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CHARACTERIZATION OF ADRIAMYCIN-RESISTANT AND RADIATION-SENSITIVE CHINESE HAMSTER CELL LINES

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Abstract—A series of cell lines derived from Chinese hamster V79 cells by selection in increasing concentrations of Adriamycin® (ADRM) was developed to study the mechanisms of drug resistance and its relationship to radiation response. Survival studies revealed that selection in increasingly higher concentrations of ADRM positively correlated with increased cellular drug resistance. Increased cellular resistance correlated positively with amplification of the hamster multidrug-resistance gene (*pgp* 1) as detected with dot blot analysis using the pCHP1 probe. Southern blot analysis of restriction endonuclease digested DNA (*Eco* RI, *Hin*d III, *Pst* I, or *Bam* HI) showed that (1) some fragments were preferentially amplified compared to others in the ADRM-resistant lines; and (2) no major gene rearrangement appeared to have occurred during the selection for greater ADRM resistance. Levels of *pgp* 1 gene expression assayed with dot blot and Northern analysis showed a parallel increase of mRNA with gene amplification and increased ADRM resistance. The amounts of the *pgp* 1 gene product, P-glycoprotein (P-gp), in the cell membrane of the ADRM-resistant cells correlated with the amount of gene amplification/expression. However, levels of P-gp only correlated with degree of drug resistance as measured by cell survival in earlier selection stages (77A and LZ-3). In later selection stages (LZ-8 and LZ-24), higher levels of ADRM resistance were achieved but levels of P-gp did not increase beyond ~20% of plasma membrane proteins. These results suggest that (1) the LZ cell plasma membrane may have a physical limit as to the amount of P-gp it can accommodate and/or there is a cellular mechanism for regulating the amount of P-gp in the plasma membrane, and (2) additional resistance mechanisms are present in LZ-8 and LZ-24 cells. Microscopic observations of intracellular drug distribution in these cell lines revealed that (1) ADRM appeared to be sequestered in cytoplasmic vesicles, and (2) the amount of sequestration (number of vesicles) exhibited correlated with the degree of drug resistance attained by the cell lines. These results suggest that drug sequestration is another mechanism of resistance in LZ cells in addition to P-gp-mediated drug efflux.

Selection of cells in a single drug [i.e. Adriamycin® (ADRM)†] can result in the acquisition of cellular cross-resistance to multiple drugs [1–3]. This phenomenon, termed multidrug resistance (MDR), has been studied extensively *in vitro* in hamster, mouse and human cell lines [4–6]. Analysis of human tumors from patients with and without prior drug treatment shows that MDR is prevalent in some types of tumors [for review, see Ref. 7]. The MDR phenotype can be produced by several different mechanisms. A common mechanism is the presence of increased levels of a 170,000 Da protein, P-glycoprotein (P-gp), in the cell plasma membrane. This protein is thought to rapidly efflux drug from the cell, thereby reducing intracellular drug levels and enhancing cell survival (i.e. producing greater drug resistance) [1–3]. The increased levels of P-gp have been shown to result from gene amplification and/or increased gene expression [4–7]. Although several genes for MDR have been described, transfection experiments suggest that the *mdr* 1 gene (designated *pgp* 1 in hamster) is sufficient to produce the MDR phenotype in cells [8, 9]. Other molecular mechanisms reported to account for the MDR phenotype include: alterations in the quantity/function of topoisomerase II [10–12], changes in the activity/levels of detoxification enzymes (i.e. glutathione *S*-transferase [13, 14] or cytochrome P450 enzymes [15]), and the compartmentalization of drug away from target sites [16, 17]. Increasing evidence suggests that the MDR phenotype can result from more than one mechanism in a particular cell line or human tumor [18–20].

Currently, it remains unclear whether cells undergoing selection for drug resistance develop multiple mechanisms of resistance sequentially or simultaneously. To identify and study the mechanisms involved in achieving ADRM resistance, we selected a series of increasingly ADRM-resistant cell lines from V79 Chinese hamster lung cells over a number of years [21, 22]. These resistant cell lines are unique among MDR lines in that they also exhibit radiation sensitivity [23, 24]. This series of increasingly ADRM-resistant cells was studied to determine the major mechanisms contributing to ADRM resistance in these cell lines. This report focuses on the role of overexpression of P-gp (amplification and expression of the *pgp* 1 gene) and sequestration of drug in vesicles. An accompanying report [25] describes a

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† Abbreviations: ADRM, Adriamycin; MDR, multidrug resistance; and P-gp, P-glycoprotein.

third mechanism of resistance present in LZ cells involving enhanced drug inactivation.

MATERIALS AND METHODS

Cell culture. V79 and 77A cells were maintained in F-10 medium (Gibco BRL, Grand Island, NY) plus 10% heat-inactivated fetal bovine serum (ICN Flow Biomedicals Inc., Costa Mesa, CA) and a 1% penicillin/streptomycin mixture (Gibco BRL). LZ cells were maintained in the same medium plus the following concentrations of ADRM (Adria Laboratories, Columbus, OH): 3 μg/mL for LZ-3, 8 μg/mL for LZ-8, and 24 μg/mL for LZ-24.

Selection of ADRM-resistant cell lines. The earliest steps in the selection of V79 cells to produce the LZ cell lines have been detailed previously [22]. Briefly, V79 cells were cultured in 0.05 μg/mL ADRM for 77 weeks to produce the cell line designated 77A. This cell line has retained its resistance to ADRM despite being cultured without drug for ~5 years [23]. A hypoxanthine guanine phosphoribosyltransferase mutant of 77A was selected and subjected to brief treatments (1–5 hr) of 20 or 50 μg/mL ADRM and colonies were isolated. Cells derived from one of these colonies were then selected by rapid stepwise increases in the concentration of ADRM in the culture medium up to 3 μg/mL thereby producing the LZ-3 cell line (early passages were originally designated LZ-632) [22]. LZ-3 cells were further selected in ADRM up to a dose of 8 μg/mL to produce LZ-8 (formerly designated LZ III N [23, 24]). Some LZ-8 cells were selected in ADRM up to 24 μg/mL to produce the LZ-24 line. For some experiments, LZ-3 and LZ-8 cells were grown in medium without ADRM to allow reversion and have been designated LZ-3R and LZ-8R.

DNA/mRNA isolation. An Invitrogen mRNA Isolation kit (Invitrogen Corp., San Diego, CA) was used according to the manufacturer's instructions to isolate mRNA for dot blot and Northern analysis.

Labeling of probe. The pCHP1 probe was labeled with [α-^{32}P]CTP (3000 Ci/mmol, ICN Biomedicals, Costa Mesa, CA) to a specific activity of approximately 2×10^8 cpm/μg using a random-primed labeling kit (Boehringer Mannheim, Indianapolis, IN).

Dot blots. Dot blots were prepared as previously described [26]. Serial dilutions of isolated DNA or mRNA were spotted onto Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL) using a 96-well Minifold II manifold (Schleicher & Schuell, Keene, NH). The applied samples were fixed to the Hybond-N using a Fotodyne DNA transfer lamp (Fotodyne Inc., New Berlin, WI) according to the manufacturer's instructions. Prehybridization, hybridization, and washing were according to standard protocol [27]. The pCHP1 probe [28] (which represents ~600 base pairs of the Chinese hamster sequence of the *pgp* 1 gene) was labeled with ^{32}P and used for the hybridizations. The amount of probe:target complex was quantified using direct beta counting on a Matrix 96™ (Packard Instruments Corp., Meriden, CT) as described [26].

Southern blots. High molecular weight DNA purified as above was digested with one of the following restriction enzymes under conditions suggested by the supplier (Gibco BRL): *Eco* RI, *Bam* HI, *Hind* III, or *Pst* I. Digested DNA was size fractionated by electrophoresis through 0.8% agarose. Southern transfers were performed essentially as described by Ausubel *et al.* [29] onto Hybond-N nylon membrane. Following hybridization with the ^{32}P-labeled pCHP1 probe, blots were washed two times with 1X SSC (20X SSC = 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0) plus 0.1% sodium dodecyl sulfate (SDS) for 15 min at 42°. This was followed by two 30-min washes in 0.1X SSC plus 0.1% SDS at 55°. Autoradiograms were obtained by exposing the filters at −70° to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with Dupont lightning plus intensifying screens. Bands on autoradiographic films were quantified using an Applied Imaging PBI Image Analyzer (Applied Imaging, Santa Clara, CA) which densitometrically measured the individual bands and quantitated in sq mm of O.D.

Quantitation of P-glycoprotein in cell membrane. P-gp was quantitated using gel scanning on a Beckman DU-8 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA) following separation of the proteins using polyacrylamide gel electrophoresis and Western blotting as described in detail [30].

Visualization of intracellular ADRM. Cytofluorescence of ADRM was visualized essentially as described [31]. Briefly, cells were subcultured into 6-well plates containing presterilized coverslips and allowed to grow for 1–2 days in growth medium in the presence of ADRM. Coverslips were removed from plates and placed cell side down onto clean microscope slides. To prevent drying, edges were sealed with molten wax or a thin coating of rubber cement. Slides were viewed with a Zeiss Axiophot microscope with excitation at 450–490 nm.

RESULTS

Survival characteristics of cell lines. To estimate the resistance of various cell lines, the $1/K_i$ values (inverse of the slope of initial portion of survival

Table 1. Values for $1/K_i$ calculated from the slope of the initial portion of the survival curves for V79, 77A, LZ-3, LZ-8 and LZ-24 after a 1-hr treatment with 0–500 μg/mL Adriamycin

Cell line	$1/K_i$
V79	0.18 ± 0.01
77A	0.90 ± 0.05
LZ-3	416 ± 20.8
LZ-8	1650 ± 82.5
LZ-24	2479 ± 137
LZ-3R	2.20 ± 0.1
LZ-8R	55 ± 2.8

Values are means ± SD, N = ≥3.

Table 2. Correlation of *pgp* 1 gene amplification, mRNA expression, and levels of P-glycoprotein in the cell plasma membrane

Cell line	Hybridization (total counts accumulated/10 min) DNA 5 μg	mRNA 2 μg	P-gp (% TMP)
V79	410	340	1.5
77A	1,010	680	2.6
LZ-3	4,050	10,890	15.5
LZ-8	6,150	13,330	19.9
LZ-24	8,100	16,280	21.9
LZ-3R	2,040	1,460	3.5
LZ-8R	2,110	2,550	8.9

Columns 2 and 3 represent the amount of hybridization with a ^{32}P-labeled pCHP1 probe determined using direct beta counting. Column 4 lists the amount of P-glycoprotein expressed as percentage of total plasma membrane protein (% TMP).

curve) were calculated from the survival curves after treatment of the cell lines with different concentrations of ADRM for 1 hr (Table 1). Selection in increasing concentrations of ADRM correlated with increased $1/K_i$ values and increased resistance as shown in column 2. Removing LZ-3 and LZ-8 from growth in drug (for 600 and 700 days, respectively) decreased the $1/K_i$ (increased sensitivity). Expressing the $1/K_i$ values of the resistant cells relative to that of V79 reveals that 77A (selected in 0.05 μg/mL) exhibited a 5-fold increase in resistance, LZ-3 (selected up to 3 μg/mL) a > 2,300 increase, LZ-8 (selected up to 8 μg/mL) a > 9,100 increase, and LZ-24 (selected up to 24 μg/mL) a > 13,000 increase in resistance to ADRM compared to the parental, wild-type V79 cells. Complete ADRM survival curves have been published for V79, 77A, and LZ-8 [22, 24].

Analysis of amplification/expression of pgp 1 *gene.* To determine if the gene for MDR was amplified in the resistant cell lines, dot blot analysis of isolated genomic DNA was performed. Hybridization was with a ^{32}P-labeled pCHP1 probe for the hamster *pgp* 1 gene with the amount of hybridization (probe:target complex) quantified using direct beta counting (Table 2) [26]. With selection in increasingly higher concentrations of ADRM, correspondingly higher levels of hybridization (indicative of *pgp* 1 gene amplification) were observed. Assuming that V79 cells contain one copy of the *pgp* 1 gene (based on *in situ* hybridization studies [32]), a figure for the relative resistance for the other cell lines can be calculated. Compared to V79, 77A contained 2.4×; LZ-3, 9.8×; LZ-8, 14.8×, and LZ-24, 19.6×. When the LZ-3 and LZ-8 cells were allowed to grow in medium without ADRM, a corresponding decrease in the amount of DNA hybridization was observed, indicating a loss of gene copy number in the absence of selection pressure.

The third column of Table 2 shows the results of dot blot analysis of mRNA hybridized with the pCHP1 probe. With selection in increasing concentrations of ADRM, increasing amounts of gene expression were observed as indicated by increasing amounts of hybridization. Gene expression increased in the following order: V79 < 77A < LZ-3 < LZ-8 < and LZ-24. When LZ-3 and LZ-8 cells were allowed to revert (600 and 719 days respectively), decreased expression was observed (compare LZ-3 with LZ-3R and LZ-8 with LZ-8R). Another indicator of *pgp* 1 gene expression is the amount of P-gp in the cell plasma membrane. The levels of P-gp in each cell line are shown in the fourth column of Table 2. The level of P-gp increased from 1.5% of the total plasma membrane protein in V79 to a maximum value of ~20% of the total plasma membrane protein in LZ-8 and LZ-24. This suggests that the amount of P-gp in the cell membrane reaches a plateau at this level. Additional stepwise selection of LZ-24 cells to growth in 100 μg/mL ADRM (LZ-100) did not increase the amount of P-gp in the plasma membrane beyond ~20% even though drug resistance was increased six times compared to LZ-3 based on K_i values (slope of survival curves; data not shown). This suggests that this may represent the maximum amount of P-gp that the LZ plasma membrane can accommodate. When the LZ-3 and LZ-8 cells were allowed to grow in the absence of ADRM (600 and 836 days, respectively), the level of P-gp in the plasma membrane decreased (see LZ-3R and LZ-8R).

If the values presented in Table 2 are expressed relative to the fold increase compared to V79 cells, the amount of relative gene amplification closely approximates the relative amount of the final percentage of P-gp present in the plasma cell membrane. Although the amount of mRNA expressed increased in parallel with higher selection in ADRM, it was higher than might be expected for the amount of P-gp found in the plasma cell membrane and the level of gene amplification.

Southern blot analysis. Southern analysis of *Eco* RI digested genomic DNA from V79 hybridized with the pCHP1 probe revealed a series of fragments ranging from 1.3 to 13 kb (Fig. 1). These same bands were present in 77A, LZ-3, LZ-8, and LZ-24. Based

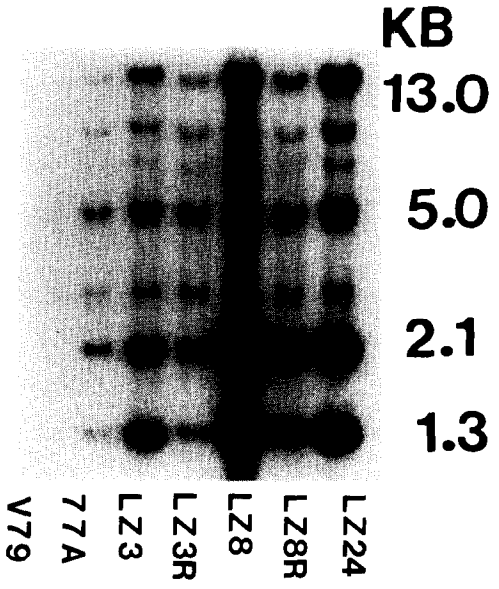

Fig. 1. Southern analysis of *Eco* RI digested genomic DNA from V79 and ADRM-resistant cell lines hybridized with the pCHP1 probe.

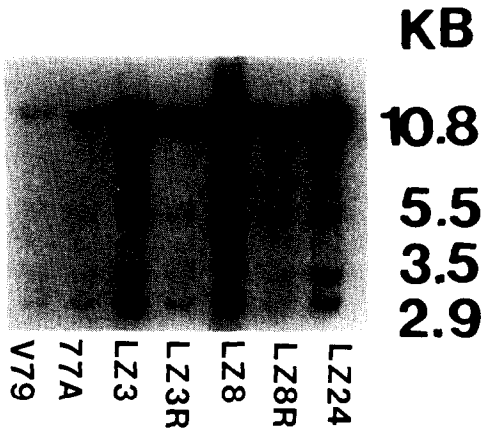

Fig. 2. Southern analysis of *Hind* III digested genomic DNA from V79 and ADRM-resistant cell lines.

on the intensity/size of the bands, amplification of gene sequences recognized by the pCHP1 probe did not occur to the same extent on all fragments. Greater amounts of hybridization were observed on the 1.3, 2.14, 5.0, and 13 kb fragments with selection in increasing concentrations of ADRM. Densitometry quantitation of the autoradiograms revealed that all pCHP1 hybridizing fragments in the drug-resistant cell lines were present in greater amounts than in V79. Table 3 shows the relative increase in the levels of these four major gene fragments for the ADRM-resistant cells compared to the amount present in V79. These same fragments exhibited decreased amounts of hybridization in LZ-3 and LZ-8 cells grown in the absence of ADRM for 600 and 719 days, respectively (see LZ-3R and LZ-8R).

Southern analysis of *Hind* III digested DNA from the various cell lines is shown in Fig. 2. Four major pCHP1 hybridizing fragments were detected in the cell lines analyzed: 2.93, 3.56, 5.5, and 10.8 kb. The relative increase in gene copy number for the 10.8 and 5.5 kb bands was determined with densitometry. The most significant increase occurred in the 10.8 kb band. This band increased as ADRM concentration increased and diminished when ADRM selection was removed (LZ − 3R = 600 days and LZ − 8R = 719). Lesser amounts of amplification were observed on the other DNA fragments, but again all pCHP1 hybridizing fragments were present in higher levels in the ADRM-resistant cells than in V79 cells.

Southern analysis of *Pst* I digested DNA revealed five pCHP1 hybridizing fragments with molecular weights of 1.17, 1.56, 2.7–2.8, 4.65, and 5.35 kb (Fig. 3). The greatest changes in the amount of amplification were observed in bands 5.35, 1.58, and 1.22 kb.

Southern analysis of *Bam* HI digested DNA detected some differences between the cell lines (Fig. 4). V79 exhibited two pCHP1 hybridizing fragments at 5.3 and 5.2 kb. Two fragments were

Table 3. Relative increase in densitometry values for ADRM-resistant cell lines compared to V79 as determined by Southern analysis of *Eco* RI digested DNA

	Relative increase in densitometry values (sq mm O.D.)			
	Eco RI digest fragment size (kb)			
Cell lines	13	5.0	2.1	1.3
V79	1.0	1.0	1.0	1.0
77A	1.78	2.25	2.94	2.45
LZ-3	6.88	5.06	10.70	13.50
LZ-8	14.98	4.91	14.40	16.50
LZ-24	20.90	9.76	19.67	24.96
LZ-3R	3.77	4.40	6.08	4.93
LZ-8R	6.86	4.40	11.30	12.89

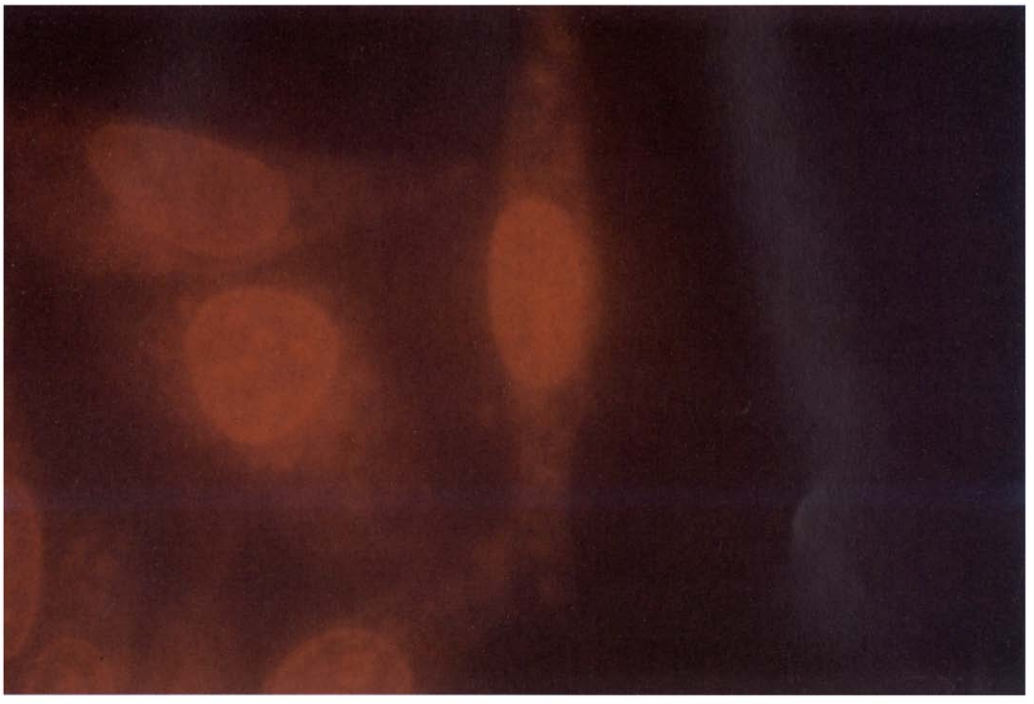

Fig. 6. Wild-type V79 cells following exposure to 1 μg/mL ADRM. Red fluorescence is from ADRM and most can be seen to be localized to the nucleus.

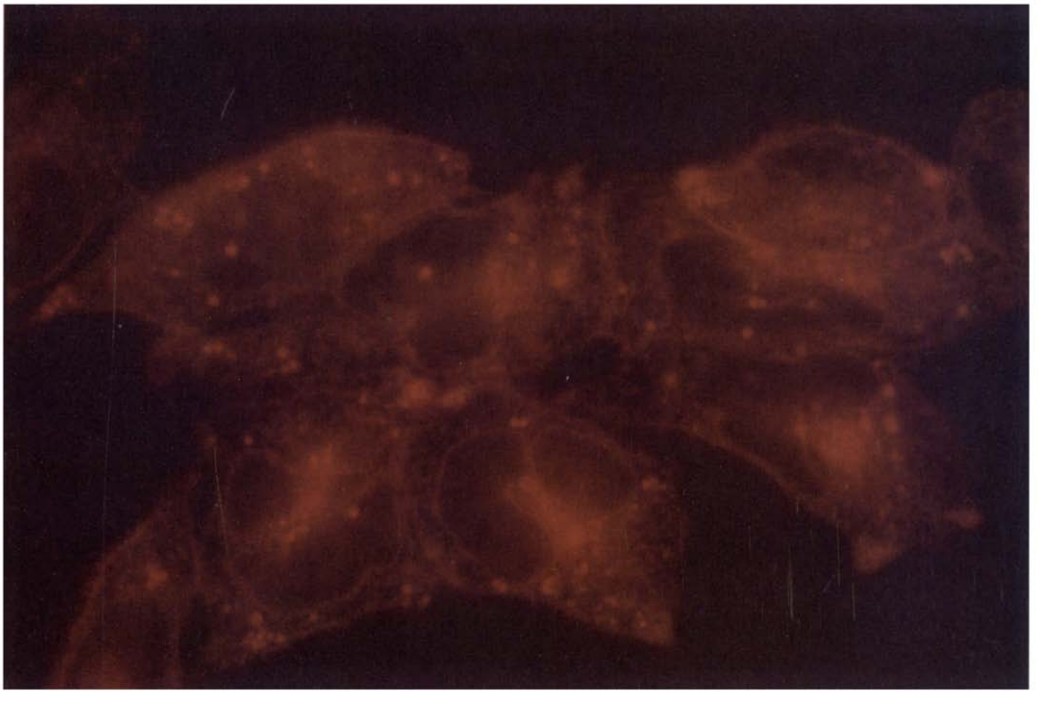

Fig. 7. LZ-8 cells following exposure to 8 μg/mL ADRM. Little ADRM (red fluorescence) is localized to the nucleus but a number of red colored vesicles can be seen in the cytoplasm of the cells.

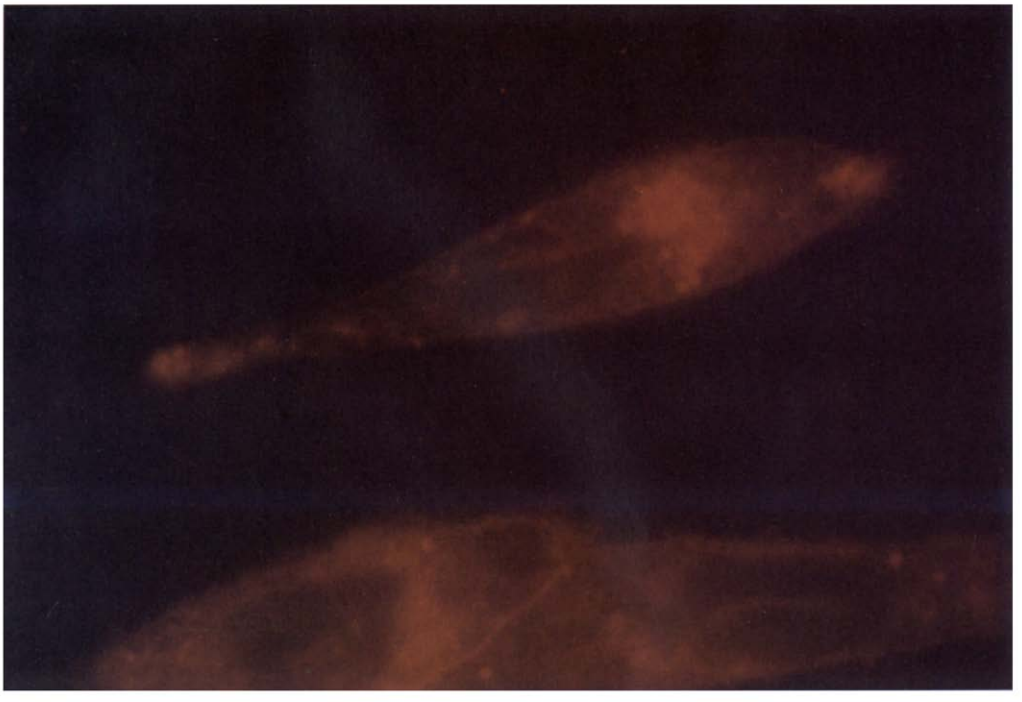

Fig. 8. LZ-24 cells following exposure to 24 μg/mL ADRM. Many red colored vesicles are localized around the nucleus and at the apical regions of the cell cytoplasm.

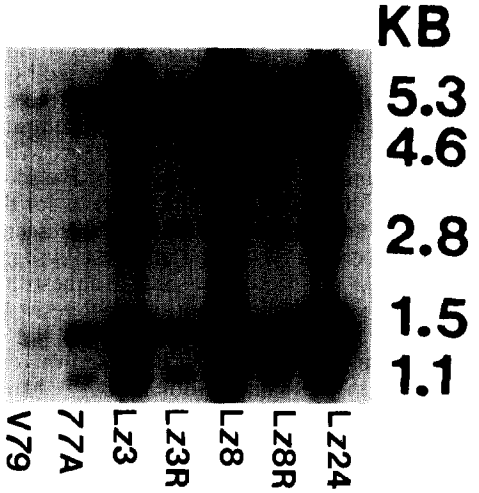

Fig. 3. Southern analysis of *Pst* I digested genomic DNA from V79 and ADRM-resistant cell lines.

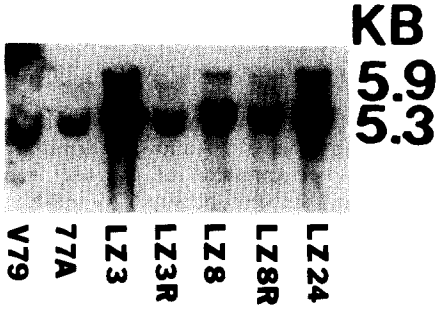

Fig. 4. Southern analysis of *Bam* HI digested genomic DNA from V79 and ADRM-resistant cell lines.

also observed in 77A but at 5.9 and 5.6 kb. Since these same sized fragments were present in LZ-3, LZ-8 and LZ-24, a change in the DNA sequence probably occurred during the selection of 77A. Based on the digestion pattern, this alteration was retained in subsequent higher selections of 77A up to LZ-24. LZ-3R (600 days without ADRM) fragments appeared virtually identical to those of 77A. Only one band, representing a fragment approximately 5.3 kb, was observed in LZ-8R (719 days without ADRM), suggesting that during reversion some sequence loss or change occurred in this cell line.

Northern analysis of mRNA was also performed with densitometry quantification of film exposures (Fig. 5). Increases in the levels of hybridization (indicative of increased expression of the *pgp* 1 gene) compared to V79 were observed for 77A and LZ-3. Similar amounts of hybridization were observed for LZ-3, LZ-8, and LZ-24 with the particular film exposure shown. Analysis of shorter film exposure intervals prohibited quantification of the V79 and

Fig. 5. Northern analysis of mRNA isolated from V79 and the ADRM-resistant cell lines.

77A samples (bands too light) but did reveal increasing levels of hybridization with LZ-3, LZ-8, and LZ-24 (data not shown). Overall, similar trends in mRNA expression were seen using the two different methods of analysis. When LZ-3 and LZ-8 were allowed to grow in the absence of ADRM for 600 and 834 days, respectively (LZ-3R and LZ-8R), decreased mRNA expression was observed. Based on molecular weight standards the size of the message was estimated to be ~4.5 kb which is consistent with other reports [1, 2, 3, 7].

Visualization of intracellular drug distribution. To determine if intracellular drug distribution was altered in the different cell lines, cells grown in the presence of ADRM were viewed with a fluorescent microscope with excitation at 450–490 nm. Since ADRM is a fluorescent anthracycline, the intracellular drug distribution can be determined without staining the cells [31]. Figure 6 shows that exposure of wild-type V79 cells to a 1 μg/mL concentration of ADRM for 1–2 hr resulted in the majority of the ADRM localizing to the nucleus with some cytoplasmic staining evident. In 77A (treated with 1 μg/mL ADRM) and LZ-3 (treated with 3 μg/mL), little or no fluorescence was visible in the nucleus and a few fluorescent vesicles became evident (data not shown). In LZ-8 (treated with 8 μg/mL) (Fig. 7), nuclear fluorescence remained diminished but more vesicles that appeared to contain ADRM (i.e. based on their specific red fluorescence) were seen. An even greater number of cytoplasmic vesicles were observed in LZ-24 (treated with 24 μg/mL) (Fig. 8) with vesicles localized to the apical regions of the cells as well as to the perinuclear regions. To determine if the difference in the number of vesicles formed in the LZ cell lines represented differences in their ability to form vesicles or was a consequence of the different concentrations of ADRM used for treatment, all cell lines were exposed to 100 μg/mL ADRM and the number of vesicles formed was quantitated visually. LZ-3 formed fewer vesicles than LZ-8 and LZ-24 (data not shown). Thus, the number of vesicles observed correlates with the level of drug resistance exhibited by the cell lines.

DISCUSSION

These studies show that selection of Chinese hamster V79 cells in increasing concentrations of ADRM resulted in increasing resistance to the drug as reflected by enhanced cell survival following drug

treatment. Depending on the level of resistance exhibited by the cell line, one or more of the following mechanisms contributes to the resistant phenotype: (1) increased *pgp* 1 gene amplification/expression producing enhanced drug efflux; (2) ability to sequester drug into cytoplasmic vesicles; and (3) increased inactivation of the cytotoxicity of the ADRM molecule [25].

P-pg-mediated enhanced drug efflux, like that observed in the LZ cell lines, is a common mechanism producing drug resistance. These studies found an overall correlation between amplification of *pgp* 1 gene (previously shown to correlate most closely with MDR) [8, 9], mRNA expression and P-gp levels in the plasma membrane. P-gp levels were observed to increase with increasing ADRM resistance in early selection stages (77A and LZ-3). However, P-gp levels reached a plateau at ~20% of the plasma membrane proteins with LZ-8 and did not increase with further selection for ADRM resistance. Thus, (1) there may be a physical limit to the amount of P-gp the plasma membrane can accommodate, and/or (2) the cell regulates the amount of P-gp in the plasma membrane. The fact that P-gp levels did not increase further, even though greater ADRM resistance was achieved in LZ-8, LZ-24, and LZ-100, suggested that additional mechanisms of resistance must account for the increased cell survival observed in these cell lines after ADRM treatment. These mechanisms of resistance appeared to develop sequentially as greater drug resistance was achieved in the LZ cell lines, i.e. P-gp enhanced efflux, followed by drug sequestration and increased drug inactivation.

Paralleling the gene amplification and higher P-gp levels in the cell membrane, increased gene expression was observed as indicated by increased levels of mRNA. The presence of higher mRNA levels than expected for the amount of gene amplification and P-gp in the cell membrane of LZ-3, LZ-8, and LZ-24 is consistent with other studies [6, 33, 34] and may have resulted from: (1) a longer half-life for mRNA, (2) production of some nonfunctional mRNA, or (3) the presence of P-gp in intracellular structures as well as the plasma membrane.

Southern analysis of the *pgp* 1 gene with four different enzymes of the ADRM-resistant cell lines revealed that, overall, the same or a very similar fragment distribution pattern was observed as in V79. This suggests that (1) the *pgp* gene was amplified in LZ cells without significant gene rearrangement of the creation of novel joints since no novel fragments were observed in contrast with some other cell lines [28] and (2) amplification occurred preferentially on some fragments compared to others as detected with the pCHP1 probe, indicating that some gene sequences are more important for drug resistance. The localization of the *pgp* 1 gene to an HSR on a marker chromosome designated M1 in LZ-3 through LZ-24 is consistent with the Southern analysis [32].

A number of reports have suggested that altered drug distribution may be a general mechanism of drug resistance but few details are known about the exact processes involved [17, 35–37]. In our studies, the number of vesicles formed in LZ cell lines in response to ADRM treatment correlated (increased) with the level of ADRM resistance exhibited by the cells. This was true regardless of whether the cells were treated with their maintenance concentration of ADRM (i.e. 3 μg/mL for LZ-3) or with a high concentration (i.e. 100 μg/mL). Based on fluorescence, the vesicles remained in cells for days after drug removal and, based on fluorescence, appeared to contain ADRM. These results suggest that the cells utilized the drug sequestration mechanism to a greater extent as drug resistance increased.

Anthracycline resistance has been shown to be associated with mechanisms other than enhanced drug efflux due to overexpression of P-gp, such as alterations in activity or amount of DNA topoisomerase II [10–12, 38] and alterations in enzymes such as glutathione-*S*-transferase (GST) [13, 14, 38]. Our previous studies have eliminated altered levels/activity of GST [39] and topoisomerase (unpublished observations) as major contributors to drug resistance in LZ cell lines so that the enhanced resistance observed cannot result from these mechanisms. Our results presented here and elsewhere [25] suggest that drug sequestration in vesicles and greater drug inactivation are alternate, important mechanisms contributing to ADRM resistance in LZ cell lines.

Analysis of fresh human tumor samples for increased P-gp levels (and by implication, amplification/overexpression of the *mdr* gene) is currently being actively pursued to improve the taxonomy of tumors with regard to prognosis [40–45]. Despite the extensive literature on the relationship between the multidrug resistant phenotype and *mdr* gene amplification in *in vitro* cell culture models, there has been no conclusive clinical evidence for the predictive validity of P-gp levels and subsequent response to chemotherapeutic agents. The reasons for this lack of correlation are not clear. However, non-P-gp-mediated mechanisms of resistance have been identified in human tumors with and without expression of P-gp-mediated resistance [46–48]. Our results detailed above and in the accompanying report [25] suggest that P-gp may have important functions other than as a transporter in the expression of drug resistance. This suggestion has important clinical implications with regard to: (1) the expectation that calcium-channel blockers would significantly reverse drug resistance may not be realized, and (2) alternate mechanisms for drug resistance in human tumor material should be sought and characterized. Such studies may result in the development of more effective therapeutic strategies.

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