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Thymidylate Synthetase Overproduction in 5-Fluorodeoxyuridine-Resistant Mouse Fibroblasts

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We describe the isolation and characterization of a series of 5-fluorodeoxyuridine (FdUrd)-resistant mouse 3T6 cell lines that overproduce thymidylate synthetase (TS) by up to 50-fold compared with the parental cells. The resistant cells were selected by growing 3T6 cells or a methotrexate-resistant 3T6 cell line (M50L3, isolated previously in our laboratory) in gradually increasing concentrations of FdUrd. Uridine and cytidine were included in the culture medium to reduce toxicity from metabolic products of FdUrd. Cells that were resistant to the drug by virtue of loss of thymidine kinase activity were eliminated by selection in medium containing hypoxanthine, methotrexate, and thymidine. M50L3 cells were found to adapt to FdUrd more readily than 3T6 cells. A number of clones were isolated that were able to grow in the presence of 3 μM (M50L3 derived) or 0.3 μM (3T6 derived) FdUrd. Several were found to overproduce TS by 10 to 50-fold compared with normal 3T6 cells. All were found to have thymidine kinase activity, although the enzyme level was significantly reduced in some clones. The overproduced TS was inactivated by 5-fluorodeoxyuridylic acid at the same concentration as the enzyme from 3T6 cells. TS was purified from the LU3-7 clone (50-fold overproducer) by affinity chromatography on methotrexate-polyacrylamide. The monomer molecular weight was about 38,000, which was the same as the molecular weight of the monomer in 3T6 cells. The overproduction trait was gradually lost (half-life, 3 weeks) when LU3-7 cells were grown in the absence of FdUrd. The overproducing cells will provide an abundant supply of TS and (very likely) its mRNA and may serve as a convenient model system for detailed studies of the regulation of TS gene expression during the cell cycle.

Thymidylate synthetase (TS) (thymidylate synthase, EC 2.1.1.45) is the enzyme that catalyzes the final reaction in the de novo synthesis of thymidylic acid. This reaction involves the reductive transfer of the methylene group from N^5,N^{10}-methylene tetrahydrofolate to the 5 position of deoxyuridylic acid to form thymidylic acid (reviewed in reference 32). TS is inhibited in a stoichiometric manner by the substrate analog 5-fluorodeoxyuridylic acid (FdUMP), which forms a covalent linkage with the enzyme at the active site (27). The enzyme can be inactivated in vivo by exposing cells to the nucleoside form of the inhibitor, 5-fluorodeoxyuridine (FdUrd), which is transported into the cell and then converted to FdUMP by the enzyme thymidine kinase (TK) (11). Inhibition of TS activity in rapidly proliferating cells leads to starvation for thymidylic acid, inhibition of DNA synthesis, and cell death. For this reason, FdUrd or similar analogs have been widely used as antineoplastic drugs (reviewed in references 6 and 8).

Proliferating cells are able to develop resistance to FdUrd by a number of mechanisms. If cells growing in FdUrd are also provided with thymidine, they are able to synthesize TMP by using the salvage enzyme TK (which is not inhibited by the drug). In the absence of thymidine, cells have been found to develop resistance to FdUrd by losing the ability to convert it to FdUMP as a result of a loss of TK activity (4, 30). Cells might also be able to develop resistance by decreasing the rate of transport of the analog into the cell, by increasing the rate of catabolism of the drug, or by synthesizing an altered TS with a decreased affinity for FdUMP.

Another possible mechanism for the development of resistance to FdUrd is the overproduction of TS. Previous studies have shown that cells are able to develop resistance to other specific enzyme inhibitors by overproducing the target enzymes. For example, cells are able to develop resistance to high concentrations of methotrexate (MTX) or *N*-phosphonacetyl-L-aspartate by overproducing the enzymes dihydro-

folate reductase (DHFR) (2, 10) or aspartate transcarbamylase (16), respectively. The mechanism for overproduction of these enzymes has been studied extensively (reviewed in reference 28).

In this paper we show that cultured mouse 3T6 fibroblasts are able to develop resistance to high FdUrd concentrations by overproducing TS by up to 50-fold over the level found in normal 3T6 cells. The overproduced enzyme is inactivated by FdUMP at the same concentration as the enzyme in 3T6 cells and appears to have the same molecular weight as that enzyme.

MATERIALS AND METHODS

Development of FdUrd-resistant cell lines. Cultures of cells were maintained in plastic petri dishes in the Dulbecco-Vogt modification of Eagle medium (GIBCO) supplemented with 10% dialyzed calf serum (Colorado Serum). A line of cells resistant to 0.3 μM FdUrd was developed from mouse 3T6 fibroblasts (29) in the following manner. Cells were plated in medium supplemented with 0.06 μM FdUrd, 1 mM uridine, and 1 mM cytidine. After the surviving cells had adapted to the drug and were growing rapidly (which took several weeks), they were grown in a modification of HAT medium (10 μM hypoxanthine, 16 μM thymidine, 10 μM MTX) to select for TK^+ cells (18). The HAT-selected cells were grown in HT medium (10 μM hypoxanthine, 16 μM thymidine) for several cell doublings to allow them to recover from the toxic effects of MTX. The cells were then adapted to gradually increasing concentrations of FdUrd (0.1, 0.15, 0.2, and finally 0.3 μM) in medium containing 1 mM uridine and 1 mM cytidine and then subjected to HAT selection again. The surviving cells were plated at low density in medium lacking drug, and a series of clones were picked with stainless steel cloning rings. Clones that grew well in medium containing 0.3 μM FdUrd, 1 mM uridine, and 1 mM cytidine and that had high levels of TS activity were studied further.

In a similar manner, a line of cells resistant to 3 μM FdUrd was developed from M50L3 cells, a line of 3T6 cells resistant to 50 μM MTX, which we isolated previously (33). M50L3 cells were grown in the absence of MTX for 2 weeks and then exposed to medium supplemented with 0.04 μM FdUrd, 1 mM uridine, and 1 mM cytidine. After the cells had adapted to the drug level, the concentration of FdUrd was increased, and the process was repeated. The cells were adapted to FdUrd at the following concentrations: 0.08, 0.2, 0.5, 1, 2, and finally 3 μM FdUrd, in medium containing 1 mM uridine and 1 mM cytidine. Cells that had adapted to 3 μM FdUrd were selected in HAT medium containing 300 μM MTX and then cloned in medium containing 3 μM FdUrd, 1 mM uridine, and 1 mM cytidine. Clones that grew well and had high levels of TS activity were used for later studies.

Cloning efficiency. Cells were seeded at a density of 400 cells per 100-mm petri dish in medium supplemented with 1 mM uridine and 1 mM cytidine and FdUrd at various concentrations. After 10 to 14 days, the colonies were fixed with 10% Formalin in isotonic saline solution and stained with hematoxylin. The number of clones per dish was normalized to the number of clones that were observed when the cells were plated in the same medium without FdUrd to give the relative cloning efficiency. Each clone contained at least 30, and usually several hundred cells.

Determination of TS, DHFR, and TK activity. Cells were grown in the absence of FdUrd for 10 to 13 days before enzyme assay. TS activity was determined by the procedure of Roberts (24) as described previously (21). This procedure measures the release of $[^3H]$water from [5-^{3}H]dUMP during the formation of TMP. DHFR activity was determined by the $[^3H]$MTX-binding assay (13, 14). TK activity was assayed by the procedure of Ives et al. (12) as described previously (13a). Enzyme activities were normalized to the amount of protein in the extract (20) to give the specific activity.

Purification of TS. MTX-polyacrylamide was prepared according to the procedure of Newbold and Harding (22). TS was purified from roller-bottle cultures of LU3-7 cells by a modification of the procedures of Dolnick and Cheng (9) and Rode et al. (26). Briefly, cell extracts were subjected to streptomycin sulfate fractionation, ammonium sulfate precipitation, and finally affinity chromatography on MTX-polyacrylamide. TS was bound to the matrix, washed extensively with buffers containing dUMP, and then eluted from the column with buffer lacking dUMP. The fractions with the highest TS activity were pooled, and the purity of the enzyme was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis according to the procedure of Laemmli (17).

TS-FdUMP complex formation. A modification of the procedure of Lockshin et al. (19) was used to form the covalent complex between TS and $[^3H]$FdUMP (18 Ci/mmol, Moravek Biochemicals). The amount of complex formed was determined by measuring radioactivity in the trichloroacetic acid-insoluble fraction. The molecular weight of the complex was determined by electrophoresis on an SDS-7.5% polyacrylamide slab gel according to the procedure of Laemmli (17). Protein bands were visualized by staining with Coomassie brilliant blue. Radioactive bands were detected by fluorography with En^{3}Hance (New England Nuclear Corp.) by a procedure described by the manufacturer. The impregnated gel was dried on Whatman no. 1 filter paper and exposed to X-ray film at −80°C.

RESULTS

Isolation of FdUrd-resistant cells. Cells resistant to high concentrations of FdUrd were isolated by serial selection in gradually increasing concentrations of the drug. Uridine and cytidine were included in the selection media to prevent toxicity from possible metabolic products of FdUrd. For example, FdUrd might be hydrolyzed to 5-fluorouracil and then converted to 5-fluorouridine. This analog can be incorporated into RNA where it prevents correct processing of rRNA (7), thereby leading to cell death.

To eliminate TK^- cells from the population of FdUrd-resistant cells, we subjected cultures to at least one cycle of selection in HAT medium

(18). We found that if we did not carry out the HAT selection virtually all of the clones we isolated had little if any TK activity.

We first attempted to isolate a line of 3T6 cells resistant to high levels of FdUrd. Unfortunately, we were never able to adapt these cells to grow in FdUrd at a concentration greater than about 0.3 μM. Nevertheless, some of these cells were found to overproduce TS by up to 20-fold (see below).

In hopes of achieving still higher levels of drug resistance and enzyme overproduction, we also subjected an MTX-resistant 3T6 cell line (M50L3, isolated previously in our laboratory) to the FdUrd selection procedure. We reasoned that these cells, which had previously developed the ability to overproduce one enzyme (DHFR), might be able to overproduce a second enzyme more readily than normal 3T6 cells. We found that the M50L3 cells were indeed able to adapt much more rapidly and to much higher FdUrd levels than 3T6 cells. For example, it took 5 months of continuous selection for the 3T6-derived cells to adapt to 0.3 μM FdUrd, whereas it took only 2 months for the M50L3-derived cells to adapt to 2 μM FdUrd.

Since the M50L3 cells were already resistant to MTX, it was more difficult to select for TK^+ cells by using HAT selection. However, our previous studies showed that the level of DHFR overproduction decreased to about 5 to 10 times that of 3T6 cells when M50L3 cells were grown in the absence of MTX for several months (33). Therefore, we delayed the HAT selection until just before the cells were cloned and increased the level of MTX in the HAT medium to 300 μM to select for TK^+ cells.

Characteristics of FdUrd-resistant clones. A series of clones was isolated from the mass culture of FdUrd-resistant 3T6 and M50L3 cells, and the clones that grew best were characterized further. To determine how well the various clones were able to survive in the presence of FdUrd, we determined the cloning efficiency in the presence of various concentrations of the drug. Figure 1 shows the results of a typical experiment. The relative level of drug resistance, as well as the doubling times in the presence and absence of drug, were determined for many of the clones (Table 1). Many of the clones were able to grow with similar kinetics in the presence or absence of FdUrd.

Several clones were analyzed for the level of TS overproduction as well as for TK and DHFR specific activity. Since the cellular levels of all of these enzymes vary considerably with changes in cell growth rate (13, 13a, 21), all determinations were made with rapidly growing cells that were harvested at subconfluent cell density. In spite of this, we still found that the enzyme specific activities varied somewhat (usually less than 25%) from experiment to experiment. TS activity was determined by assaying cell extracts for the ability to convert [5-^{3}H]dUMP to [^{3}H]water (a measure of the catalytic activity of TS in the cell extracts). Essentially the same results were obtained when we determined the level of TS overproduction by using the

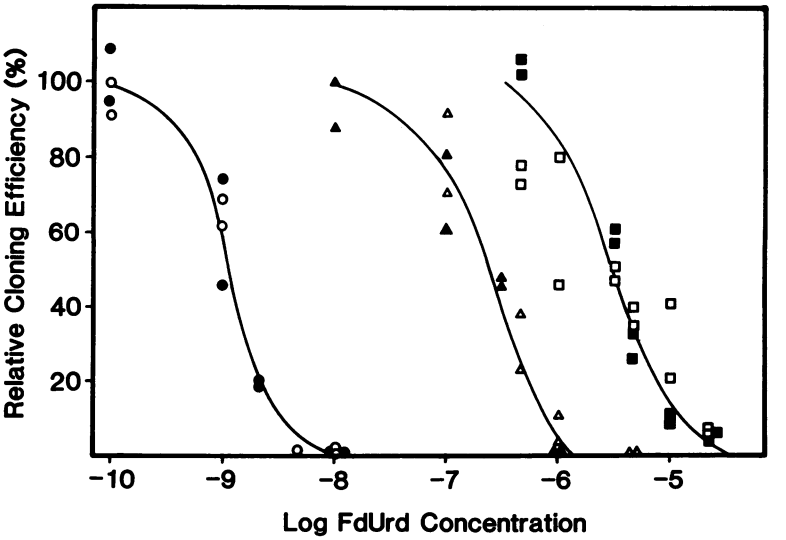

FIG. 1. Cloning efficiency of FdUrd-sensitive and resistant cell lines. The cloning efficiency of various cell lines was determined at the indicated concentrations of FdUrd and normalized to the cloning efficiency of the same cells in medium lacking drug (which ranged from 24 to 53%). Symbols: ●, 3T6; ○, M50L3; △, UH.3-3; ▲, UH.3-8; □, LU3-3; ■, LU3-7.

TABLE 1. Properties of FdUrd-resistant cell lines

Cell line	Doubling time (h)	Relative drug resistance[a]	Enzyme level[b]		
			TS	DHFR	TK
3T6	16	1	1.0	1.0	1.0
M50L3	17	1	0.71	300[c]	0.3
UH.3-3 (3T6)[d]	17 26 (in 0.3 μM FdUrd)	250	13	2.2	0.6
UH.3-8 (3T6)[d]	20 27 (in 0.3 μM FdUrd)	250	19	1.6	0.6
LU3-3 (M50L3)[d]	Not determined	3,000	34	8.4	0.2
LU3-7 (M50L3)[d]	19 22 (in 3 μM FdUrd)	3,000	51	10	0.07

[a] The relative level of resistance to FdUrd was the FdUrd concentration at 50% relative cloning efficiency normalized to that for cell line 3T6.

[b] Enzyme levels were determined after cell cultures were grown for 10 to 15 days in the absence of FdUrd. This was necessary to allow for the recovery of TS activity. Cultures of rapidly growing subconfluent cells were harvested, and the amount of enzyme activity was determined. This was divided by the amount of protein in the sample to give the specific activity. Each value was then normalized to the value obtained for 3T6 cells; each value represents the mean of at least 2 (and for LU3-7, at least 10) determinations. The specific activity of TS in exponentially growing 3T6 cells is approximately 1 nmol of TMP formed per min per mg of protein (21).

[c] Value obtained from reference 33.

[d] Parent cell line shown in parentheses.

[^{3}H]FdUMP binding assay, which determines the number of TS molecules present in the extract (data not shown). Several of the clones overproduced TS by a factor of at least 10, and one (LU3-7) overproduced TS by a factor of greater than 50 compared with normal 3T6 cells (Table 1). The clones derived from M50L3 cells and resistant to 3 μM FdUrd generally had higher levels of TS activity than clones derived from 3T6 cells, which were resistant to 0.3 μM FdUrd. It should be noted that the original M50L3 cells had approximately the same level of TS activity as 3T6 cells. Therefore, the rapidity with which the M50L3 cells developed resistance to the drug was not due to overproduction of the enzyme before the selection procedure.

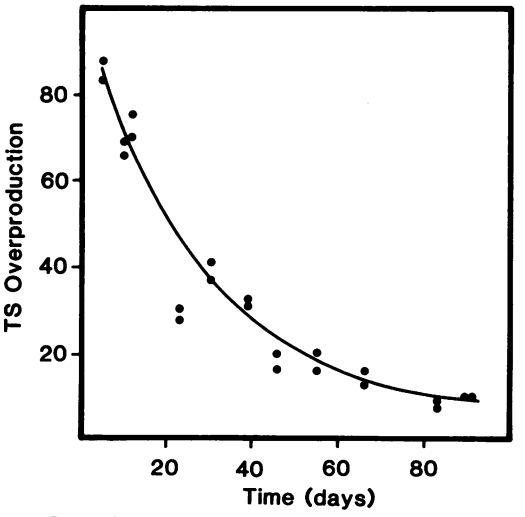

FIG. 2. Stability of overproduction trait in the absence of selective pressure. Cultures of LU3-7 cells were removed from FdUrd on day 0 and carried thereafter in the absence of drug. At the indicated times, cultures of exponentially growing cells were harvested and the specific activity of TS was determined. This was normalized to the specific activity of TS in 3T6 cells to give the value for TS overproduction.

All of the clones analyzed had reduced levels of TK, although the activity remaining in the cells was sufficient to permit survival in HAT medium. The DHFR levels in the clones derived from M50L3 cells were about 10 times greater than the level in 3T6 cells. This was expected from our previous results, which showed that the overproduction of DHFR decreased to about this level after the cells had been grown for several months in the absence of MTX. We also observed a slight (twofold) elevation of the DHFR level in the FdUrd-resistant clones derived from 3T6 cells.

To determine if the various clones would continue to overproduce TS in the absence of selective pressure, we grew cultures of LU3-7 cells in medium lacking FdUrd and assayed for TS activity at various times thereafter. The level of TS overproduction decreased with a half-life of about 3 weeks under these conditions (Fig. 2). These results also show that the actual level of TS overproduction may be somewhat greater than 50-fold in LU3-7 cells maintained in FdUrd or in cells assayed for TS activity within a few days after withdrawal from the drug. The overproduction trait was also found to be unstable in the LU3-3 clone (data not shown).

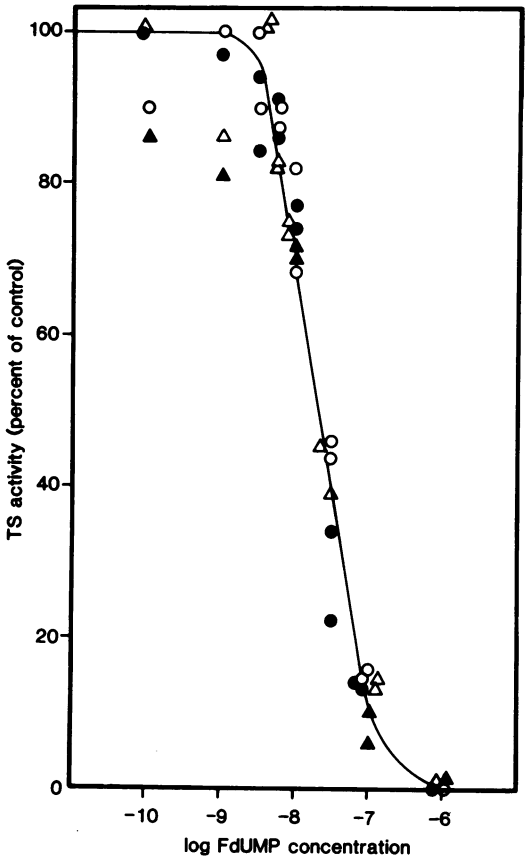

FIG. 3. Inactivation of TS by FdUMP. Cultures of exponentially growing 3T6 (○), UH.3-3 (▲), LU3-3 (△), and LU3-7 (●) cells were harvested, and cytoplasmic extracts were prepared. Approximately equal amounts of TS enzyme activity were added to TS assay mixtures containing FdUMP at the indicated final concentrations. The amount of TS activity remaining was determined by incubating the mixture at 37°C for 20 min, and determining the amount of TMP formed as described in the text. These values were normalized to the values observed in the absence of FdUMP and plotted as a function of drug concentration.

Properties of the overproduced enzyme. We conducted a preliminary characterization of the overproduced enzyme to determine if there were any obvious differences between the enzyme found in drug-resistant cells and that found in parental 3T6 cells. We first determined the ability of FdUMP to inactivate the normal and the overproduced enzyme. Both types of enzyme were inhibited to the same extent by various concentrations of the inhibitor (Fig. 3).

We compared the cytoplasmic proteins of 3T6 and LU3-7 cells on SDS-polyacrylamide gels to see if the overproduced enzyme could be detected in the crude cell extract. Figure 4, lanes 1 and 2, show that there were few significant differences in the pattern of proteins detected by this procedure, except for a protein band at about 38,000 daltons that was prominent in LU3-7 cells but not in 3T6 cells. From the intensity of the band we estimate that this protein represents beteen 0.1 and 1% of the total cytoplasmic protein.

To determine if this band corresponded to TS, we purified the enzyme from the drug resistant cells by ammonium sulfate fractionation followed by affinity chromatography on MTX-polyacrylamide. TS binds tightly to this affinity matrix in the presence of dUMP and can be eluted by washing the column with buffer lacking dUMP. The overall recovery of TS activity was usually about 20 to 30% in this simple and rapid procedure. The purity and molecular weight of TS eluted from the column were determined by SDS-polyacrylamide gel electrophoresis. Figure 4, lane 4, shows that the en-

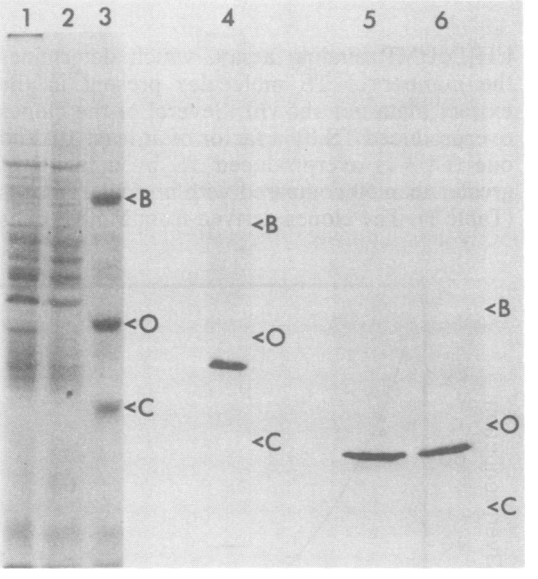

FIG. 4. Electrophoretic analysis of TS on 7.5% polyacrylamide gels. Cytoplasmic extracts of exponentially growing LU3-7 (lane 1) and 3T6 (lane 2) cells were electrophoresed, and protein bands were detected by staining with Coomassie brilliant blue. Molecular-weight markers were run in lane 3: bovine serum albumin (B), (68,000); ovalbumin (O), (45,000); chymotrypsinogen (C), (24,000). Lane 4 shows TS purified from LU3-7 cells by affinity chromatography and detected by staining. In lanes 5 and 6, extracts of LU3-7 and 3T6, respectively, were incubated with [^{3}H]FdUMP under conditions which lead to the formation of a covalent FdUMP-TS complex. Equal amounts of labeled complex (5,000 cpm) were electrophoresed, and the location of the complex was determined by fluorography. The positions of the molecular-weight markers that were run in adjacent wells and detected by staining are indicated.

zyme was nearly pure, although in many preparations there was some contamination with proteins migrating between 50,000 and 80,000 daltons. The protein had the same molecular weight as the protein band that was prominent in the LU3-7 cell extract but undetectable in 3T6 cell extracts. The molecular weight of the protein was found to be 38,000. This is the same as the molecular weight of the enzyme monomer isolated from human cells (25, 26).

To determine if the molecular weight of the overproduced TS was the same as that of the enzyme in 3T6 cells, we took advantage of the fact that TS forms a covalent complex with FdUMP. Crude extracts of LU3-7 and 3T6 cells were allowed to react with [^{3}H]FdUMP, and the molecular weight of the labeled complex was determined by SDS-polyacrylamide gel electrophoresis followed by fluorography. Figure 4, lanes 5 and 6, show that the FdUMP-TS complexes from 3T6 and LU3-7 cells comigrate, indicating that the normal and overproduced enzymes have very similar, if not identical, molecular weights. We also found that the complex comigrated with the major protein band eluted from the affinity column (data not shown), further supporting the idea that this protein band corresponded to TS.

DISCUSSION

These results show that mammalian cells are able to develop resistance to FdUrd by overproducing the target enzyme, TS, up to 50-fold compared with the parental cells. The overproduced enzyme appears to have the same molecular weight and the same sensitivity to FdUMP as the enzyme in the parental 3T6 cells, suggesting that the drug-resistant cells are simply making a much larger amount of the normal enzyme. However, a more thorough comparison of the normal and overproduced enzymes will be necessary to be certain that no alterations have occurred.

It will be interesting to determine the mechanism of TS overproduction in these cell lines. In particular, it will be important to see if TS gene amplification is responsible for enzyme overproduction, as found previously in cell lines that overproduce other enzymes (1, 31). The observation that the overproduction trait is unstable in the absence of selective pressure is consistent with the possibility that the amplified genes, if present, are located on double minute chromosomes (15). However, TS overproduction could also be due to an increase in the efficiency of the TS promoter, leading to increased transcription of the gene, or to other mechanisms.

Since FdUrd and related analogs are widely used as antineoplastic drugs, it is possible that tumor cells may be able to develop resistance to this class of drugs by overproduction of TS. However it seems more likely that the primary mechanism of resistance to FdUrd would be the loss of TK activity. Indeed, we found that it was extremely difficult to isolate cells that contain high levels of TS if we did not eliminate TK$^-$ cells from the population by HAT selection.

Other laboratories have also reported the isolation of FdUrd-resistant mammalian cell lines that overproduce TS. However, the level of overproduction has, to our knowledge, never exceeded 10-fold (3, 5, 23, 34). The higher level of overproduction obtained in our selection procedure might be due to differences in the cell lines being selected or to differences in the selection procedure (for example, the inclusion of uridine and cytidine in the selection medium or the elimination of TK$^-$ cells by HAT selection).

We are not certain why the M50L3 cells were able to develop resistance to FdUrd more readily than 3T6 cells. The M50L3 cells are not inherently more resistant to the drug due to a higher level of TS activity before selection. We did observe that the M50L3 cells had a somewhat lower TK level than the 3T6 cells. The lower TK level might confer a slightly greater level of resistance to FdUrd. However, Fig. 1 shows that the M50L3 cells appeared to be just as sensitive to FdUrd toxicity as 3T6 cells. Another possibility is that an elevated DHFR level provides a selective advantage to cells with increased TS activity. This is reasonable since an increased rate of TMP synthesis, which might result from an elevation of TS activity, could result in a lowering of the intracellular tetrahydrofolate concentration. This might lead to a reduction in the rate of purine synthesis, resulting in slower growth rates or even cell death. Overproduction of DHFR would eliminate this problem. Furthermore, this might explain why the 3T6 cells that were able to develop resistance to FdUrd also had higher levels of DHFR than normal 3T6 cells.

A final possibility is that the M50L3 cells, which had previously been selected for the ability to overproduce one protein, might be able to overproduce a second protein much more readily than normal cells. This would be especially interesting if TS overproduction were found to be the result of TS gene amplification. This might suggest that the ability to amplify genes is a selectable trait.

The TS-overproducing cell lines will be quite useful for a variety of biochemical and genetic studies. First, they will provide an abundant source of the mammalian enzyme, thereby facilitating detailed studies of the structure and mechanism of action of this enzyme. Second, they

will greatly facilitate the isolation and cloning of TS cDNA, since it is very likely that TS mRNA is far more abundant in the overproducing cells than in normal cells. The TS cDNA will be a valuable probe for TS mRNA, heterogeneous nuclear RNA, and gene sequences. Third, the overproducing cell lines may be useful model systems for detailed studies on the mechanism for regulating TS gene expression during the cell cycle. Our previous studies have shown that the amount of TS activity is quite low in 3T6 cells resting in the G_0 state of the cell cycle. When the resting cells are stimulated by serum to reenter the cell cycle, TS activity increases sharply at about the same time the cells enter S phase (21). If the overproducing cells regulate TS gene expression in the same manner as normal 3T6 cells, then it should be possible to study the content and metabolism of the enzyme and its mRNA directly and determine if TS gene expression is controlled at the level of transcription or at some other level. Such studies are not possible in normal 3T6 cells due to the extremely low levels of the enzyme and its corresponding mRNA in FdUrd-sensitive cells.

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