Effect of Adriamycin on the Cell Cycle Traverse and Kinetics of Cultured Human Lymphoblasts

Awtar Krishan and Emil Frei, III

Laboratory of Cytokinetics, Sidney Farber Cancer Center and Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

Exposure of cultured human lymphoblasts to adriamycin (ADM) (0.1 μg/ml for 24 hr or 0.5 μg/ml for 1 hr) leads to an accumulation of cells with the DNA content of late S and G2. Higher concentrations of ADM (0.5 to 10 μg/ml) inhibit cell cycle traverse. Effect of ADM on cell cycle traverse, cell growth, and incorporation of labeled precursors into DNA is dependent on drug concentration and length of exposure to ADM. Synchronized cells in G1 or G2 part of the cell cycle are less sensitive to ADM than cells in S phase. Similarly, plateau-phase cells are less sensitive to ADM than cells from log-phase cultures.

INTRODUCTION

ADM, a relatively new antitumor antibiotic of the anthracycline group (4), inhibits tumor growth in a variety of experimental animal systems (17) and in a number of categories of human neoplasia (13, 15, 16). In tissue culture cells, ADM has been shown to enter cells rapidly and bind preferentially to chromatin (18). In L1210 and HeLa cells, ADM inhibited incorporation of labeled precursors into nucleic acids (8, 14). A number of studies have shown the cell cycle-dependent lethal action of ADM. In synchronized HeLa cells, the DNA-synthetic part of the cell cycle has been shown to be the most sensitive to the lethal action of ADM (8). In CHO cells, ADM has no effect on initiation of genome replication (21), although cells in S and mitosis are most sensitive to the lethal effects of the drug (1). Similarly, in cultured human lymphoma cells exposed to ADM, cells at the S-G2 boundary and in G2 are 10-fold more sensitive than cells in late G1-S (5). An excellent review of the literature on ADM has been recently published (19).

The recent introduction and availability of flow cytometry (24) for rapid analysis of DNA/cell content (3) makes it relatively easy to follow the cell cycle traverse of large cell populations exposed to various drug concentrations (9, 11, 22, 23, 25). In this study, we have used this methodology in combination with [3H]TdR incorporation studies and viable cell count data to assess and compare the effect of various concentrations of ADM on the cell cycle traverse of asynchronous (log and plateau) and synchronized populations of human leukemic lymphoblasts of the CCRF-CEM cell line.

MATERIALS AND METHODS

Cell Cultures. Log-phase suspension cultures (1 to 2 × 10^6 cell/ml) of CCRF-CEM human lymphoblasts were grown in Eagle’s minimal essential medium (for spinner cultures), supplemented with 10% fetal calf serum, penicillin, and streptomycin. Plateau-phase cultures were obtained by growth of cultures to a maximum cell density (approximately 3.5 × 10^6 cells/ml). Cultures could be held at this cell density for approximately 48 hr after which a rapid decline in the number of viable cells was seen. Approximately 30% of cells in plateau-phase cultures (24 hr after reaching 3.5 × 10^6/ml density) were labeled after pulse labeling with [3H]TdR.

Synchronization. Cell cycle synchronization of CCRF-CEM cells was achieved by exposure to 1.0 mM IMPY for 18 hr (2, 6) followed by release for 10 hr and a 2nd exposure to 1.0 mM IMPY for 18 hr. We have described (11) the synchronized cell cycle traverse of CCRF-CEM cells after release from the IMPY-induced block. ADM hydrochloride (NSC 123127) was obtained from the Division of Cancer Treatment, National Cancer Institute. Dilutions were made in glass-distilled water immediately before addition to the cultures.

[3H]TdR Incorporation. Three to 5 ml of cells were incubated with [3H]TdR (1 μCi/ml; specific activity, 54 Ci/m mole) for 30 min. Isotope uptake was terminated by addition of cold 0.9% NaCl solution. Triplicate samples were washed twice with cold 0.9% NaCl solution and centrifuged, and the cell pellets were precipitated in cold 10% trichloroacetic acid for 30 min. Precipitates were collected on Millipore filters and washed twice with 5% trichloroacetic acid. Dry filters were placed in scintillation vials containing 10 ml scintillation fluid [4 g Omnifluor (New England Nuclear, Boston, Mass.) in 1 liter toluene]. Vials were counted in Beckman Model LS-335 liquid scintillation system.

Cells incubated with [3H]TdR (similar to those used for scintillation counting) were centrifuged, washed, and resuspended in fetal calf serum. Smears were fixed in acetic:alcohol (1:3). Rehydrated slides were coated with Kodak NTB-3 emulsion and exposed at 10° for 24 to 72 hr. Slides were developed in D-19 for 2.5 min and stained in carbol fuchsin.

Flow Cytometric Analysis. For analysis of DNA con-
tent/cell, small samples (1 ml) were removed under sterile conditions and centrifuged at 150 × g for 5 min; then cell buttons were resuspended in 1 to 5 ml of propidium iodide (0.05 mg/ml of 0.1% sodium citrate). Details of this staining method have been published elsewhere (9). Stained cells were analyzed in a Cytofluorograph Model 4801 (Bio/Physics Systems, Inc., Mahopac, N.Y.). In this instrument, a single-cell suspension of the sample is exposed in a flow chamber to a focused argon ion laser beam (488 nm). Fluorescence resulting from the laser excitation of the propidium iodide-DNA complex is collected and quantitated through a combination of appropriate mirrors, filters, and photomultipliers. The resulting electrical pulses (proportional to the DNA-providium iodide fluorescence in each cell) are collected and stored in a pulse-height distribution analyzer. Histograms generated from these data are photographed from the cathode-ray tube of storage oscilloscope. The abscissa of the histogram (generated by the pulse-height analyzer) is divided into 100 channels of increasing linear value from 0 to 100. The height of the bars indicates the number of cells recorded in each channel. Data on the number of cells recorded per channel were also simultaneously printed on paper tape.

DNA/cell frequency distribution of some representative and selected populations of CCRF-CEM cells in various phases of the cell cycle have been reported and discussed in recent papers (9–11). To facilitate comparisons of various populations, we have arbitrarily divided channels on the abscissa of the pulse-height analyzer-generated histograms into the following 5 major groups: Channels 0 to 19, cells with DNA content of less than G1 cells, normally pyknotic and dead cells; Channels 20 to 35, cells with DNA content per cell of G1 (and possibly some early S); Channels 36 to 50, cells in the process of replicating their genome (S) and with DNA content intermediate between that of G1 and G2-M; Channels 51 to 70, content of late S, G2, and M; Channels 71 to 99, cells with greater than G2 DNA content. In the latter channels (71 to 99), cell clumps of 2 or more cells are also recorded. Numbers recorded in circles on the histograms represent the percentage of the total population recorded between the above mentioned channel groups of 20 to 35 (mainly G1 DNA content); 36 to 50 (S DNA content); and 51 to 70 (late S, G2, and DNA content).

RESULTS

Log-phase CCRF-CEM lymphoblasts used in these experiments have a doubling time of 24 hr. After incubation with [3H]TdR for 24 hr, 90 to 95% of the population is labeled and the approximate cell cycle parameters (as determined by percentage of labeled mitosis curve) are: G1, 2 hr; S, 13 hr; and G2, 4 hr. Chart 1A shows DNA distribution of a log-phase culture; 56, 21, and 23% of this population has the DNA content of Channels 20 to 35 (G1-early S), 36 to 50 (S), and 51 to 70 (late S-G2-M), respectively. Pulse labeling with [3H]TdR for 30 min shows an approximate LI of 50%.

Exposure of Log-Phase Asynchronous Cultures to ADM

0.01 μg/ml. Cell counts in log-phase cultures incubated with ADM (0.01 μg/ml) for 24 hr were 80% of the control.

However, no difference was seen in the subsequent growth (up to 72 hr) of the treated and control cultures. Except for a small increase (~10%) in the number of cells with the DNA content of late S, G2-M, DNA distribution histograms of
cultures exposed to ADM (0.01 μg/ml) for 24 hr were similar to those of log-phase cultures.

0.1 μg/ml. In cultures exposed to ADM (0.1 μg/ml) for 1 or 2 hr, washed, and reincubated in fresh medium for 23 hr, viable cell counts were, respectively, 90 and 70% of the control (Chart 2). DNA distribution histograms of these cultures are shown in Chart 1, B and C and show that, depending on the length of exposure to this drug concentration, cells with the DNA content of late S-G2 are accumulated in the population. This accumulation is accompanied by a corresponding decrease in the number of cells with G1-early S DNA content.

In cultures incubated with ADM (0.1 μg/ml) for 24 hr, no increase in cell numbers was seen. DNA distribution histograms of these cultures (Chart 1D) had a predominant part of the population with the DNA content of late S-G2 (Channels 51 to 70). LI was ~30% and cpm incorporated, [3H]TdR into acid-insoluble DNA, were 48% of cells in log-phase cultures. By the 48th hr of incubation, approximately 5% of the cells were labeled and [3H]TdR incorporation was ~4% of control.

0.5 μg/ml. In cultures incubated with ADM (0.5 μg/ml) for 1 or 2 hr and subsequently washed and reincubated in fresh medium for 23 hr, cell counts (after dye exclusion) were, respectively, 40 and 35% of the control. LI were around 10% and, as seen in the DNA distribution Histograms B and C of Chart 3, an accumulation of cells with the DNA content of late S and G2 was evident. With increasing length of exposure to ADM (0.5 μg/ml), traversal of cells through the cell cycle was inhibited and, as seen in Histogram 3D (0.5 μg/24 hr), no accumulation of cells with the DNA content of late S and G2 was seen in these cultures. LI in these cultures were also very low (~2%) and cpm incorporated after pulse labeling with [3H]TdR were 6% of control. The number of viable cells had also decreased to 20% of the control, and a large number of pyknotic cells were seen in these cultures.

1.0 to 10 μg/ml. In cells exposed to higher concentrations of ADM (1.0 to 10 μg/ml for 1 to 24 hr), DNA distribution histograms showed no accumulation of cells with late S-G2 DNA content. The number of viable cells in these cultures was approximately 10% of control and labeled cells were rarely seen.

Exposure of Synchronized Cell Populations to ADM

Chart 4 shows the DNA distribution histograms and [3H]TdR incorporation data of various synchronized cell populations used in the present study. As described in "Materials and Methods," synchronized cell populations were obtained after release of cells from a 1.0 mm IMPY-induced block of DNA synthesis and cell cycle traverse. Chart 3A shows DNA distribution of a log-phase culture, and compared to this, cells exposed to a 2 × IMPY block (Chart 3B) had 70% of the population with the DNA content of G1-early S. LI was ~20% and cpm incorporated were 10% of cells from a log-phase culture. On reincubation in fresh medium, cell cycle traverse was resumed by the blocked cells in a synchronized manner and after 4 hr of incubation LI reached a peak of ~86% and cpm were 220% of control. DNA distribution histogram of this population is shown in

Chart 3C and shows an accumulation of cells with the DNA content of S. By the 8th hr of reincubation, LI had fallen to 51% and as seen in the DNA distribution histogram of Chart 3D, a major part of the population had the DNA content of late S-G2. By the 11th hr of incubation LI falls to 10%, and between the 9th and 13th hr a ~60% increase in cell counts is seen.

G1 Cells. Histogram 5A (Chart 5) shows DNA distribution in a G1-blocked population (2 × IMPY, 1.0 mm block) used in these experiments; 69% of this population had the DNA content of G1-early S, and LI was 20%, and cpm incorporated were ~10% of those in log-phase cultures. After 24 hr of incubation in fresh medium, LI was 60% and cell counts had increased 66%. Chart 5B shows DNA distribution of this population, and the remaining effects of the earlier synchronization are seen in the higher than normal percentage of cells with the DNA content of S and G2 (Channels 36 to 70).

In G1-blocked cultures incubated with ADM (0.1 and 0.5 μg/ml) for 1 hr and subsequently washed and reincubated in fresh medium for 23 hr, cell counts were, respectively, 90 and 40% of the control (Chart 6). LI was approximately 30%. Chart 5, C and D shows DNA distribution histograms of these cultures after 24 hr of incubation. An accumulation of cells with the DNA content of late S-G2 was seen in G1 cultures exposed to ADM (0.5 μg/ml) for 1 hr.

S Cells. Chart 7A shows DNA distribution histogram of the S population used for these experiments. LI in this population was 86% and a major part of the cell population had the DNA content of S. Chart 7B shows this population
A. KriShan and E. Frei, III

DNA distribution histograms (Chart 7, C and D) show accumulation of cells with the DNA content of late S and G2.

Late S-G2 Cells. G2 cells used were from cultures released after 24 hr of incubation in fresh medium. Cell counts had approximately doubled by this time and the LI was ~60%. In Chart 7, C and D, DNA distribution histograms of S-phase cultures exposed to ADM (0.1 and 0.5 µg/ml) for 1 hr and reincubated in fresh medium for 23 hr are shown. As seen in Chart 6, the number of viable cells in these cultures was much lower than that of G1 cells similarly treated and described above. Incorporation of [3H]TdR was also dimin-

Chart 3. A, log-phase control. B and C, ADM (0.5 µg/ml) for 1 or 2 hr, respectively, washed and reincubated in fresh medium for 23 hr. D, ADM (0.5 µg/ml) for 24 hr. Cnt, number of cells capable of dye exclusion after trypan blue staining, expressed as percentage of control.
Adriamycin and Cell Cycle Traverse

for 9 hr after a 2nd IMPY block. The L1 in this culture was about 30%, and a major part of the population had the DNA content of either G1 or late S-G2. DNA distribution histograms of cells from this sample (after incubation with ADM (0.1 and 0.5 µg/ml) for 1 hr, washed, and reincubated in fresh medium for 24 hr) were similar to those of S cells similarly treated and described above. However, as seen in Chart 6, the number of viable cells in these cultures was higher than those of S cells similarly treated.

**Exposure of Plateau-Phase Cultures to ADM.** A DNA distribution histogram of a plateau-phase culture (3.4 x 10⁶/ml; L1, ~30%, cpm, 44% of log-phase cultures) is shown in Chart 8B. Compared to a log-phase culture (Chart 8A), 64% of the population in this culture had the DNA content of G1-early S. Cells from this culture were incubated with ADM (0.1 or 0.5 µg/ml) for 24 hr. As shown in the data accompanying Chart 8C, after 24 hr of incubation with 0.1 µg/ml, L1 had shown a small increase (43%), cell counts were 62% of the control, and cpm incorporated were 68% of cells from untreated plateau-phase culture. A DNA distribution histogram of this population (Chart 8C) shows an accumulation of cells with the DNA content of S and G2. However, in contrast to log-phase cultures similarly treated (Chart 1D), the proportion of such cells was lower in the ADM-treated plateau-phase cultures due to the smaller number of cycling cells.

Exposure to higher concentrations of ADM (0.5 µg/ml) for 24 hr led to a reduction in the number of labeled cells (~10%). DNA distribution histograms of these cultures (Chart 8D) show that a major part of the population had the DNA content of G1-early S and no accumulation of late S and G2 cells.

**Exposure of Plateau-Phase Cultures to ADM, Followed by Reincubation in Fresh Medium at Lower Cell Density.** Plateau-phase cultures used in this part of the study were similar to those described in Chart 8B. These cultures were incubated with ADM concentrations of 0.1 and 0.5 µg/ml for 1 hr, washed twice, and reincubated in fresh medium at a lower cell density (1.0 x 10⁶/ml). Log-phase cultures of similar cell density were identically treated (washed and centrifuged). Chart 9, A and B show, respectively, the log-phase and the earlier plateau-phase culture after reincubation in fresh medium for 24 hr. A higher L1 (Chart 9B) is...
A. Krishan and E. Frei, III

DISCUSSION

Observations in this study show that the effect of ADM on cell cycle traverse and its cytotoxicity are determined by the drug concentration and by the length of exposure of cells to probably due to the partial synchronization induced by the earlier growth at the plateau phase. Chart 9, C and D, shows data as well as DNA distribution histograms of cultures exposed to ADM (0.1 and 0.5 μg/ml) in their plateau phase for 1 hr. After 24 hr of reincubation in fresh medium (at lower cell density), the LI in these cultures was between 30 to 50% (60% in control) and cell counts were between 65 and 78%. [3H]Tdr incorporation was slightly higher than the control and may be due to the larger population of cells in S and the late S part of the cell cycle.

Chart 8. A, log-phase culture; B, plateau-phase; C and D, same as B after incubation for 24 hr with ADM (0.1 and 0.5 μg/ml). CnT, number of cells capable of dye exclusion after trypan blue staining, expressed as percentage of control.

Chart 7. A, cells released from IMPY-block for 4 hr; B, same as A after 24 hr incubation. C and D, same as A after incubation with ADM (0.1 and 0.5 μg/ml) for 1 hr, a wash, and reincubation in fresh medium for 23 hr. CnT, number of cells capable of dye exclusion after trypan blue staining, expressed as percentage of control.
Adriamycin and Cell Cycle Traverse

the earlier observations of Meriwether and Bachur (14) who reported that, in contrast to daunomycin, where the intracellular drug level reaches a steady state within 20 to 60 min, uptake of ADM is continuously increasing even after an interval of 1 hr.

A number of recent studies (7, 9–12, 20, 22, 23, 25) have used impulse cytophotometry or flow microfluorometry to analyze the effect of drugs on the cell cycle traverse of cultured cells. Göhde and Dittnich (7) have used impulse cytophotometry to assess the effect of daunomycin (antibiotic closely related to ADM) on the cell cycle traverse of Ehrlich ascites tumor cells. In cells exposed to daunomycin (10 to 100 μg/ml) Göhde and Dittnich (7) reported the arrest of cells in G2. In a similar study, Linden et al. (12) have studied the effect of daunomycin on the cell cycle traverse of synchronized L-cells. By exposing G1 cells to daunomycin (1 μg/ml) they produced an arrest of cells in G1, whereas exposure of cells in S phase to daunomycin (1 μg/ml) for 4 hr had no effect on the cell cycle traverse, thus indicating that cells that had already started DNA synthesis were not prevented from completing DNA synthesis. Daunomycin treatment in late S phase or in the G2 phase resulted in the accumulation of cells in G2.

Exposure to ADM (0.01 μg/ml) leads to a small increase in the number of cells with the DNA content of late S and G2. Compared to this, higher ADM concentrations (0.1 μg/ml for 24 hr or 0.5 μg/ml for 1 hr) lead to an accumulation of cells with the DNA content of late S-G2 and, consequently, no mitotic figures are seen in these cultures. These observations are supported by those of Tobey (21), who reported no inhibition of genome replication in CHO cells exposed to ADM. A similar effect on cell cycle traverse is exercised by podophyllotoxin derivatives VM-26 and VP-16-213 (10), although podophyllotoxin by itself like colchicine, vinblastine, and vincristine allows progression of cells beyond G2 into M. A number of other chemotherapeutic agents may exercise a similar effect on cell cycle traverse and lead to an accumulation of cells with the DNA content of late S-G2 (20).

Observations in the present study show that cells in S-phase of cell cycle are more sensitive to the cytostatic and cytoidal effects of ADM. In contrast cells blocked in G1 or from populations with a mixture of G1 and G2 were less sensitive to ADM. Similarly, cells from plateau-phase cultures exposed either continuously for 1 hr (followed by washing and reincubation in fresh medium at lower cell density) had less damage done by ADM than log-phase cultures similarly treated. These observations support the earlier reports on the sensitivity of S-phase cells to lethal effects of ADM (1, 5, 8). In synchronized CHO cells, Barranco et al. (1) have also reported sensitivity of cells in early S and mitosis to ADM. Whereas in cultured human lymphoid cells, Drewinko and Gottlieb (5) described enhanced lethal effects of ADM on cells at the S-G2 boundary. These observations have an important bearing on the use of ADM in cancer chemotherapy either as a single agent or in combination with agents known to have cycle-specific action (e.g., cytosine arabinoside). Flow cytofluorometry, especially with the recent development of rapid methods for DNA per cell analysis (3, 9), is an ideal tool for analysis of such drug effects on cell cycle traverse both in vivo and in vitro.

Chart 9. A, log-phase; B, same as 88 after resuspension at lower cell density for 24 hr. C and D, same as 8B after exposure to ADM (0.1 and 0.5 μg/ml) for 1 hr, a wash, and reincubation in fresh medium (at lower cell density) for 24 hr. CnT, number of cells capable of dye exclusion after trypan blue staining, expressed as percentage of control.
ACKNOWLEDGMENTS

We are thankful to K. Paika, J. Sullivan, and P. Rivers for technical assistance; to M. Kasac, L. Adamson, and S. Daya, we are thankful for help in preparation of the manuscript.

REFERENCES

Effect of Adriamycin on the Cell Cycle Traverse and Kinetics of Cultured Human Lymphoblasts

Awtar Krishan and Emil Frei III

Cancer Res 1976;36:143-150.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/1/143

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.