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A STUDY OF THE MECHANISM OF RESISTANCE TO ADRIAMYCIN® *IN VIVO*

GLUTATHIONE METABOLISM, P-GLYCOPROTEIN EXPRESSION, AND DRUG TRANSPORT

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Abstract—A spontaneously originated murine mammary adenocarcinoma (16C), selected for its sensitivity to agents active against breast cancer in women, and one of the very few experimental solid tumor models responsive to Adriamycin® (ADR) was used to study the mechanism of induced ADR resistance *in vivo*. A resistant variant of the tumor was obtained from the explant of a regrown tumor following a dose of ADR (12 mg/kg) that caused complete tumor repression but not cure. Progressive refractoriness to ADR was observed following up to six repeated cycles of treatment, regression and regrowth. However, beyond the sixth treatment, no further degree of resistance could be obtained. The cell line so established, designated 16C/ADR^R, has a glutathione (GSH) content 1.67 times greater than the parent 16C line. Depletion of GSH by buthionine sulfoximine (BSO) enhanced the cytoxicity of ADR in both cell lines. The sensitization effect appeared to be dependent on the degree of GSH depletion, requiring a threshold level of depletion to approximately 30% of control. The resistance of 16C/ADR^R, however, appeared not to be directly related to the increased absolute GSH level *per se* since reduction of the GSH content of the 16C/ADR^R line to levels similar to that of the parent 16C line did not restore the original sensitivity to ADR. However, the activities of two important elements in the GSH detoxification system, GSH peroxidase and *S*-transferase, were found to be elevated in resistant cells by factors of 2.4 and 4.7–5.6 respectively. *In vivo* studies with a diverse spectrum of antineoplastic drugs revealed a pattern of cross-resistance consistent with the idea that elevated GSH *S*-transferase and peroxidase activities may be responsible for the decreased (2.8- to 5.3-fold) sensitivity to ADR. 16C/ADR^R exhibited cross-resistance with melphalan (MEL), but none with vincristine (VCR), vinblastine (VBL) or etoposide (VP-16). These results clearly demonstrate non-adherence by the 16C/ADR^R tumors to the well characterized multidrug resistance (*mdr*) phenotype. Further affirmation of this conclusion was obtained by immunochemical and pharmacological studies. When a monoclonal antibody prepared against the *mdr* associated, 170 kD P-glycoprotein (170 P-gp), was used, the presence of the 170 kD P-gp in both the sensitive and resistant 16C lines could not be detected, although the presence of a lower molecular weight form of P-gp could not be ruled out entirely. High performance liquid chromatographic measurement of ADR accumulation and elimination also failed to reveal any significant differences between the sensitive and resistant variants. These results, therefore, argue against the possibility of drug transport and membrane changes as the mechanism of drug resistance, and strongly implicate the increases in GSH peroxidase and *S*-transferase activities as the basis for the *in situ* selected ADR resistance of this 16C/ADR^R murine tumor.

Because of the widely held belief that the development of drug resistance is the major factor contributing to the incurability of many forms of human solid cancer, great emphasis has been placed on the understanding of the developmental process and on the mechanisms of resistance for cytotoxic agents. The anthracyclines, including Adriamycin® (ADR)†, have received the greatest attention because of their clinical usefulness and frequent involvement in pleiotropic multidrug resistance (*mdr*). Diverse types of changes were found to be associated with the development of resistance to ADR. These include: (i) increased rate of drug efflux [1–4], probably as a result of increased expression of a membrane glycoprotein [5], and (ii) alteration in glutathione (GSH) metabolism [6, 7]. However, in most, if not all, of these studies selection of the drug-resistant variant cell types were carried out *in vitro* using treatment parameters that did not always resemble the *in vivo* pharmacokinetics of the agent (e.g. peak concentration and area under the concentration–time curve). In addition, such studies typically do not take into consideration the action of any active metbabolites formed following metabolism of the agent by the body. This divergence in the nature and degree of selection pressure may account for the correspondingly divergent modes of resistance found by various investigators in different tumor systems. In order to attempt to mimic more closely the clinical conditions, in the present investigation selection was carried out *in vivo* from a murine mammary adenocarcinoma (16C) exhibiting a spectrum of sensitivity to chemotherapeutic agents

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† Adriamycin is a registered trademark of Farmitalia Carlo Erba.

similar to breast cancer in patients. The resistant variants were obtained following repeated sequences of treatment, tumor regression and regrowth, thus bearing resemblance, even if superficial, to clinical experience. Cross-resistance studies were complemented by biochemical investigations in the search for the underlying mechanism(s). Modes of resistance including glutathione metabolism drug transport, and expression of the 170 kD P-glycoprotein (170 P-gp) were investigated. The results indicate that, while changes in GSH content, drug transport and expression of 170 P-gp were probably not directly involved, an increase in glutathione *S*-transferase activity may well represent a possible mode of resistance in this tumor system.

MATERIALS AND METHODS

Drug and drug administration

Adriamycin® (ADR) was obtained from Adria Laboratory (Columbus, OH), vincristine (VCR) from LyphoMed (Rosemont, IL), VP-16 from Bristol Laboratories (Evansville, IN), melphalan (MEL) from the Burroughs Wellcome Co. (Research Triangle Park, NC), vinblastine (VBL) from Eli Lilly (Indianapolis, IN), and D,L-buthionine sulfoximine (BSO) from the Sigma Chemical Co. VBL, VP-16, and ADR were dissolved or diluted in phosphate buffer solution (PBS), pH 7.4, at appropriate concentrations and injected i.v. at 0.01 ml/g body weight. MEL was dissolved in acid ethanol (1:9 conc. HCl/EtOH) and diluted to appropriate concentrations with PBS. BSO, also dissolved in PBS, pH 7.4, was injected i.p. at 0.01 to 0.04 ml/g body weight. In most experiments, mice received a single dose or multiple doses (up to five) of 2.5 mmol/kg BSO. For multiple BSO dosing, an interval of 8 hr was allowed between each dose. An appropriate dose of ADR was given 8 hr following the last BSO dose. In all instances, controls were given the drug vehicles.

Mice and tumors

Inbred female C3H/HeJ mice were supplied by Jackson Laboratories, Bar Harbor, ME. The 16C syngeneic mammary adenocarcinoma was originally derived from a spontaneous mammary tumor in a female C3H mouse [8] and was supplied by Dr. Ian F. Tannock (University of Toronto). This solid tumor shows exquisite sensitivity to Adriamycin® [8, 9], a characteristic that is also found in many mammary carcinomas in humans, but is extremely rare among rodent solid tumors. Tumors were maintained *in vivo* by serial transplantation for up to the 10th passage. A single cell suspension was obtained by enzyme mixture digestion of excised tumors [10], and between 2 and 5×10^5 cells were inoculated into the gastrocnemius muscles of recipients in a volume of 0.05 ml.

Regrowth delay measurement

The response of the 16C tumor to treatment with ADR with or without GSH depletion by BSO was assessed by regrowth delay. Measurement of tumor size was carried out using a specially made gauge from 6 to 18 mm in increments of 1 mm. Two measurements of the thickness of the tumor-bearing leg were made at right angles to each other by fitting it into the appropriate slot. The leg diameter product was then calculated, from which tumor weight was obtained using a calibration curve.

Mice with tumors of the correct size were ear-marked and then randomized into different experimental groups, each containing six to ten mice. After appropriate treatment, they were returned to their original cages. Tumors were measured on alternate weekdays. For each tumor an individual growth curve was plotted, and the time taken to reach 4× treatment size was then determined. The geometric mean of individual values in a group was then calculated, with standard errors, and growth delays obtained by subtraction of control from treated means.

GSH measurement by HPLC

The procedures for tumor sample preparation and HPLC analysis for GSH have been described in detail previously [11, 12]. Briefly, cells or tumors were homogenized, respectively, with 200 μl or 20 vol. (w/v) of 20 mM 5-sulfosalicyclic acid. The homogenates were centrifuged for 40 sec in an Eppendorf microcentrifuge. GSH in the supernatant fraction was derivatized with the fluorescent reagent monobromobimane (mBBr, thiolyte™, Calbiochem, La Jolla, CA). An aliquot (180 μl) of the supernatant fraction was mixed with 18 μl *N*-ethylmorpholine (0.5 M in 2 mM KOH) and 2 μl of mBBr (50 mM in acetonitrile). The mixture was immediately vortexed and stored in the dark at 20° until analysis. Modular HPLC equipment from Waters Associated (Hartford, MA) was used throughout. GSH was separated by an isocratic paired-ion technique on Waters Radial-PAK reversed-phase bonded octadecylsilane (C18, 8 mm i.d.) cartridge columns. The mobile phase consisted of 23% acetonitrile in 40 mM ammonium phosphate buffer, pH 7.2, containing 5 mM tetra-butylammonium hydroxide. The effluent was monitored for fluorescence with 340 nm excitation and emission at >410 nm.

Centrifugal elutriation

The general principle of the centrifugal elutriation technique has been reported in detail previously [13]. For the separation of 16C tumor cells from host cells, a cell mixture ($1–2 \times 10^8$) suspended in approximately 20 ml of alpha-Minimum Essential Medium (alpha-MEM) was loaded into the chamber of a Beckman JE6 elutriator system at an initial rotor speed of 4000 rpm and at a flow rate of 25 ml/min. At these initial conditions, red blood cells and cell debris were eluted. In the second stage, the rotor speed was decreased in five discrete intervals to 2800 rpm, and the flow rate was increased to 41 ml/min in two intervals. A variable number (3–5) of 40-ml fractions was collected at each interval. This second stage removed most of the host cells. In the third stage tumor cells remaining in the chamber were eluted by decreasing the rotor speed to zero rpm. The volume of the cells in each fraction was determined by a Coulter Counter channelyzer system.

Enzyme assay

Cytosolic glutathione peroxidase activity was measured by the coupled assay technique of Paglia and Valentine [14] using H_2O_2 as the substrate. Glutathione *S*-transferase was assayed by a modification of the method of Hulbert and Yakubau [15] using monobromobimane as substrate. The formation of the fluorescent GSH-conjugated product was followed by HPLC as described above. GSH *S*-transferase was also assayed using 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrates [16].

Adriamycin® transport analysis

Tumor cells isolated by centrifugal elutriation were suspended in alpha-MEM complete medium, supplemented with 10% total calf serum (Flow Laboratories) at 5×10^5/ml in a Type I vial at 37° as described previously [17]. Cells were gassed with a 95% air/5% CO_2 gas mixture, and 30 min was allowed for equilibration. Cells were treated with 1 μg/ml ADR for 1 hr. At the end of drug exposure, cells were separated from drug-containing medium by centrifugation through a layer of corn oil: dibutylphthalate mixture (1:4, v/v). The washed cells were resuspended in PBS, pH 7.4, cell membrane was disrupted by sonification, and ADR was extracted with 5 vol. of ethylacetate:1-propanol (9:1, v/v). The organic extracts were evaporated to dryness *in vacuo* using a Savent speed vac concentrator (Savent Instruments, Inc., Hicksville, NY). The dry residues were redissolved in 100 μl methanol (HPLC grade) and stored sealed at −20°. Aliquots of the methanol concentrate were used for HPLC analysis.

ADR and its metabolites were separated on a Waters Nova-PAK phenyl cartridge column (8 mm i.d., 4 μm particle size). Elution was by running a linear gradient commencing, at the time of injection, from an initial condition of 25% to the final condition, at 6 min of 50% acetronitrile in formic acid, pH 4.0. The flow rate was 3.0 ml/min. Detection was by fluorescence with excitation at 550 nm and emission at >650 nm.

Preparation of P-glycoprotein from isolated tumor cells

Isolation of the membrane-bound P-glycoprotein has been described elsewhere [18]. Cell lines Aux B1 and CHRC5 were gifts from Dr. R. M. Baker (Roswell Park Memorial Institute, Buffalo, NY). Cells were hypotonically swelled in 10 mM Tris, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 5×10^7 cells/ml. Following homogenization using a motor-driven teflon pestle (Potter–Elvehjem), differential centrifugation of the cell lysate was carried out in the following steps: 300 *g*, nuclear spin; 400 *g*; mitochondrial spin; 100,000 *g*, microsomal spin. The microsomal pellet was resuspended in 10 mM Tris, pH 7.4, overlaid onto a discontinuous 16-31-45-60% sucrose gradient and spun at 100,000 *g* for 16 hr. Aliquots from the 16%/31% and 31%/45% interfaces were collected and dissolved in sample buffer containing 1% sodium dodecyl sulfate (SDS), 4.5 M urea, and 25 mM dithiothreitol to a final protein concentration of 2 mg/ml.

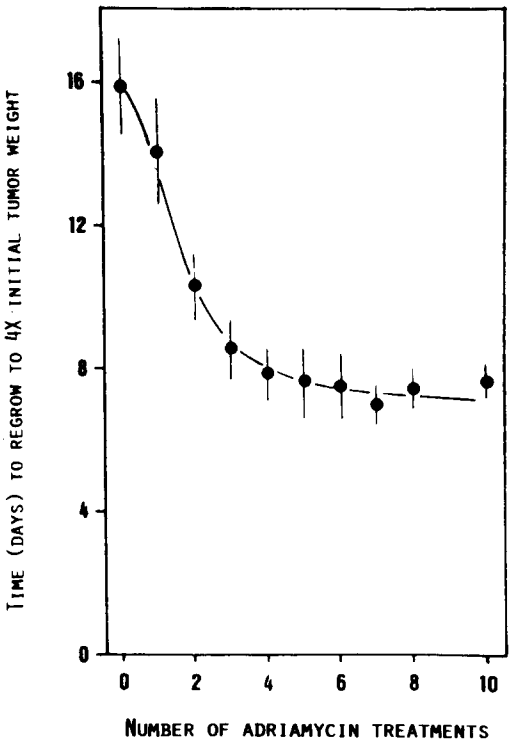

Fig. 1. Influence of the number of prior ADR treatments on the sensitivity of the retransplanted tumors to a 12 mg/kg dose of ADR. Each datum point is the mean of 6–8 tumors; error bars indicate ±2 SE.

Detection of P-glycoprotein from isolated tumor cells

Protein samples were electrophoresed on 5.6% SDS–urea polyacrylamide gels as described in Fairbanks *et al.* [19] and electrophoretically transferred to nitrocellulose [18]. The nitrocellular transfer was probed for the presence of P-glycoprotein with C219, a monoclonal antibody (Mab) prepared against P-glycoprotein [20]. Positive ab–ag reactions were visualized using a horseradish peroxidase-conjugated secondary goat anti-mouse antibody (Cooper Biochemical Co., Malvern, PA). Mab C219 was a gift of Dr. V. Ling (University of Toronto).

RESULTS

Tumor response and induction of resistance to ADR

The 16C mammary carcinoma is one of the few murine solid tumors that show sensitivity to ADR *in vivo*. A 12 mg/kg dose of ADR produced a tumor regrowth time of approximately 16 days (Fig. 1).

Resistance to ADR in the 16C tumor was induced by a protocol where tumor bearing mice were treated with a single dose of ADR (12 mg/kg). Individual tumors then underwent a typical response pattern of regression followed by regrowth. A regrown tumor was re-implanted into another group of mice which

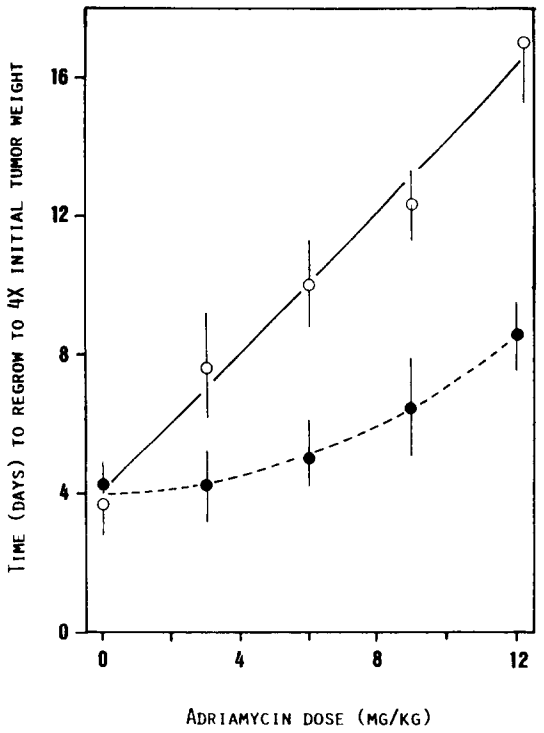

Fig. 2. Regrowth of the parent 16C tumors (○) and tumors of the resistant subline (16C/ADR^R (●) following ADR treatment. Each datum point is the mean of 6–8 tumors. Error bars indicate ±2 SE.

were then treated with 12 mg/kg ADR. This procedure (ADR treatment, tumor response, tumor reimplantation) was repeated for up to ten times. Figure 1 shows the tumor response to 12 mg/kg ADR as a function of the number of treatments each tumor group had undergone previously. It is clear that the induced resistance was both progressive and maximal. Significant resistance was evident following the second treatment and maximal resistance was reached by the sixth treatment. Figure 2 is a comparison of the response of the parent 16C tumor line with the tumor line established after treatment with six consecutive 12 mg/kg ADR doses (16C/ADR^R). The parent 16C and the variant lines demonstrated similar rates of growth, the time to reach four times the initial tumor weight for untreated tumors being 3.89 ± 0.42 and 4.05 ± 0.6 (2 SE) respectively. It is clear that 16C/ADR^R was resistant to treatment over the entire range of ADR doses evaluated. A resistance factor, calculated as the ratio of ADR doses required to yield equivalent regrowth times in the parent compared to the resistant tumor, ranged from 2.8 to 5.3.

Studies on the mechanism of drug resistance

Patterns of cross-resistance. The 16C/ADR^R line demonstrated a lack of cross-resistance to the Vinca alkaloids vincristine (VCR) and vinblastine (VBL) (Fig. 3). This finding is, therefore, in distinct contrast with the cross-resistance patterns of the multidrug resistance (*mdr*) phenotype where the development of resistance to ADR invariably led to cross-resistance with the *Vinca* alkaloids and vice versa [20]. In addition, absence of cross-resistance was also demonstrated with VP-16 (Fig. 4), a podophyllotoxin dependent on topoisomerase II for its toxic activity, suggesting that changes in topoisomerase II activity were not involved in the mechanism of resistance in this tumor line. In contrast 16C/ADR^R exhibited clear cross-resistance with the alkylating agent melphalan (Fig. 5).

Glutathione. The resistant 16C/ADR^R line was

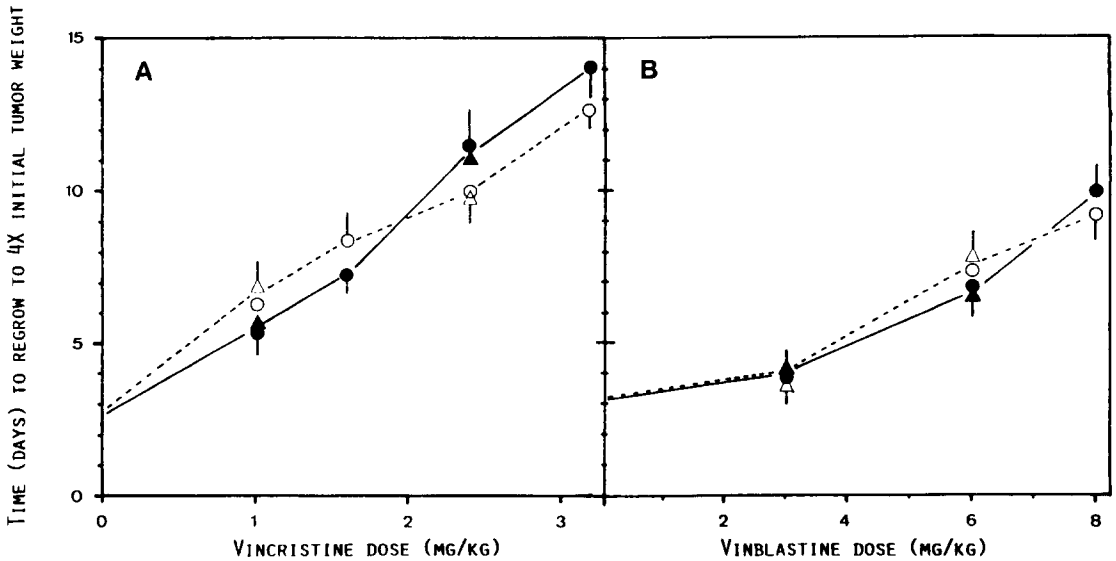

Fig. 3. Regrowth of the parent (16C; open symbols) and ADR-resistant subline (16C/ADR^R; closed symbols) following (A) vincristine and (B) vinblastine treatment. Each datum point is the mean of 6–8 tumors. Different symbols represent data from independent experiments. Error bars indicate ±2 SE.

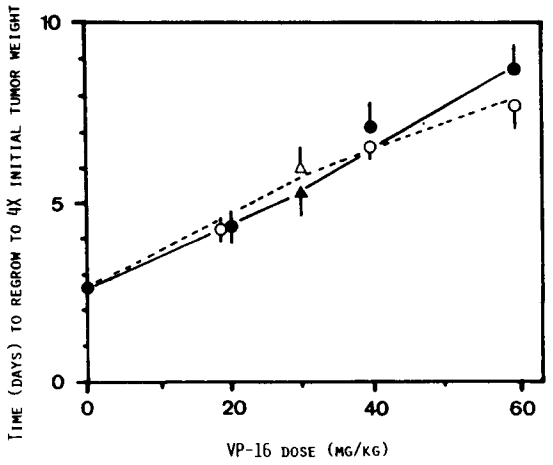

Fig. 4. Regrowth of the parent (16C; open symbols) and ADR-resistant subline (16C/ADR^R; closed symbols) following VP-16 treatment. Each datum point is the mean of 6–8 tumors. Different symbols represents data from independent experiments. Error bars indicate ±2 SE.

found to have a GSH content ~ 1.7 times higher than the parent 16C line (Table 1). Despite this difference, resistance to ADR and the absolute GSH content did not appear to be directly related. GSH depletion in the 16C/ADR^R line to levels similar to or less than that of the sensitive parent line did not restore the original sensitivity to ADR (Table 1). However, as in the case of the parent line, severe GSH depletion to below 20% did sensitize the 16C/ADR^R line to ADR cytotoxicity (Table 1). Finally, the rates of GSH depletion by BSO were similar for both lines (data not shown), suggesting similar basal rates of GSH consumption.

Glutathione peroxidase and S-transferase activity. Glutathione *S*-transferase activity was monitored by using mBBr and GSH as substrates. The formation kinetics of the conjugated product was directly followed by HPLC with fluorescence detection. For comparison, a standard assay using CDNB as substrate was also performed. Table 2 lists the GSH peroxidase and GSH *S*-transferase activities in the ADR-sensitive and -resistant 16C tumors. GSH peroxidase activity was 2.4-fold higher in the resistant tumors. Using mBBr or CDNB as substrates, GSH *S*-transferase activities were, respectively, 5.6- and 4.7-fold greater in the resistant tumors.

170 kD P-glycoprotein (170 P-gp). The possibility that over-expression of the 170 P-gp may be responsible for the observed ADR resistance of the 16C/ADR^R line also was investigated using immunochemical staining techniques (Fig. 6). The presence of 170 P-gp was not detected in the membrane preparations taken from either the 16%/31% or 31%/45% sucrose gradient interfaces from either the untreated, parent 16C tumor (Fig. 6, lanes 3 and 4) or from the ADR-resistant 16C/ADR^R tumor (Fig. 6, lanes 5 and 6). Non-specific staining of several lower molecular weight protein bands was observed in all lanes as a result of artifacts caused by overstaining. Overstaining of the horseradish peroxidase-bound goat anti-mouse secondary antibody was performed to aid in the potential visualization of grossly undetectable amounts of P-glycoprotein. However, it should be noted that the presence in small amounts of P-glycoproteins of molecular weight lower than

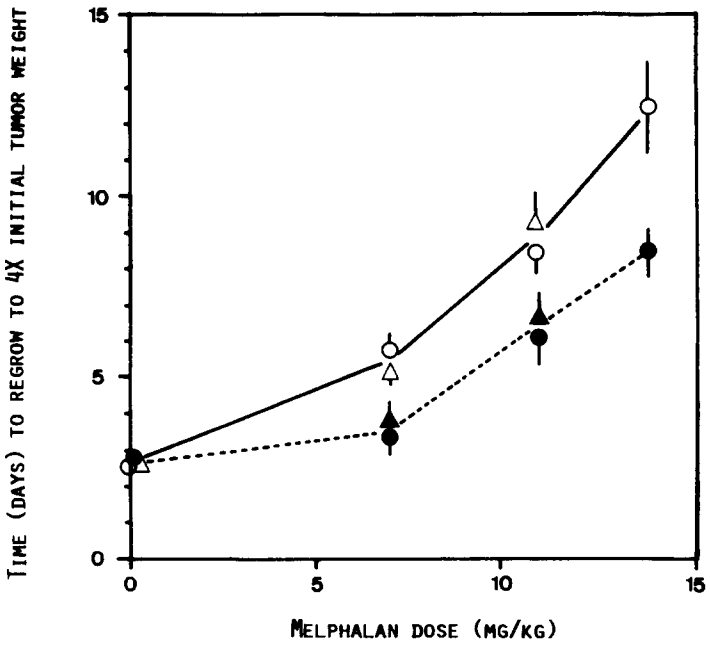

Fig. 5. Regrowth of the parent (16C; open symbols) and ADR-resistant subline (16C/ADR^R; closed symbols) following melphalan treatment. Each datum point is the mean of 6–8 tumors. Different symbols represent data from independent experiments. Error bars indicate ±2 SE.

Table 1. Regrowth time in the 16C and 16C/ADR^R tumors induced by ADR (6 mg/kg) with or without BSO

Expt	No. of BSO treatments at 8-hr intervals	GSH content at the time of ADR treatment (mmol/kg) 16C	16C/ADR^R	Regrowth time* (days) 16C	16C/ADR^R
1	0	1.32	2.25	9.1 ± 1.2†	5.3 ± 0.6
	1	0.61 (46)‡	1.01 (45)	9.4 ± 0.9	5.0 ± 1.1
	3	0.33 (25)	0.81 (36)	10.3 ± 1.7	5.9 ± 0.5
	5	0.21 (16)	0.47 (21)	12.1 ± 2.0	7.6 ± 1.4
2	0	1.21 ± 0.14	2.02 ± 0.21	8.5 ± 0.7	5.2 ± 0.8
	1	0.52 (43)	1.0 (50)	8.9 ± 1.8	5.6 ± 1.3
	3	0.36 (30)	0.67 (33)	9.5 ± 1.1	5.4 ± 0.6
	5	0.21 (17)	0.42 (21)	11.1 ± 0.9	7.2 ± 1.5

* Time to grow to 4× initial tumor weight.
† Values are geometric means of 7–9 mice ± 2 SE.
‡ Percent GSH of untreated (without BSO) tumors is given in parentheses.

Table 2. Comparison of glutathione concentrations, glutathione peroxidase and *S*-transferase activities in 16C and 16C/ADR^R cells

Cell line	Glutathione (nmol/kg)	GSH peroxidase (mmol/min/mg protein)	GSH *S*-transferase mBBr (μmol/min/mg protein)	GSH *S*-transferase CDNB (nmol/min/mg protein)
16C	1.21 ± 0.14*	1.6 ± 0.4	0.27 ± 0.03	10.2 ± 0.6
16C/ADR^R	2.02 ± 0.21	3.8 ± 0.7	1.49 ± 0.2	48.1 ± 3.3
Ratio, $\frac{16C/ADR^R}{16C}$	1.67	2.4	5.6	4.7

For details of the assay technique, see Materials and Methods.
* Mean ± SD, N ≥ 3.

170 kD cannot be ruled out completely. Positive controls were obtained with membrane samples from the well characterized multidrug sensitive and resistant cell lines, AUX/B1 and CHRC5, respectively. The synthesis of 170 P-gp, as detected by horseradish peroxidase staining, was enhanced greatly in the CHRC5 membrane sample taken from the 16%/31% sucrose gradient interface (lane 2) when compared with a similar preparation of the wild-type, drug-sensitive AUX/B1 cell line (lane 1).

Adriamycin® transport. Cellular transport of ADR was studied in the 16C parent line and the drug-resistant 16C/ADR^R line. Cells grown *in vivo* were purified by centrifugal elutriation *in vitro*. To ensure that the purified cells were representative, in terms of clonogenicity and resistance to ADR, of the original tumors *in vivo*, they were re-inoculated into mice, and the tumor take rate and sensitivity to ADR were determined. For both tumor lines the number of cells required to produce tumors in 50% of the mice inoculated was approximately 0.5×10^5 cells. The 16C/ADR^R tumors retained their full ADR resistance phenotype (results not shown). Purified cells were incubated with 1 μg/ml ADR at 37° for variable periods of time up to 4 hr. Cells were then washed rapidly by centrifugation through a layer of corn oil : dibutylphthalate. At this time, cells were either processed immediately for ADR measurement using HPLC or resuspended in ADR-free medium for a further variable period of time (up to 4 hr) before HPLC analysis. Figure 7 shows the kinetics of ADR accumulation and elimination in the drug-sensitive and -resistant 16C cell lines. For both cell types, the intracellular ADR concentration at the end of a 4-hr incubation period with ADR was roughly 22-fold greater than the extracellular drug concentration. The amounts of ADR accumulated after various times of ADR incubation were similar between the ADR-sensitive parent and the resistant variant lines (Fig. 7A), as were the rates of ADR elimination during a 4-hr post-incubation period (Fig. 7B). Furthermore, detoxification of ADR through metabolism of the parent drug was probably not an important factor in either cell line since none of the major metabolites could be detected.

DISCUSSION

We have observed, in a mouse mammary adenocarcinoma selected for ADR resistance *in vivo*, a probable mechanism of resistance to ADR related to GSH metabolism, viz. increased GSH *S*-transferase and peroxidase enzyme activities. On the other hand, the possibility that two other widely studied mechanisms may be involved, viz. increased drug efflux and increased expression of a membrane 170 kD P-glycoprotein, has been ruled out.

Resistance to drug treatment, whether at initial presentation or following relapse, frequently limits

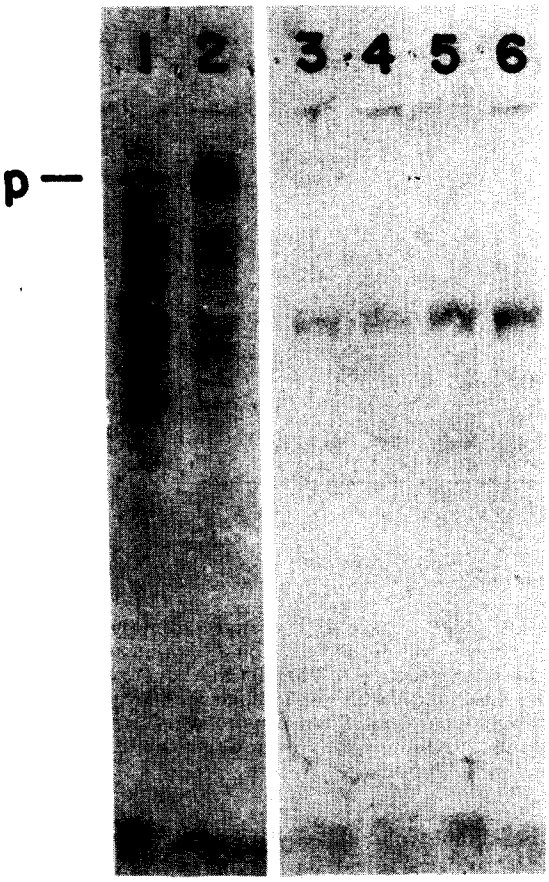

Fig. 6. Nitrocellulose blots of plasma membrane fractions isolated from CHO monolayers and freshly excised 16C tumors by means of ultracentrifugation of a 16%–60% (w/v) discontinuous sucrose gradient. Aliquots were taken from the 16%–31% and 31%/45% interfaces and probed for the presence of P-glycoprotein using the monoclonal antibody C219. The C219 antibody was raised against the P-glycoprotein from the multidrug resistant subline, CHRC5 [18] and was a gift from Dr. V. Ling. Lane 1: AUX/B1, 16%–31%; lane 2: CHRC5, 16%/31%; lane 3: 16C, untreated, 16%/31%; lane 4: 16C, untreated, 31%/45%; lane 5: 16C/ADR^R, 16%/31%; lane 6: 16C/ADR^R, 31%/45%. "p": P-glycoprotein. Equal amounts of protein were loaded onto each lane.

the success of therapy. Although several mechanisms of resistance to ADR have been identified, including those related to drug efflux and P-glycoprotein expression [1–5] and GSH metabolism [6, 7], it is unknown which of these, if any, is responsible for resistance encountered in patients. A major difficulty in extrapolating results obtained *in vitro* to the situation *in vivo* is that previous *in vitro* studies used cell lines selected specifically for ADR resistance. There are two main reasons why the mechanism of resistance may not be the same for resistance selected *in vitro* and *in vivo*. These are: (i) the *in vitro* selection procedure which generally involved prolonged exposure to incremental concentrations of ADR, bears little semblance to the *in vivo* pharmacokinetics of the agent, and (ii) ADR is metabolized *in vivo*, mostly in the liver, to active metabolites [21] not found *in vitro* [22, 23], which might operate through dissimilar mechanisms from the parent drug. Alternatively, performing the selection procedure *in vivo* has been difficult due to a lack of sensitive murine solid tumor models from which to select a resistant variant.

The 16C murine mammary adenocarcinoma used in this study is one of the very few tumor models that have shown sensitivity to ADR [8, 9]. The resistant variant established in these experiments (16C/ADR^R) was obtained only after repeated ADR treatment during which resistance to ADR increased progressively. The maximal degree of resistance obtained by this selection procedure was about 3- to 6-fold, a degree of difference in sensitivity that is relatively small in comparison with those reported for resistant cell lines selected *in vitro* (see, for example, Table 1, Ref. 20). However, it is similar to the range of sensitivities observed in tumor cell lines established directly from patients [22, 23].

This similarity in the degrees of resistance suggests that perhaps the biochemical mechanism(s) responsible for ADR resistance may also be similar. In this respect, it is of interest to note that increased ADR elimination [1–4] and over-expression of the 170 kD P-glycoprotein [5] did not appear to operate in the 16C/ADR^R line. 16C/ADR^R accumulated ADR to an extent similar to that of the parent line. Neither the parent nor the variant line expressed detectable levels of the 170 kD P-glycoprotein, although the presence of lower molecular weight P-glycoprotein cannot be ruled out. These results therefore suggest that resistance to ADR does not need to be associated entirely with changes in drug transport, a conclusion that is consistent with the findings of a number of recent studies using human tumor cell lines [24–28]. Strong evidence against the possibility of a major participation of the P-glycoprotein associated *mdr* mechanism was obtained from the cross-resistance studies. 16C/ADR^R demonstrated a complete lack of cross-resistance with the *Vinca* alkaloids VCR and VBL (Fig. 3), a feature that is in distinct contrast with the findings obtained using *mdr* cell lines (for example, see Ref. 20). Similarly, the lack of cross-resistance with VP-16 suggested that changes in topoisomerase II activity were not a major factor responsible for ADR resistance of the 16C/ADR^R tumors.

The role of GSH in the ADR resistance of the 16C/ADR^R line is somewhat less clear. Although the GSH content of the 16C/ADR^R line was ~ 1.7 times higher than the parent line, reduction of the GSH content of the resistant variant to the parental level did not restore the original sensitivity to ADR. This is not to say, however, that the changes in GSH metabolism (e.g. increase in the rate of GSH synthesis) that led to the elevated GSH levels in the resistant cells may not also be responsible for the resistance itself. For example, increases in the rate of GSH synthesis have been shown to impact the cellular response to ADR [23].

The largest difference found between the sensitive and resistant 16C lines was in the activities of the two detoxification enzyme systems, GSH peroxidases and GSH *S*-transferases. It is quite possible, therefore, that the resistance to ADR observed in

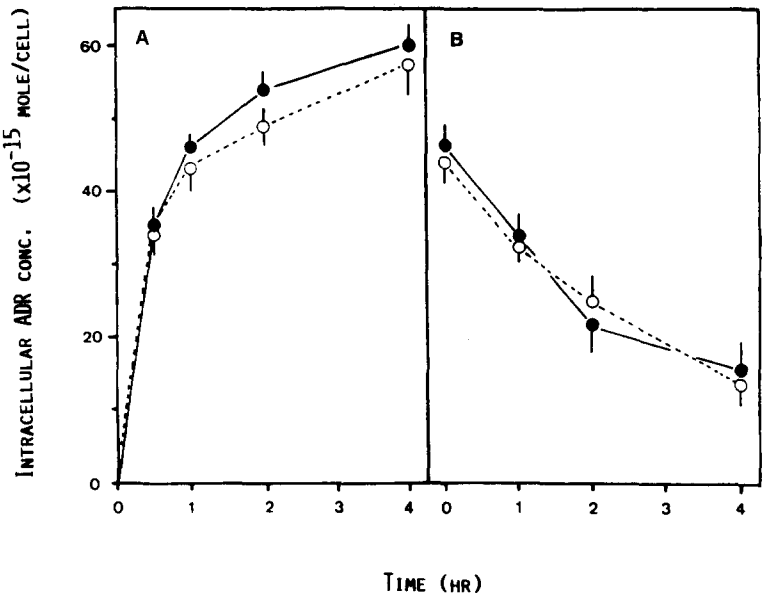

Fig. 7. Intracellular Adriamycin® concentrations in the 16C parent (○) and ADR-resistant (16C/ADR^R) subline (●) tumor cells isolated from *in vivo* solid tumors by centrifugal elutriation. (A) Amount of ADR accumulated in cells exposed to 1 μg/ml ADR for various time up to 4 hr. (B) Kinetics of ADR elimination. Cells were exposed to 1 μg/ml ADR for 4 hr, immediately washed free of ADR, and resuspended in ADR-free medium. Aliquots of cells were removed at different times afterwards for ADR assay. Data were from representative experiments. A repeat experiment gave similar results.

the 16C/ADR^R line was due to increased activity of these two enzyme systems. This conclusion would be similar to that reached in previous studies with a human breast cancer cell line (MCF-7) where resistance to ADR also was found to be associated with elevated GSH peroxidase and GSH *S*-transferase activities [6, 7]. In these investigations the resistant line, selected *in vitro*, was 192 times more resistant than the wild type and exhibited a 45-fold increase in GSH *S*-transferase activity. Although it is now clear that a large portion of the multidrug resistance observed in the MCF-7 variant was due to increased *mdr* expression, a significant remaining portion of the ADR resistance was because of changes in the glutathione redox cycle [29]. A similar conclusion has also been made recently for two ovarian adenocarcinoma cell lines derived from a patient before and after the onset of acquired drug resistance [30]. It is perhaps important to note that in the present study the 16C/ADR^R tumors exhibited considerably lower resistance factor (3- to 6-fold) than practically all *mdr* cell lines (for example, see Ref. 20). The mechanisms responsible for the ADR resistance of such low magnitude may well not be the same as those responsible for ADR resistance of much greater magnitude (e.g. >100-fold).

In conclusion, it is clear at present that the mechanism of resistance to ADR in patients is not known. Whilst there is little doubt that increased P-glycoprotein expression is important in some instances, increasing evidence has suggested that resistance in human tumors may not be due entirely to a membrane-related drug transport mechanism. The present study supports this possibility and further suggests that GSH *S*-transferases also may play an important role in ADR resistance. Indeed, the physiological role of the enzyme in detoxification, its presence as an integral part of the nuclear domain [31], and its inducibility and great adaptability due to the existence of multifarious isomeric forms [32] should logically make it a likely candidate for modification through selection.

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