 4-5 years, but the risk remains higher than normal for up to 10 years. In myeloma, where melphalan is one of the primary drugs used, the risk of developing acute leukemia within 4 years of therapy is approximately 200 times that expected (Sieber and Adamson 1975). An increased risk of secondary leukemia has also been noted in patients treated for Hodgkin's disease. In a large case-control study, the cumulative dose of mechlorethamine appeared to be the most important leukomogenic risk factor (van Leeuwen, et al. 1994).

Mechlorethamine

Mechlorethamine (nitrogen mustard, mustargen, mustine, HN_2) is a structural analog of chemical warfare agents like sulfur mustard in which the sulfur atom has been replaced by an amide to produce a less reactive substance. Mechlorethamine is used intravenously, primarily in the treatment of advanced Hodgkin's disease, almost

exclusively in combination chemotherapy (Aisenberg 1999). Mechlorethamine rapidly undergoes chemical transformation and combines with either water or nucleophilic molecules of cells, so that the parent drug has an extremely short mean residence time in the body (Calabresi and Chabner 1990). Mechlorethamine is taken up into cells by an active, carrier-mediated mechanism, that has been identified as the transport carrier for choline in L5178Y lymphoblasts (Goldenberg, et al. 1971). The major acute toxic manifestations of mechlorethamine are nausea, vomiting, and lacrimation, as well as myelosuppression. Leukopenia and thrombocytopenia limit the amount of drug that can be given in a single course (Souhami and Tobias 1998). Mechlorethamine also possesses weak immunosuppressive activity. In addition to Hodgkin's disease, mechlorethamine is approved for the treatment of lymphoma, chronic myelogenous leukemia, and chronic lymphocytic leukemia (Gerson 1998).

Chlorambucil

In cytotoxic drugs with a nitrogen mustard group, the biological efficacy is closely related to the reactivity of the functional 2-chloroethyl groups. Substitution of electrophilic groups at the amino nitrogen reduces the basicity and thus reduces the reactivity of the functional groups. A reduction of the reactivity of mechlorethamine was achieved by coupling of the nitrogen mustard group to a benzene ring, thereby increasing the chemical half-life of the drug in blood to 90 minutes in the resulting compound, chlorambucil (leukeran) (Dirven, et al. 1996). Chlorambucil, which is relatively stable in aqueous solution, is well absorbed from the gastrointestinal tract and has the distinct advantage, over mechlorethamine, of being active after oral administration. The major mechanism by which chlorambucil enters and exits cells appears to be passive diffusion (Bank, et al. 1989). Chlorambucil is extensively metabolized in the liver and its primary

metabolite, phenylacetic acid mustard, is also a bifunctional alkylating compound with activity against neoplastic cell lines (Dirven, et al. 1996).

Chlorambucil is generally well tolerated and is used alone or as a component of various chemotherapeutic regimens mainly in the treatment of chronic lymphocytic leukemia, malignant non-Hodkin's lymphomas, and advanced Hodgkin's disease. The use of high-dose chlorambucil is being explored for the treatment of chronic lymphocytic leukemia (Jaksic, et al. 1996). Chlorambucil also shows activity in hairy cell leukemia, advanced breast cancer, nonseminomatous testicular carcinoma, multiple myeloma and ovarian cancer (Gerson 1998). Chlorambucil is the slowest–acting nitrogen mustard in clinical use, and although it is possible to induce marked hypoplasia of the BM with excessive doses administered over long periods, its myelosuppressive action is usually moderate, gradual in onset but persistent. Thrombocytopenia is frequent but haemorrhagic cystitis rarely occurs. Other toxicities include gastrointestinal discomfort and azoospermia (Souhami and Tobias 1998).

Melphalan

Melphalan (phenylalanine mustard, L-PAM, alkeran) is the L-isomer of the phenylalanine derivative of mechlorethamine. It was designed in the hope that its resemblance to phenylalanine, a precursor of melanin, would facilitate its uptake in melanoma cells. Unfortunately, this has not proved to be the case in conventional doses. Melphalan is used alone or as a component of various chemotherapeutic regimens mainly in the treatment of multiple myeloma and ovarian carcinoma (Gerson 1998). Melphalan is administered either orally or intravenously (IV). Compared to oral melphalan, IV administered melphalan has markedly more predictable and greater bioavailability (Sarosy, et al. 1988). The entry of melphalan into cells is mediated by two energy-dependent transport systems, both of which are normally engaged in the transport of

amino acids (Goldenberg, et al. 1979). At high melphalan concentrations, uptake occurs predominantly by a leucine-preferring system; and at low melphalan concentrations, uptake is achieved by a system that transports alanine, serine and cystine. Thus, melphalan is actively concentrated in cells, resulting in much higher intracellular concentrations than those obtained with chlorambucil.

Most of the evidence suggests that the plasma elimination curve of melphalan is biphasic, with a $t_{1/2}\alpha$ of 6 to 10 min and a $t_{1/2}\beta$ of 40 min to 2 hrs (Tranchand, et al. 1989). Penetration across the blood-brain barrier is low and hydrolysis to dihydroxymelphalan is the main route of elimination (Jones and Matthes 1992). Melphalan's cytotoxic effects are related to its concentration and the duration of exposure. In L1210 murine leukemia, the extent of DNA cross-links increases over time, implying that melphalan quickly forms mono adducts that slowly convert to cytotoxic DNA interstrand, intrastrand, or DNA-protein links (Ross, et al. 1978). BM suppression is the dose-limiting toxicity of melphalan, including both leukopenia and thrombocytopenia. Both the severity and duration of myelosuppression are dose-dependent. At high doses requiring stem cell transplant support, the nonhematologic dose-limiting toxicity of melphalan is gastrointestinal, consisting of mucositis, nausea and vomiting, and diarrhea (Hersh, et al. 1983). Alopecia is also reported at these high doses (Selby, et al. 1987).

In addition to multiple myeloma and ovarian carcinoma, clinical studies show activity in carcinoma of the breast and testes (Fisher, et al. 1975, Wasserman, et al. 1975). Melphalan given intrathecally may also be useful in the treatment of meningeal carcinomatosis (Friedman, et al. 1994). Minimal activity has been reported in patients with malignancies such as non-small-cell lung, rhabdomyosarcoma, pancreas and colon carcinomas with IV melphalan (Sarosy, et al. 1988). High-dose melphalan (HDM) has become an established and effective salvage regimen for children with relapsed neuroblastoma, as well as an effective consolidative treatment for children with high-risk

disease (stage IV) following conventional chemotherapy (Samuels and Bitran 1995). Additionally, HDM appears to be an active agent in patients with stage II and III multiple myeloma in relapse (Vesole, et al. 1994). Interest in HDM has led to phase II trials in relapsed Hodgkin's disease (Russell, et al. 1989), breast cancer (Bitran, et al. 1995) and ovarian cancer (Juttner, et al. 1992). Finally, hyperthermic isolated limb perfusion with HDM and tumor necrosis factor α (TNF-α) has emerged as a very promising option for the management of locally advanced soft tissue sarcomas (Schraffordt Koops, et al. 1998) and *in transit* melanoma metastases (Lejeune, et al. 1998).

CYTOSINE NUCLEOSIDE ANALOGS

Cytosine nucleoside analogs function by replacing cytosine in normal cell functions because of their similarity to the naturally occurring substrate. They are prodrugs that must be activated by phosphorylation to effectively inhibit their primary enzyme targets and to incorporate into DNA chains. Cytosine nucleoside analogs are cell cycle, S-phase-dependent in their cytotoxicities, and thus are time-dependent and/or schedule-dependent in their actions (Spears 1995).

Cytarabine

Cytarabine (cytosine arabinoside, 1-β-D arabinofuranosyl cytosine, Ara-C) is one of the most effective drugs used in the treatment of hematopoietic malignancies. It is a structural analog of deoxycytidine, differing only by the presence of a hydroxyl group (OH) on the configuration at the 2' position. At the low plasma levels achieved with standard dose therapy, Ara-C transport into cells occurs by facilitated diffusion via a nucleoside carrier system in the cell membrane (Wiley, et al. 1982). At the higher plasma

levels reached during high-dose Ara-C therapy, an increasing proportion of transport occurs by simple diffusion (Woodruff and Wiley 1983). Intracellularly, Ara-C is converted to the biologically active metabolite, Ara-C triphosphate (Ara-CTP), via a three-step phosphorylation process. The first step, the conversion to Ara-C monophosphate, is performed by the pyrimidine salvage pathway enzyme, deoxycytidine kinase (dCK), and constitutes the rate-limiting step in Ara-C metabolism.

Ara-CTP competitively inhibits the normal substrate, deoxycytidine triphosphate (dCTP), exerting its lethal effects on cells by inhibition of deoxyribonucleic acid (DNA) polymerase, DNA repair and ribonucleic acid (RNA) synthesis. Its direct incorporation into DNA, where it may occupy either intranucleotide or chain terminus positions, leads to slowing of chain elongation and inhibition of ligation of newly synthesized strands (Mikita and Beardsley 1988). Ara-C has a short biological half-life. Following systemic administration, it is rapidly deaminated by CD to the inactive metabolite, uracil arabinoside, predominantly in the systemic compartments (Riva, et al. 1985).

Various mechanisms of cellular resistance to Ara-C have been described in cultured cells and in experimental animal tumor models (Capizzi, et al. 1991). These include a decrease of Ara-C membrane transport and of intracellular Ara-C phosphorylation; increased catabolism of Ara-C and its phosphorylated metabolites by CD, deoxycytidylate deaminase or 5'-nucleotidases; an increase of the cellular dCTP pool; decreased Ara-C retention; and enhanced excision of Ara-C residues from DNA. The dose-limiting toxicity of standard-dose Ara-C is myelosuppression. Other important adverse effects are gastrointestinal toxicity, nausea, vomiting and alopecia. In general, high-dose Ara-C gives more prolonged myelosuppression than standard-dose Ara-C and the incidence of the other side effects is also increased. Other common adverse effects of high-dose Ara-C include dose-limiting cerebellar toxicity, ophthalmological toxicity, hepatotoxicity and skin reactions (Stentoft 1990).

Ara-C is currently regarded as the single most important drug available for the treatment of acute myelogenous leukemia, and is given in combination with anthracyclines or thiopurines (Lister, et al. 1987). It is also active in acute lymphoblastic leukemia (Stryckmans, et al. 1987) and non-Hodkin's lymphomas (Peters, et al. 1987). During the past decade, the feasibility of administering Ara-C as a high-dose bolus infusion has become firmly established. A subset of patients with refractory or relapsed disease respond to high-dose Ara-C, administered alone or in combination with DNA topoisomerase II inhibitors, asparaginase or fludarabine (Cole and Gibson 1997). However, remissions obtained with high-dose Ara-C tend to be short-lived, and ultimately, patients relapse with highly resistant disease refractory to all subsequent forms of therapy (Estey 1996). High-dose Ara-C is also effective in leukemia with central nervous system (CNS) and meningeal localization due to Ara-C's ability to penetrate the CNS when high plasma levels of the drug are maintained (Rustum and Raymakers 1992). Finally, high-dose Ara-C is effective in the blastic phase of chronic myelogenous leukemia (Herzig, et al. 1985).

Gemcitabine

Gemcitabine (2',2'-difluorodeoxycytidine) is a new nucleoside antimetabolite of deoxycytidine that resembles Ara-C in both structure and its metabolism. The critical difference in the structures is that two fluorine atoms are appended at the 2' position of the deoxyribose sugar ring of gemcitabine. Contrary to Ara-C, gemcitabine is very active as a single agent in many solid tumors. After entering cells by a saturable, carrier-mediated process that is shared by other nucleosides, gemcitabine is phosphorylated by dCK to form the active metabolites gemcitabine diphosphate and triphosphate (Plunkett, et al. 1989). Since gemcitabine is an excellent substrate for dCK, the phosphorylation of gemcitabine is favored over that of deoxycytidine. Deactivation of gemcitabine occurs

mainly through the action of CD, which converts gemcitabine to 2',2'-difluorodeoxyuridine (Grunewald, et al. 1994).

Unlike Ara-C, gemcitabine exhibits multiple mechanisms of action. Gemcitabine triphosphate inhibits DNA synthesis by competitively inhibiting DNA polymerase and by direct incorporation into replicating DNA (Allerheiligen, et al. 1994). Incorporation of gemcitabine triphosphate is masked by one or two additional nucleotides delivered by DNA polymerase, which in turn allows the fraudulent nucleotide to be hidden and not removed by excision repair (Plunkett, et al. 1995). This is in contrast to Ara-CTP, which causes inhibition of the growing DNA strand at the point of incorporation but is easily removed by exonuclease activity. In addition, gemcitabine diphosphate is a relatively strong inhibitor of ribonucleotide reductase and thereby reduces the intracellular amount of the naturally competitive dCTP (Heinemann, et al. 1990). A decline in the amount of intracellular dCTP favors the phosphorylation of additional gemcitabine and reduces the deamination of gemcitabine monophosphate. These mechanisms, which constitute self-potentiation properties, enhance the effective accumulation and prolonged retention of gemcitabine nucleotides.

Gemcitabine is generally well tolerated. Its most common dose-limiting toxicity is myelosuppression. Mild and transient neutropenia, thrombocytopenia, and anemia have been observed. Nausea and vomiting are extremely mild and are rare compared with other cytotoxic drugs. Likewise, alopecia is extremely unusual (Carmichael 1998).

The complicated mechanisms of action with multiple cellular targets of gemcitabine suggest that resistance to this drug can be multifactorial. Aside from the mechanisms of resistance potentially shared with Ara-C based on their common metabolism, additional resistance mechanisms can be proposed for the unique targets of gemcitabine, such as increased CTP-synthetase, ribonucleotide reductase, or RNA polymerase activities (Peters, et al. 1996). However, most mechanisms remain speculative

since evidence of their implication exists only for some of these mechanisms, and then only for the resistance to Ara-C. Initial studies have shown that cell lines with acquired resistance to either Ara-C or decitabine were cross-resistant to gemcitabine (Heinemann, et al. 1988, Peters, et al. 1996).

Early phase II trials with gemcitabine have identified activity against non-small-cell lung cancer (NSCLC) and pancreatic cancer, tumor types for which gemcitabine has a license for treatment in many countries. Several phase II studies have shown promising results for the use of gemcitabine with cisplatin, carboplatin, ifosfamide, paclitaxel or radiation in the treatment of NSCLC (Dombernowsky, et al. 1998, Steward 1998). In other phase II studies, activity has been identified against breast cancer and bladder cancer, both as a single agent and in combination with doxorubicin and cisplatin, respectively. Antitumor activity has also been seen in patients with ovarian cancer, head and neck cancer, small-cell lung cancer, cervical cancer and Hodgkin's disease (Borchmann, et al. 1998, Carmichael 1998).

Decitabine

Decitabine (5-AZA-2'-deoxycytidine) is an analog of 2'-deoxycytidine in which the 5 carbon of the pyrimidine ring has been replaced by a nitrogen. It is an experimental antileukemic agent that has shown activity in the treatment of leukemia and myelodysplasia. After entering cells by a facilitated nucleoside transport mechanism (Plagemann, et al. 1978), decitabine is converted to a nucleotide through phosphorylation by dCK. Decitabine is then incorporated into DNA, where it blocks DNA methylation through the formation of covalent adducts with the enzyme DNA methyl transferase, which results in a striking inhibition of the enzyme's biochemical activity (Jüttermann, et al. 1994). Hypermethylation of DNA, which is one of the mechanisms of tumor progression, has been noted in 85% to 100% of patients with acute myelogenous

leukemia and in 50% of those with chronic myelogenous leukemia (Issa, et al. 1997). Due to its unique mechanism of action of demethylating DNA, decitabine has the potential to activate tumor suppressor and differentiation genes that have been silenced by DNA methylation in leukemic cells. Indeed, decitabine has been demonstrated to induce the *in vitro* differentiation of human leukemic cell lines (Momparler, et al. 1985) and leukemic blasts from patients (Pinto, et al. 1984). In addition, decitabine has been shown to activate the expression of the tumor suppressor genes: p16 (Merlo, et al. 1995), p15 (Herman, et al. 1996), VHL (Herman, et al. 1994) and retinoic acid receptor β (RARβ) (Côté and Momparler 1995).

The antileukemic activity of decitabine is slow to occur and is also highly schedule-dependent since decitabine is an S-phase-specific agent that has a short plasma half-life of 15-30 min due to its rapid inactivation trough CD-mediated hepatic deamination (Momparler, et al. 1997). The main toxic effects of decitabine are delayed and prolonged myelosuppression, mucositis, nausea and vomiting, and alopecia (Kantarjian, et al. 1997b). Decitabine has been shown to have comparable or superior antiproliferative effects to Ara-C in murine and human leukemia cell lines (Momparler 1990). However, cross-resistance exists between Ara-C and decitabine based on their initial common pathway of metabolization (Eliopoulos, et al. 1998b).

Clinically, decitabine has shown promising activity in chronic myelogenous leukemia (Kantarjian, et al. 1997b), acute myelogenous leukemia (Kantarjian, et al. 1997a) and myelodysplastic syndrome (Wijermans, et al. 1997) as a single agent. Decitabine has also demonstrated encouraging activity in acute leukemia when combined with topoisomerase II reactive agents such as amsacrine or anthracyclines (Schwartsmann, et al. 1997, Willemze, et al. 1997). Based on the significant myelosuppression observed with decitabine, two programs have been proposed in the setting of stem cell rescue therapy. The first program offers decitabine in combination

with busulfan and cyclophosphamide as part of a preparative regimen for allogeneic stem cell transplantation for patients with high-risk leukemia, and the second program proposes to treat patients who relapse post-allogeneic stem cell transplantation with decitabine alone followed by allogeneic stem cell rescue. Decitabine therapy has been shown to be well tolerated in the setting of allogeneic stem cell transplantation and initials results in patients relapsing after allogeneic BM transplant are encouraging (Giralt, et al. 1997).

GLUTATHIONE

BIOLOGICAL FUNCTIONS

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that serves essential functions within the cell. It is the most abundant nonprotein thiol in almost all aerobic species, occurring at intracellular concentrations of 0.5 to 10 m*M*. Under physiological conditions, GSH disulfide reductase maintains more than 98% of intracellular GSH in the reduced, thiol form (GSH). The rest is present within the cell as mixed disulfides (mainly GS-S-protein), as the disulfide, and as thioethers (Wang and Ballatori 1998).

The key functional element of the GSH molecule is the cysteinyl moiety, which provides the reactive thiol group and is responsible for the many functions of GSH. These functions include the maintenance of protein structure and function by reducing the disulfide linkages of proteins, the regulation of protein synthesis and degradation, the maintenance of immune function, the protection against oxidative damage, and the detoxification of reactive chemicals. GSH also serves as a storage and transport form of the cysteine moiety, and it functions in the metabolism of leukotriene and prostaglandin, in the reduction of ribonucleotides to deoxyribonucleotides, in the modulation of microtubule-related processes, and in bile formation (Meister and Anderson 1983).

GSH is the predominant defense against the toxic products of oxygen, particularly in the mitochondria, which is a major site for the synthesis of reactive oxygen intermediates, such as superoxide and hydrogen peroxide, that can cause lipid peroxidation and disrupt metabolic processes (Deleve and Kaplowitz 1991). The reduction of organic hydroperoxydes by GSH can be catalyzed either by selenium-dependent or independent GSH peroxidase activities, provided respectively by GSH peroxidase and GSTs of the alpha class (Wang and Ballatori 1998). GSH also plays a

major role in detoxifying many reactive electrophiles and metals by either spontaneous conjugation or by a reaction catalyzed by the GSTs.

SYNTHESIS AND DEGRADATION

GSH is synthesized in all mammalian cells, and the liver is a major site of biosynthesis. The synthesis of GSH from its three amino acid precursors L-glutamate, L-cysteine, and glycine takes place in the cytosol. In the first step of GSH synthesis, an amide linkage is formed between cysteine and glutamate catalyzed by the γ-glutamylcysteine synthetase (γ-GCS). GSH synthetase then catalyses the reaction between glycine and the cysteine carboxyl of γ-glutamylcysteine dipeptide to form GSH. γ-GCS is the rate-limiting enzyme and is controlled by a negative feedback from its end product, GSH (Richman and Meister 1975). GSH is transported out of the cell and broken down by the membrane-bound enzyme γ-glutamyltranspeptidase, which removes the γ-glutamyl moiety, and by dipeptidases, which remove the glycine moiety. The resulting amino acids can be reabsorbed and used for additional GSH synthesis (Meister and Tate 1976).

GSH S-conjugates are made intracellularly and then transported out of the cell for subsequent degradation by the same enzymes that metabolize GSH. The cysteine S-conjugates that are formed are then transported back into the cell and N-acetylated to form mercapturic acids. Mercapturic acids are released into the circulation or bile; some are eventually excreted in urine, and some may undergo further metabolism (Hinchman, et al. 1991).

GSH AND CANCER

GSH in drug resistance

GSH was identified as an important determinant in the success of cancer therapy in the 1950s by radiobiologists who found that GSH depletion sensitized cells to ionizing radiation (Alper 1956). In the 1970s, Vistica and colleagues found that L1210 murine leukemia cells with acquired resistance to melphalan had higher GSH content than parental cells. They also found that sensitivity could be almost completely restored by decreasing GSH content (Somfai-Relle, et al. 1984). Conversely, providing a source of thiols could increase resistance to melphalan. Subsequently, GSH contents have been found to be elevated in a number of cancer tissue and cancer cell lines (Zhang, et al. 1998). GSH content has been shown to play a role in determining drug resistance to a variety of chemotherapeutic agents including adriamycin (Lee, et al. 1989), mitomycin C (Xu, et al. 1994), melphalan (Kramer, et al. 1987), cyclophosphamide (Ono and Shrieve 1986), chlorambucil (Yang, et al. 1992), daunorubicin (Lutzky, et al. 1989) and cisplatin (Newkirk, et al. 1997).

Clinically, it has been shown that the median survival time was significantly longer in patients who were subjected to combination therapy with cyclophosphamide and carboplatin with tumor GSH content of <4.9 μg/mg protein compared to those with a tumor content of >4.9 μg/mg protein (Tanner, et al. 1997). After chemotherapy, the non-responders had a higher GSH concentration than responders (Cheng, et al. 1997). GSH levels have also been found to be 10-fold greater in human ovarian tumor cells obtained after development of resistance to alkylating agents than in biopsy samples obtained prior to treatment (Britten, et al. 1992). Thus, GSH may play a role in chemoresistance.

Glutathione depletion

It has been reported in many studies that the resistance of tumor cells to a number of chemotherapeutic agents could be modulated by depletion of GSH with buthionine sulphoximide (BSO), which is a specific inhibitor of γ-GCS (Zhang, et al. 1998). In parallel with depletion of GSH, it was demonstrated *in vitro* and *in vivo* that resistant cells exhibited restored sensitivity to platinum compounds and alkylating agents following BSO pretreatment (Hamilton, et al. 1985). The *in vivo* studies suggested that some selectivity for tumor cells was achievable, since the combined use of BSO and melphalan markedly enhanced the survival of OVCAR-3-bearing nude mice over that of mice treated with melphalan alone (Ozols, et al. 1987). However, phase I studies of the combination of melphalan and BSO demonstrated an increased BM toxicity with the addition of BSO (O'Dwyer, et al. 1992). Preliminary data from a phase II study of melphalan plus BSO for the treatment of metastatic melanoma demonstrated a greater inhibition of γ-GCS in the tumor and a larger degree of GSH depletion in the tumor as compared with the normal peripheral mononuclear cells (PMNCs). This suggests that, at least in this tumor type, GSH turnover is greater in tumor versus normal PMNCs and therefore that there may be some degree of selectivity on this basis between tumor and normal tissue (Chen, et al. 1998).

GLUTATHIONE S-TRANSFERASES

ENZYMES OF DETOXIFICATION

GSTs comprise a family of multifunctional isoenzymes with broad substrate specificities that catalyze the conjugation of GSH to a variety of electrophilic compounds to render them more water soluble and less toxic, and to enhance their elimination (Hayes and Pulford 1995). As such, they are an integral part of the Phase I (oxidation), Phase II (conjugation) and Phase III (elimination) system that metabolizes many endogenous and foreign compounds (Ishikawa 1992). The electrophiles that GSTs can use as substrates include xenobiotics (e.g., mutagens, carcinogens) that frequently have been activated by cytochrome P450 (P450) monooxygenases and reactive oxygen species (e.g., lipid peroxides) produced during normal metabolism or during physiologic oxidative stress (Halliwell 1991). GSTs are fairly abundant and are found in variety of aerobic organisms including vertebrates, plants, insects, and bacteria. In human liver, they comprise up to 10 % of cytosolic proteins (Whalen and Boyer 1998).

NOMENCLATURE

Several nomenclatures have been proposed over the years for the classification of GST subunits. A species-independent and class-based nomenclature, proposed for human and other mammalian GSTs, will be used here (Mannervik, et al. 1992). In this nomenclature, small letters designating the common name of the species are used as prefixes (e.g., r for rat). Single capital letter abbreviations are used to signify the different classes (e.g., A for alpha), and Arabic numerals are employed for numbering each of the

separate gene products (e.g., A1, A2, A3, etc.). The dimeric GST isoenzymes are represented by the single letter suffix followed by hyphenated Arabic numerals. Hence, the rat class alpha heterodimer formed between A1 and A3 is designated rGSTA1-3. Finally, a lower case letter following the subunit type (e.g., M1a-1b for the class mu hybrid of the 1a and 1b alleles) designates allelic variants of isoenzymes.

Rat GTSs were originally called Y-proteins and subunits were named based on their mobility as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bass, et al. 1977). Thus, the rat GST Yc_1-Yc_1 used in the present investigation is an alpha class GST and is now called rGSTA3-3.

GENE FAMILIES

At present, six gene families of cytosolic or soluble enzymes are known in man: alpha, mu, pi, theta, kappa and zeta (Ketterer 1998). A human sigma has yet to be reported, although one has been isolated from rat (Meyer and Thomas 1995). These class distinctions are based on structural differences. Within a class, different GSTs share at least 40% identity of amino acids, while they share less than 30% identity between classes (Hayes and Pulford 1995). The alpha and mu classes comprise 5 distinct genes each, named hGSTA1 to A5 and hGSTM1to M5, respectively. The theta class has 2 genes, hGSTT1 and hGSTT2; and only one gene is known so far in each of the pi, kappa and zeta classes, namely, hGSTP1, hGSTK1 and hGSTZ1 (Ketterer 1998).

In addition to the cytosolic GSTs, three membrane-bound GSTs are known. These proteins share a small degree of sequence similarity. However, they bear no discernible relationship to any of the known cytosolic enzymes with respect to sequence. Microsomal GST I is an integral membrane protein that has been characterized from both rats and

humans and appears to be involved in xenobiotic metabolism (Morgenstern and DePierre 1988). Microsomal GST II and leukotriene C_4 (LTC_4) synthase are involved in the synthesis of leukotrienes, which are potent mediators of inflammation and immunity (Jakobsson, et al. 1996, Weinander, et al. 1996).

Finally, there is one known plasmid-encoded bacterial GST that is associated with bacterial resistance to the antibiotic fosfomycin (Arca, et al. 1990). Its primary structure is not related to any of the soluble or microsomal GSTs, nor does it catalyze the addition of GSH to any of the usual electrophilic substrates used to assay the soluble enzymes.

EVOLUTION OF GSTS

It has been proposed by Pemble and Taylor based on comparisons of complementary DNA (cDNA) and genes sequences of class alpha, mu, pi and theta GSTs that a alpha/mu/pi precursor gene arose from duplication of the theta gene (Pemble and Taylor 1992). The highly conserved 3'-noncoding sequences of the mu and theta genes also suggest that the mu gene diverged from this precursor before the pi or the alpha gene. The progenitor of the theta class may be the kappa class gene (Pemble, et al. 1996). A high conservation of nucleotide sequence in the intervening sequences of the eukaryotic class mu enzymes led Pickett and co-workers to propose that gene conversion events in a cluster of mu genes may be responsible for the development of catalytic diversity in this class (Morton, et al. 1990). Divergent portions of coding exons are found, from the three-dimensional protein structure, to correspond to the xenobiotic substrate binding site (Lai, et al. 1988). Within the class alpha, the human GSTA4 is considerably diverged from GST A1, A2 and A3 since it has only about 52% amino acid sequence identity with this group compared with > 90% identity within the GST A1, A2 and A3 group (Board 1998).

The membrane-bound microsomal GSTs show no apparent sequence relationship to any of the cytosolic enzymes. However, they share evolutionary roots with enzymes in the leukotriene biosynthetic pathway, including LTC_4 synthase (Lam, et al. 1996). The GST conferring resistance to fosfomycin belong to a superfamily of metalloenzymes (Armstrong 1998).

TISSUE DISTRIBUTION

GSTs are present in all tissues of the human body. Each tissue, however, contains a unique group of GST isoenzymes that are expressed at varying levels in different tissues. The GST subunit profiles in cell types within a tissue also may vary, especially during development. For example, GSTP1-1 is highly expressed in fetal hepatocytes but it is not normally expressed by adult hepatocytes in rats or humans (Awasthi, et al. 1994). Gender-related differences also have been noted in the levels of expression of different isoenzymes within some tissues (e.g., colon) (Singhal, et al. 1992) but not in others (e.g., erythrocytes or lymphocytes) (Al-Turk, et al. 1987). The greatest total amount of GST has been found in the testis, in which the levels of all of the major GSTs are relatively high in comparison with other tissues. The liver also contains high concentrations of GSTs, particularly of the alpha class, which is consistent with its role as a major site of detoxification of xenobiotics and naturally occurring toxins (Whalen and Boyer 1998).

Knowledge of the spectrum of hGST isoenzyme expression in tissues is so far incomplete. There is a general consensus that the human liver expresses GSTs A1, A2, A4, M1, T1, T2, K1, Z1; the lung, GSTs A4, M1, M3, P1, T1 and T2; the erythrocytes, GSTs P1 and T1; the lymphocytes, GSTs P1 and M1; the colon and the kidney, GSTs A1, M1, P1 and T1; the skin, GSTs M1, M3 and P1; the brain, GSTs A4, M1, M3, P1, T1 and

Z1; the testis, GSTs A1, A4, M1, M3, P1 and T1; the prostate, GSTs P1 and T1; the spleen, GSTP1; the small intestine and the pancreas, GSTs A4 and T1; and the muscle, GSTs A4, M1, M2, P1, T1 and Z1 (Ketterer 1998). GSTM4 expression has also been detected in human tissues, with relatively high levels in the heart, brain and skeletal muscle, and with relatively low levels in the placenta, lung, kidney, and pancreas (Comstock, et al. 1993).

Microsomal GST I is found in large amounts in liver and is distributed in both the microsomal and outer mitochondrial membrane (Morgenstern and DePierre 1988).

ENZYME STRUCTURE

Cytosolic GSTs

The overall tertiary structures of all of the soluble GSTs described to date are similar. GSTs are globular dimeric proteins with one catalytic site per subunit and range in molecular weight from about 23,000 to 29,000 daltons per subunit. Monomeric subunits of a specific class of GST share 60% to 80% amino acid sequence identity, yet exhibit distinct substrate specificity (Raha and Tew 1996). Each subunit is formed by about 200 to 240 amino acids. GSTs are typically homodimers; however, heterodimers can form between some closely related members of the same class and are known to occur among alpha and mu class enzymes (Whalen and Boyer 1998). GSTs have two active sites per dimer that behave independently of one another. Each active site consists of at least two ligand-binding regions. The GSH binding site is very specific for this tripeptide, whereas the binding site for the electrophilic substrate is less specific (Danielson and Mannervik 1985)

The polypeptide chain of each GST subunit forms two domains connected by short linker regions. The N-terminal domain is composed of approximately 80 amino acids arranged in a β-sheet and three α-helices and makes most of the contacts or binding of GSH (the G site) and some of the contacts for binding of the hydrophobic electrophile (the H site). The C-terminal domain includes the remaining amino acids in 5 or 6 α-helices and makes most of the contacts for the H site. Thus, the principal intersubunit interactions occur between the N-terminal domain of one subunit and the C-terminal domain of its partner. The amino acids residues that contribute to the G site of alpha, mu, pi and sigma class enzymes are well conserved (Whalen and Boyer 1998). The most fundamental difference among the GSH binding sites of the various enzyme classes involves the interaction of the protein with the sulfur of the peptide. The theta class enzymes, thought to be the evolutionary precursor of the alpha, mu, pi and sigma class proteins, appear to utilize the hydroxyl group of a serine residue located near the N-terminus of the polypeptide to activate the sulfhydryl group of bound GSH (Board, et al. 1995). In contrast, the class alpha, mu, pi and sigma enzymes have recruited the hydroxyl group of a tyrosyl residue to act as a hydrogen bond donor to the sulfhydryl group of GSH (Armstrong 1997). The residues that comprise the H sites of GSTs are not as well conserved among the different GSTs as are those that define the G site (Whalen and Boyer 1998).

Microsomal GSTs

Considerably less is known about the structure of the microsomal GSTs. Although no high-resolution three-dimensional structure has been obtained yet, all the information obtained so far is consistent with a trimeric structure of the enzyme (Andersson, et al. 1994). In contrast to cytosolic enzymes, it seems that the trimer does not function with three independent non-interacting active sites (Sun and Morgenstern 1997).

Fosfomycin resistance GSTs

Little is known about the structure of the GST involved in bacterial resistance to fosfomycin. The active enzyme is a dimer of 16 kilodalton (kDa) subunits (Arca, et al. 1990). The primary structure of the enzyme exhibits regions of high sequence similarity to a group of bacterial dioxygenases and to glyoxalase I, which are also metalloenzymes (Armstrong 1997).

FUNCTIONS

GST-mediated GSH conjugation

All cytosolic and membrane-bound GSTs possess the ability to conjugate GSH with compounds containing an electrophilic center. A carbon, a nitrogen, or a sulfur atom can provide the electrophilic functional group. The substrates have in common a high degree of hydrophobicity, and possess electrophilic centers that undergo nucleophilic substitution, nucleophilic addition to α,β-unsaturated ketones or epoxides or, in the case of hydroperoxides, nucleophilic attack on electrophilic oxygen, resulting in reduction. The range of compounds that contain electrophilic centers is extremely large and includes carcinogens, pesticides, products of oxidative stress and therapeutic drugs. In addition these detoxification reactions, GSTs conjugate a small number of endogenous compounds to GSH as part of their normal biosynthetic pathway. Membrane-bound LTC_4 synthase enzymes conjugate leukotriene A_4 with GSH (Jacobsson, et al. 1996, Weinander, et al. 1996) to form LTC_4 (Nicholson, et al. 1993) and various cytosolic GSTs metabolize prostaglandin (PG) H_2 to PGD_2, PGE_2, and $PGF_{2\alpha}$ (Ujihara, et al. 1988).

Conjugation with GSH is not always protective and may actually activate compounds. For example, the GSH conjugation of dibromoethane, which is used in

leaded gas, forms a 2-bromo-thioether, which is subsequently transformed into a highly reactive, mutagenic, and carcinogenic intermediate, possibly an episulfonium, capable of alkylating nucleophilic sites in proteins and DNA (van Bladeren, et al. 1981). Other classes of compounds, including nephrotoxic haloalkenes, quinones, and isothiocyanates, are also converted by GSH conjugate formation to toxic metabolites (Koob and Dekant 1991).

Export of GSH S-conjugates

Once formed, the conjugates can be transported from the cell by ATP-dependent GSH *S*-conjugate efflux pumps (GS-X). GS-X pump activity is found in many organisms, including yeasts, plants, and mammals (Ishikawa, et al. 1997). The 190-kDa membrane glycoproteins mediating the transport of GSH *S*-conjugates have been identified as the multidrug resistance proteins MRP1 and MRP2. MRP1 is detected in the plasma membrane of many cell types whereas MRP2 has been localized to the apical domain of polarized epithelia. Human MRP1 and MRP2 are composed of 1531 and 1545 amino acids, respectively. They contain two characteristic ATP-binding domains and are members of the family of ABC transporters (Keppler, et al. 1998).

Physiologically important substrates of both transporters include GSH *S*-conjugates, such as LTC_4, as well as glucoronides and GSH disulfide. Both transporters have the capacity to pump drug conjugates and drug complexes across the plasma membrane into the extracellular space. The GSH *S*-conjugate of aflatoxin B_1 (AFB_1) was recently been shown to be a high affinity substrate of MRP1 (Loe, et al. 1997). In addition, several GSH and glucoronate conjugates of anticancer drugs, including cisplatinum and the nitrogen mustards melphalan and chlorambucil, are substrates for human MRP1 (Barbouin, et al. 1998, Ishikawa and Ali-Osman 1993). MRP1 also transports doxorubicin, daunorubicin, etoposide and vincristine, which are also substrates

of multidrug resistance 1 (MDR1) P-glycoprotein (Grant, et al. 1994). However, the transport mechanism is quite different for the two transporters. MDR1, but not MPR1, can transport the above-mentioned antitumor drugs in their unmodified forms (Loe, et al. 1996). Moreover, the amino acid sequence identity between MRP1 and MDR1 P-glycoprotein is only 15% (Cole, et al. 1992).

Peroxidase activity of GST

In addition to their capacity to catalyze the formation of a thioether bond between GSH and electrophilic xenobiotics, a significant number of the GST isoenzymes, particularly the members of the alpha and theta classes, also exhibit GSH peroxidase activity and catalyze the reduction of organic hydroperoxides to their corresponding alcohols. The substrates that GSTs reduce include fatty acids, phospholipid hydroperoxides and DNA hydroperoxides (Hayes and Pulford 1995). Since these compounds are generated by lipid peroxidation and oxidative damage to DNA, GSTs may help combat oxidative stress (Mannervik 1986).

Noncatalytic-binding activities

GSTs are known to bind, both covalently and noncovalently, a variety of nonsubstrate ligands (NSL) such as bilirubin, heme, hormones, and carcinogens. These NSLs appear to bind to a unique site on the enzyme, distinct from the active sites, and to act as allosteric inhibitors of enzyme activity (Boyer, et al. 1984). The function of NSL binding is not known with certainty. The binding may be important for intracellular transport of water-insoluble molecules, for sequestration of metabolic precursors for storage, and for sequestration of toxic molecules as a defense mechanism (Kamisaka, et al. 1975, Listowski, et al. 1988).

DETOXIFICATION OF ENVIRONMENTAL CARCINOGENS

Through the concerted actions of several isoenzymes, the GST supergene family provides several levels of defense against toxic chemicals. Many carcinogens are substrates for GSTs. A major group of chemical carcinogens, the polycyclic aromatic hydrocarbons (PAH), are commonly encountered in combustion products such as car exhaust fumes and cigarette smoke and are associated with several occupational cancers (Phillips 1983). PAHs (e.g., benzo[a]pyrene), which requires activation by P450 isoenzymes, are substrates for class mu and pi GSTs (Hayes and Pulford 1995).

AFB_1 is one of the most potent naturally occurring hepatocarcinogen known. It is produced as a secondary by the fungus *Aspergillus flavus* and is widely encountered as a contaminant of cereal crops and nuts in humid areas of Asia and Africa where it has been linked to the high incidence of human liver cancer (Eaton and Gallagher 1994). Like PAHs, it must be activated to the ultimate carcinogen AFB_1 *exo*-8,9-epoxide primarily by P450 enzymes before it can interact with DNA and produce genotoxic damage (Baertschi, et al. 1988). The importance of GST-mediated detoxification is suggested by the fact that the mouse, which is intrinsically resistant to AFB_1, expresses high levels of alpha class GSTs in the liver, whereas the rat, which is sensitive to AFB_1, expresses much lower amounts of these enzymes (Buetler, et al. 1992).

PROTECTION AGAINST OXIDATIVE STRESS

Increased or inappropriate generation of reactive oxygen species (ROS), often referred to as oxidative stress, can cause cell damage and even cell death via effects including GSH depletion, perturbation of intracellular calcium homeostasis, loss of DNA

integrity and peroxidation of membrane lipid (Halliwell and Aruoma 1991). Alpha, mu and pi class GSTs catalyze the conjugation with GSH of a variety of electrophiles that result from ROS activity. Substrates include α,β-unsaturated aldehydes such as 4-hydroxyalkenals and base propenals that are the products of ROS reactions and lipid peroxidation (Berhane, et al. 1994). In addition, alpha and theta class GSTs demonstrate *in vitro* peroxidase activity against substrates that include lipid hydroperoxides and products of oxidative damage to DNA such as DNA hydroperoxide and 5-hydroxymethyluracil (Ketterer, et al. 1993).

GENE STRUCTURE

Most class alpha genes isolated from the rats, mice, and humans are 11 to 15 kb in length and comprise seven exons. One notable exception is the rat GSTA5 gene that has six exons (Pulford and Hayes 1996). Whereas the human alpha class genes all are located on chromosome 6p12, in the rat, the GST A1 and A2 genes are found on chromosome 8, and the GST A3 and A5 genes on chromosome 9 (Yamada, et al. 1992). The class mu genes isolated from rats, mice, and humans are all about 5 kb and are composed of eight exons. In humans, the class mu genes are located on chromosome 1. Class pi genes from rats, mice, and humans are about 3 kb and contain seven exons. In humans, the class pi genes are located on chromosome 11 (Hayes and Pulford 1995). The rat class theta genes are about 4 kb in length, contain five exons and are located on chromosome 22 (Ogura, et al. 1994). The membrane-bound class genes form rats and humans are about 12 kb in length and contain at least three exons. In humans, the membrane-bound class genes are located on chromosome 12 (Hayes and Pulford 1995).

INDUCTION AND REGULATION OF EXPRESSION

Rodent Alpha genes

The 5'-flanking regulatory regions of the rat alpha GST A2, A3 and A5 genes have been characterized recently (Bernat, et al. 1997, Hayes and Pulford 1995, Wilce, et al. 1995). The proximal promoter region of the rat GST A2 and A5 genes contains a TATA box upstream of the transcription site. In contrast, the rat GSTA3 gene does not contain typical TATA or CAAT boxes, characteristic of genes that are widely expressed in different cell types. Computer-assisted analysis of the 5'-flanking regions has revealed the existence of several common potential transcription factor binding sites in the rat alpha GST A2, A3 and A5 genes, including antioxidant response elements (ARE) and Barbie boxes (Fotouhi-Ardakani and Batist 1999, Hayes and Pulford 1995, Pulford and Hayes 1996). The ARE is a transcription enhancer that mediates induction by diverse chemicals, including phenolic antioxidants, ROSs and polyaromatic hydrocarbons activated by P450 enzymes. Barbie box elements mediate the induction by phenobarbital. In addition, a xenobiotic response element (XRE), a transcription enhancer that responds to polyaromatic hydrocarbons (e.g., 3-methylcholanthrene), and hepatic nuclear factor (HNF) elements have been identified in the rat alpha GST A2 and A5 genes. Other potential binding sites for transcription factors identified in the rat GSTA3 gene include consensus sequences for activator protein-1 (AP-1), nuclear factor-κB (NF-κB), E box, cAMP-response-element-binding protein (CREB), SP-1 (or G/C box) and *ras*-response element (RRE).

Human alpha genes

The transcriptional regulatory control regions in the human GST A1 and A2 genes have been investigated. Like their rat homologs, the 5'-upstream sequences of the human

genes contain a TATA-box upstream of the transcription start site. The intron and exon structures of the human GST A1 and A2 genes are the same as those of their rat homologs (Suzuki, et al. 1993). However, the 5'-flanking or promoter sequences of the human genes are very different from the promoter for rGSTA2. Most importantly, the human alpha class GST genes apparently lack the ARE and XRE through which the rodent alpha genes are induced (Suzuki, et al. 1994). Positive and negative regulatory regions have also been found in the promoters of the human genes. One of the regulatory elements was identified and shown to function as an enhancer was an HNF-1 site. Other sites identified by computer analysis were AP-1 and AP-2 sites, and a glucocorticoid responsive element (GRE), responsible for induction by dexamethasone (Lorper, et al. 1996). An incomplete transcript of the human GSTA3 gene has been identified recently form a cDNA library derived from 8-9 week placenta, after unsuccessful attempts to obtain a copy of this cDNA in several adult tissues (Board 1998). This suggests that, in the human, the A3 subunit gene is under developmental regulation.

Human Pi gene

The human GST Pi gene's 5'-flanking regulatory region has been characterized. The identified transcription factor recognition sites include an NF-κB site, a consensus AP-1 binding site, and two SP-1 sites. The AP-1 site, essential for promoter activity, contains the consensus sequence for a TRE (TPA [12-*O*-tetradecanoylphorbolester-13 acetate] responsive element) and GST expression is induced by TPA or combinations of coexpressed AP-1 family proteins (v-jun, c-fos, c-jun) (Moffat, et al. 1996). The regulation of the GSTP1 gene appears to be complex. For example, the reactive oxidant hydrogen peroxide also induces GSTP1 expression through the NF-κB site. However, the antioxidant *N*-acetyl cysteine induces activity of GSTP1 through the AP-1 site, but suppresses the induction caused by hydrogen peroxide. This suggests that the effects of

oxidant stress on the GSTP1 promoter are mediated by the NF-κB site, whereas those of an antioxidant are mediated by the AP-1 site (Xia, et al. 1996). In addition, insulin induces GSTP1 expression through one of the SP-1 sites, whereas retinoic acid suppresses it via the AP-1 site.

ROLE IN CANCER

There are several lines of evidence implicating GSTs in intrinsic and acquired drug resistance. These include the following: (1) the overexpression of GTSs in cancers; (2) the overexpression of GST isoenzymes in cell lines with acquired resistance due to selection with specific chemotherapeutic agents; (3) the transfection of specific GST cDNAs conferring drug resistance to mammalian cells; (4) the demonstration that anticancer drugs are substrates for GST isoenzymes; and (5) the reversal of drug resistance through inhibition of GST isoenzymes.

Coordinate changes in expression of protective genes in drug-resistant cells

Maintenance of cellular homeostasis is a critical survival trait in tumors when exposed to anticancer drugs. Because conjugation and elimination of drugs and their metabolism is dependent upon sequential and coordinated pathways, acquired drug resistance through a gradual adaptive response would rarely be expected to be the consequence of changes in the expression of a single gene product. Tew *et al* have used a number of drug-resistant human cell lines to characterize genes that are implicated in maintaining a resistant phenotype to electrophilic drugs. Human HT29 colon cancer cells chronically exposed to ethacrynic acid (EA), a GSH and GST modulator, have acquired resistance to EA and exhibit low level cross resistance to chlorambucil, melphalan and

adriamycin (Ciaccio, et al. 1993). Using directed and random (differential display) approaches, a number of gene products were found to be overexpressed in the EA-resistant cell line, including γ-GCS, GSTP1 and MRP (Ciaccio, et al. 1996, Kuzmich, et al. 1992). An adriamycin-resistant human HL60 cell line also shows overexpression of γ-GCS, GSTP1 and MRP, suggesting the possibility that increased cellular GSH, together with GST and MRP (to conjugate and efflux the product) may be a common requirement for resistance to electrophilic drugs (Krishnamachari, et al. 1994).

GSH S-conjugate efflux pump in drug resistance

The broad substrate specificity of the conjugate export pumps MRP1 and MRP2 enables the terminal excretion of a multitude of conjugates and amphiphilic anions which are formed by a large number of relatively specific monooxygenases and transferases in phase I and phase II metabolism of endogenous and xenobiotic lipophilic substances. Overexpression of MRP has been found in many non-P-glycoprotein MDR cell lines (Zhang, et al. 1998). Transfection of MRP gene to sensitive cells could induce MDR and the transfected cells showed a decreased drug accumulation owing to an enhanced drug efflux (Grant, et al. 1994). It has also been demonstrated that drug transport in MRP-overexpressing MDR tumor cell lines could be regulated by intracellular GSH level (Versantvoort, et al. 1995). More recently, combined expression of MRP1 and GST A1-1 was shown to confer resistance to chlorambucil in human MCF-7 breast carcinoma cells, while expression of either GST A1-1 and MRP1 alone failed to confer resistance to the drug. The results demonstrate that GST A1-1 and MRP1 can act in synergy to protect cells from the cytotoxicity of chlorambucil (Morrow, et al. 1998a).

Overexpression of GST isoenzymes in tumor cells

The GST isoenzymes are induced in preneoplastic foci and in tumors isolated from organisms exposed to carcinogens or alkylating agents, and this induction occurs even after a single *in vivo* exposure to carcinogens (Buchmann, et al. 1985, Sato 1988). GSTP1 is primarily expressed in these preneoplastic foci as well as in tumors (Howie, et al. 1990). The overexpression of the pi GST isoenzyme in tumor tissues has led the identification of GSTP1 as a tumor marker in lung, colon, bladder and other human cancers (Sato 1989). Cell lines derived directly from various human tumors also overexpressed GST isoenzymes (Castro, et al. 1990). In most cases, GSTP1 is predominantly expressed with lower, but detectable, levels of the alpha and mu class GST isoenzymes. A survey of the National Cancer Institute (NCI) cancer drug screening panel of 60 cell lines showed that GST level demonstrated a strong negative correlation with alkylator sensitivity (Tew, et al. 1996). The fraction of tumors with elevated GST is at least as high as that of other tumor associated biochemical phenotypes such as mutant p53 or *ras* (Harris 1995).

Clinically, Schisselbauer *et al* reported a two-fold increase of GST activity in lymphocytes from chronic lymphocytic leukemia patients clinically resistant to nitrogen mustards over cells from untreated chronic lymphocytic leukemia patients, which was attributed primarily to the expression of the pi GST isoenzyme (Schisselbauer, et al. 1990). The work of Lewis *et al* also suggested a direct link between GST overexpression and the onset of clinical resistance. They found 2.2-fold elevation of GST activity of cell lines derived from some patients after onset of the resistance to chlorambucil, cisplatin and 5-fluorouracil (5-FU) over the cells taken from the patients prior to the treatment (Lewis, et al. 1988a). Increased levels of GSTP1 expression after chemotherapy were also linked to drug resistance in patients with ovarian carcinoma (Cheng, et al. 1997).

Drug-resistant cell line studies

The first evidence to suggest that GSTs are directly involved in drug resistance came from reports of the overexpression of the pi and alpha GST isoenzymes in drug-resistant cell lines. Overexpression of the GST alpha isoenzymes has been more strongly implicated in resistance to alkylating agents, more specifically, nitrogen mustards. Human ovarian adenocarcinoma cells derived from patients resistant to chlorambucil primarily overexpressed a GST alpha isoenzyme (Lewis, et al. 1988a). The Walker-256 rat mammary carcinoma cells resistant to chlorambucil were also shown to be resistant to other nitrogen mustards (Buller, et al. 1987, Wang and Tew 1985). Among the phenotypic differences noted in the Walker resistant cells relative to the wild-type cells, the increased expression of GSTA2 isoenzyme was detected (Buller, et al. 1987, Clapper, et al. 1993, Clapper and Tew 1989). The resistant cell line exhibited a 10- to 15-fold resistance against chlorambucil over the wild-type Walker cell line. Chinese hamster ovary cells made resistant to bifunctional nitrogen mustards were shown to express enhanced levels of the GSTA3 isoenzyme (Lewis, et al. 1988b). In another study, the same cell line, but resistant to chlorambucil, was also cross-resistant to melphalan (Hall, et al. 1994). Finally, an inverse correlation was found for melphalan and chlorambucil between both the GSH concentration and the GST activity and the number of DNA cross-links formed (Alaoui-Jamali, et al. 1992, Johnston, et al. 1990).

Overexpression of the mu GST isoenzymes has been implicated in resistance against another subset of the alkylating agents, the nitrosoureas. One study involving rat 9L gliosarcoma cells resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) attributed this resistance to an overexpression of GST mu isoenzymes (Evans, et al. 1987). In a human NSCLC cell line, overexpression of the GSTM3 isoenzyme has been shown to be responsible for this resistance (Berhane, et al. 1993). Overexpression of the pi GST isoenzyme has been linked to the MDR phenotype (Batist, et al. 1986, Cowan, et al.

1986). However, it is unclear whether GSTP1 is directly responsible for much of the drug resistance since no MDR-drug has yet been clearly established as a substrate for this isoenzyme (Tew 1994). There is a growing body of evidence that an increase in GSTP1 in drug-resistant cells and tumors may be the result of a pleiotropic stress response and may not be directly responsible for the production of the MDR phenotype.

Gene transfer studies

In general, the results of transfection and/or transduction experiments indicate that GST isoenzymes can confer drug resistance, particularly in relation to overexpression of the GST alpha isoenzymes and nitrogen mustard resistance. The relatively modest increases in drug resistance observed in GST transfected cell lines are similar to those observed clinically and represent significant changes with respect to attainment of successful therapy. Transfection and transduction of human GSTP1 cDNA into NIH 3T3 and MCF-7 (Doroshow, et al. 1995, Moscow, et al. 1989, Nakagawa, et al. 1990) conferred up to 8- and 3-fold level of resistance against EA and adriamycin, respectively. Chinese hamster ovary cells transfected with a GST pi expression vector conferred a two- to threefold level of resistance against cisplatin and carboplatin (Miyasaki, et al. 1990). Introduction of GSTP1 to a human lung cancer cell line caused modestly increased resistance to adriamycin but not to cisplatin and etoposide (Miyara, et al. 1996). Although there is a suggested link between GST pi overexpression and the MDR phenotype, some studies have shown that GSTP1 plays a minor role in multidrug resistance (Fairchild, et al. 1990, Puchalski and Fahl 1990). To date, the largest increases in GST-mediated resistance to anticancer drugs have been reported by Schecter *et al* (Schecter, et al. 1993). Transfection of MatB rat mammary carcinoma cells with a rat GSTA3 cDNA expression vector conferred up to a 30-, 16-, and 12-fold resistance to chlorambucil, mechlorethamine and melphalan, respectively, over nontransfected cells. The transfected

cells showed up to a fourfold increase in GST activity and increased expression of the GSTA3 protein. Transfection of the rat GSTA2 gene into COS cells conferred between 1.3- and 2.9-fold resistance to chlorambucil and melphalan (Manoharan, et al. 1991, Puchalski and Fahl 1990).

Because of the low level of GST expression and modest levels of resulting drug resistance following transfection of expression vectors into mammalian cells, some investigators have performed similar studies in yeast cells. Transfection of a human GSTP1 cDNA expression vector into *Saccharomyces cerevisiae* cells conferred a 2- to 10-fold resistance to adriamycin and a 2- to 5-fold resistance to chlorambucil. In addition, cells transfected with a human GST alpha cDNA expression vector became resistant to chlorambucil (up to 8-fold) and adriamycin (up to 16-fold) (Black, et al. 1990).

GST-mediated conjugation of anticancer drugs

There has been direct evidence that specific GST isoenzymes can catalyze conjugation reactions with anticancer drugs. In comparison with other GST isoenzymes, kinetic studies have shown that the mu class GSTs are primarily responsible for catalyzing the denitrosation of BCNU and nitrosoguanidinium compounds in rodents (Jensen and Mackay 1990, Smith, et al. 1989). The human GSTM3 isoenzyme has also been shown to catalyze the denitrosation of BCNU in BCNU-resistant NSCLC cell lines (Berhane, et al. 1993).

The best evidence to date that specific anticancer drugs serve as substrates for GSTs comes from the demonstration that various alkylating agents are substrates for alpha GST isoenzymes. Both chlorambucil (Ciaccio, et al. 1990) and melphalan (Hall, et al. 1994) have clearly been shown to be ligands for GST alpha isoenzymes. Mouse and human liver GST alpha and ovarian GSTP1 were demonstrated to conjugate GSH to

chlorambucil, but the alpha isoenzymes had at least a 22-fold greater affinity for the drug (Ciaccio, et al. 1990, Ciaccio, et al. 1991). Mouse liver GST alpha isoenzymes were demonstrated to conjugate melphalan (Bolton, et al. 1991) and purified cytosolic GSTs from Chinese hamster ovary cells, which were made resistant to bifunctional nitrogen mustards, were shown to conjugate melphalan and to be of the alpha class (Hall, et al. 1994). The human GSTA1 and GSTP1 isoenzymes were demonstrated to conjugate thiotepa, a trifunctional alkylating agent (Dirven, et al. 1995a). The cyclophosphamide metabolite acrolein has been shown to be a good substrate for the pi, mu, and alpha human GST isoenzymes (Berhane and Mannervik 1990), whereas conjugation of phosphoramide mustard, another metabolite of cyclophosphamide, was shown to be catalyzed by the human GSTA1 isoenzyme (Dirven, et al. 1994). Finally, conjugation of ifosfamide mustard, a metabolite of ifosfamide, was demonstrated to be catalyzed by the human GSTP1 isoenzyme (Dirven, et al. 1995b).

Inhibition of GST isoenzymes

EA, which acts as both a competitive and a noncompetitive inhibitor of alpha, mu and pi classes of GSTs, has been shown to inhibit the conjugation reaction between chlorambucil and GSH by alpha GST isoenzymes (Ciaccio, et al. 1990), and to potentiate the activity of numerous alkylating agents. EA was shown to increase the efficacy of the drug in previously resistant cells derived from rats (Walker 256 mammary carcinoma) and humans (HT-29 colon carcinoma) (Phillips and Mantle 1991). Sensitization of human colon tumor xenografts and melanoma cells to melphalan was induced by EA treatment (Clapper, et al. 1990, Hansson, et al. 1991). In a more recent study, EA, which inhibited GST activity >85% in human MCF-7 cells and MCF-7 cells resistant to 4-hydroperoxycyclophosphamide (4-HC), could sensitize the resistant but not the parental cells to 4-HC (Cheng and Waxman 1995). Clinically, the clearance of thiotepa in patients

treated with the adjuvant of EA was only half of that obtained in earlier studies using thiotepa alone (O'Dwyer, et al. 1991).

GST POLYMORPHISMS AND SUSCEPTIBILITY TO CANCER

GSTM1 gene

GSTM1 has three alleles, namely M1a, M1b and M1o. Whereas GSTM1a and M1b differ by only one base in exon 7, without apparent effect on the activity of the enzyme, GSTM1o represents a gene deletion and when homozygous, produces a null phenotype (Fryer, et al. 1993). Approximately 50% of the populations have the homozygous null genotype for GSTM1 and therefore lack the GSTM1-1 enzyme in all tissues (Board 1981). However, there are wide variations among different ethnic groups for GSTM1o, ranging from 22% to 100% (Rebbeck 1997).

Epidemiologic studies have suggested a link between the GSTM1 null genotype and the development of certain cancers with the strongest and most consistent relationships observed between smoking-dependent lung and bladder cancer. Benzo(*a*)pyrene is a major component of tobacco smoke and its carcinogenic metabolite, benzo(*a*)pyrene-7,8-diol-9,10-oxide (BDPE), is a good substrate for GSTM1 (Strange and Fryer 1997). In GSTM1-1 null individuals, higher levels of bioactivated metabolites are able to reach the lung, most likely due to the absence of detoxification of carcinogenic metabolites in the liver, where GSTM1-1 is expressed (Hussey and Hayes 1993). Two studies have also found an increased susceptibility to lung cancer when the GSTM1 null phenotype was combined with a specific allele of the cytochrome P450 gene, CYP1A1, and cigarette smoking (Kihara and Noda 1995, Nakachi, et al. 1993).

GSTM3 gene

A potentially even more important role in determining the risk of lung cancer might be played by GSTM3-3 gene, which is expressed in the lungs (Antilla, et al. 1995). Recently, a polymorphism was found in the GSTM3-3 gene as well, resulting from a 3 bp deletion in intron 6 (Inskip, et al. 1995). This mutation appears to be linked to the GSTM1a allele and thus might explain why GSTM1a and GSTM1b may play different roles in cancer susceptibility, although there are no known differences in catalytic activity between these two GSTM1-1 mutants (Widersten, et al. 1991).

GSTT1 gene

In the theta family, GSTT1, like GSTM1, has a null allele that also results from a large deletion and gives a null phenotype in the homozygous state (Pemble, et al. 1994). The population mean for prevalence of the GSTT1 null phenotype is about 20%, and the range among different ethnic groups is from 11% to 58% (Rebbeck 1997). Epidemiologic studies of the GSTT1 null genotype and disease susceptibility are more limited to date than for GSTM1o, but links with astrocytoma, meningioma, and myelodysplasia have been reported (Chen, et al. 1996). An absence of detoxification of ethylene oxide in GSTT1 null individuals has been proposed to explain the higher baseline sister chromatid exchange associated with the null phenotype (Wiencke, et al. 1995).

GSTP1 gene

Two different alleles have been identified for GSTP1-1, P1a and P1b. These alleles differ only by one nucleotide at bp 104, resulting in an amino acid substitution: valine in the GSTP1b mutant enzyme instead of isoleucine in the GSTP1a wild-type enzyme. The GSTP1b allele has been reported to have an altered specific activity and

affinity for electrophilic substrates and was reported to be associated with bladder and testicular cancer (Harries, et al. 1997).

THERAPEUTIC STRATEGIES INVOLVING GST ISOENZYMES

Inhibitors of GST enzymes

The diuretic drug EA has been used experimentally as an adjuvant for cancer chemotherapy, but the diuretic side effects were found to be too severe (O'Dwyer, et al. 1991). To reduce or eliminate the diuretic side effects, a GSH paralog, Ter199 (γ-glutamyl-*S*(benzyl)cysteinyl-*R*-(−)-phenyl glycine diethyl ester) was designed, and shown to be a specific inhibitor of the GSTP1 isoenzyme (Lyttle, et al. 994). Concomitant administration of Ter199 with a range of anticancer drugs has been found to enhance the therapeutic efficacy, both in cultured human tumor cell lines and in rodents (Morgan, et al. 1996a). A particular beneficial pharmaceutical effect of Ter199 is its potential to enhance recovery from myelosuppression in rodents treated with BM suppressing drugs (Broxmeyer, et al. 1996, Morgan, et al. 1996b). For example, rats treated with 5-FU doses that caused significant depletion of both peripheral neutrophils and platelets, recovered faster with daily administration of Ter199. In mice, Ter199 acted synergistically with granulocyte colony stimulating factor (G-CSF) in mobilizing both myeloid progenitor cells and mature neutrophils following drug exposure. In cynomolgus monkeys, Ter199 causes a similar increase in peripherally measured mature white cells. Thus, Ter199 appears to achieve two beneficial goals: sensitization of tumor cells to cancer drugs and protection of normal BM populations from side effects.

Thiol-activated alkylating agents

The elevated levels of GSTs in neoplastic tissue might also be used as an advantage in chemotherapeutic strategies. An interesting example is the development of GSH analogs as prodrugs, such as Ter286 (γ-glutamyl-α-amino-β-[(2-ethyl-*N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate)sulfonyl]-propionyl-(*R*)-(−)-phenylglycine) that release a nitrogen mustard upon activation by the GST P1 and A1 isoenzymes (Lyttle, et al. 1994). Because GST activates Ter286, and elevated GST levels are often found in drug resistant tumors, it was expected that the compound would be more toxic to cell lines that are resistant to conventional agents. This expectation has been confirmed in several cell culture experiments comparing a parental cell line with one selected for resistance to a particular agent, such as cyclophosphamide or adriamycin (Kauvar, et al. 1998). However, since GSTP1 is elevated in most primary biopsy specimens of human tumors, the clinical utility of Ter286 may not be limited to patients who have tumors that have acquired drug resistance. Moreover, the new compound has been found to be effective on tumors for which other drugs had failed, suggesting that Ter286 might have a novel spectrum of activity that would merit its inclusion in drug combinations for first line therapy (Izbicka, et al. 1997). Ter286 has also been tested for its toxicity against mouse BM which was found to be equivalent or less than that of cyclophosphamide (Kauvar, et al. 1998).

Induction of GST enzymes

Induction of phase II detoxification enzymes, including the GSTs, by either naturally occurring or synthetic agents, represents a promising chemoprotective strategy for individuals deficient in the protection afforded by these enzymes. The known chemoprotective activity of cruciferous vegetables in animal models of chemically induced carcinogenesis in combination with their ability to induce detoxification enzymes

led the establishment of a dietary intervention in individuals at increased risk for colorectal cancer (Clapper, et al. 1997). The clinical chemoprotective intervention consisted of comparing the baseline GST activities of blood lymphocytes and colon tissue from high-risk individuals with that of the same tissues two weeks following daily ingestion of concentrated broccoli supplements. Although no significant differences was observed between the GST activities of the control and treated groups following the intervention, data suggested that individuals with a personal history of colorectal cancer might benefit most from supplementation (Clapper and Szarka 1998).

A phase II enzyme inducer that currently shows great promise as a chemoprotective agent is oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione). The strongest molecular evidence available for the chemoprotective activity of oltipraz has been provided by studies which demonstrate its effectiveness in inhibiting DNA adduct formation. Oltipraz administration decreased the DNA adducts present in the liver tissue of AFB_1-treated rats by 76% (Kensler, et al. 1985), by virtue of its induction of GSTA5, by far the most effective enzyme for the detoxification of the carcinogenic metabolite AFB-*exo*-8,9-oxide (Raney, et al. 1992). Moreover, pretreatment of MCF-7 cells with oltipraz produced a 95% reduction in the formation of dibenz[a,l]pyrene adducts (Smith, et al. 1997). The ability of oltipraz to inhibit chemically induced colon cancer has also been established. Administration of oltipraz to rats 2 weeks prior to azoxymethane exposure and for the duration of the study reduced the incidence of colon and small intestinal tumors, by approximately 33% (Rao, et al. 1991). Oltipraz completely suppressed the formation of invasive carcinomas and significantly inhibited the multiplicity and incidence of non-invasive adenocarcinomas when drug exposure was confined to the period following tumor initiation (Rao, et al. 1993). Chronic exposure of rats to oltipraz beginning 3 days following azoxymethane treatment produced more than 93% reduction in tumor volume. These data suggest that in addition to its role as an anti-

initiator, oltipraz may also inhibit tumor progression. Confirmation of the effectiveness of oltipraz against established tumors may lead to its extended use as a chemotherapeutic agent for the treatment of colorectal cancer.

Engineering GST enzymes

A number of laboratories are investigating ways of developing new GST enzymes with different and perhaps more desirable catalytic properties. Such studies generally involve the generation of a library of random mutants followed by the screening of the library toward a particular substrate or active site ligand. These studies, which are just beginning, hold considerable promise for evolving catalysts with new and interesting properties. For example, by using random mutagenesis of the region encoding residues 10 to 15 of the rat GSTA3-3, Gulick and Fahl have identified mutant GSTs that conferred up to 10-fold increased resistance toward mechlorethamine relative to the wild-type GST (Gulick and Fahl 1995).

HIGH-DOSE THERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION

The indications for intensive chemotherapy followed by stem cell transplantation have greatly expanded in recent years. Worldwide, more than 30,000 stem-cell transplants are performed each year, the great majority of which are intended to treat patients with malignant disease (Applebaum 1997). Autologous stem cell transplants (ASCT) circumvent the need for matched—related or unrelated—BM donors and thus allow a more widespread utilization of the procedure. Stem cells are harvested after remission has been obtained by conventional therapy. The cells are usually then cryopreserved and reinfused after the patient has received myeloablative chemotherapy. The virtually complete absence of graft-versus-host disease, the rarity of engraftment failure, and the significantly lower incidence of cytomegalovirus infection make ASCT a relatively safe procedure (Storb, et al. 1989, Wingard, et al. 1988). In contrast, the possibility of malignant contamination of the autograft and the lack of graft-versus-tumor effect represent the most important drawbacks of autotransplantation (Horowitz, et al. 1990).

RATIONALE FOR THE USE OF HIGH-DOSE CHEMOTHERAPY

The justification for high-dose chemotherapy (HDC) is based on the correlation between the treatment dose and the degree of tumor kill, which is both tumor- and agent-specific. Since a portion of the dose-response curve is often a linear-log, a small increase in the dose may result in a large increase in tumor cell kill even in tumors resistant to conventional-dose therapy. As such, the dose-limiting toxicity of the agents used should be hematopoietic. Because of this, and the steep dose-response observed with alkylating

agents, these drugs have served as the foundation for all transplant regimens (Waselenko, et al. 1999).

MOBILIZATION AND EXPANSION OF HEMATOPOIETIC STEM CELLS

Hematopoietic cell phenotypes

Both stem cells and lineage-committed hematopoietic progenitor cells (HPCs) express the CD34 antigen (Krause, et al. 1996), though there are recent reports that hematopoietic stem cells (HSCs) may also reside outside of the $CD34^+$ cell compartment (Goodell, et al. 1997). $CD34^+$ cells constitute 1-5% of cells in adult BM and 5-10% in fetal BM. Primitive stem cells, however, lack the differentiation antigens that are present on lineage-committed progenitors and are thus $CD38^-$, $CD45^{lo}$, and $CD71^{lo}$ (Craig, et al. 1993). HLA-DR is absent or is expressed at low levels on adult stem cells but is present on fetal and neonatal HSCs (Lansdorp, et al. 1993). The Thy-1 antigen is present on all human fetal and neonatal hematopoietic cells but is only expressed on a proportion of lineage-committed progenitors in the adult (Craig, et al. 1993). Peripheral blood progenitor cells are $CD34^+$ cells and are equivalent to their marrow counterparts, i.e. they have the same capacity to form unilineage and multilineage or blast cell colonies (Brand, et al. 1988).

Hematopoietic growth factors

Interleukin-3 (IL-3) is the major multilineage stimulator with the ability to stimulate the growth of myeloid progenitors belonging to all lineages (McNeice, et al. 1989). IL-3 also induces the differentiation of myeloid stem cells and the maturation of macrophages, eosinophils and basophils *in vitro* (Lopez, et al. 1987). *In vivo*, IL-3

administration results in a multilineage response with increases in neutrophils, platelets, and reticulocytes (Lindemann, et al. 1991).

G-CSF is a lineage specific cytokine whose effect is restricted to stimulating the proliferation of colonies consisting primarily in neutrophils (Souza, et al. 1986). G-CSF also stimulates *in vitro* the biological activities of mature neutrophils such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, superoxide generation, and chemotaxis (Avalos, et al. 1990). *In vivo*, G-CSF induces a dose-dependent increase in blood neutrophils and the release of hematopoietic precursors into the blood stream (Okabe and Takaku 1986).

Granulocyte monocyte colony-stimulating factor (GM-CSF) is a cytokine acting at several levels of hematopoiesis. *In vivo*, GM-CSF stimulates the proliferation and differentiation of granulo-monocytic precursors inducing neutrophilia and monocytosis; it prolongs the half-life of mature circulating cells; recruits the non-circulating pool of neutrophils and activates several granulocyte functions (Aglietta, et al. 1990). GM-CSF also induces the release of hematopoietic precursors into the blood stream and increases the proliferative activity of erythroid and megakaryocytic precursors (Aglietta, et al. 1995).

Stem cell factor (SCF), also known as c-kit ligand, has a very low potential in stimulating proliferation of HSCs by itself. However, it synergizes with a large number of cytokines (IL-3, GM-CSF, G-CSF, erythropoietin, etc) to stimulate pluripotent stem cells, possibly acting as a survival factor (Berardi, et al. 1995). It exerts action on all myeloid pathways. *In vivo* SCF administration leads to an increase in both differentiated and primitive HPCs within the marrow (Hoffman, et al. 1993).

Flt-3 ligand (FL) is a class III tyrosine kinase receptor expressed on primitive human and murine HPCs (Matthews, et al. 1991). Similarly to SCF, FL has been shown to support the growth of HPCs and HSCs. In particular, FL induces the proliferation of

quiescent totipotent ($CD34^+$, $CD38^-$) BM cells (Shah, et al. 1996). FL causes a significant amplification of the numbers of these cells *in vitro* while this effect is not exhorted by SCF (Gabbianelli, et al. 1995).

Thrombopoietin (TPO) stimulates the proliferation and survival of megakaryocyte development. TPO has also been shown to act synergistically with erythropoietin to expand erythroid progenitors after myelosuppressive therapy (Kaushansky, et al. 1995). Recently, TPO has been reported to support the proliferation of primitive hematopoietic cells in synergy with IL-3, SCF and FL (Kobayashi, et al. 1996, Petzer, et al. 1996).

Mobilization of hematopoietic stem cells

Stem cell mobilization occurs after the administration of myelosuppressive chemotherapy. During hematopoietic recovery, there is up to a 50-fold increase in peripheral blood granulocyte-monocyte colony-forming unit (CFU-GM). The alkylating agent cyclophosphamide is the best documented and most widely used drug for HSC mobilization. Several clinical trials have demonstrated that the addition of hematopoietic growth factors results in an increased progenitor cell yield while myelotoxicity and its clinical sequelae are reduced (Haynes, et al. 1995). At present, G-CSF is the most commonly used growth factor. G-CSF increases the $CD34^+$ cell yield 4- to 6-fold in patients following high-dose cyclophosphamide (Schwartzberg 1993). GM-CSF is similar in its mobilizing activity when given following chemotherapy but is less commonly used due to the more frequent adverse events such as fever, bone pain, and hypoxemia (Bolwell, et al. 1994). Several clinical studies have shown an increase in the $CD34^+$ cell yield when SCF was given together with G-CSF (Basser, et al. 1995). Hematopoietic growth factors can also be given alone to mobilize HSCs.

Expansion of hematopoietic stem cells

There has recently been great interest in the *ex vivo* expansion of hematopoietic stem and progenitor cells for a variety of applications, including the reduction of the period of pancytopenia following myeloablative chemotherapy, the reduction of the number of HSCs required for transplantation, the expansion of selected subsets of HPCs, and the reduction of malignant cell contamination of HSCs (Pettengell and Moore 1998). Many different systems have been developed to expand HSC numbers *ex vivo* using defined media containing combinations of growth factors. Most of the developed cell expansion protocols essentially conform to one of two methods: the culture of naïve unpurified cell populations at high density in perfused systems, or the culture of enriched HSC populations at low density in static systems (Mandalam, et al. 1999). When combinations of SCF, IL-1, IL-3, GM-CSF, and erythropoietin are added to perfusion or bioreactor cultures of tumor BM, it is possible to achieve a 4- to 8-fold stimulation of long-term culture-initiating cells (LTC-ICs) (Koller, et al. 1993). Unfortunately, however, many of the growth factors intended only to induce cycling and expansion of marrow stem cells may also induce their phenotypic differentiation and lineage commitment, suggesting that they have lost their self-renewal capacity (Emerson, et al. 1995). The clinical use of growth-factor combinations that expanded committed progenitor cells, but also produced lineage commitment in pluripotent progenitors, might therefore produce the combination of early engraftment and late graft failure.

AUTOLOGOUS STEM CELL TRANSPLANTATION

Sources of hematopoietic stem cells

Aspirated BM was initially used for hematopoietic reconstitution following myeloablative therapy. However, rapid and sustained hematopoietic recovery occurs after autologous peripheral blood stem cell (PBSC) transplantation, with a shorter period of thrombocytopenia, thus improving the risk/benefit ratio of the procedure compared with BM transplantation (Schmitz, et al. 1996). Other advantages of PBSC transplantation include the avoidance of a general anesthetic for BM harvesting and the ability to offer HDC to patients with BM involvement by tumor. For these reasons, PBSC have replaced BM as a source of HSCs for transplantation in many medical centers, and high-dose treatments are now being offered to a wider range of patients. There are, however, no prospective randomized trials showing improved disease outcome in patients treated with peripheral blood progenitor cell transplantation over BM transplantation (Pettengell and Moore 1998).

Malignant contamination of autologous grafts

There is considerable concern about possible tumor contamination of HPCs collected in cancer patients. Marking studies were performed to evaluate the potential of tumor cells in BM grafts to contribute to relapse in children with malignancies who received autologous BM transplantation as part of their therapy. Genetically modified tumor cells were detected after relapse in children with neuroblastoma (Rill, et al. 1994) or acute myelogenous leukemia (Brenner, et al. 1993), unequivocally establishing that contaminating tumor cells in marrow can contribute to tumor relapses and had to be removed if the transplant procedure was to be therapeutically successful. Similar trials have now been performed in adults with myeloid leukemia (Cornetta, et al. 1996,

Deisseroth, et al. 1994), breast cancer, or multiple myeloma (Dunbar, et al. 1995). Only in chronic myelogenous leukemia have marked tumor cells been documented to contribute to subsequent relapses (Deisseroth, et al. 1994). Since most solid tumors do not express CD34, the selection of $CD34^+$ cells has been used to reduce tumor cell contamination by a factor of $10\text{-}10^4$, using immunomagnetic beads or biotin-avidin columns (Farley, et al. 1997, Shpall, et al. 1994). However, studies are required to examine the clinical efficacy of tumor cell purging methods.

Hematological malignancies

ASCT is now a recognized therapeutic option for acute myelogenous leukemia therapy. Prospective randomized studies have shown its superiority over conventional chemotherapy, with the possible reservation that none has tested high-dose Ara-C in a parallel arm (Gorin 1998). In contrast, there appears to be no advantage to ASCT for acute lymphoblastic leukemia over chemotherapy for consolidation of either high-risk or standard-risk patients in first complete remission (Finiewicz and Larson 1999).

At present, allogeneic progenitor cell transplantation provides the only curative therapy for patients with chronic myelogenous leukemia. ASCT is not indicated for chronic myelogenous leukemia because of a lack of graft-versus-tumor effect and is a reasonable approach only for chronic-phase patients lacking an allogeneic donor (Hahn and To 1999). Several phase II studies have also demonstrated the feasibility of performing ASCT in patients with chronic lymphocytic leukemia. However, while a high rate of complete responses is induced, a number of studies have reported a continuous risk of relapse in chronic lymphocytic leukemia patients treated with this modality (Waselenko, et al. 1999).

Hodgkin's disease patients with short initial remissions appear to have a clear survival advantage with HDC and autografting, with a low peritransplant mortality rate

when compared with salvage chemotherapy alone (Marshall and DeVita Jr 1999). In contrast, although disease-free survival may be prolonged in patients with relapsed or refractory low-grade non-Hodgkin's lymphoma by transplantation, it is unknown presently if overall survival is prolonged (Morrison and Peterson 1999).

In patients with multiple myeloma, ASCT does not produce a cure, and virtually all patients will relapse and have progression of their disease. However, it appears that event-free survival and overall survival may be prolonged with this approach (Kyle 1999).

Solid tumors

Breast cancer is the most common solid tumor for which HDC and ASCT is performed (Antman, et al. 1997). A number of studies have shown that HDC with ASCT results in greater cytoreduction in patients with chemotherapy-sensitive metastatic breast cancer than what is achieved with standard-dose chemotherapy, resulting in higher overall response and complete response (CR) rates. However, despite high CR rates, the survival benefit of this procedure remains unproven in either metastatic or high-risk primary breast cancer. From the few randomized trials reported in patients with metastatic or high-risk stage II and III breast cancer, no definitive conclusions can be drawn. Thus, despite the widespread use of HDC with ASCT, its indications and role in the treatment of breast cancer and potential benefit to patients are not clear (Rahman, et al. 1998).

HDC supported by ASCT is also being investigated in women with ovarian carcinoma. There is some hint of survival benefit in certain populations, such as women with limited disease or perhaps in women with more advanced disease who have not been previously treated. However, randomized trials are needed to determine whether this approach provides any advantage over standard-dose chemotherapy (Herrin and Thigpen 1999).

Finally, HDC with ASCT is being investigated in the setting of poor-prognosis or relapsed germ cell tumors (GCT). From the trials performed to date, it appears that early use of this modality results in the best outcome for poor-prognosis GCT patients. However, here also, randomized trials are needed to compare this approach and conventional dose chemotherapy (Sobecks and Vogelzang 1999).

HEMATOPOIETIC CHEMOPROTECTION

RATIONALE FOR HEMATOPOIETIC CHEMOPROTECTION

Myelosuppression and the consequent risks of infection and bleeding represent the predominant dose-limiting toxicity of many chemotherapeutic agents. While current clinical strategies for attenuating drug-induced myelosuppression are clearly useful, they are associated with a number of limitations. One strategy that is widely used in clinical oncology is the use of hematopoietic growth factors such as G-CSF. One disadvantage is that these growth factors usually affect only a single hematopoietic lineage, so that severe cytopenias in other lineages may still occur. Second, because administration of growth factors acts by hastening hematopoietic regeneration, cytopenic nadirs are not eliminated or diminished in magnitude, but simply shortened in length. A second strategy for overcoming dose limitations is the use of ASCT. While this approach allows high doses of myelosuppressive drugs to be given over several days, HDC is generally administered only once and subsequent chemotherapy is usually poorly tolerated. For these reasons, ASCT does not enable significant dose intensification over time (Sorrentino and Nienhuis 1999).

The transfer of drug resistant genes to HSCs circumvents many of these limitations (Sorrentino 1996). Transplantation of pluripotent stem cells that have been transduced with a resistance gene may protect all hematopoietic lineages. Because stem-cell progeny are protected at all developmental stages, cytopenic nadirs may be completely eliminated. This hematopoietic protection may be durable with repeated drug-treatment cycles, due to the long-lived nature and self-renewal capacity of stem cells. However, it may not be necessary to target the most primitive, long-term repopulating stem cells since early, non stem cell progenitors can maintain hematopoiesis for

prolonged periods of time. This suggests that if a clone of progenitor cells is rendered drug resistant, it may contribute to hematopoiesis for an extended period, particularly under selection pressure during drug therapy (Koç, et al. 1999a). In addition, because drug-resistant hematopoiesis may be established after engraftment, post-transplant dose intensification may be possible (Spencer, et al. 1996). Finally, since chemotherapy with alkylating agents, epipodophyllotoxins and anthracyclines is associated with a significant risk of myelodysplasia and secondary leukemia in long-term cancer survivors, overexpression of drug-resistance proteins may decrease the mutagenic and carcinogenic effects of antineoplastic agents (Koç, et al. 1999a). These advantages all have been validated in murine animal model studies, where the efficiency of stem-cell transduction is not limiting.

However, even if drug resistance gene transfer can be shown to successfully protect cells *in vivo*, there are a number of potential pitfalls. It is now clear—in part from gene transfer studies—that stem cell populations may be contaminated by tumorigenic malignant cells (Rill, et al. 1994). Drug resistance genes may therefore be transferred to tumor cells that contaminate the marrow graft and produce a drug-resistant phenotype relapse. Second, the toxicity to non-protected organs, including gut, heart, and lungs, may rapidly supervene when marrow resistance allows intensification of cytotoxic drug dosages, so that there may be little overall benefit (Hanania, et al. 1996). Finally, drug-resistant tumor cells may have an abnormally high threshold for the engagement of apoptosis (programmed cell death) resulting from mutations of tumor suppressor genes such as p53 and bcl-2, thus precluding the elimination of malignant cells (Dive 1997).

HEMATOPOIETIC STEM CELLS AS VEHICLES OF GENE TRANSFER

HSCs are rare cells found in the peripheral blood, cord blood, and BM, where they represent about 1 in 10^4 to 10^5 of the population. They are self-renewing and capable of differentiating into progeny that multiply to become the erythroid, lymphoid, and myeloid components of the blood (Wilke and Mulé 1999). In addition to their property of pluripotency, it has been shown that, *in vivo*, only a minor proportion of HSCs are cycling, and relatively small numbers of cycling stem cells contribute to the process of steady-state hematopoiesis (Dunbar, et al. 1995). Because of their potential to self-renew and generate a large population of progeny cells with new genetic material, HSCs are attractive targets for gene therapy.

RETROVIRAL VECTORS AND GENE TRANSFER INTO MURINE HEMATOPOIETIC CELLS

Retrovirus-mediated gene transfer is the most widespread method for transfer of therapeutic genes into somatic cells (Miller 1992). A number of retroviral vectors have been developed for gene transfer into human hematopoietic cells. They are generally based on the Moloney murine leukemia virus (MoMLV) backbone. Retroviral vectors are packaged into infectious particles after introduction into a retroviral packaging cell line (Miller, et al. 1993). A typical packaging cell is a cell that contains a modified retroviral genome that retains the structural genes (*gag*, *pol*, and *env*) but lacks the packaging signal (psi). Psi is required for efficient encapsidation of the vector RNA into virions (Bender, et al. 1987). The engineered retroviral vector, which is introduced into the packaging cell, is in effect the complement to the packaging cell genome and retains the psi encapsidation

and replication signals but is devoid of the structural genes. A packaging cell line to which a retroviral vector has been added is defined as a producer cell line (Morgan and Anderson 1993). The producer cell line effectively generates replication-incompetent retroviral vectors that are capable of only one round of infection. The use of split function packaging cell lines in which the *gag* and *pol* genes were encoded on a separate transcriptional unit from that which encoded the envelope gene has reduced the risk of generation of replication-competent retroviruses by homologous recombination within vector-producing clones (Markowitz, et al. 1988).

Retroviral transduction is usually performed in the presence of a polycation (e.g. polybrene™ or protamine sulfate) to reduce electrostatic interference between the virus and its target cell. Particle uptake is initiated by the interaction of the viral envelope protein with cell surface glycoproteins that act as a receptor. Different classes of viruses use different proteins for receptors. Ecotropic viruses that infect murine and other rodent cells utilize a cationic amino acid transporter, whereas amphotropic viruses, which have a broader host range that includes human cells, utilize a sodium-dependent phosphate transporter (Miller and Chen 1996). After fusion with the cell membrane glycoprotein, the virus enters the cell, where its single-stranded RNA genome is reverse transcribed into double-stranded DNA. This DNA copy is transported to the nucleus, where it is integrated into the host genome to form the provirus (Miller 1992). The provirus is then transcribed by the host cell transcriptional machinery into RNA that becomes genomic viral RNA or is multiply spliced to produce subgenomic messages from which viral proteins are produced.

The advantages of retroviral vectors are the high transfer efficiencies, precise and stable integration into the host cell genome, and the lack of further retroviral production after infection. Disadvantages include the inability to infect nondividing cells (Miller, et al. 1990), potential insertional mutagenesis, which may cause activation of cellular

oncogenes or gene inactivation (Boris-Lawrie and Temin 1994), and the possibility of recombination to produce replication-competent retrovirus. The size of the gene insert is also limited as retroviruses are only capable of packaging around 10 kb of genetic material (Naviaux and Verma 1992).

Transduction in murine hematopoietic systems is clearly facilitated by the addition of early acting hematopoietic cytokines. The use of IL-3 and IL-6 (Bodine, et al. 1989) and later SCF (Luskey, et al. 1992) was shown to enhance mouse stem cell transduction, presumably by supporting stem-cell viability and inducing cell cycle progression. Similarly, coculture of murine BM with vector-producing cells increases transduction efficiencies severalfold (Bodine, et al. 1991). Recent work has documented expansion of murine stem cells in serum-free medium containing SCF, FL and TPO (Miller and Eaves 1997). 5-FU is also routinely given 48 hours before marrow harvest from syngeneic murine BM donors because it is known to deplete the hematopoietic tissues of cycling cells and to initiate the activation of otherwise quiescent stem cells (Bodine, et al. 1991).

DEVELOPMENT AND USE OF LARGE-ANIMAL MODELS

The early successes achieved in murine models stimulated an interest in developing large-animal models to test the emerging principles for stem-cell-targeted gene transfer. Nonhuman primates including rhesus and cynomolgus macaque monkeys, as well as baboons, dogs, and sheep have been used for these studies. Early results in these animals indicated that gene-transfer efficiency, as reflected by the frequency of genetically modified cells in peripheral blood, was far lower than that achieved in the murine models. The percentage of peripheral blood cells that contained the proviral genome was generally less than 1% or 2%. The refractoriness of the stem cells in larger

animals to retrovirus-mediated gene transfer was thought to be due, at least in part, to their quiescent status (Sorrentino and Nienhuis 1999).

Early studies in primate species supported the safety of retroviral vectors for gene transfer. None of the animals that were utilized in the early experiments experienced untoward effects that could be attributed directly to the gene-transfer procedure. Injections of large amounts of replication-competent retrovirus into immunosuppressed animals failed to result in any significant disease manifestations or tissue pathology (Cornetta, et al. 1990, Cornetta, et al. 1991). However, the development of aggressive lymphomas in rhesus monkeys that had undergone complete ablation of the hematopoietic system by external irradiation and then received stem cells exposed to vector preparations contaminated by replication-competent retroviruses provided the first evidence of the pathogenicity of MLV in primates (Vanin, et al. 1994).

RETROVIRUS-MEDIATED GENE TRANSFER INTO HUMAN HEMATOPOIETIC CELLS

The success of gene transfer in the murine system has led to studies in humans. Although *in vitro* assays such as LTC-ICs have demonstrated relatively high transduction levels, the percentage of transduced cells engrafting in the BM as well as the numbers of marked cells in the peripheral blood of patients remain disappointingly low. One of the difficulties in translating results from the murine system to humans has been developing similar protocols for clinical implementation. For example, the high levels of gene transfer routinely reported for murine progenitors were often achieved by cocultivation of target cells with retroviral producers. Although transduction by cocultivation has been shown to be superior to cell-free viral supernatant (Havenga, et al. 1997), there is a risk of

contamination of cellular infusions with producer cells, which has been considered undesirable for clinical application (Gordon 1988).

In addition to low levels of gene transfer *in vitro* contributing to poor gene expression *in vivo*, it is also possible that transduced cells may be immunogenic. For example, transferred herpes simplex virus thymidine kinase (HSV-TK)-modified cytotoxic lymphocytes were eliminated after 5-8 weeks by a host-immune response to the foreign transgene product (Riddell, et al. 1996). Therefore, even though retroviral vectors themselves are relatively nonimmunogenic, cells expressing their transgene may be cleared by the host's immune system.

Altering the retroviral tropism by pseudotyping with an *env* gene derived from the gibbon ape leukemia virus (GALV) or the G protein of vesicular stomatitis virus (VSV-G) has been successful in increasing gene transfer into human HSCs (von Kalle, et al. 1994, Yang, et al. 1995). The vast distribution of the receptors for the VSV-G proteins appears to increase the vector host range. In addition, the stability of VSV-G allows pseudotyped particles to be concentrated by ultracentrifugation to titers that are higher than those of amphotropic vectors (Friedmann and Yee 1995). Recent studies have also shown improved transduction of human HSCs in plates coated with a fragment of the fibronectin molecule (Traycoff, et al. 1997).

Most clinical protocols include overnight preculture in media supplemented with IL-3, IL-6 and SCF to induce cycling. A significant concern is that this growth factor combination will act primarily to differentiate cells, not to promote self-renewal divisions of HSCs. Recent studies have shown that FL, SCF, and thrombopoietin-megakaryocyte growth and development factor (MDFD) are capable of expanding progenitor cell numbers while maintaining the reconstituting ability of these cells (Rasko, et al. 1997, Yonemura, et al. 1997).

In vivo selection is a promising strategy for overcoming the current limitations imposed by low stem cell transduction efficiencies with retroviruses. In this approach, a gene that confers a dominant selective advantage on transduced cells is incorporated into the vector, most commonly a gene conferring resistance to a cytotoxic drug. Recipients that are chimeric for transduced and unmodified hematopoietic cells are then treated with the relevant drug. This drug treatment may cause enrichment and expansion of the subset of hematopoietic progenitors and stem cells that express the transferred dominant selectable marker. Repetitive courses of drug selection would then cause the progressive enrichment of vector-expressing cells (Sorrentino and Nienhuis 1999).

ALTERNATIVE VECTOR SYSTEMS FOR STEM-CELL-TARGETED GENE TRANSFER

Lentiviral vectors

Human immunodeficiency virus (HIV), a lentivirus, has the capacity to infect quiescent cells productively (Naldini, et al. 1996). In contrast to the nucleoprotein, preinitiation complex of MLV, which requires dissolution of the nuclear membrane during mitosis to access cellular DNA, the preinitiation complex of HIV is transported through the nuclear membrane by virtue of nuclear localization signals present in the p19 matrix (MA) and Vpr accessory proteins (Bukrinsky, et al. 1993, Heinzinger, et al. 1994). Either signal will suffice to allow HIV to infect quiescent cells. Another key property of lentiviruses with respect to their ability to infect nondividing cells is their ability to form a relatively stable transcription intermediate (Zack, et al. 1992). In contrast, the MLV nucleoprotein complex disappears within 24 hours of infection of nondividing cells (Naldini, et al. 1996). A packaging genome encoding the viral Gag and Pol proteins as

well as the accessory proteins under the control of heterologous transcriptional control elements is used to generate infectious vector particles. HIV particles are readily pseudotyped with either the MLV amphotropic or VSV-G proteins. Elimination of nonessential elements, use of split packaging genomes, and the avoidance of overlap between vector and packaging sequences are steps that have been taken to improve the safety of HIV vectors. In fact, the vector preparations in current use may readily be shown to be devoid of replication-competent, infectious HIV particles.

Preliminary data suggest that human hematopoietic cells can be efficiently transduced with VSV-G pseudotyped HIV vectors. In one study, transduction of quiescent, $CD34^+$ cells with vector particles followed by culture in cytokines (IL-3, IL-6, and SCF) for 3 days resulted in expression of the HIV-vector-encoded marker protein in more than 80% of viable cells (Reiser, et al. 1996). Remaining to be determined is whether HIV vectors will transduce primitive cells capable of long-term hematopoietic reconstitution in an immunodeficient mouse model or a nonhuman primate.

Adeno-associated virus vectors

Adeno-associated virus (AAV) is a replication defective, nonpathogenic parvovirus that depends on a helper virus, either adenovirus or herpesvirus, for virus production during lytic infection (Berns and Giraud 1996). AAV particles are relatively stable, permitting their purification and concentration by physical techniques. AAV has a broad host range and does not require target cells to proliferate for successful infection (Kotin 1994). Infection of susceptible cells by wild-type AAV in the absence of a helper virus often results in the integration and persistence of a latent genome. However, no AAV vectors have yet been manufactured which maintain this capability completely (Ponnazhagan, et al. 1997a). As a gene transfer vector, AAV can accommodate about 4.7 kb of DNA insert. This size restriction limits the potential cDNA that can be employed as

a therapeutic (Muzyczka 1992). Another disadvantage of this delivery system includes difficulty in purifying the vector from contaminating wild-type AAV and helper virus during vector preparation.

There is unequivocal evidence that AAV vectors are able to transduce human clonogenic hematopoietic progenitors, although high multiplicities of infection are required, particularly when purified vector particles are used (Walsh, et al. 1994). Transduction and integration in human progenitors and LTC-IC by AAV vectors have been reported, as has the transduction of murine repopulating stem cells (Fisher-Adam, et al. 1996, Ponnazhagan, et al. 1997b). However, attempts to achieve AAV vector-mediated gene transfer into rhesus repopulating cells have been unsuccessful (Sorrentino and Nienhuis 1999). Thus, the utility for this vector system for stem-cell-targeted gene transfer remains uncertain.

TRANSFER OF DRUG RESISTANCE GENES TO HEMATOPOIETIC CELLS

Multidrug transporter P-glycoprotein

The multiple drug-resistance gene 1 (MDR1) encodes the 170-kDa P-glycoprotein (Pgp), an ATP-dependent transmembrane efflux pump for a diverse group of lipophilic compounds. Pgp consists of two intracellular ATP-binding domains and two transmembrane domains that span the membrane multiple times, forming the channel used for translocation of the cytotoxic agents. Substrates for Pgp include a variety of drugs that are typically cationic, weakly basic, and hydrophobic but are structurally unrelated. These include the anthracyclines, the vinca alkaloids, the epipodophyllotoxins, actinomycin D, and paclitaxel. Physiologic and pharmacological studies of MDR mutant cell lines have revealed that the Pgp-mediated mechanism of resistance is reduced drug

accumulation within the cell (Gottesman and Pastan 1993). Both increased efflux and decreased influx of drug have been demonstrated. A variety of other compounds are competitive inhibitors of MDR1; examples are verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressant (Cano-Gauci and Riordan 1987). MDR1 is normally expressed on the apical or lumenal surface of secretory cells in the adrenal glands, liver, colon, and kidney (Fojo, et al. 1987). Most relevant for gene therapy, MDR1 is expressed in hematopoietic cells, and the highest level of expression occurs in the most immature subsets of $CD34^+$ precursors (Chaudhary and Roninson 1991).

The first evidence that the MDR1 gene could be used for hematopoietic protection came from *in vitro* studies of retrovirally transduced murine myeloid progenitors (McLachlin, et al. 1990) and from studies in MDR1 transgenic mice (Mickisch, et al. 1991). This protective capacity was later confirmed in mice that were transplanted with retrovirally transduced stem cells (Aksentijevich, et al. 1996). A landmark MDR1 gene transfer study demonstrated *in vivo* selection of MDR1-transduced cells in mice treated with paclitaxel (Sorrentino, et al. 1992). Long-term expression of human MDR1 in repopulating stem cells was demonstrated in mice by serially transplanting MDR1-transduced BM cells in six successive cohorts of mice with paclitaxel selection (Hanania and Deisseroth 1994). Each successive transplant produced recipient mice with greater resistance to paclitaxel than donor mice. MDR1 expression was observed 17 months after the initial transduction, implying that continuous expression can be achieved during *in vivo* selection. In addition to the use of BM-derived cells as targets for gene transfer, MDR1 transduction has been achieved in murine and human hematopoietic cells mobilized from peripheral blood (Ward, et al. 1994), human cord blood (Bertolini, et al. 1996), and fetal liver cells (Richardson and Bank 1995). A model of simultaneous chemoprotection of BM and chemosensitization of tumor cells was also described by Hanania *et al* (Hanania and Deisseroth 1997). In this animal model, nude mice were

implanted with tumor cells transfected with a p53 expression vector and transplanted with MDR1-transduced hematopoietic cells. The simultaneous use of both chemoprotection and chemosensitization vectors, which provided protection of the normal cells to high doses of paclitaxel and at the same time sensitized the tumor cells to the toxic effects of the drug, resulted in levels of *in vivo* tumor reduction that were not possible when either genetic chemoprotection of marrow cells or chemosensitization of tumor cells was used alone. Finally, it was recently demonstrated that retroviral expression MDR1 can alter the response of murine HSCs to cytokine stimulation *in vitro* (Bunting, et al. 1999). Lethally irradiated mice were transplanted with very small numbers of MDR1-transduced murine stem cells that had been previously expanded *ex vivo* for 15 days with IL-3, IL-6 and SCF. Long-term reconstitution with 100% donor cells was seen in all mice injected with MDR1-transduced cells. However, the transplanted mice developed a myeloproliferative disorder characterized by an abnormal increase in peripheral blood leukocytes. Thus, MDR1-transduced stem cells can be expanded *in vitro* with hematopoietic cytokines, but the increased stem cell division frequency can lead to stem cell damage.

Several clinical trials have been initiated to test the feasibility of transferring MDR1 retroviral vectors into reconstituting hematopoietic cells obtained from cancer patients. In a trial at M.D. Anderson Cancer Center, 20 patients with either ovarian or breast cancer were transplanted with MDR1-transduced $CD34^+$ cells obtained from either BM or peripheral blood, respectively (Hanania, et al. 1996). Three to four weeks after transplant, MDR1-transduced BM cells were detected in 5 of 8 patients who received cells transduced on stromal monolayers. The frequency of transduced cells in these patients was about 1 in 1000. No marked cells were seen in any of the 10 patients who received cells transduced in suspension without stromal cell support. A second study at Columbia University has yielded similar results (Hesdorffer, et al. 1998). Some of these patients have been treated with taxol, but no enrichment in MDR1-modified cells has

been seen (Sorrentino and Nienhuis 1999). The small numbers of MDR1-transduced hematopoietic cells seen in these initial clinical trials will not be sufficient to attenuate the myelosuppression. It is clear that improved gene-transfer protocols will be necessary to confer significant protection. One area that can be optimized is the stability of the vector genome. The MDR1 cDNA is prone to rearrangements due to the presence of cryptic RNA splicing sites within the coding sequence (Sorrentino, et al. 1995). The availability of alternate MDR1 cDNAs that yield more stable retroviral vectors should provide an advantage in this regard (Galipeau, et al. 1997).

Dihydrofolate reductase mutants

Dihydrofolate reductase (DHFR) is a 22-kDa enzyme that catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of dihydrofolate to tetrahydrofolate, an essential carrier of one-carbon units in the biosynthesis of thymidylate, purine nucleotides, and methyl compounds (Blakley 1984). Antifolates such as methotrexate (MTX) and trimetrexate (TMTX) are folic acid analogs that bind tightly to the active site of DHFR, inactivate the enzyme, and therefore inhibit the generation of reduced folate (Werkheiser 1961). MTX but not TMTX is converted to polyglutamates after entry into the cell and inhibits enzymes in the purine nucleotide pathway other than DHFR, such as thymidylate synthase and transformylases. MTX resistance can originate from amplification of the wild-type DHFR gene (Alt, et al. 1978) or from mutations that either reduce MTX transport into the cell (Sirotnak, et al. 1968) or diminish the affinity of DHFR to MTX (Flintoff, et al. 1976). Quiescent HSCs are thought to be resistant to MTX because of the low requirement for reduced folate and the lack of DNA synthesis. Normal hematopoietic progenitors can also escape from the cytotoxicity of antifolates by importing thymidine and hypoxanthine from serum (Koç, et al. 1999a). In addition to the wild-type DHFR, several naturally occurring mutations of

DHFR have been characterized. A DHFR molecule with arginine substituted for leucine at position 22 (L22R) was isolated and was shown to have 1/270 the binding affinity for MTX with only a threefold decrease in dihydrofolate catalysis (Simonsen and Levinson 1983).

The first demonstration of the potential of DHFR for protection of the hematopoietic system was obtained in mice transplanted with L22R DHFR-transduced marrow (Williams, et al. 1987). Whereas MTX treatment was lethal to mice transplanted with cells transduced with a neomycin resistance gene, MTX-treatment of mice transplanted with L22R DHFR-transduced cells was associated with a significant increase in survival. Subsequent studies in murine models confirmed the ability of DHFR gene transfer to protect hematopoiesis from various antifolates and have shown that this protection confers tolerance to otherwise lethal drug doses (Corey, et al. 1990, Li, et al. 1994). However, subsequent MTX treatment failed to demonstrate convincing selection of L22R DHFR-transduced myeloid cells. Although a later study suggested that MTX could be used for selection of DHFR-transduced myeloid progenitors (Zhao, et al. 1994), this result has been called into question based on the fact that MTX does not appear to exert a cytotoxic effect within the myeloid progenitor compartment (Blau, et al. 1996). Nonetheless, a recent study has shown that the increased tolerance to high doses of MTX leads to improved curability of human breast tumor xenografts (Zhao, et al. 1997). DHFR vectors have also been shown to protect human hematopoietic cells from MTX *in vitro* (Flasshove, et al. 1995).

A number of recent advances have rekindled interest in the use of DHFR mutants for *in vivo* selection of vector-expressing cells. New active site mutations in the human DHFR gene have been discovered that have kinetic advantages for use as dominant selectable markers. One of these variants, L22Y DHFR, confers extremely high levels of cellular resistance to TMTX (Spencer, et al. 1996). In contrast to the relatively modest

increase in MTX resistance conferred by the original murine L22R variant, the human L22Y gene results in a 100-fold increase in TMTX resistance. A second advance has been the development of novel pharmacological approaches for the selection of transduced stem cells. Antifolates alone do not kill myeloid progenitors or stem cells and therefore cannot exert selective pressure on early hematopoietic cells (Blau, et al. 1996). This intrinsic resistance to antifolates is mediated by nucleoside salvage pathways, which allow stem cells to overcome drug-induced DHFR blockade by importing purine and pyrimidine precursors from the serum. By blocking thymidine transport with nucleoside transport inhibitors such as nitrobenzylmercaptopurine riboside phosphate (NBMPR-P), unmodified stem cells and progenitors become very susceptible to killing with TMTX (Allay, et al. 1997b). To test the hypothesis that treatment with TMTX and NBMPR-P can be used to efficiently select stem cells expressing a DHFR resistance vector, mice were transplanted with L22Y-DHFR-transduced BM and treated with TMTX and NBMPR-P following recovery (Allay, et al. 1998). In many mice, vector-expressing erythrocytes and granulocytes were increased from 10% to more than 80% with serial rounds of drug selection. Secondary transplant experiments confirmed that selection had occurred at the level of repopulating stem cells. If these results can be reproduced in large-animal models, this strategy may prove useful for increasing the number of transduced hematopoietic cells *in vivo*.

Methylguanine DNA methyltransferase

The human methylguanine DNA methyltransferase (MGMT) gene encodes the 207 amino acid repair protein, O^6-alkylguanine DNA alkyltransferase (AGT), which removes alkyl lesions from the O^6 position of guanine and to a much lesser degree from the O^4 position of thymidine. Expression of MGMT provides resistance to monofunctional methylating agents such as dacarbazine, temozolomide, streptozotocin,

and procarbazine (Stevens and Newlands 1993) as well as bifunctional chloroethylating agents such as BCNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloro)-3 nitrosourea (ACNU) and 3-cyclohexyl-1-chloroethyl-nitrosourea (CCNU) (Koç, et al. 1999a). These drugs are toxic to hematopoietic cells, causing prolonged BM suppression as the major dose-limiting toxicity. The primary toxicity of nitrosoureas, tetrazines, and triazines occurs by alkylation of the O^6 position of guanine (Gonzaga and Brent 1989). Direct DNA repair of the adduct by AGT is the predominant repair mechanism associated with drug resistance (Imperatori, et al. 1994). There is considerable variation in AGT expression in mammalian tissues. In humans, the liver contains the highest AGT activity and hematopoietic $CD34^+$ cells possess the lowest activity (Gerson, et al. 1996). The low activity of AGT in human hematopoietic progenitors compared with high-level expression of other drug-resistance genes such as MDR1 suggest that targeting these cells for MGMT-gene transfer may result in more dramatic protection from myelosuppression after chemotherapy than that obtained with other drug-resistance genes.

Retroviral vectors have been used to transfer the human MGMT cDNA into repopulating murine hematopoietic cells. After transplant, mice were engrafted with drug-resistant myeloid progenitors (Allay, et al. 1995) and were protected from the myelosuppressive effects of BCNU (Moritz, et al. 1995). Furthermore, repetitive *in vivo* BCNU administration increased the proportion of cells containing the MGMT provirus in mice transplanted with MGMT-transduced cells (Allay, et al. 1997a). Stem cell transfer of MGMT also protected lymphopoiesis and thereby ameliorated the severe immunodeficiency caused by BCNU treatment (Maze, et al. 1997). These protective effects on hematopoiesis significantly increased survival in heavily BCNU-treated mice (Maze, et al. 1996), showing that otherwise lethal dose increases can be achieved using this strategy. *In vitro* resistance to BCNU has also been demonstrated in human $CD34^+$ BM cells transduced with an amphotropic MGMT retroviral vector (Allay, et al. 1996).

This field has most recently focused on the use of mutant MGMT genes that encode for proteins resistant to O^6-benzylguanine (BG), an inactivator of wild-type MGMT. BG strongly potentiates the tumoricidal effects of nitrosoureas by stoichiometrically depleting cells of MGMT activity (Gerson, et al. 1993). The attractiveness of using BG to sensitize tumor cells to BCNU has been dampened by the observation that BG also sensitizes hematopoietic cells (Gerson, et al. 1996), predicting that the use of this drug combination would be limited by severe myelotoxicity. There is now evidence that this limitation can be circumvented by transfer of MGMT genes bearing single amino acid substitutions (Crone, et al. 1994) that confer resistance to BG-induced inactivation. One such mutant, G156A MGMT, has been tested in the context of a retroviral vector. Human peripheral blood $CD34^+$ cells, transduced with a G156A MGMT vector, were significantly more resistant *in vitro* to BG plus BCNU than cells transduced with a wild-type MGMT vector (Reese, et al. 1996). This vector has also been tested in a mouse transplant model, where it was shown to protect from the lethal effects of BG and BCNU administration (Davis, et al. 1997b). In these studies, one cycle of BG and BCNU increased the proportion of cells expressing mutant AGT form 30% to 60%. Moreover, infusion of G156A MGMT-transduced progenitors into nonmyeloablated mice and subsequent BG and BCNU administration generated 10- to 30-fold enrichment of G156A MGMT-expressing cells with each cycle (Davis, et al. 1997a). These results further support that BCNU is cytotoxic to untransduced quiescent cells and allows enrichment of G156A MGMT-transduced cells. Recent data also indicate that G156A MGMT-transduced hematopoietic progenitors have a clear advantage over tumor cells following treatment with BG and BCNU, providing a therapeutic benefit over that of drug resistance alone. Following infusion of G156A MGMT-transduced marrow, nude mice bearing a BCNU-resistant human tumor xenograft were treated with multiple cycles of BG and BCNU (Koç, et al. 1999b). Dose-intense BG plus BCNU therapy was lethal to

tumor-bearing control mice ($lacZ^+$ cell infused) but tolerated by the G156A MGMT mice and led to effective growth suppression of the human tumor xenograft. Thus, rather than simply making the marrow cells more resistant, the use of BG in combination with G156A MGMT gene transfer results in hematopoietic progenitors uniquely resistant to BCNU, thereby increasing the therapeutic window of this drug combination. Clinical trials in adult patients with solid tumors are just beginning at the Case Western Reserve University and should determine the feasibility of this approach (Gerson and Koç 1997).

Cytidine deaminase

CD is an enzyme of the pyrimidine salvage pathway that catalyzes the deamination of cytidine and deoxycytidine to their corresponding uridine derivatives. As mentioned previously, CD also deaminates several antitumoral cytosine nucleoside analogs including Ara-C, gemcitabine and decitabine, resulting in their inactivation. The presence of high levels of CD in liver is responsible for the short half-life of CD analogs in humans (Momparler, et al. 1972). Mature granulocytes also contain high levels of CD activity (Chabner, et al. 1974). In contrast, low levels of CD activity are found in immature hematopoietic cells (Nygaard and Sundstrom 1987). CD may be implicated in drug resistance to deoxycytidine analogs since leukemic cells with high levels of this enzyme have been detected at the time of relapse following therapy with these agents (Onetto, et al. 1987). The human CD gene has been cloned recently by two independent groups. Comparison of the two published cDNA sequences has revealed identity within the coding region except for one nucleotide deviation at position 79, resulting in either a glycine (Kühn, et al. 1993) or a lysine (Laliberté and Momparler 1994) at codon 27. The recombinant enzyme corresponding to the lysine-carrying natural variant exerts a 1.3- to 3.3-fold higher *in vitro* deamination rate of Ara-C than the glycine-carrying enzyme (Kirch, et al. 1998).

Overexpression of this gene in murine hematopoietic progenitors resulted in 90% to 100% clonogenic survival *in vitro* at concentrations of Ara-C that allowed survival of 0% to 2.5% of untransduced progenitors (Momparler, et al. 1996). Long-term expression of CD in hematopoietic tissues of CD-transplanted mice following treatment with Ara-C has also been demonstrated recently (Eliopoulos, et al. 1998a). Moreover, in these studies, increased *in vitro* resistance to Ara-C was observed in spleen and marrow cells derived from CD animals expressing high levels of CD. Thus, CD gene transfer may be useful for chemoprotection in cancer patients.

Multidrug-resistance-associated protein

As mentioned previously, MRP confers resistance to a similar group of chemotherapeutic agents as MDR1. However, in contrast to Pgp, MRP fails to extrude taxol (Breuninger, et al. 1995). MRP is frequently expressed in NSCLC (Giaccone, et al. 1996). The clinical relevance of this mechanism of drug resistance in certain tumor entities is underlined by recent investigations showing a correlation between MRP expression and treatment failure in childhood neuroblastomas (Norris, et al. 1996). Since MRP is not sensitive to Pgp inhibitors, transplantation of MRP-transduced HSCs along with MDR1 inhibitor treatment has been suggested as a method to sensitize MDR tumors while protecting the marrow. Recently, transplantation of lethally irradiated mice with MRP-transduced BM cells has been shown to reduce doxorubicin-induced leukopenia and mortality (Machiels, et al. 1999). Furthermore, *in vivo* selection of MRP-transduced cells following doxorubicin treatment was demonstrated, with better and more significant chemoprotection after the second chemotherapy cycle. These results suggest that MRP gene transfer may be useful for chemoprotection.

Aldehyde dehydrogenase

Of the multiple isoenzymes of aldehyde dehydrogenase (ALDH), class 1 and 3 ALDH are cytosolic and have been shown to confer resistance to the oxazaphosphorines such as cyclophosphamide and ifosfamide. The cDNA of ALDH-1 is 1.6kb in length and codes for a 340 amino acid protein (Hsu, et al. 1985). The cDNA of ALDH-3 is also 1.6kb, but codes for a 453 amino acid protein (Hsu, et al. 1992). Human hematopoietic progenitors ($CD34^+$) express high levels of ALDH-1, whereas differentiation is associated with a decrease in expression (Kastan, et al. 1990). Purging of BM with 4-HC, the active metabolite of cyclophosphamide and mafosfamide, selectively eliminates leukemic cells and maturing hematopoietic cells. However, neither more quiescent LTC-ICs nor hematopoietic repopulating ceils that have higher expression of ALDH are affected (Koç, et al. 1999a). ALDH-1 gene transfer provides a 4- to 10-fold increased resistance to cyclophosphamide in human HPCs (Magni, et al. 1996). Animal studies are needed to evaluate the feasibility of using ALDH-1 gene transfer for chemoprotection.

Combined expression of drug resistance genes

In order to generate broad-range drug resistance to a variety of chemotherapeutic agents, retroviral vectors that express two drug-resistance genes have been developed. Because eukaryotic cells will only translate the first gene encountered on a mRNA transcript, methods to generate expression of two proteins from one vector have been designed. The most promising approach to express multiple genes from one vector utilizes the picornavirus-derived internal ribosome entry site (IRES). The IRES is a sequence that folds into a secondary structure recognized by ribosomes, allowing the initiation of 7-methylguanine cap-independent translation of the second gene in one transcript (Morgan, et al. 1992). An advantage of these bicistronic constructs is that one promoter (the viral long terminal repeat) controls the transcription of one messenger RNA

(mRNA) transcript containing both genes. Thus, selection for cells expressing one of the genes should generate cells with higher levels of proviral mRNA, allowing expression of the second gene. However, the protein expressed from the IRES is usually translated less efficiently than the protein translated from the 7-methylguanine mRNA cap (Doroshow, et al. 1995).

A number of studies have demonstrated the feasibility of combining drug resistance mechanisms for the purpose of chemoprotection. A bicistronic vector containing the MDR1 and L22Y DHFR was transduced into human CEM cells and primary murine myeloid progenitors, resulting in increased resistance to Pgp effluxed drugs, antifolates, or their combination (Galipeau, et al. 1997). Another recently described IRES-based vector expresses both the MDR1 gene and the MGMT gene (Suzuki, et al. 1997). Transduced murine BM cells selected in vincristine showed simultaneous resistance to Pgp effluxed drugs and nitrosoureas. Finally, increased resistance to antifolates and cytosine nucleoside analogs was demonstrated in murine BM progenitor following transduction with a bicistronic vector coexpressing the F31S DHFR and CD genes (Beauséjour, et al. 1998). *In vivo* studies with murine or human hematopoietic progenitors have yet to be reported but may yield a vector with a broad range of drug resistance.

OBJECTIVES OF THE PRESENT INVESTIGATION

The goals of this study were:

1. Determine the ability of retrovirus-mediated gene transfer of GSTA3 to confer resistance to nitrogen mustards;

2. Study, in an animal model, the feasibility and potential benefit of using somatic gene transfer of rat GSTA3 to confer protection to the hematopoietic system against nitrogen mustards.

CHAPTER 2

MANUSCRIPT ONE

PREFACE

The largest increase in resistance to nitrogen mustards has been obtained following transfection of the rat GSTA3 homodimer. This chapters describes the ability of retrovirus-mediated gene transfer of the rat GSTA3 to confer resistance to nitrogen mustards in populations of murine fibroblasts, confirming and extending the findings obtained with GSTA3-transfected MatB cells by excluding potential artifacts related to clonal variability.

RETROVIRUS-MEDIATED GENE TRANSFER OF RAT GLUTATHIONE S-TRANSFERASE Yc CONFERS ALKYLATING DRUG-RESISTANCE IN NIH 3T3 MOUSE FIBROBLASTS[1]

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ABSTRACT

A major limitation to successful cancer treatment is the existence of drug resistance. While several mechanisms of drug resistance have now been well characterized, mechanisms of resistance to alkylating drugs have remained less well defined. Several experimental models of alkylator-resistance have implicated isoforms of glutathione *S*-transferase (GST), but transfection experiments using cloned isoforms of GST have yielded conflicting results. While there are several plausible explanations to these apparently contradictory findings, the issue of clonal variability potentially confounding the results of conventional transfection experiments has been raised. To address this issue properly, we have studied rat GST-Yc expression and drug sensitivity to alkylating drugs in populations of mouse NIH 3T3 fibroblasts following either transfection or transduction with an N2-based retrovirus vector. In comparison with cells treated with an antisense vector, Yc-transfected and Yc-transduced populations of NIH 3T3 cells expressed increased levels of GST-Yc mRNA (Northern blots), increased levels of immunodetectable GST-Yc (Western blot), and, respectively, 1.4- and 1.9-fold increase in total GST activity, and 6.1- and 8.3-fold increase in glutathione peroxidase activity (associated with the Yc subunit). Yc-transfected and Yc-transduced cell populations were respectively 5.8- ($p<0.001$) and 2.4-fold ($p<0.05$) resistant to chlorambucil, and 10.8- ($p<0.01$) and 5.4-fold ($p<0.001$) resistant to mechlorethamine. The range of resistance of clonal isolates from either population was 1.8- to 6.0-fold for chlorambucil, and 4.6- to 6.1-fold for mechlorethamine ($p<0.05$ or less). In contrast, these cells showed unaltered sensitivity to the antimetabolite methotrexate, a non-alkylating drug. These results clearly demonstrate that the rat GTS-Yc is able to confer alkylating drug resistance in mouse fibroblasts. The ability to confer alkylating drug resistance following retrovirus-mediated gene transfer also raises the possibility of using

GST-Yc somatic gene transfer to confer protection to the hematopoietic system in a gene therapy strategy applicable to cancer.

INTRODUCTION

A major limitation to successful treatment of cancer is the existence of intrinsic or acquired mechanisms of drug resistance in tumor cells. A wide range of cellular mechanisms may lead to drug-resistance, including: increase in DNA, protein or membrane repair; decreased drug accumulation; altered drug metabolism; and altered drug target (1). Well characterized mechanisms of drug-resistance include multidrug resistance mediated by P-glycoprotein (reviewed in (2, 3)), and resistance to DNA intercalating agents and epipodophyllotoxins resulting from altered DNA topoisomerase II activity (4, 5, 6, 7). Another clinically important form of drug resistance which is not mediated by these mechanisms is the resistance to alkylating agents. Alkylating agents have a wide spectrum of antitumor activity and are among the most commonly used anticancer agents. Gene transfer of a bacterial repair enzyme, O^6-alkylguanine-DNA alkyltransferase (ATase), has been shown to increase the resistance of mammalian cells to chloroethylating agents such as *N,N'*-bis(2-chloroethyl)-N-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) (8, 9, 10, 11). The human enzyme has also been shown to prevent the appearance of thymic lymphoma in transgenic mice treated with *N*-methyl-*N*-nitrosourea (12). While ATase might be implicated in tumor resistance to chloroethylating agents (13), it has not been implicated in the resistance to other alkylating agents (14). Another important mechanism of alkylator resistance involves the thiol glutathione and the enzymes which catalyze its conjugation to potentially toxic molecules, particularly the GSTs. Cytosolic GST in mammals have been divided according to their isoelectric points into four gene families termed alpha, mu, pi and theta encoding subunits that form homodimers or heterodimers within the same family. Although a considerable number of isoenzymes have been described in rat, mouse and man, the same multigene families are seen and there is significant

conservation in primary structure across the three species (15). There are a number of non-human (16, 17, 18, 19, 20) and human (21, 22) experimental models of drug resistance to alkylating agents in which specific GST forms are over expressed. Most of the experimental models implicate an alpha class form of GST, particularly the Yc homodimers in rat (GST 2-2 in the most recent nomenclature) which has the highest organic peroxidase activity (23, 24). GST inhibitors have also been shown to overcome the resistance of certain cell lines to alkylating agents (25, 26). Several transfection experiments have provided direct evidence for the ability of GST's to confer modest levels of resistance to alkylating agents in yeast and mammalian cells (27, 28, 29, 30, 31), while other experiments have failed to confirm consistent drug resistance (32, 33, 34).

Thus, the evidence for a role of some specific forms of GST in resistance to alkylating drugs is now very strong, however the issue of clonal variability potentially confounding the results of conventional transfection experiments has been raised (35). To circumvent this difficulty and resolve the important issue of GST's role in drug-resistance, we have studied rat GST-Yc expression and sensitivity to alkylating drugs in populations of mouse NIH 3T3 fibroblasts transfected with a GST-Yc expression vector or transduced by a retrovirus vector. Populations of Yc-expressing cells and single cell-derived clones were more resistant to chlorambucil, and mechlorethamine than cells subjected to gene transfer with control antisense constructions. The effect of GST-Yc gene transfer on the drug sensitivity of cell populations excludes potential artifacts related to clonal variability. In addition, retrovirus-mediated transfer of alkylating drug resistance raises the possibility of using GST genes to confer protection to the hematopoietic system in a gene therapy strategy applicable to certain forms of cancer.

MATERIAL AND METHODS

Cell lines. Retrovirus packaging cell lines GP+E-86 and GP+envAm-12 were obtained from Dr. Arthur Bank, Columbia University (36, 37). These cell lines are derived from mouse NIH 3T3 fibroblasts and produce ecotropic and amphotropic retrovirus envelopes respectively. Both cell lines were maintained in DMEM (Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (ibid).

Construction of plasmids pN2Yc and pN2revYc. The full length cDNA of rat GST-Yc was isolated from pPSMYc (obtained from William E. Fahl, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI)(38) by *Bam* HI and *Xho* I digestion, agarose gel electrophoresis separation and purification. The human adenosine deaminase (ADA) cDNA was then removed from the Moloney-Murine Leukemia Virus-based vector pN2stADA (39, 40) by *Bam* HI digestion and replaced by the 734 bp GST Yc cDNA in forward or reverse orientation by blunt end ligation to produce pΔN2Yc and pΔN2revYc respectively.

Generation of virus-producing cell lines. The plasmids pΔN2Yc or pΔN2revYc were cotransfected in a 10:1 molar ratio with pSV2neo (41) into GP+E-86 ecotropic packaging cells by calcium-phosphate precipitation. Stable transfectants were then selected with G418 (Geneticin, Gibco), a neomycin analogue, at 300 μg/ml (active drug) starting 48 hours post-transfection. This concentration of drug was shown to completely inhibit the growth of non transfected GP+E-86 cells (data not shown). Populations of amphotropic virus producing cells were in turn obtained by cocultivating the transfected populations GP+E-86/Yc or GP+E-86/revYc with GP+envAm-12 in a 1:1 ratio for 17 to 21 days,

similar to a previously described strategy (42). Amphotropic producing cells were then selected with hygromycin 200 μg/ml. This concentration of drug had been found to completely inhibit the growth of GP+E-86 cells but not GP+envAm-12 which were transfected with an hygromycin resistance gene in their construction (37). Single cell-derived clones of GP+E-86/Yc and GP+envAm-12/Yc were obtained by limiting cell dilutions in 24 well plates.

Determination of virus titers. Virus titers were determined by RNA slot blot analysis as described (43). Briefly, cell culture medium was conditioned for 16 hrs by subconfluent virus-producing cells, made up to 0.5% SDS, 5mM EDTA, 100 ug/ml of tRNA and 500 ug/ml proteinase K, incubated for 45 minutes at 37 °C, extracted with phenol, phenol/chloroform, and chloroform, and the ethanol precipitate of the aqueous phase was dissolved in water. Serial dilutions of these RNA preparations were blotted on Zeta Probe membrane (Biorad, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Membranes were then hybridized with a 176 bp fragment of pΔN2stADA located between the 5'LTR and the cloning site of the GST-Yc cDNA. This probe therefore detects the viral RNA of all pΔN2-derived vectors with equal efficiency. Virus titers were thus determined by comparing the intensity of signal from viral RNA in supernatants of Yc- and revYc-virus producing cells with a cell line of known viral titer, GP+E-envAm-12/ADAsc14 (40).

Southern and northern blot analysis. Genomic DNA and RNA extractions and Southern and Northern blot analysis were performed according to standard protocols (44). Genomic DNA was restricted with *Eco* RI for Southern analysis. The *Bam* HI / *Xho* I fragment of pPSMYc (containing the full length rat GST Yc) was radiolabelled by random primer (Oligo Labelling Kit, Pharmacia, Baie D'Urfé, Québec, Canada) and used

as probe for Southern and Northern blots. Hybridization was at 42 °C with 50% formamide and final washes of blots were with 1.8 x SSC and 10% SDS at 65 °C.

RNAse protection of vector-specific transcripts. The correct structure of the RNA transcript at its junction between the pN2 vector and the GST-Yc cDNA was verified by RNAse protection assay. Ten µg of total RNA from the tested cell lines were hybridized with a 193 bp ^{32}P-labeled single strand RNA probe (riboprobe) containing 120 bp complementary to the 5' end of pΔN2stYc expected full-length transcript. Unprotected single strand RNAs were then digested with 40 ug/ml *RNAse* A. Following migration in a 5% polyacrylamide gel, protected riboprobe fragments were detected by autoradiography.

Western blot analysis. Cytosol extracts were prepared by cell lysis in water and centrifugation of debris at 12,000g for 1 minute. Protein concentrations were determined according to the method of Lowry (45) using bovine serum albumin as a standard. Purified rat liver cytosolic GST (Biorad), which contains Ya (GST 1), Yb (GST 3 and 4) and Yc (GST 2) subunits was used as a positive control. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (46) using a 4% polyacrylamide stacking gel layered over a 12% resolving gel. Seventy-five µg of protein from each sample were run under 50 mA of current for 5 hrs and transferred onto nitrocellulose membrane by electroblotting (15 volts for 16 hours). Non specific binding to the membrane was blocked by incubation in 5% BSA and 0.02% sodium azide in PBS for 2 hours at room temperature. Blots were then reacted with 0.5% BSA in wash buffer (0.05M K_2HPO_4, 0.5M NaCl, 0.1% $Tween_{20}$) and a polyclonal rabbit anti-rat GST antibody for 16 hours at 4°C. The antibody used was either a 1:1000 dilution of anti-Yc (Medlabs-Biotrin International, Dublin, Ireland), a 1:250 dilution of anti-rat liver GST (kindly provided by M. Clapper, Fox Chase Institute for Cancer Research, Philadelphia)

or a 1:1000 dilution of anti-Yp (Medlabs-Biotrin International). Following three washes with wash buffer for 15, 10 and 5 minutes at room temperature, membranes were reacted with a 1:3000 dilution of goat anti-rabbit horse-radish peroxidase conjugated secondary antibody (Biorad) and 0.5% BSA in wash buffer. The sites of antibody binding were finally revealed by incubation with 0.52 mg/ml diamino benzidine and 0.04% H_2O_2 in PBS.

Enzymatic assays. Cytosolic protein extracts were prepared as described above. Total GST enzyme activity was assayed using 2-chloro-1,3-dinitrobenzene (CDNB) as a substrate (47). Selenium-independent glutathione peroxidase activity was assayed using cumene hydroperoxide (48). Assays were linear functions of protein concentration and time (for up to 5 minutes) when the amount of enzyme used resulted in an absorbance change of 0.02 to 0.04 $O.D._{340nm}$ per min for total GST and 0.01 to 0.03 $O.D._{340nm}$ for GSH peroxidase. Cells subjected to gene transfer with the sense construction were compared with cells transferred with the antisense vector but otherwise treated exactly in the same manner.

Drug sensitivity assay. Drug sensitivity assays were performed using a colorimetric assay which measures the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to an insoluble purple-colored formazan precipitate (49, 50). Cells in the logarithmic phase of growth were resuspended at a concentration of 1-5 x 10^4 cells/ml and 100 µl per well were plated in 96 well microtiter plates. Plates were incubated for 24 hours at 37°C in 5% CO_2. One hundred µl of medium containing the drug was then added to 8 wells for each drug concentration and plates were incubated for a further 72 hours. One hundred and eighty µl of medium was then removed from each well and replaced by 150 µl of medium containing 10 mM PIPES (pH 7.4) and 50 µl MTT (Sigma, St.Louis,

MO) at 2 mg/ml in PBS. Plates were then wrapped in aluminum foil paper and incubated for 4 hours at 37°C. The formazan crystals were dissolved in 180 µl DMSO (Fisher, Montreal, Quebec) and 25 µl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) by mixing on a shaker for 5 minutes. The formazan product formed by viable cells was then quantitated by measuring the optical absorbance at a wavelength 570 nm on a microplate reader (model 3550, Biorad). The concentration of drug resulting in 50% growth inhibition (IC_{50}) was determined by plotting the percentage of surviving cells vs the log of drug concentration. Again, cells treated with the sense vector were compared with cells transferred with the antisense construction. Chlorambucil and mechlorethamine were obtained from Sigma; methotrexate was obtained from Adria Laboratories, Columbus, Ohio.

RESULTS

Integrity and copy number of the GST-Yc vectors. The integrity of the vector sequence integrated in populations of virus-packaging cells was verified by Southern blot analysis following digestion of genomic DNA with restriction enzymes which cuts twice in the N2Yc and N2revYc sequence, but outside the GST-Yc cDNA. Blots were then hybridized with ^{32}P-labelled GST-Yc cDNA insert of pPSMYc. This results in a labeled fragment of predictable size independently of the integration site(s) in contrast to single "cutters" which would result in a multiplicity of fragment sizes corresponding to the diversity of integration sites present in transfected or transduced cell populations. Figure 1 shows the results of Southern blot analysis following *Eco* RI digestion; the expected 1.1 Kb Yc containing fragment is seen in both GP+E-86/Yc and GP+envAm-12/Yc. Similar analysis confirmed the absence of gross rearrangement or deletion of the vector sequence in the packaging cell line populations transfected or transduced with N2Yc and N2revYc vectors. It also revealed, as seen in fig.1, that the copy number of transferred vectors was several fold greater in the transfected ecotropic packaging cells than in the transduced (infected) amphotropic packaging cells (ethidium bromide staining of the gel before transfer showed equivalent loading of both DNAs (data not shown)).

Transcriptional activity of the vector. The transcription activity of the transferred vectors was assessed by Northern blot analysis using a full length GST-Yc cDNA probe. This demonstrated efficient expression of the expected 2.8 Kb vector mRNA transcript in GP+E-86 cells transfected with pN2Yc or pN2revYc, as well as in GP+envAm-12 transduced by the corresponding retroviral vectors (data not shown). The correct structure of vector transcripts was further verified by RNAse protection assay using a riboprobe complementary to 21 bp of N2 vector and 99 bp of GST-Yc cDNA at the 5'

junction of these sequences. Figure 2 shows the results of this assay on GP+E-86 cells stably transfected with pN2Yc or pN2revYc. As expected, there was no rat GST-Yc sense transcript in non transfected GP+E-86 or after transfection with the antisense construction. In contrast, RNA from cells transfected or infected with the sense construction protected the expected 120 bp riboprobe fragment. A second predominant fragment, approximately five nucleotides shorter than expected, was also present in these cells (Fig.2).

Virus titers. The moderate level of drug resistance conferred by GST gene expression was insufficient to allow a complete discrimination between cells expressing the transduced GST-Yc and non transduced cells (approximately 20% of Yc-transduced cells will survive at the drug concentration that would kill all control cells)(data not shown). Therefore, virus titers were determined by RNA slot blot analysis of RNA isolated from the supernatant of virus-producing cells in comparison with GP+E-envAm-12/ADAsc14 (not shown). According to this analysis, the populations of transfected GP+E-86/Yc and GP+E-86/revYc produced their vector at titers of approximately 5×10^5 and 2×10^6 viral particles per ml, respectively. Viral titers of populations of transduced GP+envAm-12/Yc and GP+envAm-12/revYc were estimated at 5×10^6 and 2×10^6 particles per ml, respectively. Viral titers of two clonal isolates from GP+envAm-12/Yc were 2×10^6 (clone # 15) and 10^6 (clone # 17) viral particles per ml.

Expression of immunodetectable rat GST-Yc. Western blot analysis was performed on cell populations and clones subjected to GST-Yc gene transfer using the following polyclonal rabbit anti-rat GST antibodies: anti-Yc, anti-rat liver GST (Ya, Yb, Yc) and anti-pi. This revealed a marked increase in the expression of immunodetectable Yc isoform in N2Yc-transfected or transduced NIH 3T3 cell populations (packaging cells)

and clones in comparison with very low levels of cross-reacting murine Yc isoform in wild-type cells or cells treated with the antisense vector (Fig. 3). In contrast, NIH 3T3-derived packaging cells expressed unchanged low levels of Yb and Ypi and no Ya cross-reacting material following gene transfer of N2Yc or N2revYc (not shown).

Total GST and GSH peroxidase activity. Cytosolic extracts from cells subjected to GST-Yc gene transfer were assayed for total GST activity and for selenium-independent glutathione peroxidase activity, the latter being associated with the Yc subunit of GST. Both the population of transfected GP+E-86 cells and a clonal isolate selected for its high titer of virus production showed a modest increase in total GST activity (1.1- to 1.4-fold) and a more important increase in glutathione peroxidase activity (5.6- to 6.1-fold) compared to cells transfected with the antisense construction (Table 1). However, only the increase in glutathione peroxidase activity of the clonal isolate reached statistical significance after three independent measurements of enzyme activity (unpaired student's t-test). Likewise, the population of Yc-transduced GP+envAm-12 cells and six randomly selected clonal isolates showed a 1.2- to 2.8-fold increase in total GST activity and a 4.8- to 11.5-fold increase in glutathione peroxidase activity compared to cells transduced with the antisense vector. The increase in total GST activity was statistically significant in five out of six GP+envAm-12/Yc clones and the increase in glutathione peroxidase was significant in the population and all six clones after three determinations (unpaired student's t-test). Parental, non transduced GP+envAm-12 cells had significantly higher (1.3-fold) total GST activity than cells transduced with the antisense vector, but their glutathione peroxidase activity was not significantly different.

Drug sensitivity studies. Drug sensitivity assays were performed using the MTT assay (49, 50). In comparison with cells subjected to gene transfer with the antisense Yc vector,

transfected GP+E-86/Yc cells (population and one clone tested) showed a 5.8- to 6.0-fold increase in the concentration of chlorambucil required to inhibit 50% of cell growth (inhibitory concentration$_{50}$ or IC_{50}) and a 4.6- to 10.8-fold increase in the IC_{50} of mechlorethamine (Table 2). These levels of resistance were highly significant after three (mechlorethamine) to five independent determinations of IC_{50} (unpaired student's t-test). Likewise, transduced GP+envAm-12/Yc cells were 1.8- to 4.3-fold (population and six randomly selected clones) more resistant to chlorambucil than GP+envAm-12/revYc cells, and 5.4- to 6.1-fold (population and one high viral titer clone) more resistant to mechlorethamine. These levels of resistance were also statistically significant. In contrast, both Yc- and revYc-modified cells demonstrated unaltered sensitivity to the antimetabolite methotrexate, a non alkylating drug.

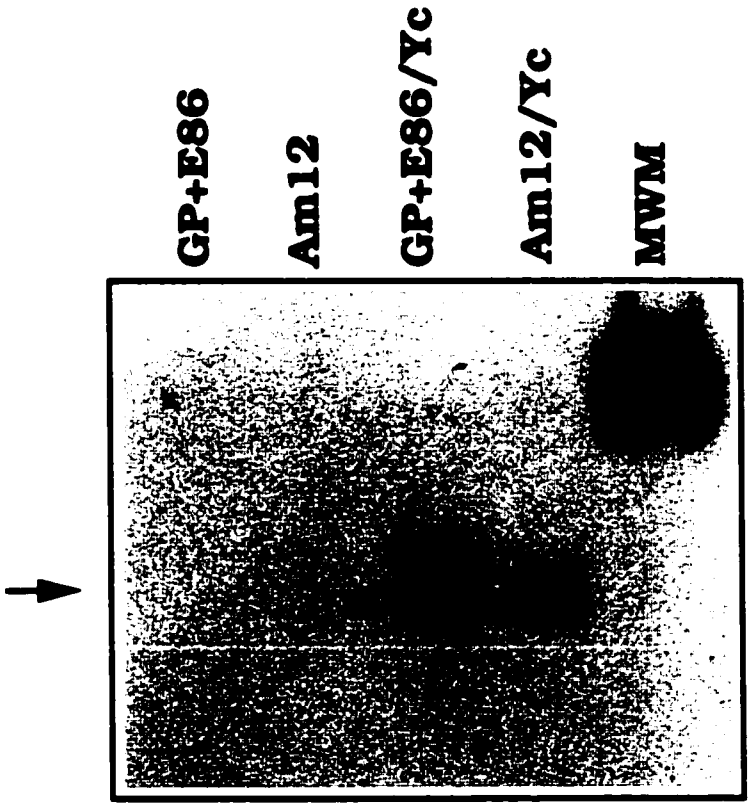

Figure 1. Southern blot analysis of GST-Yc transfected and transduced NIH 3T3 cells. Ten ug of genomic DNA from each sample was restricted with *Eco* RI, migrated on a 1.0% agarose gel, transferred to Hybond-N (Oakville, Ontario, Canada) nylon membrane and hybridized with a radiolabelled GST-Yc cDNA probe. GP+E-86: NIH 3T3-derived ecotropic packaging cells. Am12: GP+envAm-12 i.e. NIH 3T3-derived amphotropic packaging cells. GP+E-86/Yc: GP+E-86 cells transfected with the pN2Yc plasmid. Am12/Yc, GP+envAm-12 cells transduced with N2Yc virus. MWM, molecular weigth markers (presence of plasmid sequence in probe results in cross-hybridization).

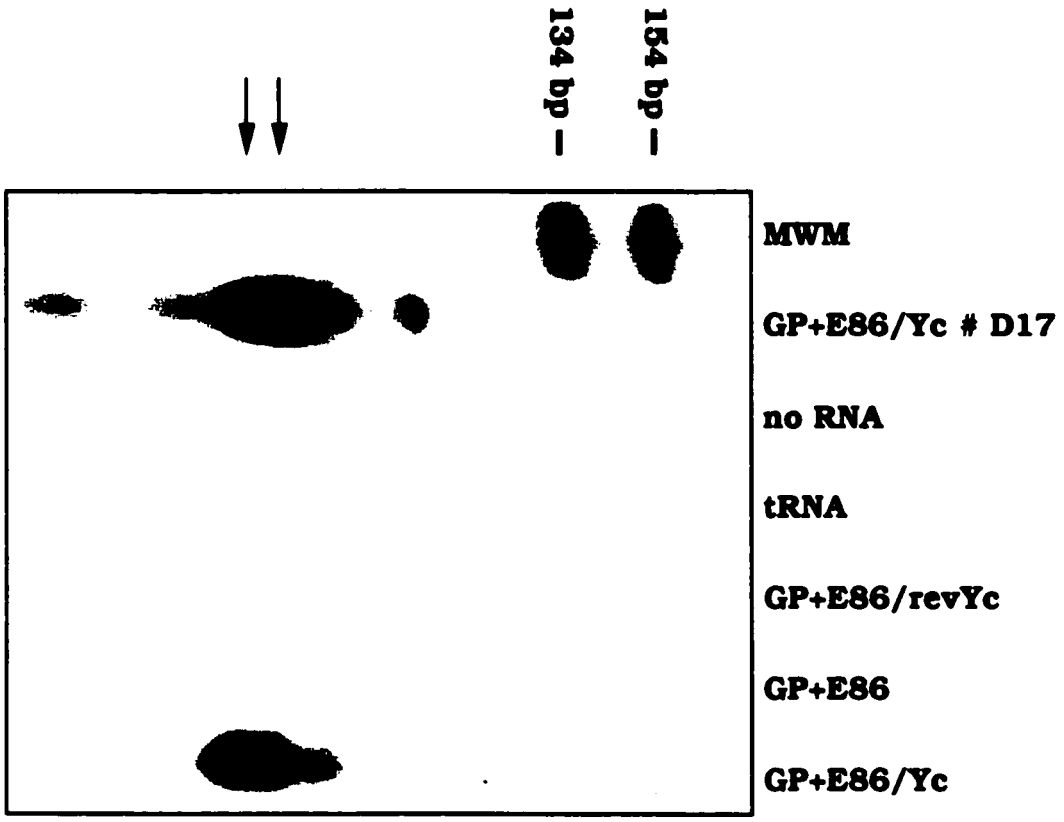

Figure 2. RNase protection of vector-specific transcripts. Ten μg of total RNA from the tested cell lines were hybridized with a 193 bp ^{32}P-labeled riboprobe containing 120 bp complementary to the 5' end of pΔN2Yc expected transcript. Unprotected single strand RNAs were then digested with 40 μg/ml *RNAse* A. Following migration in a 5% polyacrymamide gel, protected riboprobe fragments were detected by autoradiography. MWM, end-labelled molecular weight markers. The lane with no RNA verifies that the probe is completely digested in absence of protecting RNA. tRNA, yeast transfer RNA; GP+E-86/revYc refers to GP+E-86 cells transfected with the antisense construction pN2revYc; GP+E-86/Yc # D17 refers to a clonal isolate from the transfected GP+E-86/Yc cell population. Other symbols are the same as in Figure 1. 154 bp and 134 bp indicate the position of migration of markers. The upper arrow indicates the position of migration of the 120 bp full-length fragment of riboprobe protected by the primary transcript of pΔN2st ADA. The lower arrow indicates a protected fragment which is approximately 5 nucleotides shorter than expected.

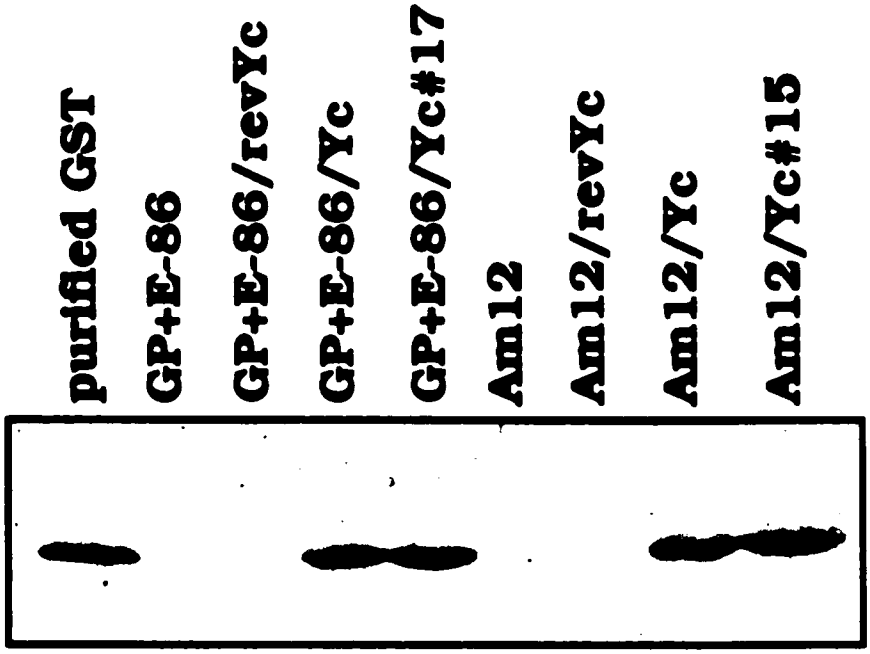

Figure 3. Western blot detection of GST-Yc gene product. Seventy-five μg of cytosolic protein from each cell line were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the membrane was reacted successively with a polyclonal rabbit anti-GST-Yc antibody, a horse-radish peroxidase conjugated goat anti-rabbit antibody, and a solution of diamino benzidine and H_2O_2 as detailled in "Material and Methods". Purified GST: purified rat liver GST. GP+E-86/Yc # 17, same clonal isolate refer to as GP+E-86/Yc # D17 in Figure 2. Am12/Yc#15, clonal isolate from the transduced GP+<u>env</u>Am12/Yc cell population. Other symbols are the same as in Figures 1 and 2.

	Total GST ± S.D. (nmol/min/mg protein)	Ratio	GSH Peroxidase ± S.D. (nmol/min/mg protein)	Ratio
GP+E-86/revYc	146.7 ± 34.7	1.0	0.83 ± 0.52	1.0
GP+E-86/Yc	199.7 ± 68.4	1.4	5.09 ± 2.84	6.1
GP+E-86/Yc#17	163.6 ± 34.4	1.1	4.65 ± 1.13	5.6 [a]
GP+env Am-12/revYc	103.7 ± 6.1	1.0	0.43 ± 0.17	1.0
GP+env Am-12/Yc	191.9 ± 63.4	1.9	3.57 ± 1.90	8.3 [a]
GP+env Am-12/Yc#2	147.3 ± 13.6	1.4 [b]	2.07 ± 0.06	4.8 [c]
GP+env Am-12/Yc#4	205.6 ± 53.6	2.0 [a]	3.26 ± 0.95	7.6 [b]
GP+env Am-12/Yc#7	144.7 ± 15.4	1.4 [a]	2.63 ± 0.71	6.1 [b]
GP+env Am-12/Yc#13	159.4 ± 33.8	1.5 [a]	2.27 ± 0.90	5.3 [a]
GP+env Am-12/Yc#15	284.8 ± 12.1	2.8 [c]	4.41 ± 0.77	10.3 [c]
GP+env Am-12/Yc#17	119.3 ± 11.9	1.2	4.95 ± 0.56	11.5 [c]

[a], $p < 0.05$; [b], $p < 0.01$; [c], $p < 0.001$ (unpaired student's t-test)

Table 1. Total GST activity and selenium-independent glutathione peroxidase activity of GST-Yc transfected and transduced cells. Total GST activity and selenium-independent peroxidase activity of cytosolic extracts were determined as previously described (47,48). Results represent the mean of three independent assays, each one performed in triplicate, with standard deviation (S.D.). Ratios of the enzyme activity of each cell line over the enzyme activity of the same cell line transfected or transduced with the antisense N2revYc vector are given. An Arabic number following GP+E-86/Yc or GP+envAm-12/Yc indicate independent clonal isolates from the corresponding cell population. Statistical values by unpaired student's t-test are indicated.

	CHLORAMBUCIL		MECHLORETHAMINE	
	IC_{50} (μM)	ratio	IC_{50} (μM)	ratio
GP+E-86/revYc	33.0 ± 12.0	1.0	3.2 ± 0.5	1.0
GP+E-86/Yc	192.O ± 66.7	5.8 [c]	34.7 ± 9.4	10.8 [b]
GP+E-86/Yc#17	199.0 ± 57.8	6.0 [c]	14.7 ± 2.5	4.6 [b]
GP+envAm-12/revYc	31.0 ± 9.6	1.0	1.6 ± 0.3	1.0
GP+envAm-12/Yc	74.6 ± 37.8	2.4 [a]	8.7 ± 2.2	5.4 [b]
GP+envAm-12/Yc#2	54.4 ± 15.9	1.8 [a]	N.D.	
GP+envAm-12/Yc#4	64.0 ± 15.4	2.1 [b]	N.D.	
GP+envAm-12/Yc#7	61.6 ± 14.5	2.0 [b]	N.D.	
GP+envAm-12/Yc#13	54.2 ± 16.5	1.8 [a]	N.D.	
GP+envAm-12/Yc#15	132.8 ± 45.7	4.3 [b]	9.8 ± 1.5	6.1 [c]
GP+envAm-12/Yc#17	87.6 ± 26.0	2.8 [b]	N.D.	

[a], $p < 0.05$; [b], $p < 0.01$; [c], $p < 0.001$ (unpaired student's t-test)

Table 2. Drug sensitivity of GST-Yc transfected and transduced cells. Drug sensitivity assays were performed using the MTT colorimetric assay (49,50). IC_{50}: inhibitory concentration 50%. Results represent the mean of three (mechlorethamine), five (chlorambucil) or six (methotrexate) independent assays, each cell lines being tested 8 times in every individual assay. Ratios of the IC_{50} of each cell line for a given drug over the IC_{50} of the same cell line transfected/transduced with the antisense N2revYc vector are given. An Arabic number following GP+E-86/Yc or GP+envAm-12/Yc indicate independent clonal isolates from the corresponding cell population. Statistical values by unpaired student's t-test are indicated.

DISCUSSION

We have used a retroviral vector to test the ability of the Yc isoform of rat GST to confer resistance to alkylating drugs. NIH 3T3-derived GP+E-86 ecotropic retrovirus packaging cells were stably transfected with a retrovirus plasmid construction designed to transcribe the rat GST-Yc cDNA in sense (pN2Yc) or antisense (pN2revYc) orientation from the virus promoter/enhancer in the 5' long terminal repeat. The resulting defective retroviruses were used to transduce NIH 3T3-derived GP+envAm-12 amphotropic retrovirus packaging cells. Southern blot analysis confirmed the presence of unrearranged vector sequence in the transfected and transduced packaging cell lines (Fig. 1). It also revealed that the copy number of the vector per cell was greater in transfected than in transduced cell populations. This is not surprising since transfected plasmids tend to integrate in concatemers of large number of plasmids (51) whereas retroviruses tend to integrate in low copy number per cell (on average one copy per cell).

Both transfected GP+E-86 and transduced GP+envAm-12 cells (populations and clonal isolates) showed efficient expression of the expected 2.8 Kb sense or antisense transcripts following transfer of N2Yc or N2revYc. A more detailed analysis of the region of the transcript corresponding to the junction between the vector sequence and the 5' end of cDNA insert in the sense construction by RNAse protection assay revealed that there was approximately equivalent amounts of two predominant protected mRNA species, one being 5 to 6 bp shorter than expected (Fig. 2). The mechanism by which this smaller transcript arose is unclear but, and may relate to the presence of a cryptic splice acceptor signals in N2-derived vectors (52). There was no evidence, however, that this alternate mRNA structure interfered with expression of the transferred gene. Efficient transcription of the vector was also reflected in the titer of virus-producing cells. Populations of transfected ecotropic packaging cells produced the vectors at 0.5-2 x 10^6

particles per ml, whereas populations of "superinfected" amphotropic packaging cells produced 2-5 x 10^6 particles per ml. Titers > 10^6 viral particles per ml will generally permit efficient gene transfer in a wide range of target cell types.

Western blot analysis demonstrated the efficient expression of immunoreactive GST-Yc isoform in packaging cells transfected or transduced with N2Yc (Fig. 3). Likewise, expression of functional enzyme following gene transfer was evident in the 1.1- to 2.8-fold increase in total cytosolic GST activity and, more clearly, in the 4.8- to 11.5-fold increase in glutathione peroxidase activities (Table 1). Most of these changes reached statistical significance (unpaired student's t-test). The slightly higher total GST activity of parental, non transduced GP+envAm-12 cells in comparison to cells transduced with the antisense vector could conceivably be due to an antisense effect on endogenous GST expression. However, the glutathione peroxidase activity was equivalent in both cell populations and there was no detectable increased in any isoform of GST on Western blot.

The most important and interesting effect of GST-Yc gene transfer was its impact on cell sensitivity to alkylating agents (Table 2). Populations of NIH 3T3-derived packaging cells transfected with the retrovirus N2Yc construction were 5.8-fold resistant to chlorambucil and 10.8-fold resistant to mechlorethamine. Likewise, populations of packaging cells superinfected with the vector but not preselected for successful gene transfer were 2.4-fold resistant to chlorambucil and 8.7-fold resistant to mechlorethamine. The range of resistance in clonal isolates from either population was 1.8- to 6.0-fold for chlorambucil and 4.6- to 6.1-fold for mechlorethamine. These changes in sensitivity to chlorambucil and mechlorethamine were all statistically significant. There was no significant difference in drug sensitivity between parental cells and cells transfected or transduced with the antisense vector, again arguing against a significant inhibition of GST expression by the antisense vector.

These results clearly indicate that the rat Yc isoform of GST is able to confer alkylating drug resistance in mouse-derived fibroblasts. The reasons for contradictory results from previous transfection experiments (27, 28, 29, 32, 33, 34) are not entirely clear, but may relate to substrate specificities of the isoenzymes that were studied or to differences in endogenous expression of GST isoenzymes or of cofactors between the cell lines that were transfected. We have obtained relatively high levels of drug resistance by transferring the Yc isoform of GST in MatB rat mammary carcinoma cells which normally express low levels of GST-Yc (31). Stably transfected cell clones expressing increased levels of GST activity and immunoreactive Yc isoform were resistant to melphalan (6 to 12-fold), mechlorethamine (10-16-fold) and chlorambucil (7 to 30-fold). Late passage transfectants showed decreased GST activity concurrent with a partial reversion toward wild type drug sensitivity. The present study extends those findings and addresses the issue of clonal variability potentially confounding the results of conventional transfection experiments (35). To circumvent this problem, we have studied rat GST-Yc expression and alkylating drug sensitivity of populations of mouse NIH 3T3 fibroblasts transfected with a GST-Yc expression vector or transduced by a Yc-retrovirus vector. The effect of GST-Yc gene transfer on the sensitivity of cell populations to alkylating agents excludes potential artifacts related to clonal variability and thus confirms and extends our earlier findings.

The ability of GST-Yc to confer alkylating drug resistance following retrovirus-mediated gene transfer supports the implication of Yc in naturally occurring resistance. It also raises the possibility of using GST-Yc somatic gene transfer to confer protection to the hematopoietic system in a gene therapy strategy applicable to cancer. Autologous transplantation of drug-resistant bone marrow cells could reduce life-threatening toxicities from subsequent courses of chemotherapy and might permit safe dose-intensification. Contaminating tumor cells could be excluded from the bone marrow or

later killed by the inclusion of a safety "suicide gene" in the vector. The feasibility of "bone marrow protection" has been demonstrated in mice using a human *MDR*1-transducing retrovirus and clinical trials are now being considered. Although the efficiency of gene transfer needs to be directly evaluated in hematopoietic cells, we have evidence for efficient gene transfer with the N2Yc vector since all 20 clones derived from single cells of transduced GP+envAm-12/Yc were found to contain at least one copy of integrated provirus and 14 of them (70%) over expressed glutathione peroxidase activity (data not shown). Despite the relatively modest, but most likely clinically significant levels of drug-resistance that appear to be mediated by GST isoenzymes, there are several potential advantages to transferring these genes into the hematopoietic system. Alkylating drugs have a broad spectrum of antitumor activity, maintain a dose-related effect through multiple logs of tumor cell kill and are in most cases limited by their hematotoxicity (53, 54, 55). Thus, they are among the most important and useful antitumor agents. While the levels of drug resistance obtained following gene transfer of GST may appear modest compared to *MDR* genes, relative resistance to alkylating agents observed clinically is of the same or lower magnitude (22), suggesting that resistance factors of 1.5- to 3.0-fold are important in chemotherapy. Finally, we have recently demonstrated that there is no detectable constitutive expression of GST-Yc in either human and rodent mononuclear bone marrow cells (56), indicating a potential benefit from heterologous gene expression. Future experiments will address the feasibility of using GST-Yc to confer drug-resistance to the hematopoietic system.

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CHAPTER 3

MANUSCRIPT TWO

PREFACE

The effect of GSTA3 retroviral transduction on the sensitivity to nitrogen mustards was found to be of variable magnitude in different cell lines, possibly in relation to differences in endogenous glutathione (GSH), in total glutathione *S*-transferase (GST) levels, or in pattern of GST isoforms expression. Given the potential applicability of the chemoprotection mediated by GSTA3 to gene therapy of cancer, this chapter examines the ability of retrovirus-mediated transduction of rat GSTA3 to confer resistance to nitrogen mustards in hematopoietic cells.

RETROVIRUS-MEDIATED GENE TRANSFER OF RAT GLUTATHIONE S-TRANSFERASE Yc CONFERS IN VITRO RESISTANCE TO ALKYLATING AGENTS IN HUMAN LEUKEMIA CELLS AND IN CLONOGENIC MOUSE HEMATOPOIETIC PROGENITOR CELLS[1]

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Running title: Retrovirus GST gene transfer in hematopoietic cells

ABSTRACT

We have recently reported that N2Yc, a Moloney-based retrovirus vector expressing the Yc isoform of rat glutathione *S*-transferase (GST-Yc), conferred resistance to alkylating agents in mouse NIH 3T3 fibroblasts (Greenbaum et al. 1994). In this report, we address the feasibility of using rat GST-Yc somatic gene transfer to confer chemoprotection to the hematopoietic system. Human chronic myelogenous leukemia K-562 cells were efficiently transduced with the N2Yc retrovirus vector and showed a significant increase in the 50% inhibitory concentration of chlorambucil (3.2 to 3.3-fold), mechlorethamine (4.7 to 5.3-fold) and melphalan (2.1 to 2.2-fold). In addition, primary murine clonogenic hematopoietic progenitor cells transduced with the N2Yc vector were significantly more resistant to alkylating agents *in vitro* than cells transduced with the antisense N2revYc vector: the survival of Yc-transduced hematopoietic colonies at 400 nM mechlorethamine and 4 μM chlorambucil was 39.4% and 42.6% respectively compared to 27.2% and 30.4% for N2revYc-transduced cells. Future experiments will determine the level of chemoprotection achievable *in vivo* following transplantation of N2Yc-transduced hematopoietic cells in mice.

INTRODUCTION

Despite significant advance in the development of cancer treatment based on the use of biological response modifiers or other forms of immunotherapy, cytotoxic chemotherapy generally remains the most effective treatment for disseminated neoplasia. However, the effectiveness of systemic chemotherapy is greatly limited by the inherent toxicity of the drugs. The therapeutic margin which exists between the dose which is effective and the one that results in intolerable toxicity is relatively narrow and often disappears with the development of drug resistance by the neoplastic cells. The principal dose-limiting tissue for many of the currently available drugs is the hematopoietic system. Therefore, attempts have been made at designing strategies that would prevent or circumvent hematopoietic toxicity. An attractive approach would be to use somatic gene transfer of drug resistance genes to confer chemoprotection to the hematopoietic system. This hematopoietic chemoprotection could reduce the toxicities and morbidity of chemotherapy, and allow safe dose intensification that might result in improved responses (Bertino 1990). Experimental evidence supports the feasibility of this gene therapy approach to cancer: hematopoietic chemoprotection has been obtained *in vivo* in mice following transplantation of bone marrow cells transduced with retrovirus vectors transducing either a mutant dihydrofolate reductase or the human multidrug resistance-1 (*MDR1*) gene encoded P-glycoprotein (Corey et al. 1990; Li et al. 1994; Mickisch et al. 1992; Podda et al. 1992; Sorrentino et al. 1992; Williams et al. 1987).

An important mechanism of drug resistance involves the thiol glutathione (GSH) and the enzymes glutathione *S*-transferases (GSTs). GSTs are multifunctional homo- or heterodimer enzymes that conjugate GSH to a wide range of xenobiotics. Their main function is to detoxify and enhance cellular efflux of potentially toxic molecules (Daniel 1993; Powis et al. 1987). Several laboratories have demonstrated an association between

increased bulk GST or specific GST isozymes with resistance to alkylating agents (Buller et al. 1987; Evans et al. 1987; Lewis et al. 1988; Robson et al. 1987; Wolf et al. 1987). In addition, transfection studies have provided direct evidence for the ability of GSTs to confer modest levels of resistance to alkylating agents (Nakagawa et al. 1990; Puchalski et al. 1990; Schecter et al. 1993). The highest level of resistance was obtained when transfecting the rat Yc homodimer (GST 2-2 in the most recent nomenclature) (Mannervick 1985) in rat mammary carcinoma cells (Schecter et al. 1993). This might be related to the high intrinsic organic peroxidase activity of that isoform (Mannervick et al. 1982). We have recently produced a retrovirus vector transducing the expression of rat GST-Yc and studied the effect of rat GST-Yc expression on sensitivity to alkylating drugs (Greenbaum et al. 1994). Yc-expressing populations and single cell-derived clones of mouse NIH 3T3 fibroblasts transfected or transduced with the GST-Yc retrovirus vector were more resistant to chlorambucil (1.8-6.0-fold) and mechlorethamine (4.6-10.8-fold) than were cells subjected to gene transfer with control antisense constructions. To address the feasibility of using rat GST-Yc somatic gene transfer to confer chemoprotection to the hematopoietic system, we have transduced human leukemia K-562 cells and primary mouse hematopoietic progenitor cells with a GST-Yc retrovirus vector. K-562 cells and clonogenic primary murine hematopoietic progenitor cells transduced with the GST-Yc retroviral vector were more resistant to alkylating agents than cells transduced with a control antisense vector. Future experiments will determine the level of chemoprotection achievable *in vivo* following transplantation of genetically modified bone marrow cells in mice.

MATERIALS AND METHODS

Cell lines and retrovirus vectors. Chronic myelogenous leukemia K-562 cells (Lozzio et al. 1975) were maintained in suspension culture in RPMI-1640 medium (Biofluids, Rockville, Md) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Burlington, Ontario, Canada) and 0.01% gentamicin (Boehringer Mannheim, Laval, Québec, Canada). Virus producing cells derived from GP+*env*Am-12 amphotropic virus packaging cells were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% FBS and 0.01% gentamicin (Markowitz et al. 1988). GP+*env*Am-12/Yc is a population of packaging cells producing the retrovirus vector N2Yc; GP+*env*Am-12/Yc#4 is a clonal isolate from GP+*env*Am-12/Yc; GP+*env*Am-12/revYc is a population of packaging cells producing the retrovirus vector N2revYc (Greenbaum et al. 1994). In these vectors, a single transcript originates from the Moloney-murine leukemia virus promoter/enhancer in the 5' long terminal repeat and includes a single insert: the rat GST-Yc cDNA in sense (N2Yc) or antisense (N2revYc) orientation.

Transduction of human leukemia cells. Exponentially growing GP+*env*Am-12/revYc, GP+*env*Am-12/Yc and GP+*env*Am-12/Yc#4 (Greenbaum et al. 1994) were sublethally irradiated (20 Gy, cobalt or cesium source) and 3-5 X 10^6 cells were plated in 20 X 100 mm diameter tissue culture dishes 6 to 24 hours prior to transduction. Three to five million exponentially growing K-562 cells were then co-cultivated in a 1:1 ratio with the irradiated virus-producing cells for 72 hours in RPMI-1640 supplemented with FBS, gentamicin, and 4 μg/ml polybrene (Sigma, Mississauga, Ontario, Canada). Four consecutive rounds of transduction were performed with the same virus-producing cell lines at weekly intervals.

Transduction of mouse bone marrow cells. Seven to 21-week-old C3H HeJ female mice (Jackson Laboratories, Bar Harbor, ME) weighing 15 to 30 g were injected intraperitoneally with 150 mg/kg 5-fluorouracil (David Bull Laboratories, Vaudreuil, Québec, Canada) 48 hours before bone marrow harvest. Fresh marrow was collected from the femurs and tibias and 3-5 X 10^6 nucleated bone marrow cells were cocultivated with an equal number of irradiated GP+*env*Am-12/revYc or GP+*env*Am-12/Yc cells for 72 hours. The cocultivation medium for murine bone marrow cells consisted of alpha minimum essential medium (α-MEM, GIBCO) supplemented with 20% FBS, 10 mg/ml bovine serum albumin (Boehringer Mannheim), 0.3 mg/ml iron-saturated human transferrin (Boehringer Mannheim), 0.01% gentamicin, 4 μg/ml polybrene, 10% conditioned medium (CM) from the murine myelomonocytic line WEHI-3B (Murphy et al. 1978) and 10% CM from the human primary bladder carcinoma cell line HTB-9 (Hoang et al. 1985).

Nucleic acid and protein analyses. Southern, Northern and Western blots were performed by standard methods as previously described (Greenbaum et al. 1994). For enzyme assays, separate cytosolic extracts were prepared for each independent assay by cell lysis in water and centrifugation of debris at 12,000 x *g* for 1 min. Protein concentrations were determined according to the method of Lowry *et al.* (Lowry et al. 1951) using bovine serum albumin as standard. Total GST enzyme activity was assayed using 2-chloro-1,3-dinitrobenzene as substrate (Habig et al. 1974) and selenium-independent GSH peroxidase activity was assayed using cumene hydroperoxide (Paglia et al. 1967). These enzyme assays were linear functions of protein concentration and time (for up to 5 min) when the amount of enzyme used adjusted to obtain a change in absorbance of 0.02-0.04 A_{340nm}/min for total GST and 0.01-0.03 A_{340nm}/min for GSH

peroxidase. Cellular GSH content was measured by a described spectrophotometric assay (Ellman 1959).

Drug sensitivity of human leukemia cells. Drug sensitivity of K-562 cells was initially assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (Greenbaum et al. 1994). Cells were tested by MTT for sensitivity to chlorambucil (Sigma), mechlorethamine (Sigma), melphalan (Alkeran, generously provided by Burroughs Wellcome, Kirkland, Quebec), and methotrexate (Adria, Columbus, Ohio).

Drug sensitivity of K-562 cells was also evaluated by continuous exposure to mechlorethamine while the cells were maintained in culture. Survival of cells exposed to no drug was compared to the survival of cells exposed to the 50% inhibitory concentration (IC_{50}) of mechlorethamine for the parent K-562 cells as determined by MTT assays (6 μM, see below). The cell culture medium was replaced by medium containing the same concentration of drug every three days. Viable cell counts were obtained by trypan blue exclusion and cells were passaged as needed at the time of medium change. The experience was stopped when the survival of parent K-562 cells exposed to mechlorethamine was less than 1% of the control culture.

Drug sensitivity of clonogenic mouse hematopoietic cells. Sensitivity of mouse bone marrow to drugs was assessed using an *in vitro* assay for clonogenic hematopoietic progenitor cells. Immediately after cocultivation with the virus producers, non adherent bone marrow cells were plated at a final concentration of 1-2 X 10^5 cells/ml in α-MEM containing 0.9% methylcellulose (Fisher Scientific, Montréal, Québec, Canada), 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol (Sigma), 0.01% gentamicin, 2 units/ml erythropoietin (Stemcell Technologies, Vancouver, British Columbia, Canada), and 1%

pokeweed mitogen-stimulated spleen conditioned media (GIBCO). Cytotoxic drugs were added to the assay mix in doses that inhibited 45% to 70% of normal hematopoietic colony growth: mechlorethamine (Sigma) 4.0 X 10^{-7} M, chlorambucil (Sigma) 4.0 X 10^{-6} M, or vinblastine (David Bull Laboratories) 7.5 X 10^{-9} M. Cultures were plated in triplicate in 1-ml aliquots in 35 mm diameter petri dishes and placed in 5% CO_2 at 37°C. The total number of hematopoietic colonies (>50 cells) was scored at day 10 to 14 of culture.

Detection of transferred sequence in individual hematopoietic colonies. Individual hematopoietic colonies grown in methylcellulose were isolated as previously described (Cournoyer et al. 1991). Crude DNA extracts from individual progenitor colonies were obtained by lysing colonies for 4 hours at 55°C in 30 µL lysis buffer consisting of 1.7 µM sodium lauryl sulfate, 0.1 mg/ml proteinase K (Boehringer Mannheim), 40 mM dithiothreitol (DTT)(Sigma), 50 mM KCl, 10 mM Tris-Cl pH 8.4, 0.1 mg/ml gelatin and 3 mM $MgCl_2$ (modified from Li *et al* (Li et al. 1988)).

A polymerase chain reaction (PCR) was used to detect the presence of integrated N2Yc provirus in crude DNA extracts from individual hematopoietic colonies. The sense synthetic oligonucleotide primer for that reaction (5'-GAGACGGCACCTTTAACCGA) was derived from the gag^+ sequence of Moloney murine leukemia virus (nucleotides 775 to 794 in ref. (Shinnick et al. 1981)); the antisense primer (5' - CTCCTTCTGCATACATGTCG) was complementary to the 5' region of the GST Yc cDNA (nucleotides 276 to 295 in ref. (Telakowski-Hopkins et al. 1985)). This PCR specifically amplifies a fragment of 611 base pairs (bp) in presence of N2Yc proviral DNA.

A second PCR was used, as previously described, to detect the presence of contaminating DNA from GP+*env*Am-12 virus producing cells which might be

responsible for the detection of N2Yc sequence in non transduced hematopoietic colonies (Cournoyer et al. 1991). This reaction amplifies a 590 bp fragment of the bacterial hygromycin B resistance (HGB^R) gene which is present in one of the plasmids used to construct the GP+*env*Am-12 packaging cell line.

For both PCR assays, 10 µl of the crude DNA extracts from individual hematopoietic colonies were amplified in a final volume of 50 µl of 50 mM KCl, 10 mM Tris-Cl pH 8.4, 0.1 mg/ml gelatin, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each oligonucleotide primer and 2.5 units of Taq polymerase (Boehringer Mannheim). $MgCl_2$ was also added at 1.5 mM for N2Yc amplification and at 3 mM for HGB^R amplification. The samples were heated at 94°C for 6 min to inactivate the proteinase K before adding the Taq polymerase and subsequently underwent 50 cycles of amplification in a thermocycler (Perkin Elmer-Cetus, Norwalk, CT), each cycle consisting of 30 sec at 94°C for denaturation, 30 sec for annealing (at 50°C for N2Yc, 55°C for HGB^R), and 1 min 45 sec at 72°C for extension. Amplified fragments were visualized after electrophoresis in 1% agarose gels containing 0.5 µg/ml ethidium bromide.

RESULTS

In order to evaluate the feasibility of using rat GST-Yc retroviral gene transfer to confer chemoprotection to the hematopoietic system, K-562 human chronic myelogenous leukemia cells and primary murine bone marrow cells were transduced with the N2Yc retroviral vector or with the control N2revYc antisense vector. The efficiency of gene transfer, the expression of the transduced GST-Yc and the impact of Yc-transduction on drug sensitivity were then evaluated as described in the "Materials and Methods" section.

Gene transfer into human leukemia cells. The absence of deletion or rearrangement in the vector sequence integrated in K-562 cells was assessed by Southern blot analysis following digestion of genomic DNA with restriction enzymes which cut twice in the N2Yc and N2revYc proviral sequences (but outside the GST-Yc cDNA). Blots were then hybridized with a ^{32}P-labeled full length GST-Yc cDNA insert isolated from pPSMYc (Telakowski-Hopkins et al. 1985). This results in the detection of a fragment of predictable size independent of the integration site. Fig. 1 shows the result of Southern blot analysis following *Eco* RI digestion; the expected 1.1-kilobase (kb) Yc-containing fragment is seen in DNA from K-562 cells transduced with either N2Yc or N2revYc.

To determine the average copy number of transferred vectors in these cells, genomic DNA from a clonal isolate of amphotropic virus-producing cells, GP+*env*Am-12/Yc#4, was digested with *Hin* dIII, a restriction enzyme which has no recognition site within the N2Yc and N2revYc sequences. This results in a multiplicity of fragments sizes detected by the radiolabeled Yc cDNA, each corresponding to one integration site in GP+*env*Am-12/Yc#4 (fig. 1). Densitometric comparison, on non saturated autoradiograms, of the intensity of these fragments with the 1.1-kb *Eco* RI fragments seen in transduced cells revealed an average of approximately 3.8, 5.6 and 4.6 vector copies

per cell in K-562/revYc, K-562/Yc and K-562/[Yc#4] cells respectively. Because there was no procedure of selection or cloning to isolate the successfully transduced K-562 cells following the four rounds of co-cultivation with irradiated virus producers, the populations of transduced cells constitute a mixture of transduced and non transduced cells. The vector copy number determined by Southern blot analysis thus represents an average that includes both the transduced and non transduced cells in the population.

Transcriptional activity of the vectors in transduced human leukemia cells. The transcriptional activity of the transferred vectors in transduced K-562 cells was assessed by Northern analysis using a full-length GST-Yc cDNA probe. This demonstrated efficient expression of the expected 2.8 kb vector messenger RNA (mRNA) transcript in K-562 cells transduced with N2Yc or N2revYc vectors (Fig. 2).

GSH content, GSH peroxidase and total GST activity of transduced human leukemia cells. Cytosolic extracts from cells subjected to GST-Yc gene transfer were assayed for GSH content, total GST activity and selenium-independent GSH peroxidase activity, the latter being associated with the Yc subunit of GST (Table 1). No significant differences in GSH content or in total GST activity were observed in K-562 cells transduced with N2Yc or N2revYc vectors. K-562 cells transduced by cocultivation with GP+*env*Am-12/Yc (K-562/Yc) or by cocultivation with the clonal isolate GP+*env*Am-12/Yc#4 (K-562/[Yc#4]) showed a 2.5 to 3.0-fold increase in GSH peroxidase activity in comparison with parent K-562 cells ($P < 0.01$, paired student's t test).

Expression of immunodetectable rat GST-Yc in transduced human leukemia cells. Western blot analysis was performed on cells subjected to GST-Yc gene transfer using the following polyclonal rabbit anti-rat GST antibodies: anti-Yc, anti-rat liver GST (Ya,

Yb and Yc) and anti-Ypi. This revealed high expression of immunodetectable Yc isoform in N2Yc-transduced K-562 cells (Fig. 3). Expression of Ya and Yb isoforms remained undetected and that of Ypi isoform unchanged following transduction with sense or antisense Yc vectors (not shown).

Drug sensitivity of GST-Yc transduced human leukemia cells. Drug sensitivity studies on transduced K-562 cells were performed using the MTT assay (Table 2). In comparison with the parent K-562 cells, N2Yc-transduced K-562 cells showed a 3.2 to 3.3-fold significant increase in the IC_{50} of chlorambucil, a 4.7 to 5.3-fold significant increase in the IC_{50} of mechlorethamine and a 2.1 to 2.2-fold increase in the IC_{50} of melphalan (significant for K-562/Yc) after 3-5 independent assays (paired Student's t test). In contrast, N2Yc- and N2revYc-transduced K-562 cells demonstrated unaltered sensitivity to the antimetabolite methotrexate, a non alkylating drug.

In addition, the survival of K-562 cells and K-562/Yc cells was assessed following repeated exposure to mechlorethamine 6 μM, the IC_{50} concentration of that drug for K-562 cells in MTT assays. After 6 days in culture, cell survival was 0.9 ± 0.3 % for K-562 cells, in comparison with 16.6 ± 2.0 % for K-562/Yc ($P < 0.01$, four independent experiments, paired Student's t test).

Gene transfer into clonogenic mouse hematopoietic progenitor cells. The PCR assays amplifying sequences of the N2Yc provirus and of the HGB^R gene were shown to be equally sensitive in detecting as little as 25 pg of GP+*env*Am12/Yc genomic DNA (i.e. the DNA content of approximately four diploid cells)(data not shown). Like in previous studies of adenosine deaminase gene transfer, we considered the amplification of proviral sequence from the DNA of an hematopoietic colony indicative of the presence of vector sequence in the hematopoietic cells themselves if HGB^R sequences could not be amplified

(Cournoyer et al. 1991). The efficiency of gene transfer into clonogenic hematopoietic cells was assessed in five independent experiments. In each experiment, ten to twenty hematopoietic colonies (predominantly granulocyte, macrophage and granulocyte/macrophage colonies) were randomly picked after 10-14 days of culture and analyzed in the PCR assays. The proportion of colonies positive for proviral sequence were as follow (with the number of colonies excluded because of amplifiable HGB^R sequence given in parenthesis): 7/9 (1), 3/9 (1), 6/6 (4), 7/8 (2), 10/13 (7). The results of this last PCR assay are shown in figure 4. The cumulative percentage of hematopoietic colonies positive for the presence of N2Yc vector sequence in absence of HGBR sequence was thus 70% (33 of 45 colonies).

Drug sensitivity of GST-Yc transduced mouse hematopoietic progenitor cells. Clonogenic murine bone marrow progenitor cells were tested for their sensitivity to mechlorethamine and chlorambucil following cocultivation with GP+*env*Am-12/Yc or GP+*env*Am-12/revYc cells using an *in vitro* assay for clonogenic hematopoietic cells. Survival of hematopoietic colonies in presence of 400 nM mechlorethamine and 4 μM chlorambucil were 39.4 ± 3.5% and 42.6 ± 2.5% (mean ± SE) respectively for bone marrow cells transduced with the N2Yc vector, in comparison with 27.2 ± 2.8% and 30.4 ± 2.7% respectively for cells transduced with N2revYc vectors respectively (Table 3). These differences were significant for mechlorethamine ($P < 0.01$) and chlorambucil ($P < 0.05$) as measured in 8 independent assays (paired Student's t test). In contrast, sensitivity of N2Yc and N2revYc-transduced clonogenic hematopoietic cells to the vinca alkaloid agent vinblastine remained unchanged.

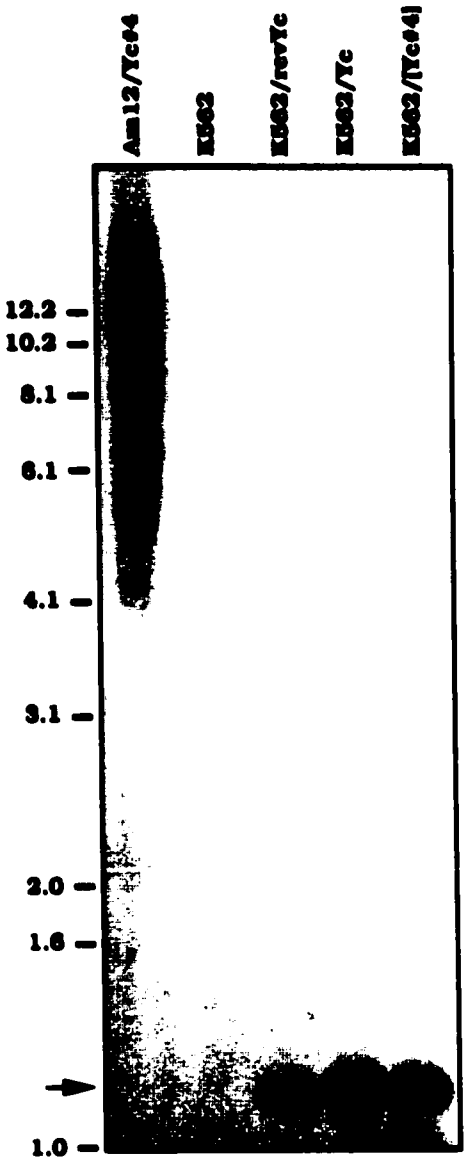

Fig. 1. Southern blot analysis of GST-Yc transduced K-652 cells. Genomic DNA from K-562 cells and derivatives were digested with *Eco*RI while DNA from GP+*env*Am-12/Yc#4 was restricted with *Hin*dIII. Ten μg of genomic DNA from each sample were migrated on a 1.0% agarose gel, transferred to Hybond-N nylon membrane (Hybond, Oakville, Ontario, Canada), and hybridized with a ^{32}P-labeled full length GST-Yc cDNA probe (approximately 10^6 CPM/ml). AM12/Yc#4 stands for GP+*env*Am-12/Yc#4. K562/revYc, K562/Yc and K562/[Yc#4] refer to K-562 cells transduced by cocultivation with GP+*env*Am-12/revYc, GP+*env*Am-12/Yc and GP+*env*Am-12/Yc#4 respectively.

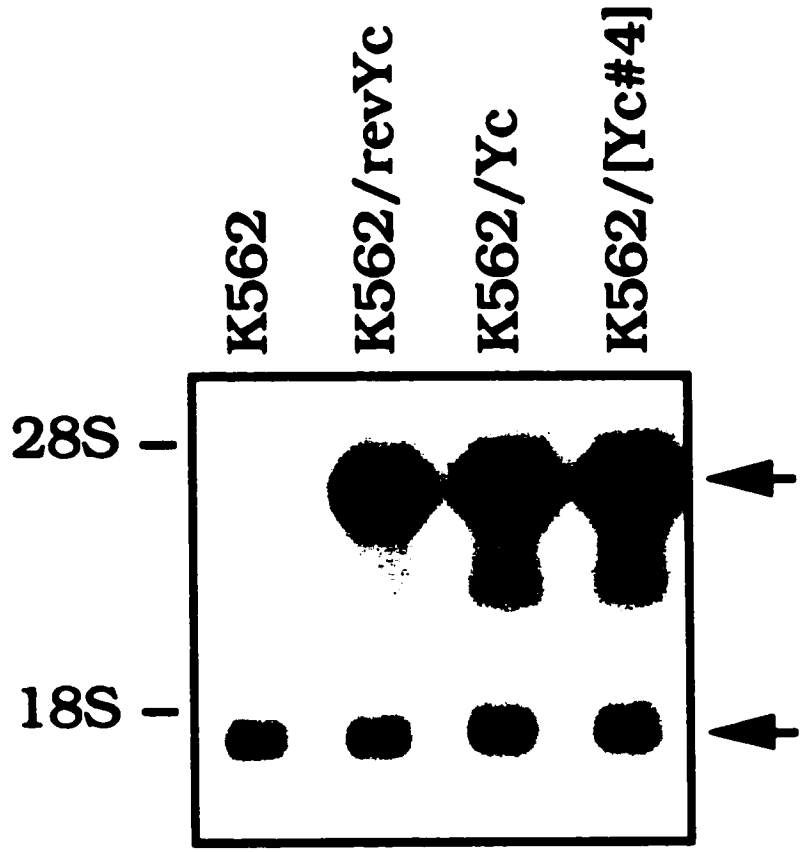

Fig. 2. Northern blot analysis of GST-Yc transduced K-562 cells. Ten μg of total RNA from each sample were migrated on a 1.0% agarose/2.2 M formaldehyde gel, transferred to Hybond-N nylon membrane, and hybridized with a ^{32}P-labeled GST-Yc cDNA probe and a ^{32}P labeled glyceraldehyde 3-phosphate dehydrogenase cDNA probe (each probe at approximately 10^6 CPM/ml). Symbols are the same as in Figure 1. Upper arrow indicates the position of migration of the 2.8 kb N2Yc vector mRNA; lower arrow indicates the position of migration of the 1.1 kb glyceraldehyde 3-phosphate dehydrogenase mRNA.

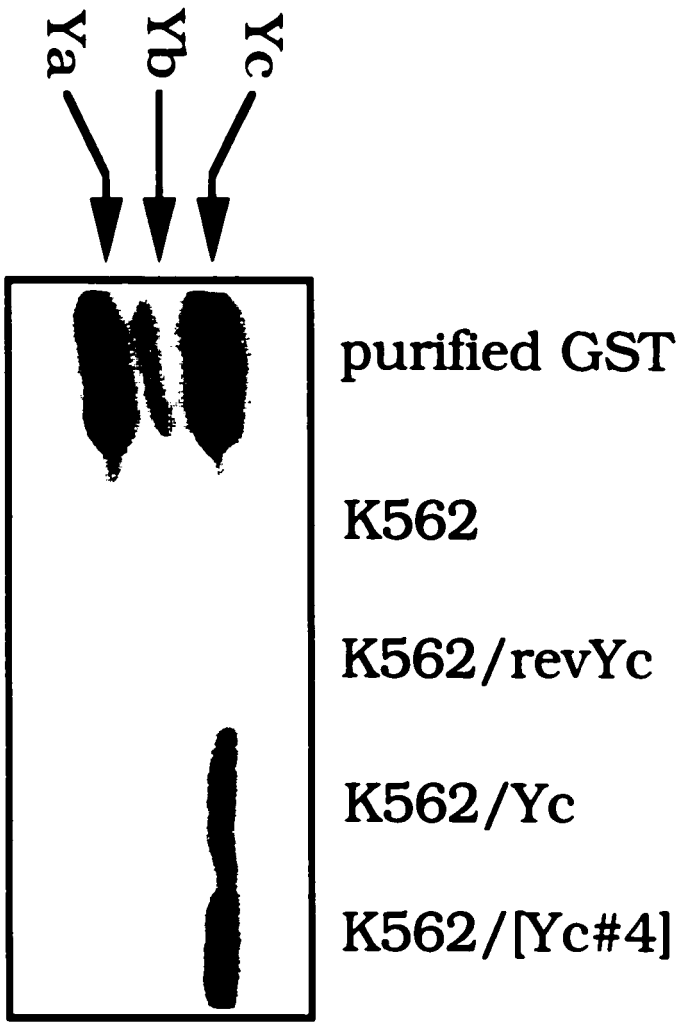

Fig. 3. Western blot detection of GST-Yc gene product in K-562 cells. Seventy-five μg of cytosolic protein from each cell line were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose; the membrane was reacted successively with a polyclonal rabbit anti-rat liver GST antibody, a horseradish peroxidase-conjugated goat anti-rabbit antibody, and a solution of diaminobenzidine and hydrogen peroxide (H_2O_2). Purified GST, purified rat liver GST. Other symbols are the same as in Figure 1. Arrows indicate position of migration of the indicated isoforms of rat liver GST.

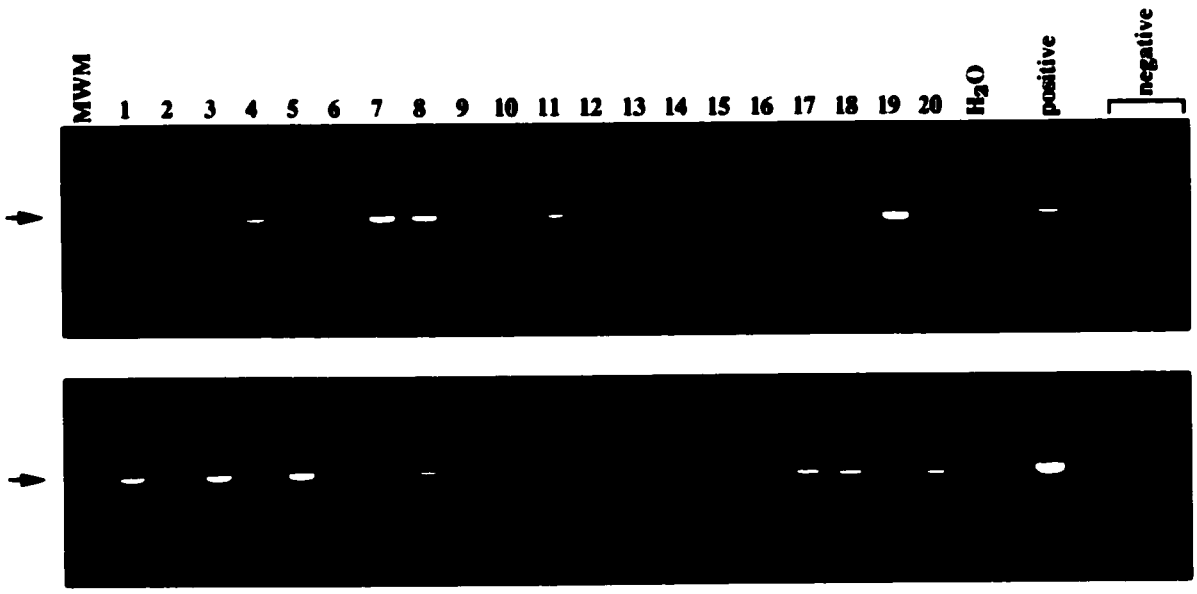

Fig. 4. PCR detection of HGB^R and N2Yc sequences. HGB^R sequences (upper panel) and N2Yc sequences (lower panel) were amplified from individual hematopoietic colonies as described in the methods. MWM, molecular weight markers; lanes 1 to 20 represent individual colonies derived from N2Yc-transduced bone marrow cells; H_2O: water i.e. no template DNA; positive: amplification of DNA from GP+*env*Am-12 for HGB^R reaction and from GP+*env*Am-12/Yc for N2Yc reaction; negative: two hematopoietic colonies from N2revYc-transduced bone marrow cells. On the original photograph, N2Yc-transduced colonies 2, 4, 7, 8, 10, 11 and 19 are positive for HGB^R sequence; the same colonies plus colonies 1, 3, 5, 6, 12, 14, 15, 17, 18 and 20 are positive for N2Yc proviral sequence.

	GSH	ratio	GSH peroxidase	ratio	total GST	ratio
K-562	22.7 ± 4.9	1.0	0.6 ± 0.2	1.0	117.3 ± 16.6	1.0
K-562/revYc	18.0 ± 4.4	0.6	0.3 ± 0.1	0.4	116.9 ± 18.4	1.0
K-562/Yc	17.0 ± 4.0	0.6	1.5 ± 0.1	2.5[a]	126.6 ± 22.0	1.1
K-562/(Yc#4)	22.0 ± 5.7	0.8	1.8 ± 0.2	3.0[a]	116.8 ± 24.3	1.0

[a] $P < 0.01$

Table 1. GSH content, selenium-independent GSH peroxidase activity and total GST activity of transduced K-562 cells. GSH content, selenium-independent GSH peroxidase activity and total GST activity of cytosolic extracts were determined as previously described (1). Results represent the mean of three independent assays, each one performed in triplicate, with standard error (SE). GSH values are expressed in nmol/mg protein and enzyme activities in nmol/min/mg protein. Ratios of the GSH content and enzyme activities of transduced K-562 cells over the GSH content and enzyme activities of untransduced K-562 cells are given. Statistical values are by paired Student's t test. Symbols for cell lines are the same as in figure 1.

	Chlorambucil		Mechlorethamine		Melphalan		Methotrexate	
	IC_{50} (μM)	ratio	IC_{50} (μM)	ratio	IC_{50} (μM)	ratio	IC_{50} (nM)	ratio
K-562	7.3 ± 0.4	1.0	5.3 ± 0.5	1.0	5.5 ± 0.3	1.0	26.4 ± 7.3	1.0
K-562/revYc	7.9 ± 0.8	1.1	5.3 ± 0.7	1.0	6.5 ± 0.8	1.2	18.2 ± 4.4	0.7
K-562/Yc	24.4 ± 1.9	3.3[c]	28.3 ± 5.3	5.3[c]	12.3 ± 0.7	2.2[a]	16.1 ± 4.0	0.6
K-562/(Yc#4)	23.4 ± 2.4	3.2[b]	25.0 ± 4.2	4.7[a]	11.3 ± 3.4	2.1	35.3 ± 9.8	1.3

[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$

TABLE 2. Drug sensitivity of human K-562 cells transduced with sense or antisense GST-Yc. Drug sensitivity assays were performed using the MTT colorimetric assay as previously described (1). Results represent the mean of 3-5 independent assays, each cell line being tested 8 times in every individual assay, with SE. Ratios of the IC_{50} of transduced K-562 cells for a given drug over the IC_{50} of untransduced K-562 cells are given. Statistical values are by paired Student's t test. Symbols for cell lines are the same as in Table 1.

	% surviving hematopoietic progenitor colonies		
	mechlorethamine	chlorambucil	vinblastine
BM/revYc	27.2 ± 2.8	30.4 ± 2.7	54.7 ± 7.4
BM/Yc	39.4 ± 3.5[b]	42.6 ± 2.5[a]	50.9 ± 6.6

[a] $P < 0.05$
[b] $P < 0.01$

TABLE 3. Percentage of surviving hematopoietic progenitor colonies in the presence of drug. Survival of bone marrow progenitor cells was assayed *in vitro* in the presence of 400 nM mechlorethamine, 4 μM chlorambucil and 7.5 nM vinblastine as described in Materials and Methods. Results represent the mean of 8 independent assays, with standard error (SE). Statistical values are by paired Student's t test. BM/revYc: bone marrow co-cultivated with GP+*env*AM-12/revYc cells; BM/Yc: bone marrow co-cultivated with GP+*env*AM-12/Yc cells.

DISCUSSION

We have recently shown that transfection and retrovirus-mediated gene transfer of rat GST-Yc into NIH 3T3 mouse fibroblasts resulted in increased *in vitro* resistance to chlorambucil (1.8-6.0-foid) and mechlorethamine (4.6-10.8-fold) (Greenbaum et al. 1994). However, the effect of GST-Yc retroviral transduction on the sensitivity to alkylating agents was found to be of variable magnitude in different cell lines, possibly in relation to differences in endogenous GSH and GST levels or pattern of GST isoforms expression ((Tew 1994) and M.G., S.L. and D.C., unpublished data). Given the potential applicability of the chemoprotection mediated by GST-Yc to gene therapy of cancer, the present report examines the ability of retrovirus-mediated transduction of rat GST-Yc to confer *in vitro* resistance to alkylating agents in hematopoietic cells.

Human chronic myelogenous K-562 leukemia cells were transduced by cocultivation with amphotropic cells producing the N2Yc sense retrovirus vector (the GP+*env*Am-12/Yc virus-producing cell population or the clonal isolate GP+*env*Am-12/Yc#4) or the N2revYc antisense vector (the GP+envAm-12/revYc cell population). Virus titers from these cell lines determined by RNA slot blot analysis are comparable and range between 2 to 5 X 10^6 viral particles/ml ((Greenbaum et al. 1994) and M.G. and D.C., unpublished results). These viral titers have been stable since the virus-producing cells were isolated. Southern blot analysis of *Eco* RI restricted genomic DNA from the transduced cell lines confirmed the presence of unrearranged vector sequence in transduced K-562 cells (Fig. 1). Densitometry analysis of Southern blots comparing the intensity of fragments containing GST-Yc cDNA sequences in *Eco* RI restricted genomic DNA from the transduced cells and in *Hind*III restricted DNA from GP+*env*Am-12/Yc#4 revealed that a relatively high average copy number of vector per cell was obtained in K-562 cells transduced by four rounds of cocultivation (3.8 to 5.6 copies/cell).

Results of Northern blot analysis likewise showed efficient expression of the expected 2.8-kb sense or antisense vector transcripts in transduced K-562 cells (Fig. 2). Western blot analysis using either anti-rat liver GST or anti-GST-Yc polyclonal rabbit antibodies similarly demonstrated efficient expression of immunoreactive GST-Yc protein in N2Yc-transduced cells (Fig. 3). Finally, transduction of functional GST-Yc enzyme was apparent in the 2.5 to 3.0-fold increase in the selenium-independent GSH peroxidase activity of N2Yc-transduced K-562 cells in comparison with the parent cells (Table 1, $P < 0.01$). There was no significant change in the total GST activity of N2Yc-transduced K-562 cells. The lack of effect of GST-Yc transduction on total GST activity in K-562 cells was most probably related to the minor contribution of the transferred GST-Yc enzyme to the total cytosolic GST activity in the transduced cells. Likewise, we have previously observed a better correlation of GST-Yc transduction with GSH peroxidase activity than with total GST activity in NIH 3T3 fibroblasts (Greenbaum et al. 1994).

Functional expression of the transferred GST-Yc was firmly established by the significant increase in alkylating drug resistance of Yc-transduced K-562 human leukemia cells (Table 2). Cells transduced with N2Yc were 3.2- to 3.3-fold resistant to chlorambucil, 4.7- to 5.3-fold resistant to mechlorethamine, and 2.1- to 2.2-fold resistant to melphalan in MTT assays (all significant changes except for melphalan resistance in K-562/[Yc#4]). In addition, K-562/Yc cells cultivated in presence of 6 μM mechlorethamine (the IC_{50} for parent K-562 cells) demonstrated a significantly higher survival after 6 days than parent cells: 16.5 % vs 0.9 % ($P < 0.01$). This indicates a significant survival advantage to Yc-transduced cells in presence of an alkylating agent.

While the above effect of GST-Yc transduction on drug resistance of K-562 were obtained following four rounds of transduction by cocultivation, cells subjected to a single round of cocultivation had an average of 2.4 copies of N2Yc vector per cell and

were 3.3-fold resistant to mechlorethamine (data not shown). The absence of significant differences in GSH peroxidase, total GST activities and in sensitivity to alkylating agents between wild-type cells and cells transduced with the antisense N2revYc vector indicates, along with our previous findings NIH 3T3 mouse fibroblasts, that there is no significant antisense inhibition of endogenous GST activity by our rat GST-Yc antisense vector in either mouse or human cells. This most probably relates to the imperfect homology (approximately 75% in the coding sequence) between the rat and human or mouse αGST cDNAs (Daniel 1993).

Given that an increase in drug resistance was observed with a relatively high copy number of vector per cell in K-562 cells, we next investigated whether sufficiently efficient gene transfer of GST-Yc could be achieved in primary hematopoietic cells to augment their tolerance to alkylating agents. Because clonal isolates of virus-producing cells can vary in their ability to support the growth of primary murine hematopoietic cells (personal communication, Dinko Valerio, IntroGene, Rijswijk, Netherlands), we chose to use the population of GP+*env*Am-12/Yc amphotropic virus producing cells to transduce primary murine clonogenic hematopoietic progenitor cells. Bone marrow cells were collected from C3H HeJ mice and co-cultivated with GP+*env*Am-12/Yc or GP+*env*Am-12/revYc virus-producing cells. PCR amplification of provirus and HGB^R sequences from crude DNA extracts of individual hematopoietic colonies indicated that the cumulative transduction efficiency was approximately 70%. Despite the imprecision of measuring transduction efficiency in small number of colonies, this transduction efficiency is moderately superior to the range of 5-50% that has previously been reported in clonogenic mouse hematopoietic progenitor cells when using similar conditions (i.e. using conditioned media as a source of hematopoietic growth factors during the cocultivation, and in absence of preselection in favour of the transduced cells) (Belmont et al. 1988; Keller et al. 1985; Magli et al. 1987; McLachlin et al. 1990). This improved

efficiency of transduction is most probably related to the high titer of vectors produced by our virus-producing cells. Finally, the sensitivity of transduced clonogenic hematopoietic progenitor cells to cytotoxic drugs was assessed in colony formation assays. The percentage of surviving N2Yc-transduced colonies was approximately 12.2% higher in presence of 400 nM mechlorethamine or 4 μM chlorambucil (significant by paired Student's t test) while it remained unchanged in vinblastine, a non alkylating agent (Table 3).

These results demonstrate that retrovirus-mediated gene transfer of rat GST-Yc confers *in vitro* resistance to alkylating agents in a human hematopoietic cell line (K-562) and in clonogenic primary murine hematopoietic progenitor cells. This further supports the feasibility of using GST-Yc somatic gene transfer to confer chemoprotection to the hematopoietic system against alkylating agents. The feasibility of *in vivo* hematopoietic chemoprotection has been demonstrated in mice using retrovirus vectors transducing a mutant dihydrofolate reductase or the P-glycoprotein encoded by the human multidrug resistance-1 (*MDR1*) gene (Corey et al. 1990; Li et al. 1994; Mickisch et al. 1992; Podda et al. 1992; Sorrentino et al. 1992; Williams et al. 1987).

Clinical trials testing *MDR-1* mediated chemoprotection in cancer patients have now been approved or are undergoing review (1994; 1995). Potential advantages to chemoprotection from alkylating agents include the broad spectrum of antitumor activity of these drugs, their constant dose-related effect through multiple logs of tumor cell kill, and their predominant and dose-limiting hematotoxicities (Chabner et al. 1989; Peters 1985; Tormey et al. 1984). Although the enzymes O^6-alkylguanine-DNA alkyltransferase and class 1 and class 3 aldehyde dehydrogenase have been shown to increase the resistance to specific alkylating agents (Bodell et al. 1986; Bunting et al. 1994; Dumenco et al. 1989; Gerson et al. 1992; Sreerama et al. 1993; Yoshida et al. 1993), GST isoenzymes mediate a broader spectrum of resistance to alkylating agents (Tew 1994).

The levels of drug resistance obtained following gene transfer of GST-Yc may appear modest compared to the resistance obtained by MDR gene transfer, but relative resistance to alkylating agents observed clinically is of the same or lower magnitude (Wolf et al. 1987), suggesting that GST-Yc mediated chemoprotection would be clinically relevant. Finally, the lack of detectable constitutive expression of GST-Yc in either human and rodent mononuclear bone marrow cells also suggests a potential benefit from heterologous gene expression (Schecter et al. 1994). Future experiments will evaluate the level of *in vivo* chemoprotection obtained following transplantation of GST-Yc transduced bone marrow cells in lethally irradiated mice.

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CHAPTER 4

MANUSCRIPT THREE

PREFACE

We have explored the possibility of conferring chemoprotection against nitrogen mustards *in vivo* following transplantation of mice with GSTA3-transduced BM cells (Appendix 1). Unfortunately, we did not observed chemoprotection from chlorambucil in mice transplanted with N2/Yc-transduced BM. Our results suggested that the moderate growth advantage conferred by GSTA3 expression from our vector was insufficient to increase the tolerance to chlorambucil in transplanted mice. To circumvent this problem, we have constructed a bicistronic retrovirus vector (pMFG-GIC) combining the expression of the rat GSTA3 and the expression of a gene conferring an apparently stronger growth advantage, the human cytidine deaminase (CD). This chapter demonstrates that retroviral gene transfer of GIC in murine fibroblast 3T3 cells confers *in vitro* resistance to nitrogen mustards and cytosine nucleoside analogs, as well as to the combination of both classes of drugs. In addition, selection of GIC-transduced cells with Ara-C or melphalan is shown to increase the level of resistance to both classes of drugs.

COEXPRESSION OF RAT GLUTATHIONE S-TRANSFERASE A3 AND HUMAN CYTIDINE DEAMINASE BY A BICISTRONIC RETROVIRAL VECTOR CONFERS IN VITRO RESISTANCE TO NITROGEN MUSTARDS AND CYTOSINE ARABINOSIDE IN MURINE FIBROBLASTS

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Running title: Nitrogen mustards and Ara-C resistance by GST-CD vector

Keywords: glutathione S-transferase; cytidine deaminase; bicistronic retroviral vector; drug resistance; nitrogen mustards; cytosine arabinoside

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ABSTRACT

The transfer of drug resistance genes into hematopoietic cells is an experimental approach to protect patients from drug-induced myelosuppression. Because anticancer drugs are often administered in combination to increase their clinical efficacy, vectors that express two drug resistance genes are being developed to broaden the spectrum of chemoprotection. We have constructed a bicistronic vector, MFG/GST-IRES-CD (MFG/GIC) coexpressing the rat glutathione *S*-transferase A3 isoform (rGST Yc_1) and the human cytidine deaminase (CD). Murine NIH 3T3 (3T3) fibroblast cells transduced with this vector were evaluated for their resistance to nitrogen mustards and cytosine nucleoside analogs. GIC-transduced polyclonal cell populations (GIC cells) demonstrated marked increases in selenium-independent glutathione peroxidase (peroxidase) and CD activities, as well as increased resistance to melphalan (2.3-fold), chlorambucil (3.4-fold) and cytosine arabinoside (Ara-C) (8.1-fold). Following selection with Ara-C, the peroxidase and CD activities of GIC cells augmented 2.6- and 2.9-fold, respectively, in comparison with unselected cells, and the resistance to melphalan, chlorambucil and Ara-C further increased to 3.7, 5.9- and 53-fold, respectively. Melphalan selection of GIC cells likewise augmented their peroxidase (2.3-fold) and CD (1.9-fold) activities. GIC cells proliferated in the simultaneous presence of melphalan and Ara-C at drug concentrations that completely inhibited the growth of untransduced cells. The growth rate of unselected GIC cells exposed to the drug combination averaged 18% that of drug-free cultures. The growth rate of GIC cells exposed to the drug combination increased to 30% of controls following Ara-C selection, and to 50% following melphalan selection. Our results suggest that retroviral transfer of MFG/GIC may be useful for chemoprotection against the toxicities of nitrogen mustards and cytosine nucleoside analogs.

INTRODUCTION

Systemic treatment with cytotoxic drugs is the basis of most effective treatments for disseminated cancers. However, the effectiveness of systemic chemotherapy is greatly limited by acute dose limiting toxicities, principally myelosuppression. Current measures to circumvent this limitation, such as the use of hematopoietic growth factor support, pharmacological rescue, and hematopoietic cell transplantation, do not entirely eliminate the hematopoietic toxicity, and may not protect from multiple cycles of high dose chemotherapy. The introduction of drug resistance genes into hematopoietic cells may increase the bone marrow tolerance to chemotherapy and may permit safer dose escalation to increase clinical efficacy (reviewed in references[1-3]). The feasibility of this approach has been validated in murine animal models for the following genes: multidrug resistance (*MDR1*),[4,5] dihydrofolate reductase (*ΔDHFR*),[6,7] methylguanine methyltransferase (*MGMT*)[8,9] and alkyltransferase (*ada*).[10] On the basis of these preclinical studies, clinical trials have begun in cancer patients with the introduction of *MDR1* and *MGMT* genes into hematopoietic progenitor cells.[11-13]

Glutathione S-transferases (GSTs) represent a family of multifunctional cellular homo and heterodimer proteins that detoxify and enhance cellular efflux of a wide range of xenobiotics and metabolites. GSTs catalyze conjugation of electrophilic compounds to the tripeptide glutathione (GSH), and are capable of binding directly to nonsubstrate lypophilic substances. Some members of the family possess significant peroxidase activity, which may enable them to detoxify lipid and nucleic acid hydroperoxides resulting from drug exposure. Overexpression of the GST alpha isoenzymes has been implicated in resistance to nitrogen mustards.[14] Our laboratory has been investigating the rat GST A3 gene (previously designated GST Yc_1), a member of the alpha family of GSTs characterized by a high organic peroxidase activity.[15] The highest level of

resistance demonstrated to nitrogen mustards, to date, has been obtained following gene transfer of the rat GST A3 isoform.[16] We have shown, in previous studies, that retroviral gene transfer of this isoform confers *in vitro* resistance to nitrogen mustards in human K-562 cells and in murine fibroblasts and bone marrow-derived hematopoietic progenitor cells.[17,18] We have also studied the human drug resistance gene CD, which catalyses the deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. We have demonstrated an increased *in vitro* resistance to the cytosine nucleoside analogs gemcitabine, decitabine and cytosine arabinoside (Ara-C) in murine fibroblasts following retroviral gene transfer of CD.[19,20] We have also shown increased *in vitro* resistance to Ara-C in murine bone marrow progenitor cells transduced with CD,[20] and we have recently demonstrated long-term expression of CD in hematopoietic cells of transplanted mice.[21]

Because anticancer drugs are usually given in combination to increase their efficacy and limit the development of drug resistance in tumor cells, designing vectors that coexpress drug resistance genes for two distinct classes of drugs should be useful for the purpose of hematopoietic chemoprotection. A number of studies have demonstrated the feasibility of combining drug resistance mechanisms for the purpose of chemoprotection. Resistance to P-glycoprotein effluxed drugs and antifolates or nitrosoureas was obtained by coexpression of *MDR*1and Δ*DHFR*[22] or *MGMT* genes.[23] Resistance to antifolates and fluoropyrimidine has been demonstrated by coexpression of Δ*DHFR* and thymidylate synthase (*TS*) genes.[24] We have recently shown resistance to antifolates and cytosine nucleoside analogs by coexpression of Δ*DHFR* and *CD* in murine fibroblasts and bone marrow progenitor cells.[25]

In the present study, we have combined the expression of the rat GST A3 (GST) and human CD genes in a bicistronic retroviral vector, MFG/GIC, in which translation of the GST cDNA is cap-dependent, whereas translation of the CD cDNA is dependent on

an internal ribosome entry site (IRES). Murine fibroblasts transduced with MFG/GIC displayed resistance to nitrogen mustards and Ara-C administered separately or in combination. Selection of transduced cells with Ara-C or melphalan augmented their levels of resistance to both classes of drugs, separately and in combination. Our results suggest that retroviral transfer of MFG/GIC may be useful for chemoprotection against the combined toxicities of nitrogen mustards and cytosine nucleoside analogs. Our results also suggest that MFG/GIC-mediated chemoprotection may be further increased following selection with either class of drugs. This may be particularly useful in order to increase the level of chemoprotection from the widely used nitrogen mustards.

MATERIALS AND METHODS

Cell lines. 3T3 mouse fibroblasts and virus-producing cells derived from GP+E-86 and GP+*env*Am-12 packaging cells[26,27] were maintained in Dulbecco's modified Eagle's medium (DMEM, BIO MEDIA, Drummondville, Quebec, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS, BIO MEDIA) and 5 μg/ml gentamicin (Boehringer Mannheim, Laval, Quebec, Canada) at 37°C in a 5% CO_2 incubator.

Vector constructions. The rat GST cDNA was inserted in the MFG expression retrovirus vector at the position of the viral *env* initiation codon. A 986 base pairs (bp) fragment comprising the last 299 bp preceding the *env* initiation codon of MFG and the complete GST cDNA (665 bp) was constructed by polymerase chain reaction (PCR) using two different templates and three amplification reactions. First, the region of MFG preceding the *env* initiation codon (3'MFG) was amplified from pMFG-tPA (obtained from R. Mulligan, Whitehead Institute, Cambridge, MA) with the sense oligonucleotide primer 5' GCCATACGCCAACCCTCTCTCCAAGC 3', and the antisense primer 5' GCTTCCCCGGCATGGCAGTCTAGAG 3', the latter including the 5' end of GST (underlined in the sequence). Second, the cDNA of GST was amplified from pPSMYc[17] using the sense primer 5' CCATCCTCTAGACTGCCATGCCGGG 3' and the antisense primer 5' CCCCATCCGCGGTTAACTGAAGATCTTAA 3', the underlined sequences corresponding to vector sequence preceding the viral *env* initiation codon of MFG, including a *Sac*II restriction site. These amplification reactions yielded products of 324 bp and 694 bp, respectively. Twenty-five ng of pMFG-tPA and 10 ng of pPSMYc were amplified in a final volume of 50 μl of 50 m*M* KCl, 10 m*M* Tris-Cl pH 8.4, 0.1 mg/ml gelatin, 0.4 m*M* each of dATP, dCTP, dGTP, and dTTP, 0.5 p*M* of each oligonucleotide primer, and 2 units of ID-Proof (*Taq*) DNA polymerase (ID Labs Biotechnology,

London, Ontario, Canada). The samples were heated at 94°C for 6 min during which 3 m*M* $MgCl_2$ was added. The samples were then amplified for 20 cycles, each cycle consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 1 min 45 sec at 72°C for extension, with a terminal extension of 7 min at 72°C. The complete 3'MFG/GST fragment was amplified with the same reaction conditions using the 3'MFG and GST PCR products as templates. The 20 base pair homology between the two templates allows an efficient amplification when using the sense primer of the 3'MFG amplification and the antisense primer of the GST amplification. Ten ng of each template were amplified for 22 cycles, the first two cycles being performed in the absence of primers. The 3'MFG/GST fragment was then digested with *Sac*II, gel purified and ligated with pBluescript II KS$^+$ (Stratagene, La Jolla, CA) that had previously been digested with *Sac*I, blunt, digested again with *Sac*II and purified on gel. The integrity of the PCR component was confirmed by sequencing (with a Pharmacia automatic DNA sequencer). Finally, the plasmids Bluescript/3'MFG/GST and MFG/tPA were digested with *Age*I + *Bam*HI; and the fragments 3'MFG/GST and pMFG were gel purified and ligated together to generate pMFG/GST (Fig. 1).

To construct pMFG/GIC, pBluescript/3'MFG/GST was digested with *Sac*II + *Bam*HI, gel purified and the 3'MFG/GST fragment was ligated with the IRES-CD fragment released and gel purified from a *Sac*II + *Bam*HI digest of pMFG-DHFR-IRES-CD.[25] The plasmids MFG/tPA and Bluescript/3'MFG/GIC were then digested with *Age*I + *Bam*HI, gel purified, and the fragments pMFG and 3'MFG/GIC ligated together to obtain pMFG/GIC (Fig. 1).

The construction of pMFG/CD has been described previously (Fig. 1).[20]

Generation of virus producing cells. The plasmid pMFG/GST was cotransfected in a 10:1 molar ratio with pSV2neo[28] into GP+*env*Am-12 amphotropic packaging cells using a

polycationic reagent (SuperFect; Qiagen, Chatsworth, CA). Stable transfectants were selected with G418 (Geneticin; Gibco, Burlington, Ontario, Canada), a neomycin analogue, at 300 mg/ml (active drug), starting 48 hr post-transfection. For ten consecutive days, GP+E-86 ecotropic packaging cells were supplied with filtered supernatant from the G418-resistant polyclonal population of amphotropic producers, supplemented with 4 μg/ml polybrene (Sigma, St-Louis, MO). The ecotropic virus producers were then co-cultivated with amphotropic packaging cells in a 1:1 ratio for 21 days. The GP+*env*Am-12/GST polyclonal population was finally isolated by selection with 200 μg/ml hygromycin.[29]

The plasmid pMFG/GIC was cotransfected in a 5:1 molar ratio with pSV2neo into GP+*env*Am-12 cells by calcium phosphate precipitation (Pharmacia, Baie d'Urfé, Quebec, Canada), and stable transfectants were selected with 300 mg/ml G418. Filtered supernatant from the G418-resistant polyclonal population was supplemented with 8 μg/ml polybrene and applied over GP+E-86 cells for three consecutive days. The ecotropic virus producers were selected for 4 days in 2.5 μ*M* cytosine arabinoside (Ara-C; Sigma), and subsequently co-cultivated with amphotropic packaging cells in a 1:1 ratio for 21 days. Again, the GP+*env*Am-12/GIC polyclonal population was selected with 200 μg/ml hygromycin (titer, 4.0×10^5 viral particles/ml).

The generation of the ecotropic CD producer clone, GP+E-86/CD3, has been described previously.[20]

Transduction of 3T3 cells and drug selection. 3T3 cells were transduced with filtered supernatant from GP+*env*Am-12/GST and GP+*env*Am-12/GIC virus producers supplemented with 8 μg/ml polybrene for three consecutive days to generate NIH/GST (GST) and NIH/GIC (GIC) polyclonal unselected populations. Filtered supernatant from

GP+E-86/CD3 virus producers supplemented with 4 μg/ml polybrene was applied on 3T3 cells for five consecutive days to generate the NIH/CD (CD) polyclonal population.

Three hundred thousand CD and GIC cells were plated in 80 cm^2 tissue culture flasks and selected with 10 μ*M* cytosine arabinoside (Ara-C) for 4 days. GST and GIC cells were selected with 25 μ*M* melphalan under the same conditions. The cells were trypsinized and plated in fresh medium following selection.

Southern blot analysis. Genomic DNA extraction and Southern blots were performed as described previously.[17] Genomic DNA samples were digested with *Sac*I, migrated on a 1% agarose gel and transferred to a nylon membrane. The *Nco*I/*Bam*HI fragment of pMFG/CD (containing the full-length CD cDNA) was radiolabeled by random primer and hybridized with the membrane. In order to determine the copy number of CD-containing vectors in genomic DNA, graded quantities of the MFG/CD plasmid were added to 10 μg of genomic DNA from 3T3 cells followed by digestion with *Sac*I.

Enzyme assays. Separate cytosolic extracts were prepared for each independent assay by cell lysis in water and centrifugation of debris at 3,300 X *g* for 30 min at 4°C. Protein concentrations were determined according to the method of Bradford with the Bio-rad Protein Assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada), using bovine serum albumin as a standard. The peroxidase assay was performed using 1.5 m*M* cumene hydroperoxide as substrate.[30] The reaction mixture was prepared fresh for each assay and consisted of 50 m*M* potassium phosphate buffer, pH 7.4, 1.0 m*M* EDTA, 1.0 m*M* sodium azide, 1.0 m*M* GSH (reduced form, Sigma), 0.2 m*M* NADPH (Boehringer Mannheim) and 1 unit/ml GSH reductase (Boehringer Mannheim). Samples (400 μl to 800 μl) were preincubated at 37°C for 5 min and then mixed with 100 μl reaction mixture and 100 μl cumene hydroperoxide in a total volume of 1.0 ml. Absorbance at 340 nm was recorded

for 5 min and the activity was expressed as nmol/NADPH oxidized/min/mg protein over the linear range. CD activity was determined by measuring the conversion of [^{3}H]cytidine to [^{3}H]uridine as previously described.[31]

MTT assay. Drug sensitivity of 3T3 cells was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[32,33] Exponentially growing cells (650 to 1,200/well) were plated in 50 µl media in 96-well microtiter plates. The outer wells of the plates were not used and were filled with 175 µl PBS to provide added humidity. Plates were incubated for 24 hr at 37°C in 5% CO_2. Medium (50µl) containing chlorambucil (Sigma) or melphalan (Sigma) was then added to 6 wells for each drug concentration and plates were incubated for another 72 hr. Ara-C (Sigma) was added immediately after cells were plated, and plates were incubated for 96 hr. Twenty µl MTT (2.5 mg/ml in PBS; Sigma) was then added to each well, and plates were incubated for 4 hr at 37°C. One hundred µl of a solution consisting of 50% (vol/vol) N,N-dimethylformamide and 20% (wt/vol) sodium dodecyl sulfate, pH 4.7, was then added to the wells. Plates were wrapped in aluminum foil and returned to the incubator. The next day, the formazan product formed by viable cells was quantified by measuring the absorbance at a wavelength of 570 nm, with a reference at 650 nm on a microplate reader. The 50% inhibitory concentration (IC_{50}) was determined by plotting the percentage of surviving cells *versus* the log of drug concentration.

Growth inhibition assay. Exponentially growing cells (35,000/well) were plated in 6-well tissue culture plates in 3 ml growth media containing either 1.0 µ*M* Ara-C, 10 µ*M* melphalan, the combination of both drugs at the same concentration, or no drug. At the end of a 96-hr exposure at 37°C in 5% CO_2, viable cells counts were obtained by trypan blue exclusion. Growth is expressed as the increase in cell number relative to the increase

that is observed with cells grown in the absence of drug: [(final number of cells – $35{,}000)^{drug} \div$ (final number of cells – $35{,}000)^{no\ drug}$] x 100%.

Statistical analysis. Statistical analysis by Student's *t*-test was performed using StatView 4.0 software (SAS Institute inc.).

RESULTS

To evaluate the feasibility of protecting cells from the combined toxicity of nitrogen mustards and cytosine nucleoside analogs, we have transduced 3T3 cells with a bicistronic retroviral vector coexpressing the rat GST and the human CD (Fig. 1). A portion of the transduced cells were then subjected to selection with 10 μ*M* Ara-C, a dose that is lethal for untransduced cells. The efficiency of gene transfer, enzyme activities and drug sensitivity were then evaluated in transduced polyclonal cell populations, before and after Ara-C selection.

Gene transfer into 3T3 cells. The integrity of the vector sequences integrated in 3T3 cells was assessed by Southern blot analysis following digestion of genomic DNA with a restriction enzyme that cuts twice within the sequences of the MFG provirus backbone. Blots were then hybridized with a ^{32}P-labeled full length CD cDNA insert. This results in the detection of fragments of a predictable size, independent of the site of vector integration. Figure 2 shows the result of Southern blot analysis following *Sac*I digestion. The expected 2.5 kb and 3.8 kb CD-containing fragments are seen in the DNA from 3T3 cells transduced with, respectively, the MFG/CD and MFG/GIC vectors. In order to determine the average copy number of transferred vectors in these cells, graded quantities of the MFG/CD plasmid corresponding to 1, 2 and 5 vector copies per cell were added to 10 μg of genomic DNA from 3T3 cells before the digestion with the *Sac*I restriction enzyme. Densitometric comparison of the signal intensity from the resulting CD-containing fragments revealed an average of approximately 1 and 4 vector copy per cell in the polyclonal populations of CD and GIC, respectively. Following selection with 10 μ*M* Ara-C, the average vector copy number per cell increased to approximately 2 and 5 in CD and GIC cells, respectively.

Peroxidase and CD enzyme activities of transduced 3T3 cells. Cytosolic extracts from cells transduced with GST, CD or GIC vectors were assayed for CD and peroxidase activities, the latter being associated with the A3 subunit of GST (Table 1). In comparison with parent 3T3 or CD cells that had no detectable peroxidase activity, the peroxidase activity of cells transduced with GST and GIC vectors increased to approximately 32 units (nmol/min/mg protein). Following selection with Ara-C, the peroxidase activity of GIC cells further increased 2.6-fold, to 83 units ($p < 0.001$). The peroxidase activity of CD cells increased minimally to 0.9 units following Ara-C selection. However, this minimal increase in peroxidase activity, most likely a non-specific stress response, did not affect the resistance of CD cells to melphalan or chlorambucil (see below).

As expected, transduction of 3T3 cells with the GST vector did not significantly alter their CD activity. In contrast, cells transduced with CD and GIC vectors showed approximately a 290- and 240-fold increase in CD activity, respectively, in comparison with parent 3T3 cells ($p < 0.05$). Following selection with Ara-C, the CD activity of CD and GIC cells further increased 2.5- and 2.9-fold, respectively ($p < 0.05$), a 725- and 690-fold augmentation, respectively, in comparison with parent 3T3 cells.

Drug resistance of transduced 3T3 cells. Drug resistance studies on transduced 3T3 cells were performed using the MTT assay (Fig. 3). Drug resistance was evaluated by determining the inhibitory concentration 50% (IC_{50}) of a drug, defined as the concentration of drug that reduced the target cells' viability by 50%. In comparison with parent 3T3 cells, GST cells were 2.6- and 3.7-fold more resistant to melphalan and chlorambucil, respectively ($p < 0.001$). Likewise, GIC cells were 2.3- and 3.4-fold more resistant to the same drugs ($p < 0.0001$). Following selection with Ara-C, GIC cells' resistance to melphalan and chlorambucil further increased 1.6- and 1.7-fold,

respectively ($p < 0.01$), a 3.7- and 5.9-fold augmentation, respectively, when compared to parent 3T3 cells. As expected, the sensitivity of unselected CD cells to melphalan and chlorambucil remained unchanged. Intriguingly however, CD cells selected with Ara-C were 1.9-fold more sensitive to melphalan ($p < 0.01$), but not to chlorambucil when compared to 3T3 cells.

3T3 cells transduced with CD and GIC vectors were 9.4- and 8.1-fold more resistant to Ara-C when compared to parent 3T3 cells ($p < 0.01$). Selection with Ara-C further increased their resistance to Ara-C 4.1- and 6.5-fold, respectively ($p < 0.05$), corresponding to a 38.5- and 52.8-fold augmentation, respectively, when compared to parent 3T3 cells. As expected, the sensitivity of GST cells to Ara-C remained unchanged.

Resistance of transduced 3T3 cells to combination of melphalan and Ara-C. To determine if 3T3 cells transduced with GIC would be protected from the simultaneous exposure to nitrogen mustards and cytosine nucleoside analogs, GIC cells were simultaneously exposed to 10 μM melphalan and 1 μM Ara-C. These drug concentrations, either individually or in combination, completely inhibited the growth of parent 3T3 cells (Fig. 4). As expected, CD cells selected with Ara-C proliferated in the presence of Ara-C only. In contrast, GIC cells proliferated in the presence of either melphalan or Ara-C, as well as in the simultaneous presence of both drugs. The growth rate of GIC cells in the presence of Ara-C averaged 56% of that observed in absence of drug ($p < 0.0001$), 26% ($p < 0.001$) in the presence of melphalan and 18% ($p < 0.01$) in the simultaneous presence of both drugs.

GIC cells selected with Ara-C displayed significantly more active growth in the simultaneous presence of melphalan and Ara-C. The growth rate of GIC cells selected with Ara-C averaged 94% of that observed in absence of drug in the presence of Ara-C,

33% in the presence of melphalan and 30% in the simultaneous presence of both drugs. Thus, following selection with Ara-C, the growth rate of GIC cells increased 1.7- fold ($p < 0.05$) in the presence of either Ara-C alone or in the combination of both drugs. However, Ara-C selection of GIC cells failed to further increase their growth rate in the presence of melphalan in the growth assay ($p = 0.12$).

Impact of melphalan selection on GIC transduced cells. The impact of selection with a lethal dose of melphalan was assessed by measuring the enzyme activities of GIC cells selected with 25 μM melphalan and their ability to grow in the simultaneous presence of 10 μM melphalan and 1 μM Ara-C.

Selection of GST and GIC polyclonal populations with melphalan increased their respective peroxidase activities to 60 and 75 units, representing a 1.8- and 2.3-fold augmentation, respectively, in comparison to unselected cells ($p < 0.001$) (Table 1). Likewise, melphalan selection augmented the CD activity of GIC cells 1.9-fold ($p < 0.05$), a 450-fold increase when compared to parent 3T3 cells. Selection with melphalan also modestly augmented the CD activity of GST cells 4-fold when compared to parent 3T3 cells ($p < 0.05$). This result is rather intriguing since, unlike GST, CD expression has not been associated to a non-specific stress response. Nevertheless, considering the levels of CD activity and Ara-C resistance obtained in CD and GIC cells, it is extremely unlikely that such a small increase in CD activity could have affected the resistance to Ara-C.

Melphalan selected GIC cells also proliferated significantly more in the simultaneous presence of melphalan and Ara-C (Fig. 4). The growth rate of GIC cells selected with melphalan averaged 94% of that observed in absence of drug in the presence of Ara-C, 59% in the presence of melphalan and 50% in the simultaneous presence of both drugs. Thus, following melphalan selection, the growth rate of GIC cells

increased 1.7- fold ($p < 0.01$) in the presence of Ara-C, 2.3-fold in the presence of melphalan ($p < 0.01$), and 2.8-fold ($p < 0.05$) in the simultaneous presence of both drugs. In contrast, GST cells selected with melphalan grew in the presence of melphalan only. Although the growth rate of GIC cells in the simultaneous presence of melphalan and Ara-C was higher for cells selected with melphalan (50%) than for Ara-C selected cells (30%), the difference observed was not significant ($p = 0.07$).

Table 1. GSH peroxidase and CD enzyme activities of transduced 3T3 cells

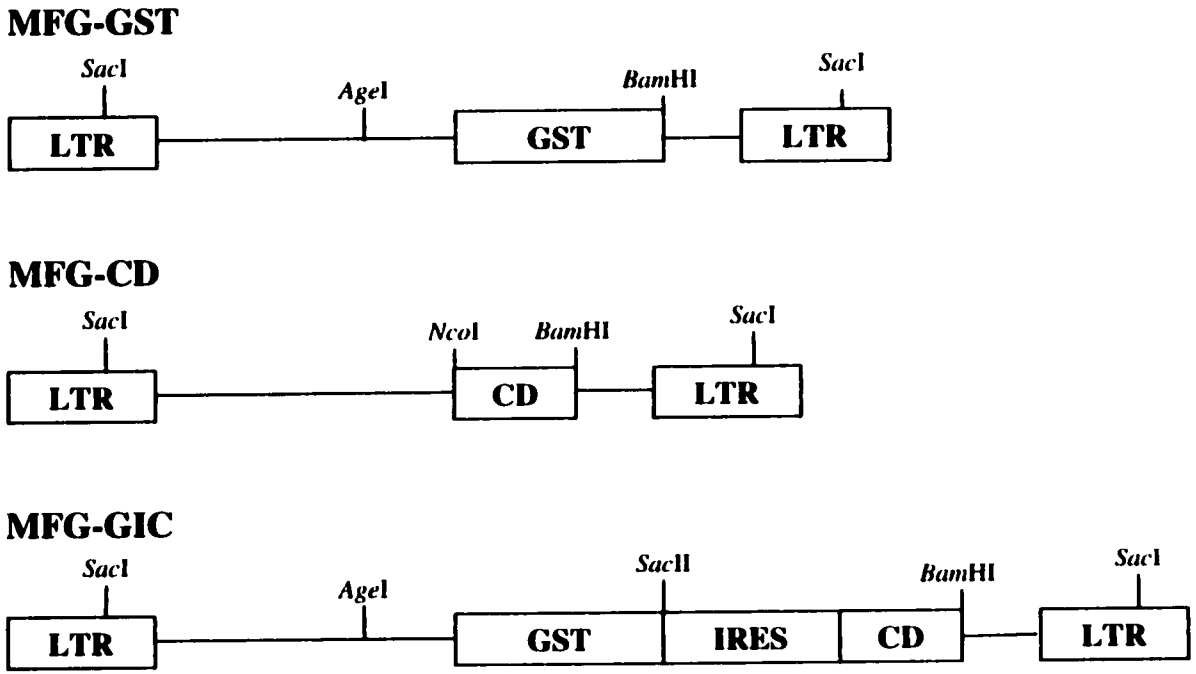
Cell line	*GSH peroxidase*	*CD activity*
3T3	0.01 ± 0.01	2.05 ± 1.11
GST	33.32 ± 4.39[a]	1.21 ± 1.21
GST(25M)	59.79 ± 2.81[b]	4.92 ± 0.60[b]
CD	0.00 ± 0.00	600.53 ± 85.45[a]
CD(10A)	0.92 ± 0.33	1488.73 ± 166.95[b]
GIC	31.78 ± 3.23[a]	488.26 ± 155.13[a]
GIC(10A)	82.80 ± 8.92[b]	1414.30 ± 43.32[b]
GIC(25M)	74.55 ± 6.33[b]	916.27 ± 67.79[b]

[a] $p<0.05$ for differences with 3T3 cells.

[b] $p<0.05$ for differences with parent transduced, unselected cells.

Results represent the mean of 3 to 6 independent assays, each one performed in triplicate, with standard error (SE). Enzyme activities are expressed in nmol/min /mg protein. GST(25M) and GIC(25M) refer to polyclonal NIH/GST and NIH/GIC cell populations preselected with 25 μM melphalan. Other symbols are the same as in figure 2.

Figure 1. Retroviral vector constructions are based on the Moloney murine leukemia virus. MFG-GST contains the rat GST A3 cDNA and MFG-CD contains the human CD cDNA. The bicistronic vector MFG-GIC contains the rat GST A3 cDNA and the human CD cDNA. In MFG-GIC, both gene products are generated from a single LTR-initiated transcript, using cap-independent translation of the second gene that is preceded by the internal ribosome entry site (IRES) of a picornavirus. The location of the *Sac*I restriction enzyme sites used in the Southern blot analysis are shown, as well as sites used for vector construction. Figure not drawn to scale.



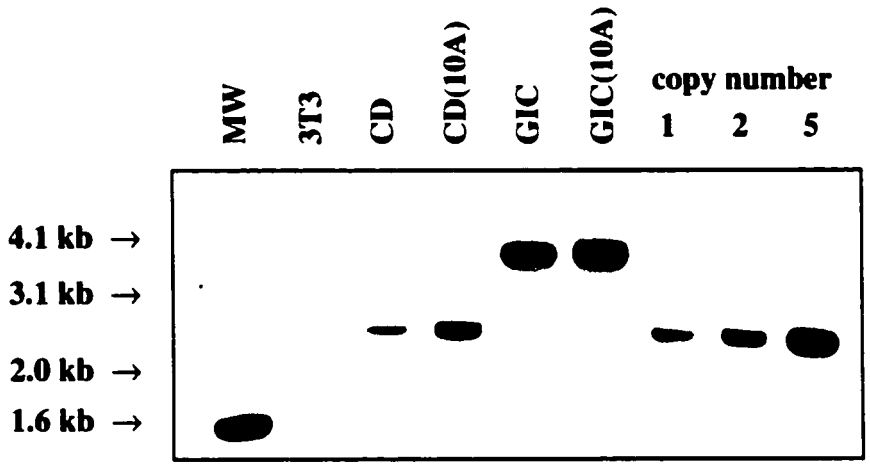


Figure 2. Southern blot analysis of transduced 3T3 cells. Genomic DNA was prepared from polyclonal populations transduced with either CD or GIC vectors. Ten micrograms from each sample were digested with *Sac*I and migrated on a 1.0% agarose gel. Following transfer to a nylon membrane, hybridization was performed with a ^{32}P-labeled full length CD cDNA probe as described under Materials and Methods. The expected fragment size is 2.5 kb for the CD vector and 3.8 kb for the GIC vector. Estimation of the copy number of CD-containing vectors was performed by adding graded amounts of pMFG-CD to 10 μg of genomic DNA from 3T3 cells followed by digestion with *Sac*I. MWM, molecular weight marker; CD and GIC, polyclonal NIH 3T3 cell populations transduced with CD and GIC vectors, respectively. CD(10A) and GIC(10A), NIH/CD and NIH/GIC cell populations selected with 10 μ*M* Ara-C.

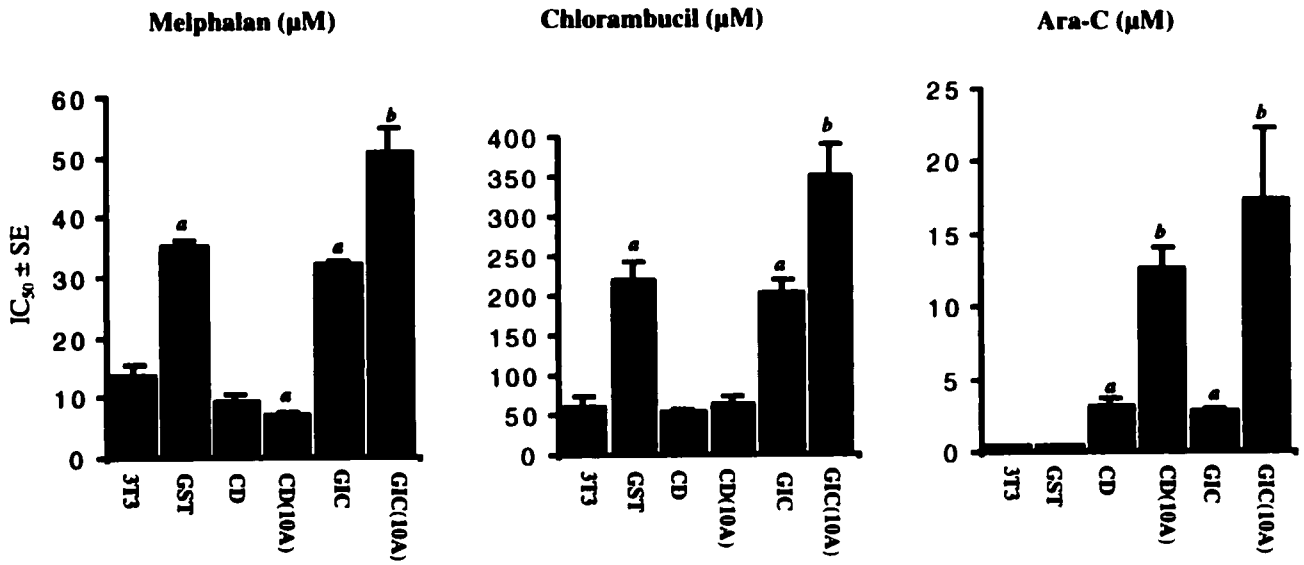


Figure 3. Drug sensitivity assays were performed using the MTT colorimetric assay as described in Materials and Methods. Results represent the mean IC_{50} of 5 to 6 independent assays, with standard error (SE) bars. Abbreviations are the same as in figure 2.

[a] $p \leq 0.01$ for differences with 3T3 cells.

[b] $p < 0.05$ for differences with transduced, unselected cells.

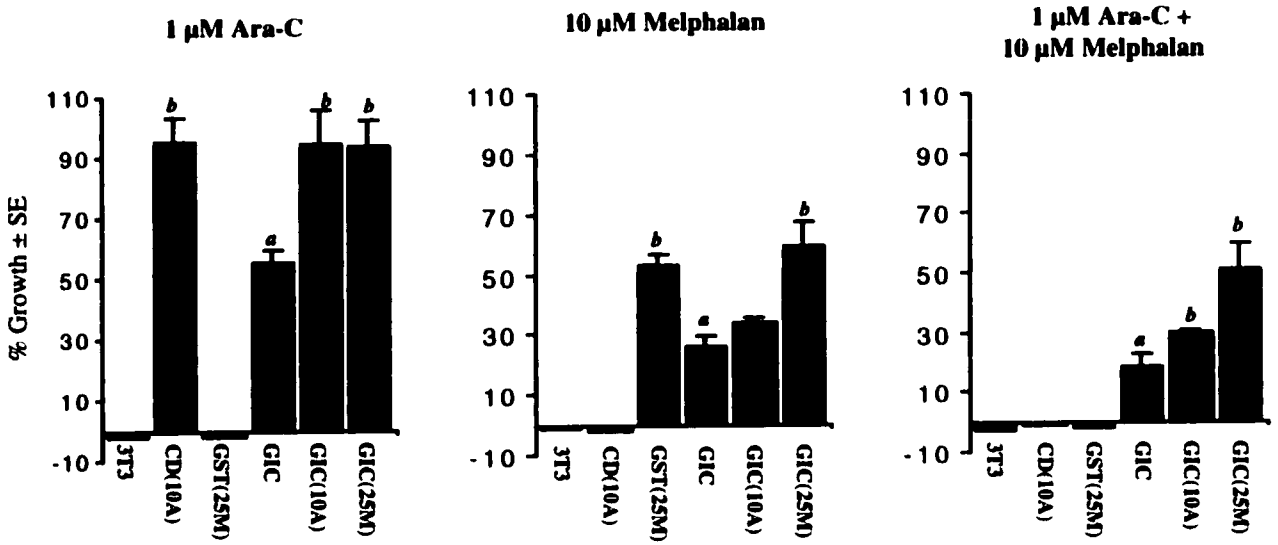


Figure 4. Growth inhibition assays comparing the effect of 1 μ*M* Ara-C and 10 μ*M* melphalan, alone and in combination, as described in Materials and Methods. Results represent the mean percent growth of 4 to 5 independent assays, with SE bars. GST(25M) and GIC(25M) refer to polyclonal 3T3 cell populations transduced with GST and GIC vectors, respectively; and selected with 25 μ*M* melphalan. Other abbreviations are the same as in figure 2.

[a] $p \leq 0.01$ for differences with 3T3 cells.

[b] $p < 0.05$ for differences with GIC cells.

DISCUSSION

We have previously demonstrated an increased resistance to nitrogen mustards in human leukemia cells and in murine fibroblast and bone marrow progenitor cells *in vitro* following retroviral gene transfer of rat GST.[17,18] However, we did not observed chemoprotection against chlorambucil in mice transplanted with bone marrow transduced with N2/GST virions (S.L., M.G. and D.C., unpublished results). A low copy number of vector (approximately 0.1copy/cell) was present in bone marrow and spleen samples of mice 6 months post-transplantation, and following two treatments with chlorambucil. These results suggested that the moderate growth advantage conferred by GST expression from our vector might have been insufficient to increase the tolerance to chlorambucil in transplanted mice. To circumvent this problem, we have constructed a bicistronic vector coexpressing GST and CD. We have shown previously that CD confers a very strong survival advantage in favor of CD-transduced hematopoietic cells *in vitro* in the presence of Ara-C.[20] Moreover, we have demonstrated long-term expression of CD in hematopoietic tissues of CD-transplanted mice treated with Ara-C, and an increased *in vitro* resistance to Ara-C in spleen and marrow cells derived from CD animals and expressing high levels of CD.[21] Together, these results suggest that *ex vivo* and/or *in vivo* Ara-C selection of transduced hematopoietic cells might be used to increase the representation of genetically modified cells in the hematopoietic system of transplanted mice. Thus, Ara-C selection might be used to increase the *in vivo* expression of a bicistronic GST-CD vector in order to achieve significant chemoprotection from the nitrogen mustards. This would be clinically significant in view of broad spectrum of antineoplastic activity of these drugs. Furthermore, combining the expressions of GST and CD would broaden the spectrum of chemoprotection conferred by the vector by simultaneously increasing the hematopoietic tolerance to both alkylating agents and

cytosine nucleoside analogs. In the present report, we demonstrate that retroviral gene transfer of GIC in murine fibroblast 3T3 cells confers *in vitro* resistance to nitrogen mustards and cytosine nucleoside analogs, as well as to the combination of both classes of drugs. Moreover, we show that selection of GIC cells with lethal doses of Ara-C or melphalan augments the level of resistance to both classes of drugs, used individually or in combination.

Southern blot analysis of genomic DNA confirmed the presence of unrearranged vector sequence in CD and GIC cells (Fig. 2). Densitometric comparison revealed the presence of a greater number of vector copies per cell in GIC- than in CD cells, before and after Ara-C selection. Nonetheless, CD and GIC cells had comparable CD enzyme activities and Ara-C resistance before and after selection (Table 1 and Fig. 3), suggesting that the IRES-driven translation of CD in our bicistronic vector was reduced in comparison to the cap-dependent translation of the monocistronic vector. Others have observed such relative decrease in IRES-driven translation.[23]

Expression of functional GST enzyme in GIC cells was demonstrated by their increase in peroxidase activity (Table 1) and resistance to melphalan and chlorambucil (Fig. 3). Likewise, functional expression of CD enzyme activity was evident in the increase in CD activity and resistance to Ara-C. The ability of our bicistronic vector to protect cells against the simultaneous exposure to nitrogen mustards and cytosine nucleoside analogs was shown by the growth of unselected GIC cells in the presence of Ara-C and melphalan at concentrations of drug that completely inhibited the growth of parent 3T3 cells (Fig.4). The degree of growth suppression observed in the presence of both drugs was greater than that observed with either drug alone, suggesting an additive effect for the cytotoxicity of both drugs.

Importantly, we were able to select GIC cells with a dose of Ara-C that was lethal for untransduced cells, supporting the hypothesis that Ara-C selection might be used to

increase the representation of GIC cells in the hematopoietic system of mice transplanted with GIC. Moreover, selection with Ara-C further increased the GST and CD enzyme activities (Table 1), the resistance to melphalan, chlorambucil and cytosine arabinoside (Fig. 3) and the growth rate of GIC cells exposed simultaneously to Ara-C and melphalan (Fig.4). These results demonstrate that the expression of GST and CD increased following Ara-C selection. Together, the increase in vector copy number, enzyme activities and drug resistance in CD and GIC cells selected with Ara-C suggests that exposure to a lethal dose of Ara-C resulted in the selection of transduced cells with higher levels of vector expression due to higher vector copy number and/or more transcriptionnally active sites of vector integration.

We have also been able to select GIC cells with a dose of melphalan that was lethal for untransduced cells. Melphalan selection augmented the GST and CD enzyme activities of GIC cells to levels comparable to the ones obtained following selection with Ara-C (Table 1). In addition, selection with melphalan also enhanced the growth rate of GIC cells in presence of Ara-C or melphalan, as well as in the simultaneous presence of both drugs (Fig. 4). These results demonstrate that, in spite of the moderate level of resistance conferred to nitrogen mustards by our bicistronic vector, it is possible to select GIC cells with a lethal dose of melphalan. Moreover, these results show that, similarly to selection with Ara-C, the selection of GIC cells with melphalan increases the expression of GST and CD as well as the resistance to nitrogen mustards and cytosine nucleoside analogs, administered individually or in combination.

The combination of GST and CD mediated chemoprotection is of potential clinical interest because nitrogen mustards and cytosine nucleoside analogs are both used in the treatment of solid tumors and lymphomas. Chlorambucil and Ara-C are part of an effective regimen for the treatment of low-grade non-Hodgkin's lymphomas;[34] high-dose melphalan and high-dose Ara-C are both active in advanced non-Hodgkin's

lymphomas;[35,36] and finally, melphalan and gemcitabine are active against ovarian and breast cancers.[37-40] In addition, Ara-C, a drug that inhibits DNA repair, has been reported to enhance the cytotoxic activity of melphalan.[41] Hence, conferring chemoprotection against the hematotoxicity produced by nitrogen mustards and cytosine nucleoside analogs could potentially allow the safe use of desirable drug combinations that might otherwise cause unacceptable hematopoietic toxicity.

Nitrogen mustards have been associated with an increased risk of development of second neoplasms, principally acute leukemia.[42] Because resistance to nitrogen mustards has been shown to correlate with inhibition of DNA interstrand cross-link formation in rat mammary carcinoma cells overexpressing GST,[16] introduction of GST into bone marrow could possibly reduce the risk of developing secondary leukemia in patients treated with these agents. Selection of hematopoietic cells expressing GIC by nitrogen mustard would probably not be desirable because of the mutagenic and carcinogenic potentials of these drugs. However, selection with cytosine nucleoside analogs could potentially increase chemoprotection to nitrogen mustards and cytosine nucleoside analogs and decrease the risk of developing secondary neoplasms associated with nitrogen mustard exposure.

In conclusion, we have shown that retroviral gene transfer of a bicistronic vector coexpressing the rat GST and the human CD conferred significant resistance to nitrogen mustards and cytosine nucleoside analogs in murine fibroblasts, which permitted cell survival in the presence of a combination of both classes of drugs. Future experiments will evaluate the potential of combining GST and CD expression for *in vivo* chemoprotection from nitrogen mustards and cytosine nucleoside analogs in mice transplanted with GIC-transduced bone marrow.

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CHAPTER 5

GENERAL DISCUSSION

EVALUATION OF EXPERIMENTAL RESULTS

The effectiveness of systemic chemotherapy is currently limited by the presence of intrinsic or acquired drug resistance in the neoplastic cells, and by the inherent toxicity of the drugs used. Myelosuppression represents the predominant dose-limiting toxicity of most chemotherapeutic agents. Current strategies to circumvent this limitation, such as the use of hematopoietic growth factor support and ASCT, do not entirely eliminate the hematopoietic toxicity, and may not protect from multiple cycles of HDC. The introduction of drug resistance genes into hematopoietic cells may increase the BM tolerance to chemotherapy and may permit safer dose escalation to increase clinical efficacy.

An important mechanism of drug resistance involves the thiol GSH and the GST enzymes. In spite of the fact that GSTP1 is the isoenzyme primarily expressed in human tumors and cell lines derived from them, overexpression of GST alpha isoenzymes has been more strongly implicated in resistance to nitrogen mustards in drug-selected cancer cell lines and in transfection studies. In fact, the largest increase in resistance to anticancer drugs has been obtained following transfection of the rat GSTA3 homodimer in rat mammary carcinoma (MatB) cells (Schecter, et al. 1993). Moreover, the most convincing evidence that specific drugs serve as substrates for GSTs exists for the alpha GST isoenzymes: both chlorambucil and melphalan have clearly been shown to be substrates for GST alpha isoenzymes.

Nitrogen mustards have a broad spectrum of antitumor activity, maintain a dose-related effect through multiple logs of tumor cell kill, and are limited by their hematotoxicity. Thus, conferring chemoprotection against the hematotoxicity produced by nitrogen mustards would be of potential clinical usefulness. While nitrogen mustards have been associated with an increased risk of development of second neoplasms,

principally acute leukemia, these late complications may potentially also be decreased by hematopoietic chemoprotection.

To determine if retrovirus-mediated gene transfer of the rat GSTA3 could be used to confer resistance to nitrogen mustards, we studied the expression of rat GSTA3 and the sensitivity to nitrogen mustards in mouse NIH 3T3 fibroblasts following either transfection or transduction of GSTA3 with an N2-based retrovirus vector (N2Yc) (Chapter 2). Populations of GSTA3-expressing cells and single cell-derived clones were more resistant to chlorambucil and mechlorethamine than were cells subjected to gene transfer with the control antisense construction. These results clearly demonstrated that the rat GSTA3 is able to confer resistance to nitrogen mustards in murine fibroblasts. Moreover, the effect of GSTA3 gene transfer on the sensitivity of cell populations to nitrogen mustards confirmed and extended the findings obtained with GSTA3-transfected MatB cells by excluding potential artifacts related to clonal variability.

To address the feasibility of using rat GSTA3 gene transfer to confer chemoprotection to the hematopoietic system, we then transduced human leukemia K-562 cells and primary murine HPCs with the N2Yc retrovirus vector (Chapter 3). K-562 cells and clonogenic primary murine hematopoietic cells transduced with the N2Yc retrovirus vector were more resistant to melphalan, chlorambucil and mechlorethamine than cells transduced with the control antisense vector. These results demonstrated that retrovirus-mediated gene transfer of rat GSTA3 confers *in vitro* resistance to nitrogen mustards in hematopoietic cells and further support the feasibility of using rat GSTA3 gene transfer to confer chemoprotection to the hematopoietic system against nitrogen mustards.

We next explored the possibility of conferring chemoprotection against nitrogen mustards *in vivo* following transplantation of mice with GSTA3-transduced BM cells (Appendix 1). Lethally irradiated female mice were transplanted with N2Yc-transduced or control N2revYc-transduced male syngeneic donor BM cells. Following hematopoietic

reconstitution, the level of *in vivo* chemoprotection achieved in these mice was investigated by administering two treatments with chlorambucil, and monitoring the ensuing level of myelosuppression. Unfortunately, we did not observed chemoprotection from chlorambucil in mice transplanted with N2Yc-transduced BM. In addition, the copy number of vector present in BM and spleen samples six months post-transplantation, and following two treatments with chlorambucil was low (approximately 0.1copy/cell). These results suggested that the moderate growth advantage conferred by GSTA3 expression from our vector was insufficient to increase the tolerance to chlorambucil in transplanted mice.

To circumvent this problem, we constructed a bicistronic retrovirus vector coexpressing the rat GSTA3 and the human CD (pMFG-GIC) (Chapter 4). We had previously shown that CD confers a very strong *in vitro* survival advantage in favor of CD-transduced hematopoietic cells in the presence of Ara-C, suggesting that Ara-C selection might be used to increase the representation of genetically modified cells in the hematopoietic system of transplanted mice (Appendix 3). Murine fibroblasts transduced with MFG/GIC displayed resistance to nitrogen mustards and Ara-C administered separately or in combination. Moreover, selection of transduced cells with Ara-C augmented their level of resistance to both classes of drugs, separately and in combination. These results suggest that retroviral transfer of MFG/GIC may be useful for chemoprotection against the toxicities of nitrogen mustards and cytosine nucleoside analogs, and that Ara-C selection might be used to increase the *in vivo* expression of a bicistronic GSTA3-CD vector.

The results obtained during the present investigation clearly demonstrated the ability of retrovirus-mediated gene transfer of the rat GSTA3 to confer *in vitro* resistance to nitrogen mustards in murine fibroblasts and primary clonogenic hematopoietic cells, as well as in human chronic myelogenous leukemia cells. Moreover, the levels of resistance

obtained following gene transfer of GSTA3 were of the same or greater magnitude than the relative resistance to nitrogen mustards observed clinically, suggesting that GSTA3-mediated chemoprotection would be clinically relevant (Wolf, et al. 1987). However, we have been unable to demonstrate nitrogen mustard resistance in rat MatB, monkey COS, Chinese hamster ovary LR73 and human promyelocytic HL-60 cells subjected to gene transfer with the N2Yc retrovirus vector (S.L., M.G. and D.C., unpublished data). Southern, Northern and Western blot analyses suggested that we were unable to confer nitrogen mustard resistance in these cell lines because of insufficient vector integration in MatB, LR73 and HL-60 cells, and because of inefficient expression of GSTA3 by our MoMLV-based retrovirus vector in COS cells. Therefore, our inability to demonstrate nitrogen mustard resistance in these cell lines appears to have been due to factors related to the method of gene transfer used. Factors that are known to decrease the efficiency of retrovirus-mediated gene transfer include a low number of receptors for retroviral particles at the cell surface (Orlic, et al. 1997) and silencing of the retroviral promoter by methylation of cytosine residues (Hoeben, et al. 1991). Other factors related to drug metabolism by the GSTs, including differences in endogenous GSH, MRP1 and total GST levels, and differences in the pattern of GST isoenzymes expression, can also influence the magnitude of the effect of GSTA3 gene transfer on the sensitivity to nitrogen mustards in different cell lines (Tew 1994).

Regarding the feasibility of using somatic gene transfer of rat GSTA3 to confer protection to the hematopoietic system against nitrogen mustards, the results obtained during the present investigation preclude any definitive conclusion. Although the results obtained in human leukemia K-562 cells and primary murine HPCs *in vitro* are encouraging, those obtained in mice transplanted with N2Yc-transduced BM are more difficult to interpret. At the moment, we can only speculate on the reasons that explain the higher number of white blood cells (WBCs) surviving the first treatment with

chlorambucil in mice transplanted with revYc-transduced BM in comparison to mice transplanted with GSTA3-transduced BM (Appendix 1). While it is improbable that the expression of GSTA3 *in vivo* is detrimental to the growth of hematopoietic cells this hypothesis cannot be excluded at this point.

If the moderate growth advantage conferred by GSTA3 expression from our N2-based retrovirus vector did preclude us, so far, from demonstrating hematopoietic chemoprotection in mice, coexpressing GSTA3 with a more strongly selectable gene may provide the means to increase the representation of cells expressing GSTA3 by administering the relevant drug to transplant recipients. In this respect, the results obtained in murine fibroblasts with our bicistronic vector coexpressing GSTA3 and CD are encouraging. Not only did we succeed in selecting GIC-transduced cells with Ara-C, but the selection also augmented the resistance to nitrogen mustards and Ara-C. We have likewise demonstrated an increased level of resistance to MTX and Ara-C, following Ara-C selection, in murine fibroblasts transduced with a retrovirus vector coexpressing a mutant DHFR and CD (Appendix 4). Moreover, we have shown long-term expression of CD in hematopoietic tissues of CD-transplanted mice treated with Ara-C, as well as increased *in vitro* resistance to Ara-C and high levels of CD expression in spleen and marrow cells derived from CD animals (Appendix 5). Together, these results support the feasibility of using Ara-C selection *in vivo* to increase the representation of genetically modified cells in the hematopoietic system of transplanted mice. Finally, we have constructed another bicistronic retrovirus vector coexpressing GSTA3 and L22Y DHFR (pMFG-GID) to confer resistance to nitrogen mustards and antifolates and increase the representation of GSTA3-expressing cells with antifolates (Appendix 2). As mentioned previously, treatment with TMTX and NBMPR-P is a promising approach to select HSCs expressing a DHFR resistance vector *in vivo* (Allay, et al. 1998). Murine fibroblasts transduced with MFG/GID displayed resistance to nitrogen mustards and TMTX *in vitro*.

Experiments in primary murine HPCs are presently underway in our laboratory to determine the level of *in vitro* chemoprotection conferred to the hematopoietic system from nitrogen mustards and cytosine nucleoside analogs or antifolates following retrovirus-mediated gene transfer of MFG-GIC and MFG-GID vectors, respectively.

SUGGESTIONS FOR FURTHER WORK

EVALUATION OF GIC- AND GID-MEDIATED HEMATOPOIETIC CHEMOPROTECTION IN VIVO

After demonstrating that retrovirus-mediated gene transfer of GIC and GID vectors confers a significant degree of chemoprotection from nitrogen mustards and cytosine nucleoside analogs or antifolates, respectively, in primary murine HPCs *in vitro*, the level of hematopoietic chemoprotection achievable *in vivo* should be evaluated in transplanted mice. To increase the representation of GIC- or GID-expressing hematopoietic cells and thus augment the likelihood of demonstrating *in vivo* chemoprotection to nitrogen mustards, several courses of Ara-C or the combination of TMTX plus NBMPR-P, respectively, should be administered to the murine transplant recipients. Once the majority of hematopoietic cells are shown to express GIC or GID vectors, the level of *in vivo* chemoprotection from nitrogen mustards could be evaluated in the transplanted mice.

EVALUATION OF THE IMPACT OF GIC AND GID GENE TRANSFER ON THE TREATMENT OF TUMOR-BEARING ANIMALS

If *in vivo* chemoprotection can be demonstrated in mice transplanted with GIC- and GID-transduced BM, the impact of this strategy on our ability to treat and eradicate tumors in animals could be investigated. Murine lymphoma and breast cancer models would be interesting to study with GIC- and GID-transplanted BM because nitrogen mustards, cytosine nucleoside analogs and antifolates are all active against these tumors.

Moreover, lymphoma and breast cancer are human tumors for which the administration of full-dose chemotherapy, and thus the patient's tolerance to the treatment, appear important to the outcome.

EX VIVO EXPANSION AND SELECTION OF GIC- AND GID-TRANSDUCED BONE MARROW PROGENITOR CELLS

One approach to overcome the current limitations imposed by low stem cell transduction efficiencies with retroviruses would be to select and expand transduced HPCs *ex vivo* before the transplantation to increase their representation in the recipient's hematopoietic system. Therefore, investigating the feasibility of expanding and selecting GIC- and GID-transduced BM progenitor cells *ex vivo* would be of potential interest. In this strategy, murine BM cells would first be subjected to gene transfer with GIC or GID virions. Transduced cells would then be selected with cytosine nucleoside analogs or antifolates, respectively, and subsequently reinfused into recipient mice. The transduction and selection would be performed in the presence of a cytokine cocktail that allows the expansion of BM progenitor cells. The feasibility of this approach has been demonstrated with human $CD34^+$ peripheral blood progenitor cells and umbilical cord blood progenitor cells transduced with a mutated DHFR cDNA (Flasshove, et al. 1998, Flasshove, et al. 1995).

COMBINATION OF GSTA3 AND MRP1 EXPRESSIONS

Because the membrane glycoprotein MRP1 mediates the transport of the GSH *S*-conjugates of nitrogen mustards outside the cell, combining the expressions of GSTA3 and MRP1 in a bicistronic retrovirus vector could potentially increase the level of chemoprotection from nitrogen mustards. MRP1 and GSTA1 have been shown previously to act in synergy to protect cells from the cytotoxicity of chlorambucil in human MCF7 breast carcinoma cells overexpressing both proteins, supporting the feasibility of using this approach to augment the degree of protection from nitrogen mustards in hematopoietic cells (Morrow, et al. 1998a). In addition, the inclusion of MRP1 in a vector would broaden the spectrum of chemoprotection since MRP1 has been shown to confer resistance to drugs that are involved in the multidrug resistance phenotype, including doxorubicin, vincristine, etoposide and mitoxantrone. Finally, GSTA3 (S.L. and D.C, unpublished results) and MRP1 (Morrow, et al. 1998b) have been independently shown to confer modest resistance (~1.5-fold each) to 4-HC, an active metabolite of cyclophosphamide. This suggests that the combined expression of MRP1 and GSTA3 could potentially confer a significant degree of resistance to cyclophosphamide, one of the most widely used drugs in chemotherapy.

COMBINATION OF GSTA3 AND γ-GCS EXPRESSIONS

Negative results of GSTs transfection experiments correlate, in some cell lines such as MCF7, with extremely low levels of transcript for γ-GCS, the rate limiting enzyme in *de novo* GSH biosynthesis (Tew 1994). This suggests that limited capacity for GSH synthesis may limit the effect of GST gene transfer. Moreover, the transfection of

the heavy and light subunits of human γ-GCS has been shown to confer modest resistance (2- to 3--fold) to melphalan (Mulcahy, et al. 1995). Therefore, combining the expressions of γ-GCS and GSTA3 in a bicistronic vector could potentially increase the level of chemoprotection from nitrogen mustards.

DEVELOPMENT OF MUTANT GSTS RESISTANT TO NITROGEN MUSTARDS

Another way to obtain a more substantial level of resistance to nitrogen mustards would be to use random mutagenesis to develop mutant GST enzymes conferring elevated level of resistance to these drugs. The feasibility of this approach has been demonstrated by the identification of mutant GSTA3 isoenzymes that conferred up to 10-fold increased resistance toward mechlorethamine relative to the wild-type GST (Gulick and Fahl 1995). In addition, the cyclophosphamide metabolites acrolein and phosphoramide mustard have been shown to be good substrates for the alpha human GST isoenzymes, suggesting that this approach might be used to develop GST alpha mutants resistant to cyclophosphamide, the most widely used bifunctional alkylating agent used in the clinic today. (Berhane and Mannervik 1990, Dirven, et al. 1994).

CLAIMS TO ORIGINAL RESEARCH

The following novel findings and observations have been demonstrated in this thesis:

1. The ability of retrovirus-mediated gene transfer of the rat GSTA3 to confer resistance to nitrogen mustards has been shown in cell populations of murine fibroblasts and clonogenic primary hematopoietic cells as well as in human chronic myelogenous leukemia cells *in vitro* (Chapters two and three).

2. The ability of retrovirus-mediated transduction of a bicistronic vector coexpressing the rat GSTA3 and the human CD cDNAs (pMFG/GIC) to confer *in vitro* resistance to nitrogen mustards and Ara-C was demonstrated in murine fibroblasts (Chapter four).

3. Retrovirus-mediated gene transfer of the rat GSTA3 and the human CD was shown to confer resistance to the simultaneous exposure to melphalan and Ara-C in murine fibroblasts (Chapter four).

4. The feasibility of selecting GIC-transduced fibroblast cells with a dose of Ara-C lethal for untransduced cells was demonstrated *in vitro*. Ara-C selection was shown to augment the expression of GSTA3 and CD as well as the level of resistance to nitrogen mustards and Ara-C, administered separately and in combination (Chapter four).

5. Similarly, *in vitro* selection of GIC-transduced fibroblasts with a lethal dose of melphalan was shown to increase the expression of GSTA3 and CD as well as the

level of resistance to nitrogen mustards and Ara-C, administered separately and in combination (Chapter four).

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APPENDIX 1

RETROVIRUS-MEDIATED GENE TRANSFER OF RAT GLUTATHIONE S-TRANSFERASE A3 FAILS TO CONFER HEMATOPOIETIC PROTECTION AGAINST CHLORAMBUCIL IN TRANSPLANTED MICE

Sylvain Létourneau, Mona Greenbaum and Denis Cournoyer

After demonstrating that retrovirus gene transfer of rGSTA3 conferred resistance to nitrogen mustards in human leukemia and murine hematopoietic progenitor cells *in vitro*, we attempted to evaluate the level of *in vivo* hematopoietic chemoprotection that could be achieved in mice transplanted with GSTA3-transduced BM.

Following lethal irradiation, twenty-one female mice were transplanted with control N2revYc-transduced male syngeneic donor BM cells and twenty-two mice received N2Yc-transduced BM cells. Of those animals, 6 revGSTA3- and 11 GSTA3-transplanted mice died following transplantation, possibly from infection or marrow engraftment failure. To assess the ability of the transduced GSTA3 to protect hematopoietic cells *in vivo*, the remaining 13 revGSTA3-transplanted (revGSTA3) and 10 GST-transplanted (GSTA3) mice were administered 20 mg/kg chlorambucil following hematopoietic recovery. We had previously shown that this dose reduced the number of leukocytes (WBCs) in the blood by 75% to 90% in non-transplanted mice. WBCs were counted before drug administration as well as 3 and 5 days later to monitor the ensuing level of myelosuppression in order to detect the nadir of the WBC count. However, contrary to what was expected, the percentage of surviving WBCs after the chlorambucil treatment was higher in mice transplanted with revGSTA3-transduced BM than in mice

transplanted with GSTA3-transduced marrow. The survival of WBCs on days 3 and 5 were 37.4 ± 4.6% (mean ± SE) and 50.6 ± 12.5%, respectively, for revGSTA3 mice, in comparison with 17.6 ± 2.3% ($p < 0.01$) and 20.3 ± 3.2% ($p = 0.05$) for GSTA3 mice (Fig. A1.1). Thus, mice transplanted with GSTA3-transduced BM were more sensitive to the chlorambucil treatment than mice transplanted with revGSTA3 BM. Several weeks later, a second injection of 20 mg/kg chlorambucil was administered to 12 revGSTA3- and 8 GSTA3-mice and the number of WBCs monitored as before. At that time, no difference in the percentage of surviving WBCs was observed between revGSTA3 mice and GSTA3 mice. The survival of WBCs on days 3 and 5 were 41.3 ± 5.5% (mean ± SE) and 37.2 ± 5.4%, respectively, for revGSTA3 mice, in comparison with 42.8 ± 6.4% and 31.8 ± 3.6% for GSTA3 mice (Fig. A1.1). In addition, the increased sensitivity observed after the first chlorambucil treatment in GSTA3-transplanted mice was eliminated after the second treatment. Likewise, the percentage of surviving WBCs returned to values similar to the ones obtained in revGSTA3-transplanted mice after both chlorambucil treatments.

Several hypotheses could explain the relative chemosensitivity observed in GSTA3 mice following the first chlorambucil treatment. First, it is possible that the quality of the graft of revGSTA3-transduced BM cells was superior to the quality of the graft of BM cells transduced with GSTA3. This would have increased the time to full hematopoietic recovery in GSTA3-transplanted mice (not evaluated in our experiments) and made them more sensitive to cell-damaging agents, particularly early on after transplantation. A graft of poorer quality in GSTA3-transduced mice could account for the fact that almost twice as many GSTA3 mice as revGSTA3 mice died following transplantation before the mice were administered any drug. A lower quality of the graft following GSTA3-transduction could be due to differences in the ability of the revGSTA3- and GSTA3-virus producing cell populations to support the growth of murine

primary progenitors during the cocultivation procedure. Such differences have been reported between clonal isolates of virus-producing cells (Dinko Valerio, IntroGene, Rijswijk, Netherlands, personal communication). Alternatively, it is possible that the expression of the rGSTA3 isoform in hematopoietic cells has deleterious effects on the growth of these cells *in vivo*. However, we have not observed any adverse effect on the formation of hematopoietic progenitor colonies *in vitro* when performing colony assays with GSTA3-transduced BM cells [Létourneau, 1996 #238]. In addition, we are not aware of any report in the literature of a detrimental effect of GSTA3 expression *in vivo* in hematopoietic cells. Thus, this is not a likely explanation. Another hypothesis that could explain the sensitivity of GSTA3 mice to the initial treatment with chlorambucil is that the *in vivo* expression of the rGSTA3 would paradoxically sensitizes hematopoietic cells to nitrogen mustards. This is, however, very unlikely since we have observed the expected opposite result of chemoprotection *in vitro* in GSTA3-transduced clonogenic hematopoietic cells.

To evaluate the number of vector copies per cell present in the transplanted mice after the two chlorambucil treatments, genomic DNA was extracted from BM and spleen samples collected 26 weeks after transplant. Southern blot analysis with a full-length cDNA GSTA3 probe revealed the presence of approximately only 0.1 copy of N2revYc or N2Yc retrovirus vector per cell in the samples analyzed (data not shown). In addition, hybridization of the blots with a cDNA pY2 probe to detect the Y-chromosome showed engraftment of male donor hematopoietic cells. Detection of the retrovirus vectors 6 months post-transplant demonstrates that murine primitive stem cells were transduced during cocultivation with virus-producing cells. However, the low vector copy number per cell obtained suggests that the moderate growth advantage conferred by GSTA3 expression from our N2Yc vector was insufficient to increase the representation of GSTA3-transduced hematopoietic cells *in vivo* following chlorambucil.

CHLORAMBUCIL-INDUCED MYELOSUPPRESSION FOLLOWING TRANSPLANTATION WITH GST-TRANSDUCED BONE MARROW

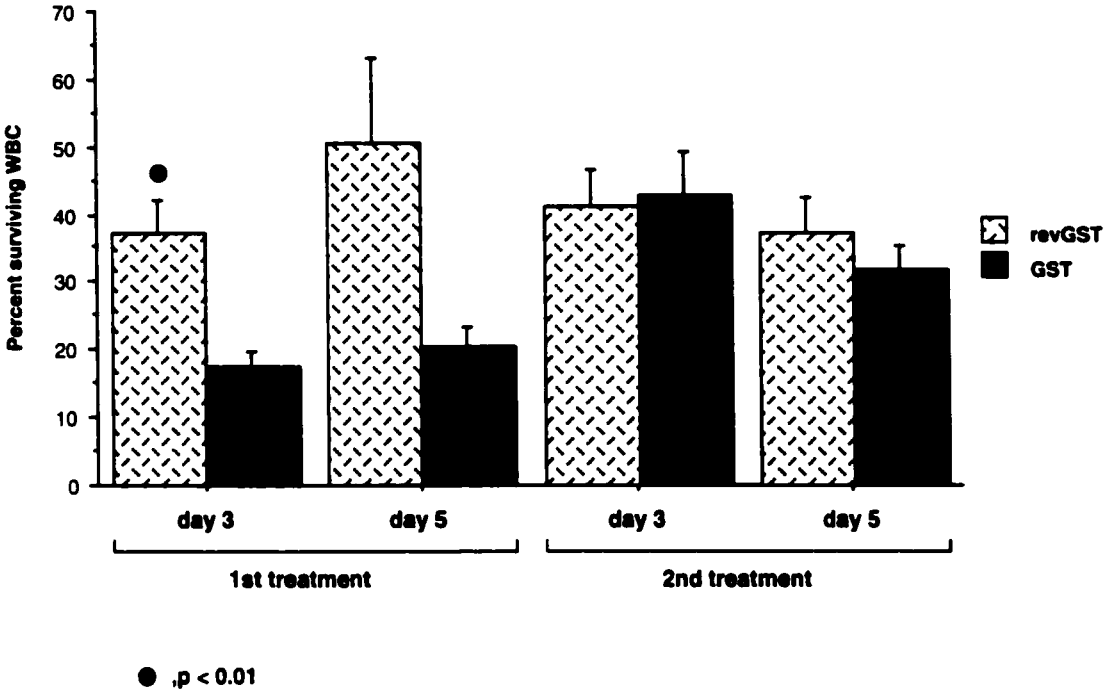

Figure A1.1. C3H HeJ donor murine BM cells were transduced with GP+*env*Am-12/revGSTA3 or GP+*env*Am-12/GSTA3 as previously described [Létourneau, 1996 #238]. Following cocultivation, the nonadherent BM cells were collected, resuspended in 150 to 200 µl 1% α-MEM, and injected via the tail vein into female syngeneic C3H HeJ recipient mice irradiated with 1200 rads (cesium or cobalt source) 4 to 5 hours earlier. Transplanted mice were injected intraperitoneally with 20 mg/kg chlorambucil. The first treatment was administered 6 to 12 weeks post transplantation. The second treatment was given 6 to 15 weeks after the first one. For WBC count determination, mice were sedated by either CO_2 or metoxyfluorane inhalation for blood collection. Blood was collected by periorbital bleeding using a heparin-treated microhematocrit capillary tubes and diluted 1:20 or 1:10 (vol/vol) in 3% acetic acid solution for erythrocyte lysis. The WBCs were counted with a hemacytometer.

APPENDIX 2

COEXPRESSION OF HUMAN MUTANT DIHYDROFOLATE REDUCTASE AND RAT GLUTATHIONE S-TRANSFERASE A3 CONFERS RESISTANCE TO ANTIFOLATES AND NITROGEN MUSTARDS IN VITRO

Sylvain Létourneau, Jean-Sébastien Palerme, Jean-Sébastien Delisle and Denis Cournoyer

Following our unsuccessful attempt to demonstrate chemoprotection from chlorambucil in mice transplanted with N2Yc-transduced BM, we constructed a bicistronic retrovirus vector that co-expresses the rat GSTA3 and the human L22Y DHFR mutant (MFG/GID). Treatment with TMTX and NBMPR-P has been shown to allow efficient selection of transduced stem cells in mice transplanted with L22Y-DHFR-transduced BM, suggesting that this combination might be used to increase the *in vivo* expression of GSTA3 in the hematopoietic system of mice transplanted with GID-transduced BM. To address the feasibility of using TMTX to select GID-transduced cells *in vitro*, murine NIH 3T3 fibroblast cells were transduced with our bicistronic vector and evaluated for their resistance to nitrogen mustards and TMTX. In comparison with untransduced cells, GID-transduced polyclonal populations demonstrated a significant increase in selenium-independent glutathione peroxidase (peroxidase) activity, as well as increased resistance to chlorambucil (1.5-fold) and TMTX (750-fold). Following selection with 25 μ*M* melphalan, the peroxidase activity of GID-transduced cells augmented 8.0-fold in comparison with unselected cells, and the resistance to chlorambucil and TMTX further increased to 3.5- and 1600-fold, respectively.

Melphalan-selected GID-transduced cells also demonstrated increased resistance to melphalan (2.2-fold). In contrast, selection of GID-transduced cells with up to 500 n*M* TMTX did not augment the peroxidase activity or the resistance to either drug. However, the selection of a mixed population of cells composed of 20% GID-transduced cells and 80% untransduced cells with 250 n*M* TMTX increased the peroxidase activity of these cells 18-fold. These results demonstrate that retrovirus gene transfer of GID confers a very high level of resistance to TMTX and a modest level of resistance to nitrogen mustards in murine fibroblasts. In addition, these results demonstrate that TMTX and melphalan can both be used to select GID-transduced cells over untransduced cells. However, only melphalan selection increases the expression of the bicistronic vector when it is applied to a population of cells that have been transduced with high efficiency. This is most likely due to the fact that the average level of expression of L22Y DHFR resulting from a single copy of the GID vector is sufficient to confer resistance to high concentration of TMTX. Thus exposure of GID-transduced cells to TMTX does not permit to select cells with more efficient expression of the vector, for instance because of multiple copies or more favorable sites of vector integration. Nonetheless, TMTX selection might prove very efficient in the context of hematopoietic cell transduction since the goal, in that case, will largely be to select a minority of successfully transduced cells among of population of predominantly non transduced cells.

APPENDIX 3

Resistance to cytosine arabinoside by retrovirally mediated gene transfer of human cytidine deaminase into murine fibroblast and hematopoietic cells

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Dose-limiting hematopoietic toxicity produced by the cytosine nucleoside analogue cytosine arabinoside (ARA-C) is one of the major factors that limit its use in the treatment of neoplastic diseases. An interesting approach to overcome this problem would be to insert a gene for drug resistance to ARA-C in normal hematopoietic cells to protect them from drug toxicity. The deamination of ARA-C by cytidine deaminase results in a loss of its antineoplastic activity. The objective of this study was to determine if gene transfer of human cytidine deaminase into murine fibroblast and hematopoietic cells would confer drug resistance to ARA-C. Retrovirally mediated transfer of the human cytidine deaminase gene into 3T3 fibroblasts resulted in efficient expression of the proviral RNA for this gene and in increased cytidine deaminase activity in cytoplasmic extracts. These cells showed marked resistance to ARA-C as determined by the effects of this drug on colony formation, cell growth, and DNA synthesis. The transfer of the human cytidine deaminase gene into murine bone marrow cells by the retroviral vector conferred a high level of drug resistance to ARA-C in clonogenic assays. These studies indicate that the cytidine deaminase gene could be used in cancer gene therapy by protecting normal hematopoietic cells against the cytotoxic effects of ARA-C and related cytosine nucleoside analogues.

Key words: Cytidine deaminase; cytosine arabinoside; drug resistance; hematopoietic cells; retroviral vector.

One of the major problems in cancer chemotherapy is the sensitivity of normal cells, such as hematopoietic cells, to antineoplastic agents, which limits the dose and frequency of drug treatments that can be safely administered to patients. Strategies that would prevent dose-limiting drug toxicities might therefore have an important impact on tumor therapy. An attractive approach to this end may be to transfer a drug resistance gene into normal bone marrow cells and thus protect them from the toxicity of anticancer agents.[1]

Replication-defective retroviruses have been efficiently used for transfer and expression of new genetic sequences into hematopoietic stem cells. Transfer of the dihydrofolate reductase gene into murine bone marrow cells has been shown to confer drug resistance to methotrexate.[2-5] Chemoprotection also has been afforded by the insertion of the human multidrug resistance (MDR) gene into murine and human hematopoietic cells.[6-9] Likewise, transfer of the Yc isoform of the glutathione-S-transferase gene provided alkylating drug resistance to murine bone marrow cells.[10]

Cytidine (CR) deaminase catalyzes the deamination of cytidine or deoxycytidine to uridine or deoxyuridine, respectively. Furthermore, this enzyme can deaminate cytosine nucleoside analogues such as cytosine arabinoside (ARA-C),[11] a potent antileukemic agent, yielding inactive uracil derivatives.[12] CR deaminase may be implicated in drug resistance because leukemic blasts from some patients with acute leukemia showed high levels of this enzyme at the time of relapse after treatment with ARA-C.[13,14] Although cell lines derived from various other tumor types have been shown to be sensitive to ARA-C *in vitro*, the marked hematotoxicity of this drug has limited its used in nonhematologic malignancies.[15] Retrovirally mediated transfer of the CR deaminase gene into normal hematopoietic cells would be expected to improve the patient's tolerance to ARA-C and other cytosine nucleoside analogues, thus improving the clinical usefulness of these drugs.

Human CR deaminase complementary DNA (cDNA) has been cloned and expressed in our laboratory and found to encode a 146-amino acid protein.[16] In a recent report, we used a plasmid expression vector containing the human CR deaminase cDNA promoted by the Moloney murine leukemia virus (MoMLV) long termi-

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nal repeat (LTR) to transfect a murine fibroblast retrovirus–packaging cell line.[17] Clones of this cell line were isolated and shown to express increased levels of CR deaminase messenger RNA (mRNA) and protein. The level of expression of CR deaminase of the clones correlated with the level of drug resistance to ARA-C. In the present study, we used the retroviral particles produced by one of these clones to transduce NIH 3T3 mouse fibroblasts. We observed an increased expression of the human CR deaminase gene as well as the transfer of the ARA-C resistance phenotype in these transduced cells. Finally, we used the viral particles from the producer clone to introduce the human CR deaminase cDNA into mouse marrow progenitor cells and found that transduced cells showed *in vitro* protection from ARA-C toxicity.

MATERIALS AND METHODS

Cell culture techniques

Cell lines were grown in Dulbecco's modified essential medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent Technologies, St Bruno, Quebec, Canada) and 5 μg/mL Gentamycin (Canadian Life Technologies), and incubated at 37°C in 7% CO_2. GP+E-86 murine ecotropic packaging cells, which are derived from National Institutes of Health (NIH) 3T3 mouse fibroblasts, were obtained from Dr A. Bank, Columbia University, New York.[18]

Clonogenic assays were performed as follows for fibroblasts cells. Aliquots of 100 cells in 1 mL of medium were placed in 12-well Costar tissue culture dishes; 18 to 20 hours later, graded concentrations of ARA-C (Upjohn Canada Ltd) were added, and the cells were maintained in culture for an additional 12 to 14 days. The wells were then stained with 0.5% methylene blue in 50% methanol, and colonies were counted. For cell growth measurements, 10^4 cells per well were plated in Costar 12-well dishes, and the next day graded concentrations of ARA-C were added. After 5 days of culture, the cells were trypsinized and counted with a Coulter ZM electronic cell counter. For DNA synthesis assays, 10^5 cells per well were plated in 12-well Costar dishes. After an overnight incubation, ARA-C was added in graded concentrations along with 0.5 μCi of ^{3}H-thymidine (20 Ci/mmol), and the incubation was continued for an additional 6 hours. The amount of radioactivity incorporated into DNA was determined after trypsinization as described previously.[16]

Vector design

The expression vector containing the human CR deaminase cDNA was constructed as described previously. Briefly, the plasmid pBluescript KSII containing the complete cDNA sequence for human cytidine deaminase[17] was used as the template for a PCR amplifying a 465-bp DNA fragment of the CR deaminase protein coding sequence using a 5'-oligonucleotide primer containing an *Nco*I linker and 3'-oligonucleotide primer containing a *Bam*HI linker.

The plasmid expression vector pMFG-tPA used in this study was obtained from R. Mulligan, Harvard University, Cambridge, Mass.[19] The CR deaminase insert was cloned between the *Nco*I and *Bam*HI sites of the vector. pMFG-LacZ was used as a control vector.

The GP+E-86 ecotropic packaging cells were cotransfected with the purified plasmid DNAs pMFG-CD (or pMFG-LacZ) and pSV2-neo using the standard calcium phosphate precipitation method as described previously.[17] Clones of cells resistant to G418 were isolated by cloning. We selected the GP+E-86-CD-3 clone for this study because it showed very high expression of CR deaminase and significant drug resistance to ARA-C.[17] The estimated viral titer of the GP+E-86-CD-3 packaging cells was about 4×10^5 infective particles/mL as determined by colony formation by 3T3 cells in the presence of 10^{-6} mol/L ARA-C.

Transduction of murine fibroblast cells

The medium of semiconfluent GP+E-86-CD-3 was replaced with fresh medium, and 18 hours later this medium was removed, centrifuged at 500 × g for 10 minutes, and the supernatant containing the retroviral particles collected. One milliliter of this medium was placed in a 25 cm^2 flask containing semiconfluent 3T3 fibroblast cells. Polybrene was added at a concentration of 4 μg/mL. After 4 hours incubation, the medium was replaced with fresh medium. The next day the cells were trypsinized, diluted 10-fold, and plated in dishes containing 10^{-6} mol/L ARA-C. After 2 weeks of selection in ARA-C, 3T3-CD3-V clones were isolated by ring cloning.

Transduction of murine bone marrow cells

Exponentially growing virus-producing GP+E-86-CD-3 or GP+E-86-LacZ cells were sublethally irradiated (20 Gy, cobalt source), and 3 to 5×10^6 irradiated cells were plated in 100-mm diameter tissue culture dishes 6 to 24 hours before transduction. Seven- to 21-week-old C3H HeJ female mice (Jackson Laboratories, Bar Harbor, Me) weighing 15 to 30 g were injected intraperitoneally with 150 mg/kg 5-fluorouracil (David Bull Laboratories, Vaudreuil, Quebec, Canada) 48 hours before bone marrow harvest. Fresh marrow was collected from the femurs and tibias, and 3 to 5×10^6 nucleated bone marrow cells were cocultivated with an equal number of irradiated virus-producing cells for 72 hours. The cocultivation medium for murine bone marrow cells consisted of alpha minimal essential medium (MEM) (Gibco, Grand Island, NY) supplemented with 20% FBS, 10 mg/mL bovine serum albumin (BSA) (Boehringer Mannheim, Dorval, Quebec, Canada), 0.3 mg/mL iron-saturated human transferrin (Boehringer Mannheim), 0.01% gentamicin, 4 μg/mL polybrene, 10% conditioned medium from the murine myelomonocytic WEHI-3B cells,[20] and 10% conditioned medium from the human HTB-9 primary bladder carcinoma cells.[21]

Drug sensitivity of clonogenic mouse hematopoietic cells

Sensitivity of mouse bone marrow to ARA-C was assessed using an *in vitro* assay for clonogenic hematopoietic progenitor cells. Immediately after cocultivation with the virus producers, nonadherent bone marrow cells were plated at a final concentration of 1 to 2×10^5 cells/mL in alpha-MEM (with no nucleosides) containing 0.9% methylcellulose (Fisher Scientific, Montreal, Quebec, Canada), 30% FBS, 1% pokeweed mitogen–stimulated spleen-conditioned medium (GIBCO), 1% BSA, 10^{-4} mol/L 2-mercaptoethanol (Sigma), and 0.01% gentamicin. ARA-C was added to the assay mix in concentrations ranging from 10^{-8} to 10^{-5} mol/L. Cultures were plated in triplicate 1-mL aliquots in 35-mm diameter Petri dishes, and placed in 5% CO_2 at 37°C. The total number of hematopoietic colonies (>50 cells) was scored at days 10 to 14 of culture.

Enzyme assay

The enzymatic assay for CR deaminase was performed as described previously.[16] Briefly, 2 to 5 × 10^7 monolayer cells were trypsinized, centrifuged, and washed once in phosphate-buffered saline, centrifuged again, and resuspended in 100 μL of 5 mmol/L Tris-HCl, pH 7.4, and 5 mmol/L dithiothreitol. The cell suspension was then subjected to three cycles of rapid freezing and thawing. The mixture was centrifuged at maximum speed in a microcentrifuge at 5°C for 15 minutes. The molarity of the supernatant (cytosol) was then increased to 50 mmol/L Tris-HCl, pH 7.4. Different dilutions of the cytosol were used in 30-minute incubation at 37°C to measure the conversion of ^{3}H-cytidine to ^{3}H-uridine.

Northern blot analysis

Total RNA was isolated from cells by a method modified from Chomczynski and Sacchi,[22] using the Biotecx Laboratories Ultraspec II RNA Isolation System kit (Biotex Laboratories, Houston, Tex). The cells were lysed with a guanidine solution, and RNA was extracted with chloroform, precipitated with isopropanol, and purified with the RNA Tack resin. Total RNA was eluted from the resin with diethylpyrocarbonate-treated TE (10 mmol/L Tris, 1 mmol/L EDTA, pH 8) and stored at −70°C. For Northern blot analysis, samples of 10 μg of RNA in loading buffer were heated at 65°C for 5 minutes and then electrophoresed in 1× borate buffer through a 1.5% agarose-borate-1.1% formaldehyde gel. After ultraviolet (UV) photography of the gel, the RNA in the gel was blotted onto a Nytran Plus nylon membrane (Schleicher & Schuell, Keene, NH) using the Turbo Blotter device (Schleicher & Schuell) with 20× SSC for neutral downward transfer for 64 hours. The membrane was then baked at 80°C for 2 hours and irradiated in a Bioslink UV linker with 0.3 J/cm^2. The probe was prepared by labeling the complete cDNA for human CR deaminase by the random prime method using the kit from Boehringer Mannheim and [^{32}P]dCTP from ICN (Mississauga, Ontario). The membrane was prehybridized in 10 mL Express Hyb solution (Clontech) at 65°C overnight and hybridized at 65°C overnight in 5 mL of Express Hyb solution containing 6.25 × 10^6 cpm of the DNA probe. The membrane was then washed once with 2× SSC and 0.1% SDS at 65°C for 15 minutes, and then twice with 0.2× SSC and 0.1% SDS at 65°C for 30 minutes. The blot was exposed to x-ray film (Kodak X-Omat) with two intensifying screens at −70°C for 5 days.

Southern blot analysis

Genomic DNA was isolated from cells with the Stratagene DNA Extraction kit. About 2 × 10^7 cells underwent lysis with a mixture of 50 mmol/L Tris-HCl, 20 mmol/L EDTA, and 2% SDS, followed by digestion with 50 mg/mL proteinase K. A saturated NaCl solution was then added, followed by centrifugation and RNAse treatment of the supernatant. DNA was precipitated with ethanol and air dried and suspended in TE (10 mmol/L Tris, 1 mmol/L EDTA, pH 8) buffer. For Southern blot analysis, 10 μg of DNA was digested with *Nco*I and *Bam*HI and separated by electrophoresis on a 1% agarose gel. After UV photography, the gel was immersed in denaturing solution (0.5 mol/L NaOH; 0.15 mol/L NaCl) for 30 minutes and then in neutralizing buffer (0.5 mol/L Tris-HCl pH 7; 1.5 mol/L NaCl) for 30 minutes. The DNA in the gel was transferred onto a Hybond-N nylon membrane (Amersham, Oakville, Ontario) using the Turbo Blotter device with 10× SSC for downward transfer for 40 hours. The membrane was then baked at 80°C for 2 hours and irradiated in a Bioslink UV linker with 0.3 J/cm^2. The membrane was probed with ^{32}P-cDNA for human CR deaminase as described above, except that the hybridization solution was 6× SSC + 2× Denhardt's solution + 1% SDS. The membrane was exposed to x-ray film with intensifying screens at −70°C for 48 hours.

Table 1. CR Deaminase Assay in Murine Cells

Cell Line	Enzyme Activity (unit/mg)
3T3	2.5 ± 0.9
3T3-CD3-V5	105.1 ± 15.4
3T3-CD3-V6	31.2 ± 7.2

Values are means ± SD; n = 3–7.
Unit, deamination 1 nmole CR/min.

Polymerase chain reaction

A PCR assay was used to verify the presence of the MFG-CD construct in transduced marrow cells. The oligonucleotides: 5'-GGT GGA CCA TCC TCT AGA CTG-3' (P1) and 5'-AGC AGC TCC TGG ACC GTC ATG-3' (P2) were used as primers in the presence of genomic DNA to amplify a specific 421-bp fragment as predicted by the DNA sequence of the pMFG-CD construct. The sense oligonucleotide P1 was 270 bp downstream from the splice acceptor region of MFG and 2 bp upstream from the start of the *env* coding region. The antisense oligonucleotide P2 was from positions 377 to 397 of the CR deaminase coding region. Genomic DNA was isolated from individual hematopoietic colonies in methylcellulose with the In ViSorb DNA Kit (ID Labs Biotechnology, London, Ontario, Canada) by cell lysis with guanidine thiocyanate, DNA adsorption on silica gel, and elution with TE buffer. For the PCR reaction, 1 ng genomic DNA was denatured at 95°C for 2 minutes and amplified for 35 cycles using ID Proof Taq DNA polymerase (ID Labs Biotechnology), each cycle consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C, and extension for 1 minute at 72°C with a terminal 5 minute extension at 72°C. The reaction mixture was separated on 2% agarose electrophoresis.

RESULTS

Enzymatic assays for CR deaminase activity were performed on the nontransduced and transduced 3T3 cells (Table 1). CR deaminase activity for the 3T3 cells was low (2.5 U/mg). However, the enzyme activity was 105.1 U/mg for the 3T3-CD3-V5 and 31.2 U/mg for the 3T3-CD3-V6 cells. This represents a 42- and 12.5-fold increase in enzyme activity for the transduced cell lines, respectively, in comparison with the 3T3 control cells.

Northern blot analysis showed the efficient mRNA expression of the transduced CR deaminase gene in both the 3T3-CD3-V5 and 3T3-CD3-V6 cells, but not in the nontransduced parental 3T3 cells (Fig 1, left panel). Two transcripts for CR deaminase of approximately 2.8 and 1.9 kb were seen in the 3T3-CD3-V5 and 3T3-CD3-V6 cell lines. These transcripts correspond to the predicted size of unspliced and spliced transcripts of MFG-CD. The higher level of expression of the 1.9-kb spliced transcript in the 3T3-CD3-V5 cells, as compared

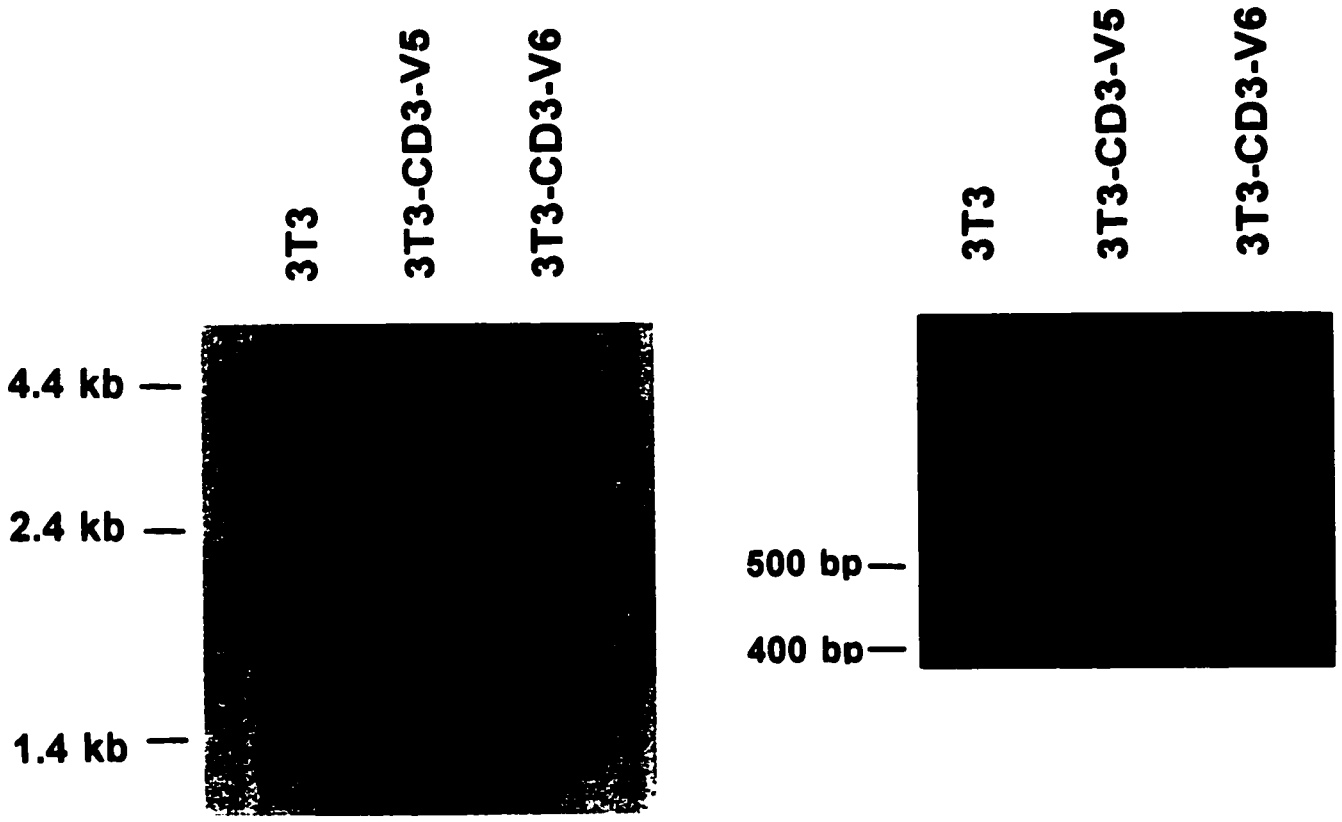

Figure 1. Northern blot hybridization (left panel) of 10 μg total RNA from each of the indicated cell lines. For Southern blot analysis (right panel) 10 μg of purified genomic DNA from the indicated cell lines was digested with *Nco*I and *Bam*HI and fractionated on 1% agarose. After transfer to a nylon membrane, hybridization was performed with ^{32}P-labeled CR deaminase cDNA probe as described in Methods for both experiments.

with the 3T3-CD3-V6 cells, may correlate with the lower enzyme activity in this latter clone.

In the Southern blot analysis, a DNA band of about 450 bp, similar to the size of the coding region of the CR deaminase cDNA, was observed in both the 3T3-CD3-V5 and 3T3-CD3-V6 cells, but not in the 3T3 cells (Fig 1, right panel). In the 3T3-CD3-V5 cells, the DNA band was of higher intensity than that in the 3T3-CD3-V6 cells, indicating that the 3T3-CD3-V5 cells may possess more copies of the transduced CR deaminase gene.

The incorporation of radioactive thymidine into DNA was used to evaluate the inhibition of DNA synthesis by different concentrations of ARA-C in the 3T3 cell lines (Fig 2). ARA-C at concentrations of 10^{-6} mol/L and 10^{-5} mol/L produced much less inhibition of DNA synthesis for the 3T3-CD3-V5 and 3T3-CD3-V6 cells as compared with the 3T3 cells. This difference in sensitivity to ARA-C inhibition was more evident at the concentration of 10^{-6} mol/L.

The effect of ARA-C on the growth of the 3T3 cell lines is shown in Fig. 3. For the 3T3-CD3-V5 and 3T3-CD3-V6 cells, ARA-C at concentrations of 10^{-6} mol/L and 10^{-5} mol/L produced less growth inhibition

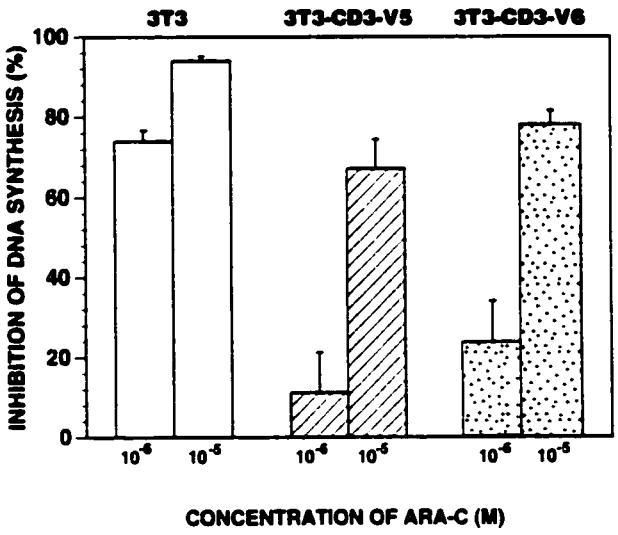

Figure 2. Inhibition of DNA synthesis by ARA-C. The cell lines were incubated with the indicated concentrations of ARA-C and radioactive thymidine for 6 hours. DNA synthesis was measured as described in Methods. The bar and the vertical line represent the mean value and SD, respectively, for five experiments.

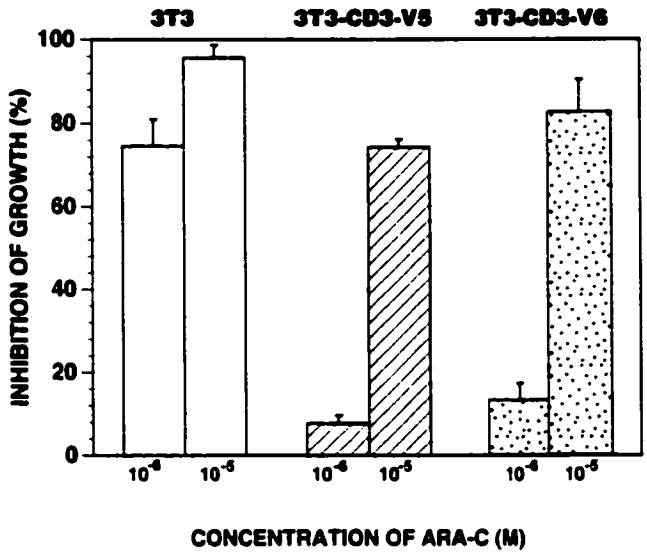

Figure 3. Growth inhibition by ARA-C. The cell lines were incubated with the indicated concentrations of ARA-C for 5 days. Cell counts were measured as described in Methods. The bar and the vertical line represent the mean value and SD, respectively, for three to five experiments.

than for the parental 3T3 cells. At the concentration of 10^{-6} mol/L, the decrease in sensitivity of the transduced cells to ARA-C growth inhibition was more evident.

A colony assay was used to evaluate the effects of ARA-C on the clonogenicity of the transduced 3T3 cells in comparison with the parental cells (Table 2). ARA-C at a concentration of 10^{-6} mol/L reduced the colony formation of the 3T3 cells to less than 1%. At this same concentration, the colony survival for the 3T3-CD3-V5 and 3T3-CD3-V6 cells was 88.6% and 74.8%, respectively. At 10^{-5} mol/L ARA-C, only the 3T3-CD3-V5 cells showed residual colony survival (17.1%).

Murine bone marrow cells were transduced by the viral producer cell lines GP+E86-LacZ or GP+E86-CD3. Using a clonogenic assay, we determined the effects of different concentrations of ARA-C on survival (Table 3). In the presence of ARA-C from 10^{-7} mol/L to 10^{-5} mol/L the percentage survival was much greater for the marrow cells transduced by the GP+E86-CD3 cells as compared with the GP+E86-LacZ cells. For marrow cells transduced by GP+E86-LacZ, treatment with ARA-C at 10^{-6} mol/L reduced the survival to less than 3% as compared with untreated cells but did not reduce survival of the marrow cells transduced by GP+E86-CD3.

In order to verify the presence of the proviral DNA in marrow cells transduced by GP+E86-CD3 cells, individual hematopoietic colonies were isolated and the genomic DNA purified. This DNA was used in a PCR reaction with specific primers to detect the presence of the proviral DNA. A DNA band of the predicted size of 421 bp was amplified from eight of 10 colonies, indicating efficient transfer of the proviral DNA (Fig 4). There were no specific MFG-CD bands detectable after PCR using DNA from marrow colonies transduced by the GP+E86-LacZ cells (data not shown).

Table 2. Effect of ARA-C on Colony Formation by Murine Fibroblasts

	Concentration of ARA-C		
Cell Line	10^{-7} mol/L	10^{-6} mol/L (% survival)	10^{-5} mol/L
3T3	83.2 ± 19.9	0.4 ± 0.9	0
3T3-CD3-V5	98.7 ± 2.9	88.6 ± 7.2	17.1 ± 13.3
3T3-CD3-V6	96.4 ± 6.2	74.8 ± 19.5	0

Values are means ± SD; n = 4.

DISCUSSION

For most antineoplastic agents, toxicities to the hematopoietic system limit the dose of drug that can be safely administered. In the case of cytosine nucleoside analogues such as ARA-C, marked myelotoxicity has limited their use to the treatment of primary hematologic malignancies where severe marrow suppression is generally part of effective therapy.

However, primary cultures of human tumors showed that ARA-C is active against lung tumors and melanoma in a colony assay.[23] In addition, the combination of ARA-C with cis-platinum showed antitumor synergy that was schedule dependent. If the optimal dose-schedule for ARA-C in combination with cis-platinum or other antineoplastic agents could be determined, there could be more use of this nucleoside analogue for the clinical treatment of nonhematologic malignancies, especially if its myelotoxicity could be circumvented.

One approach to circumvent ARA-C myelotoxicity would be to confer chemoresistance on the normal hematopoietic system. The feasibility of that approach has been demonstrated by conferring methotrexate or multidrug resistance in animal models after retrovirus-mediated gene transfer in hematopoietic stem cells.[3,4,7] This has led to the development of clinical trials in which MDR gene transfer will be used to confer hematopoietic chemoprotection to patients undergoing chemotherapy for breast cancer.[24]

We have recently cloned the human cDNA for CR deaminase.[16] CR deaminase efficiently inactivates intracellular ARA-C by deamination.[11,12] In order to evaluate the potential use of CR deaminase for chemoprotection, we constructed the retroviral vector pMFG-CD, and by transfection produced MFG-CD virions in GP+E-86 ecotropic packaging cells. We observed that these MFG-CD virus-producing cells showed markedly increased CR deaminase activity and were resistant to ARA-C.[17]

In this study NIH 3T3 mouse fibroblasts were transduced with MFG-CD virions produced by GP+E-86-CD3 packaging cells and selected in 10^{-6} mol/L ARA-C. Two of the clones isolated (3T3-CD3-V5 and 3T3-CD3-V6) were found to express increased levels of CR

Table 3. Effect of ARA-C on Clonogenic Assay for Murine Hematopoietic Cells

Viral Producer Cell Line	Concentration of ARA-C			
	10^{-8} mol/L	10^{-7} mol/L (% survival*)	10^{-6} mol/L	10^{-5} mol/L
GP+E86-LacZ	97.6 ± 9.0	75.6 ± 12.1†	2.5 ± 1.4‡	0
GP+E86-CD3	93.5 ± 9.6	98.9 ± 10.7†	102.3 ± 8.9‡	90.6 ± 15.9

Values are means ± SEM; n = 5.
*Determined by colony assay for granulocytes-macrophages.
†Difference between % survival by paired Student's *t* test ($P < 0.33$).
‡Difference between % survival by paired Student's *t* test ($P < 0.001$).

deaminase enzymatic activity (Table 1). This overexpression of CR deaminase was accompanied by a significant decrease in the degree of DNA synthesis inhibition produced by ARA-C in the CD-transduced NIH 3T3 cells; this effect was particularly marked at 10^{-6} mol/L ARA-C (Fig 2). Likewise, CR deaminase gene transfer in NIH 3T3 cells significantly reduced the inhibition of cell growth produced by ARA-C (Fig 3) and conferred drug resistance to this analogue as shown by colony formation (Table 2). Again these effects of CR deaminase transduction were especially evident at 10^{-6} mol/L ARA-C. The CD-transduced fibroblasts maintained their drug resistance to ARA-C for more than 6 months without any selecting agent in the culture medium indicating that the CR deaminase expression was stable.

To further evaluate the potential of CR deaminase gene transfer for chemoprotection of hematopoietic cells, we transduced primary mouse bone marrow cells with either MFG-CD or MFG-LacZ retroviral particles produced by the GP+E-86-CD3 or GP+E-86-LacZ packaging cells, respectively. PCR analysis of individual hematopoietic colonies grown from the transduced bone marrow cells in the absence of ARA-C showed a high efficiency of MFG-CD gene transfer into clonogenic hematopoietic progenitor cells (Fig 4). We then determined the effect of ARA-C on *in vitro* colony formation by the populations of clonogenic hematopoietic progenitor cells. At concentrations of ARA-C ranging from 10^{-7} to 10^{-5} mol/L, colony formation by the hematopoietic cells transduced with the MFG-CD vector was practically unaffected by this drug, whereas colony formation by the control LacZ-transduced cells was gradually suppressed to reach less than 1% in the presence of 10^{-5} mol/L ARA-C (Table 3). This indicates a strong *in vitro* survival advantage in favor of CR deaminase–transduced hematopoietic cells in the presence of ARA-C.

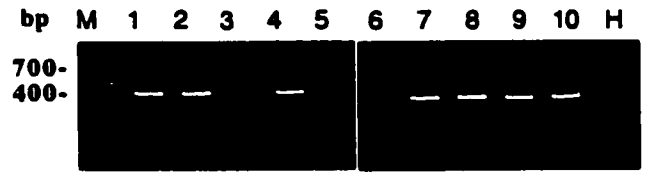

Figure 4. PCR analysis for the presence of proviral DNA in murine marrow cells. Genomic DNA was isolated from colonies of murine hematopoietic cells transduced by GP+E86-CD3 cells. A sense primer downstream from the MFG splice acceptor region and an antisense primer from the CR deaminase coding region with purified genomic DNA from the colonies were used in the PCR as described in Methods. Amplification of a specific 421-bp band indicates the presence of MFG-CD proviral DNA. Number, DNA from marrow colony assayed; M, molecular size marker; H, H_2O used as template.

The degree of hematopoietic chemoprotection conferred *in vivo* by CR deaminase transduction will need to be evaluated directly in bone marrow transplantation experiments in mice. However, the powerful *in vitro* survival advantage of CR deaminase–transduced clonogenic hematopoietic progenitor cells suggests that the degree of *in vivo* chemoprotection should be sufficient to confer drug resistance to ARA-C. The mouse marrow cells showed high survival at an ARA-C concentration of 10^{-6} mol/L (Table 3), which is in the same range as the plasma concentration of this analogue reported for patients that received a continuous intravenous infusion of ARA-C at the conventional dose.[25] These observations suggest that gene transfer of CR deaminase into human hematopoietic cells could have the potential to prevent ARA-C hematotoxicities produced by clinically effective doses of this drug.

This approach of using the CR deaminase gene for chemoprotection also could have application for other interesting deoxycytidine analogues such as 2,2'-difluorodeoxycytidine (dFdC) and 5-aza-2'-deoxycytidine (5-AZA-CdR), which are also deaminated by this enzyme.[26–28] In phase I and II studies, dFdC has shown some promising antitumor activity.[29,30] We and others have demonstrated that 5-AZA-CdR is an active antileukemic agent.[31–33]

Recently, considerable interest in 5-AZA-CdR has been generated by the reports that this analogue activates the expression of different tumor suppressor genes by demethylation.[34–37] In a preliminary study we observed that gene transfer of CR deaminase into murine fibroblasts conferred drug resistance to dFdC and 5-AZA-CdR.[38]

In summary, the present *in vitro* studies suggest that CR deaminase gene transfer could be used to confer hematopoietic chemoprotection from ARA-C and related cytosine nucleoside analogues. Future experiments will aim at evaluating the degree of *in vivo* chemoprotection obtained after transplantation of CR deaminase–transduced bone marrow cells into lethally irradiated

animals. Animals reconstituted with CR deaminase–transduced hematopoietic cells will also permit us to evaluate the feasibility of enrichment of these cells by *in vivo* selection with ARA-C, as reported for MDR.[7]

ACKNOWLEDGMENTS

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APPENDIX 4

HUMAN GENE THERAPY 9:2537–2544 (November 20, 1998)
Mary Ann Liebert, Inc.

Coexpression of Cytidine Deaminase and Mutant Dihydrofolate Reductase by a Bicistronic Retroviral Vector Confers Resistance to Cytosine Arabinoside and Methotrexate

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ABSTRACT

The transfer of a drug resistance gene into hematopoietic cells is an approach being investigated to overcome the problem of myelosuppression produced by anticancer drugs. Chemotherapeutic agents are often given in combination in order to increase their effectiveness. Consequently, there is an advantage in designing vectors for gene transfer that are capable of expressing two drug resistance genes. We have constructed a bicistronic retroviral vector, MFG-DHFR-IRES/CD, which contains the mutated human dihydrofolate reductase (DHFR) cDNA with a phenylalanine-to-serine substitution at codon 31 (F31S) and the human cytidine deaminase (CD) cDNA. Murine fibroblast and hematopoietic cells were transduced with this vector and evaluated for their resistance to methotrexate (MTX) and cytosine arabinoside (ARA-C). The transduced fibroblast cells showed high levels of resistance to MTX and to ARA-C as determined by a clonogenic assay. Using enzymatic assays, we observed a coordinate increase in resistance to MTX and DHFR enzyme activity following an ARA-C selection. In addition, MTX selection produced an increase in CD enzyme activity and ARA-C resistance. Murine hematopoietic cells transduced with the bicistronic vector also showed drug resistance to both MTX and ARA-C. Interestingly, the double-gene construct conferred an equivalent level of drug resistance compared with single-gene vectors bearing only CD or DHFR genes in the hematopoietic cells. These results demonstrate the potential of the MFG-DHFR-IRES/CD vector to confer drug resistance to both MTX and ARA-C and may have future application in chemoprotection of normal hematopoietic cells in patients with cancer.

OVERVIEW SUMMARY

Combination chemotherapy for cancer is used often to overcome the problem of drug resistance. Since most anticancer agents produce bone marrow toxicity, the combination of these agents at intensive doses can result in severe myelosuppression. An approach to overcome this problem involves the transfer into normal bone marrow progenitors cells of drug resistance genes. We have constructed a retroviral bicistronic vector capable of expressing two drug resistance genes, cytidine deaminase and a mutated dihydrofolate reductase, to protect transduced hematopoietic cells from the combined effects of cytosine nucleoside analogs and antifolate antagonists, respectively. We showed that transduced murine fibroblast and myeloid progenitor cells acquired significant resistance to cytosine arabinoside and methotrexate. Our data provide a rationale for the use of a double gene vector in an *in vivo* model in which transduced bone marrow cells will be protected against the toxic side effects of combination chemotherapy with cytosine nucleoside analogs and antifolates.

INTRODUCTION

INTENSIVE TREATMENT with most anticancer drugs produces severe myelosuppression as their major side effect. Introduction of drug resistance genes into normal bone marrow stem

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cells is an approach to confer chemoprotection (reviewed by Banerjee *et al.*, 1994a; Koc *et al.*, 1996; Rafferty *et al.*, 1996). The potential of chemoprotection is being investigated for the following drug resistance genes: multidrug resistance (MDR) (Sorrentino *et al.*, 1992; O'Shaughnessy *et al.*, 1994; Ward *et al.*, 1994), dihydrofolate reductase (DHFR) (Li *et al.*, 1994; Zhao *et al.*, 1994, 1997; Flasshove *et al.*, 1995), O^6-methylguanine DNA methyltransferase (Allay *et al.*, 1995; Moritz *et al.*, 1995), glutathione *S*-transferase (Létourneau *et al.*, 1996), multidrug-associated protein (D'Hondt *et al.*, 1997), and cytidine deaminase (CD) (Eliopoulos *et al.*, 1996; Momparler *et al.*, 1996).

Since combinations of anticancer agents are commonly used in cancer therapy to increase clinical effectiveness, it is of interest to investigate the potential of an expression vector that can confer chemoprotection to two different classes of anticancer drugs. Retroviral gene constructs in which separate genes are expressed from two independent promoters have been unreliable because of negative interactions between promoters that may lead to markedly reduced expression of one of the genes (Emerman and Temin, 1984, 1986; Olsen *et al.*, 1993). Another approach is to use the encephalomyocarditis virus internal ribosome entry site (IRES) to produce a bicistronic vector. These bicistronic vectors circumvent promoter/enhancer interference by coexpressing the two genes from a single initiated transcript (Jang *et al.*, 1989; Ghattas *et al.*, 1991; Morgan *et al.*, 1992). Bicistronic vectors containing IRES have been designed to confer drug resistance to MDR drugs and alkylating agents (Suzuki *et al.*, 1997) and MDR drugs and antifolate drugs (Galipeau *et al.*, 1997).

In this article we have designed a bicistronic retroviral vector with an IRES containing the human DHFR gene and the human CD gene. In the vector, MFG-DHFR-IRES/CD, the translation of the upstream gene (DHFR) is Cap dependent while the translation of the second gene (CD), downstream of the IRES element, is cap independent. This should allow efficient expression of both genes. DHFR confers resistance to antifolate drugs whereas CD confers resistance to nucleoside analogs such as cytosine arabinoside (ARA-C), 2',2'-difluorodeoxycytidine (dFdC), and 5-aza-2'-deoxycytidine (5-Aza-CdR) (Eliopoulos *et al.*, 1996; Momparler *et al.*, 1996). Both classes of agents are used in leukemia and solid tumor therapy (Lie and Slordahl, 1985; Shepherd, 1995; Momparler *et al.*, 1997).

Since a mutated form of the DHFR with decreased binding to methotrexate (MTX) shows greater *in vitro* chemoprotection (Banerjee *et al.*, 1994b; Ercikan-Abali *et al.*, 1996), we substituted a serine in place of phenylalanine at codon 31 (F31S) in human DHFR cDNA. Using the MFG-DHFR-IRES/CD vector, we were able to demonstrate high levels of drug resistance to both ARA-C and MTX and highly efficient gene expression in transduced cells. This bicistronic vector may be a useful tool for chemoprotection in cancer therapy.

MATERIALS AND METHODS

Cell culture and clonogenic assays

The retrovirus packaging cell lines GP+*env*AM12 and GP+E86 (a gift from A. Bank, Columbia University, New York) and the NIH 3T3 mouse fibroblast cell line were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and gentamicin (5 μg/ml) at 37°C in a 6% CO_2 incubator. The clonogenic assays were performed by adding 2 ml of medium containing 200 cells per well in Costar (Cambridge, MA) six-well culture dishes. The next day, graded concentrations of ARA-C and MTX were added for 72 hr and 12 days, respectively. In both cases, the total time of incubation was 12 days. For ARA-C assays, the dishes were rinsed and fresh medium added for the subsequent 9 days after drug treatment. For MTX assays, 10% dialyzed heat-inactivated FBS was used.

Vector construct

The pMFG-CD (Momparler *et al.*, 1996) expression vector was used as the backbone for the construction of pMFG-DHFR-IRES/CD (Fig. 1). The CD cDNA was first removed using a double digestion with *Nco*I and *Bam*HI in order to introduce the Ser-31 mutated human DHFR cDNA, which was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification and site-directed mutagenesis. The DHFR was first subcloned in pRC-CMV (Invitrogen, San Diego, CA) and mutagenesis done by PCR using four different primers. Primers used for the amplification from the new pRC-DHFR Ser-31 were 5' CCCATCGATACCATGGTTGGTTCGCTA 3' and 5' TTTCGCGGATCCTTTAATCATTCTTCTC 3', the *Nco*I and *Bam*HI restriction sites being introduced at the underlined positions. Following an *Nco*I/*Bam*HI digestion and ligation, pMFG-DHFR Ser-31 was obtained. This plasmid was then sequenced and the presence of the Ser-31 mutation confirmed (using a Pharmacia [Piscataway, NJ] automatic DNA sequencer with internal primers and fluoro-dATP).

The IRES/CD, 1084-base pair (bp) fragment was constructed by PCR using two different templates and three amplification reactions. First, the IRES fragment corresponding to the IRES element of the encephalomyocarditis virus (EMCV) was obtained by PCR amplification from pHaMAIRES (gift from S. Kane, City of Hope National Medical Center, Duarte, CA) using the following primers: 5' TACAGAGGATCC/CCGCGGTATCTGTTTAACAG 3' and 5' TCTGGGCCATATTATCATCGTG 3' (636 bp), the underlined sequences being, respectively, the *Bam*HI restriction site, the *Sac*II restriction site, and part of the 5' coding region of CD. Second, the CD

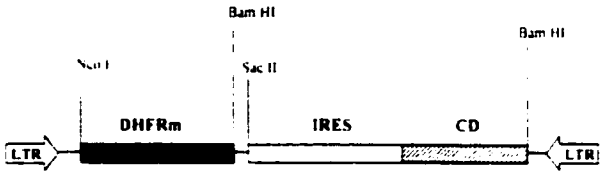

FIG. 1. Retroviral MFG-DHFR-IRES/CD vector structure is based on the Moloney murine leukemia virus. This bicistronic vector contains upstream from the IRES element the DHFR cDNA mutated (DHFRm) at codon 31 for a phenylalanine-to-serine substitution. The CD cDNA was cloned downstream from the IRES element.

fragment was obtained by PCR amplification from pBS-CD, previously described (Laliberté and Momparler, 1994), using two primers: 5′ CACGATGATAATATGGCCCAGAAGCGT 3′ and 5′ TGGGCAGGATCCGGCTGTCACT 3′ (470 bp), where the underlined sequences represent the 3′ end of the IRES fragment and the *Bam*HI restriction site, respectively. The complete IRES/CD fragment originates from a PCR amplification using as templates the amplified sequence of the IRES and CD. The 22-base pair homology between the two templates allows an efficient amplification when using the 5′ primer of the IRES amplification and the 3′ primer of the CD amplification. The IRES/CD fragment was then purified, digested with *Bam*HI, and ligated in pMFG-DHFR Ser-31 previously linearized at the *Bam*HI site. The entire amplified DHFR-IRES/CD construct was then confirmed by sequencing (by the Pharmacia automatic DNA sequencer with internal primers and fluoro-dATP).

Retroviral transduction of fibroblast cells

For three consecutive days, the medium of flasks containing $1–2 \times 10^5$ 3T3 cells was replaced for a 6-hr incubation with medium containing Polybrene (8 μg/ml) and filtered supernatant from a transfected ARA-C-resistant producer clone GP+*env*AM12 transfected with vMFG-DHFR-IRES/CD (titer, 3.5×10^3 CFU/ml). The supernatant from the parental GP+*env*AM12 cells was used for mock infection of the control 3T3 cells. Fresh medium was added between and after the three incubations. On day 5, the cells were trypsinized and the selection started. Two transduced 3T3 (t3T3) clones were selected (3T3-clone/2 and 3T3-clone/8) from among eight clones randomly isolated following a 4-day selection with 5 μ*M* ARA-C. The t3T3 MTX- and ARA-C-selected cell populations were produced from a polyclonal population of resistant cells previously selected with 500 n*M* MTX and 2.5 μ*M* ARA-C for 8 days, respectively. Both transfected and untransfected 3T3 cells have similar growth rates.

Southern blot analysis

The Southern blot was performed as described by Galipeau *et al.* (1997). The genomic DNA (5 μg) from mock and transduced 3T3 cells was digested with *Nhe*I and probed with ^{32}P-labeled CD probe.

Enzyme assays

Cytosol aliquots were prepared as follow: 2 to 5×10^7 monolayer cells were trypsinized, centrifuged, and washed once in phosphate-buffered saline (PBS). They were then centrifuged and resuspended in 100 μl of 5 m*M* Tris-HCl (pH 7.4) and 5 m*M* dithiothreitol. Cell membranes were than lysed following three sets of freezing and thawing. The resulting mixture was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant (cytosol) molarity was then adjusted to 50 m*M* Tris-HCl, pH 7.4, and the protease inhibitors leupeptin, aprotinin, and pepstatin (Boehringer Mannheim, Laval, Quebec, Canada) were added to a final concentration of 1 ng/μl. CD activity was determined by measuring the conversion of [^{3}H]cytidine to [^{3}H]uridine following a 30-min incubation at 37°C as previously described (Laliberté and Momparler, 1994). The DHFR enzyme assays were performed at room temperature with a Gilford Instruments (Oberlin, OH) 260 spectrophotometer in a reaction mixture containing 100 m*M* Tris-HCl (pH 7.0), 150 m*M* KCl, 50 μ*M* NADPH and dihydrofolate (Sigma, Oakville, Ontario, Canada) and water in a final volume of 1 ml (Schweitzer *et al.*, 1989). The reaction was started by adding 10 μl of the enzyme dilution. The DHFR enzyme activity was obtained by measuring for 25 min the decrease in absorbance at 340 nm, which correlates with the conversion of H_2-folate to H_4-folate and NADPH to $NADP^+$ (Osborn and Huennekens, 1958). The extinction coefficient used for the combined absorbance change was $12 \times 10^3\ M^{-1}$ (Bertino *et al.*, 1965).

Murine bone marrow transduction and CFU-C assays

Bone marrow cells were collected from C3H female mice (Charles River Canada, St-Constant, Quebec, Canada) treated with 5-fluorouracil (150 mg/kg) 4 days earlier. The cells were then cocultivated for 72 hr with equivalent numbers of producer cells (5×10^6) irradiated the night before with 2000 rads. The producer cells originated from a pool of GP+E86 clones transduced with viral particles collected from previously transduced GP+*env*AM12 vMFG-CD, vMFG-DHFR, or vMFG-DHFR-IRES/CD (titer, 1×10^5 CFU/ml) and resistant to 5 μ*M* ARA-C or 100 n*M* MTX (for MFG-DHFR). The viral titers of the producer cells were sufficient to transduce >90% of target cells as determined by PCR assay. Mock transduction was from parental GP+E86 packaging cells. The cocultivation medium contained bovine serum albumin (BSA, 10 mg/ml), transferrin (0.3 mg/ml) (Boehringer Mannheim), Polybrene (8 μg/ml), 10% WEHI-3B and HTB-9 conditioned medium, 20% FBS in α-MEM, and gentamicin (5 μg/ml) (Sigma). Transduced bone marrow cells were then collected and counted, and $2–5 \times 10^4$ cells/ml were plated in 1% methylcellulose in α-MEM supplemented with 30% FBS, 1% mouse spleen conditioned medium (Stem Cell Technologies, Vancouver, British Columbia, Canada), 1% BSA, and 10^{-4} *M* 2-mercaptoethanol. Drug resistance was measured as the percentage of colonies surviving in the presence of drug. Transduction rates were determined by PCR assay on colony-forming unit cells (CFU-Cs) grown in the absence of drug, using vector-specific primers. Mock-transduced cells (mock cells) were negative by PCR assay. For MTX assays, the FBS used was pretreated with thymidine phosphorylase (1 U/ml) for 4 hr at 37°C to prevent the rescue of MTX-treated cells by thymidine. The colonies were scored after 7–9 days of growth.

RESULTS

DHFR and CD enzyme activities in 3T3 cells transduced with vMFG-DHR-IRES/CD

3T3 cells transduced with vMFG-DHFR-IRES/CD showed a significant increase in DHFR and CD enzyme activities (Table 1). Without any selection, the transduced population showed a 1.3- and 100-fold increase in DHFR and CD activities, respectively, as compared with the mock 3T3 cells. After selection with ARA-C (8 days in a 2.5 μ*M* concentration), the population showed a 2- and >150-fold increase in DHFR and CD activities, respectively, as compared with mock 3T3 cells. Selection with MTX (8 days in 500 nM) produced a greater increase

TABLE 1. DHFR AND CD ENZYME ACTIVITIES OF MOCK- AND vMFG-DHFR-IRES/CD-TRANSDUCED 3T3 CELLS

Cell line	DHFR activity[a] (units/mg)	CD activity[a] (units/mg)
Mock-transduced 3T3	2.2 ± 0.5	0.3 ± 2
t3T3		
No selection	2.9 ± 0.2	30.7 ± 2.5
ARA-C selection	4.4 ± 0.1	52.9 ± 2.5
MTX selection	12.2 ± 0.5	115 ± 7
3T3-clone/2	14.8 ± 1.3	116 ± 5
3T3-clone/8	25.7 ± 1.4	359 ± 10

[a]Values presented are means ± SD of three to six determinations.

Abbreviation: t3T3, Transduced 3T3 cells.

in expression of DHFR and CD activities as compared with the population selected with ARA-C. The DHFR and CD activity showed a 5.5- and >350-fold increase, respectively, in the population selected with MTX as compared with mock 3T3 cells. Two clones were isolated from the transduced population after selection with ARA-C (4 days in a 5 μM concentration). Both clones showed a marked increase in both DHFR and CD activities (Table 1).

Drug resistance to MTX and ARA-C in transduced 3T3 cells

To determine the level of resistance to MTX and ARA-C produced by our bicistronic vector (MFG-DHFR-IRES/CD), clonogenic assays were performed on two 3T3 fibroblast clones isolated after transduction and selection with ARA-C. At a concentration of MTX that produced >90% loss of clonogenicity in the mock 3T3 cells, the transduced cells showed almost no loss of clonogenicity (Fig. 2). In addition, at a concentration of ARA-C that produced >90% loss of clonogenicity in the mock 3T3 cells, the two clones showed more than 90% survival in clonogenic assays (Fig. 2). At a higher concentration of either MTX or ARA-C, clone 8 was found to be more resistant than clone 2. This difference is associated with a higher level of CD and DHFR enzyme activities in clone 8 as compared with clone 2 (Table 1) and with a higher level of the bicistronic vector mRNA in clone 8 as determined by Northern blot (data not shown).

A transduced population of 3T3 cells was tested for drug resistance with and without preselection with either MTX or ARA-C. In a clonogenic assay, the t3T3 cells without preselection showed significant drug resistance to increasing concentrations of MTX (Fig. 3B), but not to increasing concentrations of ARA-C (Fig. 3A). Prior selection of the transduced cells with 500 nM MTX produced a significant enhancement of resistance to this antifolate and to ARA-C as compared with the unselected transduced cells. On the other hand, a 2.5 μM ARA-C selection produced only a slight enhancement of resistance to MTX and ARA-C as compared with the unselected cells. Southern blot analysis of the mock and transduced 3T3 cells showed the expected full-length 3.65 kb for the DHFR-IRES/CD fragment between the LTRs of the MFG vector, with no signs of rearrangement (Fig. 4). These results suggest that the CD gene was not deleted as a explanation for the low ARA-C resistance observed.

Chemoprotection of murine bone marrow cells

We also determined whether our bicistronic vector would confer drug resistance to both MTX and ARA-C in murine bone marrow cells and if it would be as efficient as the single-gene vectors. After cocultivation of the bone marrow cells with producer cells, we observed that more than 90% of the clonogenic progenitor cells were transduced with either vMFG-CD, vMFG-DHFR, or vMFG-DHFR-IRES/CD as determined by PCR on isolated CFU-C colonies (10 to 12 for each producer) grown without any drugs (see Materials and Methods). Clonogenic assays showed a 10- to 20-fold increase in ARA-C LD_{50} (50% lethal dose) for both MFG-CD- and MFG-DHFR-IRES/CD-transduced CFU-Cs when compared with the mock- and MFG-DHFR-transduced CFU-Cs (Fig. 5A). In a similar experiment,

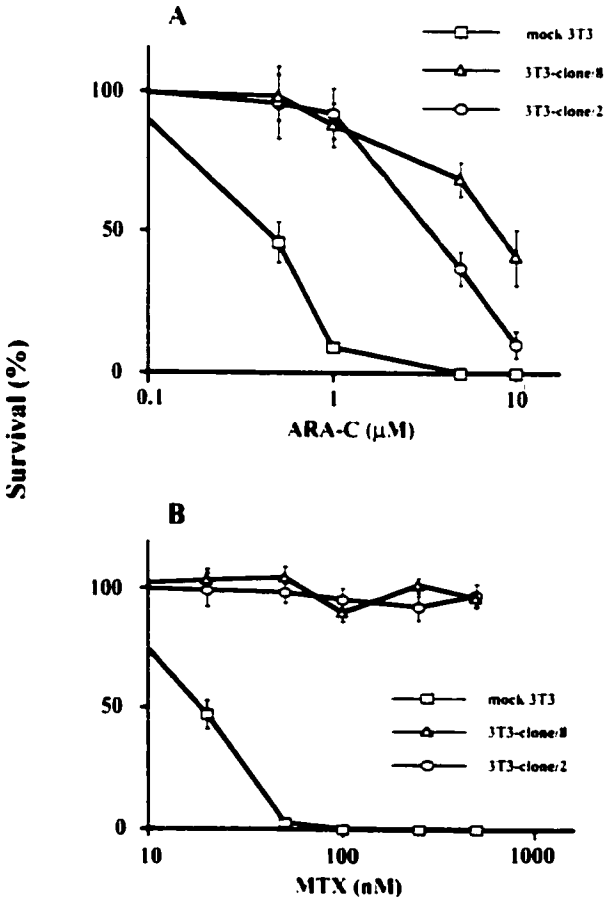

FIG. 2. Clonogenic survival in various ARA-C and MTX concentrations of two clones (3T3-clone/2 and 3T3-clone/8) that were selected for 4 days in 5 μM ARA-C from a mixed population of 3T3 cells transduced with vMFG-DHFR-IRES/CD. The cells were incubated for 3 and 12 days in the presence of ARA-C (**A**) and MTX (**B**), respectively. Colonies were counted after a 12-day incubation and percent survival is expressed as the ratio of colonies counted in the presence of drug per colonies counted in the absence of drug. The average ± SD of three or four experiments is shown.

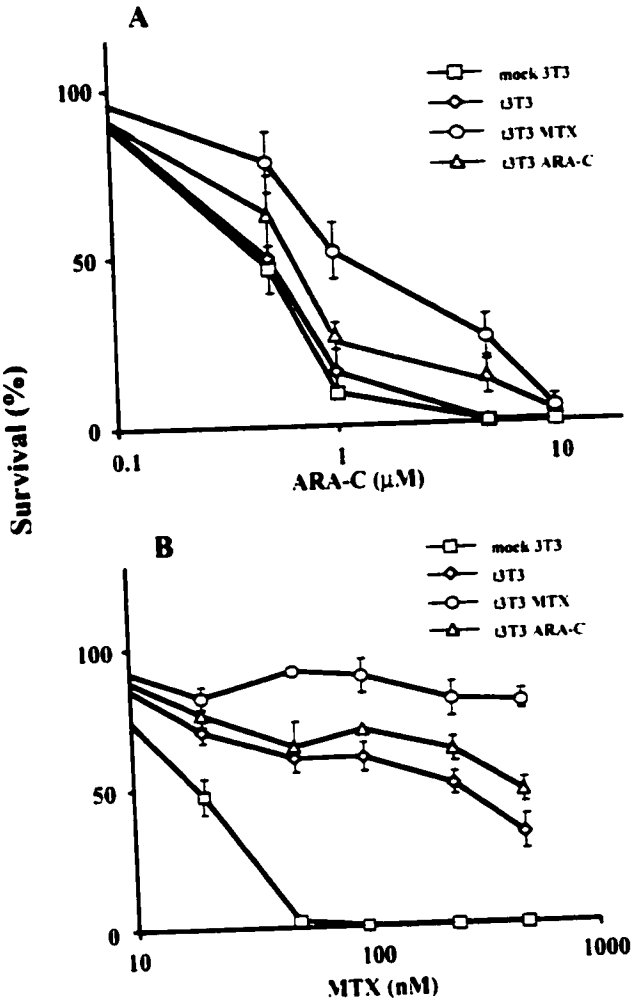

FIG. 3. Clonogenic survival in various concentrations of ARA-C and MTX of a mixed population of 3T3 cells transduced with vMFG-DHFR-IRES/CD (t3T3 cells). t3T3 ARA-C and t3TC MTX cells originate from a preselection of the mixed population with 2.5 μM ARA-C and 500 n*M* MTX for 8 days, respectively. The cells were incubated for 3 and 12 days in the presence of ARA-C (**A**) and MTX (**B**), respectively. Colonies were counted after a 12-day incubation and percent survival is expressed as the ratio of colonies counted in the presence of drug per colonies counted in the absence of drug. The average ± SD of three or four experiments is shown.

MFG-DHFR- and MFG-DHFR-IRES/CD-transduced CFU-Cs showed a >50-fold increase in MTX LD_{50} as compared with mock- and MFG-CD-transduced CFU-Cs (Fig. 5B). Furthermore, the chemoprotection conferred by the bicistronic vector was found to be as effective as that conferred by the single-gene vector.

DISCUSSION

Several different types of drug-resistant genes have been used in single-gene vectors to confer resistance to anticancer agents to overcome their hematopoietic toxicity (Banerjee *et al.*, 1994a; Koc *et al.*, 1996; Rafferty *et al.*, 1996). Since drug combinations represent a commonly used treatment, it would be useful to design vectors that confer drug resistance to two different classes of antineoplastic agents. Antifolates and cytosine nucleoside analogs are used in both leukemia and solid tumor therapy. The myelosuppression produced by these agents limits the dose intensity that can be used clinically to increase their efficacy. It has been reported that bicistronic vectors can confer in target cells *in vitro* drug resistance to MDR drugs and antifolates or alkylating agents (Galipeau *et al.*, 1997; Suzuki *et al.*, 1997). To overcome the hematopoietic toxicity problem produced by antifolates and cytosine nucleoside analogs, we have designed a bicistronic vector containing the human DHFR (F31S) and CD genes.

Our objective was to demonstrate that the MFG-DHFR-IRES/CD vector produced adequate expression of both drug resistance genes in order to confer protection against cytotoxic doses of MTX or ARA-C. Enzyme assays of cells transduced with this vector showed a significant increase in both DHFR and CD activities (Table 1). Prior selection of the transduced cells with MTX or ARA-C resulted in a greater enhancement of the activity of these two enzymes. Clonogenic assays performed on two clones of transduced cells that were selected with ARA-C showed that they were both almost completely resistant to cytotoxic concentrations of either MTX or ARA-C that reduced the survival of nontransduced cells to <10% (Fig. 2). In this study, we have shown for the first time that coexpression, from a bicistronic vector, of the human DHFR (F31S) and the human CD conferred resistance to both MTX and ARA-C.

Clonogenic assays showed that selection with MTX was seemingly more effective than selection with ARA-C with respect to the level of drug resistance (Fig. 3). The transduced cells selected with MTX showed a greater relative increase in resistance to ARA-C than the increase in resistance to MTX produced by selection with ARA-C. These findings correlate with the results of enzyme assays, which showed two- to three-fold greater increase in DHFR and CD activity after MTX selection as compared with the increase in enzyme activity after ARA-C selection (Table 1). Moreover, as compared with the

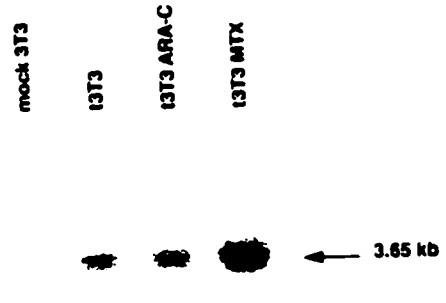

FIG. 4. Southern blot hybridization of 5 μg of genomic DNA from each indicated cell line previously digested with *Nhe*I and probed with the full-length CD DNA probe. The t3T3 ARA-C and t3T3 MTX population originate from an 8-day selection in 2.5 μ*M* ARA-C and 500 n*M* MTX, respectively. As expected, the proviral DHFR-IRES/CD fragment was revealed at the 3.65-kb level.

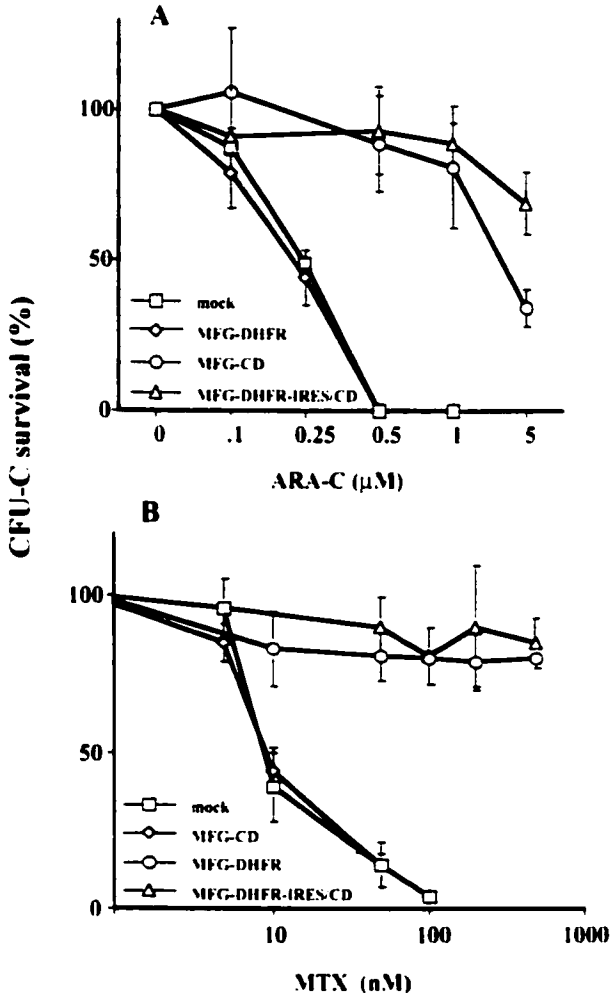

FIG. 5. Survival of transduced CFU-Cs at various concentrations of ARA-C (**A**) or MTX (**B**). Mock-, vMFG-DHFR, vMFG-CD, and vMFG-DHFR-IRES/CD-transduced murine bone marrow cells were plated in 1% methylcellulose (as described in Materials and Methods). Colonies were counted after 7 to 9 days of incubation and percent survival expressed as the ratio of colonies counted in the presence of drug per colonies counted in the absence of drug. The average ± SD of three experiments is shown.

parental 3T3 cells, there was a much greater increase in CD activity than in DHFR activity. This result suggests that it takes a higher relative increase in CD activity than in DHFR activity to obtain comparable levels of drug resistance. This could be attributed to the different mechanisms of action of these analogs and to the enhanced level of resistance produced by the mutated DHFR form. The differences in drug resistance could also be due to the different metabolism of MTX as compared with ARA-C. The reason why ARA-C was less efficient than MTX as a selection agent is not known. Our hypothesis is that the stringency of the selection used was not the same for the two drugs. The concentrations used were approximately one and five times the LD_{100} for ARA-C and MTX, respectively, in 3T3 cells. It is also suggested that the use of thymidine-free serum in the colony assays with MTX enhanced the cytotoxic action of this antifolate, since thymidine rescue could not take place.

Since the target cell for chemoprotection in cancer therapy is the hematopoietic cell, we performed gene transfer experiments with murine marrow cells. Interestingly, we found that murine bone marrow cells transduced with our bicistronic vector showed a level of drug resistance to either MTX or ARA-C similar to that of cells transduced with the single-gene vectors (Fig. 5). In a bicistronic construct, the first gene is usually expressed at a higher level (Sugimoto *et al.*, 1994, 1995). This position-dependent differential expression is even more noticeable when the size of the gene upstream of the IRES is important. The high level of drug resistance conferred on murine hematopoietic cells by our bicistronic vector indicates that we obtained efficient transduction and expression of the drug-resistance genes in these cells. It should be pointed out that in the colony assays with the murine marrow cells the drugs were present continuously, and not for a defined interval as performed in the assays with the fibroblast cells. This explains, in part, the differences in the survival curves between these different cells types (Figs. 3 and 5). Furthermore, the reason why marrow cells have a greater increase in IC_{50} to ARA-C than does the preselected population of 3T3 cells can be explained by the fact that if one compares the intrinsic resistance of mock 3T3 cells with that of the mock marrow cells, the mock 3T3 cells showed more resistance to ARA-C, even when exposed continuously to this analog (data not shown). An additional explanation is that the 3T3 cells were infected with low-titer virions as compared with the higher titer used to infect the marrow cells. This could have resulted in a greater number of proviral sequences integrated into the genome of the marrow cells as compared with the 3T3 cells and perhaps greater CD expression.

In conclusion, we have demonstrated for the first time that it is possible by gene transfer with a two-gene vector to confer chemoprotection against cytotoxic effects of MTX or ARA-C. Our results clearly demonstrate this potential by the use of bicistronic retroviral vector expressing both the DHFR (F31S) and CD genes. Furthermore, the effective IRES/CD cassette could be used in future experiments in which the DHFR gene can be replaced with other therapeutic genes.

The rationale for the use of antifolates in combination with cytosine analogs in cancer therapy is supported by the reports of a synergistic antineoplastic activity of the MTX–ARA-C combination against neoplastic cell lines (Hoovis and Chu, 1973; Edelstein *et al.*, 1975; Cadman and Eiferman, 1979; Edelstein and Valeriote, 1985). The interaction between these two agents was schedule dependent. It is the convention in cancer chemotherapy to use a combination of different drugs, each of which is active against the specific tumor, to overcome the problem of drug resistance, which develops rapidly when only a single agent is used. Current clinical protocols use MTX for the treatment of breast cancer in combination with other agents (Rivkin *et al.*, 1994). One report has shown that high-dose ARA-C shows promising activity in metastatic breast cancer (Czaykowski *et al.*, 1997). The cytosine nucleoside analog 2′,2′-difluorodeoxycytidine (Gemcitabine) also shows activity in advanced breast cancer (Possinger, 1995). We have reported that 3T3 cells containing the CD transgene showed drug resistance to both of these cytosine nucleoside analogs (Eliopoulos *et al.*, 1998). These studies suggest that advanced breast can-

cer would be an interesting target disease for a clinical protocol consisting of chemoprotection using the DHFR-IRES/CD vector and intensive therapy with antifolate drugs and cytosine nucleoside analogs. Moreover, another interesting cytosine nucleoside analog to use in combination with antifolate drugs is 5-Aza-CdR, an agent that shows interesting clinical activity in lung cancer (Momparler *et al.*, 1997) and that is scheduled to undergo a clinical trial for metastatic breast cancer.

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APPENDIX 5

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Retroviral transfer and long-term expression of human cytidine deaminase cDNA in hematopoietic cells following transplantation in mice

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The chemotherapeutic effectiveness of cytosine nucleoside analogues used in cancer therapy is limited by their dose-dependent myelosuppression. A way to overcome this problem would be to insert the drug-resistance gene, cytidine deaminase (CD), into normal hematopoietic cells. CD catalyzes the deamination and pharmacological inactivation of cytosine nucleoside analogues, such as cytosine arabinoside (Ara-C). The objective of this study was to determine if we could obtain long-term persistence and expression of proviral CD in hematopoietic cells following transplantation of CD-transduced bone marrow cells in mice. Murine hematopoietic cells were transduced with an MFG retroviral vector containing CD cDNA and transplanted into lethally irradiated mice. The recipient mice were administered three courses of 10–15 h i.v. infusions of Ara-C (75–110 mg/kg). Blood, marrow and spleen samples were obtained and analyzed for CD proviral DNA by PCR, CD activity by enzyme assay, and drug resistance to Ara-C by clonogenic assay. We detected the presence of the CD proviral DNA in most of the samples examined. Approximately 1 year after transplantation several mice showed increased expression of CD activity in these tissues and some mice displayed signs of Ara-C resistance. These data demonstrate that persistent in vivo expression of proviral CD can be achieved in transduced hematopoietic cells and indicate some potential of this gene for chemoprotection to improve the efficacy of cytosine nucleoside analogues in cancer therapy.

Keywords: *cytidine deaminase; cytosine arabinoside; gene transfer; hematopoietic cells; drug resistance; in vivo expression*

Introduction

Hematopoietic toxicity produced by intensive treatment with many anticancer drugs limits their curative potential. The transfer and expression of drug resistance genes in normal blood cells may protect them from the toxic effect of chemotherapy and permit dose escalation to increase clinical efficacy.[1]

Several investigators have inserted drug resistance genes into murine bone marrow cells to evaluate the potential of chemoprotection in mice. In these studies, the transplantation of hematopoietic cells transduced with the genes for multiple drug resistance (MDR),[2,3] dihydrofolate reductase (DHFR),[4,5] or O^6-alkylguanine-DNA alkyltransferase (alkyltransferase)[6,7] resulted in expression of these drug resistance genes and in some cases chemoprotection against the respective antineoplastic drugs. Clinical trials on chemoprotection of cancer patients with the introduction of the MDR gene into hematopoietic progenitor cells are presently under investigation.[8–11]

Our laboratory has been investigating the drug resistance gene, cytidine deaminase (CD), which codes for an enzyme that inactivates cytosine nucleoside analogues, such as cytosine arabinoside (Ara-C), 2′,2′-difluorodeoxycytidine (dFdC) and 5-aza-2′-deoxycytidine (5-AZA-CdR).[12,13] Myelosuppression constitutes a major limiting factor for the utilization of high doses of these analogues in cancer therapy.[14–17]

In previous studies, we purified, cloned and expressed the human CD cDNA.[18] We demonstrated increased resistance to Ara-C,[19,20] dFdC and 5-AZA-CdR[21,22] in murine fibroblasts following retroviral gene transfer of CD. In addition, transduction of normal murine bone marrow cells conferred *in vitro* chemoprotection to Ara-C.[20]

In the present report, we likewise used MFG-CD retroviral virions to transduce murine bone marrow cells and then transplanted these into syngeneic mice that were lethally irradiated. Following treatment with Ara-C, we detected the presence of proviral CD and increased CD activity in various hematopoietic tissues, and signs of *in vitro* drug resistance to Ara-C.

Results

Hematopoietic cells from male mice were transduced with MFG-CD or MFG-LacZ virions *ex vivo* and then transplanted into irradiated female syngeneic mice.

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Recipients were administered an Ara-C infusion at intervals of 6 or more weeks for three treatments. The peripheral blood was collected at 5 days after each Ara-C treatment. At 11 to 13 months after transplantation, the mice were killed and their blood, marrow and spleen analyzed for proviral integration and CD expression.

Detection of CD provirus in blood at different time intervals after transplantation

To establish the presence in blood of the CD transgene at different times after bone marrow transplantation, the blood was examined by PCR (Table 1). For these CD mice PCR analysis was performed on DNA isolated from blood collected 5 days after each cycle of Ara-C administered at intervals of 4–5, 6–8 and 8–10 months. The mice were killed at 11–13 months. Results showed a detectable CD provirus in at least 75% of the mice studied. The percentage of mice with CD positive blood did not diminish with time. CD proviral DNA was not detected in Lac mice (data not shown).

Detection of CD provirus in different tissues at 11–13 months after transplantation

In order to obtain evidence of long-term engraftment of transduced cells in various hematopoietic tissues, PCR reactions were performed to detect CD provirus and Y-chromosome. Figure 1 displays PCR analysis of genomic DNA from blood, bone marrow and spleen cells obtained at 11–13 months after transplantation. In seven out of nine mice, we detected the CD transgene in all three tissues. Of the other two mice, CD mouse No. 2 was positive for CD proviral DNA only in the bone marrow, whereas CD mouse No. 3 was positive in the spleen and blood. As expected, samples from Lac mice failed to yield a proviral CD signal; only Lac mouse No. 4 is included in Figure 1.

An additional PCR reaction was performed to evaluate the engraftment of male donor hematopoietic cells into the female recipients by the demonstration of the presence of the Y-chromosome. We detected this DNA fragment in all the three tissues of every CD mouse examined (Figure 1). This result further confirms that the MFG-CD positive PCR signals originated from the integrated CD gene in male donor marrow cells. Of the six Lac mice also analyzed, a positive signal for the Y-chromosome was observed in four mice (data not shown). The fact that two of the six Lac mice analyzed did not exhibit a Y-positive signal may reflect low efficiency of gene transfer, weak engraftment or shorter persistence of the MFG-LacZ transduced cells. PCR detection of the β-globin gene was also performed to verify the quality of genomic DNA isolated from each tissue. The β-globin was easily detected in the blood, bone marrow and spleen of all CD and Lac mice tested.

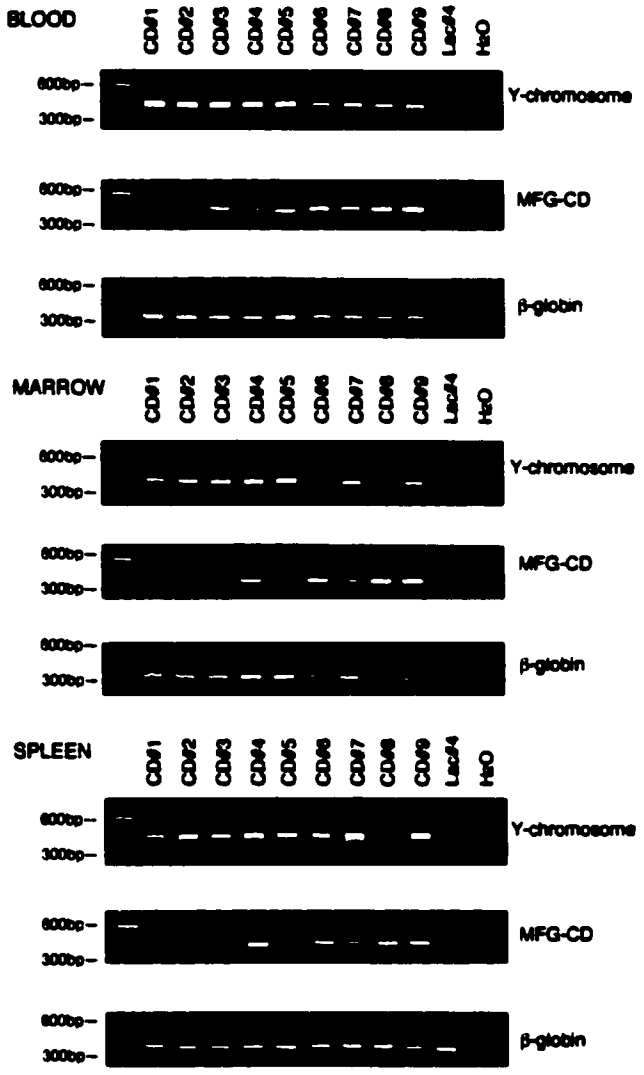

Figure 1 Detection by PCR of Y-chromosome, CD provirus, and β-globin by PCR in various tissues at 11–13 months after transplantation. PCR reactions were carried out with three different sets of primers using purified genomic DNA from blood, bone marrow and spleen cells from nine CD mice and six Lac mice at 11–13 months after transplantation. Only Lac mouse No. 4 is shown. Amplification of specific 444 bp, 421 bp and 363 bp bands indicates the presence of the donor Y-chromosome, the CD proviral DNA, and the β-globin internal standard, respectively. M, molecular size marker; H_2O, H_2O used as template.

Table 1 PCR analysis for detection of CD proviral DNA in blood cells from CD mice collected after different Ara-C treatment cycles

Ara-C treatment cycle	*CD proviral positive mice (No./total)*	*CD proviral positive mice (%)*
1[a]	3/4	75
2[a]	8/9	89
3[a]	12/15	80
3[b]	8/9	89

[a]Blood samples obtained 5 days after Ara-C treatment.
[b]Blood samples obtained 3 months after third Ara-C treatment.

Analysis of hematopoietic colonies for presence of CD provirus

Genomic DNA was prepared from a single colony or pools of two to five colonies derived from bone marrow cells harvested from mice 11–13 months after transplantation. This DNA was used for PCR analysis for detection of CD provirus, Y-chromosome and β-globin gene (Table

2). In several assays of individual colonies from CD mouse Nos 6, 8 and 9, we observed the proviral CD in 44%, 20% and 50%, respectively, of the colonies analyzed. For the pooled colonies, the positive CD signal varied from 0 to 100% for CD mouse No. 1 and CD mice Nos 4 to 7. For mouse CD No. 6, which had the highest CD enzyme activity in marrow (Figure 2), 100% of pooled colonies contained the CD proviral sequences, whereas we detected CD in 44% of the single colonies. For this mouse, 60% of individual colonies derived from spleen displayed positive PCR signals for MFG-CD (data not presented).

To determine the efficiency of engraftment of male donor cells, Y-chromosome screening was performed. For CD mouse No. 1 and CD mice Nos 4 to 7, at least 75% of pooled hematopoietic colonies scored positive by PCR for the Y-chromosome fragment. For CD mouse Nos 6 and 9, 100% and 80% of single colonies, respectively, displayed the positive Y signal. However, for CD mouse No. 8, even though we detected 20% MFG-CD positive colonies, we did not observe any Y-positive bands in the individual colonies. This occurrence was probably due to the very small amounts of DNA isolated from a single hematopoietic colony resulting in our performing PCR at the limit of detection which may have led to false negative results in some cases.

Expression of human CD enzyme activity in different tissues after transplantation

CD enzyme activity was measured in various tissues to determine if the CD provirus was capable of expression at 11 to 13 months after transplantation of transduced hematopoietic cells. As depicted in Figure 2, bone marrow cells of several CD mice exhibited increased levels of CD activity as compared with the mean value from five Lac mice. CD mouse No. 6 marrow cells displayed a striking >80-fold augmentation in enzyme activity when compared with Lac controls. Bone marrow cells from CD mouse Nos 1, 3, 4 and 8 showed a 2.9- to 5.4-fold higher CD activity than Lac mice. These differences of enzyme activity between Lac mice and CD mouse Nos 1, 3, 4, 6 and 8 were significant, $P \leq 0.05$ (Student's t test).

Table 2 PCR analysis of bone marrow-derived colonies from mice transplanted with MFG-CD transduced marrow cells

Mouse	*Colony analysis*	*% PCR positive colonies*		
		Y-ch	*CD*	*β-glob*
CD No. 6	single	100	44	89
CD No. 8	single	0	20	100
CD No. 9	single	80	50	90
CD No. 1	pooled	80	0	80
CD No. 4	pooled	100	17	100
CD No. 5	pooled	100	33	100
CD No. 6	pooled	100	100	100
CD No. 7	pooled	75	25	100

For single colony analysis, DNA isolation was performed on an individual colony, and for each mouse, five to 10 different colonies were analyzed by PCR.
For pooled colony analysis, DNA isolation was performed on a pool of two to five colonies, and for each mouse, three to six different pools were analyzed by PCR.

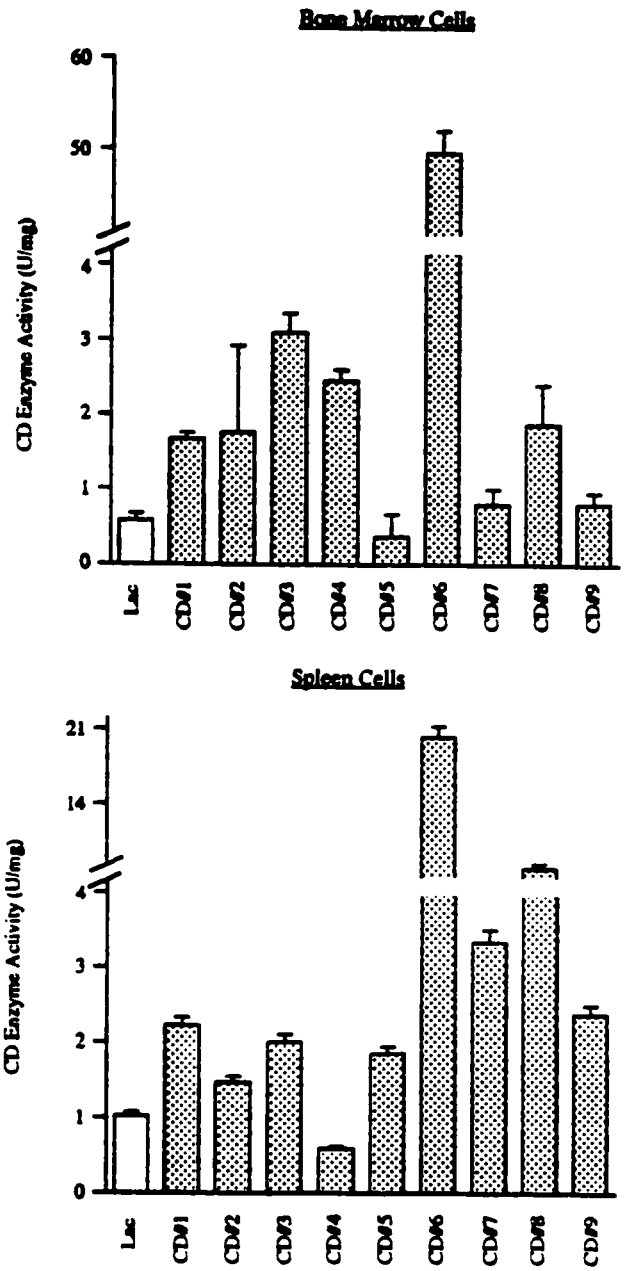

Figure 2 Expression of human CD enzyme activity in bone marrow and spleen cells at 11–13 months after transplantation. CD enzyme activity was measured in cell extracts of bone marrow and spleen cells from CD and Lac mice at 11–13 months following transplantation with transduced marrow cells. The Lac column represents the mean value from five Lac mice. For the marrow cells, $P \leq 0.05$ (Student's t test) for the differences between Lac and CD Nos 1, 3, 4, 6 and 8. For the spleen cells, $P \leq 0.05$ (Student's t test) for the differences between Lac and CD Nos 1, 6, 7 and 8. U, units (mean ± s.e.) defined as nmol cytidine deaminated per min (n = 3–6).

In Figure 2, analysis of the spleens of recipients of transduced marrow revealed that for several CD mice, the CD enzyme activity surpassed that of Lac mice. Notably, CD mouse Nos 6 and 8 showed a 20- and 8.0-fold increase, respectively, in CD activity as compared with control Lac mice. CD mouse Nos 1 and 7 showed a 2.2- and 3.3-fold elevation in CD activity, respectively, as

Table 3 Cytidine deaminase (CD) activity in blood cells

Mouse	*Blood CD activity (U/mg)*	*Relative increase*
Lac	0.4 ± 0.2	1
CD3	2.1 ± 0.3	5
CD4	4.3 ± 0.5	10
CD6	53.0 ± 5.3	130

$P \leq 0.05$ (Student's t test) for the differences between Lac and CD 3, 4 and 6.
U, Units (mean ± s.e.) defined as nmoles cytidine deaminated per min (n = 3–4).

compared to the activity in spleens from Lac mice, $P \leq 0.05$ (Student's t test).

Analysis of the blood cells showed >100-fold increase in CD activity for CD mouse No. 6 when compared with control mice (Table 3). CD mouse Nos 3 and 4 showed a five- and 10-fold rise in CD activity, respectively. Strikingly, CD mouse No. 6 displayed from 20- to 130-fold augmentation in CD enzyme activity in the different tissues analyzed when compared with the control Lac mice.

Clonogenic assay on marrow and spleen cells for Ara-C resistance

In vitro clonogenic assays were performed to evaluate drug resistance to Ara-C in bone marrow and spleen cells collected from mice 11–13 months after transplantation. At an Ara-C concentration of 0.5 μM, the clonogenic progenitor cells of several CD mice demonstrated a higher survival than those of Lac mice. A range of 2 to 6% survival in the presence of Ara-C was observed by clonogenic assays of marrow cells from CD mouse Nos 1, 3 and 4 as compared with less than 1% survival for marrow cells from Lac mice (Table 4). Notably, the marrow cells from CD mouse No. 6 (which also displayed the highest CD enzyme activity) showed 29% survival in the presence of Ara-C. All the colonies that formed in presence of Ara-C from CD mouse No. 6 showed a positive PCR signal for MFG-CD (data not shown). For colonies generated from spleen cells, 23 and 17% survival in the presence of Ara-C were noted for CD mouse Nos 4 and 6, respectively.

Table 4 Effect of Ara-C on clonogenic assays

Mouse	*% Survival in 0.5 μM Ara-C*	
	Marrow	*Spleen*
CD1	2	3
CD2	<1	ND
CD3	6	ND
CD4	2	23
CD5	<1	ND
CD6	29	17
CD7	<1	<1
CD8	ND	ND
CD9	<1	<1
Lac (mean of 6)	<1	<1

n = 3; s.e. < 10%; ND, not determined.

Discussion

The myelosuppression produced by many antineoplastic drugs has prevented the utilization of intensive doses which may improve antitumor response. For instance, the clinical effectiveness for the treatment of lung or breast cancer with cytosine nucleoside analogues, such as Ara-C[15] and the new promising agents, dFdC[16] and 5-AZA-CdR[17] is limited by their hematopoietic toxicity. The efficacy of chemotherapy with these analogues would be improved if their dose-dependent myelotoxicity could be circumvented. One way to overcome this serious side-effect would be to overexpress the gene for CD in normal hematopoietic progenitor cells to render them drug resistant.

Cytidine deaminase can deaminate cytosine nucleoside analogues and thus catalyze their conversion to pharmacologically less active uracil derivatives.[12,13] We have inserted the CD cDNA into the MFG retroviral plasmid expression vector. The cDNA for human CD contains only 438 bp[18] facilitating genetic manipulation. We succeeded in transfecting and, subsequently, transducing murine cells *in vitro*.[19,20] The transduced fibroblast cells demonstrated significantly increased expression of CD proviral RNA and enzyme activity, as well as drug resistance to Ara-C.[20] In addition, in a separate study, we observed drug resistance in these cells to other cytosine nucleoside analogues, dFdC and 5-AZA-CdR.[21,22] We also reported that clonogenic murine hematopoietic cells transduced with CD showed resistance to Ara-C.[20]

In this study, we demonstrate that CD-transduced bone marrow cells transplanted into mice can persist for more than 1 year and express CD. The persistence of the MFG-CD construct in many tissue samples after transplantation indicates the successful transduction of some pluripotent bone marrow cells capable of long-term engraftment. The detection of CD provirus by PCR in many hematopoietic colonies more than 1 year after transplantation indicates transduction and persistence of the transgene in hematopoietic progenitor cells that can survive *in vivo* and can form colonies *in vitro*.

For mouse CD No. 6, which showed the highest CD enzyme activity, 44% of individual colonies were found to be positive for MFG-CD provirus (Table 2). For this same mouse, 60% of single colonies derived from spleen displayed positive PCR signals for MFG-CD (data not presented).

The increased levels of CD enzyme activity observed in the bone marrow, spleen and blood in many mice when assayed at 11–13 months indicates long-term expression of the proviral CD gene. The low CD enzyme activity obtained in some of the tissue samples may be due to low transduction frequency and/or provirus inactivation possibly by *de novo* methylation of cytosine residues in the retroviral promoter for proviral CD.[23] Our results are in agreement with several studies utilizing other drug resistance genes that also showed long-term expression after transplantation of transduced cells into mice.[2–7]

Our investigation constitutes the first *in vivo* CD gene transfer study reported. The data presented here using the MFG-CD construct clearly demonstrate the feasibility of obtaining *in vivo* long-term persistence of CD and long-

term expression of human CD in hematopoietic cells. We plan to transplant CD transduced marrow cells into tumor-bearing mice to determine if curability with cytosine nucleoside analogues can be obtained using chemoprotection as reported by Zhao *et al*[24] for the DHFR gene and methotrexate therapy. Future improvements in vectors to increase efficiency of transduction and expression of CD in human hematopoietic cells will facilitate the use of this gene for chemoprotection to increase the therapeutic effectiveness of cytosine nucleoside analogues in cancer therapy.

Materials and methods

Cell culture of murine fibroblasts

GP+E-86 ecotropic retrovirus-packaging cells (A Bank, Columbia University, New York)[25] were grown in Dulbecco's modified essential medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent Technologies, St Bruno, Quebec, Canada) and 5 μg/ml gentamicin (Canadian Life Technologies), and incubated at 37°C with 7% CO_2.

Retroviral vector and virus-producing cell lines

The cDNA for human CD was cloned into the pMFG retroviral vector (R Mulligan, Harvard University, Cambridge, MA, USA) to yield the pMFG-CD construct, as we described previously.[19] The plasmid pMFG-CD was transfected into GP+E-86 packaging cells. Clone GP+E-86-CD3 was isolated and displayed elevated CD expression and Ara-C resistance.[19] This producer cell line had an estimated viral titer of approximately 4×10^5 infective particles/ml.[20] The pMFG-LacZ vector (R Mulligan) was similarly used to generate the clone GP+E-86-LacZ.

Donor mice and transduction of bone marrow cells

GP+E-86-CD3 and GP+E-86-LacZ cells were sublethally irradiated with 20 Gy (cobalt source) and plated at a density of 3 to 5×10^6 cells per 10 cm diameter tissue culture dish, 12 to 24 h preceding bone marrow transduction. Hematopoietic cells were harvested from the hind leg femurs and tibias of 7- to 21-week-old male C3H HeJ mice (Jackson Laboratories, Bar Harbor, ME, USA) 48 h after a single intraperitoneal injection of 150 mg/kg 5-fluorouracil (David Bull Laboratories, Vaudreuil, Quebec, Canada). Fresh nucleated marrow cells (3 to 5×10^6 cells) were layered on to each monolayer of irradiated virus-producing cells. Cocultures were incubated for 72 h in alpha minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 20% FBS, 10 mg/ml bovine serum albumin (Boehringer Mannheim, Laval, Quebec), 0.3 mg/ml iron-saturated human transferrin (Boehringer Mannheim), 5 μg/ml gentamicin (Canadian Life Technologies), 4 μg/ml polybrene (Sigma Chemical, St Louis, MO, USA), 10% conditioned medium from the WEHI-3B murine myelomonocytic cells[26] and 10% conditioned medium from the HTB-9 primary bladder carcinoma cell line.[27]

Transplantation and recipients

Following cocultivation, the nonadherent hematopoietic cells were carefully collected, concentrated by centrifugation, and 1 to 2×10^6 cells injected via the tail vein into irradiated female syngeneic recipient mice (Jackson Laboratories) exposed to 10.5 Gy irradiation 17–20 h earlier. Animals that received marrow transduced with MFG-CD were termed 'CD mice' whereas those that were transplanted with MFG-LacZ transduced cells were called 'Lac mice'. The transplanted mice were provided with 2.5 mg/ml neomycin in the drinking water starting 1 week before and until 4 weeks after transplantation.

Ara-C infusions and tissue acquisition

The mice were administered three cycles of Ara-C (Upjohn, Don Mills, Ontario, Canada) administered as a single 10 to 15 h infusion at a total dose of 75 to 110 mg/kg at intervals of 4 to 5, 6 to 8, and 8 to 10 months after transplantation. The i.v. infusions were performed as described previously.[28] Briefly, unanesthetized mice were placed in restraining cages (Scientific Products, Irvine, CA, USA) with a wood tongue blade taped to the bottom. A 25-gauge needle, attached to a butterfly and 5 cc syringe, was inserted into the lateral tail vein and immobilized to the wood blade by Transpore 3 M tape. The syringe containing sterile Ara-C in 0.45% NaCl was attached to a Harvard Model 975 infusion pump (South Natick, MA, USA) and the drug infused at a rate of 0.22 ml/h. Food pellets were placed in the restraining cage during the infusion. Five days after every treatment, peripheral blood was obtained from the tail vein. At 11 to 13 months after transplantation, nine CD mice and six Lac mice were killed to obtain the marrow and spleen. Blood was collected by cardiac puncture. These tissues were used for PCR analysis, CD enzyme activity measurements and clonogenic assays.

Clonogenic assay

Clonogenic assays were carried out using bone marrow and spleen cells. The latter cells were incubated for 7 min in 0.83% NH_4Cl to hemolyze red blood cells. The cells from the mice were plated at 10^5 cells per dish (marrow) or 5×10^5 cells per dish (spleen) in RPMI 1640 medium (Canadian Life Technologies) containing 30% WEHI-3B conditioned medium, 1 μM hydrocortisone (Sigma Chemical) and 0.36% Sea Plaque agarose (FMC BioProducts, Rockland, ME, USA). Cultures were plated in triplicate 1.5 ml aliquots in 35 mm diameter Petri dishes. Ara-C was added in graded concentrations ranging from 0 to 0.5 μM. Colonies of greater than 50 cells were enumerated after 2 to 3 weeks of culture at 37°C and 7% CO_2. Large colonies were isolated for PCR analysis.

DNA isolation and polymerase chain reaction (PCR)

Genomic DNA was prepared from blood, marrow and spleen cells as well as from individual or pooled hematopoietic colonies utilizing the IDPure Genomic DNA kit (ID Labs Biotechnology, London, Ontario, Canada). Briefly, cells were lysed with a guanidine thiocyanate solution and DNA was adsorbed on silica gel and then eluted with TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.4).

Separate PCR reactions were performed with three distinct sets of primers. To detect the MFG-CD construct, the sense oligonucleotide 5' GGT GGA CCA TCC TCT AGA CTG 3' was used with the antisense oligonucleotide 5' AGC AGC TCC TGG ACC GTC ATG 3', for the amplification of a 421 bp fragment. To verify the presence of

an internal standard, a 363 bp β-globin product was amplified using the primers 5' GAA GTT GGG TGC TTG GAG AC 3' (sense) and 5' GGA AGG TTG AGC AGA ATA GC 3' (antisense). To determine the origin of cells given that male mice served as donors for female recipients, sense primer 5' CTC CTG ATG GAC AAA CTT TAC G 3' and antisense primer 5' TGA GTG CTG ATG GGT GAC GG 3' were employed for amplification of a 444 bp Y-chromosome fragment.[29] For the PCR reaction, genomic DNA was subjected to an initial denaturation at 94°C for 2 min, followed by amplification for 40 cycles, each including denaturation at 94°C for 30 s, annealing at 57°C for 30 to 45 s, and extension at 72°C for 30 to 90 s, and a single cycle elongation step at 72°C for 5 min. The reaction mixture (25 or 50 μl) contained 1.25 units of Taq polymerase (Pharmacia, Baie d'Urfé, Quebec, Canada, or ID Proof; ID Labs). Resulting PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under UV light.

Enzyme assay

Bone marrow, spleen and blood cells were assayed for CD activity as described previously.[30] The cells (0.5 to 15 × 10^6) were washed in phosphate-buffered saline (PBS), suspended in Tris-HCl (5 mM, pH 7.4) and dithiothreiotol (5 mM), and rapidly freeze-thawed three times. The cytosolic extract was recovered in the supernatant following high speed centrifugation. For the enzyme assay, the cytosol was placed in a mixture containing 50 mM Tris-HCl and 0.5 μCi ^{3}H-cytidine (ICN Biomedicals, Irvine, CA, USA). The reaction was incubated for 30 min at 37°C and then stopped with cold HCl (0.001 N). The mix was poured on to Whatman P-81 phosphocellulose discs and the radioactivity bound assessed by scintillation counting. Under these conditions, cytosine nucleosides bind to the filter, but not the deaminated uracil nucleosides. One unit of CD activity was defined as the quantity of enzyme that catalyzes the deamination of 1 nmol of ^{3}H-cytidine per min at 37°C. Total protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

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