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Emergence of Different Mechanisms of Resistance in the Evolution of Multidrug Resistance in Murine Erythroleukemia Cell Lines

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ABSTRACT. We examined the genetic and biochemical bases for drug resistance and the order of appearance of different mechanisms underlying the increasingly more resistant murine erythroleukemia cell lines established in Adriamycin® (ADR). In the first-step low-level resistant cell line PC4-A5 (able to grow in 5 ng/mL ADR), there was a 2-fold reduction in topoisomerase IIα and topoisomerase IIβ mRNA levels, as well as topoisomerase IIα protein and activity levels as compared with the parental cell line. The topoisomerase IIα activity levels remained reduced as the cells became increasingly more resistant. In contrast, the topoisomerase II mRNA and protein levels returned to approximately the parental levels in resistant cells growing in higher drug concentrations (40–160 ng/mL). Parental cells expressed the multidrug resistance protein (MRP), but beginning with PC4-A5 MRP expression decreased and remained reduced in increasingly resistant cell lines. At high levels of ADR resistance, the cells expressed the *mdr3* gene concomitant with the appearance of vincristine resistance and energy-dependent daunomycin and vincristine efflux. Glutathione levels, internal pH, and expression of the major vault protein (MVP) remained unchanged in all cell lines. Fluorescence microscopy revealed no alterations in daunomycin distribution or vesicle numbers between the parental and resistant cell lines. Different resistance mechanisms emerge sequentially as cells become more resistant to ADR; the mechanisms are retained during the development of multidrug resistance (MDR). In intermediate-level MDR cell lines (PC4-A10 and PC4-A20), resistance involves an as yet undetermined mechanism(s). BIOCHEM PHARMACOL **54**;12:1297–1306, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; topoisomerase; leukemia

Acquisition of drug resistance by cancer cells leads to the failure of chemotherapy. One form of acquired resistance, MDR,‡ is characterized by reduced susceptibility to structurally dissimilar anticancer drugs, including those that were not used in the initial therapy (reviewed in Refs. [1–3]). Many of the most effective drugs currently in use are associated with this phenomenon, including the anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins. From observations of a large number of cell lines selected for high-level resistance, it is clear that resistance emerges in steps from low through intermediate to high levels of resistance (e.g. Refs. [4–6]). However, the mechanisms involved and their order of appearance remain to be elucidated. The majority of studies of MDR have focused on the last cell line in a series of increasingly more resistant cell lines. Much less is known about the initial low-level, drug-resistant cell lines. Identification of early appearing mechanisms of resistance might reveal potential targets for initial modulation with chemotherapy and so reduce the likelihood that a small number of low-level resistant cancer cells might survive treatment and develop into a highly drug-resistant cancer.

Recent reports have indicated that MDR is far more complex than originally thought, involving many mechanisms, possibly in combination. These include: altered transport (e.g. P-gp and MRP), altered targets (e.g. topoisomerases), modification/detoxification of the drug (e.g. glutathione conjugation), and enhanced DNA repair [1, 7–10]. P-gp and MRP appear to function as ATP-dependent drug efflux/transport pumps to reduce the effective intracellular drug concentration [11–13]. These proteins were initially identified in cell lines selected for high-level resistance in culture after exposure to a drug(s) for long durations, i.e. conditions generally more extreme than those achievable clinically. Surveys of tumor samples before

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‡ *Abbreviations:* MDR, multidrug resistance; ADR, Adriamycin®; VP-16, etoposide; VCR, vincristine; DNR, daunomycin; MEL, murine erythroleukemia; wt, wild types; P-gp, P-glycoprotein; MVP, major vault protein; topo II, topoisomerase II; MRP, multidrug resistance protein; RT–PCR, reverse transcriptase–polymerase chain reaction; glc, glucose; N_3/2DOG, 15 mM NaN_3 plus 50 mM 2-deoxyglucose; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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and after relapse suggest that P-gp and MRP may contribute to the problem of MDR, but that these proteins are unable to account for all of the drug resistance seen in the clinic [14–18]. Among other proposed contributors are the vacuolar H^+-ATPase, a number of novel high-molecular weight proteins, and LRP, the human major vault protein [19–24]. While overexpression of the latter protein is associated with poor outcome in some cancers, transfection of the *mvp* gene alone is insufficient to confer drug resistance [24].

This laboratory has described a series of MDR murine erythroleukemia (PC4) cell lines selected for resistance to sequentially increasing amounts of the anthracycline ADR (see Table 1 and Ref. [5]). The cell lines move from low-level resistance to high-level, P-gp-mediated resistance towards many drugs. The resistance mechanisms associated with low- to intermediate-level MDR may be more clinically relevant than those of highly resistant cell lines since a resistance of only 2- to 5-fold may be all that is necessary to escape the killing effects of therapy *in vivo*. Moreover, the drug concentrations employed to generate these cell lines more closely resemble those concentrations achievable *in vivo* (i.e. on the order of 10^{-7}–10^{-8} M).

Previous reports [5] on these PC4 cell lines showed that ADR and VP-16 resistance increased with each step, but that VCR resistance was not apparent until the PC4-A40 cells were obtained (see Table 1). In this cell line, a reduced DNR accumulation was noted [5]. By northern analysis, cell lines up to and including PC4-A40 (and the subclone PC4-A40c20) did not show significant expression of the *mdr* genes. By Southern analysis, PC4-A80 was the first cell line in this series to demonstrate *mdr* gene amplification [25]. We have expanded this work and examined in more detail the basis for resistance in this series of cell lines. The observed high-level MDR is multifactorial, resulting from a complex interplay between the sequential expression of previously identified mechanisms and other unidentified ones.

MATERIALS AND METHODS

Materials

All drugs and reagents were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). [^{3}H]DNR (2.6 Ci/mmol) and [^{32}P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA), [^{3}H]VCR (6.9 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and [^{3}H]VP-16 (0.79 Ci/mmol) and [^{125}I]-labeled protein-A (0.9 Ci/mmol) were from ICN (Costa Mesa, CA). All enzymes were purchased from BRL/Life Technologies (Gaithersburg, MD).

Growth of Cell Lines

The cell lines used in this study were derived by Slapak *et al.* [5]. The PC4 series of cell lines used in this work were named so as to reflect the highest level of ADR in which they were grown, i.e. PC4-A5 (formerly PC4-5) cells were grown in 5 ng/mL ADR, PC4-A10 in 10 ng/mL, etc. Cells were grown in basal medium Eagle (BRL/Life Technologies) in the absence of drug selection. The drug resistance phenotype was stable for several months without selection.

For some of the work presented in this paper, the cell line PC4-A40c20 was used instead of, or in addition to, PC4-A40, the cell line from which it was subcloned. Our initial results by northern analysis indicated little or no *mdr* gene overexpression in PC4-A40, although a reduced DNR accumulation phenotype was present. Since there was the possibility that a novel resistance-imparting efflux protein was present, we subcloned PC4-A40 to aid in its identification. All of the subclones obtained, including PC4-A40c20, demonstrated nearly the same level of resistance with little detectable overexpression of *mdr*. We found no differences between PC4-A40 and PC4-A40c20 in drug resistance or the expression levels of *mdr3*. The data on both PC4-A40 and PC4-A40c20 are presented where they were both examined.

Preparation of Topoisomerase-Containing Extracts

Cells were pelleted at 800 *g* for 3 min in the cold and then washed in ice-cold buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 4 mM $MgCl_2$; 0.5 mM phenylmethylsulfonyl fluoride; and 10 μg/mL of each of the protease inhibitors: leupeptin, pepstatin A, and aprotinin). Cells were collected by centrifugation, resuspended in 3 mL of buffer, and incubated on ice for 10 min. Cells were lysed by 10 strokes of a tight-fitting dounce homogenizer. Nuclei were separated by centrifugation at 1500 *g* for 10 min, and the nuclear pellet was transferred to an Eppendorf tube and washed a second time with buffer. The pellet was resuspended in 3 pellet volumes of buffer lacking $MgCl_2$. An equal volume of 1 M NaCl was added, and samples were vortexed and left on ice for 40 min. Samples were then centrifuged at 4° in an ultracentrifuge at 100,000 *g* for 1 hr. The resulting supernatant fluid contained active topo I and II proteins.

Assay of Topo II Activity

VP-16-stabilized cleavage complexes were measured using reagents and the protocol supplied by TopoGEN (Columbus, OH).

Western Blot Analysis

Proteins separated by SDS–PAGE were electroblotted in 25 mM Tris, 200 mM glycine, 20% methanol, pH 6.5, onto Immobilon (Millipore, Bedford, MA). Blots were probed with antibody according to the manufacturer's instructions. Anti-topo II antibodies were obtained from TopoGEN; the anti-P-gp antibody, C219, was purchased from Signet Laboratories (Dedham, MA). Bound antibody was visualized using [^{125}I]-labeled protein-A, and, when necessary,

was quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RT–PCR

Quantitative RT–PCR was performed with controls following published methods [26]. Total RNA (5 μg) was prepared [27] and subjected to oligo(dT) primed reverse transcription using Superscript II according to supplier's instructions. The first strand synthesis reaction was diluted to 50 μL, and 5 μL was used in a standard 100 μL PCR spiked with 0.25 μCi [^{32}P]dCTP cycling between 94° denaturation, 53° annealing, and 72° synthesis, 1 min each. Primers were *mdr3*: forward, CCGGACCACCATTGTGATAGCCTC (nucleotides 1863–1886) and reverse, CACAGATGCTTTTGCGAG TTGATC (nucleotides 2144–2121), product size 281 bp; *mvp* (taken from the rat *mvp* sequence): forward, GAAAT TACCACCAACTC (nucleotides 2048–2064) and reverse, TCATAGCCTCAAGCTC (nucleotides 2198–2183), product size 135 bp; β-*actin*: forward, TTGACATCCGTA AAGACC (nucleotides 782–799) and reverse, CTTGCT GATCCACATCTG (nucleotides 999–982), product size 217 bp; *topo II*α forward, ATTGAAGAACTGGAGGT TGTC (nucleotides 3563–3583) and reverse, CATCCT TCTCATCCAAACC (nucleotides 4073–4049), product size 486 bp; *topo II*β: forward, AGCAGAAGGTACAG GAGAGG (nucleotides 3856–3875) and reverse, GAGC AAAAACAGAAGCGG (nucleotides 4436–4419), product size 580 bp; *mrp* (taken from the human cDNA sequence with high homology to the mouse protein sequence) [28]: forward, CATAATGGACTATACAAGGG (nucleotides 4668–4685) and reverse, CCATGCTGTAC AAAAGACCTC (nucleotides 4769–4751), product size 102 bp. Following PCR, 50-μL samples were separated by non-denaturing PAGE, the gel was dried, and the relative amounts of radioactivity incorporated were quantitated using phosphorimaging. All primer sets produced products of the expected size when compared with double-stranded DNA size markers.

Preparation of Cytoplasts

Cytoplasts were prepared in the presence of cytocholasin B by the discontinuous Ficoll gradient method of Volloch *et al.* [29] as modified by Slapak *et al.* [30].

Drug Accumulation and Efflux

Cells were incubated at 0.5 or 1 × 10^7/mL in PBS plus 10 mM glucose (PBS/glc) or PBS plus 15 mM NaN_3 and 50 mM 2-deoxyglucose (N_3/2DOG) with various amounts of tritiated drug for 30 min at 37°. A 100-μL sample was removed, and layered onto 200 μL silicone oil (d = 1.045 g/mL) (Nye Lubricants, Inc., New Bedford, MA) over 20 μL concentrated formic acid in a microfuge tube. The tube was centrifuged at maximum speed for 20 sec and then frozen in dry ice. The frozen formic acid layer was cut off and counted by scintillation. To monitor efflux, the cells were incubated as before; then after 30 min, the cells were diluted to 1 mL in PBS, pelleted at low speed in a microfuge tube for 10 sec, resuspended in PBS (with either glu or N_3/2DOG) and incubated further at 37°. At the desired times, 100-μL aliquots were removed and pelleted through silicone oil as before. Drug accumulation into cytoplasts was monitored using a Becton–Dickinson fluorescence activated cell sorter and scanner (FACS-Scan). The ratio of DNR fluorescence to forward angle scattering (a measure of cell size) was compared to the ratio obtained for PC4-wt. This comparison is expressed as a percentage of PC4-wt.

Cell Viability Assay

Cell viability was measured by the MTT assay after 48 hr [31]. The IC_{50} is the concentration of drug that resulted in 50% decreased cell growth.

Membrane Isolation

Total cellular and plasma membranes were isolated by the method of Schurmann *et al.* [32] as modified by Martell *et al.* [33]. Protein quantitation was performed using the Bradford reagent supplied by Bio-Rad with bovine serum albumin as a standard.

Internal pH Measurements

pH_i was measured using the dye BCECF-AM (Molecular Probes, Eugene, OR) according to the manufacturer's directions.

Fluorescence Microscopy

Cells were incubated with 500 ng/mL DNR for 1 hr at 37° in basal medium Eagle. The cells were washed with PBS, resuspended in PBS/glc, and kept on ice until viewing at 100× magnification with a Zeiss Axiovert 10 fluorescence microscope. The DNR accumulated was excited at 420 nm with emission above 550 nm. Lysosomes were counted in 35–45 cells (total from two experiments) and averaged.

RESULTS

Analysis of Topo II Levels and Activity

Since reduced levels and altered activity of topo II have been associated with resistance to ADR and VP-16 [1, 7, 8], we examined topo II gene and protein expression and enzyme activity levels in the cell lines. RT–PCR was used to determine the relative topo IIα and topo IIβ mRNA levels. In the earliest selected resistant cell lines, PC4-A5 and PC4-A10, a reduced level of expression of both genes of approximately 30–50% was noted (Table 1); however, in increasingly resistant cell lines, the mRNA levels of both genes increased to levels even higher than in the parental cell line. Western blot analysis of topo IIα protein levels

TABLE 1. Resistance characteristics and topo II expression of ADR-selected MEL cell lines

	Resistance*			topo II		topo IIα mRNA	topo IIβ mRNA
Cell line	ADR	VCR	VP-16†	% Activity‡	% Protein§	mRNA Level‖	mRNA Level‖
PC4-wt	1.0	1.0	1.0	100	100	100	100
PC4-A5	4.9	1.0	8.3	38 ± 11	58 ± 11	71 ± 9.8	48 ± 15
PC4-A10	9.8	1.1	25	30 ± 16	61 ± 8.3	74 ± 19	84 ± 14
PC4-A20	15	1.5	62	42 ± 11	88 ± 5.4	128 ± 40	58 ± 20
PC4-A40	44	ND¶	74	ND	88 ± 5.1	171 ± 46	144 ± 27
PC4-A40c20	53	6.3	ND	38 ± 9.4	ND	188 ± 51	158 ± 30
PC4-A80	83	7.6	86	ND	84 ± 3.6	104 ± 17	188 ± 36
PC4-A160	180	21	133	ND	ND	ND	ND

* Fold change in IC_{50} compared with PC4-wt. The IC_{50} values for PC4-wt were: 11 ng/mL ADR, 3.7 ng/mL VCR, and 49 ng/mL VP-16.
† Slapak *et al.* [5].
‡ The topo II activity levels were measured by VP-16-stabilized cleavage complex formation. Values are means ± SD, N = ≥3.
§ The topo IIα protein levels were measured by western blot analysis (see Fig. 1). Values are means ± SD, N = ≥3.
‖ Measured by quantitation of RT–PCR products from total RNA and normalized to the level of β-actin; levels are expressed relative to PC4-wt. The topo II/actin mRNA ratios for PC4-wt were: 7.2, topo IIα; and 0.95, topo IIβ. Values are means ± SD, N = ≥3.
¶ ND = not determined.

also showed an approximately 40% drop in the amount of protein present in PC4-A5 and PC4-A10 (Fig. 1, Table 1); however, the amount of protein increased in the more resistant cell lines to levels near that in the parental cell line. The protein increase was not as great as detected in the RNA analysis.

The amount of topo IIα activity, as measured by VP-16-stabilized cleaved complex isolation, was reduced approximately 65% in PC4-A5 and remained decreased in all later cell lines (Table 1). This constant reduced activity contrasted with the apparent recovery of mRNA and protein levels in the later cell lines. As confirmation of these findings, we separately measured the ability of cell extracts to decatenate kinetoplast DNA and observed a similar decrease in topo II activity beginning in PC4-A5 and remaining in all the cell lines (data not shown).

Analysis of Internal pH and Glutathione Levels

Changes in internal pH may affect drug accumulation, resulting in possible resistance [1, 34]. All of the cell lines showed a pH_i of approximately 7.34 as detected by the use of BCECF uptake and fluorescence (data not shown). Enhanced glutathione metabolism has been noted in some resistant cell lines [1]. However, in none of the PC4 series of resistant cell lines were glutathione-*S*-transferase mRNA levels altered (data not shown). Also, glutathione (non-protein sulfhydryl) levels [35] were nearly identical in all cell lines tested, varying between 2.6 and 3.3 pmol/10^6 cells.

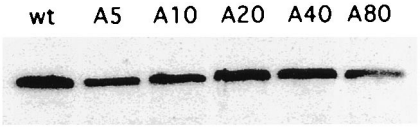

FIG. 1. Topo IIα protein levels measured by western blotting. Nuclear extracts (approximately 50 μg protein) of cell lines PC4-wt (wt), PC4-A5 (A5), PC4-A10 (A10), PC4-A20 (A20), PC4-A40 (A40), and PC4-A80 (A80) were separated by SDS–PAGE and transferred to an Immobilon membrane. Ponceau S staining verified that equal amounts of protein were transferred from all lanes. Topo IIα antibody was visualized with [^{125}I]protein A and quantitated using a Phosphorimager.

[^{3}H]DNR Accumulation in PC4-wt and Resistant Cell Lines

Changes in anthracycline accumulation in ADR selected PC4 cell lines have been detected by fluorescence studies [5], suggesting that differences in internal drug concentration could explain part of the resistance mechanism. While northern blot analysis showed overexpression of *mdr3* in PC4-A80, expression in PC4-A40 was nearly undetectable [5, 25]. Different subclones were established from PC4-A40. While the levels of ADR resistance differed slightly (10–40%), there was no change in the barely detectable expression of *mdr3*. The subclone PC4-A40c20 was selected for further studies. We evaluated cellular [^{3}H]DNR accumulation after 30 min by sedimentation of cells through silicone oil to separate bound and unbound drug. PC4-A20 accumulated the same amount of DNR as the parental cell line, while PC4-A40c20 and PC4-A80 accumulated 76 and 50% as much, respectively (Table 2).

Drug Accumulation into Cytoplasts

To determine the amount of DNR accumulated by the different cell lines without the potential interference of nuclear trapping of drug, we monitored the inherent fluorescence of DNR during accumulation into enucleated cytoplasts. Similar to the results using tritiated drug accumulation into whole cells, we observed a marked decrease in DNR accumulation in the cytoplasts from PC4-A40c20 (55%) and PC4-A80 (45%) after 30 min compared with cytoplasts from the parental cell line (Fig. 2). Cytoplasts

TABLE 2. [^{3}H]DNR accumulation and efflux in MEL cells

	Control				Energy Inhibited	
	Accumulation		Efflux†		Accumulation	Efflux†
Cell Line	(pmol/10^6 cells)	(%)*	(pmol/10^6 cells)	(%)‡	(pmol/10^6 cells)	(pmol/10^6 cells)
PC4-wt	8.85 ± 0.79	100	6.88 ± 0.88	78	12.95 ± 0.61	9.38 ± 0.31
PC4-A20	8.75 ± 0.59	99	6.36 ± 0.81	73	12.35 ± 0.66	8.67 ± 0.53
PC4-A40c20	6.73 ± 0.74	76	3.63 ± 0.5	54	13.91 ± 2.3	10.24 ± 0.26
PC4-A80	4.39 ± 0.51	50	1.57 ± 0.4	36	13.23 ± 1.1	9.90 ± 1.7

Cells were incubated at 5×10^6/mL in 50 nM [^{3}H]DNR at 37° either with glucose (control) or with N_3/2DOG (energy inhibited). After 30 min, a sample (5×10^5 cells) was centrifuged through silicone oil, and the amount of [^{3}H]DNR in the pellet was determined. For efflux studies, cells were allowed to accumulate [^{3}H]DNR for 30 min before dilution with PBS, were pelleted rapidly in a microfuge for 10 sec, and were resuspended at 5×10^6/mL in PBS/glc or PBS/N_3/2DOG lacking [^{3}H]DNR. After 30 min, a sample of these cells was centrifuged through silicone oil as before to determine the amount of [^{3}H]DNR retained. Values are means ± SD of at least three independent determinations.

* Compared to PC4-wt accumulation.

† These values represent the amount of drug retained after 30 min under efflux conditions.

‡ Percent of initial accumulation.

from cell lines displaying less resistance also showed decreased drug accumulation: PC4-A10 and PC4-A20 cytoplasts accumulated 16 and 21% less drug, respectively.

[^{3}H]DNR Efflux by ADR Selected Cell Lines

When the cells, collected by centrifugation after accumulating [^{3}H]DNR, were resuspended into medium lacking drug, the PC4-A40c20 and PC4-A80 cell lines extruded greater amounts of the drug than did the PC4-wt and PC4-A20 cells (Table 2). Analysis by thin-layer chromatography [36] showed that the extruded material comigrated with a DNR standard (data not shown), indicating that metabolism, detoxification, and/or glutathione conjugation pathways were unlikely to be involved in the observed efflux (also, see below).

To determine a rate of drug efflux from the cell lines, we evaluated retention of drug in cells incubated with different amounts of [^{3}H]DNR for 30 min. Following pelleting of the cells and resuspension into PBS/glc lacking DNR, we monitored the amount of cellular drug retained at 1-min intervals for 5 min, during which time the rate of efflux was constant (Fig. 3). The rates of [^{3}H]DNR efflux for the PC4-A40c20 and PC4-A80 cell lines were 1.8- and 3.5-fold greater than that of the parental cell line, respectively.

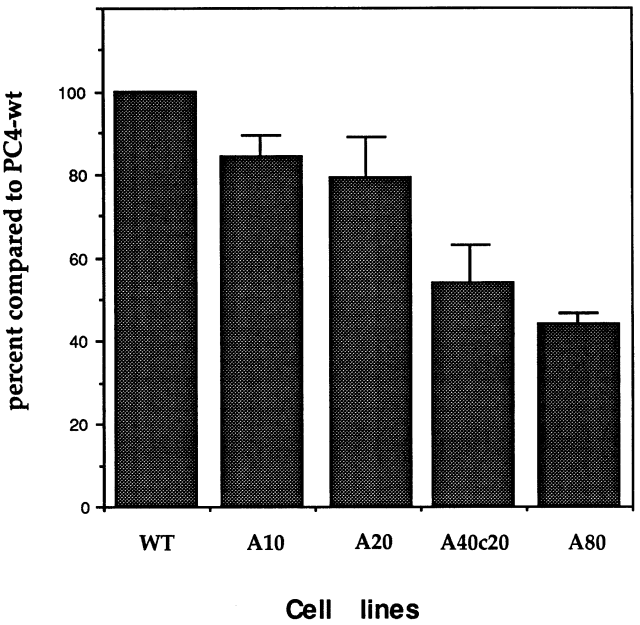

FIG. 2. DNR accumulation into cytoplasts from MEL cells. Cytoplasts were prepared and analyzed for DNR accumulation as described in "Materials and Methods." At the indicated time points, fluorescence in the cytoplasts was measured by FACScan, and the data were converted into a percentage compared with the amount of drug taken up by PC4-wt at 30 min. Fluorescence per cell was normalized for differences in cytoplast size (forward angle scattering). Each value is the mean ± SD of at least three independent determinations.

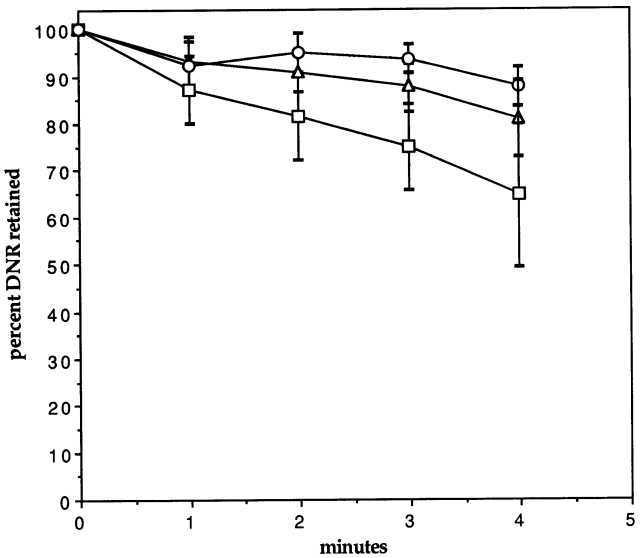

FIG. 3. Rate of DNR efflux from MEL cells. Cells were incubated with various amounts of [^{3}H]DNR for 30 min in PBS/glc before rapid centrifugation and resuspension in PBS/glc in the absence of drug. The amount of [^{3}H]DNR retained in the cells was assessed as described in "Materials and Methods." Key: (○) PC4-wt; (△) PC4-A40c20; and (□) PC4-A80. Each value is the mean ± SD of at least 3 independent determinations.

TABLE 3. Accumulation of [^{3}H]VP-16 and [^{3}H]VCR by MEL cells

Cell line	VP-16 (%)	VCR (%)	
		Control	N_3/2DOG*
PC4-wt	100†	100†	173 ± 37
PC4-A20	105 ± 9	91 ± 17	170 ± 46
PC4-A40c20	102 ± 7	69 ± 16	165 ± 38
PC4-A80	110 ± 9	36 ± 10	69 ± 14

Cells were incubated at 10^7/mL in PBS/glc (control) with 6.5 μM [^{3}H]VP-16 or 25 nM [^{3}H]VCR for 30 min at 37° before centrifugation through silicone oil. For some VCR accumulation studies, N_3/2DOG was substituted for glucose. Accumulation is expressed as a percentage compared with PC4-wt under control conditions; values are means ± SD of at least three independent determinations.

* Compared with VCR accumulation in control PC4-wt cells.

† Under control conditions, PC4-wt accumulated 1.9 pmol VP-16/10^6 cells or 187 fmol VCR/10^6 cells in 30 min.

Effect of Energy Inhibition on [^{3}H]DNR Accumulation and Efflux

Pretreating PC4-wt cells with 15 mM NaN_3 and 50 mM 2DOG in PBS reduced intracellular ATP levels to less than 5% of their initial level in 15 min (data not shown). All cell lines treated similarly with N_3/2DOG accumulated the same amount of drug, eliminating the differences seen when glucose was present. Efflux was also blocked by energy inhibition. Of the drug taken up, the same amount was retained by all cell lines during a 30-min incubation without drug in the presence of N_3/2DOG (Table 2).

Accumulation of [^{3}H]VCR and [^{3}H]VP-16 in PC4 Cell Lines

Because transport changes are seen with other drugs associated with the MDR phenotype, we examined accumulation of VCR and VP-16 in the cell lines. PC4-A40c20 and PC4-A80 accumulated 69 and 36%, respectively, as much [^{3}H]VCR as did PC4-wt or PC4-A20 (Table 3). Energy depletion with N_3/2DOG resulted in approximately the same amount of drug accumulation (about 170% of the level in the energized PC4-wt) in the parental, PC4-A20, and PC4-A40c20 cell lines. However, PC4-A80 cells consistently showed a 40% reduction in [^{3}H]VCR accumulation when compared with PC4-wt and other cell lines similarly treated with N_3/2DOG (Table 3). This observation is in contrast to DNR accumulation under energy-depleted conditions in which the PC4-A80 cell line accumulated [^{3}H]DNR to the same level as the parental cell line (Table 2). The significance of this observation is unclear, although it does suggest the presence of other non-ATP-dependent mechanisms for reducing VCR accumulation.

With [^{3}H]VP-16, we detected no altered accumulation among the different cell lines at times up to 60 min (Table 3). Varying drug concentrations did not have an effect (data not shown).

Major Vault Protein and Multidrug Resistance Protein (mrp) Gene Expression

The human major vault protein has been associated with drug resistance and with altered intracellular drug distribution in human cells [22, 24]. We examined its expression in the murine cell lines by RT–PCR. There was no change in the mvp mRNA levels in the resistant cell lines, whether expressing or not expressing *mdr3*. This finding contrasts with reports of non-P-gp expressing ADR-selected MDR human cell lines in which the major vault protein was overexpressed [22, 24].

In PC4 cell lines selected for resistance to VCR, *mrp* overexpression was observed [4]. In this series of ADR selected cell lines, we found a 25–40% decrease in the levels of *mrp* expression as compared with the parental cell line (Table 4).

mdr *Gene Expression*

Prior studies showed that *mdr1* was not expressed in any cell line [25]. The non-resistance-imparting P-gp isoform, *mdr2*, was constitutively expressed at low levels in PC4-wt and other cell lines up to PC4-A20 and at higher levels in more resistant cell lines [25]. Overexpression of *mdr3* was not seen until the PC4-A80 cell line, although expression at

TABLE 4. Expression of *mdr3*, *mvp*, and *mrp* in ADR selected MEL cell lines

Cell line	mvp mRNA*	mdr3 mRNA		mrp mRNA*
		Copies/cell	Fold change	
PC4-wt	100	0.26 ± 0.1	1.0	100
PC4-A5	131 ± 11	0.16 ± 0.1	0.61	66 ± 10
PC4-A10	109 ± 16	0.07 ± 0.05	0.25	72 ± 17
PC4-A20	105 ± 9	0.19 ± 0.05	0.74	75 ± 15
PC4-A40	96 ± 30	ND†	6.4	70 ± 13
PC4-A40c20	99 ± 37	2.6 ± 0.2	10.2	55 ± 7
PC4-A80	94 ± 36	21.6 ± 2	83.6	60 ± 1
PC4-A160	ND	120 ± 3	460	ND

* Measured by quantitation of RT–PCR products from total RNA and normalized to the level of β-actin; levels are expressed as a percentage of PC4-wt. Each value is the mean ± SD of at least three independent determinations. The mvp/actin and mrp/actin mRNA ratios for PC4-wt were 0.052, mvp and 0.49, mrp.

† ND = not determined.

TABLE 5. Modulation of ADR resistance in MDR MEL cells

Drug	Concentration*	-Fold resistance (ADR)†		
		PC4-A20	PC4-A40c20	PC4-A80
None		14 ± 1	53 ± 7	81 ± 8
Ampicillin	1 mM	14 ± 0.5	66 ± 10	118 ± 16
Chloramphenicol	0.5 μM	15 ± 3	51 ± 9	69 ± 11
Cyclosporin A	0.2 μg/mL	15 ± 0.5	19 ± 7	17 ± 7
Erythromycin	50 μM	12 ± 1	21 ± 0.8	25 ± 4
Reserpine	0.5 μM	14 ± 0.2	48 ± 1	56 ± 2
Tamoxifen	0.1 μM	13 ± 0.8	53 ± 4	70 ± 7
Trifluoperazine	1 μM	14 ± 0.9	34 ± 4	43 ± 0.0
Verapamil	1 μM	14 ± 0.2	30 ± 4	37 ± 5

* The drug concentration used in each MTT cell viability assay was the maximum amount that did not cause more than 10% killing of the cell line.

† ADR IC_{50} of the cell line in the presence of drug relative to the IC_{50} of PC4-wt in the absence of drug. For IC_{50} values, see legend of Table 1. Values are means ± SEM; each experiment was performed in triplicate at least three times.

barely detectable levels was seen in PC4-A40. We hypothesized that there was a non-P-gp efflux system operating in the PC4-A40c20 cell line but could not rule out the possible involvement of P-gp activity resulting from very low levels of expression. We used quantitative RT–PCR to determine the amount of mdr3 mRNA in each cell line [27]. The level of *mdr3* expression in cell lines up to and including the PC4-A20 was unchanged compared with the parental cell line. However, mdr3 mRNA levels were increased in PC4-A40c20, PC4-A80, and PC4-A160 cell lines by 10-, 83-, and 460-fold, respectively (Table 4). These changes in *mdr* gene expression did not correlate directly with changes in levels of ADR resistance, but did reflect the finding that increased *mdr3* expression was associated with decreased drug accumulation.

We probed western blots of plasma membrane enriched fractions with the monoclonal antibody C219, which recognizes all three P-gp isoforms. PC4-A40c20 and PC4-A80 showed an antibody-reactive protein in their plasma membranes, whereas barely detectable amounts were seen in PC4-wt and PC4-A20. However, the amounts of P-gp in the plasma membrane fractions of PC4-A40c20 and PC4-A80 were only 2- to 5-fold different (data not shown). This could be due to P-gp subcellular distribution differences and/or the presence of mdr2 protein in the plasma membrane, which precluded clear identification of the mdr3 protein.

Modulation of the MDR Phenotype

The MDR phenotype could be modulated in both the PC4-A40c20 and PC4-A80 cells through the use of known P-gp inhibitory agents (Table 5). At concentrations that reduced cell viability by less than 10%, cyclosporin A, erythromycin, trifluoperazine, and verapamil were most effective; each partially reversed ADR resistance in both the PC4-A40c20 and PC4-A80 cell lines. None of the drugs, at the concentrations tested, changed the IC_{50} values for the PC4-wt and PC4-A20 cells. Of note, reserpine, chloramphenicol, and tamoxifen appeared to affect PC4-A80 but not PC4-A40c20 cells. In no case did the level of resistance in PC4-A40c20 or PC4-A80 cells drop below that seen in PC4-A20 cells. Only cyclosporin A brought levels to about that of PC4-A20 (Table 5). With larger amounts of drugs (≥10 mM reserpine and ≥2 μg/mL cyclosporin A) (data not shown), the decreased [^{3}H]DNR accumulation into PC4-A40c20 and PC4-A80 cells was reversed, although these amounts were too toxic for susceptibility testing.

Neither buthionine sulfoximine, which inhibits the first step in glutathione synthesis by γ-glutamyl-cysteine synthase [37], nor the *mrp* inhibitor indomethacin [38], when used at a concentration that does not kill more than 10% of the cells by itself, had an effect on ADR resistance in either PC4-A40c20 or PC4-A80 cells (data not shown), indicating that glutathione-dependent detoxification pathways or mrp are unlikely to be involved in resistance.

Fluorescence Microscopic Examination of Subcellular DNR and Acidic Vesicle Distribution

We took advantage of the inherent fluorescence of DNR to establish whether significant alterations in the subcellular distribution of DNR were associated with resistance. No changes were detected in either the number of lysosomes or the distribution of DNR within the resistant cells compared with the parental cell line (Fig. 4).

DISCUSSION

MDR in tumor cells is characterized by reduced susceptibility to a variety of structurally dissimilar anticancer drugs. Resistance to anticancer agents in cell culture progresses from drug sensitivity through low-level to high-level resistance. It is difficult, if not impossible, to select, in one step, high-level MDR cell lines. Several incremental increases in drug concentration are needed to achieve high-level MDR in a variety of cell lines. Thus, high-level MDR likely involves the accumulation of a number of mutations in either one or a variety of cellular processes. The inability of

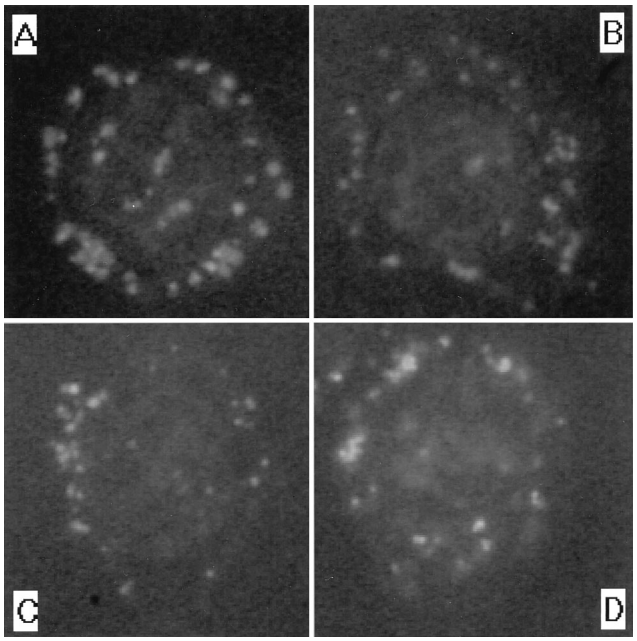

FIG. 4. DNR subcellular distribution in MEL cells. Cells were incubated for 1 hr with 500 ng/mL DNR, washed once with PBS, and resuspended in PBS before viewing at 100× magnification with an Axiovert 10 fluorescence microscope. DNR was excited at approximately 420 nm with emission above 550 nm. The number of acidic vesicles present in 35–45 cells was counted and averaged. The approximate number of acidic vesicles in each cell line was: PC4-wt, 39 ± 11 (panel A); PC4-A5, 37 ± 8 (not shown); PC4-A10, 37 ± 8 (panel B); PC4-A20, 34 ± 9 (panel C); and PC4-A80, 36 ± 9 (panel D).

most surveys of clinical MDR to explain all mechanisms of resistance [14–16] suggests that other genes and proteins may be employed to achieve MDR.

We investigated the emergence and evolution of MDR in a series of mouse erythroleukemia cell lines selected for resistance to increasing amounts of ADR. The earliest changes detected were reductions in topo IIα protein, topo IIα and topo IIβ mRNA levels, and in topo II activity levels (Table 1). Previously, a 2-fold increase in c-fos mRNA levels was identified in these early cell lines [39]. Topo II activity levels remained reduced in the sequentially selected cell lines through those with highest levels of resistance; however, both topo IIα and topo IIβ mRNA and topo IIα protein levels returned to levels comparable to those seen in the parental cell line. These observations were made on several different preparations of both protein and mRNA; activity was measured by two different methods. This finding suggests that reduced *topo II* expression, which could explain low-level drug resistance in PC4-A5, was followed by a mutation affecting drug susceptibility of topo II in higher resistant cell lines. That the mRNA levels were about twice those of parental cells is probably a reflection of the mutant protein.

Since resistance increased throughout the series, there had to be changes other than those in *topo II* and c-*fos* to account for the higher levels of resistance. We examined and ruled out mechanisms involving altered glutathione levels, *mvp* and *mrp* overexpression, and internal pH.

Decreased DNR accumulation was detected previously in PC4-A40 and PC4-A80 cells [5] and was shown to be due to active efflux in this report (Table 2). By using nuclei-free cytoplasts (Fig. 2), we could also detect a reproducible 15–20% decreased DNR fluorescence in PC4-A10 and PC4-A20 cytoplasts, as well as a much larger reduction in cytoplasts from PC4-A40c20 and PC4-A80 (Table 2). This reduction could be involved in drug resistance. Intracellular drug distribution changes [1, 22] could also explain these observations, i.e. a reduced vesicular compartment. However, there were no detectable differences in numbers of vesicles between the resistant and parental cell lines (Fig. 4). We cannot rule out completely the involvement of intracellular compartmentalization of DNR in the decreased fluorescence, since the volume of the vesicular compartment could be different or the environment within the vesicles could be altered, and thus affect DNR fluorescence. Still, how this would produce the observed drug resistance phenotype is not readily explained.

Earlier work detected no overexpression of MRP in the ADR-selected resistant cell lines as compared with the parental cells [4]. By RT–PCR we found that, beginning with PC4-A5, there was a 25–40% drop in *mrp* expression in all resistant cell lines compared with the parental cell line. Therefore, it is unlikely that MRP impacts on the resistance phenotype. Recent findings show that the chromatin structure around the *mdr3* gene in these cell lines changes early in the emergence of resistance, i.e. *mdr3* moves into a transcriptionally enriched mononucleosome H1 depleted fraction [25]. This observation could reflect larger changes in chromatin structure that occur during the acquisition of resistance. Possibly, the reduced *mrp* expression that was noted could be a result of such altered chromatin structure. In this regard, vincristine-selected PC4 cell lines also showed decreased MRP as P-gp expression appeared [4].

Two isoforms of P-gp capable of imparting the MDR phenotype exist in murine cells, *mdr1 (mdr1b)* and *mdr3 (mdr1a)*. *mdr1* Gene expression was not detected in any of the cell lines in this series [25]. By using the sensitive method of RT–PCR, we were able to detect low levels of *mdr3* expression in the PC4-A40c20 cell line, which were barely detectable by northern analysis. The 10-fold increase in mdr3 mRNA levels in PC4-A40c20 as compared with those of the parental cells was associated with the appearance of VCR resistance and an energy-dependent reduction in DNR and VCR accumulation. Characterization of efflux in PC4-A40c20 cells showed a 1.8-fold increase in the rate of efflux of [^{3}H]DNR compared with the parental cell line. Whether this amount of *mdr3* expression can account for the observed increase in efflux and resistance in PC4-A40c20 is not clear. No P-gp inhibitor could reduce resistance in PC4-A40c20 to levels approaching that seen in the precursor cell line PC4-A20, which does not express *mdr3* (Table 5). Therefore, other non-P-gp mechanisms

imparting resistance in PC4-A20 are likely to be present and active in later cell lines, including PC4-A40c20 and PC4-A80.

By RT–PCR, PC4-A80 had an approximately 8-fold increase in mdr3 mRNA levels compared with PC4-A40c20 (83-fold when compared with PC4-wt). This increase in mdr3 mRNA was not proportional to either the 2-fold increase in cellular resistance or the 2-fold increase in the rate of DNR efflux compared with PC4-A40c20. Inspection of western blots revealed that P-gp levels (mdr3 plus mdr2) in PC4-A80 were approximately 2- to 5-fold higher than in PC4-A40c20. The discrepancy between the dramatic increase in mdr3 mRNA levels and only a modest increase in drug resistance, rate of efflux, and amount of protein in the plasma membrane fractions is unexplained. It could reflect a combination of the amplification of the *mdr3* gene in PC4-A80 [25] and regulation of P-gp activity through translational and post-translational events [40–42]. The results again suggest that resistance in the cell line is not fully explained by overexpression of P-gp.

The MDR phenotype in high-level resistant cell lines is likely the product of a number of resistance mechanisms acting cooperatively. Of note, high-level ADR resistance in the PC4 cell line could not be obtained without going through a number of low-level resistant cell lines [5]. These cell lines displayed reduced topo II protein activity that remained decreased as the cells attained higher levels of resistance (Table 1). The resistance in low- to intermediate-level cell lines was not accompanied by overexpression of the *mdr*, *mrp*, or *mvp* genes (Table 4 and Ref. [4]). Moreover, resistance in PC4-A20 could not be modulated by known P-gp or MRP inhibitors in cell viability assays (Table 5), nor could these compounds reduce the level of resistance in PC4-A40c20 and PC4-A80 below that seen in PC4-A20.

Thus, the initial adaptation of PC4 murine erythroleukemia cells to treatment with ADR is to reduce *topo IIα* and *topo IIβ* and to increase c-*fos* expression. Only after selection to much higher levels of ADR resistance is P-gp overexpressed. In contrast, a study of single-step doxrubicin-resistant cells selected in relatively high levels of the drug (80 ng/mL) showed an initial activation of *mdr1* gene as the predominate mechanism of resistance [43]. These findings may relate to the different cell line and technique and/or a more transcriptionally active chromatin organization of the *mdr* gene in this cell line as compared with MEL cells [25]. The mutation(s) subsequent to those in PC4-A5 that occurred in the PC4-A10 and PC4-A20 cell lines, producing additional resistance, has not been determined. These alterations are of interest since they appeared during selection at clinically achievable drug concentrations. Together, these data support the probability that an additional, and as yet unidentified mechanism(s) contributes to low-level MDR, to which P-gp activity is later added.

The results presented here strongly suggest that factors other than P-gp and topo II are involved in the MDR seen in PC4 cell lines as they progress from drug-sensitive to high-level, P-gp-mediated MDR. These resistance mechanisms are additive and can function cooperatively. The cell may coordinately regulate these mechanisms. Identification and eventual modulation of early appearing mechanisms of resistance should have a significant impact on the chemotherapeutic treatment of cancer.

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