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INHIBITION OF DNA SYNTHESIS AND DNA FRAGMENTATION IN STIMULATED SPLENOCYTES BY THE CONCERTED ACTION OF TOPOISOMERASE I AND II POISONS

GEORGES TAUDOU,* CHRISTIANE PORTEMER,† CHRISTINE JAXEL† and MICHEL DUGUET†‡

Laboratoire d'Enzymologie des Acides Nucléiques, Université P. et M. Curie, URA 554 CNRS, 96, Bd. Raspail, 75006 Paris, France

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Abstract—Stimulated splenocytes were used as a model system to investigate the effects of topoisomerase inhibitors on normal, non-transformed, non-tumoral proliferating cells. The concerted action of camptothecin (a poison of topoisomerase I) and etoposide (a poison of topoisomerase II) lead to nearly complete inhibition of DNA synthesis in concanavalin A-stimulated splenocytes. Analysis of replicated cellular DNA after a short treatment with both drugs revealed a DNA cleavage to medium size fragments. This effect was additive, suggesting that cleavable complexes were formed independently by both topoisomerases on their respective DNA sites. In contrast, prolonged contact with both drugs was followed by degradation of the bulk cellular DNA to nucleosome size fragments, indicating that apoptosis took place in these cells. Combination of camptothecin and etoposide enhanced this phenomenon, consistent with the fact that degradation was the result of secondary events which may amplify the signal. Thus, aphidicolin, an inhibitor of eukaryotic replicases which blocks replication, also triggered DNA degradation in proliferating splenocytes.

The essential role of topoisomerases in the behavior of the cell is well established. These enzymes seem to be required mainly to solve the topological problems linked to replication, transcription and other processes concerning the DNA double helix. Briefly, their mechanism involves multiple events of breakage and rejoining of the DNA backbone, allowing the untangling of individual strands or duplexes (see Refs 1 and 2 for a review). The role of topoisomerases has been emphasized in the last 5 years by the discovery that, not only in bacteria, but also in eukaryotes, a number of therapeutic agents, inhibitors of cell proliferation, have DNA topoisomerases as a common target [3, 4]. Some of these agents seem to inhibit topoisomerization reactions by trapping the enzyme in a complex, named "cleavable complex", where the DNA is cleaved but cannot be rapidly resealed [5]. Thus, these inhibitors are useful tools not only for therapy [6], but also to elucidate the *in vivo* functions of topoisomerases [7]. Among these, epipodophyllotoxins, specific of topoisomerase II [8], and camptothecins, specific of topoisomerase I [9, 10], are especially interesting, since they do not intercalate into DNA [5, 9].

In previous studies, we have used guinea pig lymphocytes and mouse splenocytes in culture as a model system of cell proliferation [11, 12]. It should be emphasized that these are normal, non-transformed, non-tumoral cells: their proliferation can be induced simply by addition of a mitogen such as concanavalin A. By using this system, we have shown that: (i) as described for a number of tumor cells, addition of the topoisomerase II poison etoposide (an epipodophyllotoxin analog also known as VP16) to a culture of stimulated splenocytes rapidly produced cleavable complexes which can be visualized as medium-size fragments (about 50 kb) after sedimentation in the presence of a strong detergent. These complexes disappeared upon drug removal and were linked to a protein, presumably topoisomerase II. (ii) When incubation of splenocytes with etoposide was prolonged for 20 hr, the sedimentation profile was totally different: the DNA appeared degraded to unusually small fragments, involving irreversible double-strand breaks, and not associated with a protein. The same double-strand breakage was obtained when camptothecin, a topoisomerase I inhibitor which essentially produces single-strand breaks, and novobiocin, a topoisomerase II inhibitor which does not interfere with the breakage–reunion mechanism [13], were used.

Indeed, irreversible cellular DNA fragmentation was described in a variety of conditions producing the arrest of replication [14–18]. We proposed the hypothesis that the block of replication forks by topoisomerase inhibition or cleavable complex formation would induce the pathway leading to DNA degradation and perhaps cell death [11]. This hypothesis was supported by the data of several other laboratories, showing that DNA degradation and cytotoxicity induced by various topoisomerase

* Present address: Laboratoire d'Immunoallergie, Institut Pasteur, 25 rue du Dr. Roux, 75015 Paris, France.

† Present address: Laboratoire d'Enzymologie des Acides Nucléiques, Institut de Génétique et Microbiologie, URA 1354 CNRS, Université Paris Sud, 91405 Orsay Cedex, France.

‡ Corresponding author. Tel. (33) 69 41 62 16.

I and II inhibitors were dependent on DNA replication and on the phase of the cell cycle at the time of treatment [19–22]. This appeared clearly for camptothecin which blocks replication forks *in vitro* only if topoisomerase I is present [23], and whose cytotoxicity *in vivo* in yeast is dependent on DNA replication [24]. Moreover, severe DNA degradation was reminiscent of the programmed cell death called apoptosis [25, 26]. A few years ago, Kaufmann [27] and Walker *et al.* [28] found that various anticancer agents, including etoposide and camptothecin, induced apoptosis in HL 60 leukemia cells and thymocytes, while in other cell lines cytotoxicity did not seem to be related to apoptosis [29]. Recently, several authors described that cytotoxicity of topoisomerase II targeting compounds was reduced in the presence of camptothecin or other inhibitors of RNA synthesis, concluding that not only DNA, but RNA synthesis were required for lethality [30, 31].

In the present study, we have extended our earlier findings [11] by investigating the effects of the combined action of etoposide and camptothecin on splenocytes stimulated to proliferate. We found that this association enhanced the individual effect of each drug: DNA replication was blocked completely and cellular DNA was degraded to nucleosomal size, characteristic of apoptosis.

MATERIALS AND METHODS

Chemicals and drugs. Etoposide (VP16, NSC 141540) and camptothecin (NSC 94600) were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Stock solutions were made at a concentration of 10 mM in dimethyl sulfoxide (DMSO*). Aphidicolin was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and dissolved at 5 mg/mL in DMSO. Culture media were from Gibco BRL (Bethesda, MD, U.S.A.). Other chemicals were obtained from Sigma. Radiolabeled [^{3}H]thymidine and [^{3}H]TTP were from CEA (Saclay, France).

Isolation and cultures of mouse spleen cells. Isolation of splenocytes from C57/BL mice (Institut Pasteur) by spleen disruption and cultures were performed as described previously [11]. Viable cells excluding trypan blue were counted in a hemocytometer. Spleen cells were incubated at a concentration of 2×10^6 cells/mL in RPMI medium [32] containing 5% fetal calf serum (Gibco) and supplemented with 0.2 mM alanine and 100 U/mL penicillin. The concentration of concanavalin A was 2 μg/mL. Stimulated splenocytes were cultured for 48 hr, then, either directly treated by trichloroacetic acid (TCA) to measure [^{3}H]thymidine incorporation as described previously [33], or collected by centrifugation for sedimentation and electrophoretic analyses. Antitumor drugs and thymidine precursor were added to the medium at various times before the end of the culture.

Measure of DNA synthesis in permeabilized splenocytes. Cultured splenocytes were permeabilized by hypotonic treatment according to the method of Buckley and Wedner [34]. The hypotonic buffer was of the following composition: 10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 30 mM 2-mercaptoethanol and 4 mM $MgCl_2$. DNA synthesis was measured by a modification of the method of Buckley and Wedner. The reaction mixture contained 150 mM NaCl, 100 mM HEPES, pH 7.8, 21 mM $MgCl_2$, 15 mM ATP, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP and [^{3}H]TTP (1.8 TBq/mmol; 1.48 MBq/mL). The complete reaction system (0.3 mL) contained 1×10^6 cells in 0.1 mL of hypotonic buffer, 0.1 mL of DNA synthesis reaction mixture and 0.1 mL of hypotonic buffer containing 40 mM phosphocreatine and 10 μg creatine phosphokinase (Sigma). The permeabilized cells were incubated at 37° for 30 min. The reactions were terminated by the addition of 3 mL of cold 10% TCA–2% $Na_4P_2O_7$. Samples were harvested onto fiber glass filters, washed with 5% TCA–2% $Na_4P_2O_7$, rinsed with ethanol, dried and counted.

Sedimentation through alkaline sucrose gradients. The method was performed as described previously [11]. Briefly, after appropriate treatment with drugs, splenocytes (about 2×10^6 cells) were collected by centrifugation, washed in RBS buffer (30 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$), and quickly lysed in 10 mM EDTA, 1% deoxycholate and 1.5% lithium dodecylsulfate (LDS). The lysates (usually 50,000–100,000 cpm) were immediately applied to 5–20% sucrose gradients in 10 mM EDTA, 1.5% LDS, 0.2 M NaOH, and centrifuged at 23° for 16 hr at 17,000 rpm in a SW 50.1 rotor. Fractions of 200 μL were collected from the bottom, and ^{3}H radioactivity counted after TCA precipitation.

Electrophoretic analysis of cellular DNA. Splenocytes (about 2×10^6 cells), stimulated by concanavalin A and treated with drugs, were collected by centrifugation, washed twice in RBS buffer, and resuspended in 100 μL lysis buffer containing 500 mM Tris–HCl, pH 9.0, 2 mM EDTA, 10 mM NaCl, 1% SDS and 1 mg/mL proteinase K [27]. After incubation at 48° for 22 hr, samples were extracted twice with phenol, once with phenol–chloroform and finally with chloroform. About one quarter of each sample (5×10^5 cells) was loaded on 1.4% agarose gel. Electrophoresis was performed for 13 hr at 1.2 V/cm in Tris-acetate EDTA (TAE) buffer. The gel was stained with 1 μg/mL ethidium bromide and photographed under UV (254 nm).

RESULTS

Etoposide and camptothecin inhibit DNA replication in splenocytes stimulated for growth: an additive effect

In a preliminary experiment, mouse splenocytes were stimulated for growth by the addition of concanavalin A (see Materials and Methods). At the peak of DNA synthesis, cells were incubated with the drug for 1 hr, and DNA synthesis was measured as a function of drug concentration. As shown in Fig. 1A, DNA replication was severely inhibited by both etoposide and camptothecin. The latter was the most efficient inhibitor, a concentration

* Abbreviations: DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; LDS, lithium dodecylsulfate; TAE, Tris acetate EDTA.

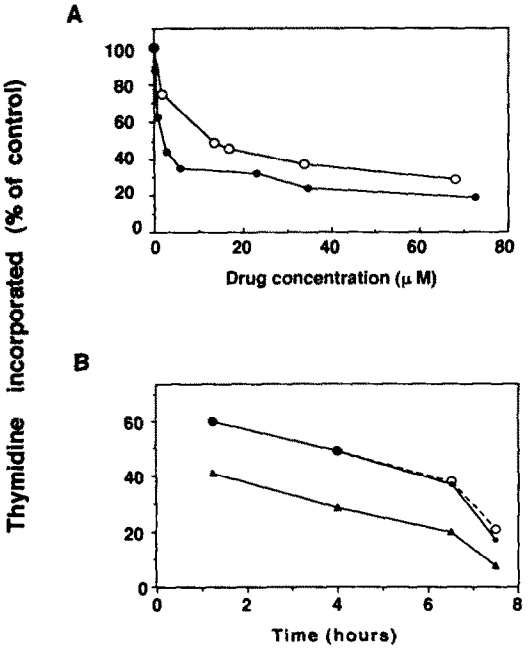

Fig. 1. Inhibition of DNA synthesis in proliferating splenocytes. (A) Dose–response experiment: 47 hr after splenocyte stimulation for growth, the inhibitor was added into the medium 15 min prior to [^{3}H]thymidine, and incubation was prolonged for 1 hr. Incorporation of the radiolabeled precursor was measured as described previously [20], and plotted against drug concentration. (○) Etoposide; (●) camptothecin. (B) Inhibition as a function of time of contact with drugs. Stimulated splenocytes were incubated for the indicated time with inhibitors and [^{3}H]thymidine incorporation was measured for the same period. (○) Etoposide (12.7 μM) alone; (●) camptothecin (2.9 μM) alone; (▲) etoposide (12.7 μM) and camptothecin (2.9 μM) together.

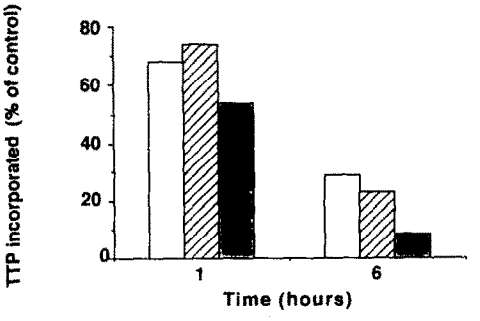

Fig. 2. Inhibition of DNA synthesis measured in permeabilized splenocytes. Stimulated splenocytes were incubated for the indicated time with inhibitors, permeabilized as described in Materials and Methods, and [^{3}H]TTP incorporation was measured. [□) Etoposide (12.7 μM) alone; (▨) camptothecin (2.9 μM) alone; (▩) etoposide (12.7 μM) and camptothecin (2.9 μM) together.

of 2.9 μM reducing [^{3}H]thymidine incorporation by 50%. Since this brief incubation with the drug did not completely abolish DNA replication even at higher concentrations (Fig. 1A), we next investigated the effects of prolonged incubation with etoposide and camptothecin separately or together. Stimulated splenocytes were incubated for various times with the drugs, and thymidine incorporation was measured during the same period (Fig. 1B). Etoposide (12.7 μM) and camptothecin (2.9 μM) concentrations were chosen to produce approximately the same extent of inhibition, which was not complete (upper curves). However, when added together, their effect on DNA replication was clearly additive and precursor incorporation was almost completely abolished after 7 hr 30 min (Fig. 1B, lower curve).

Etoposide and camptothecin do not affect the uptake of radiolabeled precursors

Various antitumor drugs have been described as modifying the cell membrane properties and permeability to nucleoside precursors [35]. In order to estimate if etoposide or camptothecin treatment could perturbate the uptake of [^{3}H]thymidine, we have measured DNA synthesis in permeabilized splenocytes as described in Materials and Methods. Figure 2 shows essentially the same result as in intact cells: DNA synthesis was severely inhibited by 6 hr incubation with the cooperation of low concentrations of etoposide and camptothecin. This suggests that drug treatment did not appreciably change the permeability of the cells to nucleotide precursors.

Effect of proliferating splenocytes treatment by etoposide and camptothecin on the integrity of replicated DNA: sucrose gradient analysis

Short-term treatment. Stimulated splenocytes were labeled and incubated for 1 hr in the presence of drugs. The size of labeled DNA was analysed in an alkaline sucrose gradient as described in Materials and Methods. The result is shown in Fig. 3. In the presence of etoposide (17 μM) or camptothecin (3.6 μM) which inhibited DNA synthesis to the same extent (about 50%, see Fig. 1A), high molecular mass DNA, visible at the bottom of the control gradient (Fig. 3a), was converted to a peak of medium size fragments (40–50 kb, fractions 8–15 of gradients 3b and 3c). Again, camptothecin was the most efficient, 3.6 μM being equivalent to 17 μM etoposide. In a previous study [11], we have shown that the fractionation observed after a short treatment of splenocytes was reversible and that the fragments were linked to a protein, presumably topoisomerase I or II, respectively, for camptothecin and etoposide. In the present study, when both drugs were added, the fractions of high molecular mass DNA completely disappear to the benefit of medium size fragments (Fig. 3d), suggesting that etoposide and camptothecin presumably form distinct and therefore additive complexes on the DNA. When low drug concentrations were used, no synergistic effect of the two drugs was observed, but again an additive effect (not shown). Finally, it should be noted that, in these short-term experiments, none of the treatments appreciably changed the amount of small size fragments visible in the control (Fig. 3a, fractions 20–23).

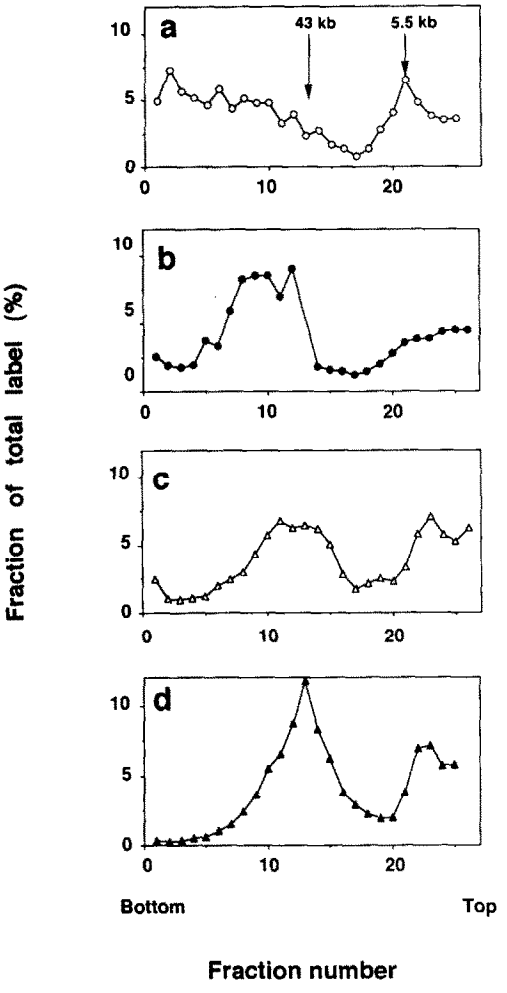

Fig. 3. Effect of short-term treatment of proliferating splenocytes with a combination of etoposide and camptothecin: an alkaline sucrose gradient analysis of replicated DNA. Splenocytes, stimulated by concanavalin A, were labeled for 24 hr (24–48 hr after stimulation) with [^{3}H]thymidine and incubated for 1 hr with drugs (47–48 hr after stimulation). The cells were lysed, immediately loaded on top of 5–20% alkaline sucrose gradients, and sedimented as described in Materials and Methods. (a) Untreated control cells; (b) incubation with 17 μM etoposide; (c) incubation with 3.6 μM camptothecin; (d) incubation with 17 μM etoposide and 3.6 μM camptothecin. Sedimentation was from right to left. The arrows indicate the positions of the 43 and 5.5 kb size markers.

Long-term treatment. Stimulated splenocytes, labeled by thymidine, were treated for 20 hr by etoposide or (and) camptothecin (see Materials and Methods), and the DNA was analysed as above. When the same range of drug concentration as in the preceding experiment was used (Fig. 4A, panels b–d), high molecular mass DNA was converted to small pieces (<5 kb), without accumulation of medium size fragments. The effect was even more dramatic when etoposide and camptothecin were used together: nearly all the DNA on the gradient

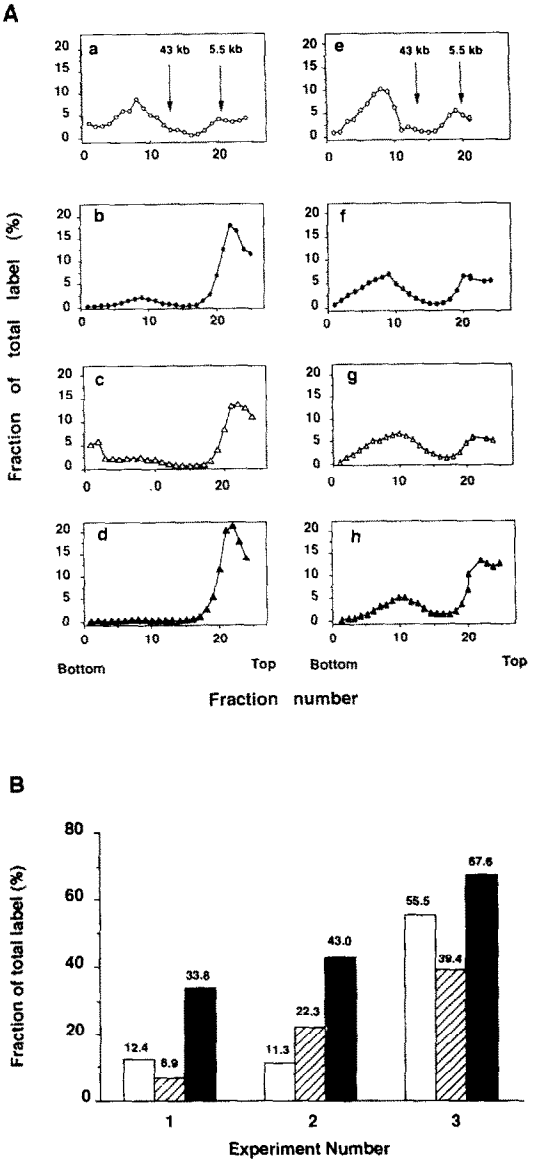

Fig. 4. Effect of long-term treatment of proliferating splenocytes with a combination of etoposide and camptothecin: alkaline sucrose gradient analysis of replicated DNA. Stimulated splenocytes, labeled for 28 hr (from 20–48 hr after stimulation) were treated for 20 hr (from 28–48 hr after stimulation) with inhibitors, lysed as described, and immediately loaded on 5–20% alkaline sucrose gradients. Sedimentation conditions were as described in Materials and Methods. (A) Sedimentation profiles. (a) and (e) untreated cells controls; (b)–(d) low drug concentrations: (b) 8.5 μM etoposide; (c) 3.6 μM camptothecin; (d) etoposide (8.5 μM) and camptothecin (3.6 μM) together. (f)–(h) very low drug concentrations: (f) 1.7 μM etoposide; (g) 0.29 μM camptothecin; (h) etoposide (1.7 μM) and camptothecin (0.29 μM) together. Sedimentation was from right to left. (B) Histogram analysis of various gradients. For each gradient, the total label of the fractions corresponding to the peak of small fragments (fractions 19–23) was determined and expressed as a fraction of the total radioactivity loaded on the gradient. (□) Etoposide; (▨) camptothecin; (□) etoposide and camptothecin. Experiment 1: 1.7 μM etoposide, 0.29 μM camptothecin. Experiment 2: 1.7 μM etoposide, 0.58 μM camptothecin. Experiment 3: 8.5 μM etoposide, 3.6 μM camptothecin.

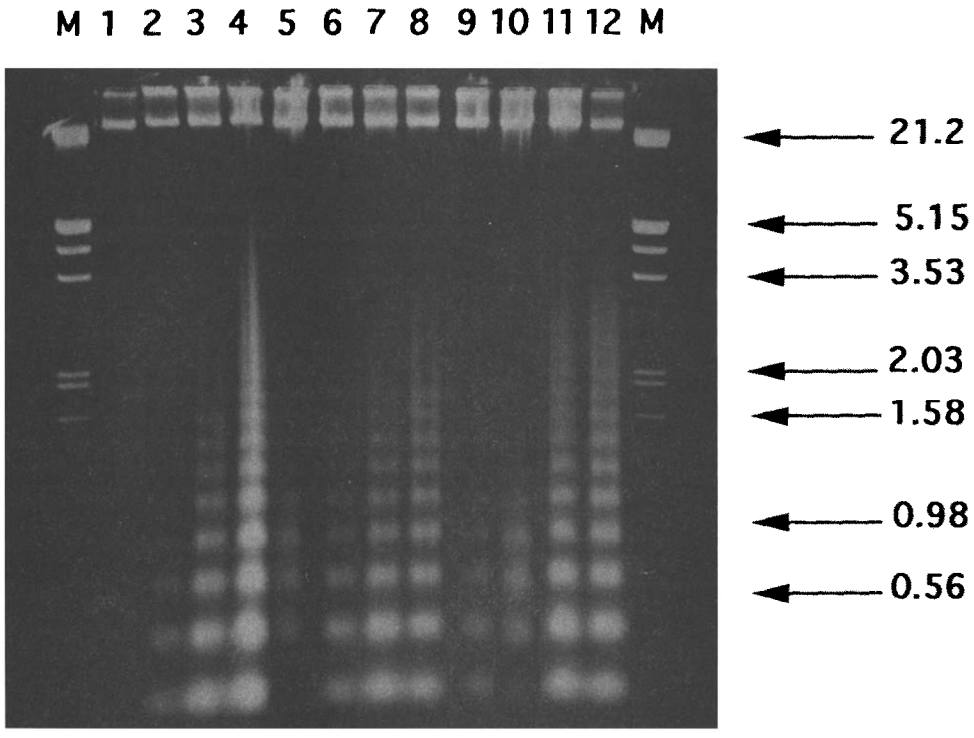

Fig. 5. Electrophoretic analysis of cellular DNA after treatment of splenocytes with etoposide and camptothecin. Splenocytes, stimulated by Concavalin A, were treated for 0–20 hr by the drugs, separately or in combination. DNA samples were prepared and electrophoresed as described in Materials and Methods. Lanes M: lambda *Eco* RI + *Hind* II digest as size markers. The length (in kb) of the fragments is 21.2; 5.15; 4.28; 3.53; 2.03; 1.90; 1.58; 1.33; 0.98; 0.83; 0.56. Lane 1 is the untreated control. Lanes 2, 5 and 9: 2 hr treatment with, respectively, 8.5 μM etoposide, 3.6 μM camptothecin, and the combination of both compounds. Lanes 6 and 10: 4 hr treatment with camptothecin and the combination of etoposide and camptothecin at the same concentrations of compounds as above. Lanes 3, 7 and 11: 6 hr treatment as above. Lanes 4, 8 and 12: 20 hr treatment as above.

was processed to small size fragments (Fig. 4A, panel d, and Fig. 4B). Even with unusually small concentrations of inhibitors (1.7 μM etoposide and 0.29 μM camptothecin), the presence of both drugs resulted in the appearance of a large amount of small fragments (Fig. 4A, panel h). Figure 4B summarizes the results of different incubations with different drug concentrations.

Effect of etoposide and camptothecin treatment of splenocytes on the integrity of cellular DNA: electrophoretic analysis

We next asked whether the severe degradation of replicated DNA revealed by sucrose gradient sedimentation was related to the phenomenon of apoptosis. Stimulated splenocytes were cultured in the absence of radioactive label and treated for various periods of time with etoposide, camptothecin or a combination of the two inhibitors at the same concentration as in the experiment of Fig. 4A (b–d). Cells were lysed, and cellular DNA was prepared as described in Materials and Methods and analysed by standard agarose gel electrophoresis. A ladder of intense bands, characteristic of nucleosomal size degradation, was actually observed in the samples treated for 20 hr (Fig. 5, lanes 4, 8 and 12), consistent with the appearance of small size fragments in sucrose gradients (Fig. 4A, panels b–d). These results suggest that not only replicated DNA but also the bulk of cellular DNA is degraded, a characteristic of apoptosis. The phenomenon was already important after only 6 hr treatment. In the case of 2 hr treatment, the majority of the DNA was not degraded, but the ladder of apoptosis was clearly visible. The high sensitivity of the electrophoretic analysis also allowed traces of apoptosis in untreated controls to be detected (Fig. 5, lane 1, see Discussion).

Treatment of stimulated splenocytes by aphidicolin also produces a fragmentation of DNA into small pieces

Splenocytes, stimulated and labeled as above, were treated for 20 hr with aphidicolin, an inhibitor of DNA replicase in eukaryotes [36]. The DNA size distribution in alkaline gradients again exhibited a peak of small size fragments (Fig. 6). The same result was obtained when the DNA was analysed in a neutral sucrose gradient (data not shown) suggesting that, as described above for topoisomerase inhibitors etoposide and campthothecin [11], this degradation was also due to apoptosis.

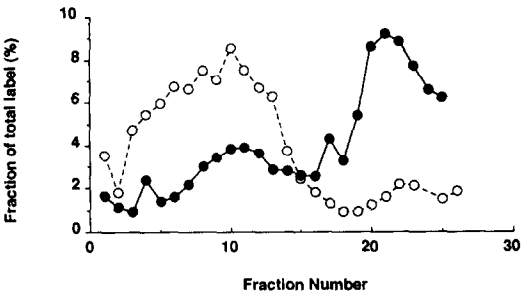

Fig. 6. Analysis of replicated DNA after long-term treatment of splenocytes by aphidicolin. Stimulated splenocytes, labeled for 28 hr (20–48 hr after stimulation), were treated for 20 hr (28–48 hr after stimulation) by aphidicolin and analysed on 5–20% alkaline sucrose gradients, as described in Materials and Methods. (○) Untreated control cells; (●) aphidicolin (14.7 μM).

DISCUSSION

The experiments described in this study show that in normal proliferating cells, DNA synthesis was inhibited by both topoisomerase I or topoisomerase II inhibitors. It is generally admitted that both enzymes are necessary during the elongation phase of DNA replication to provide the swivel required for progression of the replication forks [23]. Consistent with this idea is the finding that none of these two inhibitors alone was able to completely abolish DNA replication in splenocytes, while it is almost totally blocked by the concerted action of the two drugs. However, camptothecin appears as the most efficient inhibitor of DNA synthesis in splenocytes. This observation may be related to the prominent role of topoisomerase I as a swivelase at the fork [37], as well as in histone gene transcription.

As found for a number of tumor cell lines or transformed cells, one of the first events upon incubation with etoposide and camptothecin is to stimulate the formation of "cleavable complexes" between topoisomerases and DNA. In our system of proliferating splenocytes, cleavable complex formation was revealed by the reversible appearance of medium size protein-linked DNA fragments by LDS–sucrose gradient analysis of replicated DNA after a short (1 hr) treatment with drugs [11]. In the present study, camptothecin and etoposide would, respectively, freeze topoisomerase I and II as cleavable complexes on their respective sites. The concomitant presence of the two drugs would simply stimulate independently topoisomerase I and II complex formation, resulting in an additive effect on cleavage frequency and the disappearance of high molecular mass DNA.

The second and still not understood type of events is the pathway(s) by which the primary lesions (cleavable complexes) are processed to produce gross DNA degradation in various cells. We show here that in long-term treatments, the severe DNA degradation previously observed, which was irreversible and not protein linked [11], is clearly related to apoptosis. This phenomenon was visible after 2 hr, although not prominent, and increased from 2 to 20 hr. With the sensitivity of the electrophoretic analysis, traces of apoptosis were observed in the untreated controls. It is possible that a small fraction of cells undergo apoptosis after 48 hr culture following concanavalin A stimulation.

Similar results were obtained by Kaufmann [27] on HL 60 leukemia cells and Walker *et al.* [28] on thymocytes. In both cases, DNA degradation seemed to trigger cell death. In contrast, Bertrand *et al.* [29] found that etoposide-induced cytotoxicity in DC3F cell line was dependent on Ca, but not related to apoptosis. In the present work with non-tumoral, non-transformed splenocytes, we observed a potentiation by camptothecin of the effect of etoposide on DNA replication and on the extent of apoptosis. At first sight, this result is not consistent with the findings of several laboratories indicating that cytotoxicity induced by various topoisomerase II inhibitors is reduced by camptothecin and other RNA and protein synthesis inhibitors [28, 30, 31]. However, Kaumann found that, although the above inhibitors prevented cytotoxicity in etoposide-treated HL 60, DNA degradation was not reduced in these cells, and apoptosis was not dependent on the synthesis of new proteins [27].

Further work is needed to elucidate completely the pathways between the initial lesion and cell death, but recent works give the impression that different pathways may be involved, depending on the cell type.

Finally, the present results support our previous hypothesis that the block of replication forks by cleavable complex formation with both topoisomerases is a possible signal for DNA degradation in proliferating splenocytes. Thus, aphidicolin, which blocks replication forks, may also trigger DNA degradation in this system.

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