Inhibition of Cytotoxic T Lymphocyte–induced Target Cell DNA Fragmentation, but Not Lysis, by Inhibitors of DNA Topoisomerases I and II

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Summary

Cytotoxic T lymphocytes (CTL) kill their target cells via a contact-dependent mechanism that results in the perturbation of the target cell's plasma membrane and the fragmentation of the target cell's DNA into nucleosomal particles. The membrane disruption is presumed to be due to the action of perforin, while the DNA fragmentation is thought to be by the activation of an endogenous nuclease(s). DNA topoisomerases I and II are nuclear enzymes with inherent endonuclease activities. We have investigated their role in the CTL-induced DNA fragmentation process. We report that in CTL killing assays, the treatment of target cells with topoisomerase I and II inhibitors blocks the CTL-induced DNA fragmentation process, but not the lysis of the target cell.

CTL deliver a lethal dose of granule-associated, preformed cytotoxic components onto their target cell's plasma membrane (1–5). One component, perforin, inserts into the target's plasma membrane and forms nonspecific transmembrane pores that presumably lead to the destruction of the target by colloid osmotic lysis (1–3, 4). In addition, the "lethally hit" targets undergo a suicide phenomenon known as apoptosis (1, 5, 6), principally characterized by the fragmentation of the target's DNA (1–3, 6–9) into mono- and oligo-nucleosomal particles (7, 9, 10); single-strand lesions have also been found (11). The DNA fragmentation is thought to be mediated by the activation of a nuclease(s) endogenous to the target (1–3, 6–11). DNA topoisomerases are good candidates to mediate these insults, as the type I (topo I) and type II (topo II) enzymes generate single- and double-strand breaks, respectively, in the DNA helix, to relieve torsional stress in the cell's chromatin (12, 13). Antitumor agents that have been shown to specifically inhibit the activities of topo I (camptothecin) and topo II (4′-[9-acridinylamino]-methane-sulfon-*m*-anisidide [mAMSA] and 4′-demethyl-epipodophyllotoxin 4-[4,6,-O-thenylidene-β-D-glucopyranoside] [VM-26]) (14, 15) make it possible to study the role of these enzymes in CTL-induced apoptosis.

Materials and Methods

Generation and Preparation of Virus-specific CTL. CTL were induced by the injection of 6–12-wk-old male or female (C57BL/6 × C3H/HeSnJ)F_1 mice with lymphocytic choriomeningitis virus (LCMV),[1] Armstrong strain (10). Single cell splenocyte preparations, depleted of NK cell activity (10, 16), were prepared in warm (25–37°C) RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 2% FCS (Sigma Chemical Co., St. Louis, MO) and 25 mM Hepes (Sigma Chemical Co.), pH 7.4, and enriched for T cells (16, 17). Immediately before use, the T cells (CTL) were resuspended in RPMI 1640 containing 5% FCS (RPMI-5% FCS).

Cytotoxicity Assays. ^{51}Cr (Amersham Corp., Arlington Heights, IL) and [^{125}I]IUDR (ICN Radiochemicals, Irvine, CA) (^{125}I-DNA) release assays were performed in parallel (10). For ^{51}Cr release assays, L-929 cells were labeled with ^{51}Cr in RPMI-5% FCS (10). To 12 × 75-mm glass tubes (Fisher Scientific, Pittsburgh, PA) was added 10 μl of the desired topoisomerase inhibitor (100× concentration), dissolved in DMSO (Fluka Chemical Corp., Ronkonkoma, NY), at two times its final concentration in the assay. 1 ml of ^{51}Cr-labeled targets (10^5 cells per ml) containing 5 μg/ml of Con A (Sigma Chemical Co.) was added to these tubes. DMSO alone did not inhibit effector cell activity or cause spontaneous cell death by ^{51}Cr or ^{125}I-DNA release (data not shown). The target cells were aliquoted into 96-well plates (Costar, Cambridge, MA) at 10^4 targets per well in 100 μl and allowed to stand at room temperature (25°C) for 1–1.5 h, and the effector cells were added in 100 μl, in quadruplicate wells, for a final E/T ratio of 50:1. To parallel wells, RPMI-5% FCS was added, to serve as spontaneous release controls for each dose of drug. All wells contained Con A at a final concentration of 2.5 μg/ml. Spontaneous ^{51}Cr release from these samples was usually <5% above the spontaneous release of those samples that did not contain any inhibitors (data not shown). The E/T cell mixture was incubated at 37°C, 5% CO_2, and the medium was collected for counting after 6–10 h. For the ^{125}I-DNA targets, 10 μl of the inhibitor in DMSO was added to each assay tube (Sarstedt, Newton, NC) at two times its final concentration. 1 ml (10^5 cells/ml) of target cells containing Con A (5 μg/ml) was added and the mixture was incubated for 1–1.5 h at room temperature. 1 ml of effector cells was added to achieve a final E/T ratio of 50:1 and a final Con A concentration of 2.5 μg/ml. The cell

[1] *Abbreviation used in this paper:* LCMV, lymphocytic choriomeningitis virus.

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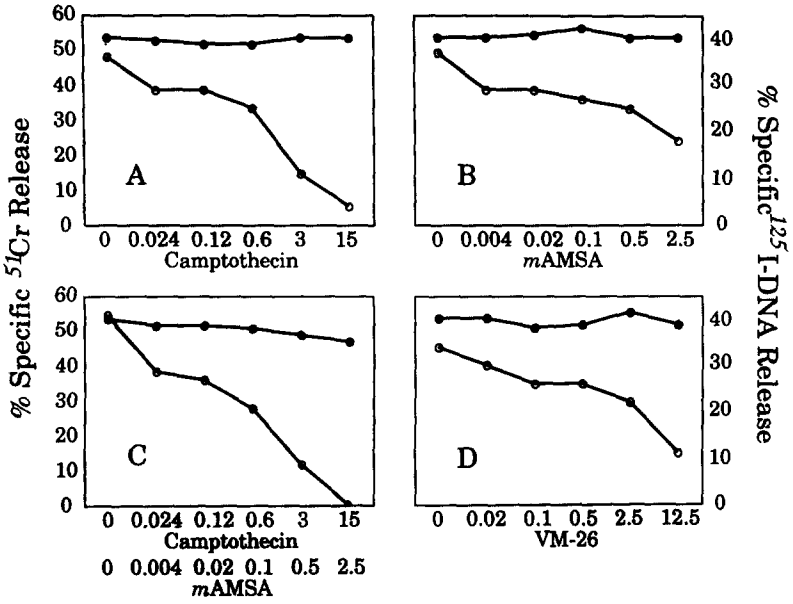

Figure 1. Target cell lysis and solubilized DNA release induced by LCMV-induced CTL in the presence and absence of topoisomerase I and II inhibitors. (*A*) Treatment of target cells with the topoisomerase I inhibitor camptothecin; (*B*) treatment of target cells with the topoisomerase II inhibitor mAMSA; (*C*) combined treatment of target cells with camptothecin and mAMSA; (*D*) treatment of target cells with the nonintercalating topoisomerase II inhibitor VM-26. ^{51}Cr release (●) and ^{125}I-DNA release (○) in the presence of varying concentrations of drug in μM. All samples were at an E/T ratio of 50:1. Camptothecin lactone (NSC-94600) and mAMSA (NSC-249992) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

mixture was incubated at 37°C, 5% CO_2 for 6–10 h. After the desired incubation period, the samples were pelleted at 500 *g*. The media from the tubes were collected, and the percent specific ^{125}I-DNA release was calculated (10). The ^{125}I-DNA release portion of the assay was performed with samples in triplicate. The spontaneous release of ^{125}I-DNA from target cells incubated in the presence of the highest concentration of inhibitor was always <5% of the release from the targets without the inhibitor (data not shown).

DNA Isolation and Electrophoresis. All ^{125}I-DNA cell pellets were pre-treated with 0.5 ml "topo lysis" buffer (18–20) containing 20 mM Tris (Research Organics, Cleveland, OH), pH 7.4, 0.15 M NaCl (Fisher Scientific Co.), 1% NP-40 (Sigma Chemical Co.), and 20 mM EDTA (Research Organics) for 10–30 min at 37°C. Total sample DNA was purified as described (21) with modifications. After topo lysis buffer treatment, an equal volume of a 9 M guanidine HCl (United States Biomedical Corp., Cleveland, OH, and Research Organics) solution containing 20 mM Tris, pH 8.0, 5 mM EDTA, was added to denature cellular proteins and to inhibit further nuclease activities, for a final concentration of 4.5 M. The samples, in guanidine HCL, were counted on a gamma counter (5500; Beckman Instruments, Inc., Palo Alto, CA). After counting to determine the levels of ^{125}I-DNA release, the respective samples, in triplicate, were pooled and incubated at 37°C overnight. The pooled samples were then extracted once or twice with chloroform/isoamyl alcohol at 24:1 (Fisher Scientific Co.), and the aqueous phase was mixed with an equal volume of isopropanol (Fisher Scientific Co.). The DNA was precipitated overnight at −20°C and isolated by centrifugation at 12,000 *g*. The purified ^{125}I-DNA was subjected to electrophoresis in a 1.5% agarose gel (Bio-Rad Laboratories, Richmond, CA), and the gel was processed for autoradiography as previously described (10).

Rationale for DNA Processing Method. Topoisomerase targeting drugs trap their respective topoisomerase in an enzyme-bridged strand-break intermediate, termed the "cleavable complex" (15). Exposure of the cleavable complex to SDS or alkali denatures the topoisomerase-DNA interaction such that a DNA strand-break occurs by virtue of the release of one DNA terminus; the topoisomerase remains covalently linked to the other terminus of the broken strand (15, 20). Treatment of the DNA with EDTA (>1 mM) (18, 19), high salt (0.5 M) (19), or high heat (>55°C) (20) induces the dissolution of the cleavable complex and allows the religation reaction of the cleaved DNA termini to occur. Thus, exposure of the drug-treated samples with the topo lysis buffer containing 20 mM EDTA, before DNA purification with the chaotropic agent, guanidine hydrochloride, prevents drug-induced DNA strand-break artifacts.

Results and Discussion

Camptothecin, mAMSA, and VM-26 are cytotoxic to all cycling cells (15). Their toxicity is directly proportional to the rate of cell proliferation (22). Drug titration assays were performed to determine the highest tolerable concentration of each drug, as assessed by spontaneous ^{51}Cr release, for our target cells. We verified that cells grown in lower serum concentrations, i.e., media containing 5% FCS (5% FCS) vs. 10% FCS, were less susceptible to drug-related cytotoxicity (22). Consequently, the target cells were cultured and assayed in 5% FCS. Initial analysis of the killing of LCMV-infected targets in 5% FCS revealed, in comparison with 10% FCS, depressed levels of DNA fragmentation, but not ^{51}Cr release (data not shown). Preliminary data suggested that this was associated with a reduced proliferative capacity of the virus-infected cells, probably due to viral interference of cellular metabolic pathways (23, 24), which could be compensated for by high serum concentrations (data not shown). To test the efficacy of the drugs in 5% FCS and under conditions free from the virus-induced interference in the target cell's metabolism, we used uninfected L-929 cells as targets with LCMV-induced CTL in a lectin-dependent (25) killing system. The lectin Con A enables CTLs to nonspecifically bind and lyse uninfected target cells.

To determine whether treatment with the topo I inhibitor, camptothecin, would prevent CTL-induced DNA fragmentation, [^{125}I]IUDR-labeled L-929 cells were preincubated with fivefold dilutions of camptothecin before CTL addition.

A parallel ^{51}Cr release cytotoxicity assay was performed to allow the comparison between cell lysis and DNA fragmentation. Fig. 1 *A* shows that camptothecin inhibited, in a dose-dependent manner, the CTL-induced DNA fragmentation in the L-929 target cells, assessed by [^{125}I]IUDR-labeled DNA (^{125}I-DNA) release. In contrast, ^{51}Cr release was not affected, indicating that the CTL were functional and could effect a perforin-like disruption (1, 4) of the target's plasma membrane. When the cell-associated ^{125}I-DNA was purified and subjected to electrophoresis (Fig. 2 *A*), the fragmentation of target cell DNA into nucleosomal particles was inhibited in a dose-dependent manner. As the concentration of camptothecin was decreased, an increasing proportion of DNA migrated as distinct bands, suggesting a processive mechanism of degradation into nucleosome core particles. Treatment with the topo II inhibitor, mAMSA, yielded similar results. Fig. 1 *B* shows that mAMSA treatment of target cells prevented ^{125}I-DNA release, but not ^{51}Cr release. Fig. 2 *B* shows that the DNA fragmentation into nucleosomal particles was also inhibited, similar to the inhibition by camptothecin (Fig. 2 *B*).

When the inhibitors were combined, camptothecin and mAMSA caused effective inhibition of ^{125}I-DNA release at ~2.5- and ~1.0-fold lower drug concentrations than that of either drug alone (Fig. 1 *C*). The autoradiogram (Fig. 2 *C*) of the electrophoresed ^{125}I-DNA samples shows an equivalent level of DNA fragmentation inhibition at a drug dose fivefold lower than either drug used alone.

A property of mAMSA is that it intercalates into the DNA while binding to topo II (15). Perturbations in DNA structure due to drug intercalation could affect the ability of the nuclease(s) to recognize the DNA substrate (26, 27). We therefore asked if VM-26, a nonintercalating topo II inhibitor (15), could also block DNA fragmentation. VM-26, like the other inhibitors, blocked ^{125}I-DNA release (Fig. 1 *D*) and fragmentation (Fig. 2 *D*), but not ^{51}Cr release (Fig. 1 *D*). Thus,

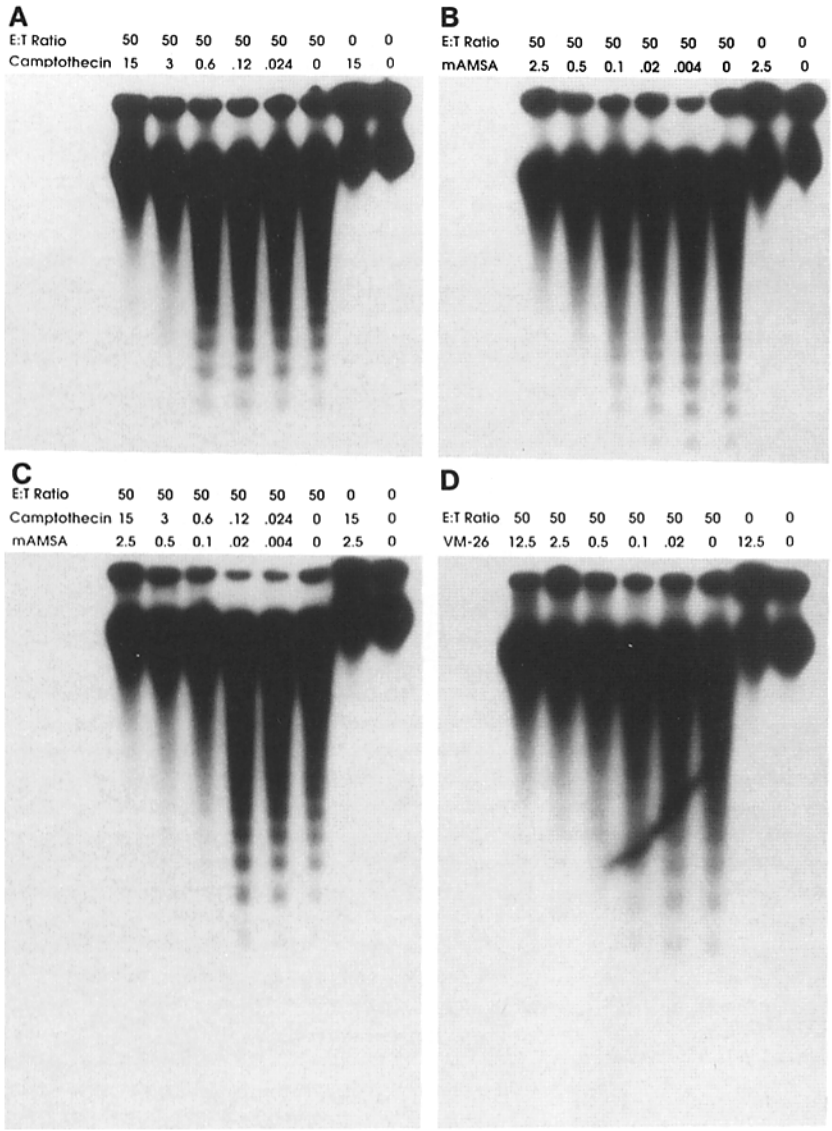

Figure 2. Autoradiograms of electrophoresed cell-associated ^{125}I-DNA samples from the experiments shown in Fig. 1 above. (*A*) Camptothecin-treated targets; (*B*) mAMSA-treated targets; (*C*) combined treatment of targets with camptothecin and mAMSA; (*D*) samples treated with VM-26. Each lane was loaded with 30,000 cpm ± 5% of sample. Gel exposures were done at −70°C with two screens for 72 h.

drug intercalation, per se, is not a requirement for the initiation in the fragmentation of the target's DNA.

To our knowledge, these results are the first to implicate distinct nuclear enzymes in the CTL-induced fragmentation of the target cell's DNA. We have also performed these experiments with the mouse mastocytoma cell line, P815, the prototype target for DNA fragmentation analysis, in short-term CTL-mediated (2–4 h) and valinomycin-mediated killing (28) assays (2 h), and have obtained results similar to those presented here (data not shown).

Reported functions for topo I and II do not include exonuclease activities that could account for the processive digestion of the target's DNA into nucleosome core particles (12, 13, 15). Topoisomerases act as swivels to relieve torsional constraints acquired as a result of transcription, replication, and packaging of the cell's DNA (12, 13, 15). These superhelical motifs are a predicament for nuclear enzyme accessibility to targeted DNA sequences (27, 29). Topoisomerases may thus play a supporting role by allowing the target cell's chromatin to be presented as a more accommodating substrate for (exo)nuclease digestion.

The experiments presented also shed light on the mechanism of target cell destruction induced by CTL. Proponents of the internal disintegration model (1–3, 6–8) of target cell killing by CTL have asserted that since the fragmentation of the target cell's DNA occurs prelytically, i.e., [^{3}H]thymidine- or ^{125}I-DNA release can be measured from NP-40 or Triton X-100 treated targets before the detectable release of ^{51}Cr from these targets, it is the fragmentation of the target cell's DNA that causes the disruption of the plasma membrane. Within our data, the fact that the significant reduction in target cell DNA fragmentation is not followed by a corresponding inhibition of ^{51}Cr release suggests that the nuclear disintegration component of CTL-mediated killing may not be required for the disruption of the target cell's membrane and subsequent target cell death. In support of this, Zychlinsky et al. (30) have recently shown that zinc, an inhibitor of endogenous nuclease activity (9), blocks DNA fragmentation, but not target cell lysis. Thus, the CTL-induced membrane perturbation may be sufficient for target cell death.

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