Elevation of Thymidylate Synthetase Activity in CCRF-CEM Cells

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SUMMARY

The basis for an elevation of thymidylate synthetase activity by methotrexate has been studied with CCRF-CEM cell cultures. In a comparison of oncolytic drug effects on three enzymes from these cells, methotrexate elevated both thymidylate synthetase and thymidine kinase activity but not alanine transaminase activity. The pattern of response by these three enzymes to cytosine arabinoside and hydroxyurea varied from the observations with methotrexate. Cytosine arabinoside and actinomycin D depressed thymidylate synthetase activity. A small but variable response to hydroxyurea was observed. The combination of actinomycin D with methotrexate resulted in a level of thymidylate synthetase activity intermediate between those observed with the individual drugs.

An elevation of thymidylate synthetase activity also occurred in cells from cultures to which substrate or product precursors of the thymidylate synthetase reaction were added. The combination of thymidine or deoxyuridine with methotrexate elevated thymidylate synthetase activity above the level observed with the individual compounds. An in vitro binding of methotrexate to thymidylate synthetase was expressed by an inhibition of enzyme activity.

Folinic acid elevated thymidylate synthetase activity and reversed methotrexate inhibition of growth and of deoxyuridine incorporation into DNA. The methotrexate-induced elevation of thymidylate synthetase activity could be modulated by high concentrations of folinic acid. The elevation of thymidylate synthetase activity by methotrexate, deoxyuridine, thymidine, and folinic acid was attributed to a stabilization of thymidylate synthetase by the formation of enzyme complexes. The predominant phase of the growth cycle of the culture presumably controls the potential for enzyme synthesis, and the action of other drugs on protein synthesis modifies thymidylate synthetase response to methotrexate.

INTRODUCTION

Thymidylate synthetase activity was elevated in leukemic leukocytes following MTX administration to patients with acute granulocytic leukemia (17). This MTX-induced elevation of thymidylate synthetase activity is of chemotherapeutic interest because of an apparent relationship between the elevation of activity and a greater recovery of the capacity to incorporate deoxyuridin into DNA by drug-intoxicated leukemic leukocytes.

The importance of thymidylate synthetase activity to DNA synthesis arises from its function in de novo synthesis of TMP. This enzyme catalyzes a reaction between DUMP and M-THFA in which TMP and dihydrofolate are the products (6).

A method for blocking the MTX-induced elevation of enzyme activity has been sought. Emphasis was placed on the use of oncolytic drugs in this effort to take advantage of any selective action of these agents on leukemic cell populations (19). The administration of cytosine arabinoside to patients with acute granulocytic leukemia depresses thymidylate synthetase activity in the leukemic leukocytes (17) and is effective in the treatment of leukemia (4).

The opposing effects of MTX and cytosine arabinoside on thymidylate synthetase activity have been investigated with CCRF-CEM cultures (19). A model system was desired that would permit more extensive investigation of the effects of these 2 drugs on thymidylate synthetase activity. The addition of MTX or cytosine arabinoside to CCRF-CEM cultures changed the level of thymidylate synthetase activity. The MTX-induced elevation of enzyme activity was blocked by cytosine arabinoside. Variation of the concentration ratios of the 2 drugs permitted the level of enzyme activity to be varied between the levels observed for the individual drugs (19).

The depression of thymidylate synthetase activity was also observed with other oncolytic drugs, but the elevation of enzyme activity was associated only with MTX (19). Thymidylate synthetase activity is also elevated in actively dividing cell populations, but under the conditions used with MTX other oncolytic agents, which also inhibited cells in the S phase, did not result in an elevation of enzyme activity.

This study presents further observations on the elevation of thymidylate synthetase activity in CCRF-CEM cultures by MTX and reports that the addition of deoxyuridine, thymidine, or F-THFA to cultures elevates thymidylate synthetase activity.

1 Supported by Research Grants CA-11148 and CA-08480 from the National Cancer Institute, NIH, and by American Lebanese Syrian Associated Charities.
2 The abbreviations used are: MTX, methotrexate; M-THFA, N5,N5'-methylenetetrahydrofolate; F-THFA, folinic acid.
3 Received February 3, 1971; accepted April 20, 1971.
MATERIALS AND METHODS

The CCRF-CEM cell line, which was used in this work, originated from the buffy coat of a blood specimen from a child with acute lymphocytic leukemia following the diagnosis of lymphosarcoma (5). The cells retain the morphology of lymphoblasts.

Eagle's minimum essential medium for suspension cultures was supplemented with L-glutamine and 10% fetal calf serum for growth of the CCRF-CEM cell line. Cultures were initiated with 40 ml of cell suspension containing approximately 5 X 10^6 cells/ml and were grown in capped plastic flasks for 20 hr at 37°C.

Deoxyuridine incorporation into DNA was assayed 20 hr after inoculation of the cultures. Two-ml aliquots of the cell suspensions were incubated for 30 min with 1 μM deoxyuridine-6-3H and 1.15 million cpm. Deoxyuridine incorporation into DNA was measured by Millipore filter techniques as described previously for thymidine (19).

Cells were collected by centrifugation and washed with Eagle's balanced salt solution. Homogenates were prepared by diluting the cell pellet in an equivalent volume of 0.01 M Tris-HCl, pH 7.2, before disruption by sonic oscillation (19). The supernatant fluid remaining after centrifugation at 30,000 X g for 25 min was stored at -20° until assayed for thymidine kinase as described earlier (18). This extract was also assayed for alanine transaminase by the conversion of uniformly labeled alanine-14C to pyruvate, which was extracted into toluene, as a dinitrophenylhydrazone, for liquid scintillation counting (13). Thymidylate synthetase was assayed by monitoring the release of tritium from dUMP-5-3H in the methylation reaction (14, 19). Protein was measured by the method of Lowry et al. (12) with Prosol standard protein solution (Standard Scientific, Carlestone, N.J.).

Eagle's minimum essential medium for spin cultures and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, N. Y. We are indebted to the American Cyanamid Company, Lederle Division, Wayne, N. J., for their generous gift of F-THFA, N7-formyltetrahydrofolate. No correction has been made in the data for the presence of the inactive optical isomer in this preparation.

RESULTS

Drug-induced Changes in Enzyme Pattern. The specificity of thymidylate synthetase response to MTX, cytosine arabinoside, and hydroxyurea was compared with the response of thymidine kinase and alanine transaminase to these drugs (Table 1). At the time of inoculation with CCRF-CEM cells, 10 μM MTX, 10 μM cytosine arabinoside, 1 mM hydroxyurea, or water was added to the cultures. After the cultures were incubated at 37°C for 20 hr, homogenates of these cells were prepared and assayed for the 3 enzymes. Thymidylate synthetase activity per g of protein was elevated by MTX, 155%; slightly lowered by hydroxyurea, 80%; and reduced by cytosine arabinoside to 42% of the control level, 100%. Thymidine kinase activity was elevated by MTX, 139%, and by hydroxyurea, 147%, and lowered by cytosine arabinoside, 45%. Alanine transaminase activity was lowered slightly by all 3 drugs. Each of these drugs has been reported to be effective as an inhibitor of cells in the S phase of the cell cycle (3, 21, 24). The variation in activity of these enzymes indicates specificity in response to the drugs.

Similar responses by thymidine kinase and thymidylate synthetase to cytosine arabinoside may result from an inhibition of RNA synthesis. A slower turnover of alanine transaminase would explain the greater retention of this enzyme activity (20, 23). MTX elevation of thymidylate synthetase and thymidine kinase could result from a derepression of their synthesis or a slowing of enzyme degradation. Enzyme stabilization by metabolites or by MTX would cause the increase in activity of thymidine kinase and thymidylate synthetase. This possibility was examined in greater detail with thymidylate synthetase by studying the response of cells incubated with substrate and product precursors of the enzyme reaction and the in vitro effect of MTX on the enzyme.

MTX Binding to Thymidylate Synthetase. Wahba and Friedkin (25) observed that MTX slightly inhibited thymidylate synthetase purified from *Escherichia coli*, and Borsa and Whitmore (1, 2) confirmed this observation with a more detailed examination of this inhibition with homogenates of L-cells. When 17 or 87 μM MTX was added directly to the assay with homogenate from CCRF-CEM cells, thymidylate synthetase activity was inhibited 35 and 75% (Charts 1 and 2). The inhibition was noncompetitive with dUMP up to 0.65 mM (Chart 1B). The MTX inhibition of thymidylate synthetase was of a mixed competitive type with M-THFA concentration up to 1.1 mM (Chart 2, A and B). At this high substrate concentration, a 33% inhibition of thymidylate synthetase activity in the control was observed relative to the optimal concentration of approximately 400

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thymidylate synthetase</th>
<th>Thymidine kinase</th>
<th>Alanine transaminase</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX (10 μM)</td>
<td>13.8, 10.8 (155)*</td>
<td>18.8, 20.8 (139)</td>
<td>50.3, 42.5 (90)</td>
</tr>
<tr>
<td>Cytosine arabinoside (10 μM)</td>
<td>1.72, 4.5 (42)</td>
<td>9.52, 5.04 (45)</td>
<td>48.7, 37.7 (83)</td>
</tr>
<tr>
<td>Hydroxyurea (1 mM)</td>
<td>6.31, 6.61 (80)</td>
<td>18.8, 23.2 (147)</td>
<td>55.0, 39.2 (90)</td>
</tr>
<tr>
<td>Control</td>
<td>8.31, 7.50 (100)</td>
<td>13.6, 15.0 (100)</td>
<td>60.6, 44.0 (100)</td>
</tr>
</tbody>
</table>

*Nos. in parentheses, mean percentage of change in enzyme activity relative to the level observed in the controls.
Elevation of Thymidylate Synthetase Activity

Chart 1. A, substrate concentration curve with dUMP for thymidylate synthetase from CCRF-CEM cells in the presence of MTX. B, reciprocal plot, 1/v versus 1/s, for Chart 1A.

Chart 2. A, substrate concentration curve with M-THFA for thymidylate synthetase from CCRF-CEM cells in the presence of MTX. B, reciprocal plot, 1/v and 1/s, for Chart 2A.

μM M-THFA. This inhibition by high concentrations of M-THFA was much less pronounced in the presence of 17 μM MTX and was absent with 87 μM inhibitor. The concentration of MTX remaining in homogenates of cells grown in the presence of 10 μM drug does not interfere with the assay of thymidylate synthetase (19).

Substrate Elevation of Thymidylate Synthetase. Deoxuridine was added to cultures at the time of inoculation, and 20 hr later the cells were collected for assay of thymidylate synthetase activity (Chart 3). An elevation of thymidylate synthetase activity that was proportional to deoxuridine concentration was observed. With 100 μM deoxuridine, a 58% increase in enzyme activity was observed.

Deoxynucleoside Substrate and Product Precursors. Cells from cultures containing 100 μM thymidine had higher levels of thymidylate synthetase activity than cells from control cultures (Table 2). Addition of thymidine plus deoxuridine resulted in further increases in thymidylate synthetase activity. MTX was more effective than thymidine, deoxuridine, or the combination, and the addition of MTX with thymidine or deoxuridine resulted in higher levels of enzyme activity than were observed for the drug alone.

Hydroxyurea blocks ribonucleotide reductase and should lead to a depletion of the deoxynucleotide pools in treated cells (11). Hydroxyurea has also been used to synchronize cell addition.
cultures by blocking their entry into the S phase of cell division (24). The experiment, which is presented in Table 3, was designed to study the effect of hydroxyurea on the elevation of thymidylate synthetase activity by MTX, deoxyuridine, and thymidine. Hydroxyurea caused an elevation of enzyme activity in these cultures where a slight decrease had been observed earlier (Table 1). Combination of hydroxyurea with MTX resulted in an additive increase in thymidylate synthetase activity. No further increase in enzyme activity occurred when deoxyuridine or thymidine was added with hydroxyurea. The combination of hydroxyurea, MTX, and deoxyuridine resulted in further elevation of enzyme activity, which was not observed when thymidine was substituted for deoxyuridine. These results are compatible with 2 types of effects associated with these compounds. Thymidine, hydroxyurea, and MTX have been used to obtain a partial synchronization of cultures by blocking progression through the S phase (6, 21, 24). A block of thymidylate synthetase degradation by MTX, deoxyuridine, and thymidine with or without reinforcement by synchronization of the cultures may explain the additive effects that were observed.

Response to F-THFA. M-THFA is required in the thymidylate synthetase-catalyzed synthesis of TMP and can be synthesized in vivo from F-THFA. F-THFA will also reverse the growth inhibitory effect of MTX. Folate is present in the cell culture media but reverses the effect of MTX much more effectively than F-THFA (7, 8). F-THFA is one of the more stable reduced forms of folate and has frequently been substituted for folate in culture media when a reduced form of the vitamin was required. The addition of F-THFA to cultures of CEM cells elevated thymidylate synthetase activity (Chart 4A). The concentration of F-THFA greatly exceeds the folate requirement for growth.

The addition of F-THFA to control cultures reduced the incorporation of labeled deoxyuridine into DNA (Chart 4A). These cultures were grown for 20 hr in the presence of F-THFA, and at the end of this period aliquots of the cultures were incubated for 30 min with deoxyuridine-6-3H. The decrease in exogenous deoxyuridine incorporation into DNA by the addition of F-THFA to control cultures presumably resulted from an increased dilution of the labeled precursor by endogenous TTP or its precursors. MTX inhibition of deoxyuridine incorporation into DNA was reversed more effectively by 100 μM F-THFA than by 1 μM, and the inhibition with 10 μM MTX plus 1 μM F-THFA was nearly as complete as with MTX alone.

The addition of 100 μM F-THFA to cultures also containing MTX, 0.1 μM to 10 μM, resulted in less stimulation of thymidylate synthetase than was observed with drug alone (Chart 4B). With 1 μM F-THFA, MTX was as effective as when added alone in elevating thymidylate synthetase activity. The effectiveness of F-THFA in blocking MIX stimulation of enzyme activity was related to the ratio of drug and vitamin concentration.

The addition of 1 and 100 μM F-THFA to control cultures slightly increased the growth rate of these cultures (Chart 4C). MIX, 0.1 to 10 μM, uniformly inhibited cell division with approximately 10% lysis of the inoculum. F-THFA failed to restore completely the inhibition of cell division observed with 10 μM MTX, although at lower drug concentrations growth of the cultures in the presence of F-THFA was equivalent to that of the unsupplemented cultures.

Response to Actinomycin D. If MTX is acting by stabilization of thymidylate synthetase, then the drug should retard the loss of enzyme activity in the presence of a blockade of enzyme synthesis. Actinomycin D, blockade of

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (μmoles TMP/hr/g protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.25</td>
<td>100</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>11.0</td>
<td>176</td>
</tr>
<tr>
<td>Thymidine</td>
<td>11.6</td>
<td>185</td>
</tr>
<tr>
<td>Deoxyuridine + thymidine</td>
<td>12.6</td>
<td>202</td>
</tr>
<tr>
<td>MTX</td>
<td>17.9</td>
<td>286</td>
</tr>
<tr>
<td>MTX + deoxyuridine</td>
<td>19.7</td>
<td>315</td>
</tr>
<tr>
<td>MTX + thymidine</td>
<td>19.6</td>
<td>314</td>
</tr>
</tbody>
</table>

Table 2
Elevation of thymidylate synthetase activity by
deoxyuridine, thymidine, and MTX

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (μmoles TMP/hr/g protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.7</td>
<td>100</td>
</tr>
<tr>
<td>MTX</td>
<td>15.3</td>
<td>143</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>13.4</td>
<td>125</td>
</tr>
<tr>
<td>Thymidine</td>
<td>12.9</td>
<td>120</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>15.6</td>
<td>146</td>
</tr>
<tr>
<td>MTX + hydroxyurea</td>
<td>17.5</td>
<td>163</td>
</tr>
<tr>
<td>Thymidine + hydroxyurea</td>
<td>12.4</td>
<td>116</td>
</tr>
<tr>
<td>Deoxyuridine + hydroxyurea</td>
<td>12.3</td>
<td>115</td>
</tr>
<tr>
<td>MTX + hydroxyurea + deoxyuridine</td>
<td>20.9</td>
<td>196</td>
</tr>
<tr>
<td>MTX + hydroxyurea + thymidine</td>
<td>17.8</td>
<td>167</td>
</tr>
</tbody>
</table>
cell cultures. The original inoculum provided 0.69 million cells/ml of medium.

The hypothesis with which these studies were initiated assumed that elevation of thymidylate synthetase activity by MTX resulted from enzyme stabilization, which retarded enzyme degradation. This hypothesis was adopted as a result of the variation in thymidylate synthetase response to inhibitors of DNA synthesis. The postulated enzyme complexes and enzyme-substrate complexes may vary in their effects on the rate of enzyme turnover. This hypothetical variation in the degradation rate of enzyme complexes could contribute to the variation in response that was observed for MTX, deoxyuridine, thymidine, and F-THFA.

The observation that MTX inhibited thymidylate synthetase indicates that the drug does form an enzyme complex. The formation of this drug-enzyme complex would be compatible with the working hypothesis. The observation that substrate precursors, deoxyuridine and F-THFA, and a product precursor, thymidine, elevate enzyme activity is also compatible.

The highest concentration of deoxynucleosides and of MTX in these studies was not a saturation level for elevation of enzyme activity. The additional increase in thymidylate synthetase activity observed with MTX in combination with deoxyuridine, thymidine, hydroxyurea and deoxyuridine, or F-THFA could result from stabilization of more enzyme by the increase in enzyme-binding substances. Thymidine did not block the effect of MTX; therefore, derepression of enzyme synthesis by the drug inhibition of the thymidine nucleotide formation appears unlikely.

The observation that MTX modulated the lowering of enzyme activity by cytosine arabinoside (19) and actinomycin D is compatible with a stabilization of thymidylate synthetase activity by MTX. Thymidylate synthetase activity fell less rapidly in cultures exposed to actinomycin D plus MTX than in the cultures to which actinomycin D alone was added.

F-THFA elevated thymidylate synthetase activity when added to the media, but at 100 μM concentration in combination with 10 μM MTX it partially blocked the elevation observed with the drug alone. A slight increase in cell number was observed under these conditions. The level of enzyme activity with 1 μM F-THFA and 10 μM MTX in combination was equivalent to the stimulation observed with MTX. With lower concentrations of MTX, when 1 μM F-THFA was reversing drug inhibition of cell division, the enzyme activity was below the level observed for MTX. F-THFA does compete with MTX uptake by some cells and could act by partially blocking drug uptake (9). MTX inhibition of deoxyuridine incorporation into DNA was reversed by 100 μM...
The failure to observe similar elevation with the tested S-phase activity results from a partial synchronization in the cell cycle F-IHFA, as would be expected from the reversal of phase could be resurrected if certain assumptions are made. The variation between the level of drug-inhibited cell division.

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active drugs may have resulted from other drug effects that alternate hypothesis. Thymidine blocks progression through enzyme stabilization. The variation between the level of observations with thymidine are also compatible with this alternate hypothesis. Thymidine blocks progression through the S phase and results in synchronization of cultures (26). The growth of CCRF-CEM cultures is inhibited by thymidine (22).

The inability to isolate I hypothesis that will satisfy all the observations suggests that the present results represent a summation of effects on enzyme synthesis and degradation and also reflect the metabolic activity of the cells in the culture. Thymidylate synthetase activity varies with the “age” or population density of the CCRF-CEM culture. When the culture is actively dividing, higher levels of thymidylate synthetase activity are observed (unpublished observation). Presumably, this reflects the increased number of cells actively synthesizing DNA. A block in enzyme degradation would result in an accumulation of enzyme activity. Drugs that block cells in the S phase would similarly shift the cell population into a state where more enzyme activity would be observed. If this S-phase population were the cells lysed by actinomycin D and cytosine arabinoside, a decrease in activity would occur with its elimination. The modulation observed with MTX in drug combinations under these conditions would result from enzyme stabilization. The variation between the level of enzyme activity observed with thymidine and MTX would then reflect the stabilization of enzyme activity and block of cell division. The varied response to F-THFA and MTX would result with an escape of some cells from MTX inhibition or decreased rate of enzyme synthesis as the cells moved out of the S phase and reduced the rate of enzyme synthesis and availability of enzyme for drug stabilization.

ADDENDUM

Reference was omitted to studies by R. Labow, G. F. Maley, and F. Maley entitled, “The Effect of Methotrexate on Enzymes Induced following Partial Hepatectomy” (Cancer Res., 29: 366—372, 1969), which reported an MTX-induced elevation of thymidylate synthetase activity in regenerating rat liver following partial hepatectomy.

REFERENCES

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