GROWTH RATE AND CELL KILL *

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The faster a cell population is proliferating, the more susceptible is it to the lethal effects of a variety of antimetabolites.[1, 2] This is believed to underlie the selective antitumor action of these antimetabolites, since the tumor cells may be proliferating more rapidly than normal cells. In order to explain why antimetabolites kill cells from a rapidly proliferating population so efficiently, several investigators have proposed that in the faster proliferating population, a greater proportion of the cells will pass through the drug-sensitive phase of the cycle and be killed during treatment.[2, 3] For this hypothesis to completely explain differences in the cytocidal action of antimetabolites, one must assume that once a cell is in the drug-sensitive phase of the cycle, it has the same probability of being killed by a given concentration of drug, regardless of the growth rate of the population from which it was derived. Thus, the sole factor limiting the rate of kill of the cells would then be the rate at which they enter the drug-sensitive phase of the cell cycle.

When the mode of action of a drug is known, its lethal effects should be related quantitatively to the degree of biochemical disturbance it produces. The kinetic hypothesis would then predict that the degree of biochemical disturbance produced by a given concentration of drug should be the same in cells in the drug-sensitive phase of the cycle regardless of whether they are drawn from rapidly or from slowly proliferating populations.

Amethopterin (Methotrexate) (MTX) is an antimetabolite whose mechanism of action is at least partially understood. By inhibiting dihydrofolate reductase, it blocks many reactions, most critical of which may be the conversion of deoxyuridylate to thymidylate. We have monitored the effect of MTX on this conversion by measuring the effect on incorporation of tritiated deoxyuridine (H^3-UdR) into DNA and have taken the blockade of H^3-UdR incorporation as a measure of the biochemical disturbance in the S-phase cells, assuming that most if not all of the incorporation is occurring in S-phase cells. In some experiments the effect on tritiated thymidine (H^3-TdR) incorporation into DNA has also been measured.

The biochemical disturbances produced by MTX have been monitored in cells from populations of varying growth rates. In the faster growing populations, the antifolate produces greater biochemical disturbances in the S-phase cells than it does in the more slowly growing populations. These findings, taken together with those of Dr. Weinstein presented in this monograph, suggest that in the faster growing population, not only would more S-phase

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cells be exposed to antifolate but each exposed S-phase cell is more severely disturbed and thus more likely to be killed.

Methods and Materials

The basic experimental design has been described previously[4] Cells from a resting culture or advanced tumor are diluted into fresh medium or inoculated into a new host. After an initial lag period, the cells proliferate rapidly (exponential or log cultures or "early" tumors) and then more slowly until the resting state is resumed or a late tumor is developed with a growth rate of zero. At intervals, when the cell population is growing at different rates, cells are harvested, treated with MTX *in vitro* for short periods, and then pulsed with H^3-UdR or H^3-TdR. Incorporation of deoxynucleoside into DNA is measured as a rate constant: amount incorporated/minute of pulse/10^6 cells. Replicate cultures are used for these determinations to keep the standard deviation below 15% of the absolute value of the rate constant. This allows comparison of rates on a logarithmic scale.[5] The rate in the presence of drug divided by the rate in the absence of drug (control rate) is the fractional incorporation rate. Thus, the fractional incorporation rate is a measure of the biochemical disturbance produced by MTX in the S-phase cells.

In some experiments, incorporation of H^3-uridine (not to be confused with H^3-deoxyuridine) into RNA and of H^3-leucine into protein have been measured. The data is expressed in the same fashion. Cell viability is measured by cloning into soft agar.[6]

Results and Discussion

Experiments in Mice

First, L1210 cells from ascitic tumors were injected into tumor-free mice. One hour later and daily thereafter six animals were sacrificed, and the L1210 cells were harvested quantitatively from their peritoneal cavities and pooled into Eagles medium with 10% horse serum. A suspension of the lymphoblasts was treated with MTX (10^{-6}M at 37° for 30 minutes) and still in the presence of MTX, were pulse labeled (30 minutes with H^3-UdR), and the fractional H^3-UdR incorporation rate was determined. Cells were studied through six transplant generations. Figure 1 summarizes the results. For three days following transplantation, the control H^3-UdR rate increased 25-fold, then declined to levels characteristic of the resting tumor. Meanwhile, the fractional H^3-UdR rate declined from .24 one hour after transplantation, to .09 on days 1–3, and then rose to .24 in the resting tumor. In other words, MTX treatment produced almost a 3-fold greater blockade of H^3-UdR incorporation in the S-phase cells from early compared to late tumors. Goldin and coworkers [7] have shown that early L1210 tumors are much more sensitive to the cytocidal effects of MTX than are late tumors.

Fractional H^3-UdR rates were also determined at concentrations of MTX ranging from 2×10^{-7}M to 1×10^{-3}M. Data from one-, two-, and three-day tumors were pooled (early tumors), as were data from tumors immediately after transplantation and six and seven days (late tumors), since no differences

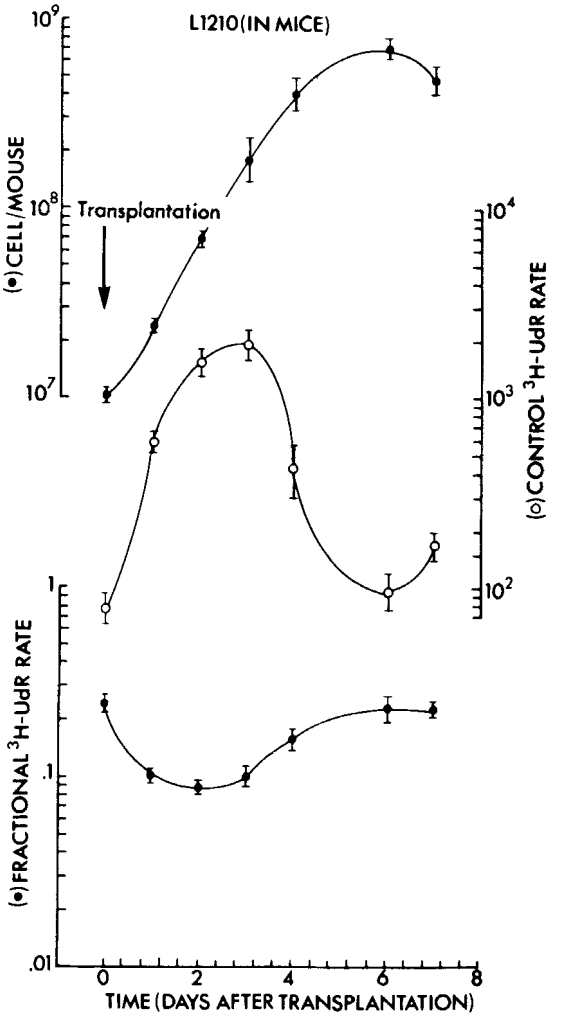

FIGURE 1. Effect of 10^{-6} M amethopterin (Methotrexate) (MTX) on incorporation of ^{3}H-deoxyuridine (^{3}H-UdR) into DNA by L1210 lymphoblasts. Cells were harvested from ascitic mice at various times after transplantation, and treated and pulsed *in vitro* over a one-hour period. Fractional ^{3}H-UdR rate = rate in presence of MTX/control rate. Vertical bars indicate one standard error of the geometric means. Control ^{3}H-UdR rate = dpm/minute of pulse/10^6 cells.

were measured within these groups. It is clear from FIGURE 2 that at all concentrations of MTX the fractional H^3-UdR rates in cells from early tumors were 2.0 to 2.7 times lower than those in cells from late tumors. Note the shape of both dose-response lines. As the concentration of MTX increased, there were decreasing increments in blockade of H^3-UdR incorporation. Bruce and colleagues [2] showed that after a certain dose of MTX had been reached, increasing

the dose did not lead to any greater kill of normal hematopoietic or AKR lymphoma colony-forming cells in mice. They suggested from these and other [3] observations that a viable portion of the target population was not in S phase at the time of treatment and therefore was insensitive to any concentration of MTX. However, the data in FIGURE 2 suggest that in the L1210 cell populations there was a residual amount of H^3-UdR incorporation into DNA—presumably by S-phase cells—which could not be suppressed by MTX no matter how high the antifolate concentration. This residual H^3-UdR incorporation probably represents DNA synthesis impervious to MTX, at least for the period of treatment applied. Such DNA synthesis might be occurring in S-phase cells refractory to the lethal effects of MTX. If so, it would be erroneous to conclude that because further doses of MTX could not increase cell kill, therefore no more cells were in S phase. Thus, it might not be necessary to invoke a "Go" state to account for MTX resistance in a cell population. On the other hand, the fact that residual DNA synthesis is detected in the presence of high MTX concentrations does not prove that the cells in which it is occurring are viable. Nevertheless, we set about to determine the prevalence of these findings, and studied cells in permanent culture.

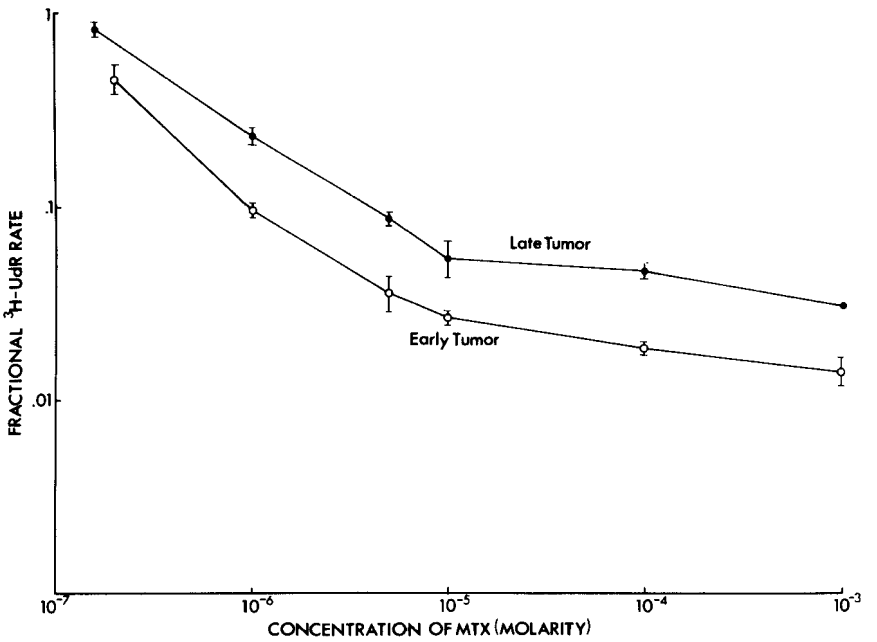

FIGURE 2. Effect of varying concentrations of MTX on incorporation of ^{3}H-deoxyuridine into DNA by L1210 lymphoblasts. (See legend to FIGURE 1 for experimental method, derivation of fractional ^{3}H-UdR rate, and significance of vertical bars.) Early tumours=one, two, and 3 days after transplantation; late tumours=one hour, six days and seven days after transplantation.

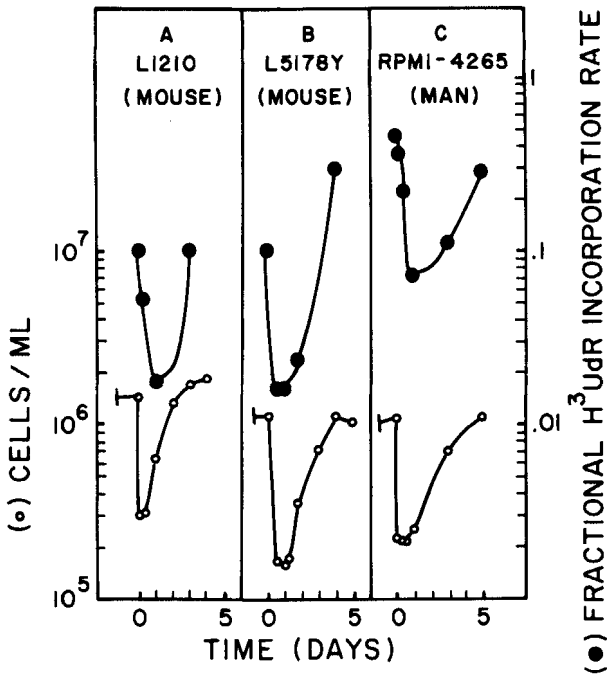

FIGURE 3. Effect of 10^{-6}M MTX on incorporation of ^{3}H-deoxyuridine (^{3}H-UdR) into DNA by cultured murine (L1210, L5178Y) and human (4265-RPMI) lymphoblasts. Cells were diluted from a resting culture into fresh medium and at various times thereafter were treated and pulsed over a one-hour period. (See legend to FIGURE 1 for derivation of fractional ^{3}H-UdR rate.)

Culture Experiments

L1210 cells from a seven-day ascitic tumor were established in culture medium 1640 with 15% fetal calf serum. At intervals during evolution of the culture, the cells were harvested and treated with MTX exactly as in the earlier experiments, and the fractional H^3-UdR incorporation rates were measured. As well, a human cell line, the 4265-RPMI cells, established in the same medium, and L5178Y mouse lymphoblasts growing in Fischer's medium with 10% horse serum were studied. FIGURE 3 shows the results from each cell line. Note that in every instance the treatment decreased the fractional H^3-UdR rate more in the rapidly proliferating cultures than in the resting cultures. Repeat experiments gave identical results. Note also that the L1210 and L5178Y mouse lymphoblasts were similar in their responses, but both were more sensitive than the human cells. Both subtypes of mouse cells were 10-fold more sensitive than the human cells to growth inhibition by MTX.[8] Thus, the observation that S-phase cells varied in their biochemical sensitivity to MTX according to the growth rate was extended to a variety of cultured cells.

More detailed studies were undertaken of the effects of MTX on the L5178Y cells. As before, the cells were sampled at various times during the evolution of

the culture and treated with 10^{-6}M MTX, but the treatment period was extended to 4½ hours. The 20-minute period of pulsing straddled the end of the drug treatment. FIGURE 4 shows that the treatment of cells from log cultures reduced H^3-UdR incorporation to a fractional rate of .0018, whereas the same treatment of resting culture cells reduced the fractional rate to .035. That is, MTX produced a 20-fold greater blockade of H^3-UdR incorporation in the S-phase cells from log cultures. This is shown as the solid line in the lower half of FIGURE 4. (The derivation of the broken line will be explained later.)

It was then found that MTX treatment blocked H^3-TdR incorporation into DNA in log culture but not in resting culture cells. It has been noted that in humans with acute leukemia responsive to MTX, the H^3-TdR incorporation rate falls following administration of the antifolate, whereas in refractory cases it rises or does not change.[9, 10] FIGURE 5 depicts the effect of 6½ hours of antifolate treatment on H^3-TdR incorporation by the L5178Y cells. Rates were measured over a 20-minute period straddling the end of the 1st, 2nd, 4th and

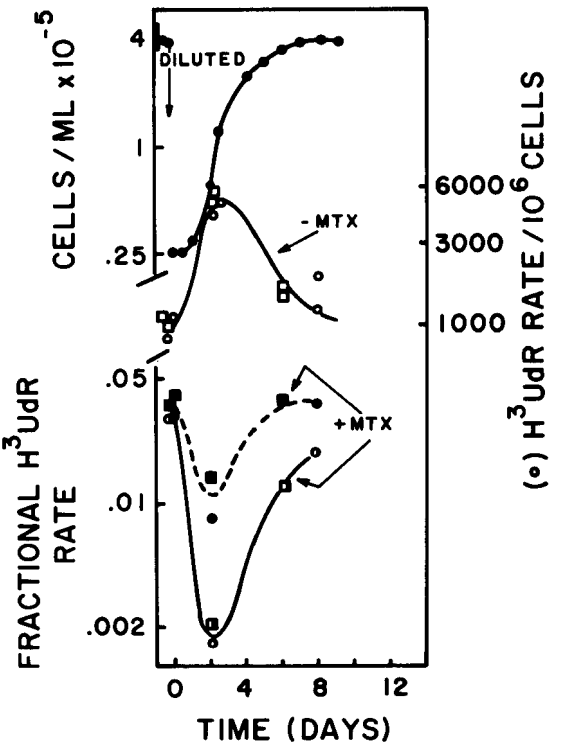

FIGURE 4. Effect of MTX on incorporation of deoxyuridine (^{3}H-UdR) by L5178Y lymphoblasts. Cells were diluted from a resting culture into fresh medium and at intervals thereafter were treated for 4½ hours with 10^{-6} MTX. A 20-minute pulse of ^{3}H-UdR was given straddling the end of the treatment to determine the fractional ^{3}H-UdR rate (See legend to FIGURE 1). The solid line connects the uncorrected, and the broken line the corrected fractional ^{3}H-UdR rates (see text). Control ^{3}H-UdR rate = dpm/minute of pulse/10^6 cells.

6½ hour of treatment. Note, in the log cells, the rapid 10-fold reduction in H^3-TdR rate. In striking contrast, this treatment had little discernible effect on H^3-TdR incorporation by resting culture cells. Coincident with these differences in effects on H^3-TdR incorporation there was exponential kill of cells in both cultures, but the cloning efficiency decreased 6.7 times faster in log compared to resting cultures. Compare the slopes of the lines in the lower half of FIGURE 5A and 5B. The decrease in H^3-TdR incorporation rate probably was not due solely to death of S-phase cells with complete cessation of DNA synthesis, because changes in the H^3-TdR incorporation rate did not parallel changes in cloning

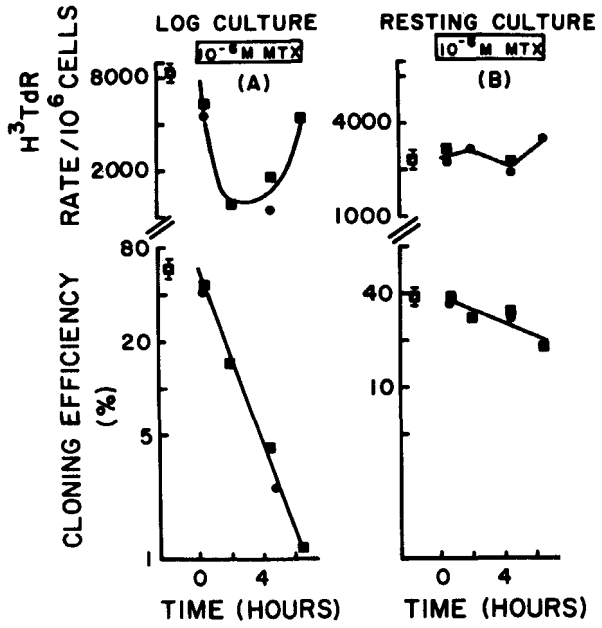

FIGURE 5. Effect of MTX on incorporation of ^{3}H-thymidine (^{3}H-TdR) into DNA and on cloning efficiency of L5178Y cells. Cells from (A) log and from (B) resting cultures were treated with 10^{-6} M MTX for 6½ hours. Cells were pulsed for a 20-minute period straddling one, two, four and six and a half hours of treatment to measure the ^{3}H-TdR incorporation rate or washed free of MTX and cloned. Controls are shown as zero time values. Two experiments are shown (● and ■); in each, controls were done in triplicate. ^{3}H-TdR rate = dpm/minute of pulse/10^6 cells.

efficiency. In fact, the H^3-TdR incorporation rate eventually rose during drug treatment, while cloning efficiency continued to decline exponentially.

In order to explain this difference in the rate of kill in kinetic terms, one would have to postulate that per unit of time of drug treatment, 6.7 times more viable cells would commit themselves to S phase in the log culture compared to the resting culture. This is quite unlikely. Watanabe and Okada,[11] using H^3-TdR, have determined radioautographically that 62% of the L5178Y cells are in S phase in the log culture and 50% in the resting culture. This represents a ratio of 1.2:1. Others have noted a similar ratio for early versus late

tumor cells *in vivo*.[12] We found that the L1210 cells from a resting tumor have a labeling index with H^3-UdR of 38%, but in the early tumors it increases to 68%—a factor of 1.8 increase. Thus, it seems quite improbable that the greater kill of early tumor versus late tumor cells or of log culture versus resting culture cells can be explained solely on the basis of the numbers of S-phase cells being exposed to drug.

We then searched for quantitative relationship between the biochemical disturbances produced by MTX and its lethal effects. If a quantitative relationship between the biochemical and cytocidal effects could be established for cultured cells, there would be more reason to believe that *in vitro* measurement of the biochemical effects of MTX on human acute leukemia cells would predict the cytocidal effects of the antifolate when it was administered to the patient. Since reliable methods for cloning malignant cells with high efficiency from patients are lacking, such an *in vitro* system might at least partially substitute for the sorely-needed cloning method.

Cultures of L5178Y cells of varying growth rate were treated with MTX at 10^{-6}M for 4½ hours. Following treatment, some cells were pulsed to measure H^3-TdR incorporation rate, while others were cloned to measure viability. The effect on H^3-TdR incorporation was expressed as logs of decrease from the control rate, and the effect on viability as logs of reduction of cloning efficiency (logs of kill). As can be seen from FIGURE 6, there is a highly significant correlation between these two parameters of MTX effect. Thus, one can predict with some accuracy what the kill will be if, for this treatment schedule, one measures the effect on H^3-TdR incorporation. For practical purposes this serves as a biochemical substitute for cloning.

Mechanism of Suppression by MTX of H^3-TdR Incorporation

Why does MTX suppress H^3-TdR incorporation into DNA in the S-phase cells most sensitive to its cytocidal effects? Probably MTX blocks *de novo* purine synthesis more effectively in these cells. Evidence for this is shown in FIGURE 7. Here, cells were treated with MTX (10^{-6}M for 4½ hours) but in the presence or absence of 2×10^{-5} M hypoxanthine, an exogenous source of purines. The suppressive effect of MTX on H^3-TdR incorporation was completely prevented by the hypoxanthine. More importantly, the lethal effects of MTX were considerably reduced. FIGURE 8 shows that at every culture growth rate tested, hypoxanthine partially prevented the loss of cloning efficiency resulting from MTX treatment. Probably this is close to the limit of the protective effect of hypoxanthine and the kill not prevented by hypoxanthine will be largely if not completely prevented by thymidine. Nevertheless, it is important to stress that the considerable protection from MTX-kill provided by hypoxanthine means that in the L5178Y cells, there is a "purineless" death as well as a "thymineless" death.

The block in DNA synthesis imposed by lack of purines complicates interpretation of the H^3-UdR incorporation data shown in FIGURE 4. Thus, the 20-fold greater blockade of H^3-UdR incorporation seen in the log cells must be due to a combination of a block in dUMP conversion to dTMP and a block in dTMP incorporation into DNA due to lack of purines. If H^3-UdR were converted to dTMP via dUMP, it would still encounter the same difficulties in being incorporated into DNA as does H^3-TdR. If we assume that it would

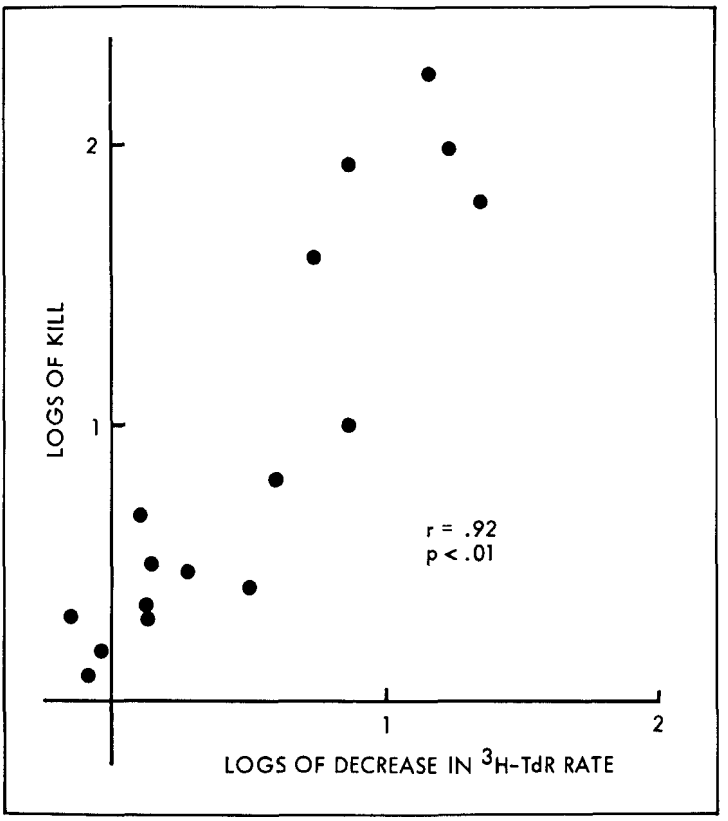

FIGURE 6. Correlation between biochemical and cytocidal effects of 10^{-6} M MTX on L5178Y cells. The ^{3}H-thymidine rates were measured over a 20-minute period straddling the end of the 4½-hour treatment period with MTX and expressed as logs of decrease from the control rate (no drug treatment). Kill was measured as logs of reduction in cloning efficiency from the control.

encounter exactly the same degree of difficulty, we can then correct the fractional H^3-UdR rate for changes in the fractional H^3-TdR rate and thus calculate the degree of suppression of H^3-UdR incorporation attributable solely to the blockade at the dUMP conversion step. When this correction is applied, the fractional H^3-UdR rates obtained are those connected by the broken line in the lower half of FIGURE 4. The calculated fractional H^3-UdR rate still remains lower in the log compared to the resting cells. Thus, even if one takes into account the effect of MTX on *de novo* purine synthesis, then there still may be a greater effect on dUMP conversion to dTMP in the log compared to the resting culture cells.

What is the mechanism of the purineless death? It may be related to the effect of the purineless state on RNA and protein synthesis. MTX suppresses H^3-uridine incorporation into RNA to a 23-fold greater degree in log cells and of H^3-leucine into protein to a 2½-fold greater degree. In fact, there is almost no suppression by MTX of H^3-leucine incorporation into the protein of resting

culture cells. These data are shown in TABLE 1 and FIGURE 8. Notice also that hypoxanthine completely prevents both of these effects of MTX and in fact appears to stimulate precursor incorporation. Perhaps the block in *de novo* purine synthesis inhibits synthesis of rapidly turning over RNA. Any protein synthesis dependent upon such rapidly turning over RNA would therefore also be curtailed. If synthesis of new dihydrofolate reductase were dependent on such RNA synthesis, then MTX inhibition of the reductase would in effect retard synthesis of new reductase. If a critical degree of inhibition were achieved, then even when extracellular MTX were removed, no new reductase

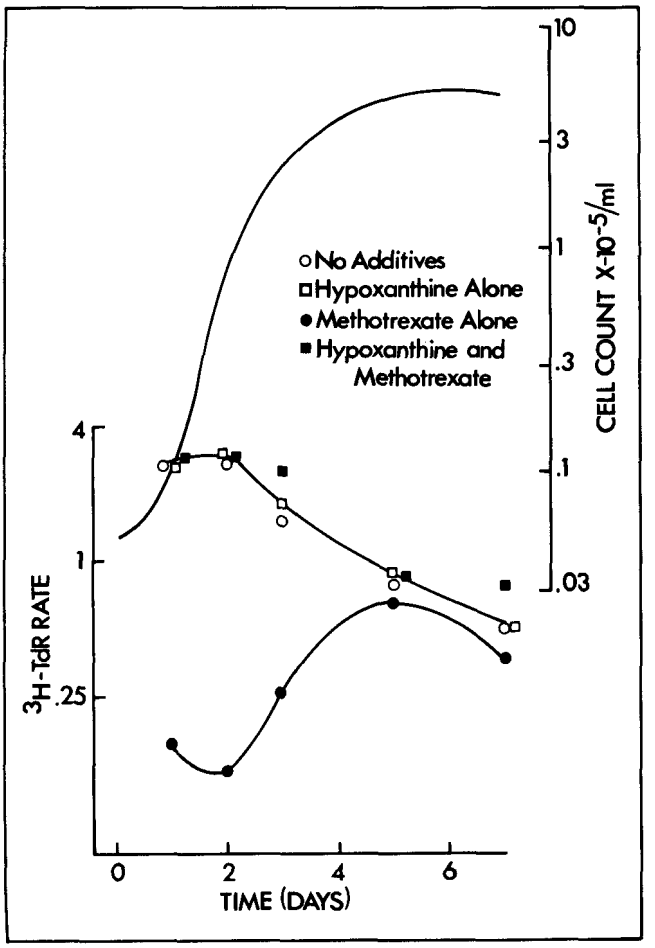

FIGURE 7. Protective effect of hypoxanthine against suppression by MTX of ^{3}H-thymidine incorporation into DNA. L5178Y cells were diluted from stock log cultures into fresh medium and at various times thereafter treated for 4½ hours with the additives shown in the figure. They were pulsed for 20 minutes straddling the end of the treatment period to determine the ^{3}H-TdR incorporation rate. ^{3}H-TdR rate = picomoles/minute of pulse/10^{6} cells.

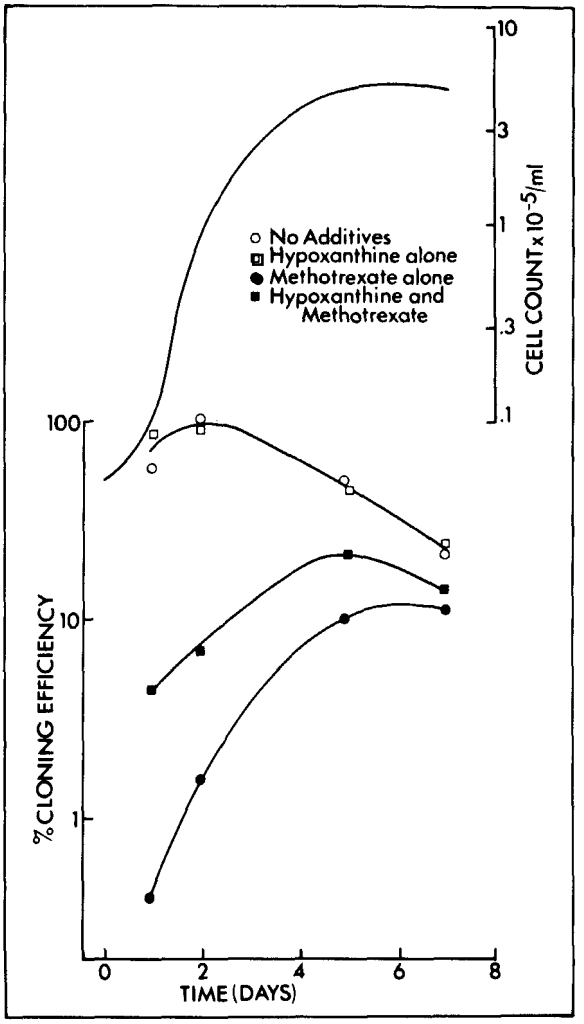

FIGURE 8. Protective effect of hypoxanthine against lethal effects of MTX on L5178Y cells. The experiment was performed exactly as in FIGURE 7 except that at the end of the treatment the cells were washed and cloned into soft agar.

could be synthesized to overcome the block imposed by this tight-binding inhibitor. The cell would then be incapable of continued replication and might even disintegrate. In either case it would, in cloning terms, be dead. Such effects might be especially likely to occur in early S-phase cells which might be actively synthesizing new reductase enzyme [13] while transporting more MTX intracellularly. Of course, this hypothesis could apply as well to some other enzyme critical to the integrity of the cells. It has previously been noted that in humans with acute leukemia receiving infusions of MTX, the best responses occur in patients in whom the reductase level of the leukemic cells fails to rise

TABLE 1

EFFECT OF MTX ON ^{3}H-URIDINE RATE IN PRESENCE AND ABSENCE OF HYPOXANTHINE

Treatment condition	^{3}H-Uridine incorporation into RNA (picomoles/min/10^6 cells ± S.D.)[a]	
	Logarithmic culture	Resting culture
Control	1.03 ± .14	.47 ± .01
Amethopterin	.021 ± .001	.22 ± .01
Control + hypoxanthine	1.72 ± .05	.54 ± .03
Amethopterin + hypoxanthine	2.45 ± .22	.52 ± .04

[a] Rates and standard deviations are averages from two separate experiments. They did not vary from each other significantly.

during treatment, while the cases refractory to treatment are those in which reductase enzyme activity rises in the surviving leukemic cells.[9]

This view of the role played by the purineless state in modulating MTX effects conflicts with the current concept of thymineless death, which is that cell death occurs following MTX treatment because of an imbalance between DNA synthesis on one hand and RNA and protein synthesis on the other. Thus, a cell is lethally affected if while DNA synthesis is inhibited, RNA and protein synthesis continue. If RNA and protein synthesis are also inhibited, the imbalance is relieved, and the cell is no longer at risk of dying. Thus, Borsa and Whitmore [14] suggest that if MTX inhibits DNA synthesis but also inhibits RNA and protein synthesis via its effects on the *de novo* purine pathway, then the cell will be prevented from dying. This appears not to be the case for L5178Y cells. Furthermore, the weight of the evidence in humans [9, 10] and in model systems [15–19] indicates that the lethal effect of MTX on both normal and leukemic cells can be directly correlated with suppression of *de novo* purine synthesis.

In conclusion, our data suggest that in assessing the sensitivity of tumors to an antimetabolite it may be necessary to study not only the kinetics of proliferation of the tumor, but also how these kinetics modulate the metabolic disturbances induced by the antimetabolite. Two species of cells might be proliferating at the same rate yet differ markedly in sensitivity to antimetabolites because of intrinsic differences in their S-phase cells, for example the difference shown in FIGURE 3 between the 4265-RPMI cells and the mouse lymphoblasts. In addition, differences in sensitivity of S-phase cells may be imposed by non-kinetic influences such as location in different body compartments, antibodies, previous exposure to chemotherapy, etc. Such superimposed differences may overshadow any differences related to kinetic influences.

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