Cytokinetic Analysis of L1210 Leukemia after Continuous Infusion of Hydroxyurea in Vivo

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ABSTRACT

Hydroxyurea (HU) was administered by continuous i.v. infusion to L1210 tumor-bearing mice (10⁶ cells, Day 0) beginning on Day 5. Doses of 1, 12, 48, 96, and 192 mg/kg/hr were infused for periods of 1 to 48 hr. Sequential changes in cell count, [³H]thymidine labeling index, grain count, DNA content distribution (determined by flow microfluorometry), and survival times were determined. The two lowest doses (1 and 12 mg/kg/hr) induced little cytotoxicity or progression delay at the G1-S boundary, and survival times were not significantly different from the control value. The effects of the higher doses of HU were dose dependent. HU (48 mg/kg/hr) effectively synchronized cells in S phase. After 24 hr, little cytotoxicity occurred, and once the infusion was ended the synchronized cells continued to progress into G2 and mitosis at a rate similar to that of unperturbed cells. Detectable synchrony was maintained through one generation. Cytotoxicity increased with infusion times longer than 24 hr. The increase in survival times with this dose of HU was equivalent to the duration of the infusion. HU (96 and 192 mg/kg/hr) was markedly cytotoxic resulting in 95% cell loss by 48 hr. The grain count decreased markedly by 4 hr after both doses, and the flow microfluorometry histograms demonstrated progression delay within 4 hr. Maximum increases in life span of 4.7 days (56%) and 5.6 days (68%), respectively, were obtained after 32-hr infusions. Toxicity occurred with infusions longer than 32 hr. These data indicate that the effects of HU in vivo are dependent on the dosage and duration of the infusion. High infusion doses are required to kill cells in vivo. Lower, noncytotoxic dose levels may effectively be used to synchronize cells in S phase.

INTRODUCTION

Synchronization of tumor cells in a discrete phase of the cell cycle may provide a means of enhancing the cell killing of subsequent phase-specific chemotherapy by potentially increasing the percentage of cells at risk. This approach has been used clinically by Lampkin et al. (6, 7) in the treatment of acute myelogenous leukemia. Ara-C⁴-induced synchrony or recruitment of the tumor cells into S phase may have potentiated the cell kill of phase-specific agents given at the appropriate time interval. A similar approach has been used with some therapeutic gain in the treatment of solid tumors (1, 8). In the clinical setting, the degree of drug-induced synchrony or

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2 Submitted in partial fulfillment of requirements for a Ph.D. dissertation, Department of Biochemistry, Boston University School of Medicine, Boston, Mass. To whom requests for reprints should be addressed.
3 The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; HU, hydroxyurea; LI, labeling index; GC, grain count; FMF, flow microfluorometry.

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1 The rate of desynchronization of the tumor cells is difficult to determine. Detailed analyses of drug effects in vivo can be determined in animal tumor models and may provide useful information for more rational clinical treatment design.

Synchronization experiments using animal tumor models have been performed with a variety of chemotherapeutic agents (2-4, 11, 14-18). In general, cytotoxic levels of drug administered by pulse injections have been required in order to achieve detectable synchrony in vivo. Synchrony with noncytotoxic levels of drug can be achieved with continuous exposure of cells to phase-specific drugs for a period at least equal to the mean cell cycle duration. This has been demonstrated for Ara-C (18) and HU in vitro (4, 17) and for Ara-C in vivo (3, 13).

Synchronization of both normal and tumor cells in vivo with multiple doses or continuous infusion of HU has been studied by several investigators (2, 11, 12, 16). In these studies, the continuous depression of [³H]thymidine uptake following HU treatment was used as an indication of a G1-S block. Other supporting cytokinetic parameters, such as cell counts or other cytotoxicity assays or DNA content distributions, were not determined. A dose-response study with continuous exposure of tumor cells to HU in vivo has not been done. Such a study would be important in order to understand the feasibility of cell synchronization in vivo by this technique.

This report describes the effects of various concentrations of HU administered for periods of 1 to 48 hr by continuous i.v. infusion to L1210 ascites tumor-bearing mice. Serial tumor cell counts were determined at intervals during and after HU infusions, and the cells were analyzed for [³H]thymidine LI and GC distribution by autoradiography. Cell synchrony was established by analyzing the DNA content distribution of the tumor cells by FMF. In addition, mean survival times were determined and related to the dosage and duration of infusion.

MATERIALS AND METHODS

Mice. Experiments were performed on male BALB/c × DBA/2 (hereafter called CD2F1) mice, weighing 20 to 25 g, that were purchased from Lab Supply Co., Indianapolis, Ind. L1210 tumor cells were maintained by weekly passage in male DBA/2 mice purchased from Charles River Breeding Laboratories Inc., Wilmington, Mass.

L1210 Leukemia Cells. L1210 ascites tumor was obtained from the Mason Research Institute, Worcester, Mass., and maintained in our laboratory by weekly passage in male DBA/2 mice. Experimental mice CD2F1 were inoculated i.p. on Day 0 with 10⁶ tumor cells diluted to a total volume of 0.1 ml with Hanks’ balanced salt solution.

Drug. HU (NSC 32065) was supplied by the Drug Development Branch, National Cancer Institute. The drug was diluted in 0.9% NaCl solution containing heparin (20 units/ml) for i.v.
continuous infusion and in 0.9% NaCl solution without heparin for i.v. push injections. Heparin was included to prevent blood clotting in the infusion catheter. HU concentration for infusion was based on the mean body weight on Day 5 of tumor growth and an infusion rate of 1 ml/24 hr. The HU infusion doses were 1, 12, 48, 96, and 192 mg/kg/hr. For i.v. push injections, HU was diluted to a total volume of 0.01 m/g body weight.

Continuous Infusion Method. The method of continuous i.v. infusion was similar to that described by M. Edelestein (3) and R. Momparler (9). The infusion system consisted of a Model 975 Harvard pump, adapted to hold twelve 3-ml syringes. The mice to be infused were housed in a specially designed infusion cage built to hold 12 mice with the tail of each mouse pulled to the outside and taped to a tongue depressor. The solution to be infused was then delivered into a lateral tail vein through a 12-inch infusion catheter and 27-gauge needle (Deseret E-Z set infusion set No. 5308).

Experimental Design. Mice were inoculated weekly with L1210 tumor cells. Randomized, untreated tumor-bearing mice were included each week to monitor the reproducibility of unperturbed L1210 tumor growth. Three to 4 control mice were analyzed daily on Days 4 to 7 for cell count, LI, and DNA content distribution of the tumor cells, and 8 to 12 mice were analyzed for survival time. On Day 5 of tumor growth, the mice were further randomized to 2 groups: (a) HU by continuous infusion; and (b) HU by i.v. push.

Drug treatment was begun on Day 5. Due to the limitation of the number of mice that could be simultaneously infused, a maximum of 5 infusion groups (9 to 12 mice per group) was processed each week. Any mouse developing a venous infiltrate was eliminated from the study. Separate groups were infused with various concentrations of HU solution containing heparin or NaCl and heparin alone for periods of 1 to 48 hr. At the end of each infusion, 2 to 3 mice were given injections of [3H]thymidine (see below), tumor cells were collected 1 hr later for LI and FMF analysis, and 7 to 9 mice per group were analyzed for survival time. Mice (9 to 12 per group) treated with HU by i.v. push were analyzed for survival time only.

LI Determination. Mice were given i.p. injections of 20 μCi [3H]thymidine (specific activity, 2.0 Ci/mmole; New England Nuclear, Boston, Mass.) diluted to a total volume of 0.2 ml with 0.9% NaCl solution. One hr later, the mice were killed, and the ascites cells were collected by washing the peritoneal cavity with Hanks' balanced salt solution and counted by hemacytometer. One \times 10^7 cells/mouse were processed for autoradiography as previously described (14). The slides were dip-coated in Kodak NTB2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) diluted 1:1 with distilled water, exposed for 6 days, developed in Kodak D-19 developer, and fixed in Kodak fixer. Five hundred cells per slide were read, and a labeling threshold of 5 grains/nucleus was used for each LI determination. Background labeling averaged <1 grain/nucleus. For GC determinations, the grains over the nuclei of 100 labeled interphase cells were counted.

FMF Analysis. An aliquot of 5 \times 10^6 cells/mouse was prepared for FMF analysis according to the method of Krishan (5). The cells were centrifuged at 300 \times g for 5 min, resuspended in a solution of propidium iodide (0.05 mg/ml in 0.1% sodium citrate) to a final dilution of 5 \times 10^6 cells/ml, and stored at 4°C for no longer than 48 hr until FMF analysis. There was no loss of reproducibility for control or treated cells within this time period. Propidium iodide (Calbiochem, La Jolla, Calif.) is a DNA-specific fluorescent stain, and the fluorescence intensity of each cell corresponds to its relative DNA content. Cells were analyzed with the Bio/Physics Cytofluorograf, Model 4800A (Ortho Instruments, Westwood, Mass.). The cytofluorograph was initially adjusted so that the control G1 peak was in Channel 80. Machine drift was monitored by analysis of the control sample prior to each experimental sample and was found to be ±2 or 3 channels.

The frequency distribution of the relative fluorescence intensity of the L1210 cells was analyzed in a Bio/Physics Model 2102 pulse height analyzer (Ortho Instruments). Fifty thousand to 150,000 cells were analyzed for each histogram. For graphic presentation, the histograms were normalized to 100,000 cells each.

RESULTS

Cell Counts. The effect of the various doses of HU infusions on the L1210 tumor cell counts is shown in Chart 1. Hr 0 is Day 5 of tumor growth. The cell counts for mice treated with NaCl solution and heparin alone (data not presented) were identical to those of the untreated controls. At the lower HU doses (1 and 12 mg/kg/hr), tumor growth was only slightly inhibited. Cell kill occurred at the 3 higher doses of drug (48, 96, and 192 mg/kg/hr) and was dose dependent. After HU (48 mg/kg/hr), the cell count decreased from an initial count of 1.6 \times 10^6 to 9 \times 10^5 cells (46%) by hr 48. The cell count decreased by greater than 95% with 48 hr of infusion at doses of 96 and 192 mg/kg/hr. The rate of cell loss was highest with HU (192 mg/kg/hr).

The cell counts from +0 to +48 after the end of HU infusions of 48 and 96 mg/kg/hr are shown in Charts 2 and 3, respec-
tively. After 24 hr of HU (48 mg/kg/hr), the cell count continued to increase slightly (Chart 2). However, after 48 hr of infusion, the cell count continued to decrease indicating that greater cytotoxicity resulted from the longer infusion time. After 24 hr of HU (96 mg/kg/hr), the cell count continued to decrease (Chart 3).

**[^H]Thymidine Labeling Studies.** L1210 cell counts as a function of time after HU infusions of 24 or 48 hr. Each point represents the mean of 2 to 3 mice; bars, S.E.

**FMF Studies.** The effect of HU (1, 12, and 48 mg/kg/hr) on the DNA content distribution of the L1210 cells is shown in Chart 6. There was little variation in histograms from separate mice at any time point, and, therefore, only one histogram per time point is shown. With the lowest 2 infusion doses, the distribution of cells in S phase after 24 and 48 hr of infusion (Chart 6, C2 and C3).

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histograms were analyzed at several intervals following 24 or 48 hr of infusion. Following HU (48 mg/kg/hr for 24 hr), the progression of cells from S phase into G2 and M is evident at +3 hr (Chart 8). At +10 hr, the size of the S phase compartment is significantly decreased and G1 is increased. At +14 hr, G1 cells have moved into S-phase. From +19 to +48 hr, there is a continued normalization of the shape of the FMF histograms.

After treatment with HU (48 mg/kg/hr for 48 hr) (data not presented), there was no evidence of progression of cells from S into G2-M. A gradual depletion of the S and G2-M regions of the FMF histograms is consistent with the continued cell loss noted after the 48-hr infusion (Chart 2).

**Survival Studies.** The survival times for mice treated for various intervals with each infusion dose of HU are shown in Chart 9. The mean survival time of the untreated tumor-bearing control mice was 8.2 days. The survival time for mice treated for 24 or 48 hr with HU (1 or 12 mg/kg/hr) was not significantly different from the control value. Twenty-four- and 48-hr infusions of HU (48 mg/kg/hr) resulted in increases in survival equivalent to the duration of the infusion. Longer increases in survival were achieved after HU (96 and 192 mg/kg/hr). Maximum increases in survival of 4.7 days (56%) and 5.6 days (68%), respectively, were obtained after 32-hr infusions of both doses. With infusion times longer than 32 hr, survival times decreased as toxicity increased.

The survival times of mice given injections of various doses (24 to 4608 mg/kg) of HU by i.v. push are shown in Chart 10. Single-dose HU treatment was ineffective at each dose level. A maximum increase in survival of 0.5 day (not significant) was achieved with HU (4608 mg/kg) by i.v. push in contrast to an increase of 5 days achieved with the same total dose delivered in 24 hr with an infusion of 192 mg/kg/hr.

**DISCUSSION**

This study demonstrates that the effects of HU administered as a continuous infusion in vivo are dependent on both the concentration and the duration of the infusion. Relatively high doses of HU (≥48 mg/kg/hr) were required to alter significantly the cell cycle kinetics of the L1210 cells from control values. Greater than 500-fold-higher doses of HU were required as compared to Ara-C to achieve equivalent perturbation effects (14).

Five infusion dose levels were evaluated. HU (1 mg/kg/hr) was virtually ineffective. HU (12 mg/kg/hr) resulted in some progression delay of cells traversing S phase after 48 hr of
HOURS OF CONTINUOUS INFUSION
OF HYDROXYUREA

Chart 4. LI as a function of HU infusion duration. Mice were given injections of \(^{3}H\)thymidine 1 hr prior to sacrifice. Each point represents the mean of 2 to 3 mice; bars, S.E.

HYDROXYUREA INFUSION DOSE

1 mg/kg/hr 12 mg/kg/hr 48 mg/kg/hr

CONTROL

HR 24

HR 48

CHANNEL NUMBER (RELATIVE DNA CONTENT)

Chart 6. L1210 relative DNA content distribution as a function of HU infusion duration. Each histogram represents 100,000 cells.

HYDROXYUREA INFUSION DOSE

96 mg/kg/hr 192 mg/kg/hr

CONTROL

HR 4

HR 8

HR 12

HR 18

HR 24

HR 48

CHANNEL NUMBER

Chart 7. L1210 relative DNA content distribution as a function of HU infusion duration. Each histogram represents 100,000 cells.

infusion as demonstrated by an increase in LI. Increases in survival time at this dose were insignificant.

HU (48 mg/kg/hr) resulted in obvious progression delay of cells in S phase on FMF analysis. Many of the cells held up in S phase continued to synthesize DNA, and LI remained above the control value. At the end of 24 hr of infusion, the cells in S phase rapidly resumed progression through G2 and mitosis as a synchronized cohort. From these histograms, it is estimated that the mean generation time of cells previously delayed in S...
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HU (96 mg/kg/hr) resulted in a transient increase in LI at 12 hr with a concurrent decrease in GC. With continued HU exposure, cells in S phase were eventually killed, and the LI decreased to <10% by Hr 48. Maximum cytotoxicity occurred after the highest dose of drug where progression delay in the G1-S region was most obvious.

Cell kill with continuous HU infusion may in part be attributed to the continued entry of cells from G1 into S phase. It is apparent from both the cell count (Chart 1) and survival data (Chart 9) that the effects of HU infusion are not "self-limiting." The continued cell kill observed with the 2 highest infusion doses may in part be due to unbalanced growth rather than to specific effects on DNA synthesis since there were few cells synthesizing DNA beyond Hr 4.

Cytotoxicity was evaluated by cell counts which may not distinguish damaged or doomed cells. A clonogenic assay might provide further information relative to the reproductive potential of HU-treated cells.

These studies indicate that high HU infusion doses effectively kill cells in vivo. The increases in survival time of 5 to 6 days obtained after 32 hr of infusion at 92 and 196 mg/kg/hr were superior to those previously reported for Li210 with optimal split-course HU therapy (10). However, the high infusion doses required to achieve significant therapeutic gains may be a limiting factor in using HU as a single agent. It may, however, be possible to exploit the synchronization properties of HU which occur at lower continuous infusion doses.

There have been few detailed reports of the effects of continuous drug administration in animal tumor models. The Ara-C data of Edelstein et al. (3) showed similar results to the data presented in this paper in that L1210 tumor cell kill (demonstrated by a decrease in leukemia colony-forming units) increased with increasing Ara-C infusion dose and also infusion duration. Similar results were obtained by Momparler (9) after infusion of 5-aza-2'-deoxycytidine.

Edelstein et al. (3) demonstrated that Ara-C infusions affected tumor cells to a greater extent than they did normal marrow. Plager (11) suggested that following HU infusion, recovery of DNA synthesis was different for various normal tissues. If there are sufficient differences in the perturbation effects of HU infusion on tumor and normal cell populations, it may be possible to time the administration of sequential therapy to damage maximally the tumor cell population.

Preliminary studies from this laboratory indicate that pulse phase is 12 to 18 hrs, which is similar to the transit rate of untreated cells. After 48 hr of infusion, many of the cells were killed by the prolonged HU exposure, and any potential advantage of cell synchronization was lost.

Marked cytotoxicity occurred with doses of HU ≥96 mg/kg/hr (Chart 1).
phase-specific therapy given at appropriate intervals after synchronizing doses of HU infusion increases the therapeutic effect.

REFERENCES


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