Expression of a Rat Glutathione-S-transferase Complementary DNA in Rat Mammary Carcinoma Cells: Impact upon Alkylator-induced Toxicity

Robyn L. Schecter, Moulay A. Alaoui-Jamali, Annie Woo, William E. Fahl, and Gerald Batist

Lady Davis Research Institute, Sir Mortimer B. Davis Jewish General Hospital and McGill University, Montreal, Quebec, Canada [R. L. S., M. A. A-J., A. W., G. B.], and McArdle Laboratory, University of Wisconsin, Madison, Wisconsin, U.S.A. (W. E. F.)

ABSTRACT

The role of glutathione-S-transferase (GST) in alkylator drug resistance has been studied in MatB rat mammary carcinoma cells. A series of GST transfected cell lines was established by using an expression vector containing the complementary DNA for the rat GST Yc gene under regulation of the SV40 early region promoter and the antibiotic resistance plasmid pSV2neo. Transfected cell lines expressing up to 4-fold higher total GST activity than in the parental wild type cell line were identified. Southern blot analysis confirmed a DNA fragment corresponding in size to the transfected GST Yc complementary DNA. Wild type MatB cells contain very low levels of Yc protein, whereas the Yc + clones showed greatly increased amounts of the Yc subunit. The effect of increased GST Yc activity on the sensitivity of the transfected clones to various cytotoxic agents was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell survival assay. The clones expressing recombiant GST Yc were more resistant to melphalan (6- to 12-fold), mechlor ethamine (10- to 16-fold), and chlorambucil (7- to 30-fold). In late passage populations of the GST Yc + clones that had been grown over a period of 14 months under continuous selection in G418, GST activity was decreased and it was paralleled by a decrease in Yc protein. These late passage clones with diminished GST Yc content also demonstrate a partial reversion toward the wild type phenotype as determined by cytotoxicity assays using melphalan, mustargen, and chlorambucil. Interstrand DNA cross-links induced by mechloethamine were significantly lower at 0, 2, and 20 h posttreatment in one of the GST Yc + clones when compared to wild type MatB cells. These studies indicate that GST Yc overexpression can confer resistance to alkylating agents and that this correlates with inhibition of DNA cross-link formation.

INTRODUCTION

In spite of the advances that have been made in the treatment of malignancies, the development of tumor cell resistance to cytotoxic drugs is a clinical phenomenon that presents a major obstacle to effective therapy. A variety of factors determine the response of tumor cells to chemotherapy. In vitro studies have demonstrated a number of mechanisms that are able to protect against the cytotoxic effects of anticancer agents. Elucidation of resistance mechanisms has relied primarily on comparative studies of sensitive and selected drug resistant cell lines.

The GSTs are a family of enzymes with a range of functions and substrate specificities. These enzymes can confer resistance to anticancer drugs through a variety of mechanisms: through conjugation of electrophilic compounds by covalent addition to the thiol-containing peptide glutathione (1); by noncovalent and covalent binding of hydrophobic compounds (2); and by detoxification of lipid and DNA hydroperoxides through an intrinsic peroxidase activity (3). A number of factors have strongly supported a direct role of GST forms in chemotherapeutic resistance, particularly for alkylating agents. Several alkylator resistant sublines have been shown to overexpress GST (Ya, Yc) activity (4-8). GST inhibitors are capable of overcoming resistance in these cell lines (9, 10). The Ya and Yc have been assigned to the same gene family, however, on the basis of their differential drug inducibility and organ distribution (1, 11) it appears likely that the expression of these two proteins is under separate control mechanisms. It has also been reported that certain members of the GST α family can be selectively induced by alkylating agents (12). There has been a limited examination of clinical specimens, but in CLL cells (13) and in specimens from ovarian and neuroblastoma cancer patients whose tumors were clinically resistant to chemotherapy there is elevated GST activity (14, 15). GSTs have been shown to catalyze the conjugation of glutathione to chlorambucil (16), cyclophosphamide (17), 1,3-bis(chloroethyl)-1-nitrosourea (18), and melphalan (19).

The critical event leading to cell killing by alkylating agents is thought to be drug-DNA interaction (20, 21). GSH may act nonenzymatically (17, 19) or through GSH-catalyzed reactions (22, 23) to compete with DNA for drug binding. GSH has been found to quench DNA monoadducts in alkylated DNA and inhibit DNA cross-link formation (24). It has also been suggested that both GSH and GST modulate other cellular processes such as DNA repair (22, 25, 26). In addition to numerous observations of alkylator resistance associated with overexpression of the α subclass of GST, involvement of an α subclass GST in the resistance phenotype was also implicated through enzyme inhibition studies. Pretreatment of chlorambucil-resistant tumor cells with the competitive GST inhibitor ethacrynic acid resulted in enhanced sensitivity to the drug in both rat and human tumor cells (21). Similarly, in chlorambucil-resistant mouse fibroblasts, inhibition of GST activity by ethacrynic acid or indomethacin significantly enhanced toxicity to chlorambucil (9). The same effect has been shown in vivo in mice carrying human tumor xenografts (27).

The introduction of isolated GST genes into lines of cultured cells has provided the opportunity to study the expression of GST and its involvement in drug resistance most directly. Transfection of α class GST into yeast cells conferred 3- to 16-fold resistance to chlorambucil (28). Low level resistance to melphalan, chlorambucil, and cisplatin was observed following transfection of the rat Ya GST cDNA into monkey COS cells (29). In the same study, reversion of transient expression in Ya + COS cell clones to a Ya − phenotype was associated with total loss of drug resistance. Transfection of the human α class GST into NIH 3T3 cells conferred a 6-fold level of resistance to 4-hydroxy-cyclophosphamide (30). However, transfection into human MCF-7 breast cancer cells of either the human α GST (31) or the rat Yc GST (32) failed to induce resistance to either melphalan, chlorambucil, or cisplatin.

We have previously reported a MLNr MatB cell line which displays several mechanisms of drug resistance. MLNr cells have increased cellular GSH levels, elevated GST activity in vitro and in vivo, where the Yc subunit is specifically elevated (8). This cell line is also cross-resistant to chlorambucil and mechlorethamine. MLNr cells have also been shown to accumulate significantly less DNA-DNA

Received 4/1/93; accepted 8/9/93.

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

1 This work was supported by a grant from the National Cancer Institute of Canada (G. B.) and by a Grant (CA-42066 from the NIH to W. F.).

2 To whom requests for reprints should be addressed, at Department of Oncology, Jewish General Hospital, 3755 Cote Ste-Catherine Road, Montreal, Quebec, Canada H3T 1E2.

3 The abbreviations used are: GST, glutathione-S-transferase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; GSH, glutathione; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; CDNB, 2-chloro-1,3-dinitrobenzene; IC50, 50% inhibiting concentration.

4900
cross-links following exposure to melphalan and chlorambucil relative to the sensitive wild type cell line (33).

To examine the importance of GST in alkylator resistance we have constructed an expression vector containing the full length coding sequence of the rat Yc subunit. Wild type MatB cells which normally express very low levels of Yc were transfected with the Yc expression vector. A number of cell lines shown to be expressing the recombinant Yc were then analyzed to determine their sensitivity to a group of alkylating chemotherapeutic agents.

**MATERIALS AND METHODS**

**Materials.** Melphalan, chlorambucil, cisplatin, and CDNB were obtained from Sigma Chemical Co. (St. Louis, MO). Adriamycin was from Adria Laboratories. Nitrogen mustard was from Merck Sharp and Dohme (Kirkland, Quebec, Canada). Reagents for biochemical analysis of GST and GSH levels were from Boehringer Mannheim (Laval, Quebec, Canada). Molecular biology reagents were obtained from BRL (Burlington, Ontario, Canada), and radioisotopes were from ICN (St. Laurent, Quebec, Canada). Blotting membranes were from Amersham (Oakville, Ontario, Canada). Immunoblotting reagents were from Bio-Rad (Mississauga, Ontario, Canada). Tissue culture reagents were from Gibco (Burlington, Ontario, Canada).

**Construction of Expression Vectors.** The rat alpha class Yc expression vector was constructed by subcloning the pGTB42 Yc cDNA (kindly provided by Dr. Cecil Pickett) into the pSM-1 expression vector. The cDNA insert is 888 base pairs long and contains 22 nucleotides of the 5'-noncoding region and a 734-base pair open reading frame. Expression of the Yc cDNA is driven by the SV40 early-region promoter. The SV40 late polyadenylation sequence from pSV2gpt is located 3' to the cDNA insertion site. The resulting plasmid (called pSVYc) was transformed into Escherichia coli HB101 and was isolated by previously described techniques (34).

**Transfection of MatB Cells.** Rat MatB cells were grown in minimal essential medium (supplemented with 1.3% sodium pyruvate, 1.3% nonessential amino acids, and 2.6% glutamine) containing 100,000 units/liter gentamycin and 10% fetal bovine serum. MatB cells (5 X 10^5/3-cm plate) were cotransfected with the pSVYc expression vector (18 μg) and pSV2neo (2 μg), using DEAE-dextran to mediate transfection (35). GST peroxidase activity was assayed by using eumene hydroperoxide as the substrate (36). Cellular GSH content was assayed for GST activity following cell lysis by hypotonic shock at 4°C. Total RNA was extracted from 0.2 X 10^6 cells by using the guanidinium isothiocyanate method according to manufacturer's suggestions (37). Total cellular protein levels were determined by the Lowry protein assay.

**Immunoblotting Analysis.** Cytosolic protein (75 μg) was subjected to electrophoresis on a 12% SDS-polyacrylamide gel and transferred overnight to nitrocellulose by electroblotting. The blots were blocked with 5% BSA/PBS and incubated overnight in 0.5% BSA containing a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. Following 3 washes, the blots were developed in PBS containing 0.5 mg diaminobenzidine/ml and 0.04% H2O2.

**Nucleic Acid Analysis.** Transfected cells were harvested and lysed in guanidinium isothiocyanate and RNA was isolated by CsCl gradient centrifugation (34). Total cellular RNA was denatured in 10 mM NaOH and applied directly onto nitrocellulose membrane according to manufacturer's suggestions (Bio-Rad). High molecular weight DNA was isolated according to standard techniques (34). For Southern analysis, genomic DNA (10 μg) was digested with XhoI and BamHI and electrophoresed on a 0.8% agarose gel, deproteinized, transferred to Hybond-N membrane, and hybridized to 32P-labeled pSVYc by the random-primer method. Hybridization was carried out in 50% formamide, 0.05 μM Na2HPO4, 1% BSA, 0.1 μm EDTA, and 5% SDS at 42°C for 18 h. The membrane was washed 3 times in 2X salt-saturated citrate, 0.1% SDS at room temperature, and 4 times in 0.1X salt-saturated citrate, 0.1% SDS at 65°C.

**Cytotoxicity Assays.** Cells were plated out in 100 μl of medium at a concentration of 2-10 X 10^3 cells/flat-bottomed well in 96-well microtiter plates. Plates were incubated for 24 h at 37°C in an atmosphere of 5% CO2. One hundred μl of media containing drug dissolved in appropriate solvent were added to triplicate wells and incubated for a further 72 h. One hundred μl of medium were then removed from the wells and 25 μl of MTT were then added to each well and incubated for 4 h. The formazan crystals were dissolved in 100 μl of isopropanol/1 N HCl (24:1). The absorbance was recorded in an enzyme-linked immunosorbent assay plate reader (Bio-Rad) at a wavelength of 570 nm. The IC50 values were determined for chlorambucil, mecloothrene, melphalan, and cisplatin. The fold resistance for MLNR cells was determined by dividing the MLNR IC50 for each drug by the IC50 value obtained for WT cells. The fold resistance of the transfected cell lines was calculated by dividing the IC50 values obtained for pSV2neo-transfected cells.

**DNA-DNA Cross-Link Studies.** DNA interstrand-cross-links were quantified by the alkaline elution assay. Exponentially growing cells were labeled with either 0.05 μCi/ml [14C]thymidine or 0.5 μCi/ml [3H]thymidine at a final concentration of 10^-6 M for two cell doublings. The labeled cells were washed twice with cold PBS and the radioactivity was chased by an addition of 16-h incubation in medium containing 10^-3 M cold thymidine. The 14C-labeled cells were treated in serum-free medium for 30 min, and treatment was then stopped by immediate chilling of cells on ice. Aliquots of cells were used immediately (0 time) or further incubated in drug-free medium for the accumulation and removal of DNA cross-links. Control or drug-treated 14C-labeled cells (0.5 X 10^6) were then mixed with H^-labeled cells (0.5 X 10^6) and irradiated with 6 Gy by using 60Co γ-rays at a dose rate of approximately 1 Gy/min, at 4°C. The combined cell suspension was immediately prepared for alkaline elution as described previously (39). Briefly, 1 X 10^6 combined H^- and 14C-labeled cells were layered on a polyvinyl chloride filter, washed with cold PBS, and lysed with 5 ml lysis solution (2% SDS, 0.025 μm EDTA), with or without 0.5 mg/ml proteinase K (pH 9.7) for 45 min. Filters were then washed twice with 2 ml 0.02 μm EDTA, pH 10.3, and the DNA was eluted with 30 ml tetrapropylammonium hydroxide-EDTA buffer (pH 12.1) containing 0.5% SDS solution, at a flow rate of approximately 0.05 ml/min, in the dark at room temperature. The 14C and 3H activities on filters and fractions were analyzed by liquid scintillation counting and DNA-DNA cross-link frequency, expressed as DNA-DNA cross-link index, was estimated as:

\[
\text{IC50 values for pSV2neo-transfected cells.}
\]

\[
\left(1 - R_0 \right)^2 - 1,
\]

where \(R_0\) and \(R\) are the final fractions of [14C] and [3H]DNA retained on the filter, respectively.

**Transport Studies.** Exponentially growing cells (2 X 10^6 cells/ml) were incubated with [chloroethyl-14C]melphalan for different periods of time at 37°C. At the end of each incubation time, 400 μl of the incubation mixture were layered onto 1 ml of Vestaflow F-50 silicone oil in microfuge tubes and centrifuged at 12,000 X g for 1 min at room temperature. The radioactivity in the medium and the cell pellet was determined as previously described (40). Nonspecific absorption of labeled drug was determined by layering 200 μl of untreated cells onto 200 μl of medium containing labeled melphalan at 4°C.
followed by immediate centrifugation as described above. Extracellular melphalan trapped in the cell pellet through the oil layer was estimated by using [carboxyl-14C]inulin as a marker for extracellular fluid.

RESULTS

Expression of GST in MLNr MatB Cells. Selection of WT cells in 10 μM melphalan (MLNr) results in overexpression of GST Yc subunit mRNA. To determine whether enhanced transcription of the GST Yc gene is responsible for the observed increase in Yc mRNA, nuclear run-on experiments were performed (Fig. 1). Drug-sensitive (WT) cells showed very low levels of initiated transcription complexes of the Yc gene, whereas MLNr cells demonstrated a much higher rate of transcription (Fig. 1A).

We have also isolated a MLNr variant subline (MLNr+) which has been grown in 10 μM melphalan for over a period of 18 months. These cells are shown to have markedly higher levels of Yc mRNA relative to MLNr cells which were isolated far earlier in the protocol which selected for resistance to melphalan (Fig. 2A). MLNr cells growing in 10 μM melphalan are 96-fold resistant to this drug based on MTT assays (Table 2), while MLNr+ cells are more resistant (183-fold) to melphalan.

Expression of Yc cDNA. The pSVYc expression vector was co-transfected with pSV2neo into rat MatB mammary carcinoma cells and selected in the presence of the neomycin analogue antibiotic G418 (5.2 units/ml). A total of 100 clones were isolated. Two clones designated R49 and M49-8 were found to have elevated GST activity. Integration of the recombinant GST Yc expression gene in the R49 and M49-8 clones was confirmed by Southern blot analysis (Fig. 3). A single band of approximately 700 base pairs was detected in the GST-transfected R49 and M49-8 cell lines but not in the WT or control pSV2neo-transfected MatB cells. This band corresponds to the expected restriction fragment size of the Yc cDNA in the pSVYc expression vector. The several other hybridizing fragments in all cell lines examined represent DNA sequences corresponding to the endogenous Yc gene.

Whole cell extracts were prepared from the various cell lines to quantitate the level of the Yc cDNA gene product. Drug-sensitive cells (Fig. 4, Lane 2) express very low levels of Yc protein. Selection in 10 μM melphalan resulted in a sharp increase in the level of this subunit (MLNr, Lane 4). Transfection of the Yc cDNA yielded 2 clones, R49 and M49-8, with significantly elevated levels of Yc protein. We observed variable stability of expression of the transfected Yc gene. Clone R49 demonstrated a significant decline in Yc protein expression.
over 18 months (early versus late), (despite the continued presence of G418 in the growth media) to a level just above that seen in the neo cells, whereas clone M49-8 does not show as appreciable a loss in expression over time (early versus late).

Biochemical Analysis of GST and Cytotoxicity. Enzyme assays were carried out to determine GST activity levels in the isolated clones. Total GST enzyme activity was studied by using the universal substrate CDNB. A range of activity levels was observed for the various clones isolated. Table 1 summarizes the data for clones R49 and M49-8 that consistently expressed higher GST enzyme activity (approximately 4-fold); MLNr cells are included for comparison. In late passage cells, the GST activity levels correspond with Western analysis of the Yc subunit. Selenium-independent organic peroxidase activity has been shown to be associated with dimerized Yc/Yc enzyme (3). Cumene hydroperoxide was used to measure enzyme activity associated with Yc expression. GSH-peroxidase activity correlates well with the increase in total GST activity which presumably results from expression of the transfected pSV2Yc plasmid. Although GST has been shown to enhance the conjugation of drugs such as chlorambucil and melphalan with GSH, spontaneous conjugation of these drugs with GSH may be a mechanism of resistance in cell lines (3). Cytotoxicity assays were carried out by using an MTT assay on MatB clones expressing recombinant GST Yc to assess their sensitivity to alkylating agents versus control cells (Table 2). Cells expressing increased GST Yc, either encoded by the endogenous gene (MLNr) or by the expression gene (R49, M49-8), showed significant resistance to the alkylating drugs examined here. Importantly, the degree of resistance was significantly reduced in revertant cell populations (R49L, M49-8L), which had significantly reduced levels of the recombinant GST Yc protein (Table 2).

For all of the drugs examined, the IC50 values were not significantly different between WT cells and WT cells transfected with pSV2neo (Table 2). These results indicate that transfection of the marker plasmid pSV2neo does not contribute to the drug sensitivity, even though this results in overproduction of the Yc subunit (Fig. 4). The level of resistance for the Yc-expressing clones ranged from 11.7- to 30.8-fold for mechiorethamine and 6.6- to 12.8-fold for melphalan, and 1.2- to 4.4-fold for cisplatin. The M49-8 clone, which expresses the highest GST activity (Table 1), was consistently more resistant to each of the drugs tested. The sensitivity of clones R49 and M49-8 were tested after an extended length of time in tissue culture (18 months). Clone R49L, which demonstrates a significant reduction in Yc expression and activity is not as dramatic, the reduction in the fold resistance is likewise smaller than that seen with the R49/R49L pair. These paired early and late passage samplings of GST were carried out to determine GST activity levels in the isolated clones. Ten µg of genomic DNA were digested with BamHI and XhoI, electrophoresed, blotted, and probed with a GST Yc cDNA fragment. kb, kilobases.

Table 1 Intracellular levels of GSH, GST, and glutathione-peroxidase activity in MatB cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GST activity (nmol/min/mg protein)</th>
<th>GSH peroxidase activity (nmol/min/mg protein)</th>
<th>GSH (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.8 ± 1.0*</td>
<td>4.7 ± 0.9</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>MLNr</td>
<td>37 ± 1.1</td>
<td>18.2 ± 1.2</td>
<td>2.12 ± 0.98</td>
</tr>
<tr>
<td>WT-neo</td>
<td>10.3 ± 1.2</td>
<td>5.7 ± 1.0</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>R49</td>
<td>28.6 ± 1.1</td>
<td>9.5 ± 0.1</td>
<td>1.16 ± 0.33</td>
</tr>
<tr>
<td>R49L</td>
<td>13.6 ± 0.5</td>
<td>7.3 ± 0.2</td>
<td>1.16 ± 0.28</td>
</tr>
<tr>
<td>M49-8</td>
<td>30.1 ± 0.9</td>
<td>12.2 ± 0.9</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>M49-8L</td>
<td>20.7 ± 1.0</td>
<td>9.5 ± 0.8</td>
<td>1.28 ± 0.28</td>
</tr>
</tbody>
</table>

* Mean ± SE.
Yc+ clones, which demonstrate a change from high GST Yc activity to low GST Yc activity and a concomitant change to greater drug sensitivity, argue against the likelihood that clonal variation explains the drug resistance results in these MatB clones expressing recombinant GST Yc.

Because melphalan is actively transported into cells by two separate amino acid transport systems, the sodium-dependent alanine-serine-cysteine-like system and the sodium-independent leucine-preferring system (41), drug transport studies were carried out to determine whether alterations in the membrane of Yc-expressing clones could account for their resistance to melphalan. The uptake of [14C]melphalan by MatB cells is shown in Fig. 5. These data show that between the two GST Yc-expressing clones and the control cells there is no significant difference in melphalan accumulation which might account for their resistance to melphalan. The uptake of [14C]melphalan in the most resistant GST Yc+ clone to see if they would be reduced because of GST Yc-catalyzed conjugation of mechlorethamine in the most resistant GST Yc+ clone to see if they would be reduced because of GST Yc-catalyzed conjugation of mechlorethamine.

DNA-DNA Cross-link Studies. The cytotoxicity and antitumor activity of bifunctional nitrogen mustards is related to their ability to undergo bifunctional addition reactions with DNA producing interstrand and intrastrand cross-links as well as DNA-protein cross-links (42, 43). DNA crosslinking, especially of the interstrand type, has been shown to correlate with cytotoxicity to nitrogen mustard derivatives (42, 43). The alkaline elution technique allows for the measurement of both interstrand cross-links and DNA-protein cross-links. DNA cross-link formation was estimated based on diminished X-ray sensitivity of cellular DNA. Single strand DNA breaks induced by irradiation produces enhanced elution. In cells that have been treated with a cross-linking agent the effect of X-irradiation is reduced and elution is retarded. Since DNA is a target for alkylating drugs, we quantitated the presence of DNA cross-links induced by mechlorethamine. Control WT-neo cells and clone M49-8L cells were exposed to 30 μM mechlorethamine for 30 min and DNA cross-linking was measured at various times following this treatment. Fig. 6A illustrates the results of this experiment. Immediately following treatment, the accumulated interstrand cross-links in M49-8L cells were significantly less than in control cells. At 2 and 20 h posttreatment, DNA cross-links measured were uniformly less in M49-8L than in WT-neo cells. When proteinase K was omitted from the lysing solution (see "Materials and Methods." Each toxicity study was performed at least 7 times in triplicate. To establish fold resistance values (shown in parentheses), the IC₅₀ value for MLNr cells was divided by the IC₅₀ value of WT cells, and the IC₅₀ values of transfectants were divided by the IC₅₀ value of the control cell line WT-neo. IC₅₀ values are expressed in μM.

<table>
<thead>
<tr>
<th>Drug</th>
<th>WT</th>
<th>MLNr</th>
<th>WT-neo</th>
<th>R49</th>
<th>R49L</th>
<th>M49-8</th>
<th>M49-8L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>0.73 ± 0.08</td>
<td>48.5 ± 5.6* (68)</td>
<td>0.79 ± 0.09</td>
<td>6.5 ± 2.0* (8.2)</td>
<td>3.7 ± 1.1* (4.7)</td>
<td>24.3 ± 7.7* (30.8)</td>
<td>18.2 ± 5.6* (23)</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>0.01 ± 0.01</td>
<td>3.5 ± 0.47* (87)</td>
<td>0.06 ± 0.01</td>
<td>0.6 ± 0.18* (10)</td>
<td>0.08 ± 0.03 (1.3)</td>
<td>0.96 ± 0.21* (16)</td>
<td>0.34 ± 0.11* (5.6)</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.26 ± 0.03</td>
<td>24.9 ± 2.2* (96)</td>
<td>0.29 ± 0.02</td>
<td>1.9 ± 0.36* (6.6)</td>
<td>0.49 ± 1.0 (1.7)</td>
<td>3.7 ± 1.1* (12.8)</td>
<td>0.52 ± 0.18 (1.8)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.26 ± 0.04</td>
<td>40 ± 9.1* (155)</td>
<td>0.25 ± 0.04</td>
<td>0.3 ± 0.04 (1.2)</td>
<td>ND*</td>
<td>1.1 ± 3.2* (4.4)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a p < 0.001.  
*b p < 0.01.  
*c p < 0.05.  
*d ND, not determined.
terials and Methods”), the amount of DNA-protein cross-links was similar between control WT-neo cells and clone M49-8 cells (data not shown).

Because extended passaging of M49-8 cells resulted in a partial revertant line with a decreased Yc level and an increased sensitivity to alkylating agents (M49-8L), alkaline elution analysis was carried out to determine whether the loss of drug resistance in M49-8L cells was associated with a diminished ability to prevent mechlorethamine-DNA cross-links (Fig. 6B). In M49-8L cells, where GST activity is significantly decreased from the early passage M49-8 cells, there is no longer a significant difference in the level of cross-links between the M49-8L and WT-neo control cells.

**DISCUSSION**

To determine the role of GST-catalyzed detoxification in diminishing DNA cross-link formation by bifunctional nitrogen mustards, we previously used a competitive inhibitor of GSTs to treat MLNnr MatB cells (33). Ethacrynic acid exposure results in an increase in DNA cross-link formation in cells treated with nitrogen mustard; this suggested that GST was playing a role in inhibiting the formation of these cross-links rather than contributing to repair of damage. In this study we examined this directly by transfecting a GST-Yc cDNA into drug-sensitive cells. We have demonstrated that stable expression of a rat Yc cDNA yields functional enzyme in MatB mammary carcinoma cells, and that these Yc-expressing cells are resistant to a variety of bifunctional alkylating agents by virtue of decreased DNA cross-link formation. Resistance to the alkylating drugs correlated with the amount of Yc protein present in the cells, both between clones as well as within a single clone. Clone M49-8, which expresses the highest level of resistance to these agents, demonstrates a higher level of GST activity than does clone R49. Partial reversion of Yc expression in both clones, although to different degrees, is mirrored by increases in the respective sensitivities of the cells to all the drugs tested. These two clones demonstrated variability in retention of the transfected gene, perhaps related to the site of integration of the expression cassette within the genome and in relation to the neomycin resistance gene. The number of integrated Yc cDNAs in the transfected cells ranges between 40 and 50 copies. The technique used for gene transfer was designed to encourage stable transfection of the Yc cDNA. Cells that replicate episomal DNA without selection will usually lose the episome over a 4-week period (29, 44). We have not observed such a phenomenon; it is therefore likely that the cDNA transferred has become incorporated into the host cell genome.

Although there is a relationship in our experiments between the concentration of the Yc subunit and the level of sensitivity to alkylating agents, for Yc to confer resistance to alkylating agents, a threshold overexpression of Yc was necessary. This was observed in an earlier published report (45), where Yc transfectants with lower levels of Yc than those achieved here were not resistant. A novel observation here is that selection of WT MatB cells transfected with pSV2neo alone in G418 results in overexpression of the endogenous Yc gene, however, to a level below that observed in Yc transfected cells that are resistant to alkylating drugs. This observation also supports the hypothesis that a minimum level of Yc protein in tumor cells is necessary to confer resistance. Selection of the parental WT MatB cell line in G418 results in equivalent overexpression of the endogenous Yc gene as that observed in pSV2neo-transfected/G418-selected cells. It therefore appears that G418 or one of its metabolites induces expression of the endogenous Yc gene.

There is significant published evidence which correlates bifunctional alkylator cytotoxicity to the formation of DNA-DNA interstrand cross-links (43, 46). Using an alkaline elution assay, we have demonstrated that in Yc-overexpressing clone M49-8, the initial cross-links which accumulated following mechlorethamine treatment are significantly less than that seen in WT-neo control cells. Furthermore, in late passage cells of the M49-8 clone which partially revert to a more sensitive phenotype, DNA cross-link formation approaches the pattern observed in WT-neo control cells.

GST Yc-conferring resistance in MatB cells is highly specific for alkylating agents. The Yc+ transfectants in the present study were not significantly resistant to Adriamycin or radiation, but rather only to the alkylating drugs shown in Table 2. This confirms and extends a previous study demonstrating that the Yc peroxidase activity has very specific and defined substrates that result from oxygen radical reactions. T47D cells transfected with Yc cDNA were resistant to cumene hydroperoxide and singlet oxygen but not to free radical-generating Adriamycin (47). According to our data, H2O2 produced by ionizing radiation is not reduced by peroxidase-mediated GST-Yc.

Transfection of a Ya cDNA into COS and 10T1/2 cells (29) conferred resistance to chlorambucil and melphanal (1.3- to 2.9-fold). These cells do not express highly constitutive levels of the Ya subunit. Similar studies using the identical Ya-expressing vector in MatB cells failed to produce any resistance to alkylating agents. Transfection of the Ya gene into MatB cells did not result in a marked elevation in Ya subunit protein. The discrepancy in results between the different cell lines may be explained by this observation and may additionally indicate a difference between the Ya and Yc subunits in terms of their conjugating affinity toward alkylating agents.

Given the fact that GSTs are enzymes requiring cosubstrates and sufficient enzyme properly localized, it is not surprising that some cells may not be effective transfection targets. The unusually low basal GST activity in MCF-7 cells may explain the consistently negative results. The lack of resistance to nitrogen mustards in a/Yc transfected MCF-7 cells may be due to the target cell examined in combination with the gene transferred. These cells may require accessory proteins which act in conjunction with Yc to exert their protective role.

The results reported here corroborate the findings of earlier reports which hypothesized an association between nitrogen mustard resistance *in vitro* and *in vivo* to the increased expression of an α class GST. We have shown that the Yc GST is specifically overexpressed in cells chronically exposed to melphanal. If enough of this protein is present in transfected cells, the cells will be protected from the cytotoxic effects of drugs in this class. This represents important additional evidence that despite the high degree of sequence homology between the Ya and Yc subunits (75%), both the regulation and substrate specificity are significantly different. The regulatory region of the Ya gene has been studied in some detail (48, 49). Regulation of the Yc gene has not been determined.

Modulating the catalytic efficiency of GST Yc by identifying specific inhibitors or protecting sensitive tissues such as the bone marrow by introducing a recombinant Yc expression gene into them are two ways in which treatment with these highly toxic compounds may improve.

We are currently isolating and analyzing the specific Yc that is overexpressed in MLNnr MatB cells. This will be compared to the rat liver Yc used in these experiments in order to determine whether there are tissue and inducer-specific forms of this GST.

**REFERENCES**


3. Jakoby, W. B. The glutathione-S-transferases: a group of multifunctional detoxifica...


Expression of a Rat Glutathione-S-transferase Complementary DNA in Rat Mammary Carcinoma Cells: Impact upon Alkylator-induced Toxicity

Robyn L. Schecter, Moulay A. Alaoui-Jamali, Annie Woo, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/20/4900

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.