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INCREASED THYMIDYLATE SYNTHETASE IN 5-FLUORODEOXYURIDINE RESISTANT CULTURED HEPATOMA CELLS*

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Abstract—A FdUrd resistant line of cultured mouse hepatoma cells has been obtained. The resistant cell line had 6- to 10-fold higher levels of thymidylate synthetase, but dihydrofolate reductase and thymidine kinase were unchanged. No impairment of FdUrd incorporation by the resistant cell line could be detected. The increased thymidylate synthetase in resistant cells had the same turnover number and I_{50} for FdUMP as the enzyme found in sensitive cells, making it unlikely that a new gene product had been obtained. Sensitive cells could be completely rescued by the addition of thymidine, suggesting that the primary mode of drug action is to diminish thymidine metabolites. Resistant cells, removed from FdUrd for several generations, did not proliferate immediately upon reintroduction of the drug; however, loss of sensitivity was much more rapid than upon initial exposure. These results are interpreted in terms of a mechanism for resistance.

FdUrd‡ and its free base, FUra, have been widely used, alone and in combination with other drugs, in the treatment of various types of malignancy [1]. The growth inhibitory effect of these chemotherapeutic agents is mediated through their common anabolite, FdUMP, which blocks DNA synthesis by inhibition of the enzyme thymidylate synthetase. Although fluorinated pyrimidines are known to be incorporated into RNA with important biological consequences [2, 3], there is much evidence that the inhibition of thymidylate synthetase is the primary basis of their antineoplastic activity [1].

Thymidylate synthetase derives its importance as a target for cancer chemotherapeutic agents from the fact that it provides the only known *de novo* pathway to the formation of dTMP, an essential precursor for DNA synthesis. In the reaction, CH_2FH_4 is oxidized to FH_2 as dUMP is methylated at the 5-position forming dTMP. Blockage of this pivotal step in DNA synthesis cannot be circumvented except by salvage of preformed thymidine via thymidine kinase. In the presence of CH_2FH_4, FdUMP forms an extremely stable, covalently bonded, ternary complex with thymidylate synthetase which effectively blocks further enzyme action [4, 5].

A major limitation to the use of FdUrd as an antitumor agent has been the development of clinical resistance. Several determinants of sensitivity to FdUrd are apparent in the overall view of the relationship between FdUrd metabolism and the target enzyme thymidylate synthetase shown in Fig. 1. An obvious mode of resistance is a decrease in the uptake or retention of the drug. Impaired cell permeability to FdUrd has been reported for a resistant strain of *Pediococcus cerevisiae* [6], as have differences in the rate of loss of intracellular FdUMP in mouse and human cell lines [7]. There are numerous reports that resistance accompanies the loss of thymidine kinase activities and, therefore, loss of the ability to form the active metabolite FdUMP [6, 8–12]. The sensitivity of thymidylate synthetase to inhibition by FdUMP is another important determinant of response, and a resistant subline of Ehrlich ascites carcinoma cells has been shown to have a presumably

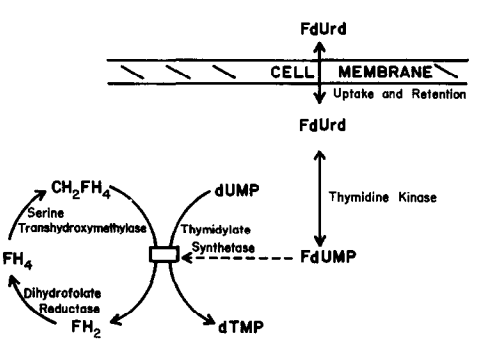

Fig. 1. Relationship between FdUrd metabolism and the target enzyme thymidylate synthetase.

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‡ Abbreviations used are: FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FH_2, dihydrofolic acid; CH_2FH_4, methylenetetrahydrofolic acid; dUMP, 2′-deoxyuridine-5′-monophosphate; and dTMP, 2′-deoxythymidine-5′-monophosphate.

altered form of thymidylate synthetase which is not inhibited by FdUMP *in vitro* [13]. Enzyme inhibition would also be influenced by levels of enzyme, cofactor and substrate. Elevated thymidylate synthetase activities have been found in FdUrd resistant variants of mouse neuroblastoma [14, 15] and in a thymidine kinase deficient Novikoff hepatoma cell line [16]. Ullman *et al.* [17] have demonstrated the requirement for reduced folate cofactors in the cytotoxicity of FdUrd, but to date no change in the level of enzymes involved in CH_2FH_4 generation has been implicated in FdUrd resistance. Recovery of P1534 ascites tumor cells after FdUMP inhibition of thymidylate synthetase has been shown to be due to a fall in the intracellular levels of FdUMP and a progressive accumulation of dUMP [18]. These studies indicate that the sensitivity of cell lines to FdUrd may be modulated by a number of determinants working independently or in concert. The role of changes in thymidylate synthetase in FdUrd resistant cultured hepatoma cells is the subject of this report.

MATERIALS AND METHODS

[5-^{3}H]dUMP (18 Ci/mmole) and [6-^{3}H]FdUMP (19 Ci/mmole) were obtained from Moravek Biochemicals, City of Industry, CA. [6-^{3}H]FUra (2.1 Ci/mmole) and [6-^{3}H]FdUrd (2.5 Ci/mmole) were from Amersham/Searle, Arlington Heights, IL. dUMP, FUra, FdUrd, FdUMP, thymidine, NADPH, ATP, folic acid and dihydrofolic acid were from the Sigma Chemical Co., St. Louis, MO. Tetrahydrofolic acid was prepared by the method of Davis [19] and stored as the methylene derivative in the presence of 75 mM formaldehyde at −70°.

Enzyme assays. Thymidylate synthetase activity was determined by the method of Roberts [20], thymidine kinase activity was measured using the procedure of Chen and Prusoff [21], and dihydrofolate reductase using the method of Kaufman and Kemerer [22]. Binding of [^{3}H]FdUMP to thymidylate synthetase was determined by the filter assay method of Santi *et al.* [23].

Cell culture. Hepa cells, the parental cell line and 5-FdUrd resistant cell lines were maintained as described before [24]. Cell numbers were determined by counting trypsin released cells in a hemocytometer.

Cell extraction. Cells were lysed by the freeze/thaw method in dry ice–acetone after release from culture dishes with 0.1% trypsin solution and centrifugation for 15 min at 1000 *g*. The extraction buffer contained 0.1 M phosphate, pH 7.4, 25% sucrose and 10 mM β-mercaptoethanol. The method of Bradford [25] was used for protein estimation.

Selection of FdUrd resistant cells. Log phase Hepa cells were transferred to media containing 4×10^{-8} M FdUrd. The culture medium was changed weekly for the following 5 weeks. Extensive cell death occurred during the first 2 weeks. By week 6 a population of FdUrd resistant cells was proliferating, as evidenced by numerous mitotic figures. The concentration of FdUrd was increased to 4×10^{-7} M in week 6. The cells adapted to this concentration within 4 weeks. Resistant cells were grown in culture medium containing 4×10^{-7} M FdUrd for an addi-

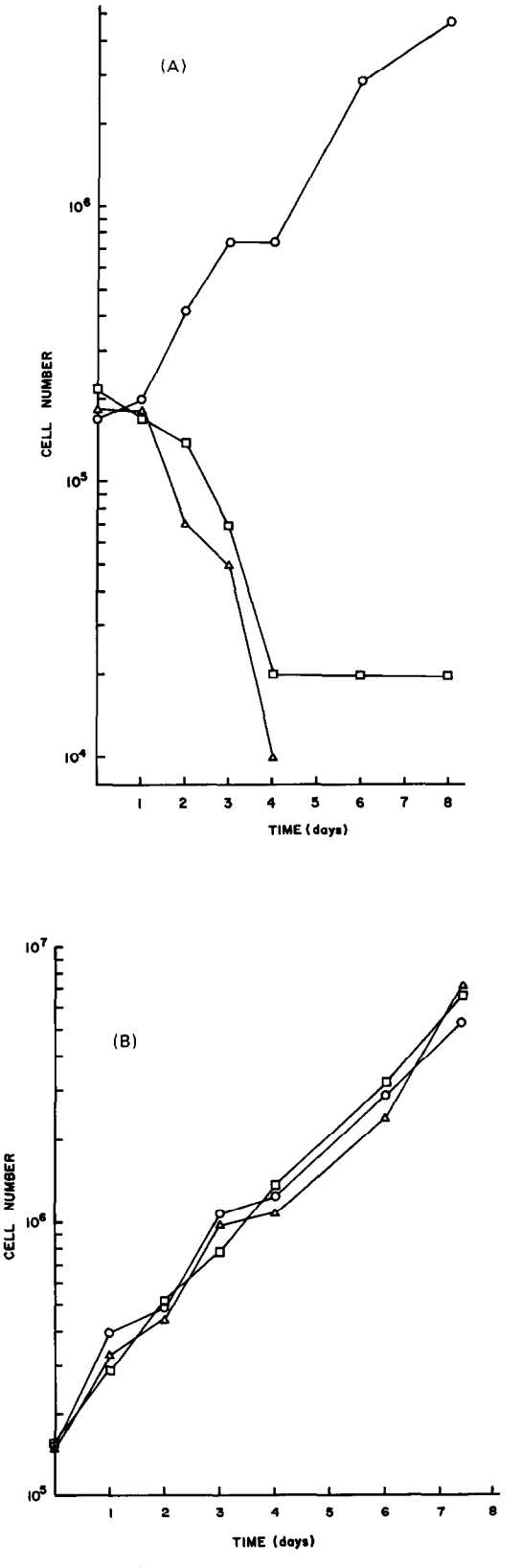

Fig. 2. Effect of FdUrd on normal (A) and resistant (B) cultured mouse hepatoma cell proliferation. Growth media contained 4×10^{-7} M (△), 4×10^{-8} M (□) or no FdUrd (○). A single dish from each treatment group was counted at the same time each day.

tional 6 weeks before initiating experiments. At this time, samples of the cell line were frozen in liquid nitrogen.

RESULTS

The proliferation of the parental line of mouse hepatoma cells in normal growth medium and in media containing FdUrd is shown in Fig. 2A. Under normal growth conditions, the cells have a 24-hr doubling time. When exposed to 4×10^{-8}M FdUrd, there was an initial period of extensive cell death which resulted in a population of resistant but non-proliferating cells. A few mitotic cells were observed suggesting that the constant cell number resulted from a balance between proliferation and death. If, however, sensitive cells were exposed initially to 4×10^{-7}M FdUrd, all of the cells were killed within approximately 5 days.

Figure 2B shows that the hepatoma cells selected for resistance to 4×10^{-7}M FdUrd proliferated with a generation time of approximately 30 hr. Their rate of proliferation was not affected by the presence of FdUrd. Identical growth curves were obtained in the presence and in the absence of FdUrd.

The FdUrd sensitivity of this mouse hepatoma cell line was tested for reversal by thymidine. It can be seen in Fig. 3 that thymidine completely prevented the effects of FdUrd on sensitive cells.

Stability of the FdUrd resistance in the hepatoma cells was tested by growing the cells in the absence of FdUrd for several generations, and then reintroducing FdUrd to the growth medium. Figure 4 shows that cells proliferated in the absence of FdUrd with a generation time of 30 hr. Initially, there was no apparent proliferation of cells upon reintroduction of either 4×10^{-8}M FdUrd or 4×10^{-7}M FdUrd. The cells exposed to 4×10^{-8}M FdUrd began to proliferate at nearly the untreated rate after 4 days, while cells exposed to 4×10^{-7}M FdUrd required approximately 6 days to regain the ability to proliferate. Thus, it appears that resistance to FdUrd is rapidly lost by the hepatoma cells, but can be reestablished by short-term exposure in a manner dependent upon drug concentration.

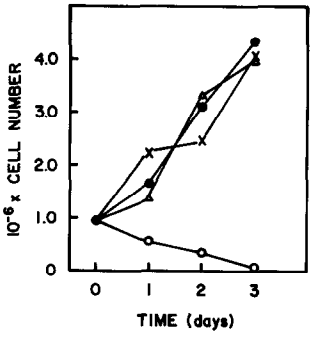

Fig. 3. Thymidine rescue of FdUrd sensitive cells. Cells of the sensitive parental line were added to growth medium containing either 4×10^{-7}M FdUrd (○), or 4×10^{-7}M FdUrd + 10^{-5}M thymidine (●) and transferred to 34 mm petri dishes. Growth media were changed daily for the duration of the experiment. Experimental controls included sensitive cells grown in normal growth medium (×) and in medium containing 1×10^{-5}M thymidine (△). A single dish from each treatment group was counted.

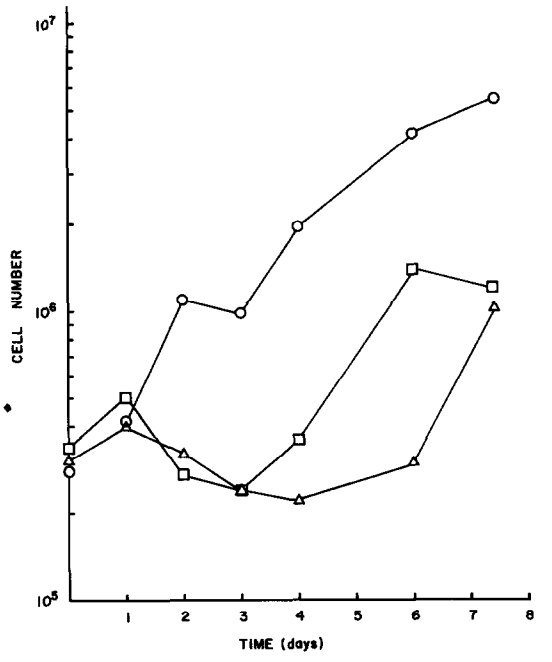

Fig. 4. Stability of FdUrd resistance in mouse hepatoma cells. Cells were added to growth media containing 4×10^{-7}M FdUrd (△), 4×10^{-8}M FdUrd (□) or no FdUrd (○) and transferred to 34 mm petri dishes. The growth media were changed daily for the duration of the experiment. The cell line used in the experiment was the parental line resistant to 4×10^{-7}M FdUrd which had been grown for 3 weeks in the absence of FdUrd. A single dish from each experimental group was counted.

Impaired incorporation of FdUrd has been observed in other systems [6]. However, Table 1 shows that the FdUrd incorporation into resistant and sensitive cells is essentially identical.

It can been seen in Fig. 5 that thymidylate synthetase activity was elevated in the FdUrd resistant mouse hepatoma cells. Activity was not detected in sensitive cells in the presence of FdUrd by the assay used, due to both the cytotoxic effects of the drug and to inhibition by residual FdUMP. However, in resistant cells which had been subcultured in medium lacking FdUrd for one generation, levels of enzyme activity 6- to 10-fold higher than in sensitive cells

Table 1. Comparison of drug uptake in resistant and sensitive cell lines*

	Resistant	Sensitive
FdUrd	11,482 ± 2504	9892 ± 2206

* Units are c.p.m./mg protein incorporated into cells washed three times. Approximately 30×10^6 cells were exposed to [6-^{3}H]FdUrd (4.5×10^6 c.p.m.) for 24 hr. After washing, cells were lysed by freeze/thaw, centrifuged at 1000 *g* for 15 min, and an aliquot of supernatant solution was counted. Values are the means ± S.D. of at least six separate determinations.

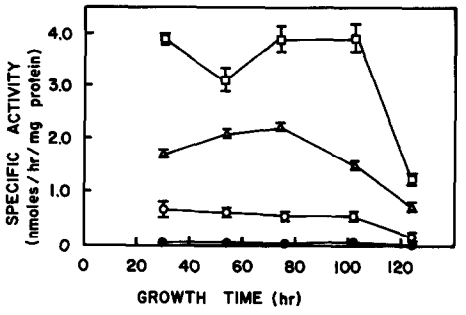

Fig. 5. Thymidylate synthetase activity in FdUrd sensitive and resistant cells. Specific activities were determined by incubation of cellular extracts with [^{3}H]dUMP for 30 min at 37°. Extracts were prepared from resistant (△) and sensitive (●) cell lines grown in the presence of 4×10^{-7} M FdUrd and from the same resistant (□) and sensitive (○) lines grown in the absence of FdUrd for 24 hr prior to extraction. Growth was initiated by subculture of cells into fresh media.

could be observed. When the resistant cell medium contained FdUrd, the enzyme activity remaining (i.e. that which was not inhibited by FdUMP) was similar to the level found in untreated sensitive cells. This suggests that sufficient additional thymidylate synthetase activity was available to compensate for that removed as a FdUMP complex, returning the effective thymidylate synthetase activity to near normal. Long-term (thirty generations) removal of FdUrd from resistant cells resulted in a return of the increased level of thymidylate synthetase to only 1.6-fold greater than sensitive cells.

It can be seen in Table 2 that, while thymidylate synthetase activity was elevated, dihydrofolate reductase and thymidine kinase activities were essentially unchanged. The catalytic properties of elevated thymidylate synthetase were the same in resistant and sensitive cells with regard to FdUMP inhibition and turnover (Table 3). Enzyme concentration was estimated by titration with [^{3}H]FdUMP. In every case, an increase of titratable enzyme paralleled the increase in enzyme activity. Thus, the higher level of thymidylate synthetase activity can be attributed to increased enzyme concentration.

DISCUSSION

There are several different ways in which cells offer resistance to the cytotoxic effects of FdUrd.

Table 3. Catalytic properties of thymidylate synthetase in sensitive and resistant cells

	Turnover number*	I_{50} (FdUMP)
Sensitive	33.7	88 μM
Resistant	34.5	66 μM

* Turnover numbers were estimated from specific activities and titrations of thymidylate synthetase cell extracts with [^{3}H]FdUMP. Turnover numbers were calculated as moles dUMP converted per min per mole of dimeric thymidylate synthetase.

One common mode of resistance is impaired drug uptake; however, uptake of FdUrd was identical in both cell lines in the current study. Since sensitive cells could be rescued by thymidine, the growth inhibitory effects of the drug can be attributed to a diminution in thymidine metabolites, and the primary mode of resistance appears to be increased levels of the ultimate target enzyme thymidylate synthetase. This enzyme activity is clearly increased in the resistant cells, while dihydrofolate reductase, a closely related enzyme shown to be increased in other mammalian cells when exposed to the antifolate methotrexate [26], was unaltered. Thymidine kinase, responsible for conversion of FdUrd to its active form, FdUMP, was also unaltered in resistant cells. Decreases in the level of this enzyme have been shown to provide resistance to the cytotoxic effects of FdUrd in other systems [6, 8–12].

Thymidylate synthetase activity increase could be due to synthesis of a different gene product, or increased levels of the same enzyme. The latter appears to be the better explanation. When cell extracts are prepared from cultures maintained in the presence of FdUrd, the level of thymidylate synthetase activity observed will reflect that amount of enzyme which is not inhibited by residual FdUMP. Such interference can be alleviated somewhat by removal of the drug from culture media for a short period of time prior to preparation of cell extracts. It must be recognized, however, that enzyme levels could be diminished during short-term drug removal since long-term removal results in a return to near normal levels. Thus, the total increased enzyme at the time the drug is withdrawn would be underestimated. Nevertheless, there appears to be a sufficient amount of additional enzyme to compensate approximately for that which is removed by FdUMP.

Table 2. Enzyme activities in FdUrd resistant and sensitive cultured mouse hepatoma cells*

	Thymidylate synthetase (x 10^{-4})	Thymidine kinase	Dihydrofolate reductase
Sensitive	0.09 ± 0.01	80 ± 13	21 ± 0.3
Resistant	0.65 ± 0.04	63 ± 13	17 ± 0.2

* Units of enzyme activity are μmoles product/min/mg protein. Cells were extracted 24 hr after removal of the drug from resistant cells. Values are the means ± S.D. of at least three separate determinations.

The presence of a different gene product in resistant cells is unlikely. *In vitro* inhibition by FdUMP is the same for thymidylate synthetase extracted from resistant or sensitive cells. Furthermore, when the enzyme was titrated with [^{3}H]FdUMP *in vitro*, increased thymidylate synthetase activity corresponded to the increase in titratable enzyme.

Resistant cells appear to produce just enough additional thymidylate synthetase to compensate for that which is lost via FdUMP complex formation and, in addition, have the capacity to respond more rapidly to a signal for more enzyme. The absence of pronounced overproduction observed with other enzyme systems [26] may be due to the necessity for maintenance of thymidylate synthetase within a rather narrow concentration range as a means of regulation. No regulatory process, other than a weak product inhibition, has been shown [27].

Thymidylate synthetase has been shown to increase in regenerating rate liver [28]. Therefore, cells contain a sufficient synthetic apparatus, and regulation of that apparatus, to provide for increased levels of the enzyme upon demand. A similar mechanism could be operative in this case but with a shorter induction period required to attain increased synthesis of the enzyme in the resistant cell line. Future investigations will be directed toward elucidation of the nature of this rapid response.

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