Methotrexate and Cytosine Arabinoside Modulation of Thymidylate Synthetase Activity in CCRF-CEM Cells

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SUMMARY

Thymidylate synthetase activity of a human lymphoblastic cell line, CCRF-CEM, was elevated by the addition of methotrexate to the culture medium and was lowered when cytosine arabinoside was added. The simultaneous addition of both drugs resulted in levels of enzyme activity intermediate between those observed for each drug alone. The level of enzyme activity could be modulated by varying the ratio of the two drugs. The ratio of enzyme activity in homogenates of cells from control or drug-treated cultures remained constant with dialysis. Changes in the rate of thymidylate synthetase turnover are suggested as the basis for the variation of enzyme activity in the drug-treated cultures.

INTRODUCTION

Combination drug therapy has as its objective the rational use of 2 or more drugs for reenforcement of the therapeutic efficacy of 1 or more of the agents. Multiple drug treatment of malignant diseases has resulted in a greater frequency of response to chemotherapy and prolongation of disease-free survival (9, 10, 14).

The present studies stemmed from the observation of a methotrexate-induced elevation of thymidylate synthetase activity in leukocytes from patients with acute granulocytic leukemia (23). The elevation of enzyme activity in leukocytes was correlated with greater in vitro incorporation of deoxyuridine into DNA. In similar studies, administration of cytosine arabinoside decreased thymidylate synthetase activity (23). These observations suggested that the methotrexate-induced elevation of thymidylate synthetase activity could be contributing to the recovery from drug intoxication by the leukemic cell and that cytosine arabinoside, by lowering thymidylate synthetase activity, might delay recovery from methotrexate intoxication. When cytosine arabinoside and methotrexate were combined in a daily schedule for the treatment of mice bearing L1210 ascites tumors, the mice survived slightly longer, as would be expected for an additive effect by the drug combination (4).

A human lymphoblastic cell line, CCRF-CEM, was selected as a model for further investigation of methotrexate and cytosine arabinoside effects on thymidylate synthetase activity. These studies show that (a) the reported effect of methotrexate and of cytosine arabinoside on thymidylate synthetase activity can be duplicated by a cell culture and (b) the level of thymidylate synthetase activity can be modulated by varying the relative concentrations of these drugs.

MATERIALS AND METHODS

The human lymphoblastic cell line, CCRF-CEM, was obtained from Dr. George Foley (8) and was grown as suspension or spinner cultures on Joklik's modification of Eagle's minimum essential medium with Earle's salts plus L-glutamine for spinner cultures supplemented with 10% fetal calf serum from Grand Island Biological Company, Grand Island, N. Y. Cultures for these studies were prepared by diluting older cultures with fresh medium to give the desired initial cell count, approximately 5 X 10^5 cells/ml. These cells were then dispersed in 40-ml aliquots and incubated at 37° for 20 hr unless otherwise indicated. This technique for preparing cultures minimized the number of steps and, therefore, the possibility of microbial contamination, but variable growth occurred from experiment to experiment as a result of different dilutions of the cultures. Each value reported, unless otherwise indicated, is the average of 3 or 4 cultures that were assayed in triplicate for thymidylate synthetase activity.

Two-ml aliquots of the cultures were added to open test tubes and incubated for 30 min at 37° with 105 μl of 4.1 X 10^-5 M 5-methylthymidine-3H containing 0.93 μCi. This concentration of thymidine saturated the rate-limiting step for its incorporation into DNA and permitted a linear rate of incorporation for more than 30 min. The incubation was terminated by adding 5 ml of ice-cold 0.9% NaCl solution and placing the tube in an ice bath. The cell suspension was washed from the tube with 0.9% NaCl solution onto a 0.45 μ MF Millipore filter disc where the cells were further washed with 5% trichloracetic acid. The filter was then dissolved in 10 ml of liquid scintillation fluid for isotopic assay. For the measurement of deoxyuridine incorporation into DNA, 4.1 X 10^-5 M deoxyuridine was substituted for thymidine in this assay. With each deoxynucleoside, the rate-limiting step was saturated, and incorporation was proportional with time for more than 30 min.

Thymidylate synthetase was assayed by measuring the release of tritium from dUMP-5^3H (21). Cells were collected by centrifugation for 5 min at 200 X g at 4°. The cell pellet
was resuspended in 2 ml of Earle's balanced salt solution and collected by centrifugation as before. The cell pellet of approximately 50 mg, wet weight, was diluted with 0.5 ml of 0.01 M Tris-HCl buffer, pH 7.2, and an homogenate, containing approximately 2 mg of protein/ml, was prepared by sonic oscillation. After the homogenate was centrifuged for 25 min at 30,000 X g, the supernatant fluid was decanted and frozen at -20° until assayed. The thawed extracts were generally assayed on the following day. The extracts were diluted with 2 volumes of 0.01 M Tris-HCl, pH 7.2, containing 1.25% bovine plasma albumin and 0.22 M sucrose, and 21.1 µl of diluted extract were incubated for 1 hr at 37° with 17.5 µl of reagent containing 0.13 mM dUMP-5-3H (70,000 cpmp), 0.001% formaldehyde, 0.17 mM dl-tetrahydrofolate, 2 mM mercaptoethanol, 0.13 M sucrose, and 0.3 M Tris-HCl, pH 7.2. At the termination of the enzyme reaction, 20 µg of unlabeled dUMP, 0.4 mg of trichloracetic acid, and 20 mg of charcoal were added for a final volume of 250 µl. After centrifugation for 10 min at 500 X g, a 100-µl aliquot of the supernatant fluid was removed for liquid scintillation counting. Protein concentration of the homogenate was measured by the method of Lowry et al. (18) against a standard curve with Pro-Sol protein solution from Standard Scientific, Carlstadt, N.J., and data are expressed in terms of milligrams of protein in the original homogenate.

RESULTS

Response of Thymidylate Synthetase to Methotrexate and Cytosine Arabinoside. A 2-fold elevation of the thymidylate synthetase activity in CCRF-CEM cells was observed 20 hr after the addition of 10 µM methotrexate to the cultures (Table 1). Cytosine arabinoside, 10 µM, reduced the enzyme activity to one-fifth of the control level. Homogenates of cells from cultures that were incubated with both drugs at 10 µM concentration contained two-thirds of the control enzyme activity. A 10-fold range in thymidylate synthetase activity was observed in this study.

Response of Thymidylate Synthetase to Various Compounds. Eleven additional agents, oncolytic drugs or compounds interfering with macromolecular synthesis, were studied for their effect on thymidylate synthetase activity (Table 2). Initially, an attempt was made to select drug concentrations that gave 95% inhibition of thymidine or deoxyuridine incorporation into DNA after a 4-hr exposure of the culture to the drug. This criterion was too severe for 5-fluorouracil (85% inhibition in 4 hr, 90 to 95% inhibition in 20 hr). Vincristine and colchicine required a longer period of time, but by 20 hr they inhibited 95% of the thymidine incorporation. With 6-mercaptopurine, a 65% inhibition was observed at 20 hr. Dexamethasone, prednisolone, and prednisone were only slightly inhibitory. Substrate concentrations of deoxynucleosides were used for the incorporation studies, and some reversal of drug action may have occurred during the 30-min incubation with substrate.

Cycloheximide and actinomycin D were extremely effective in lowering thymidylate synthetase activity. Four % of the activity that was observed in the controls remained in homogenates from these drug-treated cultures. Hydroxyurea, 6-mercaptopurine, and 5-bromodeoxyuridine had very little effect on thymidylate synthetase activity. After conversion to 5-fluoro-dUMP, 5-fluorouracil becomes a very effective inhibitor of thymidylate synthetase (6, 13, 20). Although the intracellular concentration of drug was diluted approximately 50-fold for assay, inhibition of enzyme activity probably continued.

Of the 2 mitotic inhibitors, vincristine was more effective than colchicine in lowering enzyme activity. Prednisolone was as effective as vincristine in lowering enzyme activity, while prednisone and dexamethasone were ineffective. Cytosine arabinoside less effectively lowered thymidylate synthetase activity than is shown in Chart 1. Methotrexate was the only agent that elevated thymidylate synthetase activity.

Thymidylate synthetase activity varied with the age of the culture. The inocula used for these studies were in the postlogarithmic growth phase and had low enzyme activity. Therefore, the possibility existed that the changes observed in response to methotrexate and cytosine arabinoside could be related to the "physiological" age of the cultures. If this were the case, methotrexate could fix the culture in the enzyme pattern of logarithmic growth, and cytosine arabinoside could hold the inoculum with enzyme activity of the postlogarithmic growth phase. The drug-induced variation would then be the

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thymidylate synthetase activity (µmoles TMP/hr/g protein)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.2</td>
<td>100</td>
</tr>
<tr>
<td>10 µM methotrexate</td>
<td>26.1</td>
<td>198</td>
</tr>
<tr>
<td>10 µM cytosine arabinoside</td>
<td>2.74</td>
<td>21</td>
</tr>
<tr>
<td>10 µM methotrexite + 10 µM cytosine arabinoside</td>
<td>8.40</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 1  
Effect of methotrexate and cytosine arabinoside on thymidylate synthetase activity of CCRF-CEM cells

CCRF-CEM cells were transferred to fresh medium containing 10 µM methotrexate, cytosine arabinoside, or both drugs and were incubated for 20 hr at 37°. The cells were isolated, and thymidylate synthetase activity was assayed as described in "Materials and Methods."
Modulation of Thymidylate Synthetase Activity

Table 2
Thymidylate synthetase activity of CCRF-CEM cells 20 hr after drug administration

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (µM)</th>
<th>Thymidylate synthetase (µmoles TMP/hr/g protein)</th>
<th>Drug:control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Control with ethanol</td>
<td></td>
<td>8.28</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>36</td>
<td>0.43</td>
<td>4</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>4</td>
<td>0.40</td>
<td>4</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>1000</td>
<td>8.75</td>
<td>76</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>100</td>
<td>10.21</td>
<td>88</td>
</tr>
<tr>
<td>5-Bromodeoxyuridine</td>
<td>100</td>
<td>12.31</td>
<td>107</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>100</td>
<td>2.02</td>
<td>18</td>
</tr>
<tr>
<td>Vincristine</td>
<td>1.1</td>
<td>7.48</td>
<td>65</td>
</tr>
<tr>
<td>Colchicine</td>
<td>13</td>
<td>9.26</td>
<td>80</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10</td>
<td>6.46</td>
<td>78</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10</td>
<td>5.26</td>
<td>64</td>
</tr>
<tr>
<td>Prednisone</td>
<td>10</td>
<td>8.60</td>
<td>104</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>10</td>
<td>5.61</td>
<td>49</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>10</td>
<td>19.40</td>
<td>167</td>
</tr>
</tbody>
</table>

*1% ethanol.

result of the “growth” phase of the culture and not the result of a drug effect on enzyme activity.

Chronology of Changes in Thymidylate Synthetase Activity. The chronological changes in thymidylate synthetase activity in response to methotrexate, cytosine arabinoside, and both drugs in combination are depicted in Charts 1 to 3. Suspension cultures of CCRF-CEM cells were started at zero time with cells from a spinner culture. At 4, 8, and 12 hr after initiation of the cultures, 10 µM methotrexate was added to 1 series of flasks. The cultures were harvested, thereafter, at 4-hr intervals. In the control cultures lacking methotrexate (Chart 1), the increase in thymidylate synthetase activity, which was observed following transfer of cells to fresh media, plateaued with 12.5 µmoles of dUMP converted to TMP per hr per g of protein. In this experiment, the addition of methotrexate to 1 set of controls immediately prior to harvesting the culture apparently stabilized the enzyme activity. An increase in thymidylate synthetase activity was evident within 4 hr after methotrexate addition. Enzyme activity increased to about the same level, 23 µmoles of TMP/hr/g of protein, with drug added at 4, 8, or 12 hr, and plateaued at about the same time as the control cultures to which no drug was added. Therefore, the methotrexate elevation of thymidylate synthetase activity was not the result of a decrease in the enzyme activity of control cultures.

In a similar study, 10 µM cytosine arabinoside lowered thymidylate synthetase activity (Chart 2). The control cultures, with a lower inoculum than that used in Chart 1, continued to increase in thymidylate synthetase activity throughout the course of study. The addition of cytosine arabinoside 4 hr after inoculation of the cultures was followed in 4 hr by a reduction in the rate of increase of thymidylate synthetase activity and then subsequently by a decrease in enzyme activity during the next 12 hr. A similar pattern was observed if 8 or 12 hr elapsed before the addition of cytosine arabinoside. Although the initial level of thymidylate synthetase activity was higher in the 8- and 12-hr cultures than in the 4-hr series at the time of cytosine arabinoside addition, 16 hr after drug addition the same level of enzyme activity was observed for the 3 series. The observation of an actual decrease in thymidylate synthetase activity indicates that cytosine arabinoside was not simply maintaining thymidylate synthetase activity that existed at the time of drug addition.

The simultaneous addition of cytosine arabinoside and methotrexate at 10 µM concentration blocked the increase of thymidylate synthetase activity by methotrexate (Chart 3). The reduction in thymidylate synthetase activity, which was observed with cytosine arabinoside-treated cultures, was delayed in the 8- and 12-hr series and was not observed for the
Chart 2. Thymidylate synthetase activity of CCRF-CEM cells after exposure to 10 μM cytosine arabinoside (CA) for various periods of time. Drug was added 4 (○), 8 (△), and 12 (●) hr after inoculation of cultures. Control cultures either lacked drug (•) or received drug at the time of collection (♦).

Chart 3. Thymidylate synthetase activity of CCRF-CEM cells after exposure to 10 μM methotrexate (MTX) plus 10 μM cytosine arabinoside (CA). Drug was added 4 (○), 8 (△), and 12 (●) hr after inoculation of cultures. Control cultures either lacked drug (•) or received drug at the time of collection (♦).

4-hr series in the presence of methotrexate. The cultures to which both drugs were added 8 and 12 hr after inoculation continued to increase their content of thymidylate synthetase activity in parallel with the control cultures until 12 hr after drug addition. Between the 12th and 16th hr after drug addition, enzyme activity dropped about 20% below the 12-hr post-drug addition level.

Although the drug concentrations in the preceding experiments were relatively high in comparison with other studies of cell cultures (5, 16), the concentrations were in the range that would be expected with uniform distribution in the total body water following the administration of the drugs to patients (22) and, perhaps, lower than would be expected with nonuniform distribution. In general, patients excrete a major fraction of these drugs in less than 20 hr (7, 15).

The dose-response curves of thymidylate synthetase to methotrexate, alone and in combination with cytosine arabinoside, are presented for a 20-hr study with drug concentrations ranging from 0.03 to 10 μM (Chart 4, A and B). Thymidylate synthetase activity was elevated more as the concentration of methotrexate was increased (Chart 4A). Enzyme elevation by methotrexate was reduced as the concentration of cytosine arabinoside was increased. In the corresponding plot with cytosine arabinoside (Chart 4B), the decrease in thymidylate synthetase activity was dose related. The slight stimulation of enzyme activity at 0.03 μM cytosine arabinoside was reproducible and also occurred in other studies at 0.01 μM. This stimulation may be related to the observation that, after 20 hr with 0.01 or 0.04 μM cytosine arabinoside, cultures recovered some capacity to incorporate thymidine into DNA.

Dialysis of Homogenates. Although the cell pellets from the cultures were diluted from 50- to 100-fold for assay, the possibility persisted that methotrexate increased thymidylate synthetase activity by a release from a negative feedback control. The control cultures would then have less apparent enzyme activity than a methotrexate-treated culture. Cytosine arabinoside, by blocking DNA synthesis, could permit a greater accumulation of the feedback inhibitor, which would depress the activity below the level observed in the control.

To test for these possibilities, we incubated the cultures with 10 μM methotrexate or cytosine arabinoside in spinner flasks for 20 hr. The cells were collected and assayed for concentration of cytosine arabinoside was increased. In the corresponding plot with cytosine arabinoside (Chart 4B), the decrease in thymidylate synthetase activity was dose related. The slight stimulation of enzyme activity at 0.03 μM cytosine arabinoside was reproducible and also occurred in other studies at 0.01 μM. This stimulation may be related to the observation that, after 20 hr with 0.01 or 0.04 μM cytosine arabinoside, cultures recovered some capacity to incorporate thymidine into DNA.

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**Modulation of Thymidylate Synthetase Activity**

**Table 3**
The effect of dialysis on thymidylate synthetase activity

One ml of homogenate was dialyzed for 24 hr at 4° against two 1000-ml changes of 0.01 M Tris-HCl, pH 7.2. This is representative of 3 experiments, each of which contained duplicate cultures for each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (cpm/hr/mg protein)</th>
<th>Recovery (%)</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Before dialysis</td>
<td>107,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control After dialysis</td>
<td>80,000</td>
<td>75</td>
<td>165</td>
</tr>
<tr>
<td>Methotrexate (10 μM) Before</td>
<td>284,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate (10 μM) After</td>
<td>221,000</td>
<td>78</td>
<td>176</td>
</tr>
</tbody>
</table>

**Table 4**
The effect of dialysis on thymidylate synthetase activity of CCRF-CEM cultures after cytosine arabinosae

One ml of homogenate was dialyzed for 24 hr at 4° against two 1000-ml changes of 0.01 M Tris-HCl, pH 7.2. This is representative of 3 experiments, each of which contained duplicate cultures for each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thymidylate synthetase (μmoles TMP/hr/g protein)</th>
<th>Recovery (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Before dialysis</td>
<td>19.0</td>
<td></td>
<td>77</td>
</tr>
<tr>
<td>Control After dialysis</td>
<td>14.6</td>
<td>77</td>
<td>73</td>
</tr>
<tr>
<td>Cytosine arabinoside (10 μM)</td>
<td>5.15</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>Cytosine arabinoside (10 μM)</td>
<td>3.79</td>
<td>74</td>
<td>74</td>
</tr>
</tbody>
</table>

The changes in thymidylate synthetase activity with the addition of methotrexate or cytosine arabinoside to CCRF-CEM cultures resembled the observed response in leukocytes from patients with acute granulocytic leukemia after drug administration (23). The increase in thymidylate synthetase activity was much less drug specific than the elevation of activity by methotrexate and suggests other drug combinations that could block the methotrexate-induced elevation of thymidylate synthetase activity.

The rationale for the present approach derived from the premise that oncolysis by methotrexate results from greater uptake by some sensitive cells (12, 17). Sensitivity must also be determined by the phase of the division cycle of the cell (25). A proposed biochemical basis for the oncolytic activity of methotrexate is the inhibition of dihydrofolate reductase (2, 28, 29), although this inhibition does not, per se, assure therapeutic response (22). High intermittent doses were more effective treatment of the leukemic human or mouse than lower daily doses (11, 26), which suggests, among other possibilities, a need for drug in excess of that required to establish an inhibition of dihydrofolate reductase. In support of this suggestion was the observation that the incorporation of deoxyuridine into DNA could be further depressed by adding methotrexate in excess of that required to inhibit dihydrofolate reductase (24).

Borsa and Whitmore (3) have presented strong evidence to support their contention that this "excess" drug may be required for an inhibition of thymidylate synthetase. The observation by Ngu et al. (19) of a normal rate of liver regeneration in partially hepatectomized rats with dihydrofolate reductase activity severely, if not completely, inhibited by methotrexate is not incompatible with this suggestion. These observations, with the additional evidence that cytosine arabinoside depressed thymidylate synthetase activity in the leukocytes from patients with acute granulocytic leukemia, led to the present studies.

Although cytosine arabinoside does produce the desired effect on thymidylate synthetase activity in the human and has some effectiveness in the treatment of acute granulocytic leukemia, the ideal drug combination would be with an agent with a pattern of intoxication that is dissimilar to methotrexate. Cytosine arabinoside fails to meet this last...
criteria because the pronounced thrombocytopenia, leukopenia, and mucosal ulceration, which frequently accompany treatment (1), are also associated with methotrexate intoxication.

The present results indicate that methotrexate and cytosine arabinoside do not alter thymidylate synthetase activity by changing the concentration of dialyzable, allosteric effectors, which may or may not exist. The observations on thymidylate synthetase activity are compatible with changes in the amount of enzyme present in the cells. The elevation of enzyme activity by methotrexate is not compatible with the establishment of a synchronized culture, since other drugs acting on cells in the S phase of cell division did not produce a similar elevation. An elevation of enzyme activity may occur as the result of either, or both, an increase in the rate of enzyme synthesis or a block of enzyme turnover. Other studies are compatible with the hypothesis that methotrexate acts by blocking enzyme turnover (unpublished observation).

The present work indicates for CCRF-CEM that other drugs mimic the action of cytosine arabinoside in depressing thymidylate synthetase activity after 20 hr of exposure to the agents. A nonspecific inhibition of the synthesis of thymidylate synthetase would be compatible with these observations. These 2 lines of reasoning lead to the postulation that the variation in thymidylate synthetase activity observed with various concentrations of the 2 drugs results from changes in the relative rates of enzyme synthesis and degradation. The contribution that this type of drug interaction can give to the treatment of neoplastic diseases has not been tested.

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