Increased Expression of Cytosolic Glutathione S-Transferases in Drug-resistant L5178Y Murine Lymphoblasts: Chemical Selectivity and Molecular Mechanisms

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ABSTRACT

The level of induction of three cytosolic glutathione S-transferase (GST) classes has been compared in L5178Y murine lymphoblasts resistant to either the quinone-containing compound, hydrolyzed benzoquinone mustard (HBM), or the aromatic alkylating agent aniline mustard (AM). Three established cell lines, L5178Y/HBM2, L5178Y/HBM10, and the partial revertant, L5178Y/HBMR, were 2.5-, 6-, and 2.9-fold resistant to HBM and showed 3-, 11-, and 9-fold increases in GST activity, respectively, relative to the sensitive L5178Y cell line. Western blot analysis of cytosolic proteins showed overexpression of all three cytosolic GST classes, \( \pi \), \( \alpha \), and \( \mu \), with predominance of the \( \pi \) class. Northern blot analysis demonstrated corresponding elevations in the steady-state mRNA levels of each GST class. The level of GST-\( \mu \) and -\( \alpha \) isoforms correlated more closely with HBM resistance, whereas GST-\( \pi \), the predominant isoform in these cells, paralleled enzyme activity. These findings suggested that other factors such as quinone reductase may contribute to resistance.

The AM-resistant cell line L5178Y/AM was 10-fold resistant to the alkylating agent AM, and GST activity was elevated 3.6-fold relative to the parental L5178Y cell line. Western blot analysis and Northern blot analysis provided evidence of overexpression of all three cytosolic GST classes but with marked predominance of the \( \alpha \) class. These studies provide evidence that induction of GST isoforms in drug-resistant cells may have both a nonspecific as well as a selective component. The difference in isozyme profile between HBM- and AM-resistant cell lines emphasizes how structural differences, in particular, the nature of the electrophilic signal, may influence the pattern of induction of GST isoforms.

INTRODUCTION

GSTs\(^{3}\) represent a multigene family of enzymes that catalyze the conjugation of glutathione to a broad range of electrophilic xenobiotics and carcinogens (1–3). By conjugating glutathione to various xenobiotics, GSTs appear to play a role whereby cells develop resistance to antineoplastic agents (1). Five distinct GST classes or gene families have been identified (1). The cytosolic GSTs are abundant and are classified on the basis of isoelectric point as basic (\( \alpha \) class), neutral (\( \mu \) class), and acidic (\( \pi \) class); the other two GST classes are microsomal and the recently described \( \theta \) class (4).

Two major experimental approaches have been followed to establish the role of GSTs in resistance to antineoplastic agents. The first has involved correlative studies in which elevated levels of expression and activity of GSTs have been associated with increased levels of drug resistance (1–3). This approach has also included modulation of enzyme activity by inhibitors of GST such as ethacrynic acid in order to circumvent or reverse drug resistance (5). The second approach has used transfection studies to provide direct functional evidence that GSTs cause drug resistance.

Both approaches, including the more definitive transfection studies, have provided conflicting evidence. Transfection of human GST-\( \pi \) was reported to increase resistance of Chinese hamster ovary cells (6) but not that of NIH-3T3 transfected (7) to the alkylator cisplatinum. More consistently, GST-\( \pi \) has been associated with resistance to Adriamycin (7–9) and GST-\( \alpha \) with resistance to alkylating agents (3) not only in correlative but also in transfection studies. Puchalski and Fahl (10) reported that GST-\( \alpha \) conferred resistance to chlorambucil and melphalan, whereas GST-\( \mu \) and -\( \pi \) conferred resistance to cisplatinum and Adriamycin, respectively, in stably transfected mouse C3H cells and transiently transfected COS cells. Transfection studies also supported a causal role for GST-\( \alpha \) in resistance to bleomycin in Chinese hamster ovary cells (11), and for GST-\( \alpha \) and -\( \pi \) in resistance of the yeast Saccharomyces cerevisiae to chlorambucil and Adriamycin, respectively (12). Conversely, several attempts to transfect MCF-7 human breast cancer cells with either class \( \pi \), \( \alpha \), or \( \mu \) GST have resulted in overexpression of the gene product and, occasionally, resistance to ethacrynic acid, benzpyrene, or benzpyrene epoxide but without resistance to antineoplastic agents (13–16).

This study was undertaken to investigate the chemical specificity of induction of GST isoforms in drug-resistant L5178Y murine lymphoblasts. Two related compounds were used with structural differences conferring distinct mechanisms of cytoidal action. The chloroethyli groups in AM make it an active alkylating agent, whereas in HBM, these groups are hydrolyzed and cytotoxicity is due to the chemically reactive quinone moiety. A second objective was to elucidate the molecular mechanism whereby up-regulation of GSTs is achieved in resistant cells.

MATERIALS AND METHODS

Chemicals and Molecular Reagents. Hydrolyzed benzoquinone mustard [di(2-hydroxyethyl)amino-1,4-benzoquinone] and aniline mustard [N,N-di(2-chloroethyl) aniline] were prepared as previously described (17). Polyclonal antisera for the combined rat liver Ya (\( \alpha \) class), Yc (\( \alpha \) class), and Yb (\( \mu \) class) GST isoforms was provided by Dr. Kenneth Tew (18). Polyclonal antisera for mouse GST F3 (\( \mu \) class) was obtained from Dr. C-Y. Lee (19), and that for human GST-\( \pi \) was from Dr. W. D. Henner, as described previously (9). Plasmids pGTB38 and pGTA/C4X containing cDNA for rat liver GST Ya (\( \alpha \) class) and Yb (\( \mu \) class) GST isoforms was provided by Dr. Kenneth Tew (18). Plasmid pDTD55 containing the quinone reductase cDNA were provided by Dr. Cecil B. Pickett (20, 21). Plasmid PTSS1–2 containing nucleotides 106–438 of the cDNA for human GST-\( \pi \) was obtained from Dr. W. D. Henner as reported previously (9). The cDNA probes for GST are class specific, discriminating between major GST classes but not between different isoforms within the same class. The plasmid pCHPI, with a 640-base pair cDNA for the plasma membrane protein P-glycoprotein, was from Dr. J. R. Riordan (22). [\( ^{32} \text{P} \)]dCTP used to label cDNA probes was from ICN Biochemicals and the labeling kit was from Pharmacia, Uppsala, Sweden.

Cell Lines and Cultures. The parental cell line L5178Y and HBM-resistant lines L5178Y/HBM2 and L5178Y/HBM10 and the partial revertant L5178Y/HBMR have been described previously (23). Resistant cells were obtained by growing L5178Y cells in suspension in Fischer’s medium containing 12% horse serum and HBM. L5178Y/HBM2 and L5178Y/HBM10 cell
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RESULTS

Western Blot Analysis of GST Gene Products in HBM-sensitive and HBM-resistant L5178Y Cell Lines. In a previous publication, dose-survival studies showed that L5178Y/HBMB2, L5178Y/HBM10, and L5178Y/HBM10R cells were 2.5-, 6-, and 2.9-fold more resistant, respectively, to HBM relative to sensitive L5178Y cells (23). Western blot analysis with polyclonal anti-serum against subunits of each of the 3 major cytosolic GST classes revealed protein bands ranging in size from M, 25,000 to M, 28,000 with a relative size distribution of π < α < β (Fig. 1), consistent with previous reports (27, 28). The relative levels of GST-π, -α, and -β isozymes in HBM-sensitive and -resistant L5178Y cell lines were estimated by densitometry. GST-π was increased in L5178Y/HBMB2, L5178Y/HBM10, and L5178Y/HBM10R cells approximately 2.2-, 6.4-, and 5.1-fold, respectively, relative to sensitive cells (Fig. 1a), whereas GST-μ was increased 1.6-, 3.3-, and 1.1-fold, respectively (Fig. 1b). The GST-α isozyme was detectable only in L5178Y/HBM10 cells (Fig. 1c); the absence of a detectable band for GST-α in L5178Y/HBM10 cells precluded a direct comparison of signal intensity. However, Western blot of a serial dilution of protein extract from L5178Y/HBM10 cells suggested an approximate 4-fold elevation in expression of GST-α above background in those cells (data not shown). In the partial revertant L5178Y/HBMI cells, the level of GST-μ and -α decreased to that observed in sensitive or resistant L5178Y/HBM2 cells (Fig. 1, b and c, respectively), whereas GST-π remained elevated at levels close to that of resistant HBM10 cells (Fig. 1a).

Western Blot Analysis of GST Gene Products in AM-sensitive and AM-resistant L5178Y Cell Lines. Elevated levels of all 3 GST isozymes were observed in AM-resistant cells (Fig. 2). Densitometric analysis revealed a 3-and 3.4-fold increase in expression of GST-π and -μ, respectively, relative to sensitive cells (Fig. 2a and b). The α gene product was not detected in extracts of sensitive cells (Fig. 2c, Lane 1) so the absence of a reference signal precluded a direct comparison of isozyme levels on this blot. Serial dilution of the cytosolic protein extract of AM-resistant cells was analyzed by Western blot and showed that the level of up-regulation of GST-α was approximately 12-fold in these cells (Fig. 3a). The level of GST-π and -μ isozymes in AM-resistant cells was roughly comparable to that found in L5178Y/HBM10 cells, whereas the amount of GST-α was much greater.

Fig. 1. Western blot analysis of cytosolic protein from HBM-sensitive and -resistant L5178Y cell lines; 200 μg of protein from each of the following cells were loaded per lane: Lane 1, sensitive L5178Y cells; Lane 2, resistant L5178Y/HBM2 cells; Lane 3, resistant L5178Y/HBM10 cells; and Lane 4, revertant L5178Y/HBMBR cells. Blots were probed with polyclonal antisera against the following GST antigens: (a) human GST-π (κ class); (b) mouse GST F3 (μ class); and (c) rat liver GST Ya (α class), Yb (β class), and Yc (γ class). Molecular weight standards were rabbit muscle phosphorylase B (M, 97,000), bovine serum albumin (M, 66,000), hen egg white ovalbumin (M, 45,000), bovine carbonic anhydrase (M, 31,000), and soybean trypsin inhibitor (M, 21,000). The gels in a and b were run simultaneously and for a shorter time than the gel shown in c. The assignment of GST isozymes was based on molecular weight size and comigration with rat liver GST standards.

Fig. 2. Autoradiograph and Densitometry. Autoradiograms of hybridized membranes were obtained using Kodak XAR-5 film exposed at −70°C with a DuPont Cronex Lightning-Plus intensifying screen. Levels of protein or mRNA were quantitated using a Bio-Rad model 620 video densitometer.
Northern Blot Analysis of GST Expression in HBM-sensitive and HBM-resistant L5178Y Cell Lines. Northern blot analysis of total RNA from HBM-resistant cells detected bands of approximately 1.4, 1.2, and 1.0 kilobase pairs, corresponding to GST-6, -a, and -IT mRNA, respectively. The level of mRNA for each of the 3 GST classes was elevated in HBM-resistant cells (Fig. 4). Densitometric analysis showed that the level of mRNA for GST-IT in L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBM10 cells was elevated 1.3-, 8.4- and 5.5-fold, respectively, relative to sensitive cells (Fig. 4a). A similar comparison for the level of mRNA for GST-IT showed a 2.5-, 5.7-, and 2.1-fold elevation in L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBM10 cells, respectively (Fig. 4b). The level of mRNA for GST-6 in L5178Y/HBM10 cells was clearly elevated in AM-resistant cells relative to sensitive L5178Y cells (Fig. 5). Densitometric analysis demonstrated that the level of message for GST-6 and a was increased 3.4- and 7.9-fold, respectively, relative to sensitive cells (Fig. 5, a and b). Serial dilutions of total RNA from AM-resistant cells was analyzed by Northern blot, and this demonstrated a greater than 64-fold elevation of mRNA for GST a over background (Fig. 3b).

Northern Blot Analysis of P-Glycoprotein and Quinone Reductase Expression in Sensitive and HBM- or AM-resistant L5178Y Cell Lines. Resistance to HBM was previously shown to be multifactorial with decreased drug uptake, markedly increased levels of quinone reductase, as well as elevated levels of GST and other enzymes (23). To determine if elevated levels of P-glycoprotein might contribute to reduced uptake of HBM, the level of expression of that drug extrusion pump was evaluated by Northern blot analysis. No signal was detected for mRNA for the plasma membrane protein, P-glycoprotein, on Northern blot analysis of total RNA from sensitive or resistant L5178Y cell lines (data not shown). This suggested that decreased uptake of HBM in resistant cells was unlikely due to increased drug efflux.

To investigate the molecular mechanism of the elevated activity of quinone reductase previously reported, the level of mRNA for that enzyme was determined in HBM- and AM-sensitive and -resistant L5178Y lymphoblasts. Although mRNA for quinone reductase was not detected in drug-sensitive L5178Y cells, distinct bands were observed on Northern blot analysis of RNA from resistant L5178Y/HBM10 and L5178Y/AM cells (Fig. 6). Thus, level of expression of quinone reductase, as well as that of GST isoforms, was increased in these drug-resistant cell lines.

DISCUSSION

It was previously shown that resistance to the quinone-containing compound HBM was multifactorial, with resistant cells displaying decreased drug uptake and increased activity of several enzymes including DT-diaphorase, catalase, as well as GSTs (23). Thus, the resistance profile of HBM-resistant cells resembled that of other drug-resistant cell lines.

![Fig. 3. A series of dilutions of cytosolic protein and total RNA from AM-resistant cells (L5178Y/AM) was examined by Western blot analysis (a) and Northern blot analysis (b).](attachment:image)

a. cytosolic protein from sensitive L5178Y cells, as control, and from AM-resistant cells was loaded in the following amounts: Lane 1, sensitive L5178Y cells; 250 µg; Lanes 2-13, resistant L5178Y/AM cells, 250, 200, 160, 128, 102, 82, 66, 52, 42, 34, 27, and 21 µg, respectively. The blot was probed with polyclonal antiserum against rat liver GST Ya, Yb, and Yc. b. total RNA from L5178Y cells, as control, and from AM-resistant cells was loaded in the following amounts: Lane 1, sensitive L5178Y cells, 80 µg; Lanes 2-10, resistant L5178Y/AM cells, 80, 40, 20, 13.3, 10, 8, 6.7, 5.8, and 5.1 µg, respectively. The blot was hybridized with the plasmid pGTB38 containing the cDNA for rat liver GST Ya (a class).
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resistant model cell lines reported previously from this and other laboratories (9, 29–33). Furthermore, resistance to HBM was unstable in that culture of L5178Y/HBM10 cells for 4 months in the absence of drug resulted in isolation of a partial revertant L5178Y/HBMR with a level of resistance approximating that of L5178Y/HBM2 cells (Table 1).

The level of GST enzyme activity in drug-resistant cell lines L5178Y/HBM2, L5178Y/HBM10, and L5178Y/HBMR was 3-, 11-, and 9-fold elevated, respectively, relative to that of sensitive cells (Table 1). Thus, GST enzyme activity appeared to parallel the level of HBM-resistance except for the partial revertant cell line L5178Y/HBMR in which the drop in drug resistance appeared to be greater than the decrease in enzyme activity (Table 1).

Western blot analysis of cytosolic protein from HBM-resistant cells showed elevated levels of the gene product for the cytosolic GST classes π, μ, and α and, for each, the increase was incremental and parallel to the level of HBM-resistance with the following relative levels: L5178Y/HBM10 > L5178Y/HBM2 > L5178Y cells (Fig. 1; Table 1). Furthermore, the relative increase of the GST-π isozyme was greater than that of the μ and α classes of GSTs.

Northern blot analysis of total RNA from HBMI-sensitive and -resistant L5178Y cells (Figs. 1 and 4; Table 1). By contrast, there was a more marked decrease in level of message and gene product for GST-μ and α to approximate levels observed in sensitive cells. These findings together with the sustained elevation of GST enzyme activity in the partial revertant suggest that factors other than GST, such as quinone reductase, may contribute to HBM resistance. Indeed, Northern blot analysis demonstrated elevated steady state levels of mRNA for this enzyme not only in resistant L5178Y/HBM10 cells, but also in AM-resistant cells (Fig. 6).

Unlike class π and μ isoforms which appear to be constitutively expressed in sensitive L5178Y cells, the α class GST isozyme was not detected by either Western or Northern blot analysis (Figs. 1 and 4). This may reflect limitations in sensitivity of these two techniques or,
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Table 1 Relative levels of drug resistance, enzyme activity and of gene product, and mRNA for all 3 cytosolic GST classes in drug-sensitive and -resistant L5178Y leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relativea resistance</th>
<th>Enzymeb activity</th>
<th>Gene productc mRNA†</th>
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<tr>
<td></td>
<td>π</td>
<td>μ</td>
<td>α</td>
</tr>
<tr>
<td>L5178Y</td>
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<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>L5178Y/HBM2</td>
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<td>3</td>
<td>2.2±0.1</td>
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<td>L5178Y/HBM10</td>
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<tr>
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<td>9</td>
<td>5.1±1.3</td>
</tr>
<tr>
<td>L5178Y/AM</td>
<td>10.0</td>
<td>3.6</td>
<td>3.0±0.2</td>
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a Relative resistance represents the D10 values for resistant cells treated with HBM relative to that obtained for sensitive parental L5178Y cells: the D10 (mean ± SE) for treatment of sensitive L5178Y cells with HBM was 0.56 ± 0.08 mm as published previously (23). The D10 for resistant L5178Y/AM cells treated with AM was 22.2 μM and that for sensitive cells was 2.32 μM; accordingly, the relative resistance to AM was approximately 10-fold.
b GST enzyme activity was determined using CDNB as substrate; enzyme activity for each of the resistant cell lines is expressed relative to that of sensitive L5178Y cells. GST activity in sensitive parental L5178Y cells was 2.3 nmol/min/10⁶ cells; these data have been published previously (23), except for L5178Y/AM cells. GST enzyme activity (mean ± SE) in L5178Y/AM cells was 55.1 ± 9.9 nmol/min/mg protein and that in sensitive L5178Y cells was 15.7 ± 0.7 nmol/min/mg protein; accordingly, relative enzyme activity in resistant L5178Y/AM cells was approximately 3.6-fold greater than that of sensitive cells.
c Data were obtained by densitometric analysis of Western or Northern blots and are presented relative to levels of gene product or mRNA in sensitive L5178Y cells. For Northern analysis, the data were also corrected for irregularities in RNA loading by reference to the signal obtained with β-actin. ND, not detected.
d Absence of signal in sensitive L5178Y cells for GST-α precluded a direct densitometric comparison of gene product or mRNA level in drug-sensitive and -resistant cells. In these cases, Western and Northern blot analyses were performed on serial dilutions of protein or RNA extracted from drug-resistant cells to provide an estimate of fold-elevation above background level.

† Single determination.

alternatively, suggest that α class GST expression may require an appropriate inductive signal. Overexpression of all 3 cytosolic GST classes by cells resistant to HBM suggests that induction of GSTs may be nonspecific. The preferential induction of α class GST suggests that the response may also have a class-selective component. HBM contains a quinone ring, and the association of resistance to quinone-containing antineoplasticcs and up-regulation of α class GST is consistent with previous reports in the literature of overexpression of anionic α class GST in ADR-resistant MCF-7 breast cancer (8) and P388 leukemia cells (9), as well as in mammalian (10) and yeast cells (12) transfected with GST-α.

HBM-resistant cells are not cross-resistant to AM, which functions as an aromatic alkylating agent (34). Western blot analysis of cytosolic proteins from AM-sensitive and -resistant cells showed increased levels of all 3 cytosolic GST isoforms α, μ, and α in resistant cells (Fig. 2; Table 1). However, in addition to this nonspecific response to AM, there appeared to be preferential or selective induction of the α class gene product.

Parallel alterations in the level of mRNA for each of the 3 cytosolic GSTs was observed on Northern blot analysis of RNA from AM-resistant cells (Fig. 5; Table 1). Since GST-α did not appear to be expressed constitutively in sensitive cells (Figs. 2 and 5), serial dilutions of cytosolic protein and total RNA was examined by Western and Northern blot analysis, respectively, to provide an estimate of the fold-elevation of α gene product and message (Fig. 3; Table 1). By this approach, the elevation of GST-α gene product was over 12-fold and that of the α class message was over 64-fold in AM-resistant cells relative to background. Finally, an elevated level of mRNA for quinone reductase was observed in AM-resistant cells (Fig. 6), suggesting a possible contribution of this enzyme to the drug resistance phenotype, even though AM does not contain a quinone moiety.

The finding of preferential induction of an α class GST in AM-resistant cells provides additional evidence of the role of GST-α in alkylator resistance. An electrophile-responsive element composed of two adjacent AP-1-like binding sites has been described in the 5'-flanking region of the mouse GST Yγ subunit gene and is responsible for the induction of gene expression by xenobiotics containing or acquiring an electrophilic center (35). The difference in isozyme profiles between AM- and HBM-resistant cell lines emphasizes how structural differences and, in particular, the nature of the electrophilic signal, may influence the pattern of induction of GST isoforms. In summary, in HBM-resistant cells, the level of GST-α and -isoforms correlated more closely with drug resistance, whereas GST-π, the predominant isoform, paralleled enzyme activity. These findings suggest that other factors, such as quinone reductase, may contribute to resistance. Increased levels of mRNA for all 3 GST classes and quinone reductase suggest that induction of these enzymes may be regulated at a transcriptional level. However, nuclear run-on studies and measurement of message stability would be required to validate this hypothesis. The strongest evidence for chemical selectivity was marked overexpression of the α class GST message and gene product in AM-resistant cells.

The use of gene-specific oligonucleotide probes for the various GST isoforms as described by Waxman et al. (1) offers a measure of precision previously unavailable to establish chemical specificity for the induction of various GST classes following treatment with chemotherapeutic agents or xenobiotics. Waxman et al. (1) showed that treatment of rats with cisplatin increased liver expression of mRNA for GST Yγ, but decreased that of GST Yδ1, another α class isozyme, with little or no effect on several other GSTs. The large cDNA probes used in this study do discriminate between major GST classes but not between different isoforms within the same class. Future studies correlating GST overexpression with drug resistance will likely use isoform-specific oligonucleotide probes and may serve to clarify some of the contradictory findings regarding the chemical specificity of induction of GSTs by xenobiotics.

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Increased Expression of Cytosolic Glutathione S-Transferases in Drug-resistant L5178Y Murine Lymphoblasts: Chemical Selectivity and Molecular Mechanisms

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