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# TIME COURSE OF GLUTATHIONE *S*-TRANSFERASE ELEVATION IN WALKER MAMMARY CARCINOMA CELLS FOLLOWING CHLORAMBUCIL EXPOSURE

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**Abstract**—Resistance of Walker 256 rat mammary carcinoma cells to chlorambucil has been shown to be accompanied by a specific increase in the A2-2 subunit of glutathione *S*-transferase (GST) (Buller *et al.*, *Mol Pharmacol* **31**: 575–578, 1987). Analysis of the time course of GST activity following chlorambucil exposure revealed a 7.5- and 3-fold elevation on day 7 post-treatment in Walker-sensitive (WS) and Walker-resistant (WR) cells, respectively. Flow activated cell sorting (FACS) analyses using antibodies specific for rat liver cytosolic GST supported these results and demonstrated the heterogeneous response of WS cells to chlorambucil exposure. The range of GST levels in drug-treated cells was very broad as compared to that of untreated cells. Transcripts for each class of GST ($\alpha$, $\mu$ and $\pi$) were quantified for days 1–9 post-treatment from densitometric scans of RNA slot blots. Elevations in GST $\alpha$RNA preceded increases in GST activity (day 7) in both WS and WR cells. Because fluctuations in GSTA1-1 transcripts were not observed, it was concluded that the increased expression of the $\alpha$ class must be attributed to increases in GSTA2-2 transcripts. Amplification of the GST genes in drug-treated cells was not present. These results support the role of GSTA2-2 in the detoxification of chlorambucil. The time course of the cellular response to chlorambucil suggests that the elevation of GSTA2-2 transcripts following alkylating agent exposure may represent only one component of a series of events which collectively confer protection and lead to the establishment of drug resistance.

Use of glutathione *S*-transferase (GST)§ as a biomarker of cellular protection and drug resistance continues to be supported by the repeated association of elevated GST activity with resistance of tumor cells to chemotherapeutic agents. Further characterization of the GST isozymes in drug-resistant cells has revealed a correlation between elevations in specific GST isozymes and exposure to particular classes of compounds. Resistance of carcinoma cells to Adriamycin® has been attributed, in part, to elevations in GST $\pi$ [1, 2]. Elucidation of the role of GST $\mu$ in denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) provided an explanation for increased levels of GST $\mu$ in 9L-2 cells resistant to BCNU [3]. Our previous results indicate that Walker rat mammary carcinoma cells 10-fold resistant to chlorambucil [4] possess increased levels of cytosolic GST $\alpha$ (A2-2) [5]. A 29 kDa microsomal protein which cross-reacts with cytosolic GST antibodies has also been identified in chlorambucil-resistant cell populations but not in drug-sensitive parental cells [6]. Chinese hamster ovary cells resistant to chlorambucil also exhibit elevations in GST $\alpha$ [7].

The association between elevated expression of GST $\alpha$ and cellular resistance to alkylating agents has been characterized using *in vitro* enzyme-mediated detoxification reactions. These studies have confirmed that detoxification of the chemotherapeutic alkylating agents chlorambucil and melphalan is mediated by GST $\alpha$. Chlorambucil–glutathione conjugates generated by microsomal GST [8] and mouse liver cytosolic GST [9] have been isolated by high performance liquid chromatography and identified using mass spectrometry. A comparison of the conjugating activity of GST $\alpha$, $\mu$ and $\pi$ from mouse liver revealed increased production of the monoglutathionyl chlorambucil adduct in the presence of GST $\alpha$ and minimal elevation of the adduct in reactions utilizing GST $\mu$ and $\pi$. Levels of the mono adduct were elevated 4.4-fold at 5 min after the addition of GST $\alpha$. Similar results have been obtained using melphalan as a substrate [10]. Only GST $\alpha$ catalyzed the conjugation of melphalan with glutathione to produce the monochloro, monohydroxyl derivative as the major reaction product. The substrate specificity of human hepatic GST $\alpha$ appears to be similar to that of mouse liver [11].

Results from *in vitro* conjugation studies have been supported by the reported ability of transfected GST to confer resistance to chlorambucil [12]. Both human GST $\alpha$ and $\pi$ protected *Saccharomyces cerevisiae* from the cytotoxic effects of chlorambucil but, in general, survival was greatest following

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§ Abbreviations: GST, glutathione *S*-transferase; WS, Walker cells sensitive to chlorambucil; WR, Walker cells resistant to chlorambucil; FACS, flow activated cell sorting; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; PBS, phosphate-buffered saline; 10X SSC, 1.5 M sodium chloride/0.15 M sodium citrate, pH 7; and CHO-Chl$^r$, Chinese hamster ovary cells resistant to chlorambucil.

transfection with GST $\alpha$. It should be noted that the protective effect of GST $\alpha$ may have been underestimated since the activity of $\alpha$ transfectants was significantly less than that of GST $\pi$ transfectants (103 and 597 U, respectively). COS-M6 monkey kidney cells transfected with GST $\alpha$ (A1-1) exhibited increased resistance to chlorambucil and melphalan (1.3- and 2.9-fold, respectively) [13]. In contrast, transfection of MCF-7 human breast cancer cells with GSTA2-2 did not confer resistance to either chlorambucil or melphalan [14].

The $\alpha$ class of rat GST isozymes contains two members which are designated GSTA1-1 and GSTA2-2 and are encoded by two separate genes. Sequence comparisons in the coding regions of GSTA1 and A2 cDNAs revealed 66–70% nucleotide sequence homology [15, 16] and 65% amino acid sequence homology [15]. In contrast, the sequences of the 3′ [15, 16] and 5′ [16] noncoding regions for GSTA1 and A2 were extremely divergent. These observations provided an explanation for the differential inducibility of the GSTA1-1 and A2-2 isozymes by phenobarbital [17] and supported the independent transcriptional regulation of the rGSTA1 and A2 genes.

The present study represents an extension of our previous experimentation with chlorambucil-resistant cells and reports on the molecular alterations associated with elevations in GSTA2-2. We investigated the ability of chlorambucil to increase GST $\alpha$, $\mu$ and $\pi$ transcript levels in drug-sensitive (WS) and drug-resistant (WR) cell populations and further defined the time course of this elevation. In addition, novel methodology was utilized to determine the heterogeneity of the response of individual cells to chlorambucil exposure.

## MATERIALS AND METHODS

### *Cell culture*

Walker 256 rat mammary carcinoma cell cultures were established from an ascites carcinoma. A population of Walker cells resistant to chlorambucil (WR) was derived from the sensitive parent cell line (WS) as described previously [18]. All cells were maintained as suspension cultures in Dulbecco's minimal essential medium supplemented with 4 mM L-glutamine, penicillin/streptomycin (200 U and 200 mg/L, respectively) (Sigma, St. Louis, MO) and 10% fetal bovine serum (Biofluidics, Rockville, MD). All cells were routinely tested for Mycoplasma contamination.

For the time–course experiments, cells were seeded at a density of $2 \times 10^4$ cells/mL. Mid-log cells were exposed to chlorambucil (75 $\mu$M in 100% ethanol) for 3 hr, pelleted, and resuspended in drug-free medium. [Biannual exposure of WR cells to 66 $\mu$M chlorambucil (20 $\mu$g/mL) is required to maintain cellular resistance. Maximal enzymatic induction was observed when the drug concentration was raised to 75 $\mu$M.] Because of a reported effect of cell density on GST activity [19], vehicle and chlorambucil-treated cultures were maintained at the same cell density throughout the experiment. Cells were counted and reseeded at $8 \times 10^4$ cells/mL on days 1, 4, and 7 post-treatment.

### *Enzyme assays*

At the time of harvest, cell suspensions were centrifuged at 3000 $g$ and rinsed with phosphate-buffered saline (PBS), pH 7. Cells were resuspended in PBS and sonicated on ice for 30 sec using a Sonic Dismembrator (Artek, Farmingdale, NY) at setting 6. Samples were centrifuged at 10,000 $g$ at 4° for 15 min. The resulting supernatants were assayed for GST, aldolase and lactate dehydrogenase activities. GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene as substrate [20]. Aldolase and lactate dehydrogenase activities were assayed spectrophotometrically using the appropriate diagnostic kits (Sigma). The protein concentration of each cell extract was determined using the Bradford assay (Bio-Rad, Richmond, CA).

### *Radiolabeling*

GST $\alpha$, A1 and $\mu$ isozymes were probed using rat liver cDNAs pGTB42, pGTB33 and pGTA/C48 (provided by Dr. Cecil Pickett, Merck Frosst Center for Therapeutic Research, Quebec, Canada). The rat probe for GST P (pGp5) was provided by Dr. Gerald Batist (Montreal General Hospital Research Institute, Montreal, Quebec). Chicken $\beta$-actin (pA1) was used to standardize RNA levels. All probes were labeled with [$^{32}$P]dCTP (New England Nuclear, Boston, MA) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN).

### *RNA analysis*

*Isolation.* Total RNA was extracted from WS and WR cells using a modification of the procedure by Promega (Madison, WI). Briefly, cells were washed with PBS and lysed with 4 M guanidinium isothiocyanate containing 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. RNA was extracted using 0.1 vol. of 2 M sodium acetate (pH 4.0), 1 vol. of water-saturated phenol and 0.2 vol. of chloroform:isoamyl alcohol (49:1) and precipitated twice at −20° with 1 vol. of isopropanol. The quality and purity of the isolated RNA were determined by electrophoretic separation and by comparison of the absorbances at 260 and 280 nm.

*Slot blots.* RNA samples (10 $\mu$g) were diluted to 100 $\mu$L with water, incubated with 300 $\mu$L of 6.15 M formaldehyde at 65° for 15 min, and placed immediately on ice. Samples were applied under vacuum to a nitrocellulose membrane [prewet in water and 10X sodium citrate/sodium chloride (SSC)] using a slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH). Wells were rinsed with 10X SSC, and membranes were air dried and baked at 80° for 2 hr under vacuum. Blots were prehybridized overnight at 42° in 6X SSC containing 0.25% Carnation non-fat dried milk. Hybridization of radiolabeled GST probe ($2 \times 10^6$ cpm/mL) was performed at 65° overnight in prehybridization buffer containing 5% dextran sulfate [21]. Blots were rinsed twice at room temperature (15 min each) in 2X SSC containing 0.1% sodium dodecyl sulfate (SDS), air dried, and exposed to X-ray film. X-ray films were developed and scanned using an Ultrascan densitometer with a GXL software package

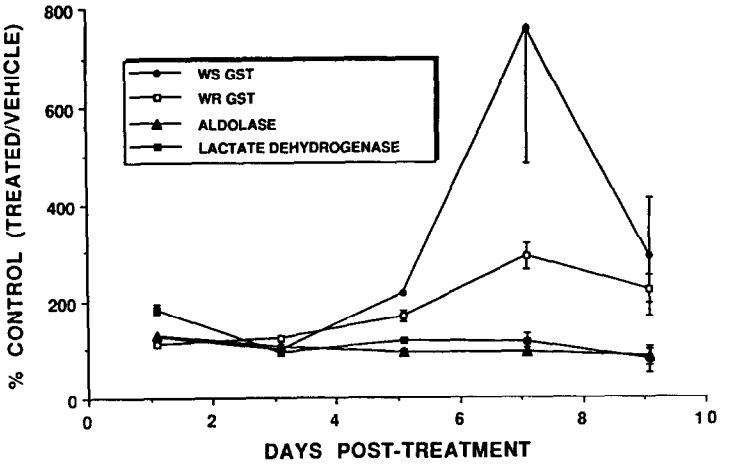


Fig. 1. GST activity in WS and WR cells at various time intervals following chlorambucil treatment (75 μM). Aldolase and lactate dehydrogenase were selected as control cytosolic enzymes. Duplicate determinations were performed on triplicate flasks and all enzymatic activities have been presented as a percentage of vehicle-treated controls ± SEM. The specific activities (±SEM) of control cells were: 4.6 ± 0.1 nmol/min/mg for WS GST; 8.6 ± 0.4 nmol/min/mg for WR GST; 329.0 ± 20 U/mg for WS adolase; and 151.5 ± 14 U/μg for WS lactate dehydrogenase.

(Pharmacia, Piscataway, NJ) to quantify hybridization. All results were expressed as a percentage of control β-actin.

*Northern blots.* RNA species were separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to Gene Screen Plus (New England Nuclear, Boston, MA) in 10X SSC by capillary action. Membranes were prehybridized, hybridized ($1 \times 10^6$ cpm/mL of radiolabeled probe), and washed according to the manufacturer's procedure. Air-dried membranes were exposed to X-ray films.

### *DNA analysis*

High molecular weight DNA was extracted from WS and WR cells by standard procedures [22]. Slot blot analysis involved application of 0.1, 0.5 or 2.5 μg of DNA to a nylon membrane (Zeta-Probe, Bio-Rad). For Southern analysis, DNA (15 μg) was digested with either *Eco*RI or *Hind*III endonuclease, size fractionated on a 1% agarose gel, and transferred to the Zeta-Probe matrix. In all cases the DNA was hybridized to either a GST or β-actin cDNA probe that was $^{32}$P-labeled as described above. Membranes were washed following standard protocol stringency conditions recommended by the manufacturer. Autoradiographs were densitometrically scanned to quantify the appropriate signals.

### *FACS analysis*

Normal goat serum was purchased from Vector Laboratories (Burlingame, CA). Fluorescein (FITC)-conjugated affinity-purified goat anti-rabbit IgG (H + L) was provided by Jackson Immunoresearch Laboratories (West Grove, PA). To remove antibody complexes, the FITC-conjugate was resuspended in $H_2O$ to a concentration of 1.5 mg/mL and centrifuged for 1 hr (89,000 *g*) at 4°. The clarified supernatant was stored at 4°. DEAE affi-gel blue gel was purchased from Bio-Rad.

*Purification of serum IgG.* Sera from control or rat liver GST antisera-producing rabbits were fractionated using ammonium sulfate (30, 50 and 70%). The IgG-containing fractions (30 and 50%) were combined, dialyzed against 0.02 M $K_2HPO_4$, pH 8.0, and chromatographed on DEAE affi-gel blue gel. The fractions containing IgG (first protein peak) were combined and concentrated against 0.02 M $K_2HPO_4$, pH 8.0.

*Staining of intracellular GST.* WS and WR cells were fixed, permeabilized, and stained by a method similar to that described by Schmid *et al.* [23] with minor modifications. Briefly, $1.5 \times 10^6$ cells were pelleted (3000 *g* at 4° in this and all subsequent centrifugations) in 12 × 75 mm polystyrene round bottom tubes (Falcon No. 2058). Cell pellets were washed (2 × 2 mL cold PBS) and resuspended in 875 μL cold PBS. Cold 2% paraformaldehyde solution (125 μL) was added, and the mixture was vortexed immediately. The cells were fixed in this solution for 1 hr at 4° and then collected by centrifugation. For permeabilization, the fixed cell pellet was resuspended in 1.0 mL of PBS containing 0.2% Tween 20, vortexed gently, and incubated for 15 min at 37°. One milliliter of 5% goat serum/PBS was added. Following gentle mixing, the cells were collected by centrifugation. The permeabilized cell pellet was resuspended in PBS containing 5% goat serum and exposed to 1 μg (1:500 dilution) of either preimmune IgG (control) or IgG from GST antisera (final volume, 100 μL). The cell suspension was incubated for 30 min at 4°, centrifuged, and washed twice with 1 mL of PBS containing 0.2% Tween 20. The cell pellets were resuspended in PBS containing 5% goat serum and 0.75 μg (1:200 dilution) of FITC

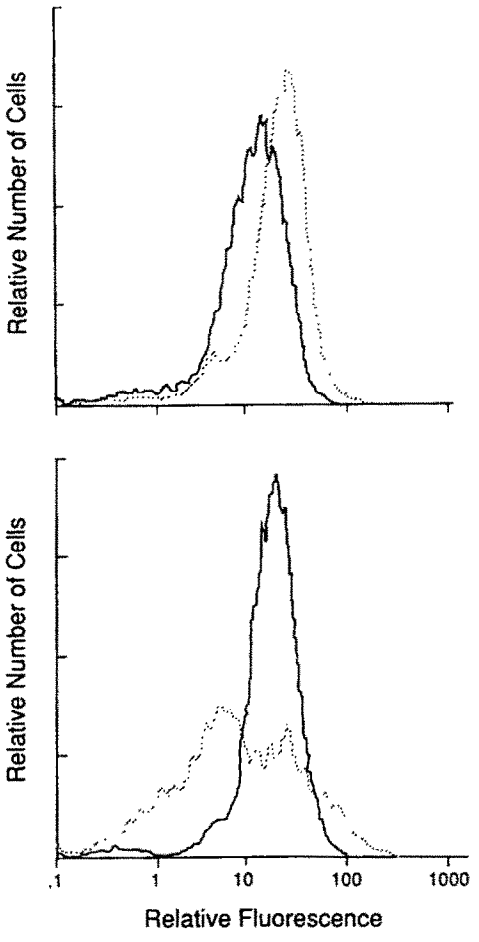


Fig. 2. Representative FACS data analyzing cellular levels of GST. Top: GST levels in untreated WS (solid line) and WR (broken line) cell lines. Bottom: A comparison of GST levels in WS cells before (solid line) and 7 days after (broken line) exposure to 75 $\mu$M chlorambucil.

goat anti-rabbit IgG (H + L) (final volume 100 $\mu$L) and incubated at 4° for 30 min. The stained cells were centrifuged, washed twice in Tween buffer, and resuspended in 200 $\mu$L of PBS containing 5% goat serum for FACS analysis.

## RESULTS

Elevation of GST activity in WR cells resistant to chlorambucil has been attributed previously to increased GSTA2-2 protein as determined by Western blot analysis [5]. To examine the time course of events leading to accumulation of the GSTA2-2 protein, WS cells were exposed to chlorambucil and GST activity was monitored for 9 days post-treatment (Fig. 1). Total GST activity started to increase on day 5 following treatment and peaked at 7.5-fold on day 7. A similar time–course response was observed in WR cells following drug exposure, except that GST activity peaked at approximately 3-fold (Fig. 1). The absence of similar alterations in lactate dehydrogenase and aldolase activities over time suggested that the observed elevation in GST activity was unrelated to cell growth.

Cell counts taken from nine independent experiments at the time the cells were reseeded (days 1, 4 and 7) revealed a significant increase in cell doubling time following drug treatment. Growth of chlorambucil-treated WS and WR cells was inhibited on day 4 by more than 90% as compared to vehicle-treated controls and remained affected for the duration of the experiment. Clonogenic assays indicate that a 2-hr exposure of Walker cells to 75 $\mu$M chlorambucil results in approximately 20% survival of sensitive cells and 90% survival of resistant cells [6].

To evaluate baseline and drug-induced changes in WS and WR cell heterogeneity with respect to GST expression, a FACS assay was developed. The staining of GST required fixation and permeabilization of cells followed by internalization of anti-rat liver GST IgG and FITC IgG. It should be noted that fluorescence detected in cells which internalized preimmune IgG (control) was not significantly different from background autofluorescence, indicating that any staining above this level in treated samples was specific for the GST antigen. Figure 2 depicts representative data from these FACS analyses. The top panel reflects the staining intensity associated with untreated WS and WR cells and indicates that WR cells have a 2-fold increase in GST antigen relative to WS cells. In contrast to the narrow distribution of fluorescence in untreated cells, the fluorescence of WS cells on day 7 following chlorambucil exposure was very heterogeneous (Fig. 2, bottom). This is reflected in the broadness of the peak representing this population. Similar heterogeneity in cellular GST was observed in resistant cells following drug exposure. FACS analyses conducted in triplicate demonstrated the emergence of two WS cell populations following chlorambucil exposure. One population exhibited GST levels equivalent to untreated cells, while the second population possessed elevated levels of GST by 7 days post-treatment. While both WS and WR cell populations had elevated GST levels as compared to the corresponding untreated population, the increase in WS cells was greater (3.0- vs 1.3-fold, WS vs WR, respectively).

Total RNA was extracted from WS and WR cells on various days post-treatment and probed for each class of GST. GST $\pi$ levels decreased in drug-treated sensitive cells over time, while GST $\mu$ levels were elevated slightly on day 5 post-treatment (Fig. 3). GST $\alpha$ was expressed maximally on day 3 post-treatment, with RNA levels peaking at 1.8-fold. WR cells responded to chlorambucil in a similar manner, with GST $\alpha$ transcripts peaking at 1.5-fold on day 5 post-treatment (Fig. 4). GST $\pi$ RNA levels remained unchanged throughout the 9-day period, while GST $\mu$ RNA levels fluctuated from day to day.

The GST $\alpha$ probe pGTB33, specific for GSTA1, was used to differentiate between A1-1 and A2-2 transcript levels. The level of GSTA1 transcripts remained unchanged in both sensitive and resistant cells following chlorambucil treatment (Figs. 3 and

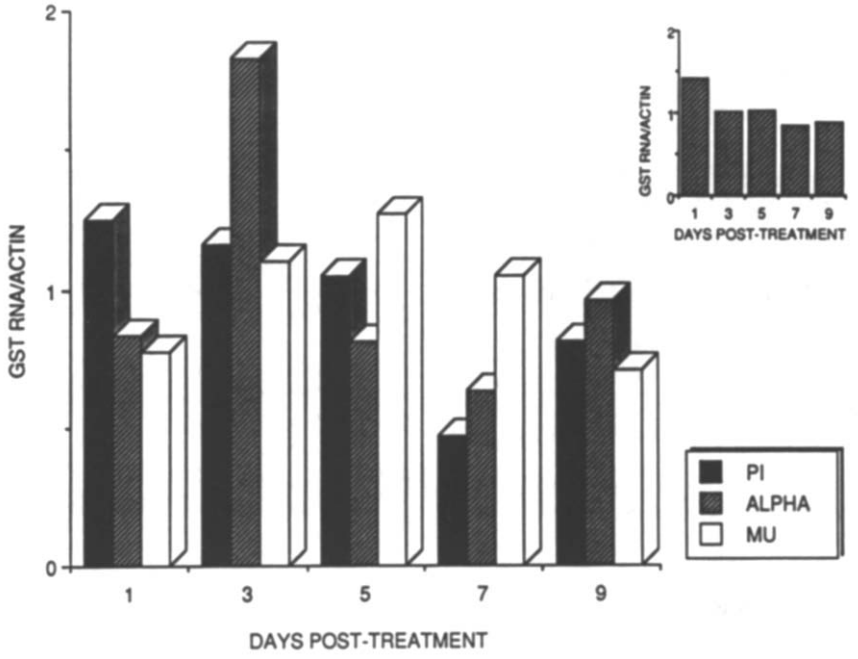


Fig. 3. Time course of GST expression in WS cells following chlorambucil exposure (75 μM). Values represent densitometric scans of total RNA hybridized with cDNA probes encoding either GST α, μ, π or A1 (graphic inset). Transcript levels were standardized to actin and expressed as a function of the corresponding RNA in vehicle-treated cells.

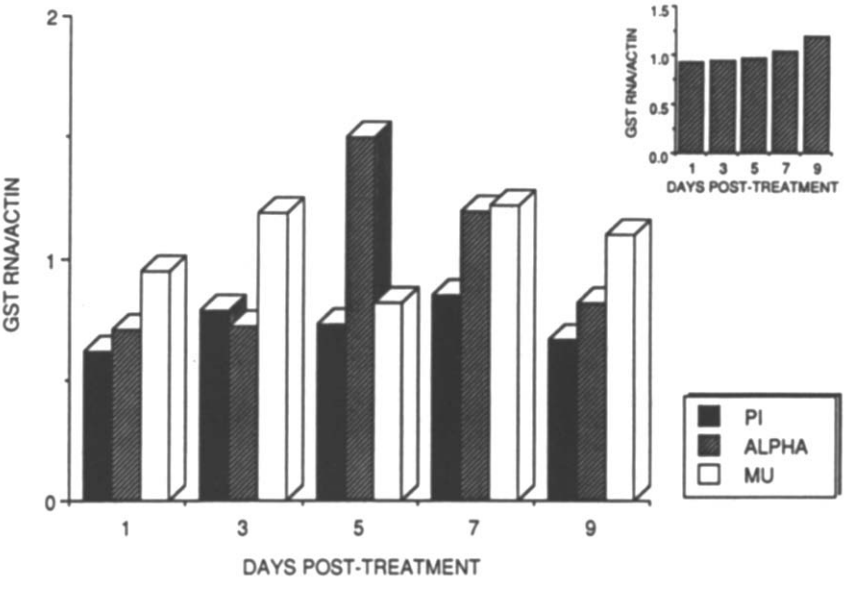


Fig. 4. Time course of GST expression in WR cells following chlorambucil exposure (75 μM). Values represent densitometric scans of total RNA hybridized with cDNA probes encoding either GST α, μ, π or A1 (graphic inset). Data have been expressed as described for Fig. 3.

4). This observation indicated that chlorambucil was specifically altering the expression of the A2-2 subunit.

Quantitation of DNA slot blots did not reveal any amplification of the GST genes in WR cells (Fig. 5). In addition, the length of the restriction fragments generated in WR cells appeared to be identical to those of WS cells as compared by Southern blot analyses (data not shown).

## DISCUSSION

Previous studies from this laboratory have reported

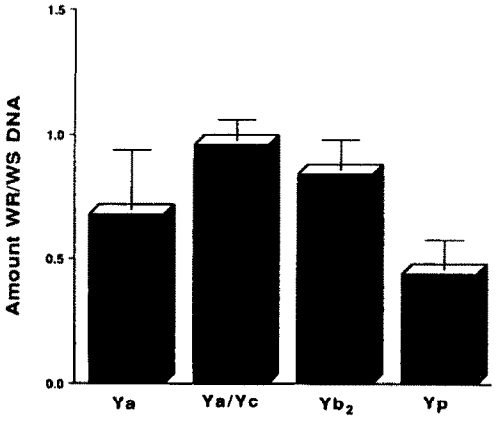


Fig. 5. Levels of the GST $\alpha$, $\mu$ and $\pi$ genes in WS and WR cells. High molecular weight DNA from both populations was hybridized with $^{32}$P-labeled GST cDNAs, and autoradiographs were quantified by densitometric scanning. Blots were stripped and reprobed using $\beta$-actin as a standard. Histograms represent the mean (±SD) of three separate determinations and are standardized to $\beta$-actin levels.

elevated levels of GST activity and the GSTA2-2 isozyme in WR cells 15-fold resistant to chlorambucil as compared to the parental WS cells [5]. The magnitude of the detoxification enzyme response elicited by drug exposure correlated with the sensitivity of cells to chlorambucil. Walker cells that had not been exposed previously to chlorambucil exhibited elevations in GST activity that were 2.5 times greater than those produced in drug-resistant (WR) cells (Fig. 1). Likewise, the fluorescent GST signal was of 3-fold greater intensity in WS cells than in WR cells treated with chlorambucil (Fig. 2). These data suggest that either WR cells possess a "memory" of chlorambucil exposure similar to an immune response, or the extent of GST elevation in WR cells is limited by their increased basal levels of activity. Alternatively, enhanced GST expression may be an initial event in the response of naive cells to chlorambucil exposure. This event may stimulate a cascade of biological reactions which prepare the cell for insult from subsequent drug exposures.

A comparison of the time required to achieve maximal elevation of GST $\alpha$ following cellular exposure to chlorambucil (7 days post-treatment) and treatment of rats with phenobarbital (16–24 hr post-treatment) [16] indicates that two independent mechanisms are responsible for the observed drug-induced changes in GST expression. The delayed response of GST to chlorambucil suggests that GST $\alpha$ may represent one component in a cascade of events which collectively confer drug resistance. The involvement of P450 in the induction of Phase II enzymes has been proposed [24] and supports a cascade hypothesis.

Our data suggest that alkylating agents differ from classical inducers like phenobarbital in that the establishment of cellular resistance involves both cell selection, as demonstrated by the FACS analyses, and enzymatic induction. Cell counts taken at the time of cell passage indicated that WS and WR cells initially respond to drug exposure in a manner which is independent of their sensitivity to chlorambucil. We do not believe that the observed elevation in GSTA2-2 is related to cell death since the isozyme was induced in the WR population where 90% of the cells survived chlorambucil exposure at 75 $\mu$M.

FACS analyses confirmed the results of the enzymatic activity assays and for the first time revealed the heterogeneity of the cellular response to alkylating agent exposure. Although these cell sorting experiments did not provide definitive evidence of cellular selection, they did identify multiple subpopulations of cells on day 7 post-treatment. Fluorescence profiles indicate that these multiple cell populations are not the result of cellular differences in baseline GST. First, untreated parent cells are more homogeneous than treated cells in their GST content. Second, the substantial overlap in the untreated and treated cell profiles suggests that the GSTs are not elevated to the same extent in all cells. Thus, the observed heterogeneity may reflect intercellular variability in cytotoxicity, in GST mRNA stability or in the concentration and integrity of the transcriptional factors required for induction of GST $\alpha$. Differences in the transcriptional and/or translational regulation of the GST $\alpha$ genes in WS and WR cells were supported by corresponding elevations in mRNA at 4 and 2 days (respectively) prior to elevations in GST activity (Figs. 3 and 4).

Elevations in GSTA2 mRNA following chlorambucil exposure are in direct agreement with previous findings of increased A2-2 protein in WR cells [5] and GST $\alpha$ protein and transcripts in chlorambucil-resistant Chinese hamster ovary cells (CHO-Chl$^r$) [7]. Unlike the present study, both GSTA1-1 and A2-2 proteins and mRNA were overexpressed in CHO-Chl$^r$ cells. Southern blot analyses linked these alterations with amplification of the GSTA1 and A2 genes. The absence of DNA amplification in WR cells is consistent with a less stable drug resistance which requires at least biannual exposure to chlorambucil in order to maintain resistance.

Induction of the GSTA2-2 but not the A1-1 isozyme following chlorambucil exposure confirmed the independent regulation of the A1 and A2 GST genes. Treatment of rats with phenobarbital causes the selective elevation of A1 mRNA [17]. The hepatic $\mu$ and $\pi$ class isozymes are preferentially elevated in mice treated with butylated hydroxyanisole [25]. Differential regulation of the GST genes has also been observed in cultured hepatocytes [26] and in human colon biopsies [27]. Three *cis*-acting regulatory elements have been identified in both rats [28, 29] and mice [30] in the 5′ noncoding region of the GSTA1 gene which controls basal and inducible transcription. Transcriptional activation of the rGSTA1 gene by planar aromatic compounds requires a single copy of the $\beta$-naphthoflavone-responsive element and a functional Ah receptor [31]. In contrast, phenolic antioxidants can interact directly with an antioxidant-responsive element to activate transcription of the rGSTA1 gene [31]. The electrophile-responsive element of the mGSTA1

gene consists of two motifs (9 base-pairs each) which are similar in sequence to AP-1 binding sites and recognized by a nuclear transactivating factor [32, 33]. The regulatory elements of the GSTA2 gene have not been elucidated. The present data suggest that either a regulatory element exists which is unique to the GSTA2 gene or the sensitivity and stimuli to which the A2 regulatory sequences respond are distinct from those of the A1 gene. The role of the Ah receptor in the cellular response to chlorambucil is not known.

The ability of alkylating agents such as chlorambucil to deplete cellular thiol pools provides an alternative mechanism by which GST $\alpha$ may respond to drug exposure. Thiol imbalance may provide the required signal for a sensitive regulatory element to respond to drug exposure with enhanced transcription of the GSTA2 gene.

In summary, the development of a FACS assay for GST has enabled us to visualize the heterogeneous response of tumor cells to alkylating agent exposure. The delayed response of GST to chlorambucil exposure suggests that this enzyme may be one component in a sequence of events which collectively confer cellular protection and drug resistance. The specificity of the GST response to A2-2 confirms the role of GST $\alpha$ in the conjugation of chlorambucil with glutathione and supports the differential regulation of the rat A1 and A2 genes. Further analysis of the stability of the A2 message in sensitive and resistant cells combined with the identification and characterization of the regulatory elements in the 5′ flanking region of the A2 gene will allow us to target therapeutic interventions to those molecular events which are critical to the establishment of drug resistance.

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## REFERENCES

1. Deffie AM, Alam T, Seneviratne C, Beenken SW, Batra JK, Shea TC, Henner WD and Goldenberg GJ, Multifactorial resistance to Adriamycin: Relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res* **48**: 3595–3602, 1988.
2. 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.
3. Smith MT, Evans CG, Doane-Setzer P, Castro VM, Tahir MK and Mannervik B, Denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea by class mu glutathione transferases and its role in cellular resistance in rat brain tumor cells. *Cancer Res* **49**: 2621–2625, 1989.
4. 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.
5. Buller AL, Clapper ML and Tew KD, Glutathione *S*-transferases in nitrogen mustard-resistant and -sensitive cell lines. *Mol Pharmacol* **31**: 575–578, 1987.
6. Clapper ML and Tew KD, Identification of a glutathione *S*-transferase associated with microsomes of tumor cells resistant to nitrogen mustards. *Biochem Pharmacol* **38**: 1915–1921, 1989.
7. Lewis AD, Hickson ID, Robson CN, Harris AL, Hayes JD, Griffiths SA, Manson MM, Hall AE, Moss JE and Wolf CR, Amplification and increased expression of alpha class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc Natl Acad Sci USA* **85**: 8511–8515, 1988.
8. Dulik DM, Colvin OM and Fenselau C, Characterization of glutathione conjugates of chlorambucil by fast atom bombardment and thermospray liquid chromatography/mass spectrometry. *Biomed Environ Mass Spectrom* **19**: 248–252, 1990.
9. Ciaccio PJ, Tew KD and LaCreta FP, The spontaneous and glutathione S-transferase-mediated reaction of chlorambucil with glutathione. *Cancer Commun* **2**: 279–286, 1990.
10. Bolton MG, Colvin OM and Hilton J, Specificity of isozymes of murine hepatic glutathione *S*-transferase for the conjugation of glutathione with L-phenylalanine mustard. *Cancer Res* **51**: 2410–2415, 1991.
11. Ciaccio PJ, Tew KD and LaCreta FP, Enzymatic conjugation of chlorambucil with glutathione by human glutathione *S*-transferases and inhibition by ethacrynic acid. *Biochem Pharmacol* **42**: 1504–1507, 1991.
12. Black SM, Beggs JD, Hayes JD, Bartoszek A, Muramatsu M, Sakai M and Wolf CR, Expression of human glutathione *S*-transferases in *Saccharomyces cerevisiae* confers resistance to the anticancer drugs Adriamycin and chlorambucil. *Biochem J* **268**: 309–315, 1990.
13. Puchalski RB and Fahl WE, Expression of recombinant glutathione *S*-transferase $\pi$, Ya or $Yb_1$ confers resistance to alkylating agents. *Proc Natl Acad Sci USA* **87**: 2443–2447, 1990.
14. Townsend AJ, Tu C-PD and Cowan KH, Expression of human $\mu$ or $\alpha$ class glutathione *S*-transferases in stably transfected human MCF-7 breast cancer cells: Effect on cellular sensitivity to cytotoxic agents. *Mol Pharmacol* **41**: 230–236, 1992.
15. Tu C-PD, Lai H-CJ, Li N-q, Weiss MJ and Reddy CC, The $Y_c$ and $Y_a$ subunits of rat liver glutathione *S*-transferases are the products of separate genes. *J Biol Chem* **259**: 9434–9439, 1984.
16. Telakowski-Hopkins CA, Rodkey JA, Bennett CD, Lu AYH and Pickett CB, Rat liver glutathione *S*-transferases. Construction of a cDNA clone complementary to a Yc mRNA and prediction of the complete amino acid sequence of a Yc subunit. *J Biol Chem* **260**: 5820–5825, 1985.
17. Pickett CB, Donohue AM, Lu AYH and Hales BF, Rat liver glutathione *S*-transferase B: The functional mRNAs specific for the Ya Yc subunits are induced differentially by phenobarbital. *Arch Biochem Biophys* **215**: 539–543, 1982.
18. Tisdale MJ and Phillips BJ, Alterations in adenosine 3′,5′-monophosphate-binding protein in Walker carcinoma cells sensitive or resistant to alkylating agents. *Biochem Pharmacol* **25**: 1831–1836, 1976.
19. Batist G, Behrens BC, Makuch R, Hamilton TC, Katki AG, Louie KG, Myers CE and Ozols RF, Serial determinations of glutathione levels and glutathione-related enzyme activities in human tumor cells *in vitro*. *Biochem Pharmacol* **35**: 2257–2259, 1986.
20. Habig WH and Jakoby WB, Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol* **77**: 398–405, 1981.
21. Johnson DA, Gautsch JW, Sportsman JR and Elder

JH, Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal Tech* **1**: 3–8, 1984.
22. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K, *Current Protocols in Molecular Biology*. Wiley–Interscience, New York, 1989.
23. Schmid I, Uittenbogaart CH and Giorgi JV, A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry* **12**: 279–285, 1991.
24. Prochaska HJ, De Long MJ and Talalay P, On the mechanisms of induction of cancer-protective enzymes: A unifying proposal. *Proc Natl Acad Sci USA* **82**: 8232–8236, 1985.
25. Stanley JS, York JL and Benson AM, Nitroreductases and glutathione transferases that act on 4-nitroquinoline 1-oxide and their differential induction by butylated hydroxyanisole in mice. *Cancer Res* **52**: 58–63, 1992.
26. Abramovitz M, Ishigaki S and Listowsky I, Differential regulation of glutathione S-transferases in cultured hepatocytes. *Hepatology* **9**: 235–239, 1989.
27. Clapper ML, Hoffman SJ and Tew KD, Glutathione *S*-transferases in normal and malignant human colon tissue. *Biochim Biophys Acta* **1096**: 209–216, 1991.
28. Telakowski-Hopkins CA, King RG and Pickett CB, Glutathione *S*-transferase Ya subunit gene: Identification of regulatory elements required for basal level and inducible expression. *Proc Natl Acad Sci USA* **85**: 1000–1004, 1988.
29. Rushmore TH, King RG, Paulson KE and Pickett CB, Regulation of glutathione *S*-transferase Ya subunit gene expression: Identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc Natl Acad Sci USA* **87**: 3826–3830, 1990.
30. Daniel V, Sharon R and Bensimon A, Regulatory elements controlling the basal and drug-inducible expression of glutathione S-transferase Ya subunit gene. *DNA* **8**: 399–408, 1989.
31. Rushmore TH and Pickett CB, Transcriptional regulation of the rat glutathione *S*-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem* **265**: 14648–14653, 1990.
32. Friling RS, Bergelson S and Daniel V, Two adjacent AP-1-like binding sites form the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. *Proc Natl Acad Sci USA* **89**: 668–672, 1992.
33. Friling RS, Bensimon A, Tichauer Y and Daniel V, Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc Natl Acad Sci USA* **87**: 6258–6262, 1990.