A Basis for Fluoropyrimidine-induced Antagonism to Methotrexate in Ehrlich Ascites Tumor Cells in Vitro

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ABSTRACT

The inhibitory effect of methotrexate on [3H]deoxyuridine incorporation into DNA is reduced as the basal rate of this reaction is inhibited by pretreatment of Ehrlich ascites tumor cells with fluoropyrimidines. This observation is a basis for fluoropyrimidine-methotrexate antagonism in anticancer regimens and supports the concept that the sensitivity of thymidylate synthesis in tumor cells to methotrexate is related, in part, to the basal rate of thymidylate synthesis from deoxyuridylate.

INTRODUCTION

The combination of fluoropyrimidines and the antifolate, MTX, has been widely used in cancer chemotherapy (7, 13, 23, 25, 29). The recent application of these drugs along with cyclophosphamide as adjuvant therapy of breast cancer is of particular potential importance (4). The biochemical rationale for the combined use of these drugs has not, however, been established. While additive effects or therapeutic synergism has been reported in vivo with some regimens (1, 3, 18), less than additive or antagonistic interactions have been observed (3, 14, 28). Recent studies suggest that sequence of drug administration may be a critical determinant of efficacy and that prior administration of FUra decreases tumor cell sensitivity to MTX. Hence, administration of MTX before FUra produces additive toxicity against Sarcoma 180 in vivo while simultaneous or prior administration of FUra resulted in therapeutic antagonism (3). Likewise, treatment of mice bearing mammary tumors with MTX plus leucovorin prior to FUra was therapeutically more efficacious than was MTX plus leucovorin given simultaneously or after FUra (14). While antagonism to fluoropyrimidines induced by MTX has been attributed to depletion of 5,10-CH2-H4-folate, the cofactor necessary for the covalent association of 5-fluorodeoxyuridine monophosphate with thymidylate synthetase (26) and an increase in intracellular dUMP which competes with and limits the interaction between 5-fluorodeoxyuridine monophosphate and thymidylate synthetase (28), a basis for antagonism to MTX induced by fluoropyrimidines has not been clarified. We now report on one rationale for antagonism between these agents related to a diminished MTX effect produced by fluoropyrimidines based upon a decreased sensitivity of dUrd incorporation into DNA to inhibition by MTX as the basal rate of this reaction is inhibited by FdUrd or FUra.

MATERIALS AND METHODS

Ehrlich ascites tumor cells were grown i.p. in CF-1 mice, harvested, and prepared for experimentation as previously described (8). Cells were suspended into a modified Eagle's medium (10), free of folates or serum, with the following electrolyte composition: 135 mM NaCl, 4.4 mM KCl, 16 mM NaHCO3, 1.1 mM Na2HPO4, 1.0 mM MgCl2, 1.9 mM CaCl2. The cell suspension was stirred continuously with a motor-driven Teflon paddle, and pH was maintained at 7.3 to 7.4 in an atmosphere of 95% O2-5% CO2 at a temperature of 37°. [3H]MTX was synthesized by Amersham/Searle (Des Plaines, Ill.). This and the nonlabeled MTX were generously supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

For determination of intracellular [3H]MTX levels, cells were separated by centrifugation and washed twice with 0.85% NaCl solution (buffered to pH 7.4) at 0°. The cell pellet was transferred to polyethylene tares and dried overnight at 60°; the pelleted cell was weighed on a Beckman LM800 automatic microbalance. The dried cells were then transferred to liquid scintillation vials, digested in 1 N KOH, and cooled; a fluor was added; and cell 3H was measured in a liquid scintillation spectrometer. Incorporation of [3H]dUrd into DNA was determined on a dilute acid extract of a perchlorate precipitate following removal of [3H]dUrd incorporated into RNA by alkaline extraction as previously described (31). Cellular dUMP levels were measured on acetic acid extracts according to the method of Myers et al. (21). Data are expressed as mean ± S.E.

RESULTS

Ehrlich ascites tumor cells were exposed to varying concentrations of FdUrd for 15 min to achieve increasing suppression of [3H]dUrd incorporation into DNA. The cells were then separated by centrifugation and resuspended into fresh media without FdUrd but in the presence or absence of 50 μM MTX. Thirty min later, when free intracellular MTX was at a steady state with extracellular drug,
["H]dUrd was added to achieve a final concentration of 0.5 μM and incorporation of "H into DNA was monitored over a 5- to 30-min interval when the rate of incorporation was constant. Cells exposed to neither FdUrd nor MTX or cells exposed to MTX alone were treated similarly. A representative experiment is illustrated in Chart 1; 50 μM MTX alone suppressed ["H]dUrd incorporation into DNA by 97%; a 15-min exposure to 0.01 μM FdUrd alone decreased the rate of ["H]dUrd incorporation by 65%; however, the subsequent addition of 50 μM MTX produced a further inhibition of only 77%. As the level of FdUrd in the initial exposure was increased to 0.1 and 1 μM, suppression of ["H]dUrd incorporation into DNA was increased to 88 and 99% of control cells, and the effect of the added MTX was decreased to an additional inhibition of only 21% or was abolished, respectively. When the inhibitory effect of FdUrd alone was less than the inhibitory effect of MTX alone, true antagonism by FdUrd could be demonstrated when the agents were combined. Hence, as seen in Chart 1, left, under these conditions, inhibition by MTX plus FdUrd was less than the inhibitory effect of MTX alone. Chart 2 is a composite of 5 similar experiments in which cells were pulsed with 2.5 to 1 μM FdUrd to achieve a wide range of basal ["H]dUrd incorporation rates after which extracellular FdUrd was removed prior to addition of MTX. The data indicate the striking decrease in the inhibition of the residual rate of ["H]dUrd incorporation into DNA by MTX as inhibition of the basal rate of ["H]dUrd incorporation by FdUrd is increased. A similar decrease in the effect of MTX was observed after pretreatment of cells with Fura.

These studies monitor the incorporation of radiolabeled dUrd into DNA; however, additional studies suggest that these observations reflect alterations in the rates of incorporation of total cellular dUrd into DNA as well since changes in the total dUMP pool within the cell as dTMP synthesis was inhibited over the short interval of these experiments was negligible. In 5 experiments performed on 5 separate days, FdUrd, MTX, or MTX plus FdUrd, under the conditions used in these experiments, increased the dUMP pool by only 16.6 ± 5.7, 18.8 ± 10.4, and 28.8 ± 7.2%, respectively, as compared to control cells. While all dUMP levels are slightly higher than in the control cells (p < 0.05; p < 0.02; p < 0.05, respectively), dUMP levels in cells treated with 1 or both drugs are not significantly different from each other (p > 0.2). Finally, these effects of fluoropyrimidines cannot be attributed to alterations in the membrane transport of MTX. Hence in 3 experiments performed on 3 different days, after exposure of cells to 1 μM FdUrd for 30 min, the initial uptake velocity and steady-state level of 50 μM ["H]MTX were not different from that of control cells (p > 0.5).

DISCUSSION

Previous studies from this laboratory support the concept that only a small fraction of cellular DHFR activity is required to maintain normal levels of cellular THF cofactors as 5,10-CH<sub>2</sub>H<sub>4</sub>-folate is oxidized to DHF in the synthesis of dTMP from dUMP (30). The capacity of DHFR to reduce DHF to THF is so great that, when cells are incubated with 10 μM DHF, intracellular DHF is so low (<10<sup>-8</sup> M) as to be undetectable (30). This is compatible with observations in the L1210 leukemia that DHF levels were not measurable in cells equilibrated with ["H]folic acid (20), and DHF can be detected in these cells only after prior incubation with MTX following exposure of cells to 5-methyl[9,3',5'-"H]tetrahydrofolate (22). Hence, since the K<sub>m</sub> for DHF is ~1 μM, it follows that FdUrd would be expected to compete with DHF for access to DHFR.

The data indicate that the inhibition of ["H]dUrd incorporation into DNA by MTX is less than the inhibitory effect of MTX alone. Chart 2 is a composite of 5 similar experiments in which cells were pulsed with 2.5 to 1 μM FdUrd to achieve a wide range of basal ["H]dUrd incorporation rates after which extracellular FdUrd was removed prior to addition of MTX. The data indicate the striking decrease in the inhibition of the residual rate of ["H]dUrd incorporation into DNA by MTX as inhibition of the basal rate of ["H]dUrd incorporation by FdUrd is increased. A similar decrease in the effect of MTX was observed after pretreatment of cells with Fura.

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DHFR must be at a very low state of saturation with respect to its substrate and only a small percentage of total enzyme activity is required to meet cellular demands for THF. Accordingly, when cells are exposed to MTX and the drug enters the intracellular compartment, the following sequence of events would be expected to occur. Initially, as MTX binds to enzyme, free drug does not accumulate within the cell since influx is rate limiting to binding (12), the ratio of free enzyme to intracellular drug is very great, and the intracellular DHF level is too low to compete effectively with MTX for the enzyme sites. Association of MTX with DHFR sites results in a small transient reduction in the rate of THF synthesis with a consequent increase in the DHF substrate level. Since the DHF level in the cell is initially so much lower than its $K_m$, the interaction between DHF and DHFR is essentially first order, and the increase in the intracellular concentration of DHF results in a proportional increase in the rate of DHF reduction as the net rate of interaction between the higher level of DHF and those enzyme sites unassociated with MTX increases. Hence, THF synthesis is initially maintained at an essentially normal rate as MTX associates with an increasing proportion of enzyme sites and DHF continues to accumulate within the cell. Ultimately, as MTX binds to the major portion of enzyme sites, the DHF concentration within the cell approaches its $K_m$, the rate of DHF synthesis no longer increases in proportion to the increase in the intracellular DHF level, and cellular THF cofactor levels fall further. Because the interaction between MTX and DHFR is not "stoichiometric" at physiological pH (2) and because cellular DHF has risen to high levels, it can compete with MTX for the few remaining DHFR sites. Finally, to achieve saturation of the enzyme sites and inhibit THF synthesis completely, high levels of free intracellular MTX, orders of magnitude above its $K_m$, are required (30).

Since the rate of generation of DHF depends solely on the rate of dTMP synthesis, as the rate of dTMP synthesis from dUMP is decreased by fluoropyrimidines, the rate of DHF generation from 5,10-CH$_2$H$_4$folate should be decreased (since intracellular DHF is not measurable under usual conditions, a further fall in the cell level in the presence of fluoropyrimidines cannot be quantitated), and the fraction of DHFR activity required to regenerate THF should be decreased. Hence, the level of free intracellular MTX required to suppress this decreasing fraction of enzyme should be increased, and the response to any given intracellular and extracellular MTX level should be decreased. The data in this paper support this formulation and indicate that this interaction is a basis for antagonism between fluoropyrimidines and antifolates. These findings are consistent with the predictions of the model proposed by Jackson and Harrap (16). Further, by diminishing depletion of intracellular THF, not only should fluoropyrimidines reduce the inhibitory effect of MTX on residual dTMP synthesis but also they may decrease MTX inhibition of THF cofactor-dependent purine synthesis. It has been suggested that MTX-FdUrd antagonism, observed in vivo and in vitro, may be due, in part, to the purine-sparing effect of fluoropyrimidines that reduces the MTX-induced depletion of deoxypurine nucleotides required for DNA synthesis (28), an effect of MTX that may be a critical determinant of its cytotoxicity (13, 15).

While there are a variety of factors that should result in antagonism between antifolates and fluoropyrimidines, additive effects in vivo have been reported (1, 3, 18). Hence, understanding of the consequences of the interaction between these agents is incomplete. The pharmacological effect of these agents in combination may be related to drug dose, host or tissue conditions, or other factors, as yet undefined. Relative effects of MTX on purine- versus pyrimidine-dependent metabolism will be determined by the MTX dose (31) and the availability of preformed nucleosides (5, 24, 27). As indicated above, a critical determinant of antitumor effectiveness may be related to the sequence of drug administration. The observation that administration of MTX prior to FUra in contrast to administration with or after FUra produces greater survival of Sarcoma 180-bearing mice than does either agent alone (3) and that the former sequence enhances survival of mice bearing the mammary tumor (14) is compatible with the expected MTX-FUra antagonism that we now report when cells are exposed to MTX during the pharmacological influence of FUra. Indeed MTX-induced antagonism to FUra seemed to be of little importance in these systems since enhanced efficacy was demonstrated when MTX was administered before FUra. In any event, because of the extensive co-administration of these agents in clinical regimens, further studies are necessary to clarify the effect of sequence and interval between administrations of MTX and fluoropyrimidines on their chemotherapeutic efficacy and the biochemical perturbations that they produce.

Finally, these observations suggest a critical relationship between the basal rate of dTMP synthesis in general and the ability of MTX to inhibit that process. Hence, dTMP synthesis during the highest rates of semiconservative DNA synthesis in S phase should be most sensitive to MTX because the highest fraction of DHFR activity is necessary to meet cellular THF cofactor demands. On the other hand, under conditions in which semiconservative DNA synthesis is slow or dTMP synthesis is required largely for DNA repair or mitochondrial DNA replication, intracellular THF cofactor pools may also be depleted as DHF is generated in the synthesis of dTMP if regeneration of THF is blocked by MTX. However, because the fraction of enzyme activity that would be necessary to maintain cellular THF synthesis under these conditions is small, complete inhibition of DHFR activity would require high levels of free intracellular MTX. Likewise, because of the slow rate of THF consumption, a long exposure to MTX would be required to deplete THF cofactor stores within the cell. THF deficiency under these conditions might produce cytotoxicity due to the consequences of impaired DNA synthesis and repair and/or a purine-deficient impairment of RNA and subsequent protein synthesis. Hence, one rationale for high-dose MTX regimens in the treatment of slow-growing solid tumors (6, 17, 19) may be the generation and maintenance of very high intracellular drug levels in cells with a very low requirement for THF synthesis. This is an end point that requires substantial levels of extracellular drug both to block entry of circulating THF cofactors into the cells and to generate
critical intracellular drug levels. The latter may be particularly difficult to achieve because of the limited capacity of mammalian cells to accumulate free intracellular MTX due to (a) the anionic nature of the MTX molecule, the negative electrochemical potential of mammalian cells, and (b) an energy-dependent process that appears to pump MTX out of the cell (9, 11).

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REFERENCES

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