Topoisomerase II-reactive Chemotherapeutic Drugs Induce Apoptosis in Thymocytes

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ABSTRACT

The effects of topoisomerase II-reactive epipodophyllotoxins etoposide and teniposide as well as amscarine on the viability of thymocytes in primary culture has been examined. All three drugs were shown to produce DNA cleavage detectable by resolving isolated DNA by pulsed field agarose gel electrophoresis. The DNA cleavage was found to have two components. The first was due to the interaction of the drugs with topoisomerase II, whereas the second component was due to endonuclease cleavage caused by the drug-induced entry of the thymocytes into programmed cell death or apoptosis. This second component of the DNA cleavage was also detected in thymocytes undergoing apoptosis following exposure to the glucocorticoid analogue, dexamethasone. The effect of the drugs on programmed cell death is dependent upon new protein and RNA synthesis, indicating that topoisomerase II has a role in the very first stages of the process. These results are discussed in terms of the use of this class of topoisomerase II-reactive drugs in chemotherapy.

INTRODUCTION

Drugs that interact with the enzyme topoisomerase II have been found to be particularly useful in the chemotherapeutic treatment of certain cancers (1–4). These include the epipodophyllotoxins VP16 and VM26, as well as m-AMSA which is an acridine derivative. All three drugs stabilize the DNA-topoisomerase II complex during strand passage preventing the resealing reaction from going to completion (1, 5–7). DNA isolated from cells treated with the drugs contains covalently attached topoisomerase II subunits and strand breaks are detectable when the protein is degraded. VM26 and VP16 do not intercalate into DNA and appear to exert their effect, both in vivo and in vitro (using purified components), by binding directly, and specifically, to the enzyme molecule (reviewed in Refs. 1 and 8), whereas m-AMSA intercalates into DNA. However, although they are effective therapeutically, the mechanism by which these drugs kill cells is still controversial (3). In general, the drugs are thought to kill proliferating cancer cells by inhibiting topoisomerase II and preventing the cells from either completing S phase or undergoing chromosome segregation at mitosis since these are two cellular processes that have an absolute requirement for the enzyme. In general, cytotoxicity correlates with drug-induced DNA cleavage (8) and increases when the drugs are administered during these phases of the cell cycle. There are, however, many exceptions (3, 9), and since other cellular processes are also dependent upon topoisomerase II activity, particularly those requiring a change in chromatin structure, the precise mechanism of cytotoxicity remains to be established.

These drugs have also become particularly useful in establishing the role of topoisomerase II in biological processes and, in particular, the role that the enzyme plays in determining and modulating chromosome structure. During a recent study of the structural organization of chromosomes we (10) used drugs to cleave thymocyte DNA at the sites of topoisomerase II binding to DNA and interpreted the results in terms of the organization of chromosomes at the level of 50- and 300-kilobase supercoiled loop domains and rosettes, respectively. Thymocytes were found to be particularly useful in these studies since the cells have a high content of topoisomerase II and maintain much of their interphase chromatin in a highly ordered state. In those experiments, the effect of the drugs on the thymocytes was restricted to short incubations (30 min or less). However, more recently, we have noticed much more extensive DNA cleavage during longer incubations which, we show here, to be caused by the topoisomerase II-reactive drug-mediated entry of the cells into apoptosis or PCD. Apoptosis is a physiological "suicide" process by which unwanted cells are removed from embryonic, developing, or somatic tissues without affecting overall tissue function (11–14). In some tissues, the process is an intrinsic part of its function. For example, apoptosis plays a crucial role in determining the levels of many, if not all, of the precursor cells in the pathways of hematopoiesis in order to balance the production of the various differentiated cell types. Apoptosis has a characteristic requirement for energy as well as RNA and protein synthesis and is recognizable different from tissue necrosis. Cells undergoing apoptosis activate or induce an endonuclease that cleaves chromatin into nucleosomes and polynucleosome chains which are observed as a characteristic ladder of DNA fragments on agarose gels (14–17). The appearance of this DNA ladder coupled with the dependence upon gene expression and protein synthesis, together with morphological criteria, are the typical markers for apoptosis and are well documented for thymocytes (13–22).

The results presented here demonstrate that topoisomerase II-reactive chemotherapeutic drugs are able to kill cells by inducing the process of apoptosis. Thus, the susceptibility of cells, particularly those involved in hematopoiesis (18, 23–25), to apoptosis would be expected to increase their sensitivity to the cytotoxic effects of this group of antineoplastic drugs. Furthermore, these observations may explain in part the increased sensitivity of certain tumor cells to this class of drugs and demonstrate that normal nonproliferating cells can also be killed.

MATERIALS AND METHODS

Preparation and Treatment of Thymocytes. Thymocytes were isolated as described previously (19). Briefly, thymuses were excised from 3-week-old (150–200 g) Sprague-Dawley rats bred at this facility, washed to remove excess blood, and minced with scissors in 10–12 ml of RPMI 1078

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3 The abbreviations used are: VP16, etoposide; VM26, teniposide; m-AMSA, amscarine [4'-acridinylamino)methanesulfon-m-aminoside]; PCD, programmed cell death; PBS, phosphate-buffered saline.
1640 tissue culture medium containing 5% fetal bovine serum. The minced thymuses were strained through gauze and the suspension was diluted 10-fold with medium to about 4 x 10^7 cells/ml. The cells were rotated at 37°C for 1 h in 10-ml aliquots in an atmosphere of 95% air and 5% CO2 before treatments. The thymocyte primary cultures were then treated with either 1 µM dexamethasone (from a 2 mM stock solution in ethanol; Sigma Chemical Co., St. Louis, MO), or 10 µM m-AMSA (from a 50 mM stock solution in dimethyl sulfoxide; Chemistry Branch, National Cancer Institute, NIH, Bethesda, MD), or 5 µM VM26 (15 mM stock solution in ethanol), or 50 µM VP16 (33.6 mM stock solution in 30% ethanol). VM26 and VP16 were obtained from Bristol Laboratories (Candiac, Quebec Province, Canada). In some experiments, the cells were also treated with 0.1 mM cycloheximide (from 8 ml of culture) were resuspended in 1.0 ml of a buffer containing 0.15 M NaCl, 2 mM KH2PO4/KOH (pH 6.4), 1 mM ethyleneglycolbis(0-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) and 5 mM MgCl2 (Sigma). Preparations of Agarose Plugs from Cells. Approximately 3 x 10^7 cells (from 8 ml of culture) were resuspended in 1.0 ml of a buffer containing 0.15 M NaCl, 2 mM KH2PO4/KOH (pH 6.4), 1 mM ethyleneglycol bis(β-aminooxy) ether)-N,N',N'-tetraacetic acid, and 5 mM MgCl2 (Sigma). In order to prevent reversal of the DNA breaks in the experiments involving treatment with topoisomerase inhibitors the m-AMSA, VP16, or VM26 were also present in this buffer at the same concentrations as in the medium. Cells were then centrifuged at 170 x g for 2 min at 4°C and resuspended in 640 µl of the same buffer. One hundred µl of the latter suspension was transferred to an Eppendorf tube, diluted to 250 µl with the same buffer, and mixed with 250 µl of molten 1.5% low melting point agarose and 50 µl of 2 mg/ml proteinase K. The mixture was pipetted into 70 x 5 x 1-mm glass molds made from microscope slides and left at 4°C for 30 min. The agarose blocks were placed in 3 ml of 10 mM NaCl-10 mM Tris-HCl, pH 9.5-25 mM EDTA-1.0% N-lauroyl sarcosine (Sigma) supplemented with 150 µl of 2 mg/ml proteinase K and incubated at 37°C for 18 h with gentle mixing on a multipurpose rotator. The incubation was followed by rinsing in three changes of 10 mM Tris-HCl, pH 8-1 mM EDTA at 4°C for 1 h each and finally suspended in 50 mM EDTA, pH 8.0. The agarose blocks could then be stored in this buffer for more than six months. About 3-mm long plugs were cut from the agarose blocks and used for electrophoresis. Each plug contained 2-5 µg of DNA.

Pulsed Field Gel Electrophoresis. Electrophoresis was carried out using a horizontal gel chamber, model 200/20 power supply and Pulsewave 760 switcher (all purchased from BioRad Laboratories, Mississauga, Ontario, Canada). The gels were run at 200 V in 0.01 M Tris-acetate, pH 8.5-0.5 mM EDTA with the ramping rate changing from T1 = 0.5 s to T2 = 10 s for the first 19 h and from T3 = 10 s to T4 = 60 s for the next 19 h, with a forward to backward ratio of 3 at 5°C, using electrophoresis grade agarose (1.5%) purchased from Bethesda Research Laboratories (Gaithersburg, MD). Under these conditions, DNA fragments ranging in size from 10 kilobase pairs to approximately 750 kilobase pairs are resolved and all DNA fragments >750-800 kilobase pairs that enter the gel migrate at the same rate. Three sets of standards with overlapping molecular weight ranges were used to determine molecular weights: the commercially available yeast chromosomes (250-1100 kilobase pairs) purchased from BioRad Laboratories (Richmond, CA), polymerized λ phage DNA (50-750 kilobase pairs) purchased from Clontech Labs Inc. (Palo Alto, CA), and a polymerized plasmid DNA (11.6-200 kilobase pairs) and/or λ DNA digest. Conventional agarose gel electrophoresis was carried out in 0.8% agarose gels in 0.04 M Tris-acetate, pH 8.5-0.002 M EDTA at 35 V for 18 h. After the gels were stained in ethidium bromide, they were photographed in UV light with a Polaroid camera using Polaroid positive-negative film 55.

Coulter Counting and Flow Cytometry. One ml of cell suspension was taken into each of 2 tubes and diluted to 10 ml with PBS. Five hundred µl were taken from one of these tubes and further diluted with 19.5 ml of PBS and counted in a model ZM Coulter Counter. The lower threshold was initially set to 15 and the upper threshold to 20 and both were incremented by 5 for each count. The range of equivalent cellular volumes was from 20 to 175 µm^3.

The second tube of thymocyte cell suspension was centrifuged at 1200 rpm for 5 min at 4°C in a Beckman J-6B centrifuge. The cell pellet was resuspended in 1.0 ml of PBS and fixed by the addition of 4 ml of -20°C absolute ethanol. These cells were stored at -20°C overnight. To prepare the fixed cells for flow cytometry they were twice centrifuged and washed in PBS as described above before being resuspended in 1.0 ml of PBS. One hundred µl of 200 µg/ml RNAse A (Sigma), which had been treated by boiling for 5 min to remove DNase, was added and the suspension was incubated at 37°C for 30 min. One hundred µl of 1 mg/ml propidium iodide (Sigma) was added and the suspension incubated for a further 30 min. The stained cell suspension was diluted 10-fold in PBS immediately before being introduced into the flow cytometer (model 50H; Ortho Diagnostic Systems).

RESULTS

Induction of Apoptosis in Thymocytes by VP16 and Dexamethasone. A characteristic feature of glucocorticoid-induced apoptosis is an increase in density of the cells which increases their migration on Ficoll gradients (20). This phenomenon also manifests itself as a decrease in cell size that can be detected with a Coulter Counter (21, 22). Other than the appearance of the nucleosome ladder of degraded DNA fragments these changes in cell density and size are the only other easily detected characteristics of the onset of apoptosis. Fig. 1 illustrates the changes in cell size determined by Coulter counting of thymocytes during a 6-h period following addition of 50 µM VP16 in comparison to control cultures that received solvent only. The actual profiles are shown on the top and a time course of the appearance of the smaller cell population is shown on the bottom of Fig. 1. Freshly isolated thymocytes have a characteristic size profile with a mean volume of approximately 100 µm^3.
TOPOISOMERASE II-REACTIVE DRUGS AND APOPTOSIS

Effects of Actinomycin D and Cycloheximide on Changes in Cell Size. Steroid-induced apoptosis of thymocytes, as well as apoptosis in other cells (13–17, 20–22, 27, 28), is dependent upon new RNA and protein synthesis which presumably reflects a requirement for new gene expression. Since the changes in cell size that were induced by the topoisomerase inhibitors were so similar to those that occur during dexamethasone-induced apoptosis, we sought to determine whether new gene expression was required for these drugs to elicit their effect or whether the interaction of the drugs with topoisomerase II was in itself sufficient to provoke the changes in nuclear and cell size. In this experiment, thymocytes were exposed for 4 h to either dexamethasone, VM26, VP16, or m-AMSA in the presence or absence of either 1 μM actinomycin D or 0.1 mM cycloheximide. At the end of the 4-h period the cells were processed for Coulter counting (Table 2). As expected the dexamethasone-induced decrease in cell size was abolished by both actinomycin D, which inhibits transcription, and cycloheximide, which inhibits translation of mRNA. Furthermore, actinomycin D and cycloheximide prevented the change in cell size that occurs in response to all three topoisomerase II-reactive drugs (Table 2), showing that for the drugs to elicit this response new genes must be expressed. Although it is known that both cycloheximide and actinomycin D can cause DNA damage (29), neither one of these compounds alone could cause sufficient damage to cause changes in cell size during the time course of these experiments. Moreover, during prolonged incubations, cycloheximide prevented the loss of cells from cultures incubated in the presence of VP16 or dexamethasone (Table 1).

Patterns of DNA Fragmentation Caused by Topoisomerase II Inhibitors and Dexamethasone. Topoisomerase II enzyme molecules are bound to DNA with a periodic spacing along the chromosome fiber. DNA extracted from thymocytes that have been treated with VM26 and m-AMSA, when resolved by pulsed field gel electrophoresis, shows a characteristic distribution of fragment sizes (10). To examine this in greater detail in relation to PCD we carried out time courses of fragmentation of thymocyte DNA following exposure to VP16 as shown in Fig. 4A using the same concentrations of the drugs that produce the changes in the size of the thymocytes that were described in Fig. 1. During 6 h of incubation with 50 μM VP16 there was an accumulation of DNA fragments of about 50 kilobases which paralleled the increase in the number of smaller cells. Only a minor amount of DNA fragmentation occurred in the presence of the same concentration of ethanol solvent (lane S), indicating that the effect is specific for VP16. A similar pattern of DNA cleavage to 50-kilobase fragments was observed after 6 h of incubation with m-AMSA and VM26 (Fig. 4B). Surprisingly, 6 h of incubation with dexamethasone also produced a similar amount of DNA cleavage to 50-kilobase fragments as shown in Fig. 4B.

To try to establish the relationship between the drug-induced DNA fragmentation and the appearance of smaller cells we examined the extent of DNA fragmentation caused by VM26, VP16, and m-AMSA in the presence of 1 μM actinomycin D which, as described above, prevents the formation of smaller cells (Table 2). As shown in Fig. 5A, actinomycin D decreased, but did not abolish, DNA fragmentation to the 50-kilobase fragments caused by a 4-h exposure to each of the drugs. In the case of VM26 actinomycin D decreased, or abolished, the...
Fig. 2. Flow cytometric 3-dimensional plots and density maps of thymocytes exposed to VP16. In both cases cell size (forward light scatter) is plotted against the integral of the fluorescence (F). A, control culture of freshly isolated thymocytes; B, 4 h of exposure to 50 μM VP16; C, 6 h of exposure to VP16.

Fig. 3. Flow cytometric 3D plots and density maps of thymocytes exposed to dexamethasone (1 μM). In both cases cell size (forward light scatter) is plotted against the integral of the fluorescence (F). A, control cultures of thymocytes exposed to 0.1% ethanol for 6 h. B, 4 h of exposure to 1 μM dexamethasone. C, 6 h of exposure to dexamethasone.

Table 2: Effects of actinomycin D and cycloheximide on topoisomerase II-reactive drug-induced apoptosis

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dex methasone</th>
<th>m-AMSA</th>
<th>VM26</th>
<th>VP16</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3446 ± 232</td>
<td>3524 ± 237</td>
<td>3160 ± 170</td>
<td>3000 ± 142</td>
<td>1610 ± 99</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>2094 ± 347</td>
<td>1700 ± 435</td>
<td>1610 ± 365</td>
<td>1442 ± 346</td>
<td>1905 ± 206</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>2149 ± 203</td>
<td>1902 ± 436</td>
<td>1984 ± 308</td>
<td>2027 ± 276</td>
<td>2147 ± 206</td>
</tr>
</tbody>
</table>

appearance of 50-kilobase fragments but had no effect on the appearance of the 300-kilobase fragments. This observation suggests that DNA fragmentation in response to the drugs has two components. In the presence of actinomycin D there is some fragmentation of DNA by m-AMSA, VP16, and VM26 as may be expected since the direct action of these drugs on topoisomerase II does not require RNA (or protein) synthesis. The second component of the fragmentation only occurs when the thymocytes become smaller (i.e., in the absence of actinomycin D). Similar results were obtained using cycloheximide as the inhibitor of apoptosis (data not shown). This conclusion is supported by the observation that the steroid-induced fragmentation of DNA to 50-kilobase fragments is completely abolished in the presence of 1 μM actinomycin D (Fig. 5B). All of the DNA cleavage seen with dexamethasone is presumably a result of the process of apoptosis and can only occur after new genes have been expressed. To try to distinguish further between DNA cleavage in response to the interaction of the drugs with topoisomerase II and DNA fragmentation occurring as a result of apoptosis we compared the patterns of DNA fragments on pulsed field gels obtained from cells exposed to VM26 for either 1 or 6 h (Fig. 6). Conventional gels were also run to determine whether the endonuclease was active at these times. During the first hour of exposure to the drug DNA was cleaved to fragments of >300 kilobases (Fig. 6A, lane 1) and there was no ladder of DNA fragments (Fig. 6B, lane 1),
Fig. 4. Size distribution of DNA fragments produced by incubation of thymocytes in the presence of 50 μM VP16, 5 μM VM26, 10 μM m-AMSA, or 1 μM dexamethasone (DEX) for the times indicated followed by plug preparation and pulsed field electrophoresis as described in "Materials and Methods." In A, cells were exposed to VP16 for 1, 2, 4, or 6 h (lanes 1, 2, 4, and 6); lane F is freshly prepared thymocytes; lane S is thymocytes exposed to solvent (0.1% ethanol) for 6 h. The positions of molecular weight markers (yeast chromosomes and λ multimers in kilobases) are shown. In B, cells were exposed to the drugs or hormone indicated for 6 h and then processed as described in A. Cell suspensions were processed in duplicate and run as adjacent lanes of the agarose gel. Lane F, fresh thymocytes; lane C, 6 h control; lane Y, yeast chromosomes; lane L, λ ladder. Ordinate, sizes of a selection of the markers in kilobases.

indicating that none of the cells have yet entered apoptosis. In contrast, by 6 h most of the cells had entered apoptosis and there was considerable DNA fragmentation producing the characteristic DNA ladder on conventional gels (Fig. 6B, lane 2). By this time the larger fragments generated by drug-induced DNA cleavage were degraded to 50-kilobase fragments or less by the endonuclease (Fig. 6A, lane 2). These data established that VM26 actually interacts with topoisomerase II during these incubations to stabilize DNA cleavage complexes. Furthermore, it is clear that the drugs do not directly activate the endonuclease. When the cells undergo apoptosis DNA is degraded to 50-kilobase fragments and subsequently to nucleosome fragments.

DISCUSSION

The results presented show that there is a marked similarity, if not identity, between the responses of thymocytes to glucocorticoids and to drugs that interact with topoisomerase II. Dexamethasone induces a decrease in cell size, decrease in the accessibility of fluorochrome to DNA, increase in DNA fragmentation, and eventual loss of cell viability which are all the hallmarks of apoptosis. The three drugs that are active toward topoisomerase II, VM26, VP16, and m-AMSA, provoke the same response with the same kinetics, suggesting that they too trigger apoptosis in thymocytes.

The mechanism by which glucocorticoid hormone induces apoptosis in thymocytes is not known. The requirement for gene expression is presumed to include the induction of an endonuclease which ultimately cleaves chromatin into the characteristic ladder of 200-base pair fragments (see Ref. 17 for a review). However, other genes are also expressed (30) including c-fos, myc, and the heat shock proteins (31, 32), and the exact sequence of events between steroid binding to its receptor and appearance of the ladder has not been elucidated. In this paper we show that drugs that specifically interact with topoisomerase II can trigger the same response. This effect of the topoisomerase II-reactive drugs requires protein and RNA synthesis, indicating that they are acting at an early step of PCD preceding the requirement for transcription, and is not the result of nonspecific DNA damage. Other cytotoxic drugs, including actinomycin D and cycloheximide at high concentrations, can cause fragmentation of chromatin into nucleosomes and polynucleosomes (29), but as shown here actinomycin D and cycloheximide cannot induce PCD.

There are three possible levels at which the drugs may act depending on the role of topoisomerase II. First, if topoisomerase II enzyme activity is required to mediate the response of the cells to steroid, then the drugs could mimic this response. Alternatively, ongoing topoisomerase II activity may be required to prevent the onset of PCD in normal cells and the
Fig. 5. Effect of actinomycin D on the size distribution of DNA fragments produced by incubation of thymocytes in the presence of topoisomerase II-reactive drugs and dexamethasone. In A, cells were incubated for 4 h with VM26, VP16, or m-AMSA in the presence (+) or absence (−) of 1 μM actinomycin D. Lanes Y and L, yeast and λ ladder markers, respectively. In B, the cells were incubated with 1 μM dexamethasone (DEX) in the presence (+) or absence (−) of 1 μM actinomycin D for 6 h. Lanes F, S, and C, the same as the equivalent lanes in Fig. 4. Ordinate, sizes of molecular weight standards in kilobases.

Drugs inhibit this reaction and allow PCD to occur. It is also possible that the drugs interfere with other normal cellular topoisomerase II activities and this is sufficiently disruptive to cause the cells to enter PCD. In addition, it cannot be ruled out that the drugs have some other intracellular target that triggers PCD.

The loss of nuclear architecture which results in the change in cell size appears to be an “all or none” response and is fairly rapid, since cells of intermediate size were not seen. This may explain why cells of increased density were not seen in previous studies using the antineoplastic drug Adriamycin which also intercalates into DNA (33), although morphologically identifiable apoptotic cells have been seen in some neoplastic cells treated with chemotherapeutic agents (34). Thymocytes must, therefore, have a mechanism for rapidly destroying chromosome structure beyond the stage that it can be repaired (the smaller, apoptotic cells cannot survive) which effectively kills the cells even though integrity and “viability” based upon dye exclusion may be maintained for 18–24 hours. Interestingly, the drug-topoisomerase II interaction alone is insufficient to trigger the change in chromatin structure that manifests itself as a change in size of the nucleus. The experiment (Fig. 5) in which the drugs were added in the presence of either actinomycin D or cycloheximide showed that, although the drugs do interact with topoisomerase II, as evidenced by DNA cleavage on the pulsed field gels, there is no change in the size of the nuclei. Thus, the effect of these drugs on chromatin structure is not equivalent to that caused by endonuclease. Only the endonuclease, induced after the cells enter PCD, can cause the amount of chromosome damage sufficient to irreversibly change the size of the nucleus and kill the cells.

Flow cytometry is being used increasingly to detect changes
in chromatin structure in a variety of cell types (35–39). For example, the accessibility of various fluorochromes to DNA changes during differentiation of Friend leukemia cells in a way that can be correlated to changes in chromatin structure (35). Damage to DNA either by digestion of isolated nuclei (36) or fixed chromatin (37) or by incubation of cells with clastogenic drugs (38) decreases the relative fluorescence of chromatin in the nuclei. A similar decrease in fluorescence was observed in the smaller cell population after 4 h of exposure to drugs that induce PCD. Although there has been sufficient DNA damage by 4 h to cause the decrease in fluorescence, no DNA has been lost from the nuclei, indicating that this reflects a change in chromatin structure. Thus, flow cytometry is a convenient way to measure the progress of apoptosis since both the decrease in cell size and change in chromatin structure can be detected and this cell population is readily quantifiable. Furthermore, it illustrates that the change in cell (i.e., nuclear) size coincides with the alteration in chromatin structure since we only see small cells with decreased fluorescence and never cells of the same size (100 \mu m^3) with decreased fluorescence or smaller cells with a normal DNA fluorescence.

The data presented here add a new dimension to the cytotoxic effects and therapeutic value of these drugs and are supported, indirectly, by evidence already in the literature. Kupfer et al. (40) have shown that depletion of intracellular ATP pools does not affect antineoplastic drug-induced DNA cleavage-complex formation, i.e., the drugs are still able to interact with the enzyme but their effect is no longer cytotoxic. This observation shows that drug interaction with topoisomerase II and the cytotoxic effect of the drugs can be uncoupled (i.e., drug interactions with topoisomerase II are not intrinsically cytotoxic) and is consistent with drug-induced apoptosis being the cytotoxic response. Moreover, it has been shown also that the cytotoxic effect of m-AMSA on a mastocytoma cell line is cycloheximide sensitive (41). Apoptosis is an ATP-dependent process and, as we have shown here, drug-induced apoptosis of thymocytes requires protein synthesis and is inhibited by cycloheximide. In addition, Jaxel et al. (42) have noted irreversible DNA fragmentation in splenocytes incubated for prolonged periods of time in the presence of VP16 which is consistent with endonuclease cleavage of DNA during apoptosis. Furthermore, it has been shown that, when a drug such as m-AMSA is removed, cleavable complex formation is reversed (2), and we have shown also that following removal of the drugs thymocytes still undergo apoptosis. Thus, continued presence of the drugs is not a prerequisite for a lethal effect.

Finally, in a recent review of the controversial areas in our understanding of the mechanism of action of topoisomerase II-reactive, antineoplastic drugs Zwelling (3) concluded that “a process dependent on ATP and distal to the stabilization of the cleavable complex must critically influence the susceptibility of treated cells to the lethal effects of topoisomerase II-reactive drugs” and “etoposide cytotoxicity can be decreased by treating cells with the protein synthesis inhibitor cycloheximide.” The evidence we have presented here demonstrates that this process (i.e., the mechanism of cytotoxicity) may well be drug-induced apoptosis. If this is the case, then it is important to determine the mechanism by which this class of antineoplastic drugs triggers the entry of cells into apoptosis. More specifically, it must be determined whether this is due to the interaction of these drugs with topoisomerase II or whether some other, as yet unknown, intracellular target is involved.

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* P. R. Walker, unpublished observations.


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