Decreased Nuclear Matrix DNA Topoisomerase II in Human Leukemia Cells Resistant to VM-26 and m-AMSA†

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ABSTRACT: CEM leukemia cells selected for resistance to VM-26 (CEM/VM-1) are cross-resistant to various other DNA topoisomerase II inhibitors but not to *Vinca* alkaloids. Since DNA topoisomerase II is a major protein of the nuclear matrix, we asked if alterations in nuclear matrix topoisomerase II might be important in this form of multidrug resistance. Pretreatment of drug-sensitive CEM cells for 2 h with either 5 μM VM-26 or 3 μM m-AMSA reduced the specific activity of newly replicated DNA on the nuclear matrix by 75 and 50%, respectively, relative to that of the bulk DNA. However, neither VM-26 nor m-AMSA affected the relative specific activity of nascent DNA isolated from the nuclear matrices of drug-resistant CEM/VM-1 cells. The decatenating and unknotting activities of DNA topoisomerase II were 6- and 7-fold lower, respectively, in the nuclear matrix preparations from the CEM/VM-1 cells compared to parental CEM cells. Western blot analysis revealed that the amount of immunoreactive topoisomerase II in the nuclear matrices of the CEM/VM-1 cells was decreased 3.2-fold relative to that in CEM cells, but there was no significant difference in the amount of enzyme present in the nonmatrix (1.5 M salt soluble) fractions of nuclei from these cell lines. Increasing the NaCl concentration used in the matrix isolation procedure from 0.2 to 1.8 M resulted in a progressive decrease in the specific activity of topoisomerase II in matrices of CEM/VM-1 but not CEM cells, which suggested that the association of the enzyme with the matrix is altered in the resistant cells. These data support the hypothesis that resistance to VM-26 and m-AMSA is directly related to the decreased activity of nuclear matrix topoisomerase II. In CEM/VM-1 cells the interaction of either VM-26 or m-AMSA with nuclear matrix topoisomerase II is specifically diminished.

Selection of human tumor cells for resistance to one natural product drug frequently results in resistance to other natural product drugs that are dissimilar in structure and apparent mechanism of cytotoxicity. This type of resistance occurs in vitro and in vivo [reviewed in Beck (1987), Endicott and Ling (1989) and van der Bliek and Borst (1989)] and is termed MDR.[1] For example, cross-resistance to the epipodophyllotoxins and DNA intercalating agents has been demonstrated in cells selected for resistance to the *Vinca* alkaloids (Beck, 1984). The most extensively characterized form of MDR (Pgp-MDR) is associated with decreased steady-state drug levels and increased expression of an integral plasma membrane glycoprotein, P-glycoprotein, which is responsible for the phenotype (Bradley et al., 1988; Pastan & Gottesman, 1988).

More recently, an at-MDR phenotype has been described. Like Pgp-MDR cells, at-MDR cells are cross-resistant to drugs that interfere with DNA topoisomerase II activity (Danks et al., 1987; Beck et al., 1987). Unlike Pgp-MDR cell lines, "pure" at-MDR cell lines do not show marked alterations in either sensitivity to the *Vinca* alkaloids, Pgp content, or drug accumulation (Danks et al., 1987; Beck et al., 1987). The only feature common to all cell lines that express the at-MDR phenotype is a reduction in drug-induced DNA "cleavage" or "breakage" (Pommier et al., 1986; Yalowich et al., 1987; Danks et al., 1988; Sinha et al., 1988; Zwelling et al., 1988; McGrath et al., 1989; Spiridonidis et al., 1989). These DNA cleavage and DNA breakage assays may be interpreted as measuring reductions in drug-stabilized topoisomerase II–DNA covalent binding. In less than half of the at-MDR lines reported, this reduction has been associated with a decrease in either amount or activity of salt-extractable topoisomerase II (Charcosset et al., 1988; Danks et al., 1988. Therefore, it is not clear that alterations in salt-soluble (nonmatrix) topoisomerase II completely describe the mechanism of at-MDR.

Evidence continues to accumulate suggesting that in proliferating cells the functional DNA topoisomerase II is that which is incorporated into the salt-insoluble nuclear matrix. DNA topoisomerase II is a major protein of the *Drosophila* nuclear matrix (Berrios et al., 1985) and is enriched at the sites of attachment of chromatin loops to the nuclear matrix (Gasser & Laemmli, 1986). At these sites, DNA topoisomerase II may be involved in the attachment of DNA loops to the nuclear matrix. This enzyme may also function enzy-

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[1] Abbreviations: MDR, multidrug resistance; Pgp-MDR, multidrug resistance associated with increased expression of P-glycoprotein and decreased steady-state drug levels; at-MDR, multidrug resistance associated with altered DNA topoisomerase II; VM-26, teniposide [4′-demethyl-9-[(4,6-*O*-(2-thenylidene-β-D-glucopyranosyl)oxy]epipodophyllotoxin]; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; CEM/VM-1, cloned CCRF-CEM subline about 50- and 15-fold resistant to VM-26 and m-AMSA, respectively; PMSF, phenylmethanesulfonyl fluoride; kDNA, kinetoplast DNA isolated from mitochondria of *Crithidia fasiculata*; dThD, (2′-deoxyribosyl)thymine; SDS, sodium dodecyl sulfate.

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matically on the nuclear matrix by regulating the DNA superhelicity that would be generated by RNA transcription along the matrix-bound DNA loops (Wu et al., 1988). In addition, matrix-bound DNA topoisomerase II activity is required for the segregation of intertwined sister chromatids during mitosis (DiNardo et al., 1984; Holm et al., 1985).

Previous studies provided evidence that the antiproliferative effects of the DNA topoisomerase II inhibitors VM-26 and m-AMSA are localized on the nuclear matrix of CEM leukemia cells (Fernandes et al., 1988). Furthermore, work from one of our laboratories (Danks et al., 1988, 1989) with the VM-26-resistant cell lines (CEM/VM-1 and CEM/VM-1-5) revealed alterations in the catalytic activity but not in the amount of 1.0 M salt extractable nuclear DNA topoisomerase II. These observations raised the following questions: Is the DNA topoisomerase II in the nuclear matrix of CEM/VM-1 cells altered in either amount or activity? If so, are these alterations associated with a decreased interaction of VM-26 and m-AMSA with matrix-bound DNA topoisomerase II in resistant cells? In this paper we address these questions and relate our findings to a common mechanism of at-MDR.

Materials and Methods

Cell Culture. Crithidia fasiculata cultures were a generous gift of Dr. Warren Ross of the University of Florida School of Medicine. The trypanosomes were propagated at 27 °C in 37 g/L brain heart infusion medium (Difco Laboratories, Detroit, MI) supplemented with 20 μg of hemin/mL, 100000 units of penicillin/L, and 100 mg of streptomycin/L. The human CCRF-CEM leukemic cell line and its at-MDR subline (CEM/VM-1) were propagated as previously described (Beck et al., 1979; Danks et al., 1987). CEM/VM-1 cells were grown in the absence of drugs, and resistance to VM-26 was stable under these conditions for at least 12 months. Cells were found to be free of mycoplasma contamination when tested with the Gen-Probe mycoplasma ribosomal RNA hybridization kit (Fisher Scientific Co., Raleigh, NC).

Chemicals and Reagents. Rabbit antiserum IID3 prepared against the 3′ end of recombinant mammalian DNA topoisomerase II was a generous gift from Dr. Leroy F. Liu of the Johns Hopkins University (Baltimore, MD). A monoclonal antibody to α tubulin was obtained from Accurate Chemical Co. (Westbury, NY). VM-26 was provided by Dr. William T. Bradner of the Bristol Myers Co. (Wallingford, CT). m-AMSA was obtained from the National Cancer Institute (Bethesda, MD). [*methyl*-^{3}H]dThd and [2-^{14}C]dThd with specific radioactivities of 7–20 and 0.056 Ci/mmol, respectively, were purchased from ICN Radiochemicals (Irvine, CA). RNase-free pancreatic DNase I with a specific activity of 2200 Kunitz units/mg of protein was purchased from Worthington Biochemical Corp. (Freehold, NJ). Low-salt buffer consisted of 10 mM Tris-HCl, pH 7, 1 mM $MgCl_2$, 10 mM NaCl, and 1 mM PMSF. High-salt buffer consisted of 10 mM Tris-HCl, pH 7, 0.6 mM $MgCl_2$, 1.5 M NaCl, and 1 mM PMSF.

Isolation of the Nuclear Matrix. The standard procedure for nuclear matrix isolation involved 1.5 M NaCl and DNase I treatment of purified nuclei as detailed previously (Fernandes et al., 1988). In the experiment that examined the effects of increasing salt concentrations on matrix topoisomerase II activity and protein content, the concentration of NaCl used in the matrix isolation procedure varied between 0.2 and 1.8 M.

Preparation of DNA Substrates. [^{3}H]kDNA was prepared by incubating cultures of *C. fasiculata* with 3.0 mCi of [*methyl*-^{3}H]dThd (50 Ci/mmol) per milliliter of culture medium. After 72 h the cells were harvested at a density of 2.5×10^8/mL, and the [^{3}H]kDNA was isolated following cesium chloride density gradient centrifugation as previously described (Englund, 1978). The final preparation has a specific radioactivity of 4.86×10^4 dpm/μg of DNA. P4 knotted phage DNA was prepared according to the method of Liu and Davis (1981) as modified by Danks et al. (1988).

Assays of DNA Topoisomerase II Activity. Nuclear matrices were prepared from logarithmically growing CEM or CEM/VM-1 cells as described previously (Fernandes et al., 1988). The matrices were washed once with high-salt buffer and once with low-salt buffer at 4 °C and then resuspended in 50 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), and 1 mM dithiothreitol. Immediately before assay the samples were sonicated with three 5-s bursts at 15 W from a Branson 200 sonifier equipped with a microtip. The [^{3}H]kDNA decatenation assay developed by Sahai and Kaplan (1986) was modified and used to measure DNA topoisomerase II activity quantitatively. The reactions were carried out for 30 min at 37 °C in the presence of 0–2 mM ATP, 50 mM Tris-HCl, pH 7.9, 150 mM KCl, 10 mM $MgCl_2$, 1.5 mM dithiothreitol, 4.9 μg of [^{3}H]kDNA, and 0–25 μg of matrix protein in a final volume of 40 μL. Reactions were terminated by the addition of 5 μL of 4.5% SDS (w/v). Following centrifugation of the samples at 16600*g* for 5 min, 20-μL aliquots of the supernatant were removed for either counting of radioactivity or for electrophoretic analysis (Fernandes et al., 1988). After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/mL) and photographed under UV illumination. Protein concentrations were determined by the method of Bradford (1976).

The unknotting activity of DNA topoisomerase II was measured under the conditions described by Liu et al. (1981) using DNA prepared from phage P4 capsids as a substrate. The reaction mixtures contained 0–4 μg of nuclear matrix protein, prepared as described above. Religation of the nicked DNA intermediates produced in the P4 unknotting assay was carried out with T4 DNA ligase (International Biotechnologies, Inc., New Haven, CT) according to the manufacturer's instructions.

Western Blotting. The matrix and nonmatrix fractions of the nuclei were prepared as described above. The fractions were boiled in electrophoresis buffer, and Western blotting was carried out as described by Danks et al. (1988). The specific reaction conditions were as follows: various amounts of nuclear matrix protein (75–300 μg) or nonmatrix protein (45–120 μg) were loaded on a 5–15% SDS–polyacrylamide gel (Laemmli, 1970). After electrophoresis and transfer of the proteins to nitrocellulose paper, immunoblotting was done with IID3 antiserum to DNA topoisomerase II (1:1000 dilution). The relative amounts of topoisomerase II seen on Western blots of matrix and nonmatrix preparations of CEM and CEM/VM-1 cells were quantitated with a Visage 110 scanner (BioImage, Ann Arbor, MI). The program driving the scanner digitized specified regions of a positive film image of the Western blot and computed the area and image intensity of bands within the given region. Image intensity was calibrated against internal computer standards and is expressed as arbitrary units relative to these standards. The topoisomerase II antibody revealed bands of 170 and 160 kDa from both the nuclear matrix and nonmatrix preparations of the CEM and CEM/VM-1 cells. The amount of immunoreactive topoisomerase II = ($\text{area}_{170\text{kDa}} \times$ intensity) + ($\text{area}_{160\text{kDa}} \times$ intensity). As a check on the accuracy of this method for quantitating topoisomerase II, Western blotting was carried out following the loading of various amounts of nuclear protein

on the electrophoresis gels. The products of the area and band intensities calculated after reaction with antisera to topoisomerase II or a monoclonal antibody to α tubulin were linear with respect to the amounts of protein loaded on the gels. Statistical analysis was carried out with the two-tailed t test.

Results

Effects of VM-26 and m-AMSA on the Association of Nascent DNA with the Nuclear Matrix of CEM and CEM/VM-1 Cells. DNA synthesis is thought to take place in the nuclear matrix, since this subnuclear structure is enriched in newly replicated DNA compared to the nonmatrix fraction of the nucleus. The appearance of newly replicated DNA in the nonmatrix fraction results from the migration of the newly synthesized matrix DNA away from the matrix-bound replication sites into the nonmatrix DNA (Pardoll et al., 1980; Vogelstein et al., 1980; Berezney & Buchholtz, 1981; Fernandes et al., 1988). Therefore, even though the newly synthesized DNA can be detected in both matrix and nonmatrix fractions of nuclei, it is the replication of DNA on the nuclear matrix that is specifically inhibited by VM-26 and m-AMSA (Fernandes et al., 1988). For these reasons, it was of interest to compare the effects of these drugs on newly synthesized DNA associated with the nuclear matrix fractions of drug-sensitive CEM cells and drug-resistant CEM/VM-1 cells. The enrichment of newly synthesized DNA in the matrix fraction was demonstrated by first prelabeling the cells for 72 h (about three doubling times) with [^{14}C]dThD in order to uniformly label the total DNA. The cells were then pulsed for 45 s with [^{3}H]dThD to label only the newly synthesized DNA. Nuclear matrices were prepared that contained $\simeq$5% of the total nuclear DNA. The specific activity of each nuclear fraction (matrix or nonmatrix) is defined as the ratio of dpm of newly synthesized DNA to dpm of total DNA.

Table I shows that while newly synthesized DNA was associated with both the matrix and nonmatrix fractions, the specific activity of nuclear matrix DNA of CEM cells was 4.3-fold higher than that of the nonmatrix DNA (6.4 ^{3}H dpm vs 1.5 ^{14}C dpm) (relative specific activity therefore equals 4.3). Similarly, the relative specific activity of nuclear matrix DNA from untreated CEM/VM-1 cells was 4.1 (4.9 ^{3}H dpm in matrix/1.2 ^{14}C dpm in nonmatrix). After a 2-h treatment with 5 μM VM-26, however, the relative specific activity of nuclear matrix preparations from CEM cells was decreased 4.3-fold (relative specific activity of 4.3 for untreated cells versus relative specific activity of 1.0 for VM-26-treated cells). Thus, VM-26 reduced the relative specific activity of the matrix fraction 4.3-fold more than that of the nonmatrix fraction. Table I also shows that m-AMSA, like VM-26, selectively decreased the amount of nascent DNA associated with the matrix fraction of CEM cells (2.2 relative specific activity). In contrast to the results from the drug-sensitive CEM cells, the relative specific activity of the drug-resistant cells was unaffected by either VM-26 or m-AMSA. Preparations from untreated, VM-26-treated, or m-AMSA-treated CEM/VM-1 cells all had similar relative specific activities (4.1, 4.0, and 4.0, respectively). Therefore, these drugs did not preferentially inhibit the association of newly synthesized DNA with the nuclear matrices of resistant cells.

The lack of a preferential effect of either VM-26 or m-AMSA on nuclear matrix DNA synthesis in CEM/VM-1 cells was not due to diminished drug uptake, since CEM/VM-1 cells accumulate at least as much VM-26 and m-AMSA at steady state as CEM cells (Danks et al., 1987; W. T. Beck and M. C. Cirtain, unpublished results; D. J. Fernandes, unpublished results). Instead, the above results provided evidence

Table I: Effects of VM-26 and m-AMSA on the Association of Nascent DNA with the Nuclear Matrices of CEM and CEM/VM-1 Cells[a]

cell line	total DNA (^{14}C dpm)	newly synthesized DNA (^{3}H dpm)	sp act. ^{3}H/^{14}C	sp act. relative[b,c]
CEM (untreated)				
matrix	1 702	10 851	6.4	4.3 ± 0.9
nonmatrix	51 177	77 276	1.5	
CEM (5 μM VM-26)				
matrix	2 104	312	0.1	1.0 ± 0.7[d]
nonmatrix	63 330	6 230	0.1	
CEM (3 μM m-AMSA)				
matrix	1 375	1 721	1.3	2.2 ± 0.4[d]
nonmatrix	29 353	18 507	0.6	
CEM/VM-1 (untreated)				
matrix	2 214	10 834	4.9	4.1 ± 1.0
nonmatrix	53 890	62 677	1.2	
CEM/VM-1 (5 μM VM-26)				
matrix	1 394	2 845	2.0	4.0 ± 0.9
nonmatrix	65 133	33 333	0.5	
CEM/VM-1 (3 μM m-AMSA)				
matrix	1 377	7 137	5.2	4.0 ± 0.6
nonmatrix	47 282	61 181	1.3	

[a] Logarithmically growing CEM and CEM/VM-1 cells were prelabeled for 72 h with [^{14}C]dThD and then incubated with either no drug, 5 μM VM-26, or 3 μM m-AMSA for 2 h. The cells were then pulse labeled for 45 s with [^{3}H]dThd after which the matrix and nonmatrix fractions of the nuclei were isolated. The pellets (nuclear matrices) were washed once with 1.5 M NaCl buffer and once with low-salt buffer. The matrix and nonmatrix fractions were precipitated in ice-cold 10% trichloroacetic acid. The RNA was solubilized in 0.5 N KOH and the DNA extracted in hot 5% trichloroacetic acid. Aliquots of the DNA extracts were removed for counting of the ^{3}H and ^{14}C labels. [b] Ratio of the specific activity of DNA in the nuclear matrix fraction to the specific activity in the nonmatrix fraction. [c] Mean ± SD (n = 4). [d] $p < 0.01$ compared to the relative specific activity of the matrix DNA from untreated CEM cells (two-tailed t test).

that VM-26 and m-AMSA interact with DNA topoisomerase II in the nuclear matrix of sensitive CEM cells and that this interaction with topoisomerase II in matrices of CEM/VM-1 cells is considerably diminished.

Comparisons of Topoisomerase II Activities in the Nuclear Matrices of CEM and CEM/VM-1 Cells. It was important to determine if the decreased interaction of VM-26 and m-AMSA with nuclear matrix topoisomerase II of CEM/VM-1 cells was related to alterations in the activity of the matrix-bound enzyme. Since the activity and content of topoisomerase II in various drug-sensitive cell lines were shown to be proliferation dependent (Heck & Earnshaw, 1986; Nelson et al., 1987; Sullivan et al., 1987), all measurements of topoisomerase II were done with nuclear matrix and nonmatrix preparations from cells in exponential growth. Two assays were used to measure the catalytic activity of DNA topoisomerase II: the P4 DNA unknotting assay and the [^{3}H]kDNA decatenating assay. The P4 unknotting assay measures the ability of type II topoisomerases to unknot the knotted DNA of P4 phage and is specific for topoisomerase II even in crude cell extracts that contain excess topoisomerase I activity (Liu et al., 1981). Topoisomerase II activity is visualized on agarose gels after electrophoresis as the formation of bands of nicked and unknotted DNA that migrate more slowly than the smear of the knotted DNA substrate. Although this assay is not strictly quantitative, it is possible to compare the relative activities of DNA topoisomerase II in drug-sensitive and -resistant cells. Figure 1, lane 4, shows the amounts of nicked and unknotted

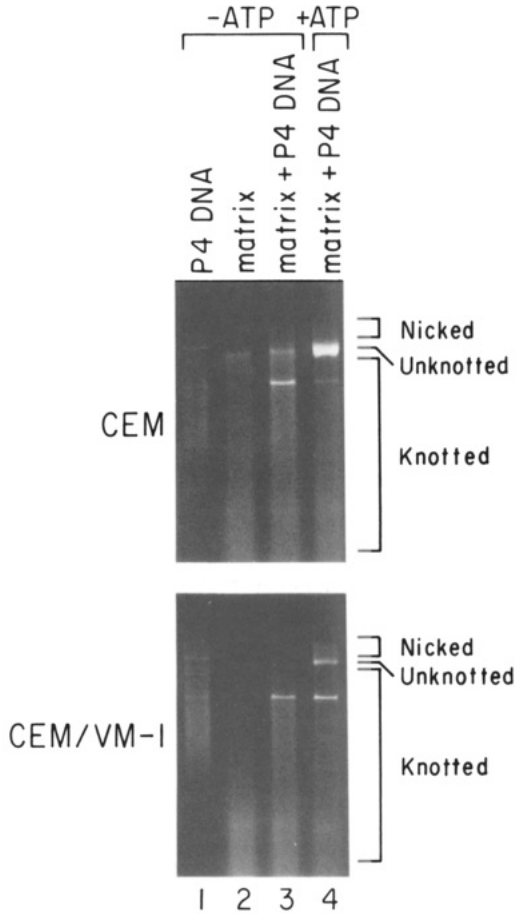

FIGURE 1: P4 unknotting activities of nuclear matrices from CEM and CEM/VM-1 cells. Nuclear matrices were prepared as described under Materials and Methods. The assays were carried out for 30 min at 37 °C. Lane 1, 0.75 μg of P4 DNA; lane 2, 4 μg of nuclear matrix protein from either CEM cells (top) or CEM/VM-1 cells (bottom); lane 3, 4 μg of matrix protein plus 0.75 μg of P4 DNA. Lanes 1–3 contained no ATP. The complete reaction mixture (lane 4) contained 4 μg of either CEM or CEM/VM-1 matrix protein, 0.75 μg of P4 DNA, and 1 mM ATP. The samples were treated with proteinase K and electrophoresed on a 0.7% agarose gel, and the DNA was visualized by ethidium bromide staining. The relative amounts of unknotted DNA in the complete reaction mixtures were quantitated with a Visage 110 scanner.

P4 DNA products that were formed with 4 μg of nuclear matrix protein from either CEM (top) or CEM/VM-1 (bottom) cells. In both CEM and CEM/VM-1 cells unknotting activity was ATP dependent, since only in the presence of ATP (lane 4) was the amount of unknotted DNA increased over the various controls (lanes 1–3). Analysis of the unknotted DNA bands with a Visage 110 scanner revealed that at equal amounts of matrix protein (4 μg) unknotting activity was 7.1-fold higher in the nuclear matrix preparation of CEM cells compared to CEM/VM-1 cells. Some nicked P4 DNA was also observed when nuclear matrices of drug-resistant cells were incubated with P4 knotted DNA and ATP (Figure 1, lane 4). That this product was nicked DNA was verified by further incubating the reaction products of the unknotting assay with 400 units of T4 ligase for 18 h. T4 ligase catalyzed the religation of the nicked DNA into closed circular, partially unknotted DNA (data not shown). These results are consistent with those of others (Zechiedrich et al., 1989) who identified nicked DNA as a reaction product of eukaryotic topoisomerase II.

DNA topoisomerase II activities in nuclear matrix preparations of VM-26-sensitive and -resistant cells were also assayed quantitatively by measuring the rate of decatenation of [^{3}H]kDNA. Reaction of DNA topoisomerase II with the catenated networks of [^{3}H]kDNA results in the release of double-stranded [^{3}H]DNA minicircles, which remain in the supernatant after centrifugation of the reaction products (Sahai & Kaplan, 1986). As shown in Figure 2, the amount of tritium in the supernatant (decatenating activity) was increased by nuclear matrix preparations of both CEM and CEM/VM-1 cells and was linear with respect to the amount of protein assayed. However, the ATP-dependent decatenating activity catalyzed by DNA topoisomerase II was considerably greater in nuclear matrices of CEM cells compared to CEM/VM-1 matrices (Figure 2). The ATP-dependent decatenating activity present in CEM nuclear matrices was also sensitive to inhibition by VM-26 (data not shown). Table II shows that the average decatenating activity of DNA topoisomerase II, determined from three separate experiments, was about 6-fold lower in nuclear matrix preparations of CEM/VM-1 cells than in CEM cells. This was comparable to the approximately 7-fold decrease in unknotting activity of nuclear matrices from drug-resistant cells (Figure 1).

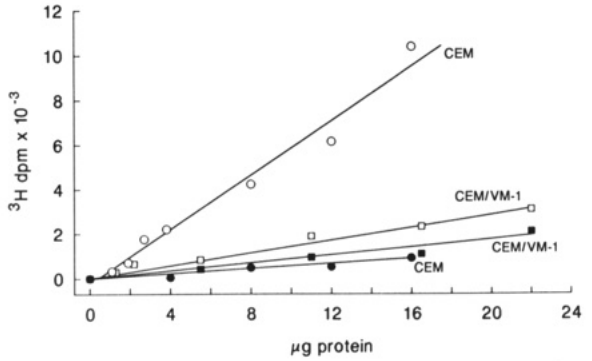

FIGURE 2: Decatenating activities of nuclear matrix preparations of CEM and CEM/VM-1 cells. Nuclear matrices were prepared as described under Materials and Methods. The matrices were sonicated and then incubated with 4.9 μg of [^{3}H]DNA for 30 min at 37 °C in the presence and absence of 2 mM ATP. The samples were centrifuged, and the radioactivity in the supernatant was counted: (○) CEM (+ATP); (□) CEM/VM-1 (+ATP); (●) CEM (−ATP); (■) CEM/VM-1 (−ATP).

Table II: DNA Topoisomerase II Activity in Nuclear Matrices of CEM and CEM/VM-1 Cells[a]

	units of topoisomerase activity/mg of protein			
cell line	expt 1	expt 2	expt 3	mean ± SD
CEM	7.1	6.7	5.0	6.3 ± 1.1
CEM/VM-1	1.2	1.6	0.6	1.1 ± 0.5[b]

[a] Nuclear matrices were isolated from 2 × 10^7 CEM or CEM/VM-1 cells and then washed with 1.5 M high-salt buffer and low-salt buffer. The nuclear matrices were sonicated, and DNA topoisomerase II activity was assayed by measuring the rate of decatenation of [^{3}H]-kDNA. One unit of DNA topoisomerase II activity is defined as the quantity of enzyme required to catalyze the formation of 1 μg of soluble product/min. [b] $p < 0.01$ compared to the mean specific activity of DNA topoisomerase II from the nuclear matrix of CEM cells.

Comparison of the Amounts of Immunoreactive Topoisomerase II in the Nuclear Matrix and Nonmatrix Fractions of CEM and CEM/VM-1 Cells. It was not possible to accurately measure topoisomerase II activity in the nonmatrix fractions of nuclei because of the high concentrations of salt (1.5 M NaCl) and DNase I in these fractions as a result of nuclear matrix preparation. However, we were able to determine the amounts of immunoreactive topoisomerase II in both the nuclear matrix and nonmatrix fractions by immu-

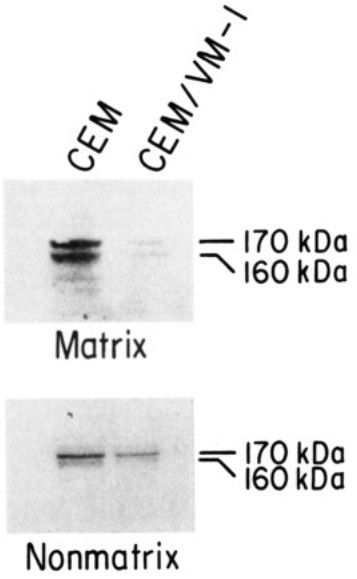

FIGURE 3: Western blots of DNA topoisomerase II in matrix and nonmatrix fractions of nuclear preparations from CEM and CEM/VM-1 cells. Equal amounts of matrix and nonmatrix protein (75 μg) from each cell line were separated on a 5–15% SDS–polyacrylamide gel and the immunoblots done as described under Materials and Methods. This figure is a representative blot of five different matrix and eight different nonmatrix preparations.

noblotting. Figure 3 is a representative Western blot that was obtained following electrophoresis of 75 μg of either matrix or nonmatrix protein from each cell line. The topoisomerase II antibody revealed bands of 170 and 160 kDa from both the nuclear matrix and nonmatrix preparations of the CEM and CEM/VM-1 cells. The 160-kDa band likely represents a partially degraded form of topoisomerase II, since its intensity increased at the expense of the 170-kDa band and as a function of the number of times the preparation was subjected to freeze–thawing. Immunoblots with native rabbit serum were negative (data not shown). It can be seen in this figure that topoisomerase II was significantly decreased in the nuclear matrix but not in the nonmatrix fraction of drug-resistant CEM/VM-1 cells. Quantitation of the relative amounts of immunoreactive topoisomerase II in 5 immunoblots of 5 different matrix preparations and 16 immunoblots of 8 different nonmatrix preparations was obtained by scanning the film positives of the Western blots with the Visage 110 scanner. The amount of topoisomerase II was (3.2 ± 0.8)-fold higher in the nuclear matrix preparations from CEM cells compared to the drug-resistant CEM/VM-1 cells ($p < 0.005$). In contrast, only a (1.2 ± 0.3)-fold difference ($p = 0.05$) was detected in the amount of salt-soluble (nonmatrix) enzyme from the CEM versus CEM/VM-1 nuclei. This is in agreement with our previous observation with 1.0 M salt extractable topoisomerase II (Danks et al., 1988).

Effect of Ionic Strength on Nuclear Matrix Topoisomerase II Activity. The data obtained from immunoblotting indicated that the nuclear matrix fraction of topoisomerase II was selectively decreased in drug-resistant CEM/VM-1 cells. In addition, results from one of our laboratories (Danks et al., 1988, 1989) provided evidence that salt-soluble topoisomerase II from CEM/VM-1 cells is altered in catalytic activity, VM-26-stimulated formation of DNA–protein complexes, and interaction with ATP compared to the parental CEM cells. Collectively, these findings suggested that the decreased amount and catalytic activity of topoisomerase II in nuclear matrices of CEM/VM-1 cells were related to an alteration in the enzyme, which impaired its incorporation into the nuclear matrix. To test this possibility, nuclear matrices were isolated from drug-sensitive and -resistant cells by DNase I treatment and extraction of nuclei with various concentrations of NaCl from 0.2 to 1.8 M. Low-salt matrices prepared from CEM and CEM/VM-1 cells with 0.2 M NaCl had similar levels of topoisomerase II activity (Figure 4A). However, nuclear matrices isolated from CEM/VM-1 cells with increasing concentrations of NaCl from 0.2 to 1.0 M had progressively lower specific activities of topoisomerase II. NaCl at concentrations up to 1.8 M did not change the specific activity of topoisomerase II in nuclear matrices of drug-sensitive CEM cells. Unlike the decrease seen in matrix topoisomerase II activity of resistant cells, no differences were observed between CEM and CEM/VM-1 cells in total matrix protein content at any salt concentration (Figure 4B). Taken together, the above data are consistent with the concept that in CEM/VM-1 cells a mutation in topoisomerase II resulted in a decreased association of the enzyme with the nuclear matrix. An alternative postulate is that the high-salt extraction of nuclei during the matrix isolation procedure induced aggregation of topoisomerase II with matrices of parental CEM cells to a greater extent than with matrices of CEM/VM-1 cells. The results shown in Figure 4A are not consistent with this proposal, since the specific activity of topoisomerase II was not significantly different in matrices from CEM cells prepared with salt concentrations between 0.2 and 1.8 M.

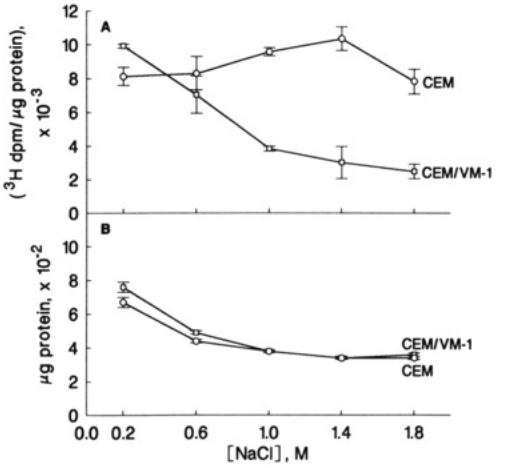

FIGURE 4: (A) Topoisomerase II activities in nuclear matrices prepared with varying salt concentrations. Nuclear matrices were prepared by DNase I digestion of nuclei followed by extraction with increasing concentrations of NaCl between 0.2 and 1.8 M. The matrices were then washed with low-salt buffer. Topoisomerase II decatenating activity was assayed in the presence of 2 mM ATP as described in the legend to Figure 2. (B) Total protein content of nuclear matrices obtained from 2×10^7 cells according to the above procedure. Values are the means ± SD: (○) CEM; (□) CEM/VM-1.

Discussion

The results presented herein are the first demonstration that the at-MDR phenotype is associated with a selective decrease in the interaction of either VM-26 or m-AMSA with nuclear matrix topoisomerase II. Other cell lines resistant to topoisomerase II active drugs have been described (Glisson et al., 1986; Pommier et al., 1986; Per et al., 1987; Yalowich et al., 1987; Danks et al., 1988; Sinha et al., 1988; Zwelling et al., 1988; McGrath et al., 1989; Deffie et al., 1989; Harker et al., 1989; Spiridonidis et al., 1989). Some of these cell lines appear to have a "pure" at-MDR phenotype, i.e., show a reduction in drug-stimulated DNA damage without changes in steady-state drug levels (Danks et al., 1987; Beck et al., 1987; Yalowich et al., 1987; McGrath et al., 1989). Although reductions

in drug-induced DNA damage in these cell lines suggested that some alteration(s) in DNA topoisomerase II were important in the expression of at-MDR, the molecular mechanism responsible for this phenotype has not been defined.

Previous work from one of our laboratories indicated that the cytotoxic target for VM-26 and m-AMSA is the DNA topoisomerase II that is tightly bound to the nuclear matrix (Fernandes et al., 1988). The present study extends these observations by providing evidence that in the at-MDR cell line CEM/VM-1 the interaction of VM-26 and m-AMSA with nuclear matrix topoisomerase II is selectively decreased. We showed that in intact CEM cells, but not in drug-resistant CEM/VM-1 cells, VM-26 and m-AMSA preferentially decreased the association of newly replicated DNA with the nuclear matrix. The effect was specific for topoisomerase II active drugs and was not seen with either cytosine arabinoside or hydroxyurea (Fernandes et al., 1988). These results are consistent with those of others who demonstrated that DNA topoisomerase II is enriched at the attachment sites of chromatin loops to the nuclear matrix (Gasser & Laemmli, 1986) and is preferentially associated with newly replicated DNA (Nelson et al., 1986; Woynarowski et al., 1988). If the functional DNA topoisomerase II is tightly bound to the nuclear matrix, then one would observe a higher frequency of strand breaks in newly replicated matrix DNA compared to bulk DNA of VM-26- and m-AMSA-sensitive cells (Chiu et al., 1989). The preferential induction of strand breaks in matrix DNA would likely account for the decreased association of nascent DNA with the nuclear matrix that we observed after treatment of intact CEM cells with either VM-26 or m-AMSA. By contrast, in the drug-resistant CEM/VM-1 cells we found a decreased amount and activity of matrix topoisomerase II and no preferential effect of either VM-26 or m-AMSA on the association of newly replicated DNA with the nuclear matrix. The decreased topoisomerase II in the nuclear matrix of CEM/VM-1 cells would likely result in fewer strand breaks in nuclear matrix DNA and decreased cytotoxicity after VM-26 or m-AMSA treatment. The selectivity of the depletion of nuclear matrix topoisomerase II in at-MDR cells is further supported by our observation that no difference between drug-sensitive and -resistant cells in the amount of immunoreactive salt-soluble (nonmatrix) topoisomerase II was found [this study and Danks et al. (1988)]. Preliminary studies indicate that this decrease in matrix topoisomerase II content and activity also occurs in an HL-60 line resistant to mitoxantrone (D. J. Fernandes and G. W. Harker, unpublished experiments).

The significant reduction in topoisomerase II activity and the amount of immunoreactive enzyme in nuclear matrices of drug-resistant cells is, nevertheless, still compatable with cell viability and growth. Earnshaw et al. (1985) and Heck and Earnshaw (1986) have provided evidence that topoisomerase II is present in mitotic scaffolds of transformed cells at levels in excess of that required for enzymatic regulation of DNA loop topology. It was also reported that human KB cells, which are cross-resistant to topoisomerase II active drugs, have increased topoisomerase I activity (Fergusen et al., 1988). They suggested that the enhanced activity of the type I enzyme may partially compensate for the decreased amount of topoisomerase II in the resistant KB cell line. Thus, it seems reasonable that at-MDR cells may still be able to proliferate at about the same rate as parental cells despite a significant reduction in the amount of nuclear matrix topoisomerase II.

Several lines of evidence support the concept that the decreased content and catalytic activity of topoisomerase II in nuclear matrices of at-MDR CEM/VM-1 cells are directly related to a mutation in the enzyme. The specific activity of topoisomerase II in nuclear matrices of CEM/VM-1 cells decreased in a linear fashion when the NaCl concentration in the incubation buffer was raised from 0.2 to 1.0 M. In contrast, the specific activity of topoisomerase II was not changed when nuclear matrices of drug-sensitive cells were incubated with concentrations of NaCl as high as 1.8 M. This concept is further supported by our recent findings that the salt-soluble topoisomerase II activity of CEM/VM-1 cells is altered in its ATP requirement, catalytic activity, and ability to form DNA–protein complexes with VM-26 (Danks et al., 1988, 1989).

Zwelling et al. (1989) have studied an at-MDR cell line (HL-60/AMSA) that is resistant to DNA intercalating agents but only minimally cross-resistant to the epipodophyllotoxins. This cell line likely contains a drug-resistant form of DNA topoisomerase II. However, many at-MDR cell lines are resistant to both classes of anticancer agents (Pommier et al., 1986; Charcosset et al., 1988; Danks et al., 1988; Spiridonides et al., 1989). The results described herein have led us to propose a common mechanism of simultaneous resistance to the intercalating agents and the epipodophyllotoxins. A plausible mechanism would be that a mutation in topoisomerase II alters its incorporation into or its association with the nuclear matrix. This would lead to a decreased interaction of the topoisomerase II active drug with the matrix-bound enzyme and fewer strand breaks in newly replicated DNA. The decreased content and activity of nuclear matrix topoisomerase II could account for the cross-resistance to various topoisomerase II active drugs with chemically diverse structures.

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