ABSTRACT

Continuous exposure of exponentially growing 9L rat brain tumor cells for 24 hr to a nontoxic dose (0.77 \textmu M) of 5-fluorouracil (5-FUra) produced a progressive increase in S-phase cells from 35% (asynchronous culture) to 70% as shown by DNA histograms based on data obtained by flow cytometry. When 9L cells were treated with the S-phase-specific agent hydroxyurea (1.3 mM) immediately after treatment with 5-FUra, a synergistic cell kill resulted. A centrifugal elutriation study confirmed that enhanced cell kill was caused in part by the S-phase synchrony produced by 5-FUra and to the S-phase specificity of hydroxyurea. A higher dose (7.7 \textmu M) of 5-FUra caused a partial G1-S block; subsequent treatment with hydroxyurea also enhanced cell kill, but the enhancement was not related to S-phase synchrony. A centrifugal elutriation study suggested that, after 24-hr treatment with 5-FUra, hydroxyurea might kill both cells in S phase, which has the greater number of clonogenic cells, and kill cells that are in other phases of the cell cycle, including cells blocked at the G1-S border that are vulnerable to the phenomenon of "thymidine death"; concomitant administration of thymidine along with 5-FUra eliminated enhanced cell kill.

INTRODUCTION

Most cytotoxic agents used to treat neoplasms both kill cells and perturb the progression of cells through the mitotic cycle; perturbations can delay DNA synthesis, block progression of the cell cycle at a certain phase, and thereby either synchronize or cause cells to accumulate at some point in the mitotic cycle. Patterns of perturbations sometimes depend on the drug dose and the length of drug treatment. Therefore, fractionated protocols with a single agent and/or a combination of several agents might be designed to take advantage of these perturbations to achieve increased cell kill or synergism.

5-FUra has been used extensively for the treatment of various cancers and, because it crosses the blood-brain barrier, has been used in combination with other drugs for the treatment of malignant brain tumors (9, 10). We have shown that a nontoxic dose (0.77 \textmu M) of 5-FUra causes 9L rat brain tumor cells to accumulate in S phase in vitro using flow cytometry and that a higher, but still relatively low, dose of 5-FUra (7.7 \textmu M) produces a partial G1-S block that causes cells to accumulate in G1 phase (21).

Bhuyan et al. (2) have shown that pretreatment of L1210 cells with a nontoxic dose of 5-FUra caused a partial S-phase synchrony and, when combined with a second S-phase-specific agent, produced synergistic cell kill. In the studies reported here, we used a low and nontoxic and a higher but still relatively low dose of 5-FUra to compare the ways in which perturbation of cells caused by 2 doses of 5-FUra affects the cytotoxicity of the S-phase-specific agent HU (3, 11, 15, 18) that was administered after 5-FUra pretreatment. We investigated cytokinetic phenomena that were the result of using the 2 drugs in combination using centrifugal elutriation.

MATERIALS AND METHODS

9L Cell Culture. 9L cells were grown in monolayer culture with CMEM consisting of MEM supplemented with nonessential amino acids, 10% fetal bovine serum, and gentamicin (50 \mu g/ml) (1, 7, 8, 21). Cell survival was determined with a CFE assay (7, 8). SFs were calculated as the ratio of the CFEs of treated cells to the CFEs of control cells. When the 2 drugs were combined, the expected additive cell kill was estimated as the product of the SFs of each drug acting alone (20).

Drugs and Treatment. Stock solutions of 5-FUra (fluorouracil injectable; Roche Laboratories, Nutley, N.J.), HU (Calbiochem-Behring, La Jolla, Calif.), and thymidine (Calbiochem) were added to exponentially growing cells at different volumes to achieve desired final concentrations. We treated 9L cells with 2 doses of 5-FUra; 0.77 \textmu M is a nontoxic dose, and 7.7 \textmu M is a higher, but still relatively low, dose of the drug as determined in other studies (21). The effect of HU given immediately after 0 to 36 hr of 5-FUra pretreatment was measured with a CFE assay in order to determine the extent of enhanced cell kill by the combination. Because of dose-response and time-response curves that showed the effect of HU on 9L cells (data not shown), HU (1.3 mM for 6 hr) was chosen as an S-phase-specific agent.

Various treatment schedules with 1.3 mM HU for 6 hr and 0.77 \textmu M or 7.7 \textmu M 5-FUra for 24 hr were tested to determine the schedule that produced the maximum cell kill. Thymidine, which is supposed to bypass the blocking effect of 5-fluorodeoxyuridine monophosphate on thymidylate synthetase, was given concomitantly with 5-FUra to determine its effect on the drug combination.

For combination treatments, medium that contained the first drug was decanted, cells were rinsed twice with MEM, and then they were refed with CMEM that contained the second drug. Drugs were administered to 9L cells between 1 and 3 days after seeding, so that drug exposure was completed during the logarithmic growth phase of cells.

Centrifugal Elutriation. The cytokinetic contribution of 5-FUra perturbation on 9L cells in the 5-FUra-HU combination treatment protocols was assessed using centrifugal elutriation. Approximately 1 x 10^6 control or 5-FUra-treated cells were injected into the Beckman elutriation system (Beckman Instruments, Inc., Palo Alto, Calif.). The flow rate of CMEM (4\textsuperscript{th}) was held constant at 32 ml/min, and the rotor speed was reduced from 2400 to 1500 rpm in 100-rpm decrements. About 85% of the injected cells were recovered in the elutriated fractions. Each fraction was monitored routinely for DNA content by FCM and plated for CFE.
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Different fractions of elutriated cells were plated into Petri dishes. When HU was administered after 5-FUra treatment, elutriated cells were plated first and then treated with HU for 6 hr at 37°C, after which cells were rinsed twice, refed with CMEM, and incubated for 14 days for CFE. Cells attached to the dishes within 1 hr after plating; no cells were lost when the medium was changed.

FCM Analysis. Approximately 1 x 10⁶ cells in suspension were fixed with 70% alcohol, stained with chromomycin A3 (Calbiochem), and suspended in distilled water for FCM analysis (5) using a FACS III flow cytometer (Becton-Dickinson, Mountain View, Calif.). Data were analyzed using Dean’s computer program (6).

RESULTS

Survival and DNA Distribution Pattern of 9L Cells Treated with 5-FUra. After a 24-hr treatment with 0.77 μM 5-FUra (35% of asynchronous 9L cells are in S phase), 70% of 9L cells accumulated in S phase (21). However, when cells were incubated in CMEM after a 24-hr treatment, they gradually became asynchronous and began to proliferate. The survival curve (Chart 1A) shows that cell kill was minimal for this dose of 5-FUra for an exposure of up to 24 hr.

After treatment with 7.7 μM 5-FUra, the number of 9L cells in S and G2-M phase decreased, and there was a relative increase in the G1 population (21). When cells were incubated in drug-free CMEM after a 24-hr treatment with 7.7 μM 5-FUra, the pattern of DNA distribution remained relatively constant up to 24 hr, and the cell number remained the same or decreased moderately for the 24-hr incubation period. Although more than 95% of cells treated for 24 hr excluded trypan blue dye, many of them were doomed to die; the SF was approximately 13% (Chart 1B).

5-FUra-HU Combination Treatment. When 0.77 μM 5-FUra was administered for 12 hr or more, subsequent administration of 1.3 mM HU for 6 hr increased cell kill markedly (Chart 1A): 11% cell survival at 24 hr compared to the expected cell survival (73% of 24 hr). This dose of HU administered alone produced approximately 22% cell kill.

Treating cells for 12 hr or more with 7.7 μM 5-FUra followed by a 6-hr treatment with HU also produced enhanced cell kill (Chart 1B); cell survival was 2% at 24 hr compared to the expected survival of 10% at 24 hr.

When the length of treatment with 5-FUra was fixed at 24 hr and when the duration of subsequent treatment with 1.3 mM HU was changed, enhanced cell kill was observed beyond the 3-hr treatment with HU (Chart 2A). After a 24-hr treatment with 0.77 μM or 7.7 μM 5-FUra, a dose of greater than 1 mM HU for 6 hr was needed to produce enhanced cell kill (Chart 2B).

Schedule Dependency of the Combination Treatment. HU (1.3 mM) was administered for 6 hr beginning at various times during and after the 24-hr 5-FUra treatment period (Chart 3). The most marked cell-killing effect was produced when HU was administered immediately after 5-FUra. When cells were incubated for 6 hr in CMEM after a 24-hr treatment with 5-FUra, enhanced levels of cell kill were lower than those produced by treatment with HU immediately after treatment with 5-FUra.

For 0.77 μM 5-FUra, the recovery of cell survival was related to the desynchronization of cells that had accumulated in S phase after treatment. For 7.7 μM 5-FUra, no correlation could be found between the recovery of survival and the DNA distribution pattern during incubation of cells in CMEM after treatment with 5-FUra.

Effect of Thymidine on the 5-FUra-HU Combination. Thymidine (10 μM), given concomitantly with 0.77 μM 5-FUra for 24 hr, did not change the cytotoxicity of 5-FUra but did prevent the enhanced cell kill of the 5-FUra-HU combination (Table 1). The same dose of thymidine given concomitantly with 7.7 μM 5-FUra for 24 hr decreased markedly the cytotoxicity of 5-FUra (to 14% cell kill) and prevented the enhanced cell kill obtained by the 5-FUra-HU combination. The DNA distributions of 9L cells that were treated with 5-FUra (0.77 or 7.7 μM) and thymidine (10 μM) for 24 hr were almost the same as control.

Centrifugal Elutriation Study. The percentages of cells in various phases of the cell cycle in each elutriated fraction of untreated, exponentially growing 9L cells and of cells treated with both doses of 5-FUra for 24 hr are shown in Chart 4. Most
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1600 and 1500 rpm, which were enriched in S-phase cells, had a higher repopulation rate than did cells in other fractions (2200 to 1800 rpm) that contained mostly G1 cells. CFEs for fractions consisting of S-phase cells (1600 and 1500 rpm) treated for 6 hr with 1.3 mM HU after elutriation decreased from 11 to 12% to 1 to 2%, and CFEs of fractions consisting of G1 cells (2200 to 1800 rpm) decreased from 3 to 7% to 0.4 to 1.0%.

DISCUSSION

We have reported that the nontoxic dose (0.77 μM) of 5-FUra produced a partial S-phase synchrony in 9L cells after a moderately long exposure (21). When these cells were treated subsequently for 6 hr with a toxic dose (1.3 mM) of HU, synergistic cell kill was obtained (Chart 3A). If synchronized 9L cells were held in CMEM before treatment with HU, enhanced cell kill was reduced (Chart 3). These results explain the synergism by S-phase synchrony reported by Bhuyan et al. (2). Cells that accumulate in S phase as a result of a decreased rate of DNA synthesis should have a different intracellular metabolism than untreated S-phase cells. Using centrifugal elutriation, we showed that HU killed more cells synchronized into S phase by 5-FUra than other unperturbed S-phase cells.

HU given after a low but moderately toxic dose (7.7 μM) of 5-

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Table 1

| First drug (24 hr) | Second drug (6 hr) | SF
|-------------------|-------------------|---
| 5-FUra (μM) | Thymidine (μM) | HU (mM) |     |
| 0.77 | 10 | 1.3 | 0.97 |
| 0.77 | 10 | 1.3 | 0.98 |
| 0.77 | 10 | 1.3 | 0.98 |
| 0.77 | 10 | 1.3 | 0.13 |
| 0.77 | 10 | 1.3 | 0.81 |
| 7.7 | 10 | 1.3 | 0.13 |
| 7.7 | 10 | 1.3 | 0.86 |
| 7.7 | 10 | 1.3 | 0.922 |
| 7.7 | 10 | 1.3 | 0.75 |

* Thymidine (10 μM) was given concomitantly with 5-FUra (0.77 μM, 7.7 μM) for 24 hr to 9L cells with or without subsequent 6-hr HU (1.3 mM) treatment.

* Mean of at least 4 samples from at least 2 separate experiments. S.E.s were within 10% of each value.

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S-phase control cells were elutriated at 1700 to 1900 rpm, but S-phase cells from the 5-FUra-treated groups were obtained at a lower rotor speed. CFEs of elutriated control cells were 60 to 70%, with little fraction-to-fraction variation (Chart 5). When each elutriated fraction was treated in Petri dishes with 1.3 mM HU for 6 hr, cells in the 1900- and 1800-rpm fraction, which was enriched with S-phase cells, had the lowest cell survival.

All elutriated fractions of cells treated for 24 hr with the 0.77 μM 5-FUra had essentially the same CFE (Chart 6), but treating cells in each fraction with HU after elutriation killed more cells in the 1800- to 1600-rpm fraction, which was enriched in S-phase cells, than in the other fraction.

Cells treated with 7.7 μM 5-FUra for 24 hr, which had a SF of 13%, were elutriated (Chart 7). Populations that elutriated at

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Chart 3. Survival of 9L cells treated with both 0.77 μM (•) or 7.7 μM (▲) 5-FUra for 24 hr and 1.3 mM HU for 6 hr on various schedules. Values are plotted at the point at which HU was administered to the time reference with the 24-hr 5-FUra treatment (—). The assay was performed immediately after the second treatment. Points, mean of 4 samples from 2 separate experiments; bars, S.E. The additive values calculated are shown (I I). 1, cells treated with 5-FUra for 6 hr, rinsed twice, treated with both 5-FUra and HU for 6 hr, rinsed twice, then treated with 5-FUra for 12 hr and assayed; 2, cells treated with 5-FUra for 24 hr, rinsed twice, incubated in CMEM for 6 hr, then treated with HU for 6 hr and assayed.

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Table 1

Effect of thymidine on 5-FUra-HU combination treatment

| First drug (24 hr) | Second drug (6 hr) | SF
|-------------------|-------------------|---
| 5-FUra (μM) | Thymidine (μM) | HU (mM) |     |
| 0.77 | 10 | 1.3 | 0.97 |
| 0.77 | 10 | 1.3 | 0.98 |
| 0.77 | 10 | 1.3 | 0.13 |
| 0.77 | 10 | 1.3 | 0.81 |
| 7.7 | 10 | 1.3 | 0.13 |
| 7.7 | 10 | 1.3 | 0.86 |
| 7.7 | 10 | 1.3 | 0.922 |
| 7.7 | 10 | 1.3 | 0.75 |

* Thymidine (10 μM) was given concomitantly with 5-FUra (0.77 μM, 7.7 μM) for 24 hr to 9L cells with or without subsequent 6-hr HU (1.3 mM) treatment.

* Mean of at least 4 samples from at least 2 separate experiments. S.E.s were within 10% of each value.

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S-phase control cells were elutriated at 1700 to 1900 rpm, but S-phase cells from the 5-FUra-treated groups were obtained at a lower rotor speed. CFEs of elutriated control cells were 60 to 70%, with little fraction-to-fraction variation (Chart 5). When each elutriated fraction was treated in Petri dishes with 1.3 mM HU for 6 hr, cells in the 1900- and 1800-rpm fraction, which was enriched with S-phase cells, had the lowest cell survival.

All elutriated fractions of cells treated for 24 hr with the 0.77 μM 5-FUra had essentially the same CFE (Chart 6), but treating cells in each fraction with HU after elutriation killed more cells in the 1800- to 1600-rpm fraction, which was enriched in S-phase cells, than in the other fraction.

Cells treated with 7.7 μM 5-FUra for 24 hr, which had a SF of 13%, were elutriated (Chart 7). Populations that elutriated at

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Chart 4. Percentages of G1-phase (●), S-phase (□), and G2-M-phase (●) 9L cells in the various fractions of cells collected by centrifugal elutriation (A, control; B, cells treated with 0.77 μM 5-FUra for 24 hr; C, cells treated with 7.7 μM 5-FUra for 24 hr). Values are generated by computer analysis of DNA histograms obtained from the cells in each fraction.

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5-FUra, which caused a partial G1-S transition block in treated 9L cells, produced increased cell kill compared to the expected additive cell kill (Chart 18). It is not possible that HU killed cells released into S phase from G1-S block caused by treatment with 7.7 μM 5-FUra, because during the drug-free interval, cells did not tend to move into S phase in a synchronized fashion.

Results of the elutriation study (Chart 7) showed that cells in S phase after a 24-hr 7.7 μM 5-FUra treatment had a higher repopulation rate than did cells in other phases of the cell cycle, even though their absolute number was small. This finding suggests that cells that could synthesize DNA, or at least could remain in S phase after a 24-hr 5-FUra treatment, were more refractory to 5-FUra cytotoxicity. The fact that a portion of these cells was killed by HU (Chart 7) might explain the enhanced cell kill obtained by the 5-FUra-HU combination at this concentration. If the elutriated fractions of 9L cells that remained in G1 phase after a 24-hr exposure to 7.7 μM 5-FUra had a lower repopulation rate because of "thymidineless death" (4, 16, 18), another 6-hr treatment with HU, a potent G1-S blocking agent (18, 19), might increase cell kill as well (Chart 7).

The cytotoxicity of 5-FUra is thought to be the result of 2 mechanisms: DNA-directed cytotoxicity that is the result of conversion of 5-FUra into 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase, depleting cells of thymidine nucleotides and thereby inhibiting DNA synthesis; or RNA-directed cytotoxicity caused by incorporation of 5-FUra into RNA. The finding that thymidine, given together with either 0.77 or 7.7 μM 5-FUra for 24 hr, eliminated both the cytotoxicity of 5-FUra and the enhanced cytotoxicity of the 5-FUra-HU combination (Table 1) indicates that the enhancement was the result of DNA cytotoxicity (12). As a matter of fact, no increased uptake of tritiated 5-FUra into RNA was observed in 9L cells treated with labeled compound on the protocol schedules.5

Centrifugal elutriation separates cells by sedimentation rate,

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5 S. Kobayashi and T. Hoshino, unpublished results.
which depends on cell density, shape, and volume; thus, elutriated cells used in this study were not separated strictly into cell cycle phases. For example, G1-phase cells obtained after treatment with 7.7 µM 5-FUra were elutriated at rotor speeds between 2200 and 1900 rpm, but G1 cells from untreated cultures were elutriated at rotor speeds of 2200 to 2100 rpm (Chart 4). This change in the sedimentation rate of treated cells may reflect subtle changes, such as an increase in the amount of RNA and/or protein, in G1 cells that are not related to "maturational age" (17).

The results reported here show that non- or moderately toxic doses of 5-FUra, which cause different perturbation patterns, used in combination with HU can cause greatly increased cell kill in vitro, and the results indicate further that some biochemical mechanism, for example, effects of HU on deoxy- and 5-fluoro-deoxyuridine monophosphate pools after 5-FUra treatment (13, 14), should be sought to explain this synergism. The effects of the same or similar combinations of 5-FUra and HU must be tested in an animal model, and any possible toxicity against normal tissue must be evaluated before this treatment regimen can be tested in a clinical setting.

ACKNOWLEDGMENTS

We thank Dennis F. Deen, Ph.D., for helpful suggestions and discussion, Kathy D. Knebel and Peter Linfoot for technical assistance with FCM and centrifugal elutriation, Beverly McGehee for preparation of the manuscript, and Neil Buckley for editorial assistance.

REFERENCES


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