

Role of thymidylate synthetase activity in development of methotrexate cytotoxicity

(citrovorum factor rescue/5-formyltetrahydrofolate/chemotherapy/thymidine/fluorinated pyrimidines)

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ABSTRACT Methotrexate (MTX) inhibition of the growth of mouse or human leukemia cells in culture was partially prevented by either thymidine (dThd) or hypoxanthine. 5-Fluoro-2'-deoxyuridine (FdUrd) also decreased the growth-inhibitory potency of MTX in the presence of small concentrations of 5-formyltetrahydrofolate (citrovorum factor) and sufficient exogenous dThd to support the synthesis of thymidylate nucleotides by salvage mechanisms. In addition, citrovorum factor-induced reversal of MTX was several orders of magnitude more efficient in the presence of both FdUrd and dThd than in the presence of dThd alone or in the absence of both nucleosides. Likewise, the presence of FdUrd (3 μ M) and dThd (5.6 μ M) completely prevented the lethality of 0.3 mM MTX to L1210 cells in culture medium supplemented with micromolar concentrations of citrovorum factor. We propose that this protection against the cytotoxic effects of MTX by dThd, hypoxanthine, and FdUrd have a common biochemical mechanism—namely, inhibition of the *de novo* synthesis of thymidylate by either a direct [FdUrd; inhibition of thymidylate synthetase (thymidylate synthase; 5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45)] or indirect (dThd and hypoxanthine; feedback inhibition by anabolites on ribonucleotide reductase and deoxycytidylate deaminase) effect. The resultant decreased rate of loss of reduced folates due to *de novo* thymidylate synthesis would allow a higher degree of inhibition of dihydrofolate reductase to be endured without damage to the cell.

Methotrexate (MTX) is an agent of considerable clinical utility in cancer chemotherapy. Its primary biochemical mode of action has been extensively studied but is still under dispute. It is a salient yet poorly understood fact that the growth inhibitory effects of MTX on some cell lines are decreased by thymidine (dThd) alone but not by purines, whereas the inhibitory effects of MTX on other cell lines are diminished in the presence of purines but are unchanged in the presence of dThd (1, 2). Likewise, the lethal effects of MTX are diminished by dThd in some cell lines and by purines in other cell lines (3, 4). Yet both dThd and a source of purines are required by all cell lines examined to date to eliminate completely MTX cytotoxicity in medium supplemented with dialyzed serum (2, 5-8). Several reports have appeared of similar observations *in vivo* (7, 9-12). Until recently, these effects have remained a puzzle in molecular pharmacology and have had no direct implications in the clinical use of MTX. This is no longer the case: the utility of combinations of MTX and dThd for treatment of human cancer is currently being tested (13).

We here advance the hypothesis that the decrease in the cytotoxic effects of MTX observed in the presence of dThd or purines is due to feedback effects of anabolites of these compounds, which lower the rate of *de novo* thymidylate synthesis [and, hence, the rate of oxidation of tetrahydrofolate pools by

thymidylate synthetase (thymidylate synthase; 5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45)] by limiting the supply of deoxyuridylylate (dUMP). Furthermore, the prediction of this hypothesis that direct inhibition of thymidylate synthetase would protect cells from MTX was confirmed. Numerous studies (14-16) have documented a partial antagonism between MTX and fluorinated pyrimidines. We now demonstrate that even high concentrations of MTX can be rendered innocuous by FdUrd under appropriate conditions.

MATERIALS AND METHODS

Sera and cell culture media were purchased from GIBCO; nucleosides, MTX, citrovorum factor (CF), and other chemicals were from Sigma. CF concentrations refer to those of the *dl* calcium salt. Cultures were established from mycoplasma-free frozen stocks at 2.5-month intervals, and all experiments were performed on cells found negative for mycoplasma by agar techniques. The origin and methods of propagation of the L1210 and CCRF-CEM cell culture lines have been described (17, 18). Cellular proliferation assays were performed by a modification (7) of described methods (19). Briefly, cells were continuously exposed to drugs and metabolites during a 48-hr (L1210 cells) or 72-hr (CCRF-CEM cells) incubation in RPMI 1640 medium (which contains 2.3 μ M folate) supplemented with 10% dialyzed fetal calf serum. Experiments on the cloning efficiency of L1210 cells were performed in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum, 10% dialyzed horse serum, and 0.25-0.4% agarose by utilizing a modification of published procedures (20). In such experiments, cells were continuously exposed to drugs and metabolites for 3 weeks. All cells that formed microscopically observable colonies (≥ 50 cells) were scored as viable.

RESULTS

Effect of dThd and Hypoxanthine. Inhibition of the proliferation of L1210 mouse leukemia cells or CCRF-CEM human lymphoblastic leukemia cells by MTX was decreased by either dThd or hypoxanthine to a small but reproducible extent (Fig. 1). The concentrations of dThd (5.6 μ M) and hypoxanthine (32 μ M) used in these experiments were those previously found optimal to support the growth of these cells in folate-deficient medium or in the presence of high concentrations of MTX (7, 21).

Inhibition of Cell Growth by FdUrd. The growth-inhibitory effects of FdUrd on L1210 cells were dramatically reversed by

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Abbreviations: MTX, methotrexate; CF, citrovorum factor (5-formyltetrahydrofolate).

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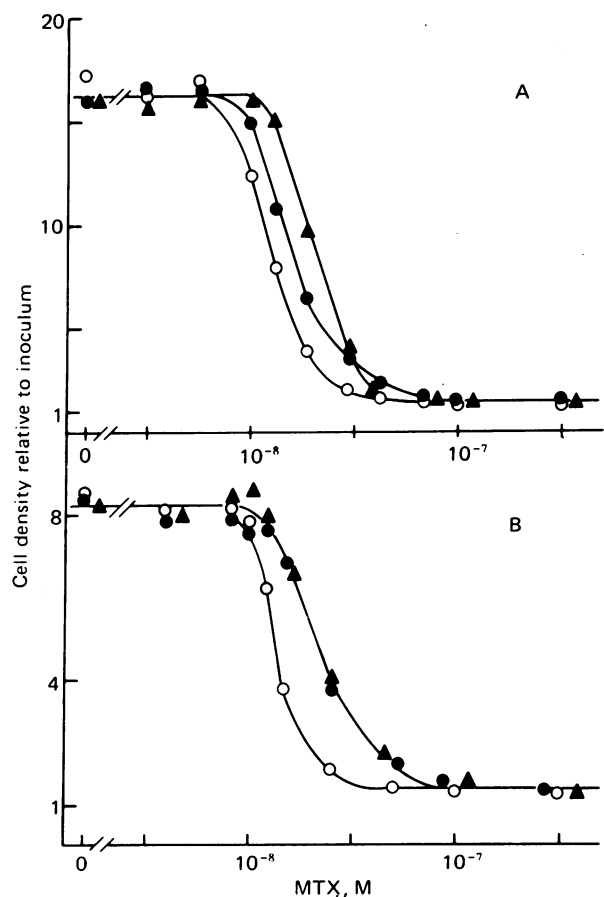


FIG. 1. Inhibition of growth of L1210 (A) or CCRF-CEM (B) cells by MTX during a 48-hr (L1210) or a 72-hr (CCRF-CEM) incubation in RPMI 1640 medium (O) or this same medium supplemented with 5.6 μ M dThd (●) or 32 μ M hypoxanthine (▲). The ordinate represents the number of cells found after incubation, expressed as a multiple of the inoculum. Each symbol represents the mean density of two (B) or four (A) cultures.

dThd; growth inhibition was observed only in the presence of 5×10^4 times higher concentrations of FdUrd than those inhibitory in the absence of dThd, suggesting that in L1210 cells FdUrd acts primarily by inhibition of thymidylate synthetase (Fig. 2).

Effects of FdUrd on Cytotoxicity of MTX. The potency with which MTX inhibited leukemic cell growth was decreased

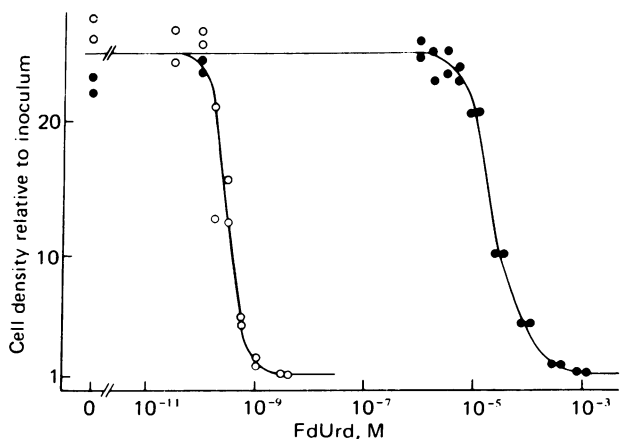


FIG. 2. Inhibition of growth of L1210 cells by FdUrd in the presence (●) or absence (O) of 5.6 μ M dThd. Each symbol represents one culture. For details, see text and Fig. 1.

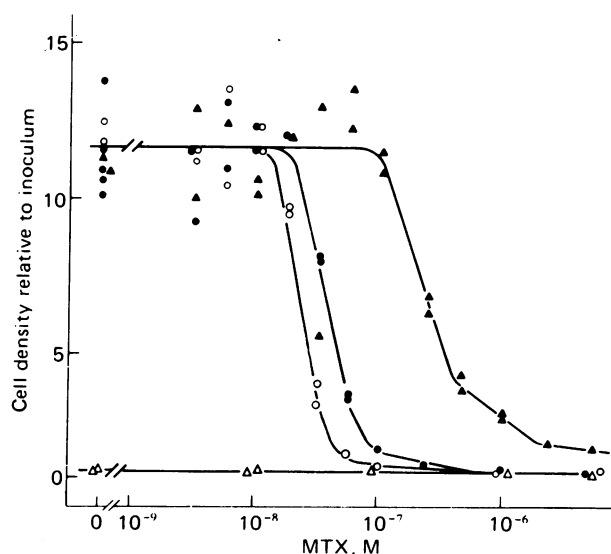


FIG. 3. Protection from growth inhibitory effects of MTX by FdUrd. L1210 cells were exposed to various concentrations of MTX alone (O) or to MTX in the presence of 5.6 μ M dThd (●), of 3 μ M FdUrd (Δ), or of 3 μ M FdUrd and 5.6 μ M dThd (\blacktriangle) during a 48-hr period of growth. CF was added to the medium of all cultures at 25 nM.

by the addition of 3 μ M FdUrd in the presence of 25 nM CF and 5.6 μ M dThd (Fig. 3). The concentration of FdUrd used in this experiment completely prevented growth in the absence of dThd (Figs. 2 and 3). In the absence of CF, the reversal of MTX inhibition by FdUrd and dThd did not appreciably exceed that caused by dThd alone (Figs. 4-6). If FdUrd and dThd were both present in the medium, reversal of the inhibitory effects of 10 μ M MTX was attained in the presence of CF concentrations several orders of magnitude lower than those required in the presence of dThd alone (Fig. 4). The concentration of FdUrd used in these experiments prevented growth in the absence of dThd at any concentrations of MTX and CF tested (data not shown). The slope of a log-log plot of the 50% inhibitory concentration of MTX versus CF concentration in

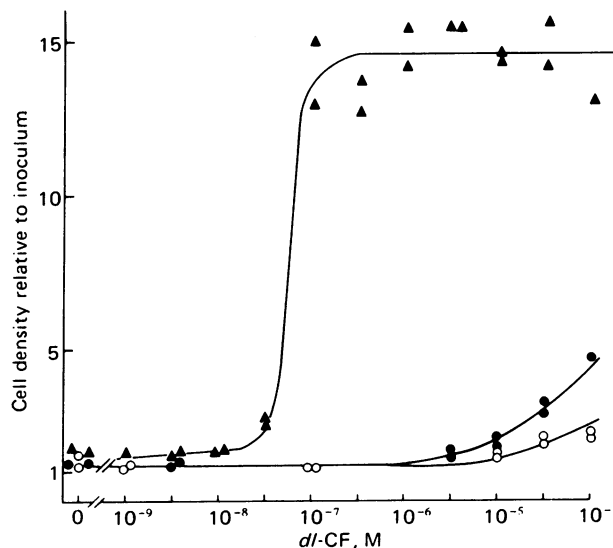


FIG. 4. Reversal of growth-inhibitory effects of MTX by CF in the presence of FdUrd. L1210 cells were exposed to 10 μ M MTX and various concentrations of CF in unsupplemented medium (O) or in the presence of 5.6 μ M dThd (●) or of 5.6 μ M dThd and 3 μ M FdUrd (\blacktriangle).

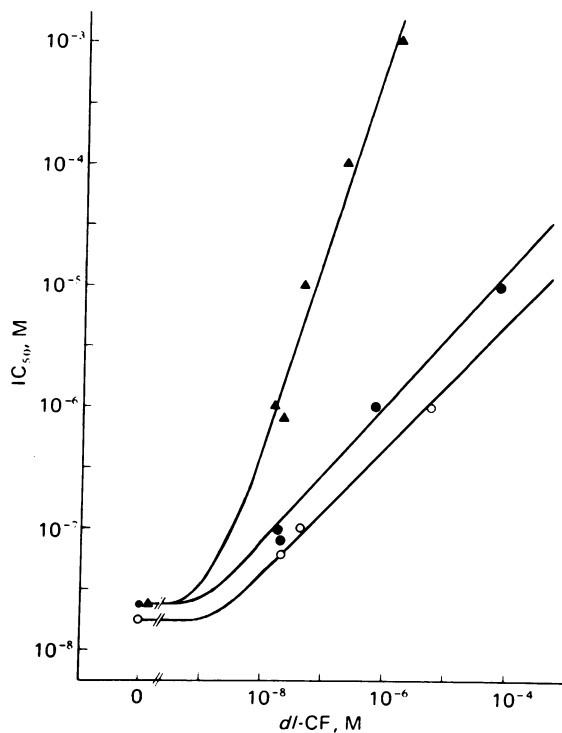


FIG. 5. Relationship between growth-inhibitory potency of MTX and CF concentration. The concentration of MTX that depressed the apparent mean growth rate was graphically estimated from a series of experiments similar to that of Fig. 4. Each symbol represents one experiment: O, L1210 cells exposed to MTX in unsupplemented medium; ●, cells exposed to MTX in the presence of 5.6 μ M dThd; ▲, cells exposed to MTX in the presence of 5.6 μ M dThd and 3 μ M FdUrd. IC_{50} , concentration of MTX necessary to inhibit growth by 50%.

the medium was not significantly different than 1.0 for cells grown in unsupplemented medium or in the presence of dThd (i.e., the potency of MTX was inversely proportional to CF concentration over the concentration range tested), but the slope was 3.5 for cells grown in the presence of both FdUrd and dThd (Fig. 5). Thus, when blockage of thymidylate synthetase was imposed on the cells, the characteristics of the reversal of MTX by CF changed from that of the competitive relationship observed in the presence of dThd alone (2, 22) to behavior more typical of end-product or "noncompetitive" reversal (2, 22). However, the theoretical characteristic of end-product reversal (2, 22)—namely, that a concentration of CF that reverses a low but toxic concentration of MTX should also reverse higher concentrations of MTX—was not observed in L1210 cells grown in the presence of FdUrd and dThd. Experiments were also performed to determine the ability of L1210 cells to form colonies in the presence of MTX under various conditions. As was the case with CF reversal of MTX-induced growth inhibition (Fig. 5), concentrations of CF in the micromolar range also completely reversed the lethal effects of 0.3 mM MTX in the presence of both FdUrd and dThd. No surviving colonies were found on plates seeded with 1000 cells and exposed to this concentration of MTX in the absence of nucleosides or in the presence of dThd alone or of FdUrd alone (Fig. 6). L1210 cells surviving 0.3 mM MTX in the presence of dThd and FdUrd formed colonies of the same size as those found on control plates not exposed to MTX (approximately 500–1000 cells per colony).

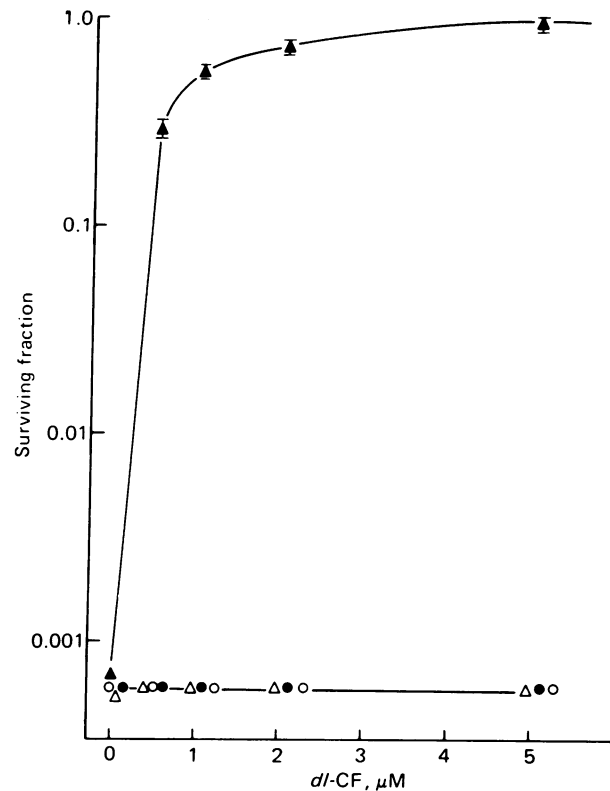


FIG. 6. Prevention of lethality of MTX by FdUrd in the presence of dThd and CF. L1210 cells (1000 cells per plate) were continuously exposed to 0.3 mM MTX in the presence of various concentrations of CF in unsupplemented medium (O) or in the presence of 5.6 μ M dThd (●), of 3 μ M FdUrd (Δ), or of 5.6 μ M dThd and 3 μ M FdUrd (\blacktriangle). After a 3-week incubation period, microscopically visible colonies were counted. Ordinate values represent plating efficiencies relative to control. Forty-nine percent of cells plated in MTX-free medium formed colonies in this experiment. Plates without colonies were plotted as having a plating efficiency of 0.0006, an upper limit value.

DISCUSSION

The data of Borsa and Whitmore on the partial protection by dThd from the cytotoxic effects of MTX on L cells (2) have been widely quoted (23–27) as evidence that inhibition of dihydrofolate reductase is insufficient to explain the cytotoxic effects of MTX and have had a lasting influence on the interpretation of investigations on MTX toxicity. If a tissue or cell line undergoes less MTX-induced toxicity in the presence of dThd, it has been interpreted as evidence that the thymidylate (TMP) biosynthetic pathway is more sensitive to MTX than is the purine biosynthetic pathway; if purines decrease the toxic effect of MTX, the converse was thought to be true. Yet there has been no direct demonstration of a differential effect of MTX on the availability of the folate cofactors necessary for TMP synthesis *vis a vis* purine synthesis, nor has there been a report of a direct inhibitory effect of MTX on any of the enzymes that synthesize TMP, formamidoimidazole carboxamide ribonucleotide, or *N*-formylglycinamide ribonucleotide that is sufficiently potent to be causal in MTX toxicity (28–31). The existence of cell lines (refs. 1, 32; Fig. 1) in which *either* dThd *or* purines decrease the potency of MTX argues that dThd or purines affect the development of the toxic effects of MTX by some mechanism other than that of the supply of the product of a growth rate-limiting pathway because, logically, both cannot be limiting at the same time.

For any cell being supplied with folates at the tetrahydrofolate reduction level, the *only* function of dihydrofolate re-

ductase is to regenerate tetrahydrofolate from the dihydrofolate formed by thymidylate synthetase from 5,10-methylenetetrahydrofolate during the synthesis of TMP. This will be the case for cells in culture growing on sufficient levels of CF and for many tissues *in vivo*, because reduced folates, principally 5-methyltetrahydrofolate, are the only forms of folate found in serum of those mammals (33–35) investigated to date. Under such conditions, dihydrofolate reductase is probably irrelevant to folate metabolism in the absence of thymidylate synthetase activity.

Existing data suggest that control of thymidylate synthetase is indirect in eukaryotic cells. The rate of *de novo* TMP synthesis does not seem to be modulated by feedback inhibition of thymidylate synthetase by the end product of the TMP biosynthetic pathway, TTP (36, 37), but rather it seems to be modulated by effects of TTP on the supply of the dUMP, the substrate of thymidylate synthetase (37). Hence, we now postulate that the availability of dUMP determines the rate of the thymidylate synthetase reaction *in vivo*. It has been shown that an exogenous supply of dThd increases the intracellular pools of TTP in L1210 cells (38). It is also known that TTP is a potent feedback inhibitor of deoxycytidylate deaminase (37, 39) and of the reduction of UDP and CDP by ribonucleotide reductase (37, 40). In eukaryotic cells these two enzymes catalyze the only reactions known to synthesize dUMP, the substrate of thymidylate synthetase.[§] Hence, a sufficiently expanded TTP pool could diminish cellular pools of dUMP which would, in turn, decrease thymidylate synthetase activity and, consequently, the oxidation of tetrahydrofolates. Diminution of cellular dUMP pools by exogenous dThd has been demonstrated recently in Novikoff hepatoma cells (37). The decreased rate of loss of tetrahydrofolates so produced would thereby allow a considerably higher degree of inhibition of dihydrofolate reductase to be endured without cytotoxicity, which we have here demonstrated (Figs. 1 and 4). In the complete absence of thymidylate synthetase activity, due either to feedback effects that eliminate dUMP pools, to mutational enzyme deletion, or to direct inhibition of thymidylate synthetase by a second drug, inhibition of dihydrofolate reductase by MTX would thus be predicted not to be inhibitory to cell growth as long as the cell has an exogenous supply of TMP and sufficient reduced cofactor (e.g., CF or 5-methyltetrahydrofolate) to allow for continued purine synthesis (Figs. 3–6).

The mechanism of selection of thymine-requiring bacteria is relevant to our proposed hypothesis: such mutants are exposed to toxic concentrations of antifolates in the presence of thymine or dThd. In the vast majority of mutant bacteria selected by this procedure, thymidylate synthetase has been deleted or substantially reduced (42). Hence, the mutant bacteria are unaffected by inhibition of dihydrofolate reductase by virtue of the fact that the tetrahydrofolate cofactor pools are not depleted by *de novo* TMP synthesis. A eukaryotic analog is known: the S-180/AT cell, which was selected for MTX resistance in the presence of dThd and has 1/30th the thymidylate synthetase activity of the parent cell line (1, 43).

A consideration of the role of thymidylate synthetase in the development of MTX cytotoxicity and of the previously discussed indirect effect of TTP on the loss of cellular folates would explain several perplexing phenomena: (i) *Partial protection by dThd against toxic effects of MTX observed in cell culture and in vivo* (refs. 1, 2, 9–13, Fig. 1).

(ii) *Partial protection by purines against toxic effects of*

MTX (refs. 1, 4; Fig. 1). The decreased toxicity of MTX observed in some cells in the presence of deoxyadenosine or hypoxanthine could be due to feedback effects of purine anabolites on the availability of dUMP. Thus, ATP and dATP are strong allosteric effectors of ribonucleotide reductase (40), and dAMP is a competitive inhibitor of homogeneous deoxycytidylate deaminase from donkey spleen (44).

(iii) *Deoxycytidine reversal of dThd-induced alterations in toxic effects of MTX* (2). In the presence of deoxycytidine, the dUMP pool would be expanded in spite of the presence of dThd, due to a reversal by dCTP of the TTP inhibition of deoxycytidylate deaminase. Such an effect has been documented with purified mammalian deoxycytidylate deaminase (39). Our hypothesis predicts that expansion of the dUMP pool, caused by any exogenous cytidine or uridine nucleoside, should eliminate the dThd-induced protection against MTX. Our experiments to date have indicated that this is the case (unpublished observations). At appropriate concentrations, deoxycytidine, deoxyuridine, cytidine, and uridine offset the effect of dThd on MTX toxicity in L1210 cells (data not shown); deoxycytidine was by far the most potent nucleoside of this series in this regard. The mechanism of these effects can be examined by using a newly developed microassay for dUMP (45).

(iv) *Competitive relationship between MTX and CF observed under most conditions* (refs. 1, 2, 8, and 10; Fig. 4). In the presence of thymidylate synthetase activity, this could be due to accumulation of folate pools in the form of dihydrofolate or dihydrofolate polyglutamates (46–48), thereby causing a direct competition between substrate and inhibitor of dihydrofolate reductase. At cellular pH, MTX is a competitive inhibitor of dihydrofolate reductase, albeit a potent one (49). The work of Jackson *et al.* (48, 50) and Meyers *et al.* (51) suggests that the dissociation constant of the MTX–enzyme complex would be compatible with such a competition.

(v) *Noncompetitive reversal of effects of MTX by CF in the presence of dThd in L cells* (2). If TTP accumulates in the presence of dThd and decreases or eliminates dUMP pools, and thereby *de novo* TMP synthesis, an exogenous concentration of CF that supplies formylated cofactor levels sufficient for purine synthesis would reverse the toxicity of any concentration of MTX. It is clear that a direct pharmacological inhibition of thymidylate synthetase is necessary to bring about a blockade of sufficient intensity to demonstrate a noncompetitive pattern of CF reversal in L1210 cells (Fig. 3–5), suggesting that there is a basic difference in the regulation of dUMP synthesis between L1210 and L cells. The extensive protection by dThd against the lethal effects of MTX seen in mice (10) and humans (13) is remarkable and not easily understood. One explanation of this phenomenon is that the dUMP pool in dividing stem cells of the gut and bone marrow is sensitive to depletion by the type of feedback effects we now postulate to be involved in Borsa and Whitmore's L cells (2). If this were the case, high-dose MTX–dThd rescue (13) or high-dose MTX–CF rescue combined with continuous low-dose dThd infusions should be effective therapy for any tumor, the dUMP pool of which was relatively unresponsive to exogenous dThd.

(vi) *Competitive reversal of MTX by CF in L cells in the presence of dThd and deoxycytidine* (2). With the addition of deoxycytidine together with dThd, dUMP pools and *de novo* TMP synthesis would be reinstated and dihydrofolate reductase would again be necessary for cell growth.

The role we postulate here that cellular thymidylate synthetase activity plays in the development of the cytotoxicity of MTX is best demonstrated by the virtual elimination of cytotoxicity and growth inhibition by MTX in the presence of FdUrd (and, of course, sufficient CF and dThd) (Figs. 3–6).

[§] A third possible pathway for the synthesis of dUMP via phosphorylation of 2'-deoxyuridine has been recently postulated (37). It would also be subject to feedback inhibition by TTP (41).

Although antagonism between fluorinated pyrimidines and MTX has been demonstrated on short-term effects such as isotope incorporation (16, 52), only limited antagonism has been observed by utilizing tumor growth (14, 15). It is now clear (Fig. 6) that the potential for a decreased tumor cell kill with combinations of MTX and FdUrd is far greater than that previously documented, if reduced folate cofactors and dThd are available to the tumor. This study also suggests that thymidylate synthetase activity will be a major determinant of the amount of CF required to rescue any given tissue during high-dose MTX chemotherapy. Thus, a tumor with high thymidylate synthetase activity might be a poor candidate for chemotherapy with fluorinated pyrimidines, but an excellent choice for high-dose MTX-CF rescue. In addition, the explanation we offer in this report for the decreased cytotoxic effects of MTX observed in the presence of dThd alone or purine alone (refs. 1-4; Fig. 1) would reconcile these observations with the concept that direct sites of inhibition other than dihydrofolate reductase need not be invoked to understand the mechanism of MTX cytotoxicity and chemotherapy.

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