Genetic Characterization of the Multidrug-resistant Phenotype of VM-26-resistant Human Leukemic Cells

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ABSTRACT

Our human T-cell leukemia line, CEM/VM-1, selected for resistance to VM-26 (teniposide), is cross-resistant to several drugs that interact with topoisomerase II, including VP-16 (etoposide), 4'-9(acridinylamino)methanesulphon-m-anside, daunorubicin, and mitoxantrone. However, in contrast to cell lines exhibiting multidrug resistance (MDR) associated with overexpression of P-glycoprotein, this line is not cross-resistant to the Vinca alkaloids, is not impaired in drug accumulation, and does not overexpress the mdr1 gene (Cancer Res., 47: 1297, 5455, 1987). More recently we found that nuclear extracts of these cells exhibit decreased topoisomerase II catalytic and cleavage activity, compared to the drug-sensitive line (Biochemistry, 1988). These results suggest that an alteration in topoisomerase II or a modulator of this enzyme may be responsible for the altered topoisomerase II-form of multidrug resistance (at-MDR). In the present work, we studied the somatic cell genetics of at-MDR. We produced hybrid cell lines by polyethylene glycol-mediated fusion of the CEM/VM-1 line with a hypoxanthine-guanine phosphoribosyltransferase-deficient, ouabain-resistant CEM line (CEMA.G1.OU1.5) that exhibits VM-26 sensitivity. Ten of the hybrid lines that grew in selective medium were randomly chosen for expansion and four were analyzed for both DNA content by flow cytometry and VM-26 sensitivity in a 72-h growth inhibition assay. The hybrid lines all contained >2x DNA compared to unfused controls, indicating that the fusions were successful. The IC50 for VM-26 in 3 of the 4 lines was the same as that of the sensitive controls, ranging from 4.7 to 7.4 x 10^-8 M, and another was 76 x 10^-8 M. These data indicate that drug sensitivity was reconstituted by the hybridization procedure. By comparison, the VM-26 IC50 values in the CEM/VM-1 cells and CEM/VM-1 x CEM/VM-1 "fusions" were 360 and 750 x 10^-8 M, respectively. To determine whether a topoisomerase II-mediated function was reconstituted in the hybrids, we measured drug-stimulated DNA cleavage ("cleavable complex formation"). Using 32P-labeled pBR322 DNA as substrate with nuclear extracts from drug sensitive cells, 100 μM VM-26 maximally stimulated DNA cleavage by 11-fold compared to no-drug controls. By contrast, 100 μM VM-26 stimulated DNA cleavage by only 7-fold in extracts from CEM/VM-1 cells. Drug stimulation of cleavage obtained with extracts from a hybrid of VM-26-sensitive and -resistant cells that expressed the drug-sensitive phenotype were also similar to those of the sensitive cells: VM-26 stimulated activity by ~11-fold, indicating that the fusion process reconstituted a topoisomerase II-mediated function. We have previously shown that although topoisomerase II activity was decreased in at-MDR cells compared to controls, immunodetection of topoisomerase II in a Western blot assay revealed no differences in enzyme amount in 1.0 M NaCl nuclear extracts from these lines. Similarly, we found here that the hybrids also had about the same amount of immunoreactive topoisomerase II as in the unfused cells. Our data demonstrate that we have reconstituted topoisomerase II activity by the cell fusion protocol and are the first to show that VM-26 resistance in human tumor cells is expressed recessively.

INTRODUCTION

We have described (1, 2) a form of MDR3 in a human T-cell leukemia line, CEM/VM-1, that is associated with an alteration in the activity of DNA topoisomerase II (at-MDR; ref. 3). This line, which was selected for resistance to the epipodophyllotoxin, VM-26, appears to have features similar to those of a VP-16-resistant Chinese hamster ovary cell line (4), but differs from an L1210 cell line selected for VM-26 resistance (5). The MDR of our cells, which are cross-resistant to a number of drugs known to stabilize topoisomerase II-DNA complexes (6), including VP-16, anthracyclines, mitoxantrone, and 4'-9(acridinylamino)methanesulphon-m-anside (1, 2), is distinguished from the MDR associated with P-glycoprotein overexpression (Pgp-MDR) in the following ways: (a) lack of cross-resistance to the Vinca alkaloids (1); (b) absence of a drug accumulation defect (1); (c) relative insensitivity to modulation of resistance by verapamil or chloroquine (2); and (d) lack of overexpression of the mdr1 gene or of its product, Pgp (2).

Because the phenotype of our CEM/VM-1 cells suggested that alteration in topoisomerase II activity might be important in the expression of at-MDR, as has been suggested in studies with similar cell lines (4, 7-10), we extensively characterized the amount and activity of DNA-topoisomerase II in nuclear extracts from these cells (3). We found that there was little difference in the amount of immunoreactive topoisomerase II in 1.0 M NaCl extracts from both the CEM/VM-1 and CEM cells. However, compared to extracts from drug sensitive cells, we did observe decreases in catalytic activities (i.e., unknotting and rate of catenation) and a reduced effect of VM-26 in stimulating DNA cleavage by nuclear extracts from the CEM/VM-1 cells (3). Our data strongly support the hypothesis that the activity of topoisomerase II or a modulator of this enzyme is altered in the CEM/VM-1 cells. Based on these results, we now use the term "at-MDR" to distinguish this form of MDR associated with alterations in topoisomerase II activity from that form associated with overexpression of P-glycoprotein (Pgp-MDR).

In contrast to the many rodent and human Pgp-MDR cell lines that exist (reviewed in ref. 11), only six cell lines have been described that originally appeared to express a "pure" at-MDR phenotype (1, 2, 10, 12, 16), and of these, only three are human lines (1, 2, 10, 15, 16). Of these at-MDR lines, only two rodent lines, one selected for resistance to VP-16-213 (12) and the other selected for resistance to 9-hydroxy-ellipticine (14), have been characterized genetically to determine how this phenotype of MDR was expressed. The cost of publication of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 To whom requests for reprints should be addressed, at Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101.

3 The abbreviations used are: MDR, multidrug resistance (resistant); at-MDR, multidrug resistance (resistant) associated with alterations in topoisomerase II activity; VM-26 (teniposide), 4'-demethylepipodophyllotoxin 9-(4,6-O-2-thenylinde-β-D-glucopyranoside; Pgp, P-glycoprotein; PEG, polyethylene glycol; S/MEM, Eagle's minimal essential medium with modified Earle's salts for suspension culture; OHA-M, selective medium for hybrid cells, S/MEM with glutamine (1 mM), 10% fetal bovine serum, ouabain (50 nM), asparagine (60 μM), and hypoxanthine (100 μM).

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MODEL

We have presented a preliminary account of this work (21).

MATERIALS AND METHODS

Chemicals and Supplies. S/MEM, L-glutamine, and trypsin blue were purchased from Gibco (Life Technologies, Inc., Grand Island, NY); fetal bovine serum was from Hyclone Laboratories (Sterile-Systems, Inc., Logan, VT). Azaserine-hypoxanthine (50%), ouabain octahydrate, Triton X-100, and PEG (approximate molecular weight, 1000) were purchased from Sigma (St. Louis, MO). Phosphate buffered saline was prepared as described (22). Teniposide (VM-26) was a gift from Bristol-Meyers (Bristol- Meyers, Wallingford, CT). Propidium iodide monohydrate was from Calbiochem (La Jolla, CA) and dimethyl sulfoxide was from Fisher Scientific (Fairlawn, NJ). Superoiled (Form I) pBR322 DNA dimer, Klenow fragment of DNA polymerase I, and HindIII restriction enzyme were purchased from Bethesda Research Laboratories (Gaithersburg, MD). [α-32P]dATP was obtained from Dupont/NEN (Boston, MA) and Hoechst dye 33258 was purchased from Polysciences, Inc. (War- lington, PA).

Cells and Culture Conditions. We used the following cell lines: the drug-sensitive parental T-lymphoblastic line, CCRF-CEM; and OHA-M selective for hybrid cells. Established hybrid cell lines were Western blot analysis, as detailed elsewhere (3), using IJD3 antisera against DNA topoisoerases II, generically provided by Dr. Leroy Liu, Johns Hopkins University. We used an antitubulin antibody to control for protein loading and transfer.

DNA Cleavage Assays. These measurements of "cleavable complex formation" were done according to the method of Liu et al. (29), but using the reaction mixture for the P4 unknotting reaction (30) containing 1 mM ATP, and are detailed elsewhere (3). Dimethyl sulfoxide (1.5–3%) had no effect on cleavable complex formation.

RESULTS AND DISCUSSION

Selection and Characterization of Cell lines for Fusion. We chose a CEM subline, CEM.AG1.OU1.5, which is hypoxanthine-guanine phosphoribosyltransferase-negative and ouabain-resistant, for fusion with CEM/VM-1 to provide hybrids that could be selected by growth in appropriate selective medium. We first determined that the CEM.AG1.OU1.5 cell line was as sensitive to VM-26 as was the parent CEM line. We also confirmed that this line was essentially uninhibited by the ouabain component of the OHA-M selective medium, but was inhibited strongly (≥90%) by the azaserine/hypoxanthine component of OHA-M. Conversely, we found that the CEM and the CEM/VM-1 cell lines were only slightly inhibited (10–20%) by the azaserine/hypoxanthine combination, whereas ouabain inhibited the growth of these cell lines by ≥90%.

Fusion and Growth of Cells in Selective Medium (OHA-M). As described in the "Materials and Methods" section, we performed fusions of the following pairs of cell lines: CEM/VM-1 × CEM.AG1.OU1.5; CEM × CEM.AG1.OU1.5; CEM.AG1.OU1.5 × CEM.AG1.OU1.5; CEM × CEM; and CEM/VM-1 × CEM/VM-1, these last three pairs being control "fusions." 

Mock fusions in the absence of PEG were also done. At 2 weeks after fusion, we found that the majority of wells seeded with cells from the fusions of CEM.AG1.OU1.5 with either CEM/VM-1 or CEM had viable colonies. In contrast, none of the wells seeded with cells from the control fusion "fusions" containing only CEM.AG1.OU1.5 yielded viable colonies, indicating that CEM.AG1.OU1.5 cells or hybrids from the fusion of these cells with each other were eliminated. A few wells from the control fusions containing either CEM/VM-1 only or CEM/VM-1 only as well as from mock fusions did show growth in the selective medium. We attribute this to either the emergence of ouabain-resistant CEM or CEM/VM-1 mutants or, for CEM/VM-1, the ability of this line to grow slowly in the 50 mM ouabain present in the selective medium (data not shown).

Ten of these colonies growing in the 96-well plates were

Growth Inhibition Assays. The concentration of VM-26 that inhibited the growth of cells by 50% (IC50) in 72 h was determined as previously described (25). Duplicate plates were also counted at 96 h, with comparable results. Similar studies were done to evaluate growth inhibition of the parent cell lines by the selective components of the OHA-M medium, ouabain and azaserine/hypoxanthine.

DNA Content. Ten 106 cells in log phase growth were evaluated for relative DNA content by flow cytometry as described by Dow et al. (26).

Preparation of Nuclear Extracts and 32P-Labeling of DNA. Nuclear extracts were prepared according to the method of Sullivan et al. (27), but with modifications as detailed in Danks et al. (3). pBR322 DNA dimer was digested with HindIII under reaction conditions recommended by the manufacturer. The linear 4.3-kilobase reaction product was then labeled at the 3' end with [32P]dATP by the Klenow fragment of DNA polymerase I, as described by Maniatis et al. (28). Final DNA concentration was measured using Hoechst dye 33258 with a fluorometer (Model TK0100; Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions. The specific activity of the DNA was approximately 1 × 107 cpm/μg DNA.

Immunodetection of Topoisomerase II. Topoisomerase II was detected in 1.0 M NaCl extracts of nuclei from CEM, CEM/VM-1, and hybrid cell lines by Western blot analysis, as detailed elsewhere (3), using IJD3 antisera against DNA topoisoerases II, generically provided by Dr. Leroy Liu, Johns Hopkins University. We used an antitubulin antibody to control for protein loading and transfer.

DNA Cleavage Assays. These measurements of "cleavable complex formation" were done according to the method of Liu et al. (29), but using the reaction mixture for the P4 unknotting reaction (30) containing 1 mM ATP, and are detailed elsewhere (3). Dimethyl sulfoxide (1.5–3%) had no effect on cleavable complex formation.
selected at random and expanded in 24-well plates in selective medium. We then transferred cells at 3 weeks postfusion from randomly selected wells to flasks with standard medium without cloning. We did this because of the rapidity with which changes in phenotype, probably attributable to chromosomal segregation events, can occur in hybrid cells (31). Indeed, homogeneously staining regions may be particularly unstable in hybrids, with consequent rapid loss of amplified gene sequences (32).

DNA Content of Fusion-derived Cell Lines. Determinations by flow cytometry of the DNA content of the parent cells and of several fusion-derived cell lines were made at 6 and 9 weeks postfusion with comparable results. Representative DNA histograms are shown in Fig. 1 and quantitated in Table 1. These results indicate that the selected lines derived from the CEM.AG1.OU1.5 × CEM/VM-1 fusion were indeed hybrids, as they contained close to twice the DNA content of each parent line. The line (F2A-3) resulting from the CEM.AG1.OU1.5 × CEM fusion also appeared to be a hybrid, although it had somewhat less DNA content than the CEM.AG1.OU1.5 × CEM/VM-1 hybrids. The infrequent lines arising from the control fusions of either CEM or CEM/VM-1 with themselves (F4AB-4 and F5A-1) had DNA contents similar to that of the parent cells, suggesting that they represented the emergence of unfused cells that were capable of growing in the selective medium. No example of the control fusion (CEM.AG1.OU1.5 × CEM.AG1.OU1.5) is given, as all of these cells died in the selective medium, as expected.

Table 1 Relative DNA content and cytotoxicity of VM-26 in normal and hybrid cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative DNA content* (at 9-week postfusion)</th>
<th>IC50 of VM-26 (μM × 10^-8) at 7-week postfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>2.0</td>
<td>5.2</td>
</tr>
<tr>
<td>CEM.AG1.OU</td>
<td>2.0</td>
<td>5.7</td>
</tr>
<tr>
<td>CEM/VM-1</td>
<td>2.0</td>
<td>360.0</td>
</tr>
<tr>
<td>CEM/VM-1 × CEM.AG1.OU1.5 fusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4AB-4</td>
<td>3.3</td>
<td>76.0</td>
</tr>
<tr>
<td>F4A-2</td>
<td>3.5</td>
<td>5.8</td>
</tr>
<tr>
<td>F4A-4</td>
<td>3.2</td>
<td>4.7</td>
</tr>
<tr>
<td>F4A-5</td>
<td>3.6</td>
<td>7.4</td>
</tr>
<tr>
<td>CEM × CEM.AG1.OU1.5 fusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2A-3</td>
<td>2.9</td>
<td>4.4</td>
</tr>
<tr>
<td>CEM/VM-1 × CEM/VM-1 fusion*</td>
<td>1.8</td>
<td>750.0</td>
</tr>
<tr>
<td>F5A-1</td>
<td>1.8</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* DNA content was determined by flow cytometry of propidium iodide-treated cells (26). Relative DNA content was approximated by measuring the distance from the left-side start of the histogram to the center of the G1 peak.

VM-26 Sensitivity of Hybrid Cell Lines. The IC50 values for growth inhibition by VM-26 were determined 7 weeks postfusion (Table 1). Values obtained for the CEM and the CEM/VM-1 lines were comparable to those that we have reported previously (1, 2). Also, the VM-26 IC50 in the CEM.AG1.OU1.5 line was virtually identical to that of the parent CEM line. The cell lines from the two control fusions (F4AB-4 and F5A-1) yielded IC50 values that are consistent with the suggestion that they were derived from unfused cells capable of growing in the ouabain-containing selective medium. The similarity of the IC50 value of the CEM.AG1.OU1.5 × CEM hybrid (F2A-3) to that of the sensitive cells indicated that the fusion of two drug-sensitive cells did not result in any change in sensitivity to VM-26.

Of the four hybrids tested that were derived from fusion of the sensitive and resistant lines, three (F1A-2, F1A-4, F1A-5) had VM-26 IC50 values that were very close to that of the sensitive parent, ranging between 4.7 and 7.4 × 10^-8 μM. The fourth cell line, F1A-1, had a VM-26 IC50 value about 10 times higher (76 × 10^-8 μM), but it was still more sensitive to this drug than were the CEM/VM-1 cells.

We interpret the results of Table 1 to indicate that the VM-26 resistance of the CEM/VM-1 cell line is expressed in a recessive manner. The higher IC50 for line F1A-1 is clearly not a manifestation of a codominant expression of VM-26 resistance; if this were the case, the other three hybrid cell lines could not be as sensitive to VM-26 as the drug-sensitive CEM lines. While this higher IC50 value may simply result from experimental variation in the assay, it does not appear to be a consequence of emergence of unfused CEM/VM-1 cells, since there were no peaks in the DNA histogram that could be assigned to such a population. It is also possible that this IC50 results from chromosomal segregation events leading to reexpression of the initially extinguished resistant phenotype in a fraction of the cell population (31, 32).

Cleavage Activity of Nuclear Extracts. The stabilization of topoisomerase II-DNA complexes by topoisomerase II-directed drugs has been shown in several studies (33–36). We evaluated the effect of VM-26 on the stabilization of complexes between
pBR322 DNA and topoisomerase II using nuclear extracts from our cell lines. This drug-induced stabilization is seen as an increase in counts precipitated by potassium-sodium dodecyl sulfate and measures covalent binding of topoisomerase II and DNA (29). As seen in Fig. 2, extracts from the VM-26-sensitive cells stimulated DNA cleavage by $\approx 11$-fold in the presence of 100 $\mu$M VM-26. By contrast, VM-26 induced DNA breakage by nuclear extracts from the CEM/VM-1 cells by $\approx 7$-fold, compared to the no drug control. Whether this nearly twofold difference in cleavage activity of the extracts can account for the nearly 40-fold resistance of the cells is not known. Since cleavage is measured in nuclear extracts in vitro in a 30-min assay and resistance is measured in whole cells "in vivo" in a 72-h growth-inhibition assay, it would seem that direct quantitative comparison is not possible. Nevertheless, we have shown for two at-MDR cell lines that the amount of drug-stimulated cleavage mediated by nuclear extracts is inversely proportional to the resistance of the cells (3). When we examined the drug-stimulated cleavage of DNA by nuclear extracts prepared from the hybrid cell line F1A-5, which was derived from fusion of CEM/VM-1 × CEM.AG.OU1.5 cells, we found that the effect of 100 $\mu$M VM-26 was similar to that produced in extracts from the drug-sensitive cells, i.e., $\approx 11$-fold increase. Clearly, the nuclear extracts from the F1A-5 hybrid were reconstituted in this cleavage activity, consistent with expression of a drug-sensitive phenotype.

Immunodetection of Topoisomerase II. In light of these results, it was important to determine the level of topoisomerase II in the hybrid cells. Using an antitopoisomerase II antiserum kindly provided by Dr. Leroy Liu, we found that there was approximately the same amount of immunoreactive topoisomerase II in 1.0 M NaCl extracts from the CEM, CEM/VM-1, and CEM.AG.OU1.5 cells (Fig. 3). It can also be seen in Fig. 3 that the amount of topoisomerase II in the hybrid cell lines was the same as or slightly more than that in the parent cell lines; there is clearly no decrease in the amount of immunoreactive topoisomerase II in the hybrids. These results are in agreement with the previous data, and indicate that by reconstituting topoisomerase II and topoisomerase II-associated functions, we have also reconstituted sensitivity to VM-26.

Conclusions. We have shown through the construction of somatic cell hybrids of our at-MDR cells and drug sensitive cells that sensitivity to the growth-inhibitory effects of VM-26 can be restored. Consistent with this finding, VM-26 stimulation of DNA cleavage (i.e., the formation of cleavable complexes) by nuclear extracts from such hybrids can also be restored to levels seen in drug-sensitive cells. Taken together, our results support the hypothesis that at-MDR is expressed in a recessive manner and likely involves an alteration in the activity of either topoisomerase II or a modulator of this enzyme.

Only six other cell lines have been described that appear to exhibit an at-MDR phenotype, and of these, only two rodent lines have been characterized genetically. In these studies, hybrids produced by fusion of drug-sensitive and -resistant cells were shown to exhibit sensitivity to either VP-16 (12) or 9-hydroxyvellipicine (14), suggesting that these resistant phenotypes were expressed recessively. Since other studies have demonstrated that the resistance of these rodent lines likely involves an alteration in the activity of topoisomerase II (4, 8), it can be inferred that resistance associated with altered topoisomerase II activity is a recessive phenotype. Our results confirm these previous findings and extend them to human tumor cells. Indeed, ours is the first demonstration in human tumor cells that resistance to a drug that interacts with topoisomerase II is expressed recessively.

We have presented evidence elsewhere demonstrating that at-MDR is very different than Pgp-associated MDR (1, 2, 37), and is due to an alteration in both catalytic and cleavage activity of topoisomerase II (3). The results presented here extend this...
distinction to the mode by which these two different forms of MDR are expressed. Studies from the laboratories of Ling (17) and of Pastan (18) revealed clearly that Pgp-MDR is expressed in a dominant or codominant fashion, and other studies showing that such cells amplify and/or overexpress the mdrl gene provide a molecular basis for this phenotype (38). By contrast, the recessive nature of at-MDR is consistent with the observation that the activity of topoisomerase II is decreased in such cells (3, 33–36). However, since we have only characterized topoisomerase II-mediated activities in nuclear extracts of our cells, we do not know yet if the at-MDR phenotype is due to an alteration in the enzyme itself or a factor that modulates it. Studies are currently underway in our laboratory to address this issue.

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