Mitoxantrone Resistance in HL-60 Leukemia Cells: Reduced Nuclear Topoisomerase II Catalytic Activity and Drug-Induced DNA Cleavage in Association with Reduced Expression of the Topoisomerase II β Isoform†

W. Graydon Harker,*,‡ D. Lynn Slade,‡ Fred H. Drake,§ and Ryan L. Parr‡

Medical Service, Veterans Affairs Medical Center, and Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah 84148, and Department of Cell Sciences, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

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ABSTRACT: Mitoxantrone-resistant variants of the human HL-60 leukemia cell line are cross-resistant to several natural product and synthetic antineoplastic agents. The resistant cells (HL-60/MX2) retain sensitivity to the *Vinca* alkaloids vincristine and vinblastine, drugs that are typically associated with the classical multidrug resistance phenotype. Mitoxantrone accumulation and retention are equivalent in the sensitive and resistant cell types, suggesting that mitoxantrone resistance in HL-60/MX2 cells might be associated with an alteration in the type II DNA topoisomerases. We discovered that topoisomerase II catalytic activity in 1.0 M NaCl nuclear extracts from the HL-60/MX2 variant, as measured by the decatenation of *Crithidia fasiculata* kinetoplast DNA, was reduced 4- to 5-fold compared to that in the parental HL-60 cells. Total cellular topoisomerase II activity in HL-60/MX2 cells was only 50% lower than that in HL-60 cells, however, because the "cytosolic fraction" of the HL-60/MX2 nuclear preparation contained high levels of decatenating activity. Antisera to calf thymus topoisomerase II defined a distinctive immunoreactive pattern of topoisomerase II proteins in crude nuclear extracts from the HL-60/MX2 cells. Both α (170 kDa) and β (180 kDa) forms of topoisomerase II were detected in the HL-60 cell extracts, but only the α form was detected in extracts from HL-60/MX2 cells. This finding was associated with the appearance of a new 160-kDa immunoreactive species in nuclear extracts from HL-60/MX2 but not HL-60 cells. Studies were designed to minimize the proteolytic degradation of the topoisomerase II enzymes by extraction of whole cells with hot SDS. Both α and β isoforms of topoisomerase II were detected in HL-60 cells extracted by this method. The HL-60/MX2 cell extracts, however, contained the 170- and 160-kDa species, but not the 180-kDa form. When nuclear extracts from the two cell types were normalized for equivalent catalytic activity, mitoxantrone inhibited the decatenation of kDNA by these extracts to an equal extent but levels of mitoxantrone-induced cleavage of ^{32}P-labeled pBR322 DNA by nuclear extracts from HL-60/MX2 cells were 3- to 4-fold lower than in comparable HL-60 extracts. Resistance to the topoisomerase II inhibitor mitoxantrone in HL-60/MX2 is associated with reduced nuclear and whole cell topoisomerase II catalytic activity, immunologically undetectable levels of the 180-kDa topoisomerase II isozyme, and reduced mitoxantrone-induced cleavage of radiolabeled DNA by topoisomerase II in nuclear extracts from these cells.

DNA type II topoisomerases are enzymes that regulate the topologic state of DNA by a process in which DNA segments are passed through transient enzyme-bridged double-strand DNA breaks. These enzymes appear to be closely associated with chromosomal structures and are thought to play a crucial role in cellular processes such as DNA synthesis, transcription, and recombination [for reviews, see Wang (1985), Vosberg (1985), and Osheroff (1989)]. Two forms of functional topoisomerase II have been identified in murine and human cells (Drake et al., 1987, 1989). The two enzyme isoforms, which are encoded by separate genes, differ in molecular mass (170 kDa vs 180 kDa) as well as other biochemical and pharmacologic properties (Drake et al., 1989; Chung et al., 1989). While proliferation and cell cycle dependent differences in expression of the 170- and 180-kDa forms of topoisomerase II (also known as p170 and p180 or topoisomerase II α and β, respectively) have been described in some cell types (Woessner et al., 1990, 1991), the respective roles that these two enzyme isoforms play in the cellular functions noted above are not known.

Type II topoisomerases have recently been identified as the intracellular targets for a number of important antineoplastic agents. These include DNA intercalating agents such as doxorubicin, amsacrine, and mitoxantrone, as well as agents that lack intercalating activity, such as etoposide and VM-26 (teniposide) [for review, see Liu (1989)]. The topoisomerase II mediated DNA strand breaks which are induced by these agents are thought to be responsible for the dose-dependent killing of human tumor cells (Zhang et al., 1990). There appears to be a rough correlation between the cellular level of topoisomerase II enzyme and the in vitro sensitivity to topoisomerase II active antitumor agents, cells displaying low levels of topoisomerase II being less sensitive to drug than cells with high enzyme levels (Davies et al., 1988; Tan et al., 1987; Potmesil et al., 1988; Smith & Makinson, 1989). Concepts regarding the role that the topoisomerase II enzymes play in determining cellular sensitivity to the antineoplastic agents will likely change as a result of the discovery of the two distinct

† Supported by the Department of Veterans Affairs Medical Research.
* Author to whom correspondence should be addressed at Medical Service 111C, Department of Veterans Affairs Medical Center, 500 Foothill Dr., Salt Lake City, UT 84148.
‡ University of Utah School of Medicine.
§ SmithKline Beecham Pharmaceuticals.

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topoisomerase II isoforms. For instance, the purified α and β enzymes have been found to differ in their in vitro sensitivity to the antineoplastic agent teniposide (Drake et al., 1989). In addition, data obtained from studies in NIH-3T3 and *ras*-transformed NIH-3T3 cells suggest that both cellular topoisomerase II α content and the ratio of the cellular levels of the α and β forms of the enzyme are important factors in determining cellular sensitivity to topoisomerase II directed agents (Woessner et al., 1990).

We have recently described a variant of the human leukemia cell line, HL-60, which was selected for resistance to the anthracenedione compound mitoxantrone (Harker et al., 1989a). The HL-60/MX2[1] cells display cross-resistance to a number of antineoplastic agents in vitro but retain sensitivity to the *Vinca* alkaloids. The multiple drug resistance (MDR) demonstrated in these cells is not associated with overexpression of the multidrug transporter, P-glycoprotein, and [^{14}C]mitoxantrone accumulation and retention are identical in the drug-sensitive and -resistant cell types. We postulated, therefore, that resistance to mitoxantrone in HL-60/MX2 cells might be related to an alteration in DNA topoisomerase II. Here we report that nuclear extracts prepared from mitoxantrone-resistant HL-60/MX2 cells contain markedly reduced levels of topoisomerase II catalytic activity when compared to the drug-sensitive HL-60 cells. The decrease in topoisomerase II catalytic activity in HL-60/MX2 extracts is associated with the absence of detectable p180 on immunoblots and the appearance of a new 160-kDa immunoreactive species which is not recognized by p180-specific antisera. In addition, while the topoisomerase II catalytic activity in crude nuclear extracts from the two cell types is equally sensitive to mitoxantrone inhibition, levels of drug-induced, topoisomerase II mediated cleavage of DNA by the enzymes in HL-60/MX2 crude nuclear extracts are much lower than in extracts from parental HL-60 cells.

Experimental Procedures

Cells and Cell Culture. The human HL-60 myeloid leukemia cell line and subcloned variant HL-60/MX2, which is approximately 35-fold resistant to mitoxantrone, were propagated as previously described (Harker et al., 1989a). For all experiments cells were grown in mitoxantrone-free medium for a minimum of 1 week prior to use and were harvested in the logarithmic phase of growth. Cultures of these cells were found to be free of mycoplasma contamination when tested with the GEN-PROBE mycoplasma hybridization kit (GEN-PROBE, Inc., San Diego, CA).

Chemicals and Reagents. Mitoxantrone was a gift from Lederle Laboratories (Pearle River, NY). Bleomycin was a gift from Bristol-Myers Co. (Evansville, IN). Doxorubicin was provided by Adria Laboratories (Columbus, OH). Dactinomycin, *m*-AMSA, etoposide, teniposide, bisantrene, daunorubicin, melphalan, cisplatin, merbarone, mitomycin C, vincristine, and vinblastine were obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. Drugs were prepared by dissolving in water, DMSO, or 0.9% NaCl solution with all dilutions made in 0.9% NaCl or water. Rabbit antiserum A17, prepared against the 3′ end of recombinant mammalian DNA topoisomerase II, was a generous gift from Dr. Leroy F. Liu of the Johns Hopkins University (Baltimore, MD). Purified p170 and p180 topoisomerase II enzymes from P388 leukemia cells and rabbit polyclonal antibody against these two enzyme isoforms were prepared as previously described (Drake et al., 1987; Chung et al., 1989). [*methyl*-^{3}H]Thymidine (sp act. 65–85 Ci/mmol) and [α-^{32}P]deoxyadenosine 5′-triphosphate (sp act. 3000 Ci/mmol), were purchased from ICN Radiochemicals (Irvine, CA). RPMI 1640 medium, L-glutamine, fetal bovine serum (FBS), plasmid pBR322, Klenow fragment of DNA polymerase I, and the restriction enzyme *Hin*dIII were purchased from GIBCO/BRL (Gaithersburg, MD). Agarose was purchased from FMC Bioproducts (Rockland, ME). Human immunoglobulin adsorbed, alkaline phosphatase linked, goat anti-rabbit IgG (heavy and light chain) was purchased from Tago, Inc. (Burlingame, CA). Reconstituted recombinant topoisomerase II from bacteriophage T2 was a kind gift from Dr. Wai Mun Huang of the University of Utah. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Chemosensitivity Testing. The in vitro sensitivity of the HL-60 and HL-60/MX2 cells to mitoxantrone and other chemotherapeutic agents was defined by utilizing a previously described modification (Harker et al., 1989a) of the MTT assay described by Mosmann (1983).

Preparation of DNA Substrates. Cultures of the protozoan *Crithidia fasiculata* (provided by Dr. Raymond Warters, University of Utah) were grown for 72 h at 27 °C in brain heart infusion medium (DIFCO Laboratories, Detroit, MI) supplemented with hemin (20 μg/mL), penicillin (1000 units/mL), and streptomycin (1000 μg/mL). The kinetoplast DNA (kDNA) was isolated from these organisms by cesium chloride density gradient centrifugation according to the method of England (1978). [^{3}H]kDNA was prepared by incubating *C. fasiculata* in culture medium containing [*methyl*-^{3}H]deoxythymidine (82.7 Ci/mmol) at a concentration of 5 μCi/mL. When a cell density of (1.8–2.2) $\times$ 10^8 cells/mL was reached (typically 48–72 h in culture), the cells were harvested and the radiolabeled kDNA was isolated as described above. The final kDNA preparations had a specific activity of (4.5–5.2) $\times$ 10^4 dpm/μg of DNA. Plasmid pBR322 DNA was purified by a standard cleared lysate procedure (Clewell & Helsinki, 1969) followed by banding in cesium chloride equilibrium gradients in the presence of ethidium bromide. The plasmid pBR322 DNA utilized for the induced cleavage reaction was purchased from GIBCO/BRL.

Preparation of Nuclear Extracts. Nuclear extracts from the HL-60 and HL-60/MX2 cell lines were prepared according to the method of Sullivan et al. (1987) as modified by Danks et al. (1988). In order to minimize proteolysis during the extraction process additional protease inhibitors (benzamidine, soybean trypsin inhibitor, leupeptin, pepstatin, and aprotinin) were prepared just prior to each experiment and added to buffers G, H, I, J, and M at the final concentrations described by Constantinou et al. (1989). The protein contents of the nuclear extracts were determined by the method of Bradford (1976). Nuclear extracts were stored at −70 °C for periods up to 30 days without perceptible loss of topoisomerase II activity. Fresh preparations served as the source for the topoisomerase II immunoblotting studies while both fresh and stored preparations were used for measurements of topo-

[1] Abbreviations: MDR, multiple drug resistance or multidrug resistance; HL-60/MX2, cloned HL-60 subline which is ~35-fold resistant to mitoxantrone; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide or amsacrine; IC_{50}, drug concentration that inhibits cell growth by 50%; TPBS, Tris phosphate buffered saline; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing Tween 20; Dox, doxorubicin; SDS, sodium dodecyl sulfate; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloroindoxyl phosphate; kDNA, kinetoplast DNA; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

isomerase II and topoisomerase I activity.

Immunochemical Detection of Topoisomerase II. Samples of fresh nuclear extracts (100 μg/lane) were electrophoresed on 7% SDS–polyacrylamide gels according to the method of Laemmli (1970). The proteins were transferred to nitrocellulose paper overnight (12–16 h at 400 mA) at 4 °C in a model TE 50 Transphor electrophoresis unit (Hoefer Scientific, San Francisco, CA). Detection of transferred proteins was performed essentially as described by Towbin et al. (1979). Blocked nitrocellulose blots were probed at 37 °C for 2 h with the anti-topoisomerase II rabbit antiserum A17 and then incubated with an alkaline phosphatase linked goat anti-rabbit antibody (to heavy and light chain determinants) for 60 min at room temperature. The blots were then developed using NBT and BCIP as the chromogenic substrates. Nuclear extract proteins from HL-60 and HL-60/MX2 cells, which had been electrophoretically separated and transferred to nitrocellulose, were also examined using the p180-specific antiserum.

Immunoblotting techniques were also used to compare total cell levels of topoisomerase II in cells extracted with hot SDS as described by Drake et al. (1989). The cell lysate proteins were electrophoretically separated by SDS–PAGE (100 μg of protein/lane) and transferred to nitrocellulose for immunological detection as described above.

Assays of Topoisomerase II Activity. The topoisomerase II catalytic activity in nuclear extract and hypotonic wash fractions of cellular preparations was assayed by the ATP-dependent decatenation of *C. fasiculata* kinetoplast DNA (kDNA). The decatenation assay was a modification of an earlier report (Marini et al., 1980). The 15-μL assay volume contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50–85 mM KCl, 5 mM EDTA, 5 mM DTT, 15% (v/v) glycerol, 1 mM ATP, 0.4–0.8 μg of kDNA, and various amounts of either crude nuclear extract or recombinant bacteriophage T2 topoisomerase II. Following incubation at 37 °C for 30 min the reaction was stopped by the addition of 5 μL of a 2.5% (w/v) SDS solution. The entire sample was loaded onto a 1% agarose gel and electrophoresed for 16 h in Tris–borate–EDTA buffer. The gel was stained in 1.0 μg/mL ethidium bromide, destained, and photographed under UV visualization.

For quantitative studies of nuclear extract topoisomerase II activity we utilized a modification of the [^{3}H]kDNA decatenation assay developed by Sahai and Kaplan (1986). The reaction mixture (40 μL) contained 50 mM Tris-HCl, pH 7.5, 85 mM KCl, 10 mM $MgCl_2$, 1.0 mM DTT, 1.0 mM EDTA, 1.0 mM ATP, 10% (v/v) glycerol, 0.5–1.0 μg of [^{3}H]kDNA, and various amounts of nuclear extract or purified T2 topoisomerase. After incubation at 37 °C for 30 min the reaction was stopped by adding 5 μL of 2.5% (v/v) SDS. The samples were centrifuged at 12000*g* for 10 min, following which 35-μL aliquots of the supernatant were removed for either scintillation counting or electrophoretic analysis. For studies evaluating the effect of drugs on kDNA decatenation by topoisomerase II in crude nuclear extracts, 4 μL of 10× drug or appropriate drug diluent replaced an equal volume of water in the reaction mixture.

Assays of Topoisomerase I Activity. The ATP-independent relaxation of plasmid pBR322 DNA by nuclear extracts from HL-60 and HL-60/MX2 cells was performed as described by Constantinou et al. (1989).

Quantitative Precipitation of the Covalent Topoisomerase II–DNA Complex. These studies, which quantify the precipitation of single-stranded, uniquely 3′-end-labeled plasmid pBR322 DNA, were performed as previously described by Nelson et al. (1984). For experiments using nuclear extracts from HL-60 and HL-60/MX2 cells the results are expressed as a ratio of the amount of DNA precipitated in the presence of drug to that precipitated without drug.

Table I: Cross-Resistance Patterns of HL-60 Leukemia Cells Selected for Resistance to Mitoxantrone

drug	HL-60 IC_{50} (μg/mL)	degree of resistance,[a] HL-60/MX2 cells
mitoxantrone	0.023 ± 0.016[b]	35
amsacrine	0.051 ± 0.030[c]	32
teniposide	0.039 ± 0.002	24
etoposide	0.361 ± 0.168	15
merbarone	7.38 ± 2.55	8
bisantrene	0.106 ± 0.064	6
daunorubicin	0.030 ± 0.006	4
doxorubicin	0.080 ± 0.044	4
dactinomycin	0.025 ± 0.011	2
vincristine	0.006 ± 0.003	1
vinblastine	0.008 ± 0.006	1
mitomycin C	0.032 ± 0.008	1
melphalan	0.383 ± 0.168	1
cisplatin	0.231 ± 0.027	1
bleomycin	5.71 ± 1.75	0.3

[a] Expressed as a ratio of the drug concentration (HL-60/MX2:HL-60) inhibiting cell growth by 50% (IC_{50}) as measured by the MTT assay. [b] Mean ± SD of 12 experiments. [c] Values are means ± SD of 3–8 experiments.

Results

Cross-Resistance Patterns of HL-60/MX2 Cells. The HL-60/MX2 cells are 35-fold resistant to mitoxantrone and display cross-resistance to many of the agents in the "classical MDR" phenotype, with the exception of the *Vinca* alkaloids vinblastine and vincristine, melphalan, and mitomycin C (see Table I). The cells are highly cross-resistant to amsacrine (32-fold) and moderately cross-resistant to tenoposide and etoposide (24- and 15-fold, respectively), agents which like mitoxantrone have been demonstrated to inhibit type II topoisomerase activity in vitro (Tewey et al., 1984). While there is also cross-resistance to other type II topoisomerase inhibitors, such as merbarone, doxorubicin, daunorubicin, and bisantrene, the levels of cross-resistance to these agents is quite low. The reason for the differences in degree of cross-resistance to these various agents is not known.

Topoisomerase II and I Catalytic Activities in Nuclear Extracts and Hypotonic Wash Preparations. One of the mechanisms of action described for the drugs to which the HL-60/MX2 cells display resistance is an inhibitory action on topoisomerase II. Thus the development of resistance might result from either alterations in cellular topoisomerase II protein content or activity or through altered drug interactions with the topoisomerase II–DNA complex. We compared the topoisomerase II catalytic activities contained in crude nuclear extracts derived from HL-60 and HL-60/MX2 cells using assays involving both unlabeled and radiolabeled forms of *C. fasiculata* kDNA. While nuclear extracts from both the HL-60 and HL-60/MX2 cells displayed topoisomerase II catalytic activity, there were differences in the amount of activity per microgram of nuclear protein. As seen in Figure 1, the catenated kDNA network was completely resolved into individual minicircles by the addition of as little as 0.125 μg of the HL-60 nuclear protein to the reaction mixture, whereas maximal decatenation was not seen with the HL-60/MX2 extract material until reaction mixtures contained 0.75–1.0 μg of nuclear protein. As described by Danks et al. (1988), kinetoplast decatenation was inhibited by the addition of larger quantities of nuclear extract protein (in our case over 2.0 μg)

Table II: Topoisomerase II Catalytic Activity in Nuclear Extracts and Hypotonic Wash Fractions from Mitoxantrone-Sensitive and -Resistant HL-60 Leukemia Cells

	topoisomerase II catalytic activity[a] (units/10^6 cells)					
	nuclear extract		hypotonic wash[b]		whole cell[c]	
experiment[d]	HL-60	HL-60/MX2	HL-60	HL-60/MX2	HL-60	HL-60/MX2
1	171.8	71.6	0	50.8	171.8	122.4
2	153.7	42.6	13.9	48.8	167.6	91.4
3	89.7	18.5	12.4	39.4	102.1	57.9
4	188.4	45.0	5.8	26.2	194.2	71.2
5	140.6	34.4	13.0	31.2	153.6	65.6
mean ± SD	148.8 ± 37.7	42.4 ± 19.3	9.0 ± 6.0	39.3 ± 10.7	157.9 ± 34.4	81.7 ± 25.9[e]

[a] Topoisomerase II catalytic activity was assayed quantitatively using the radiolabeled kinetoplast DNA decatenation assay described under Experimental Procedures. One unit of enzyme activity was defined as the amount of nuclear extract which resulted in a level of substrate kDNA decatenation representing 50% of that decatenated by the recombinant bacteriophage T2 topoisomerase II in 30 min at 37 °C. [b] The hypotonic wash represents the supernatant fluid removed following the 400*g* centrifugation of the cells out of the hypotonic buffer (buffer G under Experimental Procedures). [c] Sum of the topoisomerase II activity in the nuclear extract and hypotonic wash portions of each preparation. [d] The units of enzyme activity for each experiment is a mean of duplicate determinations. [e] Comparison of the mean values for HL-60 vs HL-60/MX2 whole-cell results by paired Student's *t* test, $p = 0.006$.

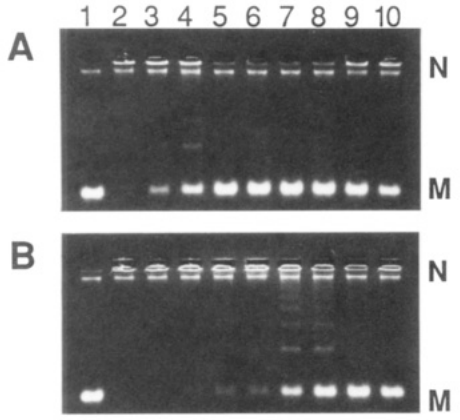

FIGURE 1: Decatenation of *C. fasiculata* kinetoplast DNA (kDNA) by 1.0 M NaCl extracts of nuclei from HL-60 (A) and HL-60/MX2 (B) cells. Various quantities of nuclear extract from the two cell types were added to reaction mixtures containing 0.8 μg of kDNA. The decatenation reaction and electrophoresis were performed as detailed under Experimental Procedures. Reaction mixtures electrophoresed in lanes 2–10 contained 0, 0.075, 0.1, 0.125, 0.25, 0.5, 0.75, 1.0, and 2.0 μg of nuclear protein, respectively. Lane 1 represents a control reaction mixture to which purified recombinant bacteriophage T2 topoisomerase II had been added. N, catenated network of minicircles; M, free minicircles.

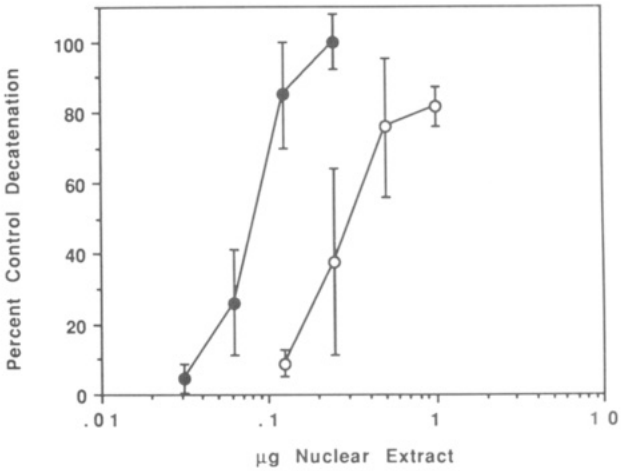

FIGURE 2: Quantitative assay of the decatenating activity of topoisomerase II in nuclear extracts from mitoxantrone-sensitive and -resistant HL-60 cells. Reaction mixtures containing ^{3}H-labeled kDNA were incubated in a final volume of 40 μL with various amounts of nuclear protein from HL-60 (●) and HL-60/MX2 (○) cells as described under Experimental Procedures. The quantity of radioactive kDNA released in reaction mixtures containing nuclear extract proteins is expressed as a percentage of that released in control reactions containing recombinant bacteriophage T2 topoisomerase II. Points represent the mean ±1 SD of 5 determinations.

from either cell type. The factor(s) responsible for this inhibition is (are) not known.

A quantitative comparison was made of the catalytic activity of the nuclear extracts from these two cell types using the tritiated kDNA decatenation assay. In preliminary studies we determined the amount of purified bacteriophage T2 topoisomerase II required in the reaction mixture to release 90–100% of the labeled kDNA. For experiments using cell extracts the amount of radiolabeled kDNA released by nuclear extract or hypowash proteins was compared to that released in control mixtures containing T2 topoisomerase II. The enzyme activity was therefore expressed as a percentage of the T2 enzyme control. For these studies, 1 unit of topoisomerase II activity was defined as the amount of nuclear extract which led to a level of substrate kDNA decatenation representing 50% of that produced by the T2 enzyme in reactions at 37 °C for 30 min. By this method we discovered that the topoisomerase II catalytic activity per microgram of protein in HL-60/MX2 nuclear extracts was 4- to 5-fold lower than in comparable HL-60 extracts (Figure 2). We also found that the hypotonic wash portion of the HL-60/MX2 nuclear preparation contained considerable quantities of kDNA decatenating activity. Topoisomerase II activity was found in the hypotonic wash of HL-60/MX2 cells irrespective of the duration of cell suspension in the hypotonic wash, the osmolality of the hypotonic solution, the use of radiolabeled versus nonlabeled kDNA substrate, or the number of protease inhibitors utilized (data not shown). Table II provides a comparison of the total cellular topoisomerase II activity (the combined catalytic activity in the hypotonic wash and nuclear extract portions) for five HL-60 and HL-60/MX2 topoisomerase II preparations. As can be seen, relatively small quantities of kDNA decatenation activity were detected in the hypotonic wash from HL-60 cells (an average of 9.0 topoisomerase II units/10^6 HL-60 cells versus 39.3 units/10^6 HL-60/MX2 cells in five separate extractions). Overall, the average total cellular topoisomerase II activity in the drug-sensitive HL-60 cells (157.9 units/10^6 cells) was found to be approximately twice that of the HL-60/MX2 cells (81.7 units/10^6 cells).

To determine if the discovery of higher quantities of topoisomerase II catalytic activity in the hypotonic wash of HL-60/MX2 cell preparations was related to a generalized "leakiness" of nuclear membranes during the hypotonic exposure, we compared the topoisomerase I catalytic activity in the nuclear extract and hypotonic wash portions of several topoisomerase II preparations from the drug-sensitive and

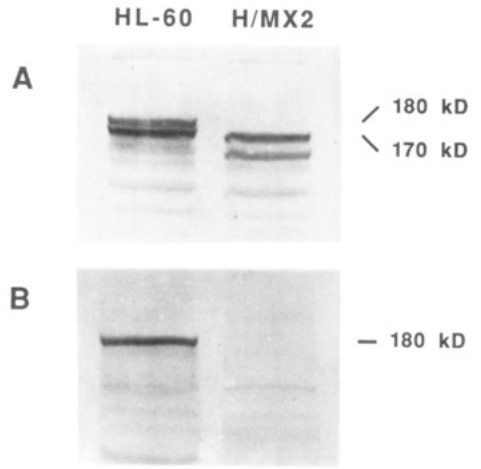

FIGURE 3: Western blot analysis of DNA topoisomerase II in nuclear extracts of HL-60 and HL-60/MX2 leukemia cells. DNA topoisomerase II was extracted from the nuclei with 1.0 M NaCl, and equal amounts of protein (100 μg/lane) were separated on 7% SDS–polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes and the immunoblots developed as described under Experimental Procedures. In panel A, the nitrocellulose blot has been developed with the A17 rabbit antiserum to recombinant topoisomerase II, which recognizes both the 170- and 180-kDa forms. In panel B, antisera specific for the 180-kDa (β) form of topoisomerase II was used to develop the blot.

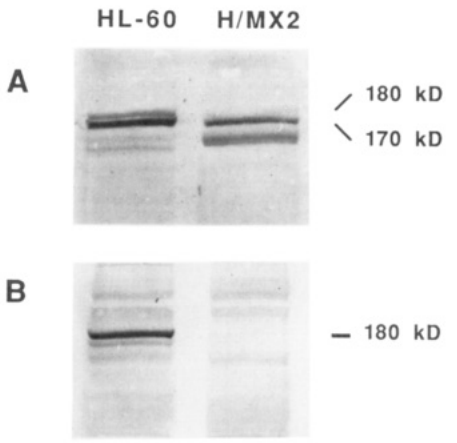

FIGURE 4: Western blot analysis of DNA topoisomerase II in whole-cell extracts from drug-sensitive and -resistant HL-60 leukemia cells. Whole-cell protein extracts were obtained from cells using the hot SDS method described under Experimental Procedures. One hundred micrograms of protein from each cell type was electrophoretically separated by SDS–PAGE, transferred to nitrocellulose membrane, and then reacted with rabbit antiserum to topoisomerase II. In panel A, the nitrocellulose blot was developed with rabbit antiserum A17, which recognizes both the 170- and 180-kDa forms of topoisomerase II. In panel B, the blot was developed with rabbit antisera specific for the 180-kDa form of topoisomerase II.

-resistant cell types. Topoisomerase I catalytic activity, as measured by the ATP-independent relaxation of supercoiled plasmid pBR322 DNA, was equivalent in the nuclear extracts from HL-60 and HL-60/MX2 cells. The addition of ≥15.6 ng of nuclear protein from either the HL-60 or HL-60/MX2 cell line resulted in the complete conversion of supercoiled pBR322 DNA to the relaxed form (data not shown). Small amounts of topoisomerase I activity were found in the hypotonic wash portions of both the HL-60 and HL-60/MX2 topoisomerase II preparations, but there was no difference in level of activity between the two cell types (data not shown).

Levels of Immunoreactive Topoisomerase II. That the levels of topoisomerase II catalytic activity in HL-60/MX2 nuclear extracts were found to be reduced compared to those in HL-60 extracts might be explained by either changes in the cellular topoisomerase II protein content or a reduction in catalytic activity per topoisomerase II molecule. Using the rabbit antiserum A17 as the primary antitopoisomerase II antibody, we measured the immunoreactive topoisomerase II levels in nuclear and hypowash preparations from both the HL-60 and HL-60/MX2 cell lines. Two prominent immunoreactive bands, which migrated at 180 and 170 kDa, were detected in the nuclear extracts from the HL-60 cells (Figure 3A). A prominent 170-kDa band was also noted in nuclear extracts from the HL-60/MX2 cells, in an amount equivalent to that seen in HL-60 extracts, but there was no demonstrable 180-kDa protein in the HL-60/MX2 nuclear extracts. Immunoblots of the nuclear extracts from the HL-60/MX2 cells did reveal the presence of a new band, however, migrating at approximately 160 kDa, which was not identified in extracts from the HL-60 cells. As noted in Figure 3a, the A17 antiserum also identified small quantities of proteins with higher electrophoretic mobility (M_r <150 000), presumably representing minor proteolytic fragments, in extracts from both the HL-60 and HL-60/MX2 cells. The pattern of protein immunoreactivity noted above (180/170-kDa bands in HL-60 and 170/160-kDa bands in HL-60/MX2) has been noted in nine enzyme preparations from these cells, using two different preparations of the A17 antiserum as well as rabbit antiserum prepared in this laboratory against gel-purified recombinant human topoisomerase II C-terminal polypeptide (data not shown). The immunoreactive pattern of the material in the hypotonic wash was also examined using the A17 rabbit antiserum. Small amounts of the 170-kDa protein were detected in the hypotonic wash portion of preps from both cell types, and a prominant 160-kDa band was invariably present in the HL-60/MX2 hypowash fraction only. There was no demonstrable 180-kDa protein species in the hypowash fractions of either cell type (data not shown).

As Aquino et al. (1990) had previously reported that the development of a doxorubicin-resistant variant of HL-60 cells was associated with enhanced Ca^{2+}-dependent proteolysis, we postulated that the absence of the 180-kDa protein and the finding of the new 160-kDa form in the drug-resistant HL-60/MX2 cells might result from proteolysis of the 180-kDa enzyme during the extraction procedure. To evaluate this possibility, we performed immunoblotting studies, using the A17 antiserum, on cellular proteins which had been extracted by the hot SDS method. In this procedure, viable cells are lysed after being washed in PBS which contained several protease inhibitors. The presence of the protease inhibitor in both the wash and extraction solutions should greatly reduce the cellular proteolysis which might occur during the wash or extraction process. The patterns of the topoisomerase II proteins detected by the A17 antiserum in cells extracted by the hot SDS method were identical to the pattern seen in the 1.0 M NaCl nuclear extractions; i.e., both 170- and 180-kDa forms were found in the HL-60 cells and 170- and 160-kDa forms in HL-60/MX2 cells (Figure 4A).

Nuclear extract, hypotonic wash, and whole-cell preparations from HL-60 and HL-60/MX2 cells were also examined using rabbit antisera with specificity for the topoisomerase II β isoform. Prominent 180-kDa bands were detected in nuclear extracts from HL-60 cells, but there was no detectable 180-kDa protein in simultaneously extracted HL-60/MX2 cells (Figure 3B). Nor was the previously described 160-kDa protein detected in the HL-60/MX2 extracts by the topoisomerase II β-specific antiserum. An identical staining pattern (i.e., 180 kDa staining in HL-60 cells, but none in HL-60/MX2 cells) was noted when cellular proteins extracted from intact HL-60 and HL-60/MX2 cells by the hot SDS method

were probed with the topoisomerase II β-specific antiserum (Figure 4B). These studies suggest that the absence of the 180-kDa form and the presence of the 160-kDa form of topoisomerase II in HL-60/MX2 nuclear extracts were not preparative artifacts and likely represent the topoisomerase II enzyme forms present in intact HL-60/MX2 cells. These findings are also consistent with our recent observations that topoisomerase II β gene expression, as determined by Northern blot analysis of total cellular RNA using topoisomerase II β-specific cDNA probes, is markedly reduced in the HL-60/MX2 cells (W. G. Harker and L. S. Slade, manuscript in preparation). Thus, it appears that the reduced topoisomerase II activity noted in the nuclear extracts from mitoxantrone-resistant HL-60/MX2 cells is associated with a marked reduction, if not virtual absence, of the β form of topoisomerase II. In addition, extracts from the mitoxantrone-resistant cells contain a new 160-kDa immunoreactive protein which is detected by the A17 antiserum, but not the topoisomerase II β antiserum. Neither the origin nor the catalytic capacity of the 160-kDa species is known at this time.

Mitoxantrone Effect on the Catalytic Activity of HL-60 and MX-2 Nuclear Extracts. The effects of several antineoplastic agents on the DNA catalytic activity of nuclear extracts from both the HL-60 and HL-60/MX2 cell lines were examined using the quantitative kDNA decatenation assay. Since HL-60/MX2 nuclear extracts typically contain 4- to 5-fold less decatenating activity per microgram of nuclear protein, the amounts of nuclear extract added to these reaction mixtures were calculated to provide comparable decatenating activity. The kDNA decatenation activity in nuclear extracts from HL-60 and HL-60/MX2 cells was equally sensitive to inhibition by mitoxantrone, with a concentration of 1.0 μM resulting in a 50% decrease in the kDNA decatenating activity in nuclear extracts from both cell types (data not shown). The catalytic activity of the extracts from these two cell types was also equally sensitive to the inhibitory action of amsacrine, a drug to which the HL-60/MX2 cells are highly cross-resistant (data not shown).

Effect of Mitoxantrone on Topoisomerase II Mediated Cleavage of DNA. We compared the effect of mitoxantrone on cleavage of radiolabeled pBR322 DNA by topoisomerase II contained in nuclear extracts from HL-60 and HL-60/MX2 cells using the quantitative induced-cleavage procedure. While the catalytic activities in nuclear extracts from these two cell types were equally sensitive to mitoxantrone, we found that the DNA cleavage activity in nuclear extracts from HL-60/MX2 cells was comparatively resistant to stimulation by mitoxantrone. The addition of increasing concentrations of mitoxantrone (0.005–0.5 μM) to reaction mixtures containing HL-60 nuclear extracts led to a progressive increase in the amount of DNA precipitated relative to reactions which contained no drug (Figure 5). By contrast, there was no stimulation by mitoxantrone of the covalent enzyme–DNA complex in reaction mixtures to which equivalent amounts of HL-60/MX2 protein had been added. Nor was mitoxantrone found to significantly stimulate the precipitation of DNA in reaction mixtures to which 4- to 5-fold more HL-60/MX2 protein had been added in order to achieve a level of topoisomerase II catalytic activity comparable to that found in the HL-60 reaction mixtures (data not shown). At concentrations above 1.0 μM mitoxantrone was noted to inhibit the DNA cleavage by nuclear extracts from both cell types. At the highest noninhibitory mitoxantrone concentration used, however, we consistently noted 3- to 4-fold less DNA precipitated by topoisomerase II in nuclear extracts from the HL-60/MX2 subclone. Thus, the topoisomerase II enzymes present in the nuclear extracts from HL-60/MX2 cells appear to be resistant to the effect of mitoxantrone on the topoisomerase II mediated cleavage of pBR322 DNA.

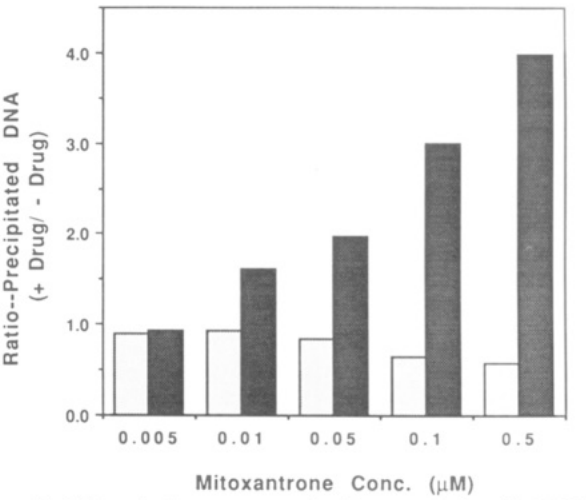

FIGURE 5: Effect of mitoxantrone on the cleavage of plasmid pBR322 DNA by topoisomerase II in nuclear extracts from HL-60 (dark hatch) and HL-60/MX2 (light hatch) cells. Reaction mixtures contained 0.1 μg of the 3′-end-labeled [^{32}P]pBR322 restriction fragment and 1.25 μg of nuclear protein from either of the two cell types. The mitoxantrone concentrations utilized in these reactions ranged from 0.001 to 1.0 μM. Shown here is a representative example ($n = 6$) of an experiment in which 1 mM ATP has been added to the reaction mixture. Data are expressed as a ratio of the amount of DNA precipitated in the presence of drug to that precipitated in the absence of drug for each cell type.

Discussion

The mechanisms responsible for the development of cellular resistance to mitoxantrone, which is a synthetic anthracenedione derivative with DNA intercalating properties, are poorly understood. The drug has been demonstrated to produce cellular protein associated DNA strand breaks in vitro as well as to stimulate DNA cleavage in the presence of purified topoisomerase II (Smith et al., 1990; Tewey et al., 1984). We have recently demonstrated that mitoxantrone can affect all of the known functions of purified recombinant bacteriophage T2 topoisomerase II, including the catalytic and ATPase activities, as well as enzyme-mediated DNA cleavage (Harker et al., 1989b). Despite the effects of this drug on topoisomerase II activity there have been no reports of the development of cellular resistance to mitoxantrone in association with an altered type II DNA topoisomerase. Murine P388 leukemia cells selected for resistance to mitoxantrone (P388/MX) display cross-resistance to several topoisomerase II active drugs, but appear to be resistant, at least in part, on the basis of altered mitoxantrone transport resulting from P-glycoprotein overexpression (Johnson & Howard, 1981; Johnson et al., 1983; W. G. Harker, unpublished experiments). Wallace et al. (1987) have reported the development of a mitoxantrone-resistant variant of the WiDr human colon carcinoma cell line which exhibits sensitivity to the *Vinca* alkaloids and does not overexpress P-glycoprotein (Dalton et al., 1988). A mitoxantrone-resistant variant of the human gastric carcinoma cell line EPG85-257 has also been described in which P-glycoprotein is not overexpressed, but these cells appear to be resistant on the basis of a complex alteration in cellular drug compartmentalization (Dietel et al., 1990). In none of these mitoxantrone-resistant cell lines has an alteration in topoisomerase II activity or content been implicated as a factor in the development of cellular resistance to mitoxantrone.

Several features of the mitoxantrone-resistant HL-60/MX2 cells, including the pattern of cross-resistance, absence of P-glycoprotein expression, and reduced levels of mitoxantrone-induced DNA strand breaks, suggested to us that altered topoisomerase II activity or sensitivity to drug might play a role in the development of resistance to mitoxantrone. Levels of topoisomerase II catalytic activity in nuclear extracts from the HL-60/MX2 cells were found to be 4- and 5-fold lower than in comparable HL-60 extracts. This appears to be related in part to altered cellular distribution of the enzyme, since large amounts of decatenating activity were consistently demonstrated in the hypotonic wash portion of the HL-60/MX2 nuclear preparations. Overall, whole-cell levels of topoisomerase II were found to be reduced by approximately 50% in the drug-resistant cells. Priel et al. (1985) previously identified topoisomerase II catalytic activity in both cytoplasmic and nuclear fractions of HL-60, K562, and U937 human leukemia cell lines, while normal peripheral blood lymphocytes contained little, if any, activity in either fraction. The authors speculated that the presence of cytoplasmic topoisomerase II activity in those rapidly proliferating tumor cells may reflect an impairment in the control of enzyme production. While topoisomerase II catalytic activity is detected in the hypotonic fraction of both HL-60 and HL-60/MX2 cells, levels of enzyme activity in that fraction are 4- to 5-fold higher in the mitoxantrone-resistant cells. These results suggest a possible "leak" of nuclear topoisomerase II into the cytoplasm during the extraction process in these cells. The finding of only small but comparable amounts of topoisomerase I activity in the hypotonic fraction from the two cell types would argue against increased permeability of the HL-60/MX2 nuclear membrane being responsible for the high levels of topoisomerase II in that fraction.

While the specific differences in subcellular topoisomerase II distribution reported here have not been noted by other investigators, Fernandes et al. (1990) have reported a decrease in DNA topoisomerase II activity and protein in the nuclear matrix fraction of CEM leukemia cells selected for resistance to VM-26. These cells, which are thought to be resistant on the basis of an altered topoisomerase II (Danks et al., 1988), displayed levels of topoisomerase II in non-matrix-associated nuclear fractions that were comparable to those of the parental CEM cells. The mechanism(s) responsible for the reduced levels of matrix-associated topoisomerase II in the drug-resistant CEM cells is (are) not known. Danks and co-workers postulate that a mutation in the topoisomerase II enzyme in these cells results in altered enzyme incorporation into or association with the nuclear matrix, thereby leading to altered drug interaction with the enzyme.

Reduced cellular topoisomerase II catalytic activity has been observed in other cell lines selected for resistance to topoisomerase II active agents (Matsuo et al., 1990; Minota et al., 1990; Deffie et al., 1989; Charcosset et al., 1988; Danks et al., 1988; Per et al., 1987; Spironidis et al., 1989; de Jong et al., 1990; Ferguson et al., 1988). Cellular p170 content has been reduced in some of these cell lines (Matsuo et al., 1990; Deffie et al., 1989), while in other models the catalytic activity is reduced in the resistant subline despite what appears to be comparable p170 enzyme content (Minato et al., 1990; Danks et al., 1988). Glisson et al. (1987) have described the presence of an altered topoisomerase II in a Chinese hamster overy cell line selected for resistance to VM-26. Despite equivalent topoisomerase II protein content and catalytic activity in crude nuclear extracts from the two cell types, purified enzyme from the drug-resistant Vpm^R-5 cells was less responsive to drug-stimulated DNA cleavage and also less stable at 37 °C than the wild-type enzyme (Sullivan et al., 1989). Similarly, Zwelling and co-workers have reported that nuclear extracts prepared from *m*-AMSA-sensitive and -resistant variants (HL-60/AMSA) of HL-60 leukemia contained equal topoisomerase II catalytic activity and protein content. When the enzymes were purified from these cells, however, marked differences were noted in their sensitivity to *m*-AMSA-induced DNA cleavage and the ATP concentrations required to achieve comparable levels of DNA cleavage (Zwelling et al., 1989).

In addition to containing reduced levels of nuclear topoisomerase II catalytic activity, the HL-60/MX2 cells are notably different from the HL-60 cells with respect to the pattern of topoisomerase II staining by Western blot analysis. Specifically, there was no demonstrable 180-kDa form of the enzyme in either nuclear extracts or hypotonic wash fractions from HL-60/MX2 cells, whereas both 170- and 180-kDa forms of the enzyme were present in nuclear extracts from the HL-60 parent. These findings were confirmed by immunoblotting studies using p180-specific rabbit antiserum to probe nuclear extracts and whole-cell extracts from the two cell types. The HL-60/MX2 cell line represents the first experimental model in which the demonstration of resistance to a topoisomerase II active agent has been associated with the finding of reduced or absent levels of the p180 enzyme. As a result, these cells may be very different from the models noted above in which drug resistance has been associated with either alterations in p170 content or sensitivity of the cellular topoisomerase II to drug-induced cleavage of DNA. Our findings regarding the p180 enzyme are difficult to interpret in the context of the prior studies, however, because in those studies the p180 isoform was not detected in either drug-resistant or -sensitive cells by the topoisomerase II specific polyclonal antiserum (Danks et al., 1988; Matsuo et al., 1990; Minota et al., 1990; Zwelling et al., 1989; Pommier et al., 1986; Sullivan et al., 1989). This is so despite the fact that Tan et al. (1988) had previously identified both p170 and p180 forms of the enzyme in nitrogen mustard sensitive and resistant Raji Burkitt lymphoma cells using polyclonal antiserum to calf thymus topoisomerase II. The demonstrated similarities in the nucleotide and putative amino acid sequences of the two forms of the enzyme (Tsai-Pflugfelder et al., 1988; Chung et al., 1989) would suggest that polyclonal antiserum developed against one of the enzyme isoforms would detect both forms of the enzyme. Our use of additional protease inhibitors during the enzyme extraction process may have facilitated our detection of the p180 isoenzyme in these studies.

The availability of type-specific antisera has led to the detection of both forms of topoisomerase II in several other leukemia and solid tumor cell lines (Chung et al., 1989; Drake et al., 1987; W. G. Harker and L. S. Slade, unpublished experiments). Drake and colleagues demonstrated both p170 and p180 enzymes in P388 murine leukemia cells selected for resistance to the intercalating agent *m*-AMSA (P388/A20) (Drake et al., 1987). Nuclear extracts from the P388 and P388/A20 cells contained equivalent p180 levels, but p170 levels were lower in the drug-resistant cells. Studies using p170 and p180 enzymes purified from the P388 and P388/A20 cells suggest that there may be differences in isoenzyme sensitivities to the antineoplastic agents. For instance, the concentration of teniposide required to inhibit the catalytic activity of the p180 form was 3-fold higher than that needed to inhibit p170 (Drake et al., 1989). The p170 enzyme was also found to be more sensitive than p180 to the effect of teniposide on the enzyme-induced cleavage of DNA. No comparisons were

reported of the drug sensitivities of either the catalytic or cleavage activities of the two forms of the enzyme from the two cell types. In a subsequent report from the same group, Woessner et al. (1990) postulated that selective loss of the most drug-sensitive form of the topoisomerase II enzyme might be sufficient to account for the cellular resistance to drug and speculated that increased drug resistance might occur as a result of a reduction in one or both forms of the enzyme. Our studies with HL-60/MX2 cells provide the first evidence that a reduction in the amount of p180 enzyme might be sufficient to explain reduced cellular sensitivity to a drug, in this case mitoxantrone.

The HL-60/MX2 cell extracts (nuclear, hypowash, and whole cell) were also noted to contain a 160-kDa immunoreactive protein species not present in comparable HL-60 extracts. The hot SDS extraction studies and immunoblotting studies with isomer-specific antisera provide evidence that the 160-kDa species does not represent a proteolytic fragment of either the p170 or p180 enzyme form. Whether or not the 160-kDa species represents a catalytically active form of the enzyme and the role that this protein plays in the resistance of HL-60/MX2 cells to mitoxantrone are unclear.

In these studies we have demonstrated that topoisomerase II catalytic activities in nuclear extracts from the mitoxantrone-sensitive and -resistant HL-60 cells are equally responsive to inhibition by mitoxantrone. The level of DNA cleavage produced by the addition of mitoxantrone to reaction mixtures containing topoisomerase II enzymes from mitoxantrone-resistant HL-60/MX2 nuclear extracts, however, was much lower than that produced by the enzymes in drug-sensitive HL-60 extracts. These results suggest that the HL-60/MX2 170-kDa enzyme is resistant to mitoxantrone stimulation of DNA cleavage, since there was no demonstrable 180-kDa enzyme in nuclear extracts from those cells. Thus, while the presence of reduced amounts of topoisomerase II catalytic activity and 180-kDa protein might adequately explain the reduced sensitivity of HL-60/MX2 cells to mitoxantrone, other alterations in the 170-kDa enzyme from these cells might contribute as well. The relative roles that the four topoisomerase II alterations demonstrated in the HL-60/MX2 cells (i.e., reduced total cellular enzyme activity; altered cellular enzyme distribution; reduced levels of the p180 isoform; and the reduced sensitivity of the p170 enzyme to mitoxantrone-induced cleavage of DNA) play in altering the cellular susceptibility to mitoxantrone is unknown. Furthermore, the drug susceptibility and enzymatic capacities of the 160-kDa species detected in HL-60/MX2 cell extracts are undefined. Studies are in progress to determine the mechanisms responsible for the reduced expression of the β form of the topoisomerase II enzyme in HL-60/MX2 cells, as well as to compare the relative drug sensitivities of the purified α forms of topoisomerase II from the drug-sensitive and -resistant HL-60 cells.

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Nucleotide-Induced Changes in the Interaction of Myosin Subfragment 1 with Actin: Detection by Antibodies against the N-Terminal Segment of Actin†

Gargi DasGupta and Emil Reisler*
Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT: The binding of myosin subfragment 1 (S-1) to actin in the presence and absence of nucleotides was determined under conditions of partial saturation of actin, up to 80%, by $F_{ab}(1-7)$, the antibodies against the first seven N-terminal residues on actin. In the absence of nucleotides, the binding constant of S-1 to actin (2×10^7 M^{-1}) was decreased by 1 order of magnitude by $F_{ab}(1-7)$. The binding of S-1 to actin caused only limited displacement of F_{ab}, and between 30 and 50% of actin appeared to bind both proteins. In the presence of MgAMP·PNP, MgADP, and $MgPP_i$ and at low S-1 concentrations, the same antibodies caused a large decrease in the binding of S-1 to actin. However, the binding of S-1·nucleotide to actin in the presence of $F_{ab}(1-7)$ increased cooperatively with the increase in S-1 concentration. Also, in contrast to rigor conditions, there was no indication for the binding of $F_{ab}(1-7)$ and S-1·nucleotide to the same actin molecules. These results show a nucleotide-induced transition in the actomysin interface, most likely related to the different roles of the N-terminal segment of actin in the binding of S-1 and S-1·nucleotide. The possible implications of these findings to the regulation of actomyosin interactions are discussed.

The generation of force and the motile action of myosin and actin are believed to involve at least two different states in which these proteins bind to each other in different orientations or have different conformations (Cooke, 1990). Clearly, the transitions between the various structural states of actomyosin must be dominated by the nucleotides which are bound to it. This perception stimulated much interest in the ATP, ADP, and nucleotide analogue induced changes in actomyosin interactions in solution and in fibers. The eventual goal of such studies is to characterize actomyosin in a state-specific manner (i.e., in relationship to particular ATP hydrolysis steps).

Solution work on acto–subfragment 1 (acto–S-1)[1] and actomyosin complexes produced ample, albeit largely qualitative

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[1] Abbreviations: S-1, myosin subfragment 1; $F_{ab}(1-7)$, affinity-purified F_{ab} fragment of polyclonal peptide antibodies raised against the first seven N-terminal residues of α-skeletal actin; AMP·PNP, adenyl-5-yl imidodiphosphate; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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