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REDUCED DNA TOPOISOMERASE II ACTIVITY AND DRUG-STIMULATED DNA CLEAVAGE IN 9-HYDROXYELLIPTICINE RESISTANT CELLS*

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Abstract—We have isolated a Chinese hamster lung cell line resistant to 9-hydroxyellipticine (DC-3F/9-OH-E) which is also cross-resistant to topoisomerase II inhibitors such as amsacrine and etoposide. In this work we have studied quantitatively both DNA topoisomerase II activity by decatenation of kinetoplast DNA and drug-stimulated DNA cleavage of pBR 322. DNA topoisomerase II activity of DC-3F/9-OH-E nuclear extract was reduced by 3.5-fold as compared to that from DC-3F (sensitive parent) nuclear extract. We also found that DC-3F/9-OH-E nuclear extracts have a reduced capacity to induce *in vitro* topoisomerase II-mediated DNA cleavage upon stimulation by etoposide and amsacrine (7- and 10-fold respectively). Besides, mixing nuclear extracts from both sensitive and resistant cells indicates that either the enzyme in resistant cells is modified or a modulating factor is associated to it. Our results suggest that the resistance of the DC-3F/9-OH-E cell line to topoisomerase II inhibitors might be due to both a reduced amount of the enzyme and its reduced ability to form the cleavable complex in the presence of drugs.

As recently reviewed, DNA topoisomerase II appears to be a target for cancer chemotherapy [1–3]. Namely, ellipticine derivatives [4], amsacrine (m-AMSA‡ [5]) and epipodophyllotoxins [6] are able to induce topoisomerase II-mediated DNA cleavage *in vitro*. These drugs also induce protein-associated DNA strand breaks in cultured cells [7–9] that seem to result from the poisoning of topoisomerase II [10, 11]. This primary effect seems to be related to the cytotoxicity of these drugs [1–3].

We have isolated a Chinese hamster lung cell line resistant to 9-hydroxyellipticine (9-OH-E), named DC-3F/9-OH-E, by adding stepwise increasing drug concentrations to the medium [12]. These resistant cells are cross resistant to topoisomerase II inhibitors including ellipticine derivatives such as NMHE, m-AMSA and epipodophyllotoxins and have a reduced capacity to form protein-associated DNA breaks upon exposure to these drugs [13]. Because of no impaired uptake of these drugs by the cells [13, 14] and taking into account that this reduced capacity was also found in isolated nuclei [13], this led us to propose that DC-3F/9-OH-E cells had a modified topoisomerase II activity [13].

Pommier *et al.* found that the DNA cleavage activity of DC-3F/9-OH-E nuclear extract was not stimulated by m-AMSA whereas that from DC-3F nuclear extract was, although no difference between topoisomerase activities in the two nuclear extracts was observed [15]. Moreover, they found a high drug-independent DNA linking activity in DC-3F/9-OH-E nuclear extract which co-purified with a topoisomerase I activity [15]. Hence, they proposed that the resistance of DC-3F/9-OH-E cells to topoisomerase II inhibitors is due to the presence of a modulating factor perhaps related to topoisomerase I [15].

In this paper, we study quantitatively the topoisomerase II-mediated DNA cleavage induced by the drugs and the topoisomerase II activity in order to further clarify their relation with the cytotoxicity previously reported [13]. We found that DC-3F/9-OH-E nuclear extracts have a reduced capacity to induce *in vitro* topoisomerase II-mediated cleavage of pBR 322 DNA ("cleavable complex") upon incubation with the epipodophyllotoxin VP-16 and m-AMSA: quantification of the assay shows a 7-fold and 10-fold reduction respectively. Moreover, we found that topoisomerase activity in DC-3F/9-OH-E nuclear extract is 3.5-fold lower than that in DC-3F nuclear extract. Finally, reconstruction experiments, i.e. mixing nuclear extracts from DC-3F and DC-3F/9-OH-E cells, indicate that either the modification of the enzyme in the resistant cells or an associated modulating factor are the two possibilities that could account for, at least in part, the resistance mechanism in addition to a reduced topoisomerase II activity.

MATERIALS AND METHODS

Cell lines. The parental hamster lung cell line DC-3F and the resistant subline DC-3F/9-OH-E were

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‡ Abbreviations used: m-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; NMHE, 2-*N*-methyl-9-hydroxy-ellipticinium; VP-16, etoposide; kDNA, kinetoplast DNA.

grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum and penicillin-streptomycin [11].

Drugs and chemicals. Etoposide (VP-26-213) was kindly provided by Dr W. T. Bradner, Bristol-Myers Company, Syracuse, New York. Amsacrine (m-AMSA; NSC 249992) was a generous gift of Dr Y. Pommier, N.C.I., N.I.H., Bethesda, Maryland. Both drugs were dissolved in dimethylsulfoxide at 10^{-2} M. VP-16 solution was prepared immediately before use and m-AMSA solution was kept frozen at −20° as stock solution. All chemicals were of reagent grade and obtained from commercial sources.

DNAs. pBR 322 DNA was prepared from an *E. coli recA F* strain (HB 101[pBR 322]) kindly provided by Dr J. Pierre, Institut Gustave Roussy, Villejuif, France, as described in Ref. 16, using lysis by alkali, without prior amplification with chloramphenicol. kDNA was prepared as previously described [17] from *Trypanosoma cruzi* pellets kindly provided by Dr G. Riou, Institut Gustave Roussy, Villejuif, France.

Nuclear extracts. Preparation of nuclear extracts was performed according to Glisson *et al.* [18] with minor modifications. About 1×10^8 exponentially growing cells (about 1.5×10^5 cells/cm^2) were trypsinized and pelleted at 400 *g* for 10 min. The following steps were carried out at 0–4° in the presence of freshly added 1 mM phenylmethylsulfonyl fluoride (from 100 mM stock in absolute ethanol). After two washings with phosphate buffered saline, the pellet was resuspended in 1 ml buffer A (10 mM Tris–HCl (pH 7.5)–1.5 mM $MgCl_2$–10 mM NaCl) and the suspension was gently stirred while 9 ml of swelling buffer (buffer A containing 1% Nonidet P-40) was added. After 10 min the nuclei were isolated by Dounce homogenization (15 strokes) and centrifugation at 600 *g* for 10 min. They were resuspended in 2 ml of buffer B (50 mM Tris–HCl (pH 7.5)–25 mM KCl–2 mM $CaCl_2$–0.25 M sucrose) and then layered on a 0.6 ml cushion of buffer B containing 0.6 M sucrose and pelleted at 2000 *g* for 10 min. The nuclei were washed with 2 ml of buffer C (50 mM Tris–HCl (pH 7.5)–25 mM KCl–5 mM $MgCl_2$) containing 0.25 M sucrose. The pellet was resuspended in 300 μl of buffer C and 30 μl of 0.2 M EDTA (pH 8.0) was added. Then, 660 μl of buffer D (80 mM Tris–HCl (pH 7.5)–1 mM dithiothreitol–2 mM EDTA–0.53 M NaCl–20% glycerol (w/v)) was added to give a final NaCl concentration of 0.35 M. After 30 min insoluble material was pelleted by centrifugation at 12,000 *g* for 30 min. Protein concentration in the supernatant (0.35 M NaCl nuclear extract) was determined immediately by the method of Bradford [19] and nuclear extracts from sensitive and resistant cells were then adjusted at the same value by dilution with a mixture of buffer C–0.2 M EDTA (pH 8.0)–buffer D (3–0.3–6.6). Bovine serum albumin was added to a concentration of 1 mg/ml (from a 10 mg/ml stock solution) and glycerol to 50% (w/v). Aliquots were stored at −20° and used within one week.

Topoisomerase II assays. Both drug-stimulated DNA cleavage activity and decatenation of kDNA were carried out in a reaction mixture containing 40 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA (pH 8.0), 30 μg/ml bovine serum albumin, 1 mM ATP.

Drug-stimulated DNA cleavage activity was assayed quantitatively by the generation of linear DNA (form III) from supercoiled (form I) pBR 322 DNA containing less than 5% of nicked DNA (form II). Five microlitres of nuclear extract was added to 20 μl of reaction mixture containing 0.25 μg pBR 322 DNA and the drug (final dimethylsulfoxide concentration less than 5%); incubation proceeded for 30 min at 37°. The reaction was stopped by the addition of 2 μl of 6% SDS and 2 μl of 1.5 mg/ml proteinase K and further incubation proceeded for 30 min at 50°. Whole samples were then analysed by electrophoresis through 0.8% agarose in 89 mM Tris-borate–2 mM EDTA (pH 8.0) containing 0.45 μg/ml ethidium bromide at 1.6 V/cm for 16 hr. After destaining, gel was photographed under UV illumination and negative film was scanned by a double beam recording Microdensitometer MKIIIC, Joyce, Loebl and Co. Ltd., U.K. Peak height of gaussian peaks was measured and percent of form III was calculated.

Decatenation of kDNA was assayed by incubating 10 μl of a dilution of nuclear extract with 40 μl of reaction mixture containing 1.7 μg of kDNA at 30°. At the indicated times, a 5 μl aliquot was taken and the reaction was stopped by the addition of 3 μl of 50 mM EDTA, 1% sodium dodecylsulfate, 30% glycerol (w/v). Whole samples were then analysed by electrophoresis through 1.2% agarose in 89 mM Tris-borate–2 mM EDTA (pH 8.0) at 4 V/cm for 2.75 hr. After staining with 0.45 μg/ml ethidium bromide and destaining, gel was proceeded as described for drug-stimulated DNA cleavage. Peak area of liberated minicircles was determined by weighing tracing paper.

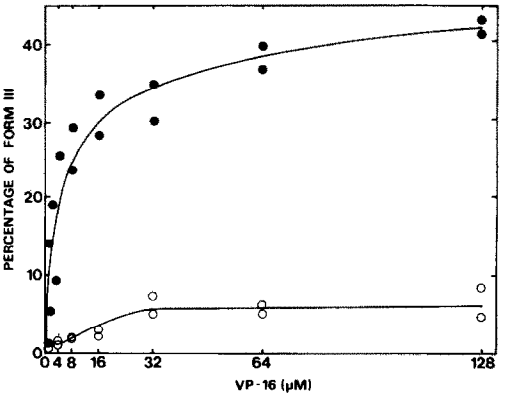

Fig. 1. Drug-stimulated DNA cleavage of pBR 322 by increasing concentrations of VP-16. Five microlitres of nuclear extract from DC-3F (●) or DC-3F/9-OH-E (○) cells was incubated with the same amount of pBR 322 DNA in a final volume of 25 μl containing the indicated VP-16 concentrations. Each determination was made twice in two completely separate experiments: nuclear extracts and pBR 322 were prepared independently. Controls (nuclear extract, no drug = 6–7%) were substracted.

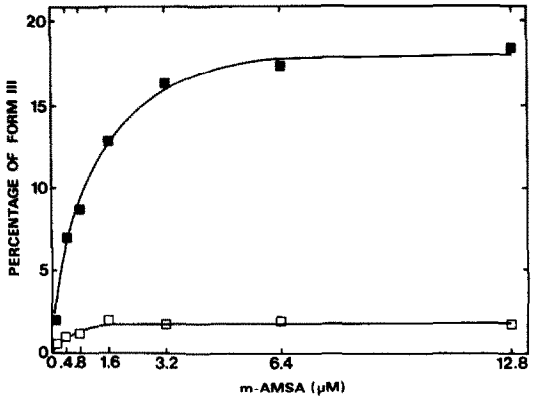

Fig. 2. Drug-stimulated DNA cleavage of pBR 322 by increasing concentrations of m-AMSA. Same conditions as in Fig. 1. ■, DC-3F; □, DC-3F/9-OH-E.

RESULTS

Drug-stimulated DNA cleavage by VP-16 and m-AMSA

Figure 1 shows that DNA cleavage of pBR 322 DNA by nuclear extracts from DC-3F and DC-3F/9-OH-E cells is stimulated by increasing concentrations of VP-16. However, at highest concentrations, this activity tends to a plateau and at 128 μM VP-16, nuclear extracts from resistant cells induce about 7-fold decrease of cleavable complex compared to the one from sensitive cells (6% of form III as compared to 42%, respectively).

Figure 2 shows that nuclear extract from DC-3F/9-OH-E cells induces less DNA cleavage of pBR 322 than that from DC-3F cells upon stimulation by m-AMSA. With both extracts, a plateau is reached above 3.2 μM at which a 10-fold difference in the amount of cleavable complex is observed. The range of concentrations of m-AMSA used to reach the plateau is approximately 1/10 of the one used with VP-16.

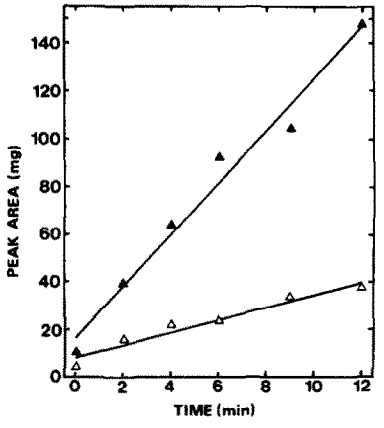

Fig. 3. Decatenation of kDNA. Ten microlitres of diluted (1/4) nuclear extract from DC-3F (▲) or DC-3F/9-OH-E (△) was incubated with the same amount of kDNA in a final volume of 50 μl. Aliquots were taken at the indicated times at which the reaction was stopped.

Decatenation of kDNA

The lower amount of cleavable complex induced by the drugs in DC-3F/9-OH-E nuclear extracts could be the consequence of a decreased topoisomerase II level. This enzymatic activity was assayed by decatenation in aliquots of the same nuclear extracts assayed for drug-stimulated DNA cleavage of kDNA.

Figure 3 shows a typical result of the first linear part of kinetics of decatenation of kDNA by nuclear extracts from DC-3F and DC-3F/9-OH-E cells. On this figure, topoisomerase II activity (as determined by the slope) of nuclear extract from resistant cells is 4-fold decreased. Three experiments were carried out independently with various dilutions of nuclear extracts: the mean value of the ratio between these two activities is 3.5.

Reconstruction experiment

In order to go further through the hypotheses previously proposed [15], we carried out a reconstruction experiment by mixing various amounts of nuclear extracts from DC-3F and DC-3F/9-OH-E in a final volume of 5 μl and assayed these mixtures for drug-stimulated DNA cleavage with VP-16.

Figure 4 shows that in the presence of 8 μM VP-16, the amount of cleavable complex decreases linearly as percentage of nuclear extract from resistant cells increases from 0 to 100%. This indicates that the ability to form the cleavable complex in sensitive cells is diluted by the one in resistant cells as percentage of nuclear extract from resistant cells increases linearly.

DISCUSSION

The data presented in this paper confirm that the DNA cleavage activity of DC-3F/9-OH-E nuclear extract stimulated by m-AMSA is reduced as compared to that from DC-3F nuclear extract, as previously shown [15]. By quantification of this effect, the present study allows new suggestions and conclusions.

Using both intercalating (m-AMSA) and non-intercalating (VP-16) agents, we found a 10- and 7-fold reduction of this activity, respectively (Figs 1 and 2). In addition, a low amount of cleavable complex is still induced by the drugs in nuclear extracts from resistant cells at concentrations of drugs which are almost not cytotoxic [13]. This suggests that either a threshold of the amount of cleavable complex is required to make it a cytotoxic event or the drug-stimulated DNA cleavage is not the only mechanism involved in cell killing as we previously proposed from a study of the protein-associated DNA strand breaks induced by topoisomerase II inhibitors in DC-3F/9-OH-E cells [13]. Furthermore, a 3-log reduction of the survival fraction was observed with 3.2 μM m-AMSA and 128 μM VP-16 [13] corresponding to the 10- and 7-fold reductions shown in this work. Hence, these quantitative results suggest that the cytotoxicity is not directly related to the drug-dependent stabilization of topoisomerase II molecules on the DNA. A cooperativity between the cleavable complexes induced by the drugs or the

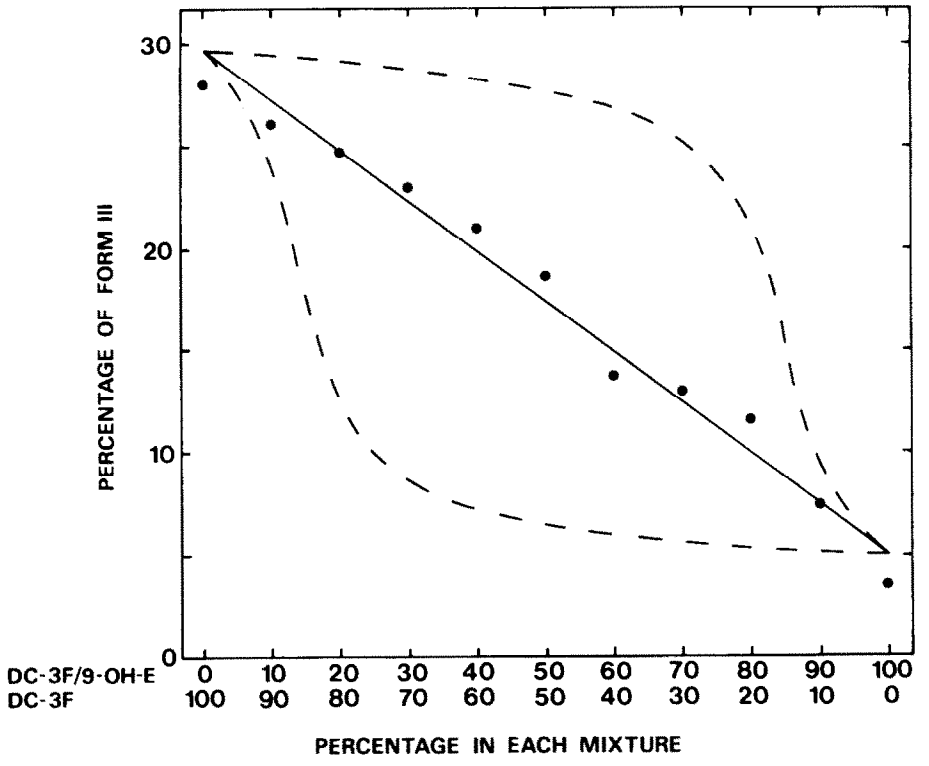

Fig. 4. Drug-stimulated DNA cleavage of pBR 322 by mixtures of nuclear extracts from DC-3F and DC-3F/9-OH-E cells in the presence of 8 μM VP-16. Final volume of mixed nuclear extracts is 5 μl in a final volume of 25 μl as in Fig. 1. Legend of dashed lines: see Discussion.

induction of secondary events seem to be required to link cytotoxicity and cleavable complex.

Two possibilities can be considered to explain the reduced ability to form the cleavable complex in resistant cells as compared to sensitive cells: (1) a reduced amount of DNA topoisomerase II, and/or (2) a reduced ability of the enzyme to form a cleavable complex in the presence of drugs. Both are consistent with the fact that the amount of cleavable complex induced by the drugs by extracts from sensitive and resistant cells reaches different plateaus (Figs 1 and 2).

Pommier *et al.* [15] reported that total extract from DC-3F nuclei contains approximately 2-fold more enzyme than that from DC-3F/9-OH-E nuclei as indicated by Western blots. This result was in contrast with the decatenation activity which was found the same as a function of the amount of protein [15]. Our data (Fig. 4 and data not shown), which allow a comparison of the slope of the linear part of the kinetics, show a 3.5-fold (mean) reduction of the decatenation activity. This result is in agreement with the immunoblotting showed in Ref. 15, within the precision of such an assay. Hence, with respect to the 7–10-fold reduction of the amount of cleavable complex in the resistant line, this 3.5-fold reduction cannot be regarded as a negligible part of the resistance.

A reduced ability of the enzyme to form a cleavable complex in the presence of drugs may be due to (a) a modification of the enzyme, (b) a modulating factor associated in an equal amount to the enzyme, and (c) a modulating factor which is present in excess. In the two latter cases, the modulating factor may be an activator which would miss in the resistant cells or an inhibitor which would miss in the sensitive cells. The result of the reconstruction experiment (Fig. 3) is not consistent with the third hypothesis. The data should be of the same type as the dashed lines shown, as example, in Fig. 3: the upper one in the case of an activator or the lower one in the case of an inhibitor present in excess. Rather, the fit is linear and is consistent with the hypotheses (a) and (b) assuming that in the second hypothesis, the enzyme of both sensitive and resistant cells can bind the modulating factor with the same affinity. Hence, the modulating factor perhaps related to topoisomerase I proposed by Pommier *et al.* seems not to be directly related to the ability to form the cleavable complex. If it was directly related, such a phenotype should be completely dominant rather than partially dominant as previously shown in hybrids between DC-3F and DC-3F/9-OH-E cells [20] in the case of an inhibiting factor present in excess. Depending on how the enzyme (or the modulating factor) expression is regulated in these hybrids, our data are consistent with the hypotheses (a) and (b) and the result on co-dominance previously observed [20]. Because both alleles of the topoisomerase II gene can be expressed independently, we cannot rule out at the present time that resistant cells could have both normal and modified enzymes.

In conclusion, the resistance of our DC-3F/9-OH-E cell line to topoisomerase II inhibitors might be

due to both a reduced (about 3.5-fold) amount of the enzyme and its reduced ability to form the cleavable complex in the presence of drugs. Purification of the enzyme of both sensitive and resistant cells should indicate whether a modification of the enzyme or the presence of a modulating factor, or both, is responsible for the reduced ability to form the cleavable complex in the resistant cell line.

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