Atypical Multidrug Resistance in a Therapy-induced Drug-resistant Human Leukemia Cell Line (LALW-2): Resistance to \textit{Vinca} Alkaloids Independent of P-Glycoprotein

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ABSTRACT

Near diploid leukemic T-cells (LALW-2), exposed to cytotoxic drugs only as a consequence of therapy administered to the donor patient, have been maintained by serial xenograft in nude mice. In comparison with the leukemic line CCRF-CEM, using a growth inhibition assay, LALW-2 cells were resistant to \textit{Vinca} alkaloids and actinomycin D (relative resistance, 200-fold or more), were slightly resistant to Adriamycin (relative resistance, 4-fold), and showed no resistance to daunorubicin or teniposide. By comparison, a vincristine-resistant CEM subline developed in our laboratory (CEM/VCR R) was resistant to all these agents by at least 30-fold. The VCR R subline served as a positive control, confirming the previously reported correlation between multidrug resistance and amplification of the P-glycoprotein gene. Comparison of CEM, CEM/VCR R, and LALW-2 cells establish that the P-glycoprotein gene was not amplified or overexpressed in the LALW-2 cells; neither could the gene product be detected by immunoblotting in extracts from these cells. The LALW-2 cells were further distinguished from CEM/VCR R cells due to the lack of increased vincristine efflux by the xenografted cells, an effect readily demonstrable in the CEM/VCR R cells. However, although LALW-2 cells efflux vincristine at the same rate as CCRF-CEM cells, the xenografted cells exhibited a reduced rate of vincristine accumulation. Uptake of daunorubicin by LALW-2 cells was not distinguished from that by CEM cells, consistent with similar 50% inhibitory dose levels for this drug in both cell populations, and differentiating both from CEM/VCR R cells. Thus, clinical resistance in this case appears to be an “atypical” form of multidrug resistance specifically distinguished by resistance to \textit{Vinca} alkaloids and actinomycin D occurring in the absence of increased amounts of P-glycoprotein and manifesting decreased drug uptake.

INTRODUCTION

The phenomenon of multidrug resistance has been associated with certain “natural product” drugs, such as the \textit{Vinca} alkaloids, anthracyclines, and epipodophyllotoxins. Rodent cell lines selected for resistance to one of these classes of drugs exhibit cross-resistance to other structurally unrelated members of the natural product group, despite there being no previous exposure to these drugs (1–4). Underlying this resistance is decreased cellular drug retention, due to increased drug efflux, which in turn has been correlated with the enhanced expression of a \textit{M}, 170,000 membrane protein, termed the P-glycoprotein (5, 6). Analogous experiments have been conducted using human cancer cells. The gene responsible (\textit{mdr}1) has been cloned and sequenced (7) and has been shown to code for P-glycoprotein (8). This gene is expressed under normal conditions in certain human tissues including kidney, liver, intestine, and adrenal gland (9) and may account for intrinsic drug resistance in tumors arising from these tissues (10).

Some observations are consistent with alterations in the amount of P-glycoprotein in response to therapy. The level of P-glycoprotein in at least one case of ovarian carcinoma increased with disease progression (11). Association between level of P-glycoprotein and disease progression was later demonstrated in two adults with acute nonlymphoblastic leukemia (12). Overexpression of P-glycoprotein may certainly occur in the absence of \textit{mdr}1 amplification (13, 14) which has not been reported in response to therapy. Most tumors express low or barely detectable amounts of \textit{mdr}1 mRNA. Drug resistance in cases of rhabdomyosarcoma and neuroblastoma has been correlated with elevated levels of MDR3 expression (9). Nonetheless, Cantwell et al. (15) concluded that classic MDR was not the major mechanism of resistance in a series of small cell lung cancer patients who proved to be cross-resistant to alkylating agents following treatment with drugs of the MDR family. Louie et al. (16) demonstrated that decreased drug accumulation was not the primary mechanism of Adriamycin resistance in ovarian cancer cells from clinically refractory patients, despite observing such a mechanism in cells with \textit{in vitro}-induced Adriamycin resistance.

We have sought to further examine clinical drug resistance using cells from an acute lymphoblastic leukemia patient who relapsed during treatment with a range of drugs including vincristine. Cells initially obtained by leukapheresis have been maintained by serial xenograft in nude mice and not further exposed to drugs. These leukemic T-cells (LALW-2) have been characterized morphologically, karyotypically, and immunophenotypically (17). The cells exhibit a t(11;14)(p13;q11) translocation, the break point for which lies within the T-cell receptor \delta chain locus and an 800-base pair break point cluster region on chromosome 11 (18). The present investigation was prompted by the observation that LALW-2 cells, maintained in short term culture, exhibited marked resistance to vincristine as well as to methotrexate by comparison with the lymphoblastoid line CCRF-CEM (19). Because resistance to vincristine was consistent with clinical induction of the MDR phenotype, the response of the cells to a range of natural product drugs was determined. In parallel studies evidence was sought for...
expression of P-glycoprotein by LALW-2 cells using sublines of CEM cells rendered resistant to vincristine or vinblastine as positive controls. Resistance by LALW-2 cells to natural product drugs could be clearly distinguished from that of the MDR sublines in terms of cytotoxicity data and evidence for the noninvolvement of P-glycoprotein in mediating this resistance. Nevertheless, the mechanism of resistance by LALW-2 cells appears to involve alterations in intracellular drug concentration.

MATERIALS AND METHODS

Chemicals and Animals. Cytotoxic drugs were purchased from Australian subsidiaries of the manufacturers as follows: vinblastine and vincristine from Eli Lilly and Co.; Adriamycin from Farmitalia; daunorubicin from May and Baker; actinomycin D from Merck Sharp and Dohme Int.; and VM-26 from Bristol Laboratories. [3H]Vincristine was purchased from Amersham Australia (Sydney) and [3H]daunorubicin from Du Pont-NEN Research Products (Sydney). Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, MA. Nucle mice (nu/nu BALB/c background) were obtained from the Australian National Science and Technology Organisation (Sydney) and maintained in a protected environment (17).

Tissue Culture and Xenograft Maintenance. CCRF-CEM, a human leukemic cell line (20), was kindly supplied by P. Slowiacek (Ludwig Institute, Sydney, Australia) and maintained as a static suspension culture at 37°C in RPMI 1640, supplemented with N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer (20 mm), l-glutamine (2 mm), penicillin-streptomycin (100 units/ml), and 10% fetal calf serum (Commonwealth Serum Laboratories). LALW-2 was maintained by serial passaging as a xenograft in nude mice (17). Cell suspensions, (4 to 8 µl) at a concentration of 10^5 cells/ml, were introduced into nude mice by direct bilateral intraocular injection. LALW-2 was maintained in short-term culture using tumor sample obtained from metastatic sites in the cervical lymph nodes. Following mincing with scalpel blades and vigorous pipeting, a single cell suspension, thus obtained, was washed twice with RPMI 1640 before being resuspended in the same medium, supplemented as described above. Interleukin 2 (Cellular Products, Inc., Buffalo, NY) at a concentration of 10% (v/v) was also added in order to achieve proliferation of LALW-2 in culture.

Drug-resistant Sublines. A CCRF-CEM subline (CEM/VCR R) was selected stepwise for resistance to vincristine essentially as described by Alam et al. (21). The initial concentration used was 5 x 10^{-4} M vincristine and after normal growth had been achieved, cells were maintained at this concentration for 8 to 10 days. Following this, the drug dose was initially increased by multiples of 2, with intervals of approximately 4 to 6 weeks between treatments. The CEM/VCR R subline was maintained at a final concentration of 6 x 10^{-5} M vincristine. Three vinblastine-resistant CEM sublines, VLB,0, VLB,0,00, and VLB,0,000, were selected for growth in 10, 100, and 1000 ng/ml vinblastine, respectively (4). Routine screening for Mycoplasma indicated that all cell lines, including drug-sensitive controls, were free of contamination.

Cytotoxicity Assays. Cells were adjusted to a concentration of 2 x 10^5 cells/ml in complete growth medium, in 25-cm² flasks. The various cell populations were then exposed to one of the following drugs at the concentrations indicated: vincristine, vinblastine (10^{-4} to 10^{-3} M); Adriamycin, daunorubicin (10^{-4} to 10^{-3} M); actinomycin D (10^{-4} to 10^{-3} M); and VM-26 (10^{-4} to 10^{-3} M). Viable cell numbers were determined in triplicate after 72 h, using phase contrast microscopy.

For each assay the ID₅₀ value was determined. A computer fitting of a spline curve was made to the data points using a log scale for molar concentration of the drug, and the concentration corresponding to 50% inhibition, was determined from the curve. The log ID₅₀ value, for a given drug and cell line was calculated by taking the average of the individual log ID₅₀ determinations from at least 3 replicate assays. t tests with 2-sided P values were used to assess the statistical significance between ID₅₀ values from different cell lines. Relative resistances for a given drug and cell line were calculated by dividing the ID₅₀ of the resistant line by that of the sensitive CEM control.

Drug Accumulation and Efflux Studies. The intracellular accumulation of vincristine and daunorubicin was determined by adjusting cells to 5 x 10^6 cells/ml in fresh growth medium and monitoring drug uptake for up to 5 h. Mean incorporation was determined for duplicate 1-ml cultures to which [3H]vincristine (8 Ci/mmol; final concentration, 12.5 nm) or [3H]daunorubicin (3.2 Ci/mmol; final concentration, 500 nm) was added. Cultures were incubated at 37°C, and incubations were terminated at the end of specified time intervals by washing cells 5 times in rapid succession in ice-cold PBS. The cell pellets were hydrolyzed at 70°C for 5 min after the addition of 3 M NaOH (100 µl). The lysate was then neutralized with 2 M HCl (200 µl) prior to adding scintillant (Insta-Gel; Packard Instruments) and counting samples. Results were expressed as pmol of drug/10^6 cells. To study the retention of [3H]vincristine, cells were incubated for 60 min at 37°C with 500 nm [3H]vincristine, prior to washing out the drug 3 times in rapid succession in prewarmed growth medium. Cells were then reincubated at 37°C in drug-free growth medium for 1 to 60 min. Incubations were terminated as described above for drug accumulation studies, and samples were processed for radioactivity determination. The amount of radioactive drug remaining in cells was expressed as a percentage of the initial 1-h drug accumulation.

Southern Blot Analysis. DNA was isolated from either tissue cultured cells or xenografts with the use of a modified phenol extraction method as described by Moir et al. (22), and 5-µg aliquots were digested with BamHI using the manufacturer’s recommended buffer. Following electrophoresis in 0.8% agarose gels using 90 mA for 4 h, gels were transferred at 30 V for 24 h to a nitrocellulose filter, fixed with 0.1 M NaHCO₃, 0.1 M NaOH, and 0.1% (v/v) acetic acid-2 mM EDTA buffer, pH 8.3, alkaline Southern transfers were performed onto GeneScreen Plus membrane, essentially as described by Chomczynski and Qasba (23).

The plasmid pCHP1 was kindly supplied by V. Ling (Ontario Cancer Institute, Toronto, Ontario, Canada), and contained a 600-base pair hamster complementary DNA coding for P-glycoprotein inserted into the EcoRI site of pUC9 (6). This insert was extracted and nick translated to a specific activity of greater than 5 x 10^6 cpm/µg. Hybridizations were carried out in a solution of 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. After hybridization, the membranes were washed twice in 2x SSC for 5 min (room temperature), followed by two 30-min washes at 65°C in 2x SSC and 1% SDS. The final washes consisted of two 30-min rinses in 0.1x SSC. Autoradiography was performed at −70°C with intensifying screens for an appropriate period of time, using Fuji new RX film.

Northern Blot Analysis. Total cellular RNA was isolated from cells using a modification of the method of Chirgwin et al. (24). Approximately 10^6 cells were washed with sterile PBS solution and, after pelleting, 7.5 ml of 4 M guanidine isothiocyanate solution (25 mM sodium citrate-0.5% N-lauroylsarcosine, pH 7.0) were added, with vigorous mixing. This mixture was then loaded onto a 2-ml bed of cesium chloride solution (5.7 M CsCl-0.1 M EDTA, pH 5.0) and centrifuged for 24 h at 25,000 rpm.

RNA (20 µg) was subjected to electrophoresis on 1% agarose gels containing 1x 20 mM morpholinopropanesulfonic acid-5 mM sodium acetate-1 mM EDTA, buffer pH 7.0, and 6% formaldehyde, according to the method of Khandjian and Meric (25). Following electrophoresis, gels were subjected to capillary transfer onto GeneScreen Plus membrane using 10x SSC as the transfer solution. Hybridization and autoradiography were as described for Southern analysis.

Immunoblot Analysis for the Presence of P-Glycoprotein. The method used was a refinement of that used by Ling et al. (3), Bell et al. (11), and Hitchins et al. (26). Cells were washed initially with PBS (4°C) and resuspended in lysis buffer (0.01 M Tris, pH 7.4) for storage at −20°C. After thawing, the suspension was homogenized in a motor-driven Teflon-glass Dounce (20 to 50 strokes at 1200 rpm) until >75% cells were disrupted as determined by light microscopy. After preliminary centrifugation (4000 x g, 10 min), the membranes were separated from the supernatant by sedimentation at 35,000 x g for 1 h. The pellet was then washed twice in PBS and stored frozen. For electrophoretic analysis, membranes were suspended in sample buffer (0.01 M Tris-
acetate, pH 7.4-1 mM EDTA-0.05 mM dithiothreitol-5 mM urea-10% sucrose-2% SDS) at 1 to 2 mg/ml and heated (100°C for 5 min) before cooling to room temperature. The sample (3 to 5 μl) was loaded on a Fairbanks (27) 5.6% polyacrylamide gel containing 0.02 mM Tris (pH 7.4)-9 mM urea-1% SDS and electrophoresed for 1 h at 100 V in a Bio Rad Mini-protein II system using a running buffer of 0.04 mM Tris-acetate (pH 7.4)-1% SDS. Separated proteins were transferred to 0.45 μm nitrocellulose using the Mini-protein II Trans blot system with transfer buffer (0.025 mM Tris-glycine, pH 8.2, containing 20% methanol) for 1 h at 100 V. The membrane was blocked in 5% skim milk in TBS, containing 0.15% Tween 1% Tween 48 h at room temperature before washing three times in the same solution containing 0.01% Tween. The membrane was incubated in a 1:500 dilution of monoclonal antibody C219 in 0.5% skim milk-TBS for 2 h at room temperature before washing as described previously and incubating in a 1:500 dilution of anti-mouse antibody-alkaline phosphatase conjugate (Dakopatts) in 0.5% skim milk-TBS for 1 h at room temperature. The filter was then washed three times before development.

RESULTS

In Vitro Drug Resistance. LALW-2, CEM, and CEM/VCR R cells were maintained in short term culture and tested for their resistance to a number of drugs belonging to the MDR family. With respect to the LALW-2 cells, cytotoxicity testing has been the subject of a separate report (19) which describes the short term growth characteristics of the cells under the influence of interleukin 2, with particular reference to the similar proliferative activity of LALW-2 and CEM cells during the first 72 h after seeding. The doubling time for the xenograft cells was 21 h, providing a basis for comparing their response to cytotoxic drugs with that exhibited by CEM cells and various resistant sublines (including CEM/VCR R). The CEM/VCR R cells, capable of growing in 6 × 10^{-4} M vincristine, displayed a typical MDR phenotype, being most resistant to the selecting drug, followed by marked cross-resistance to another Vinca alkaloid, vinblastine, and exhibiting varying levels of cross-resistance to the anthracyclines, Adriamycin D and to the epipodophyllotoxin, VM-26 (Table 1). The LALW-2 cells likewise displayed marked resistance to vincristine and vinblastine and were significantly cross-resistant to actinomycin D. Despite displaying a moderate degree of resistance to Adriamycin, however, the LALW-2 cells were completely sensitive to the structurally related drug, daunorubicin. Similarly, the LALW-2 cells were sensitive to VM-26.

Drug Accumulation and Efflux Studies. In order to characterize the nature of the observed drug resistance in the LALW-2 cells, vincristine and daunorubicin accumulation patterns were measured in the sensitive CEM line, the vincristine-resistant CEM/VCR R subline and the LALW-2 cells. [3H]Vincristine uptake into the cells was measured over a 5-h period. After this time the CEM/VCR R cells had accumulated less than 10% as much [3H]vincristine as the drug-sensitive CEM controls (Fig. 1). By comparison, the LALW-2 cells had accumulated 3 times the amount of [3H]vincristine as the CEM/VCR R cells, although still significantly less than the CEM cells. Examination of daunorubicin uptake by the various cell populations indicated that no distinction on this basis could be made between LALW-2 cells and the control CEM cell line. However, while LALW-2 cells exhibited the same daunorubicin uptake pattern as CEM cells, both vincristine- and vinblastine-resistant CEM sublines accumulated far less of the anthracycline during a 4-h incubation (Fig. 2).

Efflux of vincristine was determined after a 60-min preincubation. As would be anticipated, the various cell populations differed in their uptake of the drug after exposure to 0.5 μM [3H]vincristine (Table 2). For comparative purposes, the amount of intracellular vincristine in CEM, CEM/VCR R, and LALW-2 cell populations was expressed as the percentage of [3H]vincristine remaining at the selected time interval after 60 min pretreatment (Fig. 3). After 1 h, by comparison with the drug-sensitive control CEM cells which retained 60% of the

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Table 1 Cross-resistance patterns of LALW-2 and CEM/VCR R cells to various antitumor agents

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID₅₀ for sensitive CEM cells* (n × 10⁻⁹)</th>
<th>Relative resistance¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LALW-2</td>
<td>CEM/VCR R</td>
</tr>
<tr>
<td>Vincristine</td>
<td>2.64</td>
<td>1,920&quot;¹³&quot;³&quot;</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>8.02</td>
<td>220&quot;¹³&quot;³&quot;</td>
</tr>
<tr>
<td>Adriamycin D</td>
<td>1.13</td>
<td>4.13&quot;²&quot;³&quot;</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>53.1</td>
<td>61&quot;²&quot;³&quot;</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>10.8</td>
<td>187&quot;²&quot;³&quot;</td>
</tr>
<tr>
<td>VM-26</td>
<td>313</td>
<td>35&quot;²&quot;³&quot;</td>
</tr>
</tbody>
</table>

* Values shown for CEM cells are means of individual ID₅₀ determinations from at least 5 replicate assays, while for LALW-2 and CEM/VCR R cells values are means of 3 to 6 replicate assays.

¹ Determined by dividing the ID₅₀ for the resistant line by the ID₅₀ for the sensitive CEM cells.

² p < 0.005.

³ p < 0.05.

⁴ Not significant.
coprotein in CEM/VCR R cells, the broad band being often radiographic signal, consistent with overexpression of P-gly.

The mdrl gene can precede gene amplification (9, 14), and this suggests.

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fluorimetry and confirmed, in terms of equal distribution be.

overexpressing P-glycoprotein (12). The level of amplification
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compared to the CEM control. CEM/VCR R DNA exhibited
cation or rearrangement was apparent in LALW-2 DNA when

digested DNA preparations was hybridized to the 600-base pair

vestigate this possibility, a Southern transfer of various BamHl

amplification of the P-glycoprotein gene in

in the amount of DNA loaded, which was determined using

approximately 4-fold. The result is not attributable to variation

in the CEM/VCR R line was determined by densitometry to be

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CEM/VCR R DNA (4 and 20 fjg respectively), demonstrating overexpression of

P-glycoprotein message; Lane 5, retinoblastoma line Y79 RNA; Lane 6, LALW-

2 RNA. Arrowheads, position of the 28S and 18S ribosomal bands.

observed in Northern blots when the gene is markedly overex-

pressed (28–30). There was no evidence of P-glycoprotein expression in LALW-2 cells. The presence of intact LALW-2 mRNA was confirmed by reprobing this membrane for a constitutively expressed messenger species, namely that of the dihydrofolate reductase gene; similar amounts of the corresponding mRNA were detected for all cell types (data not shown).

P-Glycoprotein Gene Analysis. Despite being relatively resistant to vincristine, the response of LALW-2 cells to drugs of the MDR family suggested a mode of resistance different from that mediated by overexpression of P-glycoprotein. In order to investigate this possibility, a Southern transfer of various BamHI digested DNA preparations was hybridized to the 600-base pair P-glycoprotein insert of plasmid pCHP1 (Fig. 4). No amplification or rearrangement was apparent in LALW-2 DNA when compared to the CEM control. CEM/VCR R DNA exhibited gene amplification, as did three vinblastine-resistant CEM sublines. These VLB lines have been shown previously to be highly overexpressing P-glycoprotein (12). The level of amplification in the CEM/VCR R line was determined by densitometry to be approximately 4-fold. The result is not attributable to variation in the amount of DNA loaded, which was determined using fluorimetry and confirmed, in terms of equal distribution between lanes, by fluorescence in the presence of ethidium bromide. The lack of amplification of the P-glycoprotein gene in LALW-2 cells has been confirmed using other restriction digests.

Earlier studies have indicated that increased expression of the mdr1 gene can precede gene amplification (9, 14), and this possibility was investigated. A Northern transfer was performed on 20 fjg of denatured total cellular RNA extracted from LALW-2 and other cells (Fig. 5). There was an intense autoradiographic signal, consistent with overexpression of P-glycoprotein in CEM/VCR R cells, the broad band being often observed in Northern blots when the gene is markedly overexpressed (28–30). There was no evidence of P-glycoprotein expression in LALW-2 cells. The presence of intact LALW-2 mRNA was confirmed by reprobing this membrane for a constitutively expressed messenger species, namely that of the dihydrofolate reductase gene; similar amounts of the corresponding mRNA were detected for all cell types (data not shown).

Immunoblot Analysis of P-Glycoprotein. Membranes were prepared from CEM, CEM/VCR R, CEM VLB100, and LALW-2 cells, and proteins reactive with the monoclonal antibody C219 were examined by immunoblot analysis (Fig. 6). As described previously (4), the CEM VLB100 cells displayed high levels of P-glycoprotein overexpression. The CEM/VCR R cells

Table 2 Efflux of vincristine from various cell lines

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Vincristine concentration (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEM</td>
</tr>
<tr>
<td>0</td>
<td>0.754 ± 0.164*</td>
</tr>
<tr>
<td>2.5</td>
<td>0.706 ± 0.167</td>
</tr>
<tr>
<td>5</td>
<td>0.653 ± 0.162</td>
</tr>
<tr>
<td>10</td>
<td>0.682 ± 0.186</td>
</tr>
<tr>
<td>30</td>
<td>0.579 ± 0.118</td>
</tr>
<tr>
<td>60</td>
<td>0.414 ± 0.101</td>
</tr>
</tbody>
</table>

* Mean ± SE of at least four determinations.

Fig. 3. Efflux of [3H]vincristine from CEM (•), LALW-2 (○), and CEM/VCR R (▲) cells. Cells were incubated at 37°C with 500 nM [3H]vincristine for 60 min. The cells were then washed and drug efflux measured as described in “Materials and Methods.” Each point represents the mean of duplicate determinations from at least 2 experiments run on separate days; bars, SE.

Fig. 4. Southern blot analysis of the P-glycoprotein gene. DNA (5 fjg) digested with BamHI, was electrophoresed in a 0.8% gel and subjected to alkaline capillary transfer. Following hybridization with the 600-base pair insert of plasmid pCHP1, autoradiography was performed at -70°C for 20 h with intensifying screens. Lane 1, LALW-2 DNA; Lane 2, VLB10 DNA; Lane 3, VLB100 DNA; Lane 4, VLB1000 DNA; Lane 5, CEM/VCR R DNA; Lane 6, CEM DNA. The 3 vinblastine-resistant sublines (VLB10,100,1000) displayed amplification, as did the CEM/VCR R subline. The level of amplification in the CEM/VCR R DNA was quantitated as 4-fold by densitometry.

Fig. 5. Northern blot analysis of P-glycoprotein expression. Denatured RNA (20 fjg, except Lane 3, for which 4 fjg were run) was subjected to electrophoresis in a 1% agarose gel. Following transfer onto GeneScreen Plus membrane, hybridization was performed with the 32P-labeled insert of probe pCHP1, prior to autoradiography for 3 days. Lanes 1 and 2, CCRF CEM RNA; Lanes 3 and 4, CEM/VCR R RNA (4 and 20 fjg respectively), demonstrating overexpression of P-glycoprotein message; Lane 5, retinoblastoma line Y79 RNA; Lane 6, LALW-2 RNA. Arrowheads, position of the 28S and 18S ribosomal bands.

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Drug resistance associated with increased expression of P-glycoprotein has been referred to as "classic" MDR and has been well characterized (32). The CEM/VCR R subline, experimentally selected in vitro for resistance by stepwise growth in vincristine exhibited resistance to vinblastine, Adriamycin, daunorubicin, actinomycin D, and VM-26 (Table 1). This finding is typical of classic MDR (28, 29). LALW-2 cells could be readily distinguished from the CEM/VCR R subline since, despite exhibiting marked resistance to vincristine and vinblastine, the xenografted cells were as sensitive to daunorubicin and VM-26 as the CEM parent line (Lane 1). This pattern is quite distinct from classic MDR in that cells highly resistant to Vinca alkaloids normally display high levels of cross-resistance to anthracyclines and epipodophyllotoxins (32). Patterns of cross-resistance differing from classic MDR have been described. A P388 leukemia cell line made resistant to mAMSA was found to have cross-resistance to epipodophyllotoxins (VM-26 and VP-16) and some anthracyclines, while displaying sensitivity to the Vinca alkaloids (33). A similar pattern of resistance was displayed by an HL-60 line also selected for mAMSA resistance (34). Danks et al. (35) used the term "atypical MDR" to characterize a CEM subline selected for resistance to VM-26; exhibiting cross-resistance to VP-16, mAMSA, and anthracyclines; but retaining sensitivity to Vinca alkaloids. Likewise a Chinese hamster ovary epipodophyllotoxin-resistant line displayed minimal cross-resistance to vinblastine (36). A VP16-resistant Chinese hamster ovary cell line that was cross-resistant to mAMSA, mitoxantrone, and Adriamycin demonstrated altered topoisomerase II activity (37). Altered topoisomerase activity and other mechanisms of resistance have been described in cell populations variously selected using individual MDR drugs (38). Nonetheless, at least some lines refractory to Adriamycin (and with varying degrees of resistance to Vinca alkaloids) appear to be resistant as a result of reduced drug accumulation (39–41). By comparison with many lines selected using Adriamycin or epipodophyllotoxins, and irrespective of mechanism, the cross-resistance pattern displayed by the LALW-2 cells is distinct in that these cells are highly resistant to the Vinca alkaloids, but completely sensitive to daunorubicin and VM-26.

Classic MDR is linked with the overexpression of P-glycoprotein (6). The CEM/VCR R line described here meets this criterion: the cells have amplified the P-glycoprotein gene(s) (Fig. 4); and there is evidence of overexpression, at both the transcriptional (Fig. 5) and translational (Fig. 6) levels. Moreover, the CEM/VCR R line exhibits the reduced drug accumulation typical of MDR cells (Figs. 1 and 2), with an active drug efflux system clearly being operational in these cells (Fig. 3). The question of P-glycoprotein overexpression in atypical MDR lines has been specifically addressed in only a few studies, and the resultant data are confusing. Defife et al. (30) describe a cell line which appears to display classic MDR, and which overexpresses P-glycoprotein, but in which there is no evidence for the operation of an active efflux pump. In contrast, Slovak et al. (39) and Beck et al. (42) both reported no evidence of P-glycoprotein expression in cell lines which displayed atypical MDR. The former line, however, exhibited enhanced drug efflux, while the latter did not. Various investigations (43–45) have described human cell lines which display classic MDR in terms of their drug resistance profiles, despite which they do not overexpress P-glycoprotein. The cell line described by Marsh et al. (43) nevertheless displays an efflux mechanism, albeit an unusual one in that it appears to be activated only when cells are exposed to drug for prolonged periods. LALW-2 cells appear to be distinguished from all these patterns by virtue of the relevant P-glycoprotein and drug transport data. These cells exhibited neither amplification of P-glycoprotein DNA (Fig. 4) nor increased expression as determined by Northern blot (Fig. 5) or immunoblot (Fig. 6) analysis. The possibility of a subpopulation of LALW-2 cells overexpressing P-glycoprotein has been excluded by immunocytochemistry which indicated that all cells are deficient in this protein.* Moreover LALW-2 cells failed to show enhanced efflux of [*H]vincristine (Fig. 3). The resistance to vincristine displayed by LALW-2 cells has features in common with a recently described vincristine-resistant HL-60 subline, resistance in this case being associated with a distinct M, 150,000 phosphorylated protein and not with P-glycoprotein (46).

The LALW-2 cells displayed reduced vincristine uptake specifically in relation to the CEM cells (Fig. 1). Reduced uptake of vincristine by LALW-2, in comparison to CEM cells, was confirmed at a higher drug concentration by the zero time determination for efflux studies (Table 2). However, in terms of either the absolute amount of drug effluxed or patterns of relative efflux (Fig. 3), these cells were not distinguished from the CEM line by the rate of loss of intracellular vincristine. Hence more efficient efflux of the drug will not account for the decreased rate of vincristine accumulation by LALW-2 cells (Fig. 1). The data are consistent with a membrane-associated mechanism of resistance. The significance of the reduced vincristine uptake is indicated by the differential response of LALW-2 cells to vincristine and daunorubicin. As distinct from classic MDR, LALW-2 cells were sensitive to daunorubicin by comparison with CEM cells (Table 1). Accordingly, the lack of reduced daunorubicin accumulation, again by comparison with CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2), both distinguishes the cells from vincristine-resistant CEM cells (Fig. 2).
resistant sublines and further confirms the lack of P-glycoprotein activity. Thus the present study describes yet another permutation with regard to MDR, P-glycoprotein expression, and drug accumulation, distinct from those reported previously. The LALW-2 cells display an atypical drug resistance profile, fail to overexpress P-glycoprotein, and exhibit reduced drug accumulation by virtue of not enhanced drug efflux but rather by decreased drug uptake.

The mechanism of cytotoxic drug resistance in tumor cell lines is far from clear. The status of MDR in patients undergoing chemotherapy is even less clear. Examination of MDR in either a clinical setting (15) or a clinically relevant model (16) led to the conclusion that classic MDR was not a major mechanism of clinical resistance. Similarly, in the present study utilizing cells rendered drug resistant because of chemotherapy administered to the donor patient, response patterns are quite different from those predictable on the basis of in vitro-conditioned MDR cells. Rather, the resistance pattern displayed by LALW-2 cells can be related to the drugs utilized in treating the donor. Thus, the two drugs to which LALW-2 cells were unexpectedly sensitive, daunorubicin and VM-26, had not been used. Also, the cells showed differential resistance to the Vinca alkaloids, being markedly more resistant to vincristine which had been used therapeutically than to vinblastine which was not (17). Response of the cells to growth inhibition by the different drugs (Table 1) was consistent with the efficacy or otherwise of these agents when administered to mice carrying the LALW-2 xenograft (19).

Currently, much effort in many centers is being directed toward screening large numbers of tumor specimens for P-glycoprotein expression. The presumption that such surveys will adequately characterize clinical drug resistance seems questionable. (a) changes in membrane and cytoplasmic proteins apart from P-glycoprotein have been described in cells displaying the MDR phenotype; (b) the present study adds to the small but growing body of data which suggests that acquired resistance patterns may involve certain of the natural product but growing body of data which suggests that acquired clinical drug resistance may involve certain of the natural product glycoprotein expression. The presumption that such surveys toward screening large numbers of tumor specimens for P-glycoprotein expression would provide a molecular marker associated with multidrug resistance seems premature. (c) The present study describes yet another permutation with regard to MDR, P-glycoprotein expression, and drug accumulation in multi-drug resistant cell lines. Nature (Lond.), 316: 820-823, 1985.

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Atypical Multidrug Resistance in a Therapy-induced Drug-resistant Human Leukemia Cell Line (LALW-2): Resistance to Vinca Alkaloids Independent of P-Glycoprotein

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