Formation of Topoisomerase IIα Complexes with Nascent DNA Is Related to VM-26-Induced Cytotoxicity†

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ABSTRACT: Several clinically active anticancer drugs are known to interfere with DNA topoisomerase II activity. However, the importance of the individual α (170 kDa) and *β* (180 kDa) isozymes as targets of topoisomerase II-active drugs is not clear. To address this question, human CCRF-CEM leukemia cells were incubated with bromodeoxyuridine, and either the nascent DNA or bulk DNA not undergoing replication was purified by immunoprecipitation with an anti-bromodeoxyuridine antibody. The topoisomerase II isozymes that coprecipitated with either the nascent DNA or bulk DNA were analyzed by Western blotting. The α isozyme formed complexes with nascent DNA in cells pretreated with either VM-26 or mitoxantrone, while the *β* isozyme was only bound to bulk DNA. At moderately cytotoxic concentrations, VM-26 enhanced the binding of topoisomerase IIα to nascent DNA at least 5.2-fold compared to bulk DNA. However, in VM-26 resistant CEM/VM-1 cells incubated with equitoxic concentrations of VM-26, topoisomerase IIα complex formation with nascent DNA was decreased at least 5.5-fold compared to bulk DNA. Drug-induced binding of topoisomerase II*β* with bulk DNA in CEM/VM-1 cells did not correlate with cytotoxicity. Collectively, these results indicate that the formation of VM-26 stabilized complexes of topoisomerase IIα with nascent DNA are critical to the development of cytotoxicity, and that resistance of CEM/VM-1 cells to VM-26 is related to impaired formation of these complexes. The results also provide indirect evidence that topoisomerase IIα is involved in DNA replication.

Mammalian type II DNA topoisomerases are enzymes that modify the degree of DNA supercoiling within the cell (Wang, 1985; Maxwell & Gellert, 1986). They catalyze these topological changes by forming complexes with DNA, passing the double-stranded DNA helix through a transient double-strand break, and then resealing the strand break. Two isozymes of topoisomerase II with apparent molecular masses of 170 kDa (α) (Miller et al., 1981; Tsai-Pflugfelder et al., 1988) and 180 kDa (*β*) (Chung et al., 1989; Jenkins et al., 1992; Austin et al., 1993) have been identified. The α and *β* isozymes are encoded by two distinct genes that are located on chromosomes 17 and 3, respectively (Tan et al., 1992). The protein sequences of the two isozymes show 70% homology in the N-terminal domain, but considerable divergence is found in the C-terminus (Jenkins et al., 1992; Austin et al., 1993). Some immunohistochemical studies revealed that the α isozyme was primarily localized in the nucleoplasm, while the *β* isozyme was found mainly in the nucleolus (Coutts et al., 1993; Zini et al., 1994). The results obtained concerning the isozyme distribution in various nuclear subfractions were consistent with these observations. Only the α isozyme was present in the nuclear matrix which lacked nucleoli, while the high salt-soluble (nonmatrix) fraction of the nucleus contained both the *β* and α isozymes in CEM[1] cells (Fernandes et al., 1990; Danks et al., 1994). The nuclear matrix is thought to be the subnuclear site of DNA replication in eukaryotic cells (Pardoll et al., 1980; Berezney, 1984; Nakayasu & Berezney, 1989; Paff & Fernandes, 1990; Vaughn et al., 1990). In addition, the expression of the α isozyme, but not the *β* isozyme, is proliferation dependent (Woessner et al., 1991; Kimura et al., 1994). Thus, these observations also suggest that the α isozyme is involved in DNA replication.

Type II DNA topoisomerases are targets for some important anticancer drugs such as VM-26, VP-16, *m*-AMSA, and mitoxantrone (Liu, 1989; Smith et al., 1990). These drugs stabilize an intermediate in the topoisomerase II catalytic mechanism. As a result, topoisomerase-catalyzed DNA cleavage occurs, but religation of the cleaved DNA by the enzyme is inhibited (Tewey et al., 1984; Liu, 1989; Osheroff, 1989). Drake et al. (1989) showed that the catalytic activities of both topoisomerase II α and *β* were inhibited by VM-26 *in vitro*. Other studies have demonstrated that VM-26 stabilized complexes of both the α and *β* isozymes with DNA in human leukemia cells (Danks et al., 1994). However, the extent to which DNA complex formation with either isozyme contributed to cell death induced by VM-26 has not been determined.

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[1] Abbreviations: CEM, human CCRF-CEM lymphoblastic leukemia cells; CEM/VM-1, cloned subline of CEM cells about 50- and 15-fold resistant to VM-26 and mitoxantrone, respectively; VM-26, teniposide [4′-demethyl-9-[(4,6-*O*-(2-thenylidene-*β*-D-glucopyranosyl)oxy]epipodophyllotoxin]; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; mitoxantrone, 1,4-dihydroxy-5,8-bis ({2-[(2-hydroxyethyl)-amino]ethyl}amino)-9,10-anthracenedione; MTT, [3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazolium bromide]; BrdUrd, bromo-2′-deoxyuridine; dThd, 2′-deoxythymidine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Evidence continues to accumulate that the formation of topoisomerase II−DNA complexes in the proximity of DNA replication forks may be responsible for the cytotoxic effects of certain topoisomerase II-targeted agents in proliferating cells (Holm et al., 1989; D'Arpa et al., 1990; Froelich-Ammon & Osheroff, 1995). VM-26 stabilized-topoisomerase II−DNA complexes were preferentially formed on nascent DNA relative to bulk DNA (Nelson et al., 1986a; Woynarowiski et al., 1988). Results from our laboratory indicated that arrest of DNA replication fork movement through the c-myc gene occurred specifically at sites of VM-26-induced DNA cleavage (Catapano et al., 1995). Also consistent with this concept was the observation that topoisomerase II was decreased in the nuclear matrix of CEM/VM-1 cells that were resistant to multiple topoisomerase II-active agents (Fernandes et al., 1990). To address the importance of topoisomerase II α and β as drug targets, we identified the isozymes that form complexes with replicating and nonreplicating DNA in CEM cells incubated with VM-26 and mitoxantrone. The results obtained were related to the degrees of cytotoxicity induced by these agents.

MATERIALS AND METHODS

Cell Culture. Human CEM and CEM/VM-1 leukemia cell lines were provided by Drs. Mary Danks and William Beck of the St. Jude Children's Research Hospital (Memphis, TN). The cells were propagated at 37 °C under 95% air−5% CO_2 in E-MEM medium supplemented with 10% fetal bovine serum, penicillin (20 000 units/L), and streptomycin (20 mg/L).

Chemicals and Reagents. VM-26 was a gift of Bristol Myers Co. (Syracuse, NY), and mitoxantrone was a gift of Dr. John L. Nitiss of St. Jude Children's Research Hospital (Memphis, TN). The rabbit antiserum raised against a M_r 70 000 COOH-terminal peptide of recombinant HeLa topoisomerase IIα was a gift of Dr. Daniel M. Sullivan of the University of South Florida (Tampa, FL). The rabbit antiserum directed against a C-terminal peptide consisting of residues 1512−1530 of human topoisomerase IIβ was a gift of Dr. Frits Boege of the University of Würzburg, Germany. A mouse anti-BrdUrd monoclonal antibody was purchased from Becton Dickinson (San Jose, CA). Immobilized protein A (with 6% beaded agarose) and rabbit anti-Mouse IgG (H+L) chains were purchased from Pierce (Rockford, IL). Centricon-100 concentrators were purchased from Amicon (Beverly, MA). [*methyl*-^{3}H]dThd, [2-^{14}C]-dThd, and [^{3}H]BrdUrd, at specific radioactivities of 65.4 Ci/mmol, 50 mCi/mmol, and 16.9 Ci/mmol, respectively, were obtained from Moravek Biochemicals (Brea, CA). Pepstatin A, aprotinin, PMSF, benzamidine, leupeptin, soybean trypsin inhibitor, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Sources for most other chemicals and supplies were reported previously (Fernandes et al., 1990). Protein concentrations were determined using the BCA assay from Pierce (Rockford, IL) as outlined by the manufacturer. Protease inhibitor solution contained 1 mM benzamidine, 1 mM PMSF, 10 μg/mL soybean trypsin inhibitor, 50 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 μg/mL pepstatin. Nuclei extraction buffer consisted of 10 mM Tris-HCl, pH 7.0, and 2 mM $MgCl_2$ in protease inhibitor solution. Nuclei lysis buffer consisted of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS in protease inhibitor solution. DNase I digestion buffer consisted of 10 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$. TE buffer consisted of 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Isolation of Nascent DNA. Exponentially growing CEM cells (2×10^7 cells per group) were incubated at 37 °C for 72 h with 0.05 μCi/mL of [^{14}C]dThd to prelabel parental DNA. The cells were grown in radioactive free-medium for 24 h and then labeled with 50 μM [^{3}H]BrdUrd (0.5 μCi/mL) for 10 min to label nascent DNA. [^{3}H]BrdUrd labeling and all subsequent steps were performed in subdued natural light to limit photodamage to BrdUrd-substituted DNA. The cells were resuspended in fresh medium at a density of 2×10^7 cells/mL in nuclei extraction buffer. The cells were allowed to swell on ice for 5 min and were then disrupted with five strokes in a Dounce homogenizer. The lysates were layered over a solution of 45% (w/v) sucrose at 4 °C, and the nuclei were purified by centrifugation at 1900*g* for 30 min at 4 °C. Nuclei were then resuspended in nuclei lysis buffer containing 1% SDS, and the lysates were sonicated for 45 s at an intensity of 4 with a Cole Parmer 4710 ultrasonic homogenizer equipped with a microtip. The sonicates were diluted with 5 vol of TE buffer. To remove the unincorporated [^{3}H]BrdUrd and reduce the concentration of SDS in the samples, the sonicates were centrifuged through an Centricon-100 concentrator (100 000 dalton cutoff) at 1000*g*, 12 °C, for 50 min. The retentates were resuspended in 2 mL of TE buffer and subjected to a second Centricon-100 concentration. The recovery of nascent [^{3}H]-DNA and bulk [^{14}C]DNA averaged 80 ± 6% SD and 86 ± 4% SD, respectively. The samples were diluted in 1 mL of TE buffer, and NaOH at a final concentration of 0.2 N was added for 1 h at 4 °C to obtain single-stranded DNA. The samples were neutralized to pH 7.4 with HCl prior to the addition of mouse anti-BrdUrd antibody at a concentration of 5 μg/2×10^7 nuclei. Immunoreactions were carried out for 2 h at 4 °C. Rabbit anti-mouse IgG (15 μg) was added, and the samples were incubated at 4 °C for 45 min. The immunocomplexes were precipitated by addition of protein A immobilized to 6% beaded agarose. The degree of purification of nascent [^{3}H]DNA was determined by counting the ^{3}H and ^{14}C in the immunoprecipitates and supernatant fractions.

Immunoprecipitation of Nascent and Bulk DNA Containing Bound Topoisomerase II. Groups of 2×10^7 CEM or CEM/VM-1 cells were labeled with 50 μM [^{3}H]BrdUrd (0.5 μCi/mL) for 10 min. A second group of cells was labeled with 50 μM [^{3}H]BrdUrd (0.5 μCi/mL) for 10 min and then resuspended in BrdUrd-free medium for 2 h to chase the BrdUrd into bulk DNA. The cells were then incubated for 30 min with either no drug or various concentrations of VM-26 or mitoxantrone, which stabilized topoisomerase II−DNA complex formation and reduced further incorporation of residual BrdUrd into DNA to about 10% of the untreated control. Purification of BrdUrd-labeled nascent and bulk DNA by immunoprecipitation was carried out as described above. The immunoprecipitates were washed twice with TE buffer at room temperature, and the DNA−protein complexes were solubilized by heating at 90 °C for 8 min. The solubilized immunoprecipitates were concentrated by ultrafiltration as described above and then incubated in DNase I digestion buffer with 125 units of DNase I for 20 min at 37 °C. This allowed the topoisomerase II isozymes that were bound to BrdUrd−DNA to enter the 8% SDS−PAGE gel (Zwelling et al., 1989). Before the samples were loaded on

Table 1: Purification of Newly Replicated DNA by Immunoprecipitation[a]

fraction	[^{3}H]BrdUrd (dpm)	[^{14}C]dThd (dpm)	[^{3}H]/[^{14}C]	fold purification
immunoprecipitate (nascent DNA)	10 036 ± 1 384	155 ± 46	64.3	54
supernatant	13 808 ± 1 963	11 568 ± 6 438	1.2	1

[a] CEM cells were incubated with [^{14}C]dThd for 72 h and [^{3}H]BrdUrd for 10 min to label bulk and newly replicated DNA, respectively. The immunoprecipitation of newly replicated DNA from the cell lysates was carried out with an anti-BrdUrd antibody as described in Materials and Methods. The results are the means ± SD of four separate experiments.

the gel, 10 μL aliquots of each sample were removed for liquid scintillation counting in order to determine the relative amount of [^{3}H]BrdUrd-labeled DNA in each sample. Aliquots of each sample containing an equal amount of [^{3}H]-BrdUrd−DNA were loaded in each lane of the gel. Immunoblotting was carried out as described below.

Western Blotting. The proteins were transferred to a nitrocellulose membrane using a semidry transfer apparatus (Amicon) at 180 mA for 1 h. When necessary the gels were cut in segments to allow immunoblotting with both the topoisomerase II α and β specific antibodies. Immunoblotting was carried out using 1:5000 and 1:2500 dilutions of the topoisomerase II α and β antibodies, respectively. The immunoblots were digitized with a digital camera and analyzed with Gel-Pro Analyzer computer software (Media Cybernetics, Silver Spring, MD). The amounts of immunoreactive topoisomerase II isozyme was calculated as the products of the band intensity and the band area and were expressed in arbitrary units as defined by internal computer standards. The products of the areas and the topoisomerase II band intensities were linear with the respect to the amounts of protein loaded on the gel.

MTT Assay. Exponentially growing CEM and CEM/VM-1 cells were incubated with 0.1% Me_2SO/ethanol or with 1, 5, 10, 25, 50, 100, or 200 μM VM-26 in 0.1% Me_2SO/ethanol for 30 min. The cells were then washed three times with E-MEM medium, and 4×10^4 cells were dispensed into a 96-well microtiter plate. Six wells were used for each experimental condition. After a 24 h incubation at 37 °C, 100 μL of MTT solution was added to each well, and the plate was incubated at 37 °C for an additional 4 h. The plates were then centrifuged for 10 min at 500*g*. After the supernatant was removed, the formazan crystals were dissolved with 100 μL of DMSO. The absorbance of each sample was measured with a kinetic microplate reader at 450 nm. The percentage of cell survival was defined as (mean absorbance of treated wells/mean absorbance of untreated wells) × 100.

RESULTS

Purification of Nascent DNA by Immunoprecipitation. A major goal of these studies was to identify the isozymes of topoisomerase II that form complexes with DNA either at or away from the replication fork following incubation of cells with topoisomerase II-active agents. The experimental strategy was to selectively immunoprecipitate either the nascent or bulk DNA containing covalently bound topoisomerase II. Prior to initiating this work, it was necessary to evaluate the degree of purification of nascent or bulk DNA that was achieved by the immunoprecipitation method. CEM cells were incubated for 72 h with [^{14}C]dThd and then grown in radioactive-free medium for 24 h (one doubling time) to chase the radiolabel into parental DNA. The cells were then pulse-labeled with [^{3}H]BrdUrd for 10 min to label nascent DNA prior to immunoprecipitation. In addition, by resuspending a second aliquot of cells in a fresh medium and chasing the BrdUrd into high molecular weight (bulk) DNA for 2 h, it was also possible to purify the bulk DNA away from replication fork. We have previously shown that in CEM cells essentially all of the nascent DNA had migrated away from replication sites on the nuclear matrix into the bulk DNA within 2 h after a pulse−chase with [^{3}H]dThd (Fernandes et al., 1988). Thus, the only difference between nascent and bulk DNA was the distance of the DNA sequences from the replication fork. The degree of purification of the nascent DNA was determined by comparing the ratio of [^{3}H]BrdUrd/[^{14}C]dThd in the immunoprecipitate (nascent DNA) and the supernatant (mixture of parental [^{14}C]-dThd DNA and nascent [^{3}H]BrdUrd−DNA not recovered in the immunoprecipitate) fractions (Table 1). A relatively high degree of purification of nascent DNA was achieved, since this ratio was about 50-fold higher in the nascent DNA than in the supernatant fraction. Equally important, the nascent DNA fraction was essentially free of parental [^{14}C]-DNA, which allowed an accurate estimation of the amount of the topoisomerase II isozymes bound to nascent DNA in subsequent experiments. When this procedure was applied to the purification of bulk [^{3}H]BrdUrd−DNA, a 32- and 41-fold purification of bulk DNA was obtained in two separate experiments.

Binding of the α and β Isozymes of Topoisomerase II to Nascent and Bulk DNA in Cells Treated with Either VM-26 or Mitoxantrone. The formation of drug-stabilized topoisomerase II−DNA complexes protects the unstable β isoform from degradation (Danks et al., 1994). Thus, by incubating CEM cells with either VM-26 or mitoxantrone and using the above immunoprecipitation procedure to isolate the nascent or bulk BrdUrd−DNA, it was possible to identify the isozyme of topoisomerase II that was covalently bound either near or away from the DNA replication fork.

The topoisomerase II isozymes bound to nascent and bulk DNA in cells treated with or without 25 μM VM-26 were isolated by immunoprecipitation and identified by Western blotting (Figure 1). Each lane of the electrophoresis gel contained an equal amount of [^{3}H]BrdUrd-labeled DNA. No binding of either topoisomerase IIα or topoisomerase IIβ to either nascent or bulk DNA was detected in untreated control cells. In cells incubated with 25 μM VM-26, topoisomerase IIα formed stable complexes with both nascent and bulk DNA (lanes 3 and 4). VM-26 did not stabilize binding of topoisomerase IIβ to nascent DNA (lane 7). However, topoisomerase IIβ became associated with the [^{3}H]BrdUrd−DNA only after the nascent DNA was chased into the bulk DNA of the VM-26 treated cells (lane 8).

Incubation of cells with 8 μM mitoxantrone yielded results similar to those obtained with 25 μM VM-26 (Figure 2).

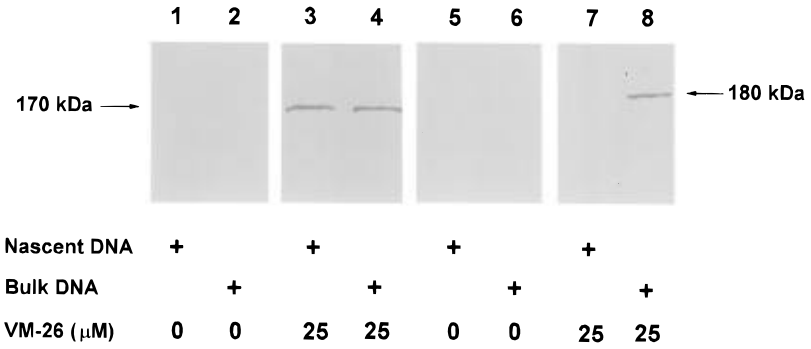

FIGURE 1: Binding of topoisomerase II isozymes to nascent and bulk DNA in CEM cells incubated with VM-26. CEM cells were incubated with [^{3}H]BrdUrd for 10 min to label newly replicated DNA or incubated with [^{3}H]BrdUrd for 10 min and then resuspended in [^{3}H]BrdUrd-free medium for 2 h to chase the [^{3}H]BrdUrd into bulk DNA. The cells were then incubated with or without 25 μM VM-26 for 30 min. [^{3}H]BrdUrd-labeled DNA was purified by immunoprecipitation with an anti-BrdUrd antibody. Samples containing equal amounts of [^{3}H]-BrdUrd−DNA were digested with DNase I, loaded on an 8% SDS−PAGE gel, and then analyzed by Western blotting. Lanes 1−4 were probed with the topoisomerase IIα specific antibody, while lanes 5−8 were probed with the topoisomerase IIβ specific antibody.

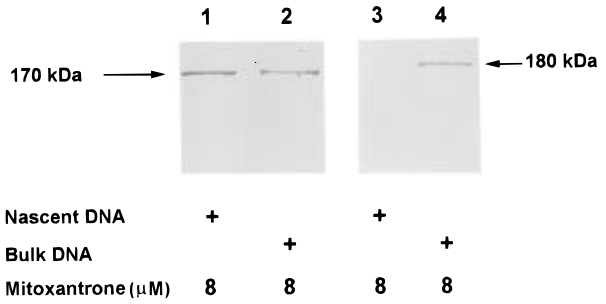

FIGURE 2: Binding of topoisomerase II isozymes to nascent and bulk DNA in CEM Cells incubated with mitoxantrone. Nascent and bulk DNA of CEM cells were labeled as described in the legend to Figure 1. The cells were then incubated with 8 μM mitoxantrone to stabilize the topoisomerase II−DNA complexes. The binding of topoisomerase II isozymes to either nascent or bulk DNA was analyzed as described in the legend to Figure 1. Each lane contained an equal amount of [^{3}H]BrdUrd−DNA. Lanes 1 and 2 were probed with the topoisomerase IIα specific antibody, while lanes 3 and 4 were probed with the topoisomerase IIβ specific antibody.

Like VM-26, mitoxantrone stabilized binding of only the α isozyme of topoisomerase II to nascent DNA (compared lanes 1 and 3) and both isozymes to bulk DNA (lanes 2 and 4).

VM-26 Preferentially Stabilizes Binding of Topoisomerase IIα to Nascent DNA Compared to Bulk DNA of CEM Cells. Previous studies in our laboratory (Fernandes et al., 1988; Catapano et al., 1995) and those of others (Nelson et al., 1986a; Woynarowiski et al., 1988; Holm et al., 1989; D'Arpa et al., 1990) provide evidence that topoisomerase II−DNA complexes formed at or near DNA replication forks are important to the cytotoxic effects of topoisomerase II-active drugs. The results shown in Figures 1 and 2 identify topoisomerase IIα as the isozyme that participates in drug-induced complex formation with newly replicated DNA. However, these results also indicate that VM-26 at 25 μM stabilized binding of topoisomerase IIα to both newly replicated and bulk DNA to a similar extent. Incubation of CEM cells with 25 μM VM-26 for 30 min reduced cell viability to about 30% of the untreated control (Figure 3), and resulted in nearly maximal complex formation of topoisomerase IIα and IIβ with total DNA (Danks et al., 1994). Thus, it was possible that preferential binding of topoisomerase IIα to either nascent or bulk DNA occurred at concentrations of VM-26 lower than 25 μM. To address this question, CEM cells were incubated for 30 min with

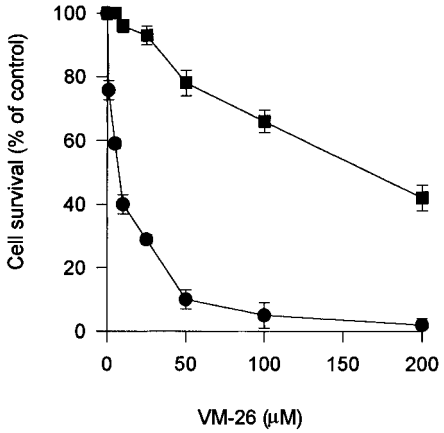

FIGURE 3: Effects of VM-26 on the viability of CEM and CEM/VM-1 cells. CEM or CEM/VM-1 cells were incubated with and without various concentrations of VM-26 for 30 min. The cells were then washed three times with drug-free medium and the effects of VM-26 on the viability of CEM cells (●) and CEM/VM-1 cells (■) was measured with the MTT assay as described in Material and Methods. Each point represents the mean of six determinations ± SD.

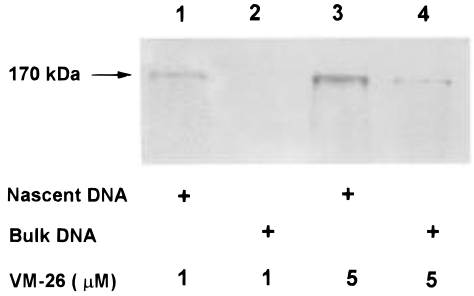

FIGURE 4: Preferential binding of topoisomerase IIα to nascent DNA in CEM cells incubated with VM-26. Nascent and bulk DNA of CEM cells were labeled as described in the legend to Figure 1. The cells were then incubated with either 1 or 5 μM VM-26 to stabilize the topoisomerase IIα−DNA complexes. The binding of topoisomerase IIα to either nascent or bulk DNA was analyzed as described in the legend to Figure 1. Each lane contained an equal amount of [^{3}H]BrdUrd−DNA. Lanes 1−4 were probed with the topoisomerase IIα specific antibody.

either 1 or 5 μM VM-26, which reduced cell viability to 75% and 60% of the untreated control, respectively. Binding of topoisomerase IIα to nascent, but not to bulk DNA, was readily detectable in cells incubated with 1 μM VM-26 (Figure 4, compare lanes 1 and 2). Scanning and computer

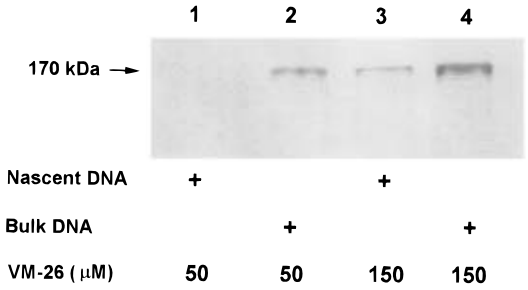

FIGURE 5: Decreased binding of topoisomerase IIα to nascent DNA in CEM/VM-1 cells incubated with VM-26. Nascent and bulk DNA of CEM/VM-1 cells were labeled as described in the legend to Figure 1. The cells were then incubated with either 50 or 150 μM VM-26 to stabilize the topoisomerase IIα−DNA complexes. The binding of topoisomerase IIα to either nascent or bulk DNA was analyzed as described in the legend to Figure 1. Each lane contained an equal amount of [^{3}H]BrdUrd−DNA. Lanes 1−4 were probed with the topoisomerase IIα specific antibody.

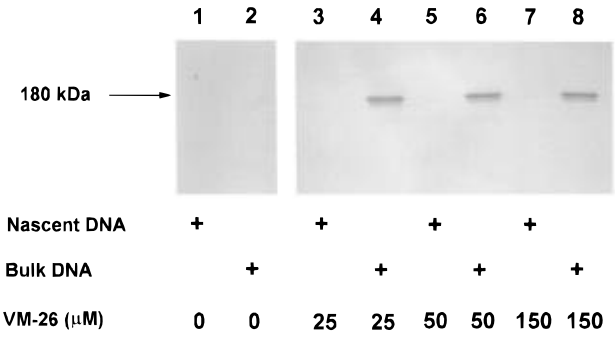

FIGURE 6: Binding of topoisomerase IIβ to bulk DNA in CEM/VM-1 cells incubated with VM-26. Nascent and bulk DNA of CEM/VM-1 cells were labeled as described in the legend to Figure 1. The cells were then incubated with either 0, 25, 50, or 150 μM VM-26 to stabilize the topoisomerase IIβ−DNA complexes. The binding of topoisomerase IIβ to either nascent or bulk DNA was analyzed as described in the legend to Figure 1. Each lane contained an equal amount of [^{3}H]BrdUrd−DNA. Lanes 1−8 were probed with the topoisomerase IIβ specific antibody.

analysis of lanes 3 and 4 of Figure 4 indicated that the binding of topoisomerase IIα was 5.2-fold greater to nascent DNA compared to bulk DNA in cells incubated with 5 μM VM-26. When the experiment was repeated, a 4.8-fold greater binding of topoisomerase IIα to nascent DNA relative to bulk DNA was observed. These results suggest that the formation of topoisomerase IIα complexes with nascent DNA was related to the cytotoxicity induced by VM-26.

Resistance of CEM/VM-1 Cells Is Related to Impaired Formation of Topoisomerase IIα Complexes with Nascent DNA. To further address the importance of topoisomerase IIα complex formation with nascent DNA to VM-26-induced cytotoxicity, the above experiment was repeated using CEM/VM-1 cells that are resistant to multiple topoisomerase II-active agents (Danks et al., 1987). Resistance of these cells to VM-26, mitoxantrone, *m*-AMSA, etc. is related to decreased drug-induced DNA cleavage, which is thought to be the result of mutations in topoisomerase IIα (Bugg et al., 1991; Danks et al., 1993) that impair its binding to DNA (Danks et al., 1989) and association with the nuclear matrix (Fernandes et al., 1990). Figure 3 shows that CEM/VM-1 cells were about 58-fold resistant to VM-26 compared to parental CEM cells. If the formation of topoisomerase IIα complexes with nascent DNA is critical to VM-26-induced cytotoxicity, then the formation of these complexes should be selectively impaired in VM-26 resistant CEM/VM-1 cells. To address this question we incubated CEM/VM-1 cells for 30 min with 50 or 150 μM VM-26, which reduced cell viability to 80% and 55% of control, respectively (Figure 3). The reduction in cell viability induced in CEM/VM-1 cells by these concentrations of VM-26 were similar in degree to that induced by 1 and 5 μM VM-26 in parental CEM cells (Figure 3). A comparison of Figures 4 and 5 reveals striking differences between CEM and CEM/VM-1 cells in VM-26-induced DNA complex formation. While the binding of topoisomerase IIα was at least 5.2-fold greater to nascent DNA compared to bulk DNA in VM-26 treated CEM cells (Figure 4), binding of the α isozyme was at least 5.5-fold less to nascent DNA than to bulk DNA in CEM/VM-1 cells incubated with equitoxic concentrations of VM-26 (Figure 5, compare lanes 3 and 4). The results indicate that the formation of topoisomerase IIα complexes with nascent DNA in CEM cells is related to VM-26-induced cytotoxicity. In addition, resistance of CEM/VM-1 cells to VM-26 is associated with impaired formation of these complexes with nascent DNA. At high concentrations (greater than 50 μM), VM-26 can kill CEM/VM-1 cells by inducing the formation of topoisomerase IIα complexes with bulk and possibly nascent DNA.

The formation of complexes of topoisomerase IIβ with nascent and bulk DNA was also examined in CEM/VM-1 cells incubated with either 0, 25, 50, or 150 μM VM-26 for 30 min (Figure 6). In the absence of VM-26, topoisomerase IIβ complex formation with either nascent or bulk DNA was not seen (lanes 1 and 2). Also, no binding of topoisomerase IIβ to nascent DNA was detected in CEM/VM-1 cells incubated with 25−150 μM VM-26 (lanes 3, 5, and 7). Although VM-26 at all of these concentrations stabilized binding of topoisomerase IIβ to bulk DNA of CEM/VM-1 cells (lanes 4, 6, and 8), a dose-response relationship was not observed between the degree of cytotoxicity produced (Figure 3) and the extent of drug-induced binding of the β isozyme to bulk DNA. The amounts of topoisomerase IIβ bound to bulk DNA in cells incubated with either a noncytotoxic concentration (25 μM) or cytotoxic concentrations (50 and 150 μM) of VM-26 were not significantly different; i.e., the relative amounts of DNA-bound topoisomerase IIβ determined by computer analysis of samples from CEM/VM-1 cells incubated with 50 and 150 μM VM-26 were 105% and 103%, respectively, of that observed at 25 μM VM-26. Therefore, resistance of CEM/VM-1 cells to VM-26 does not appear to be directly related to alterations in the drug-induced binding of topoisomerase IIβ to either nascent or bulk DNA.

DISCUSSION

In the studies described herein drug-stabilized topoisomerase II-nascent DNA complexes were purified, and the topoisomerase II isozymes bound to nascent and bulk DNA were identified with isozyme specific antibodies. The results obtained provide the first direct evidence that VM-26 stabilized complexes of topoisomerase IIα with nascent DNA are closely related to the cytotoxic effect of the drug. Muller et al. (1988) reported that topoisomerase II-induced DNA cleavage sites in plasmid DNA were similar in the presence and absence of VM-26. Since VM-26 does not appear to induce new DNA cleavage sites, the formation of complexes

of only topoisomerase IIα with nascent DNA also provides indirect evidence that topoisomerase IIα is involved in DNA replication. A second interesting observation was that VM-26 and mitoxantrone-stabilized binding of topoisomerase II*β* only to bulk DNA not undergoing replication. The formation of these complexes appear to be unrelated to drug cytotoxicity. It is clear that the VM-26 or mitoxantrone induced binding of topoisomerase II*β* only to bulk DNA was not the result of differences in the DNA sequences between the nascent and bulk DNA. The bulk [^{3}H]BrdUrd−DNA sequences that contained bound topoisomerase II*β* were previously the nascent [^{3}H]BrdUrd−DNA sequences that were chased into the bulk DNA. Thus, if the primary DNA sequence was the only determinant of drug-induced binding of topoisomerase II*β* to DNA, then complex formation of topoisomerase II*β* with both bulk and nascent DNA would have been observed.

Consistent with the finding that only topoisomerase IIα forms drug-stabilized complexes with nascent DNA is the observation that only the α isozyme is present in the nuclear matrix of CEM cells and forms VM-26-stabilized complexes with nuclear matrix DNA (Danks et al., 1994). The nuclear matrix is the subnuclear site of DNA replication in CEM cells (Fernandes et al., 1988; Paff & Fernandes, 1990) as well as in many other types of cells (Pardoll et al.,1980; Nakayasu & Berezney, 1989). Nuclear matrices are highly enriched in DNA replication fork intermediates (Paff & Fernandes, 1990) and some of the enzymes involved in replication (Collins & Chu, 1987; Tubo & Berezney, 1987; Paff and Fernandes, 1990; Fernandes et al., 1990). In contrast, some ultrastructural studies with monoclonal antibodies indicated that topoisomerase II*β* was localized primarily in nucleoli (Zini et al., 1994), which are absent in nuclear matrix preparations from most cells (Berezney, 1984; Fernandes et al., 1988). To further examine the importance of nuclear matrix topoisomerase IIα as a target for topoisomerase II-active drugs, future studies will determine whether VM-26 enhances the formation of complexes of nascent DNA with nuclear matrix topoisomerase IIα to a greater extent than with soluble topoisomerase IIα.

Simple inhibition of topoisomerase II activity is not an adequate explanation for the cytotoxic effects of the topoisomerase II-active drugs. However, VM- 26 and mitoxantrone-stabilized complexes of topoisomerase IIα with newly replicated DNA appeared to be highly cytotoxic lesions. DNA strand separation at the replication fork may be a major cellular process that disrupts the topoisomerase IIα−DNA complexes and thereby transforms these cleavable complexes into lethal double-strand breaks at the replication forks (Froelich-Ammon & Osheroff, 1995). VM-26-induced replication fork breaks have been shown to both inhibit DNA synthesis by blocking replication fork progression (Catapano et al., 1995) and by inducing the dissociation of newly replicated DNA from the nuclear matrix (Fernandes et al., 1988). Since the packaging of DNA into higher order chromatin structures is dependent upon the attachment of chromatin loops to the nuclear matrix (Nelson et al., 1986b), drug-induced dissociation of nascent DNA from the nuclear matrix would likely lead to the disorganization of DNA within the nucleus and subsequently cell death.

The interaction of the topoisomerase II-directed agent, VP-16, with nuclear matrix topoisomerase II formed the basis in part for the development of an effective clinical protocol for the treatment of hormone refractory prostate cancer (Pienta et al., 1994). Preclinical studies revealed that the combination of VP-16 with another drug that binds to the nuclear matrix, estramustine phosphate, resulted in synergistic inhibition of DNA synthesis and growth of prostate cancer cells (Pienta & Lehr, 1993).

The data presented in this study indicate that in exponentially growing CEM cells, topoisomerase II*β* is a less important target for VM-26 than topoisomerase IIα. Some investigators have reported that resistance to VP-16 was related to a decreased expression of the α isozyme (Harker et al., 1991), while others have reported that VP-16 cytotoxicity was directly related to topoisomerase II*β* expression (Brown et al., 1995; Houlbrook et al., 1995). Part of this controversy may stem from the fact that the biological endpoint of these studies was the total cellular amount of the topoisomerase II isozymes detectable by immunoblotting. Data on the extent to which the individual isozymes actually participated in drug-induced DNA complex formation with both nascent and bulk DNA would probably be more informative. In CEM and CEM/VM-1 cells sensitivity to VM-26 was not related to the total amount of topoisomerase IIα or II*β* (Danks et al., 1989; Fernandes et al., 1990), but rather to the degree of drug-stabilized topoisomerase IIα complex formation with newly replicated DNA (Figures 4 and 5). Nevertheless, it is possible that in more slowly growing cells having decreased expression of the α isozyme (Heck & Earnshaw, 1986; Woessner, 1991), drug-stabilized binding of topoisomerase II*β* with nonreplicating DNA may also lead to cytotoxicity. Since there is evidence that topoisomerase II*β* is localized in nucleoli (Zini et al., 1994) where ribosomal RNA metabolism takes place (Scheer & Benavente, 1990), it is tempting to speculate that topoisomerase II-active drugs inhibit the growth of some slowing growing tumors by interfering with ribosomal RNA maturation.

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