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Cytotoxic Effects of Hyperthermia, 5-Fluorouracil and their Combination on a Human Leukemia T-Lymphoblast Cell Line, CCRF-CEM*

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Abstract—*The cytotoxic effects of* 5-*fluorouracil (FUra), hyperthermia, and the combination of these treatments were examined in a human T-lymphoblast cell line, CCRF–CEM. Simultaneous exposure of exponentially growing CCRF–CEM cells to hyperthermia* (39 *and* 42°C) *and FUra* (10, 50, *and* 100 μ M) *for* 1 *or* 2 hr *resulted in subadditive or additive cell kill. When CCRF-CEM cells were exposed to these agents in sequence (hyperthermia → FUra and FUra → hyperthermia) for* 1 *and* 2 hr *duration additive cell kill was also observed. Enhanced cytotoxic effects were observed when a longer exposure* (4 *and* 8 hr) *to FUra* (100 μM) *followed heat* (42°C *for* 1 *and* 2 hr). *Heat exposure* (42°C, 1 *and* 2 hr) *induced a rapid decrease in the synthesis of DNA of CCRF–CEM cells, followed by a rebound increase at* 12 hr *and a new decrease at* 24 hr. *Flow cytometry demonstrated an accumulation of cells in the S phase at* 12 hr *after heat exposure, followed by a marked increase of the G + M population (maximum at* 24 hr). *The exposure time, and the sequence of administration of hyperthermia and FUra were critical determinants of cytotoxicity in this* in vitro *system and might constitute important variables of treatment when these two agents are used clinically.*

INTRODUCTION

THE POTENTIAL of using hyperthermia in combination with chemotherapeutic drugs in the treatment of cancer patients is of considerable current interest [1]. Thermal enhancement of cytotoxic effects for many antineoplastic antibiotics, alkaloids and alkylating agents has been observed in *in vitro* and *in vivo* systems (reviewed in [2–4]). At the present time, only incomplete information is available concerning the interaction of elevated temperatures and antimetabolites on tumor cell kill [5–10]. In particular, little experimental information has been reported dealing with the interaction of 5-fluorouracil (FUra) and hyperthermia [6,11]. The clinical use of FUra-heat combination may be worthwhile, especially for the treatment of patients with solid tumors sensitive to this drug, or with leukemia, in which FUra has been reported to be effective [12].

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In this study, the interaction of FUra and heat was investigated using a human leukemic T-lymphoblast cell line, CCRF–CEM, as the target cell. The effects on cell growth and viability as a function of temperature, FUra concentration, time and sequence of exposure to both agents were determined. The effects of elevated temperatures on DNA synthesis and cell cycle kinetics were studied. The results indicate that the cytotoxic effects of FUra were additive when the cells were exposed to simultaneous heating. Heat followed by FUra exposure produced enhanced cell kill depending on the time of exposure to these two agents.

MATERIALS AND METHODS

Chemicals

FUra was purchased from Sigma Chemical Co., St. Louis, MO. The media, sera, and antibiotics for tissue culture were purchased from Grand Island Biological Co., Grand Island, NY and plasticware from Corning Glass Works, Corning, NY. Noble agar was purchased from Difco Laboratories, Detroit, MI. [^{3}H] deoxyuridine (dUrd) with a specific activity of 27 μ Ci/mmol was obtained from Amersham Corp., Arlington Heights, IL. Mithramycin

was obtained from Miles Laboratories, Inc., West Haven, CT. All other chemicals were of the highest purity available, and were obtained from standard commercial sources.

Cell line

The human T-lymphoblast cell line CCRF–CEM was used in this investigation [13]. Cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere, and subcultured twice a week. The doubling time of logarithmically growing cells was 20 hr. Cells were tested periodically, using the Hoechst Stain Kit (Flow Laboratories, Inc., Rockville, MD) and found to be mycoplasma free.

Heat and drug treatment

All heat and drug treatments were performed on exponentially growing cells at an initial concentration of 1–3 × 10^5 cells/ml. Cells were heated in a specially designed water bath with ± 0.1°C accuracy. The temperature was monitored by a National Bureau of Standards thermometer (0.1°C gradations). Cell suspensions in different volumes (10–250 ml), appropriate for each experimental procedure, were placed in sterile culture vessels and immersed in the water bath. Temperatures of the cell suspension inside the incubation vessels equilibrated with the water bath temperature within 2–5 min of immersion, depending on the volumes used. When large volumes (50–250 ml) were heat-treated, small volumes of cell suspension (5–10 ml) were added to medium warmed at the experimental temperature, so that warm-up time was limited to the previously indicated time. For rapid equilibration to 37°C after treatment heated cells were put in a water bath at room temperature for a sufficient time (2–5 min) as determined in preliminary experiments. For simultaneous 37°C controls, a Metabolyte water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) with ± 0.2°C accuracy was used. The pH of the cell suspensions was adjusted to 7.4 with 0.1N HCl. Solutions of FUra were prepared in water immediately prior to use.

Doses, temperatures, times, and sequences of exposure to heat and/or FUra are indicated in the text.

Soft agar cloning

Cell viability after heat and/or drug treatment, was measured by the cloning technique of Chu and Fischer [14]. At the end of the treatment, the cells were harvested by centrifuging, washed twice, resuspended in drug-free medium supplemented with 15% horse serum, and cloned in 0.1% (w/v) soft agar. Ten ml plastic culture tubes were inoculated with a number of cells varying from 50 to 1000 depending upon the expected cell kill. After 16 days of incubation, visible colonies were counted using a Darkfield Quebec colony counter (American Optical Corp, Buffalo, NY). The average cloning efficiency for control cells was 58.1 ± 10.7% which was normalized to 100%. Each condition was cloned in quadruplicate, and all experiments were repeated at least twice.

Growth inhibition studies

Following heat and/or drug treatment, the cells were harvested, washed twice, resuspended in drug-free medium supplemented with 10% horse serum at a concentration of 2 × 10^4 ± 0.5 cells/ml and maintained at 37°C. Subsequent increase in cell number was followed for 7 days using a Coulter counter, Model B (Coulter Electronics, Inc., Hialeah, FL). The percent of cells which excluded trypan blue was also determined. More than 95% of the untreated cells excluded the dye.

To compare the effects of heat and FUra treatments on growth of cells resuspended at slightly different initial density, the numbers of trypan blue excluding cells were converted to total cell division (TCD), calculated from the formula:

$$\text{Total cell divisions} = \frac{\log_{10}(N_t/N_0)}{\log_{10}2}$$

in which N_t is the initial cell number following resuspension, and N_0 is the cell number at time t. Growth curves were generated plotting TCD as a function of time.

Incorporation of [^{3}H] *dUrd into DNA*

The effect of heat on the rate of [^{3}H] dUrd incorporation into DNA of CCRF–CEM was determined as described by Hryniuk *et al.* [15] at different time intervals (0,6,9,12, and 24 hr) following treatment. After exposure to heat, the cells were centrifuged at 300 ***g*** for 5 min, and resuspended in medium with 10% horse serum at a concentration of 2–5 × 10^6 cells/ml, for the 0 time measurement. For measurement at successive time intervals, following heat exposure, the cells were allowed to recover at 37°C. At these times, the cells were processed as previously described. Twelve-ml cell suspensions were distributed into Erlenmeyer flasks and incubated in a shaking water bath at 37°C. [^{3}H]dUrd was then added at a final concentration of 1.0 μM (specific activity 2.25 μCi/μmol). At 5-min intervals after the addition of the radiolabel, duplicate 1 ml aliquots of cell suspensions were removed from each flask and precipitated with ice-cold 5% $HClO_4$. The precipitates were washed and hydrolyzed with $HClO_4$ at 85°C.

The extractable radioactivity was counted in 15 ml Hydrofluor scintillation fluid (National Diagnostics, Sommerville, NJ) using a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Control cells incorporated [^{3}H]dUrd linearly during a 20-min exposure period. The rate of [^{3}H]dUrd incorporation into DNA of treated cells was calculated from the slope obtained by least squares linear regression and expressed as percentage of untreated cells.

Flow cytometry

Cell fluorescence and forward light scatter (1–15°C) were measured, following exposure to heat, by a fluorescence activated cell sorter (FACS IV, Becton Dickinson FACS, Sunnyvale, CA). A 520 nm and a 535 nm long pass filter were used to distinguish the mithramycin fluorescence emission (peak at 575 nm) from the laser excitation (457 nm). Flow rates were between 500 and 1000 cells per second.

To analyze heat effects on cell cycle progression, cells were stained using a mithramycin staining technique, as described by Crissman and Tobey [16]. Prior to staining, cells were fixed in cold 70% ethanol and stored at 4° until flow cytometric analysis. Aliquots of 10^6 cells were centrifuged and resuspended in an aqueous solution containing mithramycin (20 μg/ml) and 15 mM $MgCl_2$, for 1–2 hr prior to analysis.

Approximately 20,000 cells were analyzed for each sample. Histograms were normalized to contain equal number of cells for display purposes. The percentages of cells, in G_1, S and G_2 + M phases were obtained by a computer fitting method of analysis of the DNA distributions [17].

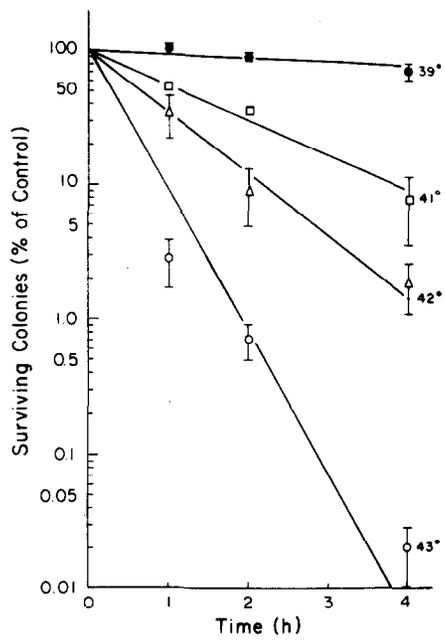

Fig. 1. Survival curves of CCRF-CEM cells exposed to hyperthermia. Cells were exposed to 39–43°C for the indicated length of time. Points, mean of at least two separate experiments; bars, standard errors. Regression lines were fitted to the data points by the method of least squares. ●, 39°C; □, 41°C; △, 42°C; 0, 43°C.

Quantitation of heat–FUra interaction

For quantitating the effects of heat and FUra combined treatment on cell survival, the ratio between the product of the survival fraction of each individual agent and the survival fraction of the heat and FUra combination was calculated. A ratio of 1.0 indicates additive effects, greater than 1.0 synergistic effects, and less than 1.0 antagonistic effects [18]. For quantitation of the effects of heat and FUra combined treatment on cell growth, the difference between the product of the per cent growth of each individual agent and the per cent growth of the heat and FUra combination at 72 hr after the end of the treatment and resuspension of cells in drug-free medium at 37°C was calculated. No difference (± 15%) indicates an additive effect, while positive or negative values indicate a synergistic or an antagonistic effect on cell growth, respectively.

RESULTS

Effects of heat alone and simultaneous heat and FUra on cell viability

The survival curves of CCFR–CEM cells exposed to temperatures of 39–43°C for 1, 2 and 4 hr are shown in Fig. 1. Survival curves are consistent with an exponential cell kill at all temperatures over the time range tested.

The effects of simultaneous treatment with FUra and heat on the survival of CCRF–CEM were measured, and the results are shown in Fig. 2.

Cells were exposed to FUra (10, 50, 100 μM) for 1–2 hr, at 37, 39 and 42°C. At 37°C, maximum cell kill values obtained by 100 μM FUra were 14.8 and 35.7% for 1 and 2 hr exposure. Heat treatment alone for both 1 and 2 hr at 39°C produced less than 15% cell kill and at 42°C, resulted in 69.7 and 85.9% cell kill, respectively. An analysis of survival data obtained by combined FUra and heat treatments is summarized in Table 1. The ratios of expected vs. observed per cent survival for combined FUra and heat treatment were between 0.8 and 1.2 at all FUra concentrations, temperatures, and exposure times tested, indicating additive effects, or possibly slightly less than additive effects.

Sequence-dependent effects of heat and FUra on cell viability and growth

The sequence-dependent effects of heat and FUra on cell survival were determined. Cells were exposed to heat (42°C) and FUra (100 μM) for

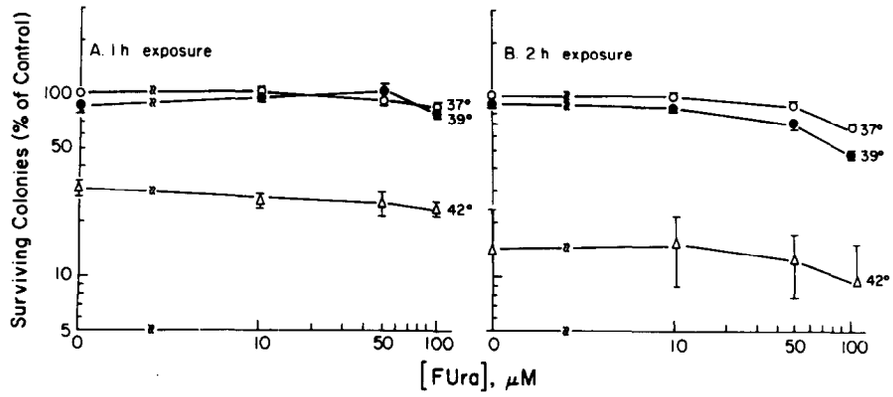

Fig. 2. Survival curves of CCRF-CEM cells simultaneously exposed to FUra and heat for 1 *(Panel A) and* 2 *(Panel B)* hr. *Points, mean of at least two separate experiments; bars, standard errors.* 0, 37°C; ●, 39°C; △, 42°C.

Table 1. *Ratios of the per cent survival expected (S_E) under conditions of additivity to the experimentally observed per cent survival (S_0) for combined FUra and heat*

FUra (μM)	Temperature	S_E/S_0* Exposure Time (hr) 1	2
10		0.9	1.0
50	39°C	0.8	1.1
100		0.9	1.2
10		1.2	0.9
50	42°C	1.1	1.0
100		1.1	1.0

* (% S_{Hyp}) (%S_{FUra}) / %$S_{(Hyp)}$ (FUra)

Table 2. *Sequence-dependent effects of heat and FUra on CCRF-CEM cell survival*

Treatment*	Surviving colonies† (% of control, 37°C)	S_E/S_0
Heat	5.1 ± 0.8	–
FUra	52.2 ± 5.8	–
FUra → Heat	2.8 ± 0.7	1.0
FUra + Heat	3.7 ± 1.1	0.7
Heat → FUra	2.6 ± 0.9	1.0

Exposure time to heat (42°C) and FUra (100μM) was 2 hr.
* +, Simultaneous administration; →, sequential administration.
† Mean ± S.E. of quadriplicate determination within a single experiment.
Similar patterns were observed in replicate experiments.

2 hr; FUra was administered first, followed by hyperthermia (after drug removal), simultaneously with, or following hyperthermia (Table 2). Single agent treatments were also performed. Additive cell kill effect was observed regardless of the sequence of administration (Table 2). When a longer exposure (4 and 8 hr) to FUra (100 μM) followed heat (42°C, and 2 hr) synergistic inhibitory effects on cell growth were also observed (Figs. 3B, C). Expected growth values 72 hr after the end of treatment were in fact 19.4 and 6% for the combinations of heat and FUra 4- and 8-hr exposure, respectively, as compared to 3.3 and 26.5% experimentally observed values. Similar synergistic inhibitory effects were noted when FUra (4 and 8 hr) followed 1 hr exposure to 42°C (data not shown).

Effects of hyperthermia on (^{3}H]dUrd incorporation into DNA

The effect of heat (42°C, for 1 and 2 hr) on DNA synthesis of CCRF–CEM cells was measured by the incorporation of [^{3}H]dUrd into acid-insoluble precipitates (Fig. 4). (^{3}H]dUrd incorporation into DNA was decreased to 65 and 70% of control values after 1 and 2 hr of heat treatment, respectively. A rebound increase to 50 and 70% above control was observed by 12 hr after 1 and 2 hr of heating. DNA synthesis decreased to 10 and 20% of control at 24 hr after heating for 1 and 2 hr.

Effects of hyperthermia on cell cycle progression

In order to possibly correlate cell killing with cell cycle effects caused by hyperthermia, the effects of heat on cell cycle progression were studied by flow cytometry. Exponentially growing cells were kept at 37°C or incubated at 42°C for either 1 or 2 hr. After treatment, the cells were returned to 37°C, and sampled for flow cytometric measurement of DNA content at various time intervals. Selected DNA histograms illustrating characteristic kinetic data are presented in Fig. 5.

A moderate accumulation of cells in the S phase occurred at 12 hr after heating, with a concurrent

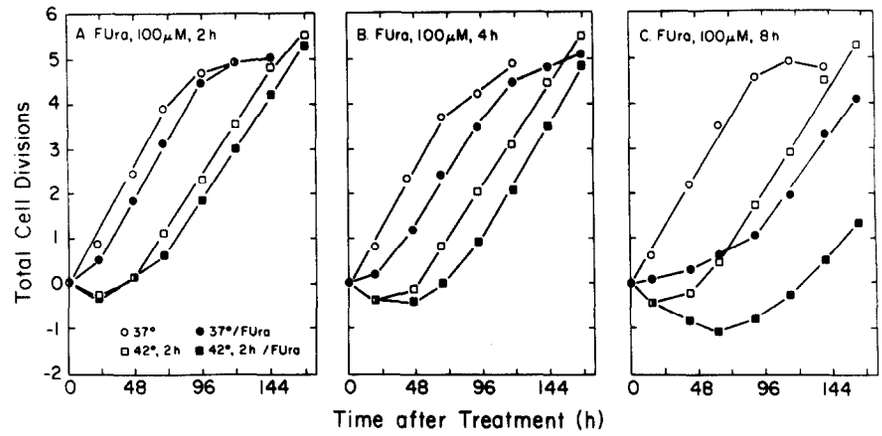

Fig. 3. Inhibitory effects of sequential heat-FUra treatments on growth of CCRF-CEM cells. Cells at a density of about 2×10^5/ml *were exposed to* 42°C *for either* 1 *or* 2 hr *then returned to* 37°C *and exposed at this temperature to FUra* (100 μM) *for various periods of time* (2, 4 *and* 8 hr). *After drug exposure, cells were washed twice, resuspended in drug-free medium at a density of* 2×10^4/ml, *and their growth followed. Growth curves were generated by plotting cell divisions (TCD) vs. time. TCD was calculated as described in Materials and Methods. Symbols used are* 0, 37°C; ●, 37°C/FUra; □, 42°C, 2 hr; ■, 42°C, 2 hr FUra.

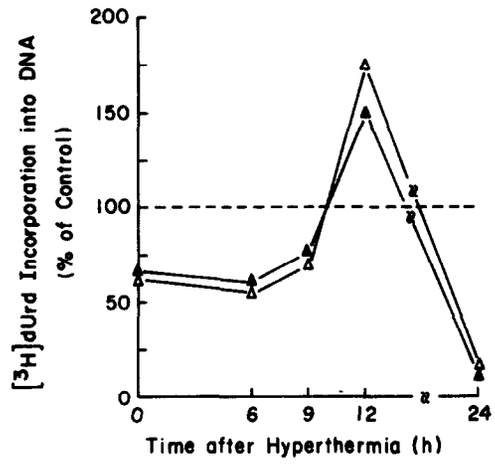

Fig. 4. Effects of hyperthermia on incorporation of [^{3}H]dUrd *into DNA of CCRF-CEM cells. Cells were exposed to heat* (42°C) *for either* 1 *or* 2 *hr. After exposure, cells were returned at* 37°C. *At indicated times* (0–24 hr) *following heat exposure,* [^{3}H]*dUrd incorporation was determined as described in Materials and Methods.* △, 42°C, 1 hr; ▲, 42°C, 2 hr.

depletion of cells, in the G_1 phase. Subsequently, cells moved into the G_2+M phase, and by 24 hr after the return to 37°C, a marked increase in the G_2+M population was observed, (45 and 60% for 1 and 2 hr heated cells, respectively). This event correlated well with the [^{3}H]dUrd data. A gradual repopulation of G_1 and S cell compartments occurred following the release from the G_2+M phase. Differences in cell cycle distributions between the 1 and 2 hr treated cells were more evident after 12 hr from the end of the treatment.

The [^{3}H]dUrd incorporation results, together with the flow cytometry data may be interpreted to show that the effect of heat is to initially cause cells to accumulate in the S-phase. They subsequently are either killed, or survivors progress into the G_2S compartment. Cell viability data as measured by the trypan blue exclusion test underestimate cell death, since this test showed that 16% cell death was caused by a 2-hr exposure at 42°C measured at 24 hr, while the 1-hr exposure at this temperature showed no appreciable lethal effect. In contrast cell kill as measured by soft agar cloning was 91 and 66% respectively for the 2 and 1 hr heat treatments at 42°C. This underestimate of cell kill may be caused by the length of time required for lethally damaged cells to lose their membrane integrity following a heat insult, the ability of 'surviving' cells to continue to proliferate during this time, and the ability of lethally damaged cells to undergo early disintegration.

DISCUSSION

Despite potential clinical importance, the interaction of FUra and hyperthermia has not been studied extensively. Previous laboratory studies by other investigators have shown no appreciable potentiation of cytotoxicity, when cells in culture were exposed for short periods of time to this drug in combination with heat [6]. In addition, no substantial therapeutic advantage was noted when mice bearing colon carcinoma 38 were treated with FUra (60 mg/kg/dose) followed by moderate heat administration (38.8–38.9°C, 45 min) for three courses [11]. However, the possibility that the effectiveness of heat and FUra combinations might be improved by specific sequencing of these agents has not been adequately assessed.

The human T-lymphoblast line, CCRF–CEM, is highly sensitive to heat, even for short exposure

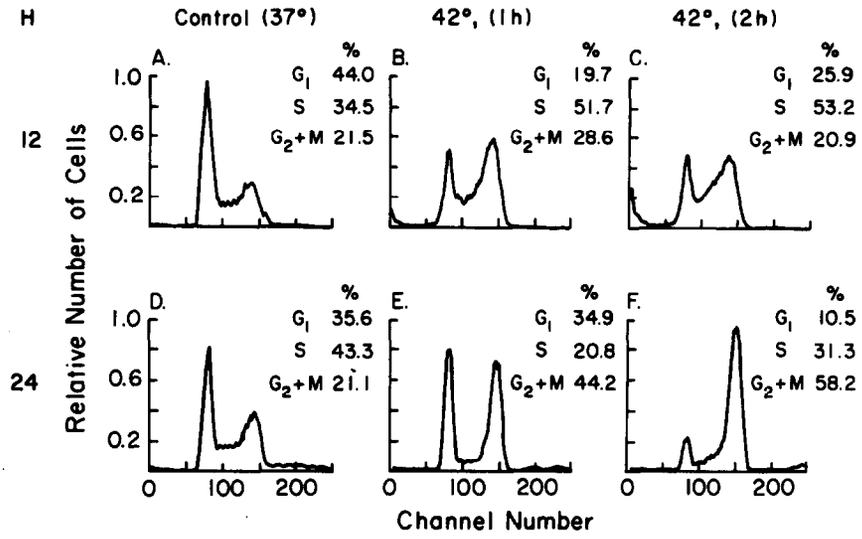

Fig. 5. DNA distributions of CCRF–CEM cells after 12 hr *and* 24 hr *following exposure to* 42°C *either for* 1 *or* 2 hr, *as compared to contemporary controls* (37°C). *Channel number, relative fluorescence intensity (DNA content).*

times as shown in Fig. 1. Sensitivity to high concentrations of FUra for short exposure times (1, 2, and 4 hr) was poor (maximum cell kill values were less than 1 log); exposure to this drug for a longer time (24 hr), however, significantly increased cell kill (maximum cell kill 3.6 log) [19]. Relative FUra ineffectiveness in this cell line has been related to deficient activation via uridine/thymidine phosphorylase pathways [20]. Hyperthermia did not potentiate FUra cell kill, when cells were exposed to these agents simultaneously. This may be due to the observed inhibitory effects of heat on DNA synthesis of CCRF–CEM cells, thus protecting cells from further cell kill by FUra, a cycle specific agent.

The flow cytometric data (as a function of time) relate to the total population (viable and nonviable cells) as described by the regrowth method at the same times. The flow cytometric data were obtained on a substantially viable cell population as demonstrated by the regrowth patterns (data not shown), viability data and also [^{3}H]dUrd incorporation data demonstrating DNA synthesis within 12 hr after treatment. Trypan blue excluding cells were present in a relatively high percentage (16%) only at 24 hr after a 2-hr exposure to 42°C.

The possibility that sublethal exposure to elevated temperatures, found to block cell cycle transit in S and G_2 and M [21–23], could lead to partial synchronization of cell growth, and thus increased sensitivity to subsequent exposure to FUra was investigated. DNA synthesis, as measured by [^{3}H]dUrd incorporation into DNA, indicated that hyperthermia causes a decrease in DNA synthesis, followed by a rebound increase 12 hr later (Fig. 4). This overshoot was found to be associated with an increased proportion of cells in S phase (Fig. 5). Subsequently, marked inhibition of DNA synthesis occurs (Fig. 4), associated with concurrent accumulation of cells in G_2 + M (Fig. 5). However, after release of this G_2 + M block, there was no further evidence of synchronization, presumably due to subsequent cell death, and gradual recovery of some of the cell population (data not shown).

Survival data (not shown) as well as growth inhibition data (Fig. 3) of the sequential heat-FUra combinations demonstrated additive effects when FUra was administered for a short time (2 hr) following heat treatment (42°C, 1 and 2 hr); prolonging exposure to FUra to 4 and 8 hr, demonstrated synergism (Fig. 3).

The exposure to FUra immediately prior to hyperthermia produced additive effects on cell survival (Table 2). Further studies of the sequence FUra → heat, based on available kinetic information, may be worthwhile. FUra, in fact may induce partial synchronization of cell cycle by blocking cells at the G_1/S boundary [24,25], and once the block is released hyperthermia may selectively kill cells in S and G_2 +M phases [26–28].

These data indicate that the interaction between elevated temperatures and FUra on human leukemic CCRF–CEM cells is complex, and not easily extrapolated to the clinical situation. Sequential administration of hyperthermia followed by FUra administration is worth exploring in patients with leukemia and lymphoma since synergistic cytotoxic effects may be attained, especially with longer exposures to FUra. Recent data from this labora-

tory indicates that FUra cytotoxicity to these cells may be markedly enhanced by pretreatment with methotrexate or leucovorin (5-formyltetrahydrofolate) [19,29]. These data have prompted a phase I study using FUra in combination with leucovorin in patients with refractory lymphoma. The possibility that hyperthermia will further increase the cytoxic effects of this FUra–leucovorin combination is being explored.

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