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Drug-induced death of leukaemic cells after G_2/M arrest: higher order DNA fragmentation as an indicator of mechanism

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Summary Many reports have documented apoptotic death in different cell types within hours of exposure to cytotoxic drugs; lower drug concentrations may cause cell cycle arrest at G_2/M and subsequent death, which has been distinguished from 'classic' apoptosis. We have analysed etoposide-induced cell death in two lymphoblastoid T-cell lines, CCRF-CEM and MOLT-4, specifically in relation to DNA cleavage as indicated by pulse-field gel and conventional electrophoresis. High (5 μM) concentration etoposide causes 50-kb cleavage of DNA that occurs at the same time as apoptotic morphology and internucleosomal cleavage. At lower concentrations (0.5–0.05 μM), sequential change may be discerned with altered gene expression being similar to that at high dose, but preceding cell cycle arrest and 50-kb cleavage. These last changes, in turn, clearly precede internucleosomal fragmentation of DNA, vital dye staining and morphological evidence cell death. The pattern of higher order fragmentation constitutes a sensitive indicator of commitment to cell death in these cells. Morphological evidence of cell death is associated with internucleosomal fragmentation in one of the lines, but the pattern of 50-kb DNA cleavage provides the clearest evidence of commonality in death processes occurring at low and high drug concentration.

Keywords: apoptosis; 50-kb DNA breakage; etoposide; leukaemia; G_2/M arrest

The term 'apoptosis' is reasonably equated with physiological cell death during immunological selection and biological development (Wyllie, 1987; Allen et al, 1993). It is also recognized that this mode of cell death is induced by certain markedly non-physiological stimuli exemplified by radiation and certain drugs and specifically including most agents used in cancer chemotherapy (Hickman, 1992; Sachs and Lotem, 1993; da Silva et al, 1996). In the laboratory, induction of apoptosis by drugs has facilitated identification of processes associated with cell death, and of these internucleosomal fragmentation of DNA has been pre-eminent (Barry et al, 1990; Stewart, 1994). This effect is induced by a multiplicity of agents thereby providing evidence of a common pathway considered to be initiated by drug–target interaction and to culminate in cell death (Lennon et al, 1991; Marks and Fox, 1991). Accordingly, internucleosomal fragmentation of DNA, usually detected as DNA ladders after conventional agarose gel electrophoresis, has been perceived as the hallmark of apoptosis (Compton, 1992). However, the biological significance accorded to internucleosomal fragmentation of DNA is changing from the perception of an early indicator (Barry and Eastman, 1992; Smith et al, 1992), to a molecular change concurrent with morphological evidence of cell death (Lazebnik et al, 1995) and denoted by Vaux and Strasser (1996) as a 'post-mortem' event. Moreover, some cell populations do not exhibit ladders after drug treatment (Bertrand et al, 1991; Hotz et al, 1992; Falcieri et al, 1993). Other studies, however, indicate the absence of ladders when cell death is not immediate, but, in response to lesser concentrations, occurs 24 h or more after the addition of the drug (Ormerod et al, 1994; Bonelli et al, 1996).

Many, perhaps most, studies of cell death induced by anticancer drugs have involved cell lines exposed to a sufficiently high drug concentration that has an immediate effect, i.e. cause cell death within 8 h or less. Both apoptic morphology and internucleosomal fragmentation of DNA may be evident, the term 'classic apoptosis' being used. Although cytotoxic drugs commonly cause G_2/M cell cycle arrest (Rao and Rao, 1976; Chow and Ross, 1987), the same agents, when used at concentrations that cause cell death within hours, result in G_1 arrest (Chen et al, 1995; Dou et al, 1995; An and Dou, 1996). G_1 arrest is intimately associated with functional p53 (Lowe et al, 1993), and the role of this gene in mediating drug-induced apoptosis is clear and has been implicated in determining response to therapy (Lowe et al, 1994; Eliopoulos et al, 1995; Rusch et al, 1995; Marks et al, 1996). However, in many instances, drug concentations in patient serum are less than those used in the laboratory. Thus, in common with others, we studied death of lymphoblastoid cells within hours of exposure to 5 μM (or more) etoposide (Catchpoole and Stewart, 1993); clinical concentrations of this drug are typically 0.05 μM and rarely exceed 0.5 μM (Mross, 1996). Different pathways to cell death, dependent upon drug concentration, have been discerned (Del Bino et al, 1991; Lock et al, 1994; Tornaletti and Pfeifer, 1995; Jordon et al, 1996). In experimental cell populations exposed to drug concentrations which cause G_2/M arrest, the mode of cell death has been perceived to be distinct from classic apoptosis and, in some such cases, the term mitotic death has been proposed (Chang and Little, 1991; Demarcq et al, 1994; Ormerod et al, 1994; Dou et al, 1995; Bonelli et al, 1996). We have sought to investigate this mode of

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cell death using two lymphoblastoid cell lines CCRF-CEM (CEM) and MOLT-4 that have been exposed to etoposide concentrations between one and two orders of magnitude less than that which causes immediate cell death. A particular focus was the emergent hypothesis that, during apoptosis, internucleosomal cleavage may be accompanied by higher order fragmentation of DNA.

Detection of DNA fragments of 50 kb or more by pulse-field gel electrophoresis (PFGE) after exposure of cells to etoposide was initially interpreted with reference to the location of topoisomerase II (Filipski et al, 1990). Subsequently, the same effect was associated with apoptosis caused by a variety of stimuli, including agents not associated with direct damage to DNA (Brown et al, 1993; Oberhammer et al, 1993; Cohen et al, 1994; Desjardins and MacManus, 1995).

In the present study, we monitored genomic fragmentation by both conventional and PFGE and examined its relationship to changes in gene expression after treatment with etoposide at both high (5 μM) and low (0.5–0.05 μM) concentration. Many cell types undergo differentiation when exposed to cytotoxic drugs at low concentration, and the contribution of induced differentiation to induction of programmed cell death remains uncertain (Warrell, 1997). Specifically in relation to T cells, progressive differentiation is associated with increased susceptibility to apoptosis and correlated with gradual loss of bcl-2 and gain of Fas expression (Salmon et al, 1994). T-cell differentiation is marked by sharp changes in the expression of purine metabolic enzymes including purine nucleoside phosphorylase (PNP) (Ma et al, 1983) and such change is induced by phorbol esters (Martinez-Valdez and Cohen, 1988). Consistent with the intimate relationship between normal and malignant lymphoid cells (Campana and Janossy, 1988), altered expression of genes associated with purine metabolism is induced in CEM cells by phorbol 12-myristate 13-acetate (PMA) (Madrid-Marina et al, 1990). In preliminary experiments, we compared PMA with etoposide and established that drug-induced changes in PNP and terminal deoxynucleotide phosphorylase preceded and were more marked than altered adenosine deaminase expression. On this basis, we have contrasted the effect of etoposide with that of PMA in relation to PNP expression and other parameters including morphological change and genomic fragmentation.

After exposure to etoposide at high concentration, changes in 50-kb cleavage of DNA, gene expression and cell cycle progression were all evident within 6 h and not only occurred simultaneously but also coincided with morphological change and altered membrane permeability. Experiments with etoposide at low concentration revealed a more informative scenario. Sequential change may be discerned, providing a means for establishing relationships between classic apoptosis and cell death after G_2/M arrest. In this context, 50-kb fragmentation of DNA is established as a sensitive indicator of commitment to the cell death/apoptotic pathway and may be clearly shown to precede internucleosomal fragmentation, which occurs coincident with morphological evidence of cell death.

MATERIALS AND METHODS

Cell biology

MOLT-4 and CEM cell lines were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Biosciences Pty, Australia) at 37°C. Cells in logarithmic growth were treated with etoposide (Sigma Chemicals, St Louis, MO, USA) in dimethyl sulphoxide (DMSO, final concentration, 0.05%), the vehicle alone being added to control cultures. Cell numbers were determined by phase-contrast light microscopy using a haemocytometer. Procedures for treatment and harvesting have been described (Catchpoole and Stewart, 1993).

Morphological analysis

Cytospin preparations were made with 100-μl (4×10^5 cells) aliquots and stained using Wright's solution (Catchpoole and Stewart, 1993). Morphological assessment was made by light microscopy. Quantification of specific lesions was made by examination of at least 150 cells per field from at least three separate experiments and the result was expressed as the mean percentage of cells exhibiting the structure specified.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA was isolated (Chomczynski and Sacchi, 1987) and reverse transcribed using Maloney murine leukaemia virus DNA polymerase and random hexanucleotide primers (Noonan et al, 1990). Gene-specific primers for PNP (sense: 5′-ATGCAGCAGGAGGGCTGAAC-3′; antisense 5′-AGGCATCAGACATGGCAGGG-3′; product length, 153 nucleotides) and glyceraldehyde-3-phosphate dehydrogenase (GAP) (sense: 5′-TGGGGAAGGTGAAGGTCGGA-3′; antisense 5′-TGGTGCAGGAGGCATTGCTG-3′; product length, 110 nucleotides) were used to co-amplify target mRNA sequences in a competitive PCR reaction essentially as described by Bordow et al (1994), including primer design to negate possible DNA contamination of the original isolate and the establishment of a cycle number corresponding to linear incorporation. A cDNA amount equivalent to 40 ng RNA was subjected to PCR for 35 cycles in a final volume of 25 μl using 1 unit of Ampli*Taq* Polymerase. The optimized reaction conditions involved an initial denaturation at 94°C: each cycle consisted of 45 s at 94°C, 45 s at 55°C and 90 s at 72°C. After PCR, aliquots (10 μl) were subjected to 12% polyacrylamide gel electrophoresis before ethidium bromide staining and photography. To quantitate product formation after competitive PCR, photographic negatives were densitometrically scanned (Hoeffer GSV300 Australian Chromatography, Sydney, Australia) and a ratio between target and control PCR products was determined for each sample.

Flow cytometry

Cell cycle distribution was determined by flow cytometry after propidium iodide staining (Deitch et al, 1982), as described previously (Catchpoole and Stewart, 1993). Using red propidium–DNA fluorescence, 10 000 events were acquired with a FACScan cytometer and analysed using LYSIS II and Cellfit software (Becton Dickinson, San Jose, CA, USA).

Agarose gel electrophoresis

Internucleosomal DNA breakage was detected using conventional agarose gel electrophoresis after isolation of DNA as follows. Cells (2×10^6) were washed in phosphate-buffered saline (PBS) by centrifugation at 10 000 ***g*** at 4°C for 5 min. Cell pellets were lysed in 200 μl of DNAzol (Molecular Research Centre, Cincinnati, OH, USA) by gently pipetting the suspension. RNAase A was added

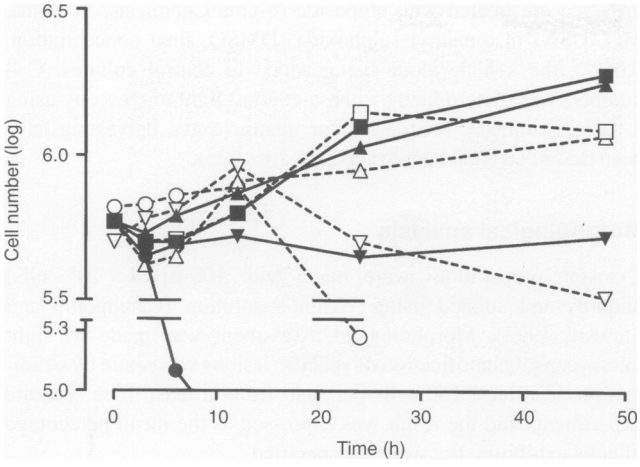

Figure 1 The relationship between etoposide concentration and growth of CEM and MOLT-4 cells. At time zero, etoposide at final concentrations of 0.05 μM (upright triangle), 0.5 μM (inverted triangle), 5 μM (circle) was added to the medium of CEM (closed symbols) or MOLT-4 cells (open symbols) and cell numbers for up to 48 h in the presence of the drug were contrasted with those in cultures receiving the vehicle alone (squares). At the highest etoposide concentration used, the numbers could not be determined beyond 24 h because of cellular debris and cytolysis

(30 μl of a 10 mg ml^{-1} stock) and the suspension incubated for 30 min at 37°C and further centrifuged for 10 min at 10 000 ***g*** at 4°C. After centrifugation, the viscous supernatant was transferred to a fresh tube. DNA was precipitated by addition of 100 μl cold 100% ethanol and pelleted by centrifugation for 30 min at 10 000 ***g*** at 4°C. The resulting DNA precipitate was washed twice with 800 μl of 95% ethanol and allowed to air dry for 30 min at room temperature before resuspending in 80 μl of 8 mM sodium hydroxide buffered with 2 μl 1 M Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; final pH 7.2) and subjected to electrophoresis in 1.8% agarose (Catchpoole and Stewart, 1993).

PFGE

Cells (5×10^6) were resuspended in 60 μl of cell suspension buffer (10 mM Tris, pH 7.2, 20 mM sodium chloride, 50 mM EDTA) and allowed to equilibrate at 50°C. The suspension was gently mixed with 37 μl of pre-warmed (50°C) '2% Clean Cut Agarose' (Bio-Rad, Hercules, CA) and the mixture immediately transfered to plug moulds (using sterile transfer pipettes) and incubated at 4°C for 20 min to expedite agarose solidification. Agarose plugs were then subjected to proteinase K digestion by the addition of 500 μl of 100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine solution containing 40 μg ml^{-1} proteinase K and incubated at 50°C overnight. Plugs were washed for 1 h (× 4) in wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA) at room temperature with gentle agitation and stored in 0.1 × wash buffer at 4°C before electrophoresis. Samples, including a DNA molecular weight standard (48.5 kb lambda ladder, Bio-Rad), were electrophoresed through 1% pulsed field gel grade agarose in a horizontal electrophoresis system (model CHEF-DR-III; Bio-Rad Laboratories). The run parameters included a ramped switch time of 50–90 s over 22 h at 6 V cm^{-1} across an included angle of 120° in a 0.5 × TBE (89 mM Tris-HCl, 89 mM sodium borate, 2 mM EDTA pH 8.3) buffer system. The buffer was allowed to recirculate continuously and the temperature maintained at 14°C using a cooling module. After staining with ethidium bromide (0.5 μg ml^{-1}) for 30 min, the gels were photographed under UV irradiation.

RESULTS

We have previously examined the response of CEM and MOLT-4 cells to etoposide in terms of internucleosomal fragmentation of DNA, cell cycle distribution and morphological change (Catchpoole and Stewart, 1993; 1995). A feature of these data was the difference between the two cell populations. In studies using 5–100 μM etoposide, CEM cells exhibited 'classic' apoptosis, whereas internucleosomal fragmentation and apoptotic bodies were not evident in the MOLT-4 population. Moreover, although the drug was equally cytotoxic in both lines, loss of membrane integrity appeared to have different kinetics. Despite these differences, there was a uniformity in response of both the CEM and the MOLT-4 populations: exposure to etoposide caused immediate apoptotic cell death as determined by morphological criteria. The response was independent of drug concentration: if this were reduced from 100 μM to 5 μM, apoptosis was still evident within 6 h. The present data indicate that this is not the case if the etoposide concentration is reduced by one further order of magnitude. Whereas exposure to 0.5 μM etoposide causes cell death in both CEM and MOLT-4 cells, this lesser concentration is associated with an altered response such that cell death is preceded by changes not evident using the high concentration range previously studied.

Growth patterns

As determined by MTT cytotoxicity assay (72-h exposure), CEM and MOLT-4 cells are equally sensitive to etoposide, the ID_{50} being approximately 0.05 μM (Catchpoole and Stewart, 1993). The determination of cell number up to 48 h after addition of the drug provided further evidence that the two lines were equally sensitive. In relation to an effect on cell number, no adverse effect was apparent in either population after the addition of 0.05 μM etoposide. Stasis in cell number over the incubation period was achieved using 0.5 μM etoposide (Figure 1) and a sharply decreased number of cells was evident after the addition of 5 μM etoposide.

Morphology

Light microscopic examination was made of cells exposed to either etoposide or PMA. In the case of the phorbol ester, no treatment-related changes were apparent in CEM (Figure 2A and E) or MOLT-4 cells (data not shown). Morphological evidence of cell death is obvious 6–12 h after exposure of CEM cells to 5 μM etoposide (Figure 2F). In contrast, light microscopic examination of cells exposed to 0.5 μM or 0.05 μM etoposide for up to 24 h revealed minimal evidence of injury and cell death. There was progressive accumulation of darkly staining nuclei indicative of dividing cells after the addition of etoposide. In addition, the drug-treated cells could be distinguished from appropriate controls because cytoplasmic vacuoles were more common in cells exposed to etoposide (Figure 2A–C and G). When incubation was continued from 24 to 48 h, a marked change was immediately obvious. Cell death and associate debris were prominent to the point of obscuring any unaffected cells. Lesions specifically associated with apoptosis were evident: condensed chromatin, apoptotic bodies and secondary necrosis (Figure 2D and H). Multipolar mitotic figures

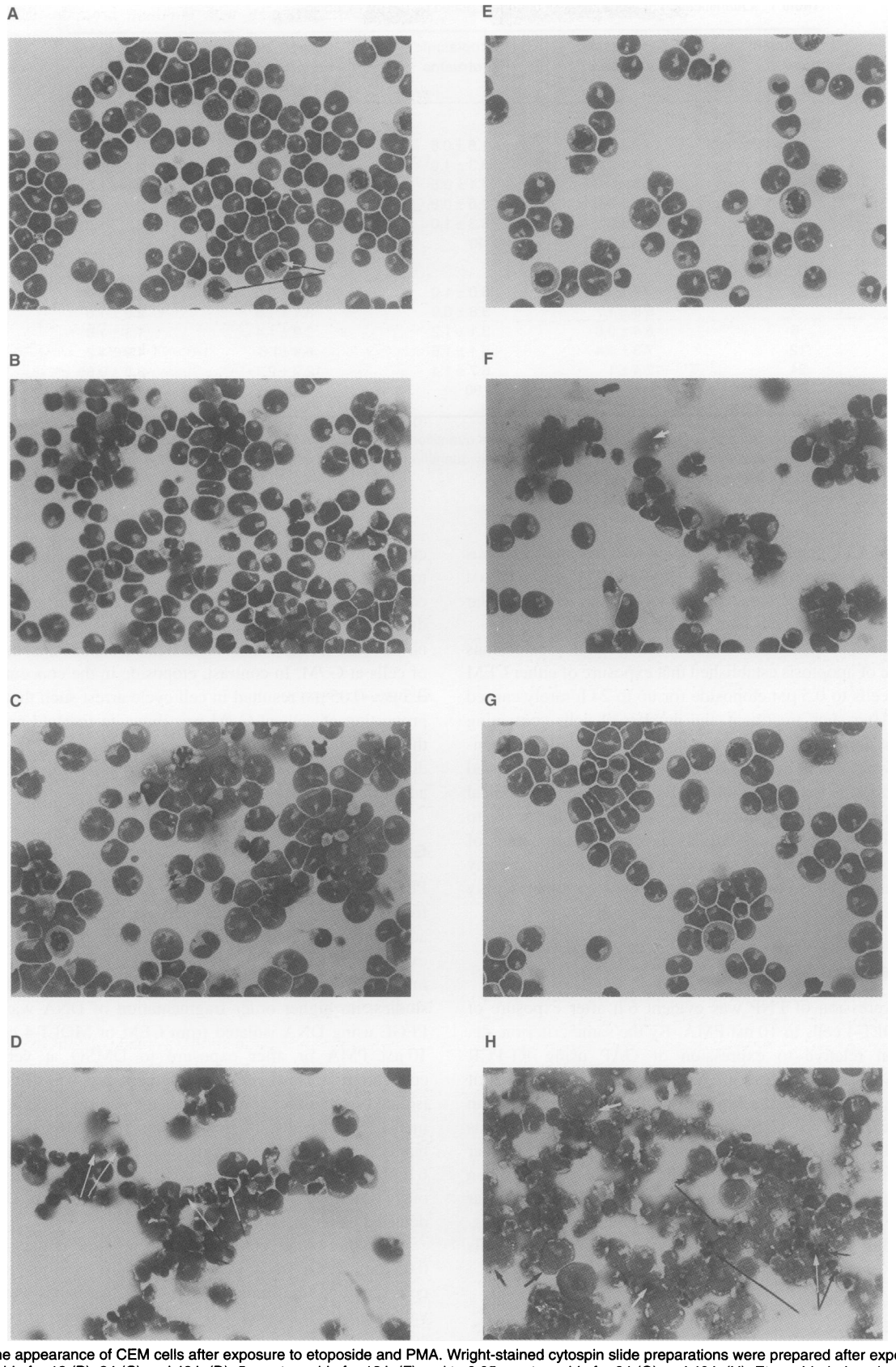

Figure 2 The appearance of CEM cells after exposure to etoposide and PMA. Wright-stained cytospin slide preparations were prepared after exposure to 0.5 μM etoposide for 12 (B), 24 (C) and 48 h (D), 5 μM etoposide for 12 h (F) and to 0.05 μM etoposide for 24 (G) and 48 h (H). Etoposide-induced morphological change may be discerned by comparison with vehicle control cells (0.05% DMSO for 24 h; A) and cells exposed to 10 nM PMA for 12 h (E). Morphological features evident include mitosis (M), cytoplasmic vacuoles (CyV) as well as change specifically indicative of cell death, including cytoplasmic protrusions (CyPr), apoptotic bodies (ApB), condensed chromatin (Ccr), primary necrosis (1°N) and secondary necrosis (2°N), these terms identifying lysis of whole cells and apoptotic bodies respectively

Table 1 Quantification of morphological change induced by 0.5 μM etoposide

Treatment time (h)	Cytoplasmic vacuoles	Cytoplasmic protrusins	Marginated condensed chromatin	Secondary necrosis
CEM				
Vehicle control	4.0 ± 0.4[a]	1.6 ± 0.6	0.9 ± 0.2	4.2 ± 1.5
3	8.4 ± 1.9	3.3 ± 1.0	1.6 ± 0.6	2.4 ± 0.4
6	8.8 ± 0.96	5.1 ± 0.6	3.1 ± 0.9	3.8 ± 1.8
12	7.5 ± 0.6	6.0 ± 0.8	4.2 ± 1.5	2.4 ± 0.6
24	21.1 ± 6.1	5.3 ± 1.0	4.7 ± 2.1	5.1 ± 1.8
48	81.3 ± 3.1	> 90[b]	> 90	55.3 ± 2.8
MOLT-4				
24	16 ± 3.1	6.0 ± 1.0	1.1 ± 0.5	2.5 ± 0.9
3	5.8 ± 1.7	3.8 ± 0.9	3.1 ± 1.5	2.9 ± 1.6
6	6.4 ± 0.6	3.1 ± 1.2	3.3 ± 1.4	1.3 ± 1.0
12	7.3 ± 1.4	5.1 ± 1.6	6.9 ±1.6	4.9 ± 1.2
24	12.4 ± 1	6.7 ± 1.4	12.7 ± 2.7	3.8 ± 0.60
48	> 90	> 90	> 90	18.7 ± 2.5

[a]The percentage of cells exhibiting each lesion was quantified as described in Materials and methods. [b]This result indicates that the result could not be precisely quantified because the majority of cells exhibited the lesion and cell debris precluded quantification.

were not seen. Cell debris limited detailed analysis and there was little distinction between preparations exposed to 0.5 and 0.05 μM etoposide. Essentially the same results were observed in similar preparations of MOLT-4 cells (data not shown).

Quantification of cells exhibiting various morphological lesions characteristic of apoptosis established that exposure of either CEM or MOLT-4 cells to 0.5 μM etoposide for up to 24 h rarely caused cell death. After such treatment, the number of cells containing vacuoles was increased, but no increase in the number of non-viable cells was evident in MOLT-4 or CEM preparations exposed to 0.5 μM etoposide for 24 h (Table 1). By comparison, the radical change in morphology consequent upon exposure of these cells to 0.5 μM etoposide for 48 h effectively precluded summation of discrete lesions: cell debris and secondary necrosis obscured many of the remaining cells, limiting quantitation to estimates only (Table 1).

Gene expression

Increased expression of PNP was evident 6 h after exposure of CEM or MOLT-4 cells to 10 nM PMA. By the same criterion, i.e. quantification relative to expression of GAP using RT-PCR (Figure 3), a marked increase (greater than three-fold in terms of the arbitary units used) in the expression of PNP was recorded in CEM cells exposed to 0.5 μM etoposide and this increase was sustained till at least 12 h after the addition of the drug. Similar results were recorded using MOLT-4 cells (data not shown). When the concentration of etoposide was reduced to 0.05 μM, a similar induction of PNP occurred. Moreover, such altered PNP expression could not be distinguished from that occurring in cells exposed to 5 μM etoposide (Figure 3).

Cell cycle

In contrast to the pattern of similarly increased expression of the marker gene PNP over the dose range 0.05–5 μM etoposide, a sharp demarcation was apparent when flow cytometry was used to assess cell cycle distribution. Earlier studies had involved 5 μM etoposide, and indicated that 3 or 6 h after the addition of the drug to the medium there was a slight increase in the proportion of G_1 cells. Even when such cultures were analysed after 24 h, cell cycle distribution within the small fraction of surviving cells exhibited no increase (relative to vehicle-treated controls) in the proportion of cells at G_2/M. In contrast, etoposide in the concentration range 0.5 μM–0.05 μM resulted in cell cycle arrest such that by 24 h the proportion of cells at G_2/M was three- to fivefold greater than in the untreated population (Figure 4). No change in cell cycle distribution of either CEM or MOLT-4 cells was evident after the addition of 0.005 μM etoposide.

Genomic fragmentation

PFGE was used to examine higher order fragmentation of DNA from CEM and MOLT-4 cells exposed to PMA and etoposide. In addition to causing differentiation (Madrid-Marina et al, 1990), the exposure of leukaemic cells to PMA has been reported to cause internucleosomal fragmentation of DNA (Ohta et al, 1995). In our studies, no higher order fragmentation of DNA was evident by PFGE using DNA isolated from CEM or MOLT-4 cells exposed 10 nM PMA or after exposure to DMSO at vehicle–control concentration. However, such fragmentation was detected in DNA isolated from either cell line after the addition of etoposide to the media. Specifically, in preparations from MOLT-4 cells, broad bands corresponding to approximately 50–150-kb were observed 6 h after the addition of 5 or 50 μM etoposide although, as previously described, internucleosomal fragmentation of DNA was not detected under the same conditions.

Over the broad etoposide concentration range studied (50–0.05 μM), PFGE data involved bands of various intensity, but corresponding to a limited size range. Most bands were 50 kb, but variation to 150 kb occurred. This was common to both lines. No consistent pattern for this slight variation in size range was apparent: no progression from 150 kb to 50 kb with increasing treatment time was recorded, and no progression over the 50–150-kb range within increasing or decreasing concentration of etoposide could be discerned. Moreover, repeat experiments indicated that identical

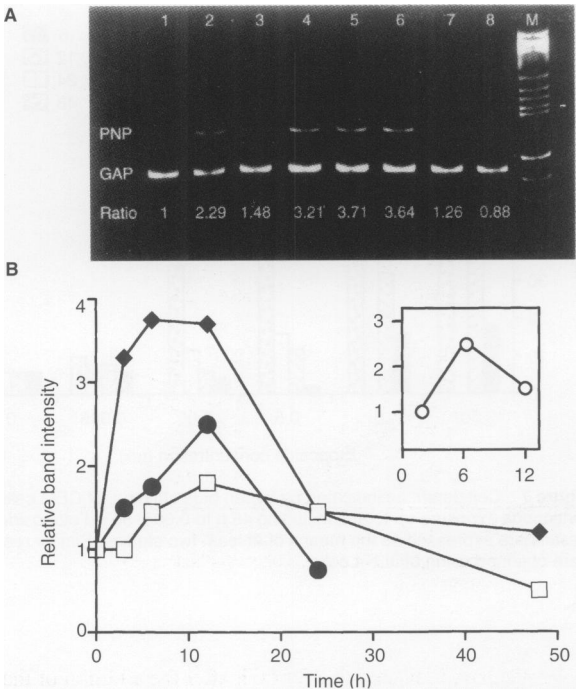

Figure 3 RNA isolated from control and drug-treated CEM cell preparations was reverse transcribed and subject to competive RT-PCR analysis as previously described (Bordow et al, 1994). (A) A representative RT-PCR gel showing changes in PNP expression (upper band) after 0.5 μM etoposide treatment in CEM after 3, 6, 12, 24 and 48 h exposure (lanes 4 to 8 respectively) compared with vehicle control (lane 1). Preparations corresponding to phorbol ester (10 nM PMA)-treated samples isolated after 6 and 12 h are in lanes 2 and 3 repectively. After densitometric analysis of the PCR products, the relative intensity shown is the ratio between the target gene PNP and the housekeeping gene GAP (lower gel band). (B) Progressive change in expression of PNP after treatment of CEM cells with 0.05 μM (■), 0.5 μM (◆) and 5 μM (●) etoposide. Each point is the mean relative expression based upon at least two experiments. The inset shows relative changes in PNP expression after 10 nM PMA treatment

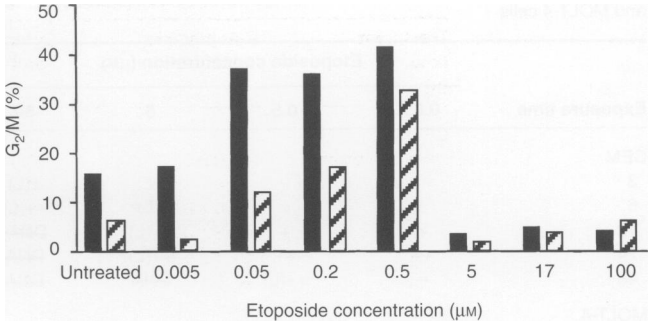

Figure 4 The proportion of cells at G_2/M after 24 h continuous exposure to etoposide. After drug treatment, CEM (■) and MOLT-4 cells (◩), cell cycle distribution was determined by flow cytometry and the proportion of G_2/M expressed as a percentage. Typical flow cytometric profiles, specifically involving 6 h exposure to 5 μM, 17 μM and 100 μM etoposide have been published (Catchpoole and Stewart, 1993). Results shown are the means of at least two experiments, except for 0.005 μM etoposide which records a single study

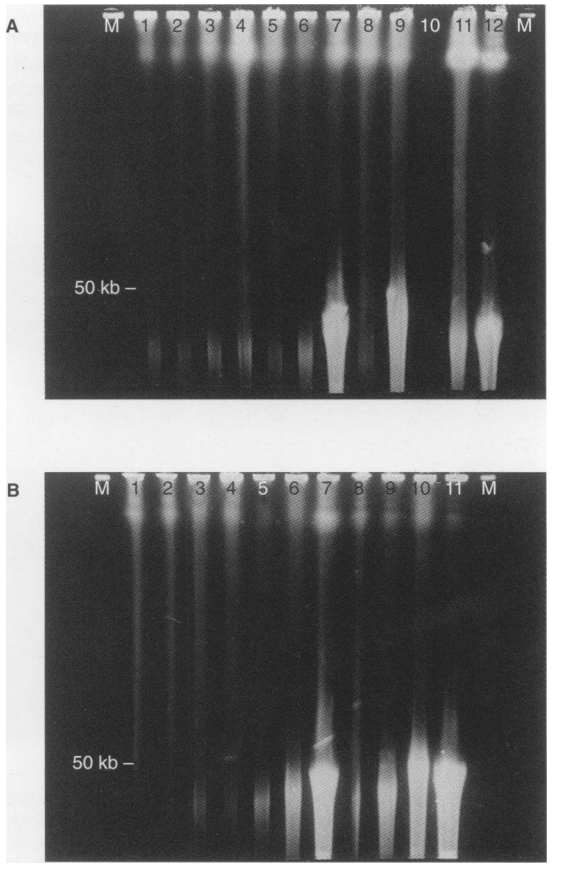

Figure 5 PFGE analysis of DNA from cells exposed to 0.05–50 μM etoposide. DNA from MOLT-4 (A) and CEM (B) cells was subject to PFGE and ethidium bromide fluorescence is presented as a photographic negative. For A, DNA was isolated from untreated cells (lane 1), cells exposed to etoposide for 3 h at concentrations of 0.05 μM, 0.5 μM and 5 μM (lanes 2–4), for 6 h at 0.05 μM, 0.5 μM and 5 μM (lanes 5–7), for 12 h at 0.05 μM and 0.5 μM (lanes 8 and 9). DNA was also isolated from cells exposed to 50 μM etoposide for 3 h and 6 h (lanes 11, 12). Preparations from CEM cells (B) correspond to untreated (lane 1), DMSO vehicle control for 24 h (lane 2), 10 nM PMA for 6 and 12 h (lanes 3, 4), 0.05 μM etoposide for 6, 12 and 24 h (lanes 5–7), 0.5 μM etoposide for 12, 24 and 48 h (lanes 8–10) and 5 μM etoposide for 6 h (lane 11). Markers were run in lanes indicated (M), with the 50-kb marker band indicated on the left of each panel

treatment conditions involved fluctuation within the size range indicated. Such variation is often (Cohen et al, 1994; Zhivotovsky et al, 1994; Weis et al, 1995) but not invariably (Brown et al, 1993; Oberhammer et al, 1993; Beere et al, 1995) seen in similar PFGE analyses.

When the various etoposide concentration/exposure time combinations were assessed, the results from CEM and MOLT-4 cells were similar (Figure 5). After exposure to etoposide at high concentration (5 or 50 μM), 50–150-kb fragmentation of DNA was evident. Such breakage was detected 6 h after the addition of the drug and a similar result was recorded up to 24 h after the addition of 5 μM etoposide. It was not possible to make PFGE analysis at such times after 50 μM etoposide because complete cellular destruction precluded recovery of DNA. Such 50-kb breakage of DNA was not evident in preparations isolated 3 or 6 h after the addition of 0.5 or 0.05 μM etoposide. It was, however, consistently detected in DNA isolated 12 h, and most markedly observed in DNA isolated 24 h after the addition of the drug at these concentrations (Figure 5). These findings were equally clear in both MOLT4 and CEM cells.

Data from multiple PFGE studies are summarized in Table 2. Among other considerations, data from both cell lines indicate that results using a single exposure time would be inadequate to assess higher-order fragmentation of DNA caused by etoposide, the 6 h

Table 2 Etoposide-induced 50 kb and internucleosomal cleavage in CEM and MOLT-4 cells

	Etoposide concentration (μM)			
Exposure time	**0.05**	**0.5**	**5**	**50**
CEM				
3	–[a]	–	±	±(L)
6	±	±	+(L)[b]	++(L)
12	+	+	++(L)	~~DNA~~[c]
24	++	++	++(L)	~~DNA~~
48	–	++(L)	~~DNA~~	~~DNA~~
MOLT-4				
3	–	–	–	+
6	–	–	+	++
12	+	+	++	~~DNA~~
24	+	±	~~DNA~~	~~DNA~~
48	Not done	++	~~DNA~~	~~DNA~~

[a]Summary results of multiple PFGE of DNA from cells exposed to etoposide are summarized using ++ to indicate the most intense band for the drug concentration used, + for consistent bands of lesser intensity, ± for results in which 50-kb bands were observed in some but not all analyses of DNA for the exposure time/etoposide concentration indicated and – to indicate non-detection of 50-kb breakage. Each result is indicative of at least two, and usually three or more analyses involving separate experiments. [b]DNA isolated from the same cells was subjected to conventional agarose gel electrophoresis and (L) indicates those protocols causing ladders; for all other samples, internucleosomal fragmentation was not detected. ~~DNA~~[c] indicates non-availability of DNA consequent upon complete cellular destruction after prolonged exposure to the drug at high concentration.

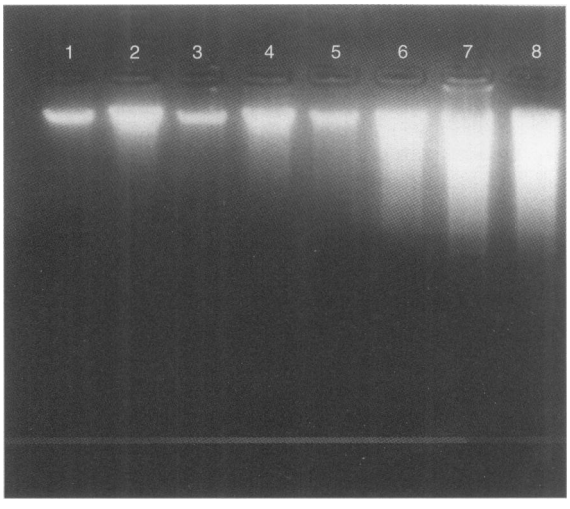

Figure 6 Analysis of etoposide-induced internucleosomal DNA fragmentation by 1.8% agarose gel electrophoresis. DNA was isolated from CEM cells after 24 h exposure to 0.05% DMSO (lane 1), 12 and 24 h exposure to 0.2 μM etoposide (lanes 2 and 3); 12, 24 and 48 h exposure to 0.5 μM etoposide (lanes 4–6) and after exposure to 5 μM etoposide for 12 and 24 h (lanes 7 and 8). Internucleosomal fragmentation formation is evident in lanes 6–8. The corresponding analyses using DNA isolated from MOLT-4 cells revealed no evidence of internucleosomal fragmentation

time point, for example, being appropriate to high concentrations but failing to detect positive results from lower drug concentrations.

Whereas PFGE analysis of DNA from CEM and MOLT-4 cells yielded similar results, the two lines differ most markedly in

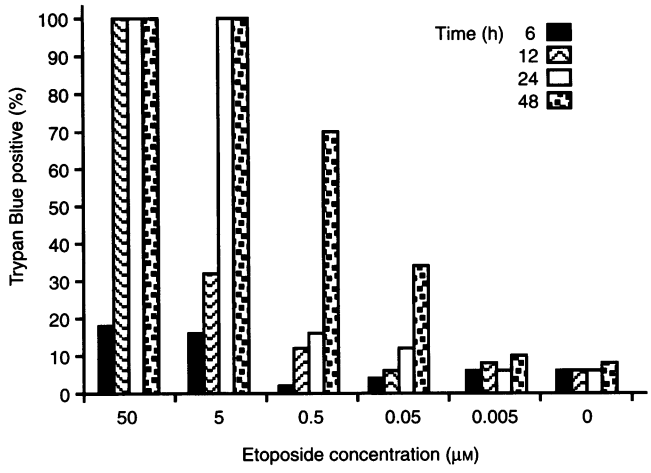

Figure 7 Cell death, as indicated by trypan blue staining, of CEM cells after continuous exposure up to and including 48 h to 0.005–50 μM etoposide. Results are expressed as the means of at least two studies. Similar results were obtained using MOLT-4 cells

relation to internucleosomal fragmentation. We have described the absence of DNA ladders after etoposide treatment of MOLT-4 cells (Catchpoole and Stewart, 1993). Under the same conditions (i.e. high etoposide concentration), the occurrence of internucleosomal fragmentation in DNA from CEM cells is coincident with 50 kb breakage, i.e. it is evident 3–6 h after treatment. In contrast, DNA ladders were not detected 3, 6, 12 or 24 h after treatment with 0.5 μM etoposide, but were evident in DNA isolated 48 h after such treatment (Figure 6). Ladders were not detected in any DNA preparations from cells exposed to 0.05 μM etoposide. To facilitate direct comparison with PFGE data, these findings have been included in the section of Table 2 corresponding to CEM cells.

Trypan blue

Delayed loss of membrane integrity is a recognized characteristic of apoptosis. In common with many of the other biological parameters studied, staining of CEM or MOLT-4 cells exposed to etoposide over a wide concentration range indicated two response patterns. At high etoposide concentrations (50 or 5 μM), approximately one-quarter of CEM cells were stained after 6 h, with the entire treated population showing uptake by 24 h. Staining of MOLT-4 cells after the same treatment was similar (i.e. immediately and progressively increasing), with all cells permeable to the dye by 48 h. A different response pattern was observed at lower drug concentrations. When etoposide concentration was decreased from 5 μM to 0.5 μM, a notable increase in trypan blue uptake was not evident before the 48-h time point, when 70% of cells were stained positive (Figure 7). A similar pattern was evident using 0.05 μM etoposide, although in this case only one-third of cells were killed by 48 h. Exposure of cells to 0.005 μM etoposide had no significant effect on viability by this criterion. Trypan blue staining of MOLT-4 cells exposed to 0.5 μM or lower concentration of etoposide yielded a result (data not shown) virtually identical to that recorded for CEM cells. In particular, positive staining of MOLT-4 cells exposed to 0.5 μM etoposide was only observed in the majority of the population after 48 h exposure, and not at earlier times.

DISCUSSION

Despite the use of DNA ladders as a 'hallmark' of apoptosis, this lesion is not observed during apoptosis induced by a variety of stimuli (Barres et al, 1992; Oberhammer et al, 1922), and specifically including cytotoxic drugs in some situations (Bertrand et al, 1991; Hotz et al, 1992; Catchpoole and Stewart, 1993; Falcieri et al, 1993). Although it is generally accepted that cytotoxic drugs elicit apoptosis via a 'final common pathway', there have been few molecular criteria apart from DNA ladders to characterize this process (Lennon et al, 1991; Marks and Fox, 1991). Relatively recently, however, higher-order fragmentation of DNA as evidenced by PFGE has been widely reported in cells during classic apoptosis (Bortner et al, 1995). Moreover, Hickman and colleagues (Beere et al, 1995) were able to distinguish between DNA breakage attributable to stabilization of topoisomerase II cleavable complex and higher-order fragmentation of DNA not associated with this mechanism. Not so readily summarized are findings on the relationship of higher-order breakage to internucleosomal fragmentation and to morphological change indicative of apoptosis. Our data, which contribute insight in relation to both these issues, were generated in the course of investigating whether a mode of cell death distinct from apoptosis could be characterized in lymphoblastoid cells exposed to etoposide at low concentration. The study was prompted by the observation that apoptotic morphology and internucleosomal fragmentation of DNA were not evident 6 or 24 h after incubation of CEM cells with 0.5 μM etoposide (Stewart et al, 1995), as distinct from positive findings using 5 μM, 17 μM or higher concentration etoposide (Catchpoole and Stewart, 1993). The findings appeared to support the conclusion reached by Ormerod et al (1994): namely that cisplatin-induced cell death of L1210 cells after G_2/M arrest should not be classified as apoptosis.

Whereas treatment of CEM and MOLT-4 with 5 μM etoposide caused cell death within hours (Catchpoole and Stewart, 1993) (see also Figure 2F) and a commensurate decrease in cell number, 0.5 μM etoposide caused a cytostatic effect (Figure 1). A growth response, involving murine fibroblast monolayers exposed to 0.1–10.0 μM etoposide, almost identical to that shown in Figure 1 was recently described by Bonelli et al (1996), although their study involved cell death at the two higher concentrations (i.e. 1.0 and 10 μM). In our study, histological evaluation indicated that despite the absence of any net decrease in total cell number (Figure 1), cell death occurred 48 h after exposure to 0.5 μM (or more) etoposide (Figure 2), the consistent cell number being attributable to some proliferative activity. Although a further decrease in drug concentration to 0.05 μM resulted in no effect on cell number by comparison with vehicle-treated controls, drug-induced cell death was again evident after light microscope examination (Figure 2).

Morphological evidence of cell death 48 h after exposure to 0.05 μM etoposide (Figure 2) indicates the limitation of cell count (Figure 1) as an immediate indicator of severe injury at low drug concentrations. Cell count does not differentiate between viable and non-viable (i.e. trypan blue positive) cells, the latter making up an increasing portion of the treated population (Figure 7). The data suggest that 48 h after the addition of 0.05 μM etoposide, dead and dying cells are still intact during phase contrast microscopy (for cell count), but rupture during cytospin preparation. Such fragmentation might give the appearance of necrosis despite the cell being commited to an apoptotic pathway.

The complex relationship between cellular proliferation, differentiation and apoptosis is indicated among other things by the differentiating effect of anti-cancer drugs at non-cytotoxic concentration (Constantinou et al, 1992; Schwartz et al, 1992; Li et al, 1995; Grant et al, 1996). Altered expression of genes associated with lymphocyte differentiation (Ma et al, 1983; Madrid-Marina et al, 1990) may have indicated a differential response in which lower etoposide doses were more closely associated with differentiation. However, the data (Figure 3) indicate that an immediately lethal concentration of etoposide (5 μM) caused an increase in PNP expression similar to that which occurred using concentrations 100-fold less. This response was similar to, if not more marked than that induced by PMA. In relation to etoposide concentration, similar induction of PNP precludes using this effect to identify different response pathways. Moreover, induction of PNP occurred within a similar time frame and there was no delay associated with lower concentrations of etoposide. Thus, a conspicuous feature of altered PNP expression is its immediacy. Of the biological parameters analysed (cell cycle progression, genomic fragmentation, dye uptake, etc.), monitoring gene expression revealed the earliest response to drug treatment.

After studying death of murine leukaemic cells induced by cisplatin, Eastman and colleagues (Sorenson et al, 1990) described two pathways: one involving a rapid, direct nuclease activation induced by very high drug concentrations and the other necessitating G_2/M arrest before cell death. This relationship appears to be common. Etoposide and similar agents have been variously reported to cause either G_1 (Chen et al, 1995; Dou et al, 1995; An and Dou, 1996) or G_2/M arrest (Rao and Rao, 1976; Chow and Ross, 1987), the difference being attributable, in the first instance, to drug concentration (Del Bino et al, 1991; Touneki et al, 1993). After the addition of 0.5 μM etoposide to CEM or MOLT-4 cells, the proportion of cells at G_2/M increased progressively in determinations made between 6 h and 24 h later, when a maximal value was recorded (data not shown). This response pattern involved a specific etoposide concentration range (Figure 4). However, although G_2/M arrest may precede drug-induced cell death in a variety of circumstances, the relationship between these effects varies. Thus, Schimke et al (1995), studying cell death after drug-induced G_2/M arrest in HeLa cells, describe aberrant mutipolar mitoses. However, we observed no such structures in the course of the present study. During drug-induced death of HeLa cells after mitotic block, internucleosomal fragmentation of DNA has been detected in some circumstances (Jordon et al, 1996) and not in others (Lock and Stribinskiene, 1996). DNA ladders were not evident during etoposide-induced death of murine fibroblasts after G_2/M arrest (Bonelli et al, 1996). More relevantly, absence of ladders was noted in L1210 leukaemic cells after cisplatin-induced G_2/M arrest (Ormerod et al, 1994). The present findings include detection of DNA ladders 48 h after the addition of 0.5 μM etoposide to CEM cells but not in MOLT-4 cells (Figure 6), although all our other findings suggest the mode of etoposide-induced cell death in these two populations is the same. In fact, the diverse observations summarized above provide no basis for establishing a relationship between G_2/M arrest and cell death. However, at least in respect of the present data, a relationship may be proposed by reference to the PFGE results.

Reviewing the description of higher-order fragmentation during apoptosis in many contexts, Cidlowski and colleagues (Bortner et al, 1995) commented that more studies were needed to determine whether higher-order fragmentation is indeed a universal

characteristic of apoptosis. As MOLT-4 cells were significant in demonstrating the limitations of DNA ladders as a molecular indicator of apoptosis (Hotz et al, 1992; Catchpoole and Stewart, 1993; Falcieri et al, 1993), detection of higher-order fragmentation in these cells after exposure to etoposide is noteworthy. Such a result was reported by Beere et al (1995) using 50 μM etoposide. It is now evident that concentrations of the drug 1000-fold less may induce the same effect in these cells, albeit after a markedly different exposure time (Figure 5). Our studies indicate no difference in sensitivity for 50-kb breakage between MOLT-4 and CEM cells. The two lines are equally sensitive to the drug [(Catchpoole and Stewart, 1993) and Figure 1] and this is consistent with virtually identical dose–response patterns for 50-kb fragmentation.

After the exposure of CEM or MOLT-4 cells to 5 μM etoposide, the appearance of 50 kb fragments (Table 2) is virtually coincident with topoisomerase II-mediated single-strand breakage, production of DNA ladders and histological evidence of cell death (Catchpoole and Stewart, 1993). In each of the lines exposed to either 0.5 μM or 0.05 μM etoposide, higher-order DNA fragmentation was detectable by PFGE after 12 h, although there was no morphological evidence of cell death at that time (Table 1, Figure 2). Under these conditions, 50-kb breakage coincided with G_2/M arrest. The inability to detect ladders 24 h after the exposure of CEM cells to 0.5 μM etoposide was a consistent finding. However, 48 h after the addition of 0.5 μM etoposide, loss of membrane integrity was clearly evident in both lines (Figure 7) and DNA ladders were evident in CEM cells (Figure 6). The separate genesis of 50 kb and internucleosomal fragments, respectively, is compatible with other reports of higher-order fragmentation in the absence of DNA ladders (Oberhammer et al, 1993; Watanabe et al, 1995) and evidence that, in thymocytes, nuclease activity mediating cleavage of DNA into large fragments is distinguished from that causing ladders (Sun and Cohen, 1994; Walker et al, 1994).

It is evident from the present findings that DNA ladders are a limited, if not an inadequate vehicle to establish a specific mode of cell death. As exemplified by MOLT-4 cells, ladders are not observed when all other criteria of apoptosis, including 50-kb breakage, are apparent. More critically, as exemplified by CEM cells, ladders are an insensitive analytical tool that are observed in a narrow range, both in terms of time and drug concentration, compared with higher-order fragmentation. The remarkably restricted dose/time distribution of ladders (L) in Table 6 suggests that at least some reports concerning absence of this lesion in death after G_2/M arrest involve missing the narrow 'window' at which DNA ladders appear. Finally, in the experimental system described here, DNA ladders are coincident with, and do not precede membrane and morphological evidence of cell death, confirming their status as a post-mortem event (Vaux and Strasser, 1996). None of these limitations apply to 50-kb fragmentation of DNA.

Higher-order fragmentation of DNA, and specifically generation of 50-kb breaks, has been associated with apoptotic cell death. The present results using 5 μM etoposide provide further evidence for such an association in the case of CEM cells and, in the case of MOLT-4 cells, indicate that such breakage may occur more generally than another indicator, namely DNA ladders. Processes associated with drug-induced apotosis have been characterized in systems exemplified by treatment of these two lines with 5 μM etoposide: cell death occurs in 4–8 h. Drug-induced cell death may also occur over a wider timeframe, and specifically after G_2/M arrest. An argument has been made that such cell death occurs by processes that distinguish it from apoptosis. Our findings mitigate caution in this regard. First, apoptotic indicators (typified by DNA ladders in this study) may be not so much absent as difficult to detect in a broader timeframe. Second, certain indicators (commitment to differentiation and 50-kb DNA breakage) were observed by us to be common to apoptotic cell death and to cell death after G_2/M arrest. Moreover, in the latter circumstances, 50-kb DNA breakage clearly precedes both DNA ladders and morphological change by light microscopy. Finally, in etoposide-treated CEM and MOLT-4 cells, 50-kb DNA breakage is best characterized as being coincident with G_2/M arrest rather than a consequence of it. This mode of cell death seems closely related to apoptosis, but may be particular to haematopoietic cells.

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