In Vivo Cell Cycle Synchronization of the Murine Sarcoma 180 Tumor following Alternating Periods of Hydroxyurea Blockade and Release

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ABSTRACT

The durations of the cell cycle intervals of the murine Sarcoma 180 tumor were determined by computer analysis of the fraction-labeled mitosis curve following tritiated thymidine administration. This tumor has a usual total cell cycle duration of 19.6 hr, a DNA synthetic time of 8.3 hr, and a growth fraction of 1. Approximately 38% of cells are in S phase at one time. Hydroxyurea (HU) infusions (i.v.) at 1.17 mg/hr into tumor-bearing mice rapidly inhibit tumor DNA synthesis. Following a 5-hr HU infusion, 58% of all tumor cells are in S phase, and maximal tumor mitotic rates after release of the HU blockade are double control rates.

HU was infused for 5 hr, followed by 7 hr of Ringer’s solution, and then another 5 hr of HU. Following this 2-cycle blockade, 70% of tumor cells are in S phase, predominantly in early S phase and at the G1-S junction. After release, peak mitotic rates are 2.5 times control. The duration of the intermitotic time of the tumor following HU infusion is less than the total cell cycle time of control tumor. Cycles of HU infusion and release, timed according to the predetermined duration of the cell cycle intervals, will synchronize significant increments of S phase or mitotic cells of the Sarcoma 180 tumor during predictable periods of time.

INTRODUCTION

Radiation therapy and many cancer chemotherapeutic agents are known to have additional tumoricidal activity during specific phases of the cell cycle (3, 11, 12, 14, 25). It has therefore been recognized that any procedure that provides an increment of tumor cells or removes normal cells from the cycle phase in which an agent is active while such therapy is administered will result in an increased therapeutic index. This knowledge has provided added impetus to studies of cycle synchronization of normal and malignant tissues.

HU is an agent which shows cell cycle specificity in its activity (1, 2, 10, 16, 17, 22, 25, 28) and offers several other advantages as a synchronizing agent. It is readily soluble in water and equilibrates rapidly throughout the body water of an animal (17, 20). It is known to block specifically new DNA synthesis by inhibiting the ribonucleotide reductase enzyme system, while largely leaving the rate of RNA and protein synthesis intact (1, 4, 10, 21, 22, 28). This DNA blockade may be promptly reversed in tissue culture by removing HU from the culture medium, and since HU is rapidly cleared from the plasma of an animal, its in vivo inhibition of DNA synthesis may also be rapidly reversed (1, 17, 20). As would be expected, HU action is limited to cells committed to DNA synthesis and is associated with unbalanced growth (7, 8, 9, 17, 22).

Partial synchronization of tumor or tissues of experimental animals has been reported on several occasions following a single, sustained HU blockade (5, 6, 10, 12, 13, 21). In these cases, HU was given i.p.; transient increases in mitotic rate in the surviving cells were shown following release from DNA inhibition, and this was probably secondary to the synchronous release of cells previously stopped in S phase. The tissue concentration of HU is known to be critical in determining whether cells will be killed or reversibly inhibited, or will entirely escape inhibition (5, 7, 8, 19). This concentration is controlled with difficulty during intermittent i.p. administration. Partially owing to this, previous experiments in animals have not demonstrated cell cycle control of any tissue for periods of time greater than the duration of 1 cell cycle. The development of a practical method for introducing and maintaining an i.v. infusion in unrestrained mice for periods of a week or more (18) has offered the opportunity of closely controlling the rate of drug administration during prolonged periods of time, of providing accurately timed pulses of DNA blockade and release, the durations of which may be dictated by the known cell cycle intervals of a tumor, and of gathering additional increments of tumor cells in synchronous fashion during each of several sequential cell cycles. This is presently reported in mice bearing the Sarcoma 180 tumor following alternating periods of HU infusion and release.

MATERIALS AND METHODS

Male, white, Swiss Ha/Icr mice, 10 to 13 weeks old and weighing between 25 and 30 g, were used. The mouse sarcoma, Sarcoma 180, was maintained by s.c. passage at 10-day intervals. For the present experiments, 5-mg tumor fragments were implanted by trocar at 2 s.c. sites, and the mice were studied 10 days later. At this time, the tumor at each site weighed between 500 and 1000 mg. Only 1 of the 2 tumors from each animal was sectioned and studied.

Infusions (i.v.) of Ringer’s solution were established in the tail vein of unanesthetized animals. The original technique for establishing this infusion (18) was modified in that the skin over the tail vein and the vein itself were punctured by a 30-gauge hypodermic needle, and polyethylene tubing of internal diameter 0.279 mm and external diameter 0.61 mm was cut to a broad point and directly threaded 1.5 to 2 cm into the vein. All experimental procedures were started 18 to 24 hr later to allow the animal time to recover from any stress of establishing the...
infusion. During the experimental period, the mice remained undisturbed in their cages with available food and the prevalent
day-night cycle.

The HU solution was prepared immediately before use. It
was infused at a rate of 1.17 mg in 0.58 ml Ringer’s solution
per hr.

\[ \text{[methyl-}^{3}\text{H]} \text{thymidine (6.7 mCi/mM)} \text{ was purchased from}
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New England Nuclear Corp., Boston, Mass.; 24 mCi (in 0.1 ml
Ringer’s solution) were injected into the i.v. infusion 0.5 hr
before sacrifice of the animal. Mice were killed by cervical
dislocation, the tumor was fixed in neutral formalin, and slides
were prepared. For autoradiographic study, the slides were
dipped into Kodak NTB-2 emulsion diluted 1:1 with water and
then exposed in a dessicant box at 5°C for 2 weeks. They were
developed in Kodak D-19, rinsed in tap water, and cleared in
Kodak rapid fixer. Slides were stained with hematoxylin and
eosin.

All slides were coded for counting so that the observer was
not aware of their source. The fraction of labeled mitoses was
determined by identifying 300 mitoses in sections taken from
several portions of the tumor and scoring each as labeled or
unlabeled. One grain per cell was the maximal control count
determined from autoradiography of tumor obtained from ani-
malms which had not been given radioactivity. Therefore, in data
obtained for the fraction-labeled mitoses curve, a cell was
considered labeled if it had 2 or more grains superimposed. The mean grain count data were derived from 100 consecutive
labeled cells chosen at random from the tissue sections. For
these data only, a cell was considered labeled if 1 or more
grains were superimposed. The tumor mitotic rate was deter-
mined by evaluating 1000 cells from each of 3 inner and 3
outer portions of 3 separate sections of the tumor (a total of
18,000 cells were counted).

In this report, numerical values are presented as the mean
± S.D.; data for each of these calculations are derived from
between 5 and 10 different tumors. The value from each tumor
was treated as a single entry for statistical purposes.

RESULTS

**Determination of the Cell Cycle Times and Growth Fraction**

**of the Sarcoma 180 Tumor.** The mean duration of the gener-
ative phase and the S phase of the Sarcoma 180 tumor has
been previously reported (15, 23, 24). Since the present in-
vestigation utilized different sized tumor implants and utilized
the tumor under different circumstances, and since previous
data were not subjected to computer analysis, these determi-
nations were repeated.

Ringer’s solution was infused i.v. into tumor-bearing mice.
Tritiated thymidine was given as a single pulse through the
infusion tubing, and the mice were killed at known intervals
during the following 22 hr. The total number of mitoses, the
number of labeled mitoses, and the proportion of cells labeled
were determined from tumor sections obtained at each time
interval. Dr. M. L. Mendelsohn kindly analyzed these data,
using methodology which he and his coworkers had published
previously (27). The curve which best fits the experimental data
was determined by computer analysis and is presented in Chart
1. The mean cell cycle values (in hr) derived from this optimal
fit with the respective coefficients of variation are: \( T_c \), 19.6
(0.4); \( T_{G1} \), 8.2 (1.0); mean duration of the DNA synthetic period,
8.3 (0.3); mean duration of the post-DNA synthetic, pre-mitotic
period, 2.3 (0.4); and the mitotic period, 0.9 (0.4). This analysis
also indicates that the tumor growth fraction is 1. The present
values for the duration of \( T_c \) and \( T_{G1} \) are appreciably longer
than those previously published.

**Time Period Required for HU Blockade and Blockade Release.** If HU were to be used for intermittent S-phase block-
ade, it would be necessary to determine an optimal infusion
rate and the time period required for this infusion rate to block
DNA synthesis and to make an estimate of tissue recovery time
after the HU infusion was stopped. Initial experiments deter-
mined that HU, when infused at a rate of 1.17 mg/hr provided
rapid blockade which dissipated within a reasonable period
after the HU was discontinued. The rate of HU infusion was
deliberately chosen so as not to completely block thymidine
incorporation by the tumor. Previous experimental observations
suggest that allowing a small amount of DNA synthesis offers
some protection to the cell from death from unbalanced growth
(19).

HU was infused for 5 hr (blockade) after which Ringer’s
solution was infused for an additional 4 hr (release). The mice
were killed during this 9-hr experimental period; tritiated thym-
idine was given i.v. 0.5 hr prior to sacrifice. Data from this
experiment are presented in Chart 2. The mean cell grain count
decreased significantly within 10 min of institution of the HU
infusion and to make an estimate of tissue recovery time
after the HU infusion was stopped. Initial experiments deter-
mined that HU, when infused at a rate of 1.17 mg/hr provided
rapid blockade which dissipated within a reasonable period
after the HU was discontinued. The rate of HU infusion was
deliberately chosen so as not to completely block thymidine
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Rapid changes in the cellular pool size of deoxyribonucleo-
tides are known to follow in vitro exposure of mouse embryo
cells to HU, and the pool size rapidly returns to normal following
removal of HU from the medium (26). However, the rate of new
DNA synthesis is only gradually restored following normaliza-
tion of the deoxyribonucleotide pools. This would suggest that
the recovery of the grain count following HU administration
takes place more rapidly than does the recovery of DNA
synthesis. One may estimate a 1- to 1.5-hr period required for

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Chart 1. The fraction of labeled mitoses in the murine Sarcoma 180 tumor
during a 22-hr period following i.v. tritiated thymidine administration. The curve
was fitted by computer analysis of the experimental data according to the method
of Takahashi et al. (27). The duration of each cell cycle interval in hr \((T)\) was
derived from this analysis and is presented in this figure with the coefficient of
variation \((CV)\).

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recovery of the grain count in the Sarcoma 180 tumor following HU administration; this would therefore be the minimal period necessary for renewed tumor DNA synthesis.

One-cycle Blockade and Release. HU was infused into tumor-bearing mice for 5 hr. Ringer's solution was infused for the following 25 hr, and the mice were sacrificed during this period. The tumor mitotic rate was determined at each time interval noted in Chart 3. The maximal mitotic rate was seen at the 8.5- to 10-hr interval following HU administration. While the 8.5- and 10-hr values did not differ significantly from one another, the 8.5-hr value is significantly greater than the 7-hr value (p < 0.01), and the 10-hr value is significantly greater than that at 11.5 hr (p < 0.05). The peak values reflect gathering of cells during HU infusion, in the mid to latter S phase. The maximal mitotic rate is twice that of control levels. A clear increment of recycled cells may be seen at 19 hr. This value is significantly greater than both the 17.5- and 20.5-hr values (p < 0.02 and 0.05, respectively). The maximal $T_i$ is approximately 12 hr; this represents an appreciable shortening of the 19.6-hr $T_c$ of control tumor.

Two-cycle Blockade and Release. HU was infused for 5 hr, then Ringer's solution was infused for 7 hr, and HU was again infused for 5 hr. The mice were killed during the 26 hr following release from the second blockade, and the tumor mitotic rate was determined (Chart 4). The maximal mitotic rate was now seen at the 12- to 13.5-hr interval and was 2.5-fold greater than control levels. The 12-hr value was significantly greater than the value at 10.5 hr (p < 0.02), and the 13.5-hr value was significantly greater than that of the 15-hr interval (p < 0.01). The peak in recycled cells was seen at 22.5 hr, and this value was significantly greater than values at the 21- and 24-hr intervals (p < 0.05 and 0.01, respectively). The maximal $T_i$ is again approximately 12 hr.

The curves from Charts 3 and 4 show an initial rise during the 4- to 5-hr interval following HU. This represents the G2-S junction. If the duration of G2 is not influenced by HU, the true tumor recovery time following the end of the HU infusion is about 1.7 to 2.7 hr.

Both the 1- and 2-cycle curves fall sharply following the 14-hr interval, which indicates passage through the G1-S junction.

The Determination of Cell Synchronization Following HU. Labeled thymidine was given in a single pulse to tumor-bearing animals, and the tumor LI ($\pm$ S.D.) was determined to be 38 $\pm$ 3% ($n = 9$), with a ML of 0.97 $\pm$ 0.4% ($n = 10$).

If the 2.3-hr duration of G2 is not affected by HU infusion, the administration of a single pulse of tritiated thymidine 4 hr following the end of the 1-cycle HU infusion should label those cells represented in the ML curve of Chart 3 during the 6.3- to 14.6-hr interval. This time interval reflects the maximal number of cells synchronized in S phase following 1-cycle HU infusion. Similarly, administration of a labeled thymidine pulse 5 hr following release of the 2-cycle blockade should label the maximal number of cells represented between the 7.3- to 15.6-hr interval of Chart 4.

Tritiated thymidine was given to mice 4 hr following 1-cycle HU and 5 hr following 2-cycle HU infusion, and the tumor LI was determined. The LI following the 1-cycle and 2-cycle experiments were 58 $\pm$ 3 and 70 $\pm$ 4, respectively.

Three-cycle Blockade and Release. There were 2 opposing considerations in planning experimental times for a third cycle
of HU blockade. First, large numbers of cells were in early S phase and at the G1-S junction following the 2-cycle blockade. A maximal release period should therefore be allowed, following the second HU infusion, for these cells to escape from S phase before reinstituting the HU blockade. However, it was also apparent that the T1 had become shorter following HU blockade; after the second HU cycle, the T1 was approximately 9 to 10 hr, and this included a mean duration of the DNA synthetic period of about 8.3 hr. If one allowed too long a release period following the second HU infusion, tumor cells would enter the following S phase and perhaps pass through it. The following infusion sequence was studied: HU, 5 hr; Ringer’s solution, 7 hr; HU, 5 hr; Ringer’s solution, 10 hr; and HU, 5 hr. This should permit approximately an 8-hr period of release following the second HU infusion. The mice were killed during the 16-hr period following the third HU infusion.

The data following 3-cycle blockade and release are presented in Chart 5. The mitotic peak at the 10.5-hr interval was 2.2 times control rates and was significantly greater than the 9- and 12-hr values (p < 0.05 and 0.01, respectively); this represents cells released from the second blockade which grew in S phase for several hr before the third HU blockade became effective. The M1 decreases significantly between the 13.5- and 15-hr periods (p < 0.05); this is the interval of the G1-S junction.

**DISCUSSION**

In the present series of experiments, significant increments of tumor cells were synchronized in their generative phase, during predictable intervals, and by each of 2 cycles of S-phase blockade and release. The durations of these cycles were chosen so as to allow the majority of tumor cells to be sequentially gathered in S phase, released into the following G2, mitotic, and G1 growth phases, and then regathered in S phase again, predominantly at the G1-S junction. The number of Sarcoma 180 tumor cells in S phase was 38% prior to HU administration, and this increased to 58 and 70% following 1- and 2-cycle HU treatment, respectively. Since this tumor has a high growth fraction, the increment in cells gathered in S phase does not represent cell recruitment from a nongenerating pool, but rather generating cells which have been made to grow in synchronous fashion. Sharp increments in mitotic activity during predictable periods of time were also noted.

The second wave of the curves of Charts 3 and 4 probably represents synchronous cohorts of cells passing through a second round of mitosis. During HU blockade, the rate of RNA and protein synthesis remains largely unchanged from normal (1, 4, 10, 21, 22, 28). It appears likely that during HU infusion proteins necessary for DNA synthesis continue to be synthesized and may be stored so that following the end of the infusion cycles of DNA synthesis will follow each other more closely. This is illustrated in the present experiments by the decrease in T1 following HU administration. As the excess initiator proteins are exhausted in each cell, normal cell cycle intervals should then be regained and the cell synchrony which followed the pulsed HU infusion will be lost.

For cycle-specific therapy to be effective, it is necessary that normal tissues, which are the usual site of therapy-limiting toxicity, be predominantly out of cycle phase with the tumor. This objective should be attainable. Studies have been carried out by other workers (6) in which single and multiple i.p. doses of HU were administered to mice bearing the S102F mammary tumor and at time intervals designed to enhance the killing of tumor cells and spare normal duodenal cells. Their results suggest that kinetic data may be useful in planning optimal intervals for 2-dose regimens of cycle-specific agents to maximize injury to tumor relative to the duodenal cells. However, their data were not sufficient to delineate the recovery pattern of tumor or duodenal cells following HU blockade so that this type of experiment could be designed with assurance.

The recovery pattern of the Sarcoma 180 tumor from HU blockade may be less complicated since most tumor cells are in the generative phase, obviating the need to consider recruitment of cells from nonproliferating pools. In addition, the duration of the S phase did not appear to be significantly influenced by HU infusion; the shortening of the T1 reflected primarily shortening of the T01.

This laboratory previously reported (19) that normal murine tissues were also partially synchronized in S phase following HU infusion. Tissues which would usually be considered to be likely sites of toxicity from cancer therapy, i.e., small intestine, tongue, bone marrow, and splenic mononuclear cells, had all completed their burst of mitotic activity and had passed through the area of the curve which reflected the G1-S junction within 8 hr following release from a single HU blockade. This would clearly separate these tissues from the 11- to 14-hr Sarcoma 180 mitotic peak which followed 2-cycle HU infusion. It appears likely that a mitotically active agent could be administered to damage tumor preferentially during this 3-hr period. However, it must be reiterated that we have limited knowledge of the recovery kinetics of the normal tissues following HU perturbation. The rate of cell recruitment from a nonproliferating compartment, the rate of maturation of these cells into cells no longer actively synthesizing DNA, and therefore independent of HU blockade, and alterations in tumor cell loss functions following repeated periods of HU blockade and release are among the incompletely understood variables which may influence the rate of differential injury to one tissue in comparison to another.
REFERENCES


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