Sequential Emergence of Distinct Resistance Phenotypes in Murine Erythroleukemia Cells under Adriamycin Selection: Decreased Anthracycline Uptake Precedes Increased P-Glycoprotein Expression

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ABSTRACT

First-step Adriamycin (doxorubicin)-resistant mutants of the murine erythroleukemia cell line PC4 were cloned from Adriamycin-containing (10 ng/ml) methylcellulose at a frequency of 3 × 10^-5. They demonstrated a 1.6- to 2.4-fold stable resistance to Adriamycin. Most were cross-resistant to etoposide, but not to vincristine, and were without enhanced expression of mdr genes, which code for P-glycoproteins. Two different murine erythroleukemia cell lines, PC4 and C7D, were passaged in suspension culture into stepwise increasing amounts of Adriamycin. No high-level resistant mutants were isolated de novo; cells initially displayed low-level resistance to Adriamycin and etoposide. Two stepwise doublings of the drug concentration were needed before PC4 cells acquired vincristine resistance, but there was no detectable overexpression of mdr or a change in drug uptake. In a subsequent doubling of Adriamycin concentration, the cells showed a further increase in resistance to all three drugs and now a decreased anthracycline accumulation. However, there was still no detectable increase in mdr expression as judged by Northern analysis of poly(A)+ enriched RNA and Western blot analysis of membrane proteins. Only after a four doubling of Adriamycin concentration did the cells demonstrate enhanced expression of mdr and P-glycoprotein. Equivalent mutants of C7D were selected, but generally at lower Adriamycin concentrations. Verapamil partially lowered resistance, but failed to restore parental susceptibility in any mutant; it caused an increased uptake in those mutants showing decreased anthracycline accumulation, including those that did not overexpress mdr. This study demonstrated different resistance phenotypes among mutants appearing spontaneously under stepwise drug selection; mutants with vincristine resistance and decreased anthracycline uptake preceded those associated with overexpression of P-glycoprotein.

INTRODUCTION

Adriamycin resistance is an important problem in clinical oncology. Most cell lines selected in Adriamycin have been examined as MDR1 cells displaying over 100-fold decreased Adriamycin susceptibility (1-3). Frequently multiresistant cells have been isolated after passage into stepwise increasing amounts of a drug at concentrations significantly higher than that achieved in vivo. Studies of such cells led to the discovery of the mdr gene family, which codes for P-glycoproteins (4, 5). These mutants display decreased intracellular drug accumulation associated with energy-dependent efflux of the cytotoxic agents (6).

The clinical importance of P-glycoprotein in acquired drug resistance has yet to be fully defined (7, 8), although studies have shown its intrinsic expression in primary tumor cells and some normal tissues (9-13). Other studies suggest that increased expression may be acquired in tumor cells during drug therapy or by selection of pre-existing mutants, which appear during relapse (9, 14). Despite these data, highly resistant cells, isolated after months of passage in vitro, appear far removed from the clinical situation in which patients may receive a drug over a few days with long intervals between treatment. Consequently, the emergence of resistant tumor cells in patients must be associated with short drug exposure times and with levels of drug far below that employed in most in vitro studies (15).

The phenotypes and accompanying mechanisms that emerge in mutants early in their progression to high-level resistance, characterized by P-glycoprotein overexpression, remain largely unknown. Intermediate cell types have been examined in other studies, but primarily to compare them with MDR mutants (16, 17). It is unclear whether particular resistance phenotypes characterize all cells progressing to higher resistance levels and what factors (e.g., drug dosage, exposure time) influence the appearance of these phenotypes. In this study, we follow the emergence of Adriamycin resistance and accompanying cross-resistance to etoposide and vincristine. We found that there was a uniform progression of phenotypes during stepwise selection with increasing amounts of drug. Only with relatively high amounts of drug were cell lines established showing high resistance levels and increased expression of P-glycoprotein. Many mutants, appearing before mdr overexpression, were stably resistant to drug concentrations above those achievable clinically. One phenotype was characterized by a decreased, verapamil-reversible, drug uptake not associated with a detectable increase in expression of P-glycoprotein.

MATERIALS AND METHODS

Drugs and Chemicals. Adriamycin was purchased from Adria Laboratories (Columbus, OH), daunorubicin was from Wyeth-Ayerst Laboratories (Philadelphia, PA), and etoposide was from Bristol-Myers Co. (Evansville, IN). Vincristine and verapamil came from Sigma Chemical Co. (St. Louis, MO). Methylcellulose was provided by Dow Chemical Co. (Midland, MI).

Cell Lines and Cultures. The MEL cell lines PC4 and C7D were grown in suspension culture at 37°C using Eagle’s basal medium containing 10% heat-inactivated fetal calf serum in a humidified, 5% CO2 atmosphere. PC4 (received from D. Houseman, Massachusetts Institute of Technology, Cambridge, MA) is a subclone of MEL cell line 745, originally derived by C. Friend, whereas the C7D line was developed in our laboratory from a s.c. tumor (18, 19). Besides different origins, the cell lines differ in their level of response to chemical induction of erythroid differentiation (19).

Mutant Derivation: MC. Logarithmic phase grown PC4 and C7D were placed in a 1.8% methylcellulose culture system (19) containing a final Adriamycin concentration of 10 ng/ml. After 5 days, plates were scored.
for colonies containing more than 20 cells, which were plucked and grown in suspension culture without drug. Except where indicated, the methylcellulose-derived mutants, referred to as PC4-MC, received no additional Adriamycin exposure. Frequency estimates are determined by the number of 5-day colonies greater than 20 cells per the number of cells that form colonies in methylcellulose without drug. With this system, the cloning efficiency of PC4 cells plated in drug-free methylcellulose without a source of colony-stimulating factor was 10%.

**Mutant Derivation: Suspension Culture.** PC4 and C7D mutants were derived in liquid culture by initially inoculating 10⁴ cells/ml in suspension cultures containing Adriamycin (5 ng/ml). After growth for 10 passages, the cultures were divided: one sample of cells was continued in medium with 5 ng/ml; another sample of cells was grown in 10 ng/ml Adriamycin. Once adapted to 10 ng/ml of drug, these cultures were again split, with one sample continued at 10 ng/ml and another increased to 20 ng/ml. In this manner, subclones were derived whose final maximum Adriamycin concentration was 5, 10, 20, 40, 80, and 160 ng/ml. These sublines are referred to as PC4-5, PC4-10, etc., or C7D-5, C7D-10, etc., whereas parental cell lines are named PC4-WT and C7D-WT. In general, cells were passaged for 10 passages in a drug concentration before being passed into the higher drug concentration. An additional C7D cell line was established (C7D-10A) by inoculating parent cells into 10 ng/ml of Adriamycin. After 3 weeks with minimal growth, a resistant cell line eventually emerged that was then easily passaged at that drug concentration. Before further studies, all cell lines were passaged in drug-free medium for a minimum of 1 month. At frequent intervals, cells were frozen in 50% fetal calf serum, 10% dimethyl sulfoxide, and stored in a liquid nitrogen freezer.

**Assay of Drug Sensitivity.** Sensitivity to Adriamycin, vincristine, etoposide, and Adriamycin/verapamil was determined by a growth inhibition assay. Both MEL cell lines grew with a 8-9-h doubling time such that 1 × 10⁴ cells/ml increase to 1 × 10⁵ cells/ml by 48 h. Thus, exponentially growing cells were seeded in suspension culture at 1 × 10⁶ cells/ml and grown in five increasing drug concentrations. After 48 h, cells were counted with a model ZBI Coulter counter. Survival was expressed as a percentage of drug-treated cells per untreated control cells, which when plotted versus log of drug concentration yielded a standard dose-response curve. The IC₅₀ was determined by calculating the linear interpolation of survival on the log of concentration. This equation was then solved for the drug concentration that gave 50% survival. Experiments were generally repeated a minimum of four times. The -fold resistances represent the ratio of the IC₅₀ of the resistant cell line to the IC₅₀ of the parental cell line.

**RNA Extraction and Northern Blot Analysis.** Total RNA was isolated using a modification of a method described previously (20). Logarithmically growing cells (5 × 10⁶) were pelleted by centrifugation, resuspended in 1 ml of PBS, and added to 5 ml of lysis buffer (0.06 M sodium acetate, pH 5.0-1.25% SDS, 65°C). Phenol (6 ml), equilibrated with 50 mM sodium acetate, was added. After 15 min of gentle stirring at 65°C, and 15 min on ice, the samples were centrifuged at 10,000 × g for 10 min at 4°C. The aqueous phase was recovered and precipitated overnight (−70°C) with 3.5 volumes of ethanol/0.1 M sodium acetate. Samples were centrifuged (10,000 × g, 60 min, 4°C) and the resulting pellets were washed with 95% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. The preparations were separated into aliquots based on absorbance determinations and reprecipitated with ethanol/sodium acetate.

After treatment with glyoxal buffer, RNA (20 µg) was separated by size via electrophoresis in 1.0% agarose at 150 V, 100 mA (21). The RNA was transferred to a nylon membrane (Gene Screen Plus; New England Nuclear, Boston, MA) by blotting with 0.75 M NaCl, 0.075 M sodium citrate buffer, then allowed to dry at room temperature.

Enrichment for polyadenylated RNA was performed essentially as described (22). Approximately 300 µg of total RNA were loaded onto a 100-ng oligodeoxynucleotide cellulose column (Collaborative Research, Bedford, MA). The total eluted polyadenylated RNA was quantified by absorbance and divided into three fractions. Samples were loaded onto a 1 % agarose gel after glyoxal treatment and subjected to electrophoresis and then transferred as described above.

**DNA:RNA Hybridization.** The murine mdr probe, pcDR1.3 (received from D. Housman, Massachusetts Institute of Technology), is a BglII fragment of ADR11, which hybridizes with all three murine mdr genes (23). This was labeled with [³²P]dCTP to a specific activity of 10⁶ cpm/µg using a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN). The membranes were prehybridized for 2 h, and then hybridized overnight in 50% formamide at 42°C as described (21). Subsequently, the blots were washed once in 50% formamide, 1% SDS, 5× SET buffer, once in 1× SET, 1% SDS; and twice in 1× SET, 0.1% SDS. Autoradiograms were obtained after exposure to Kodak XAR film at −70°C. Equal loading of RNA was assessed by staining with ethidium bromide and confirmed by rehybridizing the blots with a α-actin probe (received from G. Brawerman, Tufts University, Boston, MA).

**Isolation of Plasma Membranes and Western Blot Analysis.** Plasma membrane-enriched fractions were prepared from MEL cells by the method of Riordan and Ling (24) in the presence of 2 mM phenylmercuri sulfonfyl fluoride. Protein was quantified by the method of Bradford (25). Twenty µg/lane was resolved by SDS-polyacrylamide gel electrophoresis (26) in a 7.0% polyacrylamide gel containing 8 M urea. The samples were reduced but not heated before loading (27). The protein was transferred to Immobilon-P (Millipore, Bedford, MA) at 4°C in 25 mm Tris, 192 mM glycine/10% methanol at a constant 300 mA for 3.5 h (28). Prestained molecular weight standards confirmed quantitative transfer of higher molecular weight proteins.

**Antibody Staining.** Blotted Immobilon strips were wetted according to the manufacturer's directions before blocking for 2 h at room temperature with 5% low-fat dried milk in Tris-buffered saline. The strips were incubated with 1 µg/ml of monoclonal antibody C219 (a gift from V. Ling, Ontario Cancer Institute, Toronto, Ontario, Canada; then purchased from Centocor, Malvern, PA) at 4°C in 5% milk overnight. After extensive washing, the strips were incubated for 2 h with 1:2000 goat anti-mouse alkaline phosphatase conjugated second step (Promega, Madison, WI) and developed as per standard protocols (29). For further verification, additional strips were incubated with rabbit polyclonal antiserum (diluted 1:500) directed against murine P-glycoprotein (received from S. Horowitz, Albert Einstein College of Medicine, New York, NY) (30) and similarly developed after 1:2000 goat anti-rabbit alkaline phosphatase antibody treatment (Sigma).

**Drug Uptake Studies: Flow Cytometry.** Anthracycline accumulation was assayed using flow-cytometric analysis; daunorubicin was chosen for its superior fluorescence profile (31). Previous studies have demonstrated the validity of this method for quantifying intracellular anthracycline concentrations in parental and resistant Friend murine erythroleukemia cells (32). Cells in logarithmic growth were suspended at 1 × 10⁶/ml with PBS-glucose (1000 mg/liter) in polypropylene tubes in a shaking water bath at 37°C. Although studies have demonstrated increased uptake of daunorubicin versus Adriamycin in some parental cells (32), efflux of both drugs in MDR mutants appears to be identical (6). Preliminary studies were performed using parental cells with increasing daunorubicin concentrations (50–500 ng/ml). At each time point, there was a linear correlation between drug uptake (intracellular fluorescence) and drug concentration. All subsequent experiments were done at a final daunorubicin concentration of 100 ng/ml. At given time points, cells were rapidly diluted with ice-cold PBS, pelleted by centrifugation (0°C), and resuspended with cold PBS and kept on ice until analysis by flow cytometry. Verapamil (5 or 10 µg/ml) was added after 60 min where indicated. Similar results were found with either concentration of verapamil.

For each experiment, a Becton Dickinson FACSscan equipped with a 15-mW argon laser was calibrated using glutaraldehyde-fixed chicken RBC (Biosure; Riese Enterprises, San Jose, CA). A minimum of 1 × 10⁶ cells was analyzed after laser excitation at 488 nm for red fluorescence above 563 nm and FALS. After conversion to a linear value, mean cellular fluorescence, obtained from histogram plots of fluorescence intensity versus cell frequency, was divided by mean FALS. Linear background (divided by mean FALS) was subtracted after determining the fluorescence of the appropriate sample blank without daunorubicin (32, 33).

**Statistical Analyses.** The -fold resistances were determined by standard bioassay methods calculating the average differences and variance.
of the log values of the IC50s and then estimating the -fold resistance and 95% confidence intervals, assuming log normal distributions (34). Three-way analysis of variance was used to analyze the drug uptake data, with cell line, time, and replicate being the factors analyzed. Where significant differences were found, the individual cell lines were compared using a Newman-Keuls test for post hoc comparison (35). All statistics were calculated using software programs from Lotus (36) and Number Cruncher Statistical System (37).

RESULTS

Resistance Phenotypes of PC4 Mutants Isolated in Methylcellulose. Single-step Adriamycin-resistant mutants of the PC4 cell line were selected at a frequency of 3.1 ± 0.8 × 10⁻⁴ (range, 1.5 × 10⁻⁴ for four experiments) from methylcellulose containing 10 ng/ml of Adriamycin. Lower amounts of drug (5 ng/ml) were unable to select stable resistant mutants, whereas at a higher concentration (20 ng/ml) viable colonies could not be isolated despite increasing the cell inoculum to 10⁷/ml. In three experiments, a total of 10 subclones from 10 ng/ml were isolated for further characterization. All 10 subclones showed stable Adriamycin resistance (1.6-2.4-fold), which did not revert after 30 passages without drug. Nine of the mutants (PC4-MC-2 through 10) showed simultaneous etoposide resistance (1.6-7.2-fold), but no mutant showed detectable vincristine resistance. One mutant, PC4-MC-1, was resistant to Adriamycin alone. However, when plated into methylcellulose a second time with the same amount of Adriamycin, this mutant increased its Adriamycin resistance 2-fold (1.8- to 3.6-fold) and acquired 1.7-fold etoposide resistance, still without measurable vincristine resistance.

Effects of a Constant Adriamycin Concentration on Selection of PC4- and C7D-resistant Mutants in Suspension Culture. PC4 cells grown in liquid culture with 5 ng/ml of Adriamycin for five passages displayed approximate 2.5-fold resistance to Adriamycin and etoposide (Table 1). The resistance levels gradually increased as the cells were continued at 5 ng/ml such that by the 70th passage the cells had significantly increased their Adriamycin and etoposide resistance. PC4-5 cells never acquired detectable vincristine resistance.

Table 1  Effect of a constant Adriamycin concentration (5 ng/ml) on resistance phenotype

<table>
<thead>
<tr>
<th>Passage</th>
<th>PC4-5</th>
<th>C7D-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR*</td>
<td>VP-16</td>
<td>ADR*</td>
</tr>
<tr>
<td>p5</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>p10</td>
<td>(1.9-3.5)</td>
<td>(2.4-2.5)</td>
</tr>
<tr>
<td>p20</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>p70</td>
<td>(2.2-4.7)</td>
<td>(2.7-3.0)</td>
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<tr>
<td></td>
<td>3.3</td>
<td>3.3</td>
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<tr>
<td></td>
<td>(2.4-4.6)</td>
<td>(3.7-8.2)</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>(4.0-6.0)</td>
<td>(6.3-11)</td>
</tr>
</tbody>
</table>

* ADR, Adriamycin; VP-16, etoposide.

Adriamycin resistance to a level similar to that of C7D-5. After 10 passages at 5 ng/ml, a sample of C7D-5 cells was passed into 10 ng/ml of drug. The C7D-10 cells that emerged acquired further Adriamycin and etoposide resistance, but now, in addition, displayed low-level vincristine resistance. For comparison, parental C7D cells were directly inoculated into Adriamycin at 10 ng/ml (C7D-10A). After a considerable delay (3 weeks), a cell line emerged that was passed over 70 times at 10 ng/ml. These cells showed triple drug resistance, but at significantly lower levels compared with C7D-10. For example, they demonstrated Adriamycin resistance of 5-fold (C7D-10A) versus 10-fold (C7D-10) and vincristine resistance of 1.6-fold versus 3.1-fold. Passage of C7D-10 cells to 20 ng/ml of Adriamycin was successful at passage 12 and cell line C7D-20 was established, which showed only minimally higher drug resistance levels. Resistance increased as the cells were then passed into higher drug concentrations (Table 2). At every level up to and including 40 ng/ml, C7D cells showed greater etoposide resistance than Adriamycin resistance.

Effect of Verapamil on Drug Susceptibility. Treatment of the resistant mutants with verapamil, known to reverse resistance associated with the MDR phenotype (38, 39), failed to restore Adriamycin sensitivity to the level of the parental cell line in any of the mutants (Table 2). Verapamil pretreatment had little effect on Adriamycin cytotoxicity of the PC4-5, PC4-10, and PC4-20 mutants. However, with the PC4-40 and PC4-80 mutants, verapamil pretreatment restored the drug sensitivity to an intermediate level between the PC4-10 and PC4-20 mutants (Table 2). Likewise, verapamil treatment had essentially no effect on Adriamycin toxicity of C7D-5 or C7D-10A, but increased the susceptibility of C7D-10 cells to the same level as C7D-10A. With C7D-20 cells, verapamil treatment decreased Adriamycin resistance to a level similar to that of C7D-5.

Analysis of Daunorubicin Uptake in C7D and PC4 Mutants. The uptake of daunorubicin, as assayed by flow cytometry, was identical in the parental PC4 cells, the PC4-5 and PC4-10 cells (Fig. 1A). Minimally decreased drug accumulation was observed in PC4-20, which was not statistically significant. The addition of verapamil at 60 min had no effect on daunorubicin accumulation in the parental PC4 cells or the PC4 mutants PC4-5, PC4-10, or PC4-20 (Fig. 1A). However, the PC4-40 cells demonstrated significantly decreased daunorubicin uptake compared with the parental cell line, which was partially reversed by the addition of verapamil at 60 min. PC4-80 showed further diminished daunorubicin uptake, which was significantly less than that of the parental cells and significantly less than that of the parental cell line C7D.
of PC4-40. It was also partially reversed by verapamil addition. The C7D-5 cells reproducibly demonstrated an unexpected increased daunorubicin uptake as compared with the parental cell line (Fig. 1B). This finding was significant at P < 0.05. The C7D-10A showed no difference from wild-type cells in drug uptake, whereas the C7D-10 showed a significantly decreased drug uptake. C7D-20 displayed a further significantly decreased drug uptake when compared with the parental cells and C7D-10. The addition of verapamil at 60 ng/ml affected only C7D-10.

Analysis of mdr Expression in PC4 and C7D Mutants. Total RNA extracted from each of the PC4-MC mutants was probed by Northern analysis with the murine probe pcDR1.3. None of the 10 PC4-MC mutants displayed increased mdr expression as compared with the sensitive parental cell line. Blots exposed for longer time periods demonstrated a 4.5-kilobase mdr band as compared with the sensitive parental cell line. Blots exposed with 2.5 times more antibody C219. The same membrane-enriched fractions from PC4-WT and PC4-40 examined by Northern analysis. They still remained mdr negative (data not shown).

Analysis of mdr Expression in PC4 and C7D Mutants. Total RNA extracted from each of the PC4-MC mutants was probed by Northern analysis with the murine probe pcDR1.3. None of the 10 PC4-MC mutants displayed increased mdr expression as compared with the sensitive parental cell line. Blots exposed for longer time periods demonstrated a 4.5-kilobase mdr band in the parental cells that was not changed in any of the PC4-MC mutants. Probing the same blots with α-actin confirmed near equal RNA loading of the parental cell line and MC mutants (data not shown).

When the RNAs from mutants PC4-5, PC4-10, PC4-20, and PC4-40 were probed by Northern analysis, they showed no change from parental cell expression of mdr (Fig. 2B). Only with passage to 80 ng/ml and higher did PC4 mutants show overexpression of mdr RNA. Heavily exposed blots showed a baseline mdr expression in the parental cells that was unchanged in PC4-5, PC4-10, PC4-20, and PC4-40. To verify this result, the mdr-negative cell lines, derived in suspension culture, were passaged for 4 additional months in their respective drug concentrations, and the RNA was reextracted and reanalyzed by Northern methods; they remained mdr-negative (data not shown).

To improve the sensitivity of the Northern blot analysis, we prepared and analyzed poly(A)+ enriched RNA from PC4-WT, PC4-40, PC4-80, and PC4-160 (Fig. 3). In PC4-80 and PC4-160 cell lines, there was an easily detected increase in the previously visualized 4.5-kilobase band, as well as hybridization to a 2.0-kilobase band. This second band has been observed previously (40). The PC4-40 demonstrated only the baseline level of mdr expression, now easily detected, that was unchanged compared with the parental PC4 cell line (Fig. 3).

With the C7D mutants, a baseline equivalent mdr message was present in the C7D-WT, C7D-10, and C7D-10A. The earliest cell line with detectable overexpression of mdr was C7D-20. The expression of mdr proportionally increased with the C7D-40, C7D-80, and C7D-160 mutants (Fig. 4). The early C7D mutants that did not overexpress mdr (C7D-5, C7D-10, C7D-10A) were grown in their respective Adriamycin concentrations for an additional 4 months, and subsequently reexamined by Northern analysis. They still remained mdr negative (data not shown).

Analysis of P-Glycoprotein Expression in PC4 Mutants. Membrane-enriched fractions from PC4-WT and PC4-40 examined with monoclonal antibody C219 by the Western blot method showed a barely detectable M, 150,000 band present in each at equivalent amounts. Membranes from PC4-80 and PC4-160 demonstrated a markedly increased expression of the M, 150,000 band compared with PC4-WT (Fig. 5). For enhanced sensitivity, 2.5-fold increased amounts of membrane proteins were probed with 2.5 times more antibody C219. The same relative amounts of P-glycoprotein were observed, with PC4-WT and PC4-40 still demonstrating equivalent low levels of P-
ADRIAMYCIN RESISTANCE IN MEL CELLS

1.81
1.6–
0>
1
0.8–
0.6
0.4
0.2
0.0
15 30 45 60 75
1.0–
0.8–
0.6
1.0
0.8
0.6
45 60 75
90 105
Time (minutes)

Fig. 1. Uptake of daunorubicin in PC4 and C7D cell lines. Cells were incubated in PBS/glucose containing daunorubicin at 0.1 μg/ml. Verapamil was added at 60 min (→) to a final concentration of 10 μg/ml. At appropriate time points, cells were resuspended in drug-free PBS (°C) and cell-associated fluorescence was quantified by flow cytometry. The integrated red fluorescence (IRFL) was divided by the FALS, and background fluorescence from the appropriate sample blank was subtracted. A: PC4-WT, D; PC4-5, •; PC4-10, O; PC4-20, •; PC4-40, A; PC4-80, A. B: C7D-WT, D; C7D-5, •; C7D-10, O; C7D-10A, •; C7D-20, A.

Using three-way analysis of variance and a Newman-Keuls test for post hoc comparison, PC4-40 and PC4-80 had statistically significantly less uptake than PC4-WT (P < 0.05). C7D-5 displayed statistically significantly greater uptake than C7D-WT, whereas C7D-10 and C7D-20 had significantly less uptake than C7D-WT (P < 0.05).

glycoprotein expression. Other experiments that probed the membranes with rabbit antisera to murine P-glycoprotein gave identical results: an equivalent, barely detectable level of P-glycoprotein was present in PC4-WT and PC4-40, with marked overexpression in PC4-80 and PC4-160 (data not shown).

DISCUSSION

In contrast to most studies that have described high-level resistant mutants, we have examined the early resistant mutants that emerge when grown in low concentrations of Adriamycin. Clonal selection of first-step mutants of a murine erythroleukemia cell line PC4 was done in methylcellulose. Stable, low-level Adriamycin-resistant colonies appeared at a relatively high frequency after a single 5-day drug exposure. Nine of 10 such mutants studied exhibited low-level resistance to Adriamycin and etoposide; none showed vincristine resistance.

Progression of resistance from low-level to multidrug resistance was followed in suspension culture of two different MEL cell lines, PC4 and C7D. In both cell lines, intermediate-step mutants appeared before the isolation of high-level mutants. Mutants selected at the lowest drug concentrations (C7D-5, PC4-5, PC4-10) displayed resistance to Adriamycin and etoposide. Although mutants with Adriamycin resistance alone were isolated (PC4-MC-1, C7D-5-p5) in each instance, brief additional exposure at the same drug concentration led to cross-resistance to etoposide. These earliest stable mutants resembled mutants previously termed "atypical MDR" (41), defined by resistance to anthracyclines and epipodophyllotoxins without vinca alkaloid resistance and no decrease in drug accumulation or reversal of resistance by verapamil. Such mutants, as found in these MEL cell mutants, show no overexpression of mdr, the
family of genes that codes for P-glycoproteins (5).

When MEL cells were then passed into higher concentrations of Adriamycin, two additional stable phenotypes emerged sequentially, before overexpression of mdr. First, the cells acquired measurable vincristine resistance (C7D-10A, PC4-20) without changes in anthracycline accumulation. In the next step, the cells demonstrated higher resistance accompanied by decreased drug accumulation. These mutants showed a partial reversal of resistance by verapamil (PC4-40, C7D-10). In fact, the mutants at this stage express the full “MDR phenotype,” but without detectable overexpression of mdr RNA (Fig. 2–4). Such mutants appear to be intermediates along the way to a “classic” MDR mutant.

Reduced cellular accumulation of Adriamycin without P-glycoprotein overexpression has been described in other mammalian cell lines. An Adriamycin-resistant fibrosarcoma cell line has been reported that was P-glycoprotein negative and demonstrated decreased drug accumulation with minimal reversal by verapamil (42). McGrath et al. (40) and Marsh and Center (43) have described an HL-60 line, selected in Adriamycin, that does not overexpress mdr (40) or P-glycoprotein (43). It does demonstrate decreased Adriamycin accumulation, apparently due to efflux (44), that is reversible by verapamil (40). Others have also reported P-glycoprotein-negative Adriamycin-resistant HL-60 cells (45). Our findings are the first demonstration that mutants showing decreased anthracycline accumulation reversible by verapamil sequentially precede others with mdr overexpression. We have found no increase in mdr RNA in poly(A)+ enriched fractions and no increase in P-glycoprotein in these early mutants.

In both MEL cell lines, selection of mutants that overexpressed mdr was dependent on drug concentration and occurred as a late resistance mechanism. It did not arise de novo in a single step, but required prior steps (mutants) that had achieved lower resistance levels. Furthermore, time alone was insufficient to cause mdr overexpression, since multiple passages at lower drug concentrations failed to alter the initial mdr-negative phenotype. It is unclear why these MEL cell lines, capable of mdr overexpression, do so only as a late occurring resistance mechanism requiring passage into a particular drug concentration.

The murine genome contains a family of three mdr genes expressed differentially in a tissue-specific manner (23). In acquired drug resistance, mdr1 is sufficient to confer the multidrug resistance phenotype (46). The gene mdr2 is not associated with acquired drug resistance (5). The probe we used hybridizes to all three genes (23). It seems unlikely that increased expression of mdr was missed in intermediate mutants due to a failure of the probe to detect one or more of the known mdr gene family. Thus, we believe that decreased drug uptake in these P-glycoprotein-negative mutants may be due to the appearance of other uncharacterized efflux proteins or a mutation in a mdr gene that mediates resistance without increased expression of the gene.

The time of overexpression of the mdr gene appears to be somewhat dependent on the individual cell line. The two MEL cell lines studied were both derived from s.c. tumors from spleen implants from Friend virus-infected animals. Despite identical drug treatments, the two lines showed differences in the rate of appearance of mdr overexpression: C7D mdr mutants appeared at lower drug concentrations (20 ng/ml) and level of Adriamycin resistance (12-fold) than mutants of PC4 (80 ng/ml and 98-fold resistance).

When mdr is overexpressed (PC4-80, C7D-20), the only change from the previous mutants is further diminished drug susceptibility. Particularly striking, however, is that Adriamycin resistance more than doubles between PC4-40 and PC4-80 without significant change in etoposide or vincristine resistance. Little if any significant change in resistance occurs in C7D mutants (C7D-10 to C7D-20) coincident with initial mdr overexpression. These results suggest that the increase in P-glycoprotein is, initially at least, contributing minimally to the resistance phenotype and that other mechanisms of resistance are involved.

After the appearance of mdr overexpression, the earlier resistance phenotypes to which the cells first mutated appear to remain. For example, treatment of PC4-80 cells with verapamil restored their Adriamycin sensitivity nearly to that of the PC4-20 cells. Thus treatment modalities developed to overcome multidrug resistance based on mdr encoded P-glycoproteins, such as use of calcium antagonists or calmodulin inhibitors, may fail since cells still possess their earlier achieved resistance mechanisms.

In this study, certain distinguishable phenotypes characterized the progression of early, low-level Adriamycin-resistant mutants to multidrug resistance presumably involving more than one mechanism of resistance. An understanding of the factors that affect emergence of the early mutants and the direction and kinetics that underlie their progression to higher levels of resistance may provide insights into dealing with clinically important acquired resistance.
REFERENCES


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