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RESISTANCE OF CCRF-CEM CLONED SUBLINES TO 5-FLUORODEOXYURIDINE ASSOCIATED WITH ENHANCED PHOSPHATASE ACTIVITIES*

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Abstract—Resistance of human CCRF-CEM leukemic cells in tissue culture to 5-fluoro-2′-deoxyuridine (FdUrd) has been examined following a single drug exposure (FS sublines). In two FS sublines generated by soft agar cloning of FdUrd sensitive cells in the presence of 10 nM FdUrd, the level of drug resistance was maintained at 22- to 30-fold following 1 month growth in the absence of FdUrd. Characteristic of the FS sublines was a decreased accumulation and retention of free intracellular 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) averaging 3% of FdUrd sensitive cells, a more rapid rate of disappearance of free FdUMP and FdUMP-bound thymidylate synthase (EC 2.1.1.45, 5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase), and enhanced alkaline and acid phosphatase activities. There was no significant difference in the number of nucleoside transport sites per cell among the FS sublines and FdUrd-sensitive cells, indicating that the decreased accumulation of FdUMP in the resistant sublines was not the result of impaired FdUrd transport across the plasma membrane. The more rapid turnover of FdUMP-bound TMP synthase observed in the FS sublines was neither accompanied by a decreased stability of the TMP synthase–FdUMP–5,10-methylenetetrahydrofolate ternary complex, nor an enhanced rate of degradation of FdUrd to the less potent agent, 5-fluorouracil. In addition, the growth rates of the two FS sublines were similar to that of FdUrd sensitive cells in medium containing hypoxanthine, methotrexate, and thymidine, indicating that there was no depletion of thymidine kinase (EC 2.7.1.21, ATP:thymidine-5′-phosphotransferase) in the FS sublines. Therefore, we propose that enhanced activities of acid and alkaline phosphatases, which influence the intracellular accumulation and retention of FdUMP, are important determinants of stable FdUrd resistance in CCRF-CEM cells.

5-Fluoro-2′-deoxyuridine (FdUrd)‡ continues to be an important drug in the treatment of solid tumors of the gastrointestinal tract, liver, and breast [1]. Recent reports indicating that FdUrd is highly effective in inducing remissions following continuous hepatic artery chemotherapy of primary and metastatic cancer of the liver [2–4] have generated further interest in this compound.

The emergence of resistance to FdUrd, as evidenced by a failure in maintaining tumor remissions, is a frequent clinical problem which seriously limits the usefulness of this drug [1, 5]. Obviously, the effectiveness of FdUrd therapy could be improved if the biochemical basis for clinical FdUrd resistance in tumors was identified and ways devised to circumvent the development of resistance. Since the intracellular metabolism of fluoropyrimidines is quite complex, one would anticipate that different biochemical effects might contribute to FdUrd resistance in tumor cells.

Indeed, several biochemical effects accompanying resistance to fluoropyrimidines have been described including: (a) decreased thymidine kinase activity [6, 7] and presumably diminished formation of the FdUrd active metabolite, FdUMP; (b) a more rapid disappearance of FdUMP in resistant tumors [8–10]; and (c) changes in the target enzyme, TMP synthase, involving either impaired binding of FdUMP [11, 12] or enhanced intracellular enzyme activity [13–16]. However, the relevance of some of these observations to the therapeutic use of FdUrd was not clear. For instance, the 10- to 50-fold elevations in TMP synthase activity measured in tissue culture cells following growth for several months in greater than therapeutic concentrations of FdUrd [14, 15] would probably not occur in clinical cancers. Nevertheless, it appeared reasonable that more modest biochemical alterations could have been induced in tumor cells by limited exposure to therapeutic concentrations of FdUrd. Therefore, we thought it important to examine various biochemical para-

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‡ Abbreviations: FdUrd, 5-fluoro-2′-deoxyuridine; TMP, thymidine-5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; CMP, cytidine-5′-monophosphate; MTX, methotrexate; DTT, dithiothreitol; HPLC, high performance liquid chromatography; (*d,l*)-5,10-CH_2-H_4PteGlu, racemic mixture of the natural (*l*) and unnatural (*d*) diastereoisomers of 5,10-methylenetetrahydropteroylglutamate; SDS, sodium dodecyl sulfate; ED_{50} concentration of the compound required to inhibit cell growth 50% compared to untreated cells following a 48-hr continuous exposure; TMP synthase, 5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45, thymidylate synthase; FS sublines, FdUrd resistant CCRF-CEM leukemic cell clones isolated following a single FdUrd exposure.

meters which could relate to the development of resistance following a single FdUrd selection. In this paper we describe two cloned sublines of human CCRF-CEM leukemic cells in which stable FdUrd resistance is related to enhanced phosphatase activities that promote the more rapid turnover of free and FdUMP-bound TMP synthase.

MATERIALS AND METHODS

MTX (sodium salt) was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. DTT, dUMP (sodium salt), FdUMP (sodium salt), CMP (disodium salt), FdUrd, hypoxanthine, bovine serum albumin, dextran, and β-glycerophosphate were obtained from the Sigma Chemical Co., St. Louis, MO. [5-^{3}H]dUMP, 5-*p*-(*p*-nitrobenzyl)6-thioinosine-[^{3}H-G] (specific radioactivity of 16 Ci/mmole), [6-^{3}H]FdUrd (specific radioactivity of various batches determined to be between 10.6 and 12.1 Ci/mmole), and [6-^{3}H]FdUMP (specific radioactivity measured as 12.1 Ci/mmole) were obtained from Moravek Biochemicals, Brea, CA. All tissue culture medium, serum, and antibiotics were obtained from the Grand Island Biological Co., Grand Island, NY. Dextran–bovine serum albumin-treated charcoal suspension consisted of 5% (w/v) activated charcoal (Norit A, J. T. Baker Co., Phillipsburg, NJ), 1% (w/v) bovine serum albumin, and 0.1% dextran (average molecular weight, 4.6×10^5) in water. Coomassie blue G-250 was obtained from the Eastman Kodak Co., Rochester, NY. Agarose (Sea Kem LE) was purchased from the FMC Corp., Rockland, ME. DE-81 disks (2.5 cm diameter) were obtained from Whatman Inc., Clifton, NJ. (*d,l*)-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid [17], and converted to (*d,l*)-5,10-CH_2-H_4PteGlu by the addition of formaldehyde. ACS liquid scintillation fluid was purchased from the Amersham Corp., Arlington Heights, IL. Buffer A consisted of 10 mM Tris–HCl, pH 7.5, and 4 mM DTT. HAT medium consisted of Fischer's medium supplemented with 10% (v/v) horse serum and 100 μM hypoxanthine, 1 μM amethopterin (MTX), 5 μM thymidine, and 3 μM glycine. The concentrations of all stock drug solutions were determined spectrophotometrically.

Isolation of FdUrd resistant sublines. The CCRF-CEM human leukemic cell line was obtained from Dr. Joseph R. Bertino of the Yale University School of Medicine, and was propagated at 37° under 95% air–5% CO_2 in Fischer's medium supplemented with 10% horse serum, 100,000 units/liter of penicillin, and 100 mg/liter of streptomycin. Under these conditions, the doubling time of logarithmically growing FdUrd sensitive cells was 32 hr. Cells were checked periodically for mycoplasma with the Hoechst fluorescent staining method [18], and mycoplasma free cultures were used for subsequent experiments.

FdUrd resistant CCRF-CEM sublines were isolated by cloning in soft agarose [19] following a single exposure to FdUrd. Single-step selection was carried out by cloning 1×10^4 untreated cells per culture tube in 10 nM FdUrd, a drug concentration which produces a 3.2 log reduction in cloning efficiency. The cloning efficiency of FdUrd sensitive CCRF-CEM cells in the absence of FdUrd varied among experiments between 50 and 80%. Resistant colonies were removed from the tubes with a sterile Pasteur pipette and grown in 75 cm^2 polystyrene flasks in the absence of drug for 1 month. The FdUrd resistant sublines were designated sublines FS-1 and FS-2. The EC_{50} values of FdUrd for the FS sublines were calculated from electronic determinations of cell numbers. The FS sublines had been propagated for 1 month growth in the absence of FdUrd prior to the determination of the EC_{50} values. After confirming that FdUrd resistance was maintained in the absence of FdUrd in the FS sublines, biochemical analyses of the FS sublines were carried out. The FS sublines were subsequently maintained by reintroducing FdUrd at 10 nM to the culture medium, which is ten times the EC_{50} concentration for FdUrd sensitive cells.

Enzyme extractions and assays. The specific activity of TMP synthase has been shown to vary directly with the rate of cell proliferation [20, 21]. Thus, in order to compare TMP synthase activities in FdUrd sensitive and resistant sublines, all cultures were harvested at similar cell densities in the logarithmic phase of growth. The cells were washed once with 0.9% (w/v) NaCl solution and centrifuged, and the pellets were resuspended in 5 vol. of buffer A at 4°. The pellets were then subjected to a single freeze–thawing in a solid CO_2–methanol bath and sonicated with three 15-sec bursts of 15 W using a Branson 200 Sonifer cell disrupter equipped with a microtip. The suspension was then centrifuged at 13,000 *g* for 10 min at 4°, and the supernatant fraction was used for all enzyme assays. Protein concentrations were estimated by the Coomassie blue staining method [22].

TMP synthase activity was assayed in cell extracts for 15 min at 37° by the tritium displacement method of Lomax and Greenberg [23], as modified by Dolnick and Cheng [24] in a final volume of 40 μl. The reaction mixture was preincubated for 2 min at 37° before the initiation of the reaction by the addition of enzyme. Tritium release was linear with respect to time and protein concentration under the conditions used in all experiments. One enzyme unit is defined as the quantity of enzyme required to form 1 nmole of product per min at 37° under our assay conditions. The results were expressed as the mean ± S.D. of four determinations.

Acid and alkaline phosphatase activities were determined by measuring the rates of disappearance of [6-^{3}H]FdUMP in the 13,000 *g* supernatant fractions of CCRF-CEM cells prepared as described for the TMP synthase assay. Reactions were carried out in a final volume of 40 μl and contained the following final concentrations in the acid phosphatase assay: 1 mM [6-^{3}H]FdUMP (2.42 mCi/mmole), 25 mM sodium acetate, pH 5.8, and between 5 and 10 μl of enzyme extract containing a maximum of 100 μg protein. Alkaline phosphatase activity was assayed under the same conditions as above except that the buffer was 25 mM ammonium bicarbonate, pH 9.2. The assay mixture was preincubated for 2 min at 37° before the addition of enzyme, followed by a 10- to 30-min assay period at 37° for various samples. The

reaction mixtures were filtered through DE-81 paper disks, and the filters washed with two aliquots (12.5 ml each) of 2 mM sodium acetate, then placed in scintillation vials and spotted with 0.2 ml of 0.2 M KCl in 1 M HCl to elute the radiolabeled nucleotide from the filters. Radioactivity was measured in 10 ml of ACS scintillation fluid. One unit of enzyme activity is defined as the quantity of enzyme required to convert 1 nmole of substrate per min under our assay conditions. The acid and alkaline phosphatase catalyzed reactions were linear with respect to time and protein concentration when as much as 64 and 77%, respectively, of the substrate had been converted to product, indicating a lack of significant product inhibition. Acid and alkaline phosphatase activities in the 13,000 *g* pellet of FdUrd sensitive cells were less than 10% that in the supernatant fraction.

Measurement of rates of disappearance of free intracellular FdUMP and FdUMP-bound TMP synthase and the conversion of FdUrd to FUra. FdUrd sensitive and resistant cells were grown at a volume of 50 ml in 75 cm^2 flasks. When the cultures reached the mid-logarithmic phase of growth at a density of 5×10^5 cells/ml, either 88 nM [6-^{3}H]FdUrd (carrier-free) for determination of free intracellular FdUMP, or the various concentrations of nonradiolabeled FdUrd described below for determination of FdUMP-bound TMP synthase, were added to the flasks. After a 2-hr incubation the cells were washed twice with drug-free medium at 37°, and then resuspended in drug-free medium containing 100 nM 2′-deoxyuridine to retard further conversion of FdUrd to FdUMP. For the determination of free intracellular [^{3}H]FdUMP, 5-ml aliquots were removed from duplicate flasks at 0, 1, 2, 4, and 24 hr after resuspension of the cells in FdUrd-free medium. The cells were washed twice with a 0.9% (w/v) NaCl solution at 4° and counted. The free intracellular [^{3}H]FdUMP, but not the [^{3}H]FdUMP covalently bound to TMP synthase, was then extracted by freeze–thawing in 1 M acetic acid as previously described [25]. The acetic acid extracts were lyophilized to dryness and the powders were resuspended in 0.02 M sodium phosphate, pH 3.3. The mixture was then co-chromatographed with authentic FUra, FdUrd, and FdUMP standards on a Whatman Partisil 10-SAX HPLC column maintained at 45° and eluted isocratically at 45° with 0.02 M sodium phosphate, pH 3.3, at a flow rate of 0.8 ml/min. One-minute fractions were collected for radioactive counting in 10 ml of ACS scintillation fluid. The recovery of exogenous [^{3}H]FdUMP added to the washed cell pellets averaged 96% following the combined extraction and HPLC procedures. Results were expressed as the mean from four determinations of the number of pmoles of either FdUMP or TMP synthase per 10^9 cells, since there were no significant differences in the mean cell volumes between sensitive and any FdUrd resistant sublines. When the assays measuring free FdUMP and FdUMP-bound TMP synthase were repeated, similar results were obtained.

For the determination of FdUMP-bound TMP synthase, 88 nM FdUrd was added to the flasks of FdUrd sensitive cells. To the flasks containing sublines FS-1 and FS-2, 1.9 and 2.6 μM FdUrd, respectively, were added so that the final extracellular FdUrd concentrations achieved would be proportional to the 22- and 30-fold levels of FdUrd resistance shown in Table 1. When sublines FS-1 and FS-2 were incubated with only 88 nM FdUrd, no FdUMP-bound TMP synthase could be detected. After a 2-hr preincubation at 37°, the cells from each flask were washed once with drug-free medium and then resuspended in drug-free medium. One flask from each sensitive and resistant cell line was then harvested at 0, 1, 2, and 4 hr later. The cells were washed once with a 0.9% (w/v) NaCl solution and counted. The intracellular concentrations of free and FdUMP-bound TMP synthase were quantitated using a radiolabeled FdUMP binding assay as previously described [26]. The concentrations of free and FdUMP-bound TMP synthase were corrected for the dissociation of FdUMP from the ternary complex during the 30-min assay procedure as described [26]. Results were expressed as the mean of three determinations of the number of pmoles of FdUMP-bound TMP synthase per 10^7 cells, since there were no significant differences in the mean cell volumes among FUdR sensitive cells and the resistant sublines.

For measuring the conversion of [^{3}H]FdUrd to [^{3}H]FUra, 1 nM [^{3}H]FdUrd (carrier-free) was added to the FdUrd sensitive, FS-1, and FS-2 flasks, and at 0, 2, 4, 6, and 24 hr 0.4-ml aliquots were removed. The samples were precipitated with a final concentration of 10% (w/v) trichloroacetic acid and centrifuged. The supernatant fractions were then adjusted to pH 6.0 with NaOH prior to quantitation of the radiolabeled FUra, FdUrd, and FdUMP by HPLC as previously described [27].

Nitrocellulose filtration assay. Nitrocellulose filtration was carried out on an Amicon filtration manifold. Ternary enzyme complexes were formed by incubating 0.04 to 0.08 units of enzyme extract for 30 min at 37° in a volume of 120 μl with the following final concentrations: 0.1 μM [^{3}H]FdUMP, 0.2 mM *(d,l)*-5,10-CH_2-H_4PteGlu, 3.1 mM formaldehyde, 20 mM 2-mercaptoethanol, 100 mM NaF, and 150 mM Tris–HCl, pH 7.5. At the end of the incubation period, $MgCl_2$ (13 μl) was added to yield a final concentration of 100 mM in order to enhance protein binding to the filter. Aliquots (100 μl) of the mixtures were then pipetted onto nitrocellulose filters presoaked with 25 mM sodium phosphate, pH 7.5, and 50 mM $MgCl_2$. Mildly reduced pressure was applied to the filters, and the filters were then washed with two (15 ml) aliquots of the above buffer. Bound radioactivity was counted after placing the damp filters in scintillation vials containing 10 ml of scintillation fluid.

Measurement of the number of nucleoside transport sites in intact FdUrd sensitive and resistant CCRF-CEM cells. The number of specific nucleoside transport sites per tumor cell was determined by measuring the specific binding of 5-*p*-(*p*-nitrobenzyl)6-thioinosine[^{3}H] to washed cells as described by Cass *et al.* [28].

RESULTS

Effects of FdUrd on the growth of FdUrd sensitive and resistant sublines. Single-step FdUrd selection

Table 1. Levels of FdUrd resistance and free intracellular FdUMP*

Subline	EC_{50}† (nM) FdUrd	FdUMP†, ‡ (pmoles/10^9 cells)
FdUrd sensitive	1	26.0 ± 4
FS-1	22	1.0 ± 0.2§
FS-2	30	0.5 ± 0.1§

* Logarithmically growing CCRF-CEM cells were incubated in triplicate with FdUrd for 48 hr. The EC_{50} values were determined from a range of five concentrations of FdUrd by linear regression analyses of the data.

† Determined after 4 weeks growth of the FS sublines in the absence of FdUrd.

‡ Mean ± S.D. of four determinations after a 2-hr incubation with 10 nM [^{3}H]FdUrd.

§ Significantly different ($P < 0.01$ two tailed t-test) from FdUrd sensitive cells.

involved cloning FdUrd sensitive cells in the presence of 10 nM FdUrd, a drug concentration which produced a 3.2 log reduction in cloning efficiency in sensitive cells. FdUrd EC_{50} values obtained for cloned sublines FS-1 and FS-2 following 1 month growth in the absence of drug were 22 and 30 nM respectively (Table 1). The EC_{50} value for FdUrd sensitive cells was 1 nM. In a second outgrowth experiment, the FdUrd EC_{50} values for FdUrd sensitive cells and sublines FS-1 and FS-2 were 1, 16, and 30 nM respectively.

It was of interest to determine whether or not FdUrd resistance in the FS sublines reflected the intracellular accumulation of the FdUrd active metabolite, FdUMP. Following a 2-hr exposure to 10 nM carrier-free [^{3}H]FdUrd, the mean intracellular free FdUMP concentration (i.e. acid-soluble FdUMP not complexed with TMP synthase) of each FS subline was significantly less than the mean FdUMP concentration of FdUrd sensitive cells (Table 1). Thus, the degrees of resistance of the FS sublines were inversely related to the accumulation of FdUMP.

FdUrd transport as a determinant of resistance. It was possible that impaired transport of FdUrd into the resistant sublines FS-1 and FS-2 contributed to the decreased accumulation of FdUMP shown in Table 1. Transport across the plasma membrane of nucleosides, including FdUrd, occurs by carrier mediated (facilitated) diffusion [27, 28]. Nitrobenzylthioinosine is a specific inhibitor of nucleoside transport which binds tightly to the nucleoside transport carrier, and it has been used to quantitate the number of nucleoside transport sites on the plasma membrane [28, 29]. Scatchard analysis of the [^{3}H] nitrobenzylthioinosine binding data revealed that the resistant sublines FS-1 and FS-2 had 1.63 ± 0.18 and 1.75 ± 0.23 S.E. × 10^5 specific nucleoside transport sites per cell, respectively, which were not significantly different from that of FdUrd sensitive cells of 1.77 ± 0.23 S.E. × 10^5 sites per cell. Since there were also no differences in the mean cell volumes among FdUrd sensitive and resistant cells, the above data indicate that impaired transport of FdUrd across the plasma membrane did not significantly contribute to the decreased accumulation of FdUMP in the resistant sublines.

Thymidine kinase and phosphatase activities. Since the FS sublines accumulated less FdUMP after a 2-hr FdUrd exposure than FdUrd sensitive cells, we subsequently attempted to measure the activities of enzymes catalyzing the synthesis and degradation of FdUMP. FdUrd is an alternate substrate for thymidine kinase, which converts the nucleoside to FdUMP [7]. However, it was not possible to accurately measure thymidine kinase activity in the crude cell extracts of the FS sublines because of their elevated phosphatase activities, which rapidly degraded FdUMP to FdUrd (Table 2). NaF, β-glycerophosphate, and CMP could not be used to selectively inhibit phosphatase activity in the thymidine kinase assay. NaF at 100 mM and 1 mM β-glycerophosphate only partially inhibited the alkaline and acid phosphatases activities, respectively, and CMP inhibited thymidine kinase (data not shown). To evaluate intracellular thymidine kinase activity, sublines FS-1 and FS-2 and FdUrd sensitive cells were grown in medium supplemented with either hypoxanthine, amethopterin (methotrexate), thymidine, and glycine (HAT medium), or with HAT medium lacking thymidine (HA medium). The growth rates of sublines FS-1 and FS-2 were similar to that of sensitive cells in HAT medium, and in HA medium in which the proliferation of all the cell lines was inhibited to similar extent (Fig. 1). This assay indirectly indicated that thymidine kinase activity, which is necessary for both the utilization of exogenous thymidine and the activation of FdUrd to

Table 2. Effects of single FdUrd selection on the specific activities of acid and alkaline phosphatases*

Subline	Acid phosphatase (units/mg protein)	Alkaline phosphatase (units/mg protein)
FdUrd sensitive	9 ± 2	28 ± 3
FS-1	37 ± 6†	45 ± 3†
FS-2	16 ± 2†	49 ± 10†

* Acid and alkaline phosphatase specific activities were determined in extracts of FdUrd sensitive and resistant FS sublines harvested at equal cell densities as described in Methods. FS sublines were grown for 4 weeks in the absence of FdUrd prior to analysis. Values are means ± S.D. of four determinations.

† $P < 0.01$ compared to FdUrd sensitive cells, two-tailed t-test.

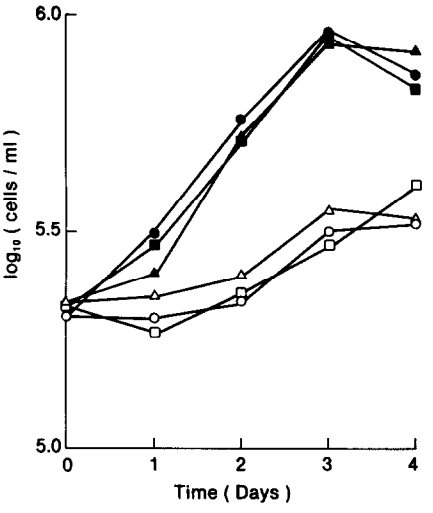

Fig. 1. Growth rates of FdUrd sensitive and resistant CCRF-CEM cells in HAT and HA media. Cells were grown in Fischer's medium containing 10% horse serum and the following additions: FdUrd sensitive cells with 100 μM hypoxanthine, 1 μM methotrexate, and 3 μM glycine (HA medium) (○); clone FS-1 in HA medium (△); clone FS-2 in HA medium (□); FdUrd sensitive cells in HA medium plus 5 μM thymidine (HAT medium) (●); clone FS-1 in HAT medium (▲); and clone FS-2 in HAT medium (■). Points, mean of duplicate cultures.

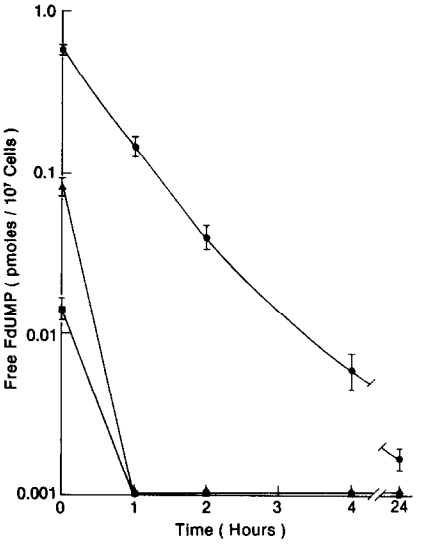

Fig. 2. Rates of disappearance of acid-soluble FdUMP. Logarithmically growing FdUrd sensitive cells (●), and resistant sublines FS-1 (▲) and FS-2 (■), were preincubated with 88 mM [^{3}H]FdUrd (sp. act. 10.7 Ci/mmole) for 2 hr. The cells were then washed twice with drug-free medium and resuspended in drug-free medium containing 100 nM 2′-deoxyuridine. FdUMP levels were determined by HPLC at 0, 1, 2, 4, and 24 hr after resuspension of the cells in drug-free medium. Points, mean of duplicate determinations ± range.

FdUMP, was not decreased significantly in these resistant sublines compared to FdUrd sensitive cells. Therefore, the markedly lower accumulation of FdUMP in the resistant sublines did not appear attributable to diminished thymidine kinase activity.

On the other hand, in the FS-1 and FS-2 sublines statistically significant increases in both alkaline and acid phosphatase specific activities were evident (Table 2). Similar results were obtained when this experiment was repeated. In the FdUrd sensitive, FS-1, and FS-2 cells, the acid phosphatase specific activities measured in the second experiment were 10 ± 1, 39 ± 7, and 17 ± 3 S.D. units/mg protein; the alkaline phosphatase activities were 29 ± 3, 50 ± 3, and 59 ± 11 S.D. units/mg protein respectively. The phosphatase activities measured in the acid and alkaline pH ranges are likely associated with two distinct enzymes. β-Glycerophosphate at 1 mM inhibited only the acid phosphatase catalyzed degradation of FdUMP and dUMP, whereas 100 mM NaF was inhibitory only to the alkaline phosphatase when FdUMP was the substrate (data not shown).

Rates of disappearance of acid-soluble FdUMP and FdUMP-bound TMP synthase. As an additional approach for evaluating intracellular phosphatase activities, the rates of disappearance of acid-soluble FdUMP and FdUMP-bound TMP synthase were measured in FdUrd sensitive cells, and in the resistant sublines FS-1 and FS-2. FdUrd sensitive cells accumulated higher levels of free FdUMP than either sublines FS-1 or FS-2 after a 2-hr preincubation with [^{3}H]FdUrd (Fig. 2). The results of this pulse-chase experiment shown in Fig. 2 indicated that a decreased retention of intracellular FdUMP contributed to the lower intracellular concentrations of FdUMP measured in the resistant sublines. Within 1 hr after resuspension of the cultures in FdUrd-free medium, free intracellular FdUMP fell to nondetectable levels in sublines FS-1 and FS-2. However, in FdUrd sensitive cells the disappearance of intracellular FdUMP was much slower and free FdUMP was detectable after 24 hr.

This persistence of FdUMP in sensitive cells was sufficient to prevent any loss of FdUMP-bound TMP synthase for at least 4 hr after resuspension of the cells in FdUrd-free medium, since the fraction of total TMP synthase containing bound FdUMP did not decrease during this time period (Fig. 3). When this assay was carried out with the two FdUrd resistant sublines, no FdUMP-bound TMP synthase was detectable following a 2-hr preincubation with 88 nM FdUrd. Therefore, sublines FS-1 and FS-2 were preincubated with 1.9 and 2.6 μM FdUrd, respectively, so that the final extracellular FdUrd concentration achieved would be proportional to the 22- and 30-fold levels of FdUrd resistance shown in Table 1. The fractions of FdUMP-bound TMP synthase in both FS sublines were similar to that of FdUrd sensitive cells after the resistant sublines were preincubated with the higher FdUrd concentrations (Fig. 3). However, despite preincubation with these higher FdUrd concentrations, the fractions of FdUMP-bound TMP synthase decreased 52 and 56% in sublines FS-1 and FS-2, respectively, within 4 hr after resuspension of these sublines in FdUrd-free medium.

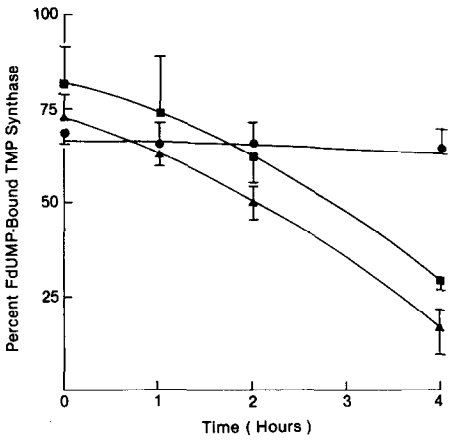

Fig. 3. Rates of disappearance of FdUMP-bound TMP synthase. Logarithmically growing FdUrd sensitive cells (●), and resistant sublines FS-1 (▲) and FS-2 (■), were incubated with 88 nM, 1.9 μM, and 2.6 μM FdUrd, respectively, for 2 hr at 37°. The cells were then washed once with drug-free medium and resuspended in drug-free medium; the concentrations of total and FdUMP-bound TMP synthase were determined at 0, 1, 2, and 4 hr. The time 0 value of 69% FdUMP-bound TMP synthase in FdUrd sensitive cells represents 2.07 pmoles/10^7 cells. Points, mean of triplicate determinations ± 1 S.D.

Degradation of FdUrd to FUra. The possibility was examined that an enhanced rate of degradation of FdUrd to the much less potent fluoropyrimidine, FUra, contributed to FdUrd resistance in sublines FS-1 and FS-2. Because of the extremely rapid equilibration of FdUrd and FUra across the plasma membrane [27], it is possible that significant amounts of these fluoropyrimidines could be lost during the cell harvesting and washing procedures prior to analysis. Thus, the concentrations of FdUrd and FUra in the tissue culture medium were measured at various time points as a means of estimating the rate of intracellular conversion of FdUrd to FUra.

In cultures of FdUrd sensitive cells, 38% (range 37–38%) of the total radioactivity in the medium was present as [^{3}H]FUra at 24 hr. Sublines FS-1 and FS-2 degraded FdUrd much more slowly than sensitive cells, with greater than 90% (range 88–93%) of the extracellular radioactivity remaining as [^{3}H]FdUrd at 2, 4, 6, and 24 hr.

Effects on TMP synthase. The specific activities of TMP synthase were measured in FdUrd sensitive cells, and in the FS sublines maintained for 4 weeks in the absence of FdUrd. All extracts were prepared from logarithmically growing cultures harvested at similar cell densities. None of the FS sublines had as much as a 2-fold difference in TMP synthase specific activity compared to FdUrd sensitive cells (Table 3).

The relative stabilities of the [^{3}H]FdUMP–5,10-CH_2-H_4PteGlu–TMP synthase ternary complexes extracted from FdUrd sensitive and resistant cell lines were compared by the nitrocellulose filtration assay, and by measuring the first-order rate constant for FdUMP dissociation from the ternary complex (k_{off}) in cell extracts. No differences among FdUrd sensitive cells and the two resistant sublines were observed in the tightness of FdUMP binding to TMP synthase as measured by the nitrocellulose filtration assay (Table 3).

There were also no significant differences among the k_{off} values for FdUMP of 3.14 ± 0.17 S.E. $\times 10^{-3}$, $3.45 \pm 0.22 \times 10^{-3}$, and $3.50 \pm 0.25 \times 10^{-3}$ min^{-1} at 37°, pH 7.5, in cell extracts of FdUrd sensitive cells and sublines FS-1 and FS-2 respectively. In summary, the above results indicate that alterations in TMP synthase did not contribute significantly to FdUrd resistance in these sublines.

DISCUSSION

In the two FS sublines that were between 22- and 30-fold resistant to FdUrd, it was found that a decreased accumulation of FdUMP, increased alkaline and acid phosphatase activities, and a more rapid rate of disappearance of free FdUMP and FdUMP-bound TMP synthase were associated with stable levels of FdUrd resistance. Following a preincubation of sensitive cells with FdUrd, there was no significant loss in FdUMP-bound TMP synthase for at least 4 hr after resuspension of the cells in drug-free medium. On the other hand, in the two FS clones having enhanced alkaline and acid phosphatase activities greater than 50% of the FdUMP-bound TMP synthase disappeared within 4 hr. This more rapid disappearance of FdUMP-bound TMP synthase was observed even though the FS-1 and FS-2 sublines were incubated with 22- and 30-fold higher FdUrd concentrations, respectively, than the FdUrd sensitive cells. FdUMP at intracellular concentrations in excess of TMP synthase are probably required to maintain inhibition of free enzyme generated by inhibitor–enzyme complex dissociation and the synthesis of new TMP synthase molecules,

Table 3. Effects of FdUrd selection on TMP synthase*

Subline	TMP synthase (units/mg protein)	[^{3}H]FdUMP bound (pmoles/unit of TMP synthase activity)
FdUrd sensitive	0.11 ± 0.01	7.3 ± 1.0
FS-1	0.10 ± 0.01	8.0 ± 0.6
FS-2	0.06 ± 0.01	7.9 ± 0.6

* TMP synthase specific activity and the binding of FdUMP to the enzyme following nitrocellulose filtration were determined as described in Methods. Logarithmically growing cultures harvested at similar cell densities were used in all assays. Values are means of four determinations ± 1 S.D. after 4 weeks growth of the FS sublines in the absence of FdUrd.

especially in the presence of expanded pools of 2′-deoxyuridine-5′-monophosphate [25]. This concept is further supported by our observation that, in FdUrd sensitive cells pulse-labeled with 88 nM FdUrd, free intracellular FdUMP in excess of TMP synthase persisted for 24 hr. However, in sublines FS-1 and FS-2 having enhanced alkaline and acid phosphatase activities free FdUMP was no longer detectable at 1 hr after FdUrd pulse-labeling.

The rates of disappearance of free FdUMP and FdUMP-bound TMP synthase are influenced by the cellular rates of FdUMP synthesis and degradation, and the dissociation of FdUMP from the 5,10-CH_2-$H_4PteGlu_n$–TMP synthase ternary complex. We propose that enhanced rates of disappearance of free intracellular FdUMP and FdUMP-bound TMP synthase were seen in the FdUrd resistant sublines primarily because the free FdUMP, including that which dissociated from TMP synthase, was more rapidly degraded by the enhanced phosphatase activities. An evaluation of the number of membrane carrier sites involved in the transport of FdUrd, thymidine kinase activities and direct measurements of the stabilities of the TMP synthase–FdUMP–5,10-CH_2-$H_4PteGlu$ ternary complexes indicated that neither the intracellular rates of FdUMP synthesis nor dissociation of FdUMP from TMP synthase were major determinants of the more rapid turnover of FdUMP-bound TMP synthase in the resistant sublines.

Mulkins and Heidelberger [7] reported that the major biochemical determinant to resistance of murine leukemic cell lines isolated following one-step mutagenesis and FdUrd selection was a deficiency in thymidine kinase activity, although measurements of phosphatase activity were not published. We were unable to accurately measure thymidine kinase activity in crude extracts of the FS sublines examined because of extensive degradation of the product, FdUMP, by phosphatases. However, the two FS sublines examined proliferated at the same rate as FdUrd sensitive cells in HAT medium, suggesting that the decreased accumulation of FdUMP in the resistant sublines was not primarily the result of a difference in thymidine kinase activities and a consequently slower rate of conversion of FdUrd to FdUMP. The possibility that any FdUrd resistant tumor could have markedly enhanced phosphatase activities that are not inhibited by NaF should be considered when enzyme assays involving phosphorylated substrates or products (for example thymidine kinase and TMP synthase) are being carried out. The activity of pyrimidine nucleoside phosphorylase, which cleaves FdUrd to FUra [30], was also not directly measured in these sublines. However, no appreciable FUra appeared in the medium from cultures of resistant sublines FS-1 and FS-2, most likely reflecting the decreased rate of FdUrd degradation as a secondary consequence of impaired intracellular accumulation and retention of FdUrd metabolites in these sublines. In addition, Mulkins and Heidelberger [7] have shown that pyrimidine nucleoside phosphorylase activity was not changed in murine L1210 and P388 leukemic cloned sublines resistant to either FdUrd, FUra, or 5-fluorouridine.

The results of this extensive biochemical examination of FdUrd resistance support those previous studies which suggested that an enhanced rate of disappearance of FdUMP was characteristic of fluoropyrimidine resistant tumors [8–10]. For instance, Klubes *et al.* [9] reported that free intracellular FdUMP was retained at detectable levels 2 days longer in the 5-fluorouracil responsive murine L1210 leukemia than in the relatively unresponsive Walker 256 rat tumor when the animals were injected with 100 and 120 mg/kg, respectively, of 5-fluorouracil. Inhibition and resumption of DNA synthesis coincided with the persistence and disappearance of FdUMP.

An enhanced rate of active metabolite dephosphorylation has also been proposed by others as the basis for the insensitivity of human tumors to cytosine arabinoside [31] and 6-thiopurines [32], and this phenomenon may represent a common mechanism of cellular resistance to agents having phosphorylated active metabolites. We are thus currently evaluating cross-resistance between FdUrd and various purine and pyrimidine antimetabolites in FdUrd resistant sublines FS-1 and FS-2.

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