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Brief Communication

Exchangeable Intracellular Methotrexate Levels in the Presence and Absence of Vincristine at Extracellular Drug Concentrations Relevant to Those Achieved in High-Dose Methotrexate-Folinic Acid "Rescue" Protocols[1]

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SUMMARY

Studies were undertaken to (*a*) assess intracellular methotrexate (MTX) levels at extracellular drug concentrations comparable to those achieved in high-dose MTX-folinic acid rescue protocols and (*b*) establish whether there is a rationale for the use of vincristine in these regimens. The data indicate that only low levels of exchangeable MTX (intracellular MTX in excess of the tightly bound fraction) accumulated in Ehrlich ascites tumor cells at high extracellular MTX concentration. For instance, the exchangeable steady-state intracellular MTX level was ~6.5 μM when the extracellular drug concentration was 85 μM. Over the interval of these experiments, exchangeable intracellular MTX did not exceed ~10 μM even when extracellular MTX was raised to 250 μM. These exchangeable intracellular MTX concentrations are comparable to those levels required experimentally to suppress (*a*) tetrahydrofolate synthesis from dihydrofolate and (*b*) tetrahydrofolate-dependent purine, pyrimidine, and amino acid synthesis in these cells *in vitro*. Vincristine (10 μM) augmented net MTX accumulation when the extracellular MTX level was 10, 100, or 250 μM. The limited capacity of cells to accumulate exchangeable intracellular MTX and the apparent role for this intracellular MTX component in achieving the metabolic effects of this agent may account for the necessity for high MTX blood levels in the treatment of some tumors and may be the basis, in part, for the enhanced chemotherapeutic efficacy of high-dose MTX regimens. These studies provide a rationale for the combined use of vincristine and MTX in high-dose MTX protocols. The addition of vincristine may permit the achievement of the level of exchangeable intracellular MTX that is required to critically inhibit tetrahydrofolate synthesis without an increase in the extracellular MTX concentration. This may permit a reduced MTX dose, diminishing the excretory load on the kidney and minimizing nephrotoxicity due to deposition of MTX in the renal tubule and interstitium.

While the data indicate that the ratio of the concentration of exchangeable intracellular MTX to the extracellular drug concentration may be very low under steady-state conditions at high extracellular drug levels, further studies are required to establish that these steady-state gradients for MTX represent nonequilibrium conditions.

INTRODUCTION

The properties of MTX[4] transport have been characterized for a number of mammalian cells in several laboratories. These studies have largely been directed towards an analysis of MTX transport at low extracellular concentrations relevant to MTX blood levels achieved in conventional drug regimens and the properties of the high-affinity membrane carrier utilized by the major circulating folate, 5-methyltetrahydrofolate (9, 10, 13, 18). However, recent demonstrations (5, 7, 21, 25) of the efficacy of high-dose MTX regimens, some of which generate extracellular drug levels orders of magnitude above the K_t[5] for the high-affinity carrier, now warrant exploration of the transport properties of MTX under these conditions. Of particular interest is the level of exchangeable intracellular MTX ("exchangeable" intracellular MTX refers to drug in excess of the tightly bound fraction) accumulated within the cell as the extracellular MTX level is increased since (*a*) there is increasing evidence that this intracellular component may be an important determinant of the efficacy of high-dose MTX regimens [this was the subject of a recent review (12)], and (*b*) earlier studies suggested on the basis of kinetic considerations that there is a limited capacity of cells to accumulate exchangeable drug (13, 16). Hence, the relationship between

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[4] The abbreviations used are: MTX, methotrexate (4-amino-N^{10}-methylpteroylglutamic acid); THF, tetrahydrofolic acid.

[5] Extracellular MTX concentration at which inflex is one-half of maximum.

the intracellular MTX level and the extracellular drug concentration follows an adsorption isotherm in which the concentration of exchangeable MTX approaches ~6.5 μM as the extracellular MTX level is increased to 12 μM in the L1210 leukemia cell (16). A similar phenomenon was observed qualitatively for the L-cell mouse fibroblast (11).

The following studies were undertaken to (*a*) quantitate exchangeable intracellular MTX levels at extracellular MTX concentrations comparable to those achieved in high-dose protocols and (*b*) determine whether there is a rationale for the combined use of MTX and vincristine in high-dose MTX regimens based upon the ability of vincristine to augment net MTX accumulation (3, 14, 30) and potentiate MTX inhibition of DNA synthesis (15) when extracellular MTX levels are low.

MATERIALS AND METHODS

Ehrlich ascites tumor cells grown i.p. in CF_1 mice were harvested and prepared for experimentation as previously described (14). Cells were suspended into a 135 mM NaCl-16 mM $NaHCO_3$-1 mM Na_2HPO_4-4 mM KCl-1.9 mM $CaCl_2$-1.0 mM $MgCl_2$ buffer. pH was maintained at 7.3 to 7.4 by passing warmed and humidified 95% O_2-5% CO_2 over the cell suspension. The final cytocrit was less than 4%. 3′,5′,9-[^{3}H]MTX was obtained from Amersham/Searle (Des Plaines, Ill.). Radiolabeled and nonlabeled MTX were purified by DEAE-cellulose column chromatography (16). Vincristine sulfate was generously supplied by Dr. Robert Hosley, Eli Lilly Research Laboratories, Indianapolis, Ind.

For determination of intracellular [^{3}H]MTX levels, cells were separated by centrifugation and washed twice in 0.85% NaCl solution at 0°; a cell pellet was then incubated until dry at 60°. The pellet was weighed on a Beckman LM800 automatic microbalance and digested in 1 N KOH, following which ^{3}H was determined on a liquid scintillation spectrometer. The exchangeable intracellular MTX level was determined from the difference between the total cell MTX level and the level of MTX that remains within the cell when cells loaded with MTX are resuspended into a large volume of MTX-free buffer. In addition, to ensure as complete as possible elimination of exchangeable intracellular drug, the buffer was changed several times during the MTX washout. Intracellular water content was determined from the difference between the wet and dry weights of a cell pellet less the [^{14}C]inulin space. The methodology has been described in detail in previous publications from this laboratory (11, 14, 16).

RESULTS

Chart 1 describes the time course of accumulation of exchangeable intracellular MTX at different extracellular drug levels in the presence or absence of 10 μM vincristine. It can be seen that net uptake of MTX rapidly falls approaching a steady state. Although the cells have not as yet reached a steady state at all 3 drug concentrations, it is clear that the level of drug accumulation within the cell is far below that of the extracellular compartment over this interval of observation. Vincristine augments the accumulation of exchangeable intracellular MTX. In the experiment of Chart 1 the percentage of increase in accumulation of exchangeable intracellular MTX by vincristine was 146, 85, and 47% at extracellular concentrations of 10, 100, and 250 μM, respectively. While the percentage of increase in exchangeable MTX induced by vincristine falls as the extracellular MTX level is increased, in 3 experiments similar to that of Chart 1, there continued to be a ~40% increase in the

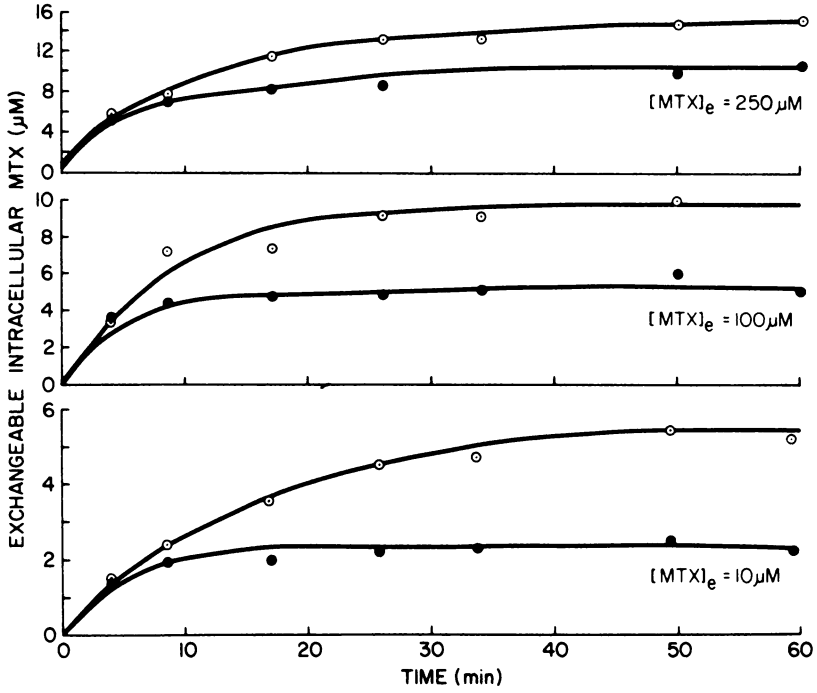

Chart 1. The time course of uptake of exchangeable intracellular MTX at 3 extracellular MTX concentrations: 10, 100, and 250 μM in the presence (○) or absence (●) of 10 μM vincristine sulfate. Exchangeable intracellular MTX levels were determined from the difference between total cell MTX and MTX that remains in the cells after resuspension of cells into MTX-free buffer as described in "Materials and Methods." $[MTX]_e$, extracellular MTX concentration.

exchangeable intracellular MTX level even when extracellular MTX was 250 μM. Chart 2 represents an experiment designed to assess the true steady-state intracellular MTX concentration. Since it is difficult to be certain that steady-state conditions are achieved in uptake experiments, an experimental technique was utilized in which the intracellular MTX level falls to the steady state. Cells were incubated with 175 μM MTX for 30 min, following which the cell suspension was diluted with fresh buffer to reduce the extracellular MTX concentration to 85 μM. Under these conditions, exchangeable intracellular MTX fell from an initial level of 7.5 μM to a steady-state concentration of 6.5 μM, indicating a distribution ratio (ratio of the concentration of exchangeable intracellular MTX to extracellular MTX) of 0.074.

DISCUSSION

Free intracellular MTX apparently plays an important role in the ability of this agent to achieve its metabolic effects in the L-cell mouse fibroblast, Ehrlich ascites tumor (11, 12, 15, 28, 29), L1210 leukemia cell (26), and human leukemia cells *in vitro* (2). Studies from this laboratory indicate that 50% inhibition of deoxyuridine or formate incorporation into DNA, or formate incorporation into RNA and protein, requires exchangeable intracellular MTX levels of ~0.3, ~1.0, and >3 μM, respectively (11, 29), a phenomenon apparently related to the requirement for free intracellular MTX to achieve cessation of THF synthesis. Hence, THF synthesis from dihydrofolic acid is completely inhibited only when exchangeable intracellular MTX levels of ~3.0 μM are achieved in the Ehrlich ascites tumor (12, 28). Other studies suggest that the level of free drug and its persistence within the cell following drug administration are important determinants of cytotoxicity *in vivo* (6, 27). This requirement for free intracellular MTX may be related (*a*) to the capacity of only a small fraction of a homogeneous high-affinity dihydrofolate reductase species to sustain normal rates of THF synthesis (12, 20) or (*b*) to the presence of an additional form of dihydrofolate reductase with a reduced affinity for MTX which continues to reduce dihydrofolic acid unless there are appreciable levels of free drug within the cell (12, 19). The apparent critical role for free intracellular MTX in the suppression of purine, pyrimidine, and amino acid synthesis suggested that the basis for the efficacy of high-dose MTX regimens that generate extremely high levels of extracellular drug may be related, in part, to their capacity to achieve the critical levels of free intracellular MTX necessary to abolish THF-dependent biosynthetic processes and to sustain this inhibition until the tumor cell is irreversibly damaged (12). We report now that the capacity of Ehrlich ascites tumor cells to accumulate exchangeable intracellular MTX is markedly limited. Indeed, exchangeable intracellular MTX levels (~10 μM) achieved over the interval of these experiments at MTX concentrations up to 250 μM were just in that critical range required to suppress THF-dependent processes in these cells (15, 28, 29). Studies from other laboratories suggest that accumulation of exchangeable MTX is also limited in human tumor cells and leukocytes at high and low levels of MTX (2, 3, 22). It is quite possible that some human tumors resistant to low-dose MTX regimens, but more sensitive to high-dose infusions, may be even more limited in their capacity to accumulate exchangeable MTX and/or have an increased rerequirement for this intracellular MTX component to abolish THF synthesis. Hence, the necessity for high extracellular MTX levels and the capacity of vincristine to augment exchangeable intracellular MTX may be critical determinants of the efficacy of some MTX regimens.

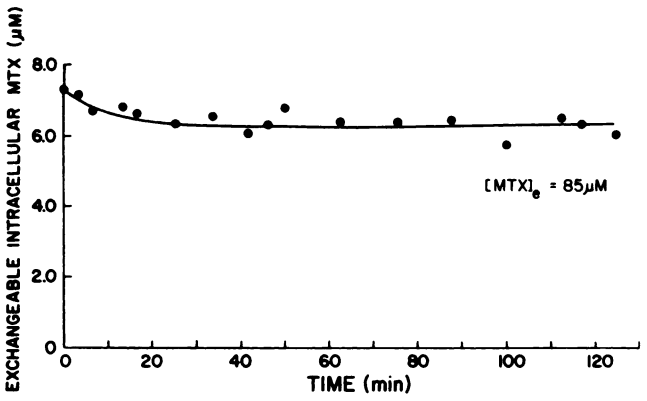

Chart 2. Analysis of exchangeable intracellular MTX under true steady-state conditions. Cells were incubated for 30 min with 175 μM MTX following which sufficient fresh buffer was added to the extracellular compartment at time zero to reduce the extracellular drug level to 85 μM so that intracellular MTX would fall to the steady state. Exchangeable MTX was measured as described in "Materials and Methods." $[MTX]_e$, extracellular MTX concentration.

While the capacity of vincristine to augment MTX accumulation with high-dose MTX therapy has been questioned (4), the data indicate that vincristine does markedly augment net MTX accumulation even at high extracellular MTX levels, an interaction that provides an important rationale for the combined use of these agents in high-dose protocols. Hence, to achieve in the absence of vincristine, an exchangeable intracellular MTX level comparable to the level achieved in the presence of vincristine would require a substantial increase in the extracellular MTX concentration. For instance, initial studies suggest that to produce a ~6 μM increase in exchangeable intracellular MTX when extracellular MTX is 100 μM would require an increase in the extracellular level to 200 μM; to again double the exchangeable intracellular level would necessitate doubling the extracellular level, requiring increases in the MTX dose of at least ~1.4 and 2.7 g, respectively.[6] On the other hand, vincristine augments the exchangeable intracellular MTX level without increasing the total MTX load. The capacity of vincristine to decrease the MTX dose may be critical to minimize toxicity to normal proliferating tissues [assuming some selectivity in the vincristine effect (14)] and, in addition, the reduced dose decreases the excretory burden on the kidneys which may be particularly vulnerable to the physical consequences of the high MTX dose due to deposition of this poorly soluble drug in the renal tubules and interstitium (24). While the level of vincristine required to augment net MTX accumulation in Ehrlich ascites tumor cells is high,

[6] Estimated on basis of an initial pulse of drug to achieve the indicated blood level in a 70-kg subject followed by a 6-hr infusion to sustain this blood level. A $t_{\frac{1}{2}}$ of 3 hr for plasma disappearance of MTX is assumed.

concentrations of vincristine 2 orders of magnitude lower (10^{-7} M) produce a 30% increase in net MTX accumulation in acute myelogenous leukemia cells (3), suggesting that vincristine levels achieved in clinical regimens can augment net MTX uptake into human tumor cells. However, further studies are required to assess (*a*) the capacity of normal and malignant human cells to accumulate exchangeable intracellular drug and (*b*) the effect of vincristine on this intracellular component.

Previous studies from this laboratory suggested that only low levels of MTX are likely to accumulate within the cell under steady-state conditions even at high drug concentrations (13, 16). This is now confirmed in the Ehrlich ascites tumor of drug levels 50 times greater than the influx K_t. While conforming to kinetic predictions, the thermodynamic implications of this observation are complex since, if the electrochemical potential for intracellular MTX is less than that of the extracellular drug under steady-state conditions, this implies uphill transport out of the cell, an energy-requiring condition. An exit pump for MTX has been proposed to account for the observation that inhibitors of energy metabolism augment the cellular accumulation of MTX (8, 9, 17). Indeed, it was the similarity between the effects of *Vinca* alkaloids and metabolic poisons on MTX uptake that led to the suggestion that vincristine acts in this content as an inhibitor of energy metabolism (14). However, further studies are required to establish that the low distribution ratios observed for MTX at high extracellular drug levels represent a nonequilibrium condition. Hence, to account for a steady-state distribution ratio for MTX (a bivalent anion) on the basis of passive forces in these studies would require a membrane potential of −33 mV (from Chart 2), a value higher than early measured membrane potentials in the Ehrlich ascites tumor (1) but too similar to recent, more accurate measurements (−24 ± 7 mV) to indicate with certainty uphill exit of MTX (23). This is currently under further study in this laboratory.

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