Etoposide-induced Cytotoxicity in Two Human T-Cell Leukemic Lines: Delayed Loss of Membrane Permeability rather than DNA Fragmentation as an Indicator of Programmed Cell Death

Daniel R. Catchpole and Bernard W. Stewart

Children's Leukaemia and Cancer Research Centre, University of New South Wales, Prince of Wales Children's Hospital, Sydney, New South Wales 2031, Australia

ABSTRACT

Features of the apoptotic response evident in glucocorticoid-treated thymocytes are not uniformly observed in cell lines exposed to anticancer drugs. The significance of such variation has been assessed by monitoring molecular and cellular processes induced by etoposide (VP-16) in the human lymphoblastoid T-cell lines CCRF-CEM (CEM) and MOLT-4 contrasted, where appropriate, with those induced by necrotizing injury. Cytotoxic concentrations of the drug were determined to be 5–100 µg/ml on the basis of tetrazolium reduction assay. The two lines were equally sensitive to VP-16; no difference in concentration of drug which inhibited cell growth by 50% with respect to control (i.e., drug free) cultures was apparent irrespective of exposure times from 3–72 h. DNA strand breaks were evident in both populations within 3 h of exposure to VP-16. Morphological change, assessed microscopically, involving nuclear condensation and cell shrinkage was qualitatively and quantitatively similar in VP-16-treated CEM and MOLT-4 cells. Flow cytometric analysis indicated that the G2/M fraction of the randomly dividing MOLT-4 population was approximately one-third that of CEM cells, but each line exhibited a decrease in this fraction 3–6 h after treatment. Despite these similarities, marked differences in the response to VP-16 were evident in the two populations. Internucleosomal fragmentation, detected electrophoretically 3 h after treatment in DNA isolated from CEM cells, was not detected under any condition in MOLT-4 DNA. Apoptotic bodies, also evident within 3 h of VP-16 treatment of CEM cells, were not readily apparent in MOLT-4 cells under the same conditions. Treatment causing necrosis resulted in trypan blue uptake within 1 h in a similar high proportion of cells from both lines. Exposure to VP-16 resulted in such a loss of membrane integrity by 6 h in CEM cells, while change in this parameter occurred only after 24 h in the case of MOLT-4 cells. The findings indicate a wide scope of difference in apoptotic response and suggest delayed loss of membrane permeability, rather than DNA fragmentation, as the clearest indicator of programmed cell death in these populations.

INTRODUCTION

VP-163, a member of the epipodophyllotoxin class of cytotoxic drugs, is an inhibitor of topoisomerase II (1–3). Drugs of this type cause strand breakage in DNA of target cells attributable to stabilization of the “cleavable complex” formed by the covalent linkage of each of the two homologous subunits of the enzyme to the 5'-phosphoryl ends of DNA (4). Such damage, evident at a level of approximately 3 strand breaks/107 base pairs, is detectable soon after exposure to the drug (5). Several lines of evidence suggest a relationship between inhibition of topoisomerase activity and the cytotoxicity of VP-16 (and similar drugs) principally from studies using cell lines derived from leukemias and lymphomas. Correlations have been established between VP-16-induced protein-linked DNA strand breaks and cytotoxicity (4, 6). Development of resistant cell populations is attributable to alterations in topoisomerase activity or structure (7, 8). However, removal of the drug allows rapid reversal of DNA strand breakage, yet exposed cells still die (9). Furthermore, monitoring cell cycle-specific variations in the respective parameters suggests a complex relationship between topoisomerase-associated strand breakage and cell death (10, 11). Exposure to VP-16 also results in endonucleolytic cleavage of DNA to a nucleosome-like pattern evident following agarose gel electrophoresis (12, 13). This change, distinguishable from that associated with topoisomerase in being irreversible and not associated with protein, is attributable to activation of an endogenous endonuclease. Although such fragmentation is considered a molecular hallmark of apoptosis (14, 15), its relationship to strand breakage mediated by topoisomerase II is not clear (16, 17).

VP-16, in common with a range of cytotoxic drugs, causes cell death by apoptosis (16, 17), a process characterized at the morphological level in lymphoid cells by margination of chromatin, nuclear envelope breakdown, blebbing of the cytoplasmic membrane, and gradual condensation of cellular remnants (18, 19). Assessed on this basis, using morphological or flow cytometric criteria, investigators have discerned differences between various lymphoblastoid cell lines in their apoptotic response. In particular, the MOLT-4 T-cell leukemia line has been described as exhibiting a lesser response than other hematopoietic populations (20–23). By comparison, apoptosis is readily apparent in another human T-cell-derived lymphoblastoid line, CEM (24).

The present study has involved investigation of response of CEM and MOLT-4 cells to VP-16, with particular reference to molecular and cellular indicators of apoptosis and the interrelationship of these criteria to cytotoxicity. We have sought to describe the process of VP-16-induced cell death by monitoring sequential change beginning from the first few hours after treatment and continuing for up to 3 days. The investigation has been restricted to drug concentrations sufficient to cause cell death. The two lines could not be distinguished in terms of their sensitivity to VP-16 as indicated by ID50 concentrations. However, markedly different response patterns were evident by reference to other indicators of cell injury. A specific feature of the present study was utilization of two independent and quantitative criteria for viability, namely, trypan blue exclusion and tetrazolium reduction, in addition to morphological evaluation. In consequence, the present observations indicate limitations inherent in using any single criterion for cell death. Although each of the differing VP-16-induced patterns of sequential change evident in the CEM and MOLT-4 cell populations might be characterized as “programmed cell death,” the data also preclude utilizing internucleosomal fragmentation of DNA as a definitive indicator of apoptosis. However, prolonged maintenance of membrane integrity following ultimately lethal cell injury appeared to be a useful criterion to distinguish these different patterns of cell death from necrosis.
MATERIALS AND METHODS

**Chemicals.** Cell culture media and supplements were obtained from ICN Biomedicals (Sydney, Australia). VP-16, MTT, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). VP-16 stocks were made up to 20 mg/ml in DMSO and stored as 100-μl aliquots at -20°C. A stock solution of MTT was made up to 2 mg/ml in phosphate-buffered saline and stored in the dark at 4°C.

**Cell Lines.** The human leukemic cell lines CEM and MOLT-4 were kindly donated by Dr. Peter Slowiakczek and Dr. Lilly Huschtscha, respectively (Department of Cancer Medicine, University of Sydney, Sydney, Australia). Cell lines were maintained as a static suspension culture at 37°C in antibiotic-free RPMI 1640 supplemented with 20 mM 4-(2-hydroxethyl)-1-piperazinethanesulfonic acid buffer, 2 mM glutamine, and 10% fetal calf serum. Cultures were passaged every 2–3 days and were prevented from reaching “confluency” (approximately 10⁶ cells/ml).

Cells were treated with 5–100 μM VP-16 by addition of the drug directly to the culture flasks and incubated at 37°C for the time indicated. When required, necrosis was induced by exposure of cells from both cell lines to 10% ethanol, 30% DMSO, or 50°C for 60 min. After all the protocols described above were completed, aliquots from treated and control cell preparations were stained with the vital dye trypan blue, and 100-μl aliquots of all cultures were subjected to cytopsin and staining according to Wright (25). Morphological change in these preparations was quantified on a “blind” basis. Slides were appropriately coded prior to microscopic examination. Cells from one evenly spread ×40 field per slide were classified as either apoptotic or normal and counted, the result being expressed in terms of the proportion of cells exhibiting apoptosis. At least 200 cells/field were counted. Classification of cells as “apoptotic” was based on reduced size, increased intensity of nuclear staining, and other morphological change as described in “Results.”

**Cytotoxicity Assay.** The chemosensitivity of the leukemic cell lines was determined by a quantitative colorimetric assay based on MTT (26). Exponentially growing cultures were adjusted to 1.5 × 10⁶ cell/ml with 100 μl being added to the wells of a 96-well microtiter plate. Stock VP-16 was serially diluted with RPMI 1640 to a concentration of 2 × 10⁻¹⁰ up to 2 × 10⁻⁶ M, and 100-μl aliquots were subsequently added to the cells. Plates were incubated at 37°C in 5% CO₂ for 72 h continuous exposure. The experiments were repeated with a starting cell concentration of 3 and 8 × 10⁵ cell/ml, and plates were only incubated for 24 and 3 h, respectively. In some instances, cells were allowed a 72-h “recovery” period in drug-free media after a 3-h VP-16 exposure. Following treatment, MTT was added to each well to a final concentration of 400 μg/ml and allowed to incubate at 37°C in 5% CO₂ for 3 h. Cells were centrifuged at 1500 rpm for 10 min, the supernatant was removed, and 150 μl DMSO were added to each well to dissolve incorporated MTT crystals. The color was enhanced by the addition of 25 μl glycine buffer (0.1 M glycine-0.1 M NaCl), and the dual A560 and A630, was determined on a Tietzert Multiscan (ICN) microtiter plate reader. Determination of ID₅₀ values and statistical analysis were undertaken as previously described (27).

**Flow Cytometry.** The cell cycle distribution of exponentially growing CEM and MOLT-4 cells following exposure to VP-16 was determined by flow cytometry. Cells (10⁷) were washed and resuspended in 1 ml phosphate-buffered saline, 100 μl 2% Triton X-100, and 50 μl 10 mg/ml RNase, subsequently stained with propidium iodide (final concentration, 13 μg/ml), and incubated on ice for 30 min. Stained cells were analyzed using a FACScan (Becton and Dickinson) flow cytometer.

**DNA Isolation and Agarose Gel Electrophoresis.** Cells (10⁷-10⁸) were suspended in 1 × SSC (425 μl) containing proteinase K (5 mg/ml, 50 μl) and lysed by adding 10% sodium dodecyl sulfate (25 μl) to each tube and gently inverting until clear but viscous. DNA was extracted twice using phenol saturated with SSC (500 μl) and 500 μl chloroform:isoamyl alcohol (24:1, v/v), mixing, and centrifuging at 12000 rpm for 5 min. The upper aqueous layer was re-extracted once with chloroform:isoamyl alcohol. Two volumes of ice-cold 95% ethanol were layered onto the extracted DNA, and the tube was inverted until a precipitate was evident, after which the sample was stored at −70°C for 1 h. The DNA was pelleted by centrifugation (12000 rpm for 30 min) and the supernatant removed. The pellet was gently washed with ice-cold 70% ethanol and dissolved in 0.1 × SSC (200 μl) overnight at 4°C.

Subsequently, the sample volume was adjusted to 500 μl using 0.1 × SSC, followed by the addition 20× SSC (31.25 μl) and an equal volume of RNase (10 mg/ml) solution. Samples were incubated at 37°C for 30 min, after which proteinase K solution (5 mg/ml, 31.25 μl) was added, and the incubation continued for a further 30 min. DNA was extracted with SSC-saturated phenol and chloroform:isoamyl alcohol as before, followed by extraction using chloroform:isoamyl alcohol only. DNA was precipitated in ice-cold 95% ethanol and incubated at −70°C for 1 h. The DNA pellet was washed with 70% ethanol, dissolved in 0.1 × SSC (200 μl), and stored overnight at 4°C. The sample concentration was determined by A₂₆₀ and A₂₈₀; the ratio of the two readings being greater than 1.5 in all cases.

DNA (10–15 μg) isolated from treated and control CEM and MOLT-4 cells was loaded onto 1.2 or 1.5% agarose gels in 89 mM Tris-HCl (pH 8.0), 89 mM boric acid, 2 mM EDTA containing 1 μg/ml ethidium bromide and electrophoresed at 50 V for 3 h. Gels were destained in H₂O for 1 h and photographed under UV light.

**DNA Strand Breakage.** The number of DNA strand breaks induced by VP-16 were quantified by the fluorescence-based DNA-unwinding assay described by Kanter and Schwartz (28).

**Statistical Analysis.** Where appropriate, the significance of differences between experimental values was determined by two-sample t test assuming equal variance.

**RESULTS**

**Cytotoxicity.** Response of the CEM and MOLT-4 cells to the cytotoxic effect of VP-16 was determined using the tetrazolium-based assay reported by Mosmann (26). After the two lines were exposed to the drug for 3 days, their response patterns were similar (Fig. 1A), with no significant difference in the ID₅₀ concentrations (5.6 × 10⁻⁸ M in CEM and 3.1 × 10⁻⁸ M in MOLT-4 cells). Additional testing of cytotoxicity was undertaken using exposure times of <3 days to determine whether such perturbation revealed a difference in response between the two lines. When viability of the respective cell populations was determined 24 h after addition of VP-16, toxicity of the drug to both cell lines was reduced by approximately 1 order of magnitude by comparison with the conventional (72-h) exposure. For this, and the result achieved when cells were exposed to VP-16 for 3 h and then maintained in fresh media for a further 72 h, the data were suggestive of MOLT-4 being slightly more sensitive (Fig. 1B, C and D), but in neither case was the difference in ID₅₀ between the two cell lines statistically significant, which is consistent with results of comparisons of the cytotoxic activity of other drugs between these two lines (29). Testing viability 3 h (Fig. 1C) and 6 h (data not shown) immediately after addition of VP-16 indicated that the drug had minimal effect, although, again, there was the suggestion of MOLT-4 being the more sensitive line.

**Trypan Blue Uptake.** The proportion of the respective cell populations permeable to trypan blue was determined at various times after addition of VP-16 to the media. When tested 3 h after such exposure to VP-16 at the highest concentration routinely used (100 μM), the proportion of cells stained by dye uptake was not significantly increased above that observed in control (untreated) preparations. Repetition of the determination 6 h after addition of the drug yielded a similar result for MOLT-4 cells (Table 1), but an increased proportion of drug-treated CEM cells was permeable to trypan blue in a dose-dependent manner. Thus, the result for 5 μM VP-16 indicated no significant change, 17 μM caused a clear increase, and the greatest proportion of stained cells, approximately one-third, was recorded after 100 μM VP-16. Increased membrane permeability of MOLT-4 cells in response to VP-16 treatment was clearly evident after 24-h exposure to the epipodophyllotoxin, but a further 24 h (48 h total) of continuous exposure of MOLT-4 cells was required to eclipse the proportion of stained CEM cells seen within 24 h of drug treatment (Table 1). There was no relationship, however, between the proportion of trypan blue-positive MOLT-4 cells and VP-16 concentration either
Fig. 1. Effect of different exposure times on the cytotoxicity of VP-16 for CEM (■) and MOLT-4 cells (□) as determined by the MTT assay. Cytotoxicity was determined following exposure to VP-16 for 72 h (A); 24 h (B), 3 h (C), and 1 h followed by 72 h of recovery in drug-free media (D). Points (bars), means (±SE) of at least 3 separate experiments.

Table 1 Patterns of change in trypan blue uptake by two human leukemic lines following cytotoxic injury by different agents

<table>
<thead>
<tr>
<th>Treatment (agent and exposure time)</th>
<th>CEM</th>
<th>MOLT-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1 ± 0.9a</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>5 μM VP16 3 h</td>
<td>8.7 ± 1.4</td>
<td>11.3 ± 0.9</td>
</tr>
<tr>
<td>6 h</td>
<td>11.7 ± 3.3</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>24 h</td>
<td>86.5 ± 1.9c</td>
<td>59.8 ± 4.0c</td>
</tr>
<tr>
<td>48 h</td>
<td>ND</td>
<td>87.2</td>
</tr>
<tr>
<td>17 μM VP16 3 h</td>
<td>8.4 ± 1.9</td>
<td>10.5 ± 2.0</td>
</tr>
<tr>
<td>6 h</td>
<td>25.6 ± 6.2c</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>24 h</td>
<td>82.0 ± 8.4c</td>
<td>44.2 ± 4.0c</td>
</tr>
<tr>
<td>48 h</td>
<td>ND</td>
<td>89.5</td>
</tr>
<tr>
<td>100 μM VP16 3 h</td>
<td>12.9 ± 2.5</td>
<td>10.1 ± 1.8</td>
</tr>
<tr>
<td>6 h</td>
<td>36.0 ± 9.9c</td>
<td>12.1 ± 1.8</td>
</tr>
<tr>
<td>24 h</td>
<td>87.8 ± 7.3c</td>
<td>44.8 ± 6.1c</td>
</tr>
<tr>
<td>48 h</td>
<td>ND</td>
<td>85.3</td>
</tr>
<tr>
<td>30% DMSO 1 h</td>
<td>88.1 ± 2.6c</td>
<td>87.6 ± 1.3c</td>
</tr>
<tr>
<td>10% ethanol 1 h</td>
<td>78.3 ± 10.0c</td>
<td>84.4 ± 7.0c</td>
</tr>
</tbody>
</table>

* Cells were treated with VP-16 and other agents at the concentration and for the duration shown.

a The percentage of trypan blue-positive cells expressed as the mean ± SE of at least three separate experiments or as a single result in the three cases for which only one determination was made.

b Significantly (*P < 0.05*) different from control.

c ND, not determined.

Uptake of trypan blue was evident in 75–85% of MOLT-4 and CEM cells 1 h after the respective populations were exposed to 30% DMSO or 10% ethanol. In response to these treatments, which were calculated to cause necrosis, no distinction in dye uptake was apparent between the two lines (Table 1).

Morphology. Within 3 h of treatment with VP-16, microscopic examination of either MOLT-4 or CEM cells revealed clear evidence of cytotoxic injury. At this stage, a similar proportion of cells from each line exhibited nuclear margination, condensation, and fragmentation with cytoplasmic convolution. In the CEM populations, cellular debris was evident and appeared to be derived from blebbing of the cytoplasmic membrane and formation of apoptotic bodies (Fig. 2). Apoptotic bodies were not evident in preparations of MOLT-4 cells after treatment with VP-16 within the concentration range tested (5–100 μM).

After 24-h exposure to a cytotoxic concentration of VP-16, preparations of both CEM and MOLT-4 cells exhibited extensive damage when examined with the light microscope. Few intact cells were seen, most remaining cellular structures exhibiting loss of cytoplasmic definition against a background of debris (data not shown). Such characteristics were initially evident in CEM cells 6 h after exposure to 100 μM VP-16, but not at this time in the equivalent MOLT-4 population, although apoptotic morphological changes (cell shrinkage, margination of chromatin) were clearly seen in both lines. Specific identification of apoptotic bodies in preparations of MOLT-4 cells 24 h after treatment was not possible because of the extent and heterogeneity of cell debris in these preparations.

Using histological preparations from both lines, we quantified drug-induced morphological changes by counting apoptotic cells evident 3 h after exposure to VP-16 at various concentrations. These determinations, including examination of appropriate controls, were undertaken on a blind basis. Both cell populations exhibited an almost linear dose response, the number of dead cells being significantly increased above control at the lowest dose used (5 μM), and the proportion increased to a maximum of 15% at the highest dose (Fig. 3A). On the
II IIIIRINO APOPTIC RESPONSES IN LEUKEMIC CELLS

Fig. 2. Morphological characteristics of CEM cells (A and C) and MOLT-4 cells (B and D) following treatment with 100 μM VP-16 for 6 h. Illustrated is a typical apoptotic change (A) by comparison with normal morphology (N) exhibited in both cell lines following exposure to VP-16, the presence of apoptotic bodies (arrows and circle) in CEM cells being contrasted with the lack of apoptotic bodies in VP-16-treated MOLT-4 preparations. Magnification: A and B, × 100; C and D, × 20.

Fig. 3. Quantitation of VP-16-induced morphological change in CEM (■) and MOLT-4 (+) cells following 3-h exposure to VP-16 at different concentrations. Procedures used to quantify the proportion of cells exhibiting apoptotic morphological change (A) and to determine numbers of apoptotic bodies (B) are described in “Materials and Methods.” Points, means of at least 3 separate experiments.

Flow Cytometry. Analysis of untreated cells indicated that the proportion of G2/M cells in the CEM line was approximately 2- to 3-fold greater than in the MOLT-4 line. Also evident within the CEM subpopulation was a fraction of tetraploid cells which is a consistent feature of this line. Following 3 h exposure to VP-16, flow cytometric analysis suggested a slight treatment-related effect on the cell cycle distribution of randomly dividing CEM cells with a decrease in the proportion of cells in G2/M phase of the cycle at the highest concentration (100 μM VP-16) used. Such an effect was not evident in MOLT-4 cells at this time but could be discerned 6 h after treatment, by which time decreases in the proportion of G2/M cells in both lines were clearly evident (Table 2).

Within 6 h of exposure of CEM cells to VP-16, marked distortion of the flow cytometric profiles was evident, specifically involving increased fluorescence corresponding to less than G1 content of DNA. This effect increased with treatment time (data not shown) and was dose dependent (Fig. 4). No such effect was evident following analysis of MOLT-4 cells. When flow cytometric analysis was undertaken 24 h or longer after application of VP-16 (≥5 μM), evident destruction of CEM cells precluded a useful profile being generated, although the MOLT-4 cells under the same conditions still exhibited a discernible G1-S-G2/M distribution (data not shown).
changes in fluorescence were quantified on the basis of appropriate standard curves using X-irradiated CEM and MOLT-4 cells. Follow fluorescent dye Hoechst H33258 to duplex DNA. Drug-induced Schwartz (28) which depends on the kinetics of DNA unwinding in treatment was assayed using the procedure developed by Kanter and cells treated with 17 /μM VP-16 and not evident at lesser concentra. The same structural change was less readily detected in DNA from cells treated at the highest concentrations, i.e., 50 and 100 /μM VP-16. 3 h, “nucleosomal ladders” could be readily seen using DNA from these cells (Fig. 5).

Evidence of internucleosomal DNA fragmentation was sought by agarose gel electrophoresis following treatment of the respective cell populations with VP-16. After CEM cells were exposed to the drug for 3 h, “nucleosomal ladders” could be readily seen using DNA from cells treated at the highest concentrations, i.e., 50 and 100 /μM VP-16. The same structural change was less readily detected in DNA from cells treated with 17 /μM VP-16 and not evident at lesser concentrations (Fig. 6A). By 6 h after the addition of VP-16, fragmentation of DNA was evident in all preparations from CEM cells (Fig. 7), and there was little difference between the respective samples in the dose range tested (5–100 /μM).

Similar analysis of DNA isolated from MOLT-4 cells revealed no evidence of ladders. Specifically, internucleosomal cleavage of DNA was not evident 3 or 6 h after treatment (Fig. 6B). Neither could such fragmentation of DNA be detected by overloading (>10 μg/well) the gels (data not shown). Agarose gel electrophoresis of DNA isolated from MOLT-4 cells subjected to more prolonged treatment revealed a smear, in addition to the main band of DNA, suggesting no specific degradation of a minor fraction of DNA from these cells (Fig. 8). No evidence of internucleosomal degradation was detected following electrophoresis of DNA isolated from either CEM or MOLT-4 cells subjected to a range of procedures which caused necrosis, including incubation at 50°C, exposure to 30% DMSO, and 10% ethanol for 60 min. Apart from the absence of ladders, there was scant evidence of DNA degradation after these treatments, even when the gel was run to detect “smearing” of high M, DNA (Fig. 9).

**DISCUSSION**

Cell death may be determined on the basis of any one of a range of criteria including morphometric, cytotoxicity, and histological change, loss of respiratory function, and failure of reproductive activity (30). Not surprisingly, quantitative assays based on such parameters yield equivalent results. Essentially the same ID₅₀ values may be derived using the MTT or a clonogenic assay (31, 32). Thus, the equivalent cytotoxicity of VP-16 in MOLT-4 and CEM cells on the basis of the MTT assay is indicative of close similarity between these T-cell populations. Concentrations >1 μM are required to cause loss of viability, and such treatments were the basis of this investigation. Of the exposure protocols assessed using the MTT assay, continuous exposure to the drug for 3 days caused the most cell death. However, lethal damage could be induced in a proportion of randomly dividing cells within 3 h of exposure to VP-16 insofar as loss of viability occurred if the exposed cells were maintained in drug-free medium for a further 3 days (Fig. 1). Sherwood et al. (33) and Kung et al. (34) previously suggested that cell killing by cell cycle phase-specific agents does not derive from their primary biochemical targets since cytotoxicity required inhibition-free growth following removal of the drug. Compared with “3-h exposure/3-day recovery” and “24 h continuous” results (Fig. 1) indicates similar levels of cell death with respect to either the CEM and MOLT-4 line, although it cannot be presumed that the same cells were affected and/or the same mechanism of cell death was operative following these different treatment regimens. No exposure protocol permitted demonstration of a difference in the cytotoxicity of VP-16 between MOLT-4 and CEM cells.

Following exposure of MOLT-4 and CEM cells to VP-16 for 3 h, strand breakage in isolated DNA (Fig. 5) is reasonably attributed to inhibition of topoisomerase II (2–4). Such topoisomerase-mediated breakage is generally considered to be an immediate effect of drugs known to inhibit this enzyme. Despite breakage being evident in MOLT-4 DNA, such molecular damage was not correlated with a greater sensitivity of this line to VP-16 (Fig. 1) nor was it indicative of internucleosomal fragmentation of DNA (Fig. 6B and 7). Likewise Bertrand et al. (35) examined the cytotoxicity of VM-26 using two human lines (HL60 and HT-29, the latter being adenocarcinoma) and noted that both showed similar loss of colony formation ability following 30-min treatment, even though initial topoisomerase II-mediated DNA break frequency was higher in the HL60 cells. Chiron et al. (36) reported a lack of correlation between sensitivity of acute myelogenous leukemia cells (both fresh samples and established lines) to VP-16 and the generation of drug-induced single-strand breaks, while Chatterjee et al. (9) reported similar findings using Chinese hamster cell lines.

Topoisomerase-mediated strand breakage and cytotoxicity of VP-16 seem to be rarely correlated (9–11), and it may be argued that drug interactions with topoisomerase II are not intrinsically cytotoxic (37). Internucleosomal fragmentation of DNA, however, has been closely, if not definitively, associated with cell death by apoptosis (14–17). Walker et al. (38) distinguished between initial VP-16-induced fragmentation of thymocyte DNA to 300-kilobase fragments, attributable to topoisomerase II, and subsequent appearance of 50-kilobase fragments in parallel with 200-base pair fragments, indicative of nuclease activation. Although the respective processes could be distinguished, it was not possible to establish whether they were causally related. Having monitored VP-16-induced endonucleolytic cleavage of DNA, Kaufmann (12) summarized evidence that such degradation is not mediated by topoisomerase II.

VP-16-induced internucleosomal fragmentation of DNA from MOLT-4 cells was not evident even in preparations isolated up to 72 h after exposure to the drug at the highest concentration used (Fig. 8). The possibility that such fragmentation might be only observed using high drug concentrations was inferred by Del Bino et al. (39) from

**Table 2: Cell cycle distribution following exposure to cytotoxic doses of VP-16**

<table>
<thead>
<tr>
<th>VP16 dose (μM)</th>
<th>Exposure time (h)</th>
<th>CEM</th>
<th>MOLT4</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>43.8*</td>
<td>40.4</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>39.7</td>
<td>45.1</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>41.9</td>
<td>44.9</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>46.6</td>
<td>42.8</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
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<tr>
<td>100</td>
<td>6</td>
<td>48.6</td>
<td>40.7</td>
</tr>
</tbody>
</table>

*The proportion of cells (as percentage) at indicated phases of the cell cycle as indicated by flow cytometry, each result being the mean of at least 3 separate experiments.
Flow cytometric data indicating that endonucleolytic cleavage induced by a number of agents, including VP-16, occurred only after a threshold concentration was exceeded. The same investigators contrasted such degradation in HL60 cells to the refractory response of MOLT-4 cells (22, 40), speculating that the difference exemplified a tissue-specific effect distinguishing myelogenous from lymphocytic leukemia lines. Clearly, such a tissue-based distinction cannot be made between MOLT-4 and CEM cells as the basis for their different response patterns observed by us. Although not observed in response to VP-16, internucleosomal fragmentation of DNA from MOLT-4 cells has been observed in response to 5-deazaacyclotetrahydrofolate (41). Accordingly, the response pattern recorded here is indicative of the diversity of pathways to cell death rather than distinguishing MOLT-4 as being totally refractory to DNA fragmentation, and to that extent, a unique or exceptional cell population.

Of the molecular and cellular events correlated with apoptosis, internucleosomal fragmentation of DNA has been characterized as a "hallmark" (15). Death of CEM, but not MOLT-4, cells may thus be described as apoptosis by this criterion. However, a series of recent observations would appear to preclude definitive utilization of endonucleolytic cleavage of DNA to characterize apoptosis. Various investigators have described an occurrence of apoptosis using morphological criteria in the absence of DNA ladders (42–46). To these phenomena, which extend from transforming growth factor β1 in hepatocytes to cell death mediated by cytotoxic T-lymphocytes, may now be added the effect of a topoisomerase II-active drug, VP-16.

Fig. 4. Flow cytometric determination of changes in cell cycle distribution in CEM (left) and MOLT-4 (right) populations following 6-h exposure to VP-16. Control profiles (A and B) are contrasted with those following exposure to VP-16 at a concentration of 5 μM (C and D), 17 μM (E and F), and 100 μM (G and H). DNA content is based on propidium iodide (PI) fluorescence intensity and expressed using arbitrary units. Arrows, regions of increased fluorescence corresponding to cells with less than G1 content.
While in no sense detracting from the significance of DNA fragmentation as a useful means of monitoring apoptosis in many situations, the data caution against determining applicability of the term apoptosis exclusively on this basis. Obviously, failure to detect DNA fragmentation may be indicative of the speed or timing of the process rather than its nonoccurrence. Such kinetic factors cannot be excluded as explaining the MOLT-4 result. It is equally notable that, when evident, the timing of DNA degradation was shown to be dependent on drug concentration (47), and manipulation of this parameter did not affect the present result (Fig. 6B).

Obviously, the failure to detect internucleosomal fragmentation in VP-16-treated MOLT-4 cells suggests a range of immediate questions in terms of various biological (e.g., the effect of VP-16 on other lines, the effect of other agents on MOLT-4 cells) and analytical (e.g., isotopic end labeling as a means of detecting ladders) considerations. These considerations, however, do not resolve the broader questions which our, and other, findings (42–46) raise. These questions include whether electrophoretic detection of internucleosomal fragmentation may be used as a definitive indicator of apoptosis and/or whether the absence of such ladders is indicative of a distinct pathway to cell death.

Flow cytometric analysis of cells undergoing apoptosis provides insight both in relation to DNA fragmentation and also to cell cycle-specific effects (48). Topoisomerase II activity varies through the cell cycle (49) and DNA of mitotic cells is hypersensitive to VP-16 (10). Such variation is not paralleled by cycle-specific patterns of cytotoxicity, with S-phase cells being the most sensitive (11). Del Bino et al. (22) used flow cytometric data to demonstrate loss of S and G2/M phase cells following 6 h of exposure to topoisomerase I or topoisomerase II inhibitors at cytotoxic concentration and also detected cells with decreased DNA content. Likewise, cells having decreased DNA content were evident in preparations of CEM cells 6 h after exposure to VP-16 (Fig. 4, left). However these cells may not necessarily be equated with the decrease in the proportion of the G2/M fraction since this fraction also decreased in the MOLT-4 preparation without a corresponding increase in cells exhibiting less than G1 DNA content (Table 2). The flow cytometric data do not indicate preferential induction of DNA degradation of S-phase CEM cells, a principle which had previously allowed HL60 cells to be distinguished from MOLT-4 (22). Difference in cell cycle distribution between the MOLT-4 and CEM populations might also be considered in relation to the absence of VP-16-induced nucleosomal ladders in MOLT-4 DNA. If such fragmentation were confined to the G2/M fraction (6%) as compared to 16% in CEM (Table 2), detection in MOLT-4 cells might be difficult 3 h after treatment. By 6 h after treatment, however, the characteristic banding pattern would be detectable if approximately one-third the amount of CEM DNA evident as ladders were present in this form in preparations from MOLT-4 cells. Accordingly, difference in cell cycle distribution would not appear to account for the difference in DNA fragmentation between the two cell lines.

Certain morphological changes caused by VP-16 treatment, condensed chromatin, nucleoid fragmentation, and cell shrinkage, were evident in both MOLT-4 and CEM cells within 3 h of treatment (Fig. 2) and were quantitatively related to drug concentration (Fig. 3A). Such morphological changes, when described as a feature of apoptosis, are usually associated with surface convolution and formation of apoptotic bodies (18, 19), as indeed was evident in CEM cells (Fig. 2), and these observations were consistent with flow cytometry data (Fig. 4). The absence (Fig. 3B), or at least low numbers, of apoptotic bodies from the MOLT-4 cells might be a basis for characterizing the pattern of cell death in this population as necrosis rather than apoptosis. However, in a comparison of apoptosis and necrosis induced in HL60 cells by low and high concentrations, respectively, of variety of stimuli (including chlorambucil, ethanol, hydrogen peroxide, and heat), Lennon et al. (21) observed that necrotizing cell injury resulted in trypan blue uptake by >80% of cells within 1 h (except for hydrogen peroxide treatment which required 2 h), whereas loss of membrane integrity was virtually undetectable for the first 6 h, and sometimes only partially for 48 h, after apoptosis-inducing injury.
Likewise, we observed uptake of the trypan blue within 1 h when necrosis was induced in either MOLT-4 or CEM cells (Table 1).

Exclusion of trypan blue by CEM cells for at least 3 h after VP-16 is consistent with morphological and molecular indicators of apoptosis in this population. However, trypan blue was excluded by MOLT-4 cells far more efficiently, uptake of the dye being evident only after 24 h. This difference between CEM and MOLT-4 cells marks a need for caution when trypan blue is used to assess viability. If examined 6 h after VP-16 treatment, trypan blue staining would convey an erroneous indication of the relative sensitivity of the two cell lines. Difference between the two lines in relation to trypan blue uptake not only concerns the time of onset of increased permeability but also dose-related response patterns (see “Results”). The complexity of the findings do not suggest a simple relationship between a drug-induced lesion and this late stage event.

Considered in relation to the associated morphological changes, DNA fragmentation is an early event in apoptosis (15, 47, 50). When monitored after anticancer drug treatment specifically, such change may occur within 3 h (12) and specifically precedes loss of membrane integrity (41). Thus, during induction of apoptotic cell death by purine nucleotide analogues, loss of membrane integrity lagged behind DNA fragmentation by 48–72 h (51). Such observations are common, perhaps even universal, and often expressed in terms of DNA fragmentation preceding loss of viability, trypan blue uptake being the determinant of viability.

Rather than an “early” event, however, DNA fragmentation is frequently characterized as a late or “final” event, particularly with reference to the biochemistry of apoptosis (16, 17, 52). This description recognizes that apoptosis may occur in response to diverse stimuli which may be presumed to act via multiple pathways and which culminate in endonucleolytic cleavage of DNA (53–55). Consistent with a mechanism involving many stimuli leading to a final event are inhibitor studies defining intermediate stages in the process (46, 53, 56). Some changes preceding VP-16-induced cell death in CEM and MOLT-4 cells are common to both lines, but changes common to both lines are evident from the outset (e.g., the immediate effect of the drug) to the final stages of cell death, with the failure of respiration in the respective lines. In parallel to these common events are marked differences which severely limit any attempt to establish simple causal and necessary relationships between any of the changes observed.

The ordering of changes observed after exposure of CEM and MOLT-4 cells to VP-16 at cytotoxic concentration provokes consideration of the term programmed cell death in the present context. If understood to indicate ordered cellular change associated with loss of viability in the absence of necrosis, the term may reasonably be applied to either MOLT-4 or CEM cells. Parenthetically, it should be noted that programmed cell death may also be used to denote the orderly elimination of particular cells during development (57, 58).
Fig. 9. Analysis by 1.2% agarose gel electrophoresis of DNA isolated from control (lane 1) CEM and MOLT-4 cells (as indicated) or cells heated to 50°C (lane 2) or exposed to 30% DMSO (lane 3) or 10% ethanol (lane 4), together with DNA isolated from CEM cells 6 h after addition of 5 μM VP-16 (lane A). Arrows, position of markers (corresponding to 1300, 1100, 872, and 603 base pairs, top to bottom).

Table 3 Patterns of response in CEM and MOLT-4 cells following exposure to VP-16

<table>
<thead>
<tr>
<th>Change</th>
<th>CEM</th>
<th>MOLT-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA strand breakage</td>
<td>3 h*</td>
<td>3 h</td>
</tr>
<tr>
<td>Nuclear condensation/disintegration</td>
<td>3 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Decrease in proportion of G2/M cells</td>
<td>3 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Failure of respiration</td>
<td>24–72 h</td>
<td>24–72 h</td>
</tr>
<tr>
<td>Internucleosomal DNA fragmentation</td>
<td>3 h</td>
<td>Not evident</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Loss of membrane integrity</td>
<td>6 h</td>
<td>24 h</td>
</tr>
</tbody>
</table>

* Earliest time at which treatment-associated change was evident in cells continuously exposed to 100 μM VP-16.

For each T-cell line, a series of changes occurs, culminating in loss of viability as indicated by respiratory failure (Table 3). When the various response patterns are thus summarized, what has been described as "classic apoptosis" (17, 59, 60) is exhibited by the CEM cells. Sequential change which culminates in cell death and which, although clearly not necrosis, fails to exhibit all features of the thymocyte apoptotic model (18, 19), exemplified by MOLT-4 cells in the present study, might usefully be characterized as "atypical apoptosis." Finally, although internucleosomal fragmentation of DNA is a significant and useful parameter in many instances, the maintenance of membrane integrity for several hours after application of a lethal cellular insult appears to be the most reliable parameter for distinguishing between apoptosis and necrosis. In order to conclude that delayed loss of membrane permeability is a more reliable indicator of programmed cell death than other measurements, such as internucleosomal DNA fragmentation, a more extensive survey of cell lines is needed.

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Etoposide-induced Cytotoxicity in Two Human T-Cell Leukemic Lines: Delayed Loss of Membrane Permeability rather than DNA Fragmentation as an Indicator of Programmed Cell Death

Daniel R. Catchpoole and Bernard W. Stewart


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