In Vivo and in Vitro Mechanisms of Drug Resistance in a Rat Mammary Carcinoma Model

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ABSTRACT

Many in vitro tumor models have been examined to help understand the precise mechanisms responsible for drug resistance. The importance of these results in vivo remains uncertain. MatB 13762 is a rat mammary adenocarcinoma cell line that can be grown both in vitro and as a solid tumor in Fischer 344 rats, thus permitting the examination of tumor cell drug resistance under both conditions. Two cell lines have been selected in vitro for resistance to Adriamycin (AdrR) and melphalan (MinR), respectively. Each subline has the following features: AdrR, increased mdr-1 messenger RNA, a high level of cross-resistance to vincristine and atypical low level resistance to melphalan and 1,3-bis(2-chloroethyl)-1-nitrosourea, decreased cellular glutathione content, and increased expression of Yc and Yp glutathione S-transferase isozymes; MinR, low level drug resistance to melphalan and cross-resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea, Adriamycin, and vincristine; increased cellular concentration of glutathione; elevated glutathione S-transferase activity; and greatly increased messenger RNA specific to the Yc and Yp glutathione S-transferase subunits. Most of the biochemical and molecular features described above are present but significantly less prominent in tumors grown in vivo. This model provides the opportunity to examine the magnitude of expression and the clinical significance of in vitro resistance in an in vivo model.

INTRODUCTION

Most metastatic or unresectable tumors are incurable. The existence or emergence of drug-resistant cells limits the effectiveness of chemotherapy. For example, the initial response of breast carcinoma to chemotherapy is limited in duration, and recurrence is inevitable. The emergence of this acquired resistance is not avoided by treating with combination chemotherapy that includes a number of drugs with different chemical structures and mechanisms of action. Cross-resistance of tumors to a number of different drugs is therefore a common clinical feature.

Many in vitro experimental models have been developed to better understand the mechanisms of drug resistance (1–5). In general, these human- and animal-derived drug-resistant cell lines express a phenotype that is characteristic for resistance to a particular class of drugs. One common experimental model of drug resistance is termed multidrug resistance (MDR). Selection of mammalian cells in vitro for resistance to any one of a limited group of lipophilic antineoplastic agents results in cross-resistance to other distinctly different members of the group. Alkylating agents are specifically not among these drugs. The MDR phenotype is associated with decreased intracellular drug accumulation resulting from energy-dependent drug efflux (6–8). A high-molecular-weight membrane-associated glycoprotein called P170 and encoded by the MDR-1 gene forms this pump. Compounds such as verapamil inhibit P-glycoprotein function and result in increased drug accumulation and sensitivity to cytotoxicity. Enhanced MDR-1 expression may be due to amplification of the gene (seen most commonly in in vitro models) or increased transcription of a single copy gene (seen in clinical specimens) or a combination of both (7, 9, 10).

Studies have shown elevated MDR-1 expression in some clinical specimens of both solid and hematological cancers including ovarian ascites cells (11), acute nonlymphoblastic leukemia (12), colon cancer (13), and breast cancer (14). However, there is not a consistent relation between the level of expression in these tissue specimens and clinical resistance to chemotherapy. While MDR-1 expression is a feature of some de novo resistant tumors (e.g., colon cancer), it is also apparently absent from others (e.g., lung carcinoma and melanoma) (15). In addition, MDR-1 expression is a common feature of a number of normal tissues with secretory or membrane transport functions (e.g., colon mucosa, adrenal gland, and blood-brain barrier), so it may play an important role in normal physiological function (16). Finally, there appears to be a relation between cellular differentiation and MDR-1 expression, such that “tumor stem cells,” the target of anticancer therapy, have lower MDR-1 expression. These findings have important potential implications for the strategy of targeting P-glycoprotein as a part of cancer therapy. Other potentially important features of multidrug-resistant cells have also been described, including (a) enhanced cellular defense against xenobiotic toxicity resulting from glutathione S-transferase (3, 17, 18), increased glutathione (18), and increased glutathione peroxidase (3, 19); (b) decreased levels or altered structure of topoisomerase II (20); and (c) increased drug metabolism to noncytotoxic products (21).

Drug resistance has also been described in a number of cell lines selected for resistance to alkylators and nitrogen mustards. Resistance to these agents has been associated with alterations in cellular glutathione content and GST activity. Both human ovarian cancer cells and a rat brain tumor cell line selected for resistance to nitrogen mustards have increased GSH concentration (22, 23). Buthionine sulfoxime, which depletes cellular GSH content, results in sensitization of tumors to melphalan in vitro and in vivo in both parenteral and resistant tumor cells (18, 23–25). Other proposed mechanisms of alkylator resistance include overexpression of specific GST isozymes, altered cellular drug accumulation and metabolism, or enhanced removal of DNA cross-links (26). Both rat mammary carcinoma cells and Chinese hamster ovary cells selected for resistance to chlorambucil have significantly higher concentrations of an α class form of GST in association with slightly increased GSH concentration (23, 27, 28). Moreover, recent reports of experiments in which full-length cDNAs of the α GST gene family transfected into yeast (29) and the π form transfected into mammalian Cos and NIH-3T3 cells (30, 31) provide direct evidence for GST’s role in alkylator and Adriamycin resistance, respectively. On the other hand, transfection
of GST into a human breast cancer cell line did not confer resistance to either alkylators or Adriamycin (32, 33). In addition, a recent study of tumor cells selected for alkylator resistance in vivo suggests that entirely different mechanisms are operative which are not even apparent in vitro (34).

The precise mechanism(s) responsible for resistance to the nitrogen mustards and the magnitude of clinical expression and functional significance of the proposed mechanisms of MDR in vivo are not certain. The studies reported here describe biochemical and molecular mechanisms associated with drug resistance in a rat mammary carcinoma cell line. MatB mammary tumors have many features consistent with the human disease. Some of these include responsiveness to natural product antineoplastics and alkylating agents, solid tumor vascularization, and metastases to regional lymphatics. This model, therefore, provides a potentially useful preclinical system in which to study drug resistance and evaluate appropriate maneuvers to circumvent it.

MATERIALS AND METHODS

Cell Growth in Vitro and In Vivo. MatB 13672 is a cell line derived from a female Fischer rat mammary tumor. Cells grow both in vitro and in vivo. WT MatB cells are grown in vitro in α MEM (Gibco) (supplemented with 1.3% sodium pyruvate, 2.6% glutamine, 1.3% nonessential amino acids) containing 10% fetal bovine serum and 100,000 units/liter gentamicin. Adriamycin-resistant (AdrR) MatB cells were selected by exposing the cells to escalating drug concentrations beginning at 10−10 M Adriamycin. The final resistant subline was established when cells were able to survive in 10−8 M Adriamycin. Cells are maintained in medium containing this concentration of Adriamycin. Similarly, a melphanal-resistant (MlnR) MatB subline was selected in escalating doses beginning at 10−8 M melphanal. Cells surviving at 10−5 M melphanal are maintained at this concentration.

Both drug-resistant sublines were passaged in drug-free media for at least 2 wk prior to use in studies of drug sensitivity, enzyme assay, and nucleic acid analysis. The WT and resistant MatB sublines were grown as solid tumors in 10–12-wk-old Fischer 344 rats. After an injection of 5 × 106 cells s.c., a solid mass is palpable within 2 wk. On postmortem examination, metastases to regional lymph nodes are present.

Drug Sensitivity Assays. Logarithmically growing cells were harvested by gently tapping the side of the flask (in fresh media) and were examined, metastases to regional lymph nodes are present. This model, therefore, provides a potentially useful preclinical system in which to study drug resistance and evaluate appropriate maneuvers to circumvent it.

Drug Sensitivity Assays. Logarithmically growing cells were harvested by gently tapping the side of the flask (in fresh media) and were plated in triplicate into 6-well plastic tissue culture dishes (Nunc) at a density of 400 cells/well in a total volume of 5 ml containing 0.35% agar, 20% fetal bovine serum, 25% 2× α MEM, and 34% α MEM. Varying amounts of drugs were added to the dishes. Melphanal was freshly prepared for each assay in acid-alcohol. The cells were incubated under 5% CO2 at 37°C for 10–14 days without changing medium. Colonies containing more than 50 cells were counted. The cellular drug resistance was expressed as the IC50 value, which is the drug dose resulting in 50% of the colonies surviving compared to the number in the untreated samples.

Drug resistance levels in vivo of MlnR cells was determined from tumor growth delay experiments. Rats received transplants of either 5 × 103 WT or MlnR cells. When the tumors were palpable, three groups of three animals each were treated with single i.v. injections of melphanal at 0.5, 1.5, or 3.5 mg/kg for WT tumor-bearing animals or 3.5, 5.0, or 6.5 mg/kg for MlnR tumor-bearing animals. Tumor size (length x width) was measured every other day following drug treatment. Six animals per group bearing either WT or AdR4 tumors were treated with a single i.v. injection of 20 mg/kg Adriamycin. Twenty-four h after treatment, tumors were removed and disaggregated in 0.05% collagenase (Sigma Chemical Co.) in Hanks’ balanced salt solution (Gibco). Viable cells were counted based on their ability to exclude the dye trypan blue. Their ability to form colonies in vivo was examined in triplicate.

Materials and Reagents. Melphanal, vincristine, and BCNU were purchased from Sigma Chemical Co. Adriamycin was purchased from Adria Laboratories. Guanidinium isothiocyanate and cesium chloride were from IBI. RNA and DNA size standards were from Boehringer Mannheim. Hybond-N membranes and deoxyctydine 5’[α-32P]pitriphosphate (3000 Ci/mmole) were from Amersham. SDS polyacrylamide gel reagents including secondary antibodies, molecular standards, and nitrocellulose membranes were from Bio-Rad.

Tissue Fractions and Enzyme Assays. Tissues were harvested immediately after sacrifice of the animals and were dissected free of necrotic or hemorrhagic material. Tissue fractions were prepared at 4°C. The tissues were weighed, homogenized with a Polytron homogenizer in a 10% solution containing 0.25 M sucrose, 150 mM KCl, and 50 mM Tris, pH 7.4. The homogenate was spun at 10,000 × g for 30 min. The remaining supernatant fluid was centrifuged at 100,000 × g for 1 h, and the supernatant (cytosol) was assayed for GST using CDNB according to the method of Habig et al. (35). Total GSH was assayed according to the technique of Elman (36) in tissue homogenized in 3% sulfoisalicylic acid.

Selenium-dependent GSH peroxidase activity was assayed using H2O2, and selenium-independent GSH peroxidase activity was assayed using cumene hydroperoxide according to the method of Paglia and Valentine (37). Protein concentrations in all fractions were determined by the method of Lowry. Student’s t test was used to assess the degree of significance between the different tissue samples assayed for enzyme activity.

Western Blotting. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli. A 4% stacking gel was layered over a 12% resolving gel. Fifteen μg of protein from each sample were allowed to stack under 100 mA of current, and the remaining running time was under 80 mA. The protein trapped in the gel was transferred onto zeta-probe membranes by electrophoretic transfer for 3 h at 50 V. The membrane was then blocked in 5% skim milk/PBS for 1 h at room temperature and subsequently reacted with the selected polyclonal antiserum (anti Y: 1:250 dilution in PBS, anti α-μ; 1:500 dilution in PBS) overnight at room temperature. Following three washes with wash buffer (0.05 M phosphate, 0.5 M NaCl, 0.1% Tween 20, pH 8.0), a goat anti-rabbit horseradish peroxidase-conjugated second antibody was added at a 1:500 dilution in PBS. The blots were then incubated with diaminobenzidine and the sites of antibody binding were revealed by a brown precipitate. Glutathione S-transferase is a multienzyme family encoded by three distinct gene families termed Yα (Ya and Yc) subunits, α (Yb1, Yb2, Yb3), and γ (Yp). Dimers of subunits from each family form the functional enzyme. Rat liver cytosol was used as a control for Ya, Yb, and Yc subunits, and purified GST γ is the positive control for the Yp GST subunit.

Isolation of Nucleic Acids and Hybridization Studies. Approximately 1 × 106 cells were grown, harvested, and washed in PBS. The cells were then resuspended in 10 ml of PBS. To that volume, NaCl and SDS were added to achieve a final concentration of 0.5 M and 0.5%, respectively. The mixture was put on ice for 10 min, and the DNA-containing aqueous phase was extracted 3 times with an equal volume of phenol equilibrated with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The remainder of the protocol was performed essentially as described (38). The DNA was verified to be of high molecular weight by agarose gel electrophoresis.

Tissue DNA was prepared by pulverizing 2–3 g of frozen tumor (cleaned free of necrotic tissue and blood) in liquid nitrogen to a fine powder. One % SDS in RSB buffer (10 mM Tris, pH 7.4-10 mM NaCl-25 mM EDTA) released DNA from nuclei. Purified DNA was further prepared according to the method of Maniatis et al. (38). The final DNA pellet was dissolved in TE to a final concentration of 2-3 μg/μl.

Total RNA was extracted from cultured cells and pulverized tumor tissue by homogenizing in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion. Twenty μg of total RNA were electrophoresed in 1% agarose/6% formaldehyde gels as described (38). Hybridization was in 1% bovine serum albumin, 0.1 mM EDTA, 0.5 M NaHPO4, 5% SDS, and 50% formamide for 18 h at 42°C. The blots were washed (15 min each time) by the addition of 1 × SSC (0.15 M NaCl-15 mM sodium citrate), 0.1% SDS at room temperature 4 times, followed by 4 washes at 65°C. For subsequent hybridizations,
membranes were boiled in 0.01 × SSC, 1% SDS 4 times for 20 min each to remove previously used probes.

DNA probes were labeled in vitro with [32P]dCTP by nick translation essentially as described (38). The rat cDNA GST probes used in these studies were the following: Yp-SalI/EcoRI insert of plasmid pGP5 (kindly provided by Dr. M. Muramatsu), and Ya/Yc and Yc-PstI inserts of plasmids pGTP38 and pGTB42 (kindly provided by Dr. C. Pickett). Hybridizations were also performed with an EcoRI-generated mdrl pCHP1 cDNA insert. The levels of gene expression were determined by densitometry of the autoradiograms.

RESULTS

Drug Sensitivity Studies. The drug sensitivities of the wild type and drug-resistant sublines determined in clonogenic assays are shown in Fig. 1. Each point represents the mean of at least 3 separate experiments. The standard error for each determination is less than 10%. The Adr* cells are 200-fold more resistant to Adriamycin than the WT cell line (Fig. 1A). The stability of resistance was determined by cloning Adr* cells passaged in drug-free media for 7 mo. After this period of time these cells displayed 166-fold resistance to Adriamycin. The Adr* cells are 230-fold resistant to vincristine (Fig. 1B). In addition, Adr* cells show 2-fold resistance to melphalan (Fig. 1C) and 2.4-fold resistance to BCNU (Fig. 1D). This is consistent with other reports of primary resistance to doxorubicin associated with cross-resistance to alkylating agents (24).

The survival curve for Mln* cells demonstrates a 10-fold level of resistance to melphalan (Fig. 1C) and 17-fold resistance to BCNU (Fig. 1D). Mln* cells appear to be marginally cross-resistant to members of other drug classes. Fig. 1A reveals that Mln* cells are 2.3-fold resistant to Adriamycin and 2-fold resistant to vincristine (Fig. 1B). There is little change in the resistance to melphalan seen in the Mln* cells over a period of 7 mo (10-fold versus 9-fold).

In Vivo Tumors. Pathological examination of a MatB tumor indicates that this tumor is a poorly differentiated mammary adenocarcinoma. Hormone receptor assays for both estrogen and progesterone are negative, similar to high grade, poorly differentiated human breast cancers. Preliminary studies of drug sensitivity of the sublines have been performed in vivo. Animals received injections of either the WT or the Mln* cells (Fig. 2). After 2 wk tumors were palpable. At this time, groups of at least 3 rats were treated with a single injection of melphalan administered i.v. via the tail vein. Tumor size was measured every other day. Using tumor growth delay it was possible to determine the fold resistance to melphalan of Mln* cells in vivo. In WT tumor-bearing rats, 1.5 mg/kg melphalan resulted in prolonged tumor growth delay or complete disappearance of the tumor over 3 wk. In Mln* tumor-bearing animals, there was a tumor response in one-third of animals treated with 3.5 mg/kg. At higher doses there was considerable toxicity, but the one surviving animal treated with 6.0 mg/kg melphalan also had disappearance of the tumor. We conclude that in vivo resistance of Mln* cells is 2–4-fold.

Preliminary studies with i.v. Adriamycin demonstrated that the dose required to affect cell growth (even WT tumors) is greater than the maximal tolerated dose of 7.5 mg/kg. Excisional tumor assays were performed to overcome this problem. In these experiments animals bearing either WT or Adr* tumors were treated with a single dose of Adriamycin (20 mg/kg) administered via the tail vein. Twenty-four h after treatment tumor cells were removed from the rats, and their ability to form colonies in vitro was examined. Resistance was measured as the surviving fraction relative to untreated controls. Tumor cells from drug-treated WT tumors formed 7.6% colonies relative to untreated cells [25.5 ± 2.56 (SE) versus 334 ± 12.9]. Adr* tumor cells treated with Adriamycin had a 73% colony survival fraction relative to untreated cells (51 ± 6.8 versus 70 ± 9.1). The Adr* subline is approximately 10-fold resistant to Adriamycin at 20 mg/kg.

Biochemical Characterization of WT and Resistant Sublines. The enzyme activities (mean ± SE) examined in cultured cells and tumor tissue derived from tumor-bearing animals are shown in Table 1. There is no apparent difference in selenium-dependent glutathione peroxidase activity between the MatB cell lines

![Fig. 1. Survival patterns of WT and resistant sublines in Adriamycin (A), vincristine (B), melphalan (C), and BCNU (D). WT: Adr*: Mel*: Values are the means of 3 experiments, with the SE less than 10% of the reported values.](image-url)

![Fig. 2. Tumor growth rate of tumors treated in vivo. WT tumor-bearing animals received no drug (○), 0.5 mg/kg (●), 1.0 mg/kg (■), or 1.5 mg/kg melphalan (▲). Mln* tumor-bearing animals received no drug (○), 3.0 mg/kg (●), 5.0 mg/kg (▲), or 6.0 mg/kg melphalan (■). Each point represents one animal; at least three animals were used per drug dose.](image-url)
Table 1 Enzyme activities in WT and resistant sublines in vitro and in vivo

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<tr>
<td></td>
<td></td>
<td>WT</td>
<td>Adr*</td>
<td>Mln*</td>
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<tr>
<td>GSH peroxidase</td>
<td></td>
<td>2.0 ± 0.8</td>
<td>2.1 ± 0.3</td>
<td>2.4 ± 0.3</td>
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<tr>
<td>Hydrogen peroxide*</td>
<td></td>
<td>0.62 ± 0.1</td>
<td>0.65 ± 0.1</td>
<td>0.80 ± 0.1</td>
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<td>Cumene hydroperoxidase*</td>
<td></td>
<td>7.9 ± 0.2</td>
<td>11.8 ± 0.5</td>
<td>16.6 ± 1.2</td>
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<tr>
<td>Glutathione S-transferase*</td>
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<td>48 ± 2.7</td>
<td>15.6 ± 1.9</td>
<td>88 ± 3.4</td>
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<tr>
<td>Glutathione*</td>
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| Enzyme                        | In vitro |          |          |          |          |
|                               |          | WT       | Adr*     | Mln*     |          |
|                               |          |          |          |          |          |
|                               |          |          |          |          |          |

The results are expressed as the means ± SE of at least three separate determinations.

- GSH peroxidase activity is 3.7-fold increased in activity in MlnR cells relative to WT cells in vitro and 1.7-fold increased in activity in a sample prepared from a MlnR solid tumor. MlnR cells in vitro have a 4.7-fold increase in GST activity, whereas activity measured in MlnR tissue is increased 2.7-fold relative to WT tissue. For AdrR cells grown both in vitro and in vivo, GST activity is increased 1.5-fold.

Glutathione was measured in cultured cells at the plateau phase of growth, since this has previously been shown to correlate best with tumors in vivo (39, 40). AdrR MatB cells have a 67% reduction in total GSH concentration relative to WT cells, whereas in MlnR MatB cells the GSH concentration is almost double. In contrast to the AdrR cells grown in tissue culture, those grown in an in vivo environment have a 2-fold increase in GSH concentration. MlnR cells in vivo have a 2.8-fold increase in GSH concentration.

Western Blot Analysis. In both AdrR and MlnR cells in vitro, there is an increase in the amount of a protein whose immunoreactivity is consistent with the Ya subunit of GST (Fig. 3). A larger protein consistent with the Yc subunit is present at extremely low levels in WT cells (not obvious from the photo reproduction), whereas the Yc subunit is increased in AdrR and even more so in MlnR cells.

WT tumor tissue, like cultured cells, expresses predominantly Ya protein. The AdrR and MlnR tissue samples have a slightly increased level of Ya protein, however. These samples also demonstrate the presence of the Yc subunit, which is present in greater amounts in the MlnR tumor sample. Thus, drug-sensitive MatB tumor cells, whether in vitro or in vivo, contain the Ya subunit as the principle glutathione transferase isoform. However, drug-resistant sublines express an altered GST pattern. This in vitro change is present but to a lesser degree in vivo. None of the sublines demonstrated the presence of a band corresponding to the Yb subunit. Northern analysis using a Yb-specific cDNA probe revealed a weak signal that was equivalent in all sublines (data not shown). Thus, Yb protein levels may not be detectable using our antisera.

Reaction of the same samples with a polyclonal antibody directed against the Yp subunit is shown in Fig. 4. In AdrR cells in vitro, Yp levels are unchanged, whereas MlnR cells demonstrate greatly increased amounts relative to WT cells. There is no significant change in the concentration of Yp protein levels in the resistant sublines in vivo versus WT tumor tissue.

Expression of MDR and GST in mRNA. Radiolabeled glutathione transferase cDNA probes were hybridized to total RNA prepared from cell lines in vitro and their corresponding solid
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Fig. 4. Western immunoblotting of cytosolic preparations of MatB cell lines (15 μg each). Lane 1 is purified human GST π. Samples were reacted with a polyclonal rabbit anti-GST π antibody.

tumors in vivo. Fig. 5A illustrates the results obtained when an α gene family probe (Ya/Yc) is used. The amount of mRNA is greatest in the MlnR cells in vitro. The signals in vitro reveal that the MlnR cells express more Ya/Yc than does AdrR tissue. These data correspond with the protein quantitation results shown in Fig. 3. The same membrane was then stripped and rehybridized with an actin probe to control for variations in RNA loading. This figure (Fig. 5B) serves as the control for all Northern analysis. When a Yc-specific cDNA is used (Fig. 6), a similar pattern is observed. Both resistant sublines in vitro have increased Yc mRNA (MlnR > AdrR). This is apparent in vivo as well, but to a lesser extent in both cell types. Densitometric quantification of the hybridizing bands demonstrates that GST-Yc is increased 14-fold in vitro versus 3-fold in vivo in MlnR cells. All of these findings coincide with the data presented in Fig. 4. In the AdrR cells Yp expression is unchanged from that in WT cells (Fig. 7). In MlnR cells Yp expression is increased 10-fold, consistent with the results of Western analysis (Fig. 4). In both resistant sublines grown in vivo, there is no change in Yp-specific mRNA levels relative to WT tissue.

To assess expression of MDR-1, the steady state level of RNA containing related sequences was analyzed with the 32P-labeled complementary DNA subclone pCHP1 (Fig. 8). AdrR cells grown in vitro express high levels of a 4.5-kilobase mRNA that hybridizes to the probe. Examination of RNA prepared from AdrR tissue also demonstrates the presence of the MDR1 transcript; however, the level of this expression is 5-fold lower than that observed in vitro; MDR-1 RNA sequences are not present in any MlnR cells.

Analysis of Gene Amplification in Wild Type and Resistant Sublines. To determine whether the Adriamycin-resistant subline has amplified the MDR-1 gene, we analyzed DNA from cells grown both in vitro and in vivo. Fig. 9 shows autoradiograms of a Southern blot analysis of DNA digested with HindIII and probed with a 680-base pair insert from the pCHP1 plasmid containing a segment of the hamster mdr-1 gene. There is no evidence of rearrangement of this gene, and a gene amplification (2-fold) is seen in AdrR cells grown both in vitro and in vivo (Fig. 9A). The actin control for this blot is shown in Fig. 9B.

The increased expression of glutathione transferases, particularly in the MlnR cells, is not associated with amplification of these genes (data not shown). Western analysis of the resistant and drug-sensitive parental cell line did not reveal any gene rearrangement (data not shown).

In Vitro/in Vivo Differences in Gene Expression. It is not possible to attribute the in vivo/in vitro differences to a dilutional effect, since the amount of stromal infiltration seen on light microscopy is extremely low (less than 5%). Endothelial cell content, determined by immunofluorescent staining for factor VIII, is even smaller (data not shown). In addition, the ratio of the amount of a specific DNA sequence (e.g., MDR-1) found in tumor relative to actin-specific DNA sequences present in both tumor and nontumor cells is constant in vitro and in vivo. This indicates that there is not a significantly greater amount of nontumor DNA present in vivo than in vitro. Therefore, there appears to be some effect on expression of the genes studied here by the microenvironment (in vitro and in vivo). Since we have previously shown that the proliferative state of some cells affects the expression of GST (41), RNA hybridizations were done in cells grown and harvested both in confluent stationary growth and in the proliferative logarithmic stage. No consistent effect to explain the in vivo/in vitro differences was seen.

DISCUSSION

We have developed an animal model of drug-resistant breast carcinoma to study the phenotype of resistant cultured cells in an in vivo environment. The MatB rat tumor is particularly well suited for this type of investigation because, unlike so many other existing ascitic tumor models, it grows in rats as a solid mass with a vascular supply and metastasizes to regional lymphatics. This model allows a direct comparison of in vitro and in vivo resistance and provides the opportunity to adapt in vitro...
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manipulations of circumventing drug resistance to an in vivo environment. The biochemical and molecular features of this model in vitro are similar to other models of natural product and alkylator resistance, which suggests that MatB may be useful as a preclinical model to study in vitro drug resistance. For the classic alkylating agents, prolonged in vitro selection pressure can produce only low levels of resistance compared with the parent line. However, extensive selection pressure using natural product antineoplastics can generate low level resistance within a short period of time and with prolonged exposure extremely high levels (up to 2000-fold in some cases) of resistance that may not exist in a clinical setting. The degrees of resistance that we have achieved for the AdrB and MlnB MatB sublines are comparable to those previously described for other cell lines resistant to similar products. These results lend support to the value of MatB as a useful model.

The in vitro studies of biochemical and molecular parameters were performed with confluent cell cultures that have been shown to more closely match in vivo preparations (39, 40). P-glycoprotein expression in AdrB MatB cells is 5-fold less in vivo relative to in vitro grown cells. The proportion of cells that are both malignant and resistant apparently has not changed in vivo, since the MDR gene remains amplified to the same extent in vivo and in vitro (2-fold) in the AdrB cells. The pattern of both MDR and GST overexpression in AdrB cells is consistent in culture and in tissue; however, the magnitude of expression in in vitro-conditioned cells is higher. The decreased expression in vivo is common to most other tumors that express low or barely detectable amounts of MDR-1 RNA. It has been previously reported that just detectable MDR expression is associated with 6-fold resistance to Adriamycin (42). A recent study showed that in vivo-selected AdrB cells accumulated 4–5-fold more drug when transferred to an in vivo environment, which also suggests a lowered MDR-1 expression (43). Other studies have shown that decreased drug accumulation was not the primary mechanism of Adriamycin resistance in ovarian cancer cells from clinically refractory patients, despite observing such a mechanism in cells with in vitro-induced Adriamycin resistance (44).

A number of studies reported elevated GSH concentration and GST activity associated with alkylator resistance (24, 28, 45–47). There is some evidence that GST catalyzes the conjugation of GSH to drug or to drug-DNA adducts in the case of alkylators (48). There is in vitro evidence that GST-μ is involved directly in the denitrosation reaction of nitrosoureas in a rat glioma cell line (49). Studies have also shown that melphalan is a substrate for GST-catalyzed conjugation with glutathione (50). Class α-GSTs have more consistently been demonstrated to be associated with cellular resistance to a number of chemotherapeutic agents. Although Ya and Yc encoding genes are

Fig. 5. Northern blot analysis. A. Ya/c expression. Denatured RNA (20 μg were run) was subjected to electrophoresis in an 0.8% agarose gel. Following transfer onto Hybond N membrane, hybridization was performed with the 32P-labeled insert of probe pGTB38 prior to autoradiography for 2 days. Arrows at left, positions of the 28S and 18S ribosomal bands; arrows at right, position of the hybridizing band. 8, hybridization with actin to standardize the amount of RNA in each lane.

Fig. 6. Northern blot analysis of Yc. Hybridization was performed with the 32P-labeled insert of probe pGTB42. Arrow at right indicates the position of the hybridizing sequence.
both members of the α family, with approximately 70% homology in the protein coding region, it has been suggested that they are members of independent gene families based on their differential drug inducibility and organ distribution (51).

Walker 256 rat breast carcinoma cells resistant to bifunctional nitrogen mustards show overexpression of the glutathione transferase Yc subunit (28, 52). Nitrogen mustard-resistant Chinese hamster ovary cells demonstrate increased expression of both Yc and Yp subunits (47). As demonstrated in this study, growth of MlnR MatB cells in vivo is accompanied by a small increase in Ya/Yc and Yp expression. The increase in GST activity in these cells (2.7-fold) is similar to the increase previously observed in human ovarian cancer cells resistant to cisplatin and chlorambucil (2.1-fold) (53). In fact, the small degree of resistance to alkylating drugs observed clinically is of the same magnitude as that demonstrated experimentally in Cos cells transfected with Ya and Yp GST isozymes (29). The low level of GST overexpression observed in MlnR MatB tumors is consistent with GST transfection data and suggests that the protective role of GST in in vivo drug resistance represents an avenue worthy of further investigation.

In MatB cells GST expression is affected differently than GSH concentration. GST expression in vivo is reduced relative to cultured cells while a 2-fold increase in GSH content seen in cultured cells is maintained in vivo, suggesting that conditions for GSH biosynthesis are more favorable in vivo than in vitro. The role of glutathione in drug metabolism in an in vivo environment has been examined in resistant tumor models. Studies with murine L1210 leukemia cells (2) and human ovarian cancer cell lines established from patients exhibiting clinical signs of drug resistance (22) indicate that resistance is related to the conversion of L-phenylalanine to its noncytotoxic derivative 4-(bis(2-hydroxyethyl)amino)-L-phenylalanine, which is associated with a 2–3-fold increase in GSH in the resistant tumor cells.

There is strong evidence that GSH peroxidase plays a role in resistance to Adriamycin and that its increase in activity in the MlnR cells may be functioning in the cross-resistance to Adriamycin observed here. Glutathione transferases Ya, Yc, and Yp all demonstrate selenium-independent GSH peroxidase activity. Increased expression of these subunits may explain the enhanced detoxification of Adriamycin-mediated cellular effects. Glutathione peroxidase is thought to detoxify peroxides resulting from oxygen radicals generated by quinone-containing compounds like Adriamycin. The mechanism of resistance in the MlnR cells to vincristine is not known, but this has previously been described in other models (54, 55). The phenotype of reciprocal cross-resistance observed in MatB cells selected in vitro in either Adriamycin or melphalan is consistent with the common clinical finding of broad cross-resistance of tumors in patients who were previously treated with any class of drugs.

Although in vitro models provide mechanistic explanations of drug resistance, the magnitude of these alterations may not necessarily be a true indication of resistance levels in vivo. MatB cells provide the opportunity to examine this problem in a more clinically relevant system. In addition to cellular factors which we have shown to be involved in drug resistance, there clearly

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**Fig. 7.** Northern blot analysis of RNA from sensitive and resistant cells using 32P-labeled pGP5 DNA. Arrow at 17# position of the hybridizing sequence.

**Fig. 8.** Northern blot analysis of P-glycoprotein expression following hybridization with the 680-base pair insert of plasmid pCHP1.
Fig. 9. Analysis by Southern hybridization of sensitive and resistant sublines cultured in vitro and grown in vivo. A. DNA was digested with HindIII and probed with the pCHP1 mdrl cDNA. Twenty μg of DNA from cultured cells and 10 μg from tissue samples were used. B. hybridization with actin to standardize the amount of DNA in each lane.

are other factors observed only in vivo that may also contribute to mechanisms of clinical drug resistance (34).

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