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Partial Synchronization of L1210 Cells by 5-Fluorouracil and Its Use in Drug Combinations[1]

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SUMMARY

When L1210 cells growing logarithmically were exposed for 8 hr to a nonlethal dose of 5-fluorouracil (FU) (0.25 μg/ml), the percentage of cells in S phase increased from 74.9% in the asynchronous culture to 93% in the FU-treated culture. This resulted in increased cell-kill by S-phase-specific inhibitors [1-β-D-arabinofuranosylcytosine (ara-C), 5-hydroxy-2-formylpyridinethiosemicarbazone] when they were added to a culture partially synchronized by pretreatment with FU. For example, 2 hr exposure to ara-C alone or ara-C plus FU (added simultaneously to asynchronous culture) gave 28.8 and 25.8% survival, respectively, compared to 6.8% survival when ara-C was added for 2 hr to the partially synchronized culture. Eight to 12 hr after FU removal, the culture became asynchronous, such that ara-C addition at this time did not result in increased cell-kill.

Cultures pretreated with FU were also highly sensitive to vincristine and Adriamycin. Adriamycin acted synergistically with FU (after 8 hr pretreatment) in killing L1210 cells.

INTRODUCTION

FU[2] is the most extensively used agent in gastric and colon cancer and is widely used in combination with other drugs for treatment of breast cancer (1, 8). In conjunction with radical mastectomy, FU plus Cytoxan and methotrexate was highly effective in the treatment of breast cancer (7). FU is also being tested in combination with L-phenylalanine mustard and methotrexate for the treatment of breast cancer (9).

We were interested in studying the lethality for cells in culture of combinations of drugs active in breast cancer. Drugs that have been reported to be active individually in treatment of breast cancer (8) and which, therefore, were selected for our study are: FU, methotrexate, VCR, L-phenylalanine mustard, and Adriamycin. In the course of these studies we observed that FU accumulated L1210 cells in S phase. Accumulation of H.Ep-2 cells at or near the G_1-S transition, after exposure to FU, had been reported by Wheeler *et al.* (20).

Our studies, reported here, show the effect of combinations that take advantage of the partial synchronization of L1210 cells obtained with nonlethal doses of FU. Parts of this study were reported previously (3).

MATERIALS AND METHODS

L1210 Cell Culture. L1210 cells were maintained in suspension culture in Roswell Park Memorial Institute 1634 medium supplemented with 0.75 mg $NaHCO_3$, 0.1 mg penicillin, and 0.05 mg streptomycin per ml, respectively, and 5% fetal calf serum (5). The cells were exposed to drug either when they were in logarithmic growth or were partially synchronized by exposure to a nontoxic dose (0.25 μg/ml) of FU. Cell survival was determined, after removing drug, by cloning in soft agar, and the procedure has been described previously (5). The cloning efficiency of L1210 cells in the soft agar medium ranged from 60 to 90%. When 2 drugs were combined, the survival expected, if the drugs acted independently and additively, was calculated by multiplying the fractional survival obtained with each drug alone (19). Two drugs acted synergistically when the observed survival value was lower than the calculated additive value.

Autoradiography. The cells were prepared for autoradiography as previously described (2).

Drugs. Adriamycin (NSC 123127), 5-HP (NSC 107392), VCR (NSC 67574), actinomycin D (NSC 33053), and FU (NSC 19893) were obtained from the Division of Cancer Treatment, National Cancer Institute. ara-C (NSC 63878) was produced by The Upjohn Company.

***In Vivo* Activity.** The antileukemic activity *in vivo* against L1210 leukemia was measured according to protocols previously published (10).

Cell DNA Content Determination. Cells were analyzed for DNA content by flow microfluorometry after washing, fixation with formaldehyde, and staining with the fluorescent dye benzoflavin, utilizing a modified Feulgen procedure (11). The frequency distribution of fluorescence emission per cell (proportional to DNA content) was measured for samples of 10^5 cells with a Cytofluorograf (Biophysics Systems, Inc., Baldwin Place, N. Y.) and analyzed with a multichannel analyzer (Tracor Northern, Inc., Middleton, Wis.). The results are expressed as a histogram representing the relative number of cells with a given DNA content.

RESULTS

Cell Survival and Cell Progression after Exposure to FU. The survival of L1210 cells exposed to FU is shown in Chart

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[2] The abbreviations used are: FU, 5-fluorouracil; VCR, vincristine; 5-HP, 5-hydroxy-2-formylpyridinethiosemicarbazone; ara-C, 1-β-D-arabinofuranosylcytosine.

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1. The results indicate that FU requires a relatively long period of contact with cells in order to exert its lethal effects. For example, at 1 μg/ml, 4 hr exposure of cells to FU were necessary before its toxic effects became apparent. At 0.25 μg/ml, FU was nontoxic during 7 hr exposure. In all further studies, unless otherwise mentioned, FU was used at 0.25 μg/ml.

The flow microfluorometry pattern obtained with cells exposed to FU (0.25 μg/ml) for 8 hr is shown in Chart 2. These results show qualitatively that FU increases the proportion of cells in S phase. Autoradiographic studies confirmed the above observation. The distribution of cells in different phases of the cell cycle is shown in Table 1. There

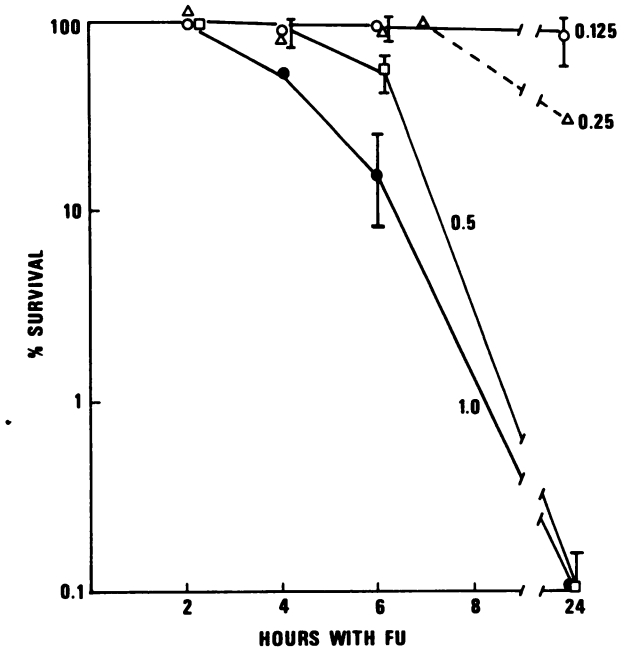

Chart 1. Dose-survival response of L1210 cells exposed to 0.125 (○), 0.25 (△), 0.5 (□), and 1.0 μg (●) of FU per ml, respectively.

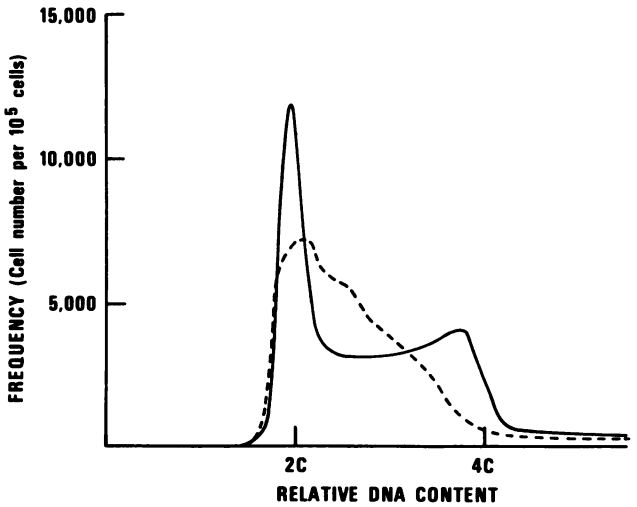

Chart 2. DNA distribution patterns of cells exposed to FU (0.25 μg/ml) for 8 hr. *Ordinate*, frequency of a given DNA content expressed in cell numbers per 10^5 cells; *abscissa*, relative flourescence emission (proportional to DNA content). The *2C* and *4C* peaks correspond, respectively, to cells with G_1 and G_2 + M DNA content. *Solid lines*, control; *dashed lines*, FU-treated populations.

Table 1

Cell distribution in FU-treated and control cultures

An exponentially growing L1210 culture was treated with FU (0.25 μg/ml) for 8 hr, after which the cell distribution was determined. S-phase cells were determined by counting cells labeled after 20-min exposure to [^{3}H]thymidine (2 Ci/mmole, 2 μCi/ml). M-phase cells were determined by counting mitotic cells. Percentage of G_1 + G_2 cells equals 100 − (% M + % S).

	% M	% S	% G_1 + G_2
Control (untreated)	3.6	78.8	17.6
FU treated	1.5	95.8	2.7

Table 2

Percentage of cells in S phase at different times after FU was removed

Hr after FU exposure[a]	% labeled cells
0	91.9 ± 1.7
1	89.5 ± 1
3	85.5 ± 3.1
8	75.4
10	73.8
12	76
24	72.8
Asynchronous culture[b]	74.9 ± 0.5

[a] Logarithmically growing, asynchronous L1210 cells were exposed to FU (0.25 μg/ml) for 8 hr. The cells were then centrifuged, washed, and resuspended in fresh medium. The cells were labeled with [^{3}H]thymidine (5 μCi/ml, 2 Ci/mmole) for 25 min.

[b] Logarithmically growing, asynchronous culture of L1210 cells was labeled as indicated above.

was a marked increase in the S-phase population and a marked decrease in the G_1 and G_2 population.

When the FU was washed off (subsequent to 8 hr contact with cells) the percentage of cells in S phase decreased (Table 2). These results show that by 8 hr after FU was removed, the percentage of S-phase cells had decreased to the level seen in the control (asynchronous) cultures.

Combination of FU with Different Cytotoxic Agents. Table 3 shows the percentage of cells surviving treatments with: (*a*) FU or other cytotoxic drugs for 2 hr (Column 4), (*b*) FU plus 2nd cytotoxic drug for 2 hr (Column 5), (*c*) FU for 8 hr (Column 6), or (*d*) FU for 8 hr followed by 2nd drug for 2 hr (Column 7).

The results allow us to draw the following conclusions.

1. FU alone (0.25 to 1 μg/ml) was not significantly lethal after 8 hr exposure (Table 3, Column 6).

2. The schedule, where cells were exposed to FU for 8 hr followed by the 2nd drug for 2 hr, was synergistic (*i.e.*, a greater than additive effect). Thus, exposure to a nonlethal level of FU for 8 hr, followed by a 2nd drug for 2 hr (Table 3, Column 7) gave much lower survivals than did simultaneous exposure to FU + 2nd drug for 2 hr (Table 3, Column 5) or the 2nd drug alone for 2 hr (Table 3, Column 4). For example, in Experiment 1, exposure to FU for 8 hr followed by ara-C for 2 hr gave 6.8% survival compared to 25.5% survival with simultaneous exposure to ara-C + FU for 2 hr. The survival expected, if ara-C and FU had acted additively, would be 27.3% (see Table 3, Footnote *h*, for calculation), which should be compared to the observed survival of 6.8%. Similar effects were seen with 5-HP, VCR, and high-specific-activity [^{3}H]thymidine. All of these agents are normally

Table 3
Survival of L1210 cells exposed to FU + other cytotoxic drugs

Experiment no.	Agent	(μg/ml)	% survival of asynchronous cells[a] Agent alone (2 hr)[c]	FU (2 hr)[d] + agent (2 hr)	% survival of partially synchronous cells[b] FU (8 hr)[e]	FU (8 hr)[f] + agent (2 hr)
1	FU	0.25	100.5 ± 4.5		94.2 ± 7.1	
	ara-C	5	28.8 ± 1.2	25.5		6.8 ± 0.5 (27.3)[h]
	[^{3}H]Thymidine[g]		38.6	25.9		4.2 (31.9)
	5-HP	30	31.9	37.6		8.9 (30)
	VCR	0.25	36.6	35.1		18.3 (34.5)
2	FU	0.25	100 ± 3.6		91.5 ± 4.7	
	Adriamycin	0.0125	96 ± 4	89.5		64.3 ± 3.4 (87.8)
	Adriamycin	0.025	88 ± 19.6	82.9 ± 6 (88)		31.2 ± 9.1 (80.5)
	Adriamycin	0.05	39.8 ± 10.6	34.3 ± 7.5 (39.8)		5.2 ± 2.8 (31.4)
3	FU	0.5	89.7 ± 0.2		104.6	
	Adriamycin	0.0125	105.6 ± 7.8	96.5 ± 1.7		55.8 (110.4)
	Adriamycin	0.025	85 ± 14.5	76.3 ± 0		29.5 (88.9)
4	FU	1.0	90 ± 2.2		82 ± 6.6	
	Adriamycin	0.0125	105.6 ± 7.8	105.3 ± 9.7		55.4 ± 2.6 (86.6)
	Adriamycin	0.025	85 ± 14.5	93.7 ± 9.1		49.6 ± 0.5 (69.7)

[a] Logarithmic cultures (≃2 × 10^5/ml) were used.
[b] Logarithmic cultures were exposed to FU for 8 hr. In experiments 1 and 2, 0.25 μg FU per ml was used, compared to 0.5 and 1 μg FU per ml in Experiments 3 and 4, respectively. The cells were exposed to FU for 8 hr in Experiments 1, 2, and 3 and for 4 hr in Experiment 4.
[c] Survival of asynchronous cells exposed to FU or other cytotoxic drugs for 2 hr.
[d] Survival of asynchronous cells simultaneously exposed to both FU and other drugs for 2 hr.
[e] Survival of asynchronous cells exposed to FU alone for 8 hr.
[f] Survival of asynchronous cells first exposed to FU for 8 hr followed by other drugs for 2 hr.
[g] [^{3}H]Thymidine (20 Ci/mmole) was added to give 3.3 μCi/ml.
[h] Numbers in parentheses, percentage survival values expected if FU and the 2nd drug acted independently and additively in combination. These values were obtained by multiplying the fractional survival obtained with each drug alone (19).

cytotoxic to cells in S phase. Therefore, the increase in cell-kill seen in the partially synchronous culture can be explained by the increase in the percentage of S-phase cells from 74.9% in an asynchronous population to 91.9% in the partially synchronized population (see Table 2).

With Adriamycin we saw clear evidence of synergism as shown in Experiment 2. For example, Adriamycin alone, at 0.025 μ/ml for 2 hr, was slightly cytocidal (88% survival). Cells exposed simultaneously to FU + Adriamycin (0.025 μg/ml) for 2 hr gave 82.9% survival (Table 3, Column 5). However, only 31.2% of cells survived (Table 3, Column 7) when Adriamycin was added to cultures partially synchronized by 8-hr exposure to FU. The survival expected, if FU (for 8 hr) and Adriamycin (for 2 hr) had acted additively, would be 80.5%. Similar synergistic effects were seen when the FU concentration was increased to 0.5 μg/ml (Experiment 3) and 1.0 μg/ml (Experiment 4).

3. There was no synergistic effect when cells were simultaneously exposed to FU + ara-c or FU + Adriamycin for 2 hr (Table 3) or up to 8 hr (not shown). In both these experiments, the cell survival obtained with ara-C or Adriamycin alone was about the same as that with FU + the 2nd drug. This suggests that the presence of FU did not sensitize the cells to ara-C or Adriamycin.

In the cultures partially synchronized by 8 hr exposure to FU, the percentage of cells in S phase decreased with time after FU was removed (Table 2). This was reflected in the decreases in percentage of cells killed by ara-C with increasing times after FU removal (Chart 3). Thus, 12 hr after FU was removed, 70% of the cells were killed by ara-C compared to 93.2% killed 1 hr after FU removal. Similar results were obtained with 5-HP and VCR and [^{3}H]thymidine. Thus, 5-HP (30 μg/ml) gave 8.9 and 37.3%

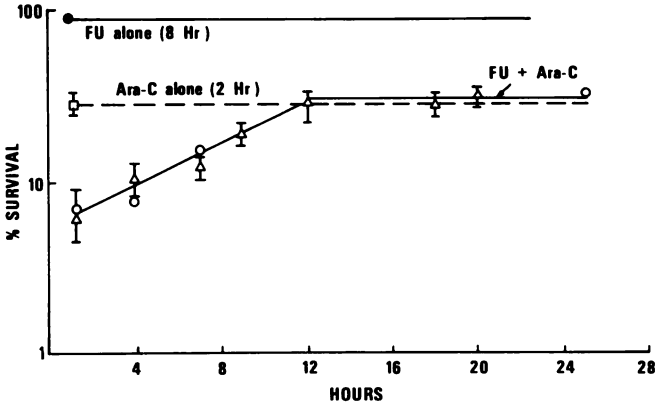

Chart 3. Cell kill with ara-C added at different times after FU removal. The culture was exposed to FU (0.25 μg/ml) for 8 hr, after which the cells were washed and resuspended in fresh medium. The cells were exposed to ara-C (5 μg/ml) for 2 hr at different times after FU removal (△, ○). The *points* are shown at the midpoint of ara-C exposure. The percentage survival of cells exposed to FU alone (0.25 μg/ml, 8 hr, ●) and ara-C (5 μg/ml, 2 hr, □) alone were 92 and 28 ± 4, respectively. Results of 2 independent experiments.

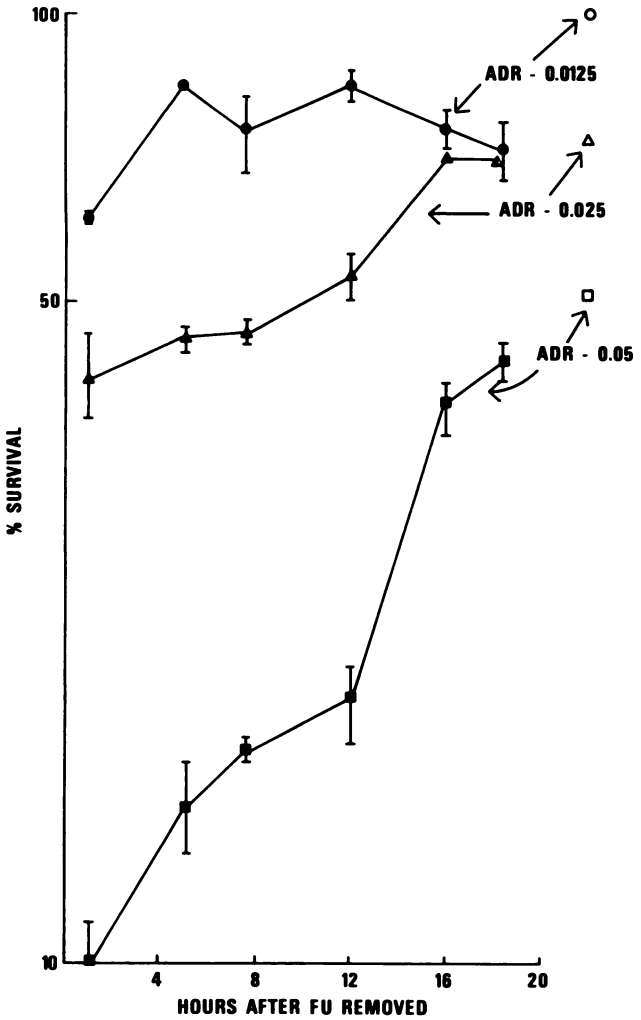

Chart 4. Cell-kill with Adriamycin (*ADR*) added at different times after FU removal. The protocol was the same as in Chart 5 except that different levels of Adriamycin were used. *Solid symbols*, cell survival with Adriamycin added to cultures pretreated with FU. *Open symbols*, at extreme right, survival values with different concentrations of Adriamycin alone for 2 hr or Adriamycin + FU simultaneously for 2 hr. ●, ○, 0.0125 μg/ml; ▲, △, 0.025 μg/ml; ■, □, 0.05 μg/ml.

survival, respectively, 1 and 25 hr after FU removal. VCR gave 18.5 and 38.5% survival, respectively, 1 and 7 hr after FU removal.

A similar decrease in percentage of cells killed by Adriamycin with increasing time after FU removal is shown in Chart 4.

Effects of FU plus ara-C on L1210 Leukemia in Mice. In order to maintain the FU concentration at a constant level for 8 hr (as was done *in vitro*), 6 injections of FU were given at hourly intervals. The last FU injection was followed 1 hr later by 1 injection of ara-C. The results showed that, under these conditions, we did not see an accumulation of S-phase cells, which might explain the additive, rather than synergistic, increase in life span (Table 4). Next, we attempted to obtain constant blood level of FU by infusing the drug. These preliminary experiments showed that infusing FU at 15 mg/kg/hr for 10 hr increased the percentage of labeled L1210 ascites cells from 64% in an untreated population to about 91% in a treated population. Further experiments utilizing this system are in progress.

DISCUSSION

Our results clearly indicated that L1210 cells in culture, exposed to a nonlethal concentration (0.25 μg/ml) of FU for 8 hr, accumulated in the S phase. Similar results were reported by Wheeler *et al.* (20) for H.Ep-2 cells. They found that FU caused partial synchronization of cells, so that within 1 hr after FU removal 80% of the cells were labeled as compared to 20% labeled cells in an asynchronous culture. During exposure to a lethal concentration of FU for 1 cell cycle period, G_2 cells and the last third of S-phase cells progressed into mitosis (20). Although we do not have any data on the progression of cells during FU exposure, and although we used a nonlethal concentration of FU, our results are not contrary to the observations of Wheeler *et al.* (20). Since for L1210 cells, the S phase and $G_1 + M + G_2$ phase are of 8- and 4-hr duration, respectively, it is possible for cells in the last third of S phase to proceed through mitosis and reenter S during the 8-hr exposure. Since we

Table 4

Effect of FU + ara-C on L1210 leukemia in vivo

Mice received i.p. injections of 10^5 L1210 cells. FU was injected i.p. in 6 hourly doses on the 2nd day. One hr after the last FU injection, ara-C was injected.

	Dose (mg/kg/dose × no. of doses)	Wt change[a] (g/mouse)	Day of death[b]	% ILS[c]	Additive % ILS[d]
Untreated control		+2.4	9.3 ± 0.6	0	
FU	5 × 6	+0.2	10.8 ± 0.7	16	
ara-C	250 × 1	+0.4	10.9 ± 1.8	17	
FU/ara-C		−0.3	12.8 ± 1.5	38	33
FU	15 × 6	+0.7	11.8 ± 0.9	27	
ara-C	250 × 1	+0.4	10.9 ± 1.8	17	
FU/ara-C		−0.6	13.3 ± 0.5	43	44

[a] Based on weights on Days 1 and 5.

[b] Mean ± S.D.

[c] % ILS, percentage increase in life span, calculated from mean survivals of control and treated groups.

[d] Percentage increase in life span expected if the drugs interact additively. It is equal to the sum of the percentage increase in life span obtained with each drug alone.

had a lower proportion of G_1 + M + G_2 cells in the FU-treated population than in the controls, this indicated that most of these cells were not blocked from progressing into S. Kovacs *et al.* (14) also saw a significant increase in the S-phase population of hepatoma 3924A cells after an injection of FU (150 mg/kg). However, the partial synchronization of cells by FU seems to be specific for certain cell lines, since it was not observed with CHO cells (our observation and Ref. 16).

Therapeutically synergistic drug combinations have been obtained when 2 drugs are combined, in which the 1st drug synchronizes cells in a certain phase followed by a 2nd drug which is most cytotoxic to cells in the synchronized phase. VCR accumulates cells in mitosis, and bleomycin is most cytotoxic to mitotic cells. Therefore, VCR followed by bleomycin was tested in the treatment of bronchogenic carcinoma (15). We took advantage of the accumulation of cells in S phase (after FU pretreatment) by following with S-phase-specific drugs. The cell kill obtained by treatment with S-phase-specific drugs, such as ara-C (6), 5-HP (6), or VCR (3), correlated well with the percentage of cells accumulated in S phase and was greater than the cell kill expected if the drugs had interacted additively. Adriamycin kills cells in all phases of the cell cycle but is most lethal to S-phase cells (4). The cell kill obtained, when 8 hr exposure to FU was followed by Adriamycin, was much greater than that expected on the basis of accumulation of cells in S phase. Thus Adriamycin alone, at 0.25 μg/ml, killed 12% of the cells as compared to 69% cells killed when Adriamycin was added to cells partially synchronized by FU. This might suggest that prior exposure to FU sensitized the cells to Adriamycin. However, when cells were exposed simultaneously to FU (0.25 μg/ml) and Adriamycin (0.025 μg/ml), the cell kill was not greater than that obtained with Adriamycin alone. We have no explanation yet for the synergistic effect of FU plus Adriamycin in these experiments. Two of the drugs used in our combination studies, namely, VCR and Adriamycin, are active against breast cancer (8). Previous studies showed that, when injected simultaneously, FU plus VCR or FU plus Adriamycin did not result in therpeutic synergism (12).

The therapeutic effect in our *in vivo* experiments (Table 4) indicated additive interaction which could be explained by the lack of accumulation of S-phase cells. Preliminary experiments to maintain constant concentrations of FU by infusing the drug are encouraging, since they show that cells accumulate in the S phase. In humans, i.v. infusion seemed to be less toxic than bolus i.v. injection of FU (18). Sadee *et al.* (17) obtained steady-state FU levels after slow i.v. or intrahepatic artery infusion in patients. Kim *et al.* (13) obtained remissions in patients with disseminated gastrointestinal carcinoma who were given mitomycin C in combination with infused FU. An attempt to transfer our results to the clinic, where FU infusion will be followed by adriamycin, may prove worthwhile.

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