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DEXAMETHASONE AND ETOPOSIDE INDUCE APOPTOSIS IN RAT THYMOCYTES FROM DIFFERENT PHASES OF THE CELL CYCLE

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Abstract—Dexamethasone and etoposide both induce apoptosis in immature rat thymocytes. We investigated the dependence of apoptosis on the phase of the cell cycle after incubation with these drugs. Cell cycle progression was followed by a combination of pulse labelling with 5-bromo-2'-deoxyuridine (BrdU), labelling fixed cells with an anti-BrdU antibody and flow cytometry. Dexamethasone had little effect on the cell cycle progression of proliferating thymocytes, while etoposide caused cell cycle arrest. Normal and apoptotic thymocytes were separated by centrifugation on discontinuous Percoll gradients into four fractions (F1–F4). It was found that both dexamethasone and etoposide induced apoptosis in cells in G0/G1 and G2/M of the cell cycle, whereas only etoposide induced apoptosis of cells in S phase. These results demonstrated that dexamethasone induced apoptosis in quiescent cells while only etoposide could induce apoptosis in cells from the proliferative compartment. Following treatment of thymocytes with etoposide, some of the proliferating thymocytes (F1) were converted to cells with intermediate size and density (F3). We have recently identified these cells as a population of preapoptotic thymocytes, at an early stage of apoptosis. These cells then further progressed to fully apoptotic cells (F4). These data support the hypothesis that normal thymocytes (F1) became apoptotic (F4) via an intermediate population (F3).

Key words: quiescent; proliferating and preapoptotic cells; bromodeoxyuridine; Percoll

In the developing thymus, during the process of negative selection > 90% of immature thymocytes die by apoptosis [1]. Diverse stimuli, such as antibodies to the T-cell receptor [2], ionising radiation [3], glucocorticoids (e.g. dexamethasone) [4] and DNA topoisomerase II inhibitors (e.g. etoposide) [5, 6] induce apoptosis in thymocytes. Although the majority of thymocytes are quiescent [7], there is also a population of proliferating cells. We wished to investigate whether these populations showed a differential sensitivity to the induction of apoptosis by dexamethasone and etoposide.

Thymocytes separated on a discontinuous Percoll gradient [8] yield four fractions (F1 to F4) [8–10]. F1 contains proliferative cells; the majority of cells in F2 are quiescent immature T-lymphocytes; F3 contains preapoptotic cells, i.e. cells at an early stage of apoptosis. We have defined these cells as preapoptotic because they are smaller in size and of higher density than the normal cells in F1 and F2. They exhibit early ultrastructural alterations associated with apoptosis, i.e. condensation of chromatin abutting the nuclear membrane, but they do not yet exhibit internucleosomal cleavage of DNA. In contrast, cells in F4 contain fully apoptotic cells based on the characteristic ultrastructure of apoptotic thymocytes and also internucleosomal cleavage of DNA [9, 10].

Thymocytes were pulse labelled with BrdU† prior to drug treatment. At different times after pulse labelling, cells were fixed and BrdU incorporation and DNA content measured by flow cytometry to study the effect of the drugs on progression through the cell cycle. Cells were also fractionated on Percoll gradients and flow cytometry used to reveal the phase of the cell cycle from which the cells became apoptotic. The data demonstrated that dexamethasone and etoposide induced apoptosis in cells from different populations. Etoposide activated apoptosis in proliferating cells, whereas dexamethasone induced apoptosis only in quiescent cells.

MATERIALS AND METHODS

Materials. RPMI 1640 medium and FBS were obtained from Gibco Ltd (Paisley, U.K.). Etoposide, dexamethasone, Percoll, minimum essential medium (MEM), PI, BrdU and goat anti-rat fluorescein conjugate were from Sigma Chemical Co. (Poole, U.K.). Anti-BrdU antibody (supernatant, ICR2 available commercially from Sera Labs Ltd, Crawley Down, U.K.) was a gift from Dr C. J. Dean (Institute of Cancer Research, Sutton, U.K.).

Cells. Thymocytes were isolated as described by Raffray and Cohen [11] from male F344 rats, 65–

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† Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; FBS, foetal bovine serum; PI, propidium iodide; FITC, fluorescein isothiocyanate; CD, cluster designation; Tween 20, polyoxyethylenesorbitan monolaurate.

85 g body weight, 4–5 weeks old (MRC Toxicology Unit). All incubations of unfixed cells were carried out at 37° in RPMI 1640 plus 10% FBS, 5% CO_2, 95% air. Thymocytes (2×10^7 cells/mL) were pulse labelled with BrdU (10 μM) for 30 min and then washed twice (200 g for 10 min). The cells were resuspended at 2×10^7 cells/mL and incubated for up to 8 hr either alone (control), or with dexamethasone (0.1 μM) or etoposide (10 μM). In some experiments, cells were treated as described above and at 3 hr separated into four populations by centrifugation on a discontinuous Percoll gradient as previously described [9]. The density of the gradients was calibrated by density marker beads. The buoyant densities at the top, 60 to 70%, 70 to 80% and 80 to 100% interfaces (fractions F1–F4, respectively) were 1.063, 1.075, 1.099 and 1.119 g/mL, respectively. The number of cells in each fraction was determined using a Coulter Counter.

Flow cytometry. Cells were analysed on an Ortho Cytofluorograf 50H equipped with an Ortho 2150 computer system and a Coherent argon-ion laser producing 500 mW at 488 nm. Data were transferred to an IBM-PC computer and the results analysed and displayed using software written by one of the authors (MGO).

Cell cycle progression. Following treatment, cells (5×10^6) were fixed in ice-cold 70% ethanol and stained to label the incorporated BrdU and the DNA [12]. Briefly, cells were treated with 2 M HCl for 27 min at 37° to produce single-stranded DNA, washed and resuspended in 200 μL labelling buffer (PBS with 0.5% Tween-20 and 10% FBS). Rat anti-BrdU monoclonal antibody (10 μL) was added and the cells were incubated at 4° for 1 hr. The cells were washed at least twice and then incubated in labelling buffer (200 μL) with goat anti-rat IgG fluorescein conjugate (10 μL) at 4° for 1 hr. Finally, the cells were washed twice and resuspended in ice-cold PBS containing PI (20 μg/mL).

In the flow cytometer, fluorochromes were excited at 488 nm and red (PI–DNA— > 630 nm) and green (fluorescein–BrdU—520 nm) fluorescence recorded. After gating on a bivariate histogram (cytogram) of the peak vs integrated area of the red fluorescence signal to remove debris and clumped nuclei from the analysis [13], a cytogram of green vs red fluorescence was displayed. The labelling index was expressed as the percentage of thymocytes which had taken up BrdU, and was estimated from this cytogram.

RESULTS

Induction of apoptosis

Both dexamethasone (0.1 μM) and etoposide (10 μM) induced apoptosis in thymocytes as assessed by agarose gel electrophoresis and flow cytometry in agreement with previous studies [4–6] (data not shown).

Cell cycle progression in unfractionated cells

Freshly isolated cells were incubated with BrdU (10 μM) for 30 min, washed and then incubated in normal medium with or without dexamethasone (0.1 μM) or etoposide (10 μM). Cells were harvested at different times, fixed and labelled with anti-BrdU and PI for flow cytometry as described in Materials and Methods. Immediately after incubation with BrdU, labelled cells were distributed throughout S phase with a labelling index of approximately 20% (Fig. 1a). Controls in the absence of antibody to BrdU (Fig. 1b) or in the absence of BrdU (Fig. 1c) showed no non-specific labelling of the cells. After 3 hr, in control cells not treated with drugs, many of the labelled cells had progressed through to late S and G2/M phases (compare Figs 1a and 2a). After 8 hr, most of the labelled thymocytes had divided and were now in G1 (Fig. 2d).

The addition of dexamethasone, immediately after labelling the cells with BrdU, had little effect on cell cycle progression (Fig. 2b and e). In contrast, the presence of etoposide (10 μM) prevented the movement of the labelled cells through S phase (Fig. 2c and f).

In all the cytograms, cells were observed with a DNA content less than those in G0/G1 (arrowed in Fig. 2d–f). Such cells were most evident at later times and after drug treatment. These cells are most probably apoptotic as they are equivalent to the cells in the hypodiploid peak in other commonly used methods to assess apoptosis [14 and references therein].

Normal and apoptotic thymocytes fractionated on discontinuous Percoll gradients

Thymocytes were incubated for 3 hr either alone, with dexamethasone (0.1 μM) or with etoposide (10 μM), and then separated on discontinuous Percoll gradients. After etoposide treatment, the percentage of cells in F1 was significantly less than in control cells. In contrast, the percentage of cells in F2 was decreased following dexamethasone treatment but not following treatment with etoposide (Table 1). These data suggested that, whilst both treatments induced apoptosis, quiescent cells from F2 were more sensitive to dexamethasone whereas the proliferative cells from F1 were more sensitive to etoposide. The percentage of cells in F3 (preapoptotic) and F4 (apoptotic) following either dexamethasone or etoposide treatment was greater than in control cells (Table 1).

In further experiments, thymocytes were pulse labelled with BrdU and incubated for 3 hr without drug or with either dexamethasone or etoposide and then separated on Percoll gradients. The flow cytometric data from control cells showed that the cells actively synthesising DNA (in S phase) were in the lightest fraction, F1. The cells in this fraction had a labelling index of approximately 50% compared to an index of approximately 15% for those in F2 (Table 2 and Fig. 3a and b). There was an absence of unlabelled cells in G2/M, showing that cells initially in G2/M at the time of labelling 3 hr earlier had since divided and moved into G1. There was an accumulation of labelled cells in G2/M and some labelled cells had moved into G1 (Fig. 3a). The majority of the cells in F2 were unlabelled in G0/G1 of the cell cycle (Fig. 3b). Cells in F3 and F4 were predominantly unlabelled and had arisen from cells in the G1 and G2 phases (Fig. 3c and d, and

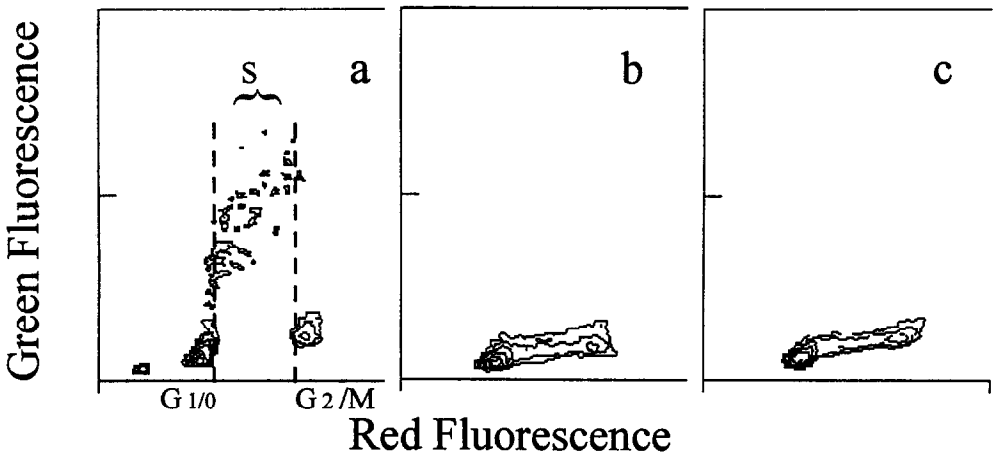

Fig. 1. Thymocytes were pulse-labelled with BrdU, incubated at 37°, fixed and incubated with rat anti-BrdU followed by FITC-anti-rat Ig; PI was added to label DNA. Cytograms of green (BrdU) vs red (PI) fluorescence were produced as described in Materials and Methods. a: Untreated cells at time 0. b: BrdU-labelled cells stained with the second antibody alone. c: cells not labelled with BrdU, but stained with both primary and secondary antibodies.

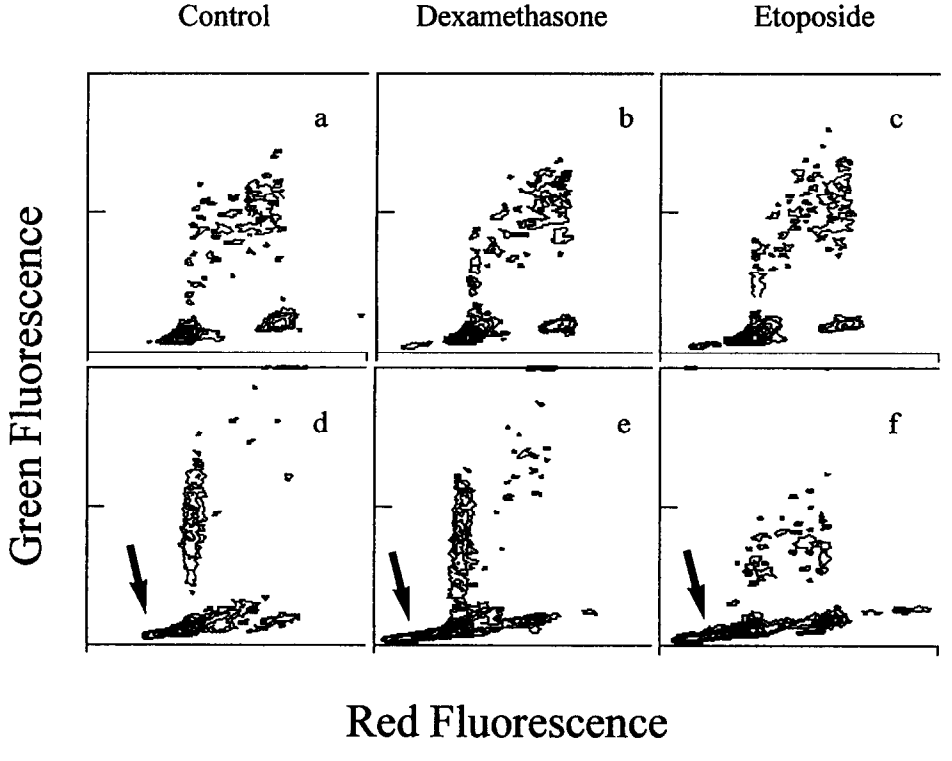

Fig. 2. Effects of dexamethasone and etoposide on cell cycle progression. Thymocytes were pulse labelled with BrdU for 30 min and then incubated for a further 3 hr (a–c) or 8 hr (d–f) either alone (a,d) or with dexamethasone (0.1 μM) (b,e) or etoposide (10 μM) (c,f). The arrows indicate cells with a DNA content less than those in G0/G1.

Table 2). These results suggested that it was quiescent cells which underwent spontaneous apoptosis.

Cells treated with dexamethasone (Fig. 3e–h) showed a pattern of labelling similar to that of control cells. The cells in F1 had continued to cycle (Fig. 3e) and the majority of cells in F2 were unlabelled in G0/G1 (Table 2 and Fig. 3f). The cells in F3 (preapoptotic) and F4 (apoptotic) had also

Table 1. Percentage of cells in fractions F1–F4 following 3 hr incubation and fractionation on Percoll gradients

Treatment	F1	F2	F3	F4
Control	25 ± 2	63 ± 2	6 ± 2	5 ± 1
Dexamethasone	19 ± 3	49 ± 3*	15 ± 3*	16 ± 1*
Etoposide	14 ± 2*	62 ± 2	13 ± 2*	11 ± 1*

Thymocytes were incubated either alone or with dexamethasone (0.1 μM) or etoposide (10 μM) for 3 hr and then fractionated on Percoll gradients as described in Materials and Methods. The number of cells in each fraction was found using a Coulter Counter and expressed as a percentage of the total number of cells from each treatment. The results are expressed as the mean ± SEM of six experiments. The results were compared to their corresponding controls using a paired *t*-test and determined to be statistically different if $P \leq 0.05$ (*).

arisen from G0/G1 and G2 (Fig. 3g and h, and Table 2).

Marked differences were observed following treatment with etoposide. The drug stopped the cells in F1 cycling: there was no movement of the labelled cells through the cycle and the unlabelled cells in G2/M remained in that phase of the cycle (Fig. 3i). Of particular interest was the observation that in cells from both F3 and F4, there were labelled cells in addition to cells from G1 and G2, demonstrating that apoptosis had occurred from all phases of the cell cycle (Fig. 3k and l, and Table 2). We have recently shown that following etoposide treatment, cells in F4 possess all the characteristic ultrastructural features of apoptosis, accompanied with internucleosomal cleavage of DNA [10].

Comparison of the intensity of green (BrdU) fluorescence of etoposide-treated cells in F1, F3 and F4 (Fig. 3i, k and l) revealed a progressive decrease through these fractions.

DISCUSSION

Our previous work has confirmed that both dexamethasone and etoposide induce a time-dependent increase in the number of apoptotic thymocytes under the conditions used in the present experiments [6, 9]. These two drugs have different modes of interaction with DNA, and recent experiments with p53 "knockout" mice suggested that the mechanism of induction of apoptosis might also be different [15, 16]. The data presented here demonstrated that, at least during the first few hr of incubation with drug, dexamethasone and etoposide affected different populations of cells.

Our experiments on untreated thymocytes showed that cells in the proliferative fraction continued to cycle over a period of 8 hr in culture (Fig. 2a and d). The cells that became apoptotic were predominantly from G0/G1 and G2/M of the cell cycle, not from S phase (Fig. 3a–d). From these data, we conclude that the quiescent, not the proliferating, cells undergo spontaneous apoptosis in culture. This behaviour reflects the fate of the quiescent immature $CD4^+/CD8^+$ cells *in vivo*, the majority of which are removed to ensure the development of a competent immune system [2, 17].

Incubation with dexamethasone had no effect on the progression of proliferative cells through the cycle (compare Fig. 2d with 2e). The pattern of apoptosis followed that of the untreated cells in that the apoptotic cells were predominantly from G0/G1 and G2/M of the cell cycle (Fig. 3e–h). The conclusion that apoptosis occurred from the quiescent population was consistent with the observation that the number of cells in fraction F2 from a Percoll gradient decreased over 3 hr, accompanied by an increase in fraction F4 (apoptotic cells) (Table 1). The physiological role of glucocorticoids in the death of thymocytes *in vivo* might explain why dexamethasone mimicked the pattern of spontaneous apoptosis seen in drug-free cultures.

Etoposide blocked the progress of the proliferating cells through the cell cycle (Figs 2c, 2f and 3i)—an observation consistent with other data from cell lines which showed a slow-down in S phase transit and a block in G2/M on incubation with etoposide and other topoisomerase inhibitors [18, 19]. Apoptosis was induced in the proliferating cells (note the reduction in fraction F1 and the increase in F4 after incubation with etoposide, Table 1) and from all phases of the cell cycle, including S phase (Fig. 3l). This result is consistent with the observation that other topoisomerase II inhibitors (teniposide, fostriecin and *m*-AMSA) caused apoptosis from the

Table 2. Labelling indices of thymocytes incubated for 3 hr and then fractionated on Percoll gradients

Treatment	Unfractionated	F1	F2	F3	F4
Control	25 ± 3	52 ± 7	15 ± 5	5 ± 1	4 ± 1
Dexamethasone	19 ± 1	53 ± 5	16 ± 5	8 ± 1	5 ± 1
Etoposide	20 ± 3	39 ± 4*	17 ± 7	29 ± 3*	25 ± 2*

Thymocytes were incubated either alone or with dexamethasone (0.1 μM) or etoposide (10 μM) for 3 hr and fractionated on Percoll gradients. The labelling index was expressed as the percentage of cells which had taken up BrdU and was determined in the different fractions as described in Materials and Methods. The results are expressed as the mean ± SEM of three experiments. The results were compared using a paired *t*-test and determined to be statistically different if $P \leq 0.05$ (*).

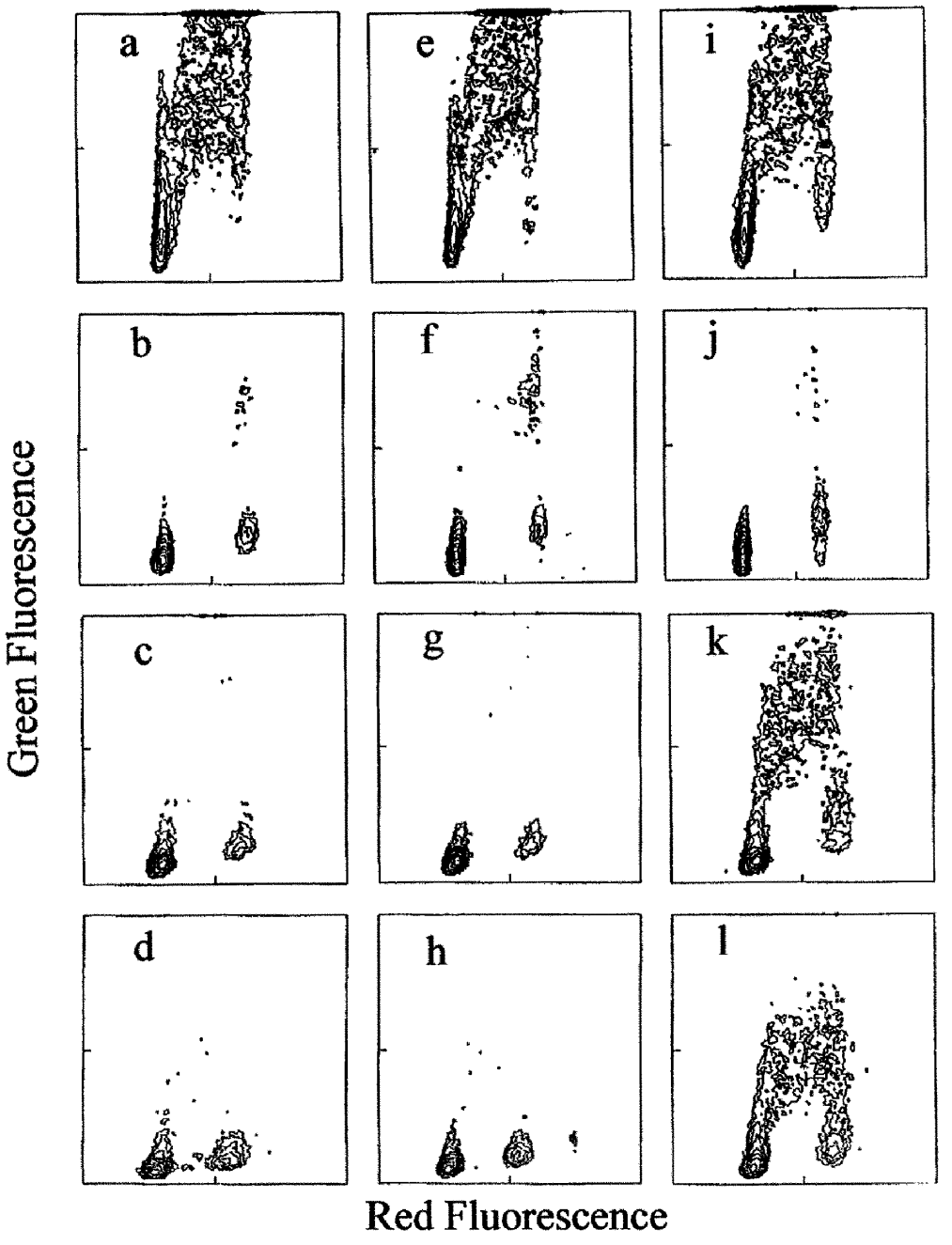

Fig. 3. The effect of dexamethasone and etoposide on the pattern of BrdU labelling in different subpopulations of thymocytes. Thymocytes were pulse labelled with BrdU as described in Materials and Methods and incubated for 3 hr, either alone (a–d), with dexamethasone (0.1 μM) (e–h) or with etoposide (10 μM) (i–l). The cells were then separated on Percoll gradients into four fractions of increasing density, F1 (a,e,i), F2 (b,f,j), F3 (c,g,k) and F4 (d,h,l) The four fractions were fixed and incubated with rat anti-BrdU followed by FITC-anti-rat Ig; PI added to label DNA. Cytograms are of green (BrdU) vs red (PI) fluorescence. In this figure, low numbers of labelled cells may not be revealed by the contour plot.

S phase in HL60 cells [20–22]. The sensitivity of proliferating cells to topoisomerase II inhibitors is not unexpected in view of the importance of the enzyme in DNA replication [23], chromatin condensation [24, 25] and chromosomal segregation [23]. The levels of topoisomerase II are also greater in proliferating than in quiescent cells [23].

Our data apparently contradict those of Bruno *et al.* [26] who reported that prednisolone (a glucocorticoid), camptothecin (a topoisomerase I

inhibitor) and teniposide all triggered apoptosis in rat thymocytes selectively in cells in G0 of the cycle. However, Bruno *et al.* [26] studied unfractionated thymocytes and used an acridine orange stain to detect a decrease in DNA content and a sensitivity to denaturation of the DNA in apoptotic cells. The fraction of proliferating cells in their system was smaller than that used in this study and the loss of cells from this compartment may have been more difficult to detect. Undoubtedly, etoposide also causes apoptosis in the quiescent cells (see discussion below) and it is also possible that teniposide is more effective in causing apoptosis in this compartment.

In our present experiments, there was no evidence that etoposide induced apoptosis in quiescent cells. However, at higher doses of drug and longer times of incubation, etoposide must induce apoptosis in this population since the percentage of apoptotic cells exceeds that found in the proliferating compartment at the start of incubation [6, 15]. Whether etoposide triggers apoptosis in quiescent cells by reacting with the low levels of topoisomerase II present or whether it acts on another target remains to be determined.

It has been found that p53 expression is not required for apoptotic death in untreated thymocytes in culture [15] or for the induction of apoptosis by glucocorticoids [15, 16]. In contrast, the absence of p53 prevents apoptosis in irradiated cells or cells incubated with etoposide [15, 16]. While the mechanism by which p53 participates in the induction of apoptosis is now known [27], it is known to have an important function in the regulation of the cell cycle [28, 29]. Our data showing that etoposide and dexamethasone affect different populations of thymocytes may explain, in part, the results observed with the p53 "knockout" mice.

We have recently shown that the cells in the F3 fraction from a Percoll density gradient represent a preapoptotic population of thymocytes, i.e. cells at an early stage of apoptosis [9]. These cells can be distinguished from the apoptotic cells in F4 by a number of criteria including morphology, size and density [9]. The present study showed that, after incubation with etoposide, a significant number of cells in both F3 and F4 was labelled with BrdU. Thus these cells had arisen directly from the proliferative cells in F1. In contrast, after incubation with dexamethasone, the cells in F3 and F4 were unlabelled. The similarity of the labelling patterns in F3 and F4 supports our identification of the F3 population as preapoptotic [9].

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