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Hydroxyurea-induced Cell Death as Related to Cell Cycle in Mouse and Human T-Lymphoma Cells[1]

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ABSTRACT

The association between DNA precursor synthesis, cell cycle perturbations, and cell death caused by the anticancer drug hydroxyurea was investigated in mouse and human T-lymphoma cells. Hydroxyurea inhibits the enzyme ribonucleotide reductase, leading to decreased deoxyribonucleoside triphosphate pools and an accumulation of cells in early *S*-phase of the cell cycle. We wished to clarify the mechanism of cell death caused by hydroxyurea in concentrations that can be obtained therapeutically.

At a 60-μM concentration of the drug, giving 25% growth inhibition during 24 h, no increase in the number of dead cells was observed as determined by cell flow calculations and density gradient centrifugation. However, the removal of hydroxyurea led to 10–30% cell loss during the following 12-h period. In parallel, there was an increase in DNA precursor levels and a rapid progression of cells through *S*- and G_2 phases of the cell cycle. The isolated dead cells showed no overrepresentation of any cell cycle phase.

The results demonstrate that, although the toxic effects of low concentrations of hydroxyurea are minimal, the drug-induced unbalanced growth state can cause substantial cell death during a posttreatment period.

INTRODUCTION

There have been many studies characterizing biochemical effects of the DNA synthesis inhibitor HU[3] (1). It is generally agreed that its mechanism of action is mediated via inhibition of ribonucleotide reductase, producing a depletion of the necessary DNA precursors (2–5). HU has also been used successfully as an anticancer drug (1). The question of how the inhibition of DNA synthesis, caused by a lack of deoxyribonucleoside triphosphates, results in cell death is, however, essentially unanswered, although some studies have tried to address this issue (6–9). These studies were performed with high concentrations of HU for short duration and relatively indirect methods to determine DNA synthesis and cell killing.

Here we report the effects of low concentrations of HU on mouse S49 T-lymphoma and on human CEM T-lymphoblastoid cells with regard to cell cycle perturbations and cell loss as determined by cell flow calculations and density gradient centrifugation procedures (12). These two cell lines have been used as model systems for T-lymphocyte functions and their DNA precursor metabolism has been extensively characterized (13). The effects of low concentrations of HU on S49 cell growth and deoxyribonucleoside triphosphate levels have recently been described (14). Here, we extend these studies and compare the cell-biological effects of HU on S49 cells and human CEM cells with the aim of explaining the mechanisms of cell loss associated with drug treatment.

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[3] The abbreviations used are: HU, hydroxyurea; EB, ethidium bromide.

MATERIALS AND METHODS

Cell Culture. Mouse S49 wild type cells and CEM cells were grown in suspension culture in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated horse serum. The general properties and details of cell culture conditions have been described earlier (10, 11, 13, 14). The number of cells was determined microscopically in a Bürker counting chamber. The proportion of dead cells was calculated using dye exclusion with trypan blue. Hydroxyurea (Sigma) was dissolved in distilled water and used fresh.

Reversal of the HU-induced growth inhibition was obtained by centrifugation of the cells at room temperature, washing the cells once with complete medium without HU, and then resuspending the cells in the original volume of complete medium without HU. The control cells were treated according to the same washing procedure.

Density Separation. The density separation procedure used in this study has been described in detail previously (12). At the time indicated the cell suspension containing $20–40 \times 10^6$ cells was centrifuged at $250 \times g$ for 5 min. The cell pellet was resuspended in 0.250 ml 0.9% NaCl and 0.1 ml of this cell suspension was mixed with a Percoll (Pharmacia, Uppsala, Sweden) gradient (one part Percoll + one part 0.9% NaCl used for the S49 cells and four parts Percoll + six parts 0.9% NaCl used for the CEM cells; methocel was added to a final concentration of 0.2%) and centrifuged in a Beckman JA-20 rotor head at 15.000 rpm for 15 min at 4°C. The shape of the gradient was determined by running density marker beads (Pharmacia) in parallel. The gradient was harvested in 20 fractions of 0.5 ml. The recovery varied between 85 and 95%. The shape of the gradient was nonlinear (Fig. 1). S49 and CEM cells were almost homogeneous in respect to the cellular density with a mean density of 1.070 and 1.050 g/ml, respectively. These cells correspond to living cells as determined by dye exclusion test. The dead cells were found in the first two fractions (Fig. 1).

Cell Cycle Composition. The proportion of G_1, *S*, and G_2+M cells were determined by quantitative cellular DNA measurements using flow cytometry as described (16). Before fixation in ice-cold ethanol of density gradient separated cells, cells were diluted 1:3 with Tris-NaCl-EDTA-buffer (Tris, 0.1 M; EDTA, 0.005 M; pH 7.5) and washed one time. Fixed cells were further washed with Tris-buffer before treatment with RNase (1.0 mg/ml) and 0.5% pepsin solution (1000 E/g, pH 2.0) in order to obtain nuclei. Nuclei were stained with EB (2.5×10^{-5} M) and the DNA content of these nuclei was determined by a flow cytometer [ICP 11; Phywe, West Germany (now Ortho Instruments, Westwood, MA)]. The percentages of G_1, *S*-phase, and G_2+M cells were calculated from the areas of the DNA histograms assuming a Gaussian distribution of G_1 and G_2+M maxima and attributing the remaining part to the *S*-phase.

Calculation of Cell Cycle Flow and Loss. The cell cycle flow was calculated based on the total number of cells and the cell cycle composition as described previously (15) assuming a growth fraction of 1.0. Furthermore, at unbalanced cell flow, a minimum value of cell loss can be accurately estimated (15).

Determination of Deoxyribonucleoside Triphosphate Levels. Cells were harvested at various times after removal of HU by centrifugation at ambient temperature and washed with chilled, isotonic phosphate-buffered saline. Nucleotides were extracted with 0.6 M trichloroacetic acid and neutralized with tri-*n*-octylamine:freon as described (17). Deoxyribonucleoside triphosphates were determined by high-performance liquid chromatography after prior oxidation of ribonucleotides (18, 19).

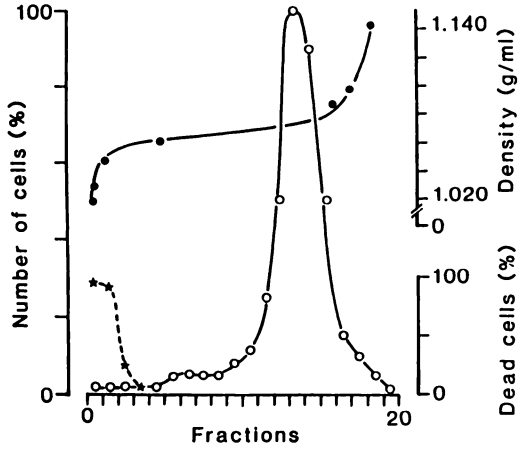

Fig. 1. Cellular density of untreated S49 cells. The first two fractions contain the dead cells while fractions 10 to 20 contain the living cells. The fraction containing the highest number of cells was labeled as 100%. The results are a representative example of five experiments. O, number of cells in each fraction; *, dead cells; ●, shape of the gradient.

RESULTS

Cell Growth. Cell growth of the untreated S49 and CEM cells was exponential up to 60 h (Fig. 2) with a doubling time of 15 and 25 h, respectively, under the culture conditions used. In the presence of a 60-μM concentration of HU the growth rate of these cells was diminished by about 25% (Fig. 2).

When the medium of both the control and the HU-treated cells was replaced after 24 h, by fresh medium without HU, the increase in the number of cells stopped or even decreased slightly for at least 10 to 12 h for both control and HU-treated cells (Fig. 2). Twelve h later the number of cells was approximately doubled.

Cell Cycle Distribution and Cell Flow. The presence of HU for 24 h decreased the proportion of G_1 cells 10–15% with a corresponding increase of *S*-phase cells, both in the S49 cell and CEM cell culture (Fig. 2). The absence of increase in cell number after removal of HU can be due either to a complete inhibition of cell division or to cell death connected with cell growth, where the number of cells produced equal the number of cells lost. In order to distinguish between these possibilities we determined the cell cycle distribution at various times after removal of HU. The results are shown in Fig. 2. When HU was removed, the proportion of *S*-cells rapidly decreased, with a corresponding increase in the proportion of G_2, M, and G_1 cells as a result of continuous cell flow through the *S*- and G_2+M phase (Table 1). Thus, since the proportion of G_1 cells increased while the total cell number was unchanged, cell division had occurred in connection with cell death. At 24 h after removal of HU the cell cycle distribution was more or less normalized. This dramatic change in cell cycle distribution of the HU-treated cells was not found in the untreated cells. The stop in cell growth of the untreated cells after the washing procedure was due to a complete inhibition of cell flow through all cell cycle stages (Table 1).

Cell Death. The magnitude of cell death was determined by two different methods. The first one was based on the cell flow calculation method described recently (15) and in the second one we used a density gradient, exploiting the fact that dying or dead cells decreased their cellular density (see "Materials and Methods"). Based on cell flow calculation there was a total cell loss of about 10 and 30% of the number of S49 and CEM cells, respectively, up to 10 h after removal of HU (Fig. 3). The number of dead cells obtained from the density gradient separation increased from 1 to about 7% for the S49 cells 6 h after removal of the drug. For CEM cells elevated amounts of dead cells of both control and HU-treated cells were found immediately after the washing procedure (Fig. 3). However, the number of dead cells declined and reached normal control values 10 h later. Twenty-four h after removal of HU there was still an elevated amount of dead S49 cells of about 3% as compared to control.

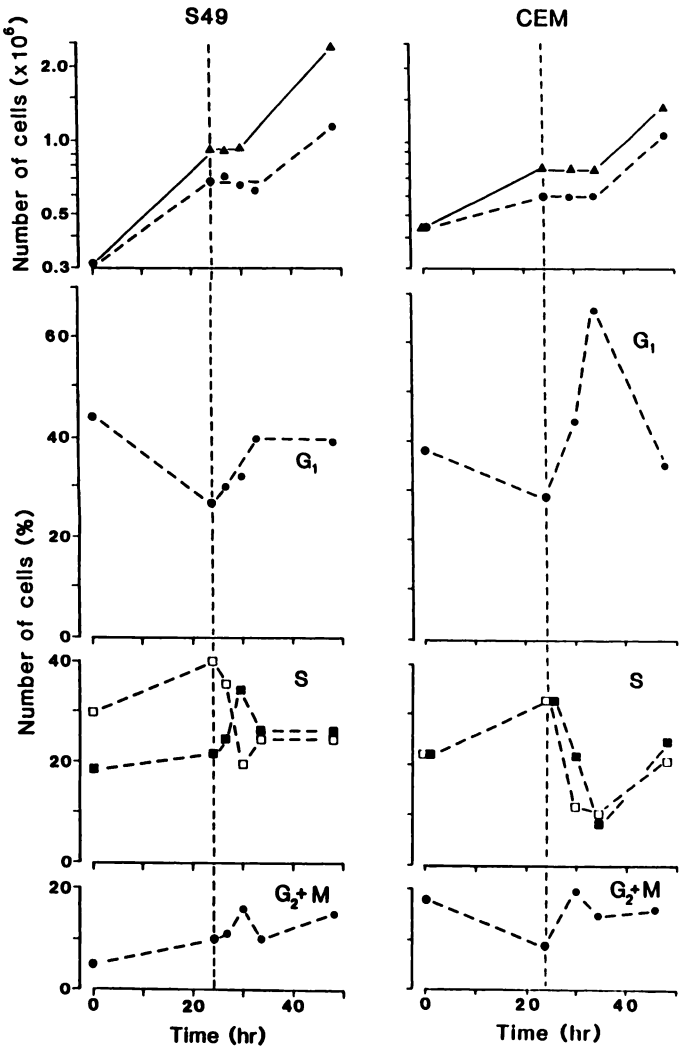

Fig. 2. Total number of cells and percentages of G_1, *S*, and G_2+M cells of untreated and HU-treated cells. At time 0 HU was added to one culture (●) and one culture was untreated (▲). *Dotted line* at 24 h, removal of the drug. The *S*-phase was subdivided into two parts denoted as early (□) and late (■). The results shown are from a typical experiment (n = 3).

Table 1 *Mean cell outflow from G_1, S-phase, and G_2 + M of untreated and HU treated CEM cells during the following time periods: 0–24, 24–34, and 34–48 h.*

At 24 h the cells were centrifuged and resuspended in fresh drug free medium. For further details, compare the growth curves in Fig. 2.

	Cell outflow rate (%/h)					
	Control			60 μM HU		
Phase	0–24 h	24–34 h	34–48 h	0–24 h	24–34 h	34–48 h
$G_1 \rightarrow S$	13	2	15	6	0	15
$S \rightarrow G_2$ + M	14	0	25	9	14	35
G_2 + M $\rightarrow S$	25	0	53	42	10	12

Cell Cycle-related Cell Death. The cell cycle composition of the dead and living cells 3 to 6 h after removal of HU was determined and the results are shown in Table 2. There were no significant differences, as determined by Student's *t* test,

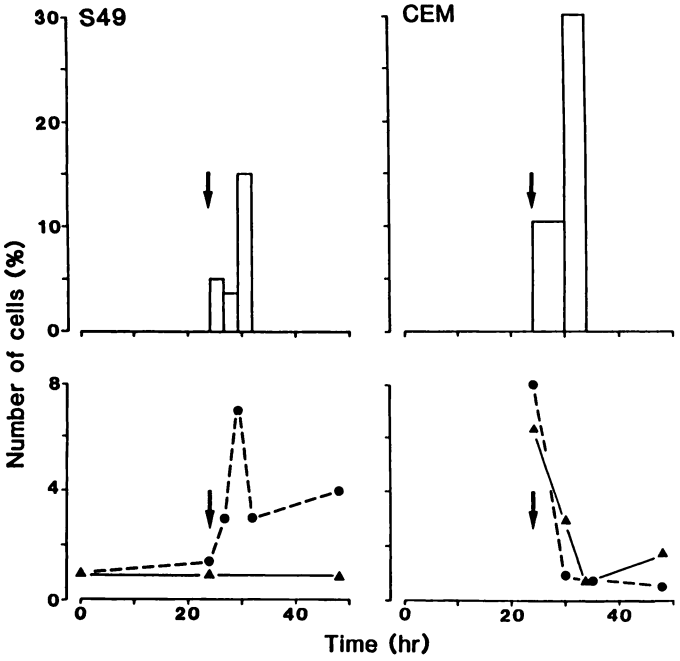

Fig. 3. The number of dead cells after removal of HU obtained by cell flow calculation (*top*) and density gradient centrifugation (*bottom*). The number of cells were expressed as a percentage of the number of cells at the time of removal of the drug, at time (*arrows*). (▲) untreated control, (●) HU-treated cells. The results shown are from a typical experiment ($n = 3$).

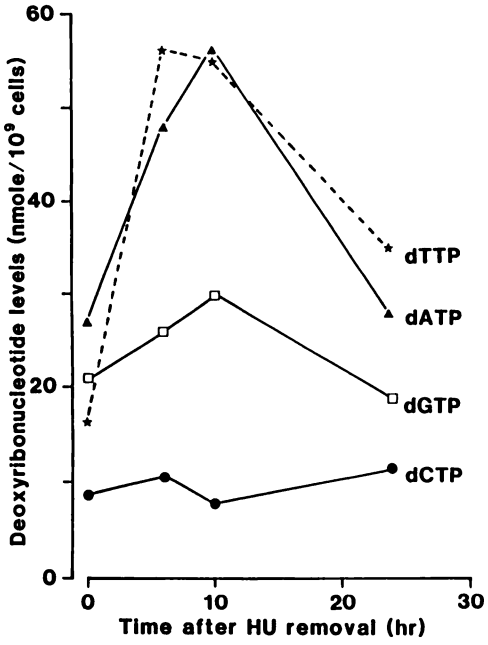

Fig. 4. Deoxyribonucleoside triphosphate levels in CEM cells after treatment with HU. The cells were treated for 24 h with 60 μM HU before the drug was removed and the cells were incubated for various times before harvested and deoxyribonucleotide levels determined as described in "Materials and Methods."

Table 2 *Cell cycle composition of dead and living untreated control cells and of cells 10 h after removal of HU*

Mean value ± SE of three experiments.

Cells	Percentage		
	G_1	*S*	G_2 + M
Control			
S49 cells			
Dead	45 ± 9	43 ± 5	12 ± 7
Living	39 ± 10	51 ± 7	10 ± 4
CEM cells			
Dead	53 ± 3	41 ± 3	6 ± 1
Living	54 ± 1	36 ± 3	10 ± 2
HU-Treated			
S49 cells			
Dead	53 ± 3	36 ± 3	11 ± 1
Living	39 ± 2	51 ± 6	10 ± 1
CEM cells			
Dead	60 ± 6	36 ± 5	11 ± 3
Living	50 ± 5	38 ± 3	16 ± 2

between dead and living cells either in the untreated cells (S49, $P = 0.4$; CEM, $P = 0.6$) or in the HU-treated S49 ($P = 0.2$) or CEM ($P = 0.6$) cells.

Deoxyribonucleoside Triphosphate Levels. The effects of low concentrations of HU on the levels of the four deoxyribonucleoside triphosphates in S49 cells have been reported earlier (14). We find here similar relative changes in the DNA precursor pools in CEM cells, *i.e.*, a 2- to 3-fold decrease in dATP and dGTP pools after treatment with 60 μM HU for 24 h. In CEM cells there was also a marked decrease in the dTTP pool to about 30% of what is found in exponentially growing untreated cells (data not shown).

In Fig. 4 the deoxyribonucleoside triphosphate pool changes in CEM cells after removal of HU is shown. As expected there was an increase in the dATP, dTTP, and dGTP levels, approximately 2- to 3-fold, 6 to 10 h after removal of HU. No significant change in the dCTP pool was observed. Thus, the levels found after 10 h were very similar to those found in exponentially growing untreated cells (19) and the lower pools observed 24 h after removal of HU were most likely due to the high cell density in the culture.

DISCUSSION

Treatment of S49 cells with low concentrations of HU leads to a reduction of the intracellular purine deoxyribonucleoside triphosphate pools (14). This is most likely the only effect of HU at this concentration of biological importance. The decreased level of DNA precursors is correlated with an increase in the duration of early *S*-phase of the cell cycle, but due to a concomitant shortening of the G_2 phase, the overall growth rate is only somewhat decreased (14).

Although the absolute levels of the four deoxyribonucleoside triphosphates are different in CEM cells, the changes produced by 60-μM concentrations of HU were similar to those described for S49 cells. However, there was a more pronounced decrease in the dTTP pool, and this could be due to less active salvage of deoxyuridine from the medium. This latter mechanism has been shown to be responsible for maintaining the dTTP pool in HU-treated cells (20). We find here with direct methods that addition of 60 μM HU leads to very little cell loss when S49 or CEM cells were grown in the presence of the drug. Earlier studies, using 50- to 100-fold higher HU concentrations have come to similar conclusions, although in this case the cytotoxic effects were measured with a cloning efficiency test (7, 9). At these high concentrations HU inhibition leads to accumulation of short DNA fragments and adjacent single-stranded regions in the vicinity of replication origins in the *S*-phase cells. The presumed cytotoxic mechanism then involves incomplete repair of the DNA lesions and subsequent chromosome fragmentation (1, 9). However, the conclusion of several of these studies has been that there is no direct correlation between the concentration-dependent induction of HU cell death and the abnormal DNA synthesis observed (1, 9). Therefore this theory of *S*-phase-specific cell death caused by HU may only be the cyto-

toxic mechanism in a minority of the cells under special conditions.

The unexpected finding in this study was that 6 to 10 h after the removal of low noncytotoxic levels of HU from S49 or CEM cells, a substantial cell loss occurred. The amount of cell loss was determined by two different methods, which measure different phases in the "death processes." From cell flow calculations, cells already broken down were determined, while using density gradient centrifugation, dead cells still present in the cell population were studied. The cell flow calculation method has some limitations; *i.e.*, cell loss can only be calculated in cell populations showing perturbated growth. In spite of this limitation, we think that this method, particularly in combination with other methods, is appropriate for studying cell loss quantitatively. In cell flow calculations (for details see Ref. 15) we assumed that the cell outflow from G_1 was zero during the first 10 h after removal of HU. If, however, there is an outflow, the amount of dead cells will increase. Thus, the magnitude of cell death obtained from both these methods is likely to be minimum. In summary, we observe a cell death during the first 10 h after removal of HU amounting to 15–20% for S49 cells and approximately 30% for CEM cells.

The dead cells isolated by centrifugation showed no overrepresentation of any of the cell cycle phases. This implies that the marked changes in cell cycle distribution up to 10 h after removal of HU was not due to loss of cells from a certain part of the cell cycle. However, since the number of isolated dead CEM cells was much lower than that calculated, it is possible that a cell cycle phase-dependent loss of cells occurred, but that these cells disintegrated rapidly. In the case of the S49 cells good correlation between the two methods was found. The elevated number of dead CEM cells found just after removal of drug must be due to the washing procedure, since a corresponding increase also was observed in the control culture. Thus, we do find differences between the two cell lines in sensitivity to HU and to the manipulation employed, which emphasizes the danger of extrapolating from observations made with one type of cell.

Deoxyribonucleoside triphosphate levels increased after removal of HU approximately to the levels found in unperturbed CEM cells. Due to the fact that DNA precursor pools change during the cell cycle, it is difficult to make accurate comparisons with untreated cells, but the availability of higher deoxyribonucleotide levels most likely explains the rapid progress of cells through *S*-phase after removal of HU. The total length of *S*-phase in both types of cells in this situation corresponds to a shortening of *S*-phase by approximately 60% as compared with untreated washed cells and by about 30% when compared with HU-treated cells before removal of the drug. Our current hypothesis for the mechanism of cell loss observed after HU treatment is that this rapid progression through the cell cycle leads to various mistakes in macromolecular synthesis and assembly, which immediately or at different times during the next cell cycle is manifested as cell death. Further experiments are required to substantiate this hypothesis but it is likely that this mechanism is of importance in patients treated with HU as an anticancer drug. The half-life of HU is short in the body and therefore the induction of this type of unbalanced growth state in various cells seems very possible in the therapeutic situation.

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