Lethal and Sublethal Effects of Hydroxyurea in Relation to Drug Concentration and Duration of Drug Exposure in Sarcoma 180 In Vitro

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SUMMARY

The lethal and sublethal effects of hydroxyurea were studied in Sarcoma 180 in vitro in relation to drug concentration and drug exposure duration using cloning methods, radioautography, and flow microfluorometry. It was shown that postperturbation changes in radioautographic labeling intensity reflected real changes in the rate of DNA synthesis in individual cells. The data suggest that both the lethal and sublethal effects of hydroxyurea are dependent on the rate of DNA synthesis. These findings have important implications for the interpretation of DNA content distributions under perturbation conditions and for the development of drug treatment regimens that are based on cell kinetics.

INTRODUCTION

We have reported previously that the percentage of labeled mitosis curves and labeling indices in Sarcoma 180 in vitro are dependent on grain-counting threshold and emulsion exposure duration (26, 27). It was suggested from these studies that S phase was not sharply demarcated and that lightly labeled cells that were demonstrated in the percentage of labeled mitosis curve trough region at low counting threshold and prolonged emulsion exposure represented cells synthesizing DNA at low rates during much of what would otherwise be called the G1 phase of the cell cycle. These conclusions were based on the premise that there is a correlation between radioautographic labeling intensity following pulse [3H]TdR exposure and the rate of nuclear DNA synthesis. In the studies reported here, a comparison of the sequence of changes induced by hydroxyurea in radioautographic labeling intensity with the sequence of postperturbation changes in the DNA content distribution as determined by flow microfluorometry confirms that radioautographic labeling intensity in individual cells provides a measure of the relative rate of DNA synthesis.

Given that there are variations in the rate of DNA synthesis with cell cycle progression, several questions arise in addition to that relating to the sharpness of S-phase boundaries per se. In particular, are "cycle stage-specific" agents such as hydroxyurea more effective against cells that are synthesizing DNA rapidly than against slowly synthesizing cells? That is, does cycle stage specificity really imply DNA synthesis rate dependence? Is the partition between drug lethality and sublethal damage also dependent at least in part on DNA synthesis rate? Studies on the effects of hydroxyurea concentration and drug exposure duration on cell cloning in soft agar were carried out in parallel with corresponding radioautographic and flow microfluorometric studies. In this paper the 3 sets of data are considered together in relation to the above questions.

MATERIALS AND METHODS

All studies were carried out in Sarcoma 180 (Foley strain CCRFII, supplied by American Type Culture Collection, Rockville, Md.) grown in vitro in Earle's Medium 199 (Flow Laboratories, Rockville, Md.) supplemented with 5% fetal bovine serum; glutamine, 2 μmoles/ml; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. Cultures were grown at 37° in a 5% CO2 atmosphere. Cells were grown in monolayer in 250-ml plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.) containing 10 ml of medium, at an initial concentration of 1 x 10⁶ cells/ml.

Cloning Studies. Two-day-old log-phase cultures were incubated with hydroxyurea at final concentrations of 0.05, 0.5, 5.0, and 50 μM for either 1, 4, or 12 hr. The drug was then removed by rinsing each flask 4 times with Hanks' basic salts solution. Cultures were then refed with Medium 199. Control flasks were included for each time point and were treated identically with respect to rinsing and refeeding. At intervals, cells from duplicate flasks were collected by incubation with 0.25% trypsin (Flow Laboratories), stained with trypan blue, and counted. Harvested cells were diluted to a concentration of 1 x 10⁶ cells/ml, and 0.1 ml was introduced into each of 5 replicate 25-ml tissue culture flasks (Falcon Plastics) containing 4.5 ml of Earle's Medium 199 modified as described above but with a fetal bovine serum concentration of 30%. Flasks were incubated undisturbed for 1 hr to allow cells to settle and attach to the flask. Then 0.5 ml of warmed 3% agar solution was added to each flask. After 10 days of incubation, flasks were rinsed once with 0.85% NaCl solution and stained for 1 hr with 10% stock Giemsa solution. Macroscopic colonies generally ranged from 2.5 to 5 mm in diameter. In the absence of

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2 The abbreviation used is: [3H]TdR, tritiated thymidine.

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perturbation, 70 to 80% of log-phase cells commonly formed observable colonies. Cloning efficiency was calculated as colonies counted per flask per 100 cells inoculated per flask; the mean was computed for 5 replicate flasks at each time point for each experiment. Viable cell number at each time point after the introduction of drug was computed as total cell number at time $t \times$ cloning efficiency at time $t$. Both total and viable cell numbers at time $t$ were divided by total cell number at time zero to obtain normalized values for a given experiment. Each experiment was performed in triplicate, and the data reported represent the mean ± S.E. for normalized data from the 3 experiments.

**Radioautographic Studies.** Two-day-old log-phase cell cultures were incubated with hydroxyurea at final concentrations of 5 or 50 mM for either 2 or 12 hr and were harvested at intervals. Cultures were exposed to $[^3]$HTdR (specific activity, 2 Ci/mmol; New England Nuclear, Boston, Mass.) at a final concentration of 0.05 μCi/ml during the 30 min prior to sample collection. Radioautographs were prepared as previously described (27). Emulsion exposure duration was 4 days. Nuclear grain counts were obtained for 500 cells at each time point. Only those grains lying directly above or within 1 μm of the cell nucleus were counted. A local background grain count was obtained for each cell and was applied to each nuclear grain count, as previously described. The average background in these studies was 1 grain/cell.

**DNA Content Distribution Studies.** Cells exposed to 5 or 50 mM hydroxyurea for 2 or 12 hr were harvested at intervals. Cells from 2 replicate flasks were pooled, fixed in 70% ethanol, and stained with mithramycin (3) at a final concentration of 100 μg/ml and a final cell concentration of $1 \times 10^6$ cells/ml in each sample. Nuclear fluorescence was measured with a Los Alamos cell sorter. Data were recorded, stored, and subsequently displayed with a DEC 11/40 computer system, using software developed at the Los Alamos Scientific Laboratory, Los Alamos, N. M. All DNA content distributions were obtained at constant photomultiplier current and amplifier gain settings. At least 30,000 cells were measured in each sample. All DNA content distributions were normalized with respect to total cells analyzed per sample.

**RESULTS**

**Cell Cloning Studies.** The effects of the concentration of hydroxyurea and the duration of drug exposure on cell viability are shown in Chart 1, cell viability being defined by the ability to form colonies in soft agar, as described under "Materials and Methods." Cell viability decreased with increasing drug concentration. Loss of viability occurred early, i.e., within the 1st 2 to 4 hr, and was not progressive with time. Drug concentration appeared to be the major factor in determining the loss of cell viability, whereas duration of exposure to hydroxyurea did not appear to play a significant role. Cell survival curves following 1-, 4-, and 12-hr drug exposures were comparable for all sets of corresponding drug concentrations (in Chart 1, compare $A_2$, $B_2$, and $C_2$).

In order to rule out the possibility of drug inactivation in vitro, medium from cells exposed to hydroxyurea for 12 hr was added to fresh cells, and its effects were compared with those of spent medium containing no hydroxyurea. Twelve hr later, the hydroxyurea-containing medium proved as effective in killing fresh cells as it was originally. This would suggest that the failure of hydroxyurea to continue killing cells during prolonged exposure was due not to drug inactivation but to changes in the cells themselves.

Total cell number was affected by increasing doses of hydroxyurea to a much lesser degree than viable cell number (Chart 1, $A_2$, $B_2$, and $C_2$). Chart 1 shows that lethally damaged cells persisted for 24 hr or longer. There was no increase in total cell number during the 1st 12 hr at all concentrations of hydroxyurea sufficient to produce a decrease in the viable cell number (i.e., drug concentrations $\geq 0.5$ mM), suggesting that the cells that survived the drug did not continue to divide during this interval.

Ninety-five to 98% of the cells excluded trypan blue throughout the course of these studies in both the control and drug-treated populations. Thus, trypan blue exclusion did not reflect the hydroxyurea-induced loss of reproductive integrity that was demonstrated in the cloning assays.

**Radioautographic Studies.** Results of the radioautographic studies are shown in Chart 2. When the controls for the 2-hr (Chart 2A) and 12-hr (Chart 2B) studies are compared, it is apparent that a change of medium alone affected population labeling characteristics. While the minimum labeling index did not change following a change of medium, labeling intensity was reduced for up to 10 hr (Chart 2A). A fall in labeling intensity was also observed in all the cells exposed to hydroxyurea. This was observed at 2 hr in both the short-exposure (Chart 2, $A_2$, and $A_3$) and long-exposure studies (Chart 2, $B_2$ and $B_3$) prior to any change of medium. Thus, the initial reduction in labeling intensity is attributable specifically to hydroxyurea exposure and not to the medium change in the 2-hr or 12-hr exposure studies.

The effects of hydroxyurea on the labeling index at low grain count thresholds were more variable than its effects on labeling intensity. In the short-term drug exposure study at the 5 mM drug concentration, there was no significant fall in labeling index at the >1-grain/cell threshold at 2 hr compared with a drop from 0.52 to 0.38 at the >5-grain threshold (Chart 2A). At the comparable 2-hr time point in the 12-hr study, a >60% fall in labeling index was observed at all thresholds. The multithresholded labeling indices shown in Chart 2 represent background corrected data.

In the short exposure study, an increase in labeling intensity became apparent at 6 hr and peaked at 8 to 10 hr. This was more pronounced after exposure to 5 mM hydroxyurea (Chart 2A) than after exposure to the 50 mM concentration (Chart 2B). The labeling index at the >1-grain threshold increased only slightly at 6 to 10 hr.

With prolonged exposure to hydroxyurea, both the >1-grain threshold labeling index and the labeling intensity were depressed for up to 10 hr. There was an increase both in the low-threshold labeling index and in labeling intensity between 10 and 12 hr, despite the continued presence of the drug (Chart 2, $B_2$ and $B_3$).

**Flow Microfluorometry.** The effects on the DNA content distribution of a 2-hr exposure to hydroxyurea at 5 and 50 mM concentrations are shown in Chart 3. There was no
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Chart 1. Effects of hydroxyurea on cell number and cell viability in relation to drug concentration and drug exposure duration. A1, total cells as a function of time in control cultures and in those exposed to 0.05, 0.5, 5, and 50 mM hydroxyurea for 1 hr; B1, 4-hr exposure data; C1, 12-hr exposure data. Corresponding viable cell numbers, as determined by colony-forming ability, are shown in A2, B2, and C2, respectively. For discussion, see text.

striking effect on the DNA content distribution during the drug exposure period (Chart 3, B1 and C1). There was a slight broadening of the postmitotic peak at 4 hr at the 5 mM concentration (Chart 3B1). At 6 hr a wave of cells appeared in the early S region (Chart 3B2) that broadened as it traversed the mid-S region over the next several hr (Chart 3B4) and appeared in the premitotic region at 12 hr (Chart 3B5). An increase in the postmitotic peak was observed at 16 hr (Chart 3B6), reflecting the division of some of these cells. By 24 hr the DNA content distribution appears to have returned nearly to normal, but the premitotic peak remained more prominent than in the controls (Chart 3B7).

At the 50 mM concentration, the broadening of the postmitotic peak was not observed until 6 hr (Chart 3C1). A wave...
of cells in the early S region became obvious at 8 hr (Chart 3C1). Its progress through the cell cycle was slower than that observed at the 5 mM concentration, as evidenced by the failure of late S and of premitotic cells to divide and replenish the postmitotic peak at 16 hr at the higher drug concentration (Chart 3C3).

When the radioautographic data are considered together with the flow microfluorometric data, it becomes apparent that there is a correlation between cell labeling intensity and the rate of DNA synthesis. At the 5 mM drug concentration, the increase in labeling intensity at 6 to 10 hr (Chart 2A2) was paralleled by the appearance of a population of cells that traversed the S region of the DNA content distribution rapidly (Chart 3, B3 to B5). That is, the increase in [3H]TdR incorporation coincided with a demonstrable, independently measured increase in the rate of DNA synthesis. When
the data obtained at the 50 mM concentration are compared with those obtained at the 5 mM concentration, it is apparent that the increase in labeling intensity at 6 to 10 hr was less pronounced (in Chart 2 compare A2 and A3). Similarly, in the flow microfluorometric studies, the population of cells that were recruited following hydroxyurea exposure traversed the cell cycle more slowly than at the lower drug concentration (in Chart 3 compare B2 and B3), supporting the relationship between cell grain count and DNA synthesis rate.

The effects on the DNA content distribution of a 12-hr exposure to hydroxyurea at the 5 and 50 mM concentrations are shown in Chart 4. During the 12-hr exposure to hydroxyurea, there were no significant changes in the DNA content distribution (Chart 4, B1 to B5 and C1 to C5), despite the fact that there were pronounced changes in the low-threshold labeling indices and labeling intensity over the same period (Chart 2, B2 and B3).

The DNA content distributions at 24 hr (Chart 4, B7 and C7) suggested recovery sequences following prolonged drug
exposure that were similar to those observed following the 2-hr drug exposures, but data sampling was too infrequent at late time points to draw firm conclusions. A separate study was carried out to elucidate the kinetic recovery patterns following prolonged exposure to hydroxyurea. The results are shown in Chart 5. Following exposure to the 5 mM concentration, the recovery sequence appears to have been quite similar to that observed following the 2-hr exposure. A wave of cells traversing the S region appeared at 16 hr (Chart 5B5), 4 hr after drug removal. Cells accumulated in the late S and premitotic region 8 to 10 hr after drug removal (Chart 5, B5 and B6). At 12 to 24 hr after drug removal, there was still a significant cell fraction remaining in the premitotic region (Chart 5, B7 to B10).

Following a 12-hr exposure to the 50 mM concentration, the onset of recovery was delayed until 22 hr, i.e., until 10 hr after drug removal (Chart 5, C4). Once begun, however, the recovery patterns appeared to follow a sequence and time
Chart 5. Late effects of a 12-hr exposure to hydroxyurea on the DNA content distribution. A, to A, control cultures (medium change at 12 hr); B, to B, 5 mM hydroxyurea (12-hr exposure); C, to C, 50 mM hydroxyurea (12-hr exposure). For discussion, see text.

course like those observed following shorter drug exposures (Chart 5, C, to C).

DISCUSSION

Hydroxyurea Lethality. The lethal and sublethal effects of hydroxyurea have been studied extensively in mammalian cell systems. Hydroxyurea has been shown to inhibit DNA synthesis (1, 6, 8–10, 12, 18–22, 24, 28, 29, 34, 35) and to kill cells selectively in S phase in vitro and in vivo (6, 8, 10, 12, 19, 28, 29). On this basis hydroxyurea has been classified kinetically as a cell cycle phase-specific agent.

The patterns of DNA synthesis in Sarcoma 180 in vitro have been studied previously in some detail by radioautographic methods in which [3H]TdR was used (26, 27). In this system both cell labeling indices and cell labeling intensity were dependent on relative radioautographic exposure and
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The mid-S cells appeared labeled even at low relative exposure and/or high-grain-count thresholds. As the efficiency of detection of radioautograph labeling was increased, progressively more lightly labeled cells were found throughout much of the rest of the cell cycle, and the "duration of S phase" required corresponding upward revision. The effects of increasing [3H]TdR dosage have been shown to be comparable to those of increasing emulsion exposure duration (17).

In the present study, the lethal effects of hydroxyurea in Sarcoma 180 in vitro have been shown clearly to be drug concentration dependent, a phenomenon that has been observed with this drug in other cell systems (12, 19, 28). In view of the [3H]TdR labeling patterns in Sarcoma 180, the possibility comes to mind that there may be some correlation between radioautographic labeling intensity and susceptibility to the lethal effects of hydroxyurea. That is, mid-S cells that are synthesizing DNA rapidly and would exhibit moderate to heavy radioautographic labeling under a variety of [3H]TdR labeling conditions might also be susceptible to the lethal effects of hydroxyurea over a broad range of drug concentrations. Cells found earlier and later in the cycle that are synthesizing DNA at low rates may label lightly or not at all, depending on relative [3H]TdR exposure. Such cells might be subject to sublethal damage at low concentrations of hydroxyurea and might be killed only at higher drug concentrations.

There are several lines of evidence to support the concept of DNA synthesis rate-dependent drug lethality. (a) The present study indicates that hydroxyurea produces a prompt fall in [3H]TdR labeling intensity in individual cells. This has also been observed in other systems, both in vitro (29) and in vivo (21). In the present study, the reduction in labeling intensity of the most heavily labeled cells was observed consistently both at the 5 mM and the 50 mM drug concentrations (Chart 2). (b) As noted above, the initially heavily labeled cells can be localized to the mid-S region of the cell cycle (26, 27). (c) Observations in synchronized cell populations indicate that cells in mid-S are more sensitive to the lethal effects of hydroxyurea than are cells in early and late S (12-14, 29). It is reasonable to conclude from these collected observations that the most heavily labeled cells are the most susceptible to the lethal effects of hydroxyurea.

Radioautographic Labeling Intensity and the Rate of DNA Synthesis. The extension of the concept of S-phase specificity to denote DNA synthesis rate dependence is based on the critical premise that [3H]TdR pulse radioautographic labeling intensity reflects the rate of DNA synthesis in individual cells. This premise has been the subject of some controversy. Baserga and Malamud (2) held that [3H]TdR incorporation is affected by so many factors other than the rate of DNA synthesis that inferences regarding the latter should not be drawn from radioautographic grain counts. It may well be that potentially uncontrolled variables such as local [3H]TdR availability, endogenous thymidine pool size and synthetic rate, and radioautographic processing conditions may affect the absolute grain counts over individual cells and increase the variability in observed grain counts among cells that might otherwise be comparable kinetically. The critical question is whether such effects are of sufficient magnitude to invalidate inferences regarding relative rates of DNA synthesis that might be drawn from observed instudy differences in radioautographic cell labeling intensity.

In studies designed to explore [3H]TdR labeling intensity in relation to relative position in S, it has been shown that the incorporation of [3H]TdR is maximal in mid-to-late S (7, 11, 23, 30, 33). Can one infer from this that the rate of DNA synthesis is higher in mid-to-late S? In the studies of Dörner et al. (7), 5-fluorodeoxyuridine was used to block endogenous thymidine synthesis, removing the potential effects of this variable on radioautographic labeling intensity. In this study it was possible to equate increased mid-S [3H]TdR incorporation with a higher rate of DNA synthesis. Schaer et al. (23) showed that peak [3H]TdR incorporation in mid-S was accompanied by similar patterns of incorporation of [3H]thymidine, [3H]cytidine, and [3H]deoxyadenosine. The authors concluded that [3H]TdR incorporation should provide a reasonably good measure of the rate of DNA synthesis in individual cells.

These conclusions have also been borne out in drug and radiation perturbation studies. Painter (16) showed that following irradiation the reduction in specific activity of labeled thymidine incorporated into DNA as a function of radiation dose was paralleled by reductions in the specific activities of incorporated labeled orotic acid and deoxyguanosine. Denby and Smith (5) showed that the decrease in radioautographic labeling intensity that followed the irradiation of cultured L-cells was accompanied by an independently measured rate of DNA synthesis that was lower than that of controls. Similarly, Terasima and Tolmach (31) showed that the decrease in labeling intensity in irradiated cells was accompanied by a division delay that was due at least in part to a prolongation of the DNA-synthetic period.

In the present drug study, following a 2-hr exposure to hydroxyurea, there was a sharp reduction in radioautographic labeling intensity per cell, followed several hr later by a brisk rise in [3H]TdR incorporation per cell both at intermediate and at high drug concentrations (Chart 2). Hydroxyurea is also known to inhibit the incorporation of [32P]phosphate (24, 35) and labeled deoxyctydine, deoxyadenosine, and deoxyguanosine (1, 20, 35), indicating that the reduction in [3H]TdR incorporation reflects a true decrease in the rate of DNA synthesis. In this study, the increase in cell labeling intensity per cell at 6 to 10 hr (Chart 2 A2 and A3) coincided temporally with the appearance of a wave of cells traversing the S region of the DNA content distribution rapidly (Chart 3, B2 to B3 and C2 to C3), clearly demonstrating the relation between radioautographic labeling intensity and the rate of DNA synthesis in individual cells.

To be sure, one must exercise great care in the general interpretation of changes in radioautographic labeling intensity under perturbation conditions. This is especially true when the perturbing agent exerts selective effects on the endogenous TdR synthesis pathway. Following 5-fluorouracil administration, an increase in [3H]TdR incorporation can be observed (4, 15). However, even under these circum-
stances, when cells are damaged sufficiently to reduce the overall rate of DNA synthesis, the incorporation of exogenous [\(^3\text{H}\)]TdR is also reduced. Thus, for example, Myers et al. (15) found that, following an initial brief stimulation of [\(^3\text{H}\)]TdR incorporation by 5-fluorouracil, the subsequent temporal pattern of [\(^3\text{H}\)]TdR incorporation into DNA paralleled that of [\(^3\text{H}\)]deoxyuridine incorporation. Similarly, there is a lower rate of exogenous [\(^3\text{H}\)]TdR incorporation into DNA in pernicious anemia, despite the fact that [\(^3\text{H}\)]TdR bypasses the metabolic block in this disorder, reflecting a defect in overall DNA synthesis rate (32).

There are ambiguities that might arise in the interpretation of in vitro changes in radioautographic labeling intensity under perturbation conditions. These are illustrated in the present study by the reduction in labeling intensity following a change of medium alone in the control cells (Chart 2A). It is possible that freshly added fetal calf serum provided an excess of cold thymidine that might have reduced the rate of [\(^3\text{H}\)]TdR incorporation, but did not materially affect the rate of DNA synthesis. Alternatively, the reduction in labeling intensity may reflect a reduction in DNA synthesis rate induced somehow by the change of medium. In earlier studies, changes of medium were shown to affect the DNA content distribution (27), supporting the latter interpretation. In the present study, no striking medium change effects could be identified in the DNA content distribution (in Chart 3 compare A to A). Clearly, this problem requires further study. For the present we would emphasize that this is a problem that is likely to arise only in studies performed in vitro, where one can include appropriate controls to distinguish drug effects from potential medium change effects, as in the present study.

Sublethal Effects. The present studies suggest that the sublethal effects of hydroxyurea may also be DNA synthesis rate dependent. As noted earlier, in Sarcoma 180 in vitro, there are low rates of [\(^3\text{H}\)]TdR incorporation throughout much of what would otherwise be considered “G,” “G,” and “G.” Sinclair (28, 29) showed that cells in G and G that survived exposure to hydroxyurea were, nonetheless, prevented from initiating active DNA synthesis during and shortly after the period of drug exposure. Does the reversible G block reported by Sinclair represent sublethal suppression of initially low rates of DNA synthesis? The 12-hr drug exposure studies suggest that this might be the case. Although there was a sharp drop in labeling index and radioautographic labeling intensity during the period of drug exposure, lightly labeled cells persisted (Chart 2, B and B), indicating that the rate of DNA synthesis was sharply reduced but not abolished. Despite these radioautographic changes, the DNA content distribution remained unchanged during the period of drug exposure (Chart 4, B to B and C to C). It can be shown on theoretical grounds that, if the rate of cell progression through the cycle is reduced, the DNA content distribution remains unaltered only when the rate of cell progression is reduced proportionally at every point in the cell cycle (25). Presumably, then, lightly labeled, slowly synthesizing G and G cells underwent reductions in the rate of DNA synthesis that were proportional to the reductions in the DNA synthesis rates of the heavily labeled mid-S cells. The absence of additional cell kill with prolonged drug exposure (Chart 1) is also consistent with this view. Together with the labeling data (Chart 2), it suggests that slowly synthesizing cells that were protected from the early lethal effects of hydroxyurea underwent further suppression of their initially low rates of DNA synthesis; this, in turn, may have protected them from cumulative damage during subsequent prolonged drug exposure.

Interpretation of the DNA Content Distribution under Perturbation Conditions. Our data illustrate several important points regarding the interpretation of DNA content distribution data under perturbation conditions. The accumulation of cells in a particular region of the distribution may represent any 1 of several different kinetic processes: (a) a transient increase in the rate of cell cycle traverse (synchronization and/or recruitment); (b) a reduction in the rate of cell cycle traverse with protracted sequestration of cells in a particular region of the DNA content distribution; and (c) irreversible damage with complete arrest of cell cycle progression. It would appear that all of these phenomena are reflected in our data at various times after drug exposure.

An examination of the 2-hr drug exposure flow microfluorometry data between 12 and 24 hr suggests that the accumulation of cells in the late S-premitotic region at 12 hr represents the summation of several different kinetic processes. It is evident from both the flow microfluorometry studies and the radioautographic data that there was a wave of recruited cells that reached the premitotic region at 12 hr (Charts 2A and 3B to B). Presumably, it was this subpopulation that went on to divide, contributing to the reconstruction of the postmitotic peak of the DNA content distribution 16 hr after exposure to the 5 mM drug concentration (Chart 3B).

It is apparent that a prominent premitotic peak was still present at 16 hr (Chart 3B). Presumably, many of the cells remaining in the premitotic region at 16 hr were members of a growth-retarded subpopulation the kinetic recovery of which was not apparent until 24 hr (Chart 3B). The presence of this growth-retarded subpopulation is more easily discerned at the 50 mM drug concentration, where there was a persistent accumulation of cells in the S and premitotic regions and a failure to replete the postmitotic peak at 16 hr (Chart 3C).

In all likelihood, the cells that persisted in the premitotic region of the DNA content distribution at 24 hr (Chart 3, B and C) were lethally damaged cells that had been rapidly proliferating and were in mid-S at the time of initial drug exposure. However, the late S-premitotic peak at 24 hr is not sufficient to account for all of the nonviable cells present at 24 hr, the latter comprising some 50 to 80% of the population at that time at the 5 mM and 50 mM concentrations (Chart 1). Furthermore, since 60 to 70% of the cells exhibited moderate to heavy radioautographic labeling between 6 and 10 hr (Chart 2, A and A) while only 20 to 50% of the cells were viable during this time interval, it would appear that some cells that participated in the recruitment phenomenon and may have gone on to divide once were nonetheless destined to die.

Our data suggest that there is no single point or single phase in the cell cycle at which cells are blocked. Rather, cells are affected at multiple points in the cell cycle, and
the effects vary in magnitude and degree of reversibility at different points in the cycle. The data are consistent with the concept that drug damage occurs in graded fashion, varying with initial drug concentration and the rate of DNA synthesis in individual cells at the time of drug exposure. Sublethal damage that is expressed as interference with cell progression through the cycle cannot be described in simple stop and go terms but in terms of a continuous spectrum of rate changes.

A better understanding of the interactions of hydroxyurea with heterogeneous mammalian cell populations and more detailed comparisons with other "cycle phase-specific" agents and "nonspecific" agents may lead to the development of more realistic approaches to the optimization of drug scheduling in cancer chemotherapy.

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