Pharmacological, Molecular, and Cytogenetic Analysis of "Atypical" Multidrug-resistant Human Leukemic Cells[1]

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ABSTRACT

We previously described the cross-resistance patterns and cellular pharmacology of a human leukemic cell line, CEM/VM-1, selected for resistance to the epipodophyllotoxin teniposide (M. K. Danks *et al.*, Cancer Res., *47:* 1297–1301, 1987). Compared to CEM/VLB$_{100}$, which is a well characterized "classic" multidrug-resistant (MDR) cell line, the CEM/VM-1 cells display "atypical" multidrug resistance (at-MDR) in that they are cross-resistant to a wide variety of natural product antitumor drugs, except the *Vinca* alkaloids, and they are not impaired in their ability to accumulate radiolabeled epipodophyllotoxin. We have extended our characterization of this at-MDR cell line in the present study. In comparison to CEM/VLB$_{100}$ cells, we found that CEM/VM-1 cells are not cross-resistant to either actinomycin D or colchicine. Verapamil and chloroquine, which enhance the cytotoxicity of vinblastine in CEM/VLB$_{100}$ cells, had little or no ability to do so in the CEM/VM-1 cells. Membrane vesicles of the two resistant sublines were examined for overexpression of the MDR-associated plasma membrane protein (P-glycoprotein, M_r 170,000 protein, or 180,000 glycoprotein) by photoaffinity labeling with the vinblastine analogue *N*-(*p*-azido[3-^{125}I]salicyl)-*N'*-β-aminoethylvindesine. We were unable to visualize the MDR-associated protein in the CEM/VM-1 membranes with this photoaffinity probe under conditions in which the P-glycoprotein was readily seen in the membranes of CEM/VLB$_{100}$ cells. Furthermore, no hybridization of the pMDR1 complementary DNA was seen in slot-blot analyses of the RNA from at-MDR cells, indicating that the *mdr* gene coding for P-glycoprotein is not overexpressed as is the case in the classic MDR cells. However, cytogenetic analysis indicated that the CEM/VM-1 cells contained an abnormally banded region on chromosome 13q, suggesting that a gene other than *mdr* may be amplified in these cells. Thus, despite the two cell lines having approximately equal degrees of resistance to epipodophyllotoxins, our data indicate that the mechanism(s) responsible for at-MDR is different from that for classic, P-glycoprotein-associated MDR.

INTRODUCTION

MDR[3] refers to the phenomenon by which mammalian tumor cells selected for resistance to one "natural product" anticancer agent display a broad cross-resistance to a variety of other natural product drugs of dissimilar structure and action (1–4). These agents include *Vinca* alkaloids, anthracyclines, and epi-

podophyllotoxins. The pharmacological basis for MDR appears to be a reduced steady-state accumulation of drugs (1, 5), which has been attributed to either their diminished uptake (6) or decreased retention (5, 7).

We have described recently an "atypical" MDR human leukemic cell line selected for resistance to the epipodophyllotoxin, VM-26 (8). This cell line, CEM/VM-1, also displays a broad cross-resistance to multiple natural product drugs and is about as resistant and cross-resistant to epipodophyllotoxins and doxorubicin as is our well-characterized MDR line, CEM/VLB$_{100}$. There is, however, one notable difference in cross-resistance patterns between the two cell lines: the CEM/VM-1 cells are as sensitive to the *Vinca* alkaloids (VLB and vincristine) as are the parent CEM cells (8). Of considerable interest, this cell line also accumulates at steady-state nearly as much [^{3}H]etoposide (8) and [^{3}H]vincristine (9) as does the parental line.

The present study extends our initial investigations of CEM/VM-1 cells and describes other pharmacological features and molecular and cytogenetic studies designed to further characterize this atypical MDR cell line. Preliminary accounts of some of this work have been presented (9, 10).

MATERIALS AND METHODS

Chemicals and Supplies. Minimal essential medium (with Earle's salts) was purchased from Gibco (Grand Island, NY), and fetal bovine serum was obtained from Hyclone Laboratories, Inc. (Logan, UT). Vindesine and VLB were from Eli Lilly and Co. (Indianapolis, IN) and Natural Products Branch, National Cancer Institute (Bethesda, MD); daunorubicin was from Adria Laboratories (Columbus, OH); actinomycin D, colchicine, podophyllotoxin, CLQ, verapamil, and 6-mercaptopurine were purchased from Sigma Chemical Co. (St. Louis, MO); methotrexate was from Lederle Laboratories (Wayne, NJ); and BCNU and VM-26 were from Bristol-Myers Laboratories (Wallingford, CT). Actinomycin D was dissolved in 50% ethanol and then diluted in 0.9% NaCl solution. All other drugs were prepared in 0.9% NaCl solution. [α-^{32}P]dATP was purchased from Dupont/NEN (Boston, MA), and [^{125}I]NASV was synthesized as described previously (11, 12). All other chemicals and supplies were obtained from commercial sources.

Cells and Culture Conditions. The cell lines used in these studies were the drug-sensitive CCRF-CEM (CEM) and two drug-resistant variants: CEM/VLB$_{100}$, selected for primary resistance to the *Vinca* alkaloid, VLB (13); and CEM/VM-1, selected for primary resistance to the epipodophyllotoxin, VM-26 (8). Cells were grown and maintained as described previously (8, 13), and the resistant lines were treated once a week with maintenance concentrations of drug (110 nM VLB or 100 nM VM-26). Drugs were removed from cultures 7 to 10 days before an experiment. We have now carried some CEM/VM-1 cells for more than 8 mo in the absence of VM-26 with no loss of resistance. Fresh cultures were brought up from storage in liquid nitrogen over 3- to 6-mo intervals. All cells were routinely tested for *Mycoplasma* contamination and were found to be free of this microorganism.

Drug Studies. Drug cytotoxicity was assayed in a 48-h growth inhibition assay, as described previously (14). The degree of resistance is

derived by dividing the IC_{50} of the drug-resistant cell line by that of the drug-sensitive line.

Membrane Preparations. Cell membrane vesicles were prepared by a modified nitrogen cavitation method (15). Briefly, cells in the midlog stage of growth were harvested, washed in cold phosphate-buffered saline solution, and resuspended in "vesicle buffer" (containing 10 mM Tris-HCl, pH 7.4; 0.25 M sucrose; 0.2 M $MgCl_2$; 1 mM EDTA; and 2 mM phenylmethylsulfonyl fluoride). The cell suspension was then placed in a nitrogen cavitation apparatus and exposed to N_2 at 400 psi for 20 min. After release of the pressure, which disrupts the cells, the suspension was diluted in Tris-buffered sucrose. Remaining whole cells and large debris were removed by centrifugation. The supernatant was then layered over a cushion of 35% sucrose (buffered with 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) and centrifuged in a swinging-bucket rotor at 16,300 × g for 45 min. The membrane band was then removed and pelleted at 100,000 × g. This pellet was resuspended in Tris-buffered sucrose [10 mM Tris-HCl (pH 7.4)-0.25 M sucrose-2 mM phenylmethylsulfonyl fluoride] and stored at −80°C in small aliquots until used for labeling with [^{125}I]NASV.

Labeling of Membrane Proteins. The resistance-associated glycoprotein, P-gp, was specifically labeled in membrane vesicle preparations with the photoaffinity probe, [^{125}I]NASV, as described (11). After labeling, the membrane preparations were solubilized, and their proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11).

RNA Expression. Total cellular RNA was analyzed for expression of the *mdr* gene using the pMDR1 probe (16), kindly provided by Dr. Igor Roninson (Center for Genetics, University of Illinois Medical Center, Chicago, IL). The pT1 probe for the α-tubulin gene (17) was provided by Dr. Peter Houghton (St. Jude Hospital). Briefly, RNA was prepared according to the method of Chirgwin *et al.* (18), denatured, and adsorbed onto duplicate nitrocellulose filters using a Minifold II slot-blot apparatus (Schleicher and Schuell, Keene, NH). Probes were labeled with ^{32}P by a modification of the primer extension method (19). One of the filters was then hybridized with the "P-glycoprotein"-specific pMDR1 plasmid, and the duplicate was hybridized with the α-tubulin-specific pT1 plasmid. Filters were baked, hybridized, and washed by standard procedures (20), except that dextran sulfate was omitted from the hybridization buffer, and radioactivity was detected by autoradiographic exposure of X-ray film.

Karyotypic Analysis. Harvesting, slide preparation, and chromosome analysis with G- or Q-banding techniques were performed as previously described (21). A minimum of 25 banded cells was analyzed per specimen, with results expressed according to International System of Human Cytogenetic Nomenclature recommendations (22).

RESULTS

Cross-Resistance Properties of CEM/VM-1 Cells. We showed previously that the CEM/VM-1 cells were cross-resistant to a variety of drugs in the "MDR phenotype" except the *Vinca* alkaloids (8). We have extended those studies, and the results are shown in Table 1. It is clear that these cells are also sensitive to vindesine, another *Vinca* alkaloid, as well as two other tubulin binding drugs, colchicine and podophyllotoxin. Of considerable interest, CEM/VM-1 cells are less cross-resistant to daunorubicin (~7-fold) than they are to doxorubicin (~80-fold; Ref. 8). These cells are also sensitive to the cytotoxic actions of both actinomycin D and BCNU, agents that interfere with DNA synthesis and repair, respectively. By contrast, the CEM/VLB$_{100}$ cells display the "classic" pattern of cross-resistance, which includes cross-resistance to the "natural products," VM-26, vindesine, actinomycin D, colchicine, and daunorubicin. Finally, there is little or no cross-resistance of either cell line to the antimetabolites, methotrexate and 6-mercaptopurine.

Effects of Verapamil and CLQ on Drug Cytotoxicity in CEM/VM-1 Cells. We and others have shown that both verapamil (14, 23) and CLQ (24, 25) can enhance the cytotoxicity of *Vinca*

Table 1 *Resistance and cross-resistance properties of CEM/VLB$_{100}$ and CEM/VM-1 cells*

Determined in a 48-h growth inhibition assay (14).

	CEM IC$_{50}$ (M × 10^{-8})	Degree of resistance[a]	
		CEM/VLB$_{100}$	CEM/VM-1
VM-26	6.49 ± 6.25[b]	24.5	53.5
Vindesine	0.15 ± 0.17[c]	1186	1.2
Actinomycin D	5.68 ± 7.66	49	2.4
Colchicine	4.57 ± 2.92	20	0.9
Podophyllotoxin	5.55 ± 3.01	0.8	2.5
Daunorubicin	2.89 ± 3.01	44	6.6
BCNU	2050[d]	0.5	1.5
Methotrexate	1.40 ± 0.88	2.1	3.3
6-Mercaptopurine	330 ± 236	1.0	1.6

[a] Relative to CEM cell controls.
[b] Mean ± SD of 10–15 experiments.
[c] Unless indicated otherwise, values are means ± SD of 3–5 experiments.
[d] Mean of 2 experiments.

Table 2 *Effects of verapamil and chloroquine on the cytotoxicity of vinblastine in three cell lines assessed in a 48-h growth inhibition assay (14).*

The fold-decrease in cytotoxicity was determined by dividing the IC_{50} of the untreated cells by that of the verapamil- or CLQ-treated cells. See "Materials and Methods" for details.

Cell line	VLB IC$_{50}$ (nM)	+10 μM Verapamil		+50 μM CLQ	
		VLB IC$_{50}$ (nM)	Fold decrease	VLB IC$_{50}$ (nM)	Fold decrease
CEM	3.4	0.46	7.5	2.2	1.6
CEM/VLB$_{100}$	343	3	114	41	8.4
CEM/VM-1	3.9	0.42	9.3	4.0	<1.0

alkaloids and anthracyclines in MDR cell lines such as CEM/VLB$_{100}$. It can be seen in Table 2, however, that the effects of verapamil and CLQ are limited to the classic MDR cells and that these modulators have little or no ability to enhance the cytotoxic activity of VLB in the CEM/VM-1 cells.

Photoaffinity Labeling of M_r 180,000 Glycoprotein with [^{125}I]-NASV. As can be seen in Fig. 1, a P-gp doublet was labeled specifically by [^{125}I]NASV in the CEM/VLB$_{100}$ cells, as indicated by the fact that it could be blocked by an excess of unlabeled VLB. In the CEM/VM-1 cells, however, there was no specifically labeled protein that could be identified by this reagent.

***mdr* Gene Analysis of CEM/VM-1 Cells.** The data in Fig. 1 show that at-MDR cells do not overexpress the "marker" protein for MDR. These results were confirmed by our analysis of the at-MDR cells for expression of the *mdr* gene. Shown in Fig. 2 is a slot-blot of total RNA extracted from CEM, CEM/VLB$_{100}$, or CEM/VM-1 cells, hybridized with a probe for the *mdr* gene (pMDR1; Ref. 16) and with a complementary DNA for the α-tubulin gene (pT1; Ref. 17) as a control. It is clear from Fig. 2 that only the CEM/VLB$_{100}$ cells overexpress the *mdr* gene; CEM/VM-1 cells do not. These results confirm the previous biochemical and pharmacological studies indicating that at-MDR cells are truly "atypical" and are resistant to many natural product drugs except the *Vinca* alkaloids by a mechanism that includes neither the *mdr* gene nor its apparent product, P-glycoprotein.

Cytogenetic Analysis. Chromosomal banding analysis revealed the presence of numerous clonal karyotypic alterations in both the parental and at-MDR cell lines (Fig. 3). The modal chromosome number of the parental CEM cell line was near-triploid ($n = 87$) with approximately 46% of the cells displaying chromosome numbers between 86 and 90. The karyotype of the CEM cell line (Fig. 3A) was characterized primarily by numerical chromosome alterations (most notably chromosome gains), although clonal structural alterations (including 4 Umars) were also observed. Identifiable structural alterations included:
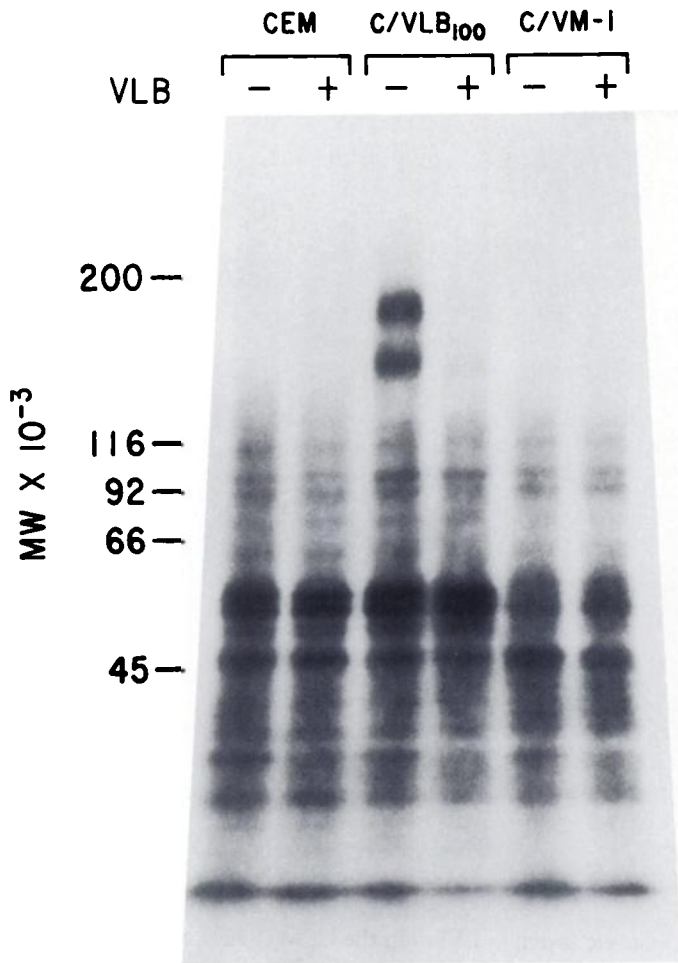
Fig. 1. Photoaffinity labeling of P-glycoprotein by [^{125}I]NASV. Cell membranes were prepared as described in "Materials and Methods." Membranes were labeled with 2.5 × 10^{-8} M of the photoaffinity analogue of VLB, [^{125}I]NASV, in the absence or presence of 50 μM nonradioactive VLB, as described previously (11). Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by fluorography, as described (11).
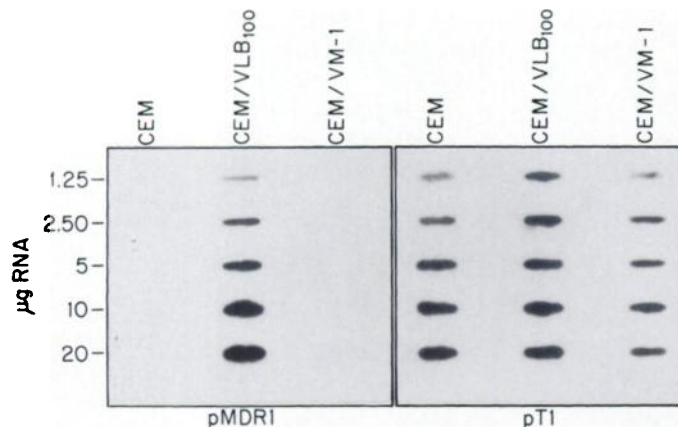

Fig. 2. Analysis of RNA for expression of the *mdr1* gene. The indicated amounts of total RNA from CEM, CEM/VLB₁₀₀, and CEM/VM-1 were applied to nitrocellulose as described in "Materials and Methods." Hybridization with the *mdr1* and α-tubulin probes was for 19 h. The autoradiograms were exposed for 70 h and at −80°C with intensifying screens. See "Materials and Methods" for details.

dup(1)(p32→p36);del(4)(q31);del(6)(q13);t(9:?)(p22:?). In addition to these identifiable alterations, the CEM cell line also displayed a large unidentifiable metacentric marker chromosome (approximately the size of a chromosome 3) and three

Umars approximately the size of a D-group chromosome (Fig. 3A).

The CEM/VM-1 subline was similar in many respects to the parental CEM cell line. However, several chromosomal rearrangements unique to the resistant subline were identified (Fig. 3B). The modal chromosome number of the CEM/VM-1 line, like the CEM parental cell line, was near triploid ($n = 93$), with 60% of cells displaying a chromosome number between 83 and 93. In contrast to the parental CEM cell line (which displayed few polyploid cells), approximately 20% of cells from CEM/VM-1 were polyploid. The CEM/VM-1 cell line displayed several structural chromosome alterations common to the parental CEM cell line, including dup(1)(p32→p36);t(9;?)(p22;?) and three D-group-sized Umars. Structural alterations unique to the resistant subline included: del(1)(p22);del(7)(q32);-del(8)(p12);del(9)(p22);ABR(13)(q14) (Fig. 3B). Of particular interest was the finding of an ABR in CEM/VM-1 cells, since ABRs have been associated with amplified chromosomal domains (3). Finally, an average of 9 Umars characterized the CEM/VM-1 subline, in contrast to 4 Umars in the drug-sensitive CEM parent.

Cytogenetic analysis of the CEM/VLB₁₀₀ subline revealed unique chromosomal abnormalities that differed from those of the CEM/VM-1 cells, and those data will be presented elsewhere.[4]

DISCUSSION

We have shown here and elsewhere (8, 9) that CEM/VM-1 cells selected for resistance to the epipodophyllotoxin, VM-26, express a pattern of broad cross-resistance to several classes of natural product drugs, but in contrast to classic MDR cells, are sensitive to the *Vinca* alkaloids, colchicine, and actinomycin D. Compared to the parent line, these cells are unaltered in their ability to accumulate [^{3}H]etoposide (8) or [^{3}H]vincristine (9). Furthermore, VLB cytotoxicity in the CEM/VM-1 cells cannot be potentiated substantially by either verapamil or CLQ. These results differ from those with the classic MDR cells, which display decreased drug accumulation (5) and modulation of VLB cytotoxicity by both verapamil (14) and CLQ (24). Consistent with the findings is the fact that the CEM/VM-1 cells do not overexpress the MDR marker protein, P-gp, or its mRNA, both of which are commonly associated with classic MDR (26, 27). Moreover, the karyotype of the CEM/VM-1 cells differs from those of both the CEM and CEM/VLB₁₀₀ cells. We conclude from these studies that at-MDR is a distinct entity from classic MDR.

Karyotypic abnormalities have been reported in many different types of drug-resistant cell lines, including those expressing MDR (28–30). Homogeneously staining regions and double minutes are common abnormalities in such cells and are associated with gene amplification (29). ABRs, which are chromosomal regions of amplified DNA that are not uniform in their staining properties, are sometimes difficult to distinguish from homogeneously staining regions (31). Trent *et al.* (30) have shown recently that anthracycline resistance in six human tumor cell lines was associated with abnormalities of chromosome 7, and in three of these lines, ABRs were found on this chromosome. Indeed, in that study, approximately 60% of all chromosome 7 alterations involved the distal long arm (30). In this regard, it is of interest that the *mdr* gene has been localized
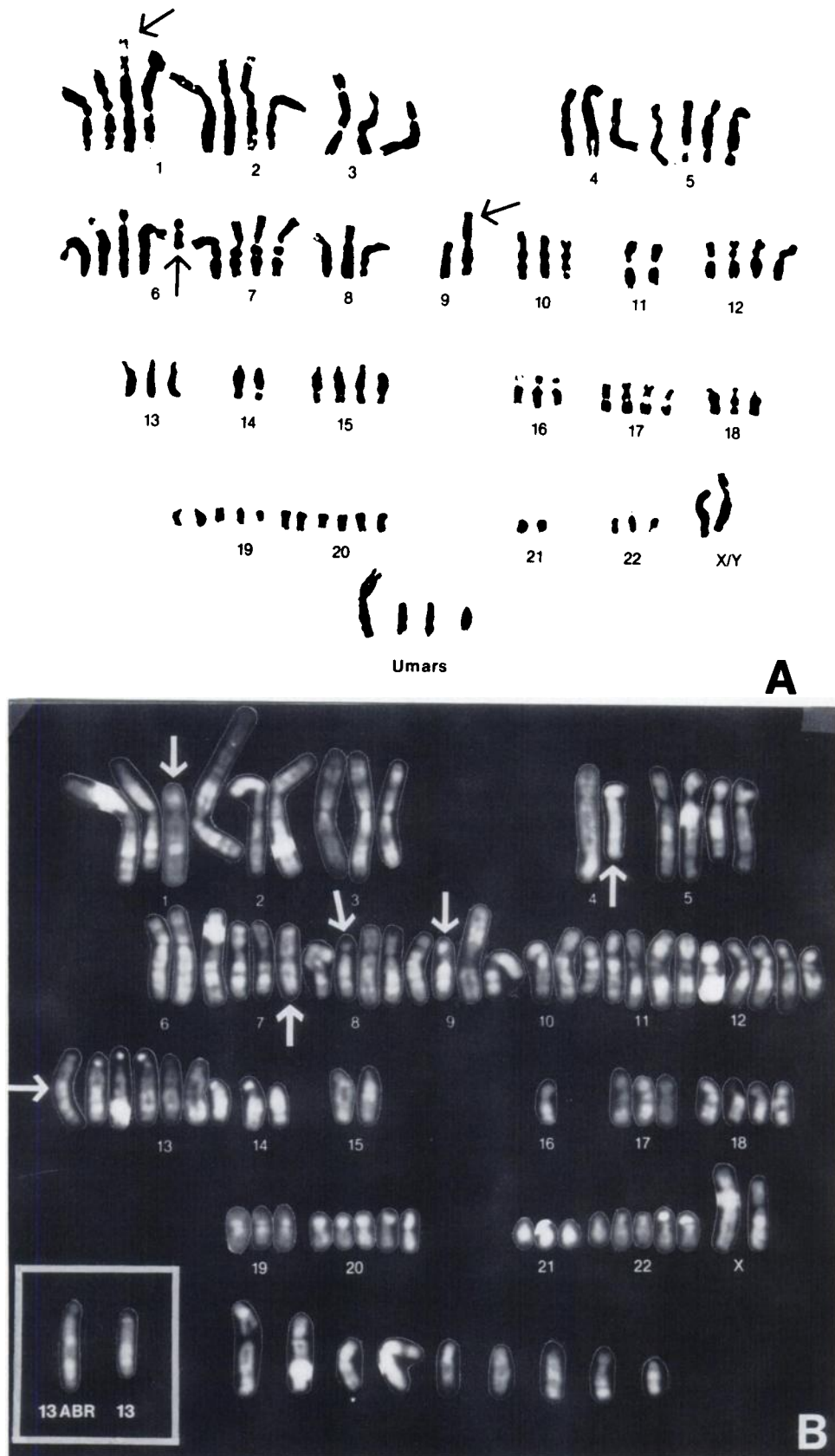
Fig. 3. Cytogenetic analysis of CEM (A) and CEM/VM-1 (B) cells. A, G-banded karyotype of the parental CEM cell line. Arrows, structural alterations including dup(1)(p32→36),del(6)(q13);t(9;?)(p22;?), and four Umars. B, Q-banded karyotype of VM-26-resistant cell line, CEM/VM-1. Arrows, clonal structural chromosome alterations unique to the CEM/VM-1-resistant subline including del(1)(p22),del(7)(q32);del(8) (p12),del(9)(p22),ABR(13)(q14). Inset, 13qABR from a second cell.

within this region in human cells (32). The ABR found in the at-MDR CEM/VM-1 cells likely represents an area of amplified DNA, but its location on chromosome 13q distinguishes it from the classic MDR cell lines (30). We do not yet know if there are in fact overexpressed proteins in the CEM/VM-1 cells that would be consistent with gene amplification.

While [^{125}I]NASV did not label specific bands in the CEM/VM-1 cells, this compound labeled M_r ~180,000 and M_r ~145,000 doublets in the CEM/VLB$_{100}$ cells. These data with the classic MDR cells are consistent with previously reported results (11). The lower band may be a breakdown product of the upper (P-gp) band, despite the fact that inhibitors of proteolysis were present during all stages of the membrane preparation procedure, as it is not always seen.[5] Peptide analysis should reveal any relationship between these two bands.

We have generated other results to indicate that at-MDR differs from classic MDR. For example, we have found that fluorescent agents such as anthracyclines are accumulated primarily in discrete cytoplasmic compartments in the CEM/VM-1 cells, and this distribution appears different from that in either the CEM/VLB$_{100}$ or the CEM cells (9). These results suggest that drug-target interactions may be altered in at-MDR. Furthermore, preliminary results of alkaline elution analyses revealed that the DNA of the CEM/VM-1 cells is relatively resistant to single-strand breaks induced by VM-26, VP-16, or 4'-[(9-acridinyl)amino]methanesulphon-m-anisidide, compared to both the CEM and CEM/VLB$_{100}$ cells (33). These data suggest, but do not prove, that the CEM/VM-1 cells are likely to be altered in topoisomerase II (34) or its modifying activity (35). Studies are currently in progress to examine these possibilities.

We believe that our studies of at-MDR may have clinical implications because of the spectrum of drugs against which these CEM/VM-1 cells are cross-resistant. It is likely that cells displaying characteristics of at-MDR will be present in clinical specimens, as has already been demonstrated for classic MDR (36, 37). However, also like classic MDR, it is not yet known whether the presence of at-MDR "markers" will correlate with clinical drug resistance.

Finally, our studies provide clear evidence that alternative (i.e., atypical) mechanisms to confer an "MDR" phenotype exist. Additional evidence appears to support this notion. For example, two other human leukemic cell lines, HL-60 and K562, selected for resistance to 4'-[(9-acridinyl)amino]methanesulphon-m-anisidide (38) or etoposide (39), respectively, display patterns of cross-resistance and drug accumulation similar to those of our CEM/VM-1 cells. Moreover, a doxorubicin-resistant human MCF-7 breast cancer cell line has been described (40) that appears to display MDR, but resistance in this line appears to be coupled to expression of a novel form of anionic glutathione transferase. It remains to be determined, however, whether any of these cell lines express the mdr gene or the P-glycoprotein, so it is not yet clear whether they display classic or alternative forms of MDR. Another doxorubicin-resistant human small cell lung cancer line has been described recently that apparently does express a "nonclassic" form of MDR (41). These cells exhibit a broad cross-resistance to a variety of natural product compounds, including the $Vinca$ alkaloids, colchicine, and etoposide, but P-glycoprotein could not be detected by immunological methods. Thus, the data suggest that there may indeed be alternative mechanisms leading to an MDR phenotype.

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[5] A. R. Safa, unpublished observations.

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