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Topoisomerase-Specific Drug Sensitivity in Relation to Cell Cycle Progression

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The nuclear enzyme DNA topoisomerase II catalyzes the breakage and resealing of duplex DNA and plays an important role in several genetic processes. It also mediates the DNA cleavage activity and cytotoxicity of clinically important anticancer agents such as etoposide. We have examined the activity of topoisomerase II during the first cell cycle of quiescent BALB/c 3T3 cells following serum stimulation. Etoposide-mediated DNA break frequency in vivo was used as a parameter of topoisomerase II activity, and enzyme content was assayed by immunoblotting. Density-arrested A31 cells exhibited a much lower sensitivity to the effects of etoposide than did actively proliferating cells. Upon serum stimulation of the quiescent cells, however, there was a marked increase in drug sensitivity which began during S phase and reached its peak just before mitosis. Maximal drug sensitivity during this period was 2.5 times greater than that of log-phase cells. This increase in drug sensitivity was associated with an increase in intracellular topoisomerase II content as determined by immunoblotting. The induction of topoisomerase II-mediated drug sensitivity was aborted within 1 h of exposure of cells to the protein synthesis inhibitor cycloheximide, but the DNA synthesis inhibitor aphidicolin had no effect. In contrast to the sensitivity of cells to drug-induced DNA cleavage, maximal cytotoxicity occurred during S phase. A 3-h exposure to cycloheximide before etoposide treatment resulted in nearly complete loss of cytotoxicity. Our findings indicate that topoisomerase II activity fluctuates with cell cycle progression, with peak activity occurring during the G_2 phase. This increase in topoisomerase II is protein synthesis dependent and may reflect a high rate of enzyme turnover. The dissociation between maximal drug-induced DNA cleavage and cytotoxicity indicates that the topoisomerase-mediated DNA breaks may be necessary but are not sufficient for cytotoxicity and that other factors which are particularly expressed during S phase may be important as well.

Type II DNA topoisomerases are ATP-dependent enzymes which catalyze the breakage and reunion of duplex DNA, allowing a second segment of DNA to pass through the break site (1, 23). They are required for the segregation of DNA following the conclusion of replication (4) and appear to be required at mitosis (12). During the breakage-reunion cycle a covalent phosphotyrosyl bond between enzyme and DNA is formed at the 5′ terminus of each strand. A number of commonly used anticancer agents such as intercalating agents and epipodophyllotoxins can stabilize this DNA protein intermediate, and convincing evidence exists that this interaction does mediate their cytotoxicity (15, 16, 25). A better understanding of the enzyme would be useful in improving the efficacy of these drugs. In addition, these topoisomerase-specific drugs have become useful probes in understanding the fundamental functions and control mechanisms of the enzyme.

Topoisomerase II activity and content varies as a function of the proliferative status of the cell (5, 19, 29, 30). Proliferating cells exhibit a much higher enzyme content and activity than cells which are quiescent (29) or terminally differentiated (30). This fluctuation in enzyme activity correlates with respect to sensitivity to the DNA cleavage effects of topoisomerase-specific antitumor drugs. Data obtained with a human lymphoma cell line (2) suggest that variations in sensitivity to the cytotoxic effects of epipodophyllotoxin drugs may occur during the cell cycle as well. To better understand the regulation of topoisomerase II activity during the cell cycle and its relationship to drug sensitivity, we examined BALB/c 3T3 cells during the first cell cycle following serum stimulation of quiescent cells. This cell line (A31) was selected as a model because it is a nontumorigenic mouse embryo fibroblast line whose growth regulation has been extensively characterized (3, 11, 22). Its proliferation can be reversibly blocked by serum deprivation or density arrest, whereupon the cell enters a G_0-like state. These cells resume proliferation synchronously upon addition of fresh serum.

In this study we examined serum-stimulated BALB/c 3T3 cells for sensitivity to the DNA-cleaving activity and cytotoxicity of the epipodophyllotoxin etoposide. Our results show that a marked increase in the drug-induced cleavage activity occurs during the G_2/M phase and that this correlates with an increase in topoisomerase II content. Maximal cytotoxicity, however, occurs during S phase. The rise in topoisomerase II content was aborted by the addition of cycloheximide.

MATERIALS AND METHODS

Materials. Cell culture medium and fetal calf serum (FCS) were from GIBCO Laboratories. Etoposide was supplied by Bristol-Myers Co. Tetrapropylammonium hydroxide was obtained from RSA Corp. Radioisotopes and Aquassure were from ICN Pharmaceuticals or New England Nuclear Corp. General lab supplies were from Fisher Scientific Co. All other materials were reagent grade from Sigma Chemical Co., Bio-Rad Laboratories, or Pharmacia Fine Chemicals.

Cell culture. BALB/c 3T3 (A31) were grown at 37°C in a monolayer in alpha minimal essential medium (αMEM) supplemented with 10% FCS, 3 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). To generate cultures that could respond to serum stimulation and syn-

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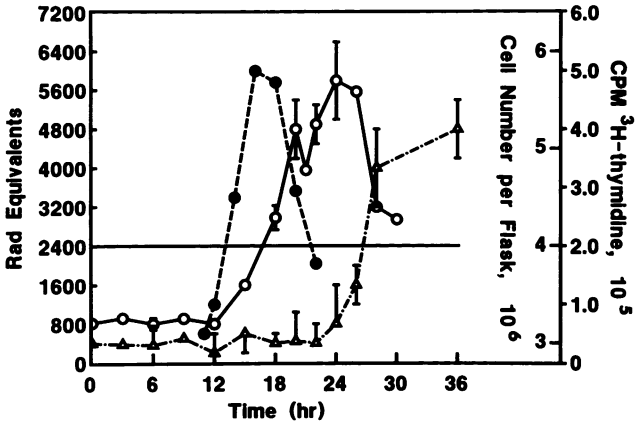

FIG. 1. Induction of DNA synthesis, drug sensitivity, and population doubling after serum stimulation of quiescent BALB/c 3T3 cells. BALB/c 3T3 (A31) cells were seeded at a density of 2×10^5 cells per 25-cm^2 tissue culture flask in 8 ml of αMEM supplemented with 10% FCS (GIBCO) and allowed to grow to confluence for 7 days at 37°C. After 7 days in culture, the confluent A31 monolayer had growth arrested and entered the G_0 phase. The cells were then stimulated to progress through the cell cycle by replacing the conditioned medium with fresh αMEM containing 20% FCS at time zero. Cell cycle progression was monitored by flow cytometry (data not shown) and by the incorporation of ^{3}H-thymidine (●) (20 μCi/ml, 37 Ci/mmol; New England Nuclear) at 37°C for 30 min into trichloroacetic acid-insoluble material. Cell numbers (△) were determined throughout the cell cycle with a Coulter counter, and cell viability was determined by trypan blue dye exclusion. Drug sensitivity (○) was assayed in vivo by drug-mediated DNA break frequency by the alkaline elution technique (as described in Materials and Methods) after a 1-h exposure to 40 μM VP-16 at 37°C for 1 h. The values shown are means ± the standard deviation (n = 4). The horizontal line indicates a sensitivity comparison of proliferating cells at log phase.

chronously enter the cell cycle, A31 cells were grown to confluence from initial seeding without replacing the conditioned medium for 7 days.

Alkaline elution. The alkaline elution technique (13) was used to assay the frequency of single-stranded DNA breaks upon drug challenge. Experimental cells were labeled with ^{14}C-thymidine and treated with etoposide at 37°C for 1 h. Human leukemia cells (CCRF-CEM), ^{3}H-labeled and irradiated with either 250 rads (low-DNA-break-frequency assay) or 2,500 rads (high-DNA-break-frequency assay) by the ^{137}Cs source, were included as internal standards. The internal controls served as a relative calibration to correlate the frequency of drug-induced DNA breaks to that of an equivalent radiation effect and to adjust for time-dependent variances (10). Cells were lysed on a polyvinyl chloride membrane (Millipore Corp.) with sodium dodecyl sulfate (SDS)-proteinase K solution (2% SDS, 20 mM disodium EDTA, 0.5 mg of proteinase K per ml). DNA was then eluted with elution buffer (375 mM tetrapropylammonium hydroxide [pH 12.1], 20 mM EDTA). One rad equivalent is approximately equal to 9×10^{-10} single-stranded DNA breaks per nucleotide (13) or simply to 16 to 20 breaks per cell (7).

Gel electrophoresis and immunoblotting. Gel electrophoresis was carried out at 200 V for 3.5 h in 7.5% polyacrylamide gels with 0.1% SDS. The stacking gel contained 4.5% polyacrylamide and 0.1% SDS. The electrophoresis buffer consisted of 50 mM Tris borate (pH 8.3)–1 mM disodium EDTA–0.1% SDS. After electrophoresis, gels were soaked in 50 mM Tris borate (pH 8.3)–1 mM disodium EDTA–20% methanol for 20 min. Proteins were then transferred from gels to nitrocellulose membranes by electrophoretic blotting for 5 h in the same buffer. The gel and the second nitrocellulose membrane were stained separately as references to ensure adequate protein transfer. After transfer, membranes were soaked in TTBS (20 mM Tris [pH 7.4], 500 mM NaCl, 0.05% Tween 20) with 3% gelatin and then rinsed with TTBS alone. Membranes were incubated overnight at room temperature with rabbit antiserum to calf thymus topoisomerase II (diluted 1:1,000 into TTBS containing 1% gelatin), washed twice with TTBS, and rinsed once with TBS (20 mM Tris [pH 7.4], 500 mM NaCl). Immunocomplexes were reacted with ^{125}I-labeled protein A (~10^5 cpm/ml in TTBS containing 1% gelatin) at room temperature for 4 h. After two rinses with TTBS and one rinse with TBS, membranes were dried and autoradiographed. Some membranes were used for color immunoblot (Bio-Rad) by washing off the ^{125}I-protein A with 100 mM glycine-HCl (pH 2.2)–20 mM magnesium diacetate–50 mM KCl at room temperature for 1.5 h (14), reprobed with antiserum to topoisomerase II, followed by alkaline phosphatase-conjugated second antibody, and then developed into a color immunoblot in buffer C (15 mg of Nitro Blue Tetrazolium per 100 ml, 0.7% *N*,*N*-dimethylformamide, 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml, 1 mM Mg_2Cl, and 100 mM $NaHCO_3$, pH 9.8) (Bio-Rad manual).

RESULTS

Quiescent A31 cells, when incubated with fresh serum, re-entered the cell cycle in a highly reproducible and synchronous manner (Fig. 1). DNA synthesis began approximately 12 h after serum stimulation and peaked approximately 3 to 6 h later. Mitosis occurred approximately 27 h after serum stimulation. These data are similar to those obtained by others (3, 24). To assess drug sensitivity throughout the cell cycle, cells were exposed to etoposide for 1 h at various times after serum stimulation and DNA cleavage was assayed by the alkaline elution technique. Etoposide (40 μM) caused 680 ± 120 rad equivalents of strand breaks in quiescent A31 cells. When compared to logarithmically growing A31 cells (solid line, Fig. 1), it was apparent that quiescent cells have a markedly diminished sensitivity to the DNA-cleaving effect of the drug. Approximately 12 to 15 h after serum stimulation, however, an increase in drug sensitivity was noted which peaked at a time corresponding to the beginning of mitosis. At the peak of drug sensitivity, 21 h after serum stimulation, drug sensitivity was about 7-fold higher than in the quiescent cells and approximately 2.5-fold higher than that of asynchronous cells in log phase. The incorporation of the drug, as assayed by measuring the uptake of ^{3}H-etoposide (31) into the cells, did not account for the changes in drug sensitivity (data not shown). Thus the sensitivity of serum stimulated A31 cells to the DNA cleaving effects of etoposide varied as a function of the cell cycle, reaching its zenith during G_2/M phase.

To determine whether DNA synthesis was required for the increase in drug sensitivity observed in G_2/M phase, cells were exposed to the DNA polymerase inhibitor aphidicolin 6 h following serum stimulation. This resulted in virtually complete inhibition of thymidine incorporation throughout the period of aphidicolin exposure (Fig. 2A, solid bars). However, when aphidicolin-inhibited cells were exposed to etoposide, DNA cleavage, as measured by alkaline elution, increased as it did in control cells (Fig. 2A, open bars). In

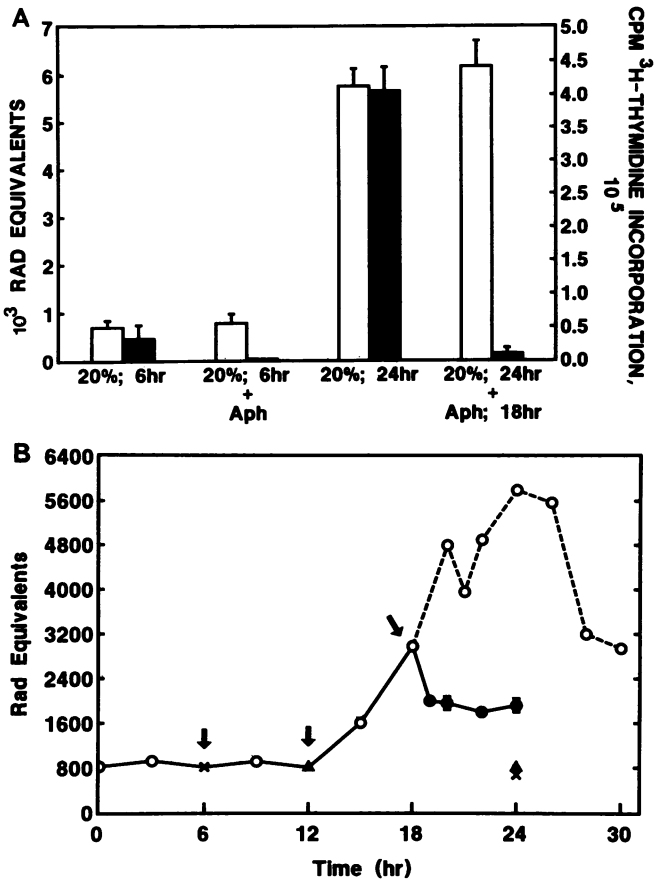

FIG. 2. Effects of aphidicolin and cycloheximide on the induction of drug sensitivity. (A) Aphidicolin (17 μM) was added 6 h after serum stimulation, and strand breaks were assayed 1 or 18 h after addition of aphidicolin. Etoposide was added in the final hour of each experiment. Open bars represent drug-induced DNA break frequency, and closed bars indicate thymidine incorporation. (B) Cycloheximide (50 μM) was added 6, 12, or 18 h after stimulation (arrows). The drug-induced DNA damage in controls is shown by a dashed curve (○). Cells treated at 6 and 12 h were assayed at 24 h only (the drug sensitivities are represented as × and ▲). The 18-h group (●) was assayed every hour after the addition of cycloheximide. The values shown are means ± the standard deviation (n = 3).

contrast, protein synthesis was required for the increase in drug sensitivity to occur. Cells treated with cycloheximide (50 μM) beginning at 6 or 12 h following serum stimulation did not show the expected increase in drug sensitivity when assayed at 24 h (× and ▲, respectively, in Fig. 2B). Furthermore, when cells were treated with cycloheximide 18 h after serum stimulation, the increase in drug sensitivity was rapidly aborted (Fig. 2B, closed circles). Previous studies have indicated that cell killing by etoposide is dependent on topoisomerase-mediated DNA cleavage and that, in general, cytotoxicity increases with the frequency of DNA breaks (17, 25). It was thus of interest to compare the variations in strand breakage observed in serum-stimulated A31 cells with the cytotoxic effects of etoposide. Very little cytotoxicity was observed in A31 cells until 12 h following serum stimulation (Fig. 3). Maximal cell killing occurred 18 h after serum stimulation, but between 21 and 23 h there was again a reduction in cytotoxicity. Incubation of the cells with cycloheximide between 18 and 21 h nearly completely eliminated drug-induced cytotoxicity. Cycloheximide also protected log-phase A31 cells from etoposide-mediated cytotoxicity (data not shown). Thus, maximal sensitivity to the cell-killing effect of etoposide occurs during a period which corresponds to the S phase and approximately 6 h before maximal drug-induced DNA cleavage.

Previously obtained evidence indicates that the intracellular content of DNA topoisomerase II is an important factor in determining the sensitivity to the DNA-cleaving effects of etoposide (29). The striking differences in drug sensitivity observed throughout the cell cycle and the effects of cycloheximide suggested that topoisomerase content may vary during the cell cycle in A31 cells and that protein synthesis was required for the G_2/M peak in sensitivity. To clarify this issue, topoisomerase content was examined in whole-cell lysates from quiescent, serum-stimulated, and cycloheximide-treated cells by immunoblotting with polyclonal serum obtained from a rabbit immunized to purified calf thymus topoisomerase II (Fig. 4B). Topoisomerase II is represented by a 168-kilodalton band. No band was detected with preimmune serum (Fig. 4A). Enzyme content was first detected about 12 h following serum stimulation and increased gradually. It reached its peak at 20 and 24 h following serum stimulation. Topoisomerase II activity determined by decatenation or drug-induced DNA cleavage in vitro showed the same magnitude of differences (data not shown). Significant loss of the 168-kilodalton band was observed when cells were exposed to cycloheximide for 3 to 6 h (Fig. 4B, lanes 8 and 9) beginning 18 h after serum stimulation.

DISCUSSION

Topoisomerase II has, in recent years, emerged as a major intracellular target for clinically important anticancer agents such as intercalating agents (26) and epipodophyllotoxins (25). Current evidence suggests that independent of direct interactions with DNA (25, 27), these drugs stabilize the

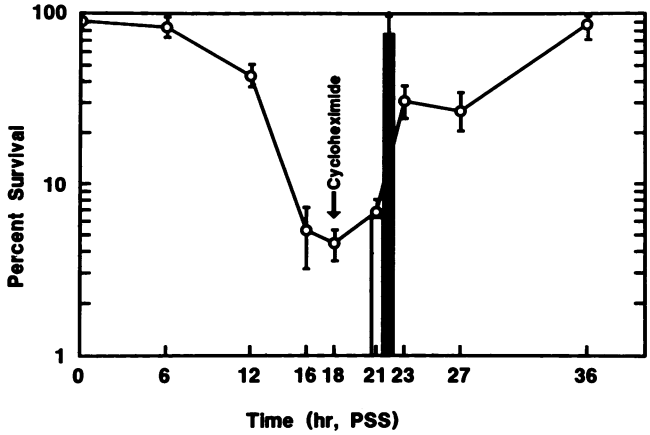

FIG. 3. Effect of etoposide on cell survival. Serum-stimulated A31 cells were treated at different time points with etoposide at 37°C for 1 h, washed twice with warm medium for 10 min, and then trypsinized. Viable cells, as determined by trypan blue dye exclusion, were seeded quantitatively into 100-mm plates with 10 ml of medium and incubated at 37°C and in 5% CO_2 over 7 to 10 days. Colonies were stained with 2% crystal violet in methanol. Percent survival was determined by comparison to dimethyl sulfoxide-treated controls. Results shown are from cells treated with 10 μM etoposide. Some cells were treated with 50 μM cycloheximide for 3 h before challenge with etoposide (closed bar, cycloheximide-treated group; open bar, control group). The arrow indicates the time cycloheximide was added. PSS, Post-serum stimulation.

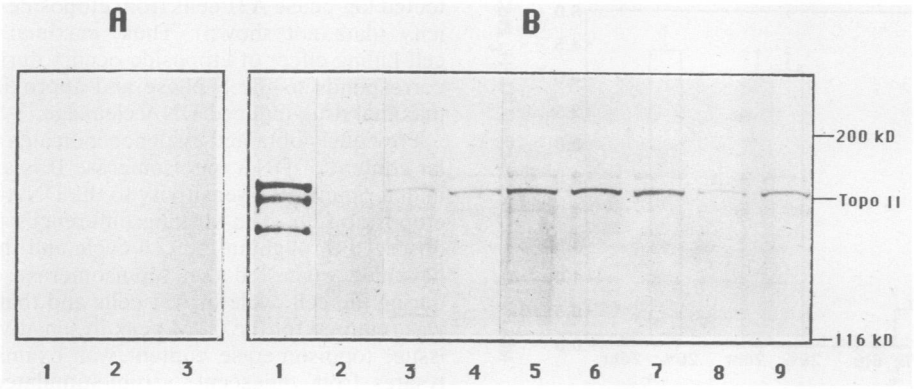

FIG. 4. Quantitation of topoisomerase II levels by immunoblotting with rabbit preimmune serum (A) and rabbit antiserum to calf thymus topoisomerase II (B). Whole-cell lysate (5×10^5 cells per lane) was prepared from quiescent A31 cells (A, lane 1; B, lane 2) and from serum-stimulated cells at 12 h (lane 3), 18 h (lane 4), 20 h (lane 5), 24 h (A, lane 2; B, lane 6), or 26 h (lane 7) after stimulation. Lane 8, Whole-cell lysate from serum-stimulated cells which had been treated with 50 μM cycloheximide for 6 h beginning 18 h after stimulation. Lane 9, Whole-cell lysate from serum-stimulated cells which had been treated with 50 μM cycloheximide for 3 h beginning 21 h after stimulation. In lane 3 (A) and lane 1 (B), 1 μg of column-purified human topoisomerase II was used as the internal standard.

DNA-protein intermediate in a form which has been referred to as a "cleavable complex" (15). The intracellular events which follow cleavable-complex formation are not well elucidated. However, a variety of evidence indicates that this lesion is required for cytotoxicity to occur. The present study sheds further light on the status of topoisomerase II during the cell cycle and how this influences drug sensitivity. The decreased drug sensitivity of quiescent cells when compared to those which are logarithmically growing is consistent with the observation of others that topoisomerase activity is stimulated in the regenerating liver of partially hepatectomized mice (5), in mitogenized lymphocytes (30), and in quiescent mouse fibroblasts stimulated by epidermal growth factor (19). Topoisomerase content was not examined in these latter studies. However, Sullivan et al. (29) found that the enzyme content of quiescent Chinese hamster ovary fibroblasts was markedly decreased compared to that of proliferating cells. This corresponded well with the sensitivity to the DNA cleavage activity and cytotoxicity of etoposide. Topoisomerase content and drug sensitivity have not been examined in detail, however, as a function of the cell cycle. A preliminary report by Estey et al. (8) with logarithmically growing HeLa cells synchronized by brief exposure to nitrous oxide suggested an increase in DNA cleavage by the intercalating agent 4′-(9-actidinylamino)methanesulfon-m-anisidide during the period immediately before mitosis. We have confirmed their finding with the epipodophyllotoxin etoposide. By demonstrating a similar phenomenon in the BALB/c 3T3 line, a cell line under more normal growth regulation, our work suggests that this phenomenon may be a general one. Furthermore, we have demonstrated that the increase in DNA-cleaving activity by a drug is associated with a rise in topoisomerase content as assayed by immunoblotting. On the other hand, since the first G_1 period following serum stimulation may not, in all respects, reflect subsequent ones in growing cells, we are less certain of the importance of the relatively low drug sensitivity during this period.

It is reasonable to ask what purpose is served by the increase in topoisomerase content just before mitosis. Elegant immunolocalization studies by Earnshaw and Heck (6) and Gasser et al. (9) clearly demonstrate that topoisomerase II is a major structural component of metaphase chromosomes. Indeed, it has been estimated that this enzyme represents 1 to 2% of the total mitotic chromosomal protein. Presumably, the enzyme plays an important role in maintaining the highly condensed structure of mitotic chromosomes. Interestingly, studies with mutant yeast cells which bear a temperature-sensitive topoisomerase II indicate that the inhibition of cell cycle traverse or mitotic spindle formation protects the mutant cells at the restrictive temperature (12).

The studies with cycloheximide considered together with our immunoblot data clearly indicate that protein synthesis is required for the rise in enzyme content observed during G_2/M phase. In addition, the data strongly suggest that enzyme degradation occurs as well, since there was a rapid decrease in topoisomerase content following protein synthesis inhibition. The effect was too rapid to be accounted for by simple changes in cell cycle distribution (18, 28). The fact that we have observed a similar phenomenon in logarithmically growing cells indicates that there is probably a continuing dynamic equilibrium between synthesis and degradation in proliferating cells. This phenomenon had not been demonstrated previously and is somewhat surprising. It suggests that enzyme content may be an important regulatory element in cell cycle progression.

As noted above, the cytotoxicity of intercalating agents and epipodophyllotoxins generally corresponds well with cleavable-complex formation. However, upon examination at various phases of the cell cycle, a clear dissociation of the two is observed. The increased sensitivity of the cells during late S phase is similar to that observed by Barlogie et al. (2) in synchronized human lymphoma cells exposed to etoposide and in synchronized HeLa cells treated with m-AMSA (E. Estey, personal communication). There is good reason to believe that topoisomerase II plays an important role in DNA synthesis. A study by Nelson et al. (21) indicates that the enzyme is intimately associated with newly replicated DNA and support the concept proposed by Noguchi et al. (22) that topoisomerase II is a component of the multienzyme "replitase" complex which has been proposed to coordinate DNA synthesis. Furthermore, studies with yeast cells indicate that the enzyme is required for the segregation of daughter DNA molecules at the conclusion of the S phase (30). However, the drug-stabilized cleavable complex may not be directly involved in either the initiation or the elongation process of DNA synthesis (data not

shown), and the effects of etoposide on macromolecular synthesis could be a secondary response to the drug (20). The impact of drug-induced cleavable-complex formation on DNA synthesis remains to be determined if this is the basis for the sensitivity of S-phase cells. It should be noted, however, that other explanations are possible. For instance, chromatin is maximally decondensed during S phase. Although both intercalating agents and epipodophyllotoxins can alter the strand passage activity of topoisomerase II in nuclear extracts, their effects in vivo are unknown. It is readily conceivable that either an increase or a decrease in strand passage would have deleterious effects on the ability of cells to prepare for mitosis. On the other hand, if the drug effect results in alterations in DNA topology during S phase, the expression of certain critical genes may be induced or inhibited in an untimely fashion, resulting in cell death. At the present time, therefore, there is no clear explanation for the increased sensitivity during late S phase. However, the observation provides a focus for future studies to elucidate the mechanism by which cleavable-complex formation causes cell death.

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