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Decreased Sensitivity to Adriamycin in Cadmium-Resistant Human Lung Carcinoma A549 Cells

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ABSTRACT. Cross-resistance presents an obstacle in cancer chemotherapy. Cadmium is a potential carcinogen whose exposure has been shown in epidemiological and laboratory experiments to cause lung cancer. Cadmium also induces various forms of resistance in human lung carcinoma cells. This resistance may be shared by antineoplastic agents, which should be a concern for chemotherapy of cadmium-induced lung cancer. In the present study, two subpopulations of human lung carcinoma A549 cells with a different magnitude of resistance to cadmium toxicity were shown to have a parallel resistance to the cytotoxic action of Adriamycin (ADR), an important anticancer drug. Several factors were examined to investigate the mechanism(s) for the cross-resistance, including cellular metallothionein and glutathione (GSH) concentrations, glutathione *S*-transferase activity, *mdr1* expression, and antioxidant enzyme activities including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Only cellular GSH content was elevated consistently in the cadmium/ADR-resistant cells relative to the cadmium/ADR-sensitive cells. Treatment with buthionine sulfoximine, a specific inhibitor of GSH synthesis sensitized both cell lines to ADR only when the cellular GSH levels were depleted to about 5% of control. This BSO treatment, however, did not affect cell viability. Further study revealed that the cadmium/ADR-resistant cells have a greater capacity in recovery of cellular GSH content following BSO treatment. The results demonstrate that cross-resistance to ADR exists in cadmium-resistant human lung carcinoma A549 cells, and enhanced GSH synthesis capacity, rather than elevated levels of cellular GSH, may be related to this resistance. BIOCHEM PHARMACOL 53;5:747–754, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. A549 cells; Adriamycin; cadmium-resistance; cross-resistance; glutathione

ADR† is a widely used anticancer agent that is highly effective against a broad spectrum of cancers. However, the development of drug resistance is a major limiting factor to its clinical application. The antitumor activity of ADR is believed to be due primarily to its ability to intercalate DNA. ADR has also been demonstrated to generate oxidative stress by undergoing redox cycling in the presence of molecular oxygen. Correspondingly, several mechanisms of ADR resistance have been identified in tumor cells *in vitro*. Among them, overexpression of the multidrug resistance MDR1 glycoprotein, elevated cellular GSH content, and enhanced antioxidant enzyme activities are well recognized [1–4].

Cadmium is a widely distributed environmental contaminant that has been demonstrated to cause numerous toxic effects in humans and animals, including severe respiratory and renal diseases [5]. Experimental and epidemiological studies have shown that cadmium is also a respiratory carcinogen [5]. Neither the mechanism of cadmium toxicity nor its mechanism of carcinogenicity is well understood, although several factors are associated with resistance to cadmium toxicity. Enhanced metallothionein synthesis and decreased cadmium accumulation are two well-known mechanisms of cadmium resistance. Recently, elevated GSH content and enhanced antioxidant enzyme activities were also identified as possible mechanisms [6–8].

It is possible that the protective mechanisms elicited by cadmium are also responsible for cross-resistance to ADR. For example, studies have demonstrated that cadmium induces *mdr1* expression in human renal carcinoma cells [9], and MDR1 glycoprotein is a mechanism responsible for resistance to ADR in some lung carcinoma cells [10, 11]. In non-MDR1-related drug resistance, GSH or the heavy metal chelator metallothionein is commonly associated with resistance to ADR [2, 3, 12, 13]. Therefore, understanding the cadmium resistance mechanisms that may be shared by anticancer agents in lung carcinoma cells could lead to improved chemotherapeutic treatment of lung can-

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† *Abbreviations:* ADR, Adriamycin; BSO, L-buthionine-(*S,R*)-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's Modified Eagle's Medium; DTNB, 2,4-dinitrofluorobenzene; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione-*S*-transferase. MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; SOD, superoxide dismutase; and SSA, sulfosalicylic acid.

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cers, particularly those induced by cadmium. In the present study, we selected two subpopulations of human lung carcinoma A549 cells with varying cadmium sensitivities to determine whether the mechanisms of cadmium resistance correlate with drug resistance to the anticancer agent ADR.

MATERIALS AND METHODS

Materials

Cadmium chloride was obtained from Fisher Scientific, Inc. (Chicago, IL), and ADR was obtained from the Sigma Chemical Co. (St. Louis, MO). McCoy's 5A medium and DMEM were purchased from GIBCO, BRL (Grand Island, NY), and FBS was from Hyclone (Logan, UT). Dissociation medium was prepared to pH 7.0 and consisted of 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM HEPES. The bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, IL). All other chemicals were purchased from either Fisher, Sigma, or Aldrich Chemical (Milwaukee, WI). All reagents were at least analytical grade.

Cell Culture

A549 human lung carcinoma cells were routinely propagated at 37° under normoxia and 5% CO_2 conditions in McCoy's 5A medium supplemented with 10% FBS. Clones were established as previously described [14]. Subcultures for experiments were established by removing the cells from monolayer stock cultures with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) and plating in 35-mm tissue culture dishes in 2 mL total volume culture medium. Tissue culture supplies were purchased from Corning Glass Works (Corning, NY).

Cadmium and ADR Sensitivities

Cadmium sensitivity was characterized by a long-term survival assay as previously described [15]. Briefly, approximately 500 cells were plated in 60-mm dishes in 5 mL medium. Cells were grown for 24 hr before adding 15 μL $CdCl_2$ solution to yield final concentrations ranging from 0 to 40 μM. After a 13-day incubation, medium was removed from each dish, and the colonies were rinsed with dissociation medium before being stained with 0.5 mL crystal violet in MeOH. The dishes were gently rinsed with tap water, and then colonies were enumerated as a function of cadmium concentration. A short-term microculture tetrazolium (MTT) assay was employed to determine the ADR cytotoxicity. In 96-well microplates, 2.5×10^4 cells per well were incubated in 0.1 ml culture medium for 36 hr before adding 10 μL ADR solution to each well. Following 12 hr of drug treatment, medium was removed and each well was rinsed with 0.1 mL dissociation medium. Then 0.1 mL fresh culture medium was added to each well, and cells were incubated for an additional 48 hr. The medium was removed and cells were incubated with 90 μL DMEM, containing no phenol red or FBS, and 10 μL MTT solution (~2 mg/mL) for 4 hr. After the DMEM and MTT were removed, the remaining formazan blue crystals were dissolved in 75 μL absolute isopropanol containing 0.04 N HCl. Absorbance at 540 nm was measured using a Bio-Tek Instruments model EL311 microplate reader. For BSO treatments, cells were incubated for 12 hr post seeding before treatment with BSO at a final concentration of 1.0 mM for 24 hr. The BSO either was removed prior to 12 hr of ADR treatment or was present concomitantly with ADR.

Cellular GSH Concentration

Total GSH was determined by the DTNB–GSH reductase recycling assay described by Tietze [16]. Briefly, approximately 2×10^5 cells were plated in 35-mm dishes in 2 mL culture medium. At 12, 24, 26, or 48 hr, the medium was removed and the cells were rinsed with 2 mL cold PBS. After incubating the cells in 0.5 mL of 5% SSA for 20 min at room temperature, the SSA solution was removed from the culture dish for cellular GSH determination. Then 0.5 mL of 0.1 N NaOH was added to the dish for protein determination. Cellular GSH content was measured as described [16]. Total protein content was determined using the Pierce BCA protein assay reagents as described by Smith *et al.* [17] with bovine serum albumin as the standard.

Enzyme Assays

Cellular enzyme extracts were prepared as previously described [15]. The assays for the following enzyme activities have been described previously [15], except for GST.

CATALASE. The enzyme activity was determined by the method described by Aebi [18]. Specific activity is expressed as micromoles H_2O_2 per minute per milligram of protein as described by Nelson and Kiesow [19].

GSH PEROXIDASE. The enzyme activity was determined by the method described by Flohe and Gunzler [20]. Specific activity is expressed as nanomoles NADPH per minute per milligram of protein.

GSH REDUCTASE. The enzyme activity was determined by the method described by Carlberg and Mannervik [21]. Specific activity is expressed as nanomoles NADPH per minute per milligram of protein.

SOD, TOTAL. The total enzyme activity was determined by the method described by Sun *et al.* [22] with some modification [15]. Bovine liver CuZnSOD was used as the standard. Specific activity is expressed as units per milligram of protein.

GST. Total enzyme activity was measured by the method of Babig *et al.* [23]. Briefly, 0.5 mL of 0.2 M KH_2PO_4 (pH

6.5) containing 2 mM EDTA, 0.3 mL ddH_2O, 50 μL of 20 mM GSH prepared in ddH_2O, and 50 μL of 20 mM CDNB prepared in 95% ethanol were combined in a 1.0-mL cuvette. The reaction was initiated by adding 100 μL cell fraction, and the change in absorbance at 340 nm was monitored for 3 min at 25°. Specific activity is expressed as nanomoles CDNB per milligram of protein.

Southern and Northern Analyses

The relative amounts of MDR1 mRNA and gene in exponentially growing cells were analyzed by northern and Southern blot assays, respectively. Cells were seeded in 100-mm dishes containing 10 mL culture medium at 8×10^6 cell/dish. For mRNA analysis, at 24 hr post seeding 50 μL saline or ADR to a final concentration of either 0.5 μM (A549-Cd/ADR^R) or 0.1 μM (A549-Cd/ADR^S) was added to each dish for 12 hr. The cells were then harvested. Briefly, total RNA was isolated for northern analysis using the RNAzol B method (Cinna/Biotecx, Friendswood, TX) and quantified spectrophotometrically. For Southern analysis, cells were harvested at 24 hr post seeding, and genomic DNA was isolated by phenol-chloroform extraction and then quantified spectrophotometrically. The RNA or DNA was then subjected to a 1% denaturating agarose gel and transferred to a GeneScreen Plus membrane (New England Nuclear, Boston, MA). Hybridization and wash procedure were conducted by the method described by Church and Gilbert [24]. The probe for MDR1 was obtained following *Eco*RI digestion of the full-length cDNA of human MDR1 from plasmid pHDR5A purchased from the American Type Culture Collection (Bethesda, MD) (Catalog No. 61360). The probe was labeled with [^{32}P]dCTP using the random-prime method of Feinberg and Vogelstein [25]. After autoradiography, the northern analysis membrane was stripped and rehybridized with human β-actin cDNA to ensure integrity of the RNA sample and to confirm that equal amounts of RNA had been loaded onto all lanes. Autoradiographic images were scanned and analyzed using the MCID system from Imaging Research Inc. (Ontario, Canada).

Statistical Analysis

Data were analyzed initially by one-way ANOVA. Student's *t*-test was employed for further determination of the significance of differences. Differences between treatments were considered significant at $P < 0.01$. The data are presented as the means ± SD from triplicate cultures for each treatment.

RESULTS

Characterization of Cadmium-Resistant Cells

A long-term survival assay was used to determine the cadmium sensitivities of two A549 subpopulations. As shown in Table 1, the LC_{50} value of the cadmium-resistant (A549-Cd^R) cells was approximately 5-fold higher than that of the cadmium-sensitive (A549-Cd^S) cells. Recent studies have shown that cadmium resistance in these cells is related to cellular GSH content [26, 27]. In addition, antioxidant capacity may also affect cadmium resistance [8, 28, 29]. Therefore, the level of GSH and the activities of GSH peroxidase, GSH reductase, catalase, and SOD were determined in the two A549 subpopulations to elucidate possible mechanisms responsible for the differential cadmium sensitivities. As shown in Tables 1 and 2, among the factors examined, only GSH was elevated in the A549-Cd^R cells, which is likely responsible for the enhanced cadmium resistance [15, 26, 27].

Cross-Resistance to Adriamycin in Cadmium-Resistant Cells

To determine whether cross-resistance to ADR occurs in the A549-Cd^R cells, an MTT assay was employed. As

TABLE 1. Characterization of cadmium-sensitive (A549-Cd^S) and cadmium-resistant (A549-Cd^R) cells

	A549-Cd^S	A549-Cd^R
$CdCl_2$ sensitivity, LD_{50} (μM)	6.9	36.8*
Metallothionein, basal (μg/mg protein)	1.11 ± 0.09	1.16 ± 0.11
Metallothionein, induced (μg/mg protein)	2.53 ± 0.47	2.53 ± 0.40
SOD (U/mg protein)	1.4 ± 0.2	1.3 ± 0.1
Catalase (μmol H_2O_2/min · mg protein)	43.9 ± 2.5	42.2 ± 8.5
GSH peroxidase (nmol NADPH/min · mg protein)	9.7 ± 1.7	11.2 ± 0.7
GSH reductase (nmol NADPH/min · mg protein)	103.7 ± 16.5	114.3 ± 16.0

For measurement of cellular basal and induced metallothionein cells were seeded at 5×10^6 cells in 20 mL medium per flask for 24 hr or for 12 hr followed by a 12-hr treatment with 10 μM $CdCl_2$, respectively, and then harvested. For enzyme activities, depending on the enzyme to be measured, between 500,000 and 750,000 cells were seeded in 5 mL medium/60-mm dish for 24 hr prior to being harvested for the respective enzymatic assay. Each value is the mean ± SD from triplicate samples.

* Significantly different from A549-Cd^S value, $P < 0.01$.

TABLE 2. Cellular GSH content in Cd/ADR-sensitive (A549-Cd/ADR^S) and Cd/ADR-resistant (A549-Cd/ADR^R) cells

	Total GSH content (nmol/mg protein)	
	A549-Cd/ADR^S	A549-Cd/ADR^R
Basal content		
Culture time		
12 hr	148.3 ± 0.1	135.7 ± 18.9
24 hr	228.8 ± 19.2	283.5 ± 16.3*
36 hr	191.3 ± 12.9	343.4 ± 33.7*
48 hr	163.0 ± 4.7	220.3 ± 10.6*
BSO Effects		
Treatment time (culture time)		
24 hr (36 hr)	19.4 ± 1.9	23.4 ± 7.4
36 hr (48 hr)	9.8 ± 0.2	9.5 ± 0.2
Recovery after 24-hr BSO treatment		
Time post BSO removal (culture time)		
4 hr (40 hr)	14.7 ± 1.9	26.2 ± 8.5*
8 hr (44 hr)	38.3 ± 6.2	73.2 ± 20.4*
12 hr (48 hr)	85.0 ± 6.9	135.4 ± 16.9*
Recovery in the presence of ADR		
Time post BSO removal (culture time)		
4 hr (40 hr)	16.1 ± 0.4	18.0 ± 4.0
8 hr (44 hr)	24.6 ± 10.6	32.4 ± 15.3
12 hr (48 hr)	77.5 ± 16.1	117.9 ± 7.6*
Depletion in the presence of BSO and ADR		
Time post 24 hr BSO treatment (culture time)		
4 hr (40 hr)	11.4 ± 1.2	10.9 ± 3.4
8 hr (44 hr)	7.6 ± 3.4	8.1 ± 3.9
12 hr (48 hr)	10.2 ± 0.5	9.4 ± 0.1

For measurement of basal cellular GSH content, cells were seeded at 250,000 cells in 2 mL medium per 35-mm dish, and harvested at various times post seeding. For BSO treatment, cells were seeded at the same cell density for 12 hr, and then 10 μL BSO was added to final concentration of 1 mM in the culture. After 24 hr of BSO treatment, 10 μL ADR to a final concentration of 1 μM was added concomitantly with the BSO. Cellular GSH was measured at various times following 24-hr BSO treatment. Each value was taken from triplicate samples (N = 3) and at least two experiments were performed; each value represents the mean ± SD.

* Significantly different from A549-Cd/ADR^S, $P < 0.01$.

shown in Fig. 1, the MTT assay demonstrated that the LC_{50} values for ADR were 0.27 ± 0.05 and 1.56 ± 0.31 μM for the A549-Cd^S and A549-Cd^R cells, respectively. The A549-Cd^R cells were thus 5-fold more resistant to ADR than the A549-Cd^S cells, which is the same magnitude of difference observed for the cadmium sensitivity.

Alteration of Adriamycin Sensitivity by Cellular GSH Modulation

As indicated in Table 2, GSH levels in the A549-Cd/ADR^R cells were much higher than in the A549-Cd/ADR^S cells after the first 12 hr of culture. Previous studies have shown that depletion of cellular GSH content sensitizes the A549 cells to cadmium [27]. To determine whether the elevated GSH level is also responsible for ADR resistance, the effect of cellular GSH depletion on ADR sensitivity was determined. As shown in Table 2, treatment of the Cd/ADR^S and Cd/ADR^R cells with 1.0 mM BSO for 24 hr depleted about 80% of the total cellular GSH content, e.g. to 19.4 ± 1.9 and 23.4 ± 7.4 nmol/mg protein in the sensitive and resistant cells, respectively. However, treatment with ADR following GSH depletion by this BSO treatment

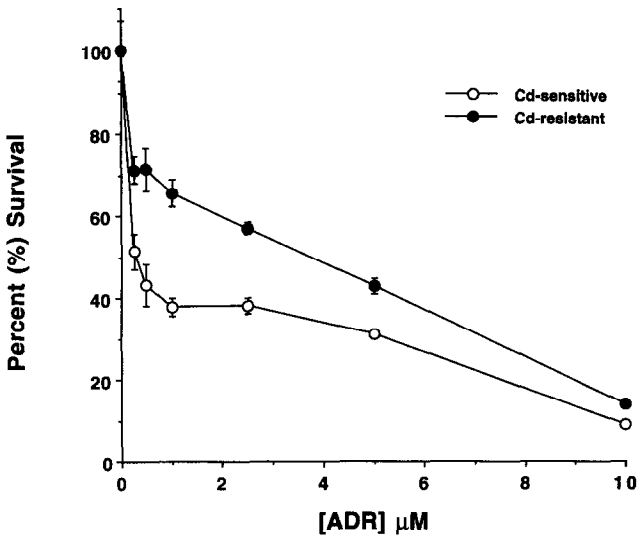

FIG. 1. ADR cytotoxic response in cadmium-sensitive and cadmium-resistant sublines of A549 cells determined by short-term survival MTT assay. The cells were seeded at a cell density of 25,000/well for 36 hr before treatment with various concentrations of ADR for 12 hr. Tetrazolium reduction was measured 48 hr after ADR removal. Each point is the mean ± SD from triplicate cultures.

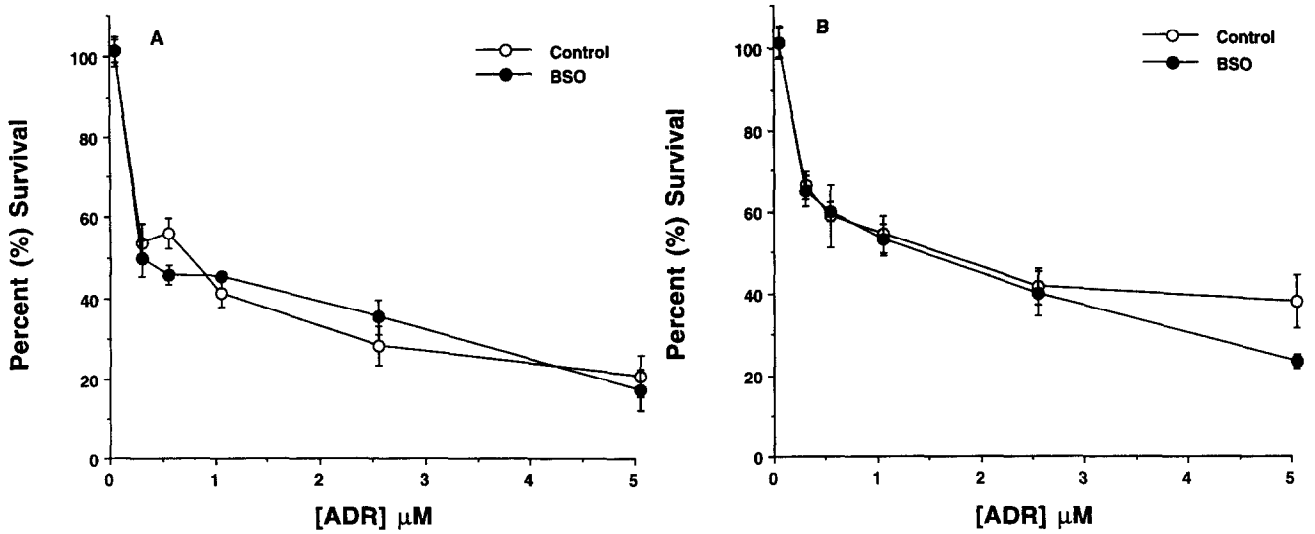

FIG. 2. Cytotoxic response of Cd/ADR-sensitive (A) and Cd/ADR-resistant (B) subpopulations of A549 cells to sequential BSO and ADR treatment, as determined by short-term survival MTT assay. The cells were seeded at a cell density of 25,000/well for 12 hr, and then pretreated with 1 mM BSO for 24 hr. The BSO was removed prior to 12-hr ADR treatment. Tetrazolium reduction was measured 48 hr after removal of BSO and ADR. Each point is the mean ± SD from triplicate cultures.

did not alter significantly the ADR sensitivity in either of the subpopulations (Fig. 2 A and B). Cellular GSH levels were further depleted to 5% of the respective control levels when the BSO treatment was continued for an additional 12 hr, and remained at the same depleted levels in the concomitant presence of 1.0 μM ADR during the last 12-hr BSO treatment (Table 2). The GSH levels were 9.8 ± 0.2 and 9.5 ± 0.2 nmol/mg protein prior to ADR treatment, and were 10.2 ± 0.5 and 9.4 ± 0.1 at the end of the ADR treatment in the Cd/ADR^S and Cd/ADR^R cells, respectively. Under this condition, i.e. BSO pretreatment for 24 hr followed by BSO cotreatment with ADR for 12 hr, the ADR sensitivity was increased markedly, and a similar level of sensitivity was attained in the Cd/ADR^S and Cd/ADR^R cells (Fig. 3). The LC_{50} values were 0.103 ± 0.02 and 0.148 ± 0.03 μM in the Cd/ADR^S and Cd/ADR^R cells, respectively. The 36-hr BSO treatment alone did not affect the cell viability.

Recovery of GSH Content Following Depletion by BSO

To examine the reason for the differential sensitizing effects of BSO pretreatment alone and the pretreatment followed by cotreatment with ADR, the recovery of cellular GSH content was measured for 12 hr after the 24-hr BSO treatment. As shown in Table 2, the Cd/ADR^R cells displayed a significantly faster rate of GSH recovery following the 24-hr BSO treatment than the Cd/ADR^S cells. During the 12-hr post BSO removal, the GSH recovery rate in the Cd/ADR^R cells was 9.6 nmol/hr compared with 5.4 nmol/hr in the Cd/ADR^S cells. ADR attenuated the rate of GSH recovery in both subpopulations, to 7.5 nmol/hr in the Cd/ADR^R cells and 4.6 nmol/hr in the Cd/ADR^S cells. When the cells were cotreated with BSO and ADR for 12 hr following the 24-hr BSO pretreatment, the cellular GSH concentrations remained at the same depleted level in the Cd/ADR^S and Cd/ADR^R cells.

Other Factors Affecting ADR Cross-Resistance

Other factors possibly involved in the Cd/ADR cross-resistance were also examined. GST has been implicated in resistance to ADR in various tumor cells [30, 31]. Total

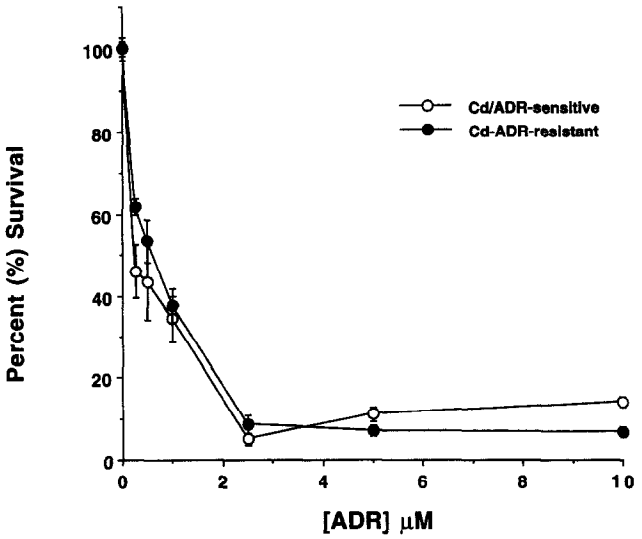

FIG. 3. Cytotoxic response of BSO-pretreated Cd/ADR-sensitive and Cd/ADR-resistant subpopulations of A549 cells to concurrent BSO and ADR treatment, as determined by short-term survival MTT assay. The cells were seeded at a cell density of 25,000/well for 12 hr, and then were pretreated with 1 mM BSO for 24 hr prior to 12-hr concomitant treatment with 1 mM BSO and ADR at various concentrations. Tetrazolium reduction was measured 48 hr after removal of BSO and ADR. Each point is the mean ± SD from triplicate cultures.

GST activity was measured from cytosolic extracts using CDNB as the substrate. The GST activities were 73.3 ± 6.7 and 71.6 ± 4.8 nmol CDNB/mg protein in the Cd/ADR^S and Cd/ADR^R cells, respectively. The gene and mRNA levels for MDR1 were also compared between the two cell lines by Southern and northern analyses, respectively. As shown in Fig. 4, there was no difference in the amount of DNA probed by *mdr1* between the Cd/ADR^S and Cd/ADR^R cells, suggesting that *mdr1* gene amplification is not a factor for the cross-resistance. In addition, determination of the relative amount of mRNA for MDR1 in the two cell lines revealed that *mdr1* expression was not induced by ADR in either the Cd/ADR^S or Cd/ADR^R cells (data not shown).

DISCUSSION

The phenomenon of cross-resistance among anticancer agents has been investigated extensively. Clinically, understanding cross-resistance is critical to the development of useful and effective anticancer chemotherapeutic regimens. Very few studies, however, have examined whether cross-resistance exists between carcinogens and anticancer agents. Many biochemical alterations occur during the development of resistance to xenobiotics and also in the process of xenobiotic-induced carcinogenesis. It seems reasonable to expect that in some instances these alterations would affect the actions of anticancer drugs. In the present study, cross-resistance between cadmium and ADR was observed in A549 cells. The magnitude of difference in the ADR sensitivities between the two populations was similar to that of the cadmium sensitivities. Decreased sensitivity to ADR has been demonstrated in human prostate carcinoma cells selected for cadmium resistance in which elevated cellular metallothionein content was responsible for the cross-resistance [32]. However, our studies showed that there is no difference in the basal or induced levels of metallothionein between the A549-Cd/ADR^R and A549-Cd/ADR^S cells. Thus, metallothionein does not appear to function in the Cd/ADR cross-resistance in A549 cells. In addition, no differences were found in any of the antioxidant enzymes including SOD, catalase, GSH peroxidase and GSH reductase between the A549-Cd^S and A549-Cd^R cells, suggesting that none of these factors was likely involved in the Cd/ADR cross-resistance.

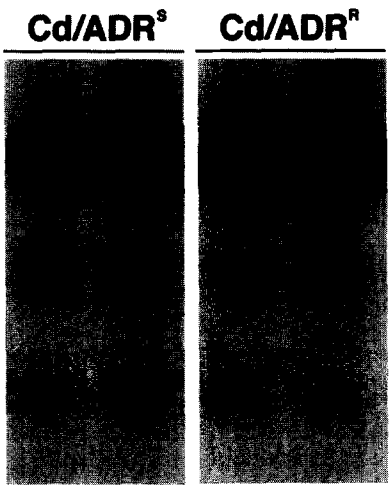

FIG. 4. Southern blot analysis of *mdr1* in exponentially growing Cd/ADR-sensitive (Cd/ADR^S) and Cd/ADR-resistant (Cd/ADR^R) A549 cells. No difference was observed in the gene levels of *mdr1* between the subpopulations.

Cellular GSH has been demonstrated to be involved in ADR resistance [13]. Depletion of cellular GSH content using BSO sensitizes numerous tumor cells to ADR [2, 3, 30, 33]. Therefore, the role of cellular GSH in the Cd/ADR cross-resistance was examined in the present study. Treatment with 1.0 mM BSO for 24 hr depleted cellular GSH to a similar low level in the Cd/ADR^S and Cd/ADR^R cells. This treatment did not alter the ADR sensitivity in either the Cd/ADR^S or Cd/ADR^R cells. However, both subpopulations were significantly sensitized to ADR when they were treated with this drug in the concomitant presence of BSO following the 24-hr BSO treatment. Under these conditions, cellular GSH was depleted to about 5% of control levels in both subpopulations. This result suggests that although the Cd/ADR^R cells contain higher levels of GSH, this may not be directly related to the resistance.

It has been shown that the kinetics of GSH status may be more relevant to the sensitization to ADR than the steady-state level of GSH [34]. In the present study, a faster rate of GSH recovery was found in the Cd/ADR^R cells than in the Cd/ADR^S cells. GSH homeostasis is regulated by several enzymatic processes, including degeneration of extracellular GSH and importation of precursor amino acids, *de novo* synthesis, oxidation-reduction and conjugation. Our previous studies have shown that there is no difference between the sensitive and resistant cells in the activity of γ-glutamyltranspeptidase (GGT), which simultaneously breaks down extracellular GSH and transports its precursor amino acids into the cell [15]. Similarly, there was no difference in the activities of GSH reductase (Table 1), which regenerates the reduced form of GSH from the oxidized form, and GST, which conjugates GSH to various substrates. In contrast, γ-glutamylcysteine synthetase (γ-GCS), which catalyzes the rate-limiting step in *de novo* GSH synthesis, is overexpressed in the Cd/ADR^R cells relative to the Cd/ADR^S cells [15]. The γ-GCS overexpression is likely responsible for the enhanced rate of GSH recovery following GSH depletion in the resistant cells. Cellular GSH levels continued to be depleted, however, to about 5% of the total levels throughout an additional 12-hr BSO treatment in both subpopulations. Under these conditions, both subpopulations were significantly sensitized to ADR. Moreover, the same level of ADR sensitivity was attained between the Cd/ADR^R and Cd/ADR^S cells. These results indicate that depletion of cellular GSH content below a threshold level is required for ADR sensitization to occur in A549 cells. This threshold is likely related to the subcellu-

lar compartmentalization of GSH among the cytoplasm, mitochondria, and nucleus. In most cells, the mitochondrial and nuclear GSH pools comprise approximately 10–20 and 0.5–1.5% of the total cellular GSH, respectively. Studies have demonstrated that the cytoplasmic GSH is depleted most rapidly, while the mitochondrial and nuclear pools are more resistant to depletion by BSO or other depleting agents [35, 36]. It appears that only a small fraction of the total cellular GSH pool is necessary for cytoprotection against ADR. The differences in the GSH synthesis capacity and the rate of recovery following GSH depletion are likely related to the differential cytotoxic responses to ADR between the two subpopulations.

Other factors possibly involved in the Cd/ADR cross-resistance including GST activity and *mdr1* expression were also compared between the Cd/ADR^S and Cd/ADR^R cells. No difference was found in the total GST activity between the Cd/ADR^S and Cd/ADR^R cells, suggesting that GST does not function in the Cd/ADR cross-resistance. Similarly, no difference was detected in gene levels of *mdr1*, indicating that gene amplification of *mdr1* is not involved. Moreover, expression of MDR1 mRNA was not induced by ADR in either subpopulation.

References

1. Volm M, Mattern J and Pommerenke EW, Time course of MDR gene amplification during *in vivo* selection for doxorubicin-resistance and during reversal in murine leukemia L 1210. *Anticancer Res* **11:** 579–586, 1991.
2. Russo A and Mitchell JB, Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione and modulation. *Cancer Treat Rep* **69:** 1293–1296, 1985.
3. Dusre L, Mimnaugh EG, Myers CE and Sinha BK, Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. *Cancer Res* **49:** 511–515, 1989.
4. Benchekroun MN, Pourquier P, Schott B and Robert J, Doxorubicin-induced lipid peroxidation and glutathione peroxidase activity in tumor cells selected for resistance to doxorubicin. *Eur J Biochem* **211:** 141–146, 1993.
5. Goyer RA, Toxic effects of metals. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Eds. Amdur MO, Doull J and Klaassen CD), 4th Edn, pp. 634–637. Pergamon Press, New York, 1991.
6. Singhal RK, Anderson ME and Meister A, Glutathione, a first line of defense against cadmium toxicity. *FASEB J* **1:** 220–223, 1987.
7. Kang YJ and Enger MD, Glutathione is involved in the early cadmium cytotoxic response in human lung carcinoma cells. *Toxicology* **48:** 93–101, 1988.
8. Kostic MM, Ognjanovic B, Dimitrijevic S, Zikic RV, Stajn A, Rosic GL and Zivkovic RV, Cadmium-induced changes of antioxidant and metabolic status in red blood cells of rats: *In vivo* effects. *Eur J Haematol* **51:** 86–92, 1993.
9. Chin K-V, Tanaka S, Darlington G, Pastan I and Gottesman MM, Heat shock and arsenite increase expression of the multidrug resistance (*MDR1*) gene in human renal carcinoma cells. *J Biol Chem* **265:** 221–226, 1990.
10. Scagliotti GV, Michelotto F, Kalikatzaros G, Leonardo E, Cappia S, Gubetta L, Borasio P and Pozzi E, Detection of multidrug resistance associated P-170 glycoprotein in previously untreated non-small cell lung cancer. *Anticancer Res* **11:** 2207–2210, 1991.
11. Reeve JG, Rabbitts PH and Twentyman RR, Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69. *Br J Cancer* **60:** 339–342, 1989.
12. Satoh M, Cherian MG, Imura N and Shimizu H, Modulation of resistance to anticancer drugs by inhibition of metallothionein synthesis. *Cancer Res* **54:** 5255–5257, 1994.
13. Richardson E and Siemann DW, Thiol-related mechanisms of resistance in a murine tumor model. *Int J Radiat Oncol Biol Phys* **29:** 387–392, 1994.
14. Enger MD, Hildebrand CE, Walters RA, Seagrave JC, Barham SS and Hoagland HC, Molecular and somatic cell genetic analysis of metal resistance mechanisms in mammalian cells. In: *Molecular and Cellular Approaches to Understanding Mechanisms of Toxicity* (Ed. Tasjjian AH), pp. 38–62. President and Fellows of Harvard College, Cambridge, 1984.
15. Hatcher EL, Chen Y and Kang YJ, Cadmium resistance in A549 cells correlates with elevated glutathione content but not antioxidant enzymatic activities. *Free Radic Biol Med* **19:** 805–812, 1995.
16. Tietze F, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem* **27:** 502–522, 1969.
17. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke MN, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
18. Aebi H, Catalase *in vitro*. *Methods Enzymol* **105:** 121–126, 1984.
19. Nelson DP and Kiesow LA, Enthalpy of decomposition of hydrogen peroxide by catalase at 25°C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Anal Biochem* **49:** 474–478, 1972.
20. Flohe L and Gunzler WA, Assays of glutathione peroxidase. *Methods Enzymol* **105:** 114–121, 1984.
21. Carlberg I and Mannervik B, Glutathione reductase. *Methods Enzymol* **113:** 484–490, 1985.
22. Sun Y, Oberley LW and Ying L, A simple method for clinical assay of superoxide dismutase. *Clin Chem* **34:** 497–500, 1988.
23. Babig WH, Pabst MJ and Jakoby WB, Glutathione S-transferases. *J Biol Chem* **249:** 7130–7139, 1974.
24. Church GM and Gilbert W, Genomic sequencing. *Proc Natl Acad Sci USA* **81:** 1991–1995, 1984.
25. Feinberg AP and Vogelstein B, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132:** 6–13, 1983.
26. Kang YJ and Enger MD, Cadmium cytotoxicity correlates with the changes in glutathione content that occur during the logarithmic growth phase of A549-T27 cells. *Toxicol Lett* **51:** 23–28, 1990.
27. Kang YJ and Enger MD, Effect of cellular glutathione depletion on cadmium-induced cytotoxicity in human lung carcinoma cells. *Cell Biol Toxicol* **3:** 347–360, 1987.
28. Manca D, Richard AC, Trottier B and Chevalier G, Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride. *Toxicology* **67:** 303–323, 1991.
29. Shiraishi N, Uno H and Waalkes MP, Effect of L-ascorbic acid pretreatment on cadmium toxicity in the male Fischer (F344/NCr) rat. *Toxicology* **85:** 85–100, 1993.
30. Meijer C, Mulder NH, Timmer-Bosscha H, Peters WHM and De Vries EGE, Combined *in vitro* modulation of adriamycin resistance. *Int J Cancer* **49:** 582–586, 1991.
31. Waxman DJ, Glutathione-S-transferases: Role in alkylating agent resistance and possible target for modulation chemotherapy: A review. *Cancer Res* **50:** 6449–6454, 1990.
32. Webber MM, Rehman SMM and James GT, Metallothionein

induction and deinduction in human prostatic carcinoma cells: Relationship with resistance and sensitivity to Adriamycin. *Cancer Res* **48:** 4503–4508, 1988.
33. Lai GM, Moscow JA, Alvarez MG, Fojo AT and Bates SE, Contribution of glutathione and glutathione-dependent enzymes in the reversal of adriamycin resistance in colon carcinoma cell lines. *Int J Cancer* **49:** 688–695, 1991.
34. Lee FYF, Siemann DW and Sutherland RM, Changes in cellular glutathione content during adriamycin treatment in human ovarian cancer—A possible indicator of chemosensitivity. *Br J Cancer* **60:** 291–298, 1989.
35. Edgren M and Revesz L, Compartmentalised depletion of glutathione in cells treated with buthionine sulphoximine. *Br J Radiol* **60:** 723–724, 1987.
36. Jevtovic-Todorovic V and Guenthner TM, Depletion of a discrete nuclear glutathione pool by oxidative stress, but not by buthionine sulfoximine. *Biochem Pharmacol* **44:** 1383–1393, 1992.