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ENZYME PATTERN DIRECTED CHEMOTHERAPY

THE EFFECTS OF COMBINATIONS OF METHOTREXATE, 5-FLUORODEOXYURIDINE AND THYMIDINE ON RAT HEPATOMA CELLS *IN VITRO**

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Abstract—The effects of methotrexate (MTX), 5-fluorodeoxyuridine (FUdR) and thymidine on the growth of four rat hepatoma lines were examined *in vitro*. In the rapidly growing 3924A and Novikoff lines, MTX exerted a marked anti-purine effect, as indicated by its continued toxicity in the presence of thymidine. Addition to the slower growing hepatoma lines 8999R and 8999S of 200 μM thymidine was able to sustain growth for 48 hr in the presence of MTX. However, at a lower thymidine concentration (20 μM) the MTX toxicity toward the 8999R and 8999S lines was not prevented, even in presence of hypoxanthine. Similarly, thymidine at 20 μM was able to protect the 3924A and Novikoff cell lines completely from the toxic effect of 10 μM FUdR, but this thymidine concentration did not protect the 8999R and 8999S lines. The failure of thymidine to protect the latter hepatoma lines was attributed to the rapid breakdown of thymidine in these cells. This interpretation was supported by experiments where addition of 5-diazouracil, an inhibitor of the rate-limiting enzyme of thymidine catabolism (dihydrothymine dehydrogenase), to the cultures resulted in an increased degree of rescue from FUdR by 20 μM thymidine. The combination of MTX and FUdR treatment in the slow growing hepatomas showed summation, but the two agents were less than additive in the rapidly growing lines. The results of these drug combination studies are interpreted in the context of the patterns of competing enzymes characteristic of the various tumor cell lines.

Extensive studies on the comparative biochemistry of rat liver and hepatomas have demonstrated that the patterns of hepatic enzyme activity show characteristic changes in neoplasia. If chemotherapy could be devised to direct cytotoxicity selectively toward the enzyme patterns of a particular target cell, this should provide a greatly improved basis for antitumor drug specificity. The present work describes studies in which selectivity of action of antimetabolites toward four cultured rat hepatoma cell lines was shown to be influenced by the balance of competing pathways in folate and pyrimidine metabolism.

Tetrahydrofolate cofactors are required for the *de novo* biosynthesis both of purines and of thymidylate, and the antifolate drug MTX† antagonizes both these functions [1–4]. However, although MTX invariably exerts an anti-thymidylate effect, the anti-purine effect appears to vary in severity in different cell types. Purine alone never prevented the toxic effect of a lethal dose of MTX, but the combination of a purine plus thymidine did so in every case in mammalian fibroblasts, lymphoblasts, and sarcoma tissue culture cell lines [3]. However, the response to MTX plus thymidine (without purine) was markedly different in various cell lines. In the L5178Y mouse lymphoma, the toxicity of 1 μM MTX was just as great in the presence of 40 μM thymidine as in its absence; in these cells, growth was apparently limited by lack of purines. In other lines, however, thymidine was able to cause a partial and temporary reversal of the MTX toxicity. This was most clearly seen in the Yoshida sarcoma which, in the presence of 10 μM MTX and 40 μM thymidine, continued cell division at 70 per cent of the control rate for 48 hr. Measurements of deoxynucleoside triphosphate pools in MTX-treated cells confirmed the interpretation of the growth inhibition studies [3]. In all five cell lines studied, 24-hr exposure *in vitro* to a lethal concentration of MTX caused a decrease in cellular dTTP. In all cases, dCTP and dATP pools were increased or not significantly altered. The effect of MTX upon dGTP levels varied; in the L1210 leukemia cells or the Yoshida sarcoma cells (both lines in which cell division continued for a time in the presence of MTX plus thymidine), dGTP levels were unchanged or increased. By contrast, in the L5178Y cells, where thymidine had virtually no effect on MTX toxicity, a marked reduction was seen in the dGTP pool, confirming that in this line the drug had a pronounced anti-purine activity.

In experiments with animals, it has been suggested that differences of this kind may be the result of the varying degrees of operation of purine salvage in different cells [4]. With cells in culture, growing in purine-free medium, the varying anti-purine effect of MTX possibly reflects differences in the compartmentation of tetrahydrofolate cofactors between the thy-

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† Abbreviations used in the text: MTX, methotrexate (amethopterin); and FUdR, 5-fluorodeoxyuridine.

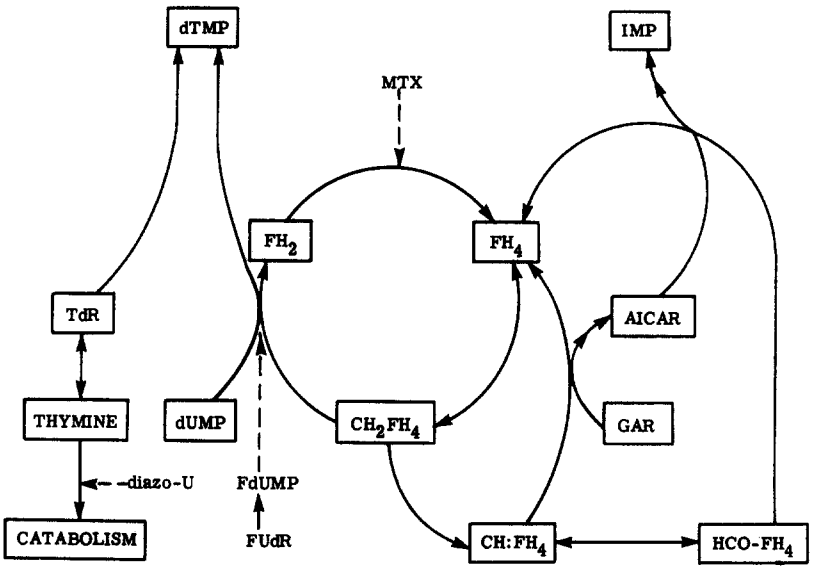

Fig. 1. Schematic metabolic map showing sites of action of the antimetabolites used in the present study. Abbreviations: TdR, thymidine; FH_2, dihydrofolate; FH_4, tetrahydrofolate; CH_2FH_4, $CH{:}FH_4$ and $HCO\text{-}FH_4$, 5,10-methylene, 5,10-methenyl- and 10-formyl tetrahydrofolates; GAR, glycinamide ribotide; AICAR, 5-amino-4-imidazole carboxamide ribotide.

midylate and purine biosynthetic pathways. The critical metabolic branch point lies in the utilization of 5,10-methylenetetrahydrofolate, which is a substrate for thymidylate synthetase (EC 2.1.1.b) and for 5, 10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), which leads to purine biosynthesis (Fig. 1). The earlier study [3] showed that the ratio of activities of 5,10-methylenetetrahydrofolate dehydrogenase to thymidylate synthetase showed a significant correlation with the extent of protection by thymidine from MTX toxicity. The present paper extends these observations to a series of cultured rat hepatoma lines of different growth rates, and analyzes further factors which influence the response of cells to MTX or FUdR in the presence of thymidine.

MATERIALS AND METHODS

Methotrexate was purchased from Lederle, Pearl River, N.Y., and 5-fluorodeoxyuridine, 5-diazouracil, thymidine, tetrahydrofolate, dUMP, NADP and hypoxanthine from Sigma Chemical Co., St. Louis, MO. Tissue culture supplies were the product of Grand Island Biological Co. (Grand Island, N.Y.) or of Flow Laboratories (Rockville, Md.).

Cell culture. The rat hepatoma cell lines were maintained in McCoy's medium 5A, supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The Novikoff cells were subcultured three times weekly, the 3924A twice weekly, the 8999R weekly, and the 8999S twice monthly. The latter two lines were fed with fresh medium at twice weekly intervals. Doubling times of the cells are given in Table 1. Further properties of the cultured hepatoma cell lines are described elsewhere [5]. Cultures were checked at regular intervals for mycoplasma contamination.

Inhibition studies. Effects of drugs were studied by measuring growth inhibition in the continuous presence of the agents, which were added 4 hr after initiation of the cultures. After 48 or 72 hr in the presence of the drug, triplicate cultures were counted in a haemocytometer, and results were expressed as per cent of the mean control cell count (controls were grown in quadruplicate). Isobol diagrams were constructed by the method previously described [5, 6].

Enzyme assays. Approximately 5×10^7 cells were collected, washed in balanced salt solution, and resuspended in 1 ml of 0.05 M Tris chloride buffer, pH 7.4. The cell suspensions were ultrasonically lysed at 0°, (Branson sonifier, position 6, 20 sec) and particulate material was removed by centrifugation at 50,000 *g* for 15 min. Thymidylate synthetase was measured in the supernatant fraction by the method of Lomax and Greenberg [7] and 5,10-methylenetetrahydrofolate dehydrogenase according to Ramasastri and Blakley [8].

Utilization of thymidine. Comparative rates of synthetic and catabolic utilization of thymidine were measured by examining the rates of conversion of [^{14}C]thymidine into DNA and CO_2 as described by Ferdinandus *et al.* [9].

RESULTS

The effects of MTX and FUdR on the growth of the four hepatoma lines in culture are shown in Fig. 2. The rapidly growing lines, 3924A and Novikoff, were about five times more sensitive to MTX than the more slowly growing 8999R and 8999S. 3924A was also the most sensitive of the lines to FUdR; the Novikoff hepatoma cells, like the 8999R and 8999S cells, were comparatively resistant to this agent. The apparent ID_{50} concentrations, i.e. the drug levels whose continuous presence in the culture medium reduced the cell count to half of the control value over the stated times, were: Novikoff, MTX 12 nM, FUdR 340 nM (48 hr); 3924A, MTX 15 nM, FUdR

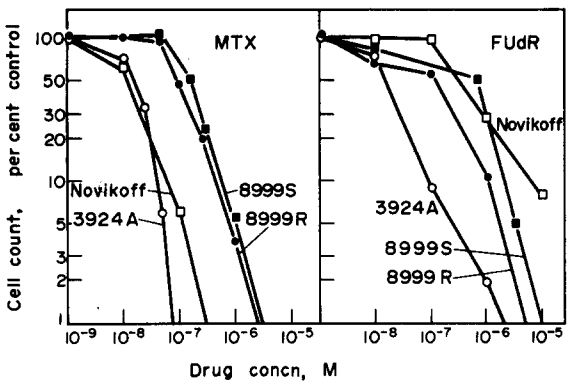

Fig. 2. Log-log dose–response curves for the four hepatoma lines growing *in vitro*. Cultures were established in the presence of the indicated concentrations of the drugs (MTX or FUdR) and cultures were counted at the following times: Novikoff, 48 hr; 3924A, 72 hr; 8999R, 84 hr; and 8999S, 120 hr.

16 nM (72 hr); 8999R, MTX 95 nM, FUdR 120 nM (84 hr); and 8999S, MTX 170 nM, FUdR 680 nM (120 hr). A more useful comparison of relative intrinsic sensitivity to growth inhibition is the normalized ID_{50}, which corrects for differences in growth rate between the cell lines. This parameter is defined as the drug concentration which doubles the log phase doubling time (i.e. halves the growth rate constant). Based on the experiments plotted in Fig. 2, and similar experiments, the normalized ID_{50} concentrations, calculated as previously described [10], were: Novikoff, MTX 29 nM, FUdR 1200 nM; 3924A, MTX 30 nM, FUdR 71 nM; 8999R, MTX 105 nM, FUdR 150 nM; and 8999S, MTX 120 nM, FUdR 180 nM. The molecular basis for these inter-strain differences in intrinsic drug sensitivity is at present unknown.

Earlier work [1–4] has demonstrated that different cell lines exhibit qualitative, as well as quantitative, differences in response to MTX. In particular, the balance between the anti-purine effects and anti-thymidylate effects shows wide variation. The anti-purine action of MTX may be measured by the growth inhibition in the presence of MTX and thymidine. Table 1 indicates that all four cell lines were extensively inhibited in the presence of 1 μM MTX plus 20 μM thymidine. In the Novikoff and 3924A lines, 20 μM thymidine plus 100 μM hypoxanthine gave complete protection from MTX toxicity. The fact that thymidine alone did not protect the cells from MTX, nor did hypoxanthine (results not shown) but that thymidine and hypoxanthine together conferred virtually complete protection suggests that in these lines MTX has a marked anti-purine effect, as well as anti-thymidylate activity. By contrast, in the 8999R and 8999S lines, 20 μM thymidine plus 100 μM hypoxanthine gave very poor protection from MTX (Table 1). However, 200 μM thymidine (a concentration which is toxic to most cell lines) resulted in considerable, though still far from complete, protection. From these results it appears that, in the presence of MTX and 20 μM thymidine, growth of Novikoff and 3924A cells is limited by the availability of purines, but that growth of the 8999R and 8999S cells is limited primarily by the lack of thymidine.

Comparison of the growth of the four hepatoma lines in 1 μM MTX plus excess (200 μM) thymidine (Table 1) indicated a correlation with the ratio of the activities of 5,10-methylenetetrahydrofolate dehydrogenase to thymidylate synthetase. The latter enzyme showed a positive correlation with the growth rate (i.e. correlated inversely with doubling time). The dehydrogenase was slightly more active in the slowly growing 8999 cells. A high activity of thymidylate synthetase, by channelling tetrahydrofolate cofactors to dihydrofolate, which in the presence of MTX cannot be reduced, thus decreases the potential of the cell for purine biosynthesis in the presence of MTX. The 5,10-methylenetetrahydrofolate dehydrogenase, which competes with thymidylate synthetase for the same tetrahydrofolate cofactor, opposes this depletion of the tetra-hydrofolate pool (Fig. 1).

The high requirement for thymidine of the 8999R and 8999S hepatoma lines suggested that these cells might be catabolizing thymidine. It was previously shown in solid tumors [9, 11] that the ability to degrade thymidine, relative to liver, was decreased, but still measurable, in the medium growth rate hepatomas, such as 8999. The catabolic activity was further reduced to an extremely small fraction of the original liver activity in the rapid hepatomas, such as 3924A. Table 2 shows the results of such experiments in the cultured hepatoma lines 3924A and 8999R. It may be seen that the 8999R cells retained part of the ability to degrade thymidine, but in 3924A the balance between the competing pathways of thymidine utilization was much more heavily in favor

Table 1. Reversal by thymidine and hypoxanthine of methotrexate toxicity to hepatoma cells *in vitro**

Cell line	Doubling time (hr)	Thymidylate synthetase (TS) (units/mg protein)†	5,10-Methylene-tetrahydrofolate dehydrogenase (units/mg protein)†	Ratio DH/TS‡	Cell count at 48 hr		
					TdR (20 μM)	TdR (200 μM)	TdR (20 μM)† Hx (100 μM)
Novikoff	10	0.41	4.0	9.8	4	5	91
3924A	15	0.22	4.4	15.7	13	16	96
8999R	39	0.11	7.3	66.3	16	36	24
8999S	81	0.07	6.8	97.5	12	49	27

* Culture medium contained 10^{-6} M methotrexate plus thymidine (TdR) and hypoxanthine (Hx) as indicated. Cell counts are presented as per cent of untreated controls.

† Enzyme units are nmoles substrate converted/min at 37°.

‡ Ratio of 5,10-methylenetetrahydrofolate dehydrogenase/thymidylate synthetase.

Table 2. Opposing routes of utilization of thymidine in cultured hepatoma cells*

Pathway	Cell line 8999R	3924A
[^{14}C]TdR into DNA (nmoles/hr/g)	6.0	20.6
[^{14}C]TdR into CO_2 (nmoles/hr/g)	0.35	0.08
DNA path/CO_2 path	17	259

* Experimental method was as described by Ferdinandus *et al.* [9]. Extracellular thymidine concentration used was 2.3 μM.

of biosynthesis. It is possible, therefore, that the poor ability of thymidine to rescue the 8999R and 8999S hepatoma lines from MTX was due to the thymidine breakdown in these cells.

The results summarized above suggest, therefore, that the fate of cells in the presence of MTX and thymidine may rest upon the balance between two sets of opposing pathways: first, the ratio of thymidylate synthetase to 5,10-methylenetetrahydrofolate dehydrogenase, and second, the ratio of biosynthetic to catabolic utilization of thymidine. Since a correlation of this nature is not sufficient to establish a causal relationship, further experiments were carried out in which selective inhibitors were used to modify artificially the ratio of the competing activities. Thymidylate synthetase may be inhibited by 5-fluorodeoxyuridine, which is converted in the cell to its 5′-monophosphate. Table 3 shows the result of an experiment of this kind. MTX (1 μM) plus thymidine 10 μM) were extremely toxic to the Novikoff cells (cell count 2 per cent of control at 48 hr). When FUdR was also administered, growth was increased to 25 per cent of the controls. Pretreatment with FUdR plus thymidine 4 hr before the addition of MTX to the cultures further decreased the toxic effect. This time-dependent antagonism of the anti-purine effect of MTX by FUdR suggested that the latter agent, by inhibiting thymidylate synthetase, was decreasing the rate of depletion of tetrahydrofolate cofactors in the cell.

Table 4. Effect of 5-fluorodeoxyuridine and thymidine on growth of hepatoma cells in culture

Compounds added	Cell count at 48 hr as per cent control: Novikoff	3924A	8999R	8999S
FUdR (10 μM)	13.5	0.4	0.6	0.9
FUdR (10 μM) + TdR (20 μM)	84	80	1.3	1.7
FUdR (10 μM) + TdR (20 μM) + 5-diazouracil (200 μM)	81	78	41	38

In a further series of experiments, we examined whether the balance between the opposing pathways of thymidine utilization could be altered by selective inhibition. Since the anti-purine effect of MTX would be an unwanted complication in this study, we utilized instead FUdR, which exerts primarily an anti-thymidylate effect. Results are shown in Table 4. As we observed with MTX, the FUdR toxicity was essentially prevented by 20 μM thymidine in the 3924A and Novikoff lines, but the thymidine was almost completely without effect in 8999R and 8999S. However, if we also added to the cultures 5-diazouracil, a potent and irreversible inhibitor of dihydrouracil dehydrogenase [12] and of thymidine degradation, thymidine was now able to confer a partial protection from FUdR. Table 5 shows the result of experiments with the 8999R cells with other pyrimidines. Uridine gave a small amount of protection from FUdR plus thymidine, but was much less active than 5-diazouracil. The small effect of uridine appeared to be antagonized by cytidine (Table 5). The slight stimulation of growth by cytidine (culture 4), though repeatable, is as yet unexplained.

Finally, experiments were conducted to investigate the interactions of the anti-hepatoma effects of MTX and FUdR in the absence of added nucleosides. Representative experiments with the 3924A and

Table 3. Effect of inhibition of thymidylate synthetase on the ability of thymidine to block MTX toxicity in cultured Novikoff hepatoma cells*

Culture	Compounds added at time of initiation	Compounds added 4 hr after initiation	Cell count at 48 hr (per cent control)
1			100
2	MTX (1 μM)		1
3	MTX (1 μM) + TdR (20 μM)		2
4	MTX (1 μM) + TdR (20 μM) + Hx (100 μM)		78
5	FUdR (1 μM)		13
6	FUdR (1 μM) + TdR (20 μM)		89
7	MTX (1 μM) + FUdR (1 μM)		7
8	MTX (1 μM) + TdR (20 μM) + FUdR (1 μM)		25
9	MTX (1 μM) + TdR (20 μM)	FUdR (1 μM)	12
10	FUdR (1 μM) + TdR (20 μM)	MTX (1 μM)	37

* Stationary suspension cultures were initiated at 4 × 10^4 cells/ml. Ten ml cultures were set up in triplicate, except controls which were quadruplicates, and counted visually. Abbreviations: Hx, hypoxanthine; and TdR, thymidine.

Table 5. Effects of pyrimidine nucleosides on FUdR toxicity in cultured 8999R hepatoma cells*

Culture	Compounds added at time of initiation	Cell count at 80 hr (per cent control)
1		100
2	FUdR (10 μM) + TdR (20 μM)	1
3	UR (100 μM)	61
4	CR (100 μM)	136
5	FUdR (10 μM) + UR (100 μM)	0
6	FUdR (10 μM) + TdR (20 μM) + UR (100 μM)	12
7	FUdR (10 μM) + TdR (20 μM) + CR (100 μM)	3
8	FUdR (10 μM) + TdR (20 μM) + CR (100 μM) + UR (100 μM)	3

* Cultures were set up in triplicate, except controls which were quadruplicates. Abbreviations: TdR, thymidine; UR, uridine; and CR, cytidine.

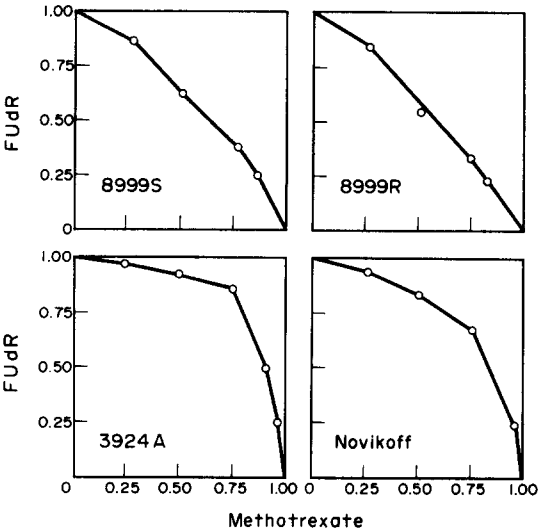

Fig. 3. Isobolograms showing the interactions between effects of MTX and FUdR. Reference doses of each agent were apparent ID_{50} concentrations measured at the times listed in Fig. 1. Figures on the axes indicate fractions of these reference drug doses. The plots thus show the fractional dose of one of the drugs required to give a constant (ID_{50}) response in the presence of the indicated fractional dose of the other drug.

8999R lines are summarized in Table 6. In the 8999R, the two agents showed near therapeutic summation, but the same combination was markedly infra-additive in the 3924A. Complete results for all four hepatomas are given in isobologram form in Fig. 3. In all experiments, in the rapidly growing Novikoff and 3924A the combined effect of MTX and FUdR was considerably less than additive, but the two agents almost showed summation in the slower growing 8999R and 8999S hepatoma cultures.

DISCUSSION

The central difficulty of cancer chemotherapy—the fact that biochemical differences between a tumor and its host are chiefly quantitative ones— has made the design of selectivity a pressing challenge for the design of anti-cancer drugs. A problem has been that existing drugs were designed to strike at one of the most prominent biological features of tumors, their proliferative nature. Perhaps for this reason, in part at least, cancer chemotherapy is at present most effectively used against rapidly growing tumors. The most frequently occurring malignancies, however, particularly solid tumors of low growth fraction, proliferate much more slowly than the normal cell renewal compartments of the body. As a result, most drugs are more toxic to intestinal epithelium or bone marrow than to such tumors. Nevertheless, biochemical studies in this laboratory and elsewhere [13, 14] have shown that the quantitative biochemical changes of malignant tissue, considered as an integrated pattern, are specific to neoplasia, and as such are distinguishable from the biochemical pattern of fetal or developing tissues, or of non-malignant hyperplasia. In principle, it should be possible to use a knowledge of tumor biochemistry and enzymology to direct drug action specifically against malignant cells.

The present study has attempted to show how patterns of competing enzymes may determine selectivity of drug action. In the four hepatoma cell lines studied, the toxicity of MTX in the presence of excess thymidine correlated inversely with the ratio 5,10-methylenetetrahydrofolate dehydrogenase/thymidylate synthetase. The greater the thymidylate synthetase activity, the greater the anti-purine effects. Since thymidylate synthetase activity correlated with growth rate, whereas the dehydrogenase activity did not differ greatly among the four lines, this combination was most toxic to rapidly dividing cells. Hyrniuk [2], working with L5178Y lymphoma cells in different

Table 6. Interaction of the effects of MTX and FUdR against cultured hepatoma cells

Cell line	Duration of experiment (hr)	MTX concn (nM)	FUdR concn (nM)	Cell count as per cent of untreated control in the presence of: MTX (a)	FUdR (b)	MTX plus FUdR Predicted* (c)	MTX plus FUdR Measured (d)
3924A	72	20	20	39	45	17.5	34
8999R	84	100	100	48	57	27	29

* Assuming summation; i.e. c = (a × b)/100.

proliferative states, has also emphasized the correlation between growth rate and purineless death. We believe that the enzymes competing for methylene FH_4 provide, at least partly, a molecular basis for this observation. Supporting this interpretation are the studies in which the ratio of competing enzymes was altered with FUdR, with a resulting decrease in the anti-purine effect of MTX. It is striking that a quantitative difference in thymidylate synthetase activity of about 6-fold (between the Novikoff and 8999S lines) appears to make a difference between purineless death in a few hours (in Novikoff) and the ability to continue cell division at half the control rate for 48 hr (in 8999S).

Unlike the MTX + thymidine combination, which is most toxic to rapidly dividing cells, the combination MTX + FUdR was relatively more effective against the slowly dividing 8999R and 8999S cells. The difference was only a relative one as the two agents gave additive results in the slow hepatoma lines, but less than additive effects in the rapidly dividing lines; however, the latter had a greater intrinsic sensitivity to MTX, so the combination has no overall advantage against the slowly dividing cells.

The ability to degrade thymidine appears to be primarily, though not exclusively, an hepatic function. Although this function has been almost completely lost in the rapidly growing hepatomas, in common with most rapidly dividing cells, traces of this function remain in the more slowly growing, better differentiated hepatomas. This residual thymidine catabolic activity has a potent effect on drug sensitivity. Concentrations of thymidine which would normally, in the presence of a purine, protect the cells from MTX are inadequate to do so in the 8999R and 8999S cells. The effect is most pronounced with the combination FUdR plus thymidine. Under conditions where the rapidly dividing hepatomas are almost completely protected by the thymidine from the FUdR, the 8999R and 8999S cells are completely killed. Here again, a quantitative difference in the balance of competing pathways appears to give rise to an all-or-none difference in drug response. Again, our interpretation of the effect is supported by experiments in which the ratio of the competing activities was artificially altered.

This last example shows how specialized biochemical functions reflecting the tissue of origin of a tumor may be used to direct the toxicity of another agent, which alone is toxic to both malignant and non-malignant proliferating cells. Another example of this approach is described in Ref. 5. This concept of chemotherapy is one which should be most effectively employed with well differentiated tumors, including some of the slow growing solid tumors which at present respond so poorly to chemotherapy.

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