Biochemical Pharmacology, Vol. 38, No. 5, pp. 787–793, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

ROLE OF GLUTATHIONE AND ITS ASSOCIATED ENZYMES IN MULTIDRUG-RESISTANT HUMAN MYELOMA CELLS*

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(*Received* 3 *March* 1988; *accepted* 25 *July* 1988)

Abstract—Multidrug resistance (MDR) is a phenomenon associated with the emergence of simultaneous cross-resistance to the cytotoxic action of a wide variety of structurally and functionally unrelated antineoplastic agents. The present study was undertaken to determine if 8226 human myeloma cells possessing the MDR phenotype had an increased ability to resist the intercalating drug doxorubicin (DOX) via glutathione-based detoxification systems. Glutathione *S*-tranferase (GST) was isolated by affinity chromatography, and the enzyme activity was assessed using 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as substrates. There was no difference in overall GST activity between the sensitive and resistant cells. Using a cDNA probe (pGTSS1-2) for the human placental, anionic GST isoenzyme, no overexpression of mRNA for this isoenzyme was noted in the resistant line. When glutathione peroxidase activity (GSH-px) was assessed using either H_2O_2 or cumene hydroperoxide as substrate, again there was no difference in enzyme activity. Non-protein sulfhydryl (NPSH) levels were found to be elevated significantly in the resistant 8226/DOX40 subline (19.2 ± 0.1 nmol NPSH/10^6 cells) as compared to the drug-sensitive parental subline 8226/S (11.6 ± 1.9 nmol NPSH/10^6 cells) ($P < 0.001$). In addition, when the 8226/DOX40 cells were cultured in medium without doxorubucin, there was a consistent decline in NPSH values reaching a steady state identical to that of the 8226/S cells. However, the decrease in NPSH level was not accompanied by a change in the level of doxorubicin resistance as assessed by colony-forming assays. Depletion of glutathione by D,L-buthionine-*S*,*R*-sulfoximine had no effect on doxorubicin sensitivity in either subline. Thus, it appears that GSH-based detoxification systems are not causally involved in maintaining the MDR phenotype in 8226 human myeloma cells; rather they appear to comprise an epiphenomenon associated with the resistance selection procedure.

Multidrug resistance (MDR) is a phenomenon associated with the emergence of a pattern of cross-resistance to the cytotoxic action of a wide variety of structurally and functionally unrelated antineoplastic agents. These agents are usually derived from natural sources and MDR has been observed both in tumor cells *in vitro* and in clinical specimens [1–3]. Furthermore, we have demonstrated recently the presence of P-glycoprotein in patients with drug-resistant multiple myeloma and non-Hodgkin's lymphoma [4]. Available evidence suggests that a decrease in drug accumulation in the resistant tumor cell population, perhaps mediated by the P-glycoprotein, accounts for the level of resistance observed in the MDR cells. Although in most instances the degree of drug accumulation and the level of resistance are correlated, accumulation alone may not entirely explain the phenomenon of MDR. Indeed, several studies have reported the lack of a direct correlation between drug accumulation and cytotoxicity [5–7].

This suggests that other mechanisms, in addition to drug accumulation, may play a role in maintaining the MDR phenotype. These alternate mechanisms could involve: (1) changes in subcellular distribution of drug to reduce drug concentrations at critical cellular targets, (2) alterations in the target molecule itself, (3) differences in repair capabilities, or (4) changes in the ability of the resistant cell to detoxify the drug. Such mechanisms could operate independently or in concert with the known alterations in drug accumulation.

Recent observations suggest that glutathione (GSH)-based detoxification mechanisms may be overexpressed in resistant tumors. For example, elevations in the activities of the enzymes glutathione-*S*-transferase (GST; EC 2.5.1.18) and glutathione peroxidase (GSH-px; EC 1.11.1.9) have been reported in a human breast carcinoma cell line which exhibits the MDR phenotype [8]. Such elevations could enable cells to resist antineoplastic agents by direct detoxification and elimination before the drug can exert a cytotoxic effect on the critical cellular target. Indeed, several non-MDR cell lines are known to contain increased levels of GSH and its

* Supported in parts by Grants CA17094, CA23078, and CA43043 from the National Institutes of Health, Bethesda, MD 20892.

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‖ 1987–88 Sterling-Winthrop Pharmacology/Toxicology Fellow.

associated enzymes as an integral component of their resistance to antineoplastic agents [9–15].

This led us to ask the question: was the elevation in non-protein sulfhydryl (NPSH) involved in maintaining the level of drug resistance in the 8226/DOX line or was it elevated in response to some selective pressure encountered in the creation of this line? Normally the resistant 8226 cells are maintained in the presence of doxorubicin (DOX). If the cells are removed from drug, would there be a subsequent decline in the NPSH content in these cells and, if so, would the level of resistance be altered? Furthermore, if the increased NPSH content of the resistant cells was contributing to the MDR phenotype, then there should be a concomitant increase in the sensitivity of these cells to the cytotoxic action of DOX as these levels decline.

The purpose of the present study was to determine if the GSH system, consisting of GSH and the two enzymes GST and GSH-px, contributed to the level of resistance observed in a DOX-treated 8226 human myeloma cell line which exhibits the MDR phenotype.

MATERIALS AND METHODS

Cell line. The RPMI 8226 human myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD). It was originally derived from the peripheral blood of a 61-year-old male with multiple myeloma [16]. The cells for the current study were maintained in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% penicillin (100 units/ml), 1% streptomycin (100 μg/ml) and 1% (v/v) L-glutamine ("complete medium") (Grand Island Biological Co., Grand Island, NY). Cells were kept at 37° in a 5% CO_2–95% air atmosphere and subcultured every 6 days.

RPMI 8226 cells were selected for resistance to DOX (Adria Laboratories, Columbus, OH) by gradually increasing DOX exposure over a period of 10 months. These cells display a typical MDR phenotype characterized by increased amounts of P-glycoprotein and cross-resistance to a wide variety of anticancer drugs including vinca alkaloids and other anthracyclines [17]. Two variants of this cell line were used. The 8226/DOX6 subline was selected for resistance by gradually exposing the cells to 6×10^{-8} M DOX, a concentration six times the initial selection concentration (1×10^{-8} M), whereas the 8226/DOX40 cells were selected by gradually increasing the drug concentration to 4×10^{-7} M DOX.

Cytotoxicity studies. The *in vitro* cytotoxic effect of DOX on sensitive and resistant 8226 cells was studied using colony-forming assays in soft agar [18]. The cells were exposed to DOX for 1 hr at 37° in complete medium. Following drug exposure, the cells were washed twice with cold PBS*, pH 7.4, and plated at a density of 2×10^5 cells/ml onto 35 mm tissue culture dishes (Falcon; Becton Dickinson Labware, Oxnard, CA) containing 0.3% agar (Difco Laboratories, Detroit, MI) in complete RPMI medium. Triplicate plates were incubated under 10% CO_2 and 90% humidified air to facilitate colony formation. Colonies >60 μm diameter in size were counted by an automated image analyzer (FAS-II Omnicon; Baush & Lomb, Rochester, NY) 10–14 days after plating [19]. The mean lethal dose, D_o, which is defined as the dose of drug required to reduce cell survival to $1/e$ (37%) of the original value, as well as the quasithreshold dose, D_q, a measure of the shoulder of the survival curve, were determined for the 8226 cell lines.

In addition to the clonogenic assays, a colorimetric assay utilizing the tetrazolium salt, 3–4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) was used to assess the cytotoxicity of a 1-hr exposure to doxorubicin [20]. Data were expressed as percent survival of control cells calculated from the absorbance (540 nm) corrected for background absorbance. The surviving fraction of cells was determined by dividing the mean absorbance values of the doxorubicin-treated samples by the mean absorbance values of untreated control samples.

NPSH assay. The NPSH levels in 8226/S, 8226/DOX6, and 8226/DOX40 were measured according to the method of Sedlak and Lindsay [21]. A total of 4×10^6 cells was washed twice with cold PBS, pH 7.4, and transferred to a microcentrifuge tube where they were lysed by sonication (model 250 Branson Sonifier, Danbury, CT). Protein was precipitated with 5% sulfosalicylic acid. The cell lysate was centrifuged at 12,000 *g* for 5 min, and a 1-ml aliquot of the supernatant fraction was transferred to a tube containing 0.2 M Tris buffer and 0.02 M EDTA, pH 8.9. To each tube, 100 μl of 0.01 N 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in absolute methanol was added. The contents were mixed, and the absorbance was measured at 412 nm. The concentration of NPSH in the sample was determined by comparing the optical density reading to a standard curve constructed using reduced glutathione. NPSH levels were measured 5 days post cell passage in all instances.

Depletion of GSH by BSO. D,L-Buthionine-*S*,*R*-sulfoximine (BSO) (Chemical Dynamics Corp., South Plainfield, NJ) was dissolved in sterile PBS, pH 7.4, immediately prior to use. The cytotoxicity of BSO alone in 8226/S and 8226/DOX40 cells was determined by the MTT dye assay. 8226 Cells were preincubated for 24 hr in RPMI medium containing 1 mM BSO prior to doxorubicin cytotoxicity assays and NPSH determinations. BSO concentration was maintained at 1 mM during doxorubicin exposure. Following drug exposure, the cells were washed twice with PBS and resuspended in RPMI medium containing 0.1 mM BSO prior to doxorubicin cytotoxicity determination using the MTT assay.

GST isolation. Glutathione-*S*-transferase enzymes were isolated based on the procedure of Mannervik and Guthenberg [22] using an affinity column consisting of *S*-hexylglutathione coupled to epoxy-activated agarose (Sigma Chemical Co., St. Louis, MO). Cytosol from 10^8 sensitive or 10^8 resistant 8226 cells was prepared by suspending cells in 5 ml of a hypo-

* PBS, phosphate-buffered saline, consisted of 137 mM NaCl, 2.65 mM KCl, 7.66 mM Na_2HPO_4 and 138 mM KH_2PO_4.

tonic lysis buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 mM Tris–HCl, pH 7.4, and 25° for 45 min. The cells were then dounce homogenized by hand (Kontes Co., Vineland, NJ). The cell lysates were centrifuged at 4000 *g* for 1 hr at 4° (L8-70 M ultracentrifuge, Beckman Instrument Co.). The resulting supernatant fraction, containing the cytosolic fraction, was then applied to the affinity column and eluted with 10 mM Tris–HCl, 1 mM EDTA, pH 7.8. GST isoenzymes were removed from the column with 5 mM glutathione in the elution buffer. Enzyme activity was measured according to the method of Habig *et al.* [23] using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH as substrates. Protein was measured by the method of Lowry *et al.* [24].

Cellular RNA expression. RNA was purified from 8226/S and 8226/DOX40 cells using guanidium isothiocynate and cesium chloride gradient centrifugation [25]. Dot blot analysis of total RNA was performed as described [26] using a cDNA probe, pGTSS1-2 (the gift of Drs W. D. Henner and T. C. Shea, Dana Farber Cancer Institute), which hybridizes to a 322 base pair (bp) region of a human anionic glutathione-*S*-transferase isoenzyme. The pGTSS1-2 probe was oligolabeled with [^{32}P]ATP (50 μCi dATP (5 μl; 3000 Ci/mmol; ICN Radiochemicals, Irvine, CA) according to the method of Feinberg and Vogelstein [27].

GSH-px assay. Glutathione peroxidase activity was assayed using H_2O_2 or cumene hydroperoxide (Aldrich Chemical Co., Milwaukee, WI) as substrate by the method of Reddy *et al.* [28].

RESULTS

NPSH. The 8226/DOX40 contained 19.2 ± 0.1 nmol NPSH/10^6 cells which was significantly higher than in the 8226/S subline ($P < 0.001$, Student's *t*-test). NPSH levels in the 8226/DOX6 subline were not statistically different from the 8226/S line (Table 1). There was no difference in cell size or protein content between these three sublines that could have accounted for the increased NPSH levels observed in the resistant cells.

Importantly, once the resistant cell line was removed from DOX exposure, the NPSH level in the 8226/DOX40 line began to fall (Fig. 1). After 3 months in drug-free medium, NPSH levels reached a steady state identical to that found in the 8226/S cells.

Table 1. Non-protein sulfhydryl content in 8226 cells*

Subline	NPSH content (nmol/10^6 cells) −BSO	+BSO†
8226/S	11.6 ± 1.9‡	ND§
8226/DOX6	12.6 ± 0.5	
8226/DOX40	19.2 ± 0.1‖	1.15 ± 0.8

* NPSH was measured 5 days post-splitting by the method of Sedlak and Lindsay [21].
† 1 mM BSO.
‡ Results are means ± SD of six experiments.
§ Not detected.
‖ $P < 0.001$ by *t*-test comparison to 8226/S.

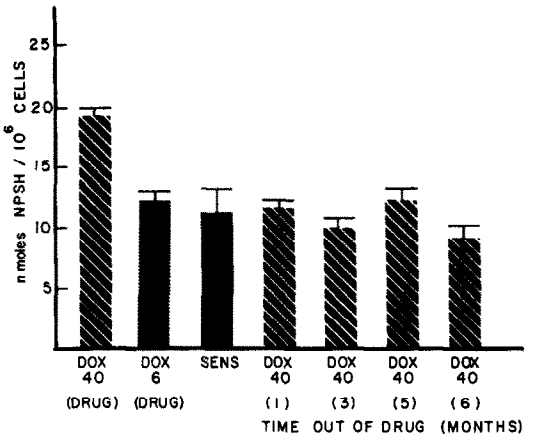

Fig. 1. NPSH levels in 8226 cells. NPSH levels were determined spectrophotometrically using the procedure of Sedlak and Lindsay [21]. There was a significant increase ($P < 0.001$, Student's *t*-test) in the NPSH content of the 8226/DOX40 cells. Doubling times were identical as were the cell cycle transit times between the sensitive and resistant cells. NPSH levels were measured 5 days post-splitting and are presented as the mean ± SD of six measurements.

BSO by itself was relatively non-cytotoxic in both the 8226/S and 8226/DOX40 cells. A concentration of 1 mM reduced the percent survival from 100% to approximately 85% (data not shown). Concentrations of 0.1 mM or less were non-toxic under the conditions employed in these studies. Following a 24-hr exposure to medium containing 1 mM BSO, NPSH values in the 8226/DOX40 cells were reduced by 94% while those of the drug-sensitive parental line were reduced below our detectable limits (Table 1).

As demonstrated in Fig. 2, depletion of glutathione by BSO had no effect on doxorubicin sensitivity

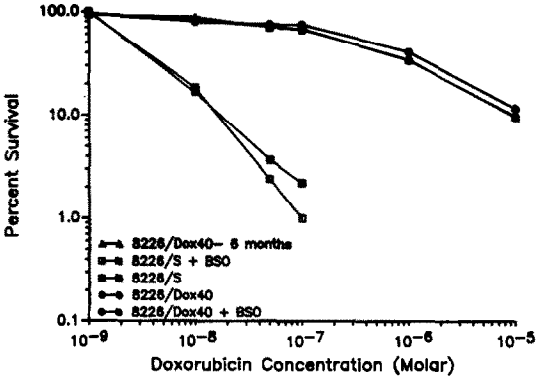

Fig. 2. Cytotoxic effects of doxorubicin on 8226 cells as measured by the MTT assay following a 1-hr drug exposure *in vitro*. BSO alone had relatively minimal cytotoxic activity (<15%). Key: (▲——▲) 8226/DOX40-6 months in drug-free medium; (□——□) 8226/S + BSO; (■——■) 8226/S without BSO; (○——○) 8226/DOX40 without BSO; and (●——●) 8226/DOX40 + BSO. Points are the means of six replicates. Note that survival curves for 8226/DOX40 + BSO and 8226/DOX40-6 months completely overlapped.

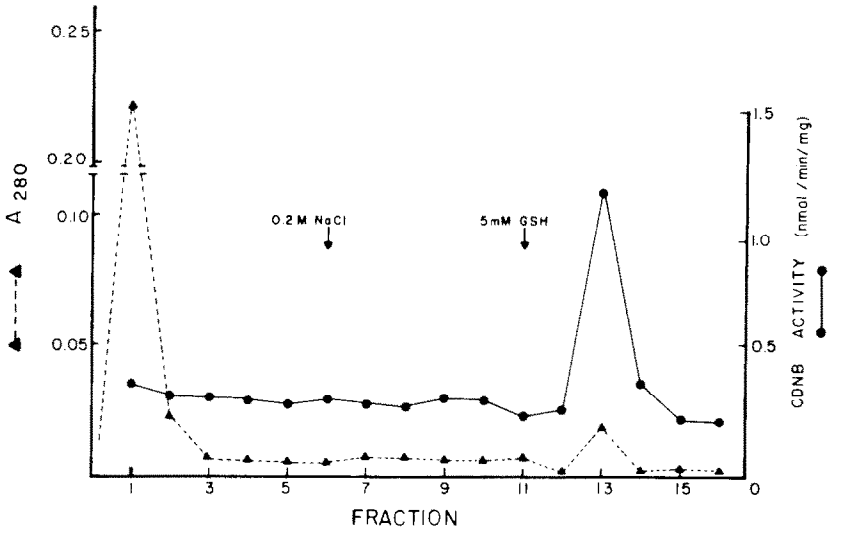

Fig. 3. Representative profile for the isolation of GST from 8226 cells using affinity chromatography. Sample was applied at pH 7, and the column was eluted with 0.05 Tris buffer, pH 7.8, followed by 0.2 M NaCl. GST was removed with 5 mM GSH in Tris buffer. Key: (▲----▲) protein elution, 280 nm; and (●——●) GST activity measured using CDNB and GSH as substrates.

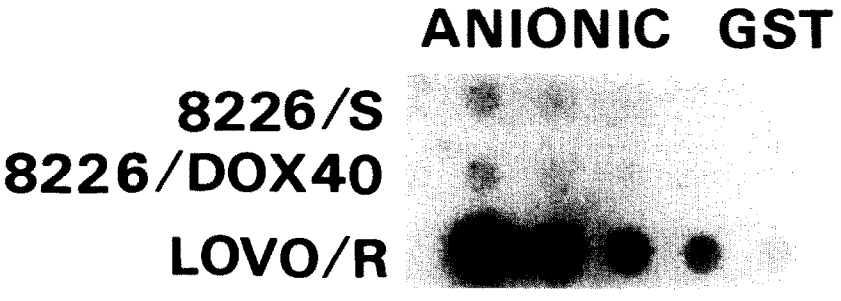

Fig. 4. Dot blot analysis of anionic GST mRNA levels in 8226 myeloma cells. Serial 2-fold dilutions of total RNA (10, 5, 2.5, 1.25 and 0.612 μg) were applied to a filter. After hybridization with pGTSS1-2 cDNA probe, the filter was exposed for 36 hr at −80°.

in the 8226 cells. There was no discernible difference among 8226/DOX40 cells maintained in the presence of doxorubicin, the same cell line maintained in drug-free medium for 6 months, or those in which BSO had been utilized to deplete glutathione. These findings were identical with results obtained using the clonogenic assay. In addition, BSO had no effect on doxorubicin sensitivity in the 8226/S line.

GST activity. Glutathione-*S*-transferases are a family of cytosolic enzymes and were isolated from sensitive and resistant sublines of 8226 by affinity chromatography. When the cytosolic fraction was applied to the glutathione affinity column, the various GST isoenzymes were eluted as a single peak of activity when assayed with CDNB and GSH (Fig. 3). In contrast to the findings with NPSH levels, this "bulk" GST activity was similar in all three cell lines. Thus, there was no correlation between the level of DOX-resistance and overall activity in any of the 8226 cells (Table 2).

To determine whether the anionic form of GST was elevated even though overall enzyme activity was equal, further tests were conducted using the cDNA probe pGTSS1-2, which is specific for this isoenzyme. Very little expression of RNA for this isoenzyme was noted in either the drug-sensitive or -resistant sublines, and there was no discernible difference between them for this message as assessed by dot blot analysis (Fig. 4). This was in contrast to

Table 2. Glutathione-*S*-transferase activity in 8226 cells

Subline	Fold-resistance	Enzyme activity* (nmol/min/mg)
8226/S	1	1.26 ± 0.14†
8226/DOX6	6	1.27 ± 0.21
8226/DOX40	40	1.17 ± 0.25

* GST was isolated and purified from 8226 resistant and sensitive cells, 5 days post-splitting, by the method of Mannervik and Guthenberg [22]. Activity was assessed by the method of Habig *et al.* [23] using 1 mM GSH and 1 mM CDNB as substrates.

† Results are means ± SD of four experiments.

Table 3. Glutathione peroxidase activity in 8226 cells*

GSH-px substrate	GSH-px activity (nmol/min/mg) 8226/S	8226/DOX40
Hydrogen peroxide (0.25 mM)	2.16 ± 1.12	2.80 ± 0.88
Cumene hydroperoxide (1.2 mM)	3.67 ± 1.14	3.23 ± 0.38

* GSH-px activity was measured by the method of Reddy *et al.* [28] using H_2O_2 or cumene hydroperoxide as substrate. Results are the means ± SD of four to five experiments.

a human colon carcinoma cell line, LOVO, which displayed a higher level of expression of this isoenzyme.

GSH-px activity. Using 0.25 mM H_2O_2 as the substrate, the 8226/DOX40 line possessed 2.80 ± 0.88 nmol/min/mg GSH-px activity as compared to 2.16 ± 1.12 nmol/min/mg in the 8226/S line. Organic peroxidase activity was measured using 1.2 mM cumene hydroperoxide as the substrate. The 8226/DOX40 cells displayed GSH-px activity equivalent to that of the 8226/S cells using either substrate. Again, no correlation was found in the GSH-px activity between the sensitive and the MDR-8226 myeloma cells (Table 3).

Cytotoxicity. Even when the 8226/DOX40 cells were removed from the presence of drug for up to 5 months, there was no significant change in the level of resistance from that of 8226/DOX40 cells maintained in the presence of drug. This can be clearly seen from the overlapping survival curves of these two cell populations (Fig. 5). In both cell lines, DOX produced identical quasithreshold (D_q) values of 1×10^{-7} M. For reference, this compares to a D_q of 8×10^{-9} M for the drug-sensitive 8226/S line. The mean lethal dose (D_o) was 70 nM for 8226/DOX40 (in drug) versus 63 nM for 8226/DOX40 (out of drug for 5 months). For reference, the D_o in 8226/S cells was 1.5 nM.

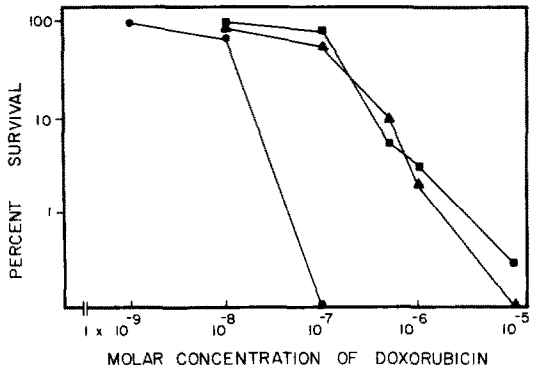

Fig. 5. Effect of doxorubicin on soft agar colony formation of sensitive (8226/S), resistant (8226/DOX40), and resistant cells maintained in drug-free medium for 5 months (8226/DOX40-5 mo) following a 1-hour drug exposure *in vitro*. Points are the means of three experiments. Key: (●) 8226/S; (■) 8226/DOX40; and (▲) 8226/DOX40-5 mo.

DISCUSSION

Elevations of GSH and GST have been reported in several cancer cell lines exhibiting resistance to various alkylating agents. Adams *et al.* [10] found that, following cyclophosphamide treatment in mice, there was a depletion of both GSH content and GST activity in the bone marrow. There was a subsequent rebound of both which correlated with the ability of the animals to survive a normally lethal dose of the drug [10]. Wang and Tew [11] reported increased glutathione-*S*-transferase activity in a Walker 256 rat mammary carcinoma cell line which is resistant to bifunctional alkylating agents; and Suzukake *et al.* [12] demonstrated that depletion of GSH in melphalan-resistant L1210 mouse leukemia cells sensitizes these cells to the cytotoxic action of the drug. Although alkylating agents are not commonly associated with the cross-resistance patterns seen in MDR, they do help illustrate that thiol systems may be elevated in response to various anticancer agents. In addition, Rice *et al.* reported elevations in GSH levels in CHO cells selected for resistance to DOX [14], but it is not known if these cells displayed the MDR phenotype or if the increased GSH levels directly contributed to the development of drug resistance.

Batist *et al.* [8] have described a variant of the human breast cancer cell line MCF-7 which also possesses the MDR phenotype. The resistant cell line which they described was selected by gradually increasing exposures to DOX in a manner similar to that employed in selecting the 8226 MDR cells. The MCF-7 cell line displays a 45-fold increase in GST activity, although there is minimal elevation in GSH content as compared to their drug-sensitive parental line. In contrast, the myeloma cells with MDR do not have increased glutathione enzyme activities. NPSH levels, of which GSH represents greater than 95% [29], were elevated significantly in the 8226/DOX40 line as compared to the 8226/S line, and yet there was no correlation between the level of drug resistance and GST activity in these cells. 8226/DOX6 cells which were selected using a lower concentration of DOX did not possess significantly elevated NPSH levels compared to the sensitive subline. Hamilton *et al.* [13] reported findings similar to these in a human ovarian carcinoma cell line which is 100-fold resistant to DOX. They found only a 1.5-fold elevation in GSH in the DOX-resistant subline as compared to the sensitive cells. This value is consistent with the NPSH elevation observed in our 8226/DOX40 cells. Like the current findings, Ham-

ilton *et al.* did not observe increases in GSH-px, GSH-reductase, or GST enzyme activities in the resistant lines. However, depletion of GSH by buthionine sulfoximine partially restored drug sensitivity to the resistant ovarian cancer cell line. This indicates that elevated thiols were contributing to the DOX resistance in these cells, although BSO did not restore drug sensitivity to the level found in the parental line nor did it have any effect on vincristine cytotoxicity [13]. Depletion of GSH by buthionine sulfoximine had no effect on doxorubicin sensitivity in either the 8226/S or 8226/DOX40 cell lines.

There are at least three groups of human GST isoenzymes as characterized by isoelectric focusing. They are referred to as basic ($pI > 7.5$), neutral ($pI \sim 6.5$), or acidic ($pI < 5.5$) [30]. Each isoenzyme appears to be coded for by different genes [31, 32]. By far, the majority of transferases belong to the basic class and are found in most adult tissues, although there are marked inter-individual differences in the exact isoenzyme patterns. Of interest, the anionic forms are predominant in the human placenta and are found in most fetal tissues [30]. The increase in GST activity observed by Batist *et al.* [8] in resistant MCF-7 cells was due to the overexpression of a particular anionic isozyme. In human colon tissue the placental GST appears to comprise a marker of neoplastic differentiation [33]. In addition to increases in the anionic form of GST, other isoenzymes may also be differentially overexpressed. Buller *et al.* [9] have demonstrated an increase in a basic GST isozyme in nitrogen mustard resistant Walker 256 rat mammary carcinoma cells.

In the present study, total GST enzyme activity, using CDNB and GSH as the substrates, was not elevated but this assay could not discriminate the various isoenzymes of GST. Thus, it was conceivable that, although results of the CDNB study did not indicate an elevation of GST activity in the resistant 8226 cells, there may have been a shift in the isoenzyme pattern towards an overexpression of the anionic form. However, results obtained with the pGTSS1-2 cDNA probe indicated no increase of RNA expression for the anionic GST isoenzyme compared to the sensitive cells and, thus, support the findings of the biochemical assays. Although this finding does not necessarily reflect the precise level of enzyme activity in the cell, it does indicate that there is no increase in the transcription of the message for the anionic isoenzyme. Total glutathione transferase activity in both the sensitive and resistant sublines is extremely low and, in fact, represents some of the lowest levels reported in a human tumor cell line. These low levels may represent further evidence that GST is not playing a major role in protecting the 8226 cells from doxorubicin.

Glutathione transferases, especially anionic forms, have been shown to express an organic peroxidase activity [8, 31]. Such an activity could serve to protect the cell from oxidative damage by reducing any peroxide metabolites to less reactive alcohols [34]. Doxorubicin has been demonstrated to produce oxygen free radicals and H_2O_2 as a result of a redox cycling of its quinone function [35]. Thus, glutathione peroxidase could play a vital role in protecting the cell from DOX damage. Using either H_2O_2, which measures selenium-dependent GSH-px activity, or cumene hydroperoxide, which measures organic peroxidase activity, there was no difference in GSH-px between 8226/DOX40 and 8226/S cells. Similarly, Hamilton *et al.* [13] found no increased GSH-px activity in MDR human ovarian carcinoma cells. In contrast, Batist *et al.* [8] found a substantially increased elevation in organic peroxidase activity in the DOX-resistant MCF-7 cells. All resistant cell lines were selected for resistance to DOX; thus, the differences noted may be tissue or tumor specific. Di Ilio *et al.* [36] have, in fact, demonstrated that GSH and associated enzymes are increased in breast cancer compared to normal breast tissue.

The 8226/DOX40 cells possessed a significant elevation in NPSH levels over the drug-sensitive subline, yet there was no concomitant increase in either GST activity or GSH-px activity. In addition, the 8226/DOX6 cells did not have significantly elevated NPSH levels but retained 6-fold resistance to the drug. Both colony-forming assays and MTT dye assays demonstrated no change in the level of resistance even when the 8226/DOX40 cells were maintained in DOX-free medium for up to 5 months. Thus, the level of resistance is stable in the face of declining NPSH levels. Such findings suggest that the increased NPSH content in the 8226/DOX40 line is an epiphenomenon associated with the resistance selection procedure, possibly to an oxidative stress placed on these cells by exposure to DOX.

In summary, the present study has shown that glutathione-based detoxification systems were not involved in maintaining the level of drug resistance observed in the 8226 human myeloma cell lines which display multidrug resistance. These findings do not rule out the possibility that other pathways which were not investigated may be elevated in the resistant cells. For example, elevations in superoxide dismutase, catalase, or DT diaphorase activities may protect the cells from oxidative damage from drugs such as doxorubicin, and it is possible that such enzymes may be elevated in the resistant 8226 lines. Although the glutathione system is implicated in maintaining the resistance observed in other MDR and non-MDR lines, our results show that it is not a universal feature of the MDR phenomenon.

REFERENCES

1. Gerlach JH, Kartner N, Bell DR and Ling V, Multidrug resistance. *Cancer Surv* **5**: 25–46, 1986.
2. Bell DR, Gerlach JH, Kartner N, Buick RN and Ling V, Detection of P-glycoprotein in ovarian cancer: A molecular marker associated with multidrug resistance. *J Clin Oncol* **3**: 311–315, 1985.
3. Gerlach JH, Bell DR, Karakousis C, Slocum H, Kartner N, Rustum YM, Ling V and Baker RM, P-Glycoprotein in human sarcoma: Evidence for multidrug resistance. *J Clin Oncol* **5**: 1452–1460, 1987.
4. Dalton WS, Durie BGM, Salmon SE, Grogan TM, Scheper RJ and Meltzer PS, Multidrug resistance in multiple myeloma: Detection of P-glycoprotein in clinical specimens and reversal of resistance with verapamil. *Blood* **70** (Suppl 1): 245a, 1987.
5. Bellamy WT, Dorr RT, Dalton WS and Alberts DS, Direct relation of DNA lesions in multidrug resistant human myeloma cells to intracellular doxorubicin concentration. *Cancer Res* **48**: 6360–6364, 1988.

6. Marsh W, Sickeri D and Center MS, Isolation and characterization of adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* **46**: 4053–4057, 1986.
7. Kessel D and Corbett T, Correlations between anthracycline resistance, drug accumulation and membrane glycoprotein patterns in solid tumors of mice. *Cancer Lett* **28**: 187–193, 1985.
8. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE and Cowan KH, Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* **261**: 15544–15549, 1986.
9. Buller AL, Clapper ML and Tew KD, Glutathione *S*-transferases in nitrogen mustard-resistant and -sensitive cell lines. *Mol Pharmacol* **31**: 575–578, 1987.
10. Adams DJ, Carmichael J and Wolf CR, Altered mouse bone marrow glutathione and glutathione transferase levels in response to cytotoxins. *Cancer Res* **45**: 1669–1673, 1985.
11. Wang AL and Tew KD, Increased glutathione-*S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat Rep* **69**: 677–682, 1985.
12. Suzukake K, Petro BJ and Vistica DT, Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. *Biochem Pharmacol* **31**: 121–124, 1982.
13. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McCoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34**: 2583–2586, 1985.
14. Rice GC, Bump EA, Shrieve DC, Lee W and Kovacs M, Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: Some applications to radiation and drug resistance *in vitro* and *in vivo*. *Cancer Res* **46**: 6105–6110, 1986.
15. Bozzi A, Mavelli I, Mondovi B, Strom R and Rotilio G, Differential cytotoxicity of daunomycin in tumor cells is related to glutathione-dependent hydrogen peroxide metabolism. *Biochem J* **194**: 369–372, 1981.
16. Matsuoka Y, Moore GE, Yagi Y and Pressman D, Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc Soc Exp Biol Med* **125**: 1246–1250, 1967.
17. Dalton WS, Durie BGM, Alberts DS, Gerlach JH and Cress AE, Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. *Cancer Res* **46**: 5125–5130, 1986.
18. Salmon SE and Hamburger A, Primary bioassay of human tumor stem cells. *Science* **197**: 461–463, 1977.
19. Salmon SE, Young L, Lebovitz J, Thompson S, Einspahr J, Tony T and Moon TE, Evaluation of an automated image analysis system for counting human tumor colonies. *Int J Cell Cloning* **2**: 142–160, 1984.
20. Carmichael J, DeGraff W, Gazdar A, Minna J and Mitchell J, Evaluation of a tetrazolium-based semiautomatic colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* **47**: 936–942, 1987.
21. Sedlak J and Lindsay RH, Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**: 192–205, 1968.
22. Mannervik B and Guthenberg C, Glutathione transferase (human placenta). *Methods Enzymol* **77**: 231–235, 1981.
23. Habig WH, Pabst MN and Jakoby WB, Glutathione *S*-transferases: The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
24. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
25. Maniatis T, Fritsch EF and Sambrook J (Eds.), *Molecular Cloning: A Laboratory Manual*, p. 187. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
26. Davis LG, Dibner MD and Battey JF (Eds.), *Basic Methods in Molecular Biology*, p. 129. Elsevier, New York, 1986.
27. Feinberg AP and Vogelstein B, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13, 1983.
28. Reddy CC, Tu CD, Burgess JR, Ho CV, Scholz RW and Massaro EJ, Evidence for the occurrence of selenium-independent glutathione peroxidase activity in rat liver microsomes. *Biochem Biophys Res Commun* **101**: 970–978, 1981.
29. Reed DJ and Fariss MW, Glutathione depletion and susceptibility. *Pharmacol Rev* **36**: 25S–33S, 1984.
30. Mannervik B, The isoenzymes of glutathione transferase. *Adv Enzyme Regul* **57**: 357–417, 1985.
31. Warholm M, Guthenberg C and Mannervik B, Molecular and catalytic properties of glutathione transferase from human liver: An enzyme efficiently conjugating epoxides. *Biochemistry* **22**: 3610–3617, 1983.
32. Rhoads DM, Zarlengo RP and Tu C, The basic glutathione-*S*-transferases from human livers are products of separate genes. *Biochem Biophys Res Commun* **145**: 474–481, 1987.
33. Kodate C, Fukushi A, Narita T, Kudo H, Soma Y and Sato K, Human placental form of glutathione *S*-transferase (GST-π) as a new immunohistochemical marker for human colonic carcinoma. *Jpn J Cancer Res (Gann)* **77**: 226–229, 1986.
34. Wendel A, Glutathione peroxidase. In: *Enzymatic Basis of Detoxification* (Eds Jakoby WB and Habig WH), pp. 333–353. Academic Press, New York, 1980.
35. Bachur N, Gordon SL and Gee MV, A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res* **38**: 1745–1750, 1978.
36. Di Ilio C, Sacchetta P, Del Boccio G, LaRovere G and Federici G, Glutathione peroxidase, glutathione *S*-transferase and glutathione reductase activities in normal and neoplastic human breast tissue. *Cancer Lett* **29**: 37–42, 1985.