Topoisomerase Inhibitors Induce Irreversible Fragmentation of Replicated DNA in Concanavalin A Stimulated Splenocytes†

Christine Jaxel,‡ Georges Taudou,§ Christiane Portemer,‡ Gilles Mirambeau,‡ Jacques Panijel,§ and Michel Duguet*,‡

Laboratorie d'Enzymologie des Acides Nucléiques, UA 554 CNRS, Université Pierre et Marie Curie, 96 Bd. Raspail, 75006 Paris, France, and Service de Physiopathologie de l'Immunité, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France

Received June 24, 1987; Revised Manuscript Received September 16, 1987

ABSTRACT: Etoposide, a nonintercalative antitumor drug, is known to inhibit topoisomerase II. Its effects have been tested in concanavalin A stimulated splenocytes, a system of cell proliferation in which topoisomerase II is induced. The primary effect of etoposide was a strong inhibition of DNA synthesis and the production of reversible DNA breaks, presumably associated with topoisomerase II. However, prolonged (20 h) contact with the drug resulted in a secondary fragmentation by irreversible double-strand breaks that yielded unusually small DNA fragments. Surprisingly, the same effect was obtained with novobiocin, which does not produce topoisomerase II associated DNA breaks. Moreover, long-term treatment with camptothecin, a specific inhibitor of topoisomerase I which is known to induce *single-strand* breaks in vitro and in vivo, also produced *double-strand* breaks and DNA fragmentation into small pieces. These findings suggest that prolonged treatment of proliferating splenocytes by etoposide and other topoisomerase inhibitors induced DNA fragmentation by a mechanism that does not directly involve topoisomerases.

Stimulation of splenocytes by mitogens such as concanavalin A is a useful system for studying the molecular events that take place during the transition from resting to proliferative state. We have previously shown that in a similar system of lymphocytes stimulated by con A[1] the activity of type II topoisomerase was considerably increased and correlated to DNA replication (Taudou et al., 1984). These findings confirmed our initial results showing that topoisomerase II was induced during liver regeneration (Duguet et al., 1983), and suggested that this enzyme is required for DNA replication. This can be extended to all of the systems of cell proliferation so far tested (Riou et al., 1985; Heck & Earnshaw, 1986; Sullivan et al., 1986; Markovits et al., 1987) except one (Tricoli et al., 1985). Furthermore, several independent works with topoisomerase I and II mutants in yeast (Uemura et al., 1984), with antibodies raised against topoisomerases (Heck & Earnshaw, 1986), with specific inhibitors (Nelson et al., 1986; Newport, 1987), and with in vitro replication systems (Yang et al., 1987) emphasize the role of type II topoisomerase in both the elongation of DNA replication and the segregation of daughter chromosomes.

Topoisomerases are enzymes that change the topology of DNA by a mechanism involving transient breaks of the phosphodiester bonds [see Wang (1985) for a review]. Recent studies in vivo and in vitro have shown that eukaryotic topoisomerase II was the target of a variety of antitumor drugs (Tewey et al., 1984; Pommier et al., 1985). In vitro, these drugs trap the enzyme in a reversible complex with DNA (called the cleavable complex), preventing the rejoining step of the reaction mechanism. Treatment of the complex with a detergent results in a DNA single-strand or double-strand break. In addition, the catalytic activity of the topoisomerase was inhibited. In vivo, topoisomerase II associated DNA breaks have also been observed after drug treatment, and it has been suggested that this fragmentation was related to the cytotoxic activity of the drugs. Among these, epipodophyllotoxins (VP16 and VM26) appear to be specific for topoisomerase II, since they do not interact with the DNA itself, although they are able to trap the topoisomerase in a covalent complex with DNA (Chen et al., 1984). Short-term incubation in vivo with these drugs produces *reversible* topoisomerase II associated DNA strand breaks (Loike & Horwitz, 1976; Chen et al., 1984).

In the present work, we have tested the effects of etoposide (VP16) and other topoisomerase inhibitors on proliferating splenocytes in conditions of prolonged contact with the drug, which presumably better reflect the in vivo activity of the drugs: we found that long-term treatment of stimulated splenocytes with low concentrations of the drugs stopped DNA replication and, in contrast with short-term incubations, produced an *irreversible* fragmentation of the DNA.

Materials and Methods

Isolation and Cultures of Mouse Spleen Cells. C57/BL mice (Institut Pasteur) 6–12 weeks old, were sacrificed by cervical dislocation. The spleens were removed aseptically and minced in phosphate-buffered saline (PBS, pH 7.2). Debris was removed by filtration through nylon sieves, and the cell suspension was washed in PBS. Viable cells excluding trypan blue were counted in a hemocytometer. Cells were suspended at a concentration of 2×10^6 cells/mL in RPMI 1640 medium (Moore et al., 1977) containing 5% decomplemented fetal calf serum (Gibco) and supplemented with 0.2 mM alanine and 100 units/mL penicillin. Cultures and concanavalin A stimulation were as previously described (Taudou et al., 1977).

Partial Purification of Topoisomerase II from Splenocytes. The 48-h-stimulated splenocytes (2×10^8 cells) were washed twice in 10 mL of 30 mM Tris-HCl, pH 7.5, 10 mM NaCl,

† This work was supported by grants from the Association pour la Recherche contre le Cancer (ARC).

* Author to whom correspondence should be addressed.
‡ Université Pierre et Marie Curie.
§ Institut Pasteur.

[1] Abbreviations: con A, concanavalin A; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; NP40, Nonidet P40; DTT, dithiothreitol; LDS, lithium dodecyl sulfate; kDNA, kinetoplast DNA; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-0095$01.50/0

and 3 mM $MgCl_2$ (RBS buffer) containing 1 mM PMSF and lysed in 10 mL of RBS containing 0.25 M sucrose, 0.2% NP40, and 1 mM PMSF. After centrifugation, the nuclear pellet was washed, and the nuclear extract was prepared as previously described (Taudou et al., 1984). The extract, dialyzed against 50 mM potassium phosphate, pH 7.0, 100 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, and 0.5 mM PMSF (buffer A), was applied to a 0.35-mL phosphocellulose column equilibrated with buffer A. The column was extensively washed with buffer A and developed with buffer A containing 1 M NaCl. Topoisomerase II active fractions were monitored by the decatenation assay (Duguet et al., 1983). One unit of activity is defined as the amount of enzyme necessary to decatenate 50% of the 0.3-μg input of kinetoplast DNA in 30 min at 37 °C.

Preparation of Kinetoplast DNA (kDNA). kDNA was prepared from *Trypanosoma cruzi* as described by Riou and Yot (1977).

Decatenation Assay. Decatenation was performed as previously described, in the presence of ATP (Duguet et al., 1983). kDNA and etoposide were preincubated at 37 °C for 5 min, and the reaction was initiated by addition of 3 units of topoisomerase II from splenocytes. After 10 or 30 min at 37 °C, the reaction was terminated by addition of 50 mM EDTA and 30-min treatment at 50 °C in the presence of LDS and proteinase K, and the products were analyzed in 1% agarose gels as previously described. Precise determination of the inhibition constant was made in initial velocity conditions (10-min incubations) by measurement of the amount of free minicircles from the densitometer scanning of gel negatives (Jaxel et al., unpublished data).

Sucrose Gradient Sedimentation. Cell cultures (1.5×10^6 cells) were centrifuged, washed in RBS buffer, and lysed in 10 mM EDTA, 1% deoxycholate, and 1.5% lithium dodecyl sulfate (LDS). LDS was used in place of sodium dodecyl sulfate owing to its stronger detergent effect and to prevent precipitation during the sedimentation. The lysates were immediately applied to 5–20% sucrose gradients in 10 mM EDTA and 1.5% LDS and centrifuged at 23 °C for 16 h at 17000 rpm in an SW50 rotor. Fractions of 200 μL were collected from the bottom of each tube. For each fraction, the TCA-precipitable radioactivity was measured and the sucrose concentration determined by refractometry. Alkaline sucrose gradients were performed with the same procedure, except that the lysis and gradient buffers contained 0.2 M NaOH. KCl precipitation was performed on each fraction as described by Trask et al. (1984).

Size of the DNA Fragments. DNA molecular weight markers ranging in size from 1.44 to 43.7 kb were centrifuged in parallel with the cell lysates. The collected fractions were analyzed by gel electrophoresis to identify the different markers along the gradient.

Enzymes, Drugs, and Chemicals. Restriction endonucleases *Bam*H1, *Sph*1, and *Pst*1 were obtained from Boerhinger (Mannheim). Etoposide (VP16, NSC 141540) was a generous gift of Dr. J. C. David (Rennes, France); camptothecin (NSC 94600) was from the National Center Institute (Bethesda, MD); etoposide and camptothecin were dissolved in dimethyl sulfoxide at a concentration of 10 mM. Novobiocin and PMSF were from Sigma Chemical Co. [^{3}H]Thymidine was from CEA (Saclay, France).

RESULTS

Effect of Etoposide on DNA Synthesis. Mouse splenocytes were stimulated for growth by the addition of 2 μg/mL concanavalin A. As expected, DNA synthesis, measured by [^{3}H]thymidine incorporation, was inhibited by VP16. Inhibition occurred at low (0.4 μM) concentrations of the drug and reached 50% with 2.2 μM VP16 (data not shown). These results were consistent with the report of Zhang et al. (1986), who found that VM26 treatment of splenocytes inhibited the incorporation of [^{3}H]thymidine into the detergent-soluble fraction of DNA.

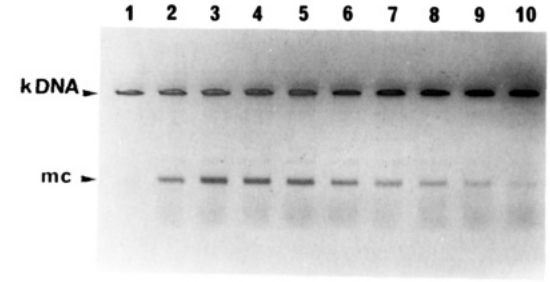

FIGURE 1: Effect of etoposide on topoisomerase II from splenocytes. Kinetoplast DNA (kDNA) was incubated with partly purified topoisomerase II in the presence of increasing concentrations of etoposide, as described under Materials and Methods. The products of incubation were analyzed by agarose gel electrophoresis. (Lane 1) kDNA control; (lanes 2–10) incubation with 0 (2), 3.4 (3), 6.8 (4), 13.6 (5), 27.2 (6), 54.4 (7), 108.8 (8), 217.6 (9), and 435.2 μM etoposide (10). mc: minicircles.

Effect of Etoposide on Type II Topoisomerase from Splenocytes. We next examined whether a correlation existed between the strong inhibition of DNA synthesis observed upon VP16 treatment and the sensitivity of topoisomerase II to the drug. As shown in Figure 1, the enzyme was inhibited by etoposide. More precise measurements of the initial velocity of the reaction (10-min incubations) gave an inhibition constant (K_i) of 42.5 μM. This value is similar to that obtained with topoisomerase II from other organisms (Jaxel et al., unpublished results; Riou, 1986), indicating that the enzyme from splenocytes is not especially sensitive to the drug. This inhibition constant is about 20 times higher than the concentration sufficient to inhibit 50% DNA synthesis. The difference can be explained by the fact that the cell may concentrate the drug (Pommier, personnal communication).

Long-Term Treatment of Splenocytes by Etoposide: Effect on Replicated DNA. Mouse splenocytes, stimulated by concanavalin A, were labeled and treated for 20 h with various concentrations of etoposide. The labeled DNA was analyzed by sedimentation through a sucrose gradient. As shown in Figure 2, treatment with increasing concentrations of the drug produced an accumulation of small DNA fragments. In parallel, the broad peak of high molecular weight DNA was reduced. This effect was visible after treatment with only 1–2 μM drug, that is, in the same range of concentrations which inhibited DNA synthesis. With 12.7 μM VP16, most of the labeled DNA was in the form of small fragments. The sedimentation profiles in neutral and alkaline gradients (Figure 2) are nearly identical, suggesting that the DNA breaks were mainly double-strand breaks. The size of the low molecular weight fragments was estimated to be 3–5 kb: this result is not in agreement with previous studies of Loike and Horwitz (1976), Ross et al. (1984), and Chen et al. (1984) with Hela or adenocarcinoma cells. These authors found that VP16 treatment produced 30–40-kb DNA fragments. However, they used high drug concentrations (up to 100 μM), and the cells were analyzed shortly after brief drug treatment (30 min to 1 h). In comparable experiments, Ross et al. (1984) found that these VP16-induced breaks were due to topoisomerase II inhibition and that DNA fragmentation reversed after drug removal. The reversibility of the extensive DNA fragmentation

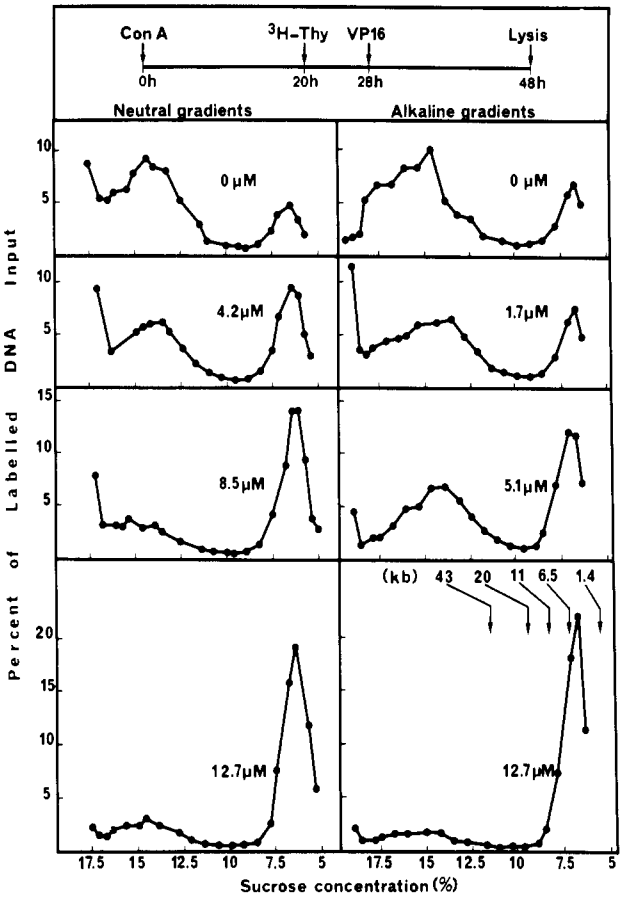

FIGURE 2: Sucrose gradient sedimentation of replicated DNA from etoposide-treated splenocytes. The upper panel indicates the schedule of the various additions. Conditions of sedimentation are described under Materials and Methods. The concentration (μM) of etoposide used is indicated on each panel. The size (in kilobases) of DNA markers is indicated on the lower right panel. Sedimentation was from right to left.

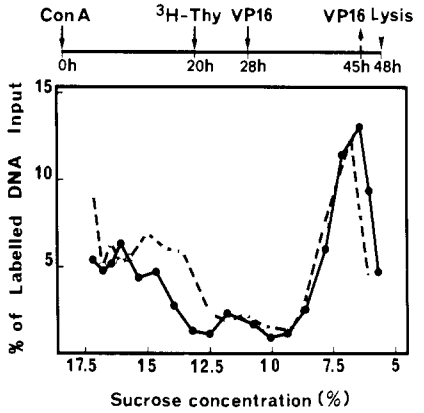

FIGURE 3: Sucrose gradient sedimentation of replicated DNA after recovery from etoposide treatment of splenocytes. Various additions were made at the times indicated in the upper part of the figure. (○) 3-h recovery; (●) control experiment in the continuous presence of the drug.

observed in our conditions was checked in stimulated splenocytes treated for 17 h in the presence of 8.5 μM VP16 and by incubation of the cells for an additional 3 h without drug. The sedimentation profile of Figure 3 shows that only a minor part of the labeled DNA was resealed in these conditions. This result suggests that the majority of the small DNA fragments was not produced by a mechanism analogous to the well-characterized topoisomerase II mediated strand breaks. Alternatively, a repair mechanism could account for the partial reversibility observed. In this case, topoisomerase II would

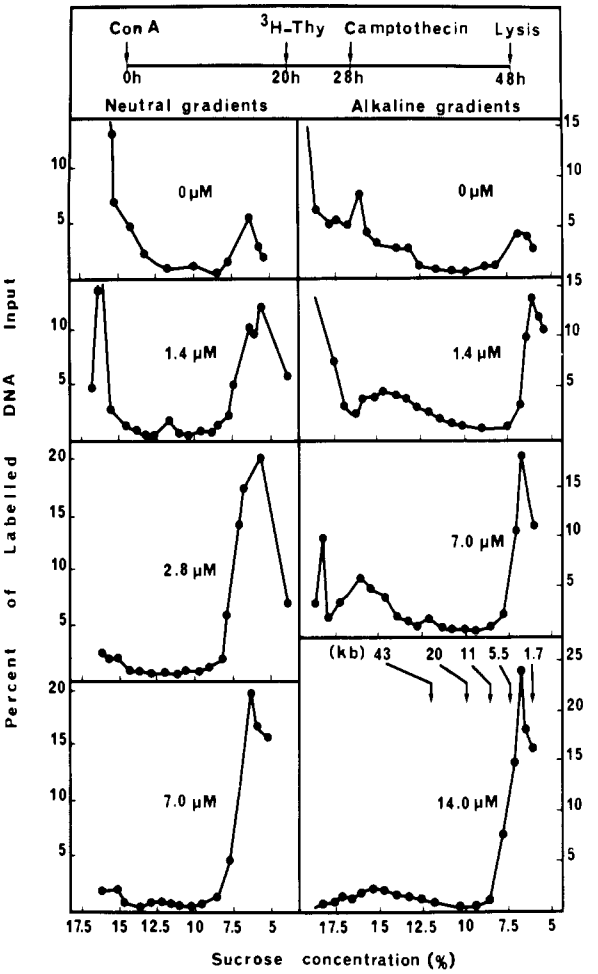

FIGURE 4: Sucrose gradient sedimentation of replicated DNA from camptothecin-treated splenocytes. Conditions are identical with those of Figure 2. Camptothecin concentrations (μM) are indicated on each panel.

not be involved in the reversion. This possibility was further supported by the fact that the small labeled fragments were not precipitable by the KCl assay of Trask et al. (1984) (not shown).

Effect of Novobiocin and Camptothecin on Stimulated Splenocytes. In order to determine whether the above observations are specific for VP16 treatment, we used the antibiotic novobiocin, which is known to inhibit eukaryotic topoisomerase II (and other ATP-utilizing enzymes) without interfering with the breakage–reunion mechanism. Prolonged incubation of splenocytes with relatively low concentrations of novobiocin (16.4–49.2 μM) also inhibited DNA synthesis (by 50–90%) and also produced small DNA fragments of about the same size (not shown).

The same experiments were performed with the topoisomerase I inhibitor camptothecin, which has been shown to trap topoisomerase I in a cleavable complex with DNA, producing topoisomerase I associated single-strand breaks (Hsiang et al., 1985; Mattern et al., 1987). We found that very low (1 μM) concentrations of camptothecin inhibited DNA synthesis in splenocytes. Moreover, long-term (20 h) incubations of splenocytes with camptothecin again produced fragmentation of the DNA (Figure 4) in a dose-dependent manner. Surprisingly, these fragments were formed by *double-strand* breaks and had a low molecular weight (3–4 kb), as observed with etoposide or novobiocin. However, very low doses (1.4–7 μM) of camptothecin produced intermediate-sized (50 kb) fragments only visible in alkaline gradients (Figure 4, right

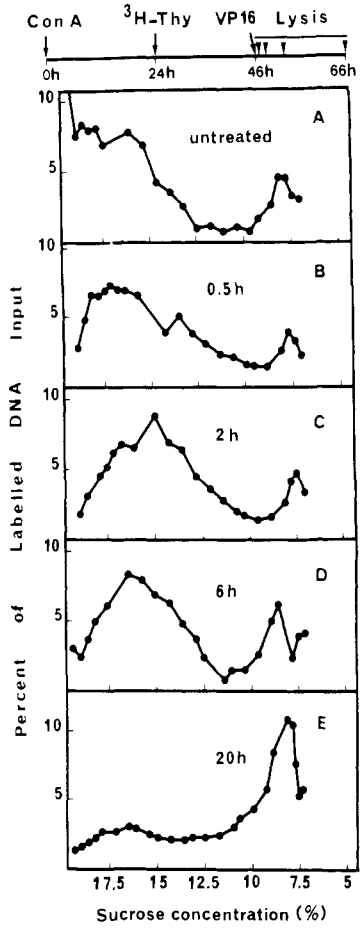

FIGURE 5: Time course of DNA fragmentation during etoposide treatment; alkaline sucrose gradients. The conditions of sedimentation are those of Figure 2. (A) Untreated control; (B) 0.5, (C) 2, (D) 6, and (E) 20 h of contact with etoposide.

panels), indicating that they were formed by single-strand breaks, presumably associated with topoisomerase I.

Short-Term Effect of Etoposide on Stimulated Splenocytes. In order to confirm that the primary effect of VP16 treatment was the production of topoisomerase II associated breaks, we have analyzed the DNA of splenocytes after a short contact with the drug. Cells were stimulated by con A, labeled with [^{3}H]thymidine for 22 h, and treated for 30 min with 12.7 μM VP16. In long-term experiments, this concentration induced a major conversion of the high molecular weight DNA into small fragments (Figure 3). In this short-term experiment, alkaline sucrose gradient sedimentation (Figure 5A,B) indicated that the amount of small fragments remained identical with that of the untreated control. Furthermore, high molecular weight DNA, visible at the bottom of the gradient in the control experiment (Figure 5A), was converted into an intermediate-sized form of about 50 kb (Figure 5B,C). The breaks associated with the appearance of these fragments were totally *reversible*: after a 30-min contact with the cells, VP16 was removed, and the splenocytes were incubated for an additional 2 h. In this case, the sedimentation profile of the labeled DNA was identical with that of the control (not shown). Moreover, the fragments produced after 30-min contact with the drug were precipitable by the KCl assay. This precipitation was abolished when the fragments were incubated with proteinase K, indicating that they were tightly associated with a protein, presumably topoisomerase II (not shown).

Production of Small DNA Fragments as a Function of Time of Contact with Etoposide. Stimulated splenocytes were treated by 12.7 μM VP16 for 30 min, 2 h, 6 h, or 20 h. The DNA was analyzed by alkaline sucrose gradient sedimentation. The results (Figure 5) indicated that, during the first 2 h of incubation with the drug, high molecular weight DNA was converted into intermediate-sized fragments. At this time, production of small fragments was already visible, but clearly appeared after 6 h. Finally, after 20-h incubation, most of the DNA was converted to small fragments, as already described.

Discussion

The experiments described in this paper were based on the assumption that in splenocytes topoisomerase II is the specific target of etoposide, as reported for other cell types. Indeed, the strong inhibition of DNA synthesis and the protein-associated reversible DNA double-strand breaks that we observed after a brief contact with the drug were consistent with this assumption.

An unexpected finding was the detection of a secondary breakage of the DNA upon prolonged incubation of S-phase splenocytes with etoposide. Although this fragmentation resulted from double-strand breaks, it seems that this breakage does not directly involve topoisomerase II. This opionion is based on the following observations: (i) Only a minor proportion of the breaks were resealed after drug removal. (ii) The size of the fragments (3–5 kb) and their progressive appearance between 2 and 20 h of contact are not consistent with topoisomerase II action. (iii) The small fragments are not tightly associated with proteins. (iv) Novobiocin, which does not promote topoisomerase II associated breaks, produced the same DNA fragmentation as VP16 in long-term incubations. A somewhat similar situation has been reported by Villeponteau et al. (1986), who found that novobiocin treatment of embryonic erythrocytes produced double-strand DNA breaks resulting in small fragments. (v) Camptothecin, a specific topoisomerase I inhibitor, when used in prolonged incubations, again blocks DNA synthesis in splenocytes and again produces small DNA fragments at very low concentrations. Moreover, although camptothecin-induced reversible cleavage is known to produce single-strand breaks, the fragmentation we observed again involved double-strand breaks.

The important question raised by our results is to find an explanation for the similar effects of the three topoisomerase inhibitors etoposide, novobiocin, and camptothecin on DNA synthesis and on the fragmentation of replicated DNA in splenocytes.

Taking into account the recent findings on the prominent role of topoisomerases in DNA replication, an attractive hypothesis could explain our results: more than the trapping of topoisomerases on the DNA, the inhibition of their enzymatic activities during the elongation process of chromosomal replication may induce the secondary fragmentation observed. The following remarks support this hypothesis: (i) Etoposide and camptothecin strongly reduce the catalytic activities of topoisomerase II and I, respectively, by trapping them in a complex with DNA. (ii) Novobiocin inhibits topoisomerase II without formation of the cleavable complex. We know that topoisomerase II is required in the vicinity of the replication fork during elongation; at this stage, it can be substituted by topoisomerase I, as recently shown in yeast mutants (Uemura et al., 1984) and for SV 40 DNA replication in vitro (Yang et al., 1987). However, it is not excluded that the constitutive replication rate in splenocytes requires the presence of both enzymes, since elongation is inhibited by camptothecin alone as well as by etoposide alone. Thus, in the replication complex, the two topoisomerases may act to stimulate the replicase, presumably by their swivel activity. They also trigger the

variations of chromosome structure necessary for replication. Then, as a consequence of reduced topoisomerase activity, the entire process would be blocked: the replication forks would be arrested, and newly replicated DNA would not be stabilized into a protecting chromatin structure. As the DNA synthesis observed in our experiments takes place in splenocytes after stimulation by a mitogen, the uncoupling of replication from the other events leading to mitosis may generate a mechanism of degradation of the exposed DNA. The recent findings of Estey et al. (1987), who suggest that the frequency of topoisomerase II mediated cleavage in Hela Cells is not correlated with the cytotoxic effect of mAMSA, support our interpretation. It should also be emphasized that earlier works on the effect of etoposide (Kalwinsky et al., 1983), camptothecin (Gallo et al., 1970; Horwitz & Horwitz, 1971), and also azaellipticine (Vilarem et al., 1984) in replicating mammalian cells are in good agreement with our results: they all induce the inhibition of DNA synthesis, a strong fragmentation of the DNA, and a low percent of survival.

Further experiments are needed to know whether these observations are limited to topoisomerase inhibitors and to determine how they can be related to cytotoxicity. In that regard, Brox et al. (1984) have shown that treatment of lymphocytes by aphidicolin (a DNA polymerase α inhibitor) produced DNA breaks and cell death. On the other hand, Ucker (1987) has recently shown that the cytotoxic effect of T lymphocytes and glucocorticoids in target cells involved a common pathway that induced DNA degradation. Finally, it is important to note that the long-term effect of etoposide we have described may be related to the in vivo mode of action of the drug, since inhibition of DNA synthesis, irreversible fragmentation of replicated DNA, and cytotoxicity occur in the same range of drug concentrations.

ACKNOWLEDGMENTS

We thank M. Gabillot and G. Riou for the gift of *Trypanosoma cruzi* pellets, A. M. Lotti for kDNA preparation, and M. Nadal, Y. Pommier, and J. Sobczak for valuable discussions.

REFERENCES

Brox, L., Hunting, D., & Belch, A. (1984) *Biochem. Biophys. Res. Commun. 120*, 959–963.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560–13566.

Duguet, M., Lavenot, C., Harper, F., Mirambeau, G., & De Recondo, A. M. (1983) *Nucleic Acids Res. 11*, 1059–1075.

Estey, E., Adlakha, R. C., Hittelman, W. N., & Zwelling, L. A. (1987) *Biochemistry 26*, 4344–4349.

Gallo, R. C., Wang-Peng, J., & Adamson, R. H. (1970) *JNCI, J. Natl. Cancer Inst. 46*, 789–795.

Heck, M. M. S., & Earnshaw, W. C. (1986) *J. Cell Biol. 103*, 2569–2581.

Horwitz, M. S., & Horwitz, S. B. (1971) *Biochem. Biophys. Res. Commun. 45*, 723–727.

Hsiang, Y.-H., Hertzberg, S. H., & Liu, L. F. (1985) *J. Biol. Chem. 260*, 14873–14878.

Kalwinsky, D. K., Look, A. T., Ducore, J., & Fridland, A. (1983) *Cancer Res. 43*, 1592–1597.

Loike, J. D., & Horwitz, S. B. (1976) *Biochemistry 15*, 5443–5448.

Markovits, J., Pommier, Y., Kerrigan, D., Covey, J. M., Tilchen, E. J., & Kohn, K. W. (1987) *Cancer Res. 47*, 2050–2055.

Mattern, M. R., Mong, S.-M., Bartus, H. F., Mirabelli, C. K., Crooke, S. T., & Johnson, R. K. (1987) *Cancer Res. 47*, 1793–1798.

Moore, G. E., Gerner, R. E., & Franklin, H. A. (1977) *JAMA, J. Am. Med. Assoc. 199*, 519–524.

Nelson, W. G., Liu, L. F., & Coffey, D. S. (1986) *Nature (London) 322*, 187–189.

Newport, J. (1987) *Cell (Cambridge, Mass.) 48*, 205–217.

Pommier, Y., Schwartz, R. E., Zwelling, L. A., & Kohn, K. W. (1985) *Biochemistry 24*, 6406–6410.

Riou, G., & Yot, P. (1977) *Biochemistry 16*, 2390–2396.

Riou, G., Gabillot, M., Barrois, M., Breitburd, F., & Orth, G. (1985) *Eur. J. Biochem. 146*, 483–488.

Riou, J. F. (1986) Ph.D. Thesis, Université Pierre et Marie Curie, Paris.

Ross, W., Rowe, T., Glisson, B., Yalowick, J., & Liu, L. F. (1984) *Cancer Res. 44*, 5857–5860.

Sullivan, D. M., Glisson, B. S., Hodges, P. K., Smallwood-Kentro, S., & Ross, W. E. (1986) *Biochemistry 25*, 2248–2256.

Taudou, G., Wiart, J., Taudou, B., & Panijel, J. (1977) *Ann. Immunol. (Paris) 128C*, 1065–1077.

Taudou, G., Mirambeau, G., Lavenot, C., Der Garabedian, A., Vermeersch, J., & Duguet, M. (1984) *FEBS Lett. 176*, 431–435.

Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182–9187.

Trask, D. K., DiDonato, J. A., & Muller, M. T. (1984) *EMBO J. 3*, 671–676.

Tricoli, J. V., Sahai, B. M., McCormick, P. J., Jarlinski, S. J., Bertram, J. S., & Kowalski, D. (1985) *Exp. Cell Res. 158*, 1–14.

Ucker, D. S. (1987) *Nature (London) 327*, 62–64.

Uemura, T., & Yanagida, M. (1984) *EMBO J. 3*, 1737–1744.

Vilarem, M. J., Gras, M. P., Primaux, F., & Larsen, C. J. (1984) *Biochimie 66*, 591–599.

Villeponteau, B., Pribyl, T. M., Grant, M. H., & Martinson, H. G. (1986) *J. Biol. Chem. 261*, 10359–10365.

Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665–697.

Yang, L., Wold, M. S., Li, J. J., Kelly, T. J., & Liu, L. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 950–954.

Zhang, L. H., Mui, S. C., & Strauss, P. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5871–5874.