Mechanism of Growth Inhibition by 5-Fluorouracil. Reversal Studies with Pyrimidine Metabolites *in Vitro** (34041)

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The fluoropyrimidine, 5-fluorouracil (FU), has proved to be one of the more useful agents available for treating patients with advanced cancer. Possible mechanisms accounting for the cytotoxicity of FU include (1) inhibition of the formation of thymidylate (TdRP), blocking DNA synthesis (1–3); (2) synthesis of malfunctioning RNA due to the replacement of uracil (U) by FU (4, 5); and (3) inhibition of pyrimidine nucleotide synthesis (6, 7), with resultant interference in the production of RNA or DNA, or both.

In the reversal studies reported here, the effects of U, uridine (UR), orotic acid (OA), and thymidine (TdR) on the toxicity of FU toward Ehrlich ascites tumor cells, strain ELD, in tissue culture were investigated.

The results suggest that FU kills ELD cells by blocking the formation of TdRP.

Materials and Methods. The Ehrlich ascites tumor cell line ELD (8) was obtained as a mouse ascites line from Dr. L. Révész in 1961. Shortly thereafter, the cells were isolated in tissue culture by one of us (R.C.). The original ELD cells had 46 chromosomes; the cells in the tissue culture line employed in the present experiments have 48 chromosomes.

The cells were grown in Leibovitz' medium L-15 (9) with 8% fetal calf serum plus bicarbonate or carbon dioxide, or both, as needed to maintain pH at 7.4 to 7.6. Falcon T-30 disposable culture flasks were used throughout.

Replicate cultures were inoculated with 0.5 ml of stock cell suspension (approximately 10^6 cells as determined by hemacytometer) from a log-phase culture. After 24-hr incubation to allow formation of a monolayer, test substances were added to the culture (total volume, 5 ml); these were FU, U, UR, OA, and TdR.

The cultures were examined daily (100× magnification) for approximate cell counts and detection of cytotoxicity or loss of adherence to the flasks. Subcultures were made by gently scraping the cells into the medium, removing sufficient medium and suspended cells to leave approximately 10^6 cells, and then adding fresh medium and test substances (total volume, 5 ml). Subculturing was maintained for 3 to 4 weeks for continuously growing cultures to verify attainment of constant growth. Cultures or subcultures showing toxicity sufficient to prevent continuous growth were observed until no viable cells remained.

Short-term exposure to FU was carried out as follows: ELD cells were transferred to a 15-ml centrifuge tube and incubated in 5 ml of medium for 1 to 3 hr in the presence of FU. At the end of the FU exposure, the cells were rapidly washed three times in fresh medium, then incubated for 1 hr in 5 ml of additional medium to remove nonmetabolized FU. This medium was centrifuged off and, after a final rinse, the cells were suspended in 5 ml of medium and transferred to culture flasks.

Results. The patterns of cell growth produced by exposure to FU, pyrimidine metabolites, or both, are shown in Fig. 1.

ELD cells grew continuously in FU $\leq$ 0.015 μg/ml (Table I). Cytotoxicity (macrocytosis, multinuclearity, irregularity of cytoplasmic outline) was apparent after 24 hr growth in 0.03–0.15 μg/ml of FU; cells usually divided once or twice before dying. At FU $\geq$ 0.5 μg/ml, early cytotoxicity was more

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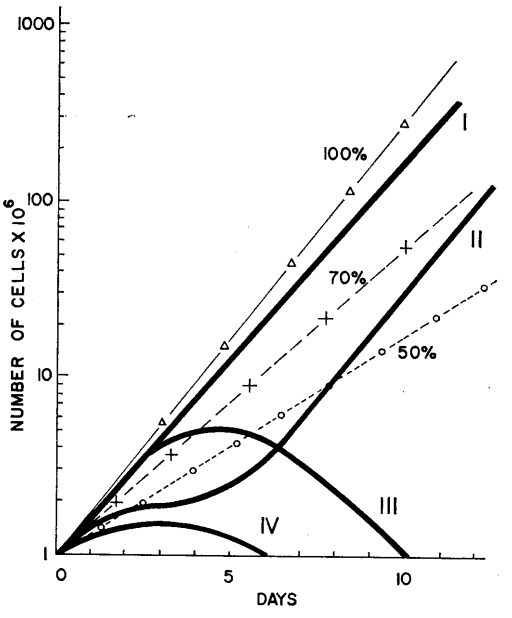

FIG. 1. Schematic representation of cell-growth rates and patterns produced by exposure to FU, pyrimidine metabolites, or both. Exponential growth rates are shown for reference. The 70% and the 50% lines were calculated from the control (100%) values and do not represent actual experimental findings. Control—△—△; 70% of control (doubling time, 1.4 times control)--×--×; 50% of control (doubling time, 2.0 times control) --○--○. Experimental growth patterns are indicated as follows (heavy lines): I, exponential growth at 70–100% of the control rate; II, initial growth rate lower than control rate, followed by an increase to approximately the control rate; III, initial growth rate at the control rate or lower, followed by cell death; IV, no growth, or doubtful growth followed by cell death.

pronounced and little or no cell division took place.

Continuous exposure to FU. An FU concentration of 0.05 μg/ml was selected for initial studies. This minimal but uniformly lethal concentration was chosen in an attempt to confine the major effects of FU to the most sensitive metabolic locus. The identity of the site of action of FU might then be inferred from the effects of normal pyrimidine metabolites on FU cytotoxicity.

Table II shows the growth rate of ELD cells in medium containing FU plus either U, UR, or OA. None of these metabolites exerted a protective effect against FU toxicity, even at 5000 times or more the molar concentration of FU.

Table III shows the growth rate of ELD cells in medium containing FU plus TdR. TdR at 2.5–10 μg/ml did have a protective effect against the toxicity of FU at 0.05 μg/ml, permitting continuous cell growth. Maximum protection was found at a TdR concentration of 5 μg/ml. (TdR alone at 50 μg/ml was toxic to ELD cells.) However, when the FU concentration was ≧ 0.5 μg/ml, TdR had only a minimal protective effect; all ELD cells died after 1 or 2 subcultures. Further testing showed that the slight protection exerted by TdR against the toxicity of FU at 0.5 μg/ml was not increased by the addition of U, UR, or OA.

Short-term exposure to FU. In contrast to the conditions of continuous exposure to fixed concentrations of FU *in vitro*, as in the above experiments, FU is usually administered to cancer patients or tumor-bearing animals by intravenous or intraperitoneal injections at daily (10) or weekly (11) intervals. Since FU is rapidly catabolized by the liver or excreted in the urine, or both (12), there are intermittent peaks (lasting about 1 hr) of high extracellular FU interspersed with long-

TABLE I. Effect of Continuous Exposure to Fluorouracil on Cell Growth.

FU (μg/ml)	Cell growth[a]	
	Initial[b]	Final[c]
—	+++	+++
0.005	+++	+++
0.015	+++	+++
0.03	++	0
0.05	++	0
0.15	++	0
0.5–5	±	0

[a] In Tables I–IV, initial and final determinations of cell growth are indicated as follows: Growth at 70% to 100% of control rate, +++; growth at 50% to 69% of control rate, ++; definite growth at <50% of control rate, +; questionable growth, ±; no growth, 0.

[b] Determined after 3–5 days in culture.

[c] Determined after 21–28 days in culture.

TABLE II. Effect of Uracil, Uridine, and Orotic Acid on Cell Growth in Medium Containing Fluorouracil (0.05 μg/ml).

U (μg/ml)	UR (μg/ml)	OA (μg/ml)	Cell growth[a] No FU Initial[b]	No FU Final[c]	FU Initial[b]	FU Final[c]
—			+++	+++	++	0
0.05–50			+++	+++	++	0
250			+++	+++[d]	++	0
	0.05–5		+++	+++	++	0
	50		+++	+++	++	0
	500		+++	++	++	0
		0.05–50	+++	+++	+++	0
		500[e]	+++	++	+++	0

[a] Basis of estimating cell growth shown in footnote *a* to Table I.
[b] Determined after 3–5 days in culture.
[c] Determined after 21–28 days in culture.
[d] Cells abnormally large.
[e] OA precipitates at 500 μg/ml in this medium.

er intervals (23 hr to 7 days) of no extracellular FU. In the following group of experiments, the effect of TdR was tested again under conditions approximating those *in vivo*.

Table IV shows the rate of growth of ELD cells when they were exposed to FU for periods of 1 to 3 hr, followed by continuous incubation in medium containing TdR, 5 μg/ml. In contrast to the results with continuous FU exposure, in these brief exposures higher FU concentrations (5–50 μg/ml) were required to inhibit ELD cell growth: 5 μg/ml for 1 hr strongly inhibited cell growth; 5 μg/ml for 2 hr and 50 μg/ml for 1 hr were $\geq LD_{100}$. These higher FU concentrations fall within the range of those produced during the treatment of ELD ascites tumors in mice (10–25 mg/kg daily). TdR exerted a definite protective effect against brief exposure to FU. Growth inhibition was not observed when, after exposure to FU at $\leq$ 50 μg/ml for 1 hr, or at 5 μg/ml for 2 hr, the cells were transferred to medium containing TdR, 5 μg/ml. TdR protected less completely against the toxic effects of FU at 50 μg/ml for 2 or 3 hr or at 500 μg/ml for 1 or 2 hr (Table IV). These latter FU concentrations or periods of exposure, or both, are, however, considerably greater than those produced in the treatment of tumor-bearing mice. All TdR-protected cultures survived and ultimately resumed normal growth.

TABLE III. Effect of Thymidine on Cell Growth in Medium Containing Fluorouracil (0.05 μg/ml).

TdR (μg/ml)	Cell growth[a] No FU Initial[b]	No FU Final[c]	FU Initial[b]	FU Final[c]
—	+++	+++	+++	0
0.05	+++	+++	+++	0
0.5	+++	+++	+++	±
2.5	+++	+++	+++	+++
5	+++	+++	+++	+++
10	+++	+++	+++	+++
50	+++	±	+++	±
500	+++	0	+++	0

[a] Basis of estimating cell growth shown in footnote *a* to Table I.
[b] Determined after 3–5 days in culture.
[c] Determined after 21–28 days in culture.

Discussion. FU and its derivatives inhibit several enzymes important in nucleic acid metabolism: FU inhibits uridine phosphorylase (6); 5-fluorouridine (FUR) inhibits uridine kinase (6); 5-fluorodeoxyuridylate (FUdRP) inhibits thymidylate synthetase

TABLE IV. Effect of Short Exposure to FU Followed by Continuous Exposure to Thymidine (5 μg/ml) on Cell Growth.

FU Concn. (μg/ml)	FU Exposure (hr)	Cell growth[a] No TdR Initial[b]	No TdR Final[c]	TdR Initial[b]	TdR Final[c]
—	—	+++	+++	+++	+++
0.5	1	+++	+++	+++	+++
5	1	+	+++	+++	+++
50	1	±	0	+++	+++
500	1	±	0	++	+++
5	2	+	0	+++	+++
50	2	±	0	++	+++
500	2	±	0	+	+++
50	3	±	0	++	+++

[a] Basis of estimating cell growth shown in footnote *a* to Table I.

[b] Determined after 3–5 days in culture.

[c] Determined after 21–28 days in culture.

(2). FU is also incorporated in RNA (4, 5). The significance of these findings relative to the *in vivo* antitumor action of FU is disputed. The most generally held hypothesis, that of Heidelberger and co-workers (1–3), is that the antitumor action of FU is due to the inhibition of thymidylate synthetase by the FU metabolite, FUdRP. Goldberg and co-workers (6), on the other hand, observed that FU produced a depression of RNA synthesis in an FU-sensitive line of the L-1210 mouse leukemia but not in an FU-resistant subline. They attributed the effect of FU to inhibition of uridine phosphorylase.

Previous tissue culture studies have supported the importance of a block in thymidylate production as a mechanism of action for 5-fluorodeoxyuridine (FUdR) but not for FU. Rich *et al.* (13) found that deoxyuridine (UdR) and TdR protected H.Ep.1 cells in tissue culture against FUdR but not against FU.

Our findings indicate that TdRP formation is the step most sensitive to FU. The toxicity of both continuous exposure to low FU concentrations and of brief exposure to higher FU concentrations was counteracted by adding TdR to the medium containing ELD cells. Inasmuch as the conditions of brief exposure approximate those produced during treatment of tumor-bearing mice with FU, it is reasonable to infer that the inhibition of TdRP synthesis also plays a major role in the antitumor activity of FU against ELD cells *in vivo*.

Our results thus differ from those of Rich and co-workers (13) with H.Ep.1 cells. This disparity may stem from a difference in the mechanisms of FU cytotoxicity in the two cells lines, but it seems equally likely that the difference in results is due to variations in experimental design. Rich and co-workers studied the effects of continuous exposure to FU on the growth of H.Ep.1 cells in tissue culture, using protein formation at 7 days as the measure of growth. For reversal studies, they selected an FU concentration (1 μg/ml) sufficient to block protein formation, whereas we used an FU concentration of only 0.05 μg/ml. We did not measure protein formation in our cultures; however, since ELD cells exposed to 0.05 μg/ml of FU usually divided once or twice before growth stopped, a considerable increase in protein during the first few days of culture seems probable. The onset of growth inhibition was much more rapid at FU concentrations of 0.5–5 μg/ml and the formation of protein was presumably minimal. These latter conditions correspond more closely to those used by Rich and coworkers. TdR did exert partial protection against the toxicity of 0.5–5 μg/ml of FU, permitting some initial cell growth, but growth stopped after 4–8 days and the cells began to slough from the flask. It may be that ELD cells, and possibly H.Ep.1 cells, lack the capacity to completely satisfy their TdRP requirements from extracellular TdR, and consequently cannot survive in the presence of enough FU to completely block TdRP synthesis.

Further work will be necessary to determine whether our findings with ELD cells constitute a special case or are generally applicable. However, preliminary findings for another tissue culture-adapted strain of Ehrlich ascites tumor are similar to those for ELD cells.

Summary. Continuous exposure to FU at a concentration of 0.05 μg/ml was uniformly toxic for a tissue culture strain of Ehrlich ascites tumor, ELD. Of the normal pyrimidine metabolites tested in the presence of FU, neither U, UR, or OA exerted a protective effect against FU toxicity. However, TdR did have a protective effect at a concentration of 5 μg/ml, permitting normal growth of ELD cells. At higher FU concentrations, TdR had less protective action. Brief (1–3 hr) exposures to FU at high concentrations (5–50 μg/ml) produced toxicity which could be counteracted when the FU exposure was followed by cell washing and continuous exposure to TdR. These findings suggest that TdRP formation is the step most sensitive to FU.

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1. Bosch, L., Harbers, E., and Heidelberger, C., Cancer Res. **18**, 335 (1958).

2. Hartmann, K-U. and Heidelberger, C., J. Biol. Chem. **236**, 3006 (1961).

3. Heidelberger, C., Kaldor, G., Mukherjee, K. L., and Danneberg, P. B., Cancer Res. **20**, 903 (1960).

4. Gros, F., Gilbert, W., Hiatt, H. H., Attardi, G., Spahr, P. F., and Watson, J. D., Cold Spring Harbor Symp. Quant. Biol. **26**, 111 (1961).

5. Lowrie, R. J. and Bergquist, P. L., Biochemistry **7**, 1761 (1968).

6. Goldberg, A. R., Machledt, J. H., Jr., and Pardee, A. B., Cancer Res. **26**, 1611 (1966).

7. Sköld, O., Ark. Kemi **17**, 51 (1960).

8. Hauschka, T. S., Grinnell, S. T., Révész, L., and Klein, G., J. Natl. Cancer Inst. **19**, 13 (1957).

9. Leibovitz, A., Am. J. Hyg. **78**, 173 (1963).

10. Ansfield, F. J., JAMA **190**, 686 (1964).

11. Jacobs, E. M., Luce, J. K., and Wood, D. A., Cancer **22**, 1233 (1968).

12. Mukherjee, K. L. and Heidelberger, C., J. Biol. Chem. **235**, 433 (1960).

13. Rich, M. A., Bolaffi, J. L., Knoll, J. E., Cheong, L., and Eidinoff, M. L., Cancer Res. **18**, 730 (1958).

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