Augmentation of the Intracellular Levels of Polyglutamyl Derivatives of Methotrexate by Vincristine and Probenecid in Ehrlich Ascites Tumor Cells

David W. Fry, Jack C. Yalowich, and I. David Goldman

Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298

ABSTRACT

The intracellular accumulation of poly-γ-glutamyl derivatives of methotrexate was evaluated in the presence of vincristine or probenecid (agents which raise the intracellular level of free methotrexate) in Ehrlich ascites tumor cells. The results show that both intracellular methotrexate and its metabolites are increased by these agents and that, in the presence of L-glutamine, polyglutamate derivatives are increased by a higher percentage than is methotrexate. From 1 to 50 μM vincristine increased the levels of polyglutamate derivatives from 25 to over 300%, whereas methotrexate was raised from 25 to 80%. Similarly, 50 to 200 μM probenecid increased methotrexate polyglutamate derivatives from 31 to 88%, whereas methotrexate was raised from 0 to 30%. A determination of the bound fraction of drug indicated that the proportion of dihydrofolate reductase bound with methotrexate polyglutamates increased in the presence of these agents. Efflux studies showed that over 90% of the large pools of intracellular methotrexate polyglutamates produced by these agents was retained for at least 1 hr in the absence of extracellular methotrexate, whereas the majority of intracellular methotrexate exited the cell. These studies (a) indicate that vincristine and probenecid may be potentially useful for selectively increasing methotrexate polyglutamates in tumor cells and (b) introduce another basis for synergism observed between alkaloids and methotrexate.

INTRODUCTION

Previous studies from this and other laboratories have demonstrated rapid synthesis of MTX3 polyglutamate derivatives within mammalian cells (6, 9, 11, 16-18, 23, 24) and their rapid replacement of MTX bound to dihydrofolate reductase (6, 10, 18, 24). Further, these derivatives accumulate to levels far in excess of the dihydrofolate reductase binding capacity (6, 10, 18, 24). Since these derivatives of MTX are inhibitors of dihydrofolate reductase (14, 23), interest in the polyglutamate derivatives of MTX has been stimulated by potentially important pharmacological consequences of this biochemical transformation. Since intracellular drug in excess of the dihydrofolate reductase binding capacity is required to inhibit enzyme activity, the extent to which MTX polyglutamates are synthesized and retained within cells may determine the extent and duration of suppression of dihydrofolate reductase activity following the decline in the extracellular monoglutamate level. These factors may be critical elements in drug cytotoxicity and selectivity heretofore not appreciated. Likewise, network thermodynamic simulations (22) indicate that differences in the net rate of decline of MTX and MTX polyglutamate derivatives from mammalian cells cannot be accounted for on the basis of the cellular membrane transport properties for MTX alone. This raises the possibility that the net rate of decline of intracellular antifolate after cells are exposed to this agent in vivo is determined largely by the rate of net loss of MTX polyglutamates and/or the relative rates of their synthesis and hydrolysis within the intracellular compartment.

Some factors that influence the rate of formation of MTX polyglutamate derivatives have been described (6, 24). For instance, accumulation is enhanced by the addition of glutamine or glutamate to the medium and also by increasing the extracellular MTX level presumably based upon the concurrent increase in the free intracellular MTX concentration, the substrate for MTX polyglutamate synthetase. Hence, agents that increase the level of free intracellular MTX have the potential for enhancing synthesis of the polyglutamate derivatives. It has been known for some time that the Vinca alkaloids augment accumulation of free intracellular MTX in vitro (4, 7, 8, 25) and that the interaction between these drugs results in enhanced inhibition of thymidylate synthesis by MTX (12). More recently, probenecid was shown to enhance net accumulation of free intracellular MTX also apparently by blocking exit of the drug more effectively than drug entry into the cell (19). In this paper, studies are described which evaluate the extent to which the Vinca alkaloid or probenecid augmentation of net MTX transport is associated with enhanced accumulation of MTX polyglutamate derivatives.

MATERIALS AND METHODS

Materials. [3', 5'-3H]MTX was obtained from Moravek Biochemicals (City of Industry, Calif.) and purified by DEAE-cellulose chromatography (13). Probenecid was obtained from Sigma Chemical Co. (St. Louis, Mo.), and vincristine was from Flow Laboratories, Inc. (Rockville, Md.). All other chemicals used were reagent grade. Authentic standards of 4-NH2-10-CH3-PteGlu2 and Glu3 were kindly supplied by Dr. C. M. Baugh.

Cells, Media, and Incubation Techniques. Ehrlich ascites tumor cells were grown in male BALB/c x DBA/2 F1 mice (Sprague-Dawley, Madison, Wis.) and passed weekly by i.p. inoculation of 0.2 ml of undiluted ascitic fluid. Cells were harvested after 7 to 10 days and washed twice in 0.85% NaCl solution to remove erythrocytes.
were suspended in 136 mM NaCl-4.4 mM KCl-16 mM NaHCO3-1.1 mM KH2PO4-1 mM MgCl2-1.9 mM CaCl2 buffer. The pH was maintained at 7.4 by passing warm and humidified 95% O2-5% CO2 over the cell suspension. The suspension was stirred with a Teflon paddle in specially designed flasks inserted into a 37° water bath. Transport fluxes were stopped by injection of the cell suspension into 10 volumes of 0.85% NaCl solution at 0°. The cell fraction was immediately separated by centrifugation (500 X g for 2 min) and washed twice with 0.85% NaCl solution. One half of the washed pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare, and dried overnight at 70°. The second half of the pellet was extracted with 1 ml of 10% TCA and used for analysis of intracellular MTX and its polyglutamate derivatives (see below). The dry pellets were weighed on a Cahn Model 4700 electrobalance (Cahn Instruments, Paramount, Calif.), placed in scintillation vials, and incubated in 0.2 ml of 1 M KOH for 1 hr at 70°. The digest was then neutralized with 0.2 ml of 1 N HCl, and the solution was incorporated into 3 ml of Readi-Solv (Beckman Instruments, Inc., Irvine, Calif.). Radioactivity was determined in a Beckman LS-230 scintillation spectrometer, and counting efficiencies were established with [3H]- or [14C]toluene internal standards.

**Analysis of Intracellular MTX and Its Polyglutamate Derivatives.** TCA extracts (see above) were neutralized by adding 0.175 ml of 1 M KOH and 0.35 ml of 1 M K2HPO4 (pH 7.0) to 0.7 ml of the sample. Analyses were performed with an Altex model 332 gradient liquid chromatograph equipped with a Model 210 injector and a 5-µm particle detector. Intracellular polyglutamates were quantitated by evaluating the digest was then neutralized with 0.2 ml of 1 N HCl, and the solution was incorporated into 3 ml of Readi-Solv (Beckman Instruments, Inc., Irvine, Calif.). Radioactivity was determined in a Beckman LS-230 scintillation spectrometer, and counting efficiencies were established with [3H]- or [14C]toluene internal standards.

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**Results**

**Effect of Vincristine on the Intracellular Accumulation of MTX and MTX Polyglutamate Derivatives.** Chart 1 is a chromatogram of the TCA extract from control cells (a) and those exposed to 10 µM vincristine (b) in the presence of 5 µM [3H]MTX and 5 mM glutamine. Peaks 6, 5, and 4 coeluted with standards of MTX, 4-NH2-10-CH3-PteGlu2, and Glu3, respectively. Although standards of the higher polyglutamate derivatives were not available, the radioactivity in Peaks 1 through 5 was reduced by 95% and appeared quantitatively in Peak 6 after treatment with hog kidney conjugase (2). Based upon their elution pattern, these data suggest that Peaks 1 through 5 represent 4-NH2-10-CH3-PteGlu2 to Glu2, respectively. Chart 2 is a time course of the effect of vincristine on accumulation of MTX polyglutamate derivatives in the presence or absence of glutamine. In the absence of glutamine, only a low level of MTX polyglutamates accumulated which remained constant after 2 hr. The addition of 10 µM vincristine produced a 2-fold increase in MTX polyglutamate derivatives, which was, again, constant after 2 hr. Glutamine (5 mM) alone enhanced the rate of accumulation of MTX polyglutamates as reported previously (6), and net uptake continued over the interval of observation. In the presence of both glutamine and vincristine, however, there was a marked augmentation in the intracellular MTX polyglutamate level with rapid accumulation continuing over the 4-hr interval of observation.

**Effect of Vincristine Concentration on Intracellular MTX Polyglutamate Levels.** When the vincristine concentration was increased from 1 to 50 µM, there was an increase in both the intracellular MTX and MTX polyglutamate level (Table 1). At high concentrations of vincristine, the relative increase in intracellular MTX polyglutamate levels was much greater than that...
for the monoglutamate. The MTX polyglutamate content was increased from 25 to 300%, whereas MTX was increased by only 25 to 80% over a vincristine concentration range of 1 to 50 μM.

**Effects of Probenecid on MTX Transport and Accumulation of MTX Polyglutamate Derivatives.** As reported previously, probenecid produces a concentration-dependent inhibition of MTX influx and efflux (19). Since at lower concentrations of probenecid efflux is inhibited to a greater degree than influx, there is an increase in the exchangeable intracellular concentration of MTX. As illustrated in Table 2, when Ehrlich ascites tumor cells were exposed to probenecid, the increase in intracellular MTX was also associated with a marked augmentation of intracellular MTX polyglutamate derivatives.

**Intracellular Accumulation of Total and Bound MTX and MTX Polyglutamates in the Presence or Absence of Vincristine or Probenecid and the Retention of These Metabolites in Drug-free Media.** Since a major factor that determines the contribution of polyglutamates to the cytotoxicity of MTX is the extent to which these metabolites bind to dihydrofolate reductase, total amounts of intracellular and bound drug were determined in cells exposed to 5 μM [3H]MTX for 3 hr in the presence or absence of 10 μM vincristine or 200 μM probenecid and after a 1-hr incubation in drug-free media. The dihydrofolate reductase binding capacity for MTX in these cells was approximately 2.0 nmol/g, dry weight, of cells. Table 3 (top) shows that after the 3-hr incubation, the levels of both total intracellular MTX and its polyglutamates were increased by vincristine and probenecid as shown earlier. In the control, about 52% of the dihydrofolate reductase was bound with MTX and 48%, with polyglutamates (bottom). In cells exposed to vincristine and probenecid, the portion of enzyme bound with polyglutamates increased to 59 and 62%, respectively. After cells were exposed for 1 hr to drug-free media, 76% of the intracellular MTX exited the control cells, and 89 and 94% exited in the vincristine- and probenecid-treated cells, respectively. In contrast, MTX polyglutamates declined only 4 to 7% under all conditions, indicating they are retained to a much greater extent than is MTX. After the efflux period, a greater percentage of the enzyme was bound with polyglutamates. In the control, 77% of the bound drug was metabolites, and this increased to 89 and 92% in cells exposed to vincristine and probenecid, respectively, suggesting that as the free level of MTX declined, polyglutamates replaced MTX on the enzyme.

**DISCUSSION**

These studies demonstrate that vincristine and probenecid increase the intracellular accumulation of MTX polyglutamate derivatives. Several possibilities exist which may explain this phenomenon. The first and most plausible is that, since these agents increase the intracellular concentration of MTX, polyglutamate levels may be increased simply by mass action. Indeed, it has been demonstrated previously that an increase in the dose or extracellular concentration of MTX results in an increase in the intracellular accumulation of MTX polyglutamates (6, 23). Other explanations must not be disregarded, however, until additional data are obtained. Since the rate of intracellular accumulation of MTX polyglutamates would be the sum of synthesis, degradation, and efflux of these derivatives, an increase could result from a direct stimulation of the polyγ-glutamyl synthetase or an inhibition of the hydrolysis or efflux of these metabolites.

The enhanced accumulation of MTX polyglutamates by vincristine and probenecid results in a higher proportion of dihydrofolate reductase to which these metabolites are bound, indicating that the increased levels of these derivatives are
Augmentation of Methotrexate Polyglutamates by Vincristine and Probenecid

Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>Total intracellular drug (nmol/g, dry wt, of cells)</th>
<th>Drug bound to dihydrofolate reductase (nmol/g, dry wt, of cells)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vincristine</td>
</tr>
<tr>
<td>MTX (3 hr)</td>
<td>3.90 ± 0.54 (48.9)</td>
<td>5.16 ± 0.42 (40.3)</td>
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<tr>
<td>MTX (efflux)</td>
<td>0.92 ± 0.26 (19.6)</td>
<td>0.55 ± 0.05 (7.0)</td>
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<tr>
<td>PG (3 hr)</td>
<td>4.08 ± 0.23 (51.1)</td>
<td>7.63 ± 0.91 (59.7)</td>
</tr>
<tr>
<td>PG (efflux)</td>
<td>3.77 ± 0.32 (80.4)</td>
<td>7.30 ± 0.71 (93.0)</td>
</tr>
</tbody>
</table>

REFERENCES


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2535

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