Cell Cycle Stage Dependent Variations in Drug-Induced Topoisomerase II Mediated DNA Cleavage and Cytotoxicity†

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ABSTRACT: The DNA cleavage produced by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) in mammalian cells is putatively mediated by topoisomerase II. We found that in synchronized HeLa cells the frequency of such cleavage was 4–15-fold greater in mitosis than in S while the DNA of G_1 and G_2 cells exhibited an intermediate susceptibility to cleavage. The hypersensitivity of mitotic DNA to *m*-AMSA-induced cleavage was acquired relatively abruptly in late G_2 and was lost similarly abruptly in early G_1. The susceptibility of mitotic cells to *m*-AMSA-induced DNA cleavage was not clearly paralleled by an increase in topoisomerase II activity (decatenation of kinetoplast DNA) in 350 mM NaCl extracts from mitotic cells compared to similar extracts from cells in G_1, S, or G_2. Furthermore, equal amounts of decatenating activity from cells in mitosis and S produced equal amounts of *m*-AMSA-induced cleavage of simian virus 40 (SV40) DNA; i.e., the interaction between *m*-AMSA and extractable enzyme was similar in mitosis and S. The DNA of mitotic cells was also hypersensitive to cleavage by 4′-demethylepipodophyllotoxin 4-(4,6-*O*-ethylidene-β-D-glucopyranoside) (etoposide), a drug that produces topoisomerase II mediated DNA cleavage without binding to DNA. Thus, alterations in the drug–chromatin interaction during the cell cycle seem an unlikely explanation for results in whole cells. Cell cycle stage dependent fluctuations in *m*-AMSA-induced DNA cleavage may result from fluctuations in the structure of chromatin per se that occur during the cell cycle. Alternatively, they may reflect fluctuations in enzyme activity occurring during the cell cycle that were masked by cell cycle stage dependent differences in extractability at 350 mM NaCl either of the enzyme or of substances that influence enzyme activity. Surprisingly, cell cycle stage dependent differences in *m*-AMSA-induced DNA cleavage did not correlate with differences in the susceptibility to the cytotoxic effects of the drug. In fact, cells in S were most sensitive to these effects. These results are an exception to the previously observed parallel between the susceptibility of mammalian cells to drug-induced DNA cleavage and the susceptibility of the cells to drug-induced cytotoxicity and indicate the complexity of any relationship between the two phenomena.

Antitumor acridines such as 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA)[1] and epipodophyllotoxins such as etoposide interact with mammalian topoisomerase II, an enzyme that alters DNA three-dimensional structure (Liu, 1983; Wang, 1985). In isolated biochemical systems these compounds produce topoisomerase II mediated DNA cleavage (Nelson et al., 1984; Minford et al., 1986; Chen et al., 1984; Ross et al., 1984). The same compounds produce protein-associated DNA cleavage in whole cells and their isolated nuclei. The biochemical characteristics of topoisomerase II mediated DNA cleavage in isolated biochemical systems are identical with those of protein-associated DNA cleavage in whole cells and nuclei (Glisson et al., 1984; Pommier et al., 1984; Zwelling et al., 1981). This suggests that drug-stimulated protein-associated DNA cleavage in whole cells and nuclei is mediated by topoisomerase II. Thus, the frequency of such DNA cleavage in whole cells may reflect the activity of the enzyme.

We and others have shown that both drug-stimulated, topoisomerase II mediated DNA cleavage and topoisomerase II activity are greater in proliferating mammalian cells than in quiescent cells of the same type (Duguet et al., 1983; Taudou et al., 1984; Zwelling et al., 1987; Sullivan et al., 1986). We have now quantified *m*-AMSA and etoposide-stimulated DNA cleavage in HeLa cells synchronized at different cell cycle stages. We have also quantified *m*-AMSA- and etoposide-stimulated cleavage of SV40 DNA in extracts made from HeLa cells during these same cell cycle stages and have correlated these measurements with measurements of that enzyme activity extractable at 350 mM NaCl. In some mammalian cells the frequency of drug-stimulated protein-associated DNA cleavage parallels susceptibility of the cells to the cytotoxic effects of the drug (Pommier et al., 1986; Glisson et al., 1986a,b; Bakic et al., 1986). Accordingly, we have compared the sensitivity of HeLa cells in mitosis, G_1, S, and G_2 to the cytotoxic effects of *m*-AMSA.

Experimental Procedures

Cells, Cell Synchrony, and Drug Treatments. HeLa cells were grown as monolayer cultures in 100-mm culture plates at 37 °C in 5% CO_2 in McCoy's modified medium 5A (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum and a penicillin–streptomycin mixture.

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[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; etoposide, 4′-demethylepipodophyllotoxin 4-(4,6-*O*-ethylidene-β-D-glucopyranoside); SV40, simian virus 40; N_2O, nitrous oxide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Cells were harvested for subculturing with a 0.05% trypsin solution. To estimate the fraction of cells actively synthesizing DNA, 1 mCi mL^{-1} [^{3}H]thymidine (6.7 Ci $mmol^{-1}$, New England Nuclear, Boston, MA) was added to cells for 30–60 min prior to harvesting, and the labeling index was determined by autoradiography.

Several different methods were used to obtain synchronized populations of cells in order to minimize the possibility that results would be attributed to biologic phenomena when, in fact, they represented artifacts induced by a particular synchronizing technique. In most experiments, mitotic cells were obtained by partially synchronizing asynchronously growing HeLa cells in S phase with a single excess thymidine block (2.5 mM) for 24 h. The thymidine block was removed by washing and incubating the cells in regular medium. The cells were then placed in a chamber filled with N_2O at a pressure of 85 psi and were incubated at 37 °C for 15 h. The rounded and the loosely attached mitotic cells were selectively detached by gentle pipetting (Rao, 1968). The procedure yielded a population with a mitotic index of ≥97%. Because the mitotic block induced by N_2O is reversible (Rao, 1968), incubation of N_2O-blocked mitotic cells under normal culture conditions (without N_2O) yielded highly synchronized populations of cells in G_1 and, subsequently, S phase. In some experiments, colcemid (0.05 μg mL^{-1} for 15 h) replaced N_2O as the means by which mitotically pure cells were obtained (mitotic index >97%). In other experiments mitotic cells were obtained by selectively detaching cells from a population that had received a single thymidine block followed by 9 h of growth after removal of the block. Such selective detachment resulted in a population with a mitotic index of 60–70%. In order to compare results obtained with selective detachment as the means to accrue mitotic cells with those obtained with N_2O for the same purpose, cells were incubated in N_2O for 9 rather than 15 h. This briefer incubation yielded a population that also had a mitotic index of 60–70%.

Pure populations of cells in S phase and G_2 were also obtained with a double thymidine block (Bootsma et al., 1964; Rao & Engelberg, 1966). Asynchronously growing cells were subjected to a single thymidine block as described above for 16 h. Nine hours after the block was removed, cells were subjected to a second thymidine block for 16 h. The labeling index of cells 3 h after removal of this second block was 85%. These cells were defined as S-phase cells. Cells in G_2 were defined as cells that had been removed from the second thymidine block 8–10 h previously. Such cells had a labeling index of 10% and a mitotic index of 10%.

Both *m*-AMSA (NSC 249992) and etoposide were kept frozen as 0.01 M stock solutions in 100% dimethyl sulfoxide. The drugs were constituted within 30 min of use at appropriate concentrations in dimethyl sulfoxide and added directly to culture dishes. Controls received appropriate amounts of dimethyl sulfoxide. All treatments were for 30 min at 37 °C following which the culture dishes were placed on ice and the cells were harvested with trypsin.

DNA Alkaline Elution. The methodology of DNA alkaline elution has been previously described (Zwelling et al., 1981). Asynchronously growing HeLa cells were radiolabeled for 4 days with 0.02–0.03 μCi mL^{-1} [^{14}C]thymidine (51 mCi $mmol^{-1}$, New England Nuclear, Boston, MA). A separate culture of asynchronously growing HeLa cells was radiolabeled with 0.02 μCi mL^{-1} [^{3}H]thymidine (6.7 Ci $mmol^{-1}$, New England Nuclear, Boston, MA) for 3 days and irradiated with X-rays (300 or 2000 rads) on ice. The elution rate of the DNA of these cells (internal standard cells) served to normalize the elution rate of cells labeled with [^{14}C]thymidine. Calculations were performed as previously described (Zwelling et al., 1981). Drug-induced DNA cleavage frequencies were expressed in rad equivalents, the amount of X-ray-induced DNA cleavage producing an elution rate comparable to that observed in drug-treated cells. In initial experiments both a low-sensitivity assay and a high-sensitivity assay were used. The former more accurately quantifies break frequencies greater than 500 rad equiv while the latter is more accurate at lower break frequencies. After initial experiments established the general range of break frequencies, later experiments were done with the low-sensitive assay.

Uptake of [^{14}C]-m-AMSA. Cells without radiolabeled DNA were exposed to 0.5, 0.2, or 0.1 μM [^{14}C]-*m*-AMSA (19.6 mCi $mmol^{-1}$, SRI International, Menlo Park, CA) for 30 min at 37 °C. Cells were concurrently exposed to ^3H_2O (1 mCi mL^{-1}, New England Nuclear, Boston, MA). Cells were then centrifuged through Versilube F-50 silicone oil (General Electric Co., Waterford, NY) in a microcentrifuge tube at 12000*g* for 2 min. The bottom of the tube was cut off, and the cell button was dispersed and solubilized. Intracellular drug was expressed in molar terms by dividing the amount of radioactivity ([^{14}C]-*m*-AMSA) in the pellet by the cell volume quantified with ^3H_2O. This method is essentially that of Vistica (1979) as modified by Zwelling et al. (1982).

Colony Formation Assays. Cells were grown and synchronized exactly like the cells used for assays of DNA cleavage. All cell populations were treated with 0.5 μM *m*-AMSA for 30 min at 37 °C, which was approximately the D_{37} dose for asynchronously growing HeLa cells. After treatment the cells were removed from their plates and counted on a Coulter counter to determine their concentration. Cells were then replated at 100, 1000, and 5000 cells in 2 mL per 35-mm plate, each in triplicate. After 9–12 days of growth the resultant colonies were fixed with 10% formalin, stained with crystal violet, and counted with a dissecting microscope.

Preparation of Cellular Extracts. Between 1.5×10^7 and 4×10^7 cells in either mitosis, G_1 (obtained 0.5 and 3 h respectively following removal from N_2O-induced synchrony), S, or G_2 (obtained 3 and 8 h respectively following removal from the double thymidine block) were used to prepare cell extracts. After being washed, cells were resuspended (4×10^7 cells mL^{-1}) in a buffer containing 1 mM KH_2PO_4 (pH 6.4), 5 mM $MgCl_2$, 350 mM NaCl, 1 mM EGTA, 0.1 mM dithiothreitol, 0.1 phenylmethanesulfonyl fluoride, and 10% glycerol. We have previously used this buffer to extract topoisomerase II activity from isolated nuclei (Minford et al., 1986). Cells were disrupted by sonication with a Heat Systems Ultrasonics cell disrupter (Heat Systems Ultrasonics Inc., Farmingdale, NY) at a setting of 7. Three 20-s pulses were used for sonication, each interspersed with a 20-s interval during which the cells were cooled at 4 °C. Phase-contrast microscopy indicated that sonication invariably resulted in disruption of >95% of the cells. The sonicate was then centrifuged at 100000*g* for 60 min at 4 °C in a Beckman LK-50 ultracentrifuge. The supernatant was stored at −70 °C. The amount of protein in the supernatant was determined with the Bio-Rad assay (Bradford, 1976).

Assessment of Topoisomerase II Activity. Topoisomerase II activity was quantified by testing the ability of extracts to decatenate ^{3}H-labeled kinetoplast (k) DNA, isolated from [^{3}H]thymidine-labeled *Crithidia fasciculata* as described by Simpson and Simpson (1974). Decatenation of kDNA is specific for topoisomerase II activity (Marini et al., 1980). Reactions were for 30 min at 37 °C in 100 μL containing 0.37

μg of kDNA, various amounts of cellular extract, 10 mM Tris (pH 7.6), 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 5 mM ATP, 30 μg mL^{-1} bovine serum albumin, and 0.5 mM dithiothreitol. After 30 min, 50-μL aliquots from each reaction mixture were centrifuged at 12000*g* for 5 min. The top 40 μL ("supernatant") was carefully removed from the bottom 10 μL ("pellet"). The radioactivity in the "supernatant" (which contained decatenated kDNA) and the "pellet" (which contained the remaining kDNA starting material) was then quantified (Sahai & Kaplan, 1986). The remaining 50 μL from each reaction mixture was electrophoresed through a 1% agarose gel following the addition of 5 μL of 10% SDS and 5 μL of 1 mg mL^{-1} proteinase K to each reaction mixture. The radioactivity in the wells of the gel (which contained the remaining [^{3}H]kDNA starting material) and in the lanes of the gel (which contained decatenated [^{3}H]kDNA) was quantified.

Assessment of Cleavage of Exogenous DNA. The ability of whole-cell sonicates to cleave DNA was tested with closed circular supercoiled viral SV40 DNA (form I; Bethesda Research Laboratories, Bethesda, MD) as substrate. Reaction mixtures contained 0.23 μg of SV40, amounts of extract from each cell cycle stage that contained equal amounts of decatenating activity, and doses of *m*-AMSA or etoposide ranging between 0 and 100 μM in a volume of 20 μL containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, and 0.1 mM EDTA. The concentration of dimethyl sulfoxide, the solvent in which the drugs were dissolved, was kept constant in each reaction mixture. A mixture containing SV40 DNA and the restriction enzyme *Eco*RI (Bethesda Research Laboratories, Bethesda, MD) was included in order to generate linear DNA (form III) as a marker for the migration distance of SV40 cleaved at one site in both strands. Reactions were for 30 min at 37 °C. Most reactions were stopped with SDS and proteinase K. In some experiments reactions were stopped by the addition of EDTA to a final concentration of 45 mM (Liu et al., 1983). Topoisomerase II mediated DNA cleavage can only be generated by denaturation of the enzyme with alkali or detergents such as SDS. EDTA will chelate Mg^{2+} and stop ongoing topoisomerase II mediated reactions without generating cleaved DNA. Thus, these alternative methods of stopping reactions can distinguish topoisomerase II mediated DNA cleavage from nuclease-mediated DNA cleavage. Following electrophoresis through a 1% agarose gel that contained ethidium bromide (0.5 μg mL^{-1}), the amounts of cleaved DNA (forms II plus III) were quantified by densitometric scanning of a photographic negative of the gel.

Results

m-AMSA-Induced DNA Cleavage during the Cell Cycle. Figure 1 illustrates the results of a typical experiment in which DNA synthesis (labeling index) and *m*-AMSA-induced DNA cleavage were quantified in cells at various times after removal from N_2O-induced mitotic synchrony. Drug-induced DNA cleavage was greatest in cells treated immediately after removal from N_2O (mitotic cells). Such cells had a mitotic index > 95%. The DNA of cells treated in late G_1 (7 h after removal from N_2O) was only 10% as sensitive as that of mitotic cells to *m*-AMSA-induced cleavage. The lowest cleavage frequency (about 15-fold lower than that in mitotic cells) occurred at a time when the greatest number of cells were in S phase. Coincident with entry into G_2, the susceptibility of the cellular DNA to *m*-AMSA-induced cleavage rose but did not approach the level of susceptibility exhibited by the DNA of mitotic cells.

Table I illustrates the results of numerous experiments in which *m*-AMSA-induced DNA cleavage was determined in mitotic and G_1 cells (0.5 and 3 h, respectively, after removal from N_2O) and in S and G_2 cells (3 and 8–10 h, respectively, after removal from double thymidine block). Results confirmed the hypersensitivity of mitotic DNA to *m*-AMSA-induced cleavage. The amount of ^{14}C present in the detergent solution used to lyse the cells comprised 40–70% of the total ^{14}C radioactivity following lysis of mitotic cells compared to less than 20% following lysis of G_1, S, or G_2 cells (data not shown). This probably resulted in part from a considerably higher frequency of DNA double-stranded breaks in mitotic compared to interphase cells. Thus, our measurements of single-stranded breaks are likely an underestimate of the frequency of *m*-AMSA-induced cleavage in mitotic cells relative to interphase cells.

We next determined whether the hypersensitivity of DNA to *m*-AMSA-stimulated cleavage persisted as cells left mitosis and entered G_1. The drug-induced DNA cleavage frequency in cells 1, 2, or 3 h after removal from N_2O (mitotic indices

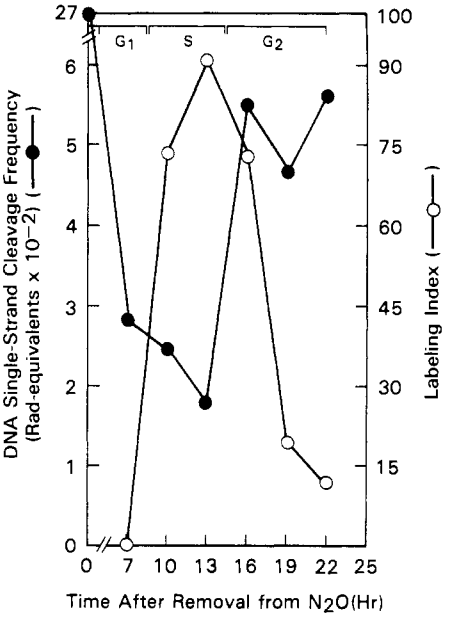

FIGURE 1: Labeling index and *m*-AMSA-induced DNA cleavage following removal of synchronized mitotic cells from N_2O. Asynchronously growing HeLa cells were synchronized in mitosis with N_2O. At the indicated times following removal from N_2O, cells were treated with *m*-AMSA (0.1 μM for 30 min at 37 °C). The DNA single-strand cleavage frequency (closed circles) was then quantified by alkaline elution. At each time an aliquot of cells not exposed to *m*-AMSA was taken for determination of labeling index (open circles).

Table I: DNA Cleavage Produced by *m*-AMSA in HeLa Cell Populations in Different Cell Cycle Stages[a]

cell cycle stage	DNA single-strand cleavage frequency (rad equiv), mean ± SEM	no. of determinations
mitosis	2472.9 ± 346.0	5
G_1	872.7 ± 238.7	5
S	559.4 ± 437.2	7
G_2	928.7 ± 376.9	7

[a] To obtain mitotic and G_1 cells, asynchronously growing HeLa cells were synchronized in mitosis with N_2O. Cells were defined as mitotic and G_1-phase cells 0.5 and 3 h respecitively after removal from N_2O. To obtain S and G_2 cells, asynchronously growing HeLa cells were synchronized in S phase with a double thymidine block. Cells were defined as S-phase (labeling index 85%) 3 h after removal from the double thymidine block. Cells were defined as G_2-phase cells (labeling index 10%, mitotic index 10%) 8–10 h after removal form the double thymidine block. Treatments with *m*-AMSA were at 0.1 μM for 30 min at 37 °C. DNA single-strand cleavage frequency was quantified by alkaline elution.

Table II: Effect of N_2O or Colcemid on *m*-AMSA-Induced DNA Cleavage[a]

	DNA single-strand cleavage frequency (rad equiv)				
	cells removed from thymidine and allowed to progress to mitosis		cells remaining in thymidine unable to progress to mitosis		
expt	$+N_2O$	+colcemid	control	$+N_2O$	+colcemid
1	2425	2212	130	378	98
2	2396	2440			
3			387	398	

[a] Asynchronously growing cells were treated with a single thymidine block (see Experimental Procedures). Some of the cells were washed and placed in fresh medium while others remained in thymidine. Aliquots from both sets of cells were then placed in N_2O or colcemid for 15 h (see Experimental Procedures). A portion of the cells remaining in thymidine was not exposed to N_2O or colcemid ("control"). Treatments with *m*-AMSA were at 0.1 μM for 30 min at 37 °C. DNA single-strand cleavage frequency was quantified by alkaline elution (see Experimental Procedures).

20, 10, and <5%, respectively) was 100, 50, and 30%, respectively, of the DNA cleavage frequency seen in cells immediately after removal from N_2O (data not shown). Thus, the hypersensitivity of DNA to *m*-AMSA-induced cleavage was limited to the period of mitosis and very early G_1 and declined abruptly as cells proceeded into G_1.

In experiments designed to examine the change in susceptibility of DNA to *m*-AMSA-induced cleavage as cells passed from G_2 into mitosis, cells were synchronized in S phase with a double thymidine block. The block was reversed, and the cells were then placed in N_2O for 10–12 h, which is the duration of S plus G_2 in HeLa cells (Rao & Engelberg, 1966). The N_2O prevented cells that had entered mitosis from leaving. After treatment with *m*-AMSA, nonadherent cells (mitotic cells: mitotic index 70–80%) were selectively detached from adherent cells (cells in late G_2: mitotic index 10–15%). These experiments indicated that the DNA of the nonadherent, mitotically enriched cells was 2–3-fold more sensitive to *m*-AMSA-induced cleavage than the DNA of the adherent, late G_2 cells (data not shown). Thus, the acquisition of the hypercleavable biochemistry of DNA was a relatively abrupt, mitotic-specific phenomenon.

The high frequency of *m*-AMSA-induced DNA cleavage observed in mitotic cells could have resulted from prolonged exposure to N_2O. However, *m*-AMSA induced a high frequency of DNA cleavage in mitotic cells regardless of whether colcemid or N_2O was used to induce mitotic synchrony (Table II). Furthermore, a 15-h exposure of cells maintained in S phase to N_2O or colcemid resulted in an *m*-AMSA-induced DNA cleavage frequency comparable to that seen in S-phase cells unexposed to N_2O or colcemid (Table II). Thus, neither N_2O nor colcemid per se altered the susceptibility of cellular DNA to *m*-AMSA. Finally, the *m*-AMSA-induced DNA cleavage frequency in mitotically enriched cells (mitotic index 60–70%) obtained with the use of selective detachment was about the same as that observed in cells with a similar mitotic index obtained with N_2O (data not shown).

Uptake of [^{14}C]-m-AMSA. The hypersensitivity of the DNA of mitotic cells to *m*-AMSA-induced cleavage could have resulted from an enhanced ability of mitotic cells to accumulate the drug. However, after exposure to 0.1 μM [^{14}C]-*m*-AMSA the amount of intracellular drug was the same in mitotic and in asynchronously growing cells (data not shown). The latter consist primarily of cells in S (45%), G_1 (40–45%) and G_2 (10%) (Rao & Engelberg, 1966). Exposure to higher doses produced on the average slightly higher intracellular amounts of *m*-AMSA in mitotic cells, but the differences between asynchronously growing cells and mitotic cells in this regard were minimal compared with the differences in drug-induced DNA cleavage between the two cell types (data not shown).

Biochemical Basis for the Hypersensitivity of Mitotic Cells to m-AMSA-Induced DNA Cleavage. The DNA cleavage induced by *m*-AMSA in whole cells (including asynchronously growing HeLa cells; Zwelling et al., 1987) that is presumably mediated by topoisomerase II is characteristically protein concealed; i.e., cleavage is only detected if protein-denaturant treatment is used (Zwelling et al., 1981). The *m*-AMSA-induced DNA cleavage detected in mitotic cells was also protein concealed (data not shown), suggesting that the DNA cleavage produced in mitotic cells after exposure to *m*-AMSA was also mediated by topoisomerase II.

Etoposide is another drug that interacts with purified mammalian topoisomerase II and that, presumably as a consequence, produces protein-associated DNA cleavage in whole cells (Chen et al., 1984; Ross et al., 1984). The frequency of etoposide-induced DNA cleavage was 3–8-fold greater in mitotic than in S-phase cells (data not shown). Thus, the DNA of mitotic cells is hypersensitive to both *m*-AMSA- and etoposide-induced cleavage. Unlike *m*-AMSA, etoposide does not bind to DNA (Ross et al., 1984). Thus the cell cycle stage dependent variations in drug-induced DNA cleavage reflected cell cycle stage dependent variations in the drug–topoisomerase II–DNA interaction rather than alterations in the drug–DNA interaction.

We next quantified decatenating (i.e., topoisomerase II) activity in extracts prepared by sonicating mitotic, G_1-phase, S-phase, and G_2-phase cells (defined as in Table I) in a 350 mM NaCl–containing buffer. This NaCl concentration was chosen because the DNA of nuclei extracted with 350 mM NaCl is resistant to *m*-AMSA-induced cleavage (Filipski et al., 1983). Thus, that topoisomerase II extracted with 350 mM NaCl is presumably the topoisomerase II that interacts with *m*-AMSA. Results using both the centrifugation assay and the gel assay (see Experimental Procedures) indicated that any excess of enzyme activity extracted from mitotic cells relative to interphase cells at this NaCl concentration could not fully account for the differences in drug-induced DNA cleavage observed in mitotic cells relative to G_1, S, or G_2 cells (compare Figure 2 with Table I). Furthermore, differences in the amount of *m*-AMSA-stimulated SV40 DNA cleavage between mitotic and S extracts (compared by use of amounts of extract that produced equivalent amounts of decatenation of kDNA) were trivial compared to the differences in *m*-AMSA-stimulated cleavage seen between mitotic and S-phase cells (compare Figure 3 with Table I). As expected, if extract-mediated drug-stimulated DNA cleavage resulted from a drug–topoisomerase II interaction (Liu et al., 1983), cleavage was not detected if EDTA rather than SDS and proteinase K was used to stop the reaction between SV40 DNA, *m*-AMSA, and extract (data not shown).

m-AMSA-Induced Cytotoxicity at Different Cell Cycle Stages. In several cellular systems manipulations that affect the quantity of *m*-AMSA-induced, topoisomerase II mediated DNA cleavage affected in parallel fashion the drug's cytotoxic potency (Pommier et al., 1986; Glisson et al., 1986a,b; Bakic et al., 1986). We determined the phase in the cell cycle when cells were most susceptible to the cytotoxic effects of *m*-AMSA. Following a 30-min exposure to *m*-AMSA, the colony-forming ability of S-phase cells was 5% of that observed for untreated S-phase cells (Figure 4). The *m*-AMSA

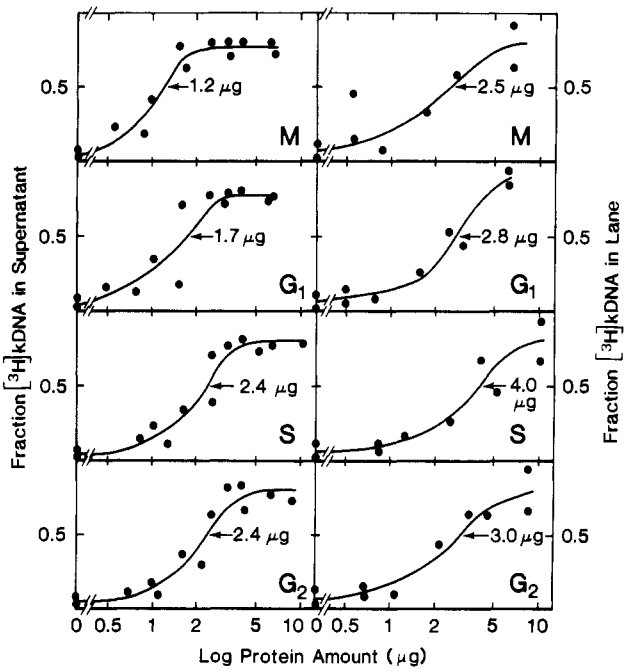

FIGURE 2: Decatenating activity of cellular extracts from HeLa cells in various cell cycle stages. The various cell populations were obtained as described in Table I. Extracts were prepared as described under Experimental Procedures. The "centrifugation assay" (left half of figure) and the "gel assay" (right half of figure) were carried out as described under Experimental Procedures. That amount of extract required to produce 50% decatenation of the [^{3}H]kDNA starting material was taken as the measure of topoisomerase II activity in the extract.

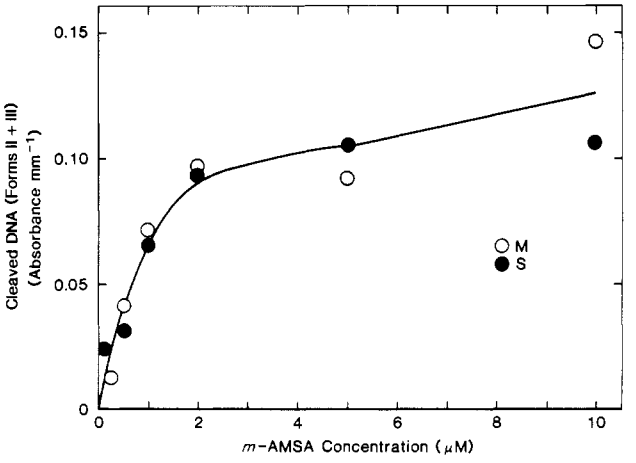

FIGURE 3: Production of DNA cleavage by *m*-AMSA with cellular extracts from mitotic and S-phase HeLa cells. Mitotic and S-phase cells were obtained as described in Table I. Extracts were prepared as described under Experimental Procedures and were compared by use of amounts of protein that produced equivalent amounts (80%) of decatenation of kDNA (lesser amounts of decatenating activity did not result in readily detectable cleavage in either extract). Reaction conditions were as described under Experimental Procedures. Nicked DNA (form II) and DNA cleaved in both strands (form III) were quantified by densitometric scanning of the photographic negative of an agarose gel that was prepared in ethidium bromide (0.5 μg mL^{-1}). The use of ethidium bromide permitted the resolution of form II DNA and form I^0 DNA (relaxed closed circular DNA).

treatment reduced colony formation to 35% of control values in mitotic cells (Figure 4). In general, there was no correlation between *m*-AMSA-induced DNA cleavage and *m*-AMSA-induced cytotoxicity (compare Table I and Figure 4). We noted that in the absence of drug the colony-forming efficiency of cells in mitosis or G_1 was lower than the colony-forming efficiency of cells in S or G_2 (25 and 42%, respectively, for the former and 77 and 100%, respectively, for the latter). These differences may have reflected the use of N_2O to obtain cells in mitosis and G_1 in these experiments, because N_2O is known to cause the formation of multipolar mitotic spindles, a potentially lethal lesion (Brinkley & Rao, 1973). Accordingly, an experiment was performed in which all the cell populations were derived from an original N_2O-synchronized population (as had been done in the experiment illustrated in Figure 1). The colony-forming efficiencies of all untreated cells were approximately 25%. The addition of *m*-AMSA reduced colony-forming efficiency most extensively in cells that had been removed 9 h previously from N_2O, a time corresponding to early S phase (Figure 1).

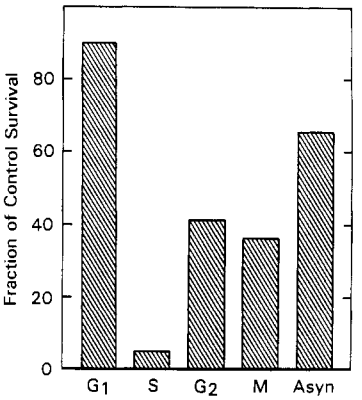

FIGURE 4: *m*-AMSA sensitivity of HeLa cell populations at different cell cycle stages. Synchronizing conditions were as described in the legend to Table I. Treatments with *m*-AMSA were at 0.5 μM for 30 min at 37 °C. After treatment, cells were replated at various low cell densities (100, 1000, and 5000 cells per plate) and were allowed to form colonies over 9–12 days. Colony-forming efficiencies of untreated cells were 42% in G_1 phase, 77% in S phase, 100% in G_2 phase, 25% in mitosis, and 72% in asynchronously growing (see text).

Disappearance of m-AMSA-Induced DNA Cleavage. If cytotoxicity and protein-associated DNA cleavage are mechanistically related, a lower frequency of drug-induced DNA cleavage might be expected to be associated with greater cytotoxicity in S phase if the DNA cleavage effect persisted longer following drug removal in S phase than in mitosis. However, when we compared the rates of disappearance of DNA cleavage in S-phase cells and in mitotic cells at various times following a 30-min *m*-AMSA treatment, DNA cleavage disappeared somewhat more quickly in S-phase cells than in mitotic cells during the 30-min period immediately following removal from *m*-AMSA (data not shown). When cells were left in drug-free medium for up to 2 h following removal from *m*-AMSA, there was no appreciable difference in the amount of DNA cleavage remaining in cells treated in S phase as compared with those treated in mitosis (data not shown). (In order to produce an equivalent initial DNA cleavage frequency in the two cell populations, S-phase cells were exposed to a 5-fold higher concentration of *m*-AMSA than were mitotic cells.)

Discussion

As measured by DNA alkaline elution, the sensitivity of HeLa cell DNA to *m*-AMSA-induced cleavage varied during the cell cycle. Cleavage was 4–15-fold greater in mitotic cells than in cells in S and was intermediate in cells in G_1 or G_2 (Figure 1 and Table I). For reasons noted above, these findings are unlikely to represent artifacts resulting from the manipulations used to obtain the various cell populations (Table II). Similarly, they are unlikely to result from differences in intracellular *m*-AMSA accumulation.

DNA cleavage in *m*-AMSA-treated cells is believed to be mediated by topoisomerase II (Minford et al., 1986). The *m*-AMSA-stimulated DNA cleavage that we observed was protein-concealed, consistent with the cleavage being topoisomerase II mediated. Thus, cell cycle stage dependent fluctuations in *m*-AMSA-induced DNA cleavage in whole cells are likely to be secondary to alterations in the enzyme (topoisomerase II), the substrate (chromosomal DNA), or the interactions of either with the drug.

Work with yeast has suggested that topoisomerase II is critically important in mitosis because it is necessary for chromosome segregation (Holm et al., 1985; DiNardo et al., 1984). Although the hypersensitivity of mitotic cells to drug-induced topoisomerase II mediated DNA cleavage may also reflect the importance of the enzyme during mitosis in HeLa cells, we were unable to unequivocally establish a correlation between topoisomerase II activity in 350 mM NaCl sonicates of mitotic cells and this hypersensitivity (Table I and Figure 2). Similarly, the interaction between drug and available topoisomerase II activity appeared comparable in mitotic and S sonicates (Figure 3); i.e., comparable decatenating activity produced comparable cleavage. The discrepancies between results in sonicates and results in whole cells could be due to differences in extractability of the enzyme at 350 mM NaCl at different cell cycle stages. Alternatively, these discrepancies could result from the extraction in sonicates from some but not other cell cycle stages (a) of condensing or decondensing substances that could affect measured decatenating activity or (b) of proteins, e.g., kinases that regulate topoisomerase II activity (Ackerman et al., 1985; Sander et al., 1984; Sahyoun et al., 1986). Finally, it is possible that other pools of topoisomerase II in addition to that extracted at 350 mM NaCl interact with *m*-AMSA, and it is these pools that fluctuate during the cell cycle. In this regard although no differences in 2 M extractable topoisomerase II activity were observed between mitotic, G_1-phase, and S-phase mouse embryo fibroblasts (Tricoli et al., 1985), the drug reactivity of that enzyme that remains associated with chromosome scaffolds even at ≥2 M NaCl (Earnshaw et al., 1985) is unknown.

Etoposide is a compound that like *m*-AMSA is believed to interact with topoisomerase II (Chen et al., 1984; Ross et al., 1984) but, unlike *m*-AMSA, is not believed to bind to DNA (Ross et al., 1984). Because the DNA of mitotic cells was hypersensitive to etoposide- and well as *m*-AMSA-induced cleavage, cell cycle stage dependent alterations in the interaction between drug and DNA appear to be an unlikely explanation for the results we observed in whole cells. It remains possible that cell cycle stage dependent alterations in chromatin structure per se account for the hypersensitivity of mitotic DNA to topoisomerase II reactive compounds. Changes in chromatin structure have been proposed to underlie the enhanced *m*-AMSA-stimulated DNA cleavage seen following pretreatment of L1210 cells with antimetabolites such as cytosine arabinoside, hydroxyurea, and 5-azacytidine (Zwelling et al., 1984; Minford et al., 1984).

Although the biochemical mechanism underlying the hypersensitivity of mitotic DNA to *m*-AMSA-induced cleavage thus remains unclear, we were surprised by the failure of this hypersensitivity to translate into a hypersensitivity of mitotic cells to the cytotoxic effects of *m*-AMSA (Figure 4). This failure did not appear to result from differences in the rates of disappearance of drug-induced DNA cleavage. Previous work has generally revealed a parallel between alterations in protein-associated, drug-stimulated DNA cleavage and alterations in drug-induced cytotoxicity. This has been true whether the alterations in cleavage have been putatively due to alterations in chromatin structure (as in the case of antimetabolites) or to alterations in the interaction of drug with topoisomerase II (as in the case of pairs of sensitive-resistant cell lines; Estey et al., 1986; Glisson et al., 1986a,b). On the other hand, we had previously demonstrated that *m*-AMSA-induced DNA cleavage was more enhanced in proliferating human brain tumor cells compared with their quiescent counterparts than was evident in similar populations of human fibroblasts. However, proliferating cells of both cell types showed approximately the same relative increase in *m*-AMSA-induced cytotoxicity relative to their quiescent counterparts (Zwelling et al., 1987). This work makes it clear that the relationship between drug-induced, topoisomerase II mediated cleavage and drug-induced cytotoxicity is complicated. It is likely that supravening events are requisite to translate the former into the latter.

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A Study of Ligand Binding to Spleen Myeloperoxidase†

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ABSTRACT: The ligand binding properties of spleen myeloperoxidase, a peroxidase formerly called "the spleen green hemeprotein", were studied as functions of temperature and pH, using chloride and cyanide as exogenous ligands. Ligand binding is influenced by a proton dissociable group with a pK_a of 4. The protonated, uncharged form of cyanide binds to the unprotonated form of the enzyme, while chloride ion binds to the enzyme when this group is protonated. In both cyanide and chloride binding, the pH-dependent change in the apparent ligand affinity is due to a change in the apparent association rate with pH. The proton dissociable group on the enzyme involved in ligand binding has a ΔH value of about 8 kcal·mol^{-1}. The present results suggest that this ionizable group is the imidazole group of a histidine residue located near the ligand binding site.

The presence of a green peroxidase in bovine spleen, with optical absorption properties somewhat similar to those of myeloperoxidase isolated from granulocytes, was first reported by Davis and Averill (1981), who named the enzyme "green heme peroxidase". The enzyme was called "the spleen green hemeprotein" in the paper of Babcock et al. (1984). Resonance Raman data indicate that the structure of the chromophore of the spleen green hemeprotein is identical with that of myeloperoxidase, an iron chlorin (Babcock et al., 1984; Sibbett & Hurst, 1984; Ikeda-Saito et al., 1985). The optical absorption, electron paramagnetic resonance, and ligand binding properties of the spleen enzyme are very similar to those of myeloperoxidase (Ikeda-Saito, 1985). Magnetic and natural circular dichroism studies (Sono et al., 1986b) have further indicated that the spleen green hemeprotein and myeloperoxidase have common prosthetic group environments and endogenous ligands to the central iron. These data have established that the spleen enzyme can be used as a more readily obtainable model to investigate the active center and its environment in myeloperoxidase (Ikeda-Saito, 1985). In their initial paper, Davis and Averill (1981) reported the spleen green hemeprotein as a monomeric peroxidase having a molecular weight of 57 000 with substrate specificity different from that of myeloperoxidase. Recent studies, however, have revealed that the spleen enzyme consists of two heavy subunits (M_r 6×10^4 with a single prosthetic group per subunit) and two light subunits (M_r 1.5×10^4) forming a tetramer of M_r 1.5×10^5, indistinguishable from granulocyte myeloperoxidase (Ikeda-Saito, 1986), and that the spleen enzyme also exhibits catalytic activities identical with those of myeloperoxidase (Ikeda-Saito, 1985). Together, these findings have led to the

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