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Characterization of a Chlorambucil-Resistant Human Ovarian Carcinoma Cell Line Overexpressing Glutathione *S*-Transferase μ

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ABSTRACT. Ovarian carcinoma cells 10-fold resistant to the alkylating agent chlorambucil (CBL) were isolated after repeated exposure of the parent cells to gradually escalating concentrations of the drug. The resistant variant, A2780(100), was highly cross-resistant (9-fold) to melphalan and showed lower-level resistance to other cross-linking agents. The resistant A2780(100) cells had almost 5-fold higher glutathione *S*-transferase (GST) activity than the parental A2780 cells with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The π-class GST(s) was the major isoform(s) in both cell lines. However, the resistant A2780(100) cells had at least 11-fold higher GST μ as compared with the parental cells, in which this isoform was barely detectable. A significant induction of GST μ was observed in A2780 cells, but not in the resistant cells, 18 hr after a single exposure to 100 μM CBL. The induction of GST μ by CBL was both time- and concentration-dependent. Assays of the conjugation of CBL with GSH showed that the human μ-class GST had 3.6- and 5.2-fold higher catalytic efficiency relative to the π- and α-class GSTs, respectively. This difference was reflected in the relatively higher (about 6-fold) efficiency of CBL conjugation in A2780(100) cells as compared with the parental cells. These results have demonstrated for the first time a near-linear correlation between CBL resistance and overexpression of μ-class GSTs and suggest that this overexpression maybe responsible, at least in part, for the acquired resistance of ovarian carcinoma cells to CBL, and possibly the other bifunctional alkylating agents. Consistent with this hypothesis, we found evidence for decreased formation of DNA lesions in A2780(100) compared with the drug-sensitive A2780 cells after exposure to CBL. BIOCHEM PHARMACOL **58**;4:693–702, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. glutathione *S*-transferase; chlorambucil; ovarian carcinoma; drug resistance

The emergence of drug resistance is a major reason for the failure of cancer chemotherapy, and, in many cases, the biochemical basis for its development is still obscure. Selection of stably resistant tumor cell lines in culture has led to identification of the multidrug resistance gene (*MDR-1*) and its product, P-glycoprotein, and the multidrug resistance-associated protein (MRP), proposed to mediate active transport of cytotoxic agents from cells [1, 2]. The multidrug resistance phenotype results in resistance to many commonly used antitumor agents, including the anthracyclines and the *Vinca* alkaloids, but, in general, not to alkylating drugs such as the nitrogen mustards. Alkylating agents are used routinely in the therapy of a variety of solid tumors as well as leukemias and lymphomas [3], and resistance to these drugs is also commonly observed. Therefore, a better understanding of the mechanisms of resistance to these agents is critical for developing more successful chemotherapeutic regimens.

The different alkylating drugs generate different types of DNA adducts, but the major cytotoxic lesion induced by the nitrogen mustards appears to be interstrand cross-links generated from the primary alkyl base adduct, 7-alkylguanine (reviewed in Ref. 4). Thus, enhanced repair of the initial alkyl adduct by the base excision repair pathway or of the DNA cross-link may result in resistance to the alkylating agent. There is some evidence that drug-resistant cells have a greater capacity to recognize and repair DNA damage [5]. However, multiple steps may affect the formation of the cytotoxic DNA lesions. These include intracel-

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lular uptake, e.g. melphalan is actively taken up using the transporter for neutral amino acids [6], and metabolism of the drug, e.g. cyclophosphamide is converted to its active species [7]. The potential for detoxification of the free intracellular drug or its reactive metabolite, e.g. by conjugation with GSH through the reaction catalyzed by GSTs,* is also important. In addition, for drugs such as CBL and melphalan, where there is a delay between formation of drug–DNA monoadducts and formation of DNA cross-links, it has been suggested that GSH and GSTs also may be involved in DNA monoadduct repair [8].

The GST superfamily is composed of three major (α, μ, and π) and two minor (θ and σ) classes of cytosolic isozymes, along with a membrane-bound or microsomal isozyme (see Ref. 9 for a review). GST-catalyzed conjugation with GSH has been demonstrated for the nitrogen mustard drugs CBL and melphalan [10, 11], although whether such conjugation contributes significantly to the overall drug detoxification has been questioned recently [12]. The fact that overexpression of various GST isozymes has been observed in a number of tumor cell lines selected *in vitro* for resistance to DNA alkylating chemotherapeutic agents [8, 13] suggests that GSTs may be involved in the mechanisms of resistance to these drugs. This possibility is consistent with studies showing that the depletion of GSH, the substrate for GST, by pretreatment with buthionine sulfoximine or treatment with GST inhibitors can potentiate the cytotoxicity of alkylating drugs [8, 14]. However, mixed results have been obtained when drug-sensitive cell lines were transfected with GST isozymes. For example, no drug resistance was seen in MCF-7 human breast cancer cells transfected with GST isozymes [15], whereas transfection of rat α-class isozyme into rat mammary tumor cells conferred resistance to both CBL and melphalan [16]. Thus, the role of GSTs in acquired resistance towards alkylating drugs remains to be defined.

To examine the potential involvement of GSTs in alkylating drug resistance of tumor cells, an ovarian carcinoma cell line resistant to CBL and highly cross-resistant to melphalan has been isolated. CBL was chosen because its cellular uptake occurs by simple diffusion [17], and also, despite extensive oxidation of its side chain, it does not require tissue-specific metabolism to an active form [18]. Furthermore, increases in GST activity have been seen in lymphocytes from CBL-resistant chronic lymphocytic leukemia patients [19]. We now report that CBL resistance in the A2780 variant is associated with specific activation of the GST μ isozyme and propose that the highly efficient mechanism for detoxification of CBL by GST μ in the resistant cells is responsible, at least in part, for the observed CBL resistance.

* *Abbreviations*: CBL, chlorambucil; GST(s), glutathione *S*-transferase(s); CDNB, 1-chloro-2,4-dinitrobenzene; MMS, methyl methanesulfonate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; PCR, polymerase chain reaction; and BSO, L-buthionine-[*S, R*]-sulfoximine.

MATERIALS AND METHODS

Materials

CBL, melphalan, MMS, and BSO were purchased from the Sigma Chemical Co. Mitomycin C (Mutamycin), cisplatin (Platinol-AQ), and BCNU (BiCNU) were obtained from Bristol Laboratories. Vinblastine was purchased from Lymphomed, and doxorubicin (Adriamycin RDF) from Adria Laboratories.

Isolation of the CBL-Resistant Variant

A2780 human ovarian carcinoma cells, provided by Dr. K. J. Scanlon, were cultured routinely in antibiotic-free RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (HyClone). Isolation of a CBL-resistant line was initiated by treating A2780 cells with 20 μM CBL for 1 hr at 37° in serum-free medium in a 5% CO_2 incubator. This concentration resulted in the killing of more than 90% of the cells. The CBL treatments were repeated continuously after recovery of the cells, and the CBL concentration gradually increased to 100 μM over the course of 6 months. The resistance of the cell line obtained at this time, A2780(100), was maintained by treating approximately once every week for 1 hr with 100 μM CBL in serum-free medium.

Cytotoxicity Assays

Drug sensitivity was determined by clonogenic assays. Cells (1500–2000 per well) were plated in 6-well tissue culture dishes, and then after 24 hr were washed and exposed for 1 hr at 37° to a range of concentrations of cytotoxic agent in serum-free RPMI medium. The agents tested were CBL, melphalan, MMS, mitomycin C, cisplatin, BCNU, vinblastine, and doxorubicin. For UV irradiation (254 nm), cell monolayers were washed twice before exposure to UV radiation (fluence 37.9 μW cm^{-2}) for the required length of time. Complete growth medium containing 10% serum was then added to each well. After 6–8 days, colonies were air-dried, stained with crystal violet, and counted using an automated Artek model 880 colony counter (Imaging Products International). The IC_{50} values (defined as the concentration required for 50% reduction in colony number compared with untreated controls) were determined from concentration–percentage of cell survival curves. The sensitivity of A2780(100) cells to CLB was also determined after preincubation with BSO to deplete intracellular GSH [20]. Preliminary experiments showed that a 24-hr incubation with 200 μM BSO resulted in a 90% reduction in the intracellular GSH level (data not shown). Following the BSO incubation, cells were treated with CBL in serum-free medium as described above.

Purification of GST Isozymes

Actively growing A2780 and A2780(100) cells were treated for 1 hr with 1–100 μM CBL in serum-free RPMI or

with an equivalent concentration of solvent (0.1% acid alcohol) alone. After 4–18 hr, cells were washed with PBS and isolated by scraping. Purification of the 'total GSTs' fraction from sensitive and resistant cell extracts was according to a method described previously [21]. Human liver and lung tissues used for the purification of GST isozymes were obtained from the autopsy service of The University of Texas Medical Branch. The use of human tissues was approved by the Institutional Review Board. Individual isozymes from human liver and lung were separated by isoelectric focusing on an LKB-8100 column using ampholines in the pH range of 3.5 to 10.0, and a 0–50% (w/v) sucrose density gradient as described previously [22]. The purity of the isozymes was ascertained by SDS–PAGE, and their immunological cross-reactivity only with the antibodies raised against their respective classes was ascertained by western blot analysis [22]. The polyclonal antibodies specific against the α, μ, and π class human GSTs were the same as used in our previous studies [21].

Determination of GST Enzyme Activity

The total GST activity with CDNB as substrate was determined by the method of Habig *et al.* [23]. GST activity with CBL was determined by quantitating the resultant CBL–GSH conjugate. The reaction mixture for the assays (100 μL) contained 1 μg of enzyme protein, 2 mM [^{3}H]GSH (labeled on glycine, specific activity 240 mCi/mmol; DuPont NEN Research Products), and 100 μM CBL in 100 mM potassium phosphate buffer, pH 7.4. The reaction mixtures were preincubated for 5 min at 37° before starting the reaction by the addition of CBL and then incubated for an additional 30-min period at 37°. The reaction was terminated by the addition of 100 μL of cold (4°) methanol and centrifugation to remove precipitated protein. The CBL–GSH conjugate product was separated by TLC [4 hr in butanol:acetic acid:water (4:2:2)] and visualized with 0.2% ninhydrin reagent. The areas corresponding to authentic CBL–GSH conjugate were scraped, and the radioactivity was quantitated by liquid scintillation counting. The corresponding areas from control incubations containing no GST enzyme also were scraped and counted. The rate of enzymatic conjugation was determined by subtracting the rate of the nonenzymatic reaction.

The enzyme activity was measured in duplicate at various concentrations of CBL (10–200 μM) and a fixed concentration of GSH (2 mM). The K_m and k_{cat} values were determined from double-reciprocal plots. One unit of GST activity was defined as the amount required for the conjugation of 1 μmol substrate/min. GST activities were statistically evaluated with a *t*-test ($P = 0.05$).

Determination of Intracellular GSH Levels

The intracellular GSH concentration in both cell lines was determined as described previously [16]. For determination of relative GSH levels in cells following exposure to CBL, a Bioxytech GSH-400 assay kit was used (Oxis International, Inc.). Cells were treated for 1 hr with 100 μM CBL as described previously, harvested at times 0–48 hr later, and homogenized in the presence of 5% metaphosphoric acid. The homogenates were centrifuged at 12,000 *g* for 10 min at 4°, and GSH levels were assayed in the supernatant fractions. The Bioxytech kit method is based on the formation of thioester substitution products between SH-containing substances and 4-chloro-1-methyl-7-trifluoromethylquinolinium metasulfate and mercaptans. Under alkaline conditions, the substitution product obtained with GSH is converted into a chromophoric thione with maximal absorbance at 400 nm [24].

Determination of DNA Lesion Frequency by Quantitative PCR

A2780 and A2780(100) cells were incubated with various concentrations of CBL (0–600 μM) for 1 hr, and then incubated in drug-free medium for 3 hr. DNA was isolated from frozen cell pellets (3×10^6 cells) with the QIAamp® DNA isolation kit (Qiagen) and quantitated by ethidium bromide fluorescence [25]. Quantitative amplification of the β-globin target sequence was performed in a GeneAmp PCR System 2400 with the GeneAmp XL PCR kit (Perkin-Elmer) as described previously [25]. Lesion frequency = ln A_d/A_o, where A_d is the amplification at a particular dose, d, and A_o is the level of amplification in non-damaged controls.

Quantitation of Interstrand DNA Cross-Links

Parental A2780 cells and the A2780(100) resistant variant were labeled with either 0.05 μCi/mL of [^{14}C]thymidine (specific activity 51.7 mCi/mmol) or 0.5 μCi/mL of [^{3}H]thymidine (specific activity 20 Ci/mmol). The cells labeled with [^{14}C]thymidine were treated for 1 hr with 100 μM CBL, then washed three times with PBS, and incubated in drug-free medium for various times. Next, control or CBL-treated [^{14}C]thymidine-labeled cells (0.5×10^6) were mixed with [^{3}H]thymidine-labeled cells (0.5×10^6) and irradiated, at 4°, with 4 Gy using a ^{60}Co γ-ray source and a dose rate of 1 Gy/min. The frequency of DNA–DNA cross-linking was estimated by alkaline elution as previously described [26].

Western Blot Analysis

A2780 and A2780(100) cells were treated for 1 hr with 100 μM CBL or solvent alone in serum-free medium, and cell extracts were prepared 18 hr later. Western blots were probed with antibodies to DNA repair proteins obtained as indicated: Ku 80 (Dr. D. Chen, Los Alamos National Laboratory), ERCC4 (Dr. M. P. Thelen, Los Alamos National Laboratory), DNA polymerase β (Dr. S. H. Wilson, National Institute of Environmental Health Sciences), and topoisomerase II (Dr. E. Schneider, New York

TABLE 1. Sensitivity of A2780 and A2780(100) human ovarian carcinoma cells to cytotoxic agents

Cytotoxic agent		IC_{50}* A2780	IC_{50}* A2780(100)	Fold resistance†
CBL	(μM)	6.7	64.3	9.6
Melphalan	(μM)	12.4	110	8.9
Cisplatin	(μM)	24.3	139	5.7
BCNU	(μM)	34.3	94.2	2.7
Mitomycin C	(μM)	1.2	2.5	2.2
MMS	(mM)	1.4	2.2	1.6
UV	(J/m^2)	5.9	9.1	1.5
Doxorubicin	(μM)	0.25	0.35	1.5
Vinblastine	(μM)	0.46	0.50	1.1

*IC_{50} values (the concentration required for 50% reduction in colony number compared with untreated controls) were obtained by clonogenic assay as described in Materials and Methods. Each value represents the mean of at least three independent experiments.

†Ratio of the IC_{50} of each agent in A2780(100) to that in A2780.

State Department of Health). Antibodies to *N*-methylpurine DNA glycosylase, AP-endonuclease, and O^6-alkylguanine-DNA alkyltransferase were produced in the Mitra laboratory. Specific bands were detected by enhanced chemiluminescence (ECL; Amersham) and visualized by exposure to Hyperfilm-ECL (Amersham).

RESULTS

Drug Sensitivity of A2780 and A2780(100) Cell Lines

The sensitivity of the parental line A2780 and the resistant variant A2780(100) to a 1-hr pulse of CBL or to exposure to a series of other cytotoxic agents was determined by clonogenic assay, and the results are summarized in Table 1. A2780(100) was found to be 9.6-fold resistant to CBL when compared with A2780 (IC_{50} values of 64.3 and 6.7 μM, respectively) and highly cross-resistant to melphalan (8.9-fold). Clonal cell lines isolated from both A2780 and A2780(100) showed a similar differential sensitivity to the selecting agent CBL (data not shown). A2780(100) was cross-resistant to other bifunctional cross-linking agents: cisplatin (5.7-fold) and, to a lesser extent, BCNU (2.7-fold) and mitomycin C (2.2-fold), but only slightly resistant to MMS, UV, and doxorubicin, and not resistant to vinblastine. The cross-resistance profile of the A2780(100) cells was similar to that found previously in a mammary adenocarcinoma cell line initially selected for melphalan resistance [8]. Preincubation of A2780(100) cells with BSO to deplete intracellular GSH was found to sensitize the resistant line to CBL (IC_{50} 10.8 μM). This result suggests that a GSH-dependent detoxification method is an important factor in the CBL resistance of A2780(100).

GST Activity and Isozymes in A2780 and A2780(100) Cell Lines

Total GST activity, with CDNB as the substrate, was 4.7-fold higher in extracts from the resistant A2728(100) cells as compared with those of parental cells (626 and 133 mU/mg protein, respectively, Table 2). When the CBL-sensitive A2780 cells were exposed to 100 μM CBL, a small but significant increase in GST activity was observed 18 hr after treatment. The activity in the resistant cells was not increased further upon CBL exposure.

To accurately assess the status of GST isozymes in the parental A2780 and resistant variant A2780(100), 'total GSTs' were purified from these cells by GSH-affinity chromatography. The preparations of 'total GSTs' were apparently free from any non-GST proteins when visualized in Coomassie-stained SDS–PAGE gels (Fig. 1A). The recovery of the purified GSTs from both of the cell lines was similar (> 95%, Table 2). The total amount of GST activity recovered after GSH-affinity chromatography from A2780(100) cells was 4.6-fold higher as compared with the parental cells (613 and 133 mU/mg protein, respectively, with CDNB as substrate, Table 2). These data are consistent with the results showing higher GST activity in the crude cellular extracts of the resistant cells. GSTs purified from the parental cells showed only a single band corresponding to 22.5 kDa (Fig. 1A) and confirmed to be GST π by western blot analysis (Fig. 1D). Total GSTs obtained from the resistant cells showed the presence of a second, major band of about 26 kDa in addition to that of 22.5 kDa (Fig. 1A) and identified as a μ class GST (Fig. 1C).

TABLE 2. GST activity in A2780 and A2780(100) human ovarian carcinoma cells

	Crude extract (nmol CDNB/min/mg cell protein)*	GSH affinity purified 'total GSTs' (nmol CDNB/min/mg cell protein)†	GSH affinity purified 'total GSTs' (nmol CBL/min/mg cell protein)*†
A2780	133 ± 12 (8)‡	133 ± 5 (4)	1.39 ± 0.50 (4)
A2780 + CBL§	161 ± 7‖ (6)	163 ± 5 (5)	ND¶
A2780(100)	626 ± 20‖ (4)	613 ± 61 (5)	7.10 ± 0.63 (5)
A2780(100) + CBL§	592 ± 11 (5)	557 ± 54 (5)	ND

*Activity with CDNB and CBL was determined as described in Materials and Methods.

†A total of 10–29 mg protein from crude extracts of the cells was used for the purification of GSTs, and the values were normalized for 1 mg protein in the crude extracts.

‡Data represent means ± SD (number of determinations).

§Exposure to CBL (100 μM) was for 1 hr, as described in Materials and Methods, and extracts were prepared 18 hr later.

‖Statistically different from control untreated A2780 cells ($P < 0.05$).

¶ND, not determined.

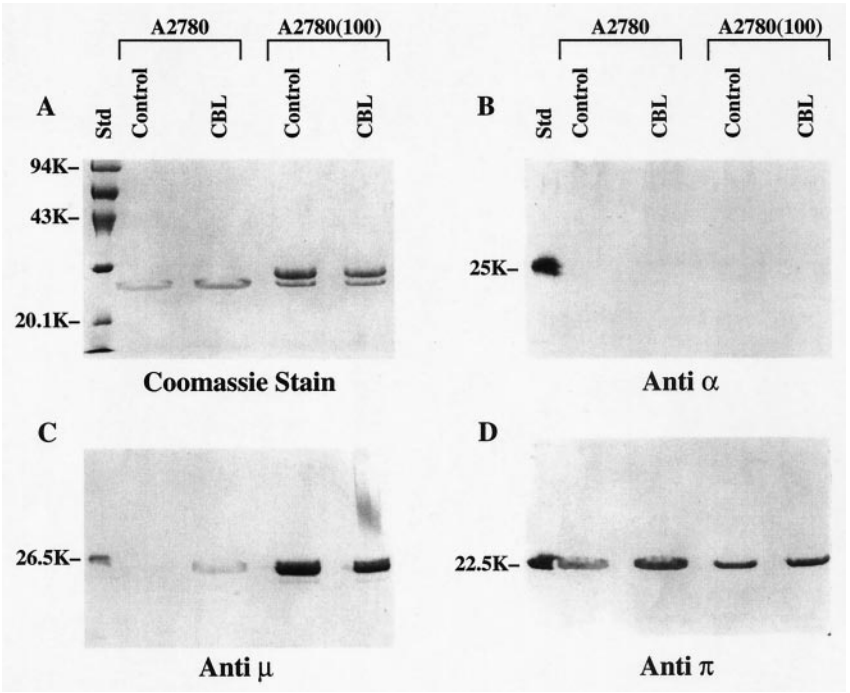

FIG. 1. Analysis of 'total GSTs' purified from A2780 and A2780(100) cell lines. (A) SDS–PAGE of GSH-affinity column-purified 'total GSTs' from A2780 and A2780(100) cell lines. Electrophoresis was carried out as described in Materials and Methods, and the gel was stained with Coomassie blue. Western blots were probed with antibodies raised against the α (B), μ (C), and π (D) classes of GST isozymes. The first lane contains protein size markers in panel A, and the respective positive control GST isozyme in panels B, C, and D. GSH-affinity column-purified protein (5 μg) from A2780 and A2780(100) cells (control) or from CBL-treated A2780 and A2780(100) (CBL) was added to each lane. CBL treatment (100 μM) was for 1 hr in serum-free medium at 37°, and cell extracts were prepared 18 hr later.

Time- and Concentration-Dependence of GST μ Induction

Western blot analysis showed that GST μ was barely detectable in control A2780 cells, but the band corresponding to this isozyme was easily observed 18 hr after a single exposure to 100 μM CBL (Fig. 1C, lane 3). Densitometric scanning of the western blot showed that the level of induction was approximately 4-fold. The level of GST μ in the resistant A2780(100) cells was about 11-fold higher than that in the parent cells (Fig. 1C, lane 4). However, unlike the parent cells, the resistant cells did not show any further activation of GST μ when exposed to CBL (Fig. 1C, lane 5). GST α was not detectable in any of the cell extracts either before or after CBL treatment (Fig. 1B). The level of expression of GST π was similar in A2780 and A2780(100) and showed a slight increase in both cell lines following CBL exposure (Fig. 1D). Therefore, these results demonstrated that exposure of A2780 cells to CBL caused a preferential induction of GST μ, and also that A2780(100) variants selected after chronic exposure to CBL have a higher basal level of expression of this class of GST.

GST μ, and to a lesser extent GST π, were induced in a time-dependent manner after a single exposure of A2780 cells to 100 μM CBL (Fig. 2, A and C). The maximal level of GST μ was achieved 18 hr after CBL treatment. The

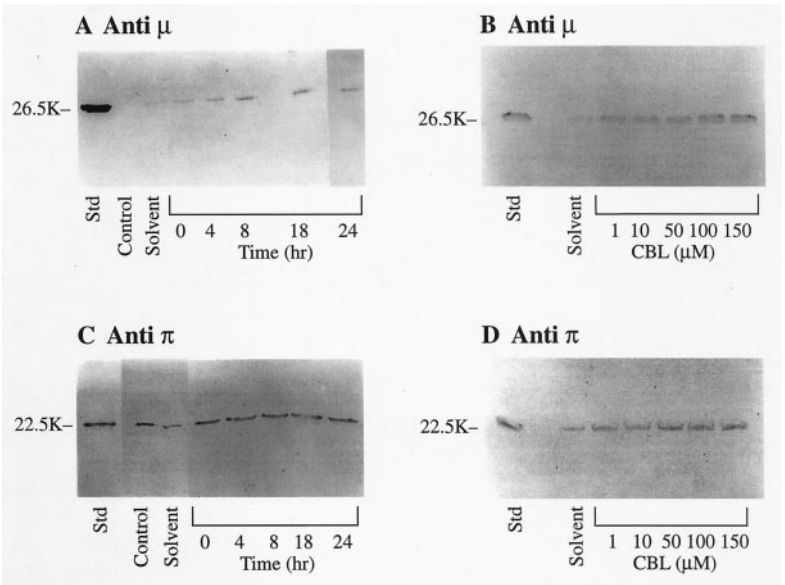

FIG. 2. Western blot analysis of the time-dependence and CBL concentration-dependence of activation of GST in 'total GSTs' purified from control and CBL-treated A2780 cells. CBL treatment was for 1 hr in serum-free medium at 37° as described in Materials and Methods. Blots were probed with antibodies raised against the μ-(A, B) and π-(C, D) classes of GST isozymes. The first lane in each panel contains the respective positive control GST isozyme. *Gels* (A, B) contain 15 μg and (C, D) 5μg GSH-affinity column-purified sample per lane. (A, C) Time-dependence. Samples from control and acid alcohol solvent-treated cells are indicated. Remaining lanes contain purified cell extracts prepared at the indicated time after 100 μM CBL treatment. (B, D) Concentration–dependence. The lane with a sample prepared from acid alcohol solvent-treated cells is indicated, and the remaining lanes contain purified samples prepared 18 hr and after a 1-hr treatment with the indicated concentration of CBL.

TABLE 3. Catalytic efficiency (k_{cat}/K_m) with CBL of human GST isozymes and total purified GSTs* prepared from A2780 and A2780(100) cells

GST isozyme source	K_m† (μM)	k_{cat}‡ (sec^{-1})	k_{cat}/K_m (10^3 M^{-1} sec^{-1})
Human liver GST α	63	0.21	3.3
Human liver GST μ	59	0.77	13.1
Human lung GST π	67	0.32	4.8
A2780 cells (total purified GSTs)	77	0.23	3.0
A2780(100) cells (total purified GSTs)	56	0.96	17.1

*GSTs were purified by GSH-affinity chromatography, and activities with CLB were determined as described in Materials and Methods.

†Each value represents the mean of two independent determinations. The experimental values were within 5% of each other.

‡Calculated on the basis of the molecular masses (kDa) of the purified isozymes: GST α, 50; GST μ, 52; GST π, 45; and A2780 and A2780(100) GSTs, 50.

level of induction of both GST μ and GST π was CBL concentration-dependent, reaching a maximum after exposure to 100 μM (Fig. 2, B and D). Whereas GST μ was barely detectable in control A2780 cells (Fig. 2A, lane 2), a small activation of GST μ was observed 18 hr following a 1-hr treatment with the solvent used to dissolve CBL (Fig. 2A, lane 3, and Fig. 2B, lane 2). However, the induction achieved by CBL exposure was clearly markedly higher at this time point (Fig. 2A, lane 7, and Fig. 2B, lanes 3–7).

Catalytic Efficiency of GST Isozymes with CBL as Substrate

The increase in expression of μ-class GSTs in the CBL-resistant line was associated with a 5.1-fold increase in GST activity, with CBL as substrate (Table 2). It is evident from the data presented in Table 3 that human liver μ class GSTs were more efficient at catalyzing the conjugation of CBL to GSH than the α-class GSTs of liver and the GST π of lung. The catalytic efficiency of human liver GST μ, as judged by k_{cat}/K_m (Table 3), indicated that the human liver μ class GSTs were 3.6- and 5.2-fold more efficient in the conjugation of CBL to GSH as compared with lung GST π and hepatic α-class GST isoenzymes, respectively. A direct comparison of the efficiencies of the 'total GSTs' purified from the A2780 and A2780(100) cells in catalyzing the conjugation of CBL to GSH showed that the purified fraction from the resistant cells had an almost 6-fold higher catalytic efficiency than did the parental cells (Table 3). These results demonstrate that CBL-resistant cells may be able to detoxify CBL more efficiently than the parental cells and are consistent with the idea that the induction of GST μ may contribute towards more efficient detoxification of CBL and be responsible for the resistance of A2780(100) cells towards this drug.

Effect of CBL on Intracellular GSH Levels in A2780 and A2780(100) Cell Lines

The intracellular levels of GSH, 25.6 ± 0.6 and 25.2 ± 1.8 nmol/mg protein in A2780 and A2780(100), respectively, were similar in both cell lines. After treatment with 100 μM CBL, GSH levels in both cell lines decreased within the first 3 hr to 47 and 59% of control in A2780 and A2780(100), respectively (Fig. 3). At later time points, the GSH in A2780 cells remained depleted, reaching a value of 39% of control at 48 hr. In contrast, the GSH in A2780(100) cells began to increase by 6 hr following treatment and reached control levels after 18 hr (Fig. 3). The initial depletion of GSH following CBL exposure is consistent with the hypothesis that CBL is detoxified in both cell lines by conjugation with GSH in a reaction catalyzed by GSTs. In addition, the data suggest that the parental A2780 cells have a limited capacity to resynthesize GSH when compared with the drug-resistant A2780(100) cells. An elevated GST activity as well as enhanced GSH synthesis capability may be necessary to enable the A2780(100) cells to support efficient GST-catalyzed detoxification of CBL.

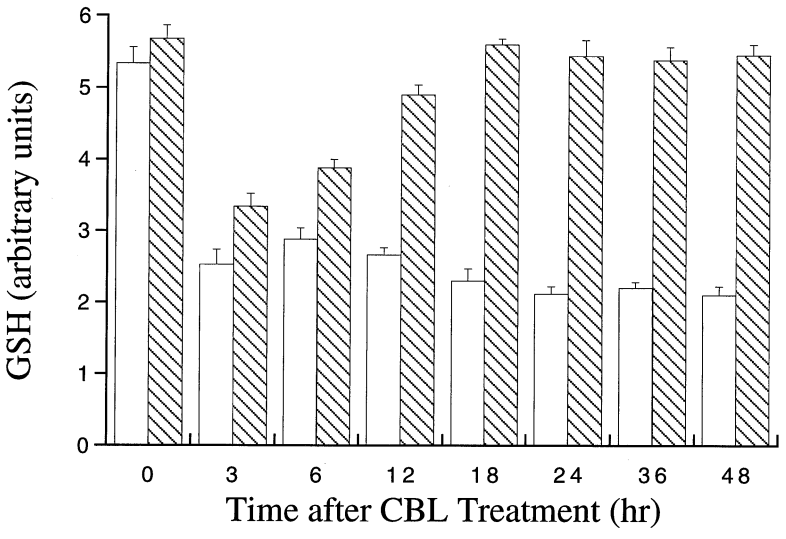

FIG. 3. Effect of CBL treatment on intracellular GSH levels in drug-sensitive A2780 (open bars) and drug-resistant A2780(100) (hatched bars). CBL treatment (100 μM) was for 1 hr in serum-free medium at 37°, and intracellular GSH was determined at the indicated time after treatment as described in Materials and Methods. Values represent means ± SD (N = 3).

TABLE 4. Interstrand cross-links in A2780 and A2780(100) human ovarian carcinoma cells after treatment with CBL*

Time after CBL treatment (hr)	Cross-link index ($\times 10^3$)	
	A2780	A2780(100)
0	3 ± 1	2 ± 1
2	75 ± 30	70 ± 20
4	155 ± 10	135 ± 10
6	330 ± 59	200 ± 59†
20	195 ± 30	80 ± 20†

*Cells were exposed to 100 μM CBL for 1 hr, washed, and then incubated in drug-free medium for the indicated times. Values represent means ± range of two independent experiments.

†Statistically different from A2780 cells ($P < 0.05$).

Quantitation of DNA Lesions Following Exposure of A2780 and A2780(100) Cell Lines to CBL

If CBL can be detoxified more efficiently by the GST μ induced in the resistant cells, it would be expected that this would result in a decrease in the formation of DNA lesions in the resistant compared with the sensitive cells. An indicator of the presence of DNA lesions in cells is the ability of a genomic DNA segment to support PCR amplification, since DNA sequences containing DNA polymerase-blocking or -terminating lesions will not be amplified in this assay [25]. We analyzed, by quantitative PCR, the total DNA damage in a fragment of the β-globin gene in both sensitive and resistant cell lines, 3 hr following exposure to a range of concentrations (0–600 μM) of CBL. Cells treated with increasing concentrations of CBL showed a linear increase in damage, and a 3.6-fold increase in DNA lesion frequency was observed in the sensitive compared with the resistant cells (data not shown). In additional experiments, the DNA cross-link index in sensitive and resistant cells was determined by an alkaline elution procedure. A2780 and A2780(100) cells were exposed to 100 μM CBL for 1 hr, and then incubated in the absence of drug for up to 20 hr. Cross-links were induced in both cell lines, reaching a maximum at 6 hr following CBL treatment (Table 4). At both 6 and 20 hr following exposure to CBL, there was a significantly decreased formation of cross-links in A2780(100) compared with A2780.

At the 3-hr time point used in the quantitative PCR experiments, it is likely that many of the lesions detected are DNA monoadducts, since the formation of DNA cross-links after CBL treatment of A2780 cells was a delayed process (Table 4). The results of this experiment suggest that there is a reduction in formation of DNA monoadducts in the resistant cells and are consistent with an extremely effective conjugation of CBL with GSH, and hence CBL detoxification in the A2780(100)-resistant cell line. In addition, the cross-link data are also consistent with the hypothesis that the elevated levels of GSH resynthesis in A2780(100) cells allow for enhanced DNA monoadduct detoxification. However, it is also possible that the decreased DNA cross-linking found in the resistant cells could result, in part, from an enhanced rate of cross-link removal by a DNA repair process. Therefore, it was imporant to determine whether various DNA repair proteins were constitutively overexpressed in the resistant cells or up-regulated after exposure of either cell line to a single concentration of CBL. However, western blotting data did not conclusively demonstrate an elevation in any of the DNA repair pathways analyzed in the resistant variant (data not shown).

DISCUSSION

A CBL-resistant variant of the human ovarian carcinoma cell line A2780 was developed by repeated exposure to escalating concentrations of CBL. The 10-fold CBL-resistance of the variant was accompanied by an 11-fold induction in GST μ, suggesting a near-linear correlation between these two parameters. Kinetic studies of GSTs purified from the parental cells and the resistant variant revealed that the resistant cell extracts were about 6-fold more efficient in catalyzing the conjugation of CBL with GSH. In addition, there was decreased formation of DNA lesions in A2780(100) compared with the drug-sensitive A2780 cells after exposure to CBL. These results are consistent with the hypothesis that the total GSTs present in the resistant cells can conjugate and therefore detoxify intracellular CBL more effectively. This may, at least in part, account for the resistance of A2780(100) cells to CBL.

A correlation between GST μ overexpression and nitrogen mustard resistance has not been described previously. In our study, the sensitive and resistant variants had comparable levels of GST π and no detectable GST α, but the resistant cells had remarkably higher levels of GST μ than the parental cells. In fact, the higher level of GST activity in the resistant cells can be accounted for completely by the overexpression of GST μ. Our results showing that the μ-class GSTs were the most efficient of the major human GSTs at catalyzing the reaction with CBL, and the fact that the resistant line could be sensitized to CBL by GSH depletion, also support the hypothesis that the overexpression of GST μ and the detoxification of CBL by conjugation with GSH are responsible for the observed CBL resistance.

Previous studies by Ciaccio *et al.* [27] have suggested that the α-class GSTs of human liver are more efficient in catalyzing the conjugation of CBL with GSH as compared with GST π isolated from the human ovary. These studies, however, did not investigate the activity of μ-class GSTs with CBL. In the present study, human lung GST π showed slightly higher activity as compared with human liver GST α. The apparent differences in the relative activities of GST π and α with CBL in the present studies and those by Ciaccio *et al.* may arise due to the different origin of GST π (ovarian GST π in the previous study vs lung GST π in the present study), or to differences in the protocols for the purification of the enzyme.

The relative predominance of π-class GSTs has been noted previously in malignant ovarian epithelial tissue and in a number of other human ovarian carcinoma cell lines [28 , 29]. Increased levels of GST π expression have been linked to drug resistance in ovarian carcinoma [30]. However, it is noteworthy that a single exposure of the parental A2780 cells to 100 μM CBL specifically induced GST μ with only a small effect on constitutive GST π. In addition, repeated exposure of A2780 cells to CBL over a 6-month period also resulted in stable and selective high-level expression of GST μ, with no change in the level of GST α or induction of GST π. Therefore, it appears likely that GST μ was specifically up-regulated in A2780 cells in response to the chemical stress brought about by CBL treatment. Furthermore, it is likely that the A2780(100) variant cells were selected because of their constitutive overexpression of GST μ, which was able to counteract the repeated exposure to CBL. This also may account for the cross-resistance of these cells to melphalan. Recent studies have suggested that GSTs of all three major classes may contribute almost equally to the detoxification of intracellular melphalan [31].

The present study does not provide any information as to which of the five μ-class genes [9] are expressed in A2780 cells and which are up-regulated during the development of resistance to CBL. In six other ovarian carcinoma cell lines, low level expression of GSTM2 and M3 was observed, but there was no detectable expression of GSTM1 [29]. It is noteworthy that in approximately 50% of the Caucasian population the gene for GSTM1 is deleted, presumably due to unequal crossing over [32]. In the current study, the purified human liver GST μ, shown to have high catalytic efficiency with CBL (Table 3), is class GSTM1–1, and the GST μ antibody used for western blotting was raised against a mixture of μ-class GSTs that are immunologically similar.

Since the primary function of GSTs is believed to be the detoxification of electrophilic compounds by catalyzing their conjugation with GSH, these enzymes have been implicated in the resistance of cancer cells to chemotherapeutic agents. Previous studies have suggested that overexpression of the α-class GSTs may be a factor in cells selected for resistance to CBL and other nitrogen mustards [13, 33] and that GST π plays a role in cisplatin and mitomycin C resistance [34, 35]. However, the results of transfection studies with GSTs neither firmly establish nor refute the role of GSTs in drug-resistance towards the alkylating cross-linking agents. A low level of resistance to CBL, melphalan, and cisplatin cytotoxicity has been demonstrated in mouse C3H/10T1/2 and COS monkey kidney cells transfected with the α- or π-class GSTs [36], and transfection of intrinsically drug-resistant colon cancer cells with antisense GST π increased sensitivity to melphalan and cisplatin [37]. In contrast, MCF-7 cells transfected with GST α-, μ-, or π-class isozymes did not develop resistance towards the alkylating drugs CBL and melphalan [38 , 39]. Previously, it has been speculated that overexpression of the GST class μ isozyme in a rat glioma cell line could be associated with resistance to BCNU [40]. The denitrosation of BCNU by GST is highly isozyme-specific, with catalysis most efficient by the GST μ-class. It is noteworthy that our CBL-resistant cells showed a moderate level (2.7-fold) cross-resistance to BCNU. Also of interest is the 3-fold cross-resistance to *trans*-stilbene oxide (data not shown), a substrate known to be absolutely specific for GST class μ [41].

Consistent with the hypothesis that the elevated GST μ and the increased capacity to synthesize GSH in A2780(100) serve to detoxify CBL and DNA monoadducts and reduce DNA damage, we found that the total lesion frequency 3 hr after CBL exposure, and the formation of DNA cross-links 6 hr following CBL treatment, were significantly less in the resistant compared with the sensitive cells. Overexpression of GST α in rat mammary carcinoma cells has been associated previously with resistance to CBL and melphalan and with inhibition of cross-link formation [16]. However, there remains the possibility that enhanced cross-link removal also contributes to the observed decrease in DNA adducts in the A2780(100) resistant cells. Persistence of DNA cross-links with time is an important factor determining cell sensitivity to bifunctional alkylating agents [42]. However, in the current study, we could not detect, by western blotting, a consistently significant difference between sensitive and resistant lines in the levels of several DNA repair proteins. Additionally, the observation that A2780(100) cells display only low level resistance to MMS and UV (1.6- and 1.5-fold, respectively) suggests that neither classical base nor nucleotide excision repair pathways are involved in the mechanism of resistance.

The results of the many studies cited above suggest that if GSTs play a role in the mechanisms of resistance of cells to alkylating agents, these mechanisms are not uniform. They may depend, for example, on the origin of the cells, the constitutive GST expression and composition of the cells, the GSH concentration in the cells, and the agent selected for developing resistance. These previous results, taken together with those of the present study, suggest that cancer cells may respond to toxic alkylating agents by induction of GST as part of an adaptive response mechanism [9]. However, enhanced GST-mediated detoxification and the induction of specific GSTs isozyme(s) in these cells are selective. Further studies are needed to address these questions and to understand the possible role of GST μ in resistance to DNA cross-linking drugs.

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