Altered DNA Topoisomerase II Activity in Chinese Hamster Cells Resistant to Topoisomerase II Inhibitors

Yves Pommier, Donna Kerrigan, Ronald E. Schwartz, Judith A. Swack, and Alison McCurdy

Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT

Most DNA intercalators and epipodophyllotoxins inhibit mammalian topoisomerase II by trapping the enzyme within DNA cleavage complexes that can be detected in cells as protein-associated DNA strand breaks. We have characterized previously a line of Chinese hamster cells (DCSF/9-OHE cells) the resistance of which to the cytotoxic effect of intercalators and etoposide is associated with a reduced formation of protein-associated DNA strand breaks. In the present study, topoisomerasees of these cells were compared to those of the parental sensitive cells (DCSF). NaCl extracts (0.35 m) of isolated DC3F/9-OHE nuclei did not form 4'- (9-acridinylamino)methanesulfon-m-anisidide-induced DNA-protein linking, whereas DCSF nuclear extracts did. In addition, DC3F/9-OHE nuclear extract had an unusually high level of DNA linking activity in the absence of 4'- (9-acridinylamino)methanesulfon-m-anisidide. Topoisomerases II from DC3F/9-OHE and DCSF nuclei appeared similar qualitatively. DC3F/9-OHE nuclear extract had approximately twice less DNA linking activity in NaCl extracts (0.35 M) of isolated DC3F/9-OHE nuclei did not form 4'- (9-acridinylamino)methanesulfon-m-anisidide. Topoisomerase II molecules than did DCSF nuclear extract but similar topoisomerase II activity. Topoisomerase I activities appeared also similar in sensitive and resistant cells. However, part of DCSF/9-OHE topoisomerase I copurified with a DNA linking activity which was not present in DCSF nuclei. This unusual DNA linking activity was not sensitive to the stimulatory effect of 4'- (9-acridinylamino)methanesulfon-m-anisidide.

INTRODUCTION

Numerous anticancer drugs as diverse as DNA intercalators and epipodophyllotoxins induce topoisomerase II-mediated cleavage of DNA in vitro (1–5) and protein-associated DNA strand breaks in mammalian cells in culture (6–8). There is good evidence that the protein-associated DNA strand breaks result from topoisomerase II-mediated cleavage of DNA and therefore that the two effects are comparable (2).

In the course of studying the cytotoxic role of drug-induced protein-associated DNA strand breaks we have used a subline of Chinese hamster cells (DCSF) resistant to DNA intercalators and epipodophyllotoxins (9). This cell line (DCSF/9-OHE) had been made resistant to 9-hydroxyellipticine by continuous exposure to the drug (10). Resistant cells (DCSF/9-OHE) have a reduced capacity to form protein-associated DNA strand breaks upon exposure to m-AMSA, 2-methyl-9-hydroxyellipticinium, and etoposide. The fact that nuclei isolated from DCSF/9-OHE cells also produced fewer protein-associated DNA strand breaks than did those from DCSF cells led us to postulate that the DCSF/9-OHE cells had modified topoisomerase II activity (9).

In the present study we have further investigated the nature of the topoisomerase II modifications in DCSF/9-OHE cells. Three mechanisms have been considered: (a) modified topoisomerase II with a reduced sensitivity of the enzyme to DNA intercalators and etoposide; (b) reduced amount of topoisomerase II; and (c) reduction of topoisomerase II sensitivity to drugs in relation with the presence of a nuclear activity which could regulate topoisomerase II. The results suggest that this last possibility is most likely because DCSF/9-OHE cells showed a modest quantitative difference in topoisomerase II but an unexpectedly large difference in a topoisomerase I.

MATERIALS AND METHODS

Cells and Materials. Chinese hamster lung cells DCSF and DCSF/9-OHE were maintained in monolayer cultures in Eagle's minimal essential medium supplemented with 10% heat inactivated fetal calf serum plus 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (GIBCO Laboratories, Chagrin Falls, OH), and penicillin streptomycin (9, 10). The resistance phenotype of DCSF/9-OHE cells has been stable in culture in the absence of 9-hydroxyellipticine for more than 1 year (10). Mouse leukemia L1210 cells were grown in suspension culture in RPMI 1630 medium supplemented with 15% fetal calf serum, 1 mM glutamine, and penicillin streptomycin (7). Exponentially growing cells were used in all experiments.

m-AMSA (NCSC 249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Etoposide (VP16-213) was a generous gift from the Bristol-Meyers Company (Syracuse, NY). m-AMSA and VP16 were dissolved in dimethyl sulfoxide at 10 mM. The m-AMSA stock solution was kept frozen at −20°C, whereas the VP16 stock solutions were prepared immediately before use.

SV40 DNA (unlabeled and labeled with [3H]thymidine), pBR322 DNA, Hind III and EcoRI restriction endonucleases, T4 polynucleotide kinase, and agarose were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Calf alkaline phosphatase was purchased from New England Biolabs; [α-32P]adenosine-5'-triphosphate and Triton X-100 were purchased from New England Nuclear Corp., Boston, MA. Sephacryl S-400 and DNA-cellulose (denatured DNA) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and Enzo Biochemicals, Inc., New York, NY, respectively. kDNA was a generous gift from Dr. Paul Englund (Department of Pharmaceutical Chemistry, Johns Hopkins School of Medicine, Baltimore, MD).

Preparation of Nuclear Extracts. The method has been described previously by Minford et al. (2). It derives from that of Filipski et al. (11). Approximately 10⁶ cells were used. Chinese hamster cells were scraped in nucleus buffer (150 mM NaCl: 1 mM KH₂PO₄: 5 mM MgCl₂: 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid: 0.2 mM dithiothreitol: 10% (v/v) glycerol: 0.1 mM phenylmethylsulfonyl fluoride, pH 6.4) and centrifuged in a Beckman J-6B centrifuge at 1200 rpm for 10 min at 4°C. The cells were washed by centrifugation twice in nucleus buffer at 4°C. The cell pellets were resuspended in 10 ml nucleus buffer at 4°C and then mixed with an additional 90 ml nucleus buffer containing 0.3% Triton X-100. The cell suspension was mixed gently by rotation for 10 min at 4°C, divided in two 50-ml tubes, and then centrifuged at 1400 rpm for 10 min at 4°C. The two nucleus pellets were washed once in Triton-free nucleus buffer, centrifuged again, and resuspended in 1 ml nucleus buffer at 4°C containing 0.35 mM NaCl (final concentration). The salt extraction was performed by gentle rotation for 30 min at 4°C. The nuclei were then spun at 1800 rpm for 20 min at 4°C. The supernatants (approximately 2 ml of salt soluble material) were collected into two Eppendorf tubes and centrifuged again at 12,000 × g for 10 min at 4°C to remove any nuclei or insoluble material. The nuclear extracts were then pooled and used immediately. One to 2 mg of protein in approximately 2 ml were usually obtained per extraction.

Received 12/4/85; revised 2/24/86; accepted 3/12/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed at National Cancer Institute, Building 37, Room 5A19, Bethesda, Maryland 20892.

2 The abbreviations used are: m-AMSA, 2-methyl-9-hydroxyellipticinium; kDNA, kinetoplast DNA; TGT, 50 mM Tris-HCl (pH 7.4): 150 mM NaCl: 5 mM EDTA: 0.25% gelatin: 0.05% Tween 20; SV40, simian virus 40.

Downloaded from cancerres.aacrjournals.org on April 30, 2017. © 1986 American Association for Cancer Research.
Purification of DNA Topoisomerases. DNA topoisomerases I and II were purified as described previously (2). The temperature was kept between 0°C and 4°C during all of the purification steps. Nuclear extracts (2 ml) were loaded onto a 200-ml Sephacryl S-400 column. The column was eluted at a pump speed of 0.2 ml/min with nucleus buffer containing 0.35 M NaCl. Fifteen-min fractions (3 ml) were collected for 15 h. Each of these fractions was then assayed for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) and for DNA linking activity in the absence and presence of m-AMSA using the filter binding assay described below. The fractions exhibiting m-AMSA-dependent DNA linking activity have been shown to contain topoisomerases II and I (2). These fractions (usually 5 to 6) were then pooled, diluted with nucleus buffer without NaCl in order to adjust the final NaCl concentration to 0.5 M and loaded onto a DNA cellulose column (1 ml packed denatured DNA cellulose). The flow-through was collected, and the column was eluted twice with 1 ml 0.35 M NaCl nucleus buffer and twice with 1 ml of 0.5 M NaCl nucleus buffer. Each of these fractions was assayed for protein concentration and m-AMSA-dependent DNA linking activity. Typically the m-AMSA-dependent activity was retained on the column at 0.15 M NaCl and eluted mostly in the first 1-ml 0.35 M NaCl nucleus buffer elution fraction (2). This active fraction was loaded onto two 10-ml 15–40% glycerol gradients in 0.35 M NaCl nucleus buffer. Gradients were centrifuged in a SW 40 rotor at 35000 rpm for 68 h at 4°C (ω2 = 3.29 × 105) in a Beckman L8-80 ultracentrifuge. Gradients were collected from the bottom of the tubes in 25 0.4-ml fractions. One of the gradients in the same run was loaded with a mixture of molecular weight marker proteins in order to determine the sedimentation characteristics of the fractions of interest. Each fraction was assayed for protein concentration, filter binding activity, and topoisomerase activity.

Filter Binding Assay of DNA-Protein Linking. The assay was performed as described previously (2, 4). Briefly, protein samples were incubated with 50 ng of 3H-SV40 DNA (3000 dpm) for 20 min at 37°C in 0.1 to 0.2 ml nucleus buffer in the absence or presence of m-AMSA. Reactions were stopped by adding 1 ml of 20 mM Na2EDTA (pH 10) at 4°C. This reaction mixture was then added to 2 ml of the EDTA solution on a polyvinyl chloride filter (Millipore type BS, 25-mm diameter, 2-μm pore size) mounted in a 25-mm Swinnex holder. The solution was allowed to flow out through the filter without applied suction. Filters were then washed with 3 ml of 2 M NaCl, 0.2% sarkosyl, NL 30, 40 mM EDTA, pH 10 (LS10). The effluents and filter washes were collected separately and mixed with 10 ml aquassure (New England Nuclear Corp., Boston, MA), and radioactivity was determined by liquid scintillation counting. Radioactivity of the filters was determined by filtering the in the presence of 1 M HC1, followed by treatment with NaOH, as described previously (12).

Agarose Gel Electrophoresis. Reactions were performed in 30 to 40 μl nucleus buffer and stopped by adding sodium dodecyl sulfate (1% final concentration) and proteinase K (0.5 mg/ml final concentration). Samples were loaded into a 1% agarose gel in Tris-acetate buffer (4). Electrophoresis was carried out at 2 V/cm for 16 h. Gels were stained in 1 μM ethidium bromide for 45 min and destained in 1 mM MgSO4 for 30 min before being photographed under UV light.

DNA Sequencing Gel Electrophoresis. The method used was derived from that of Maxam and Gilbert (13). PBR322 DNA was 5'-end-labeled at the Hind III restriction site with polynucleotide kinase and [γ-32P]ATP. In order to obtain DNA labeled only at one end, a second restriction digestion of the 32P-labeled DNA was performed with EcoR1 (14). This digestion creates a 39 base pair Hind III-EcoR1 fragment, which did not interfere with the detection of relatively long topoisomerase II-induced DNA fragments. This DNA was used for the detection of topoisomerase II cleavage sites on the 5'-labeled DNA strand. Sequencing gels consisted of 6% polyacrylamide, 7 μm urea gels in Tris-borate-EDTA buffer. Electrophoresis were performed in Tris-borate-EDTA buffer for 2 h at 70 W and 50–60°C. Gels were transferred to Whatman 3 MM paper, dried, and exposed for autoradiography. Films were scanned with a Beckman densitometer.

Protein Gel Electrophoresis and Immunoblotting. Polyacrylamide gels (7.5%) were run at 25 mA for 2 h in a circulating water bath at 4°C. The parts of the gels that were used for protein visualization were cut and stained with 0.1% Coomassie brilliant blue R250 in 45% (v/v) methanol and 10% methanol acetic acid. Destaining was in 45% methanol and 10% acetic acid.

Proteins were electrophoresed on to nitrocellulose paper (0.2 μm pore size) for 16 h at 60 V and 4°C using a Bio-Rad Trans-Blot cell according to the method described by Towbin et al. (15). The nitrocellulose paper was dried in air. For visualization of protein markers on the nitrocellulose sheet, the sheet was stained with 0.2% Ponceau S and 3% salsulofluorocetic acid in 3% trichloroacetic acid. The stained sheets were destained by soaking in 5% acetic acid. Immunobinding was performed as follows. The nitrocellulose sheets after rehydration were incubated in 10% normal goat serum overnight at room temperature. After washing with water, the sheets were incubated with the indicated dilution of rabbit L1210 topoisomerase II antibody in TGT for 1 h at room temperature followed by overnight at 4°C. The nitrocellulose sheets were washed 3 times with TGT and incubated with biotinylated, affinity purified, goat anti-rabbit IgG (H+L) (ABC kit) diluted 1:200 in TGT for 2 h at 37°C. The paper was washed 3 times with TGT. It was then incubated with a freshly prepared solution of avidin DH (2 drops = 88 μl) and biotinylated horseradish peroxidase (2 drops = 88 μl per 10 ml of TGT) for 10 min at 37°C. The paper was washed three times in TGT and three times in phosphate buffered saline. Peroxidase was localized by incubating the sheets for 30–45 s in a freshly prepared solution of 3,3'–diaminobenzidine (0.6 mg/ml):0.02% H2O2 in phosphate buffered saline. The reaction was stopped by rinsing the sheets with water.

RESULTS

Fractionation of Nuclear Extracts from DC3F and DC3F/9-OHE Cells. Nuclear extracts from DC3F and DC3F/9-OHE cells had different DNA binding activities (Fig. 1, columns on the left). In the absence of drug, and for the same protein amount (0.2 μg), DC3F/9-OHE extract had a higher DNA binding activity than DC3F extract. However DNA binding activity of DC3F/9-OHE extract was not increased by m-AMSA.
TOPOISOMERASE MODIFICATIONS IN RESISTANT CELLS

AMS. This is in contrast to results obtained with L1210 (2) and DC3F nuclear extracts the DNA linking activity of which was increased by m-AMS. Since m-AMS-dependent DNA linking activity has been shown to be due to topoisomerase II, the absence of such an activity suggests that topoisomerase II is either absent or not sensitive to m-AMS in DC3F/9-OHE nuclear extract.

The nuclear extracts were fractionated immediately by gel filtration with Sephacryl S-400 at 4°C. The protein profiles of the DC3F and DC3F/9-OHE nuclear extracts were similar (Fig. 1, B and D). The DNA binding profiles, however, were different (Fig. 1, A and C). In the case of the DC3F nuclear extract there was little DNA binding activity in the absence of m-AMS and a clear m-AMS-induced DNA binding activity in fractions 25 to 30. Such a DNA binding profile is similar to that of similarly fractionated L1210 cell nuclear extract (2). In the case of DC3F/9-OHE nuclear extract a drug-independent DNA linking activity was found in fractions 26 to 30. Two DNA linking activities were therefore present in DC3F/9-OHE nuclear extract; one was m-AMS-inducible (fractions 26 to 30) and was eluted at the same position as that of DC3F nuclear extract. The other had a high drug-independent DNA linking activity (S value = 4.9) was similar to that of topoisomerase II (16, 17).

The m-AMS-independent DNA linking activity of DC3F/9-OHE nuclear extract (fractions 31 to 34 of the gel filtration shown in Fig. 1C) was concentrated to a volume of 0.5 ml using Centricon 30. The concentrated mixture was loaded onto a 15-40% glycerol gradient in the same run as that of the m-AMS-inducible DNA linking activity. The gradient was collected from the bottom of the tube and each fraction was assayed for topoisomerase activity in the presence of 1 mM ATP (Fig. 2B). Only one peak of topoisomerase activity was found (fractions 13 to 17). The S value of this topoisomerase activity (S = 4.9) was similar to that of the second peak of topoisomerase activity found in the m-AMS-inducible DNA linking activity (compare A and B in Fig. 2) and identical to that of topoisomerase I (17).

Fig. 2. DNA topoisomerase activities of glycerol gradient fractions obtained from DC3F/9-OHE salt extracts. In A, fractions 26 to 30 from the Sephacryl S-400 gel filtration shown on Fig. 1 were pooled, further purified by DNA cellulose affinity chromatography (see "Materials and Methods" and text), and loaded onto a 15-40% glycerol gradient in 0.35 M NaCl nucleus buffer. In B, fractions 31 to 34 were pooled, concentrated down to 0.5 ml (Centricon 30), and loaded onto a 15-40% glycerol gradient in 0.35 M NaCl nucleus buffer. Gradients were spun at 35,000 rpm for 68 h at 4°C, and 0.4-ml fractions were collected from the bottom of the tubes. DNA topoisomerase activities were assayed by reacting 0.4 µg SV40 DNA (29% supercoiled) with 10 µl of the indicated fractions for 30 min at 37°C in nucleus buffer (50 mM final volume). Reactions were performed in the presence of 1 mM ATP and were stopped by adding NaDSO4 and proteinase K (1% and 0.5 mg/ml final concentrations, respectively). Reaction products were run in 1% agarose gels. Pool I, fractions 14 to 17 from A. Pool II, fractions 9, 10 from A. Pool III, fractions 13 to 16 from B.

Fig. 3. ATP dependence of the topoisomerase activities and m-AMS-induced DNA-cleaving activities of pools I, II, and III. SV40 DNA (0.4 µg) was reacted in 30 µl nucleus buffer at 37°C with 5 µl of pool II (lanes 3 and 6), pool I (lanes 4 and 7), or pool III (lanes 5 and 8) in the absence (lanes 3-5) or presence of 10 µM m-AMS (lanes 6-8). No ATP was added. Reactions were stopped after 30 min as indicated in Fig. 2. Lanes 1 and 2, native and linear SV40 DNA, respectively. S, R, and L, migration position of supercoiled, relaxed, and linear SV40 DNA, respectively.

that of topoisomerase I (17).

Topoisomerase and DNA-linking Activities of the Pooled Glycerol Gradient Fractions from DC3F/9-OHE Cells. Fractions 14 to 17 from the glycerol gradient which had been loaded with the m-AMS-inducible DNA linking activity (gradient A on Fig. 2) were pooled as pool I. Fractions 9 and 10 from the same gradient were pooled as pool II (gradient A on Fig. 2). Fractions 13 to 16 from the glycerol gradient which had been loaded with the drug-independent DNA linking activity (gradient B on Fig. 2) were pooled as pool III. Each of these three pools was assayed for topoisomerase activity and ability to induce DNA strand breaks and DNA-protein links in the absence or in the presence of m-AMS.

DNA topoisomerase activity was assayed in the absence of ATP (Fig. 3). Pool II did not induce DNA relaxation under those conditions, whereas both pools I and III did. The same samples run in the presence of ethidium bromide showed that...
the bands migrating at the form II position corresponded to nicked DNA molecules in the case of pool II, closed circular molecules in the case of pool I, and an equal mixture of nicked and closed circular molecules in the case of pool III (data not shown). In the presence of m-AMSA, pool II produced DNA molecules migrating at the form III position (full length linear DNA molecules; Fig. 3, lane 6). Neither pool I nor pool III produced increased DNA cleavage in the presence of m-AMSA (Fig. 3, lanes 7 and 8; ethidium bromide gel not shown).

The DNA linking activity of each pool was also tested by filter binding assay in the absence or presence of m-AMSA (Fig. 4). In the absence of drug both pools I and II had a comparable DNA linking activity which was detectable only at concentrations equivalent to 40 µg protein. Pool III had a higher DNA linking activity than the two other pools in the absence of drug (Fig. 4, left panel). m-AMSA enhanced only the DNA linking activity of pool II (Fig. 4, right panel).

In summary, pool II contained: (a) an ATP-dependent DNA topoisomerase activity which sedimented in glycerol gradient as topoisomerase II; (b) a DNA linking activity which was enhanced by m-AMSA; and (c) a DNA cleavage activity which was also enhanced by m-AMSA. These three properties are altogether consistent with the presence of DNA topoisomerase II in pool II (1, 2). Pool I contained: (a) an ATP-independent DNA linking activity; and (b) a weak DNA linking activity which was not m-AMSA sensitive. The topoisomerase activity of pool I sedimented in glycerol gradients as topoisomerase I, which is altogether consistent with the presence of topoisomerase I in pool I. Pool III was analogous to pool I in that (a) its topoisomerase activity was not ATP-dependent, (b) its glycerol gradient sedimentation was similar, and (c) it did not induce DNA cleavage in the presence of m-AMSA. Pool III, however, differed from pool I because of its unusually high DNA linking and nicking activities.

It seemed, therefore, that DC3F/9-OHE nuclear extract contained two different activities which could account for the results obtained with the whole extract, a drug sensitive topoisomerase II and a topoisomerase I associated with a high DNA linking activity. The identity between DC3F/9-OHE and DC3F topoisomerase II was further investigated by studying their DNA cleavage pattern in the presence of m-AMSA and teniposide.

**DNA Cleavage Patterns of DC3F and DC3F/9-OHE Topoisomerases II.** Topoisomerase II was isolated from DC3F (sensitive) cells by the procedure described for mouse leukemia L1210 cells (2). This procedure is identical to that described above for the isolation of pool II from DC3F/9-OHE cells. ATP-End-labeled pBR 322 DNA (0.1 µg) was reacted for 30 min at 37°C with either of the two topoisomerases II in the absence or presence of m-AMSA. The densitometer scanning of the autoradiography of the gel is shown in Fig. 5. Each of the enzymes produced very few cuts in the absence of drug (not shown). m-AMSA induced DNA breaks at positions 50, 62, 76, 78, 102, and 132 mm (Fig. 5). The exact sequence localizations of the DNA cleavage sites were not determined precisely because of the relatively long distance between these sites and the 32P-labeled Hind III DNA terminus. However, the relative intensity of cleavage was similar in the case of DC3F and DC3F/9-OHE topoisomerasers II. Etoposide (VP16) induced also DNA breaks in the presence of either enzyme but at positions 20, 25, 32, 50, 78, 95, and 102 mm of the gel (data not shown).

The cleavage patterns induced by etoposide and m-AMSA were different, although some of the breaks occurred at the same locations. The two topoisomerases produced, however, comparable DNA cleavage patterns for a given drug. This result is in agreement with the previous results that DC3F/9-OHE topoisomerase II was sensitive to the drugs to which the cells were resistant. It appeared therefore that DC3F/9-OHE cells had either less topoisomerase II or that a modulating factor of topoisomerase II was present in DC3F/9-OHE nuclear extract, since the DNA linking activity of the whole extract was not sensitive to m-AMSA.

**Sodium Dodecyl Sulfate Gel Electrophoresis and Immuno-blotting of DC3F and DC3F/9-OHE Nuclear Extracts, Pool II and Pool III.** Proteins were run in 7.5% polyacrylamide gel, transferred to nitrocellulose, and tested for their reactivity to rabbit antibodies against L1210 topoisomerase II (Fig. 6). Total extracts from DC3F and DC3F/9-OHE nuclei gave a similar pattern in the presence of preimmune serum (Fig. 6, lanes 1 and 2). The high background reactivity of the preimmune serum is, in part, due to the relatively low titer of the rabbit topoisomerase II antiserum. In the presence of immunized serum, total extract from DC3F nuclei showed consistently a darker staining in the Mr 170,000 region of the gel (compare Fig. 6, lanes 5 and 6). Densitometer scanning of the negatives showed a 2- to 3-fold denser staining. Pool II showed no detectable antibody staining with preimmune serum (Fig. 6, lane 3), although several bands were visible between Mr 150,000 and Mr 170,000 on the Coomassie blue staining of a similar gel. Topoisomerase II antiserum stained these bands (Fig. 6, lane 7). Pool III showed two prominent bands in the presence of preimmune serum (Fig. 6, lane 4), one at approximately Mr 95,000 and another slightly fainter at Mr 55,000. The same pattern was observed by Coomassie blue staining of a comparable gel and in the presence of topoisomerase II antiserum (Fig. 6, lane 8). These results showed that: (a) pool II contained only proteins of molecular weights between 170,000 and 150,000 which bound rabbit antiserum against mouse L1210 topoisomerase II; (b) pool III contained two protein species of molecular weight 95,000 and 55,000, respectively and that neither of these proteins was reactive to topoisomerase II antiserum; and (c) total extract from DC3F nuclei contained approximately 2-fold more Mr 170,000 proteins that selectively bound topoisomerase II antiserum than that from DC3F/9-OHE nuclei. This last result led us to estimate further the topoisomerase II and I activities of the salt extracts from DC3F and DC3F/9-OHE nuclei.

**Topoisomerase II Activity in 0.35 M NaCl Extracts from DC3F and DC3F/9-OHE Nuclei.** The ATP-dependent decatenation of
TOPOISOMERASE MODIFICATIONS IN RESISTANT CELLS

Fig. 5. Densitometer scanning of the DNA cleavage patterns of topoisomerase II from DC3F and DC3F/9-OHE cells. 32P-end-labeled pBR322 DNA was reacted with 10 μl of either DC3F (A) or DC3F/9-OHE topoisomerase II (B) in 40 μl nucleus buffer for 30 min at 37°C in the presence of 10 μM m-AMSA. Reactions were stopped by adding sodium dodecyl sulfate and proteinase K (1% and 0.5 mg/ml final concentrations, respectively). After an additional 30-min incubation at 37°C, the DNA was precipitated with ethanol over-night at −20°C, resuspended in 3 μl Maxam Gilbert loading buffer, and loaded onto 6% polyacrylamide gel:7 M urea in Tris-borate-EDTA. Electrophoresis was for 2 h at 70 W in Tris-borate-EDTA buffer. The gel was then dried, and autoradiography was performed. Film scanning was performed in a Beckman densitometer.

Fig. 6. Immunoblotting of sodium dodecyl sulfate-polyacrylamide gel of DC3F and DC3F/9-OHE nuclear extracts. Twenty μg of 0.35 M NaCl extracts from DC3F (lanes 1 and 5) or DC3F/9-OHE nuclei (lanes 2 and 6) and 5 μg of pool II (lanes 3 and 7) and pool III (lanes 4 and 8) were run into a 7.5% polyacrylamide gel, electrotransferred to a nitrocellulose sheet, and assayed for their reactivity to the serum of a rabbit before (lanes 1 to 4) and after immunization with L1210 topoisomerase II (lanes 5 to 8). Right, molecular weight markers.

Fig. 7. Topoisomerase II activity of 0.35 M NaCl extracts from DC3F and DC3F/9-OHE nuclei. Nuclei were isolated from DC3F and DC3F/9-OHE cells. These nuclei were incubated for 30 min at 4°C in 0.35 M NaCl nucleus buffer and then spun down (see "Materials and Methods"). The supernatants were then collected (salt extracts) and assayed for their decatenation activity. kDNA (0.2 μg) was reacted for 30 min at 37°C in 20 μl decatenation buffer in the absence (open symbols, dashed lines) or presence of 1 mM ATP (filled symbols, solid lines) and various amounts of DC3F (○, △) or DC3F/9-OHE (▲, ▲) nuclear extract. Reactions were stopped by adding sodium dodecyl sulfate and proteinase K (1% and 0.5 mg/ml final concentrations, respectively). Reaction products were run in 1% agarose gels. The gels were stained with ethidium bromide and the minicircle and kDNA bands were cut and counted for 3H radioactivity.

DISCUSSION

Comparison between 0.35 M NaCl extracts from DC3F and DC3F/9-OHE nuclei showed that the DNA linking activity of DC3F/9-OHE nuclear extract was not stimulated by m-AMSA, whereas that from DC3F nuclear extract was. The differential sensitivity of nuclei from DC3F and DC3F/9-OHE cells (9) extract that decatenated 50% of the kDNA was found to be approximately 8.5 μg in both DC3F and DC3F/9-OHE salt extracts.

Topoisomerase I Activity in 0.35 M NaCl Extracts from DC3F and DC3F/9-OHE Nuclei. Topoisomerase I relaxes supercoiled DNA in the absence of ATP, whereas topoisomerase II requires ATP. This differential property of the two enzymes was used to titrate the topoisomerase I activity of the nuclear extracts. The ATP-independent relaxation of supercoiled SV40 DNA by various amounts of DC3F and DC3F/9-OHE nuclear extracts was followed by agarose gel electrophoresis. Both DC3F and DC3F/9-OHE salt extracts had similar topoisomerase I activities. In both extracts, 0.38 μg was the lowest protein amount that fully relaxed supercoiled SV40 DNA (Fig. 8.
TOPOISOMERASE MODIFICATIONS IN RESISTANT CELLS

could therefore be found in the nuclear extracts. An additional finding was the presence of a high drug-independent DNA linking activity in DC3F/9-OHE nuclear extract. Three possible modifications could have occurred in DC3F/9-OHE nuclear extracts: (a) selection of a drug-resistant topoisomerase II; (b) reduced amount of topoisomerase II; and (c) a drug-induced modification of nuclear factors which modulate the sensitivity of topoisomerase II to drugs. We will discuss these three possibilities subsequently.

The first fractionation step of DC3F/9-OHE nuclear extract by gel filtration (Fig. 1) showed that an m-AMSA-inducible activity could be identified in DC3F/9-OHE nuclei. Further purification of this activity led to the isolation of a cluster of Mr 150,000-170,000 proteins that reacted to L1210 topoisomerase II antibodies and migrated in glycerol gradients at the position of mammalian topoisomerase I (16). Moreover these proteins exhibited both ATP-dependent DNA topoisomerase activity and m-AMSA-inducible DNA linking and breaking activity. The sequencing of the DNA cleavage sites showed a pattern similar to that of DC3F topoisomerase II. It is therefore clear that DC3F/9-OHE cells possessed a drug sensitive topoisomerase II.

Quantitative comparison between DC3F and DC3F/9-OHE topoisomerase II was performed in two ways. The ATP-dependent DNA decatenation assays showed no difference between topoisomerase II activities of the two nuclear extracts, whereas the immunoblotting assays showed only a 2-fold reduction in topoisomerase II molecules extracted from DC3F/9-OHE nuclei than from DC3F nuclei. Although the discrepancy between the results of the two assays is not clear, one can conclude that, even if DC3F/9-OHE nuclei had less topoisomerase II than DC3F nuclei, this difference did not exceed 2-fold. This quantitative difference is far less than the difference in drug sensitivity of the two cell lines (9) and seems unlikely to explain the high degree of resistance of DC3F/9-OHE cells.

The last possibility is therefore that DC3F/9-OHE topoisomerase II is modulated in such a way that its trapping by topoisomerase II inhibitors is reduced in total nuclear extract. The nature of the modulating factor is yet unknown. However, the presence of a drug-insensitive DNA linking activity (pool III) in DC3F/9-OHE nuclear extract is unusual and could thus be involved in this modulation. No such activity is found in DC3F (Fig. 1) or in similarly fractionated L1210 nuclear extracts (2). This DC3F/9-OHE DNA linking activity copurified with a topoisomerase I activity and showed two main protein bands in sodium dodecyl sulfate-polyacrylamide gel at Mr 95,000 and Mr 55,000 molecular weight. None of these proteins reacted to L1210 topoisomerase II antibodies. The molecular weight of previously isolated mammalian topoisomerase I is approximately 95,000 (17), which suggests that the Mr 95,000 proteins are topoisomerases I or closely related enzymes. The Mr 55,000 band could therefore correspond to the protein(s) exhibiting high DNA linking activity. This would be in agreement with the results that a Mr 55,000 topoisomerase I with a high DNA linking activity has been isolated from chick embryo erythrocytes (20).

In conclusion, it appears that the resistance of Chinese hamster DC3F/9-OHE cells to topoisomerase II inhibitors is not due to the absence of drug sensitive topoisomerase II but rather to the presence of a modulating factor perhaps related to topoisomerase I.

ACKNOWLEDGMENTS

We wish to thank Dr. P. Englund for providing us with kinetoplast DNA and Dr. K. W. Kohn for his support and valuable discussion during the course of this study. Thanks also are expressed to Madie Tyler for typing the manuscript.

REFERENCES

TOPOISOMERASE MODIFICATIONS IN RESISTANT CELLS


Altered DNA Topoisomerase II Activity in Chinese Hamster Cells Resistant to Topoisomerase II Inhibitors

Yves Pommier, Donna Kerrigan, Ronald E. Schwartz, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/6/3075

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.