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DNA DAMAGE, POLY (ADP-RIBOSYL)ATION AND APOPTOTIC CELL DEATH AS A POTENTIAL COMMON PATHWAY OF CYTOTOXIC DRUG ACTION

DAVID I. MARKS* and RICHARD M. FOX

Department of Haematology and Medical Oncology, Royal Melbourne Hospital, P.O. 3050, Parkville, Victoria, Australia

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Abstract—Although various anti-cancer drugs have widely differing primary modes of action, the mechanisms of cell death appear similar but are not well understood. To investigate this problem we exposed cultured human leukemic T-lymphoblasts to 1-hr pulse doses of an alkylating agent (mafosfamide) and a topoisomerase II inhibitor (etoposide) that cause delayed cell death. The effects of these drugs on nucleotide content, poly (ADP-ribosyl)ation and DNA strand breakage were assessed. Both drugs caused DNA strand breakage, and although the pattern differed, this seemed to be the major mechanism by which cells were killed. The degree and time course of the NAD and ATP depletion that mafosfamide and etoposide caused were similar. Both drugs caused a nadir in cellular nucleotide levels 2 hr after exposure but between 2 and 6 hr there was a partial recovery. This correlates with the time course of the DNA damage they caused and appeared to result from poly (ADP-ribosyl)ation. Both drugs were shown to cause apoptotic cell death associated with endonucleolytic DNA fragmentation. We suggest that DNA damage, as a primary or secondary effect, associated with poly (ADP-ribosyl)ation and apoptotic cell death may be a common pathway of cytotoxic drug action.

The biochemical actions of various cytotoxic agents have been intensively investigated but comparatively little is known about the precise way in which they kill normal and malignant cells. Alkylating agents and topoisomerase II inhibitors such as epipodophyllotoxins are considered to have quite different mechanism of action. Roitt [1] was the first to show that the treatment of cells with alkylating agents specifically induces NAD depletion. Alkylators cause DNA strand breakage which appears to kill cells via poly (ADP-ribosyl)ation, which causes NAD and ATP depletion [2]. The aim of this study was to determine whether this is a potential common mechanism for cytotoxic drug action, i.e. to establish if other classes of cytotoxic drugs kill cells by causing DNA damage and secondary nucleotide depletion.

The epipodophyllotoxin etoposide has the ability to induce single and double DNA strand breaks and DNA protein cross-links [3]. This is mediated through interaction of the drug with topoisomerase II to form cleavable complexes. The covalent binding of topoisomerase II to DNA is associated with DNA strand cleavage but how this is related to cell death is uncertain. The DNA strand breaks induced by some topoisomerase II inhibitors are quite transient after drug removal but cells still die [4]. This suggests that the breaks may elicit secondary lethal events. DNA damage is known to cause NAD depletion but this has apparently not been reported as a mechanism of etoposide cytotoxicity.

In this report we have examined a number of parameters in the first 24 hr following a 1-hr pulse of cultured T-lymphoblasts to various cytotoxic agents at doses which cause delayed cell death. This type of drug exposure was chosen to enable us to study various biochemical and cellular parameters in cells still viable but programmed to die. These parameters included nucleotide content and DNA strand breakage. For these experiments, mafosfamide (an *in vitro* active form of cyclophosphamide) was used as a standard alkylating agent, as cyclophosphamide is the most common clinically used alkylator. The effects of mafosfamide were compared with those of etoposide, doxorubicin and methotrexate. All drugs caused DNA strand breaks, although the time course, type and extent of these differed. We show that a delayed lethal pulse exposure to nucleotide etoposide causes significant NAD and ATP depletion, comparable to the depletion induced by the cyclophosphamide analogue mafosfamide; we postulate that these changes may be important in the mediation of its cytotoxicity. In addition, the mode of cell death in cytotoxic drug-treated CCRF-CEM (CEM†) cells was assessed. This was done morphologically and by measuring DNA fragment size with agarose gel electrophoresis. All drugs were shown to cause apoptosis 24 hr after exposure but also some necrosis, depending on dose.

MATERIALS AND METHODS

Cultured tumor cells. Exponentially growing cultures of T-lymphoblastic leukemia CEM [5] cells were grown in RPMI 1640 medium (Flow

* Corresponding author: Dr Marks, Bone Marrow Transplant Coordinator, MRC/LRF Leukaemia Unit, Department of Haematology, Hammersmith Hospital, Du Cane Rd, London W12 0NN, U.K.

† Abbreviations: CEM, CCRF-CEM; 3-ABA, 3-aminobenzamide; % D, percentage double-stranded DNA remaining; PARS, poly (ADP-ribose) synthetase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitroguanidine.

Laboratories, Irvine, U.K.) supplemented with 10% v/v fetal calf serum, penicillin and streptomycin and were maintained at 37° in a humidified atmosphere of 7% CO_2–93% air. The cells grew in suspension culture with a doubling time of 20–24 hr. Cell viability was determined by the ability of cells to exclude 0.5% Trypan blue.

Exposure of tumor cells to cytotoxic drugs. Tumor cell lines of high viability (greater than 90%), in exponential growth phase, were grown in 650 mL plastic tissue culture flasks as described above. The culture medium was changed 24 hr before all experiments.

A fixed volume (0.5 mL) of culture medium containing varying concentrations of different cytotoxic drugs was added and mixed thoroughly. After incubation of cells at 37° for 1 hr the cell cultures were centrifuged (200 *g*, 10 min), the supernatant discarded and the cells washed once then resuspended in fresh medium.

In addition, experiments were performed to see if 2 mM 3-ABA and 1–4 mM nicotinamide (inhibitors of the enzyme PARS) mitigated the cytotoxicity of mafosfamide and etoposide.

HPLC. To measure NAD and ATP content, about 5×10^6 cells were pelleted, extracted with ice-cold perchloric acid and their NAD and ATP content measured by HPLC as previously described [6, 7]. Nucleotide content was expressed as a percentage of control. Control NAD and ATP contents were 204 ± 5 and 737 ± 15 pmol/10^6 cells, respectively.

Estimation of possible nucleotide leakage from cells. To establish that the lowered cellular adenine nucleotide levels were due to NAD and ATP consumption rather than leakage through potentially damaged cell membranes, the following study was performed. Log-phase CEM cells were incubated with [^{14}C]adenosine (1 million cpm per million cells) at 37° for 24 hr. The cells were then washed to remove isotope from the culture medium. Mafosfamide and etoposide (50 μM) were added in separate experiments to CEM cells then washed off 1 hr later. At various time points after the drug exposure the cells were centrifuged and the radioactivity of the supernatant medium compared with that of the labelled cells, allowing for the percentage of radiolabel (estimated) that leaks out of cells after cytotoxic exposure.

Measurement of PARS level. The method of Berger *et al.* [8] was used to measure PARS level: cells were rendered permeable to an exogenously supplied nucleotide and then the uptake of [^{3}H]-NAD at 30° was measured.

Fluorometric assay of DNA strand breakage. Approximately 5×10^6 cells were pelleted then processed in triplicate samples according to the fluorometric method of Birnboim and Jevcak [9]. The % D value obtained from the control (unexposed) cells was corrected to 100 and the % D value of the exposed cells was expressed as a percentage of this.

Light microscopy. After cytotoxic exposure, cells were washed twice in RPMI 1640, then pelleted (150 *g*, 5 min). Cell specimens were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate, resuspended in 1:1 horse serum:saline for 2 hr (4°)

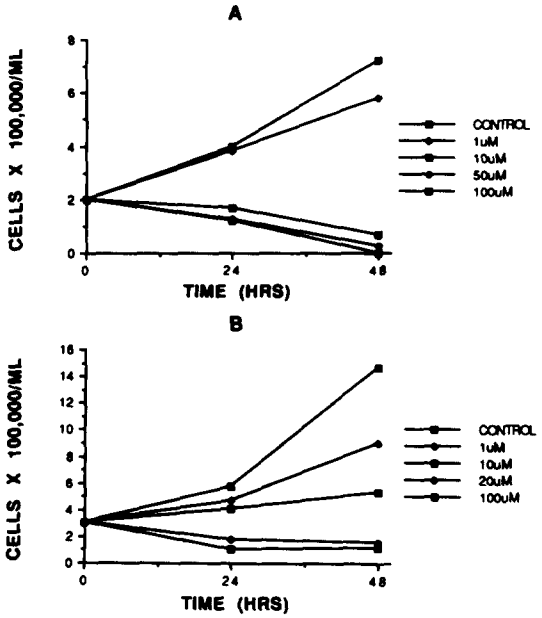

Fig. 1. Growth curves of CEM cells in log-phase after a 1-hr exposure to mafosfamide (A) and etoposide (B) at concentrations ranging from 1 to 100 μM (compared with control). Number of cells × 100,000/mL vs time (hr). Mean of three samples from representative experiments.

and then stained with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Each slide had three or more cell clumps; scoring of cells was made from several areas in order to avoid possible biases after centrifugation from the differential density of cells. At least 200 cells were counted per specimen.

DNA extraction and agarose gel electrophoresis. About 1×10^7 cells were centrifuged (200 *g*, 10 min) to form a pellet and after protein digestion the DNA was extracted twice with phenol:chloroform (50:50) and precipitated with 3 M sodium acetate and 100% ethanol. It was then dissolved in Tris–EDTA (pH 8). DNA samples were subjected to horizontal electrophoresis on a 400 mL 1.8% agarose gel block at 75 V for 4 hr. The running buffer contained 90 mM Tris, 90 mM boric acid and 2 mM EDTA. DNA was stained with ethidium bromide (3 μg/mL in 0.7 mM EDTA, pH 7). About 5 μg of DNA was added to each well after mixing with 0.2 volume loading buffer (0.25% Bromophenol blue, 40% w/v sucrose in water and 0.1% sodium dodecyl sulphate). DNA fragments were sized by simultaneously running a track of pGEM DNA (Boehringer Mannheim, Bielefeld, F.R.G.) diluted in Tris–EDTA (pH 8) mixed with 0.2 volume loading buffer.

Chemicals. KH_2PO_4 and perchloric acid (70%) were of analytical grade and were obtained from BDH Chemicals (Kilsyth, Australia). Sodium phosphate, magnesium chloride, urea, sodium hydroxide and glucose were also from BDH. HPLC-grade methanol and water were purchased from Mallinckrodt (KY, U.S.A.). All nucleotide standards were from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and were of analytical grade. Mesoinositol,

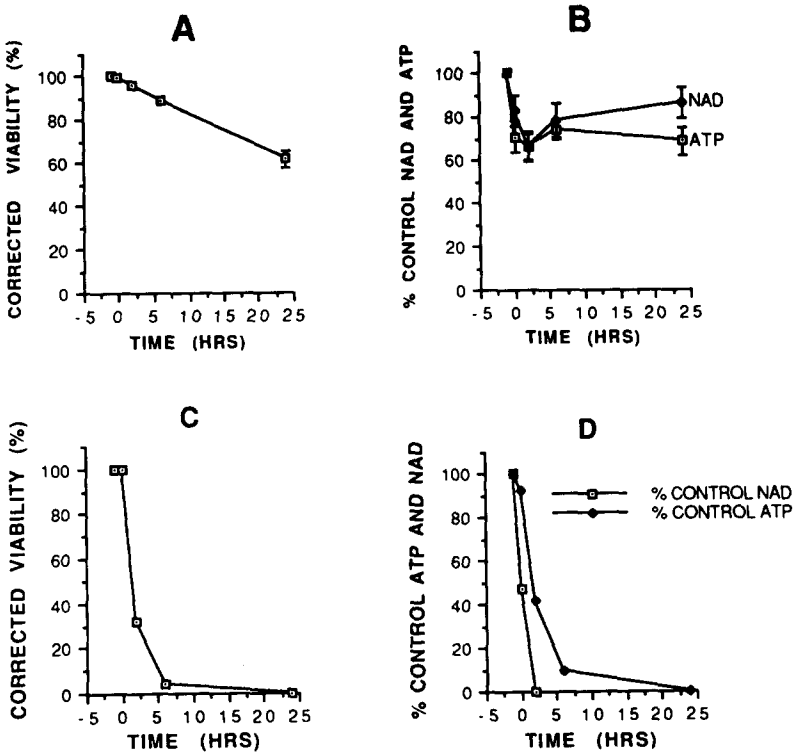

Fig. 2. A–D: CEM cells were exposed to mafosfamide, 50 μM for 1 hr (A,B) and 1 mM for 1 hr (C,D). A, C: Corrected viability (% control) vs time after exposure (hr) (−1 = before exposure, 0 = immediately after 1 hr exposure to cytotoxic agent). B, D: NAD and ATP content (% control) vs time after exposure (hr). Control NAD and ATP contents were 204 ± 5 and 737 ± 15 pmol/10^6 cells, respectively. All points represent the mean of 3–6 observations involving at least two experiments. A,B: error bars indicate standard error. Where error bars are not visible, the standard error is less than the vertical height of the point on the curve.

sodium dodecyl sulphate, mercaptoethanol and ethidium bromide were also from Sigma. Etoposide (Vepesid) was obtained from Bristol-Myers (Crow's Nest, Australia) and mafosfamide (ASTA Z 7654) was from Asta-Werke (Bielefeld, F.R.G.). Doxorubicin (Adriamycin) was from Farmitalia Carlo Erba (Milan, Italy) and methotrexate was from David Bull (Victoria, Australia). Millipore filters were from Millipore (Bedford, MA, U.S.A.). [8-^{14}C]Adenosine (49.8 mCi/mmol) and [^{3}H]NAD (3.0 Ci/mmol) were from Amersham (U.K.).

RESULTS

Drug-induced growth inhibition and cytotoxicity

Exponentially growing CEM cells were exposed to cytotoxic drugs of different classes: an alkylating agent (mafosfamide), a topoisomerase II inhibitor (etoposide), an antitumor antibiotic (doxorubicin) and an antimetabolite (methotrexate) for 1 hr over a wide range of concentrations. Pulse rather than continuous exposures were chosen to induce biochemical lesions which could evolve with time independent of further drug exposure. Pulse exposure also mimics the common clinical use of these drugs. The effects of these drugs on cell growth and viability was monitored by counting using a hemocytometer and Trypan blue staining for up to 96 hr. This method was used rather than clonogenic assays because we wanted to look specifically at lethal cell events and not proliferative capacity. Figure 1A and B demonstrates the effects of mafosfamide and etoposide on cell growth at 24 and 48 hr.

Mafosfamide at 50 μM and etoposide at 20 μM were lethal but caused minimal cytotoxicity during the first 24 hr after drug exposure (Figs 2 and 3). However, after the first 24 hr there was progressive cell death with time and based on Trypan blue uptake as a measure of viability, all cells were dead by 96 hr. These doses, which we have termed delayed lethal doses, were chosen for further experiments to allow measurement of various biochemical parameters for several hours after drug exposure on cells that were viable but programmed by the brief drug exposure to die. Similarly determined delayed lethal doses for doxorubicin and methotrexate were 25 and 100 μM, respectively (data not shown). Much higher (i.e. rapidly lethal) concentrations of mafosfamide and etoposide were also studied to establish if these doses exacerbated the biochemical abnormalities seen at lower doses (Figs 2 and 3).

Effect of drug exposure on DNA strand breakage and nucleotide content

Etoposide is known to cause protein-associated DNA strand breakage but the mechanism by which

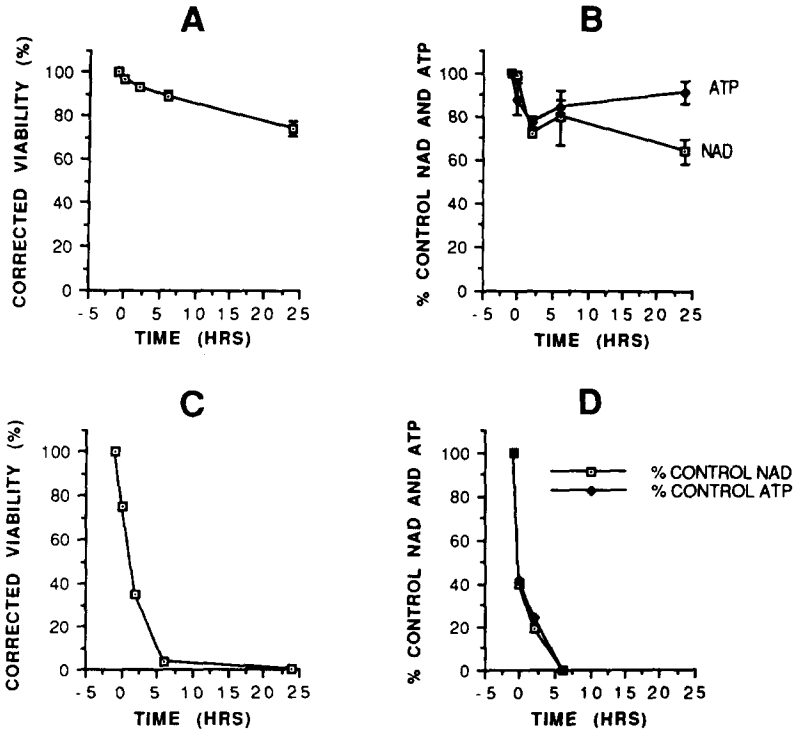

Fig. 3. A–D: CEM cells were exposed to etoposide, 20 μM for 1 hr (A,B) and 400 μM for 1 hr (C,D). A,C: Corrected viability (% control) vs time after exposure (hr). B,D: NAD and ATP content (% control) vs time after exposure (hr) (−1 = before exposure, 0 = immediately after 1 hr exposure to cytotoxic agent). A,B: error bars indicate the standard error of at least three observations.

this causes cell death is not apparent. We considered that the DNA strand breakage induced by etoposide might, in a manner similar to the primary DNA damage induced by alkylating agents or radiation, lead to poly (ADP-ribosyl)ation and subsequent depletion of NAD and ATP. We therefore compared the extent and time course of DNA strand breakage induced by delayed lethal concentrations of etoposide and mafosfamide in CEM cells (Fig. 4A and B). Mafosfamide (50 μM) induced DNA damage immediately after exposure; after this strand breaks continued to accumulate but at an apparently slower rate. In contrast, the time course of DNA strand breakage induced by etoposide (20 μM) was different. Etoposide-induced damage, which was somewhat greater than that caused by mafosfamide, appeared to be partially repaired in the first 2 hr after drug exposure.

Both mafosfamide and etoposide, at these concentrations, caused nucleotide depletion. Mafosfamide (50 μM) caused rapid initial NAD depletion (30% in 2 hr); between 2 and 6 hr there was no further depletion (in fact there was a small recovery) and finally, it stabilized at 24 hr with viable cells having lost 40% of their initial NAD content. The course of ATP depletion was similar except that, at 24 hr, ATP levels had recovered to 90% of normal. It is emphasized that these nucleotide measurements were assessed per viable cell: all these cells were lethally damaged and would die in the next 72 hr. Cells unable to exclude Trypan blue could still contain intact nucleotide although the amount would be small.

When CEM cells were exposed to 20 μM etoposide, the time courses of cellular viability and nucleotide content (Fig. 3A and B) initially shared some similarities with those produced by mafosfamide. However, at 24 hr, NAD levels recovered to 85% of normal, while ATP stabilized at 65% of control.

Higher, rapidly cytotoxic concentrations of mafosfamide (1 mM) and etoposide (0.4 mM) which caused rapid cell death (>90% in 24 hr) resulted in rapid, profound and irreversible nucleotide depletion (Figs 2C, D and 3C, D, respectively). These results suggest that, for mafosfamide- and etoposide-treated cells, nucleotide depletion is associated with cell death. However, the mechanisms of cell death at high drug concentrations may be different to those at lower drug concentrations.

The damage to DNA caused by a 1 hr exposure of 25 μM doxorubicin has a similar time course and pattern to that produced by mafosfamide but the degree of damage (maximum of 25% single strand breaks at 2 hr) is somewhat less (Fig. 5A). However, the relationship between DNA damage and changes in nucleotide content was quite different. Doxorubicin caused a transient, minor fall in NAD content (which rapidly recovered to >100% of control) while ATP content actually rose slightly. Although doxorubicin damages DNA, the damage it causes

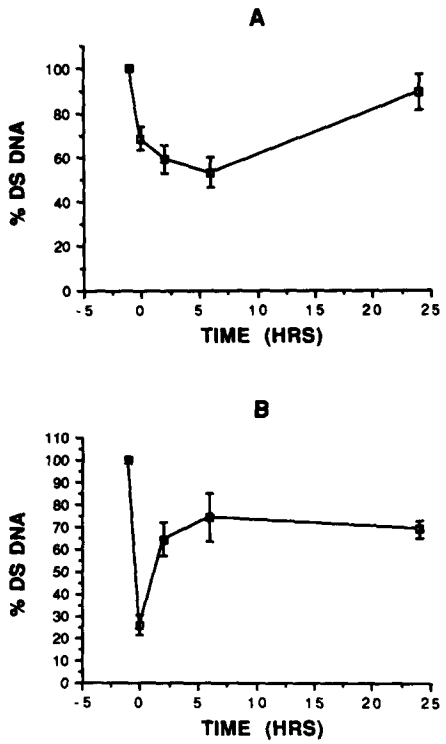

Fig. 4. DNA strand breakage was measured in CEM cells for the first 24 hr after a 1 hr exposure to a cytotoxic drug. % D is expressed as a percentage of control (untreated) cells. (A) Mafosfamide (50 μM): % D vs time after exposure (hr). (B) Etoposide (20 μM): as above. All points represent the mean of at least four observations. Error bars indicate standard error.

must be qualitatively different to mafosfamide-induced damage. The DNA strand breaks caused by doxorubicin do not produce marked nucleotide depletion. At this dose nucleotide depletion does not appear to be an important part of doxorubicin cytotoxicity.

Methotrexate (100 μM) caused a modest (25%) reduction in NAD content 2 hr after exposure but no decrease in ATP levels. This was associated with 30% DNA strand breaks immediately after exposure (0 hr) but no detectable DNA damage at 2 and 6 hr after exposure. DNA strand breaks were increased at 24 hr but this was not associated with nucleotide depletion. At this dose methotrexate is unlikely to kill cells by damaging DNA and depleting them of nucleotides.

Leakage of radiolabelled adenosine from cells

Two hours after a 1 hr exposure to 50 μM mafosfamide the culture medium contained 2.4% of the total radioactive label (Table 1). This was less than for control (unexposed) cells (3.3%) and showed that membrane damage and subsequent nucleotide leakage was not the cause of the 25% reduction in NAD and ATP levels present 2 hr after drug exposure. This was also true for 50 μM etoposide; 2 hr after drug exposure the culture medium contained 2.8% of the radioactive label (Table 1).

Measurement of PARS levels

Approximately 1×10^6 CEM cells were exposed to mafosfamide and etoposide for 15 or 60 min, and then [^{3}H]NAD uptake was measured and compared with control (unexposed) cells. After 15- and 60-min exposures, neither mafosfamide nor etoposide significantly increased the PARS activity of CEM cells compared to control (Table 2). However, after a 2 hr exposure to delayed lethal concentrations of mafosfamide and etoposide, the enzyme activity level was increased by 52 and 82%, respectively. This period of enzyme activation corresponds to the nadir in nucleotide levels caused by these two drugs. Control PARS activity was 4.71 ± 0.81 pmol/min/10^6 cells (SE, N = 3).

Effect of PARS inhibitors on cytotoxicity

Further cytotoxicity experiments were performed in the presence of PARS inhibitors. Because of the time course and extent of NAD and ATP depletion seen with both mafosfamide and etoposide, we postulated that stimulation of the enzyme PARS might be involved in the mediation of the cytotoxicity of the two drug's blocking this enzyme might alter their cytotoxic effect. 3-ABA was added to the CEM cells following one of three schedules: with the drugs, after a 1-hr exposure, or both during and after exposure. 3-ABA did not inhibit the cytotoxic effect of either drug at 24 or 48 hr. 2 mM 3-ABA was not cytotoxic to CEM cells but higher doses were (data not shown).

Nicotinamide, at a concentration range of 1 to 4 mM sufficient to inhibit PARS but which does not affect CEM cell growth, was added before and after drug exposure and had no effect on mafosfamide and etoposide cytotoxicity during the first 72 hr.

Evidence of apoptotic cell death

Although a number of cytotoxic agents kill cells by causing apoptosis, there have been few studies that have examined both morphological changes and patterns of DNA fragmentation in cultured cells following cytotoxic exposure. In parallel with light microscopic studies, cytotoxic drug-treated cells were harvested and their DNA extracted for electrophoresis.

Cells following drug treatment were cytologically classified into one of six categories. The most common classifications were normal, apoptotic or necrotic. A small number of cells were both apoptotic and necrotic, undergoing mitosis or else it was not possible to classify them (<4%). Apoptotic cells had crescentic margination of nuclear material at the periphery of the nucleus and although the cell size appeared reduced the plasma membrane was intact. Necrotic cells had ruptured membranes and it was not possible to identify normal cellular structures (Fig. 6). Untreated cultured (control) cells were always >90% viable by Trypan blue exclusion; of these control cells 1–7% were morphologically apoptotic.

Neither drug, at its delayed lethal concentration, caused increased apoptotic cell death 4 hr after

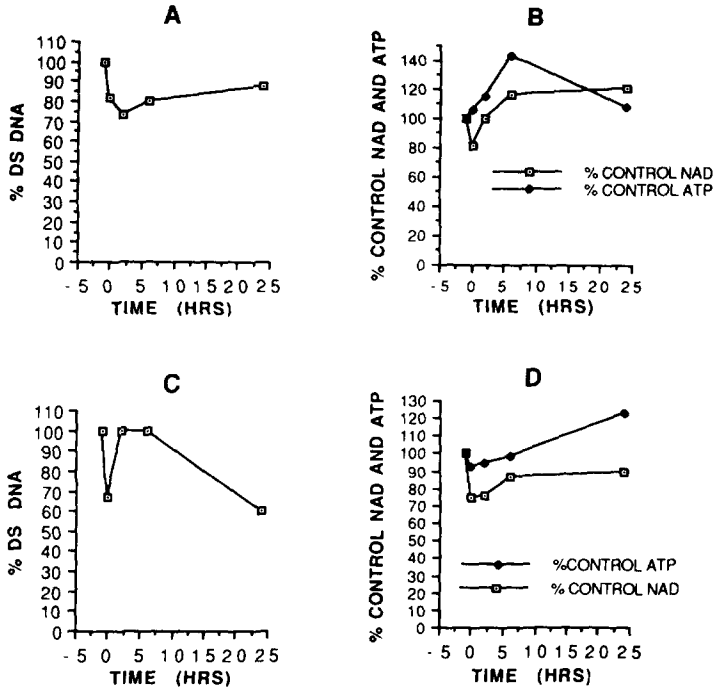

Fig. 5. DNA strand breakage and nucleotide content were measured for the first 24 hr after a 1 hr exposure to 25 μM doxorubicin (A,B) and 100 μM methotrexate (C,D). A,C: % D vs time after exposure (hr). B,D: NAD and ATP content (% control) vs time after exposure (hr). All points represent the mean of at least four observations.

Table 1. Effect of mafosfamide and etoposide on PARS activity

Duration of exposure (min)	Mafosfamide (% control PARS activity)	Etoposide
15	113	99
60	98	109
120	152	182

Log-phase CEM cells were exposed to 50 μM mafosfamide and 20 μM etoposide for 15–120 min then [^{3}H]NAD uptake was measured (see Materials and Methods) All values represent the mean of at least two experiments of triplicate samples. Control PARS activity was 4.71±0.81 pmol/min/10^6 cells.

exposure. There were significant increases in the percentages of apoptotic cells ($P < 0.001$, Table 2) 24 hr after exposure. At 25 μM mafosfamide, the majority of dead cells were apoptotic but higher doses appeared to favour necrotic cell death. At 100 μM only 10% of dead cells were apoptotic ($P < 0.001$). However, increasing the dose of etoposide did not significantly alter the number of dead cells with the features of apoptosis (39% vs 64%).

When CEM cells were exposed to pulse (1 hr) incubations with methotrexate (50–200 μM) and doxorubicin (12.5–50 μM) there were increased numbers of apoptotic cells at 24 hr. As the dose was increased significantly greater numbers of cells died by necrosis (data not shown).

Both mafosfamide and etoposide, at their delayed lethal concentrations, caused endonucleolytic DNA cleavage in CEM cells 48 hr after exposure (Fig. 7). The resultant DNA fragments which were multiples of 180 base pairs in length formed "nucleosomal ladders". This pattern of fragmentation was not apparent 24 hr after exposure.

DISCUSSION

The major finding in this study is that pulsed administration of the topoisomerase II inhibitor etoposide and the alkylating agent mafosfamide, at concentrations causing delayed cell death, cause similar patterns of DNA strand breaks, adenine nucleotide depletion and apoptotic cell death. In both cases, there was an associated increase in PARS enzyme activity after 2 hr drug exposure. Even though the addition of 3-ABA did not affect the cytotoxicity of etoposide, the stimulation of poly (ADP-ribosyl)ation and the subsequent nucleotide depletion may contribute to it. The mechanism of topoisomerase II inhibition and subsequent DNA damage induced by etoposide have been documented but the secondary effects of this DNA damage have not yet been studied. Even if initial DNA damage is repaired, the nucleotide depletion it causes may have serious consequences for the cell.

It has long been postulated that a major mechanism of alkylating agent-induced cytotoxicity is nucleotide

Table 2. Morphological changes in CEM cells 4 and 24 hr after exposure

		% Cells			
		4 hr		24 hr	
	Dose (μM)	Apoptosis	Necrosis	Apoptosis	Necrosis
Control		7	8	1	3
	25			16	9
Mafosfamide	50	10	1	8	29
	100			4	38
	10			3	5
Etoposide	20	7	10	10	12
	40			14	8
	12.5			8	4
Doxorubicin	25			10	8
	50			8	18
	25			8	4
Methotrexate	50			12	5
	100			10	15

After exposure, CEM cells were fixed and stained for light microscopy (Materials and Methods). Cells (200–500 per sample) were counted and classified. The exposure time for all drugs was 1 hr. There was no change in the proportion of apoptotic cells 4 hr after exposure. All drugs, at their delayed lethal doses, resulted in the percentage of apoptotic cells at 24 hr being significantly higher than control ($P < 0.001$).

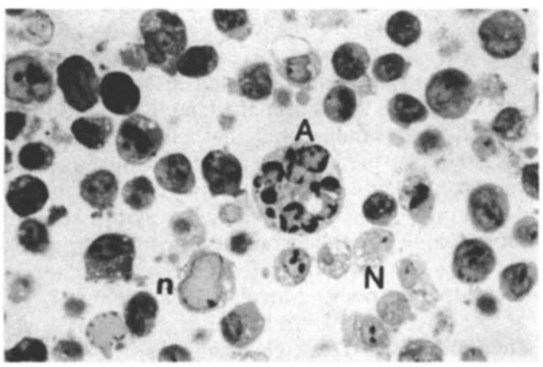

Fig. 6 Photograph of CEM cells 24 hr after a 1-hr exposure to 50 μM mafosfamide. Apoptotic (A), normal (n) and necrotic (N) cells are indicated (×600, stained with osmium tetroxide).

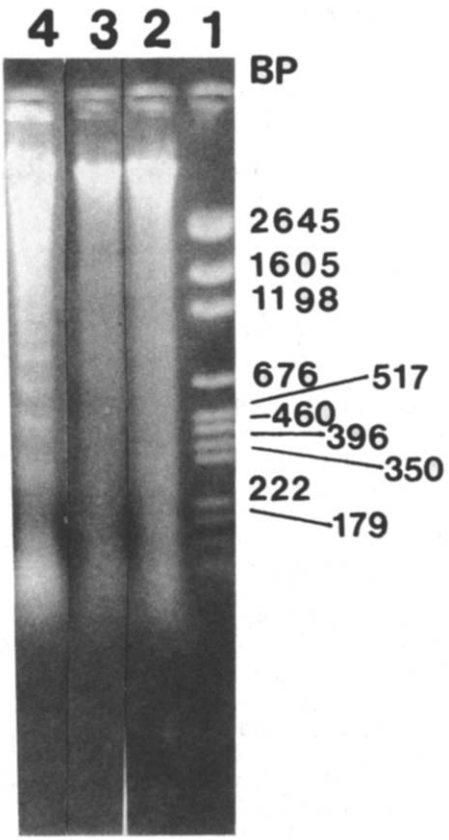

Fig. 7. DNA agarose gel electrophoresis. CEM cells were exposed to 50 μM mafosfamide for 1 hr and their DNA extracted 48 hr later. DNA (5–10 μg) was run on a 1.8% agarose gel at 75 V for 4 hr with a running buffer of Tris, boric acid and EDTA. Track 1, pGEM DNA markers; 2 and 3, control (untreated) CEM cell DNA; and 4, 48 hr after a 1-hr exposure to 50 μM mafosfamide. Marker fragments seen are (in order): 2654, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 and 126.

(NAD and ATP) depletion due to the consumption of NAD by stimulation of poly (ADP) ribosylation at sites of DNA strand breakage. Skidmore *et al.* [10] treated L1210 mouse leukemia cells with 1 hr pulses of *N*-methyl-*N*-nitrosourea and found that minimum NAD levels occurred reproducibly at 2 hr with full recovery at 8 hr. The final 30% of NAD was only lost at very high doses of alkylating agent. They correlated the time course of this NAD depletion and recovery with PARS activity. However, they did not measure DNA strand breakage or ATP levels, nor did they make it clear that these doses of cytotoxic drugs were lethal.

In contrast, Jacobson *et al.* [11] exposed 3T3 cells to a different alkylating agent, MNNG, and found that the total NAD pool could decrease by up to 50% in 1 hr but that, unlike in the present study,

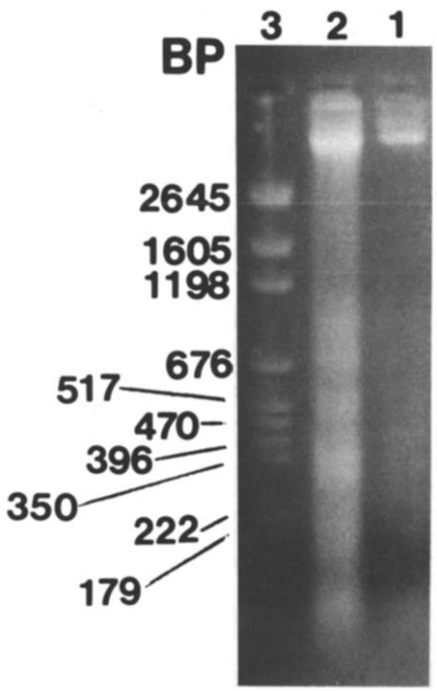

Fig. 8. DNA agarose gel electrophoresis. CEM cells were exposed to 25 μM etoposide for 1 hr and their DNA extracted 48 hr later. DNA (5–10 μg) was run on a 1.8% agarose gel at 75 V for 4 hr with a running buffer of Tris, boric acid and EDTA. Track 1, control (untreated) CEM cell DNA; 2, 48 hr after a 1-hr exposure to 25 μM etoposide; and 3, pGEM DNA markers. Marker fragments seen are (in order): 2645, 1605, 1198, 676, 517, 460, 396, 350, 222 and 179.

this had no effect on ATP levels. In our studies, mafosfamide and etoposide initially caused a parallel depletion of NAD and ATP but 6 hr after exposure the levels of the two nucleotides drifted apart. After 3 hr of MNNG exposure, although the cells were unable to form colonies they did regain normal NAD levels within 8 hr of removal of MNNG from the medium. It was not stated that this dose of MNNG was lethal but the inability of the cells to form colonies suggests this to be the case. Berger *et al.* [12] showed in glucocorticoid-susceptible lymphoid cells that following PARS activation by steroids NAD depletion occurred followed by ATP depletion and finally loss of cellular viability. In our experiments, NAD and ATP levels fell in parallel and major loss of viability occurred after 24 hr.

There was further evidence for the involvement of poly (ADP-ribosyl)ation in the cytotoxic effects of the two drugs. Increased PARS activity was not found following 15- and 60-min exposures to etoposide and mafosfamide but there was a markedly elevated enzyme activity after 2 hr of exposure. The temporal association of this increase with an initial nadir in the nucleotide levels and the proof that nucleotides were not leaking from cells suggests that poly (ADP-ribosyl)ation plays an important role in the cellular injury caused by these two drugs. Although doxorubicin and methotrexate caused some DNA damage within the first 24 hr, this did not appear to result in significant nucleotide depletion in CEM cells. In some cell systems PARS inhibitors such as 3-ABA and nicotinamide have potentiated anticancer drug cytotoxicity. However, this effect has been heterogeneous [13]. In this study, 3-ABA and nicotinamide did not protect CEM cells exposed to etoposide and mafosfamide.

Skidmore *et al.* [10] exposed mouse leukemia cells to γ radiation and found that the elevation in PARS activity lasted for less than 5 min, emphasizing the transient nature of the phenomenon in some cell systems. Similarly, Oleinick and Evans [14] found that, after γ irradiating V79-379 cells, the major peak in PARS activity lasted less than 10 min. Wielckens [15] measured levels of the polymer, poly (ADP) ribose, in Ehrlich tumor ascites cells exposed to triazaquonum and found that the half-life was less than 1 min. Stimulated PARS activity has been shown to be the cause of alkylator-induced nucleotide depletion in many cell systems [10, 13]; we confirmed this in CEM cells following mafosfamide exposure.

It is also noteworthy that, after delayed lethal doses of mafosfamide and etoposide, there was partial recovery of NAD and ATP levels between 2 and 6 hr. It has not been determined whether this partial recovery in nucleotide levels was due to increased NAD synthesis or reduced activity of poly (ADP-ribosyl)ation or NAD glycohydrolase with time after drug exposure. This pattern was different to the profound and rapid fall in NAD and ATP seen with high doses of mafosfamide and etoposide.

A further finding was that all cytotoxic drugs assessed, at their delayed lethal concentrations, caused a degree of apoptotic cell death. The percentage of non-viable cells with apoptotic morphology depended on the cytotoxic drug dose, with higher doses of drugs (except etoposide) tending to cause necrosis. Morphological changes were not evident until 24 hr after exposure while endonucleolytic DNA cleavage was not seen until 48 hr. The majority of the (primary) DNA damage and nucleotide depletion occurs much earlier than this; at the time when there is increased activity of PARS.

While it seems that many causes of DNA damage will subsequently result in endonucleolytic DNA degradation and apoptosis, the mechanism linking these events is not clear. In fact, in apoptosis, an active process, ATP levels are generally maintained, at least initially. However, the dependence of apoptosis on active protein and RNA synthesis has not been demonstrated in all apoptotic cell models. We have shown that these drugs cause two distinct modes of cell death. In some cells there may be massive nucleotide depletion leading to necrosis while other cells (with presumably preserved ATP levels) may undergo apoptosis. Measurement of total nucleotide content does not reveal what is occurring in the individual cell. Measurement of single cell nucleotide levels (and correlation of this with morphological changes) may help clarify this question. The other complicating factor is that apoptotic cells may subsequently become necrotic.

Although glucocorticoid-induced lymphocyte death is associated with both stimulation of poly

(ADP-ribosyl)ation and apoptotic DNA degradation there are few detailed time course studies that have examined the link between the two processes. Wielckens and Delfs [16] exposed S49.1 lymphoma cells to 0.1 μM dexamethasone. Apoptotic cell death was delayed for 24 hr but at this time there was already NAD depletion to 60% of control and elevation of PARS level to 80% above control. In addition, benzamide increased dexamethasone lymphocytotoxicity several-fold and cell death was earlier suggesting that poly (ADP-ribosyl)ation had a role in the repair of the DNA damage caused by apoptosis. Berger *et al.* [17], however, found that at a higher dose of dexamethasone (1 μM) the viability of S49.1 cells decreased to 50% at 24 hr, and that NAD and ATP fell in parallel.

There are a number of other potential interactions between apoptosis and poly (ADP-ribosyl)ation. When lymphoid cells are exposed to steroids (the best documented model of apoptosis), synthesis of a nuclear endonuclease is induced. This may fragment the cellular DNA resulting in activation of poly (ADP-ribosyl)ation and subsequent nucleotide depletion [17–19]. The *in vitro* attachment of poly (ADP-ribose) chains to endonucleases is known to inhibit their function [19]. Alternatively, nucleotide depletion following poly (ADP-ribosyl)ation may trigger a calcium flux activating an endonuclease responsible for the specific DNA degradation of apoptosis. Further studies, with specific inhibitors of both processes, are required to elucidate further the possible link between these two forms of programmed cell death. The fact that doxorubicin and methotrexate caused a degree of apoptosis apparently without stimulating poly (ADP-ribosyl)ation suggests that each of these two processes can operate independently of the other.

Thus, despite widely differing primary modes of action certain anti-cancer drugs may induce secondary physical and metabolic events that have much in common. DNA damage and subsequent poly (ADP-ribosyl)ation, nucleotide depletion and endonucleolytic DNA cleavage may represent such a unifying mechanism.

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